CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

FACULTY OF FORESTRY AND WOOD SCIENCES

DEPARTMENT OF FOREST PROTECTION AND ENTOMOLOGY

Evolutionary Ecology of Termites

Evoluční ekologie termitů

Dissertation thesis

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2019

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"I declare that I am the sole author of this dissertation on *Evolutionary Ecology of Termites* using the cited literature and consultations and recommendations of the supervisor. I agree with the publication of the dissertation under Act No. 111/1998 Coll. on Higher Education Institutions, as amended, regardless of the outcome of its defence"

In Prague on …… 2020

Mgr. Petr Stiblík

"Prohlašuji, že jsem disertační práci na téma *Evolutionary Ecology of Termites* vypracoval samostatně s použitím uvedené literatury a na základě konzultací a doporučení školitele. Souhlasím se zveřejněním disertační práce dle zákona č. 111/1998 Sb. o vysokých školách v platném znění, a to bez ohledu na výsledek její obhajoby."

V Praze dne …… 2020

Mgr. Petr Stiblík

Acknowledgements / Poděkování

The last years delivered me a very important first-hand experience expressed in an old proverb: “A friend in need is a friend indeed!”

Therefore, I would like to express here my very special thanks to my friends as follows: to Honza Šobotník for his great leadership of my studies and his true friendship and reliability; to Jirka Synek, Franta Jůna, Patrik Soukup and Michael Kotyk for not letting me die in PNG; to Petr Táborský and Vláďa Koucký for not letting me die in Malawi, to Smolíček for being my best man; to my whole family for their infinite support and especially to my parents for their love.

Of course, the most special thanks belong to my wife, who accompanied me not only to Brazil, Thailand and PNG, but supported me for the entire period of our relationship, although she experienced very complicated times with me.

Besides above mentioned, I thank to all my collaborators which I admire for their work and enthusiasm. Last but not least, I must acknowledge also FFWS, CULS for providing material and financial support.

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Během posledních let jsem měl několikrát možnost na vlastní kůži pochopit staré přísloví: ”V nouzi poznáš přítele.”

Proto bych na tomto místě rád poděkoval všem mým přátelům, zvláště pak: Honzovi Šobotníkovi za jeho upřímnost, skvělé školitelství a že jsem se na něho mohl vždy spolehnout; Jirkovi, Frantovi, Patrikovi a Zubovi, že mě nenechali zdechnout na Papui; Táborákovi a Čubarakovi, že mě nenechali zdechnout v Malawi; Smolíčkovi za Smolíčka; celé mojí rodině za nekonečnou podporu a lásku, mým rodičům pak obzvláště.

Ty nejvřelejší díky však patří mojí skvělé ženě Pavlínce, která mě nejenom doprovázela na některých expedicích, ale především mi svou láskou a důvěrou byla vždy oporou, ačkoli to se mnou často nemívá lehké.

Kromě výše zmíněných patří mé díky též všem mým kolegům, které upřímně obdivuji za jejich práci a nasazení. V neposlední řadě je třeba zmínit i Fakultu Lesnickou a Dřevařskou při České Zemědělské Univerzitě v Praze za finanční a materiální podporu mého výzkumu.

Abstract / Abstrakt

Abstract

Termites are shaping the tropical and subtropical terrestrial ecosystems in many ways and on many levels, but their main contribution lies in their feeding strategy. They feed on dead plant tissues in various stages of decomposition, which is a very recalcitrant matter hard to digest. This is the reason why termites had established a broad range of associations with symbiotic microbes in their guts. Some termite species are also associated with microbes that grow in their nests, but the prevalence of these associations remains largely unknown. All these relationships underwent a long and delicate co-evolution, which is of intense scientific concern over a century, but the knowledge of their evolutionary ecology is still insufficient.

Here, I present the latest scientific progress in both, termite phylogeny and termite microbial associations. Thanks to the recent studies, the cladistic relationships between the termite families are solved for a sole exception of Rhinotermitidae + Serritermitidae, although the β-taxonomy sometimes doesn't reflect the clades and should be updated. I present here my contribution to the research in the field of termite molecular phylogeny of family Kalotermitidae and subfamily Apicotermitinae (Termitidae), and in the field of internal and external termite associated microbial community evolutionary ecology.

Abstrakt

Termiti ovlivňují suchozemské tropické a subtropické ekosystémy mnoha způsoby a na mnoha úrovních. Jejich hlavní přínos však spočívá v jejich potravní strategii. Živí se odumřelými rostlinnými pletivy v různém stádiu rozkladu, což je velice těžko stravitelný materiál. Z toho důvodu si termiti vytvořili vzájemně prospěšné vztahy s mnoha mikroskopickými organismy obývajícími jejich střevo. Někteří termiti si dokonce vytvořili symbiotické vztahy i s mikroby obývajícími jejich hnízda, avšak rozšíření těchto vztahů zůstává velikou neznámou. Přestože jsou termiti a jejich mikroskopičtí symbiotičtí společníci objektem zájmu vědy po více než století, naše znalosti jsou v jejich společné evoluční ekologii stále nedostatečné.

Ve své disertační práci tak přibližuji nejnovější vědecký pokrok v oblasti termití evoluční historie s ohledem na jejich symbiotické mikrobiální komunity. Díky nejnovějším kladistickým studiím již známe příbuzenské vztahy mezi všemi čeleděmi termitů s výjimkou vzájemného vztahu mezi čeleděmi Rhinotermitidae a Serritermitidae, avšak taxonomii čeledí Kalotermitidae a Termitidae bude třeba revidovat. Proto zde předkládám výsledky současného výzkumu molekulární fylogeneze čeledi Kalotermitidae a podčeledi Apicotermitinae, na kterém jsem měl tu čest se podílet. Zároveň jsou tyto nové poznatky z fylogeneze termitů uváděny v souvislosti s evoluční ekologií vnitřních i vnějších termitích symbiotických společenstev.

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Final thesis assignment form

CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Forestry and Wood Sciences

Ph.D. THESIS ASSIGNMENT

Mgr. Petr Stiblík

Forestry Engineering
Forest Protection and Game Management

Thesis title

Evolutionary Ecology of Termites

Objectives of thesis

Introduction to the topic:
Termites (Blattodea: Termitoidea) are one of the most important ecological factors on the Earth among the animals, and are referred to as ecosystem engineers for their importance (Jouquet et al. 2006). Through their activities, they affect the surrounding environment in a very complex way – they recycle dead plant tissues, aerate the soil, transport huge volumes of matter, thus fundamentally increasing the heterogeneity of the environment, etc. and due to their ability to degrade up to 100% of the local production of complex biopolymers, cellulose and lignin, they are probably responsible for the cessation of coal seam formation in the Tertiary (Engel et al. 2009).
Termites show a breathtaking complexity of eusocial arrangement (Howard and Thorne 2011) comparable to bees or ants, however, many partial problems are solved in different ways. They are an internal group of cockroaches, and the sister clade are cockroaches of the genus Cryptocercus (Lo et al. 2000; Inward et al. 2007). Cryptocercus is a social roach living in small families with parental care in rotten wood, which is at the same time their food source. These facts predestined termites most interesting properties, the eusociality and xylophagy, which combined resulted in effective occupation of a huge niche. They are able to feed on the most abundant biomaterial on Earth – cellulose and lignin.
However, the lignocellulose is not digested by termite gut enzymes directly. Termites use a broad range of gut symbionts to extract the nutrients from the organic matter (Breznak and Brune 1994; Brune 2014). Although the communities of gut symbionts (bacteria and protists) are quite well studied, the evolution of the relationship is not sufficiently explained, like the brisk switch from protists in “lower termies” to bacteria in “higher termites”. Moreover, although it is known that termites can grow ecto-symbiotic fungi Termitomyces, the phenomenon of environmental external symbionts of termites is not studied at all. Except the Macrotermitinae fungal gardens, there are anecdotal mentions about bacterial gardens in Sphaerotermitinae (Garnier-Sillam et al. 1989; Mueller et al. 2005; Genise et al. 2010). Tight collaboration of termites with external microbes might be completely overlooked phenomenon across all termite families, but it can be fully understood only from the evolutionary perspective based on solid phylogenetic hypotheses.

Goals of the thesis:
1. Bring new insights in the evolution of termites with unresolved phylogeny.

2. Search for patterns in co-evolution of termites with gut microbes.
3. Test whether there are environmental microbes associated to termite activities and digestion.
4. Describe the externally associated microbial communities.

Methodology

Although the phylogeny of termites might seem resolved thanks to work of Thomas Bourguignon, there are several uncertain points in the evolution. I will focus my efforts to disentangle irregularities in the phylogeny of the "lower termites" Kalotermitidae and the soil-feeding "higher termites" of the sub-family Apicotermitinae. These data can be later used for mapping and investigation of various evolutionary phenomenons.

More importantly, I will focus my investigation on relationships of termites with various environmental microbes, especially to test the hypothesis of ecto-symbiotic environmental microbial communities of termites.

To test this hypothesis, I will use already collected material of termites, their food-source and non termite environment control. It is actually termite workers bodies, wood pieces infested by termites and the pieces of the same wood, but not infested by termites yet. Isolation of total DNA and amplification of specific DNA locuses (bacterial 16S and fungal ITS2) will result in sequencing library for Next-Generation Sequencing. Sequenced reads will be identified and processed through Phyloseq analysis to discover any patterns accompanying the sample types. This way, I will identify microbial communities connected to termite activities, especially the ones occurring very often in the infested wood, but little in termite bodies and very little in the not infested wood.

Additional samples will be collected during expeditions with Dr. Šobotník, who plans unique field experiments for his GAČR project. Experimental sterile baits of wood and organic rich soil substrate will be placed in the tropical forests across the world to collect unprecedented dataset of organic substrate infested and not infested by termites during half-year exposure. Hals of the baits will be covered by fine mesh, disallowing termites to encounter the food source, but still allowing the microbes to get in. This way the random environmental decomposing bacteria and fungi will be separated from the ones associated to termites. The communitiy analyses will be done in the lab the same way mentioned above

Time schedule of the research:

1st semester – Data and knowledge acquisition from the literature. Preparation for the fieldwork.

2nd semester – DNA isolation, laboratory workflow setting. Field expeditions.

Gabon/Cameroon – Field expedition for experimental samples as a team member of an ongoing GAČR project.

Peru/Ecuador – Field experiments on termite associations with environmental microbial communities within the framework of tropical agriculture and agroforestry.

3rd semester – Laboratory analyses of termite associations with environmental microbial communities. Additional field works.

4th semester – In silico analyses of termite associations with environmental microbial communities. Composition of first scientific article manuscripts.

5th semester – Analyses of termite associations with environmental microbial communities within the framework of tropical agriculture and agroforestry.

6th semester – Final analyses and composition of scientific article manuscripts.

7th semester – Composition of scientific article manuscripts and of the dissertation thesis

8th semester – Dissertation thesis compositing

The proposed extent of the thesis

100-200

Keywords

Termites, Ecology, Evolution, Social insects, Apicotermitinae, Symbiosis

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Recommended information sources

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Expected date
2020/21 WS – FFWS – State Doctoral Examinations

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Foreword

"I look at the geological record as a history of the world imperfectly kept and written in a changing dialect. Of this history we possess the last volume alone, relating only to two or three countries. Of this volume, only here and there a short chapter has been preserved, and of each page, only here and there a few lines."

Charles R. Darwin (1859)

Since the primeval attempts to record all life forms on Earth, after Aristoteles, Carl Linnaeus was probably the first taking this goal seriously and systematically in his 10th edition of *Systema Naturae* (1758). However, nowadays his effort might look romantically naïve, it was the cornerstone in a way of thinking about the biological species and diversity. Later on, influenced by Charles Lyell and his *Principles of Geology* (1830), Charles Darwin realised, that the biodiversity known from geological record surely is just a tiny fragment of all life forms ever existed on the Earth. Thanks to his book *On the Origin of Species* (1859), the concept of biological species as stable, changeless and given units, finally fell apart. Moreover, Darwin debated on factors limiting otherwise indefinite number of existing life forms and he comes with the theory that the environmental factors played probably minor role in biodiversity compare to mutual relationships between the species. This I see as an important point, as no life form, the less the Eukaryotes, is a self-standing island, and with growing species or individual number, the complexity of the system grows even more rapidly.

Though, the number of species is truly not indefinite, it is much greater than we have recorded so far. Moreover, the total number estimates of global biodiversity still range widely from a few millions up to tens of millions (Erwin 1982; May 1988; Chapman 2009; Mora *et al.* 2011; Costello *et al.* 2013; Locey & Lennon 2016; Stork 2018). It brings me back to Carl Linnaeus, who might think, that his work will be completed by his follower in maybe few decades, but instead, he opened Pandora's box, still employing thousands of scientists around the world, even after almost 300 years of Linnaeus first attempts.

Actually, the only outcome common for all above mentioned studies is, that, thanks to insects, arthropods comprise majority of all eukaryotic diversity. In respect to great number of species still not discovered, Robert May (1988) pointed out, that the economic progress of 20th century horribly limits the time available for global biodiversity research and for the nature conservation.

Luckily, in 1993 international effort for saving global biodiversity was incarnated during ratification of *Convention on Biological Diversity*. Currently, most of the States became parties of the convention, except for Holy See and USA (www.cbd.int). This convention commits the parties to protect the natural heritage and prevent unnecessary destruction of natural ecosystems. Of course, such effort must be supported with scientific research. Although it was shown that investing in natural conservation pays back (Balmford *et al.* 2002; Sumaila *et al.* 2017), we still do not invest enough.

During the present massive efforts to describe all life forms on the Earth using the latest molecular technology and approaches, we have to ask ourselves, whether the goal is only to satisfy our curiosity, or if it might provide us more important knowledge. According to some (Vermeij & Leighton 2003), global diversity actually does not mean anything without knowledge of complex systems the living things create on the local scale. And truly, just the plain record of existing species, newly discovered or already extinct, would be incredibly boring without the knowledge of their function in the ecosystem. Honestly, does anyone think, that the public would invest trillions of US dollars into biological research simply to get a really thick book with complete record of species existing in our epoch?

Thus, the biological research focuses mainly on interactions, from metabolic pathways, over the symbiosis between host and microbiota, to the whole species populations and pest invasions. In many experiments or incidents we can clearly show, how exclusion of sole biological element influences the whole ecosystem in unpredictable ways (e.g. Ripple & Beschta 2003; Mao *et al.* 2005). In my opinion, such research must be delivered to the public in understandable narratives, although the benefits to public, well-hidden in the complexity of the ecosystems, may be sometimes hard to explain.

In 2015, I got the opportunity to see things I was only taught about in school, but I was not able to imagine, at all. Exploitation of natural resources

in irreparable way, uneducated approach to the environment and nature, agriculture that does not respect climatic and pedological conditions, terrible wasting of resources intended for nature conservation and sustainable agriculture, poverty deepened by greed of corporations using greed of the leaders of the developing countries, that all together and even more collaborating in concert on the doom of the richest ecosystems on our planet. Not only in my opinion, the only way out of this machinery consuming our natural heritage is better education of the population and the new technologies. Partially, the education of the population in developing countries can be delivered by inclusion of the local communities into research projects taking place close to their homes. Perfect example is Binatang Research Centre established by prof. Vojtěch Novotný in Papua New Guinea, which I had the honour to collaborate with.

Finally, I participated on research of termites (Blattodea: Termitoidea) for last six years. My work brought me around the world to investigate not only termite diversity, but mainly their different ecological strategies and relationships with their outer and inner environments. Research of termites gave me the rare opportunity to work on extremely interesting organisms under complicated conditions together with my friends. On the following pages, I would like to present part of the knowledge I gathered during my doctoral studies.

Introduction

"If humanity depends so completely on these little creatures that run the Earth, they also provide us with an endless source of scientific exploration and naturalistic wonder."

Edward O. Wilson (1987)

1. Global biomass and its cycle

While the humankind is currently trying to invent technologies for sustainable circular economy, the nature is based on this mechanism since ever. Almost all organic matter on the Earth is directly or indirectly derived from plants tissues thanks to their ability to incorporate the atmospheric carbon during photosynthesis (Witt *et al.* 1961). Most of the carbon incorporated in complex organic polymers of plant tissues is latter available as a food source for decomposers, mostly soil-dwelling organisms or microbes (Condron *et al.* 2010). Majority of these organisms, in both measures, biomass and biodiversity, are bacteria and fungi. They interact with micro-fauna, meso-fauna and macro-fauna in highly complex food-web systems that determine the turnover of organic matter and associated nutrients in the environment (Coleman & Wall 2007; Wardle 2002). While majority of above-ground organic matter is consumed by animals, decomposition of organic matter in the soil is driven primarily by the activities of bacteria and fungi and only 10–15% can be directly attributed to the actions of soil fauna (Hopkins & Gregorich 2005). However, the importance of fauna should not be underestimated (Evans *et al.* 2011; Schowalter 2017).

Even the soil nutrient cycle is significantly promoted by the macrofauna (Bignell & Eggleton 2000; Condron *et al.* 2010; Dahlsjö *et al.* 2014). Invertebrate decomposers of plant organic matter comprise of Annelida (Dahlsjö *et al.* 2014), Mollusca (Suzuki *et al.* 2003; Xu *et al.* 2001), Nematoda (Smant *et al.* 1998), Coleoptera (Kukor *et al.* 1988), and Blattodea (Lo *et al.* 2000) including termites (Dahlsjö *et al.* 2014; Watanabe *et al.* 1998).

Thanks to wood-feeding strategy, termites are mainly known as pests of timber causing damages over $70 billion USD worldwide every year (Bradshaw *et al.* 2016; Su 2002, 2019). However, termites should be rather known as

significant drivers of nutrient cycle in the tropics as they consume over 50% of global terrestrial cellulose production and 100% in some ecosystems (Engel *et al.* 2009). Without intensive work of their mandibles, the plant tissues would not be grinded into small particles and the decomposition by microbes would take much longer. Termites can feed on sound wood, grass, leaf-litter, decayed organic matter and even soil. On the other hand, even termites would not be able to digest the organic matter without complex symbiosis with microbial community. The digestion of plant tissues in termites is aid by microbes in concert with innate cellulases (Lo *et al.* 2003; Ohkuma & Brune 2011). This example reveals that the fundament of nutrient cycle on Earth is based on tight and complex collaboration between vast number of different organisms.

It is often said that tropical rain forests host majority of animal and plant species on the Earth, however this information is mainly based on the breath-taking life diversity in Amazon rainforest (Haffer 1969; Smith *et al.* 2014), which were quite well-studied in last decades compared to, for scientists still impermeable and dangerous, locations of central Africa due the unstable geopolitical situation from the turn of the millennium (Reyntjens 2011). Who knows, what biological treasures might be still hidden there? And will we be able to discover them before we will make them extinct?

The massive efforts for description of all living forms on the Earth started in 1758 thanks to Carl Linnaeus, who published the first overview of known species in hierarchically organized system which we are using till today. However, Linnaeus himself estimated the final number to a few hundreds of thousands and believed the job will be finished in next generation of his followers. But his followers lost this belief very soon and the estimates of species in all taxonomy kingdoms started to grow quickly.

Since the iconic research of Terry L. Erwin, there is no doubt that the planet Earth is the world of insects (Erwin 1982; Wilson 1987), especially in terms of biodiversity and ecosystem services. Although Erwin's estimation of insects species richness was probably by far overestimated, it still easily exceed any other macroscopic organism on Earth (Fig.1) (May 1988; Stork 2018).

Region	Predicted % of world's plant species	Number of vascular plants	Number of arthropod species	Number of insect species	Number of beetle species
Australasia	13.10	52,728	890,799	720,521	196,515
Afrotropical	17.73	71,363	1,205,639	975,179	265,971
Central America	11.18	45,000	760,240	614,918	167,713
Indo-Malayan	13.36	53,774	908,479	734,822	200,416
North America	2.10	8,453	142,800	115,503	31,502
Neotropics	29.46	118,577	2,003,279	1,620,348	441,935
Oceanic	3.54	14,249	240,720	194,706	53,104
Palearctic	9.53	38,358	648,040	524,165	142,961
Total	100	402,500	6,799,996	5,500,163	1,500,118

Fig.1 Plant and arthropod species distribution on Earth (Stork 2018)

For a long time, the colossal diversity of insects is put in context to functional coevolution with angiosperm plants, and *vice versa*, the angiosperm plants success since Mesozoic is put in context to insect activity, although it is probably not the only reason for their dominance (Engel 2015; Grimaldi & Engel 2005; Hu *et al.* 2008; Labandeira *et al.* 1994). Undoubtedly, thanks to photosynthesis plants make up by far the majority of biomass on Earth and the ecosystem production depends on them (Fig.2). About 80% of global biomass is estimated to plants, 10% to bacteria and the remaining 10% to all other life-forms, including animals (Bar-On *et al.* 2018). Arthropods make up about 50% of the total animal biomass (≈1Gt C of ≈2Gt C) and the organisms consuming over 50% of cellulose production (and 100% in some ecosystems (Engel *et al.* 2009)), the termites, are estimated to (≈0.05Gt C) which is equal to 0.007% of total global biomass (Sanderson 1996).

The terrestrial ecosystems are responsible for production of over 75% of global biomass (Bar-On *et al.* 2018), but not all of these ecosystems are equally productive. Although the results of existing studies usually varies a lot (Clark *et al.* 2001), the primary net production of tropical forests is estimated up to 35% of global terrestrial primary production (Melillo *et al.* 1993). It can be compared only to savannas and both reach far beyond other terrestrial ecosystems (Field *et al.* 1998). Such high production of tropical forests is based on rapid nutrient cycle thanks to high decomposition rates in stable warm and humid conditions (Nye 1960; Vitousek & Sanford 1986).

The decomposition rates in tropical forests are significantly promoted by termites whose abundance easily exceed 1000 individuals /m^2 (Bignell & Eggleton 2000; Eggleton *et al.* 1996) and on the global scale is comparable to that of ants or humankind (Bar-On *et al.* 2018; Hölldobler & Wilson 1990; Sanderson 1996). Termite activity in tropics is so eminent, that they are called *ecosystem engineers* (Bignell & Eggleton 2000; Freymann *et al.* 2008; Holt & Lepage 2000; Jouquet *et al.* 2006, 2011; Sugimoto *et al.* 2000).

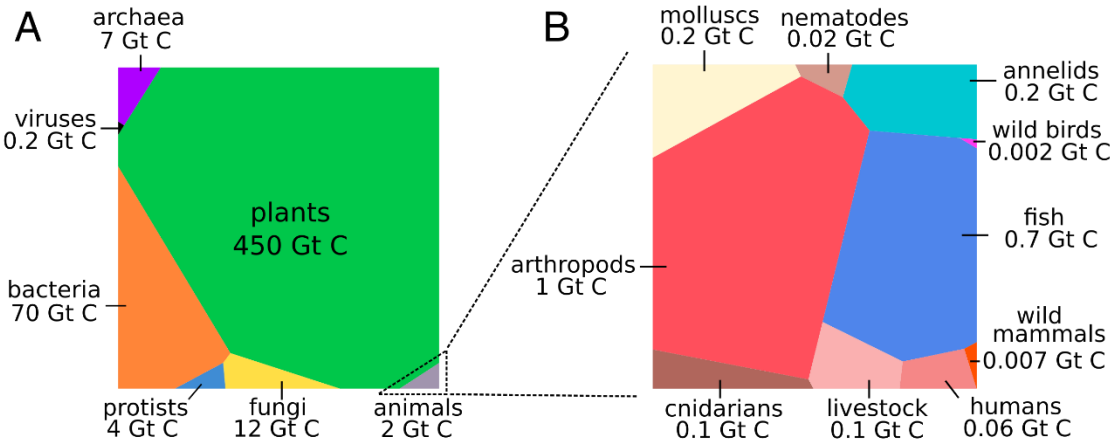

Fig.2 The biomass distribution on earth (Bar-On *et al.* 2018)

2. Who are termites?

Termites are eusocial insects inhabiting in enormous abundances tropical and sub-tropical terrestrial ecosystems. Although the adult imagoes possess wings and compound eyes, majority of termite individuals are in fact blind unsclerotised juveniles. They feed on dead organic matter of all types, from sound-wood, grass, lichens or leaf-litter, over decayed wood and humus to organic patches in soil or nest material of other termites. Adaptation of termite intestines to digest the most abundant organic matter on Earth, the lignocellulose, allowed them to occupy a huge niche. However, termites are also soft-bodied individuals vulnerable to desiccation and cold, therefore their geographical distribution is limited (Eggleton 2000; Roisin 2000; Engel *et al.* 2009).

Termites used to be considered as independent order Isoptera (Iso – the same, pteron - wing), but the progress in molecular phylogeny confirmed, what

has been intensively debated for last hundred years (Cleveland 1924, 1925, 1934; Donovan *et al.* 2000; Kambhampati 1995, 1996; Kambhampati *et al.* 1996; Kambhampati & Eggleton 2000). From the evolutionary point of view, termites are eusocial cockroaches, as they evolved within the insect order Blattodea in Jurassic at least 150Mya ago during the Pangea brekup. The closest extant relatives of termites are social wood-feeding cockroaches of genus *Cryptocercus* (Blattodea: Cryptocercidae) (Bourguignon *et al.* 2015; Inward *et al.* 2007). The common ancestor of termites (currently classified as epifamily Termitoidea) and *Cryptocercus* was probably wood-feeding cockroach living in small families inside dead logs in the warm and humid forests (Lo *et al.* 2007). The ancestor harboured plentiful microorganisms including flagellates in its gut, being over evolutionary times able to perform better in concert with their hosts, leading to the community switches (Bourguignon *et al.* 2015, 2018; Brune 2014; Ohkuma 2008). Contrary to termites, *Cryptocercus* never achieved the ecological dominance

Termites are often labelled as "lower" or "higher", which is a practical distinction of two different termite groups based on microbial gut communities, life-style and food. "Lower" termites comprise all termite families except for the phylogenetic crown group, Termitidae – the "higher" termites. "Lower" termites are wood-feeders or grass-feeders hosting symbiotic flagellates in their hindgut. Most of the "lower" termite species live in rather small colonies (except Mastotermitidae and some Rhinotermitidae) and often feed on sound wood, which makes them pests, known as dry-wood termites (Kalotermitidae) or subterranean termites (Rhinotermitiade). In contrast, the "higher" termites lack any flagellates in the hindgut and they mostly feed on more decayed organic matter, soil, sometimes also fungi or lichens. The variability in the food source is reflected in the species diversity, as "higher" termites comprise about 85% of termite generic diversity (Kambhampati & Eggleton 2000; Krishna *et al.* 2013). Their colonies can be much larger than those of "lower" termites in both, size and number of colony members.

As hemimetabolous insects, termites and *Cryptocercus* exhibit a classical developmental pathway leading from the egg to the winged imago (alate) through a number of successive immature instars, characterized by the progressive growth of wing rudiments (called wing, buds, or pads). While

Cryptocercus lives in small families, where the couple nurtures its offspring, termites are eusocial, and the offspring tends their parents. The eusociality is usually defined as a colony life-style, where the tasks are distributed between castes and moreover, the reproduction is reserved for one of these castes (Wilson 1971).

In termites, the royal pair establishes their colony and raises the first cohort of workers and one or even more soldiers. Since then, the workers are taking care about the colony tasks as foraging, cleaning, nest construction, brood care, and other castes care, while the royal pair reproduces only. The specialised defensive caste, the soldier, deals with threats of various nature, and often act also as food scouts. Together with the feeding specialization, the colony defence strategies probably led to fast diversification of various species in termites.

3. Termite colony organization

As termites prefer to stay unobserved, their presence in the environment is usually recognized thanks to constructions they build or destroy. The majority of the colony life takes place in enclosed system of galleries forming more or less centralized nest. It can be several meters of thin tunnels inside a dead branch sheltering lower hundreds of individuals, as well as a house-sized complex structure visible from long distances and inhabited by millions of termites. Such huge nests astonish scientists for decades, as the construction precision and the functionality are unprecedent for such tiny blind creatures.

Some termites simply construct their nest as tunnels in their food source, e.g. dry-wood termites of family Kalotermitidae. The nest later grows into complex system of bigger chambers for tens of individuals connected with short narrow tunnels used as bottlenecks for effective defence of the colony. In “dry-wood” termites, the faeces accumulate as pellets somewhere, and are often released from the timber, and such sign should alert the house owner to take a vigorous action.

However, many termites are dwelling in the ground substrate. Ground dwelling is an effective strategy how to reach new food sources or construct invisible but huge underground nests, from which the termites can forage

through the gallery system to remote food source. Such nests are hard to study, but they can have decentralised structure and host millions of individuals, like the ones of the significant pest genus *Reticulitermes* (Rhinotermitidae).

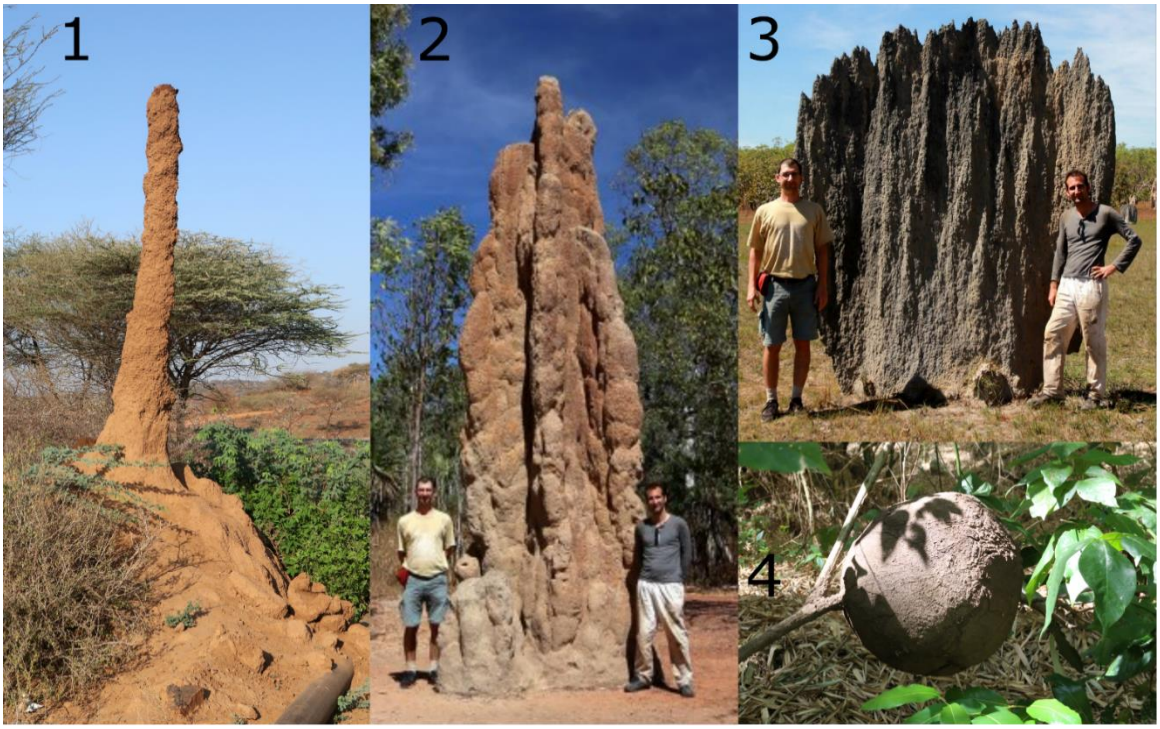

Fig.3 Termite nests - overview of incredible structures
1- *Macrotermes jeanneli* nest is the pile of ground under the chimney, which can be up to 8m high
2- *Nasutitermes triodiae* cathedral nest inhabited by millions of individuals
3- *Amitermes meridionalis* known as compass termite for constructing nests in north-south direction
4- Typical arboreal nest of South American *Nasutitermes*
Presented with permission of ©Jan Šobotník

Except for dwelling, termites can also construct novel structures from debris or their saliva and faeces. These are well known in fungus-growing termites like *Macrotermes jeanneli* (Grassé, 1937) (Termitidae: Macrotermitinae), in cathedral termite *Nasutitermes triodiae* (Froggatt, 1898) (Termitidae: Nasutitermitinae), in magnetic/compass termite *Amitermes meridionalis* (Froggatt, 1898) (Termitidae: Termitinae) or in South American *Nasutitermes* with their huge arboreal spherical nests (Fig.3). The structure is so complex that it may even have ventilation system, specialised chambers for particular tasks, like egg deposit, brood care, food storage and many bottlenecks for effective nest protection against intruders (Korb 2011). All these nests are inhabited by millions of individuals and also by the queen, which

is strongly physogastric to be able of producing over hundred billion eggs a year (Nutting 1969). Therefore, the queen is not able to move and so she is fully dependent on others care and spends her life in so-called royal chamber, well defended structure inside the nest.

The ontogeny of a termite individual is based on species-specific ontogenetic pathways regulated by genetic, colonial and environmental factors to maximise the colony gains. There are two basic ontogenetic pathways:

- *linear*, where workers (or *pseudergates*) are temporarily-specialised labour caste and usually later continue development into nymphs or soldiers;
- *bifurcated*, where many individuals lose the ability to from wings as they decide to become true workers in the early phases of their development. These mostly stay sterile.

The linear ontogeny allows for progressive (egg-to-imago direction), stationary (no change), and regressive (reduction of wing pads) moults (Roisin 1990). The workers can grow and moult in bigger workers or they can undergo stationary or even regressive moults in some cases. Under certain circumstances, workers can even moult into reproductive individuals, so-called ergatoids. For "lower" termites you may often find the term "pseudergate" or "false worker", which are usually workers possessing wing pads and not excluded from reproduction (Roisin & Korb 2011).

Interestingly, compared to the best-known eusocial insects, the holometabolous ants and bees, all termite colony members but kings and queens are immature individuals, and all of them but soldiers can moult into the next instar according to the species-specific ontogenetic pattern. This brings termites the unprecedent plasticity in colony organization and responsivity to sudden changes in their environment. For example, termite colony which consumed all available food in the dead branch on the living tree, or got threatened by bigger and more aggressive rival termite colony, can switch majority of the colony members into adult alates and escape to many other places, where the alates can start new colonies (Evans *et al.* 2009). Such strategy does not exist in eusocial Hymenoptera, as the workers are largely sterile all females (Hölldobler & Wilson 1990; Wilson 1971).

On the other hand, termites can also have the bifurcated ontogeny, is more rigid, especially in the "higher" termites (Roisin & Korb 2011). Linear and bifurcated ontogenetic pathway differ in existence of "true" workers in the apterous ontogenetic line (Roisin & Korb 2011). In linear ontogenetic pathway, only the soldiers and functional reproductives cannot fly off the maternal nest, while pseudergates/workers are still in the general attempt to moult into alate imagoes and swarm (Fig.4). However, their nestmates often prevent them from that by biting off the growing wings (Roisin 1994).

Bifurcated ontogenetic pathway, on the other hand, implies the presence of true workers, characterized by early and irreversible deviation from the egg-to-imago pathway (Fig.5) (Watson *et al.* 1977). The bifurcated ontogenetic pathway occurs in *Mastotermes,* Hodotermitidae, some Rhinotermitidae (all Rhinotermitinae, *Coptotermes, Heterotermes* and *Reticulitermes*) and all Termitidae (Roisin & Korb 2011)

The linear ontogeny seems to be ancestral based on similarity to cockroach ontogeny and the bifurcated ontogeny evolved in several basal groups including *Mastotermes*, the sister group to all remaining termites. In spite of controversial opinions on the evolution of termite castes, all we can confirm are repeated switches between both systems (Legendre *et al.* 2008; Miller 1969; Noirot & Pasteels 1987; Shellman-Reeve 1997).

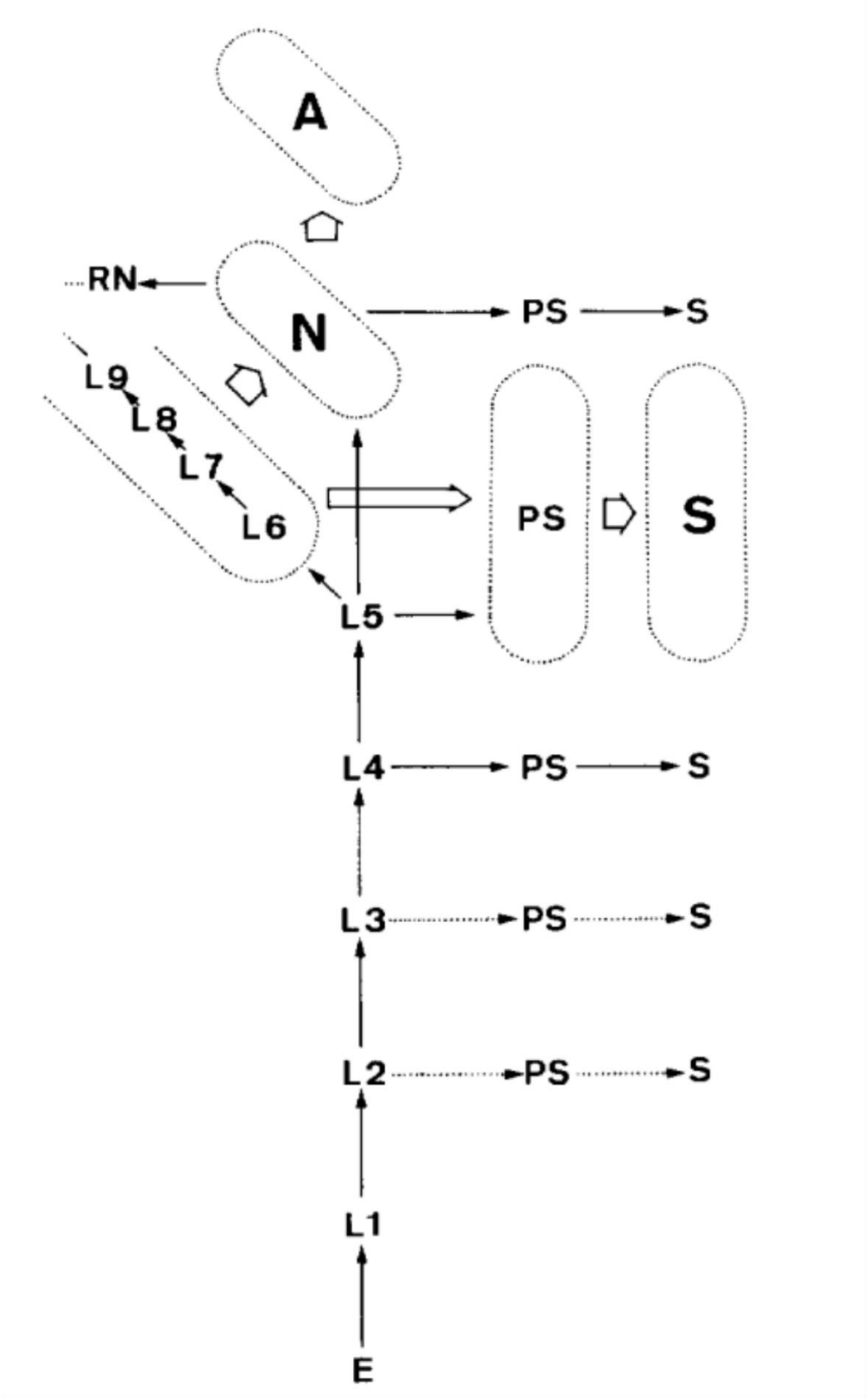

Fig.4 Linear ontogenetic pathway of *Prorinhotermes* (Roisin 1988)
The "false" workers are expressed as larvae instars. Each arrow means moulting
E-egg; L-larvae instar; PS-pre-soldier; S-soldier; N-nymph; A-alate;
RN-individual after regression moulting

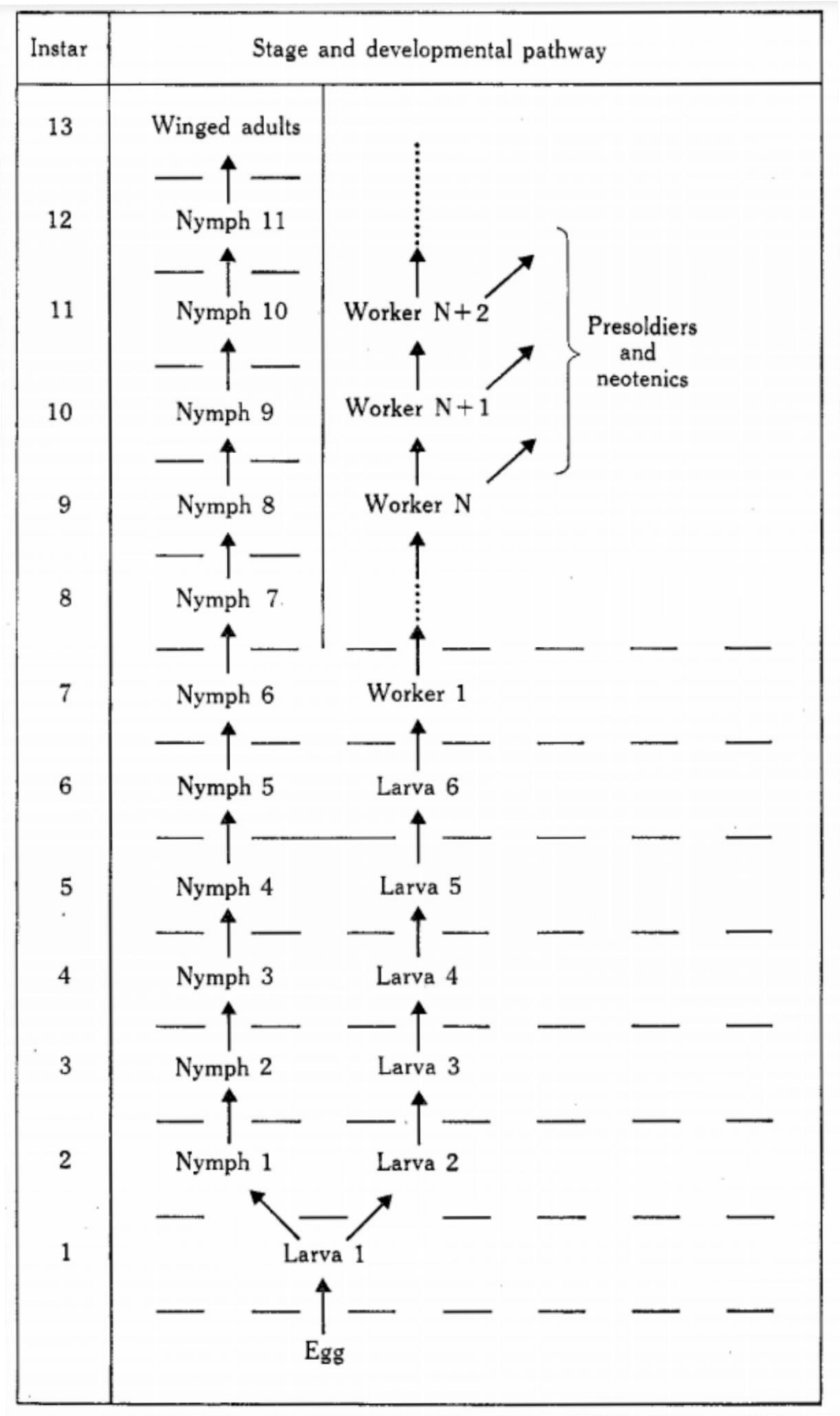

Fig.5 Bifurcated ontogenetic pathway of *Mastotermes darwiniensis* (Watson *et al.* 1977)

For purposes of this thesis, workers are functional caste irrespectively of their ontogenetic origin. They consume the organic matter and process it in their guts, so their bodies are darker than those of larvae. We could compare workers to the guts of the whole colony, as workers process the food and share either the regurgitate from both ends of digestive tube (proctodaeal or stomodaeal trophallaxis), or larvae and reproductives (rarely soldiers) by high (Noirot & Noirot- Timothée 1969; Sillam-Dussès *et al.* 2012; Scheffrahn *et al.* 2017). Apart of the crucial nutrition function, workers are also responsible for nest constructing, nest cleaning, brood care and sometimes even for active defence.

The workers can also moult into pre-soldiers and subsequently into soldiers. Soldiers are specialised individuals devoted to colony protection, possessing sclerotized cuticle and mandibles adapted to fight against the colony enemies. The diversity of soldier caste is the most diversified of all, and the usual source of characters for the species determination. Except for apparent enlarged mandibles (but reduced in Nasutitermitinae) and sclerotized head, soldiers also often possess defensive glands secreting repellent, poisonous or anti-healing liquids to poison or repeal the opponents. Soldier is a juvenile but terminal instar (Roisin & Korb 2011), which can't moult again and can reproduce in Archotermopsidae only (Thorne 1997; Thorne *et al.* 2003).

Nymphs are individuals with distinct wing pads progressing on the way to the alate imagoes. They are nutritionally independent in linear systems, while fully dependent in bifurcated, surviving on cannibalism when isolated from workers (Grassé 1984).

4. Termites as tropical ecosystem engineers

Termites move vast amounts of matter and run the soil bioturbation. They maintain the environment heterogeneity, bring the dead plant material back into the cycle, and locally are able to consume up to 100% of organic matter production (Bignell & Eggleton 2000; Dahlsjö *et al.* 2014; Evans *et al.* 2011; Fox-Dobbs *et al.* 2010; Freymann *et al.* 2008; Holt & Lepage 2000; Rouland-Lefèvre 2000). The termite-related decomposition of lignocellulose prevents since the rise of the "higher" termites some 60Mya ago formation of coal deposits, as the woody materials disappear before it could fossilise (Buček *et al.* 2019; Engel *et al.* 2009). Termites are also important CO_2 and methane producers (Sugimoto *et al.* 2000), virtually warming up the Global temperatures. Termites have also been shown to mitigate the droughts of the tropical forests due the ongoing climate change (Ashton *et al.* 2019).

As termites feed usually on dead plant tissues, they should not be referred as herbivores, but rather as saprophages (Bignell 2016; Cornaby 1977; Franzluebbers 2014; Siebers *et al.* 2015), however, they might act like herbivores when collecting leaf-litter, grass or feeding on lichens (Krishna *et al.* 2013). Termites are able to dissimilate a major proportion of the cellulose (74–99%) and hemicellulose (65–87%) components of lignocellulose they ingest (Ohkuma 2003). Such effectivity attracted attention of applied research, but no applicable outcome reached the broader usage, so far (Auer *et al.* 2017; Fujita & Watanabe 2010), in spite of ambitious current efforts (Marynowska *et al.* 2020). There is no evidence of termite feeding specializing on to particular plant taxa, as termites focus rather on the stage of decomposition, which is mirrored in their digestive system (Donovan *et al.* 2001a; Noirot 1995, 2001; Sands 1998). Based on food particles, mandible and gut anatomy, Donovan (2001) classified termites into following ecological feeding groups.

- Group I – termites feeding on sound or slightly rotten wood or grass, which is reflected in relatively simple digestive tract and wood-feeding type of mandibles. All "lower" termites belong into this category.

- Group II – termites feeding on wood, grass, microepiphytes or leaf-litter, having more complex digestive tract and wood-feeding type of mandibles. All wood-feeding "higher" termites belong into this category.

  Sometimes a special feeding Group IIa for fungus-growing Macrotermitinae may be considered.
- Group III – termites feeding on highly decayed wood, humus and other more or less decomposed organic matter, having soil-feeding type of mandibles and more complex digestive tract.
- Group IV – termites feeding on soil with relatively high inorganic content, having soil-feeding type of mandibles and extremely long and intricate digestive tract.

Although Donovan's classification allows for quick identification of feeding group, measurements of stable isotopes in termite body content distinguished only between two feeding groups of termites, merging Group I with Group II into wood-feeders and Group III with Group IV into soil-feeders (Bourguignon *et al.* 2011). While wood-feeding strategy is widespread across termite phylogeny, soil-feeders occur in family Termitidae, only. On the other hand, as family Termitidae make up over 75% of termite species diversity (Krishna *et al.* 2013) and soil feeding is their major strategy, it is estimated that over 60% of all termite species are actually soil-feeders (Brauman *et al.* 2000; Kambhampati & Eggleton 2000; Krishna *et al.* 2013).

Termites thrive on the organic matter in tropical forests and other tropical and subtropical terrestrial ecosystems, and play dominant role in the decomposing cascade returning the nutrients from dead plant matter into soil, making them available to the new plant growth (Fig.6) (Bourguignon *et al.* 2011; Coventry *et al.* 1988; Liu *et al.* 2015; Swift 1977). These ecosystem services are of prime importance at the natural sites, but also at crop-fields (Black & Okwakol 1997; Evans *et al.* 2011; Jouquet *et al.* 2011; Kaiser *et al.* 2017). Although lignocellulose is truly abundant matter, its decomposition is a difficult task. The biopolymer structure made of sugars (cellulose, hemicelluloses) embedded into an amorphous lignin matrix made the plant matter resistant and recalcitrant to the digestion (Cragg *et al.* 2015;

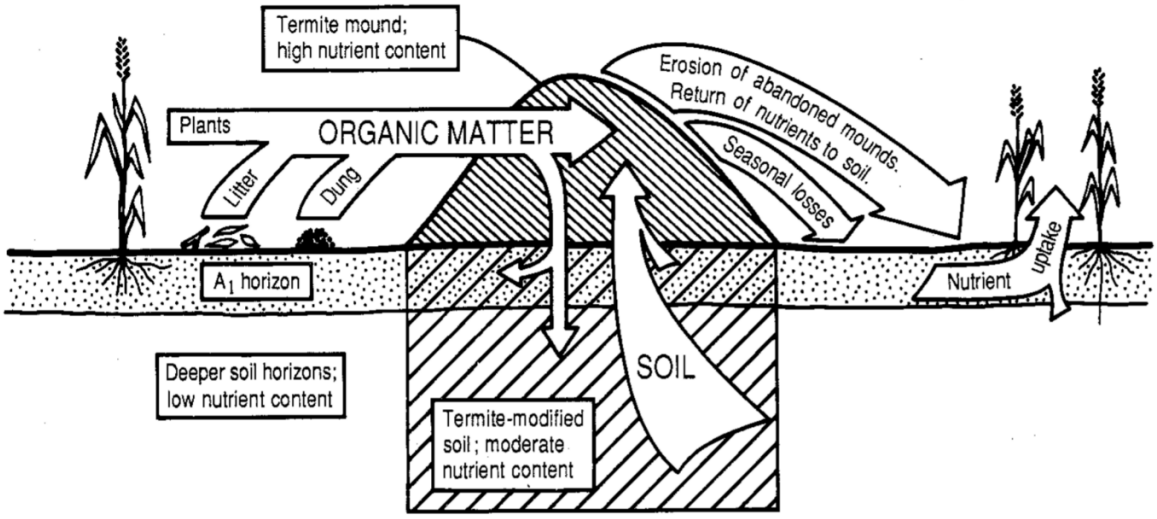

Fig.6 Nutrient flow through termite activity (Coventry *et al.* 1988)

Zimmermann & Brown 1971). There are, of course, many other organisms ingesting similar material as termites do (e.g. free-living fungi and bacteria, earthworms, many beetles, some moths or some cockroaches), but termites surely digest more of organic matter than all others combined (Hérault *et al.* 2010). This efficiency probably led to termination of coal production since Tertiary (Engel *et al.* 2009) as only a little of organic matter is left for carbonization.

Termites dominate the macrofauna diversity of tropical soils and play a role equivalent to earthworms (Eggleton *et al.* 1995). They search for patches of decayed organic matter in soil and use the remaining nutritional value to prosper. Contrary to earthworms, termites are more effective thanks to their mass activities, and so occupy most of the niche. Often, soil-feeding termites feed on nests of other termites or ants, because the faeces of wood-feeding termites used for nest construction are still more nutritionally valuable food compared to the bare soil (Bourguignon *et al.* 2013).

Except for the involvement in organic matter decomposition, termites serve to the ecosystems also other ways. Termites alter both the chemical and physical structures of the habitat not only through decomposition of organic matter (Bignell & Eggleton 2000; Donovan *et al.* 2001b), but also through bioturbation (Jouquet *et al.* 2011), soil-atmosphere gas exchange (Galbally *et al.* 2010) and formation of soil bio-structures (Decaëns *et al.* 2002).

Bioturbation is done by massive movement of soil counted in tons per hectare and year (Whitford & Eldridge 2013) from place to another and the tunnels termites leave behind allow both, water and air, to efficiently penetrate the soil. Moreover, as termites are vulnerable to desiccation, they fully depend on constant moisture in their environment. Therefore, they also transport a massive amount of water, often from very deep sources (over 30m), like the desert termite *Psammotermes hybostoma* Desneux, 1902 (Rhinotermitidae) (Grassé 1984). Worth to mention again, many termites construct huge epigeal nests, which may be of a small house size, and are common especially in tropical grasslands. Especially in arid areas, these mounds are sought-after habitat for plants (Fig.7) as the mounds concentrate organic nutrients and moisture (Bonachela *et al.* 2015). This way, termites are creating oasis for many plant and animal species during the dry seasons. In a global scale termites help to mitigate the impacts of global climate change on their ecosystems (Ashton *et al.* 2019; Bonachela *et al.* 2015).

Fig.7 Plants spatial distribution on termite mounds in savanah (Bonachela *et al.* 2015)

Termite digestion and symbioses

"The obvious efficiency in degrading lignocellulosic or humic matter, together with the high level of biodiversity within the guts and among the termites, may serve to provide new strains of microorganisms and alternative concepts for a technical treatment of recalcitrant xenobiotics bound to organic soil matter."

Andreas Brune (1998)

1. Digestive tract

Termite digestion is one of the main reasons of their evolutionary success as it allowed them to dominate the niche of dead plant matter decomposers. As termites are eusocial insects, the tasks in the colony are divided between specialised individuals. Compared to soldiers, in which the defensive gland may occupy more than 1/3 of body weight (Waller & La Face 1987), the majority of worker bodies is filled by gut. This fact intuitively leads to suggestion that digestion is a task of workers, but it is only partially true. Although termite soldiers cannot chew the feeding substrate due to the defensive modifications of mandibles, they often participate at the colony stomach, digesting the particular food provided by workers. The full dependence of soldier upon the worker nutrition evolved repeatedly in the so-called "white-gutted" soldiers that evolved repeatedly in several soil-feeding taxa (Scheffrahn *et al.* 2017).

Termite gut consists, similarly to all other insects, of three main parts: foregut (stomodeum), midgut (mesenteron) and hindgut (proctodeum), differing in embryologic origins (Chapman *et al.* 2013). The least derived intestines of wood-feeding termites are similar to that of cockroaches of genus *Cryptocercus* which is closely related to them and also feed on dead wood (Bourguignon *et al.* 2015; Buček *et al.* 2019; Inward *et al.* 2007), but the new feeding niches of family Termitidae caused adaption of whole digestive system to new substrates. The food is chewed and processed by the mandibles, masticated with the digestive enzymes from the labial gland and swallowed. The worker mandibles are more robust, with richer dentition and large molar plate in wood-feeders, while having fewer long teeth and barely some molar plate in soil

feeders (Donovan *et al.* 2000; Ahmad 1950). The food goes to a simple crop and proventriculus, where it is further grinded into micro particles (20–100 μm; Brune & Ohkuma 2011). While the armature of proventriculus is well sclerotized in "lower" termites, it is reduced in "higher" termites (Donovan *et al.* 2000), which may be due to softer and breakable food properties. The proventriculus enters the midgut by the esophageal valva.

The midgut is the primary source of digestive enzymes in insects (Chapman *et al.* 2013), however, in termites it is usually a simple tube, but still able to produce series of digestive enzymes including cellulases in the "higher" termites, while the same enzymes come from labial glands in "lower" termites (Tokuda *et al.* 1997, 2002, 2009). Otherwise, the midgut is rather constant across termites with the exception of caeca, which is noticeably present in some "lower" termites (Noirot & Noirot-Timothée 1969).

Some group-defining characters originate from *in situ* configuration and coiling of the gut as noted by Sands (1995, 1998). The midgut ends by the proctodeal valve with no special modification among "lower" termites and basal Termitidae. However, this gut part, the mixed segment, is of complicated structure in advanced Termitidae, in which it is formed by groups-specific way of midgut / hindgut overlaps, at the place of Malpighian tubules junction. (Bignell 2011; Donovan *et al.* 2000; Noirot & Noirot-Timothée 1969). The close proximity of posterior hindgut to mixed segment is associated with extremely high pH in P1 in subfamily Termitinae and the coiling of the whole gut is cause of extremely various pH values (Bignell 2011; Brune & Kühl 1996). Moreover, the Malpighian tubules attached at the junction of midgut with hindgut reduced their number from 8 or more in "lower" termites to 4 or fewer in Termitidae (Donovan *et al.* 2000).

The hindgut has 5 segments labelled as P1-P5 (Fig.8). The P1 is short and narrow in "lower" termites and Macrotermitinae, but is variable in length and dilatations among other "higher" termites. The P2 is of great size variation in termites and is known as enteric valve (Donovan *et al.* 2000; Noirot & Noirot-Timothée 1969). The enteric valve is highly variable across the Termitidae and its function is somewhat obscure. In soil-feeders it is especially complicated and taxon-specific (Bourguignon *et al.* 2013) and probably helps to separate clay particles, which have abundant soil organic matter associated with them, from silica (sand) particles, which are inert. The enteric valve may ensure that clay particles bounded to organic matter stay in the hindgut longer than silica particles (Bignell 2011; Donovan 2002). The structure of enteric valve is of special importance in soil-feeding soldierless species taxonomy (Bourguignon *et al.* 2013) or feeding group identification (Donovan *et al.* 2001a).

The P3 (paunch) part is always dilated as the fermentation takes place there and, moreover, it is inhabited by the richest community of bacteria and also flagellates in the case of "lower" termites. Sometimes the P3 segment bears diverticulum distinctly different from those of enteric valve. The P4 (colon) segment is always of considerable length as it coils in the loop of midgut and continues to P5 (rectum). In "lower" termites and Macrotermitinae the P4 makes only a single coil, but in remaining Termitidae the colon may be extended and forming extra loops. P5 is the rectum which is of general organization and constant in all termites. However, important variations occur, which seem related both to the phylogenetic position and the biology of the species (Noirot & Noirot-Timothée 1977).

In general, advanced Termitidae exhibit more complex gut morphology compared to "lower" termites (Fig.8). All the changes in concert with the acquisition of new symbiotic partners led to more effective digestion of the new feeding substrates (Brune & Ohkuma 2011; Eggleton 2011; Ohkuma & Brune 2011).

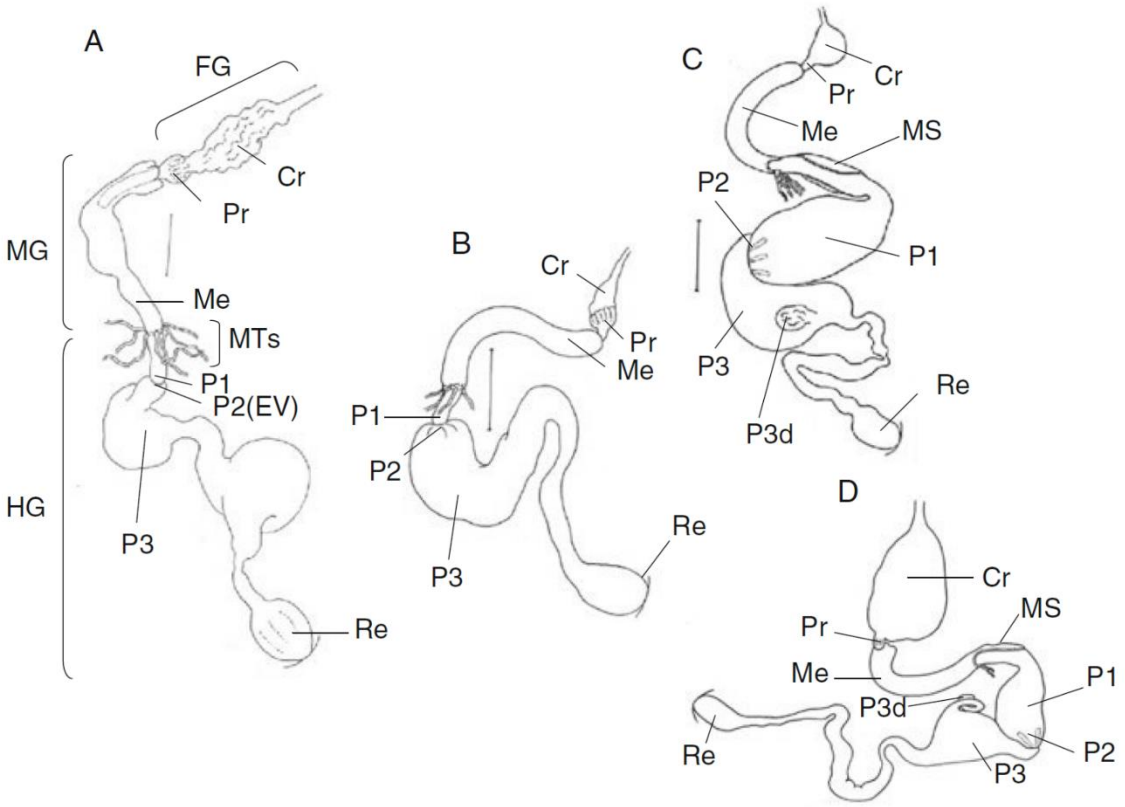

Fig.8 Digestive tract of different termites feeding groups (Sands 1998).
A: *Hodotermes* (group I grass-feeder); B: *Coptotermes* (group I wood-feeder); C: *Cubitermes* (group IV soil-feeder); D: *Ophiotermes* (group IV - soil-feeder); Cr, crop; EV, enteric valve; FG, foregut; HG, hindgut; MG, midgut; Me, mesenteron; MS, mixed segment; MTs, Malpighian tubules; P1, P2, P3, proctodaeal segments; P3d, diverticulum; Re, rectum

2. Symbioses

Termites are not able to digest organic matter by themselves. Although their activity significantly promotes the organic matter turnover, the main part of digestion and matter breakdown must be actually done by symbiotic organisms – protists, bacteria and fungi (Breznak 2000; Brune 2014; Brune & Ohkuma 2011; Inoue et al. 2000; Rouland-Lefèvre 2000; Schuurman 2005). Microbial communities inhabiting the hindgut of termites are reaching densities up to 10^{11}cells/mL (Ohkuma & Brune 2011). So-called "lower" termites uniting all families except Termitidae established symbiotic relationship with cellulolytic flagellates (Watanabe & Tokuda 2009). This symbiosis is so tight that removal of flagellates from the intestines of termite leads to its death (Cleveland 1924). The relationship established roughly 180Mya ago (Bourguignon *et al.* 2015; Brune 2014; Ohkuma 2008) and the flagellates are transferred vertically among the nestmates by the proctodeal trophallaxis, coprophagy or by consuming dead nestmates, which are the main drivers shaping the flagellates community structure (Abdul Rahman *et al.* 2015; Noda *et al.* 2007). Worth to mention that during the ecdysis of termite individual, which happen several times in its lifetime, the individual loose the whole symbiotic community, as the hindgut with Malpighian tubules are of ectoderm origin, so it is removed during ecdysis completely (Chapman *et al.* 2013). Therefore, the vertical transfer of the symbiotic communities via proctodeal trophallaxis is crucial for the survival.

2.1. Flagellates

The species richness of flagellates in termite gut is much richer than thought a decade ago (Gile *et al.* 2011, 2013; Ohkuma & Brune 2011; Radek *et al.* 2014, 2018; Tai *et al.* 2015). Majority of termite flagellates belong to phylum Parabasalia (Hypermastigida and Trichomonadida) and the rest to phylum Preaxostyla (Oxymonadida), seemed to be represented by 3 orders so far (Brune & Dietrich 2015; Noda *et al.* 2012). Interestingly, the number of flagellate species inhabiting gut of single termite species is decreasing in from basal to more lately diverged species of "lower" termites. In relation to horizontal transfer of symbiotic flagellates we might hypothesize, that better specialised flagellates of termite gut outcompeted the less successful ones (Noda *et al.* 2012; Radek *et al.* 2018).

Although the function of the symbiotic flagellates is described as cellulolytic (Watanabe & Tokuda 2009), the flagellates also harbour their own symbionts of not well studied contribution to the digestive processes – bacteria and archaea (Noda *et al.* 2003, 2005). These prokaryotes may be extra- or intra-cellular, but always in tight connection and specific to flagellate host (Dolan 2001; Hongoh 2011; Waidele *et al.* 2017). Among described functions of these prokaryotes belongs motion (Dolan 2001; Wenzel *et al.* 2003), hydrogen utilization (Inoue *et al.* 2007) and nitrogen fixation (Hongoh *et al.* 2008). In spite of our limited knowledge of this complex nutritional network between termites, symbiotic flagellates and their symbiotic prokaryotes, I may only hypothesize about the evolutionary drivers and functionality of such relationship. Interestingly, this complex system of "lower" termites guts was substitute in "higher" termites (family Termitidae) by bacteria and fungi approximately 60Mya ago, when Termitidae evolved (Bourguignon *et al.* 2015, 2017).

2.2. Bacteria

The composition of bacterial community in "lower" and "higher" termites vary, but the evolution of bacterial communities in the gut of termite ancestor of family Termitidae led to their crucial digestive function (Brune & Dietrich 2015; Brune & Ohkuma 2011). Prokaryotes participate on the carbon, nitrogen and energy requirements of termites. Acetogenesis by hindgut prokaryotes supports up to 1/3 of the respiratory requirement and N2-fixing and uric acid-degrading microbes can have a significant impact on termite N economy (Breznak 2000).

Although the bacterial communities in termite gut were of scientific concern for a long time, only minor fraction could be cultivated and further studied. The major progress into the studies was brought in the beginning of our millennium by methods of next-generation sequencing (NGS), allowing parallel sequencing of the whole bacterial community (Ohkuma & Brune 2011). Particularly the gene for 16S rRNA of the prokaryotic small ribosomal subunit is used for the description of the community.

Interestingly, some bacterial clades inhabiting intestines of Termitidae are not inhabiting any other environment on Earth and evolved together with the family Termitidae and its feeding strategies, where they reached outstanding prosperity and diversity (Ohkuma & Brune 2011). Moreover, thanks to NGS it

is possible not only to compare bacterial communities between Termitidae species (Otani *et al.* 2014) or populations of the species (Reid *et al.* 2014), but also easily compare the bacterial communities between different parts of single gut (Köhler *et al.* 2012; Tokuda *et al.* 2000) or between castes of a single species (Otani *et al.* 2014) and so observe the different importance for digestion of organic substrate in workers or for the digestion of nutrient rich substrate delivered by workers to other castes.

In total, phyla Spirochaetes, Bacteroidetes, Firmicutes, Elusimicrobia and Candidate phylum Termite Group 3 (TG3) represent together 80% of the bacterial community in the guts (Hongoh 2011; Hongoh *et al.* 2005) and its exact proportion mirror the feeding habits and gut anatomy of the host (Mikaelyan *et al.* 2017).

Spirochaetes are the most typical and most abundant inhabitants of termite hindgut, present in all termite species (Hongoh 2011). Their proportion in bacterial community may reach easily over 50% in wood-feeding termites, while their abundance decrease with other feeding substrates (Brune 2014; Dietrich *et al.* 2014; Hongoh *et al.* 2006; Köhler *et al.* 2012; Makonde *et al.* 2013; Paster *et al.* 1996). They play a role in symbiosis with flagellates of "lower" termites (Inoue *et al.* 2007), but can be found attached to wooden particles (Mikaelyan *et al.* 2014) or gut wall (Tokuda *et al.* 2001). Spirochaetes participate on fermentation, hydrogen production and reductive acetogenesis.

Another highly abundant bacterial phylum of termite hindgut are Bacteroidetes, prospering mainly in fungus growing and soil-feeding "higher" termites, but also as symbionts of protists in the gut of "lower" termites (Dietrich *et al.* 2014; Noda *et al.* 2005, 2009; Otani *et al.* 2014). Together with Firmicutes make up major share of bacterial community of fungus-growing termites (Otani *et al.* 2016).

Former TG1 group, currently Elusimicrobia, are phylum prospering in the "lower" termites hindgut mostly from the association with flagellates, and in contrast to previous phyla are less abundant and diverse among Termitidae (Abdul Rahman *et al.* 2015). Some of them are termite specific, but in general may be found in other insect intestines (Colman *et al.* 2012) and elsewhere (Herlemann *et al.* 2007). Fibrobacteres and TG3 are probably the substantial

part of the hindgut microbiota in wood-feeding Termitidae, often found in association with wooden particles (Mikaelyan *et al.* 2014).

2.3. Archaea

Even though brief, the overview of termite intestinal digestive symbionts would not be complete without mentioning Archaea. Surprisingly, these prokaryotes were not classified as independent kingdom till the 1977, although they are very different from bacteria (Woese & Fox 1977). In termite guts they represent rarely up to 10% of prokaryotes and they are responsible mainly for methanogenesis, living attached to the hindgut wall or to other members of the gut community (He *et al.* 2013; Tholen & Brune 2000).

2.4. External symbioses

Although all termites benefit from the crucial symbiosis with gut microbiota, some adopted also digestive symbionts from outer environment – ectosymbionts. This phenomenon is known in termites of sub-families Macrotermitinae and Sphaerotermitinae, the first one well known, the later waiting to be explored. Sphaerotermitinae are known to create bacterial gardens in their underground nest, where they grow bacteria on imported organic matter, which is probably later consumed by the workers (Garnier-Sillam *et al.* 1989), but further investigation is needed.

In contrast to Sphaerotermitinae, the relationship of Macrotermitinae, the fungus growing termites, with the fungi of Basidiomycota genus *Termitomyces* (Agaricomycetes: Lyophyllaceae) is quite well studied (Aanen *et al.* 2002, 2009; Aanen & Eggleton 2005; Otani *et al.* 2014; Rouland *et al.* 1992; Rouland-Lefèvre 2000). The termites are constructing complex epigeal or underground nests with intricate ventilation system, to maintain the inner conditions favourable and stable for their fungal gardens (Korb 2011). They are moving enormous amounts of organic matter into their nests as a feeding substrate for their fungus. This system is similar to that of *Atta* (Hymenoptera: Formicidae), but the ants developed their "agriculture system" by approximately 20Mya latter than termites (Buček *et al.* 2019; Hölldobler & Wilson 2010). Termite workers are foraging for dead plant matter which they immediately consume and fully overeat return to the nest, where they defecate the pre-digested matter into the fungal garden combs. This product is inoculated by the fungus, which later profits on the continuous supply of lignocellulose in very

stable and protected environment of the nest. Once the fungi grow enough, it produces the sexual reproductive fruiting body of mushroom (basidiocarp) above the nest, which is still connected to the fungal gardens by its pseudorhiza (Fig.9).

Termites, in return, consume the asexual spores (nodules) of the fungus produced in the nest and also the matter in the fungal garden after *Termitomyces* degraded it enough, so they can digest the leftovers of lignocellulose degraded by the fungus (Poulsen *et al.* 2014; Poulsen 2015; Rouland-Lefèvre 2000; Um *et al.* 2013). Worth to mention, *Macrotermes* (Termitidae: Macrotermitinae) nutrient income originate mainly from the degraded organic matter in fungal gardens, but the other genera of Macrotermitinae feed rather on the nodules of the fungus (Hyodo *et al.* 2003). In more detail, the service provided by the fungus can be summarized as:"

- Termitomyces is an additional protein-rich food source (mainly the fungal nodules);
- Termitomyces has a role in lignin degradation, which facilitates the access to cellulose;
- Termitomyces decreases the C/N ratio of foraged products by metabolising carbohydrates;
- Termitomyces provisions cellulases and xylanases to work synergistically and/or complementarily with endogenous termite enzymes"

(Bignell 2000).

Fig.9 *Termitomyces* fungus connected to *Pseudacanthotermes* fungal garden
Basidiocarp of *Termitomyces* connected to fungal garden by its pseudorhiza.
Presented with permission of ©Aleš Buček

Interestingly, genus *Termitomyces* comprise of more than 30 species (Kirk *et al.* 2001; Mossebo *et al.* 2017), but Macrotermitinae comprise of approximately 370 species (Krishna *et al.* 2013). Therefore, after logical consideration, one species of *Termitomyces* is used by more species of Macrotermitinae. On the other hand, the termites are not always using the same species of *Termitomyces* either (Nobre *et al.* 2011), but the colony is always maintaining only one fungal strain for its life-time (Aanen *et al.* 2009). In contrast to alates of *Atta* ants, which are taking the inoculum of the symbiotic fungus from their original nest along with them for the nuptial fight (vertical transmission) (Chapela *et al.* 1994; Mikheyev *et al.* 2010), termites must usually acquire new fungus for each colony from the environment (horizontal transmission) (Nobre *et al.* 2011). It explains the partial synchronization between the basidiocarps of Termitomyces growth and the swarms of Macrotermitinae (Johnson *et al.* 1981). The first worker of the fresh royal pair must collect not only organic matter, but also the inoculum of fungal symbiont in its gut, to bring it into initial fungal garden. Nevertheless, there are two known cases of vertical transmission of the symbiotic fungus among termites. The male alates of *Macrotermes bellicosus* Smeathman, 1781 and the female alates of all *Microtermes* (Termitidae: Macrotermitinae) are taking the inoculum of *Termitomyces* along with them from the original nest. Surprisingly, it didn't lead to symbiotic specificity of termite species to fungal strain, as the switches are quite common even for vertically transmitting species (Korb & Aanen 2003).

Termite Evolution and Taxonomy

Formerly, self-standing insect order Isoptera (Hexapoda: Insecta) included all termites which are traditionally categorized as "lower" or "higher" termites. However, termites evolved as an inner group of cockroaches (order Blattodea) from a social wood-feeding roach approximately 170Mya ago and since that time they reached of double species diversity than the remaining cockroaches (Bell *et al.* 2016; Bourguignon *et al.* 2015; Buček *et al.* 2019; Krishna *et al.* 2013). Therefore, currently living termites are included in epifamily Termitoidea (Blattodea) which is categorized into nine families (Krishna *et al.* 2013; Lo *et al.* 2000; Xiao *et al.* 2012) (the order follows evolutionary divergence in time according to Bourguignon *et al.* (2015) and Buček *et al.* (2019):

> Mastotermitidae, Stolotermitidae, Archotermopsidae, Hodotermitidae, Kalotermitidae, Stylotermitidae, Rhinotermitidae, Serritermitidae, and Termitidae.

The species of all the families except for Termitidae are categorized as "lower" termites and the remaining species of family Termitidae are called "higher" termites.

Thanks to the latest progress in molecular phylogeny, the evolutionary history of termites and cockroaches was reconstructed in formerly unimaginable precision (Bourguignon *et al.* 2015, 2016a, 2017; Buček *et al.* 2019). The sister clade of all termites comprises of cockroaches of genus *Cryptocercus* Scudder, 1862 (Blattodea: Cryptocercidae), which live in small families and feed on the dead wood (Inward *et al.* 2007). Except for feeding on recalcitrant wood matter, *Cryptocercus* species share with "lower" termites also (i) an obligate, rich and unique hypermastigid and oxymonadid fauna in the hindgut, (ii) horizontal transfer of these symbiotic flagellates through proctodeal trophallaxis, (iii) the long lasting biparental care of offspring or (iv) vibroacoustic alarm communication (Cleveland 1934; Klass *et al.* 2008; Stiblik *et al.* unpublished; Thorne 1990). Therefore, there is truly no doubt about existence of a common ancestor of *Cryptocercus* (Fig.10) and termites in their evolutionary history.

In contrast to "higher" termites, phylogenetic analyses revealed that "lower" termites are not a monophyletic group as the families evolved gradually. The taxonomy also uses the terms Euisoptera, which is a monophyletic clade within Termitoidea (formerly Isoptera) comprising all families except for Mastotermitidae, or term Neoisoptera, which is a monophyletic clade sister to Kalotermitidae comprised of Stylotermitidae + Rhinotermitidae+Serritermitidae+Termitidae (Fig.11) (Bourguignon *et al.* 2015).

Euisoptera are characterized by absence of some cockroach characters still present in Mastotermitidae, like absence of symbiotic *Blattabacterium* (Flavobacteriia: Flavobacteriales: Blattabacteriaceae), absence of ootheca and loss of ovipositor and anal lobe on the hindwing (Engel *et al.* 2009; Krishna *et al.* 2013). Neoisoptera are defined as termites with fontanelle on their heads. The fontanelle is an important structure in termite systematics and biology and it serve as the opening to the frontal gland, which produces defensive secretions and is therefore highly developed in soldiers. Its function in imagoes is unknown. The fontanelle occurs on the frons with its position varying from above, between, and below the eyes (Krishna *et al.* 2013).

For further introduction into living termite families and their cladistic relationships, I will rely on the recent phylogenetical studies using full-mitochondrial genomes and transcriptomes presented by Thomas Bourguignon and his collaborators (Bourguignon *et al.* 2015, 2016a, 2017; Buček *et al.* 2019). The extinct families known only from the fossil record are off the topic in this thesis. For relevant information check Krishna *et al.* (2013).

Fig.10 *Cryptocercus garciai* Burnside, Smith and Kambhampati, 1998
Cockroaches of genus *Cryptocercus* living in biparental families are the closest living relatives of termites (epifamily Termitoidea). Presented with permission of ©Troy Bartlett

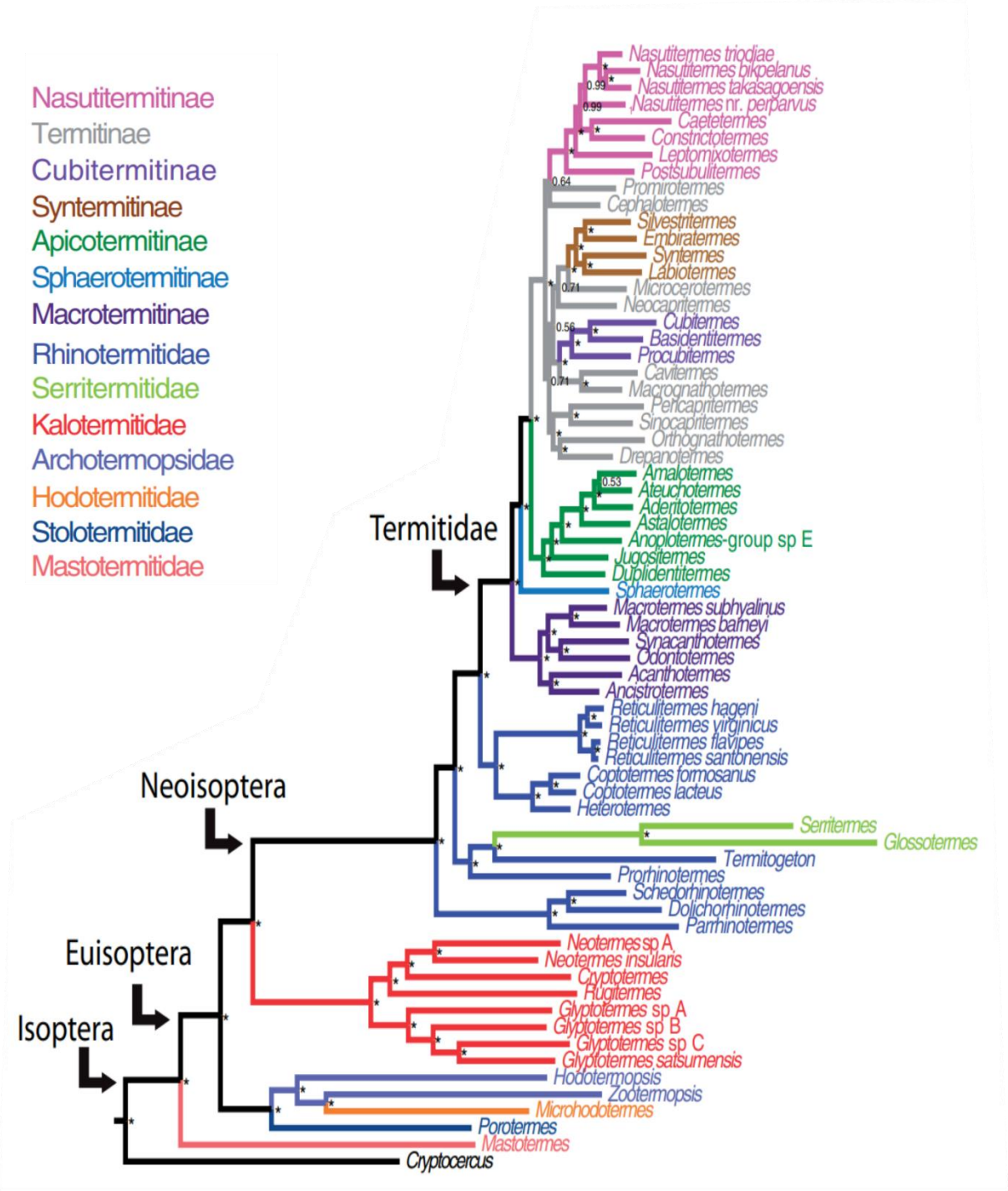

Fig.11 Phylogenetic tree of termites (Bourguignon *et al.* 2015)
Phylogenetic topography based on 66 full-mitochondrial genomes and reconstructed using Bayesian method. Family Termitidae is illustrated with its subfamilies

1. Mastotermitidae

Family Mastotermitidae is a sister clade to all other living termite families as it diverged approximately 150Mya ago (Bourguignon *et al.* 2015). Recently, this family is represented only by one species *Mastotermes darwiniensis* Froggatt, 1897, inhabiting Australian region of the Earth (Krishna *et al.* 2013), where they feed on sound wood and became a significant pest (Howick *et al.* 1975).

The main features differing Mastotermitidae from other termites are presence of anal lobe in the hind wing, rudiment of female ovipositor, and laying eggs in ootheca, which is exceptional among termites. Moreover, *M. dawrwiniensis* is the only termite species having *Blattabacterium* in the gut, which is characteristic common with cockroaches (Krishna *et al.* 2013; Lo & Eggleton 2011). Abdomen of workers is brightly white as there are deposits of fat. The number of antennomeres is often over 20, which is a primitive feature compared to most of the termites with lower number of antennae fragments.

Mastotermes are relatively large termites with size over 1cm in all castes (except from young larvae, of course) and over 3cm in alate imagoes. The colonies usually comprise of several thousands of individuals and several reproductives (Howick *et al.* 1975). Moreover, they create complex nest, use chemical alarm communication, and their ontogeny is of bifurcated type, which is usually considered as advanced feature (Delattre *et al.* 2015; Goodisman & Crozier 2002, 2003; Howick *et al.* 1975). Interestingly, *M. dawrwiniensis* has very special sperm possessing over 100 flagella, a feature exceptional among all animals (Baccetti & Dallai 1978). Fig.12 presents a sole soldier, while the Fig.13 shows the soldier compared to workers.

Fig.12 *Mastotermes darwiniensis* Froggat, 1897, soldier
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Fig.13 *Mastotermes darwiniensis* Froggat, 1897, soldier and workers
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2. Stolotermitidae

Together with Archotermopsidae and Hodotermitidae, Stolotermitidae make up a monophyletic clade within Euisoptera sister to all other Euisoptera families (Fig.11) (Bourguignon *et al.* 2015; Buček *et al.* 2019). The clade Stolotermitidae + Archotermopsidae + Hodotermitidae diverged approximately 130Mya ago and the recent findings suggest sister position of Stolotermitidae to Archotermopsidae + Hodotermtiidae. Moreover, the Archotermopsidae are probably paraphyletic with Hodotermitidae nested in.

Stolotermitidae is a small family comprised of ten living species of two genera, *Porotermes* and *Stolotermes* inhabiting southern hemisphere in Africa, Australia, and South America. They can be recognized thanks to dorsoventrally flattened heads of soldiers with (coloured or not) rudimental eyes, well-developed teeth and prolonged labrum. Although *Porotermes adamsoni* (Froggat, 1897) (Fig.14) attacks also living trees, the remaining species of Stolotermitidae rather feed on damp wood in which they establish small colonies of a few hundred individuals. Their ontogeny framework is of linear type. Species of Stolotermitidae are currently rather endemic to specific biotopes of southern hemisphere, which is actually unique among all termites, as they are the only family with a distribution that is of a classic austral disjunction (Grimaldi & Engel 2005).

3. Archotermopsidae

Compared to Stolotermitidae, Archotermopsidae inhabit rather northern hemisphere of the Earth, as their distribution is Nearcit, Palearctic and oriental. We recognize six species in three genera: *Archotermopsis, Hodotermopsis*, and *Zootermopsis*. Their colonies are very small reaching up to lower hundreds of individuals. Their ontogeny is linear which they share with Stolotermitidae, but is in contrast to Hodotermitidae (Roisin & Korb 2011).

Typically, the soldiers have two marginal teeth on right mandible and three marginal teeth on the left one. Not flattened head of soldiers bear two visible rudimental eyes and the labrum is diminished compare to Stolotermitidae (Fig.15) (Krishna *et al.* 2013).

Fig.14 *Porotermes adamsoni* (Froggatt, 1897), soldier and workers
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Fig.15 *Hodotermopsis sjostedti* Holmgren, 1911, soldier
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4. Hodotermitidae

Hodotermitidae position in termite phylogeny is surely very close to Archotermopsidae (Bourguignon *et al.* 2015; Engel *et al.* 2009), however recent study explaining in detail relationships among termite families not belonging to Neoisoptera is waiting to be published. Nevertheless, according to Bourguignon *et al.* (2015) Hodotermitidae are sister to genus *Zootermopsis* and nested within Arcotermopsidae making it paraphyletic taxon (Fig.11).

Hodotermitidae comprise of 21 living species in three genera: *Anacanthotermes, Hodotermes*, and *Microhodotermes,* which inhabit Ethiopian, Oriental, and Palearctic region. They prefer arid biotopes and feed on grass. Their underground nests are complex and huge structures dwelled in soft sandy ground inhabited by several tens of thousands of individuals, which is in sharp contrast to their closest living relatives. Hodotermitidae caste system include true workers and soldiers in apterous lineage of ontogeny starting after 2nd moult and therefore their ontogenetic framework fits the bifurcated type, what is also in contrast to their closest relatives. Interestingly, there is sexual dimorphism among workers and of *Hodotermes*, while all workers and soldiers are males in *Anacanthotermes* (Roisin & Korb 2011).

Moreover, Hodotermitidae feed on grass which they collect from the open space during massive irregular raids. Such strategy is unique among "lower" termites and termites are well-adapted to it. The workers have bit sclerotized cuticle, which is visible in their dark colouration. This is surely to protect their soft bodies against desiccation while moving in open space. Moreover, workers and soldiers possess eyes for better orientation in open space (Fig.16).

Their ecology also influenced their defensive strategies. Beside workers foraging irregularly, soldiers are guarding the entrances to the underground nest ready to fight the intruder. Interestingly, Hodotermitidae lack any vibroacoustic or chemical alarm communication, feature present in all other termites. Most probably, the vibroacoustic signals would not spread in soft ground anyway and the chemical alarm is useless in open space of savannahs (Stiblik *et al.* unpublished).

Fig.16 *Hodotermes mossambicus* (Hagen, 1853), soldier and worker
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5. Kalotermitidae

Kalotermitidae are recognized as monophyletic taxon for a long time (Krishna 1961) and novel analyses confirmed their position a sister group to Neoisoptera (Fig.11) (Bourguignon *et al.* 2015; Buček *et al.* 2019; Inward *et al.* 2007). They comprise of approximately 450 species in 21genera which makes them to most diverse termite Family, except from Neisoptera. The most diverse genera are *Cryptotermes*, *Glyptotermes* and *Neotermes* together comprising majority of Kalotermitidae species.

Although Kalotermitidae phylogeny seemed to be of genera forming monophyletic clades (Thompson *et al.* 2000), this has been recently put in question (Cintulová 2018). Therefore, the efforts for new reliable Kalotermitidae phylogeny are currently running (Stiblik *et al.* in press).

Kalotermitidae are well-known as dry-wood termites causing considerable economic damages to wooden structures and goods. On the other hand, only

handful of Kalotermitidae species are pests, but thanks to their biology are usually widespread and invasive, like *Cryptotermes brevis* (Walker, 1853) (Su & Scheffrahn 2000). Compared to other termites, Kalotermitidae lives and feed exclusively in the dead wood and try to avoid soil, as they are specialist on dead branches on living trees. They perfectly fit into definition of so-called one-piece nesters (Abe 1987), thus termites feeding and living in a sole piece of wood. This strategy led to their diversification and spread across the Globe. Among other termites, Kalotermitidae alate imagoes are good fliers able to overcome long distances and find the suitable and solitary food source in the canopy. Moreover, their colonies established in a single branch allowed them to travel for much longer distances drifting over the ocean.

They are slowly dwelling in the sound wood creating system of galleries with bigger chambers connected by short narrow tunnels, perfect for colony protection. The sound wood they consume ensure them sufficient protection, but in case of intrusion the soldiers guard the bottlenecks in the gallery system with their massive mandibles and sclerotized heads. Some termites, like *Eucryptotermes* (Fig.17) or *Cryptotermes* possess phragmotic head with only short mandibles, so they can effectively plug the bottleneck site and prevent any intruder to pass by. Moreover, it has been shown, that Kalotermitidae are able to actively avoid a clash with other insects dwelling in the wood, like subterranean termites or ants (Evans *et al.* 2009).

Kalotermitidae exhibit typical linear ontogeny, as their workers/preudergates are totipotent individuals able to moult into soldiers or became neotenics or alate imagoes (Legendre *et al.* 2008). Workers and soldiers are usually quite uniform with few exceptions like *Neotermes cubanus* (Snyder, 1922), where two different soldier castes may be found (Fig.18). The colonies usually comprise of few hundreds of individuals, but it strongly depends on the size of the food source. Once the food source is consumed, the pseudergates moult into alates and leave the nest to search for a new food source.

Fig.17 *Eucryptotermes breviceps* Constantino, 1997, soldier and worker
Example of phragmotic head with short mandibles to plug a hole
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Fig.18 *Neotermes cubanus* (Snyder, 1922), soldiers and workers
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6. Stylotermitidae

Two minor termite families remain to be introduced and for both the cladistic position is debated for longer time. At first, I focus on Stylotermitidae, which is an understudied family with a single living genus *Stylotermes* comprising about 45 species (Krishna *et al.* 2013). However, most of these species were described in China in seventies and eighties of past century and revision of the material seems more than complicated and therefore, there are strong concerns about validity of these specie descriptions. Moreover, not more than 10 different species of Stylotermitidae were significantly collected in past decade, although there were candid efforts by me and my colleagues to do so.

Family Stylotermitidae belongs to Neoisoptera thanks to the presence of Neoisoptera synapomorphy, the fontanelle on the head of soldiers and imagoes. Even for experienced termitologist is quite easy to misidentify *Stylotermes* with members of Kalotermitidae, as the workers and soldiers look very similar. But, *Stylotermes* soldiers have smooth narrow mandibles with sharp inner edge and no teeth, which is not common in Kalotermitidae, plus there is the fontanelle on the head of *Stylotermes* (Fig.19).

In recent studies, Stylotermitidae seems to be a sister clade to all other Neoisoptera (Buček *et al.* 2019; Engel *et al.* 2009) and the appearence and biology similar to Kalotermitidae support this position. However, Stylotermitidae are recently included in only one cladistic study using molecular phylogenetic methods (Buček *et al.* 2019) and future studies with more material of Stylotermitidae could move with their current position.

Biology of Stylotermitidae is similar to that of Kalotermitidae, as both creates rather small colonies on living trees. The specialization of *Stylotermes* focused on the border line between dead and fresh wood. Therefore, it may be found inside the thin range between the living tissues of the tree and the dead parts, like the places where the dead branch breaks off from the rest of the tree, in particular.

7. Serritermitidae

Family Serritermitidae is the second species poorest family among termites living only in South America. It comprises of only three species in two genera: *Glossotermes* and *Serritermes* (Krishna *et al.* 2013). The family Serritermitidae is remarkable for its soldier defense through frontal gland dehiscence (Cancello & DeSouza 2005; Šobotník *et al.* 2010). The known species share an unusual linear ontogeny resulting in all-male pseudergates (Barbosa & Constantino 2017; Bourguignon *et al.* 2009). While *Glossotermes* is feeding on rotten red wood and making colonies of thousands of individuals, *Serritermes* is a minute termite of few hundreds individuals per colony with unique strategy among "lower" termites (Fig.20). *Serritermes serrifer* (Hagen and Bates, 1858) is a specialised nest inquiline of genera *Cornitermes* (Termitidae: Syntermitinae) living there in shaded part of hard nest wall, feeding on grass leftovers, dwelling its own galleries completely separated from *Cornitermes* host species (Sillam-Dussès *et al.* 2020).

In molecular phylogenetic studies *Serritermes* + *Glossotermes* always make up a monophyletic clade (Bourguignon *et al.* 2015; Buček *et al.* 2019; Lo *et al.* 2004). While traditional cladistic study suggested Serritermitidae as sister clade to Termitidae (Engel *et al.* 2009) and molecular phylogenetic methods based on few selected genes as a sister clade to Rhinotermitidae + Termitidae (Legendre *et al.* 2008), the most recent studies based on termite full-mitochondrial genomes and transcriptomes nested Serritermitidae among paraphyletic Rhinotermitidae (Bourguignon *et al.* 2015; Buček *et al.* 2019) (Fig.11 & Fig.21). According to analysis of whole mitochondrial genomes Serritermitidae would be a sister clade to genus *Termitogeton* (Fig.11) (Bourguignon *et al.* 2015; Lo *et al.* 2004), but transcriptome based phylogeny placed Serritermitidae as sister group of subfamily Rhinotermitinae (Buček *et al.* 2019) (Fig.21), however, the relationships between Rhinotermitidae subfamilies also differ in these molecular studies and therefore we will focus on Rhinotermitidae phylogeny next.

Fig.19 *Stylotermes* sp., soldier and worker
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Fig.20 *Serritermes serrifer* (Hagen and Bates, 1858), soldier and workers
Notice the frontal gland in soldier (yellow) and the serrate mandibles
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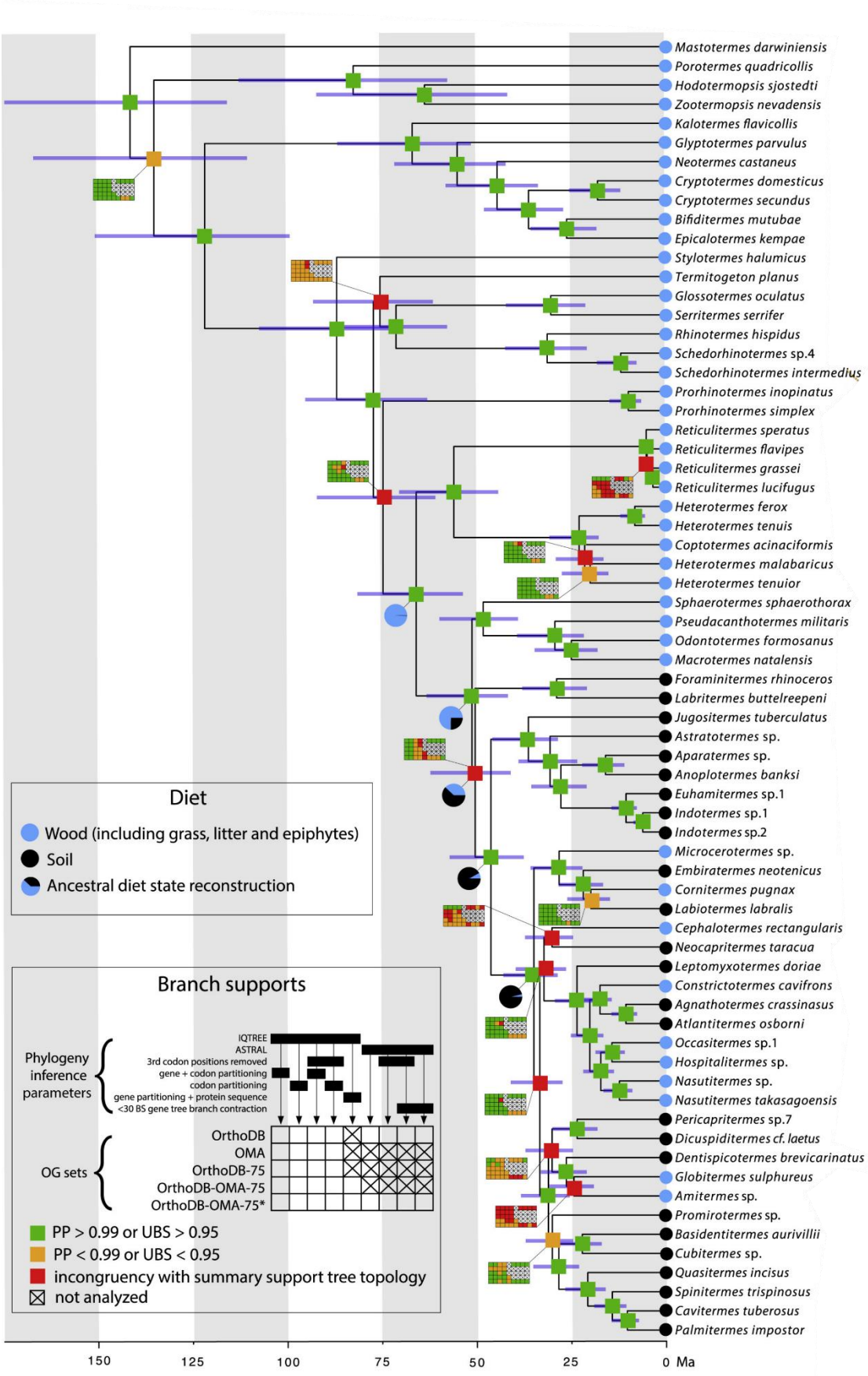

Fig.21 Phylogenetic tree of termites (Buček *et al.* 2019)
Phylogenetic topography based on transcriptomes and reconstructed using Bayesian method.

8. Rhinotermitidae

Family Rhinotermitidae was surely the most tangled riddle in termite phylogeny investigated intensively in last several decades (Donovan *et al.* 2000; Eggleton 2001; Kambhampati *et al.* 1996; Kambhampati & Eggleton 2000; Lo *et al.* 2004; Thorne & Carpenter 1992). It comprises of over 300 species in 12 genera. Their position was uncertain and their monophyly started to be questioned, as they actually lack any solid synapomorphy (Kambhampati & Eggleton 2000). However, thanks to modern molecular and computing techniques, we can be pretty sure, that Rhinotermitidae are paraphyletic group comprised of several monophyletic clades and with Serritermitidae nested within (Bourguignon *et al.* 2016a; Buček *et al.* 2019; Wang *et al.* 2019).

A monophyletic clade among Rhinotermitidae are forming: *Reticulitermes* + *Heterotermes* + *Coptotermes*. This clade diverged approximately 70Mya ago (Bourguignon *et al.* 2015; Buček *et al.* 2019; Legendre *et al.* 2008; Lo *et al.* 2004; Ohkuma *et al.* 2004) and recently was doubted rarely (Legendre *et al.* 2008). It is usually considered as a more advanced clade of Rhinotermitidae and also as a sister clade to family Termitidae (Buček *et al.* 2019; Engel *et al.* 2009; Lo *et al.* 2004; Ohkuma *et al.* 2004). While Reticulitermes forms a monophyletic clade sister to Heterotermes + Coptotermes as it was suggested many times, Heterotermes is probably paraphyletic with Coptotermes nested inside (Bourguignon *et al.* 2016a; Buček *et al.* 2019) (Fig.21), which is a novel finding compared to previous studies (Engel *et al.* 2009; Lo *et al.* 2004). The position of remaining genera of Rhinotermitidae is of continuous discussion. Although, the subfamily Rhinotermitinae was suggested as a monophyletic clade comprising of *Schedorhinotermes* + *Dolichorhinotermes* + *Rhinotermes* + *Parrhinotermes* (Wang *et al.* 2019), there is still debated exact position of *Psammotermes*, *Prorhinotermes, Termitogeton* and the inner Serritermitidae inside Rhinotermitidae. The need of Rhinotermitidae taxonomical status reconsideration is obvious.

In general, Rhinotermitidae are called subterranean termites, as they usually construct nests in the ground and forage for food elsewhere, however, e.g. *Prorhinotemes* focus on wood-pieces in separated sites, like mangroves. Otherwise, many species of this family focus on sound wood and are causing considerable damages on wooden structures across the world as they also

tend to be invasive and spread by humans (Chouvenc *et al.* 2011; Evans *et al.* 2019; Su 2002; Su & Scheffrahn 2000; Vargo & Husseneder 2009). They bet on huge numbers and often synchronize their swarms, which is an incredible spectacle of millions alate imagoes covering the sky. Their ontogeny is linear in *Prorhinotermes* and *Termitogeton* (Hanus *et al.* 2006; Parmentier & Roisin 2003), but other genera exhibit bifurcated ontogeny often with polymorphic soldier castes. The soldiers always possess fontanelle, which is connected to frontal gland, the reservoir of defensive secretion. In *Coptotermes*, this reservoir occupy over 1/3 of whole body weight (Waller & La Face 1987). There are also many modifications in soldier morphology in Rhinotermitidae. While some species bet on extremely big fontanelle and sharp cutting mandibles (Fig.22), others try to deliver the toxic frontal gland secretion via specialised prolonged labrum or attach to the opponent by piercing mandibles and extend the time-span for toxins delivery (Fig.23).

The colonies are well hidden underground, often with decentralized structure and several reproductives. These systems may be inhabited by many millions of individuals (Lee *et al.* 2019; Patel *et al.* 2020). Often, they can survive in very arid conditions as they can effectively reach the water sources from underground. In particular, desert termite *Psammotermes hybostoma* Desneux, 1902 can dwell in depths over 30m to ensure water supply to its colony (Grassé 1984).

As in all “lower” termites, workers feed on more or less decayed wood matter and the digestion is aid by symbiotic flagellates, however, the biodiversity of flagellates Rhinotermitidae gut is the lowest observed among “lower” termites (Noda *et al.* 2012; Radek *et al.* 2018).

Fig.22 *Coptotermes testaceus* (Linnaeus, 1758),soldier and worker
Note the relatively huge opening of frontal fland - the fontanelle on soldier head. © Jan Šobotník

Fig.23 *Dolichorhinotermes* sp., two soldier castes
© Jan Šobotník

9. Termitidae

Family Termitidae comprise more than 75% of all termite species diversity and about 85% of all termite genera (Kambhampati & Eggleton 2000; Krishna *et al.* 2013), so the numbers are over 2000 of species in about 240 genera. Their raise to ecological dominance started approximately 60-50Mya ago in Africa (Bourguignon *et al.* 2015, 2017; Buček *et al.* 2019).

Except for the species diversity, family Termitidae reveals the highest ecological diversity occupying many new niches compared to "lower" termites. It may be thanks to the novelty of constructing nests from organic matter, soil, feces and saliva, which opened to "higher" termites the new feeding niches, as the nest material usually go first through the gut (Eggleton & Tayasu 2001) and so Termitidae adopted to thrive on plant and fungal material in any decomposition stage, including degraded detritus in soil or fungal nodules. While all other families of "lower" termites belong into feeding Group I., Termitidae represent the other 3 feeding groups (Donovan *et al.* 2001a). Moreover, their gut is no more inhabited by flagellates, but they adopted new bacterial communities to aid their digestion.

The ontogenetical pathway is always bifurcated, rigid and uniform. The caste system and sexual polymorphism is the most advanced in this family (Noirot & Pasteels 1987; Roisin 2000). The workers are strictly apterous and different from the larvae and nymphs. Neotenic reproductives are very rare and the death of king or queen usually inevitably leads to colony extinction. The distribution of Termitidae is more dependent on warm and humid climate of tropical regions, as they usually do not prosper in colder climates (Eggleton 2000).

Last but not least, the variability of defensive strategies mirrored in the morphology of soldier caste, including its complete loss, also helped termites to dominate new niches.

Nowadays, species of Termitidae are categorized into 8 subfamiles: Sphaerotermitinae, Macrotermitinae, Foraminitermitinae, Apicotermitinae, Termitinae, Cubitermitinae, Syntermtinae and Nasutitermitinae.

9.1. Sphaerotermitinae and Macrotermitinae

Although the full mitochondrial genomes-based phylogeny suggested Macrotermitinae as sister clade to all remaining Termitidae (Fig.11) (Bourguignon *et al.* 2015, 2017), the latest transcriptome based phylogeny put them as sister to Sphaerotermitinae in separate clade (Fig.21) (Buček *et al.* 2019). This is actually in agreement with a known fact that both of these subfamilies are gardeners and wood-feeders. As Macrotermitinae are the notoriously known fungus-growers, Sphaerotermitinae grow bacterial communities in their gardens (Garnier-Sillam *et al.* 1989). Both of these cases are a nice example how termites dealt with the loss of symbiotic flagellates from their gut and the comparison reveals which strategy was more successful. While Sphaerotermitinae comprise of sole species *Sphaerotermes sphaerothorax* (Sjöstedt, 1911) endemic to central Africa, Macrotermitinae includes over 350 species in 12 genera (Krishna *et al.* 2013) and spread over Africa and Asia.

Sphaerotermes sphaerothorax lives in Congo basin and creates unique underground nests. It is a spherical bald structure placed in bigger underground chamber with only 2 exits located opposite each other. Moreover, the centre of nest is hollow and serves as colony toilet. The whole underground nest is literally hanging underground on the tree roots sourcing nutrients from the colony toilet (Fig.24).

Macrotermitinae are the only subfamily having both, multiple soldier and worker castes, although not always all present. They forage over long distances to collect material into their nests and the workers are usually accompanied by minor soldiers, while major soldiers are guarding the nest. Some even forage in open space, like *Macrotermes carbonarius* (Hagen, 1858). They may be of small size (*Microtermes* or *Ancistrotermes*) , but some of the biggest termites also belong into the subfamily (*Acanthotermes* or *Macrotermes*).

The nest may be inhabited by millions of individuals what places high demand on the queen, which is extremely physogastric any laying tens of eggs per minute (Fig.25).

Fig.24 *Sphaerotermes sphaerothorax* (Sjöstedt, 1911), nest
© Jan Šobotník

Fig.25 *Macrotermes michealseni* (Sjöstedt, 1914), inhabitants of the royal chamber
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9.2. Foraminitermitinae

The position of subfamily Foraminitermitinae is uncertain in every phylogenetical study conducted so far (Bourguignon *et al.* 2015, 2017; Buček *et al.* 2019; Engel *et al.* 2009; Inward *et al.* 2007), however, nowadays it seems that this surely monophyletic clade comprised of 3 genera with 10 species is a sister clade to all other Termitidae except from the Macrotermitinae + Sphaerotermitinae clade. Foraminitermitinae inhabit central Africa and south-east Asia

Interestingly, they are true soil-feeders, which makes them the first termites inventing this feeding strategy among living termite species approximately 50Mya ago (Buček *et al.* 2019). Little is known about their biology, as the species are of minute size and hard to find and observe. Although the soldiers (Fig.26) may superficially resembles that of *Apicotermes*, the close relationship was excluded thanks to position of fontanelle (Krishna 1963).

Fig.26 *Foraminitermes valens* (Silvestri, 1914),soldier and workers
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9.3. Apicotermitinae

The Apicotermitinae are a subfamily of soil-feeding termites that play an important role in soil processes like bioturbation and organic matter cycling in tropical rainforests and savannas (Bourguignon *et al.* 2013, 2016b; Jones & Eggleton 2011). Several lineages of Apicotermitinae are characterized by the absence of the soldier caste. These are the members of monophyletic *Astalotermes*-group and paraphyletic *Adaiphrotermes*-group in Africa and the *Anoplotermes*-group in the Neotropics (Sands 1972), all very abundant and locally making up more than 30% of the termite species diversity (Dahlsjö *et al.* 2015, 2020; Eggleton *et al.* 1995, 2002). Soldiered species comprise of the African *Apicotermes*-group and the Oriental *Speculitermes*-group however, the species of *Speculitermes*-group are tending to lose soldiers as *Indotermes* (Fig.27) reveals extremely low proportion of soldiers (roughly 1 to 1000 workers) and to find soldier of *Euhamitermes* (Fig.28) is almost impossible.

The Apicotermitinae are one of the most diverse subfamilies of Termitidae, and probably the most understudied, because they include many soldierless species that can only be distinguished morphologically by tedious dissections of the worker digestive tract (Bourguignon *et al.* 2013; Noirot 2001; Sands 1972) or, in the close future, using molecular bar-coding.

Over 200 species in 52 genera of Apicotermitinae have been described, with diversity hotspots located in Africa and South America (Bourguignon *et al.* 2016b; Constantini *et al.* 2020; Krishna *et al.* 2013), but many species are still awaiting formal description. Therefore, also the phylogeny and historical biogeography remained unclear and the results of the latest progress are presented later in this thesis.

Fig.27 *Indotermes* sp., soldiers and workers
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Fig.28 *Euhamitermes* sp., two very rare soldier and workers
© Jan Šobotník

9.4. Syntermitinae

The subfamily Syntermitinae is exclusively neotropical group of wood or soil-feeding termites. It comprise of about 100 species in 15 genera and they are easily recognisable thanks to functional biting mandibles and conspicuous frontal pore (Krishna *et al.* 2013) (Fig.29). Moreover, they are one of the rare cases of neotenic reproductives production in "higher" termites (Fougeyrollas *et al.* 2015; Myles 1999). They may be of a considerable size and their presence is often obvious thanks to epigeal mounds, but many dwell complex underground nests as well. During the nest construction, they move enormous amounts of soil and organic matter and creates oasis in arid South-American Cerrado (Fig.7). In this respect, Syntermitinae are of similar ecological importance as Macrotermitinae, which never reached Neotropics.

The phylogenetic position is still questioned, although it seems , that subfamily Syntermitinae is a monophyletic clade surely nested within paraphyletic Termitinae and probably sister to cosmotropical wood-feeding *Microcerotermes*-group (Bourguignon *et al.* 2015, 2017; Buček *et al.* 2019). Therefore, Syntermitinae are either independent case of soil-feeding strategy evolution, or the wood-feeding strategy was reacquired (Buček *et al.* 2019).

Fig.29 *Embiratermes neotenicus* (Holmgren, 1906)
Soldier, workers and neotenic reprodictives (with eyes and wingpads)
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9.5. Nasutitermitinae

This subfamily, although very various in morphology and nesting or feeding habits, is very easily recognized by soldiers greatly reduced mandibles and distinctly prolonged head into narrow nasus (Fig.30). Thank to this adaptation, the soldiers are able to spray toxic and repellent product of frontal gland over a long distance. The workers are quite uniform with age-dependent size, but the soldiers may be di- or even tri-morphic (Krishna *et al.* 2013). It is also quite common that the soldiers are considerably smaller than workers. Despite to reduced mandibles, the labial gland is sometimes well-developed and its function is debated (Constantino & Costa-Leonardo 1997).

The subfamily Nasutitermitinae is cosmotropical, very abundant and super-species-rich encompassing about 600 living species in 77 genera (Krishna *et al.* 2013). The species feed on full range of organic matter from wood to soil and some even on lichens and micro-epiphytes (Fig.31). They nest in the underground, in typical spherical or variously shaped arboreal nests (Fig.3), or as inquilines of other termites. Some species are foraging in open space, and thus their coloration is unusually black. Also in this case the original feeding strategy at the time of clade divergence was probably soil-feeding, and the wood-feeding was reacquired (Buček *et al.* 2019).

Nasutitermitinae probably originated in Africa about 30Mya ago, but is still uncertain (Bourguignon *et al.* 2017). Despite its huge diversity, Nasutitermitinae monophyly is widely agreed (Bourguignon *et al.* 2015, 2017; Buček *et al.* 2019; Engel *et al.* 2009; Inward *et al.* 2007; Legendre *et al.* 2008; Miura *et al.* 2000) and the recent studies agree on quite close kinship with Termitinae genera *Cephalotermes* and *Neocapritermes*. Otherwise, the position among paraphyletic Termitinae is highly uncertain.

Fig.30 *Anhangatermes* sp., soldiers and worker
The narrow duct of the fontanelle is visible in the head
© Jan Šobotník

Fig.31 *Hospitalitermes* sp., foraging in open-space
© Jan Šobotník

9.6. Cubitermitinae

Cubitermitinae are exclusively true soil-feeders endemic to Sub-Saharan Africa and encompassing over 150 species categorized into 26 genera (Krishna *et al.* 2013). They form a monophyletic clade within Termitinae and diverged approximately 25-30Mya ago (Bourguignon *et al.* 2017; Buček *et al.* 2019). They may be recognized according to "cubic" head of soldiers with "V" shaped labrum and typical workers with distinct diverticulum on P3 hindgut segment (Fig.32). They prosper in both, arid savannahs and rain forest, and their presence is distinctly marked by "fungi-shaped" nest (Fig.33).

They dominate the soils of Congo basin with surprisingly high population and species of genus *Cubitermes* are some of the most studied soil-feeding Termitidae. The focus on this group may be due to their high abundance and conspicuous mounds, compared to the belowground nests of soldierless soil-feeders. The ecological effects of Cubitermitinae are mainly seen in the old-growth lowland forests of Africa where their abundance and biomass reach maximum. They have been shown to increase pH in acidic soils, as well as the content of organic carbon and water. (Šobotník & Dahlsjö 2017).

Fig.32 *Cubitermes* sp., soldier and workers
Notice the transparent cuticule and the visible intestines
© Jan Šobotník

Fig.33 *Cubitermes* nest
The architecture perfectly protects against heavy rains, foods or direct sun
© Jan Šobotník

9.7. Termitinae

Phylogeny of subfamily Termitinae is one of the most debated topics among termitologists. The group is surely paraphyletic as the monophyletic subfamilies Syntermitinae, Nasutitermitinae and Cubitermitinae are nested within, so the taxonomy definitely deserves revision. But first, reliable cladistic analyses based on multi-omics approach must be conducted, as the latest results still differ considerably (Bourguignon *et al.* 2015, 2017; Buček *et al.* 2019). However, the subfamily comprise of more than 630 species in 61genera and the morphological variability of soldiers is the greatest among termites, so there is no wonder they were found paraphyletic (Deligne *et al.* 1981; Krishna *et al.* 2013).

Although the common ancestor of Termitinae was probably soil-feeder, the swap to wood-feeding is quite common within the subfamily. As a perfect example may serve *Microcerotermes* or *Amitermes*, which are clearly unrelated genera feeding on sound wood and having cosmotropical distribution (Bourguignon *et al.* 2017; Buček *et al.* 2019; Krishna *et al.* 2013).

Interestingly, it may be due to soldier caste morphological diversity and incredible variability of defensive strategies that all the 61 genera were put in one paraphyletic group. It is a strange coincidence that the soldiers of Termitinae usually have long and slender mandibles, which tend to evolution of snapping mandibles. Snapping mandibles allow to accumulate elastic energy, that can be release when triggered in. Moreover, compared to other types of mandibles, the snapping can be done repeatedly in seconds. It may be so powerful, that the opponent is smashed or thrown away. Snapping mandibles may be either symmetrical or asymmetrical with slightly different defensive capabilities (Šobotník & Dahlsjö 2017). While the snapping of symmetrical mandibles provides a hit by both to the sides, asymmetrical snapping mandibles deliver a forward blow from the left mandible only, but it is the fastest movement in animal kingdom (unpublished data).

The evolution of Termitinae defensive strategies reached obscure mechanisms, where e.g. termite soldier can snap and cut within a single movement (Fig.34), or the defenders use explosive backpacks on the basis of fusion exothermic reaction (Fig.35) (Šobotník *et al.* 2012, 2014).

Fig.34 *Orthognathotermes* sp., soldiers and workers
The long elbowed slender mandibles can cut a head in a single snap
My favourite termite :-)
© Jan Šobotník

Fig.35 *Neocapritermes taracua* Krishna and Araujo, 1968, bomber termite
Workers have bacpacks with blue protein, which can rapidly react with saliva, if needed
Older soldiers may have the protein as well, but they are better in close combat
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Aims of the thesis

- Bring new insights in the evolution of termites with unresolved phylogeny.
- Search for patterns in co-evolution of termites with gut microbes.
- Test whether there are environmental microbes associated to termite activities and digestion.
- Describe the externally associated microbial communities.

Publications

The following pages introduce my research in already accepted and published scientific publications – research articles. The first publication of the list brings the annotated full mitochondrial genome of *Cryptotermes havilandi* Sjöstedt, 1900 (Kalotermitidae) and so it contribute to resolving phylogeny of Kalotermitidae, as recently this family showed up as unexpectedly tangled riddle in termite taxonomy (Cintulová 2018). Although in general the phylogeny of termites might seemed resolved (Bourguignon *et al.* 2015; Buček *et al.* 2019) the detailed look into the termite families discover several unresolved nodes and taxonomical questions. Thus, my article bringing the whole annotated mitochondrial genome is an important shard for reliable Kalotermitidae phylogeny.

Apart of the increased pressure for highly accurate phylogenetic topology of Kalotermitidae, also the evolutionary history of subfamily Apicotermitinae provides hidden knowledge to be investigated. Therefore, I joined research activities searching for the relevant dated phylogeny of these interesting soil-feeding termites, as it is presented in the second article.

The third publication focus on metagenome-assembled genomes (MAG) of termite gut. Detailed analysis led to great dataset of MAG's and identified bacterial clades specific to termites. The evolution of termite gut symbionts is extremely thrilling due to brisk switch from broad range of protists in "lower termites" to endless range of bacteria in "higher termites". This paper is an important contribution to future analyses of termite or others insect gut

The fourth and fifth articles focus on non-random relationships of termites with environmental microbes, fungi and bacteria, respectively. Symbiosis of termites with external microbes is known for a long time as termites of subfamily Macrotermitinae create fungi gardens inside their nests and they actively grow fungus *Termitomyces* which is specific to termite nests (Krishna *et al.* 2013; Mossebo *et al.* 2017; Rouland-Lefèvre 2000). For a long time, other termite relationship with environmental microbes was not known except for anecdotal mention of *Sphaerotermes sphaerothorax* creating bacterial gardens inside their nests (Garnier-Sillam *et al.* 1989). Therefore, it was motivating to search for more symbiotic relationships between termites and microbes from

the environment. The search was successful as for three species of neotropic termites *Coptotermes testaceus*, *Heterotermes tenuis* and *Nasutitermes octopilis* were discovered tight relationships with both, fungi and bacteria.

List of articles presented in this thesis:

1. Complete mitochondrial genome of the dry-wood termite *Cryptotermes havilandi*
Authors: **Petr Stiblik**, Pierre Dieudonné Akama and Jan Šobotník
Journal: **Mitochondrial DNA Part B**
Status: Accepted manuscript

2. Molecular phylogeny and historical biogeography of Apicotermitinae (Blattodea: Termitidae)
Authors: Johanna Romero Arias, Arthur Boom, Menglin Wang, Crystal Clitheroe, Jan Šobotník, **Petr Stiblik**, Thomas Bourguignon and Yves Roisin
Status: Submitted manuscript

3. Phylogenomic analysis of 589 metagenome-assembled genomes encompassing all major prokaryotic lineages from the gut of higher termites
Authors: Vincent Hervé, Pengfei Liu, Carsten Dietrich, David Sillam-Dussès, **Petr Stiblik**, Jan Šobotník and Andreas Brune
Journal: **PeerJ**
Status: Published
DOI: doi.org/10.7717/peerj.8614

4. Termites host specific fungal communities that differ from those in their ambient environments
Authors: Tomáš Větrovský, Patrik Soukup, **Petr Stiblik**, Kateřina Votýpková, Amrita Chakraborty, Iñaki Odriozola Larrañaga, David Sillam-Dussès, Nathan Lo, Thomas Bourguignon, Petr Baldrian, Jan Šobotník and Miroslav Kolařík
Journal: **Fungal Ecology**
Status: Published
DOI: doi.org/10.1016/j.funeco.2020.100991

5. Termites are associated with external species-specific bacterial communities
Authors: Tomáš Větrovský, Patrik Soukup, **Petr Stiblik**, Kateřina Votýpková, Amrita Chakraborty, Iñaki Odriozola Larrañaga, David Sillam-Dussès, Nathan Lo, Thomas Bourguignon, Petr Baldrian, Jan Šobotník and Miroslav Kolařík
Journal: **Applied and Environmental Microbiology**
Status: Accepted manuscript
DOI: doi.org/10.1128/AEM.02042-20

1. Complete mitochondrial genome of the dry-wood termite *Cryptotermes havilandi*

Complete mitochondrial genome of the drywood termite *Cryptotermes havilandi* (Isoptera: Kalotermitidae)

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ABSTRACT

We report the first complete mitochondrial genome of an important pest of timber, the drywood termite *Cryptotermes havilandi*. The gene content and synteny of the mitochondrial genome of *C. havilandi* is identical to that of other termite species reported to date. It is composed 13 protein-coding genes, two ribosomal RNA genes, and 22 transfer RNA genes. Our phylogenetic tree, that includes the mitochondrial genomes of 14 species of Kalotermitidae, including *C. havilandi*, resolves the phylogenetic position of *C. havilandi* within Kalotermitidae.

Main text

Cryptotermes havilandi Sjöstedt, 1900 (Isoptera: Kalotermitidae) is an important pest of structural lumber and sheltered wood (Su and Scheffrahn 2000). Although it is now distributed across the tropical and subtropical regions, *C. havilandi* originated from Africa, and has been introduced outside its native range largely by the intermediary of human transportation (Evans 2011, Evans et al. 2013). It is now invasive in various Caribbean islands, Guiana, Surinam, Brazil, Madagascar, the Comores, and India (Evans et al. 2013). Despite its economic importance, the mitochondrial genome of *C. havilandi* has not been sequenced yet. Here, we provide the first complete mitochondrial genome sequence of a *C. havilandi* extracted from the sample CAM101 collected on 7th of April 2015 in an abandoned wooden house in northern Cameroon, Africa (N04°42'25" E009°43'08"), by the authors. The sample CAM101 is available in Petr Stiblik (stiblik@fld.czu.cz) collection at Czech University of Life Sciences, Prague, Czech Republic in both states, as an 80% ethanol voucher sample and in RNAlater preservative.

We sequenced *C. havilandi* (Genbank: MW208858) mitochondrial genome using Illumina HiSeq2000. The genome was assembled using the clc suite of programs as described by Bourguignon et al. (2015). The total length of the complete mitochondrial genome of *C. havilandi* is 15,559bp. As in other mitochondrial genomes of termites previously sequenced (Cameron and Whiting 2007, Cameron et al. 2012, Bourguignon et al. 2015, 2016, 2017, Wu et al. 2018, Wang et al. 2019), the mitochondrial genome of *C. havilandi* is composed of 13 protein-coding genes (following the order: *nad2,*

cox1, cox2, atp8, atp6, cox3, nad3, nad5, nad4, nad4l, nad6, cytb, and *nad1*), two ribosomal RNA genes (*rnl* and *rns*) and 22 transfer RNA genes (following the order: *Ile, Gln, Met, Trp, Cys, Tyr, Leu$^{(UUR)}$, Lys, Asp, Gly, Ala, Arg, Asn, Ser$^{(AGN)}$, Glu, Phe, His, Thr, Pro, Ser$^{(UCN)}$, Leu$^{(CUN)}$,* and *Val*). The GC-content is 34%. Our results confirm that termite mitochondrial genomes are stable in gene content and preserved their synteny.

To shed light on the phylogenetic position of *C. havilandi* within the Kalotermitidae, we reconstructed a phylogenetic tree that included all mitochondrial genomes of Kalotermitidae sequenced to date, including the mitochondrial genome of *C. havilandi*, and three outgroups: *Zootermopsis angusticolis* (Isoptera: Archotermopsidae), *Porotermes adamsoni* (Isoptera: Termopsidae) and *Coptotermes sepangensis* (Isoptera: Rhinotermitidae) (Figure 1). All genes were aligned separately using MAFFT v. 7.3 (Katoh and Standley 2013), concatenated, and the phylogenetic tree was reconstructed using MrBayes v. 3.2.1 (Ronquist et al. 2012). The parameters of the phylogenetic analysis were set as described by Bourguignon et al. (2017). Overall, our phylogenetic tree confirms the monophyly of *Cryptotermes*, within which *C. havilandi* is nested.

The genus *Cryptotermes* includes several invasive species that cause major economic losses in the world (Evans et al. 2013). Surprisingly, very few studies have used molecular markers to study the population genetics of *Cryptotermes* species. In this paper, we provide the mitochondrial genome of one of the most important termite pest. The new mitochondrial genome presented here will help to understand how the major termite pests have been introduced around the world.

Disclosure Statement

The authors report no conflict of interests.

Author contribution

PS and JS conceived the study, carried out bioinformatics analyses and wrote the paper. PA facilitated the fieldwork in Cameroon.

Funding

We are grateful to Raphael Onana and to the people of village Ebogo II for their hospitality and help in biological material collection. This work was supported by the Ministry of Education, Youth and Sports of the Czech Republic under OP RDE grant EVA4.0 (no. CZ.02.1.01/0.0/0.0/16_019/0000803) and by the of Faculty of Tropical AgriSciences under Internal Grant IGA (project number 20205014).

Acknowledgment

The authors thank Thomas Bourguignon for his valuable insights.

Data availability statement

The data that support the findings of this study are available in GenBank at https://www.ncbi.nlm.nih.gov . The mitochondrial genome of *Cryptotermes havilandi* has been deposited in GenBank under the accession number: MW208858

Data are also accessible at:

https://mfr.osf.io/render?url=https%3A%2F%2Fosf.io%2F4ykjp%2Fdownload

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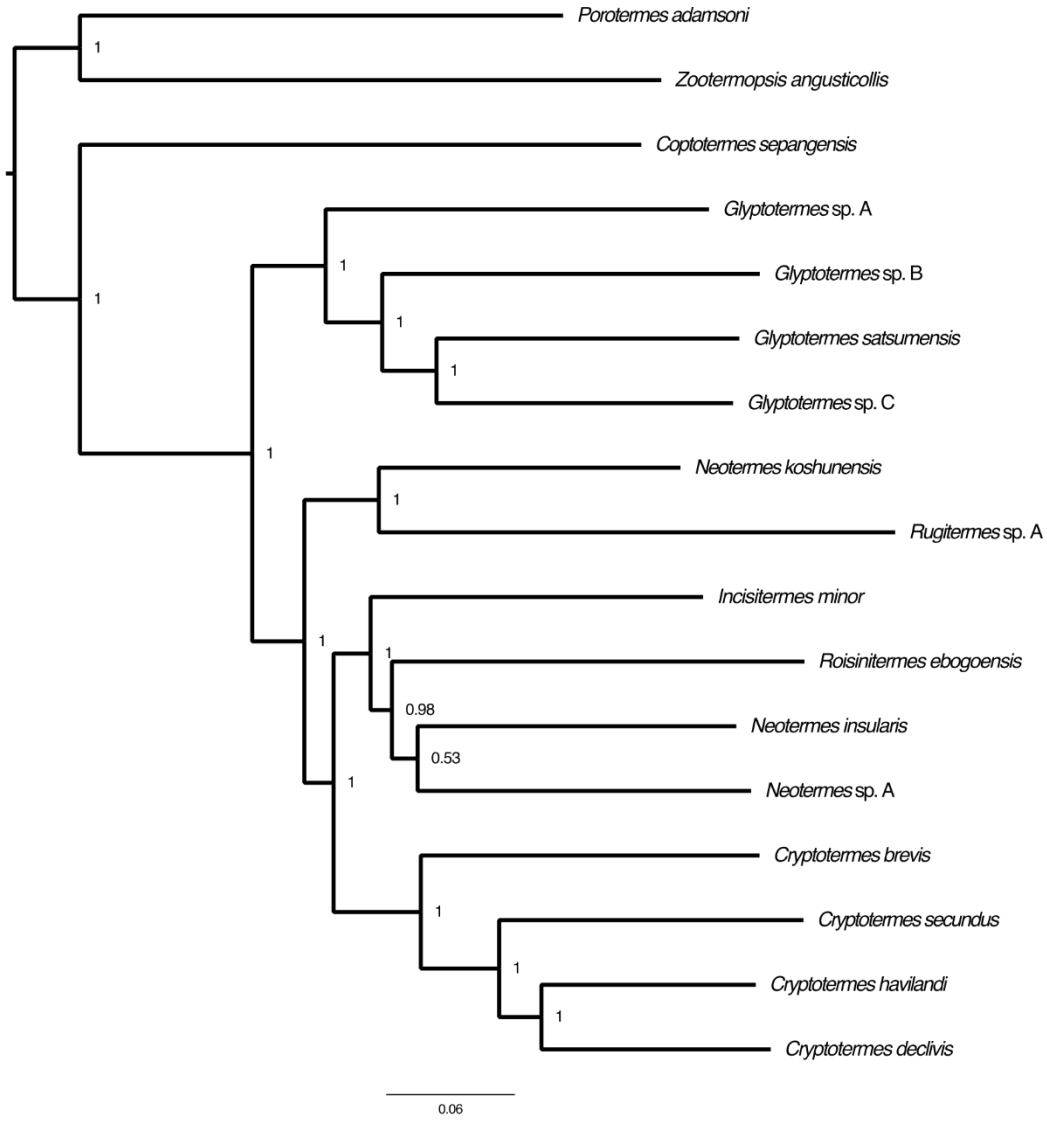

Figure 1. Bayesian phylogenetic tree of all species of Kalotermitidae sequenced to date. Numbers in nodes state for posterior probabilities and the scale indicates 6% genetic variation for its length.

2. Molecular phylogeny and historical biogeography of Apicotermitinae (Blattodea: Termitidae)

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Molecular phylogeny and historical biogeography of Apicotermitinae (Blattodea: Termitidae)

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Abstract

Soil-feeding termites are abundant in tropical regions and play an important role in soil bioturbation and in the organic matter cycle. The Apicotermitinae are arguably the most diverse lineage of soil-feeding termites, but they are also the most understudied, probably because many species are soldierless, which makes identification difficult, and because their cryptic lifestyle prevents easy sampling. Although the backbone of the termite phylogenetic tree is now well-resolved, the relationships among representatives of Apicotermitinae are still largely unknown. Here, we present phylogenetic trees inferred from 113 mitochondrial genomes of Apicotermitinae representative of the group diversity. Our analyses confirm the monophyly of the Apicotermitinae and the basal position of soldiered taxa, within which two lineages of soldierless species are nested. We resolved, with high support, the position of Asian genera as sister group of a clade comprising the monophyletic neotropical *Anoplotermes*-group and a small African clade including *Adaiphrotermes* and an undescribed genus. Our trees cast light on the intergeneric and interspecific relationships within Apicotermitinae and reveal the polyphyly of several genera, including *Ruptitermes*, *Astalotermes* and *Anoplotermes*. Biogeographic reconstructions revealed two dispersal events out of Africa, one to the Oriental realm and one to the Neotropical realm. Overall, the timing of Apicotermitinae diversification and dispersal, following the Eocene-Oligocene boundary, matches that found for other groups of Neoisoptera.

Keywords: Humivorous, Isoptera, mitochondrial genome, systematics, molecular clock

1. Introduction

The Apicotermitinae are a subfamily of soil-feeding termites that play important roles in soil bioturbation and organic matter cycling in tropical rainforests and savannas (Jones and Eggleton, 2011, Bourguignon et al., 2016). Several lineages of Apicotermitinae are characterized by the absence of the soldier caste. Soldiered species encompass the African *Apicotermes*-group and the Oriental *Speculitermes*-group (Grassé and Noirot, 1954; Sands, 1972). Soldiers are, however, rare and often unknown in species of the *Speculitermes*-group. Soldierless species comprise the *Astalotermes*-group in Africa and the *Anoplotermes*-group in the Neotropics (Sands, 1972), both of which are very abundant and can locally make up more than 30% of the termite species diversity (Eggleton et al., 1995, 2002; Bourguignon et al., 2011, 2016; Dahlsjö et al., 2015, 2020; Nduwarugira et al., 2017).

The Apicotermitinae are one of the most diverse subfamilies of Termitidae, and the most understudied, probably because they include many soldierless species that can only be distinguished morphologically by tedious dissections of the worker digestive tract (Grassé and Noirot, 1954; Sands, 1972, 1998; Noirot, 2001; Bourguignon et al., 2016). To date, 224 species and 52 genera of Apicotermitinae have been described, with diversity hotspots located in Africa and South America (Krishna et al., 2013; Bourguignon et al., 2016; Constantino, 2020; Roisin, 2020). However, the actual diversity of the group is much larger, and many species, still awaiting formal description, have been informally labelled as morphospecies in ecological surveys (e.g., Eggleton et al. 1995, 2002; Davies, 2002; Bourguignon et al., 2011; Nduwarugira et al., 2017).

The first comprehensive phylogenetic study of termites was based on a combination of morphological characters and genetic markers, including two mitochondrial genes (COII and 12S) and one nuclear gene (28S) (Inward et al., 2007). This study supported the monophyly of Apicotermitinae, which were retrieved as the sister group of a clade composed of all other Termitidae except the fungus-growers (Macrotermitinae) and the two small subfamilies Sphaerotermitinae and Foraminitermitinae (Inward et al., 2007). This phylogenetic position was later confirmed by molecular phylogenies inferred from complete mitochondrial genomes and transcriptomes (Bourguignon et al., 2015, 2017, Bucek et al., 2019). In addition, the phylogenetic tree of Inward et al. (2007) suggested that (1) the African soldiered taxa are paraphyletic to a clade composed of the soldierless lineages and the Asian (soldiered) Apicotermitinae; (2) the Oriental *Speculitermes*-group is monophyletic; (3) the Neotropical *Anoplotermes*-group is monophyletic; and (4) the Oriental *Speculitermes*-group, the Neotropical *Anoplotermes*-group and the African soldierless *Adaiphrotermes* form a monophyletic group sister to all other African soldierless taxa. This tree topology implies two independent losses of soldiers in Apicotermitinae, and two independent dispersal events between continents, with unclear directionality. Complete mitochondrial genome phylogenies confirmed that Asian and Neotropical taxa are closer to each other than to most African soldierless genera (Bourguignon et al., 2017), but, because of their insufficient sampling, poor characterization of some described genera (e.g., *Astalotermes*,

Anenteotermes), and uncertain identifications, the history of Apicotermitinae remains unclear. In addition, most relationships among African and Neotropical soldierless taxa were unresolved by Inward et al. (2007), and several genera (e.g., *Aderitotermes*, *Astalotermes* or *Anoplotermes*) appeared as polyphyletic in the phylogenetic trees of Bourguignon et al. (2017).

In this study, we used 113 mitochondrial genomes of Apicotermitinae species to reconstruct robust phylogenetic trees including most described genera, well-characterized by anatomical features. Using these trees, we tested previous phylogenetic hypotheses regarding the relationships among major Apicotermitinae clades and provided a timeframe for their evolution. We also investigated the historical biogeography of Apicotermitinae and determined the number of independent losses of soldiers. Our analyses clarify the taxonomy of Apicotermitinae and pave the path to future taxonomic revisions of non-monophyletic genera, such as *Astalotermes* or *Anoplotermes*, and provide a framework to study the anatomical evolution of the subfamily.

2. Material and methods

2.1. Sampling

Termite sampling was conducted in Burundi (n = 7), Cameroon (n = 28), Ivory Coast (n = 18), Kenya (n = 1) and French Guiana (n = 13) (Table S1). For each sample, we collected specimens in RNA-later® or in 100% ethanol for genetic analyses, and in 80% ethanol for morphological analyses. Samples collected in RNA-later® and 100% ethanol were temporarily stored at a temperature ranging from -20°C to 4°C, and shipped to the Czech University of Life Sciences or to the Okinawa Institute of Science and Technology, where they were stored at -80°C until DNA extraction. Samples collected in 80% ethanol are stored at the Université Libre de Bruxelles and the Czech University of Life Sciences. In addition to the 67 samples collected in this study, we also obtained the full mitochondrial genome sequences of 43 samples of Apicotermitinae from GenBank (Bourguignon et al. 2015, 2017) and reconstructed mitochondrial genomes from transcriptome sequences of three species (Bucek et al., 2019) (Table S1).

Species identifications were based on morphological and anatomical characters, which included the worker digestive tube configuration, the shape of the gizzard and enteric valve armature, as described in Romero Arias et al. (2020). We also re-examined the voucher material of samples sequenced in previous studies and whose phylogenetic position appeared inconsistent. In a few cases, we found that the voucher samples contained a mixture of two species. We labelled these samples with both species names. Revised species identifications are detailed in Supplementary Appendix A (see also Table S1).

2.2. DNA extraction and sequencing

Whole genomic DNA was extracted from head and thorax of three to five workers using the DNeasy Blood & Tissue extraction kits (Qiagen). Because DNA extracts were sequenced at different periods of time, two different approaches were used. For the first approach, the complete mitochondrial genome was amplified in two long-PCR reactions with the TaKaRa LA Taq polymerase, using primers previously designed for termites (Bourguignon et al., 2015). The concentration of both long PCR fragments was

determined using a Qubit 3.0 fluorometer, and the two fragments were mixed in equimolar concentration. Libraries were prepared with the NEBNext Ultra II DNA Library Prep Kit (New England Biolabs) and sequenced with Illumina MiSeq. For the second approach, whole genomic DNA libraries were directly prepared with the aforementioned NEB kit and sequenced using Illumina HiSeq4000.

2.3. Assembly and annotation of mitochondrial genomes

Paired-end reads were quality-assessed using FastQC v0.11.7 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and adapter sequences were removed with Trim Galore v0.4.5 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) using default settings. Mitochondrial reads were identified using the mitogenome of *Astalotermes murcus* (accession no. KY224676) as a reference and assembled using GetOrganelle v1.5.1 (Jin et al., 2019). Each resulting assembly graph was inspected with Bandage v0.8.1 (Wick et al. 2015) and mitochondrial genome sequences were manually circularized when necessary. Control regions were discarded from the final assemblies as they provide limited phylogenetic information and are difficult to accurately assemble with short reads. We used the MITOS2 Webserver with the invertebrate genetic code and the protein prediction method of Donath et al. (2019) to annotate the two rRNA genes, 22 tRNA genes, and 13 protein-coding genes. Other parameters were set on default settings. Annotated genomes are deposited in GenBank (accession numbers to come). In total, we generated 67 new mitochondrial genome sequences, mostly from African species (54). Forty-eight mitochondrial genomes were complete, and 19 mitochondrial genomes were nearly complete because of ambiguous circularization. The mitochondrial genomes from Bourguignon et al. (2015), that included 60 non-Apicotermitinae termites and eight non-termite polyneopteran insects, were used as outgroups (Table S2). Therefore, the final data set comprised 181 mitochondrial genomes, including 113 genomes of Apicotermitinae.

2.4. Sequence alignment

We aligned separately each of the two rRNA genes, 22 tRNA genes, and 13 protein-coding genes using MAFFT v7.300b (Katoh et al. 2002, 2013) with default settings. Protein-coding genes were aligned as protein sequences and back-translated into nucleotide sequences using PAL2NAL (Suyama et al. 2006). rRNAs and tRNAs were aligned as DNA sequences. The 37 aligned genes were concatenated and partitioned into five partitions: one for each codon position of the combined protein-coding genes; one for the combined 12S and the 16S rRNA genes; and one for the combined tRNA genes. We found no clear evidence of mutational saturation for the third codon positions of the protein-coding genes (I_{SS}=0.572, $I_{SS.C}$Sym=0.809) using the Xia's method implemented in DAMBE (Xia et al. 2003; Xia and Lemey 2009) and therefore retained the third codon positions in our phylogenetic analyses.

2.5. Phylogenetic inference

We used RAxML version 8.2.4 (Stamatakis, 2014) to reconstruct a maximum-likelihood phylogenetic tree. We used the GTR+G model for each partition. Bootstrap values were estimated from 1000 replicates. We used MrBayes version 3.2 (Ronquist et al. 2012) to reconstruct a Bayesian phylogenetic

tree. The analysis was run with four chains (three hot and one cold), and we estimated posterior distributions using Markov chain Monte Carlo (MCMC) sampling drawn every 5000 steps. The chain was run for a total of 10 million steps, with the first 1 million steps discarded as burnin, as suggested by inspection of the trace files using Tracer v1.5 (Rambaut and Drummond 2009). We used a GTR model with gamma-distributed rate variation across sites (GTR+G) for each partition. The analysis was run in triplicate to insure convergence of the chains and check for consistency. Node support was estimated using Bayesian posterior probabilities.

2.6. *Molecular dating*

We estimated time-calibrated trees using BEAST2 version 2.4.4 (Bouckaert et al. 2014). We performed the analyses with and without third codon positions to assess the influence of third codon positions on time estimates. The trees were reconstructed using an uncorrelated lognormal relaxed clock to model rate variation among branches, with single model for each partition, allowing different relative rates. A Yule speciation model was used as tree prior. We used a GTR+G model of nucleotide substitution for each partition. The chains were run for 500 million steps and were sampled every 10,000 generations to estimate the posterior distribution. We discarded the first 50 million steps as burn-in, as suggested by inspection of the trace files using Tracer v1.5 (Rambaut and Drummond 2009). A total of 13 fossils were used as minimum age constraints (see Table S3). We determined soft upper bounds using phylogenetic bracketing (Ho and Phillips 2009). Each calibration was implemented as exponential priors of node time. The analyses were run in triplicate to insure convergence of the chains and check for consistency.

2.7. Reconstruction of ancestral distribution

The ancestral distribution of Apicotermitinae was reconstructed using the ace function of the R package APE version 5.0 (Paradis and Schliep 2018). We used the Maximum Likelihood model described by Pagel (1994) and an equal-rates of transition. Sampling locations were used to assign each tip to one biogeographic realm. Apicotermitinae are distributed across three biogeographic realms, as described by Holt et al. (2013): Afrotropical, Neotropical, and Oriental. We reconstructed ancestral distribution on the maximum-likelihood tree, the Bayesian tree, and the two time-calibrated trees.

3. Results

3.1. Molecular phylogeny

Our phylogenetic trees fully supported the monophyly of Apicotermitinae (Figs 1 and S1-S3). African soldiered taxa (the *Apicotermes*-group) formed a paraphyletic assemblage, composed of two or three lineages, within which a clade composed of Asian genera and African and Neotropical soldierless taxa was nested (Figs 1 and S2-S3). This latter clade was divided into four lineages, fully supported in all analyses: (I) the African soldierless species, with the exclusion of *Adaiphrotermes* and Genus F, was retrieved as sister to the other three lineages, (II) the Asian *Speculitermes*-group was sister to the last two lineages, (III) the African genera *Adaiphrotermes* and Genus F, and their sister group, (IV) the Neotropical soldierless *Anoplotermes*-group.

Discrepancies among analyses were found for the position of soldiered lineages. More precisely, the position of the clade including Genus C + (*Hoplognathotermes* + *Labidotermes*) was variable among analyses (Figs 1 and S1-S3). Similarly, the position of species within the *Astalotermes*-group and the *Anoplotermes*-group was variable. The relationships among taxa of *Astalotermes*-group were often weakly supported, and several genera were retrieved as polyphyletic, i.e. *Astalotermes*, *Anenteotermes* and *Astratotermes*. Within the neotropical *Anoplotermes*-group (clade IV), the relationships among genera were weakly supported, and many species, referred to as *Anoplotermes*-group sp., lie on long branches and belong to undescribed genera (Fig. 1 and S1-S3). The genus *Ruptitermes* appears polyphyletic, the arboreal *R. arboreus* being broadly separated from the other species of the genus.

3.2. Divergence time estimation

The time-tree reconstructed with third codon positions included yielded older age estimates (Fig. S1), up to 10.9 million years (My) older than the analysis with third codon positions excluded (Fig. 1). The ranges given hereafter encompass the results of both analyses, with and without third codon positions. We estimated that the most recent common ancestor of Apicotermitinae lived 39.5–48.6 million years ago (hereafter Ma) (95% HPD: 34.7–53.2 Ma), during the middle Eocene. The most recent common ancestor of the soldierless Apicotermitinae + *Speculitermes*-group was estimated at 34.9–44.2 Ma (95% HPD: 30.8–48.5 Ma). The split between the *Speculitermes*-group and their sister group was dated at 31.7–41.6 Ma (95% HPD: 27.7–45.9 Ma), during the early Oligocene. The Neotropical *Anoplotermes*-group diverged from its African sister lineage (*Adaiphrotermes* + Genus F) 28.0–38.0 Ma (95% HPD: 24.4–42.0 Ma). The age estimates of cladogenesis for the current taxonomic groups are summarized in Table 1.

3.3. Ancestral distribution

We reconstructed the ancestral distribution of Apicotermitinae on the four phylogenetic trees generated in this study and found entirely congruent results (Figs S4-S7). We found that the Apicotermitinae originated in the African realm, and dispersed from there twice: once to the Oriental realm, where they gave rise to the *Speculitermes*-group, and once to the Neotropical realm, where they gave rise to the *Anoplotermes*-group.

4. Discussion

4.1. Phylogenetic relationships and systematics

Our findings are in partial agreement with those of Inward et al. (2007) and Bourguignon et al. (2017). For instance, we confirm the paraphyly of the *Apicotermes*-group, which is composed of several basal lineages, closely matching the subgroups proposed by Noirot (2001) on the basis of digestive anatomy: the *Labidotermes* subgroup, comprising also *Hoplognathotermes* (+ *Acutidentitermes*, not sequenced), is characterized by a simple enteric valve armature wholly enclosed within the P2 section of the hindgut, which probably represents an ancestral condition; the *Apicotermes* subgroup, including also *Allognathotermes* + *Duplidentitermes* and *Coxotermes* + *Heimitermes*, possesses very sophisticated enteric valve armatures protruding into the paunch; and the *Trichotermes* subgroup, including

Jugositermes and *Phoxotermes* (+ *Rostrotermes*, not sequenced), displays an enteric valve with six sclerotized plates bearing numerous, variously developed spines, which also penetrate into the paunch. Noirot (2001) made a fourth subgroup for *Eburnitermes* and *Machadotermes*, which were not sequenced. These two genera possibly constitute another basal lineage. Finally, some new taxa are known from the worker only, although their anatomy places them in the *Apicotermes*-group: this is the case of the new Genus C, whose mt-DNA confirmed distant affinities with *Labidotermes* and *Hoplognathotermes*. Another such taxon is the new genus labelled "*Kaktotermes*" (*nomen nudum*) by Donovan (2002), which still awaits sequencing.

The phylogeny of Inward et al. (2007) featured a large clade comprising, on the one hand, the *Astalotermes*-group (including all African soldierless taxa except *Adaiphrotermes*, without deeper resolution), and on the other hand, an unresolved clade including *Adaiphrotermes*, which appeared paraphyletic, the Asian taxa (with soldiers known in all genera, but often very rare) and the neotropical taxa (all soldierless, not further resolved). Here, we confirm the *Astalotermes*-group as monophyletic and resolve its sister clade with a strong support: Asian genera (*Indotermes* + *Euhamitermes*) now branch out first, as sister group to a soldierless clade including the neotropical taxa, confirmed as monophyletic (= the *Anoplotermes*-group clade), and an African branch composed of *Adaiphrotermes* plus a distinctive new African genus here called Genus F (*Adaiphrotermes*-group clade).

Inward et al. (2007) suggested that the loss of the soldier caste occurred only once in the evolution of the Apicotermitinae, but in view of the best supported phylogeny, this hypothesis cannot account for the presence of soldiers in Asian taxa. Unless soldiers have been reacquired in Asian taxa, which seems unlikely, their loss must have occurred at least twice: once at the origin of the *Astalotermes*-group (clade I on Fig. 1), and once at the origin of the clade composed of the *Adaiphrotermes*-group and the *Anoplotermes*-group (clades III+IV on Fig. 1). Note that missing taxa might in the future cast additional light on soldier loss events, when their phylogenetic position is ascertained: according to Noirot (2001), the soldiered genus *Firmitermes* possesses a digestive anatomy reminiscent of soldierless species, whereas the soldierless genus *Skatitermes* anatomically matches the *Apicotermes*-group. In addition, soldiers are very rare or even unknown in some Asian species (especially in the genus *Speculitermes*), which suggests that they may have rarefied to the point of disappearing completely several times.

Thus far, most generic descriptions of Apicotermitinae have been written in the absence of a solid phylogenetic background. Some genera are characterized by conspicuous apomorphies, such as the hypertrophied sclerotization of cushion 1 of the enteric valve in *Ateuchotermes* (Sands, 1972), whereas others mostly accommodate species that do not display particular diagnostic features. For instance, as Sands (1972: 51) himself admitted, *Astalotermes* was difficult to define because this genus "occupies a transitional position between others with more primitive and more specialized characters". Not surprisingly, this genus came out of our study as polyphyletic. Likewise, *Astratotermes* —basically, *Astalotermes* with enteric valve scales ending in tiny points— was defined on characters of poor

phylogenetic significance and ended up polyphyletic as well. The situation is even more caricatural in the Neotropics, where the genus *Anoplotermes* lumps all soldierless species that have so far not been considered eccentric enough to deserve a transfer to another genus. All those genera are now in need of an in-depth revision. The present phylogeny will constitute a useful framework to revise the whole subfamily and identify characters of phylogenetic interest.

This work also yielded less intuitive results. For instance, the tiny *Anenteotermes nanus* now appears distant from the equally tiny *An. polyscolus* and other species with a bilateral enteric valve armature — *An. cnaphorus*, *An.* sp. A (CIVT120), and probably *An. cherubimi*, recently described (Scheffrahn and Roisin, 2018) and awaiting sequencing. In the Neotropics, the arboreal open-air forager *Ruptitermes arboreus* was known to be slightly different, on anatomical grounds, from other species of the genus which are ground-dwelling litter feeders (Acioli and Constantino 2015). Our results now show that *R. arboreus* has been wrongly assigned to this genus, being closer to *Tetimatermes* than to other *Ruptitermes* species.

4.2. Time frame of Apicotermitinae evolution

As suggested by Inward et al. (2007) and Bourguignon et al. (2017), our results support the African origin of Apicotermitinae. The molecular dating analyses with and without third codon positions yield age estimates diverging by up to 10.9 My. Likely, this difference is caused by the high base compositional heterogeneity at third codon positions which can influence the estimation of divergence times (Shong et al., 2010; Zheng et al., 2011). However, time estimates of our tree with the third codon position excluded are similar to those of other phylogenetic trees (Bourguignon et al., 2015, 2017; Bucek et al., 2019). For instance, our estimation of the most recent ancestor of Apicotermitinae diverged by less than 5 My from those time-trees (Bourguignon et al., 2015, 2017; Bucek et al., 2019). According to both molecular clocks, Apicotermitinae cladogenesis was initiated during the Eocene 39.5–48.6 Ma (95% HPD: 34.7–53.2 Ma) when rainforests were more extended than nowadays. Nevertheless, most clades originated after the Eocene-Oligocene transition (about 34 Ma). This event may be compatible with a timeframe in which the atmospheric concentration of carbon dioxide dropped (Pagani et al., 2005), global temperatures decreased, and the megathermal rainforests retracted to low latitudes (Morley, 2011). Thus, this climate change led to some species extinctions and created refuges in relicts of equatorial forests that could have driven speciation events. The age estimates of our molecular clock analysis without the third codon positions match with past climatic transitions and forest distributions that may have led to the diversification of the Apicotermitinae lineage. According to our results, migratory movements of the ancestors of the *Speculitermes* and *Anoplotermes* groups occurred in two separate occasions out of the Afrotropical realm. The first dispersal event occurred 21.0–31.7 Ma (95% HPD: 16.2–35.9 Ma, without 3rd codon position) or 29.6–41.6 (95% HPD: 23.6–45.9 Ma, with 3rd codon position) and gave rise to the Oriental soldiered species while the sister lineage remained in the Afrotropical realm. Following the second dispersal event, 24.0–28.1 Ma (95% HPD: 20.8–31.9 Ma, without 3rd codon position) or 34.2–

Acknowledgements

We thank J. Migliore for fruitful discussions about sequence alignment methods. We are grateful to K. Dosso, T. Coulibaly and C.S. Tra Bi for their help during fieldwork in Ivory Coast and also to the people of Ebogo II village for their kind company and support during sample collection in Cameroon. We thank Régis Vigouroux and the staff of the Laboratoire Environnement for receiving us at the HYDRECO research station (Petit Saut, French Guiana) during fieldwork. S. Chérasse helped editing the language of early drafts. We thank the OIST sequencing section for assistance with DNA sequencing. This work was supported by a grant from the Belgian National Fund for Scientific Research F.R.S.-FNRS (PDR T.0065.15) to YR, a grant from the Van Buuren and Jaumotte–Demoulin fund to JRA, by subsidiary funding to OIST, and by the Internal Grant Agency of the Faculty of Tropical Agrisciences CULS (20205014).

Table 1. Estimation dates for the major and basal Apicotermitinae clades (Ma) with all sites included and without third codon positions. The differences (**δ**) of node ages are included.

crown clade	description	without Third codon positions		with Third codon positions		δ node ages
		node ages	95% HPD	node ages	95% HPD	
I	*Astalotermes*-group	30.7	27.0-34.8	40.5	36.8-44.8	9.8
II	*Speculitermes*-group	21.0	16.2-25.6	29.6	23.6-34.8	8.6
III	*Adaiphrotermes* + genus F	23.0	18.4-27.7	32.2	27.5-37.3	9.2
IV	*Anoplotermes*-group	24.0	20.8-27.2	34.2	30.8-37.9	10.2

Figure caption

Fig. 1. Bayesian phylogenetic chronogram of Apicotermitinae inferred from mitochondrial genomes, with third codon positions excluded. The scale bar is given in millions of years. Node bars represent the 95% HPD intervals for the ages. Nodes are labelled with symbols representing posterior probabilities and bootstrap support for all analyses (1/100% = black; <1/100%= gray) and with red squares when the topology differ among analyses. Pie charts close to the nodes show the inferred relevant ancestral shifts of biogeographic distributions on the map: Afrotropical, Oriental and Neotropical realms. Wide bars indicate current distribution of species. Dotted boxes with roman numbers indicate the crown clades: I *Astalotermes*-group, II *Speculitermes*-group, III *Adaiphrotermes* + genus F and IV *Anoplotermes*-group. Tip circles represent soldiered (dark brown), soldierless (light brown) species and unknown soldier caste presence (yellow). Names of species include colony code and scientific name, respectively.

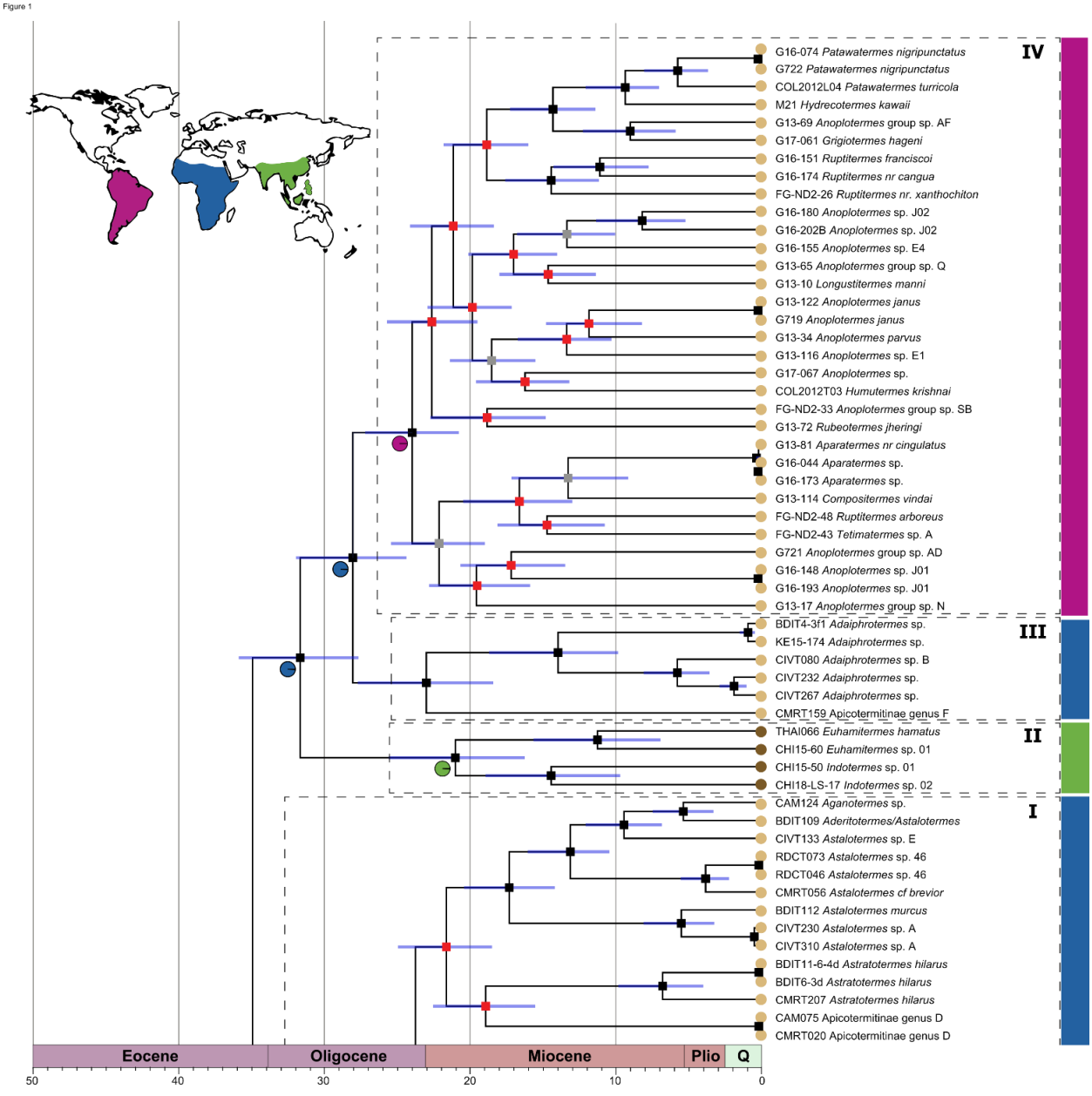
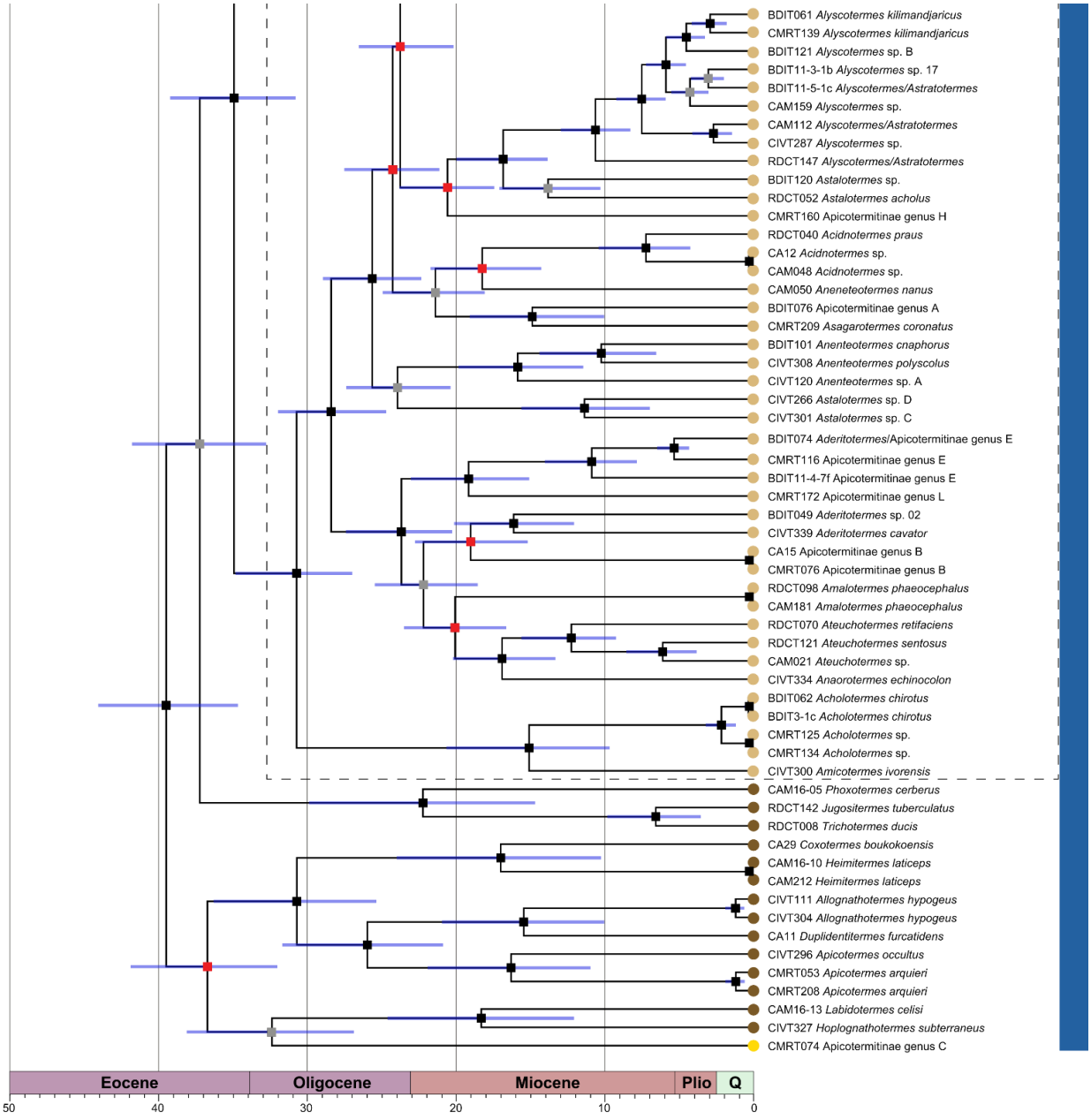


Figure 1
G16-074 Patawatermes nigripunctatus
G722 Patawatermes nigripunctatus
COL2012L04 Patawatermes turricola
M21 Hydrecotermes kawaii
G13-69 Anoplotermes group sp. AF
G17-061 Grigiotermes hageni
G16-151 Ruptitermes franciscoi
G16-174 Ruptitermes nr cangua
FG-ND2-26 Ruptitermes nr. xanthochiton
G16-180 Anoplotermes sp. J02
G16-202B Anoplotermes sp. J02
G16-155 Anoplotermes sp. E4
G13-65 Anoplotermes group sp. Q
G13-10 Longustitermes manni
G13-122 Anoplotermes janus
G719 Anoplotermes janus
G13-34 Anoplotermes parvus
G13-116 Anoplotermes sp. E1
G17-067 Anoplotermes sp.
COL2012T03 Humutermes krishnai
FG-ND2-33 Anoplotermes group sp. SB
G13-72 Rubeotermes jheringi
G13-81 Aparatermes nr cingulatus
G16-044 Aparatermes sp.
G16-173 Aparatermes sp.
G13-114 Compositermes vindai
FG-ND2-48 Ruptitermes arboreus
FG-ND2-43 Tetimatermes sp. A
G721 Anoplotermes group sp. AD
G16-148 Anoplotermes sp. J01
G16-193 Anoplotermes sp. J01
G13-17 Anoplotermes group sp. N
BDIT4-3f1 Adaiphrotermes sp.
KE15-174 Adaiphrotermes sp.
CIVT080 Adaiphrotermes sp. B
CIVT232 Adaiphrotermes sp.
CIVT267 Adaiphrotermes sp.
CMRT159 Apicotermitinae genus F
THAI066 Euhamitermes hamatus
CHI15-60 Euhamitermes sp. 01
CHI15-50 Indotermes sp. 01
CHI18-LS-17 Indotermes sp. 02
CAM124 Aganotermes sp.
BDIT109 Aderitotermes/Astalotermes
CIVT133 Astalotermes sp. E
RDCT073 Astalotermes sp. 46
RDCT046 Astalotermes sp. 46
CMRT056 Astalotermes cf brevior
BDIT112 Astalotermes murcus
CIVT230 Astalotermes sp. A
CIVT310 Astalotermes sp. A
BDIT11-6-4d Astratotermes hilarus
BDIT6-3d Astratotermes hilarus
CMRT207 Astratotermes hilarus
CAM075 Apicotermitinae genus D
CMRT020 Apicotermitinae genus D
IV
III
II
I
Eocene
Oligocene
Miocene
Plio
Q
50
40
30
20
10
0


BDIT061 Alyscotermes kilimandjaricus
CMRT139 Alyscotermes kilimandjaricus
BDIT121 Alyscotermes sp. B
BDIT11-3-1b Alyscotermes sp. 17
BDIT11-5-1c Alyscotermes/Astratotermes
CAM159 Alyscotermes sp.
CAM112 Alyscotermes/Astratotermes
CIVT287 Alyscotermes sp.
RDCT147 Alyscotermes/Astratotermes
BDIT120 Astalotermes sp.
RDCT052 Astalotermes acholus
CMRT160 Apicotermitinae genus H
RDCT040 Acidnotermes praus
CA12 Acidnotermes sp.
CAM048 Acidnotermes sp.
CAM050 Aneneteotermes nanus
BDIT076 Apicotermitinae genus A
CMRT209 Asagarotermes coronatus
BDIT101 Anenteotermes cnaphorus
CIVT308 Anenteotermes polyscolus
CIVT120 Anenteotermes sp. A
CIVT266 Astalotermes sp. D
CIVT301 Astalotermes sp. C
BDIT074 Aderitotermes/Apicotermitinae genus E
CMRT116 Apicotermitinae genus E
BDIT11-4-7f Apicotermitinae genus E
CMRT172 Apicotermitinae genus L
BDIT049 Aderitotermes sp. 02
CIVT339 Aderitotermes cavator
CA15 Apicotermitinae genus B
CMRT076 Apicotermitinae genus B
RDCT098 Amalotermes phaeocephalus
CAM181 Amalotermes phaeocephalus
RDCT070 Ateuchotermes retifaciens
RDCT121 Ateuchotermes sentosus
CAM021 Ateuchotermes sp.
CIVT334 Anaorotermes echinocolon
BDIT062 Acholotermes chirotus
BDIT3-1c Acholotermes chirotus
CMRT125 Acholotermes sp.
CMRT134 Acholotermes sp.
CIVT300 Amicotermes ivorensis
CAM16-05 Phoxotermes cerberus
RDCT142 Jugositermes tuberculatus
RDCT008 Trichotermes ducis
CA29 Coxotermes boukokoensis
CAM16-10 Heimitermes laticeps
CAM212 Heimitermes laticeps
CIVT111 Allognathotermes hypogeus
CIVT304 Allognathotermes hypogeus
CA11 Duplidentitermes furcatidens
CIVT296 Apicotermes occultus
CMRT053 Apicotermes arquieri
CMRT208 Apicotermes arquieri
CAM16-13 Labidotermes celisi
CIVT327 Hoplognathotermes subterraneus
CMRT074 Apicotermitinae genus C
Eocene
Oligocene
Miocene
Plio
Q
50
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3. Phylogenomic analysis of 589 metagenome-assembled genomes encompassing all major prokaryotic lineages from the gut of higher termites

Phylogenomic analysis of 589 metagenome-assembled genomes encompassing all major prokaryotic lineages from the gut of higher termites

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ABSTRACT

"Higher" termites have been able to colonize all tropical and subtropical regions because of their ability to digest lignocellulose with the aid of their prokaryotic gut microbiota. Over the last decade, numerous studies based on 16S rRNA gene amplicon libraries have largely described both the taxonomy and structure of the prokaryotic communities associated with termite guts. Host diet and microenvironmental conditions have emerged as the main factors structuring the microbial assemblages in the different gut compartments. Additionally, these molecular inventories have revealed the existence of termite-specific clusters that indicate coevolutionary processes in numerous prokaryotic lineages. However, for lack of representative isolates, the functional role of most lineages remains unclear. We reconstructed 589 metagenome-assembled genomes (MAGs) from the different gut compartments of eight higher termite species that encompass 17 prokaryotic phyla. By iteratively building genome trees for each clade, we significantly improved the initial automated assignment, frequently up to the genus level. We recovered MAGs from most of the termite-specific clusters in the radiation of, for example, Planctomycetes, Fibrobacteres, Bacteroidetes, Euryarchaeota, Bathyarchaeota, Spirochaetes, Saccharibacteria, and Firmicutes, which to date contained only few or no representative genomes. Moreover, the MAGs included abundant members of the termite gut microbiota. This dataset represents the largest genomic resource for arthropod-associated microorganisms available to date and contributes substantially to populating the tree of life. More importantly, it provides a backbone for studying the metabolic potential of the termite gut microbiota, including the key members involved in carbon and nitrogen biogeochemical cycles, and important clues that may help cultivating representatives of these understudied clades.

Subjects Bioinformatics, Ecology, Genomics, Microbiology
Keywords Metagenome-assembled genomes, Gut microbiology, Higher termites, Bacteria, Archaea, Phylogenomics, Metagenomics, Spirochaetes, Fibrobacteres, Bathyarchaeota

How to cite this article Hervé V, Liu P, Dietrich C, Sillam-Dussès D, Stiblik P, Šobotník J, Brune A. 2020. Phylogenomic analysis of 589 metagenome-assembled genomes encompassing all major prokaryotic lineages from the gut of higher termites. **PeerJ 8:e8614**
DOI 10.7717/peerj.8614

INTRODUCTION

Termites (Blattodea: Termitoidae) are eusocial insects that have predominantly and successfully colonized tropical and subtropical areas across the world. One of the keys to this success is their rare ability to degrade lignocellulose, a very abundant but recalcitrant complex carbon substrate (*Cragg et al., 2015*). As major decomposers, termites play an important role in carbon cycling (*Yamada et al., 2005*; *Dahlsjö et al., 2014*; *Liu et al., 2015*; *Griffiths et al., 2019*). Lignocellulose digestion by termites is attributed to the presence of a specific microbiota colonizing the different gut compartments of the host (*Brune, 2014*). Even though termites produce endogenous cellulases in the labial glands and/or midgut (*Tokuda et al., 2004*; *Fujita, Miura & Matsumoto, 2008*), the digestive processes in the hindgut are the result of microbial activities.

"Lower" termites feed almost exclusively on wood, whereas "higher" termites (Termitidae family) diversified their diet and extended it from wood to plant litter, humus, and soil (*Donovan, Eggleton & Bignell, 2001*). Higher termites represent the most diverse and taxon-rich clade and form about 85% of the termite generic diversity (*Krishna et al., 2013*). Their gut morphology is more complex than that of the basal clades, and is characterized by the presence of a mixed-segment and an enlarged proctodeal segment P1. Moreover, the gut displays strong variations in pH and oxygen partial pressure along the anterior–posterior axis, which creates microenvironments within the gut (*Brune, 2014*).

Termites harbor a specific and complex gut microbiota (*Brune & Dietrich, 2015*; *Bourguignon et al., 2018*). Over the last decade, numerous studies targeting the 16S rRNA gene have cataloged the prokaryotic diversity of the termite gut microbiota. By analyzing the structure and composition of these microbial communities, the roles of host taxonomy (*Dietrich, Köhler & Brune, 2014*; *Abdul Rahman et al., 2015*), host diet (*Mikaelyan et al., 2015a*), and microenvironments found in the different gut compartments (*Mikaelyan, Meuser & Brune, 2017*) have emerged as the main factors shaping the termite gut microbiota. These studies have also highlighted patterns of dominant taxa associated with specific diet and/or gut compartment (*Mikaelyan, Meuser & Brune, 2017*). For instance, Spirochaetes tend to be the dominant phylum in the gut of wood/grass feeders, whereas their abundance is lower in litter, humus, and soil feeders, in which Firmicutes are much more abundant. The accumulated 16S rRNA gene reads have revealed the existence of termite-specific clusters among both bacterial and archaeal phyla (e.g., among Fibrobacteres, Clostridia, Spirochaetes, and Euryarchaeota).

All these studies focusing on the 16S rRNA gene have helped microbiologists in answering the question "who is there?," but the following questions "what are they doing?" and "who is doing what?" remain open. Attempts to answer the latter questions have been made, for example, by analyzing different fractions of the gut content of *Nasutitermes* spp., which led to the identification of fiber-associated cellulolytic bacterial taxa (*Mikaelyan et al., 2014*), or by focusing on the diversity of individual functional marker genes, such as *nifH* (*Ohkuma, Noda & Kudo, 1999*) or formyl-tetrahydrofolate synthetase (*Ottesen & Leadbetter, 2011*). The latter approach, however, is problematic because the organismal origin of the respective genes is often obfuscated by frequent horizontal gene transfers

Table 1 Recovery of metagenome-assembled genomes (MAGs) from the 30 termite gut metagenomes analyzed in this study. The host termite, its mitochondrial genome accession number, dietary preference, and the originating gut compartments are indicated. *C* crop (foregut), *M* midgut, *P1–P5* proctodeal compartments (hindgut). The sample codes used for the metagenomes are the combination of host ID and gut compartment.

Termite species	ID	Mitogenome	Diet	Number of MAGs						
				C	M	P1	P3	P4	P5	Total
Microcerotermes parvus	Mp193	KP091690	Wood	– [a]	–	1	1	4	–	6
Nasutitermes corniger	Nc150	KP091691	Wood	0	1	3	6	9	1	20
Cornitermes sp.	Co191	KP091688	Litter	–	–	32	22	7	–	61
Neocapritermes taracua	Nt197	KP091692	Humus	–	–	6	70	11	–	87
Termes hospes	Th196	KP091693	Humus	–	–	6	64	27	–	97
Embiratermes neotenicus	Emb289	KY436202	Humus	–	–	45	52	21	–	118
Labiotermes labralis	Lab288	KY436201	Soil	–	–	66	72	31	–	169
Cubitermes ugandensis	Cu122	KP091689	Soil	0	0	5	5	3	18	31

Note:
[a] Not sequenced.

between prokaryotes. Thus, it has been suggested that genome-centric instead of gene-centric approaches are much more relevant for elucidation of soil or gut microbiotas (*Prosser, 2015*). Unfortunately, the number of available isolates of termite gut microbiota and their genomes (*Zheng & Brune, 2015*; *Yuki et al., 2018*) are low compared to those from other environments. However, modern culture-independent methods, namely metagenomics and single-cell genomics have recently allowed the generation of numerous metagenome-assembled genomes (MAGs) and single-amplified genomes (SAGs), respectively, from uncultivated or difficult to cultivate organisms (*Albertsen et al., 2013*; *Woyke, Doud & Schulz, 2017*). MAGs are becoming increasingly more prominent in the literature (*Bowers et al., 2017*) and populate the tree of life (*Parks et al., 2017*). Additionally, MAGs offer the opportunity to explore the metabolic potential of these organisms and to link it with their ecology.

To date, only a limited number of MAGs and SAGs of uncultured bacteria have been recovered from the guts of higher termites; these represent termite-specific lineages of Fibrobacteres (*Abdul Rahman et al., 2016*) and Cyanobacteria (*Utami et al., 2018*). Here, we applied a binning algorithm to 30 metagenomes from different gut compartments of eight higher termite species encompassing different feeding groups to massively recover hundreds of prokaryotic MAGs from these samples. After quality filtering, all these MAGs were taxonomically identified within a phylogenomic framework and are discussed in the context of insect gut microbiology and symbiosis.

MATERIALS AND METHODS

Metagenomic datasets

To cover a wide range of microbial diversity, we used 30 metagenomic datasets representing the main gut compartments (crop, midgut, P1–P5 proctodeal compartments of the hindgut) and main feeding groups present in higher-termites (see Table 1).

Eight species of higher termites, identified by both morphological criteria and analysis of the mitogenome, were considered: *Cornitermes* sp., *Cubitermes ugandensis*, *Microcerotermes parvus*, *Nasutitermes corniger*, *Neocapritermes taracua*, *Termes hospes* (*Dietrich & Brune, 2016*), *Labiotermes labralis*, and *Embiratermes neotenicus* (*Hervé & Brune, 2017*). Field experiments were approved by the French Ministry for the Ecological and Solidarity Transition (UID: ABSCH-CNA-FR-240495-2; permit TREL1902817S/118). Processing of the termite samples and DNA extraction and purification were described previously (*Rossmassler et al., 2015*). Metagenomic libraries were prepared, sequenced, quality controlled, and assembled at the Joint Genome Institute (Walnut Creek, CA, USA). DNA was sequenced using Illumina HiSeq 2000 or Illumina HiSeq 2500 (Illumina Inc., San Diego, CA, USA). Quality-controlled reads were assembled and uploaded to the Integrated Microbial Genomes (IMG/M ER) database (*Markowitz et al., 2014*). Accession numbers and information about these 30 metagenomes can be found in Table S1.

Genome reconstruction

For each metagenomic dataset, both quality-controlled (QC) and assembled (contigs) reads were downloaded from IMG/M ER in August 2017. To obtain coverage profile of contigs from each metagenomic assembly, the QC reads were mapped to contigs using BWA v0.7.15 with the bwa-mem algorithm (*Li & Durbin, 2009*). This generated SAM files that were subsequently converted into BAM files using SAMtools v1.3 (*Li et al., 2009*). Combining coverage profile and tetranucleotide frequency information, genomes were reconstructed from each metagenome with MetaBAT version 2.10.2 with default parameters (*Kang et al., 2019*). Quality of the reconstructed genomes was estimated with CheckM v1.0.8 (*Parks et al., 2015*). Only MAGs that were at least 50% complete and with less than 10% contamination, were retained for subsequent analyses. These MAGs have been deposited at the Sequence Read Archive (SRA) under the BioProject accession number PRJNA560329; genomes are available with accession numbers SRR9983610–SRR9984198 (Table S2). Additionally, the MAGs have been deposited at the NCBI's Assembly Database under the accessions WQRH00000000–WRNX00000000 (Table S2).

For each MAG, CheckM was also used to extract 16S rRNA gene sequences as well as a set of 43 phylogenetically informative marker genes consisting primarily of 29 ribosomal proteins (PF00466, PF03946, PF00298, PF00572, PF00238, PF00252, PF00861, PF00687, PF00237, PF00276, PF00831, PF00297, PF00573, PF00281, PF00673, PF00411, PF00164, PF00312, PF00366, PF00203, PF00318, PF00189, PF03719, PF00333, PF00177, PF00410, PF00380, PF03947, PF00181), nine RNA polymerase domains (PF04563, PF04997, PF00623, PF05000, PF04561, PF04565, PF00562, PF04560, PF01192), two tRNA ligases (TIGR00344 and TIGR00422), a signal peptide binding domain (PF02978), a translation-initiation factor 2 (PF11987) and a TruB family pseudouridylate synthase (PF01509). Finally, CheckM was also used for a preliminary taxonomic classification of the MAGs by phylogenetic placement of the MAGs into the CheckM reference genome tree.

Phylogenomic analysis

In order to improve the initial CheckM classification, genome trees were built for each clade of interest (from kingdom to family level). Using this initial CheckM classification and when available, the 16S rRNA gene classification, genomes of closely related organisms and relevant outgroups were manually selected and downloaded from NCBI and IMG/M ER. These genomes were subjected to a similar CheckM analysis to extract a set of 43 single-copy marker genes, to translate them into amino acid sequences, and to create a concatenated fasta file (6,988 positions). For each clade of interest, the amino acid sequences from the MAGs, their relatives, and outgroups were aligned with MAFFT v7.305b and the FFT-NS-2 method (*Katoh & Standley, 2013*), and the resulting alignment was filtered using trimAl v1.2rev59 with the gappyout method (*Capella-Gutierrez et al., 2009*). Smart Model Selection (*Lefort, Longueville & Gascuel, 2017*) was used to determine the best model of amino acid evolution of the filtered alignment based on Akaike Information Criterion. Subsequently, a maximum-likelihood phylogenetic tree was built with PhyML 3.0 (*Guindon et al., 2010*). Branch supports were calculated using a Chi2-based parametric approximate likelihood-ratio test (aLRT) (*Anisimova & Gascuel, 2006*). Finally, each tree was visualized and edited with iTOL (*Letunic & Bork, 2019*). Following the procedure described above, a genome tree containing only the MAGs generated in the present study was also built and visualized with GraPhlAn version 0.9.7 (*Asnicar et al., 2015*).

Placement of MAGs in a 16S rRNA-based phylogenetic framework

All 16S rRNA gene sequences recovered from the respective bins were classified using the phylogenetic framework of the current SILVA reference database (SSURef NR 99 release 132) (*Quast et al., 2013*). The database was manually curated to extend the taxonomic outline of all relevant lineages to genus level by linking the taxonomy to the termite-specific groups to that of the DictDb v3 database (*Mikaelyan et al., 2015b*). 16S rRNA gene sequences contained in the MAGs were aligned with SINA version 1.2.11 (*Pruesse, Peplies & Glöckner, 2012*) and imported into the reference database. Sequences longer than >100 bp were added to the reference trees using the parsimony tool of ARB version 6.0.6 (*Ludwig et al., 2004*). If none of the MAGs in a cluster contained a 16S rRNA gene longer than 100 bp, or if the placement of the 16S rRNA genes in the bin conflicted with the results of the phylogenomic analysis (indicating a contamination), the phylogenomic classification was used.

Estimation of the relative abundance of the MAGs in each metagenome

For each metagenome, raw reads were mapped against MAGs using BWA (*Li & Durbin, 2009*) with default parameters. Unmapped reads and reads mapped to more than one location were removed by using SAMtools (*Li et al., 2009*) with parameters: F 0x904. Reads mapped to each MAGs were summarized using the "pileup.sh" script (BBmap 38.26) (*Bushnell, 2014*). The relative abundance of each MAG was calculated as the total number of reads mapped to a MAG divided by the total number of reads in the corresponding metagenome sample, as described in *Hua et al. (2019)*. Similarly, the MAG coverage was

estimated by multiplying the mapped reads by the read length and dividing it by the MAG length.

Statistical analyses

Statistical analyses were performed with R version 3.4.4 (*R Development Core Team, 2019*), and data were visualized with the *ggplot2* (*Wickham, 2016*) package. Correlations between quantitative variables were investigated with Pearson's product moment correlation coefficient.

RESULTS AND DISCUSSION

Metagenomes and MAGs overview

Metagenomic reads were generated from the P1, P3, and P4 proctodeal compartments of the gut of the two termite species *E. neotenicus* and *L. labralis*. These six metagenomes were combined with 24 previously published metagenomes from the gut of higher termites (*Rossmassler et al., 2015*) in order to obtain data encompassing different gut compartments from eight species of higher termites feeding on different lignocellulosic substrates ranging from wood to soil (Table 1). Metagenomic binning of these 30 termite gut metagenomes yielded 1,732 bins in total (Table S1). For further analysis, we selected only those bins that represented high-quality (135 bins, >90% complete, and <5% contamination) and medium-quality (454 bins, >50% complete, and <10% contamination) MAGs (Table 1; Table S1). The present study focused on these 589 MAGs, which showed on average a 38.6-fold coverage (Table S2).

The number of MAGs recovered from the different metagenomes did not show a Gaussian distribution. Instead, we found a significant and positive relationship between the number of metagenome-assembled reads and the number of MAGs recovered ($r = 0.85$, $p < 0.0001$), indicating that assembly success and sequencing depth were important predictors of genome reconstruction success (Fig. 1). This is in agreement with benchmarking reports on metagenomic datasets (*Sczyrba et al., 2017*) and underscore that a good quality assembly is a prerequisite for high binning recovery, which is important to consider when designing a metagenomic project for the purpose of binning. A significantly higher number of assembled reads and of MAGs recovered was observed in the current dataset compared to the *Rossmassler et al. (2015)* dataset (Wilcoxon test, $p < 0.005$), highlighting the importance of this new dataset (Fig. 1).

MAGs taxonomy and abundance

We investigated the phylogenomic context of the 589 MAGs. An initial automated classification of the MAGs using CheckM and when available, the taxonomic assignment of the 16S rRNA gene, identified representatives of 15 prokaryotic phyla (Table S3). Initially, 142 MAGs (24% of the dataset) remained unclassified at the phylum level, and key taxa of the termite microbiota, such as Fibrobacteres and *Treponema*, were absent or only poorly represented. This is partly explained by the lack of representative genomes for certain taxa in the reference genome tree provided in the current version of CheckM (e.g., only one Fibrobacteres genome and one Elusimicrobia genome, and an

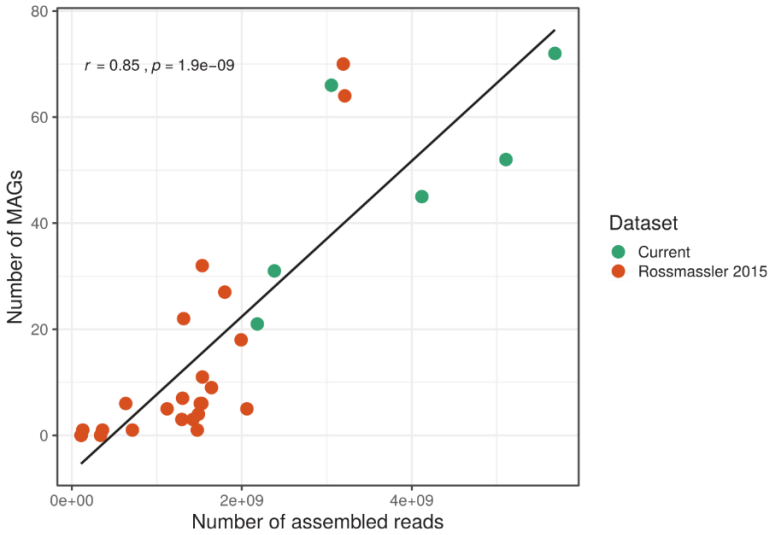

Figure 1 Relationship between the number of MAGs recovered and the number of assembled reads in the respective metagenomes. The linear regression line and the Pearson correlation coefficient (*r*) are shown for the entire dataset. Full-size DOI: 10.7717/peerj.8614/fig-1

absence of Bathyarchaeota and Kiritimatiellaeota genomes). New tools incorporating larger databases, such as GTDB-Tk (*Parks et al., 2018*; *Chaumeil et al., 2019*), will probably resolve such issues.

We improved the taxonomic resolution of the classification by iteratively constructing genome trees for each clade of interest that included all recently published reference genomes. This approach allowed the successful classification of all 589 MAGs, in some cases down to the genus level (Table S2). Thirty-eight MAGs were from the archaeal domain, and 551 MAGs were from the bacterial domain, which together represented a total of 17 prokaryotic phyla (Fig. 2). The taxonomic diversity of MAGs recovered is broadly representative of that observed in previously published 16S rRNA surveys, suggesting good taxonomic coverage of termite-associated prokaryotes from the different gut compartments and host diets (Figs. S1 and S2).

The MAG taxonomy was further refined by placing all 16S rRNA genes recovered from the bins into the phylogenetic framework of the current SILVA reference database, which allowed classifying most of the MAGs down to genus level (Table S2). When we compared the taxa represented by the MAGs to the distribution of the corresponding taxa in amplicon libraries of the bacterial gut microbiota of a representative selection of higher termites that were classified using the same framework (*Lampert, Mikaelyan & Brune, 2019*), we found a high level of congruence between the datasets. The MAGs represented 15 of the 19 bacterial phyla in the amplicon libraries that comprised >0.1% of all reads, including all core phyla (represented in >80% of all host species) with the exception of Verrucomicrobia (Fig. 3). A high representation in the amplicon libraries of the taxa represented by MAGs was confirmed at all taxonomic ranks down to the genus level

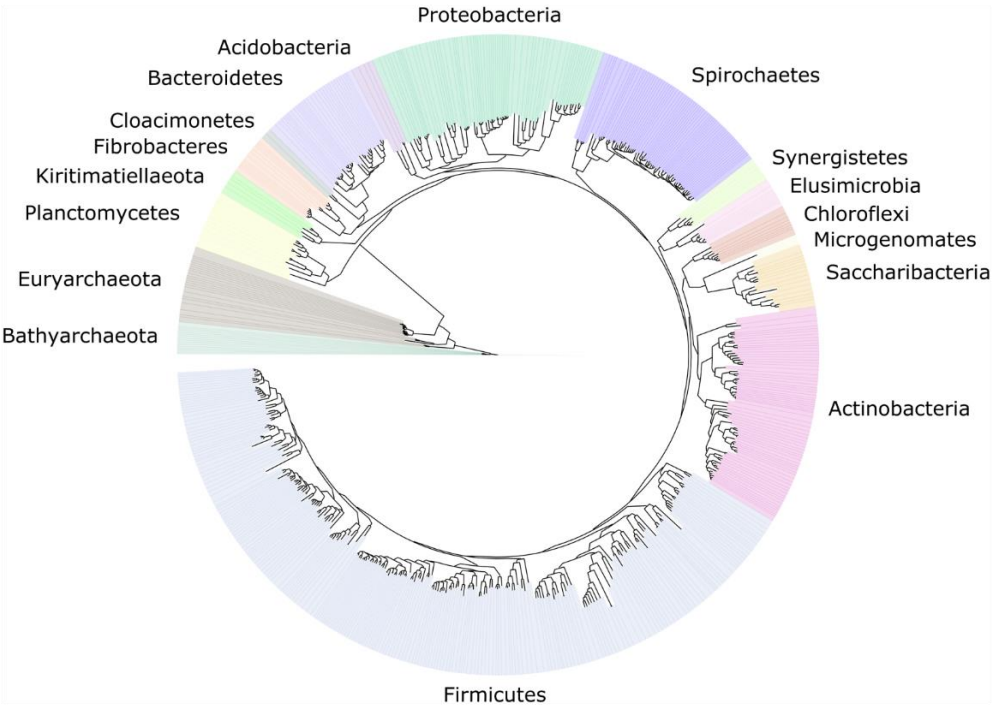

Figure 2 Distribution of the 589 MAGs among bacterial and archaeal phyla. This maximum-likelihood tree was inferred from a concatenated alignment (amino acids) of 43 protein-coding genes (6,801 positions) using the LG+G+I model of evolution. Full-size DOI: 10.7717/peerj.8614/fig-2

(Table S4), underscoring that the present dataset covers the majority of lineages that colonize the higher-termite gut.

We computed the relative abundance of each MAG. These abundances ranged from 0.005% to 4.03% (Table S2), with a mean value of 0.19%. These values indicated that the present dataset includes major taxonomic groups of the termite gut microbiota, which was confirmed when we looked at the taxonomic distribution of the MAGs. Considering the MAG relative abundance and not only their presence within samples, we could observe an effect of the host diet on the taxonomic distribution (Fig. 4). Indeed, similarities were observed when we compared taxonomic patterns of the MAG relative abundance with previously published 16S rRNA gene amplicon-based surveys (*Abdul Rahman et al., 2015*; *Mikaelyan, Meuser & Brune, 2017*). For instance, Spirochaetes were the most abundant phylum within the wood-feeding termite *N. corniger*, and their proportion decreases along the humification gradient, being less abundant in the gut of humus feeders and litter feeders and even less abundant in soil feeders, in the favor of other phyla such as Firmicutes. Fibrobacteres were preferentially abundant within wood- and litter-feeder samples (Fig. 4). Interestingly, a significant and negative relationship between the number of metagenome-assembled reads in a sample and the MAG relative abundances within this sample ($r = -0.33$, $p < 0.0001$) was observed across all the samples. This could be partly explained by the fact that increasing sequencing depth would increase the number of metagenome-assembled reads and thus allow the

Phylum	MAGs			16S rRNA amplicon libraries						
					Average abundance (%)					
	Total number	with 16S gene	Rel. abd. (%)	Core	All	Wood	Litter	Humus	Soil	Fungus
Acidobacteria	4	2	0.4	•	0.5	0.7	0.4	0.8	0.4	0.2
Actinobacteria	71	22	3.0	•	3.1	2.0	1.8	2.1	5.2	3.9
Bacteroidetes	33	5	2.7	•	17.5	7.6	22.1	18.5	12.1	28.7
Chloroflexi	8	5	0.1		0.2	0.0	0.1	0.3	0.8	0.0
Cloacimonetes	2	2	0.2		0.4	0.0	0.2	1.6	0.2	0.0
Deferribacteres	–	–			0.3	0.0	0.0	0.1	0.1	1.1
Elusimicrobia	9	2	0.4	•	0.8	0.1	1.4	1.9	0.3	0.5
Epsilonproteobacteria	–	–			1.5	0.3	0.7	0.0	0.2	6.1
Fibrobacteres	13	2	6.6	•	3.2	8.5	6.8	0.6	0.1	1.1
Firmicutes	237	73	9.2	•	33.5	13.1	26.0	41.0	56.2	28.7
Fusobacteria	–	–			0.2	0.1	0.0	0.0	0.1	0.7
Kiritimatiellaeota	5	3	0.3		0.3	0.1	0.2	0.3	0.9	0.1
Microgenomates	1	1	0.6	•	0.6	0.1	0.1	0.2	0.4	2.3
Planctomycetes	12	8	0.7		1.0	0.0	0.1	0.0	0.6	3.7
Proteobacteria	67	23	9.8	•	7.8	3.5	8.0	7.7	9.4	10.6
Saccharibacteria	15	12	0.1	•	2.2	0.4	3.1	1.9	0.8	5.2
Spirochaetes	68	10	3.0	•	24.5	62.1	27.5	21.1	8.1	4.7
Synergistetes	6	1	0.3	•	1.5	0.8	0.5	1.2	3.2	1.5
Verrucomicrobia	–	–		•	0.4	0.1	0.3	0.2	0.6	0.6
Bacteria	**551**	**170**	**36.7**		**100**	**100**	**100**	**100**	**100**	**100**
Bathyarchaeota	15	10	1.2							
Euryarchaeota	23	6	0.5							
Archaea	**38**	**16**	**1.7**							
Total	**589**	**187**	**38.4**							

Figure 3 Phylum-level representation of MAGs among the bacterial gut microbiota of higher termites. The average abundance of the corresponding lineages in 16S rRNA amplicon libraries of higher termites from different diet groups is shown for comparison. Core lineages represented in at least 80% of these samples are marked. For an interactive spreadsheet resolving each lineage to genus level, see Supplemental Information (Table S4). Full-size DOI: 10.7717/peerj.8614/fig-3

binning of sequences from less abundant organisms. However, since quantity of metagenome-assembled reads and relative abundance are not independent variables, it also implies that MAG relative abundances can not be directly quantitatively compared between samples but only within a single sample. Thus, proportions of taxa within a sample using relative abundance can be used to describe such sample.

Archaea

The archaeal domain was represented by members of the phyla Euryarchaeota and Bathyarchaeota (Fig. 5; Fig. S3). Euryarchaeota were represented by 23 MAGs that were classified as members of the genera *Methanobrevibacter* (family *Methanobacteriaceae*; three MAGs) and, *Methanimicrococcus* (family *Methanosarcinaceae*; three MAGs), and members of the family *Methanomethylophilaceae* (16 MAGs), nine of them in the genus *Candidatus* Methanoplasma. MAGs assigned as Euryarchaeota encompassed three (*Methanobacteriales*, *Methanosarcinales*, and *Methanomassiliicoccales*) of the four orders of methanogens found in termite guts (*Brune, 2019*); *Methanomicrobiales* were absent from the present dataset. This genomic resource will be extremely valuable for a better understanding of the genomic basis of methanogenesis in the termite gut and more generally for investigating the functional role of archaea in arthropod guts. Indeed, Euryarchaota have been found to be present in virtually all termite species investigated (*Brune, 2019*), and a global 16S rRNA gene survey has revealed that this phylum is the most abundant archaeal clade in arthropod-associated microbiota (*Schloss et al., 2016*). Bathyarchaeota were represented by 15 MAGs, which formed a termite-specific cluster,

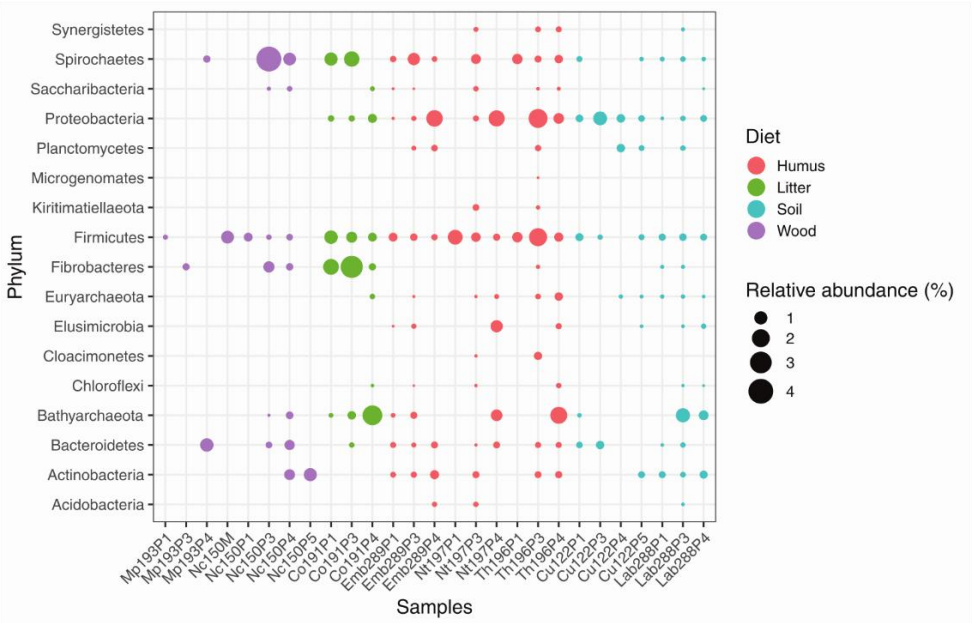

Figure 4 Relative abundance of the MAGs from different phyla among the respective metagenomes. Circle size indicates the relative abundance of the MAGs among the respective metagenome sample; color indicates host diet. To estimate the relative abundance of each MAG, the total number of reads mapped to a MAG was divided by the total number of reads in the corresponding metagenome sample.
Full-size DOI: 10.7717/peerj.8614/fig-4

with Bathyarchaeota reconstructed from sediments of the White Oak River (WOR) estuary (NC, USA) as next relatives (*Lazar et al., 2016*) (Fig. 5). Bathyarchaeota is a lineage formerly known as Miscellaneous Crenarchaeota Group (MCG), which has been reported to occur in the gut of soil-feeding termites (*Friedrich et al., 2001*). To date, MAGs of Bathyarchaeota have been mostly derived from aquatic environments (*Zhou et al., 2018*). Here, we identify the members of the termite gut lineage as Bathyarchaeota and provide the first genomes from this environment. Interestingly, Bathyarchaeota MAGs were particularly abundant in humus-, litter-, and soil-feeding termites (Fig. 4); a genomic characterization, combined with analyses of their abundance and localization, should shed light on the metabolic potential of these organisms and their functional role in termite guts.

Firmicutes

Firmicutes was by far the phylum with the highest number of MAGs, but also the phylum with the highest average relative abundance (33.5%) in 16S rRNA gene-based surveys (Fig. 3). The 237 MAGs (40% of the total dataset) represented three classes (*Bacilli*, *Clostridia*, and *Erysipelotrichia*) and ten families, including four members of *Streptococcaceae* (*Bacilli*) and three members of *Turicibacteraceae* (*Erysipelotrichia*). *Clostridia* was the most diverse and rich class (229 MAGs), in which *Ruminococcaceae* (95 MAGs), *Defluviitaleaceae* (67 MAGs), *Lachnospiraceae* (four MAGs), *Peptococcaceae*

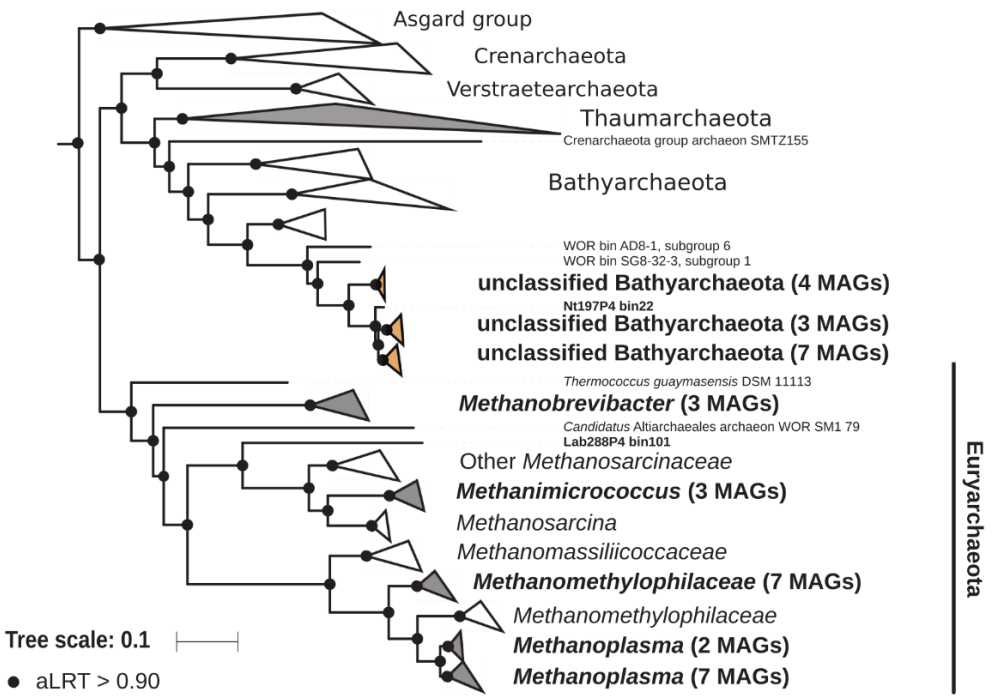

Figure 5 Phylogenomic tree of the archaeal domain. This maximum-likelihood tree was inferred from a concatenated alignment of 43 proteins (6,682 positions) using the LG+G+I+F model of amino-acid evolution. Branch supports were calculated using a Chi2-based parametric approximate likelihood-ratio test. Names in bold included MAGs recovered in the present study. Clusters shaded in brown consist exclusively of MAGs from termite guts and clusters shaded in gray contain genomes from termite guts. The Asgard group was used as outgroup. Full-size DOI: 10.7717/peerj.8614/fig-5

(16 MAGs), *Christensenellaceae* (nine MAGs), *Eubacteriaceae* (two MAGs), Family XIII *incertae sedis* (six MAGs), and Clostridiales vadinBB60 group (22 MAGs) families were identified. These high numbers of *Ruminococcaceae* and *Defluviitaleaceae* MAGs were reflected by high relative abundances of these two families in 16S rRNA gene-based surveys (15.9% and 3.1% for the *Ruminococcaceae* and *Defluviitaleaceae*, respectively; Table S4). Interestingly, among the *Defluviitaleaceae*, the genomes were mainly recovered from the P1 compartment (53 MAGs, i.e., 79% of the family members) whereas *Ruminococcaceae* were predominantly recovered from the P3 compartment (59 MAGs, i.e., 62% of the family members). Further studies should investigate the potential metabolic specialization of these two families in relation to the gut physicochemical properties.

A fourth class-level lineage could not be further classified for lack of reference genomes. In a recent global 16S rRNA gene-based survey, it has been suggested that many novel lineages of Firmicutes in insect-associated metagenomes are hidden (*Schulz et al., 2017*). Our present study confirms this idea but our genome trees also provide evidence of new lineages. Here, we report the first genomes of uncultured termite-specific lineages (Table S4) that were already detected in previous 16S rRNA gene-based surveys (*Bourguignon et al., 2018*). For example, the phylogenomic tree of the most abundant

family *Ruminococcaceae* (Fig. S4) showed various termite-specific clusters, including a cluster of 18 MAGs closely related to *Sporobacter termitidis* isolated from *Nasutitermes lujae* (*Grech-Mora et al., 1996*). *Lachnospiraceae*, *Ruminococcaceae*, *Turicibacteraceae* (previously classified as *Erysipelotrichaceae*), and *Defluviitaleaceae* (previously classified as *Lachnospiraceae*) have been reported among the dominant taxa in termite guts (*Mikaelyan, Meuser & Brune, 2017*), but most of them remain uncultivated and/or with few representative genomes. As such, many questions regarding their ecology and metabolism remain open. With 237 Firmicutes MAGs recovered from different gut compartments and from hosts with different diets, the present study provides the material for further genomic exploration of the role of these bacteria in plant polysaccharide degradation, based for instance on CAZyme distribution (*Lombard et al., 2014*). Since diet has been shown to be the main factor shaping gut community composition in higher termites (*Mikaelyan et al., 2015a*), one might hypothesize the existence of different arsenals of lignocellulolytic enzymes, potentially reflecting the host diet specificity (balance between cellulose, lignin, and hemicelluloses). More generally, Firmicutes and especially *Ruminococcaceae* are also abundant and metabolically important in rumen systems (*Svartström et al., 2017*; *Söllinger et al., 2018*; *Stewart et al., 2018*). At a broader scale, our dataset will allow comparative studies between intestinal tract microbiota of ruminants and phytophagous or xylophagous invertebrates, which would allow a better understanding of plant polysaccharide degradation across the tree of life.

Actinobacteria

Actinobacteria was the second most abundant phylum with 71 MAGs, including members of the classes *Acidimicrobiia*, *Actinobacteriia*, *Coriobacteriia*, and *Thermoleophilia* (Fig. S5). Eleven families were represented, namely *Propionibacteriaceae* (12 MAGs), *Promicromonosporaceae* (three MAGs), Clostridiales *incertae sedis* (16 MAGs), OPB41 (16 MAGs) *Cellulomonadaceae* (seven MAGs), *Frankiaceae* (one MAG), *Sanguibacteraceae* (four MAGs), *Microbacteriaceae* (two MAGs), *Nocardioidaceae* (two MAGs), *Acidimicrobiaceae* (one MAG), *Nocardiaceae* (one MAG), and *Conexibacteraceae* (one MAG). Among these 71 MAGs, 36 were recovered from humus feeders, 33 from soil feeders but only two from wood feeders, which suggests a higher prevalence in termites with a more humified diet. This phylum is known to be present and of significant abundance in both the nest (*Sujada, Sungthong & Lumyong, 2014*) and gut of termites (*Le Roes-Hill, Rohland & Burton, 2011*), but to be more abundant in the nest (*Moreira et al., 2018*). This was for instance the case for the families *Acidimicrobiaceae*, *Nocardiaceae*, *Promicromonosporaceae*, *Microbacteriaceae*, *Nocardioidaceae*, and *Propionibacteriaceae*, which were more abundant in the nest than in the gut of workers or soldiers of *Procornitermes araujoi* (*Moreira et al., 2018*). Therefore, one of the key questions regarding this phylum concerns their effective role in lignocellulose degradation in the termite guts. Are they just present in the surrounding environment of the termite and thus sometimes transit from the gut or are they actively involved in food digestion? The MAGs obtained in the present study will allow to address such questions by

evaluating gene expression of these organisms using metatranscriptomic data from higher termites (*He et al., 2013*; *Marynowska et al., 2017*).

Spirochaetes

The phylum Spirochaetes was represented by 68 MAGs from wood-, soil-, litter-, and humus-feeding termites. It has long been known that Spirochaetes are a diverse and important lineage in termite gut (*Paster et al., 1996*; *Lilburn, Schmidt & Breznak, 1999*), especially because of their involvement in reductive acetogenesis (*Leadbetter et al., 1999*; *Ohkuma et al., 2015*) and in hemicellulose degradation (*Tokuda et al., 2018*). In terms of abundance, Spirochaetes are among the dominant phyla in termite guts (Fig. 3) and may represent more than half of the bacterial relative abundance in some species (*Diouf et al., 2018a*). Three Spirochaetes orders, namely *Brevinematales* (one MAG), *Leptospirales* (four MAGs) and *Spirochaetales* (59 MAGs), were identified (Fig. 6; Fig. S6). In the latter order, 54 MAGs recovered from the P1, P3, and P4 compartments of wood-, litter-, humus-, and soil-feeding hosts were assigned to the termite-specific cluster *Treponema* I (*Ohkuma, Iida & Kudo, 1999*; *Lilburn, Schmidt & Breznak, 1999*) and represent the first genomes of this cluster from higher termites. Indeed, to date only two *Treponema* I genomes are available, and both were recovered from isolates, namely *Treponema azotonutricium and Treponema primitia*, from the hindgut of the lower termite *Zootermopsis angusticollis* (*Graber, Leadbetter & Breznak, 2004*). Thus, our dataset significantly expands the genomic resources for this taxonomic group. Subclusters of this clade have been identified on a dedicated genome tree (Fig. 6). The genome tree topology is in agreement with a previous phylogenomic Spirochaetes study (*Gupta, Mahmood & Adeolu, 2013*). Regarding Spirochaetes classification, our tree topology suggests that the genus *Treponema* could be elevated at least to the family rank due to the presence of distinct *Treponema* clusters (Fig. 6). This observation is also in agreement with the recent Genome Taxonomy Database, which now proposes a *Treponemataceae* family and a *Treponematales* order (*Parks et al., 2018*; *Chaumeil et al., 2019*).

Fibrobacteres

Members of the phylum Fibrobacteres are abundant in the hindgut of wood-feeding higher termites (Fig. 3) (*Hongoh et al., 2006*), where they have been identified as fiber-associated cellulolytic bacteria (*Mikaelyan et al., 2014*). Here, 13 members of the Fibrobacteres phylum were recovered from the P1, P3, and P4 compartments of wood-, litter-, humus-, and soil-feeding termites. These genomes encompass the three orders, namely *Chitinispirillales* (*Sorokin et al., 2016*), previously known as TG3 subphylum 1 (*Hongoh et al., 2006*, five MAGs), *Chitinivibrionales* (previously known as TG3 subphylum 2; two MAGs), and *Fibrobacterales* (six MAGs). While a previous study of termites guts had already provided MAGs of *Chitinivibrionaceae* and *Fibrobacteraceae* and documented their fiber-degrading capacities (*Abdul Rahman et al., 2016*), the present study provides the five first genomes of the termite-associated members of *Chitinispirillaceae* (Fig. 7).

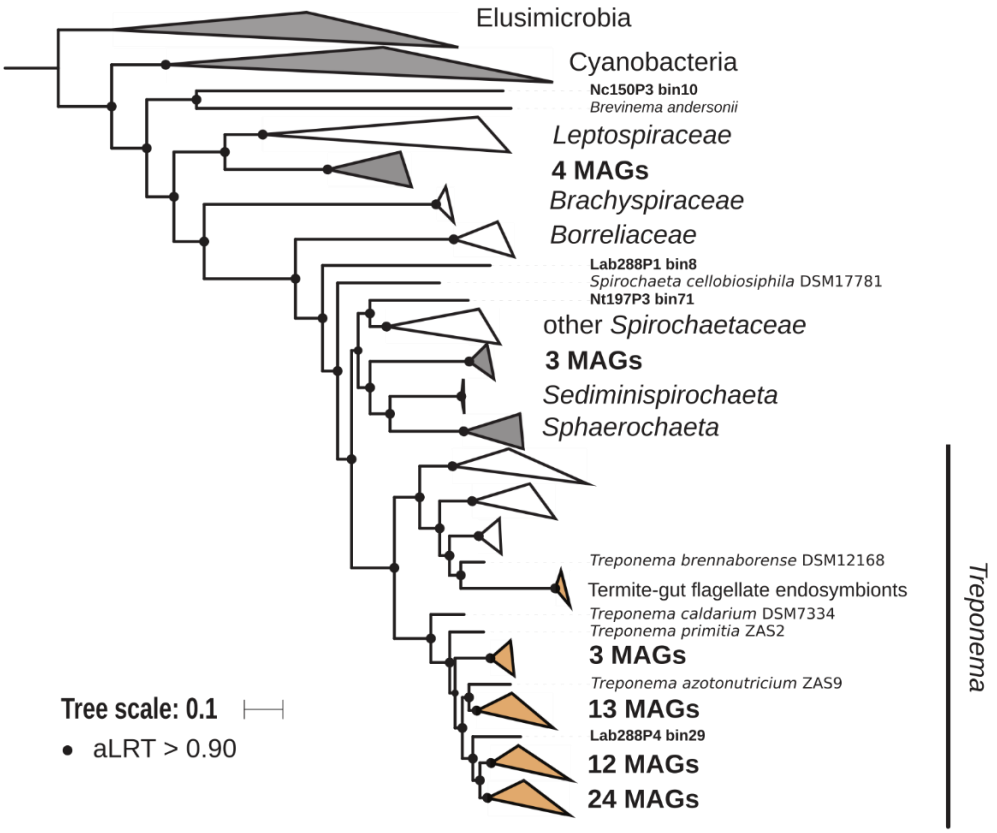

Figure 6 Phylogenomic tree of the Spirochaetes phylum. This maximum-likelihood tree was inferred from a concatenated alignment of 43 proteins (6,741 positions) using the LG+G+I+F model of amino-acid evolution. Branch supports were calculated using a Chi2-based parametric approximate likelihood-ratio test. Names in bold included MAGs recovered in the present study. Clusters shaded in brown consist exclusively of genomes from termite guts and clusters shaded in gray contain genomes from termite guts. Elusimicrobia and Cyanobacteria were used as outgroup.

Full-size DOI: 10.7717/peerj.8614/fig-6

Phylogenomic analysis indicates that the MAGs classified as Fibrobacterales represent a termite-specific cluster among *Fibrobacteraceae* that comprises *Candidatus* Fibromonas termitidis and forms a sister group to the genus *Fibrobacter* (Fig. 7; Fig. S7). This is in agreement with a previous study that identified the same lineage (but classified as family *Fibromonadaceae*) by 16S rRNA gene-based and phylogenomic analyses (*Abdul Rahman et al., 2016*). None of the MAGs fell into the genus *Fibrobacter*, which was absent also in all 16S rRNA gene-based surveys of termite gut microbiota (*Hongoh et al., 2006*; *Mikaelyan et al., 2015b*; *Bourguignon et al., 2018*). Members of this genus have been isolated from the gastrointestinal tracts of mammals and bird herbivores (*Neumann, McCormick & Suen, 2017*), where they are potentially involved in cellulose and hemicellulose degradation (*Neumann & Suen, 2018*). This suggests co-evolutionary patterns among different Fibrobacteres clades within animal hosts with a lignocellulose-based diet.

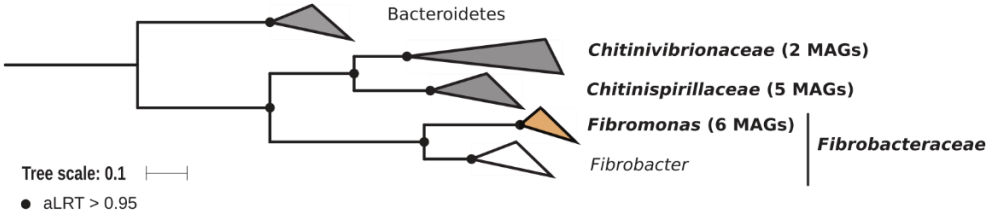

Figure 7 Phylogenomic tree of the Fibrobacteres phylum. This maximum-likelihood tree was inferred from a concatenated alignment of 43 proteins (6,516 positions) using the LG+G+I+F model of amino-acid evolution. Branch supports were calculated using a Chi2-based parametric approximate likelihood-ratio test. Names in bold included MAGs recovered in the present study. Clusters shaded in brown consist exclusively of genomes from termite guts and clusters shaded in gray contain genomes from termite guts. Bacteroidetes were used as outgroup. Full-size DOI: 10.7717/peerj.8614/fig-7

Proteobacteria and Bacteroidetes

Sixty-seven MAGs of Proteobacteria belonging to *Alphaproteobacteria* (23 MAGs), *Gammaproteobacteria* (20 MAGs), and *Deltaproteobacteria* (24 MAGs) were recovered from all hindgut compartments of litter-, humus-, and soil-feeding termites. Among the *Deltaproteobacteria*, seven orders were identified, namely *Desulfobacterales* (four MAGs, all assigned to *Desulfobulbus*), *Desulfovibrionales* (five MAGs, all *Desulfovibrionaceae*), *Desulfuromonadales* (one MAG), *Myxococcales* (five *Myxococcaceae* and four *Polyangiaceae*), *Adiutricales* (one MAG), *Syntrophobacterales* (one MAG), and MBNT15 group (two MAGs). *Desulfovibrionaceae* (*Desulfovibrionales*) members of gut and termite-gut clusters have been found to be highly prevalent in termite guts (*Bourguignon et al., 2018*). Similarly, we identified three *Desulfovibrionaceae* MAGs that form a monophyletic clade and two *Desulfovibrionaceae* MAGs that fall into a cluster of gut-associated genomes (Fig. S8). This family, among others, is composed of various sulfate-reducing bacteria; this functional group has already been identified in different termite species (*Kuhnigk et al., 1996*). Thus, these MAGs could provide new genomic resources to further investigate this metabolism in the termite gut.

Our dataset comprises 33 MAGs of Bacteroidetes (Fig. S9), including members of the families Cluster V (four MAGs), RC9 gut group (six MAGs), *Paludibacteraceae* (two MAGs, both assigned to the *Paludibacter* genus), *Rikenellaceae* (nine MAGs), *Marinilabiliaceae* (one MAG), and *Prolixibacteraceae* (one MAG). These Bacteroidetes were found in the P1, P3, and P4 compartments and in wood-, litter-, humus-, and soil-feeding termites. In Blattodea guts, different clusters of *Alistipes* (Bacteroidetes) have been found in a 16S rRNA gene survey (*Mikaelyan et al., 2015b*). Two MAGs from *L. labralis* belonging to the *Rikenellaceae* family and closely related to *Alistipes* have been identified. Additionally, among Bacteroidetes, four MAGs, all originating from P4 compartments, fall into the Cluster V family that contains symbionts of flagellates from guts of lower termites (*Hongoh et al., 2008b*; *Yuki et al., 2015*). We also recovered two MAGs assigned to *Paludibacter*; *Paludibacter propionicigenes* and *Paludibacter jiangxiensis* are both strictly anaerobic, propionate-producing bacteria isolated from rice paddy field (*Ueki et al., 2006*; *Qiu et al., 2014*). Propionate is produced by fermenting bacteria in the

gut of termites (*Odelson & Breznak, 1983*); these bacteria utilize glucose generated by cellulose degradation to form succinate and propionate (*Tokuda et al., 2014*). *P. propionicigenes* might be involved in nitrogen fixation, as *nifH* transcripts assigned to this species are the most abundant in the gut of the wood-feeding beetle *Odontotaenius disjunctus* (*Ceja-Navarro et al., 2014*).

Saccharibacteria, Synergistetes and Planctomycetes

Fifteen MAGs of *Candidatus* Saccharibacteria (also known as candidate division TM7) were reconstructed from the P1, P3, and P4 compartments of wood-, litter-, humus-, and soil-feeding termites (Fig. S10). Most of them originated from humus feeders (11 MAGs), especially from the P3 compartment (eight out of these 11 MAGs). Similarly, six MAGs of Synergistetes, all belonging to the *Synergistaceae* family that contains a termite/cockroach cluster (*Mikaelyan et al., 2015b*), were recovered from the P3 and P4 compartments of humus- and soil-feeding termites (Fig. S11). Both Saccharibacteria and Synergistetes were recently highlighted as numerically important clades of the termite gut microbiota, with some OTUs being present in the gut of the majority of 94 termite species collected across four continents (*Bourguignon et al., 2018*). They were also contributing to the core microbiota of higher termites (Fig. 3). Genomic analysis of these MAGs should help in understanding the roles of these bacteria in termite gut and also provide clues for designing successful isolation media to study their physiology.

Twelve MAGs were assigned to the phylum Planctomycetes, including four to the class *Phycisphaerae* (and among them two classified as *Tepidisphaerales* CPla-3 termite group), one to class vadinHA49 and seven to the class *Planctomycetia* (all classified as *Pirellulaceae*) (Fig. S12). These MAGs were recovered from the P3, P4, and P5 compartments and were restricted to humus- and soil-feeding termites. The recovery of Planctomycetes was expected, especially from the *Pirellulaceae* family, which also contains termite/cockroach clusters (*Mikaelyan et al., 2015b*). Interestingly, we found three MAGs from the P4 and P5 compartments of *C. ugandensis*, with one 16S rRNA gene sequence assigned to the Rs-B01 termite group, described in a previous study investigating the gut microbiota of the same termite species (*Köhler et al., 2008*). When such 16S rRNA gene information is available, it will allow the direct linkage between prokaryotic taxonomy and potential metabolisms.

Other phyla

Nine members of the phylum Elusimicrobia were identified, including members of the class *Endomicrobia* (eight members) and *Elusimicrobia* (one member) (Fig. S13). These were found in all hindgut compartments and were restricted to humus- and soil-feeding termites. Currently, only three complete genomes of Elusimicrobia from insect guts are available: *Elusimicrobium minutum* from the gut of a humivorous scarab beetle larva (*Herlemann et al., 2009*), and *Endomicrobium proavitum* (*Zheng & Brune, 2015*) and *Candidatus* Endomicrobium trichonymphae (*Hongoh et al., 2008a*) from the termite gut. Here, we provided nine additional genomes from the guts of humus- and soil-feeding termites.

The Chloroflexi phylum was represented by eight MAGs (all *Dehalococcoidia)*, including seven belonging to the family *Dehalococcoidaceae* and one to the family *Dehalobiaceae*, found exclusively in the P3 and P4 compartments of humus- and soil-feeding termites (Fig. S10). Their function in termite gut remains unclear, but Chloroflexi, including *Dehalococcoidia*, were found to be enriched in lignin-amended tropical forest soil (*DeAngelis et al., 2011*), where oxygen concentration and redox potential are highly variable, as in the termite gut (*Brune, 2014*). Therefore, their ability to use oxygen as final electron acceptor and their potential involvement in lignin degradation could be investigated by comparative genomics.

Minor phyla were also present in our dataset. Two MAGs assigned as Cloacimonetes (Fig. S14) and five MAGs assigned as Kiritimatiellaeota were recovered from the P3 compartment of the two humus-feeding termites *N. taracua* and *T. hospes* (Fig. S15). Kiritimatiellaeota have been reported to be present in the digestive tract of various animals (*Spring et al., 2016*). The few clones obtained from termite guts, which had been tentatively classified as uncultured Verrucomicrobia, were mostly obtained with planctomycete-specific primers (*Köhler et al., 2008*), underscoring the potential biases in amplicon-based studies toward certain taxa. Similarly, one MAG of Microgenomates (also known as candidate division OP11), which probably represents a lineage of Pacebacteria that was discovered only in a recent amplicon-based analysis but occurs in the majority of termites investigated (*Bourguignon et al., 2018*), was reconstructed from the P3 compartment of *T. hospes* (Fig. S10).

Finally, four MAGs classified as Acidobacteria were reconstructed from either the P3 or P4 compartments of humus- and soil-feeding termites (Fig. S16), which show a moderately alkaline or circumneutral pH in comparison to the highly alkaline P1. Of these four genomes, two were assigned to the M1PL1-36 termite group within the family *Holophagaceae* and one to the *Acidobacteriaceae* family. Acidobacteria can represent a significant fraction of the termite gut microbiota, especially in wood-feeding termites (*Hongoh et al., 2005*; *Wang et al., 2016*; *Bourguignon et al., 2018*). In the gut of higher termites, this phylum is present in the core microbiota (Fig. 3). Moreover, *Holophagaceae* and *Acidobacteriaceae* have been reported to be present in moderately acidic lignocellulosic substrates, such as peatland soil (*Schmidt et al., 2015*) and decaying wood (*Hervé et al., 2014*). Genomic analysis should help us in identifying the metabolic potential of these MAGs for lignocellulose degradation.

Phyla not represented by MAGs

Several bacterial phyla and one archaeal phylum containing prominent taxa that have been identified in previous 16S rRNA gene surveys of termite guts were not represented among the MAGs recovered in the present study. They include Cyanobacteria (class *Melainabacteria*; *Utami et al., 2018*), Lentisphaerae (*Köhler et al., 2012*; *Sabree & Moran, 2014*), Verrucomicrobia (*Wertz et al., 2012*), and Thaumarchaeota (*Friedrich et al., 2001*; *Shi et al., 2015*). Also intracellular symbionts of termite tissues, such as *Wolbachia* (Proteobacteria) (*Diouf et al., 2018b*) were not recovered. Possible reasons are a low relative abundance and/or a high phylogenetic diversity of the respective lineages.

Although larger metagenomes should improve the chances of their recovery in the medium- and high-quality bins, targeted single-cell based approaches have proven to be quite effective in recovering these genomes (*Ohkuma et al., 2015*; *Yuki et al., 2015*; *Utami et al., 2019*).

CONCLUSIONS

The 589 MAGs reported here represent the largest genomic resource for arthropod-associated microorganisms available to date. We recovered representatives of almost all major prokaryotic lineages previously identified in 16S rRNA gene amplicon-based surveys of the gut of higher termites from the metagenomes. This provides the foundations for studying the metabolism of the prokaryotic gut microbiota of higher termites, including the key members involved in carbon and nitrogen biogeochemical cycles, and important clues that may help in cultivating representatives of these understudied clades.

ACKNOWLEDGEMENTS

The authors thank all JGI staff, particularly their project manager Tijana Glavina del Rio, for their excellent service. The technical assistance of Katja Meuser is highly appreciated. We also thank the three anonymous reviewers and the editor for their helpful and constructive comments.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This study was funded by the Deutsche Forschungsgemeinschaft in the collaborative research center SFB 987 (Microbial Diversity in Environmental Signal Response) and by the Max-Planck-Gesellschaft. The work conducted by the U.S. Department of Energy Joint Genome Institute, a DOE Office of Science User Facility, is supported by the Office of Science of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231. Petr Stiblik and Jan Šobotník were supported by grant "EVA4.0," No. CZ.02.1.01/0.0/0.0/16_019/0000803 financed by OP RDE. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
Deutsche Forschungsgemeinschaft in the collaborative research center SFB 987 (Microbial Diversity in Environmental Signal Response).
Max-Planck-Gesellschaft.
U.S. Department of Energy Joint Genome Institute, a DOE Office of Science User Facility.
Office of Science of the U.S. Department of Energy: DE-AC02-05CH11231.
OP RDE "EVA4.0": CZ.02.1.01/0.0/0.0/16_019/0000803.

Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Vincent Hervé conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Pengfei Liu performed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Carsten Dietrich conceived and designed the experiments, performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- David Sillam-Dussès performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Petr Stiblik performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Jan Šobotník performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Andreas Brune conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

Field Study Permissions

The following information was supplied relating to field study approvals (i.e., approving body and any reference numbers):

Field experiments were approved by the French Ministry for the Ecological and Solidarity Transition (UID: ABSCH-CNA-FR-240495-2) (TREL1902817S/118).

Data Availability

The following information was supplied regarding data availability:

The data are available at BioProject: PRJNA560329. Genomes are available at: SRR9983610–SRR9984198. The MAGs are available at the NCBI's Assembly Database: WQRH00000000–WRNX00000000 (Table S2).

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.8614#supplemental-information.

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4. Termites host specific fungal communities that differ from those in their ambient environments

Fungal Ecology 48 (2020) 100991

Contents lists available at ScienceDirect

Fungal Ecology

journal homepage: www.elsevier.com/locate/funeco

Methodological Advances

Termites host specific fungal communities that differ from those in their ambient environments

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ARTICLE INFO

Article history:
Received 20 November 2019
Received in revised form
12 August 2020
Accepted 17 August 2020
Available online xxx

Corresponding Editor: Peter Biedermann

Keywords:
Mycobiome
Symbiosis
Coptotermes
Heterotermes
Nasutitermes
Yeast
Moulds

ABSTRACT

Termites are important plant biomass decomposers. Their digestive activity typically relies on prokaryotes and protozoa present in their guts. In some cases, such as in fungus-growing termites, digestion also relies on ectosymbiosis with specific fungal taxa. To date, the mycobiome of termites has yet to be investigated in detail. We evaluated the specificity of whole-termite associated fungal communities in three wood-feeding termite species. We showed that the whole-termite fungal community spectra are stable over diverse environments, regardless of the host species, and differ markedly from the wood in which they nest. The core mycobiome is similar to that found in other ecologically related insects and consists of a narrow spectrum of common filamentous fungi and yeasts, known for their stress tolerance and their ability to decompose plant biomass. The observed patterns suggest that a number of fungal strains may have a symbiotic relationship with termites, and our results set the stage for future investigations into the interactions between fungi, termites, and their other gut microbiota.

1. Introduction

Dead plant materials are mostly made of lignocellulose, the most common polymer on Earth, which relatively few metazoan taxa are able to significantly decompose (Lo et al., 2003). All key taxa consuming dead plant tissues, such as ruminants, earthworms and insects, largely rely upon a rich microbial consortium, which possess the necessary metabolic pathways for lignocellulose decomposition (Watanabe and Tokuda, 2010; Brune and Dietrich, 2015). Termites process this cellulose far more efficiently than other decomposers (Brune, 2014) and their dominance in tropical ecosystems makes them key players at a global scale (Bignell and Eggleton, 2000; Bar-On et al., 2018; Griffiths et al., 2019). While early branching termite lineages ("lower" termites) feed exclusively on wood or grasses, the "higher" termites (i.e. the crown family Termitidae) consume a variety of plant materials irrespective of decomposition status, and a majority of these taxa are soil-feeders (Jouquet et al., 2006; Krishna et al., 2013). To digest cellulose, termites rely on their own endogenous cellulases (Watanabe et al., 1998), in combination with microbial cellulases in their guts. Lower termites depend primarily on flagellate protozoa, with some contribution by prokaryotes, while "higher" termites lack cellulolytic flagellates completely and depend on bacteria and archaea for

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https://doi.org/10.1016/j.funeco.2020.100991
1754-5048/

cellulose decomposition (Brune and Ohkuma, 2011; Hongoh, 2011; Brune and Deitrich, 2015).

In herbivorous or detritovorous insects, both prokaryotes and fungi are generally thought to form core taxa of the gut microbiome, despite the fact that most studies have considered prokaryotes only (Gurung et al., 2019; Ravenscraft et al., 2019). Both types of microorganism can act as nutritional symbionts assisting with digestion, detoxification and essential nutrients synthesis, or as protective symbionts (Dillon and Dillon, 2004; Gurung et al., 2019). Other roles, such as the effect on the host cells physiology and interactions with other microbes can be expected, as is known in mammals (Lai et al., 2018). The core gut mycobiome of wood feeding insects covers a relatively narrow set of ubiquitous yeasts and filamentous fungi such as *Candida*, *Mucor*, *Aspergillus*, *Penicillium*, *Alternaria* or *Trichoderma* (Pérez et al., 2003; Rojas-Jiménez and Hernández, 2015; Ziganshina et al., 2018) and the same taxon spectrum is reported not only in other insects (Moraes et al., 2001; Fredensborg et al., 2020), but also in mammals (Lai et al., 2018).

Associations between termites and fungi have so far been considered in two categories: firstly, interactions that affect the discovery and consumption of food or its nutrient value, but which fall short of mutualism; secondly, the cultivation of fungus-combs (*Termitomyces* spp.) by fungus farming Macrotermitinae (Lenz et al., 1991; Rouland-Lefèvre, 2000). However, apart from a few studies reporting common yeasts and filamentous fungi found in termite guts (Prillinger et al., 1996; Prillinger and König, 2006), the fungi associated with termites are yet to be systematically investigated. The only studies which have compared the microbiota of termite guts and termite ambient environments showed that fungal assemblages of guts differ markedly from nest walls or food nodules in litter and humus feeding termites (Menezes et al., 2018; Moreira et al., 2018). Based on the current knowledge of insect microbiomes, we hypothesized that fungi, which are a neglected part of the termite gut microbiome, form predictable communities and have stable interactions with their hosts.

We compared the specificity of body associated fungal communities (i.e. fungi in gut and on exoskeleton) in three ecologically similar species, *Heterotermes tenuis*, *Coptotermes testaceus* (both lower termites, Rhinotermitidae) and *Nasutitermes octopilis* (higher termites, Termitidae: Nasutitermitinae), which can be simultaneously collected from the same large wood item. We examined the mycobiomes of whole termite bodies as a proxy for termite gut mycobiomes, which enabled us to analyse large sample sizes, necessary for statistical testing. We hypothesized that fungal communities are similar in termites with a similar diet, and more alike in the genera *Heterotermes* and *Coptotermes* compared to *Nasutitermes*, as *Coptotermes* is nested within the genus *Heterotermes* (Bourguignon et al., 2016; Buček et al., 2019). We examined fungal communities using high-throughput sequencing of ITS2 metabarcodes of termite bodies, their food source (narrow termite galleries), and intact control wood near to areas where termites were feeding. The patterns described below are based on repeated samples from the same log, usually of multiple species from the same trunk, which allowed us to test for termite species and colony-level specificity of the associations.

2. Material and methods

2.1. Study site and sampling

The samples were collected in November 2014 in Nouragues Nature reserve (French Guiana; N 04°05′, W 52°41′). Large wood items were inspected for the presence of two "lower" termite species, *Coptotermes testaceus* (Rhinotermitidae) with a preference for sound white wood, *Heterotermes tenuis* (Rhinotermitidae) preferring red-rot wood, and one "higher" termite species, *Nasutitermes octopilis* (Termitidae: Nasutitermitinae), having no clear specialisation for the wood-decomposition degree.

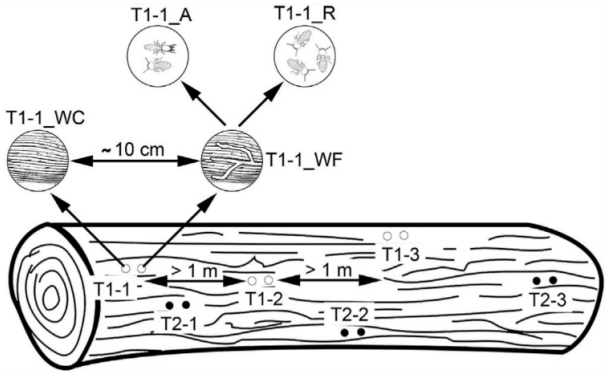

Fig. 1. Sampling scheme. Termites were collected in foraging galleries, and workers and soldiers were used for voucher sample in 80% ethanol (_A), while 10 workers for RNAlater sample (_R). Samples of foraging galleries (_WF) and control wood (_WC; roughly 10 cm from the closest termite gallery) were also stored in RNAlater. Up to three sample sets were collected from the same log, with a distance at least 1 m from each other. If more than a single focal termite (T1 and T2) was found in the same log, both were treated independently.

A single sample set comprised of three samples: (1) 10 workers from a single foraging party (2) their feeding substrate (approx. 1 cm^3 piece of wood containing gallery), and (3) the control sample (approx. 1 cm^3 of wood roughly 10 cm away from the closest termite gallery) (Fig. 1). Two or three sample sets, collected 1 m away from each other, were taken from the single wood log. Visually healthy workers were collected and narrow termite galleries with minimal amounts of frass were selected. Samples were firstly stored in RNAlater® solution at −20 °C within 12 h following collection, and shipped to Prague where they were stored at −80 °C until DNA extraction. In total, 82 samples sets (*Coptotermes*: n = 28, *Heterotermes*: n = 31, *Nasutitermes*: n = 23) originated from 23 trunks were studied. Storage in RNA later® solution caused hardening of termite bodies preventing gut dissection. Thus, as extraction of the intact intestine was impossible, we used whole termite bodies as a proxy for the study of intestinal microbiota.

2.2. DNA extraction and PCR amplification

Total DNA was extracted using Macherey-Nagel NucleoSpin® Soil kit with following modifications. Each termite sample was homogenized together in 500 μL of SL1 Lysis buffer, 100 μL of SX enhancer buffer and two sterilized steel beads (3 mm diameter) using a Mixer Mill MM 400 for 2 min, set on 30 Hz. Sample lysis by using a vortex was shortened to 2 min. The wood samples were mechanically crushed to small pieces, placed in a 2 mL tube with five steel beads, frozen in liquid nitrogen for 1 min and ground in a Mixer Mill Retsch MM 400 for 10 min at 30 Hz; 550 μL of SL2 extraction buffer was added to the homogenized material and the grinding was repeated once more. Sample lysis was extended to 10 min.

PCR amplification of the fungal ITS2 region from DNA was performed using gITS7 (5'-GTGARTCATCGARTCTTTG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Ihrmark et al., 2012; Tedersoo et al., 2015), each of them was barcoded in three PCR reactions per sample. The PCR reactions contained 2.5 μL of 10 × buffer for DyNAzyme II DNA Polymerase, 0.75 μL of bovine serum albumin (20 mg/mL), 1 μL of each primer (0.01 mM), 0.5 μL of PCR Nucleotide Mix (10 mM each), 0.75 μL of polymerase (2 U/μL DyNAzyme II DNA polymerase), and 1 μL of template DNA.

PCR was performed by using an Eppendorf Mastercycler® (Eppendorf AG, Hamburg, Germany) nexus cycler. The PCR cycling parameters were 94 °C—5 min (1 cycle), 94 °C—45 s, 56 °C—35 s, 72 °C—30 s (40 cycles), final extension at 72 °C—10 min. PCR triplicates were combined and purified using MinElute PCR Purification Kit (Qiagen GmbH, Hilden, Germany) according to the manual provided and eluted in 20 μL. Paired-ends amplicon reads were sequenced on Illumina MiSeq sequencer (Illumina Inc., USA) using V2 chemistry producing 2 × 250 bp output.

2.3. Data processing

Raw fungal ITS paired-end sequences were joined using fastq-join software (Aronesty, 2011) and demultiplexed, filtered and trimmed using the pipeline SEED 2 (version 2.1.05) (Větrovský et al., 2018). Low-quality sequences (mean Phred quality score < 30) and all sequences with mismatches in barcodes were removed from the dataset. After the quality filtering, all fungal sequences were extracted from the joined sequences using ITSx (v 1.0.11) (Bengtsson-Palme et al., 2013) to acquire complete ITS2 region. All ITS2 sequences shorter than 40 bp were discarded, yielding a dataset of 3 967 992 fungal ITS2 sequences (length distribution 40—395 bp, avg. 175 bp). The dataset was clustered into operational taxonomic units (OTUs) using UPARSE implementation in USEARCH version 8.1.1861 (Edgar, 2013) with 97% similarity threshold (109 476 fungal chimeric sequences were excluded during this step). A total of 10 742 fungal OTUs (without singletons) were obtained during the clustering step. To reduce the influence of contaminations and minimize the effects of barcode hopping all OTUs with up to 4 reads were discarded, which resulted in 2857 OTUs used for further analysis.

The most abundant sequence from each cluster was used as a representative sequence for taxonomic classification. Fungal sequences were classified based on BLAST best hit against the UNITE database, version 7.2 (Koljalg et al., 2013). The functional guild of each fungal OTU was assigned based on the FUNGuild database (Nguyen et al., 2016). For alpha diversity estimation, all fungal samples were resampled to 909 sequences. Diversity indices were estimated using SEED 2 version 2.1.05. The abundances of sequence reads were plotted on the phylogenetic tree constructed using NCBI molecular data via phyloT (Letunic, 2015) and the iTOL visualisation tool (Letunic and Bork, 2019). Data were deposited in the MG-RAST database under accession number mgp91984 and in NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA639228. Processed data (extracted ITS2 reads) were deposited in the GlobalFungi Database (https://globalfungi.com, Větrovský et al., 2020).

2.4. Statistical analysis

To test the null hypothesis of no difference between termite body, gallery and wood (control) fungal community composition, PERMANOVA analysis (Anderson, 2001) was performed with *adonis()* function of *vegan* package (Oksanen et al., 2018) in R (R Core Team, 2018). Euclidean distance on Hellinger-transformed fungal composition (i.e. Hellinger distance matrix) (Legendre and Gallagher, 2001) was used as response matrix, and sample type (body, gallery or wood) was used as fixed explanatory variable. Since observations were paired within triplets (the three sample types were sampled in each triplet), which, in turn, were nested in logs, the permutations were constrained to occur within triplets, using the variable triplet as blocking factor (or strata). To visualize the results, non-metric multidimensional scaling (NMDS) was performed in two different ways. In the first way, raw community data was ordinated by their fungal composition. This NMDS plot shows all the variability in the dataset. In the second way, community data was first regressed against triplet and log effects (i.e. the effect of spatial variability due to the experimental design was removed from the data) and, then, the residualized distance matrix was ordinated using NMDS as suggested by Anderson et al. (2017). This plot shows the variability in the dataset, once the effect of triplet and log has been taken into account.

To test the null hypothesis of no effect of sample type and termite species on fungal diversity (measured with Chao 1 index, Shannon-Wiener diversity index, and Pielou's evenness), linear mixed effect models were fitted using the function *lme()* from the R package *nlme* (Pinheiro et al., 2018). The interaction between termite species and sample type was fitted as the fixed part of the model, and, a random structure of the form ~1|triplet/log was included in each model to account for the fact that measurements were grouped in triplets, which, in turn, were nested in logs. Tukey *post-hoc* tests were performed using the function *lsmeans()* of the R package *lsmeans* (Lenth, 2016).

To identify the fungal OTUs contributing to the separation between termite bodies, galleries and wood, partial redundancy analysis (partial RDA) was used (Legendre and Legendre, 2012) for each termite species separately. Separating the communities by termite species allowed checking whether similar OTUs contribute to the separation between sample types in the three termite species. In each RDA, Hellinger-transformed fungal OTU composition was used as response matrix, sample type was used as fixed explanatory factor, and the analysis was conditioned with the effect of the log and triplet. 1% of the OTUs with highest loadings to the ordination axes RDA1 and RDA2 in the three partial RDAs were depicted in triplots (Legendre and Legendre, 2012).

Lastly, to test and quantify the effect of termite species and log identity on fungal mycobiome composition, variation partitioning was performed based on RDA (Legendre and Legendre, 2012). Variation in Hellinger-transformed fungal OTU composition of termite bodies and galleries was partitioned in the effect of termite species and log identity. Since the number of body and gallery samples per species was not equal, *Coptotermes* and *Heterotermes* were randomly subsampled to balance the design, which makes the hypothesis testing more robust to the presence of heterogeneous group dispersions (Anderson and Walsh, 2013). The partial effect of each fraction (i.e. the effect of a fraction —e.g. species— once the effect of the other fraction —e.g. log identity— has been taken into account) was tested using a permutation test in partial RDA results.

3. Results

3.1. Fungal diversity

The diversity of fungal OTUs was significantly higher in termite bodies of all three species than in their galleries and intact wood, and was also significantly different between termite species. The estimated number of OTUs (Chao-1 estimate) in termite body samples, counted from the resampled dataset, ranged from 26 to 221 with an average 92—101 per species. Estimated OTU numbers and diversity indices were at least two times lower in termite galleries and in control wood. The fungal communities from termite bodies were significantly more even than termite galleries and control wood samples (Fig. 2).

3.2. Fungal community composition

The wood control and galleries were dominated by Basidiomycota followed by Ascomycota while there was an obvious shift to the dominance of Ascomycota over Basidiomycota in the termite bodies, with the addition of Mucoromycotina and Chytridiomycota

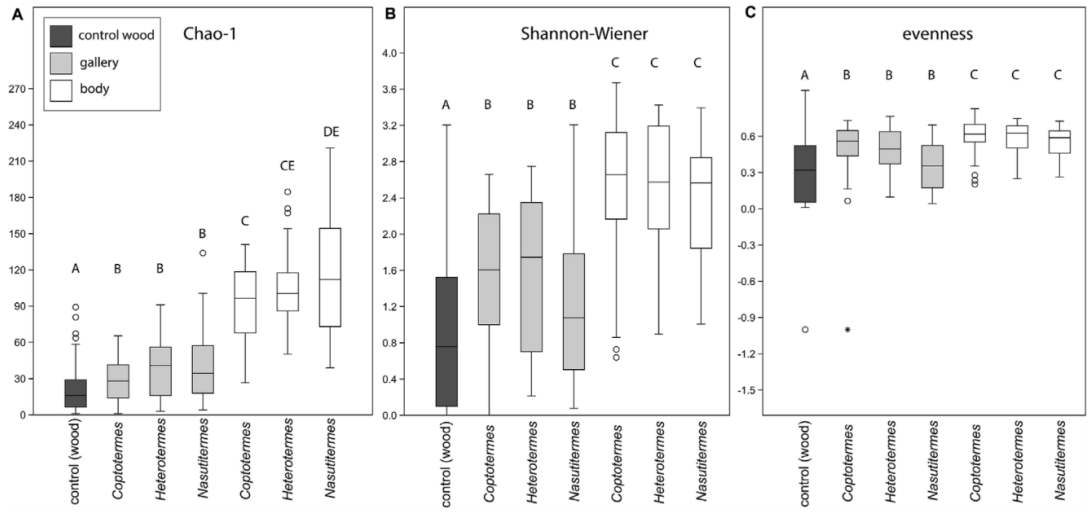

Fig. 2. Alpha diversity indices: Chao-1 index **(A)**, Shannon-Wiener index **(B)** and evenness **(C)** calculated from the fungal OTUs found in termite bodies, their galleries and insect-free wood controls. Data from *Coptotermes testaceus*, *Heterotermes tenuis*, *Nasutitermes octopilis* are shown. Groups sharing a letter are not significantly different (Tukey HSD *post-hoc* tests, $p < 0.05$).

members (Fig. 3A). A significant diversity of fungal taxa unidentified at the phylum level was recovered for all three treatments. At the finer taxonomic scale, 25 fungal orders were most abundant (Figs. 3B and 4). Among the most abundant orders, Mucoromycotina GS23 (artificial group, see Fig. 3 for definition), Eurotiales, Hypocreales, Ophiostomatales and Saccharomycetales were typically associated with termite bodies, whereas Chaetosphaeriales, Auriculariales and partially also Corticiales were associated with wood and galleries. Wood was also marked by the high abundance of Polyporales (Figs. 3 and 4, Supplementary Table 1). Finally, the members of the order Hymenochaetales were abundant in all variants. The majority of the fungal taxa identified to the ecological guild were predicted to be saprotrophs, a combination of mixed trophic modes (mostly saprotrophs and pathotrophs) and pathotrophs. Saprotrophs and pathotrophs were more abundant in termite bodies (frequency of reads in saprotrophs - 39-23%; pathotrophs - 5–11%) than in galleries and wood (22-11%; 0.1–3%). Those taxa belonged mostly to plant pathogens, with the small fraction of insect pathogenic fungi (0.05–0.25%) dominated by *Metarhizium* spp. and *Lecanicillium* spp. (Supplementary Table 2).

Multivariate analysis of the raw OTU dataset did not clearly separate samples by their types, but showed that the intestinal mycobiota of all three termite species is rather homogeneous and similar, in comparison to the very heterogeneous communities colonizing their galleries and wood controls ($k = 3$ dimensions, final stress = 0.24, Fig. 5A). By contrast, once the spatial variability due to the experimental design (i.e. the effect of log and triplet identity) is removed ($k = 3$ dimensions, final stress = 0.25, Fig. 5B), body samples clearly separate from galleries and controls. The NMDS revealed a high stress value indicating that 2D graphical

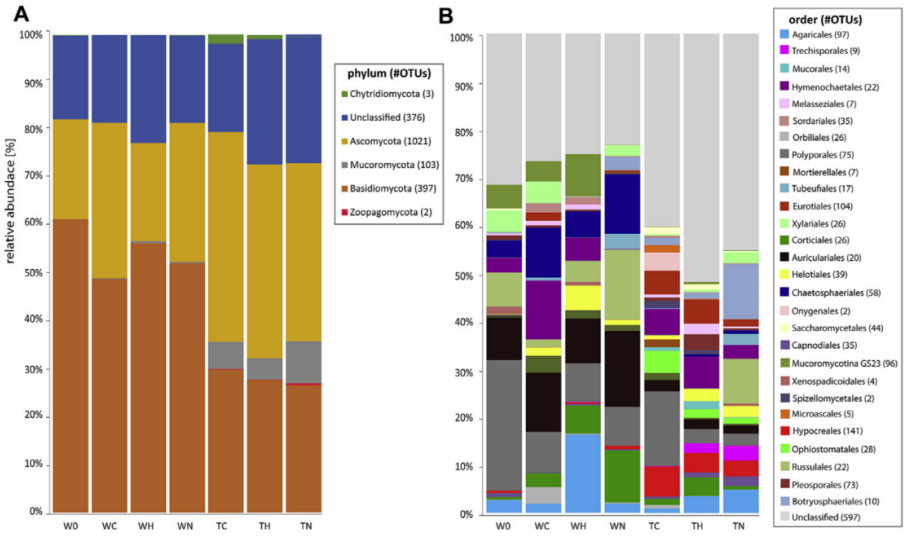

Fig. 3. Relative abundance of sequence reads classified at the phylum **(A)** and order **(B)** level. Only the orders with higher relative abundance (≥1% of reads in at least one sample type) are shown in Fig. 3B. Data from the wood control (W0), bodies (T) and galleries (W) of the termites *Coptotermes testaceus* (C), *Heterotermes tenuis* (H), and *Nasutitermes octopilis* (N) are shown. Artificial order Mucoromycotina GS23 was created for OTU285 (see Table 1) and OTUs withsimilarity ≥95%.

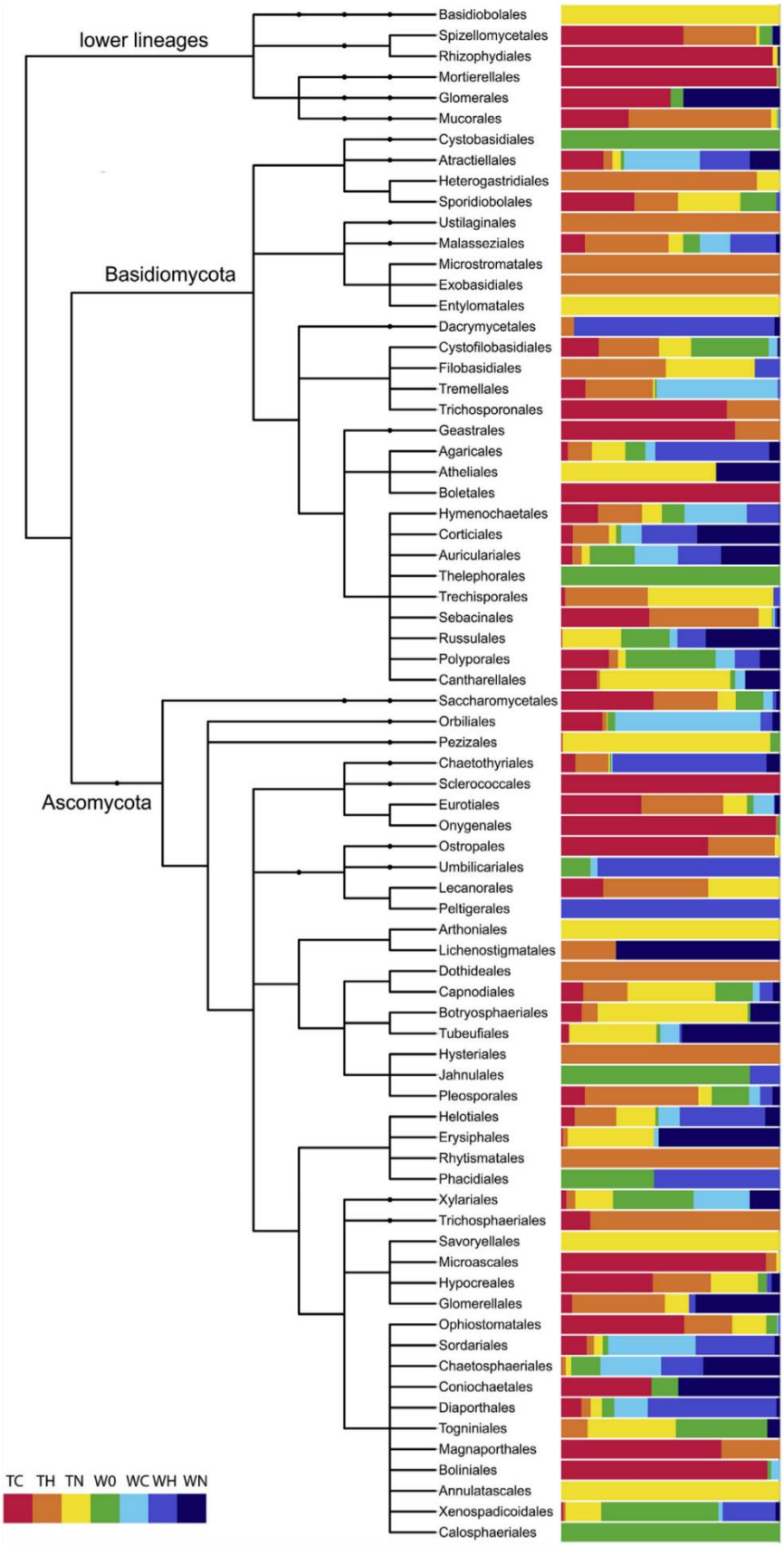

Fig. 4. Relative abundance of sequence reads classified at the order level. Data from the wood control (W0), bodies (T) and galleries (W) of the termites *Coptotermes testaceus* (C), *Heterotermes tenuis* (H), and *Nasutitermes octopilis* (N) are shown. Abundant orders, which reached at least 1% abundance in one fungal community were selected for presentation.

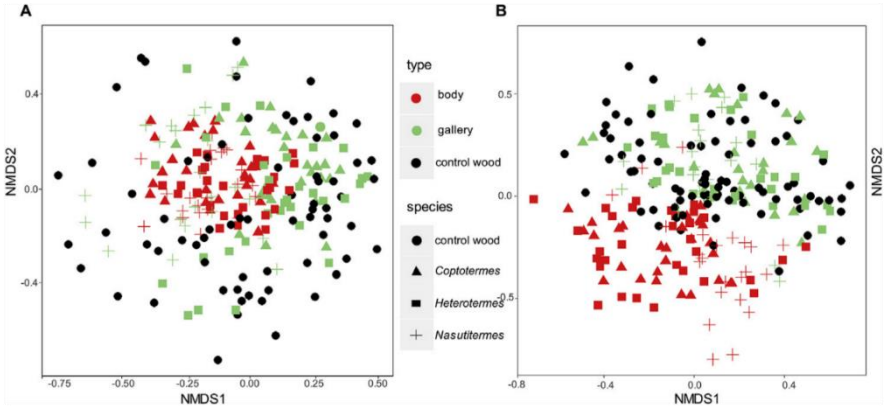

Fig. 5. Non-metric multidimensional scaling (NMDS) ordination of the sampling units by their fungal OTU (≥97% identity) composition based on ITS2 rRNA gene metabarcoding. **(A)** Ordination of raw fungal OTU composition (k = 3 dimensions, final stress = 0.24); **(B)** ordination of residualized fungal OTU composition (i.e. the effect of log and triplet identity removed; k = 3 dimensions, final stress = 0.25). Sample type significantly affects fungal community composition (PERMANOVA: permutations = 999, *P*-value = 0.001, R^2 = 0.044).

representation only roughly corresponds with the underlying data. However, the observed patterns were confirmed by the PERMANOVA analysis which showed that fungal communities from termite bodies were significantly different from galleries and controls (permutations = 999, *P*-value = 0.001).

Constrained RDA analysis with the removed effect of the sampling design revealed a clear separation of samples based on their type in all three termite species. The first axis of RDA (RDA1) separates termite bodies from galleries and controls, whereas the second axis (RDA2) separates galleries from controls (Fig. 6). As opposed to the unconstrained ordination (NMDS, Fig. 5), the constrained ordination (Fig. 6) distinguishes between the fungal compositions of galleries and controls. OTU 12, 20 and 34 are consistently positively associated with termite bodies in all three species. A further 13 OTUs are associated with two termite species (Table 1). The fungal genera linked with termite bodies (i.e. with high negative RDA1 axis loadings, Fig. 6), in all three termite species includes a narrow spectrum of filamentous ascomycetes (*Trichoderma*, *Penicillium, Scytalidium*, *Hawksworthiomyces, Lasiodiplodia*), a few basidiomycete genera (*Malassezia, Phlebia*, *Hyphodontia*, *Corticium, Wrightoporia* etc.), a single but abundant taxon from Mucoromycotina and a chytrid species from the genus *Spizellomyces* specifically associated with *Coptotermes* and *Heterotermes* (Table 1, Supplementary Table 3). Fungal genera linked with galleries include mostly wood saprobes from Basidiomycota (*Resinicium*, *Hyphodontia*, and unidentified genera), the very abundant genus *Chaetosphaeria*, and other wood inhabiting ascomycetes (*Pseudolachnella*, *Orbilia*, *Calonectria*, etc.). Genera linked with wood were *Auricularia*, *Porotheleum* and numerous, mostly unidentified, genera of Polyporales, Auriculariales and Agaricomycetes but also various wood roting ascomycota (*Hypoxylon*, *Kretzschmaria*, *Camarops, Cordana, Chaetosphaeria*) (Fig. 6, Supplementary Table 3).

The fungal community composition of termite bodies and galleries was significantly affected by both termite species and log identity. Total explained variation in gallery mycobiomes (0.118) was more than the half (0.261) of the explained variation in body mycobiomes. Accordingly, the variations explained by only termite species, only log and the shared fraction (i.e. the fraction that cannot be clearly attributed to either species or log) were more than double in termite bodies compared with galleries (Fig. 7).

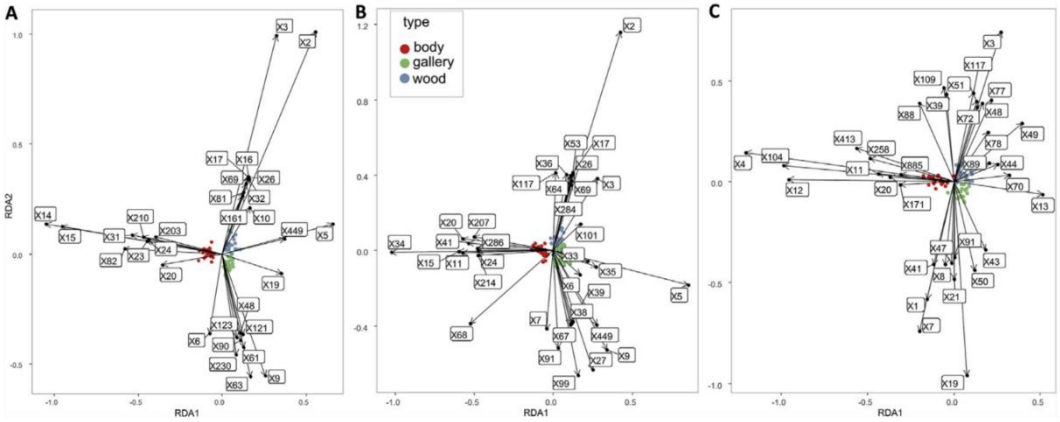

Fig. 6. Partial RDA triplots showing the partial effect (i.e. once the effect of log and triplet identity has been taken into account) of sample type on fungal OTU composition for **(A)** *Coptotermes*, **(B)** *Heterotermes* and **(C)** *Nasutitermes*. Sample type significantly affects fungal community for *Coptotermes* (Permutation test of RDA: permutations = 999, P-value = 0.001), *Heterotermes* (permutations = 999, *P*-value = 0.001) and *Nasutitermes* (permutations = 999, *P*-value = 0.001). One percent of OTUs (labelled as X14 etc.) with the highest fit to RDA1 or RDA2 are depicted (see Table 1 for further details).

Table 1
Fungal OTUs specifically associated with termite bodies. First 30 OTUs with the highest fit to RDA1 axis (see Fig. 6) are presented for each termite species. Taxonomic identity of OTUs is based on the Blast similarity search against NCBI GenBank and UNITE database. Abbreviations: A – Ascomycota, B – Basidiomycota, C – Chytridiomycota, M – Mucoromycotina.

Coptotermes testaceus					*Heterotermes tenuis*					*Nasutitermes octopilis*				
OTU #	RDA axis loadings		Best Hit description		OTU #	RDA axis loadings		Best Hit description		OTU #	RDA axis loadings		Best Hit description	
	RDA1	RDA2	Similarity/Coverage [%]	Taxon		RDA1	RDA2	Similarity/Coverage [%]	Taxon		RDA1	RDA2	Similarity/Coverage [%]	Taxon
14	−1.05	0.14	94.8/100 (KJ654590)	*Phlebia* sp. (B)	34	−1.02	−0.01		unidentified	4	−1.20	0.14	88.1/93.5 (HM162185)	*Podospora* sp. (A)
15	−0.95	0.12		unidentified	15	−0.60	−0.01		unidentified	104	−0.98	0.08	87.1/100 (AB846969)	GS23 sp. (M)[b]
82	−0.58	0.02	99.5/98.5 (HM771021)	*Hawksworthiomyces* sp. (A)	20	−0.58	0.06	91.2/89.5 (AY762623)	*Scytalidium lignicola* (A)	12	−0.96	0.01	100/100 (GU945354)	*Lasiodiplodia citricola* (A)
31	−0.54	0.09	100/100 (AY857228)	*Trichoderma harzianum* (A)	11	−0.57	−0.01	90.4/96.7 (KU214528)	*Arthrographis* sp. (A)	413	−0.56	0.17	97/100 (GU054100)	*Wrightoporia tropicalis* (B)
210	−0.47	0.08	98.3/100	*Spizellomyces punctatus* (C)[a]	41	−0.54	0.04	90.4/76	Auriculariales sp. (B)	258	−0.48	0.12		unidentified
23	−0.45	0.06	(AY997092)	unidentified	68	−0.53	−0.39	(UDB014156)	unidentified	11	−0.44	0.04	90.4/96.7 (KU214528)	*Arthrographis* sp. (A)
24	−0.44	0.06	91.3/44 (UDB028178)	*Hyphodontia pilaecystidiata* (B)	207	−0.50	0.07	99.6/100 (AY743636)	*Malassezia restricta* (B)	20	−0.37	0.02	91.2/89.5 (AY762623)	*Scytalidium lignicola* (A)
203	−0.40	0.08	100/100 (AY154939)	*Trichoderma spirale* (A)	286	−0.48	0.01	92/98 (GQ272617)	*Scytalidium ganodermophthorum* (A)	171	−0.31	−0.02	100/100 (EU401550)	*Trichoderma orientale* (A)
20	−0.35	−0.05	91.2/89.5 (AY762623)	*Scytalidium lignicola* (A)	24	−0.48	0.00	91.3/44 (UDB028178)	*Hyphodontia pilaecystidiata* (B)	885	−0.30	0.04	84.4/100 (AB846969)	GS23 sp. (M)[b]
285	−0.35	0.05	92.9/93.8 (AB846959)	GS23 sp. (M)[b]	214	−0.48	−0.03	87.8/100 (AB846969)	GS23 sp. (M)[b]	183	−0.30	−0.01	90.5/81 (AB846975)	GS23 sp. (M)[b]
286	−0.34	0.04	92/98 (GQ272617)	*Scytalidium ganodermophthorum* (A)	31	−0.45	−0.03	100/100 (AY857228)	*Trichoderma harzianum* (A)	121	−0.27	0.17		unidentified
22	−0.32	−0.17	87.6/72.9 (FJ231021)	*Penicillium curticaule* (A)	210	−0.44	0.12	98.3/100 (AY997092)	*Spizellomyces punctatus* (C)[a]	179	−0.27	0.04	94.8/100 (KT951335)	*Agaricus candidolutescens* (B)
197	−0.32	0.05	99.4/100 (KJ174211)	*Trichoderma koningiopsis* (A)	149	−0.42	−0.01	89.8/90.3 (AB846969)	GS23 sp. (M)[b]	256	−0.26	0.02	86.3/56.7 (KM103946)	Fungi sp. (unidentified)
41	−0.32	0.12	90.4/76 (UDB014156)	Auriculariales sp. (B)	145	−0.34	−0.03		unidentified	118	−0.26	−0.01	88.3/95.7 (UDB013022)	Sordariales sp. (A)
12	−0.32	0.06	100/100 (GU945354)	*Lasiodiplodia citricola* (A)	22	−0.32	0.03	87.6/72.9 (FJ231021)	*Penicillium curticaule* (A)	59	−0.25	0.04		unidentified
154	−0.31	0.02	90.7/100 (GQ272617)	*Scytalidium ganodermophthorum* (A)	629	−0.31	0.00	98.6/100 (KU164491)	*Malassezia restricta* (B)	156	−0.24	−0.07	100/19.5 (GQ280589)	*Calonectria leguminum* (A)
38	−0.30	0.13		unidentified	85	−0.30	−0.07	100/100 (HM770996)	Helotiales sp. (A)	1923	−0.24	−0.05	100/98.8 (EU280098)	*Trichoderma citrinoviride* (A)
28	−0.30	−0.02	86.9/100 (JX857794)	*Corticium* sp. (B)	203	−0.29	0.06	100/100 (AY154939)	*Trichoderma spirale* (A)	6	−0.24	0.01	86/47.6 (DQ826552)	*Resinicium monticola* (B)
34	−0.30	0.04		unidentified	248	−0.28	0.04	100/100	*Boeremia exigua* (A)	32	−0.24	−0.01		unidentified
158	−0.28	0.00	89.2/97.4 (GQ272617)	*Scytalidium ganodermophthorum* (A)	202	−0.27	0.00	(GU237707)	unidentified	229	−0.23	0.03	99.6/100 (UDB014090)	Trechisporales sp. (B)
257	−0.27	−0.02	88.6/100 (KU295549)	GS23 sp. (M)[b]	257	−0.26	−0.02	88.6/100 (KU295549)	GS23 sp. (M)[b]	34	−0.22	0.02		unidentified
152	−0.26	0.03		unidentified	259	−0.26	0.00	100/98.1 (JN626104)	*Penicillium mallochii* (A)	25	−0.22	−0.04	85.6/96.1	*Cordana terrestris* (A)
94	−0.26	−0.22		unidentified	302	−0.26	−0.01	99.5/100	*Verticillium leptobactrum* (A)	172	−0.21	0.03	(HF677173)	unidentified
133	−0.25	0.03	92.9/90.4 (EF127890)	*Hawksworthiomyces lignivorus* (A)	223	−0.25	0.00	(KF472157)	unidentified	199	−0.21	0.04	86/92.6 (KU975068)	*Pseudoproboscispora* sp. (A)

(*continued on next page*)

Table 1 (*continued*)

Coptotermes testaceus					*Heterotermes tenuis*					*Nasutitermes octopilis*				
OTU #	RDA axis loadings		Best Hit description		OTU #	RDA axis loadings		Best Hit description		OTU #	RDA axis loadings		Best Hit description	
	RDA1	RDA2	Similarity/Coverage [%]	Taxon		RDA1	RDA2	Similarity/Coverage [%]	Taxon		RDA1	RDA2	Similarity/Coverage [%]	Taxon
267	−0.25	0.00	94.3/98.1 (AF033470)	*Penicillium sclerotigenum* (A)	946	−0.25	−0.01	93.7/100 (AB846969)	GS23 sp. (M)[b]	82	−0.21	0.07	99.5/98.5 (HM771021)	*Hawksworthiomyces* sp. (A)
30	−0.24	0.03	90.9/43.4 (JX675137)	*Gymnoascus* sp. (A)	12	−0.25	−0.03	100/100 (GU945354)	*Lasiodiplodia citricola* (A)	7	−0.20	−0.74	99/100 (KT224922)	unidentified
509	−0.24	0.03	99.4/100 (DQ109528)	*Trichoderma lieckfeldtiae* (A)	83	−0.25	−0.01	99.3/100 (GQ272617)	*Scytalidium ganodermophthorum* (A)	88	−0.20	0.39	100/100 (HM148090)	*Cladosporium exasperatum* (A)
402	−0.23	0.03	91.1/93.2 (AB846969)	GS23 sp. (M)[b]	122	−0.24	−0.06	99.3/100 (AY273308)	Ascomycota sp. (A)	299	−0.18	0.04	100/19.4 (GQ280589)	*Calonectria leguminum* (A)
399	−0.22	−0.04	90.8/62.5 (AB846970)	GS23 sp. (M)[b]	14	−0.23	0.00	94.8/100 (KJ654590)	*Phlebia* sp. (B)	425	−0.18	0.04	86.6/79.2 (KY687694)	GS23 sp. (M)[b]
207	−0.22	0.05	99.6/100 (AY743636)	*Malassezia restricta* (B)	234	−0.23	0.03	99.3/100 (FR682163)	*Malassezia* sp. (B)	190	−0.18	0.04	88.7/100 (JX545187)	GS23 sp. (M)[b]

[a] — 98% sequence similarity with the type of *Spizellomyces punctatus* NR_111 189.
[b] — no reliable ITS2 sequence similarity to named taxa. The best hits (≤90%) are various *Mucoromycotina* spp. (e.g. HQ406814, LC189046).

4. Discussion

4.1. Termite associated mycobiome

Fungal communities of galleries and intact wood overlapped and differed from termite communities, which also overlapped with each other (Figs. 3–6). The termite mycobiota is likely to be a mix of fungi present on insect cuticular tissues (mostly from the mouthparts and pathogenic fungi present on the body surface), fungi present in the gut, and possibly fungi present internally in other organs or in the haemolymph. In our study, the fraction of insect pathogenic fungi was higher in termites than in their galleries, but their overall abundance was very low and did not contribute to the separation of the studied sample types (Table 1, Supplementary Table 2). Intestinal fungi appear to dominate the termite mycobiome. Whether fungi occur in other internal organs (i.e. haemolymph, gonad rudiments) is unclear. The presence of fungi on termite exterior cuticles could potentially reduce the differences between the termite and gallery communities, due to the fact that termites are in close contact with their galleries. Despite this limitation, we found statistically significant differences between both communities.

In our study, representatives of the Saccharomycetales, Malasseziales, Eurotiales, Hypocreales and Mucoromycota common in whole termites, and much less frequent in galleries and wood (Fig. 3, Supplementary Table 1), can be considered as typical members of termite mycobiome. Two previous studies quantified termite associated fungi using ITS metabarcoding. They found Eurotiales, Trichosphaeriales and Pleosporales (Menezes et al., 2018) together with Hypocreales (Moreira et al., 2018) are associated with guts and much less abundant in surrounding environments. This is in line with our results, including the presence of Trichosphaeriales and Pleosporales, which were rare in our study, but typically present in termite bodies (Fig. 3, Supplementary Table 1).

Yeasts, i.e. species from Saccharomycetales, Malasseziales and Trichosporonales, are the best studied fungi in "lower" termites (Prillinger et al., 1996; Prillinger and König, 2006) and the insect gut in general (Blackwell, 2017; Stefanini, 2018). Genera frequently found in our study, *Candida*, *Debaryomyces*, *Pichia*, *Cryptococcus*, and *Trichosporon*, are known as typical termite gut inhabitants (Prillinger et al., 1996; Prillinger and König, 2006). At the species level (i.e. OTUs with ≥99% similarity) we identified several taxa already known as intestinal symbionts of various insects (e.g. *Candida haemulonis*, *Candida parapsilosis* (Suh et al., 2007; Bozic et al., 2017), *Candida elateridarum* (Suh and Blackwell, 2004), *Malassezia restricta* (Zhang et al., 2003), *Metschnikowia pulcherrima* (Woolfolk and Inglis, 2004) and *Trichosporon insectorum* (Fuentefria et al., 2008) (Supplementary Table 1). Surprisingly, yeasts (with the exception of *Malassezia*) did not contribute to the statistical separation of gut and gallery associated fungal communities, when the effect of sampling design was removed (Fig. 6). This was partially due to the high inter-sample variability of yeast communities, but also because of their consistent occurrence (although in very low abundances) in the galleries.

The statistical separation of the whole termite mycobiota in our study was mostly due to the differences among representatives of ubiquitous genera of plant endophytes and saprobes including *Mucoromycotina* spp., *Trichoderma*, *Hawksworthiomyces* and *Penicillium* (Table 1). Data on termite gut associated filamentous fungi are scarce (for review see König et al., 2006; Prillinger and König, 2006). The genera *Trichoderma*, *Penicillium*, *Aspergillus* and *Alternaria* (Hendee, 1935; Rajagopal et al., 1979, 1981; Varma et al., 1994; Jayasimha and Henderson, 2007), together with numerous *Mucoromycotina* spp. (Zoberi and Grace, 1990), were already

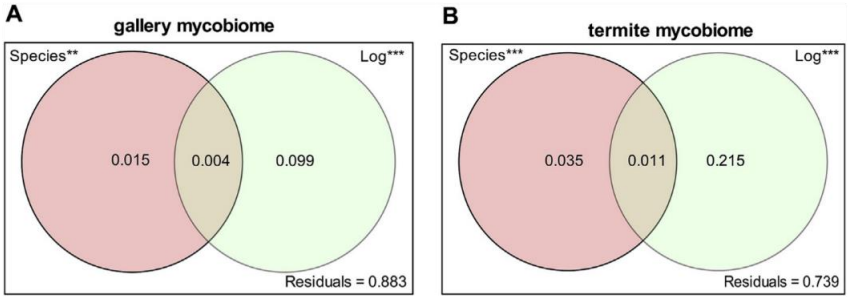

Fig. 7. Venn diagram of RDA variation partitioning of fungal OTU composition in **(A)** termite galleries and **(B)** whole termites. Numbers are adjusted R^2 values. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

reported from termite guts, which corresponds to our results (Table 1). Interestingly, a similar spectrum of genera (i.e. *Penicillium*, *Trichoderma*, *Fusarium*, *Cladosporium*, *Aspergillus*, *Rhizopus*, and *Mucor*) is also present in the guts of other plant biomass feeders such as isopods (Kayang et al., 1996), *Tenebrio molitor* (Fredensborg et al., 2020), wood feeding beetles (Rojas-Jiménez and Hernández, 2015; Mohammed et al., 2018; Ziganshina et al., 2018), cockroaches (Salehzadeh et al., 2007), bark beetles (Pérez et al., 2003) and grasshoppers (Idowu et al., 2009). It is also worth mentioning the abundant presence of *Spizellomyces* sp., identified in more than 50% of *C. testaceus* and *H. tenuis* samples, but absent in *N. octopilis*. *Spizellomyces* is a genus of zoosporic fungi living in soil, or as plant pollen, or fungal parasites (Wakefield et al., 2010), and its association with termites calls for further studies.

Although previous works studied the presence of various fungi in termites (Prillinger et al., 1996; König et al., 2006; Jayasimha and Henderson, 2007; Santana et al., 2015; Menezes et al., 2018; Moreira et al., 2018), our study is the first large and systematic comparison targeting the fungal communities using a statistically robust dataset of termites, their environments, and their feeding substrates. In agreement with previous studies (König et al., 2006; Prillinger and König, 2006), our results indicate that the termite intestine is inhabited by ubiquitous environmental fungi. We showed that the termite associated community is distinct and relatively homogeneous and stable over diverse environments and termite species, compared with termite galleries and control wood. In addition, termite galleries represent a specific habitat which significantly differs from wood in fungal community composition. This is similar to results found in studies of humus and litter feeding termites, where intestinal fungal communities differed substantially from communities of feeding nodules and/or nest walls (Menezes et al., 2018; Moreira et al., 2018) suggesting that termites possessed a host-defined intestinal mycobiome.

Strong environmental filtering appears to allow a relatively small number of fungi to grow freely and persist inside termites, across different species, after being taken up from the environment (soil galleries used for foraging, or the wood upon which termites feed). Vertical transmission of fungi by termites may also occur, although our results do not provide a clear answer on this. Both modes of symbiont acquisition, or their combination, can result in the observed stability of the intestinal communities across various collection dates, termite populations and species. In addition, the galleries themselves host specific fungal communities, which are more similar to intact wood and less affected by the termite species that form the galleries (Figs. 6 and 7).

Interestingly, the effect of termite species on fungal community composition in whole termites was very low (Fig. 7), which shows that different termites shape their fungal communities in a similar way. This is in contrast to patterns found with bacteria (Colman et al., 2012; Bourguignon et al., 2018; Chouvenc et al., 2018; Menezes et al., 2018; Moreira et al., 2018), which are more host specific. This is partly explained by the fact that many termite-associated bacteria are highly co-evolved vertically transmitted obligate symbionts (of termites or associated protists), whereas most identified fungi are presumably facultative associates, frequently existing as environmental fungi. Higher OTU diversity in whole termites in comparison to galleries and control wood is another feature constantly shared among different termite species (Fig. 2). This pattern is expected if we consider that the intestine itself is highly compartmentalised, which results in an increase in microbial diversity (Mikaelyan et al., 2017).

The core mycobiome of the termite gut is composed of plant biomass decomposers (see below), which are stress tolerant, fast growing and sporulating. It is likely that they are pre-disposed to live in the environment of the termite gut, which is characterized by harsh microaerobic conditions, steep gradients of oxygen and hydrogen, and activity of strong hydrolytic enzymes (König et al., 2006). Furthermore, it is possible that such features allow these fungi to live not only in termites, but also in taxonomically distant insect plant biomass feeders. The apparent stability of the fungal community between different termites could be considered evidence for symbiosis. Although it appears likely that the fungi we identified are able to live and grow in the termite gut, it is also possible that the origin of some strains is from the digested material, but were nevertheless detected using our methods.

4.2. Ecological role of gut associated fungi

Both the presence and the ecological role of fungi in the termite gut have been poorly studied so far. However, fungi have generally not been considered as an important part of the termite holobiont (Slaytor, 1992; Brune and Dietrich, 2015; Peterson and Scharf, 2016) and their presence is usually ignored. It has been shown that the intestinal fungi were not essential for *Nasutitermes exitiosus* survival in the lab experiments (Eutick et al., 1978), but similar studies in other termite species are needed to confirm their facultative status. More insight into their ecological functions has been provided with the transcriptome data. The fungal contigs from nymphoid neotenic (i.e. the developmental stage fed mostly by proteinaceous labial gland secretions) intestinomes in *Reticulitermes* spp. represent 10.2% of the fraction of non-termite origin (Dedeine et al., 2015). Another study showed that 18% of all carbohydrate-active enzymes in *Coptotermes formosanus* transcriptomes were of fungal origin, similar to bacteria (24%) but not to protists (6% only) (Zhang et al., 2012). In *Reticulitermes flavipes* symbiont libraries (gut content only), fungi represent 7% of the non-animal fraction of the reads (protists 71%, prokaryotes 21%) (Tartar et al., 2009). Little is known concerning fungal gut biomass.

In the termites *Zootermopsis angusticollis* and *Neotermes castaneus*, 10^7-10^8 yeast cells per millilitre of gut content were found, which is comparable to the number of flagellates, and similar, or two orders of magnitude lower, than the numbers found in bacteria (König et al., 2006; Prillinger and König, 2006). This suggests that fungi may actively proliferate in termite guts and they might be an important part of the lignocellulolytic machinery as proposed by Zhang et al. (2012).

Ecologically, yeast are typical inhabitants of the insect gut, including termites (Blackwell, 2017; Stefanini, 2018), and they can extracellularly decompose cellulose, hemicellulose and xylans, thus contributing to wood digestion (Schäfer et al., 1996; Prillinger and König, 2006). Interestingly, the dominant fungal strains identified in our study, especially *Trichoderma* and *Penicillium*, are well known for their ability to degrade cellulose, hemicellulose, and lignin, and are often used in biotechnology (de França Passos et al., 2018). Significant lignocellulosic activities have also been reported in *Phlebia*, *Hyphodontia*, *Scytalidium* (Eriksson et al., 2012), *Hawksworthiomyces* (De Beer et al., 2016) and *Lasiodiplodia* (Félix et al., 2018). Such strong enzymatic activities were shown *in vitro* directly in the strains from termites (Tarayre et al., 2015). This information, together with published transcriptomic data, reinforces the idea that, in termites, fungi may contribute to the degradation of lignocellulose and hemicellulose (Tartar et al., 2009). In addition, detoxification ability, which is well known in fungi, was also found in the yeasts from termites (Molnar et al., 2004) and therefore toxin degradation could be another important role of the intestinal fungal symbionts. However, further characterisation of the real contribution of fungi to food-processing in termites still remains to be undertaken.

Acknowledgements

We are grateful to the Agence Nationale de la Recherche Investissement d'Avenir for the Nouragues travel grant which allows us to collect the samples in French Guiana, to the Czech Science Foundation (project no. 16-05318 S), and Czech University of Life Sciences Prague (project CIGA 20184303). NL is supported by an Australian Research Council Future Fellowship.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2020.100991.

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5. Termites are associated with external species-specific bacterial communities

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Termites Are Associated with External Species-Specific Bacterial Communities

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ABSTRACT All termites have established a wide range of associations with symbiotic microbes in their guts. Some termite species are also associated with microbes that grow in their nests, but the prevalence of these associations remains largely unknown. Here, we studied the bacterial communities associated with the termites and galleries of three wood-feeding termite species by using 16S rRNA gene amplicon sequencing. We found that the compositions of bacterial communities among termite bodies, termite galleries, and control wood fragments devoid of termite activities differ in a species-specific manner. Termite galleries were enriched in bacterial operational taxonomic units (OTUs) belonging to *Rhizobiales* and *Actinobacteria*, which were often shared by several termite species. The abundance of several bacterial OTUs, such as *Bacillus*, *Clostridium*, *Corynebacterium*, and *Staphylococcus*, was reduced in termite galleries. Our results demonstrate that both termite guts and termite galleries harbor unique bacterial communities.

IMPORTANCE As is the case for all ecosystem engineers, termites impact their habitat by their activities, potentially affecting bacterial communities. Here, we studied three wood-feeding termite species and found that they influence the composition of the bacterial communities in their surrounding environment. Termite activities have positive effects on *Rhizobiales* and *Actinobacteria* abundance and negative effects on the abundance of several ubiquitous genera, such as *Bacillus*, *Clostridium*, *Corynebacterium*, and *Staphylococcus*. Our results demonstrate that termite galleries harbor unique bacterial communities.

KEYWORDS *Coptotermes*, ectosymbionts, *Heterotermes*, *Nasutitermes*, symbiosis

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Termites harbor diverse communities of microbes in their hindguts that participate in lignocellulose digestion, nitrogen metabolism, and other functions (1–4). Gut microbes have been coevolving along with termites for tens of millions of years, and many species are found nowhere else other than in the termite gut (3–5). Consequently, termite gut microbial communities are unique in terms of composition, differing substantially among species (6–8) and differing from the communities present in soil, wood, and termite nest material (9, 10).

In addition to the microbes present in their guts, some termite species are known to partner with mutualistic symbionts that grow outside of their bodies, which we define here as "external symbionts." All species of Macrotermitinae cultivate the

Citation Soukup P, Větrovský T, Stiblik P, Votýpková K, Chakraborty A, Sillam-Dussès D, Kolařík M, Odriozola I, Lo N, Baldrian P, Šobotník J, Bourguignon T. 2021. Termites are associated with external species-specific bacterial communities. Appl Environ Microbiol 87:e02042-20. https://doi.org/10.1128/AEM.02042-20.

Editor Gladys Alexandre, University of Tennessee at Knoxville

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Received 20 August 2020
Accepted 16 October 2020
Accepted manuscript posted online 23 October 2020
Published

zam-aem/zam00221/zam0204d21z | xppws | S=5 | 11/24/20 | 8:08 | 4/Color Fig: 2,3,4 | ArtID: 02042-20 | DOI:10.1128/AEM.02042-20 | CE: KGL-smg

macroscopic fungus *Termitomyces* within their nests (11–13). *Termitomyces* species are only associated with fungus-growing termites (11–13) and, due to their prevailing horizontal transmission, have undergone a number of switches between species in this group (14, 15). Another putative example of nutritional external symbiosis is that between *Sphaerotermes sphaerothorax*, the only known species of Sphaerotermitinae, and bacteria of unknown taxonomic composition that are found inside specialized combs forming the core of *Sphaerotermes sphaerothorax* nests (16). No other nutritional external symbionts are known to be associated with termites.

Termites are known to host externally associated symbiotic microbes that exhibit antifungal properties. Termites primarily feed on wood, sometimes in an advanced stage of decomposition, or on soil (17, 18), both of which are inhabited by a large number of microbes. In addition, termites are social insects that live in densely populated nests, potentially facilitating the transmission of diseases (19). Some termites harbor in their nests *Streptomyces* bacteria that display antifungal properties (20–22). External symbiotic *Streptomyces* are not specific to termites but are recruited from the soil surrounding the fecal nest and become abundant in termite-managed environments (22).

The diversity of microbes externally associated with termites is unlikely to be limited to a handful of external symbionts with nutritional and defensive functions. Termite activities are expected to have a significant effect on the composition of surrounding microbial communities. For example, termites produce antifungal and antimicrobial compounds that they release from their salivary glands and fecal pellets (23–27). Saliva and fecal fluids are used as building material (28), and their biocide properties prevent microbial colonization of the nest and galleries, which remain free of visible fungal overgrowths (21, 29). Termites also tunnel into wood and move vast amounts of soil (30–32), facilitating the spread of microbes and fungi (33). Lastly, termites maintain microclimatic conditions within their nests and galleries (28), potentially favoring the growth of certain microbes while suppressing that of others. In consequence, the microbial communities colonizing termite nests and galleries are expected to differ from those of termite-free environments.

Several studies have shown that the bacterial communities thriving on termite-modified materials differ from those of soil or wood (34–38). However, these studies provided only limited insight into the composition of bacterial communities and no insight into the specificity of termite-bacterium associations. The few studies based on high-throughput sequencing approaches, which allow taxonomic identification of bacteria, provided conflicting results, either suggesting that microbial communities of termite nests are similar to those of the surrounding soil (9) or showing that the fungal combs of each Macrotermitinae species host unique bacterial communities (39).

In this study, we used high-throughput sequencing of 16S rRNA gene fragments to compare the bacterial communities of termite bodies, termite galleries, and control wood samples devoid of termite activities. We worked on the following three wood-feeding termite species abundant in French Guiana lowland tropical rainforests: *Coptotermes testaceus* (Linnaeus, 1758), *Heterotermes tenuis* (Hagen, 1858) (both Rhinotermitidae), and *Nasutitermes octopilis* Banks, 1918 (Termitidae: Nasutitermitinae). Using this data set, we determined the influence of termites on the surrounding bacterial communities and also identified both bacterial lineages with reduced abundance in the presence of termites and bacterial lineages externally associated with termites.

RESULTS

Bacterial diversity. We analyzed a total of 258 samples of termite bodies, galleries, and wood controls in foraging areas of 10 colonies of *C. testaceus* and *N. octopilis* and 11 colonies of *H. tenuis*. After quality filtering and removal of chimeras, we obtained an average of 20,685 sequences of the V4 region of the bacterial 16S rRNA gene for each of the 258 samples. 16S rRNA gene sequences were clustered into 4,864 operational taxonomic units (OTUs) (3% sequence dissimilarity) represented by at least five sequences (see Table S1 in the supplemental material). The three diversity indices, Chao1,

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Evenness, and Shannon-Wiener, were significantly higher for samples of termite galleries than for wood controls and termite bodies (Fig. 1). Chao1 indicated that termite bodies hosted the poorest bacterial communities ($P < 0.05$), with no significant differences among termite species (Fig. 1). Evenness and Shannon-Wiener diversity indices were the smallest for *H. tenuis* bodies, followed by *C. testaceus* bodies, and *N. octopilis* bodies ($P < 0.05$) (Fig. 1).

Comparison of bacterial communities in termite bodies, termite galleries, and termite-free wood controls. We found no significant difference among wood controls associated with *C. testaceus*, *H. tenuis*, and *N. octopilis* (Table 1) and, therefore, pooled wood controls together to investigate phylum composition. The samples of termite galleries and wood controls had similar bacterial community composition at the phylum level (Fig. 2). The dominant phylum was *Proteobacteria*, which on average made up over 40% of the bacterial reads of termite galleries and wood controls. *Acidobacteria* and *Actinobacteria* were also abundant and made up, on average, a minimum of 10% of the bacterial sequences of termite galleries and wood controls. In comparison to termite galleries and wood controls, *Proteobacteria*, *Acidobacteria*, and *Actinobacteria* were rare in termite bodies. Instead, the bacterial communities of *C. testaceus* and *H. tenuis* bodies were heavily dominated by *Bacteroidetes*, which, on average, made up more than 75% of the bacterial reads. BLAST searches assigned most reads of *Bacteroidetes* in *C. testaceus* bodies to "*Candidatus* Azobacteroides" and "*Candidatus* Armantifilum," while the *Bacteroidetes* reads of *H. tenuis* bodies mostly belonged to "*Candidatus* Azobacteroides." The bacterial communities of *N. octopilis* bodies were dominated by *Spirochaetes* and *Fibrobacteres*, which, on average, made up 59.6% and 18.3% of the bacterial reads, respectively. BLAST searches showed that the 16S rRNA gene sequences of *Spirochaetes* and *Fibrobacteres* in *N. octopilis* bodies were mostly assigned to *Treponema* and putatively to *Fibrobacter*, respectively. The permutational multivariate analysis of variance (PERMANOVA) yielded significant differences among groups ($F = 22.33$; $P < 10^{-6}$), including significant differences among termite species ($F = 14.773$; $r^2 = 0.075$; $P < 10^{-5}$) and among sample types (body, gallery, and control wood) ($F = 34.636$; $r^2 = 0.175$; $P < 10^{-5}$). Figure 3 shows the nonmetric multidimensional scaling (NMDS) plot calculated for all samples and presents the bacterial communities of *C. testaceus*, *H. tenuis*, and *N. octopilis* bodies as three disjunct clusters. Termite galleries, as well as wood controls, also clustered by termite species, although these clusters were more diffuse and largely overlapped. Pairwise PERMANOVA indicated that the bacterial communities associated with *C. testaceus*, *H. tenuis*, and *N. octopilis* bodies significantly differed from each other (Table 1). Similarly, the bacterial communities of termite galleries significantly differed among termite species and significantly differed from the corresponding wood controls in the case of *C. testaceus* and *N. octopilis* but not in the case of *H. tenuis*, for which a Bonferroni correction made the comparison only marginally significant (Table 1). Bacterial communities from bodies of *C. testaceus*, *H. tenuis*, and *N. octopilis* significantly differed from communities colonizing termite galleries and wood controls in all cases (Table 1).

Identification of termite-associated bacteria. We carried out redundancy analysis (RDA) and considered OTUs from the 0.25th and 99.75th percentiles (Fig. 4). With this approach, we identified 97 bacterial OTUs associated with termites, or partly excluded by termites, of which many were independently identified for two or three of the studied termite species (see Table S2 in the supplemental material). Of the 47 bacterial OTUs detected to have nonrandom associations with *C. testaceus* (Fig. 4A), 14 OTUs were body-associated bacteria and made up 68.1% of the bacterial community of *C. testaceus* bodies; 18 OTUs were enriched in termite galleries, making up 28.3% of the bacterial 16S rRNA gene sequences in termite galleries and 14.2% of the bacterial 16S rRNA gene sequences in wood controls; and 15 OTUs were partly excluded by *C. testaceus*, making up 24.8% and 3.2% of the bacterial 16S rRNA gene sequences in wood controls and termite galleries, respectively. *H. tenuis* and *N. octopilis* provided similar results. Of the 48 bacterial OTUs considered for *H. tenuis* (Fig. 4B), 15 OTUs were

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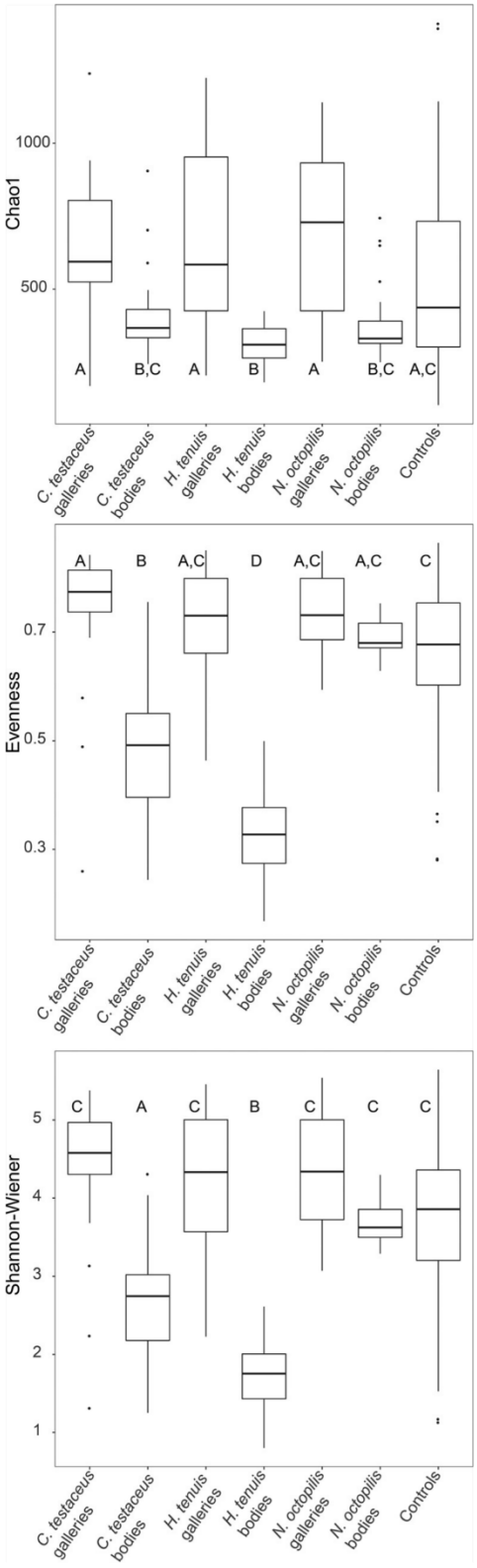

FIG 1 Box plot showing three diversity indices (Chao1, Evenness, and Shannon-Wiener) calculated for the bacterial communities associated with the bodies and galleries of the termites *Coptotermes testaceus*,

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TABLE 1 Results of the pairwise PERMANOVA analysis

Compared groups	*F* value	r^2 value	*P* value	Adjusted *P* value
C. testaceus bodies vs *H. tenuis* bodies	46.411	0.449	$<10^{-5}$	$<10^{-3}$
C. testaceus bodies vs *N. octopilis* bodies	88.668	0.626	$<10^{-5}$	$<10^{-3}$
H. tenuis bodies vs *N. octopilis* bodies	50.945	0.476	$<10^{-5}$	$<10^{-3}$
C. testaceus galleries vs *H. tenuis* galleries	2.256	0.038	$<10^{-4}$	0.003
C. testaceus galleries vs *N. octopilis* galleries	2.425	0.044	$<10^{-5}$	$<10^{-3}$
H. tenuis galleries vs *N. octopilis* galleries	1.901	0.033	$<10^{-3}$	0.022
C. testaceus galleries vs *C. testaceus* controls	2.929	0.052	$<10^{-5}$	$<10^{-3}$
H. tenuis galleries vs *H. tenuis* controls	2.057	0.033	0.002	0.07
N. octopilis galleries vs *N. octopilis* controls	3.443	0.062	$<10^{-4}$	$<10^{-3}$
C. testaceus bodies vs *C. testaceus* galleries	34.076	0.387	$<10^{-5}$	$<10^{-3}$
H. tenuis bodies vs *H. tenuis* galleries	22.625	0.274	$<10^{-5}$	$<10^{-3}$
N. octopilis bodies vs *N. octopilis* galleries	25.984	0.333	$<10^{-5}$	$<10^{-3}$
C. testaceus bodies vs *C. testaceus* controls	27.334	0.336	$<10^{-5}$	$<10^{-3}$
H. tenuis bodies vs *H. tenuis* controls	19.262	0.243	$<10^{-5}$	$<10^{-3}$
N. octopilis bodies vs *N. octopilis* controls	25.762	0.331	$<10^{-5}$	$<10^{-3}$
C. testaceus controls vs *H. tenuis* controls	1.036	0.018	0.365	1
C. testaceus controls vs *N. octopilis* controls	1.631	0.03	0.011	0.409
H. tenuis controls vs *N. octopilis* controls	1.537	0.027	0.025	0.891

body-associated bacteria and made up 80.8% of 16S rRNA gene sequences of *H. tenuis* bodies; 17 OTUs were gallery-associated bacteria, making up 27.7% of the bacterial community of termite galleries and 11.3% of the bacterial community of wood controls; and 16 OTUs were partly excluded by *H. tenuis*, making up 24.7% and 6.7% of the 16S rRNA gene sequences of the control and gallery samples, respectively. Lastly, of the 45 bacterial OTUs considered for *N. octopilis* (Fig. 4C), 15 were body-associated bacteria and made up 60.3% of the termite bacterial community, 15 OTUs were gallery-associated bacteria and made up 25.6% of the bacterial community of *N. octopilis* galleries and 9.2% of the bacterial community of wood controls, and 15 OTUs were partly excluded by *N. octopilis* and made up 34.9% of the bacterial 16S rRNA gene sequences of wood control samples and 1.4% of the bacterial 16S rRNA gene sequences of *N. octopilis* galleries (see Table S2).

DISCUSSION

In this study, we sequenced the bacterial communities associated with three termite species, *C. testaceus*, *H. tenuis*, and *N. octopilis*. We demonstrated that termite galleries host the most species-diverse bacterial communities, while termite bodies comparatively host species-poor bacterial communities. We found that the composition of bacterial communities differs among termite bodies, termite galleries, and wood controls devoid of visible termite activities in a species-specific manner. We also identified 97 abundant bacterial OTUs that are predominantly associated with termite bodies (referred to as body-associated bacteria), termite galleries (referred to as gallery-associated bacteria), or control wood samples (referred to as gallery-depleted bacteria). Consequently, our results show that termites not only shape the bacterial communities inside their gut (6, 7, 40) but also those in their environment.

The bacterial diversity indices calculated for the bodies of *C. testaceus* and *H. tenuis* closely match those previously calculated for the related species *Coptotermes niger* (6). Similarly, the bacterial diversity indices of *Nasutitermes octopilis* bodies closely match those of *Nasutitermes corniger* and *Nasutitermes takasagoensis* (6). These results indicate that our estimations of bacterial diversity are robust and reproducible. In addition, these results also suggest that the phylogenetic relationships among termites are predictive of the diversity of their bacterial communities.

FIG 1 Legend (Continued)
Heterotermes tenuis, and *Nasutitermes octopilis* and with wood controls. Boxes indicate the first and third quartiles. The horizontal lines crossing boxes are medians. Whiskers indicate the 5th and 95th percentiles, and black dots are outliers. Groups that do not share at least one capital letter are significantly different (Tukey honestly significant difference [HSD] *post hoc* test, $P < 0.05$).

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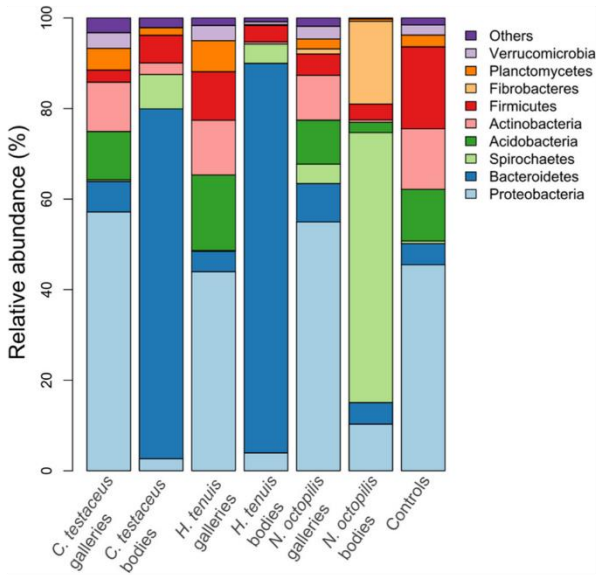

FIG 2 Relative abundance of bacterial phyla associated with the bodies and galleries of the termites *Coptotermes testaceus*, *Heterotermes tenuis*, and *Nasutitermes octopilis* and with wood controls.

The bacterial communities associated with termite galleries are more diverse than those found in termite bodies. Most OTUs found in termite bodies correspond to gut bacterial lineages identified in previous studies (5–7, 40), indicating that the majority of bacterial OTUs associated with termite bodies are gut specialists. The termite gut is a highly specialized habitat, with extreme physicochemical properties, in some species having a pH of >12 (41), and is largely populated by bacteria found nowhere else (3–5). Although termite gut hosts among the most diverse communities of microbes found in insects (42), the presence of a strong environmental filtering, preventing the colonization of most bacterial species, might explain the low bacterial diversity observed in termite guts when compared with that of termite galleries and wood controls.

We independently identified the 14 to 15 dominant body-associated bacterial OTUs for each of the three termite species (Fig. 4; see also Table S2 in the supplemental material). These OTUs made up 60.3 to 80.8% of the total bacterial 16S rRNA gene sequences and were, in most cases, known to be associated with termite guts. For example, the dominant gut symbiotic OTUs in *C. testaceus* were classified as "*Candidatus* Azobacteroides" and "*Candidatus* Armantifilum," two bacterial lineages known to be associated with termite gut protists (43, 44). "*Candidatus* Azobacteroides" was also the dominant gut symbiotic OTU in *H. tenuis*. In *N. octopilis*, which belongs to Termitidae, the only termite lineage that lost their gut protists (4), the dominant gut symbiotic OTUs were assigned to the *Spirochaeta* (*Spirochaetes*) and *Fibrobacter* (*Fibrobacteres*) genera. BLAST searches showed that our 16S rRNA gene sequences from these two genera corresponded to *Treponema* and the *Fibrobacteres* sequences previously found in the gut of other species of *Nasutitermes* (45, 46). Therefore, while our taxonomic identifications were imprecise in some cases, they matched bacterial taxa known to occur in termite guts and highlight the overwhelming dominance of a few bacterial groups.

We found that the bacterial communities associated with termite galleries are specific to termite species and differ from those of termite bodies and wood controls. These results concur with previous studies that found that bacterial communities associated with nests differ from those of surrounding soil and wood samples (7, 34, 37, 38). Exclusion experiments have also shown that termites influence the bacterial communities in wood pieces (33). Importantly, our results show that the differences

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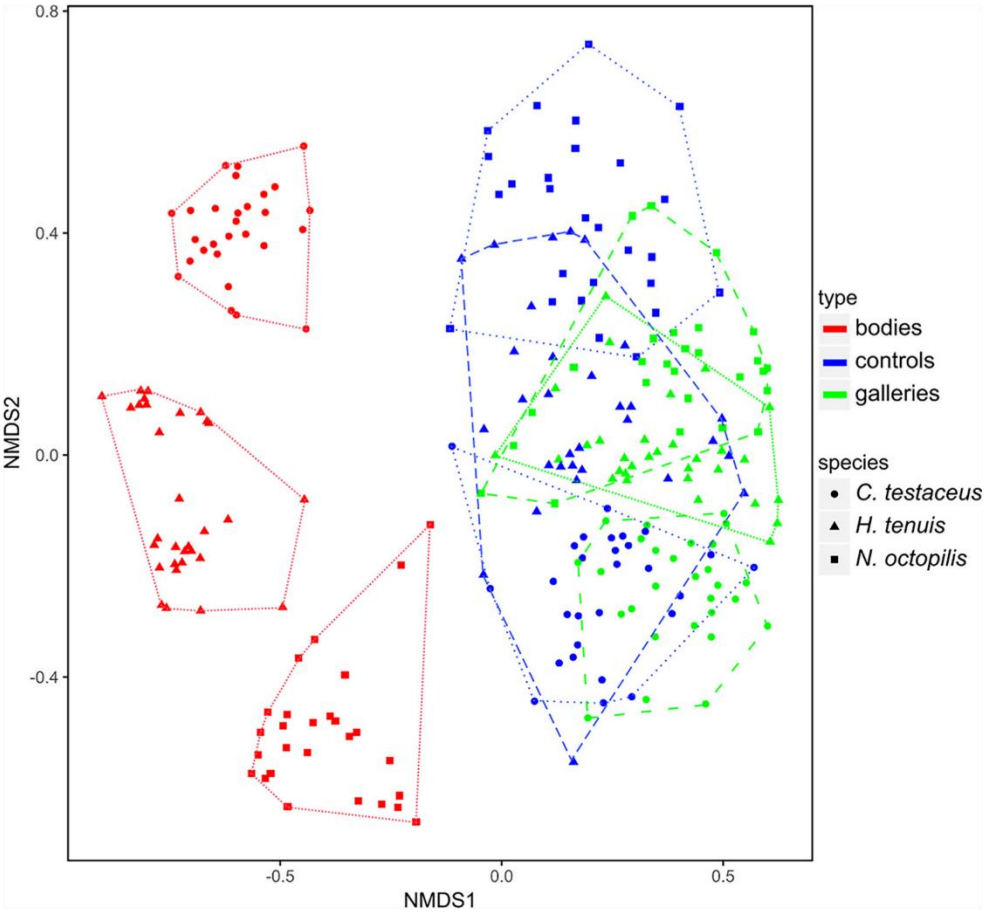

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FIG 3 Nonmetric multidimensional scaling of bacterial communities associated with the bodies and galleries of the termites *Coptotermes testaceus*, *Heterotermes tenuis*, and *Nasutitermes octopilis* and with wood controls.

between galleries of different termite species and wood control samples are subtler than those found for gut bacterial communities, suggesting that the gallery-associated bacteria are loosely associated with termites. This raises the possibility that termites established a symbiotic relationship with the bacterial communities associated with their galleries in the absence of strict coevolution between the two partners as is possibly common for many host-symbiont associations (47), including external symbionts of termites (21, 22).

The identification of the main gallery-associated bacterial OTUs confirmed their loose association with termites. We independently identified 15 to 18 bacterial OTUs classified as gallery-associated bacteria for each of the three termite species (Fig. 4; Table S2). These OTUs made up 25.6 to 28.3% of the 16S rRNA gene sequences of termite galleries. However, in contrast to body-associated bacterial OTUs, many gallery-associated bacterial OTUs were shared among termite species, and out of 28 OTUs identified as gallery-associated bacteria, 8 were shared by all three termite species, and 6 were shared by two termite species. In addition, gallery-associated bacterial OTUs were also present in wood controls, albeit in significantly lower abundances (only 9.2 to 14.3% of the 16S rRNA gene sequences). These results suggest that termite gallery-associated bacteria are recruited from the surrounding environment as has been shown for *Coptotermes formosanus* and its externally associated symbiotic *Streptomyces* bacteria (22). Lastly, we also found body-associated bacterial OTUs in termite galleries that probably originated from DNA of dead or inactive bacterial cells. One such OTU is

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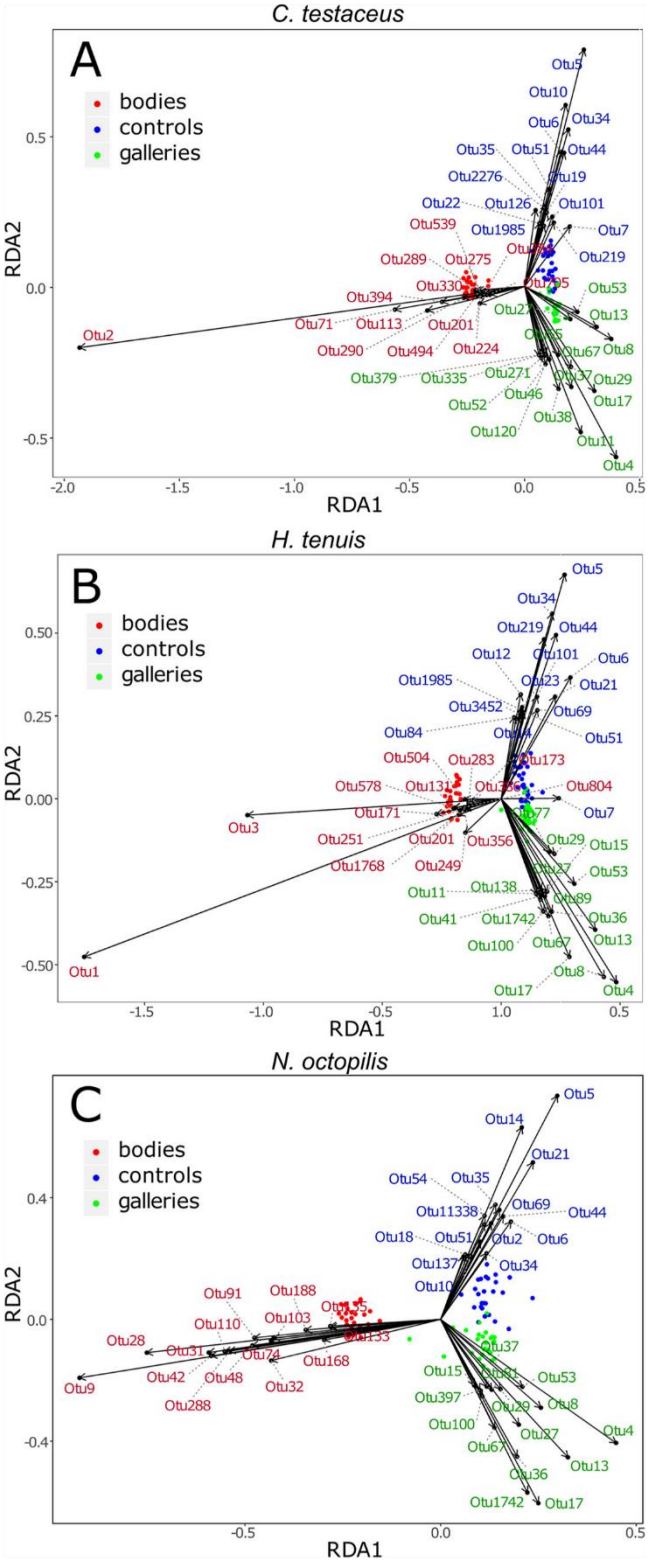

FIG 4 Partial redundancy analysis of bacterial communities associated with termite bodies and galleries and with wood controls. *Coptotermes testaceus* (A), *Heterotermes tenuis* (B), and *Nasutitermes octopilis* (C). Taxonomic identification of OTUs is provided in Table S1 in the supplemental material.

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"*Candidatus* Azobacteroides," a bacterium known to be the intracellular symbiont of termite gut protists (43) and therefore clearly unable to live outside of the termite gut.

The gallery-associated bacterial OTUs identified in this study mostly belonged to *Proteobacteria* and *Actinobacteria*, which are known to dominate the nest bacterial communities of several Termitidae species (48). A total of 18 OTUs belonged to *Proteobacteria*, including seven OTUs assigned to *Rhizobiales*, five of which were identified as gallery-associated bacteria for each of the three termite species investigated in this study. Many *Rhizobiales* are able to fix atmospheric nitrogen and have developed symbiotic associations with plant roots (49). Whether they represent a source of nitrogen for termites, supplementing the low levels of nitrogen found in the wood they consume, remains to be determined. We also identified four gallery-associated bacterial OTUs belonging to *Actinobacteria*, but none of them belonged to *Streptomyces*. Therefore, unlike those previously found for *C. formosanus* (21, 22), *Streptomyces* spp. do not appear to be important gallery-associated bacteria of *C. testaceus*, *H. tenuis*, or *N. octopilis*. Several factors might be at the origin of the lower prevalence of *Streptomyces* in our study compared to that found in *C. formosanus* (21, 22), including the differences among the studied ecosystems (i.e., tropical rainforest of French Guiana versus urban parks in Florida) and the sampling approach, based on visually located wood items colonized by termites (French Guiana) and carton material sampled in bucket traps (Florida). However, because the low prevalence of *Streptomyces* was shared among the three studied termite species, it is unlikely for termite phylogenetic relationships to be at the origin of this pattern. Further studies are required to decipher the exact role of gallery-associated bacteria.

Several bacterial OTUs were partly excluded from termite galleries. The 15 or 16 gallery-depleted bacterial OTUs that we identified for each termite species made up 24.7 to 34.9% of the 16S rRNA gene sequences in control wood samples but only 1.4 to 6.7% of the 16S rRNA gene sequences in termite galleries. These results are indicative of the ability of termites to reduce the growth of some microbes in their direct environment, possibly through the production of antimicrobial and antifungal compounds, as has been shown in several termite species (21, 29). External symbionts of termites are also known to produce antimicrobial compounds (20, 21), and it is possible that some of the gallery-associated bacteria that we identified have this function. Finally, the microclimatic conditions of termite galleries might also play a role in shaping bacterial communities and reduce the abundance of gallery-depleted bacteria.

As is the case for gallery-associated bacteria, a large fraction of the 27 gallery-depleted bacterial OTUs were identified to have reduced abundance in the galleries of more than one termite species, including five gallery-depleted bacterial OTUs with reduced abundance in the galleries of the three studied termite species and nine gallery-depleted bacterial OTUs with reduced abundance in the galleries of two of the three studied termite species. Many of the gallery-depleted bacterial OTUs belong to ubiquitous genera, often found in soil and wood, but that are also known to include animal pathogens, at least on a facultative basis. This includes, among others, OTUs belonging to the genera *Bacillus*, *Clostridium*, *Corynebacterium*, and *Staphylococcus*. Whether they are excluded because they represent potential threats to termite colonies remains to be determined. Fungus-growing termites actively exclude fungal *Pseudoxylaria* pathogens from their *Termitomyces* fungus garden (20, 50). Alternatively, modification of the physical and chemical properties of the direct environment of termites, including that of their galleries (28), potentially affects bacterial community composition by promoting the growth of some bacteria at the cost of others. Additional investigations are required to determine how termites affect their neighboring bacterial communities. Our results show that as termites host specific microbial communities inside their guts, specific microbial communities grow in their galleries.

MATERIALS AND METHODS

Study site and sampling. The fieldwork took place in November 2014 in the Nouragues Nature Reserve (French Guiana; 04°05′N; 52°41′W). All samples were collected within 50 m of the network of

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paths of the Nouragues Research Station. The full sampling area was about 100 ha. We collected samples of the following three species: *Coptotermes testaceus*, *Heterotermes tenuis*, and *Nasutitermes octopilis*. Upon encountering one of these species, we collected one series of samples, all collected in the same wood log, consisting of three termite samples (between 10 and 15 workers each), together with three samples of their feeding substrates (approximately 1-cm^3 piece of wood containing thin galleries) and three control samples (approximately 1 cm^3 of wood at least 10 cm away from the closest termite galleries). Sterile vials and flame-sterilized forceps were used for the sampling. Sample replicates were distant by more than 1 m. Occasionally, for small logs, only two samples of each type were collected. All samples were preserved in RNAlater, stored at −20°C within 8 h following collection, and shipped to Prague where they were stored at −80°C until DNA extraction. In total, we sampled wood with foraging parties belonging to 10 colonies of *C. testaceus* and *N. octopilis* and 11 colonies of *H. tenuis*.

DNA extraction and PCR amplification. Total DNA was extracted using the Macherey-Nagel NucleoSpin soil kit. For each termite sample, we homogenized whole individuals, including guts (hereafter termed "bodies"), of up to 10 workers using two sterile steel beads (3-mm diameter) and a Mixer Mill MM 400 set on 30 swings per second for 2 min. We carried out extractions as per the manufacturer's protocol, except for the lysis step that was shortened to 2 min of vortexing. Wood samples were placed in a sterile 2-ml tube, frozen in liquid nitrogen, mechanically crushed with five sterile steel beads for 1 min at 30 swings per second, and grinded with a Mixer Mill Retsch MM 400 for 10 min. Following the first grinding step, we added 550 μl of SL2 extraction buffer to the homogenized material and repeated the grinding with the same settings. The lysis by vortexing was extended to 10 min, and precipitation of contaminants was carried out with 100 μl of SL3 buffer. Lysate was filtered with 650 μl of supernatant. Silica membrane was dried for 3 min in a centrifuge. Finally, we added 50 μl of SE buffer to the silica membrane and centrifuged for 45 s to elute the DNA. Each sample was handled with flame-sterilized forceps.

PCRs were performed using the Thermo Scientific DyNAzyme II DNA polymerase kit. We used the universal primers 515F and 806R targeting the V4 region of the 16S rRNA gene (51), combined with an original combination of index reads. The PCRs contained 2.5 μl of 10× buffer for DyNAzyme II DNA polymerase, 0.75 μl of bovine serum albumin (BSA) (20 mg/ml), 1 μl of each primer (0.01 mM), 0.5 μl of PCR nucleotide mix (10 mM each), 0.75 μl of polymerase (2 U/μl DyNAzyme II DNA polymerase), and 1 μl of template DNA. DNA concentration ranged between 10.3 and 41.4 ng/μl. PCRs were performed using an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany) nexus cycler, with the following settings: initial denaturation at 94°C for 3 min; 30 cycles of 94°C for 45 s, 50°C for 1 min, 72°C for 45 s; and a final extension step at 72°C for 10 min. We carried out three independent PCR amplifications for each sample, combined the three replicates, and cleaned them using the MinElute PCR purification kit (Qiagen GmbH, Hilden, Germany). Pooled PCR products were mixed in equimolar concentration and paired-end sequenced with an Illumina MiSeq sequencer (Illumina Inc., USA) using the V2 chemistry to produce 250-bp paired-end reads. Sequence data are available on MG-RAST.

Data filtering. Raw paired-end reads were joined using fastq-join (52) and demultiplexed, filtered, and trimmed using SEED v2.1 (53). Sequences with a mean Phred quality score of <30, as well as sequences with mismatches in barcodes or ambiguous bases, were discarded. We also discarded all bacterial sequences shorter than 200 bp or longer than 350 bp. A total of 5,863,706 bacterial sequences were obtained after initial quality filtering.

OTU clustering and classification. Sequences were clustered into operational taxonomic units (OTUs) (3% sequence dissimilarity) using UPARSE implemented in USEARCH v8.1.1861 (54). Chimeric sequences were identified during clustering to OTUs using the UPARSE algorithm, and a total of 526,949 sequences were excluded from downstream analyses. To reduce the influence of contamination and to minimize the effect of barcode hopping (55), all OTUs with fewer than five reads were discarded. We also used previous Illumina run data to estimate the number of reads that potentially hopped among samples for all OTUs and removed those reads.

The most abundant sequence from each OTU was used as a representative sequence for taxonomic classification. Representative sequences were classified with the RDP classifier from the RDPTools software v2.0.2 using the 16S rRNA gene reference database (56). Classification was verified using RDP release 11 update 5, accessed on 30 September 2016 (57), which provided the closest BLAST hit for each OTU. We used rrnDB v5.4 (58) to estimate the relative abundance of each OTU, considering the variable number of 16S rRNA gene copies per bacterial genome as explained in Větrovský and Baldrian (59).

Diversity of bacterial communities in termite bodies, termite galleries, and wood controls. We carried out all statistical analyses using a subsample of 3,000 sequences per sample. We used the Chao1 (60), Evenness (61), and Shannon-Wiener (62) indices to characterize the bacterial diversity of termite bodies, termite galleries, and wood controls. The values of the three diversity indices were estimated using SEED v2.1 (53) and visualized using the R package ggplot2 (63). To test the null hypothesis of no effect of sample type and species on diversity indices, linear mixed effect models were fitted using the function lme() implemented in the R package *nlme* (64). A factor with seven levels, created by combining termite species and sample types, was fitted as the fixed part of the model, and a random structure of the form *~1|triplet/log* was included in each model to account for the fact that measurements were grouped in triplets, which, in turn, were nested in logs. Pairwise comparisons among groups were performed with Tukey *post hoc* tests using the function lsmeans() of the R package *lsmeans* (65).

Comparison of bacterial communities in termite bodies, termite galleries, and wood controls. We visualized the relative abundance of bacterial phyla for each sample type (body, gallery, and wood control) using the R package *ggplot2* (63). To test whether bacterial community composition differs among termite bodies, termite galleries, and wood controls, we performed PERMANOVA (66) using the

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adonis function from the R package *vegan* (67). The response matrix was calculated using the Euclidian distance on Hellinger-transformed bacterial composition, which resulted in a Hellinger distance matrix, commonly used as a measure of resemblance (68). We used sample type (body, gallery, and wood control) as the explanatory variable. Since samples were collected in series of dependent triplets (or sometimes doublets) coming from a single log, with each triplet comprising three dependent samples (one termite body sample, one gallery sample, and one wood control sample) collected near to each other, the permutations were constrained to occur among samples of the same triplets, which were used as a blocking factor. As such, we used the formula "termite-species*sample-type," and the strata was set to "data$triplets." We compared termite species and sample types (body, gallery, or wood control) using pairwise PERMANOVA implemented in the pairwiseAdonis R package (69). We used Bonferroni corrections to adjust *P* values. Significance was assessed using 99,999 permutations.

We visualized the data set using nonmetric multidimensional scaling (NMDS) implemented with the metaMDS function of the R package *vegan* (67). NMDS analysis was carried out using community data regressed against logs and triplets. This procedure removed the effect of spatial variability inherent to the experimental design.

Identification of termite-associated bacteria. To identify the bacterial OTUs contributing to the separation between termite bodies, termite galleries, and wood controls, we used partial redundancy analysis (partial RDA) (61). Each termite species was considered separately. For each RDA, we used Hellinger-transformed bacterial OTU composition as a response matrix and sample type as fixed explanatory factor. The effects of triplets and wood logs were removed by using logs and triplets as conditioning factors in the partial RDA (see reference 61). We focused our efforts on the identification of the main bacterial OTUs and considered those belonging to the 0.25th and 99.75th percentiles. Identified OTUs were classified in one of the following three categories: body-associated bacteria (OTUs predominantly found in termite guts), gallery-associated bacteria (OTUs predominantly found in termite galleries), and gallery-depleted bacteria (OTUs predominantly found in control wood samples). Note that generalist OTUs, showing a random distribution pattern, with no preference for termite bodies, termite galleries, or control wood samples, are not considered further.

Data availability. The sequence data generated in this study are deposited in MG-RAST under accession number mgm4904347.3.

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SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 1.1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

ACKNOWLEDGMENTS

We thank the staff of the Nouragues Research Field Station (CNRS, French Guiana) for logistic help during field work. We also thank Olivier Delattre for his help during field sampling.

This work was financially supported by the Czech Science Foundation (project no. GAČR 16-05318S), the project EVA4.0 (CZ.02.1.01/0.0/0.0/16_019/0000803), the Internal Grant Agency of Faculty of Tropical AgriSciences (no. 20205014) and Faculty of Forestry (no. 20184303), by a Nouragues travel grant "Investissement d'Avenir" from the Agence Nationale de la Recherche, and by OIST core funding.

D. Sillam-Dussès, J. Šobotník, and T. Bourguignon conceived the study and carried out the fieldwork. P. Soukup, P. Stiblik, K. Votýpková, and A. Chakraborty performed the lab experiments. T. Větrovský, M. Kolařík, and I. Odriozola analyzed the data. P. Soukup and T. Bourguignon wrote the paper with significant input from other coauthors. This study was supervised from inception to completion by J. Šobotník.

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Discussion

1. Evolution of termites

The complete resolving of termite phylogeny with ultimate resolution and dating would allow us to reconstruct the evolution of particular morphological, ecological or behavioural traits. This would be very useful for identification of particular evolutionary drivers, like historical climate changes, continents drift or even human activity. Such knowledge would also help us to better foreseen the impacts of ongoing climate and land-use changes. Therefore, two studies presented within this thesis focused on termite phylogeny.

Sequencing of mitochondrial genomes is currently a very effective way how to reconstruct termite evolutionary history (Bourguignon *et al.* 2015, 2016a, 2017). As the fully resolved and detailed phylogeny and related classification of dry-wood termites (Kalotermitidae) is still not available, here I present the full mitochondrial genome of *Cryptotermes havilandi* (Sjöstedt, 1900), native to Congo basin, which became one of the major invasive timber pests across the globe (Evans *et al.* 2013; Su & Scheffrahn 2000). Many timber pests from family Kalotermitidae are actually imported invasive species in new environments (Evans *et al.* 2013), the misidentification with original dry-wood termites could lead to inappropriate treatment of infested structures and therefore to loss of money invested in wood protection. Deeper knowledge of evolutionary history and exact taxonomy of Kalotermitidae is crucial to applied research for its effort to invent effective methods of wood goods and structures protection. The mitogenome of *C. havilandi* is an important part of ongoing effort to resolve Kalotermitidae phylogeny and taxonomy, and at the same time it extends the mitochondrial genome database for quick and precise bar-code identification. In the future, the pest control will likely use simple methods of molecular bar-coding to properly identify the exact pest species and apply appropriate treatment against it.

Contrary to dry-wood termites, in which the proper identification is still relatively quickly possible using the soldier caste characteristics, most of the species of soil-feeding Apicotermitinae lack soldier caste and therefore the identification is performed according to the structure of digestive tract, which is by far more complicated and time consuming compared to molecular bar-

coding (Donovan *et al.* 2000; Noirot 2001; Sands 1972, 1998). The study of molecular phylogeny and historical biogeography of Apicotermitinae used an unprecedented set of mitochondrial genomes representing all the main clades, but still not covering the whole undiscovered biodiversity of the group, not even on the generic level.

In agreement with the previous phylogenetical studies of termites (Bourguignon *et al.* 2015; Buček *et al.* 2019; Inward *et al.* 2007), we found the Apicotermitinae as monophyletic clade within Termitidae, which originated in Africa. We recognized 6 monophyletic clades among Apicotermitinae:

I – *Apicotermes*-group *sensu stricto*

II – *Jugositermes*-group

III – *Astalotermes*-group

IV – *Speculitermes*-group

V – *Adaiphrotermes*-group

VI – *Anoplotermes*-group

Previously recognized *Apicotermes*-group comprising all soldiered species (*Apicotermes*-group, *Jugositermes*-group, and *Speculitermes*-group) is paraphyletic, as it was suggested already by others (Bourguignon *et al.* 2015; Buček *et al.* 2019), but the basal lineages are closely matching the description made by Noirot (2001) based on anatomy of hindgut. Also, the *Anoplotermes*-groups *sensu lato* is paraphyletic and comprises the remaining three groups. Interestingly, the *Astalotermes*-group comprising of all African Apicotermitinae soldierless taxa (except *Adaiphrotermes*-group) is truly a monophyletic clade, although the cause of soldier caste loss is so far not understood. We can anyway show the evolutionary trajectory on the Asian *Speculitermes*-group. Genera *Indotermes* and *Euhamitermes* are soldiered termites and a sister group to the monophyletic *Adaiphrotermes*-group + *Anoplotermes*-group, which are completely soldierless. While *Indotermes* reveals extremely low proportion of soldiers (roughly 1 to 1000 workers), in *Euhamitermes*, a soldier was collected by our team in 3 samples out of 40, and several species of the genus are considered soldierless.

There are probably two main reasons for the loss of soldiers in soil feeding termites: (i) soil is nutritionally poor source and therefore soil-feeding termites

do not invest their energy into production of soldiers, moreover if (ii) soil is not a limited and delineated source compared to wood piece (branch or trunk) and therefore there is no need to protect it. This is probably a general pattern of ecological impact on soil-feeding termites evolution, as it may be observed in other termite groups, where wood-feeding and soil-feeding species occur (Ahmad 1976; Deligne *et al.* 1981; Miller 1984; Sands 1972).

Apicotermitinae clearly evolved in Africa and we can recognize two colonization events from there. The first was the *Speculitermes*-group migrating to Asia via land bridges, the second was the *Anoplotermes*-group colonizing South America probably by rafting, what is the sole case of oversea migration among all soil-feeding termites, nevertheless very successful as they dominate the soil fauna of Amazonian rain forest (Martius 1997). The absence of soldiered termites in South America is a fact leading to the conclusion, that the loss of soldier caste in Apicotermitinae took place in Africa, however, it still does not answer the question, how many times it actually happened? Thanks to the phylogenetic position of *Speculitermes*-group, we might come up with a conclusion that the loss of soldier caste happened probably twice independently.

2. Evolution of termite microbial associations

As explained in the introduction of my thesis, termites are fully dependent on the symbiotic relationships with microbes and therefore their evolution is tightly intertwined with evolution of these symbionts. Three out of five studies presented in my thesis deals with termite relationships with microbes of both, termite gut and termite outer environment.

Although the research on gut microbiome of termites has been going over a century (Brauman *et al.* 2000; Breznak 2000; Brune 1998; Buscalioni & Comes 1910; Cleveland 1924, 1925; Comes 1910; Imms 1919; Koidzumi 1921; Slaytor 2000), the gained knowledge is still answering rather the question "What is there?", than the question "What is it doing?". Thanks to many studies (Brune & Dietrich 2015; Köhler *et al.* 2012; Mikaelyan *et al.* 2014; Ohkuma & Brune 2011; Tokuda *et al.* 2001) we can exactly say, what microbiota is inhabiting particular part of the hindgut, but we are not fully able

to assign the particular functions to a specific clade, as the majority of the microbial community is not possible to cultivate and study separately. In general, we know what is going on in termite gut thanks to microbial community (Brune 2014; Brune & Ohkuma 2011; Ohkuma 2003; Slaytor 2000), but we usually do not know, who is responsible for a given function, in particular.

However, modern culture-independent methods like metagenome-assembled genomes (MAGs) or single-amplified genomes (SAGs) allow us to study even uncultivated organisms in detail (Albertsen *et al.* 2013; Woyke *et al.* 2017). Thanks to reconstructed genomes of particular members of the microbial community it can be suggested which metabolic pathways can eventually be expressed by the given microbe. In a study of termite metagenome-assembled genomes we focused mainly on reconstruction of MAGs from different hindgut compartments of 8 termite hosts.

In total, 589 MAGs were successfully reconstructed. Although we succeed to reconstruct majority of prokaryotic phyla known from 16S metabarcoding studies (Köhler *et al.* 2012; Utami *et al.* 2018; Wertz *et al.* 2012), some like Cyanobacteria or Verrucomicrobia were not reconstructed. This might be due the high cleaning threshold of reconstructed MAGs.

38 of reconstructed MAGs belong to domain Archaea of which 15 belong to phylum Bathyarchaeota, which are associated in termite-specific cluster among the whole phylum. The remaining 23 MAGs belong to phylum Euryarchaeota, which is known for methanogenesis in arthropod-associated microbial communities (Brune 2019; Schloss *et al.* 2016). Our MAGs of Archaea from termite guts represent a novel and an important backbone knowledge for further metabolic studies of microbial communities in termite gut.

The most of reconstructed MAGs belong to the bacterial phylum Firmicutes, which also appeared to be relatively the most abundant one. We confirmed, that the recent studies based on 16S sequencing probably did not revealed the whole diversity of the phylum (Schulz *et al.* 2017) and we brought evidence of new Firmicutes lineages including the genomes of termite-specific lineages previously detected by others (Bourguignon *et al.* 2018). We also confirmed, that Spirochaetes are major player in wood degradation of "higher" termite gust, as it was the relatively most abundant phyla among wood-feeders. In total, the presented set of 589 MAGs is currently the largest

genomic resource for subsequent studies of functional importance of arthropod gut microbiomes.

Apart of internal symbionts of termites, I focused also on possible external symbionts, excluding fungus growing termites, as mentioned previously (Bignell 2000; Garnier-Sillam *et al.* 1989; Rouland-Lefèvre 2000). There are several studies describing the effect of termites to environmental microbial community (Chouvenc *et al.* 2013, 2018; Fall *et al.* 2004, 2007; Jouquet *et al.* 2005, 2011), but no study focused directly on external symbiotic relationships, except for the notoriously-known fungus-growing termites Macrotermitinae (Termitidae). Our studies on ectosymbiosis with environmental bacteria and fungi are based on novel sampling method, where we collected termites, their actual food source and a control from the same food source, but devoid of signs of termite activity. Sequencing the microbial communities from such samples resulted in ITS2 gene and 16S gene datasets allowing us to compare the presence and relative abundances of particular OTUs amongst the samples.

Historically, the case of Macrotermitinae - *Termitomyces* symbiosis is the best-known and well-studied example of ectosymbiosis (relationship taking place out from the body), however, there also are sparse mentions of termites depending on fungi to aid partial decomposition of their food (Kirker *et al.* 2012; Rouland-Lefèvre 2000), which clearly deserved a further investigation. During their evolution, termites might have established facultative relationships with many environmental microbes for two main reasons. (a) Termites may take the advantage of partially decomposed wood; (b) Termites might avoid toxic secondary metabolites of plant tissues leaving its degradation upon the symbiotic partner. Such facultative relationship with environmental fungi helping termites to digest and/or detoxify the wood may even explain the question of termite global disproportional abundances across tropics (Bourguignon *et al.* 2017). I therefore decided to explore this understudied field and checked the pattern of co-occurrence between termites and environmental fungi and bacteria.

The fungal OTUs from the termite galleries overlapped with those from intact wood, but they were still significantly different. Moreover, both communities significantly differed from those of termite bodies. Although we

confirmed that the mycobiome of termite galleries differ from that of intact wood, the results suggested that both are dependent rather on wood species than on termite species, dwelling the galleries. However, the presence of termites in the wood alters the fungal communities and further investigation is needed as the phenomenon is documented also from our termite breeds, where abandoned wood is quickly covered by unspecific moulds. Interestingly, our experiments showed that there is higher fungal OTU diversity extracted from termite bodies, than from their galleries or intact wood. However, the mycobiota of termites in our experiments actually originated from two different environments: from the intestines and from the cuticular surface including mouthparts, which we were not able to recognize, as the termites were homogenized as whole. Nevertheless, we recognized specific termite gut mycobiome and debated the service provided by the gut yeasts, in particular. As we found out, the termite mycobiome was rather stable across termite species, which suggest rather free association dependency on environmental fungal community. This is in contrast to bacterial communities of the gut which are transferred vertically and thus species specific.

As I showed previously, although the bacterial community of the termite hindgut was studied intensively over past few decades, the knowledge of external bacterial community remains unexplored, including the case of bacterial farmers Sphaerotermitinae (Termitidae). However, it doesn't mean that there is a lack of any knowledge at all (Chouvenc *et al.* 2013, 2018). We know that termites live in a warm humid environment highly beneficial for microbial pathogens, but termites do not considerably suffer by entomopathogenic fungi or bacteria, at all. Moreover, huge amounts of money were spent in pest control research directed to use entomopathogens against termites without any sign of success (Chouvenc *et al.* 2011; Rosengaus *et al.* 2011). The reason is that termites keep stable bacterial community within their nest and galleries, which probably help them to suppress pathogens, without any known mechanism behind (Shinzato *et al.* 2005; Visser *et al.* 2012). The use of antimicrobial bacteria might help them considerably. At the same time, the bacteria can also help them to decompose or detoxify the feeding substrate.

In our research for bacterial ectosymbionts, we used the same samples as in the fungal study. We found out, that there are not only termite-specific gut bacterial communities, but also termite-specific bacterial communities in the galleries, both differing from those of intact wood. Interestingly, the bacterial communities of termite galleries are more diverse, than those of termite guts in both, the OTU diversity and the higher taxonomic level diversity. Moreover, the differences between galleries of different termite species and wood control samples are subtler than those found for gut bacterial communities of different termite species, suggesting that the gallery-associated bacteria are rather loosely associated with termites. This raises the possibility that termites established a mutually beneficial relationships with the bacterial communities already associated with their galleries in the absence of strict coevolution between the two partners as is possibly common for many host-symbiont associations (Moran & Sloan 2015), including external symbionts of termites (Chouvenc *et al.* 2013, 2018).

In both studies searching for ectosymbiotic relationships between termites and microbes very important fact was confirmed. Termites are specifically manipulating with the microbial communities of their galleries, as we found out that particular members of the microbial communities were actively suppressed while others were significantly supported by termite activity. Although this is not a proof of symbiosis, it shows that termites vigorously shape the bacterial community of their environment and therefore the efforts for new entomopathogenic microbes in pest biocontrol research are useless, as showed by Chouvenc *et al.* (2011).

Conclusions

My thesis focus on termite ecology and evolutionary history. The goals are mainly to bring the up-to-date knowledge on the progress in termite phylogeny and also the latest progress in termite ecology, which are two parts of the same story. We already know enough, to be sure that there is still much more to be discovered in the "World of Termites". We need to learn how to benefit from the knowledge how termites mitigate the impacts of the ongoing climate change, but also how to use their extraordinary ability to efficiently process any organic matter. Understanding their ecosystem services might help us to develop sustainable agriculture practices in the tropical countries and consequently protect the natural heritage, which is getting under unbearable pressure as we all witness.

Obviously, termites can alter their environment in many ways and on many levels. Thanks to molecular based phylogenetic studies we may estimate the drivers of their evolutionary ecology and test our hypotheses experimentally, as presented in this thesis. I showed how termites evolved and what knowledge gaps we still need to fill in their phylogeny. More importantly, I showed the ability of termites to modify the microbial communities in both, their guts and outer environment. Although further studies are needed, we might be pretty sure that termite external associations with microbial communities are crucial not only to termites, but also to the whole ecosystems and thus to the humankind as well.

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