Evolutionary history of the widespread association between placozoans and diverse *Rickettsiales* symbionts

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Abstract

Rickettsiales are an enigmatic clade of strictly host-associated bacteria, of which some are very common in aquatic habitats. Despite a wealth of sequencing-based observations, the hosts for many of these marine *Rickettsiales* are still unknown. The first molecular data from two closely related placozoan species points to *Rickettsiales* as abundant symbionts in these simple and early-branching animals. Given the electron microscopy observations of intracellular and Rickettsia-like-organisms in all placozoans examined, we systematically characterized the microbiomes of placozoan's with a focus on the *Rickettsiales*. Using single-animal metagenomics, we detected a consistent association with *Rickettsiales* from the *Midichloriaceae* and *Rickettsiaceae*, as well as several other commonly associated bacteria, including representatives from additional intracellular groups such as *Chlamydiota* as well as *Margulisbacteria*. Our comparative phylogenetic analyses of placozoans and their symbiotic *Rickettsiales* revealed a long evolutionary entanglement, with host species-specific replacements with other *Rickettsiales* clades in several placozoans. The common association across all placozoan lineages suggests a beneficial role. As placozoans gain more and more attention as an emerging model across the life sciences, it is imperative to consider the *Rickettsiales* symbionts, as they are commonly known as potent manipulators of their hosts' cellular and organismal biology in other associations.

Introduction

In the bacterial realm, there are several clades that are fully adapted to a host-dependent lifestyle, i.e., members of these clades have only been characterized as symbionts of other organisms. One of these enigmatic clades is the order *Rickettsiales*, alphaproteobacterial symbionts that occur intracellularly across a wide range of host animals. They are generally regarded as parasites and cause a variety of diseases in humans, livestock and wild animals[1,2]. While many terrestrial *Rickettsiales* are well-characterized be it as human and livestock pathogens or as insect symbionts, our understanding of their common association with marine invertebrates remains limited.

A key host group to marine *Rickettsiales* are the placozoa, a phylum of globally distributed, benthic invertebrates that emerged very early in animal phylogeny[3–5] and where all recent lineages appear to be associated with intracellular bacteria. This widespread association between placozoans and intracellular bacteria was initially described from microscopy data and later also found in genomic data that indicated these symbionts are *Rickettsiales*[6–11]. With their very simple and two-layered bauplan that lacks morphological characters, the placozoans were long considered a phylum-of-one. Recent molecular-based taxonomic approaches, first on mitochondrial 16S rRNA genes (haplotypes), and later using comparative genomic analyses of placozoan genomes and the diversity patterns across other early branching metazoans such as Cnidaria and sponges, led to the recognition of four placozoan orders and three families based on four described genera, *Trichoplax*, *Hoilungia, Polyplacotoma, Cladtertia*[3,12]. The linked analysis of mitochondrial haplotypes indicated a large diversity of cryptic species (the haplotypes) that are morphologically indistinguishable within these genera[3,9,13,14]. The molecular data for associated *Rickettsiales* however so far only came from two of the most closely related placozoan lineages (haplotypes H1 and H2), both from the genus *Trichoplax*[11,15]. In H1 and H2, two closely related *Rickettsiales* from the family '*Ca*. Midichloriaceae', '*Ca*. Grellia' and '*Ca.* Aquarickettsia', were identified[9–11]. The nature of the association between these two *Midichloriaceae* symbionts and their placozoan hosts is not fully resolved, as their genomes appear to contain mutualistic and parasitic traits[16].

In this study, we focused on understanding the microbiomes of placozoans with an emphasis on intracellular symbionts and specifically *Rickettsiales*, by systematically sampling metagenomic data across the placozoan tree of life. In total, we analyzed 109 individuals from eight sampling sites, covering eight placozoan species (haplotypes) that represent three of the four major lineages of placozoans[3]. We combined a reconstruction of host phylogenies with a systematic profiling of the general microbiome of each individual, based on reconstruction of full-length 16S rRNA genes. We then focused on the *Rickettsiales* symbionts, analyzed their phylogenetic relations as well as their distribution across host haplotypes and performed last common ancestor reconstructions to recapitulate the evolutionary history between placozoans and *Rickettsiales* symbionts. We found an unexpected diversity with four different clades of *Rickettsiales* symbionts linked to host haplotypes with host population specific association patterns across sites.

Results & discussion

Placozoans are associated with a large diversity of putative symbionts

To describe the microbiome of placozoans across a broad biogeographic host range, we used single animal DNA extractions to generate ultra-low input shotgun DNA libraries from 109 host individuals for eight host mitochondrial 16S rRNA genotypes (host haplotypes) sampled in eight globally distributed locations (Figure 1b, Figure 1c, Table S1). We

identified host haplotypes by reconstructing full-length mitochondrial 16S rRNA gene sequences and inferring phylogenetic relationships between our samples and reference sequences (Figure 1, Figure 2)[3]. For the description of the associated microbiomes, we reconstructed full-length symbiont 16S rRNA genes from the metagenomic data and defined genus-level symbiont clades (clusters) by clustering the sequences at 95% sequence identity. This resulted in 77 genus-level symbiont clusters that we analyzed for their phylogenetic background and relations and for which we estimated the relative abundances in each host individual (Extended Data, Table S2). For the final description of the symbiont community composition, we excluded clusters with two types of distribution patterns: those that are likely human or flow cell contamination based on literature searches, and singletons, i.e., clusters that were only detected a single time across all samples (Table S2). After the singleton and contamination removal, 63 bacterial genus-level clusters remained, and several were commonly associated with placozoan hosts.

By analyzing these 63 clusters using phylogenetic reconstructions of the placozoan phylotypes and their best hits in public databases, we could detect three groups of clusters: i) *Rickettsiales* symbionts (four clusters), ii) other, putative endosymbionts (six clusters, from four bacterial families) and iii) a variety of bacteria for which the lifestyle and association with placozoans remains unclear (53 clusters, 24 bacterial families). Some of these 24 families had a broad geographic or taxonomic range of hosts, but occurred only in few individuals, which might be a sign for a non-specific association, sequencing bias or even contamination (e.g., members of the *Flavobacteriaceae*), others seemed to be specific to certain haplotypes, which could indicate a specific association (the *Teraskiellaceae*). However, fully understanding the association between these clusters and placozoans needs further investigation with more samples, more sites or imaging data. The remaining ten clusters have a very high likelihood of being placozoan endosymbionts with an intracellular lifestyle: we detected two clusters of *Simkaniaceae* and one clade of *Coxiellaceae*, both known to occur solely as intracellular symbionts of diverse eukaryotes[17,18]. In addition, we detected two clusters of *Endozoicomonadaceae* which are common symbionts of marine invertebrates[19–21]. While there is a high chance that these are yet unknown intracellular symbionts of placozoans, this still needs corroboration with microscopy-based detection, e.g., via fluorescence in situ hybridization using taxon-specific probes, as they could also be associated to common food items. In addition, we detected '*Ca*. Ruthmannia' symbionts in three placozoan haplotypes from four locations. The '*Ca*. Ruthmannia' symbionts are members of an enigmatic phylum, the *Margulisbacteria*, and have been previously characterized with genomic, expression and microscopy data in the H2 haplotype[11]. Interestingly, their association with placozoans appears patchy but more common than anticipated[22], as they were repeatedly present across haplotypes and sampling sites. Finally, we also detected four clusters of *Rickettsiales* symbionts, including phylotypes that belong to the previously described '*Ca*. Grellia' and '*Ca*. Aquarickettsia' symbionts[10,11,23]. [11]

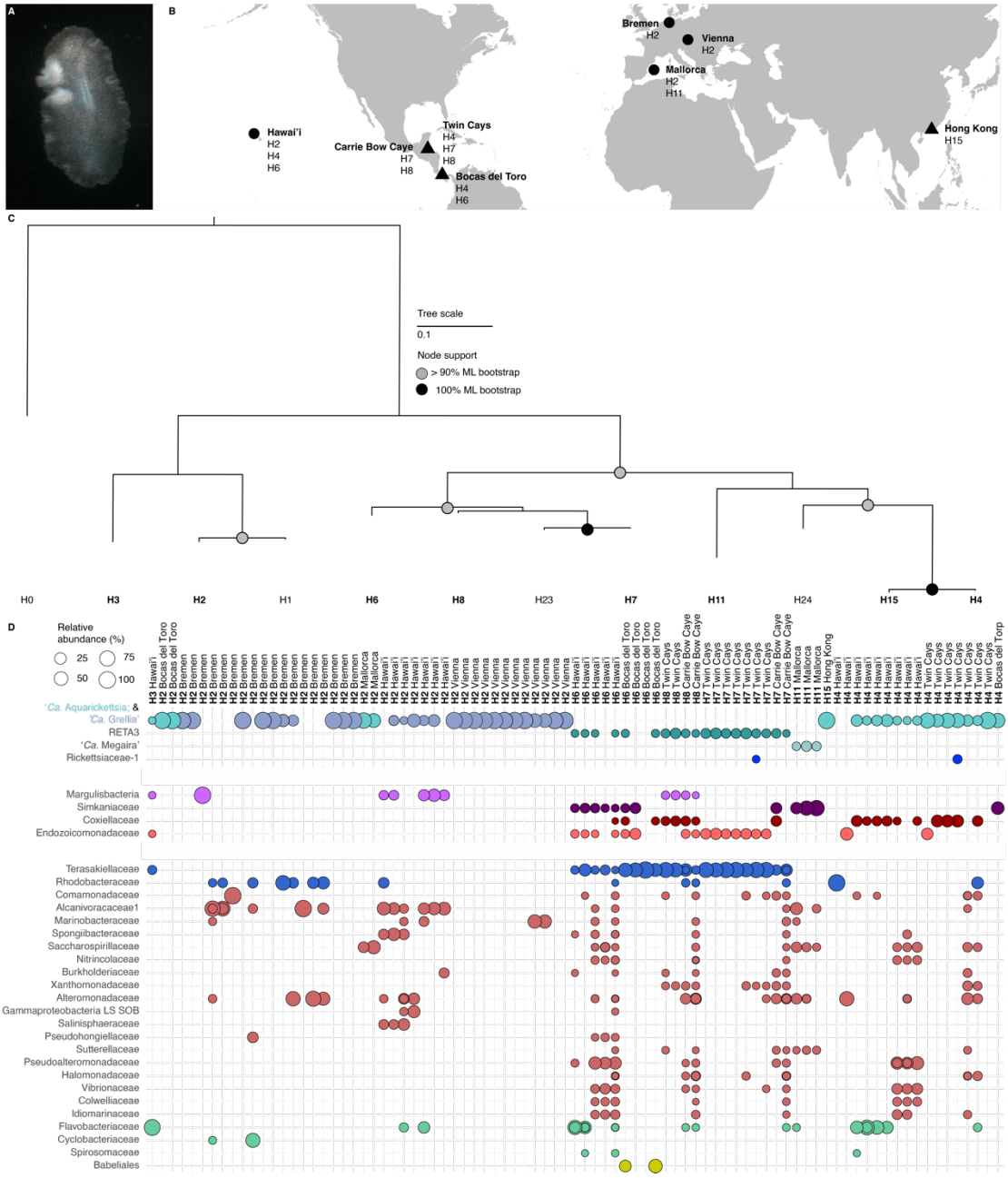

Figure 1 | Placozoan individuals are associated with diverse microbiomes. A: Microscopy image of a H2 individual. B: World map showing the sampling sites of different haplotypes. Samples from aquaria are shown by circles, samples from the natural environment are shown by triangles. C: Maximum-likelihood trees of the host m16S rRNA gene phylogeny including one individual per host haplotype. Haplotypes that were analyzed in this study are highlighted in bold. The scale bar indicates 10% estimated sequence divergence. Nodes with ultrafast bootstrap support > 90% are highlighted. D: Relative abundances of *Rickettsiales* symbionts and families from associated bacteria per host individual as estimated with EMIRGE.

Our microbiome characterization revealed a so far undescribed diversity of bacteria that are associated with placozoans, in varying degrees of specificity and distribution across sites. For many of them, the type of the association remains unclear, but, given their phylogenetic background, ten phylotypes are very likely intracellular endosymbionts. Strikingly, whilst most bacterial representatives of the orders of bacteria that were overall detected were

associated with a few individuals/haplotypes, the association with *Rickettsiales* symbionts is omnipresent across the diversity of placozoan hosts. In addition to the *Rickettsiales*, at maximum two other endosymbiotic groups were detected in a given individual, but it remains to be shown if they overlap in certain cell types, or if they are cell-type specific and do not co-occur, as the '*Ca.* Grellia' and '*Ca.* Ruthmannia' in H2[11].

Diversity, distribution and evolutionary history of *Rickettsiales* symbionts

Given the widespread and consistent association between placozoans and *Rickettsiales* phylotypes, we next explored the hypothesis that the Rickettsiales are linked to patterns of placozoan evolution. To this end we excluded the clade Rickettsiaceae-1 from our downstream analyses, as this phylotype was detected sporadically in only two individuals from two different haplotypes and in relatively low abundance. This is in contrast to the widespread and high abundance association between the placozoans and all other *Rickettsiales* phylotypes. For the remaining and widespread assocations, our clustering of 16S rRNA genes at 95% sequence identity revealed three phylogenetic clusters. The most widespread cluster contained sequences that could be attributed to the described genera '*Ca.* Aquarickettsia' (described from haplotype H1)[15], and '*Ca.* Grellia' (described from haplotype H2)[11]. Given the highly conserved 16S rRNA gene in *Rickettsiales*, the sequences belonging to '*Ca.* Aquarickettsia' and '*Ca.* Grellia' were grouped into one cluster in our genus-level clustering approach at 95% identity, but they are bona-fide genera based on amino acid sequence identities of their genomes[11]. Thus, and given their distinct phylogenetic separation (Figure 2), we will discuss them as separate taxa from here on. Both taxa belong to the *Midichloriaceae* and are a sister clade that forms the sister to '*Ca.* Bandiella'. In addition to the '*Ca.* Aquarickettsia' and the '*Ca.* Grellia' symbionts, we also detected two other clades of *Rickettsiales* symbionts that have not been described in placozoans yet. The first one, here referred to as RETA3 also belongs to the *Midichloriaceae*. The other one does not belong to the *Midichloriaceae*, its members belong to the '*Ca.* Megaira' within the *Rickettsiaceae*. Interestingly, all *Rickettsiales* symbionts that we detected in placozoans are closely related or even phylogenetically intermixed with symbionts of other marine invertebrates, especially corals for which the association with '*Ca.* Aquarickettsia' has been studied extensively (Figure 2)[10,23].

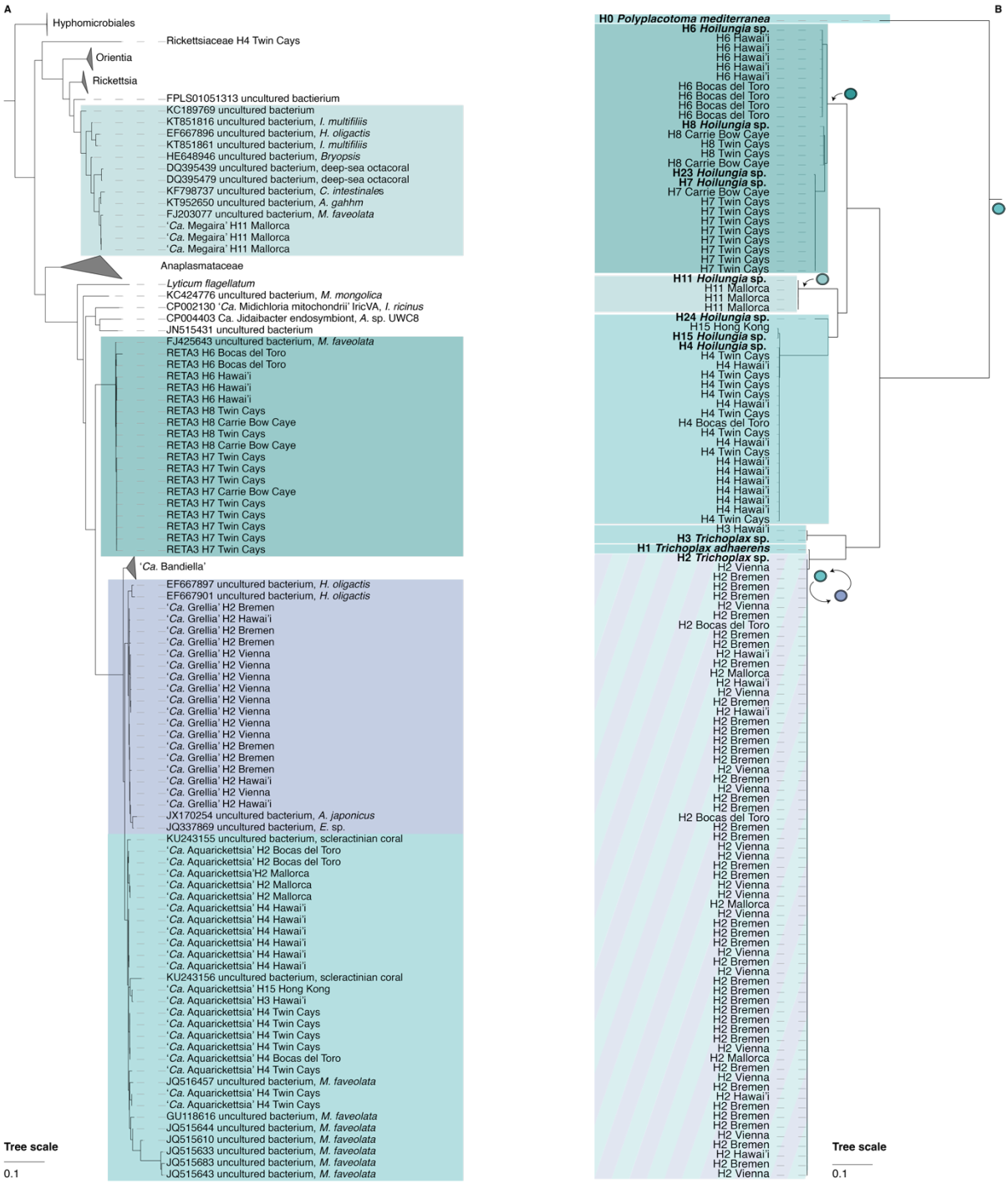

Figure 2 | Phylogeny of placozoan associated *Rickettsiales* and their distribution and evolutionary history across the host diversity. A: 16S rRNA Phylogeny of the *Rickettsiales* symbionts and close relatives. The scale bar indicates 10% estimated sequence divergence. Colored boxed highlight the different *Rickettsiales* symbiont clades. B: m16S rRNA Phylogeny of the host individuals. References are highlighted in bold. The scale bar indicates 10% estimated sequence divergence. Colored boxed highlight the predicted association with the different *Rickettsiales* symbiont clades based on a maximum-likelihood model. Additionally, predicted events of symbiont replacements are marked as the respective nodes.

Strikingly, all haplotypes from all sampling sites and nearly all individuals were associated with a *Rickettsiales* phylotypes. Besides being widespread, the association between the

different *Rickettsiales* clusters appears also highly specific to certain haplotypes and/or sampling locations. Members of '*Ca*. Aquarickettsia' were found in most haplotypes (H1, H2, H3, H4 and H15). Members of '*Ca*. Grellia' were found in distinct populations of the H2 haplotype from Bremen, Vienna and Hawai'i. Members of '*Ca*. Megaira' were distinctly found in H11 individuals and members of the RETA3 clade were found exclusively in the monophyletic clade of H6, H7 and H8 haplotypes. Given such a specific association pattern, we performed last common ancestor modeling in order to get an understanding of how the observed patterns might have evolved. Based on this, '*Ca*. Aquarickettsia' was likely the original symbiont and the association has been inherited to many recent haplotypes (Table S3 and S4). However, it was likely replaced twice in the *Hoilungia* clade, once with '*Ca*. Megaira' in haplotype H11 and once with RETA4 in the monophyletic clade formed by haplotypes H6, H7 and H8. Additionally, it seems, that '*Ca*. Grellia' replace the '*Ca*. Aquarickettsia' in three site specific populations of the H2 haplotype (Bremen, Hawai'i and Vienna), whilst '*Ca*. Grellia' is found in H2 populations from Bocas del Toro and Mallorca. Since the m16S rRNA host phylogeny does not resolve the relations between populations, it remains unclear whether the replacement with '*Ca*. Grellia' happened individually for the three populations or whether there is a shared last common ancestor of these three, that underwent the symbiont replacement and inherited it to the subpopulations. Also, the replacements with the '*Ca*. Megaira' and the RETA3, we cannot be 100% sure that the estimated acquisitions did happen as predicted. At least for the RETA3, it appears that the association is widespread in the clade of haplotypes H6, H7 and H8, which is supporting a symbiont switch at the last common ancestor. The modeled replacement with '*Ca*. Megaira' in haplotype H11 is based on a single population, thus it remains unclear how widespread the association with '*Ca*. Megaira' symbionts actually is. These analyses highlight that the association with *Rickettsiales* is a widespread and likely ancestral feature of the phylum Placozoa.

Conclusion

In this study, we provide the first overview of the placozoan associated microbiome. Besides the previously characterized association with members of the *Rickettsiales* and *Margulisbacteria*, we show that other intracellular symbionts such as *Endozoicomonadaceae*, *Simkaniaceae* and *Coxiellaceae* potentially form intracellular associations with placozoans. Additionally, our data identifies a variety of microbes that are somehow associated with placozoans and it remains to be discovered whether some of these are symbiotic associations.

Besides providing an overview over the diversity of putative placozoan symbionts, we found an abundant, widespread and specific association with *Rickettsiales* symbionts. Based on our reconstructions, this association with a *Rickettsiales* partner is likely an ancestral trait of all modern placozoans. Interestingly, it appears that placozoans could exchange symbionts

with other marine invertebrates as members of all four clades of placozoan symbionts were phylogenetically intermixed with symbionts from other marine invertebrates (Figure 2, Cnidaria, Porifera and Ctenophora)[10,11]. A recent study predicts that the 'Ca. Aquarickettsia' symbionts of corals are horizontally transmitted, which could indicate that they still have a free-living life stage although this might not be viable for longer time frames[11,16,23]. This could be also the source of acquisition and how placozoans picked up the new symbiont. Given likely symbiont switches and the observation that all *Rickettsiales* symbionts of placozoans have been found to reside in the rER lumen [6–8], placozoans could serve as a model to understand how intracellular, and even intra-organelle symbioses are established.

Similar to observations from many corals, we found that the association of placozoans with *Rickettsiales* is extremely widespread. Given such a widespread and potentially ancient association it remains to explored whether these symbionts are classical *Rickettsiales* parasites or whether this association could be of a more commensalistic/mutualistic nature. Genomic and transcriptomic evidence could not clearly answer this question yet, since the genomes of 'Ca. Grellia' and 'Ca. Aquarickettsia' symbionts show mutualistic as well as parasitic traits[10,11,16]. *Rickettsiales* are often characterized as energy parasites that rely on their host for ATP supply[24,25]. In contrast to an energy parasitic lifestyle, the '*Ca.* Grellia' symbionts of placozoans, possess genes for an ADP/ATP translocase, but they do not express it. Instead, they were expressing an ATP-Synthase, which indicates they generate their own pool of ATP[11], suggesting a more benign or commensalistic/mutualistic lifestyle. It however remains unclear whether such a focus on single traits can qualify a parasitic association, given that no overall metabolic budgets for the symbiosis are available. 'Ca. Aquarickettsia' symbionts from corals also encode for an ATPase and likely rely on themselves for ATP generation, rather than taking it from the host[10], however this has yet to be tested with expression data. In addition, it appears that both 'Ca. Grellia' and 'Ca. Aquarickettsia' are auxotroph for amino acids and nucleotides, yet, they might also supplement their hosts diet with some vitamins such as riboflavin that placozoans cannot synthesize themselves[11]. To fully understand the nature of the association, we need more insights into host and symbiont biology and their metabolic interactions.

Placozoa have gained attention as a model system to study central topics in current zoological research, such as metazoan evolution, developmental biology and tissue formation [3,26,27]. Given the diverse microbiomes that are associated with, as well as the likely ancestral, widespread and specific association with *Rickettsiales* symbionts across modern placozoans, these microbial partners should be taken into account in many research approaches. Most *Rickettsiales* are master manipulators of host cellular and organismal biology, and the *Midichloriaceae* with two secretion systems and with a plethora of predicted effectors will be no exception, and e.g., could interfere with early development during sexual reproduction[11,28]. On the other hand, the exceptional association of several

intracellular symbionts with remarkable integration into host cell organelle make placozoans an ideal model to study host-microbe interactions. Here, the available single cell atlas from several lineages of placozoa[29] will provide the host contexts to better understand crucial elements in the biology of the enigmatic intracellular *Rickettsiales* such as how they stabilize their intracellular niches and how they manipulate or support their hosts.

Material & methods

Sample collection, processing and metagenomic sequencing

108 individuals of placozoans were sampled at various field sites (Table S1). Placozoans were identified using a dissection microscope and afterwards kept in culture in 400 ml glass beakers. The culture medium was 34.5‰ artificial seawater and cultures were fed weekly with $2x10^6$ cells ml^{-1} of *Isochrysis galbana*. Cultures were kept at 25°C and a 16:8 hours light:dark cycle. DNA was extracted from single individuals with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions except the following exceptions: the proteinase K digest was performed overnight, elution volumes were halved, all samples were eluted twice reusing the first eluate and all elutions were performed after ten minutes of waiting prior to centrifugation. Library construction, quality control and sequencing were performed at the Max Planck Genome Centre (Cologne, Germany). If needed, DNA concentration was increased using the MinElute PCR Purification kit (Qiagen). Libraries for Illumina sequencing were prepared using the Ovation Ultralow Library Systems Kit (NuGEN) according to the manufacturer's instructions. The libraries were size selected by agarose gel electrophoresis. The quality and quantity of selected fragments were analysed by fluorometry. Paired end reads of 100, 150 or 250 bp length were sequenced on an Illumina HiSeq 4000 sequencer (Table S1). In addition, we included three previously published placozoan metagenomes into our analysis. They were downloaded from the NCBI short read archive (accession numbers SRR5311040, SRR5934055 and SRR5934125).

Assembly of host marker genes, phylogenetic inference and identification of host haplotypes

Mitochondrial 16S rRNA genes of all specimens were assembled by adapting the phyloFlash pipeline to operate on a custom m16S rRNA gene reference database and predict m16S rRNA genes from assembled sequences[30]. The detected m16S gene sequences were aligned to m16S rRNA gene sequences of previously identified specimens using mafft-xinsi v7.407[3,31–33]. Maximum-likelihood based phylogenies were calculated using IQ-TREE, including automatic selection of the best suited model and generation of 1000 ultrafast bootstrap replicates[34]. The sequences of *Trichoplax* H0 was used to root the phylogenies. Host haplotypes were defined based on the phylogenetic relations to the previously identified specimens[3].

Symbiont clade definition and quantification

16S rRNA genes were assembled from the metagenomic libraries using phyloFlash. using the –all option and in addition specifying the read length. For subsequent analyses, we only considered sequences that were i) assembled with SPAdes, ii) longer than 1000 bp and iii) did not contain more than 20 ambiguous bases. The resulting sequences were clustered at 95% sequence similarity using usearch v10.0.240[35]. We used the SINA search and classify algorithm to add the 16S rRNA gene sequences of close relatives from the SILVA database v132 that shared at least 90% sequence similarity for each of our assembled symbiont sequences[36]. All assembled sequences and the SILVA database hits were aligned using mafft-xinsi and a phylogenetic tree was calculated from the resulting alignment using FastTree v2.1.1[37]. The abundances of all cluster were quantified across all metagenomic libraries using EMIRGE v.0.61.1 following the standard workflow for custom EMIRGE databases[38]. Subsequently, we excluded cluster that appear to be contaminations or appeared only once (Table S5) and we merged cluster that were phylogenetically intermixed. After contamination removal and merging of cluster, we normalized the relative abundances of the remaining clades to 100% (Table S2).

Phylogeny of all symbionts and their relatives

Sequences of all initially detected clades were used to obtain sequences from closely related bacteria from the SILVA and the RefSeq public databases[39]. For SILVA, we used the SINA search and classify algorithm to obtain up to 10 relatives for each sequence that shared at least 99% and 95% sequence similarity for each of our input sequences. In addition, we also screened the RefSeq database using BLAST implemented in Geneious v11.1.5 to obtain the ten most similar 16S rRNA genes[40]. Duplicated sequences were removed from the collection of sequences of the symbionts' relatives. The resulting sequence collection was aligned using mafft-xinsi and a phylogenetic tree was calculated using IQ-TREE including automatic selection of the best suited model generation of 1000 ultrafast bootstrap replicates.

Analyses and plotting of symbiont community composition

The analyses of symbiont community composition were performed in R v4.41 unless differently stated. During the analyses, the following packages were used: (https://github.com/sdray/ade4), tidyverse[41], ggplot2 from the tidyverse package, maps (https://www.rdocumentation.org/packages/maps), mapdata (https://www.rdocumentation.org/packages/mapdata).

Last common ancestor analysis of the *Rickettsiales* symbionts

In order to determine the evolutionary history of the association of *Rickettsiales* symbionts with placozoans, were performed with pastml[42]. We used MAP and DOWNPASS prediction methods for a maximum-likelihood and a maximum-parsimony based estimate, respectively.

Data availability

Raw metagenomic sequences as well as host and symbiont marker genes generated in this study will be deposited in the European Nucleotide Archive (ENA) upon peer-review submission and are currently available upon request.

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Author contributions

AM and HRGV conceived the study and AM analyzed data with input from HRGV. HRGV, NL, MGH and BH acquired placozoan specimens and generated metagenomic data. AM analyzed the data and wrote the manuscript together with HRGV, with input from all co-authors.

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