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ORIGINAL ARTICLE

Evolutionary transition in symbiotic syndromes enabled diversification of phytophagous insects on an imbalanced diet

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Evolutionary adaptations for the exploitation of nutritionally challenging or toxic host plants represent a major force driving the diversification of phytophagous insects. Although symbiotic bacteria are known to have essential nutritional roles for insects, examples of radiations into novel ecological niches following the acquisition of specific symbionts remain scarce. Here we characterized the microbiota across bugs of the family Pyrrhocoridae and investigated whether the acquisition of vitamin-supplementing symbionts enabled the hosts to diversify into the nutritionally imbalanced and chemically well-defended seeds of Malvales plants as a food source. Our results indicate that vitamin-provisioning Actinobacteria (*Coriobacterium* and *Gordonibacter*), as well as Firmicutes (*Clostridium*) and Proteobacteria (*Klebsiella*) are widespread across Pyrrhocoridae, but absent from the sister family Largidae and other outgroup taxa. Despite the consistent association with a specific microbiota, the Pyrrhocoridae phylogeny is neither congruent with a dendrogram based on the hosts' microbial community profiles nor phylogenies of individual symbiont strains, indicating frequent horizontal exchange of symbiotic partners. Phylogenetic dating analyses based on the fossil record reveal an origin of the Pyrrhocoridae core microbiota in the late Cretaceous (81.2–86.5 million years ago), following the transition from crypt-associated beta-proteobacterial symbionts to an anaerobic community localized in the M3 region of the midgut. The change in symbiotic syndromes (that is, symbiont identity and localization) and the acquisition of the pyrrhocorid core microbiota followed the evolution of their preferred host plants (Malvales), suggesting that the symbionts facilitated their hosts' adaptation to this imbalanced nutritional resource and enabled the subsequent diversification in a competition-poor ecological niche.
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Introduction

The evolutionary success of herbivorous insects and their diversification into a wide range of ecological niches is closely connected to the diversification of their host plants (Ehrlich and Raven, 1964). Herbivores and plants engage in an evolutionary arms race, with plants continuously evolving novel chemical defenses or imbalanced nutritional composition to reduce herbivore attacks, and insects adapting by developing strategies to overcome defenses and nutritional challenges. Thus, the diversification of terrestrial plants opened up a multitude of ecological niches, permitting the adaptive radiation of herbivorous insects (Farrell and Mitter, 1994). This is probably best exemplified by the coevolution between butterflies and their host plants, with the diversification of several lepidopteran lineages following their adaptation to a particular group of chemically well-defended plants (Ehrlich and Raven, 1964), for example, Pieridae butterflies on their Brassicales host plants (Wheat *et al.*, 2007).

However, host plant selection and exploitation as a nutritional resource are not only determined by the metabolic capabilities of the insects themselves, but also their associated microbiota (Douglas, 2009;

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Hosokawa *et al*., 2007). Microbial symbionts can confer important ecological traits to their hosts, including contributions to digestion (Breznak and Brune, 1994; Warnecke *et al*., 2007; Lundgren and Lehman, 2010), detoxification (Dowd 1989; Genta *et al*., 2006) and nutrient provisioning (Borkott and Insam, 1990; van Borm *et al*., 2002). Consequently, such symbiotic interactions can have a crucial role in the evolutionary diversification of herbivorous insects by facilitating expansion into novel ecological niches (Moran, 2007; Janson *et al*., 2008). Accordingly, expansion of the host plant range and/or an increased diversification have been observed in gall midges after the acquisition of fungal symbionts (Joy, 2013). Furthermore, the replacement of an ancestral beta-proteobacterial symbiont in sharpshooters (Cicadellidae) with *Baumannia*—a symbiont with a comparatively large genome (686 kb) encoding for pathways to produce vitamins and cofactors in addition to amino acids—likely facilitated the shift from phloem sap as the main nutrient source to the even more nutritionally imbalanced xylem sap (Takiya *et al*., 2006). However, despite the wealth of information that is available on the benefits microbes can provide to their insect hosts, the role of symbionts in driving the diversification of insects and their expansion into novel ecological niches remains poorly understood (Janson *et al*., 2008).

Within the megadiverse insect order Hemiptera, the infraorder Pentatomomorpha contains over 12 500 species (Schaefer, 1993; Schuh and Slater, 1995; Henry, 1997), many of which harbor beneficial symbionts that contribute significantly to host fitness (Muller, 1956; Huber-Schneider, 1957; Schorr, 1957; Abe *et al*., 1995; Fukatsu and Hosokawa, 2002; Kikuchi *et al*., 2009; Tada *et al*., 2011; Salem *et al*., 2013). Interestingly, symbiotic syndromes (that is, identity and localization of the symbionts) vary greatly among Pentatomomorpha, indicating frequent transitions during the evolutionary history of this group. The most common symbiont-bearing organs across the superfamilies Lygaeoidea, Coreoidea and Pentatomoidea are specialized sacs or tubular outgrowths, called crypts or gastric ceca, in the posterior region of the midgut that harbor beneficial symbionts belonging to the gamma- or beta-proteobacteria (Glasgow, 1914; Miyamoto, 1961; Buchner, 1965; Fukatsu and Hosokawa, 2002; Prado and Almeida, 2009; Hosokawa *et al*., 2010; Kikuchi *et al*., 2011a,b). However, several other symbiotic syndromes occur across Pentatomomorpha, including paired or unpaired bacteriomes with intracellular symbionts in some Lygaeoidea (Kuechler *et al*., 2012; Matsuura *et al*., 2012), as well as more complex microbial communities in midgut regions devoid of crypts (in Pyrrhocoroidea; Sudakaran *et al*., 2012). Thus, evolutionary transitions in symbiotic syndromes must have occurred repeatedly in Pentatomomorpha. Although such transitions are expected to have major implications for the functionality of the symbioses, the evolutionary consequences of changes in pentatomomorphan symbiotic syndromes remain enigmatic.

Among pentatomomorphan bugs, the Pyrrhocoridae appear to be exceptional with regard to both the localization of the symbionts and the microbiota composition. Previous studies on *Pyrrhocoris apterus* and *Dysdercus fasciatus* revealed that they harbor a distinct and stable microbiota consisting of obligate and facultative anaerobes including Actinobacteria (*Coriobacterium glomerans* and *Gordonibacter* sp.), Firmicutes (*Clostridium* sp.) and Gamma-Proteobacteria (*Klebsiella* sp.). These bacteria are localized in the ventricose region (M3) of the midgut (Haas and König, 1987; Sudakaran *et al*., 2012; Salem *et al*., 2013), which is the main region for the digestion of the ingested food particles (Silva and Terra, 1994; Kodrík *et al*., 2012). Concordantly, the midgut crypts of *Pyrrhocoris apterus* and *Dysdercus fasciatus* are reduced in size and do not contain any symbiotic microbes (Glasgow, 1914; Sudakaran *et al*., 2012). Similar crypt morphologies have been reported for other genera such as *Antilochus* and *Probergrothius*, suggesting that the M3-associated microbial community may be widespread among Pyrrhocoridae (Glasgow, 1914; Rastogi, 1964; Bentz and Kallenborn, 1995; Singh and Singh, 2001; Goel and Chatterjee, 2003). In *Pyrrhocoris apterus* and *Dysdercus fasciatus*, the gut microbiota was found to be vital for growth and survival of the host (Salem *et al*., 2013), through the supplementation of B vitamins by the dual actinobacterial symbionts *C. glomerans* and *Gordonibacter* sp. (Salem *et al*., 2014). As the predominant food source of Pyrrhocoridae, that is, seeds of the plant order Malvales (Kristenová *et al*., 2011), is deficient in B vitamins, this symbiont-mediated nutritional upgrading plays an important role by allowing the hosts to exploit a nutritionally inadequate diet (Salem *et al*., 2013).

In this study, we aimed at elucidating the ecological and evolutionary implications of a major transition in symbiotic syndromes. Specifically, we tested the hypothesis that the evolutionary transition to a characteristic midgut core microbiota enabled the diversification of pyrrhocorid bugs on the nutritionally imbalanced diet of Malvales seeds. To this end, we characterized the microbiota across 25 species of Pyrrhocoroidea (22 Pyrrhocoridae and three Largidae species) through a combination of 454 pyrosequencing and quantitative PCR. In addition, we reconstructed a dated phylogeny of the hosts through calibration with the fossil record and compared it with a distance dendrogram of microbial community profiles, as well as strain-level phylogenies of the two vitamin-provisioning actinobacterial symbionts. The results allow us to assess the distribution of the characteristic M3 midgut microbiota across bugs of the superfamily Pyrrhocoridea and to identify the evolutionary origin of this symbiotic syndrome. Subsequently, a comparison with the age of Malvales plants allowed us to test the

hypothesis that the acquisition of a specific microbiota preceded the bugs' diversification on this nutrient-deficient food source. Furthermore, host–symbiont co-cladogenetic analyses shed light on the evolutionary stability and maintenance of the characteristic core microbiota in Pyrrhocoridae. Taken together, the results provide novel insights into the evolutionary transitions and ecological relevance of symbiotic microbial communities in the diverse insect order Hemiptera.

Materials and methods

Insect sample collection and DNA extraction
For characterizing the microbial community and reconstructing symbiont and host phylogenies across the Pyrrhocoroidea superfamily and outgroup taxa, live adult specimens of Pyrrhocoridae (22), Largidae (3), Lygaeidae (2), Oxycarenidae (1) and Rhopalidae (1) were collected from their respective habitats across four different continents (Supplementary Table S1). Bugs were killed and preserved in 70% ethanol until further analysis, and at least one individual per species was kept in ethanol as a voucher specimen. Before DNA extraction, samples were surface sterilized by rinsing with sterile Millipore water, 1% sodium dodecyl sulfate, and then again sterile Millipore water (Billerica, MA, USA). Up to six complete specimens per bug species (or fewer, if less than seven individual specimens were available), were homogenized under liquid nitrogen with sterile pestles. For the Japanese bug specimens, however, only the already dissected midgut was available and used instead of whole individuals. DNA was extracted using the MasterPure DNA Purification Kit (Epicentre Technologies, Madison, WI, USA) according to the manufacturer's instructions. An additional lysozyme incubation step (30 min at 37 °C; 4 µl of 100 mg ml^{-1} lysozyme, Sigma-Aldrich, St Louis, MO, USA) was included before proteinase K digestion to break up Gram-positive bacterial cells (see Sudakaran *et al.,* 2012). Individual extracts were used for quantitative PCR (qPCR) analysis, as well as for PCR and sequencing of host and symbiont genes for phylogenetic analysis. Pooled DNA extracts from each species were used for 454 pyrosequencing of the associated bacterial communities.

Reconstruction of the host phylogeny
The phylogenetic relationships among the Pyrrhocoridae, its sister family Largidae and outgroup taxa were reconstructed using PCR amplification and sequencing of two mitochondrial (cytochrome oxidase I and II) and one nuclear gene (18S ribosomal RNA (rRNA)) for all host species using primers listed in Table 1. PCR was performed in a total reaction volume of 12.5 µl, containing 1 µl of template DNA, 1 × PCR buffer (20 mM Tris-HCl, 16 mM $(NH_4)_2SO_4$ and 0.01% Tween 20), 2.5 mM $MgCl_2$, 240 µM dNTPs, 0.8 µM of each primer and 0.5 U of Taq DNA polymerase (VWR International GmbH, Darmstadt, Germany). Cycle parameters were as follows: 3 min at 94 °C, followed by 35 cycles of 94 °C for 40 s, 55 °C for 40 s and 72 °C for 40 s, and a final extension step of 4 min at 72 °C. PCR products were then sequenced bidirectionally on an ABI 3730xl capillary DNA sequencer (Applied Biosystems, Foster City, CA, USA). Protein-coding sequences (COI and COII) were curated and then aligned based on their amino-acid translation in Geneious Pro 5.4 (Biomatters, Auckland, New Zealand), whereas partial 18S rRNA sequences were aligned using the SINA aligner (Pruesse *et al.*, 2012). The individual alignments were concatenated and used for phylogenetic reconstruction with maximum likelihood algorithms (ML) and Bayesian Inference (BI), respectively. An ML tree was computed with FastTree 2.1 using the GTR model, and local support values were estimated with the Shimodaira–Hasegawa test based on 1000 resamplings without reoptimizing the branch lengths for the resampled alignments (Price *et al.*, 2010). For BI (computed using MrBayes 3.1.2; Huelsenbeck and Ronquist, 2001), the data set was partitioned into the three genes, with six substitution types for the CO genes (GTR model), and one for the ribosomal gene (F81 model). Owing to saturation in substitutions, third codon positions were excluded from the analysis for the two mitochondrial genes (COI and COII). The analysis was performed with four chains and a temperature of 0.2 for 10 000 000 generations, and we confirmed that the standard deviation of split frequencies was consistently below 0.01. Trees were sampled every 1000 generations, and a 'burn-in' of 1000 was used (= 10%). We computed a 50% majority rule consensus tree with posterior probabilities for every node.

Dating of the host phylogeny
Divergence time estimations for the Pyrrhocoroidea superfamily were inferred using BEAST v1.8.0 (Drummond and Rambaut, 2007), by testing various substitution models and parameter settings (see Supplementary Table S2 and S3) on a fixed input tree (the BI tree, see above). Two fossil calibration points were used: (i) two *Dysdercus* fossils from Florissant beds in Colorado (37.0–33.1 million years ago (mya)) (Scudder, 1890), and (ii) a *Pyrrhocoris tibialis* fossil from Rott-am-Siebengebirge in Germany (28.5–23.8 mya) (Statz and Wagner, 1950). A hard upper boundary for the age of the root was set to 160 mya, based on to the age of the oldest Pentatomomorpha fossil, as well as the estimated age of the Pentatomomorpha infraorder (~152.9 mya, Upper Jurassic) (Li *et al.*, 2012; Misof *et al.*, 2014). Evaluation and comparison of model parameters were performed using Tracer v1.5 (Drummond and Rambaut, 2007). The maximum

Table 1 Primers used for the characterization (PCR, cloning/sequencing, 454 pyrosequencing) and quantification (qPCR) of Pyrrhocoroidea and their associated microbiota

Target	*Target taxon*	*Target gene*	*Primer name*	*Primer sequence (5′–>3′)*	*Fwd./rev.*	*Length*	*Use*	*Reference*
Hosts	Heteroptera	18S rRNA	Pyr18S_2F	GGGAGGTAGTGACAAAAAATAACG	Fwd.	24	1	Li *et al.*, 2005
	Heteroptera	18S rRNA	Pyr18S_4F	ATCCTTTAACGAGGATCTATTGG	Fwd.	23	1	Li *et al.*, 2005
	Heteroptera	18S rRNA	Pyr18S_3R	ACATACTTGGCAAATGCTTTCGC	Rev.	23	1	Li *et al.*, 2005
	Heteroptera	18S rRNA	Pyr18S_4R	GTTAGAACTAGGGCGGTATCTG	Rev.	22	1	Li *et al.*, 2005
	Heteroptera	COI	C1-J-2183-F	CAACATTTATTTTGATTTTTTGG	Fwd.	23	1	Li *et al.*, 2005
	Heteroptera	COI	TL2-N-3014-R	TCCAATGCACTAATCTGCCATATTA	Rev.	25	1	Li *et al.*, 2005
	Heteroptera	COI	C1-J-2530-F	GGAGTAATTCTAGCCAACTC	Fwd.	20	1	Li *et al.*, 2005
	Heteroptera	COI	C1-N-2609-R	GAATACTGCTCCTATGGATA	Rev.	20	1	Li *et al.*, 2005
	Heteroptera	COII	TK-N3796_rev	ACTATTAGATGGTTTAAGAG	Rev.	18	1	Simon *et al.*, 1994
	Heteroptera	COII	TL-J3033_fwd	TCTAATATGGCAGATTAGTGCA	Fwd.	20	1	Simon *et al.*, 1994
Symbionts	*Coriobacterium*/*Gordonibacter*	16S rRNA	Cor-2F	GGTAGCCGGGTTGAGAGACC	Fwd.	20	2	Kaltenpoth *et al.*, 2009
	Eubacteria	16S rRNA	rP2	ACGGCTACCTTGTTACGACTT	Rev.	21	2	Weisburg *et al.*, 1991
	Eubacteria	16S rRNA	M13F	TGTAAAACGACGGCCAGT	Fwd.	18	2	Boutin-Ganache *et al.*, 2001
	Eubacteria	16S rRNA	M13R	GGAAACAGCTATGACCATG	Rev.	19	2	Boutin-Ganache *et al.*, 2001
	Clostridium	16S rRNA	Clostridium_1050-fwd	CTCGTGTCGTGAGATGTTGG	Fwd.	20	3	Sudakaran *et al.*, 2012
	Clostridium	16S rRNA	Clostridium_1248-rev	GCTCCTTTGCTTCCCTTTGT	Rev.	20	3	Sudakaran *et al.*, 2012
	Klebsiella	16S rRNA	Klebsiella_250-fwd	CAGCCACACTGGAACTGAGA	Fwd.	20	3	Sudakaran *et al.*, 2012
	Klebsiella	16S rRNA	Klebsiella_453-rev	GTTAGCCGGTGCTTCTTCTG	Rev.	20	3	Sudakaran *et al.*, 2012
	Gordonibacter	16S rRNA	D.fas_Egg.2R_qPC	CCGTATCTCAGTCCCAATGT	Rev.	20	3	This study
	Gordonibacter	16S rRNA	Act-2F	GCGAACGGGTGAGTAACAC	Fwd.	19	3	This study
	Coriobacterium	16S rRNA	Gray519F	CAGCMGCCGCNGTAANAC	Fwd.	18	3	This study
	Coriobacterium	16S rRNA	Cor-1R	ACCCTCCCMTACCGGACCC	Rev.	19	3	Kaltenpoth *et al.*, 2009
	Eubacteria	16S rRNA	Gray28F	GAGTTTGATCNTGGCTCA	Fwd.	19	4	Ishak *et al.*, 2011
	Eubacteria	16S rRNA	Gray519R	GTNTTACNGCGGCKGCTG	Rev.	18	4	Ishak *et al.*, 2011

Abbreviations: Fwd., forward; qPCR, quantitative PCR; Rev., reverse; rRNA, ribosomal RNA.
Use: (1) amplification and sequencing of host genes, (2) cloning/sequencing of Coriobacteriaceae symbionts, (3) qPCR and (4) 454 sequencing.

clade credibility (consensus) tree was inferred with TreeAnnotator using a burn-in of 5,000 and a posterior probability limit of 0.5 (Drummond and Rambaut, 2007). The consensus tree was visualized with FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/), including highest posterior density (HPD) intervals.

Characterization of microbial community profiles
Bacterial tag-encoded FLX amplicon pyroseqencing (bTEFAP) was performed to characterize the microbial community composition of members belonging to Pyrrhocoridae, Largidae and outgroup taxa. DNA was sent to external service providers (Research & Testing Laboratories, Lubbock, TX, USA, or MR DNA Lab, Shallowater, TX, USA), and amplification was achieved using the 16S rRNA primers Gray28F and Gray519R (Table 1) (Ishak *et al.*, 2011, Sun *et al.*, 2011). Sequencing libraries were generated through one-step PCR with 30 cycles, using a mixture of Hot Start and HotStar high-fidelity *Taq* polymerases (Qiagen, Valencia, CA, USA). Sequencing extended from Gray28F, using a Roche 454 FLX instrument (Branford, CT, USA) with Titanium reagents and procedures. All low-quality reads (quality cut-off = 25) and sequences < 200 bp were removed following sequencing, which left between 1990 and 30 361 sequences per sample for subsequent analysis.

Processing of the high-quality reads was performed using QIIME (Caporaso *et al.*, 2010b). Sequences were clustered into operational taxonomic units (OTUs) using multiple OTU picking in combination with chimera checking using usearch (Edgar, 2010) followed by cdhit (Fu *et al.*, 2012) with 97% similarity cut-offs. For each OTU, one representative sequence was extracted (the most abundant) and aligned to the Greengenes core set (available from http://greengenes.lbl.gov/) using PyNast (Caporaso *et al.*, 2010a), with the minimum sequence identity set to 75%. Taxonomy was assigned using the Ribosomal Database Project (RDP) classifier (Wang *et al.*, 2007), with a minimum confidence to record an assignment set to 0.80. An OTU table was generated describing the abundance of bacterial phylotypes within each sample (Supplementary Table S4). The table was then manually curated by removing low-abundance OTUs (< 0.1% in each of the samples) and through BLASTn of the representative sequences (see Supplementary Data S1) against the NCBI and RDP databases. To visualize the results, OTUs with the same genus-level assignments were combined based on the BLASTn results. The genus-level table was used to construct heatmaps using the R package 'gplots (heatmap.2)'. For beta-diversity analysis and UPGMA clustering, the raw OTU table was subsampled to the depth of 1500 sequences per sample, and distance matrices were calculated using Bray–Curtis and Jaccard metrics. For visualization, two-dimensional principal coordinate analysis plots and dendrograms based on UPGMA clustering were constructed based on the beta diversity distance matrices.

Quantification of core microbes
qPCRs were performed for the four dominant bacterial symbionts in Pyrrhocoridae (*Coriobacterium glomerans*, *Gordonibacter* sp., *Clostridium* sp. and *Klebsiella* sp.), using specific 16S rRNA primers (Table 1) on a RotorgeneQ cycler (Qiagen, Hilden, Germany) in final reaction volumes of 25 µl, containing 1 µl of template DNA (usually a 1:10 dilution of the original DNA extract), 2.5 µl of each primer (10 µm) and 12.5 µl of SYBR Green Mix (Rotor-Gene SYBR Green kit, Qiagen). Standard curves were established using 10^{-8}–10^{-2} ng of specific PCR product as templates for the qPCR. A NanoDrop 1000 spectrophotometer (Peqlab Biotechnology Limited, Erlangen, Germany) was used to measure DNA concentrations for the templates of the standard curve. PCR conditions were as follows: 95 °C for 5 min, followed by 35 cycles of 60 °C for 30 s, 72 °C for 20 s and 95 °C for 15 s; then a melting curve analysis was performed by increasing the temperature from 60 °C to 95 °C within 20 min. The efficiencies of all four quantitative PCR assays were confirmed to be > 99%. Based on the standard curves, the 16S copy numbers of the four dominant symbionts could be calculated for each individual bug from the qPCR threshold values (Ct) by the absolute quantification method (Lee *et al.*, 2006, 2008), taking the dilution factor and the absolute volume of DNA extract into account. The absolute 16S copy numbers were log transformed and then used to visualize the quantitative differences of the bacterial symbionts across different host genera using box plots.

Symbiont (Coriobacteriaceae) strain-level phylogenies
In order to reconstruct strain-level phylogenies for the two actinobacterial symbionts *Coriobacterium* and *Gordonibacter*, we followed two different strategies, based on (i) the bTEFAP sequencing data alone, and (ii) a combination of bTEFAP sequences and Sanger sequencing of cloned 16S rRNA amplicons. For the first approach, OTUs were picked individually for each host species, using the parameters as described above. This was necessary to conserve differences among symbiont strains that were below 3% sequence divergence, which would be lost in the combined OTU picking strategy used for assessing the general microbial community composition in Pyrrhocoridae. For each OTU, the longest representative sequence was extracted, and sequences affiliated with the family Coriobacteriaceae were extracted after RDP and BLAST classification (see Supplementary Data S2). The resulting representative sequences were aligned to reference sequences of all Coriobacteriaceae type strains obtained from the RDP (Cole *et al.*, 2014) using the SINA aligner (Pruesse *et al.*, 2012), and phylogenetic relationships were computed using ML as described for the reconstruction of the host phylogeny.

For the second approach, the bTEFAP data were complemented by a cloning/sequencing approach in order to obtain longer and hence more informative

reads for phylogenetic analysis. For this purpose, PCR amplifications of the 3′-region of the 16S rRNA of both Coriobacteriaceae symbionts (that is, *Coriobacterium glomerans* and *Gordonibacter* sp.) were carried out with the primers Cor-2F and rP2 using a Biometra thermocycler (Analytik Jena, Jena, Germany) in total reaction volumes of 12.5 µl containing 1 µl of template DNA, 1 × PCR buffer (20 mM Tris-HCl, 16 mM $(NH_4)_2SO_4$ and 0.01% Tween 20), 2.5 mM $MgCl_2$, 240 µM dNTPs, 0.8 µM of each primer and 0.5 U of Taq DNA polymerase (VWR International GmbH). Cycle parameters were as follows: 3 min at 94 °C, followed by 35 cycles of 94 °C for 40 s, 68 °C for 40 s and 72 °C for 40 s, and a final extension step of 4 min at 72 °C. PCR products were cloned into *Escherichia coli* using the StrataClone PCR Cloning Kit (Stratagene, Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. Transformed *E. coli* cells were grown on LB agar containing 10 mg ml^{-1} ampicillin and 2% 5-bromo-4-chloro-indolyl-β-d-galactopyranoside (X-gal) (Sigma-Aldrich) for blue/white screening. Colony PCR was performed on eight randomly selected transformants for each insect host with vector primers M13F and M13R (Table 1) using the above-mentioned reaction mix and cycling conditions, except that an annealing temperature of 55 °C was used. PCR products were checked for the expected size on a 1.5% agarose gel (130 V, 30 min) and purified using the peqGOLD MicroSpin Cycle Pure Kit (Peqlab Biotechnologies GmbH, Erlangen, Germany) before sequencing with M13 primers. Nearly full-length *Coriobacterium glomerans* and *Gordonibacter* sp. 16S rRNA sequences from different Pyrrhocoridae were obtained by combining the short sequences from OTUs picked for each individual species with bTEFAP and the sequences obtained by PCR/cloning for the respective OTUs. In cases with multiple *Coriobacterium* (for *Scanthius aegypticus, Scanthius obscurus, Pyrrhocoris apterus, Pyrrhocoris sibiricus* and *Dysdercus fasciatus)* or *Gordonibacter* OTUs (for *Scanthius aegypticus* and *Dysdercus fasciatus*) per host species, the most abundant OTU was chosen, and the identity of bTEFAP and cloned sequences was confirmed in the overlapping region to reduce the risk of chimera formation. Sequence alignment and phylogenetic tree reconstruction using ML and BI were done as described above.

Cophylogenetic analysis of host and symbiont

To test for codiversification between hosts and their Coriobacteriaceae symbionts, the phylogenies of the two Coriobacteriaceae symbionts (based on the bTEFAP data alone or the combination with cloning/sequencing data) were compared with the host phylogeny using Treemap 3 (Page RDM, 1995) and Parafit (Legendre *et al.*, 2002). In Treemap, the positions of taxa were randomized on the host and symbiont trees (1000 replicates), and the number of observed codiversification events was compared with the resulting distribution of codiversification events in the randomized data set. For Parafit analysis, a host distance matrix was computed using the R package 'Ape (cophenetic.phylo)' based on the phylogenetic tree, and symbiont distance matrices were computed in BioEdit 7.0.5.3 (Hall, 1999) based on the concatenated alignments. Permutation tests (1000 replicates) were run as implemented in ParaFit (Legendre *et al.*, 2002). In order to assess the possible obscuring effect of interspecific predation on co-phylogenetic patterns, we repeated the analyses after omission of known carnivorous host taxa (*Antilochus* spp., *Dindymus lanius*) that may have acquired the Coriobacteriaceae symbionts horizontally via feeding on heterospeficic pyrrhocorid bugs.

Results

Host phylogeny and divergence time estimates

To elucidate the evolutionary origin of the Pyrrhocoridae–microbiota association, the phylogenetic relationships across bugs of the Pyrrhocoroidea superfamily were reconstructed. The combination of partial 18S rRNA, COI and COII gene sequences resolved most of the taxonomic relationships within the Pyrrhocoroidea (Figure 1b and Supplementary Figure S1), and divergence time estimations based on two fossil calibration points and a hard lower boundary for the root age yielded consistent age estimates for selected nodes of interest across a range of different substitution models (GTR+I+G, HKY+G, HKY+I+G, TN93+G, TN93 +I+G) and parameter settings (Supplementary Table S2 and S3). Omitting the *Pyrrhocoris tibialis* fossil calibration point did not affect age estimates, whereas omitting either the *Dysdercus* fossil calibration or the root boundary resulted in significantly increased age estimates (Supplementary Table S3). Based on the tracer analysis of effective sample sizes and marginal likelihood values, the TN93+I+G model with two codon partitions for the protein-coding genes (1+2, and 3), estimated base frequencies, and a relaxed uncorrelated lognormal clock model yielded the most robust results.

The phylogenetic analyses revealed an estimated age of 135.7 mya for the superfamily Pyrrhocoroidea (95% HPD interval: 104.4–159.9 mya, Figure 1b). The families Pyrrhocoridae and Largidae formed monophyletic sister taxa that split about 125.4 mya (95% HPD interval: 92.9–154.6 mya; Figure 1b). Within the Pyrrhocoridae family, the genus *Probergrothius* diverged from the common ancestor of all other taxa about 86.5 mya (95% HPD interval: 63.1–109.3 mya). Around 81.2 mya (95% HPD interval: 58.4–102.2 mya), the clade *Dindymus+Antilochus* split from the group comprising *Dysdercus, Dermatinus, Scanthius, Pyrrhocoris,* the unknown pyrrhocorid taxon, and *Cenaeus*.

Microbial community composition of pyrrhocorid bugs

The microbiota of several species of Pyrrhocoridae ($n = 22$) and Largidae ($n = 3$), as well as outgroup taxa

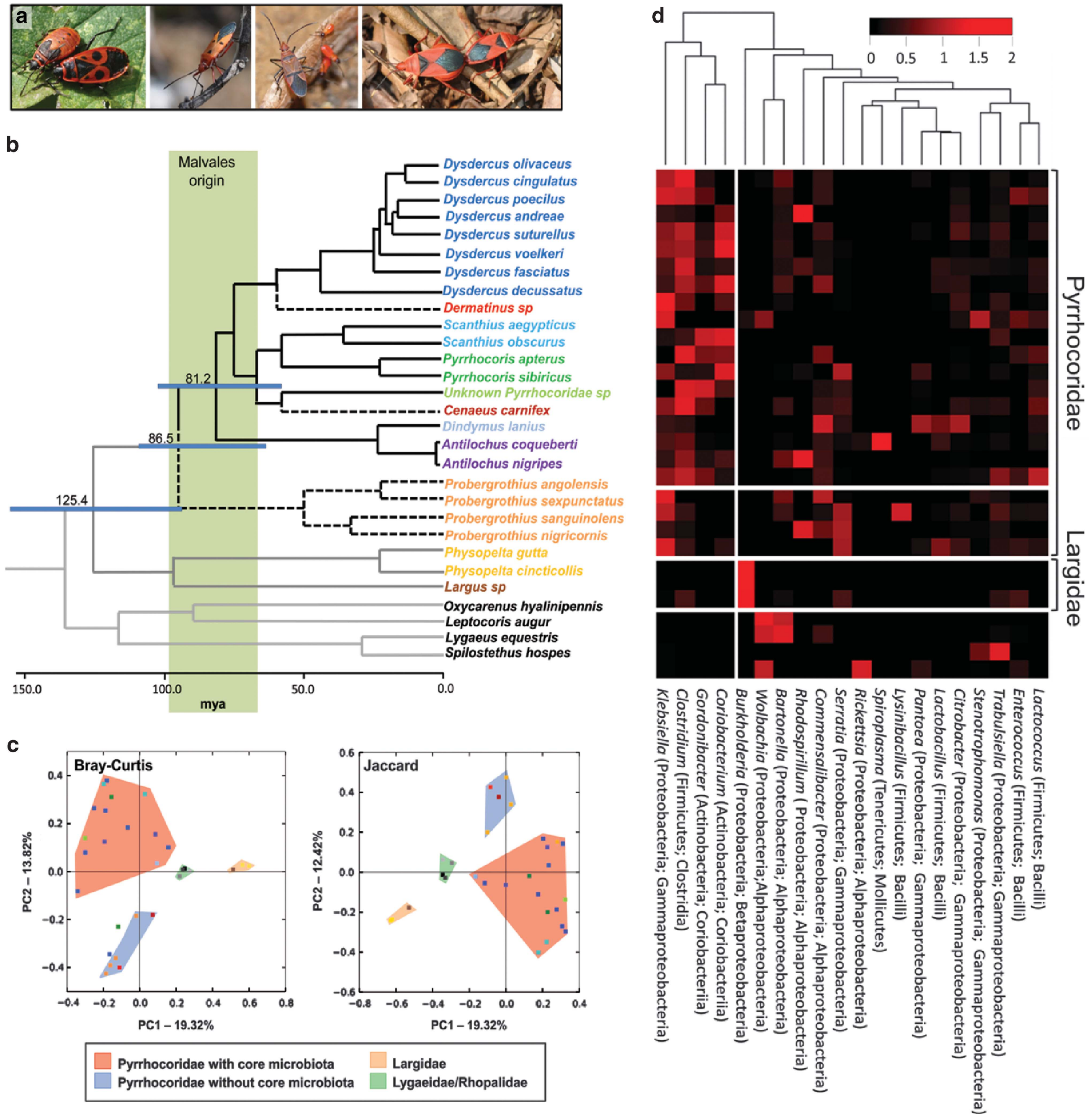

Figure 1 Dated host phylogeny and microbiota profile of 22 species within the family Pyrrhocoridae as well as outgroups (Largidae, Lygaeidae, Oxycarenidae and Rhopalidae). (**a**) Photographs of selected Pyrrhocoridae host species: adult and fifth instar nymph of *Pyrrhocoris apterus*, adult *Dysdercus cingulatus*, adult and nymphs of *Dysdercus fasciatus,* and a mating pair of *Probergrothius sanguinolens* (from left to right). (**b**) Phylogenetic relationships of the hosts (Pyrrhocoridae $n = 22$, Largidae $n = 3$, Lygaeidae $n = 2$, Oxycarenidae $n = 1$, Rhopalidae $n = 1$ species), reconstructed using partial 18S rRNA, cytochrome oxidase I and cytochrome oxidase II gene sequences. Divergence time estimates were derived using BEAST analyses (TN93+I+G model). Selected node ages are shown in mya with 95% HPD interval bars. Dashed branches represent pyrrhocorid taxa without the characteristic core microbiota. The green colored bar indicates the estimated origin of the host plant order Malvales (72–96 mya) (Wang *et al.*, 2009). (**c**) 2D Principal Coordinate Analysis (PCoA) showing the clustering of host species based on their microbial community profiles using Bray–Curtis (left) and Jaccard (right) distance matrices, respectively. Colors for individual samples correspond to the coloring of taxa in **b**. (**d**) Relative abundance of microbial taxa as obtained from 454 pyrosequencing of 16S rRNA amplicons (305,179 reads in total), represented as a heatmap based on log-transformed values. OTUs were combined on the genus level for better visualization, and only genera that amount to >1% of the microbial community in at least one of the host species are displayed. The dendrogram above the heatmap represents the clustering of microbial taxa according to their distribution and abundance across host species. Note the distinct clustering of the four core microbial taxa associated with Pyrrhocoridae.

($n = 4$) were characterized using 454 amplicon pyrosequencing of bacterial 16S rRNA (bTEFAP), which yielded a total of 305 179 sequences. After removing singletons, chimeric sequences and OTUs below 0.1% abundance, the sequences were clustered into 356 OTUs. Bray–Curtis and Jaccard clustering of the host species based on their bacterial community profiles revealed a well-defined cluster containing the genera *Dysdercus*, *Scanthius*, *Pyrrhocoris*, *Dindymus, Antilochus* and the unknown Pyrrhocoridae species, a second cluster with *Probergrothius*, *Dermatinus* and *Cenaeus*, and separate clusters for members of the Largidae family and the outgroup composed of other Pentatomomorphan bugs (*Oxycarenus hyalinipennis*, *Leptocoris augur*, *Lygaeus equestris* and *Spilostethus hospes*), respectively (Figure 1c). UPGMA dendrograms of the bacterial communities associated with the host species computed using the beta diversity matrices (Bray–Curtis and Jaccard) yielded qualitatively similar topologies with the major groupings being largely identical (Supplementary Figure S2).

Combining OTUs on the genus level revealed that the microbiota of Pyrrhocoridae bugs was dominated by four core bacterial taxa: *Coriobacterium glomerans, Gordonibacter* sp. (Actinobacteria), *Clostridium* sp. (Firmicutes) and *Klebsiella* sp. (Proteobacteria) (Figure 1d). These taxa were consistently present across Pyrrhocoridae in abundances ranging from 10^4 to 10^8 16S rRNA gene copies per individual (Figure 2), with the exception of the genera *Probergrothius*, *Dermatinus* and *Cenaeus,* which lacked the Coriobacteriaceae symbionts (Figures 1d and 2). Furthermore, although OTUs associated with the genera *Clostridium* and *Klebsiella* were present in most species of these three host genera, qPCR assays specific for the Pyrrhocoridae-associated *Clostridium* and *Klebsiella* OTUs were negative for all samples except two of the *Probergrothius* specimens, indicating that the *Clostridium* and *Klebsiella* OTUs associated with these three genera differ from those of the other Pyrrhocoridae (Figure 2). Thus, the host genera *Probergrothius*, *Dermatinus* and *Cenaeus* lacked the microbiota that is characteristic for other Pyrrhocoridae, which is also reflected in their separate clustering in the principal coordinate analyses (Figure 1c).

The microbiota of members of the family Largidae (the sister taxon to the Pyrrhocoridae) was dominated by *Burkholderia* and completely lacked the Pyrrhocoridae-associated core microbes (Figures 1d and 2). Similarly, the core microbiota was absent from other pentatomomorphan outgroup species: the microbiota of both *Oxycarenus hyalinipennis* (Oxycarenidae) and *Leptocoris augur* (Rhopalidae) was dominated by *Wolbachia* sp. and *Bartonella* sp., whereas the Lygaeidae species *Lygaeus equestris* and *Spilostethus hospes* contained consortia of *Trabulsiella* sp. and *Stenotrophomonas* sp. *(L. equestris),* or *Wolbachia* sp. and *Rickettsia* sp. *(S. hospes)*, respectively.

Phylogenetic analysis of the Coriobacteriaceae symbionts

The Pyrrhocoridae core microbiota contains two actinobacterial symbionts that were previously shown to be essential for growth and survival in *P. apterus* and *D. fasciatus* through the supplementation of B vitamins (Salem *et al.*, 2013, 2014). The phylogeny of both Coriobacteriaceae symbionts was reconstructed based on the set of short read sequences from bTEFAP representing the Coriobacteriaceae symbiont OTUs from each pyrrhocorid species (Figure 3). In addition, we amplified and sequenced the symbionts' 3'-region of the 16S rRNA gene sequences from 13 different host species, as well as only *Coriobacterium glomerans* from *Dysdercus decussatus,* and only *Gordonibacter* sp. from two *Probergrothius* species (that is, *P. nigricornis* and *P. sanguinolens*). Subsequently these sequences were combined with the bTEFAP representative sequences to enhance the resolution of the phylogenetic tree (Supplementary Figure S3). The phylogenetic analyses revealed that the symbiotic *Coriobacterium glomerans* and *Gordonibacter* sp. strains form two distinct monophyletic clades within the family Coriobacteriacae, which is consistent with a single acquisition event for each symbiont and subsequent host–symbiont coevolution (Figure 3 and Supplementary Figure S3). A possible exception are the *Gordonibacter* symbionts of the basal pyrrhocorid genus *Probergrothius*, which group within the monophyletic symbiont cluster in the combined phylogeny (Supplementary Figure S3), yet fall outside when only the bTEFAP sequences are considered (Figure 3). Interestingly, several host species contained two or more dominant OTUs for one or both of the actinobacterial symbionts. Specifically, more than one *Coriobacterium* OTU was observed for *Scanthius aegypticus, Scanthius obscurus, Pyrrhocoris apterus, Pyrrhocoris sibiricus* and *Dysdercus fasciatus*, whereas two or more *Gordonibacter* OTUs were detected for *Scanthius aegypticus* and *Dysdercus fasciatus*. For *Pyrrhocoris apterus* and *Dysdercus fasciatus*, the occurrence of two distinct *Coriobacterium* sequences, respectively, was previously confirmed using cloning and sequencing (Kaltenpoth *et al.*, 2009). Thus, the multiple *Coriobacterium* and *Gordonibacter* OTUs observed here for several host species likely reflect true symbiont microdiversity rather than 454 sequencing artifacts. Although at present we cannot exclude the possibility that the multiple Coriobacteriaceae sequences found in individual Pyrrhocoridae species represent divergent 16S rRNA copies within the same symbiont genome, the presence of multiple distinct strains seems much more likely given the high degree of similarity of the two 16S rRNA copies (99.72%) in the sequenced genome of *C. glomerans* isolated from *P. apterus* (Stackebrandt *et al.*, 2013).

In addition to the occurrence of multiple OTUs within individual host species, the incongruence of the phylogenies of both Coriobacteriaceae symbionts

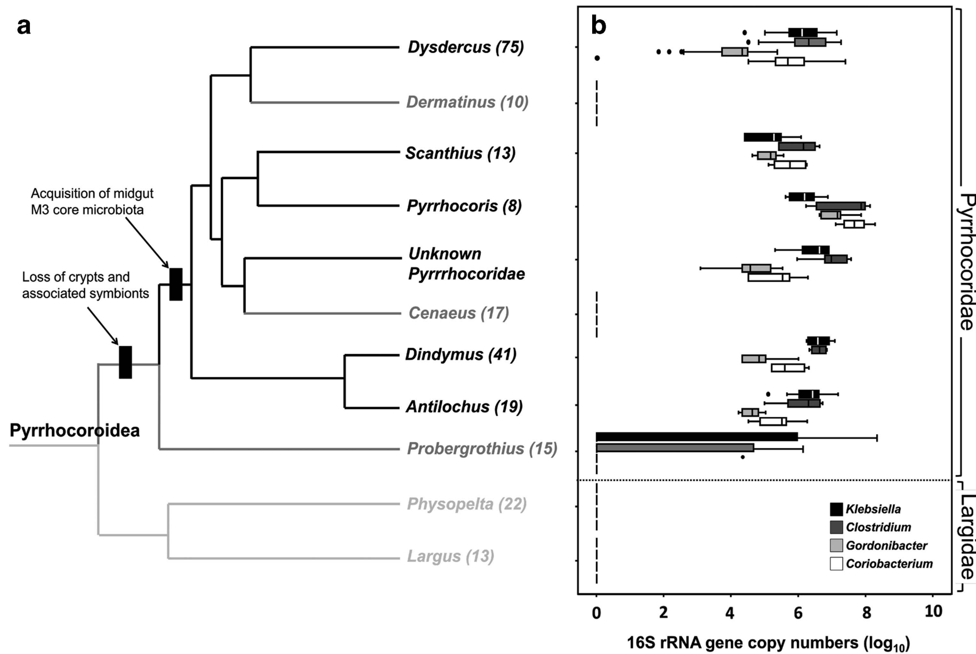

Figure 2 Evolutionary transitions in symbiotic syndromes in Pyrrhocoroidea, and abundance of core microbial taxa. (**a**) Schematic phylogeny of Pyrrhocoridae and Largidae genera (adapted from Figure 1b). Pyrrhocoridae taxa with core microbiota are given in black, those taxa without the core microbiota are in dark gray, and the Largidae with crypt-associated symbionts are in light gray. Reconstructed evolutionary transitions in symbiotic syndromes are indicated on the phylogeny. Numbers of validly described extant species are given behind each genus name (from Hussey, 1929). (**b**) Abundance of the four core symbiont taxa (*Coriobacterium glomerans*, *Gordonibacter* sp., *Clostridium* sp. and *Klebsiella* sp.) across multiple specimens of the nine different genera of Pyrrhocoridae (*Dysdercus* ($n=37$), *Dermatinus* ($n=2$), *Scanthius* ($n=5$), *Pyrrhocoris* ($n=7$), *unknown Pyrrhocoridae* ($n=6$), *Cenaeus* ($n=5$), *Dindymus* ($n=6$), *Antilochus* ($n=7$) and *Probergrothius* ($n=23$)) and two genera of Largidae (*Physopelta* ($n=4$) and *Largidae* ($n=1$)). Abundance was assessed as 16S rRNA gene copy numbers using qPCR, based on one to six replicate individuals per host species, which were then combined on the genus level. Lines represent medians, boxes comprise the 25–75 percentiles and whiskers denote the range.

with the Pyrrhocoridae host phylogeny (*Coriobacterium glomerans*: Parafit: $P=0.974$, TreeMap: $P=0.345$; *Gordonibacter* sp.: Parafit: $P=0.978$, TreeMap: $P=0.449$) (Supplementary Figure S3) suggest horizontal exchange of symbionts between host species. Given the symbiont microdiversity observed in the bTEFAP data, we paid special attention to avoid the generation of possible chimeric sequences when combining bTEFAP reads and sequences obtained after cloning of PCR amplicons. Owing to the high similarity of different symbiont strains, however, the possibility of chimera formation for the symbionts of individual host taxa cannot be completely ruled out, which may hinder accurate co-phylogenetic analyses. To exclude the possibility that co-phylogenetic patterns were additionally obscured by interspecific predation among pyrrhocorid bugs resulting in transient Coriobacteriaceae being picked up in the bTEFAP sequences, we repeated the analyses after excluding known predatory taxa (*Antilochus* spp., *Dindymus lanius*). Although some symbiont taxa clustered according to their host genera (particularly *Pyrrhocoris* and *Scanthius* for *Gordonibacter*, and *Dysdercus* for *Coriobacterium*), the tests for co-cladogenesis remained nonsignificant. Thus, although the Pyrrhocoridae maintain a specific microbiota, horizontal transmission between co-occurring species apparently played an important role during the evolution of this symbiosis. As the Coriobacteriaceae symbionts are localized in the same region of the midgut and can be co-transmitted both vertically and horizontally (Kaltenpoth *et al*., 2009), we also tested for co-cladogenesis of the two symbiont lineages. Randomization of phylogenetic trees or distance matrices and subsequent statistical evaluation, however, yielded no evidence for co-cladogenesis between *Coriobacterium glomerans* and *Gordonibacter* sp. strains across host taxa (Parafit: $P=0.898$, TreeMap: $P=0.251$).

Discussion

In this study, we characterized the microbiota associated with bugs of the hemipteran families Pyrrhocoridae and Largidae, and investigated the origin and evolutionary dynamics of the host–microbiota association on both the community and strain level. The results provide insights into an evolutionary transition from individual crypt-associated symbionts to a more complex microbiota that is localized in the insect's midgut. This transition coincided with the evolution of the hosts' preferred food plants and preceded the major radiation of pyrrhocorid bugs, highlighting the

possible importance of the microbial community in adapting to novel ecological niches.

Nutritional contributions of the core microbiota associated with pyrrhocorid host

Members of the Pyrrhocoridae are predominantly phytophagous, feeding on seeds of the plant order Malvales, with a few notable exceptions such as *Probergrothius angolensis*, which feeds on seeds of the ancient gymnosperm *Welwitschia mirabilis* (Wetschnig and Depisch, 1999). Despite being phylogenetically distant, these host plants share similar phytochemical defenses, particularly cylcopropenoic fatty acids (CPFAs) (Allen *et al*., 1967). These compounds are known to be toxic to insects

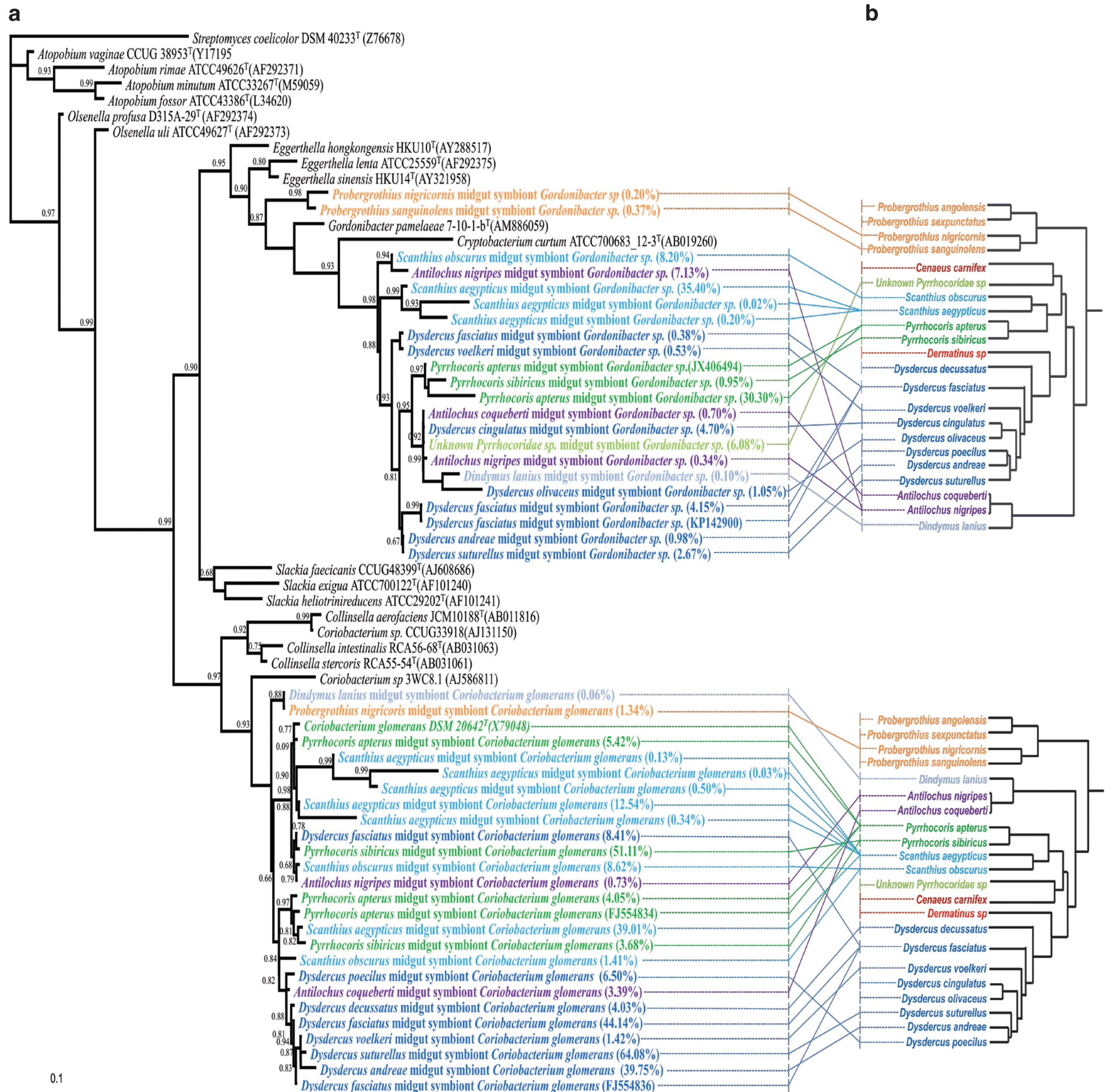

Figure 3 Cophylogenetic analysis of (**a**) the dual actinobacterial symbionts (*Coriobacterium glomerans* and *Gordonibacter* sp.) and (**b**) their Pyrrhocoridae hosts. The symbiont phylogeny is based on partial 16S rRNA bTEFAP sequences from OTUs picked for each individual species (see Supplementary Data S2). Colors for individual symbiont strains correspond to the coloring of host taxa in Figure 1b. For each Coriobacteriaceae OTU, the relative abundance (in relation to the respective host's complete microbial community) is given in brackets behind the strain designation. Host–symbiont associations are shown by connecting lines. Values at the nodes represent local support values from the FastTree analysis (GTR model).

because of the inhibition of fatty acid desaturation (Allen *et al.*, 1967). Interestingly, the noxious effects of CPFAs are particularly problematic under B vitamin starvation conditions, as artificial supplementation of vitamins partly abolished the adverse effects of CPFAs in rats (Schneider *et al.*, 1968). As the seeds of Malvales plants are known to be deficient in B vitamins (Whitsitt, 1933), the combined effect of vitamin deficiency and CPFAs likely poses severe nutritional challenges to insects attempting to exploit this food source.

In previous studies, we have shown that *P. apterus* and *D. fasciatus*, two members of the Pyrrhocoridae family, harbor a stable and specific midgut microbiota that is dominated by four microbial taxa: Actinobacteria (*Coriobacterium glomerans*, *Gordonibacter* sp.), Firmicutes (*Clostridium* sp.) and Proteobacteria (*Klebsiella* sp.), irrespective of the geographical origin or laboratory diet of the bugs (Sudakaran *et al.*, 2012; Salem *et al.*, 2013). Our present characterization of the microbiota associated with 22 different species belonging to nine genera of Pyrrhocoridae using 16S rRNA amplicon pyrosequencing and quantitative PCR revealed the presence of the same dominant bacterial taxa across six (*Dysdercus*, *Scanthius*, *Pyrrhocoris*, *Dindymus, Antilochus* and the unidentified specimen) out of the nine investigated genera of Pyrrhocoridae, but absence from the other three genera as well as all of the non-Pyrrhocoridae outgroup taxa, including the sister family Largidae (Figures 1 and 2).

The presence of a consistent core microbiota across most Pyrrhocoridae suggests that the symbionts are likely vital to the hosts' fitness. Concordantly, experimental removal of the actinobacterial symbionts by egg surface sterilization had a strong negative effect on the fitness of the host as indicated by severely reduced survival during development and strongly impaired fecundity of adult individuals (Salem *et al.*, 2013). Furthermore, a recent study that combined fitness assays of Actinobacteria-deprived and control bugs on an artificial diet with transcriptome sequencing of the host, and genomic analysis of one of the Coriobacteriaceae symbionts (*C. glomerans*) revealed that these symbionts supplement the nutrition of the host with limiting B vitamins (Salem *et al.*, 2014), which may also mitigate the toxic effects of the plants' CPFAs in the bugs' gut (Schneider *et al.*, 1968). Thus, the Coriobacteriaceae symbionts are tightly integrated into the hosts' metabolism and play an important role for nutrient provisioning and, possibly, detoxification (Salem *et al.*, 2014). The third core microbial taxon, *Clostridium* sp., is affiliated with the Lachnospiraceae (Firmicutes), whose members are anaerobic fermenters. Their production of butyric acid can serve as a nutrient source to the host (Meehan and Beiko, 2014) and/or reduce the abundance of bacterial pathogens in the insect gut by stimulating mucin and antimicrobial peptide production (Hamer *et al.*, 2008). Given that many *Clostridium* species have cellulolytic capabilities (Lynd *et al.*, 2002), the symbiont could also contribute to cellulose digestion. However, as Lachnospiraceae have to our knowledge not yet been functionally described as symbionts of insects other than Pyrrhocoridae, their contribution to host fitness remains speculative. The fourth symbiont, *Klebsiella* sp., belongs to the Enterobacteriaceae (Gamma-Proteobacteria). *Klebsiella* are facultative anaerobes that are associated with diverse eukaryotic organisms (Bagley 1985; Podschun and Ullmann, 1998). Some strains are capable of fixing atmospheric nitrogen to be utilized by plants (Cakmakci *et al.*, 1981), as well as insects (leaf-cutter ants; Pinto-Tomas *et al.*, 2009), so the Pyrrhocoridae-associated *Klebsiella* may also be involved in the nitrogen metabolism of their hosts. Taken together, the symbiont-provided benefits may enable the pyrrhocorid hosts to successfully exploit a nutritionally inadequate food source (the seeds of Malvales plants) that is unpalatable to many other insects, because of the low concentrations of available B vitamins and the presence of toxic CPFAs.

Evolutionary origin of the Pyrrhocoridae–microbiota association

The M3 core microbiota of the Pyrrhocoridae was distinctly different from that of their closest relatives, the Largidae, which harbored more or less a monoculture of *Burkholderia* (Figure 1d). Related *Burkholderia* symbionts have been described from the midgut crypts of several other bug taxa in the superfamilies Coreoidea and Lygaeoidea (Kikuchi *et al.*, 2011a,b). As Largidae also possess well-defined midgut crypts (Glasgow, 1914), a crypt localization seems very likely for their *Burkholderia* symbionts. The absence of *Burkholderia* or any other dominant symbiont taxon (Figure 1d), as well as the simple structure of the midgut crypts in the basal pyrrhocorid genus *Probergrothius* (Rastogi, 1964; Goel and Chatterjee, 2003) suggests that the Pyrrhocoridae lost their crypt-associated symbionts soon after the evolutionary split from the Largidae, which occurred around 125.2 mya ago (Figures 1a and 2). The core microbiota consisting of *Coriobacterium glomerans, Gordonibacter* sp.*, Clostridium* sp. and *Klebsiella* sp., however, was not established before *Probergrothius* split off from the rest of the pyrrhocorids in the late Cretaceous (81.2–86.5 mya) (Figures 1 and 2). Thus, the pyrrhocorid symbiosis with its extracellular core microbiota is younger than most of the known intracellular nutritional mutualisms in insects, such as the aphid—*Buchnera* (80–150 mya; Von Dohlen and Moran 2000), cockroach—*Blattabacterium* (135–250 mya; Bandi *et al.*, 1995), planthopper—*Vidania* (> 130 mya; Urban and Cryan, 2012) and Auchenorrhyncha—*Sulcia* symbioses (260–280 mya; Moran *et al.*, 2005). Importantly, however, the estimated age of the Pyrrhocoridae core microbiota coincides with the inferred origin of their host plant order Malvales

(72–96 mya, Wang *et al*., 2009; Figure 1). Although at present, host and symbiont contributions toward exploitation of the diet cannot be completely disentangled, the increased diversity in pyrrhocorid bugs after the acquisition of the M3-localized anaerobic core microbiota suggests an important contribution of the symbionts toward the adaptation of their hosts to Malvales seeds as a nutritional resource and the subsequent diversification of Pyrrhocoridae (Figure 2).

Our results indicate that some pyrrhocorid genera apparently lack the characteristic microbiota. As mentioned above, *Probergrothius* likely diverged from the rest of the Pyrrhocoridae before the acquisition of the core microbiota. Although this genus shows the occurrence of *Clostridium sp*., the symbiont differs from the taxonomically related OTUs of the other pyrrhocorids, and the two actinobacterial symbionts are entirely lacking in *Probergrothius*. Despite the absence of these symbionts, some of the *Probergrothius* species successfully exploit Malvales seeds, whereas others feed on *Welwitschia* seeds (Wetschnig and Depisch, 1999; Goel and Chatterjee 2003), which contain CPFAs as well (Aitzetmuller and Vosmann, 1998). Assuming that the switch to Malvales host plants already occurred at the base of the Pyrrhocoridae, it likely coincided with the transition from crypt-associated symbionts to an anaerobic midgut community, whereas the acquisition of the characteristic core microbiota only happened secondarily after the split from *Probergrothius.* The latter transition likely represented a key adaptation to exploit the Malvales ecological niche, as is reflected in the much greater diversity in the pyrrhocorid clade without *Probergrothius* (see Figure 2). How bugs in the genus *Probergrothius* fulfill their dietary requirements of B vitamins and prevent the toxic effects of CPFAs is currently unknown. In this context, however, it is noteworthy that one of the Lygaeidae outgroup taxa, *Oxycarenus hyalinipennis*, co-exists in the same environment as several other Pyrrhocoridae and also feeds on Malvales seeds (Saxena and Bhatnagar, 1958). Despite sharing the same ecological niche, *O. hyalinipennis* harbors a completely different microbiota comprising mainly *Wolbachia* sp. and *Bartonella* sp., suggesting that different adaptations have evolved independently to cope with the nutritional challenges associated with feeding on Malvales seeds.

In contrast to the primary lack of the core microbiota in *Probergrothius* spp., *Cenaeus carnifex* and *Dermatinus* sp. lost the core microbiota secondarily (Figure 1b). Although the Pyrrhocoridae predominantly feed on Malvales seeds, some species including members of the genera *Cenaeus, Pyrrhocoris, Antilochus* and *Dindymus* have been reported to predominantly (*Antilochus* and *Dindymus*) or occasionally (*Cenaeus* and *Pyrrhocoris*) utilize dead insects or other animals as a food source (Ahmad and Schaefer, 1987, Socha, 1993). A carnivorous supplementation of an otherwise phytophagous diet could provide essential nutrients (including B vitamins) and thereby relax the selective pressures to maintain a nutrient-supplementing microbial community. This scenario could explain the loss of symbionts in *Cenaeus*. However, no information on the feeding biology of *Dermatinus* is available. Thus, the scenario in which symbionts were lost following a shift to a partially or completely carnivorous diet remains speculative. In this context, however, it is interesting to note that although the genera *Antilochus* and *Dindymus* have been characterized as predominantly carnivorous (Ahmad and Schaefer, 1987; Jackson and Barrion, 2002; Kohno *et al*., 2004; Ari Noriega and Huay Lee, 2010), they retained the core microbiota in qualitatively and quantitatively similar composition as other Pyrrhocoridae genera (Figures 1 and 2). Although it is possible that the core bacteria in *Antilochus* and *Dindymus* represent transient associates acquired via feeding on sympatrically occurring pyrrhocorid bugs, their numerical abundance implies a contribution to host fitness. Thus, the link between the carnivorous supplementation of the predominantly phytophagous diet and the secondary loss of symbionts in some Pyrrhocoridae requires further investigation, which should particularly focus on the functional role the gut-associated microbial communities have in different Pyrrhocoridae genera.

Mixed transmission mode and partner specificity

In insect–microbe interactions, functionally relevant and vertically transmitted microbial symbionts are expected to co-evolve and co-diversify with their host, resulting in the congruence of host and symbiont phylogenies. Such patterns are well-documented in several insects harboring primary intracellular endosymbionts (Moran *et al*., 2008), as well as in some tightly integrated extracellular symbioses (for review, see Salem *et al*., 2015). More recently, changes in microbial community profiles have also been suggested to mirror the evolutionary relationships among host taxa in symbiotic associations that remain stable over long evolutionary timescales (Sanders *et al*., 2014). In this study, the gut microbiota of the Pyrrhocoridae family remains both quantitatively and qualitatively stable across most host species. However, multiple discrepancies between the host phylogeny and the symbiont relationships on the community and strain level (*C. glomerans* and *Gordonibacter* sp.), with overall no statistical support for co-cladogenesis, strongly imply horizontal transmission of symbionts between heterospecific hosts (Figure 3 and Supplementary Figure S3). In addition, multiple co-occurring strains of the two actinobacterial symbionts were detected in several host taxa. It is well documented in *Pyrrhocoris apterus* that although the symbionts are predominantly transmitted vertically from mother to offspring through egg smearing, symbiont-deprived

bugs readily acquire the microbes horizontally from conspecifics through coprophagy in the laboratory (Kaltenpoth *et al.*, 2009). A second possible explanation for the horizontal exchange of symbionts could be the tendency among some Pyrrhocorid bugs to occasionally prey on other co-occurring pyrrhocorid species. Similar findings of a predominantly vertical transmission with a low rate of horizontal transmission (that is, mixed transmission mode) in a range of other insects challenge the traditional view of a strict vertical transmission of most insect mutualists and instead highlight the importance of mixed transmission modes across a wide range of host taxa (Ebert, 2013). Examples for this mixed transmission mode include burying beetles (Kaltenpoth and Steiger 2014), fruit flies (Aharon *et al.*, 2013), beewolves (Kaltenpoth *et al.*, 2014), termites (Schauer *et al.*, 2012) and bumblebees (Koch and Schmid-Hempel, 2011).

Major transitions in symbiotic syndromes in the hemipteran infraorder Pentatomomorpha

Symbiotic associations with bacteria have been extensively studied in the hemipteran infraorder Pentatomomorpha, and the diversity of symbiont taxa, localizations and transmission routes provide interesting insights into evolutionary transitions in symbiotic syndromes. Many Pentatomomorpha possess specialized crypts in the posterior midgut M4 region that harbor primary symbionts (Glasgow, 1914) (Figure 4). Given the occurrence of midgut crypts and associated symbionts across four of the five pentatomomorphan superfamilies, they likely represent the ancestral localization of symbionts in the Pentatomomorpha excluding the fungivorous Aradoidea (Figure 4). In the superfamily Pentatomoidea, most crypt-associated symbionts are vertically transmitted Gamma-Proteobacteria that contribute to the nutrition of their hosts (Abe *et al.*, 1995; Hosokawa *et al.*, 2005; Prado *et al.*, 2006; Kikuchi *et al.*, 2009; Kaiwa *et al.*, 2010,Kaiwa *et al.*, 2014) (Figure 4). By contrast, bugs belonging to the Coreoidea superfamily and several families within the Lygaeoidea harbor *Burkholderia* symbionts in their midgut crypts. In some cases, such as in the bean bug *Riptortus pedestris,* every host generation acquires these symbionts *de novo* from the environment (Kikuchi *et al.*, 2007) (Figure 4). Several other families in the Lygaeoidea have secondarily lost the crypt-inhabiting symbionts, and some evolved bacteriomes that house a distinct clade of Gamma-Proteobacteria (Kuechler *et al.*, 2012; Matsuura *et al.*, 2012; Figure 4).

Based on the occurrence of crypt-associated *Burkholderia* symbionts in Coreoidea and Lygaeoidea, previous studies hypothesized that the *Burkholderia* symbiosis originated in the ancestor of these two sister taxa (Figure 4) (Kikuchi *et al.*, 2011a). However, our findings of highly abundant *Burkholderia* in all three investigated Largidae species, together with previous reports on complex and well-defined midgut crypts in this family (Glasgow, 1914; Miyamoto, 1961), suggest that the association with *Burkholderia* is more ancient than previously thought, with a likely origin at the base of the clade comprising the Pyrrhocoroidea, Coreoidea and Lygaeoidea (Figure 4). Under this scenario, *Burkholderia* symbionts that are localized in midgut crypts also constituted the ancestral state for the Pyrrhocoroidea, with a subsequent loss of crypts and *Burkholderia* in the Pyrrhocoridae. Interestingly, although seed feeding is widespread among pentatomomorphan bugs, the transition to an anaerobic microbiota in the midgut lumen appears to be confined to the Pyrrhocoridae. As discussed above, the specialized nutritional challenges associated with the switch to the Malvales host plants may have favored this evolutionary transition in symbiotic syndromes.

Evolutionary transitions in symbiotic associations have been described repeatedly across different insect taxa (Bennett and Moran, 2013; Hansen and Moran, 2014; Koga *et al.*, 2013). Evidently, extracellularly localized and transmitted symbionts are more flexible than intracellular associations, because they provide more opportunities to replace less beneficial symbionts (Salem *et al.,* 2015). Nevertheless, even replacements of obligate intracellular symbionts have been described in several Auchenorrhynchan lineages (Bennett and Moran, 2013; Koga *et al.*, 2013; Koga and Moran, 2014), as well as in aphids (Koga *et al.*, 2003) and weevils (Toju *et al.*, 2013). In the Pyrrhocoridae, the symbiotic association transitioned from specialized crypt-associated symbionts to a midgut-localized bacterial community. Several transitions between extracellular symbionts harbored in crypts or in the lumen of the midgut and intracellular symbionts housed in bacteriomes have been observed in related hemipteran families (Kikuchi *et al.*, 2011a,b; Kuechler *et al.*, 2012; Matsuura *et al.*, 2012; Bennett and Moran 2013; Hansen and Moran 2014), but their ecological and evolutionary implications remain poorly understood. Our results suggest that changes in the symbionts' localization and identity can allow for the insect host to invade a novel ecological niche and subsequently diversify. Future studies on the functional relevance of microbial symbionts during such transitions in symbiotic syndromes may reveal general principles in the ecological factors and evolutionary constraints that underlie niche expansions and lineage diversifications.

Conclusions

This study provides the first comprehensive characterization of the microbiota associated with different species of Pyrrhocoridae and yields novel insights into the evolution of this mutualistic symbiosis. The evolution of the specific core microbiota in Pyrrhocoridae during the late Cretaceous followed the origin

Host taxon		Symbiotic Localization	Bacterial Symbiont		References
Superfamily	Families		Phylum	Genus/Species	
Lygaeoidea	Blissidae; Geocoridae; Lygaeidae; and Oxycarenidae	Paired and unpaired bacteriomes attached to different parts of ovaries or midgut	γ-Proteobacteria	'*Candidatus* Schneideria nysicola'; '*Candidatus* Ischnodemia utricula'; '*Candidatus* Arocatia carayoni'; '*Candidatus* Kleidoceria schneideri'	Kuechler *et al* 2012; Matsuura *et al* 2012
	Berytidae; Blissidae; Pachygronthidae; and Rhyparochromidae	Midgut crypts	β-Proteobacteria	*Burkholderia sp.*	Kikuchi *et al* 2011a
	Malcidae	---	---	---	Kikuchi *et al* 2011a
Coreoidea	Alydidae; Coreidae; and Stenocephalidae	Midgut crypts	β-Proteobacteria	*Burkholderia sp.*	Kikuchi *et al* 2011a
	Rhopalidae	---	---	---	Kikuchi *et al* 2011a
Pyrrhocoroidea	Pyrrhocoridae	Midgut M3 region	Actinobacteria, Firmicutes, and γ-Proteobacteria	*Coriobacterium glomerans*; *Gordonibacter sp.*; *Clostridium sp.*; *Klebsiella sp.*	This study
	Largidae	Midgut crypts	β-Proteobacteria	*Burkholderia sp.*	This study
Pentatomoidea	Acanthosomatidae; Cydnidae; Parastrachiidae; Pentatomidae; Plataspidae; Scutelleridae; And Urostylidae	Midgut crypts	γ-Proteobacteria	'*Candidatus* Rosenkranzia clausaccus'; '*Candidatus* Ishikawaella capsulata'; '*Candidatus* Benitsuchiphilus tojoi'; *Erwinia sp.*	Hosokawa *et al* 2005, 2012; Kikuchi *et al* 2009; Kaiwa *et al* 2010, 2014; Prado *et al* 2006
Aradoidea	Aradidae	---	---	---	

Figure 4 Symbiotic syndromes (i.e., symbiont identity and localization) in bugs of the infraorder Pentatomomorpha. The superfamily-level phylogenetic relationships of the hosts were obtained from earlier studies based on the 18S rRNA (Xie *et al.*, 2005) and whole mitochondrial genomes (Hua *et al.*, 2008). The gray bar indicates the previously hypothesized evolutionary origin of crypt-associated *Burkholderia* in stinkbugs (Kikuchi *et al.*, 2011a,b), whereas the black bar denotes the revised suspected origin of this association, based on the discovery of *Burkholderia* symbionts in Largidae (this study).

of their preferred host plants (Malvales), suggesting that the symbionts were instrumental in allowing the host to adapt to and diversify in this ecological niche. Pyrrhocorid bugs represent one of the few established and experimentally amenable systems that can be used to address fundamental questions on the interactions of multiple bacterial symbionts within the gut of an insect host. The exploration of the symbionts' functional importance and the molecular basis of host–symbiont interactions may shed new light on the role microbial partners had for the evolutionary success of insects.

Data accessibilty

18S rRNA, COI and COII gene sequences of Pyrrhocoridae, Largidae and outgroup taxa along with near full-length 16S rRNA gene sequences of the Coriobacteriaceae symbionts across several species of Pyrrhocoridae were deposited in the NCBI database, and accession numbers are listed in Supplementary Table S1. The OTU table and corresponding representative sequences describing the occurrence of bacterial phylotypes across different Pyrrhocoroidea and outgroup taxa are given in Supplementary Table S4 and Supplementary Data S1. The Coriobacteriaceae representative OTU sequences from the bTEFAP analysis of individual pyrrhocorid species used for the phylogenetic analyses are given in Supplementary Data S2. The 454 pyrosequencing data (raw sff files) are available from the NCBI SRA database under the accession number SRP050243.

Conflict of Interest

The authors declare no conflict of interest.

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