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Phylogenetic Evidence for Ancient and Persistent Environmental Symbiont Reacquisition in Largidae (Hemiptera: Heteroptera)

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ABSTRACT

The insect order Hemiptera, one of the best-studied insect lineages with respect to bacterial symbioses, still contains major branches that lack comprehensive characterization of associated bacterial symbionts. The Pyrrhocoroidea (Largidae [220 species] and Pyrrhocoridae [~300 species]) is a clade of the hemipteran infraorder Pentatomomorpha. Studies on bacterial symbionts of this group have focused on members of Pyrrhocoridae, but recent examination of species of two genera of Largidae demonstrated divergent symbiotic complexes in these putative sister families. We surveyed the associated bacterial diversity of this group using paired-end Illumina sequencing and targeted Sanger sequencing of bacterial 16S rRNA amplicons of 30 pyrrhocoroid taxa, including 17 species of Largidae, in order to determine bacterial associates and the similarity of associated microbial communities among species. We also used molecular data (4,800 bp in 5 loci, for 57 ingroup and 12 outgroup taxa) to infer a phylogeny of the host superfamily, in order to trace the evolution of symbiotic complexes among Pentatomomorpha species. We undertook multiple lines of investigation (i.e., experimental rearing, fluorescence *in situ* hybridization microscopy, and phylogenetic and coevolutionary analyses) to elucidate potential transmission routes for largid symbionts. We found a prevalent and specific association of Largidae with *Burkholderia* strains of the plant-associated beneficial and environmental clade, housed in midgut tubules. As in other distantly related Heteroptera, symbiotic bacteria seem to be acquired from the environment every generation. We review the current understanding of symbiotic complexes within Pentatomomorpha and discuss means to further investigate the evolution and function of these symbioses.

IMPORTANCE

Obligate symbioses with bacteria are common in insects, particularly Hemiptera, in which various forms of symbiosis occur. However, knowledge regarding symbionts remains incomplete for major hemipteran lineages. Thus, an accurate understanding of how these partnerships evolved and changed over millions of years is not yet achievable. We contribute to our understanding of the evolution of symbiotic complexes in Hemiptera by characterizing bacterial associates of Pyrrhocoroidea, focusing on the family Largidae. Members of Largidae are associated with specific symbiotic *Burkholderia* strains from a different clade than *Burkholderia* symbionts in other *Burkholderia*-associated Hemiptera. Evidence suggests that species of Largidae reacquire specific symbiotic bacteria from the environment every generation, which is a rare strategy for insects, with potentially volatile evolutionary ramifications, but one that must have persisted in Largidae and related lineages since their origin in the Cretaceous Period.

The success of the species-rich insect order Hemiptera can be tied directly to bacterial symbionts, which allowed the ancestor of this lineage to exploit nutrient-limited diets of plant tissues such as xylem and phloem (1). Three lineages, i.e., Sternorrhyncha, Auchenorrhyncha, and Coleorrhyncha, are exclusively herbivorous, and nearly all constituent species remain associated with obligate, maternally inherited, intracellular symbionts (2–4). In contrast, the ancestor of a fourth lineage, Heteroptera, achieved independence from obligate symbionts at some point while transitioning from an herbivore to a predator (the evolution of the trophic strategies of Heteroptera is summarized in reference 5). However, two diverse clades of Heteroptera have secondarily re-evolved herbivory; together, they represent more than 60% of heteropteran diversity (6). One radiation constitutes the Pentatomomorpha, most members of which possess large populations of extracellular symbiotic bacteria in blind tubules of the posterior midgut called ceca (7, 8).

In stink bugs and allies (Pentatomoidea), symbiotic bacteria comprise various unrelated lineages of gammaproteobacteria, primarily vertically transmitted via egg smearing, coprophagy, or codeposition in a jelly or capsule (9–12). In contrast, all examined cecum-possessing members of the superfamilies Lygaeoidea and Coreoidea are symbiotically partnered with members of *Burkholderia* (Betaproteobacteria) that are acquired by early instar nymphs directly from soil (13, 14), although some vertical transmission occurs in some lineages (e.g., Blissidae [15, 16]). Cecum-possessing species of both groups have a symbiont-sorting organ at the junction of the third and fourth midgut sections, which blocks the passage of food and allows selective passage of specific

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bacteria (17). Despite a diet of comparatively nutrient-rich parts of plants, such as seeds, fruits, and new buds, the function of obligate symbionts is known or suspected to be nutritional, e.g., supplementing amino acids in Urostylididae (12) or recycling uric acid during diapause in Parastrachiidae (18), and hosts tend to face moderate to severe fitness deficits when deprived of symbionts (19–21). *Burkholderia* symbionts have been shown to enhance host innate immunity (22) or to confer resistance to the insecticide fenitrothion (23), and symbiont gene knockout experiments have demonstrated crucial components for effective symbiotic colonization, such as flagellar motility (17) and genes responsible for the synthesis of polyhydroxyalkanoates (24), but no specific nutritional function has been determined.

Largidae and Pyrrhocoridae together constitute the superfamily Pyrrhocoroidea, containing ~520 extant species (25, 26). Members of Pyrrhocoridae are associated with a characteristic microbiota that is housed not in ceca but in the third section of the midgut (28). This microbiota includes two obligate actinobacterial species, *Coriobacterium glomerans* and *Gordonibacter* sp., which are passed on via smearing of eggs (21, 28, 29). Elimination of symbionts by egg sterilization results in host death, and symbionts are thought to supplement a specialist diet of seeds of the plant order Malvales with B vitamins (30). Members of Largidae also feed on seeds and most species are generalists, although a small number may be associated preferentially with Euphorbiaceae (6, 31, 32). Early microscopic studies observed Gram-positive bacteria within ceca of members of Largidae (7, 33, 34), and recently Sudarakaran et al. discovered that representatives of the largid genera *Largus* and *Physopelta* are associated with *Burkholderia* (35). Another study found *Burkholderia* of the plant-associated beneficial and environmental (PBE) clade in ceca with *Physopelta* spp. (36). All other known heteropteran *Burkholderia* symbionts belong to a clade called the stinkbug-associated beneficial and environmental (SBE) clade, with the exception of Blissidae, in which *Burkholderia* isolates may belong to any of three described clades (15, 16).

In this study, we aimed to identify symbionts across Pyrrhocoroidea, with a focus on the family Largidae, and to determine the relationships of those symbionts to others through an Illumina bacterial 16S rRNA amplicon survey and targeted full-length 16S rRNA gene sequencing. Because alternative scenarios could explain the evolution of symbiotic complexes in Pentatomomorpha, depending on contradicting phylogenetic relationships of Pyrrhocoroidea (e.g., recent molecular phylogenies infer their closest relatives as Coreoidea and Lygaeoidea [37], Alydidae [38], Coreoidea [39], or Lygaeoidea [40]), we reconstructed a phylogeny of Pyrrhocoroidea to determine the evolutionary history of this lineage and also to allow for testing of concordance among host and symbiont phylogenies. We sought evidence to determine the method of acquisition of Largidae symbionts through experimental rearing, fluorescence *in situ* hybridization (FISH) microscopy, culturing, and investigation of patterns of symbiont and host phylogenies. We summarize our results and the current knowledge regarding the evolution of bacterial symbiont complexes in pentatomomorphan Heteroptera.

MATERIALS AND METHODS

Rearing of *Largus californicus*. Adult specimens were captured from Lytle Creek in San Bernardino National Forest (San Bernardino County, CA, USA) in June 2015, enclosed in plastic containers with dry unsterilized soil from the campus of the University of California, Riverside (not from the original habitat), and held at room temperature. Specimens were provided with grapes, cabbage, and water in a vial plugged with cotton. An egg batch (approximately 70 eggs) was divided, and approximately one-half of the eggs were subjected to DNA extraction, using the spin-column-based protocol for purification of DNA from animal tissues with the Qiagen DNeasy blood and tissue kit (with overnight incubation). Approximately 15 of the eggs were extracted before washing and another 15 were extracted after washing with distilled water, which was also extracted. The other one-half of the eggs were allowed to hatch, which they did after 2 weeks (on 28 July 2015); 2 days after hatching, five first instar nymphs were pooled for DNA extraction. The cecum-containing region of the midgut of a field-caught adult specimen was also dissected, and DNA was extracted. The presence of bacteria in DNA extracts was analyzed through PCR with universal and genus-specific bacterial 16S rRNA primers (see Table S1 in the supplemental material).

Culturing. The cecum-containing region of the midgut of one field-caught adult specimen of *L. californicus* was dissected away from other gut tissue and macerated with an Eppendorf pestle for 2 min in 200 μl of phosphate-buffered saline (PBS) (pH 7.4). An inoculating loop was used to spread the resulting cloudy homogenation liquid on a Luria-Bertani (LB) agar plate, and the plate was incubated for 3 days at 37°C. Several representatives of the dominant colony morphotype were analyzed by using colony PCR with universal bacterial primers (Table 1), and the resulting sequences were queried against the National Center for Biotechnology Information (NCBI) nucleotide database with nucleotide-nucleotide BLAST after cleaning and sequencing of PCR products, to confirm the identity of the bacterial isolate.

Florescence *in situ* hybridization. After anesthetization at −20°C for 3 min, gut tissue from live specimens of *Largus californicus* was dissected and stored separately in acetone, as were ~10 whole eggs from the unwashed one-half of the egg batch, for whole-mount microscopic preparations. We followed the protocol described previously (41), fixing tissue with Carnoy's solution overnight and staining gut tissue with 4′,6-diamidino-2-phenylindole (DAPI) for DNA labeling and with two oligonucleotide probes, i.e., a Cy-5-labeled universal bacterial probe (EUB-338; biomers.net) (42) and a Cy-3-labeled *Burkholderia*-specific 16S rRNA oligonucleotide (Burk129; Integrated DNA Technologies) (14) (purified with high-performance liquid chromatography [HPLC]), for specific bacterial symbiont staining. Confocal microscopy was performed with a Leica TCS SP5 confocal microscope, using 405-, 543-, and 655-nm lasers for visualization of DAPI, Cy-3, and Cy-5, respectively.

Sampling and DNA extraction. Individual specimens of all available Pyrrhocoroidea species (from the worldwide ethanol collection of Heteroptera in the Weirauch laboratory; details are presented in Table S1 in the supplemental material) and two outgroup taxa (a total of 32 species, including 13 genera of Pyrrhocoroidea) were surface-sterilized with a 1% bleach solution for 2 min and rinsed with 100% ethanol before removal of the abdomen from the thorax. Due to the fragility and deformation of dehydrated internal tissue with long-term ethanol preservation, we sampled all tissue from the abdomen, which included the majority of gut tissue. The tissue was removed with sterile forceps, and the resulting material was homogenized with a bead beater for 3 min at 30 Hz, after the addition of 100 μl of 0.1-mm glass beads and one 2.38-mm metal bead. Forceps were washed with ethanol, flame sterilized, and washed with a 10% bleach solution before and after each extraction. After the addition of 10 μl of 800 U/ml proteinase K, each sample was incubated for 24 h at 55°C before DNA extraction.

Host phylogeny. A total of ~4,800 bp of host DNA, consisting of two mitochondrial protein-encoding genes (COI and COII) and three rRNA genes (16S, 18S, and 28S), was amplified from DNA extracts with the primers listed in Table 1. PCR products were cleaned with Bioline Sure-Clean and submitted to Macrogen for Sanger sequencing. Chromatographs were edited in Sequencher v4.8 and aligned with MAFFT (E-INS-i strategy). A RAxML maximum likelihood phylogeny was reconstructed

TABLE 1 PCR primers and conditions

PCR use	Name	Sequence	Primer annealing temperature (°C)[a]	Cycle no.	Reference
Bacteria					
16S primers for Illumina sequencing	799F-mod3	5′-CMG GAT TAG ATA CCC KGG-3′	52	35	69
	1115R	5′-AGG GTT GCG CTC GTT G-3′			44
HPLC-purified primers to complete Illumina sequencing construct	F	5′-CAA GCA GAA GAC GGC ATA CGA GAT CGG TCT CGG CAT TCC TGC-3′	58	15	44
	R	5′-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC G-3′			44
Universal bacterial 16S primers	27F	5′-AGA GTT TGA TCM TGG CTC AG-3′	57	35	70
	1492R	5′-CGG TTA CCT TGT TAC GAC TT-3′			
Full-length PBE-specific PCR, first half	Burk16SF	5′-TTT TGG ACA ATG GGG GCA AC-3′	50	35	15
	BurkR	5′-TGC CAT ACT CTA GCY YGC-3′			71
Full-length PBE-specific PCR, second half	Burk3-Mod	5′-CGG CGA AAG CCG GAT-3′	50	35	71
	PBE822R-mod	5′-CTW CGT TAC CAA GYC AAT GAA GR-3′			70
Host					
16S rRNA	16sa	5′-CGC CTG TTT ATC AAA AAC AT-3′	48	35	72
	16sb	5′-CTC CGG TTT GAA CTC AGA TCA-3′			
18S rRNA	18S 1f	5′-TAC CTG GTT GAT CCT GCC AGT AG-3′	48	35	27
	18S 5r	5′-CTT GGC AAA TGC TTT CGC-3′			
28S D3-D5 rRNA	D3Fa	5′-TTG AAA CAC GGA CCA AGG AG-3′	48	35	73
	D5Fa	5′-CGC CAG TTC TGC TTA CCA-3′			
COI region 1	LCO-1490	5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′	48	35	74
	HCO-2198	5′-TAA ACT TCA GGG TGA CCA AAA-3′			
COI region 2	C1-J-2183	5′-CAA CAT TTA TTT TGA TTT TTT GG-3′	45	35	75
	TL2-N-3014	5′-TCC AAT GCA CTA ATC TGC CAT ATT A-3′			

[a] Primer annealing was performed at the indicated temperature for 45 s. All denaturation was performed at 94°C for 30 s, and extension was performed at 72°C for 60 s.

after partitioning based on gene and codon positions for protein-encoding genes and designation of the two included taxa of Pentatomoidea as an outgroup. Relevant sequences from GenBank were included for a maximally inclusive phylogeny of Pyrrhocoroidea (57 taxa and at least 20 genera). We noted that published sequences from *Dindymus lanius* in GenBank clustered closely with those from *Antilochus*, in contrast to our own sequences from three species of *Dindymus*, including *Dindymus lanius*; therefore, we excluded the former sequences from our analysis, in case of misidentification. A Bayesian analysis was performed with BEAST v1.8.0, using the same partitions (with the generalized time-reversible [GTR] model for 18S and 28S rRNA and the Hasegawa-Kishino-Yano [HKY] model for 16S rRNA and each codon partition of COI and COII), and was run for 100,000,000 generations, with sampling every 100,000 generations, in three separate runs (43). After confirmation that the effective sample size of each parameter in the Bayesian analysis was much greater than 200 and a check for convergence, the first 10% of trees for each run were removed and summarized using Tree Annotator v1.8.0, to obtain the maximum clade credibility tree with posterior probabilities (43). Sampled representatives were imaged with a Leica Microsystems system and entered into the Plant Bug Planetary Biodiversity Inventory database with Arthropod Easy Capture; specimen data are available via the Heteroptera Species Pages (http://research.amnh.org/pbi/heteropteraspeciespage).

Illumina 16S rRNA amplicon sequencing and analysis. Gut tissue from a subset of 17 taxa was subjected to Illumina amplicon sequencing of an ~300-bp fragment of the bacterial 16S rRNA gene using 799F and 1115R primers with barcodes for multiplexing (44), intended to minimize amplification of chloroplast DNA (45); PCR was conducted in triplicate (35 cycles, with annealing at 52°C) with 5Prime HotMasterMix. Triplicate PCR products were pooled and cleaned with the Ultraclean PCR clean-up kit (MoBio, Carlsbad, CA). Illumina adaptors were added to templates via PCR using 1 μl of cleaned product with HPLC-purified primers (15 cycles, with annealing at 58°C) (Table 1). Eighteen microliters of PCR product for each sample was normalized with a 96-well SequalPrep normalization plate, and 5 μl of each normalized sample was pooled and assessed for quality with a 2100 Bioanalyzer (Agilent, Santa Clara, CA), at the Institute for Integrative Genome Biology at the University of California, Riverside.

Samples were multiplexed on an Illumina MiSeq system with a MiSeq v3 reagent kit, using paired-end sequencing (2 × 300 bp). Paired-end reads were assembled, trimmed (reads with ambiguous bases or aberrant lengths were removed), and demultiplexed using mothur v1.35.1 (46). Sequences were aligned to the Silva v4 reference alignment, checked for chimeras with the UCHIME algorithm (using the most abundant sequences as the reference), and assigned to operational taxonomic units (OTUs) at a 97% identity level, and OTUs were classified using a Bayesian classifier; all procedures were implemented in mothur (46). Jaccard and Bray-Curtis community dissimilarity metrics and weighted and unweighted UniFrac distance matrices (47) were computed after rarefication of the data set to 2,066 reads per sample (the smallest number of reads in any sample after filtering), and bacterial communities were clustered in principal-coordinate analysis (PCoA) ordination plots visualized with Plotly.

For visualization of OTU abundance with a heat map, we removed all OTUs that together represented <1% of the total data set and representatives of any OTUs that constituted less than 0.5% of the total reads in a sample (together <2.5% of total reads after trimming). The most common representative of any OTU that was unclassified at the genus level was queried against the NCBI nucleotide database using blastn and manually curated to the lowest level possible, based on the highest scoring hit to known organisms. Putative chimeric OTUs (based on high levels of blastn hit identity to two distantly related bacterial lineages) were removed. OTUs with identical top hits were combined for visualization purposes. Read abundance was plotted on a logarithmic scale after division by 10 (equivalent to $\log_{10}$ reads − 1), using the ggplot2 package in R.

Targeted full-length 16S rRNA PCR and phylogeny. Full-length 16S rRNA sequences of *Burkholderia* were retrieved from two transcriptomes of *Largus californicus* that were sequenced recently as a part of the Hemipteroid Orders Tree of Life project. Based on these sequences, we modified existing primers to amplify full 16S rRNA sequences of *Burkholderia* with two sets of PCR with a combination of PBE-specific primers and *Burkholderia*-specific primers (Table 1). PCR products were cleaned with Bioline SureClean, and sequences were processed as described for host genes. A set of 272 sequences of 16S rRNA from environmental and insect-associated isolates of *Burkholderia*, as well as named *Burkholderia* species and 3 *Pandoraea* outgroups, was downloaded from GenBank. This data set was used to reconstruct a tree for the genus, including full-length 16S rRNA sequences of dominant *Burkholderia* strains obtained from Largidae samples via PCR and representative *Burkholderia* reads (300 bp) of less dominant OTUs from the Illumina data set acquired from each sample in which they were present (see Fig. S4 in the supplemental material). Taxa were pruned using Mesquite v3.04 (48) for easier visualization, retaining close relatives of newly sequenced bacterial 16S rRNA genes, representatives of other insect-associated lineages, and named species. The resulting data set was realigned, and a phylogeny was reconstructed (see Fig. 3).

A cophylogenetic analysis of *Burkholderia* and host phylogenies was performed with TreeMap 3 (49) and ParaFit (50). A maximally reconciled set of branching patterns of host and symbiont phylogenies was visualized with TreeMap 3. Patristic distances were calculated with the cophenetic function in the ape package in R, and the resulting phylogenetic relationships were tested for correlation of bacterial and host phylogenies via the statistical test implemented in ParaFit, with 10,000 permutations.

Accession number(s). Newly acquired nucleotide sequences have been deposited in GenBank under accession numbers KX523359 to KX523485, and newly acquired 16S rRNA sequences have been deposited in GenBank under accession numbers KX527603 to KX527621 (see Table S1 in the supplemental material). Raw multiplex data are available in the NCBI Sequence Read Archive (SRA) (accession number SRP078165).

RESULTS

Gut morphology, rearing, and culturing. As described previously for congeners (Fig. 1F), the gut morphology of *Largus californicus* (adult pictured in Fig. 1A) consists of five morphologically distinct midgut sections (Fig. 1B). The third section is the largest and is followed by a constricted region (Fig. 1B, right inset), homologous to symbiont-sorting organs described for other Heteroptera (17). Ceca are short and numerous in the fifth midgut section, consisting of two rows of tubes (Fig. 1B and C), and tend to be closely associated with the distal part of the Malpighian tubules *in situ*. FISH microscopy with a universal bacterial 16S rRNA probe highlighted a high density of bacteria in the ceca (Fig. 1D), which also stained with a genus-specific probe for *Burkholderia* (Fig. 1E). Egg batches did not contain any prominent codeposited substance (Fig. 1G), and FISH with a universal bacterial 16S rRNA probe did not result in visible staining of any bacteria (data not shown). However, first instar nymphs (Fig. 1I) were observed to probe remains of hatched eggs (Fig. 1H).

Sequencing of PCR products amplified with general bacterial primers (Table 1) from DNA extracts from the isolated cecum-containing region (Fig. 1C) produced a chromatographic sequence matching that of *Burkholderia* (see Table S1 in the supplemental material). DNA extracts of unwashed and washed eggs, first instar nymphs, and the wash from washed eggs produced no products when assayed with PCR with *Burkholderia*-specific primers. When assayed with general bacterial primers, both sets of eggs and first instar nymphs produced bands that, when sequenced, were 100% matches to *Rickettsia* spp. (see Table S1). The *Burkholderia* symbiont was apparently easily cultured on LB plates, as all representatives of the dominant small yellow colonies after plating of homogenized ceca were identical in sequence to each other and 100% identical to the PBE clade *Burkholderia australis* isolated from sugarcane roots (GenBank accession number JQ994113.1) (51).

Bacterial associates of Pyrrhocoroidea. After quality control, we recovered a total of 319,021 paired-end Illumina reads (average of 18,766 reads per sample). We found a highly prevalent association of *Burkholderia* in gut extracts of all Largidae samples, except for one specimen of *Stenomacra tungurahuana* (L40). We also recovered a previously described Pyrrhocoridae-associated *Clostridium* strain exclusively in each of our Pyrrhocoridae samples, although at a <0.5% level in a *Dysdercus* species from Australia (L23) and *Dindymus lanius* (L47) (OTUs 6, 10, 19, and 23 in Data Set S1 in the supplemental material). The presence of a *Gordonibacter* sp. (OTUs 11 and 28) was also observed exclusively in all Pyrrhocoridae samples except for *Dindymus lanius* and in only 2 reads of nearly 20,000 in *Dindymus pulcher* (<0.5% of reads for *Probergrothius* and *Dysdercus* sp. from Australia). We observed OTUs assigned to *Coriobacterium* only in the two sampled *Dysdercus* species, at a <0.5% level (OTU 64 in Data Set S1).

The principal-coordinate analysis plot displayed in Fig. 2 is that of a subsampled distance matrix based on abundance-weighted UniFrac distances (47) of OTUs and represents >45% of the variance in the data with the two plotted axes. Principal-coordinate analysis of bacterial communities based on other distance measurements similarly resulted in separate clusters of Largidae and Pyrrhocoridae, although with much less representation of variation, in all but the phylogenetically independent Bray-Curtis community dissimilarity matrices (see Fig. S3 in the supplemental material).

Phylogenetic relationships of Pyrrhocoroidea. Our phylogeny reconstructed Pyrrhocoroidea as sister to Coreoidea and Lygaeoidea (Fig. 2; also see Fig. S1 in the supplemental material) with a bootstrap support value of >85%. We recovered monophyletic Largidae with moderate support and a sister group relationship between the subfamilies Physopeltinae and Larginae. The two predatory genera of Pyrrhocoridae, i.e., *Antilochus*, whose members are specialist predators of other Pyrrhocoridae (52), and *Dindymus*, whose members possess variable trophic strategies, with some being specialist predators of mollusks (53), do not appear to be most closely related to each other, although some nodes of sister groups are not well supported. The results of the Bayesian analysis (see Fig. S2 in the supplemental material) were largely congruent with the maximum likelihood phylogeny, although with some nodes having relatively higher or lower support values. Despite ethanol preservation, we observed well-developed ceca when we sampled tissue from the unidentified female pyrrhocoroid specimen L54 (near *Ectatops* or *Saldoides*), comparable to those seen in *Largus*.

Phylogenetics of *Burkholderia* associates. A phylogeny of *Burkholderia* symbionts based on full-length 16S rRNA sequences (when available) was not concordant with host phylogenies (Fig. 3, top). There was no evidence of codiversification (ParaFit; $P = 0.5129$). Five species of Largidae from geographically distant areas (Argentina, Colombia, Costa Rica, Mexico, and the United States) harbored the same or very similar strains of *Burkholderia*, which closely matched the 16S rRNA sequences of PBE clade *Burkholderia*, including those of curated elite commercial inoculants used in agriculture for nodulation of legume crops in Brazil, housed in the SEMIA *Rhizobium* Culture Collection (e.g., 100% identical to *Burkholderia* SEMIA 6385 [GenBank accession number FJ025136.1] and SEMIA 6382 [GenBank accession number AY904775.1], iso-

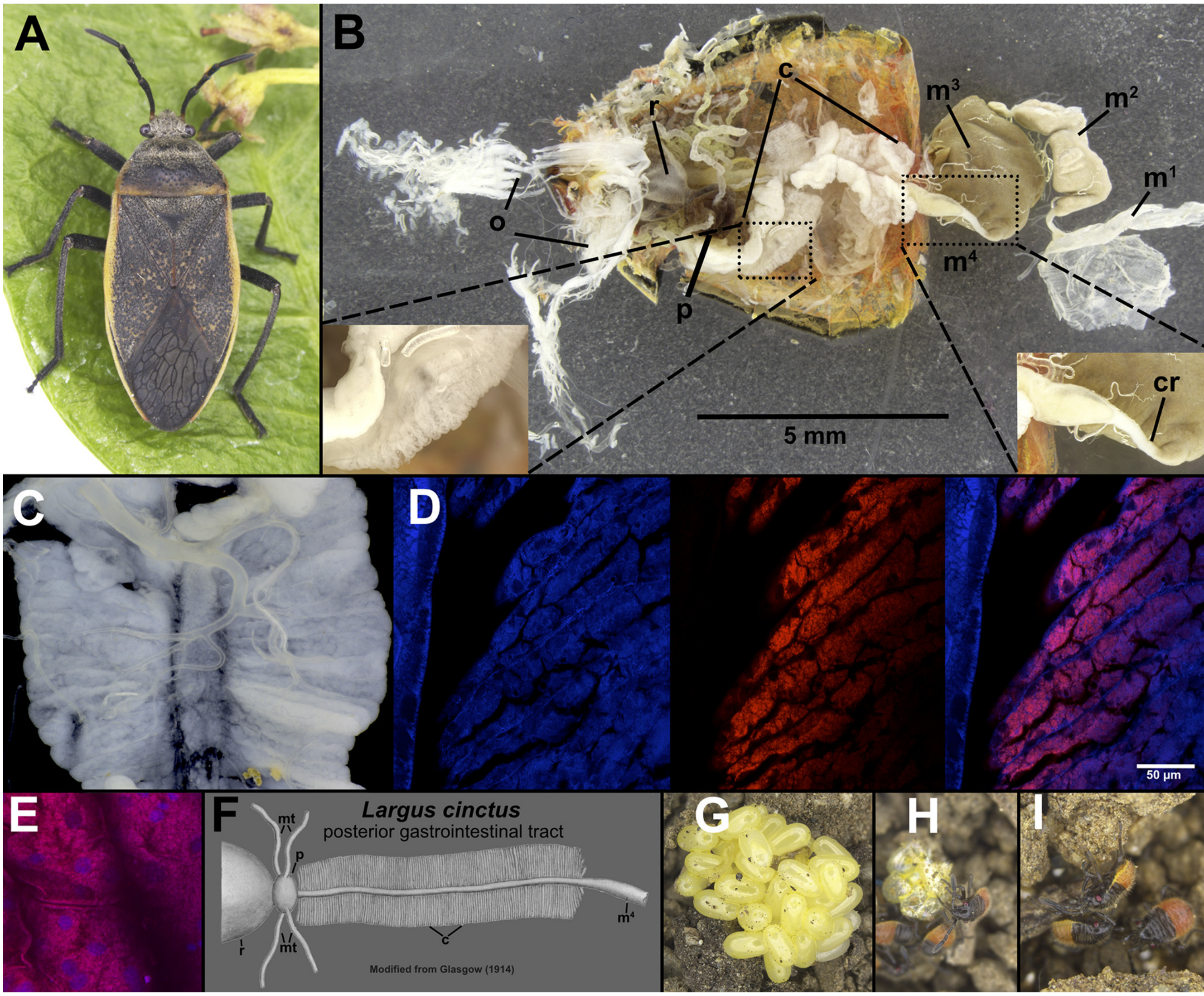

FIG 1 (A) *Largus californicus* adult female (magnification, ×3.5). (B) Gut morphology of *L. californicus* (magnification, ×7). (Left inset) Magnified posterior midgut ceca (magnification, ×20). (Right inset) Magnified view of the constricted region between the fourth and fifth midgut regions (magnification, ×13). (C) Ceca dissected away from other parts of the gastrointestinal tract (magnification, ×150). (D) FISH micrographs of ceca stained with DAPI (blue) and a Cy-5-labeled universal bacterial probe (red) for 16S rRNA (magnification, ×170). (Left) 405-nm laser. (Middle) 655-nm laser. (Right) Merged. (E) Merged FISH micrograph of cecal tissue stained with DAPI (blue) and a Cy-3-labeled *Burkholderia*-specific probe (red) (magnification, ×450). (F) Drawing of *Largus cinctus* posterior gastrointestinal tract (modified from reference 7). (G) Egg batch after removal of about one-half of the eggs (magnification, ×6). (H) First instar nymph of *L. californicus* probing an egg batch with the labium (magnification, ×6). (I) First instar nymphs of *L. californicus* (magnification, ×6). c, ceca; cr, constricted region; $m^{\#}$, indicated section of the midgut; mt, Malpighian tubules; o, oviduct; p, pylorus; r, rectum.

lated from *Piptadenia gonoacantha* and *Mimosa caesalpiniifolia* roots, respectively [54]).

A phylogeny utilizing data for all newly sequenced *Burkholderia* strains (Fig. 3, orange and a subset of red) and relatives present in GenBank showed that all Largidae-associated strains of *Burkholderia* belonged to the described PBE group (Fig. 3). Insect associates are closely related to species that are known nodulating bacteria of legumes and particularly of Mimosoideae (*Burkholderia diazotrophica*, *Burkholderia caribensis*, *Burkholderia phymatum*, and *Burkholderia tuberum* [55]) or other plant-associated nitrogen-fixing species (*Burkholderia tropica* and *Burkholderia heleia* [56]). All other non-Largidae heteropteran-associated *Burkholderia* strains fall within the SBE clade, with the exception of some of the symbiont strains isolated from Blissidae (Fig. 3, green). A tree sampling additional *Burkholderia* representatives, including environmental isolates, is shown in Fig. S4 in the supplemental material.

DISCUSSION

***Burkholderia* transmission method.** Environmentally acquired obligate symbiosis with bacteria is common in edaphic or marine habitats, where it occurs between nitrogen-fixing bacteria and legumes or bioluminescent bacteria and squids, respectively (57). Environmental acquisition of obligate symbionts is rare in insects, however, and the *Burkholderia* association with Heteroptera may be the oldest stably maintained such symbiosis in insects, as the

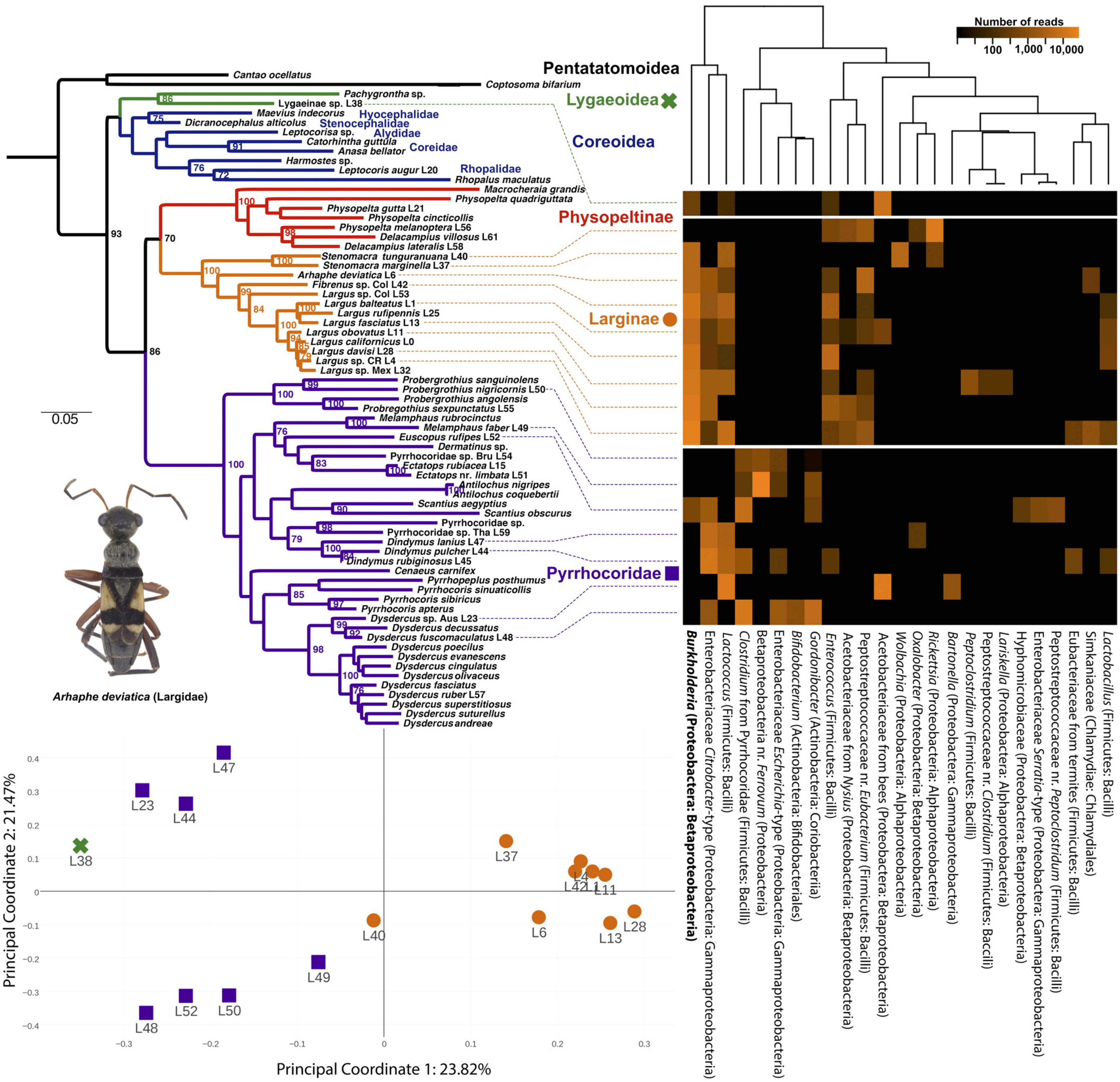

FIG 2 (Top left) Maximum likelihood phylogeny of Pyrrhocoroidea. Bootstrap values of >70% are displayed on the branches, and the numbers of nucleotide changes are indicated by branch lengths, with the scale bar corresponding to a mean of 0.05 nucleotide substitution per site. Pentatomoidea was constrained as the outgroup. (Right) Heat map of bacterial OTUs from specimens indicated in the phylogeny, with brightness corresponding to the $\log_{10}$ number of Illumina reads divided by 10 or $\log_{10}$ reads after subtraction of 1. The dendrogram at the top of the heatmap represents the clustering of OTUs based on their shared presence in samples. (Bottom left) PCoA plot of abundance-weighted UniFrac distances. Green X, Lygaeoidea; orange circles, Largidae: Larginae; purple squares, Pyrrhocoridae.

ancestor of *Burkholderia*-associated Heteroptera has been dated as originating ~130 million years ago (38). Such associations can evolve toward pathogenicity of symbionts as horizontal acquisition selects for pathogenic or cheating phenotypes (58), and similar systems exhibit policing by hosts to punish symbionts that do not participate (59). The gut ceca of Largidae and other Pentatomomorpha could provide a mechanism to manage symbionts, as each individual cecum could be modulated.

Although it has not been shown experimentally, all available evidence supports environmental reacquisition of *Burkholderia* by new generations of species of Largidae. While data are limited to a set of several pooled samples (~35 individuals in total) arising from one egg batch, the lack of evidence of *Burkholderia* in or on eggs or in laboratory-reared first instar nymphs and the lack of any sort of cophylogenetic signal are in agreement with horizontal transmission. Booth (60) noted that nearly all first instar nymphs

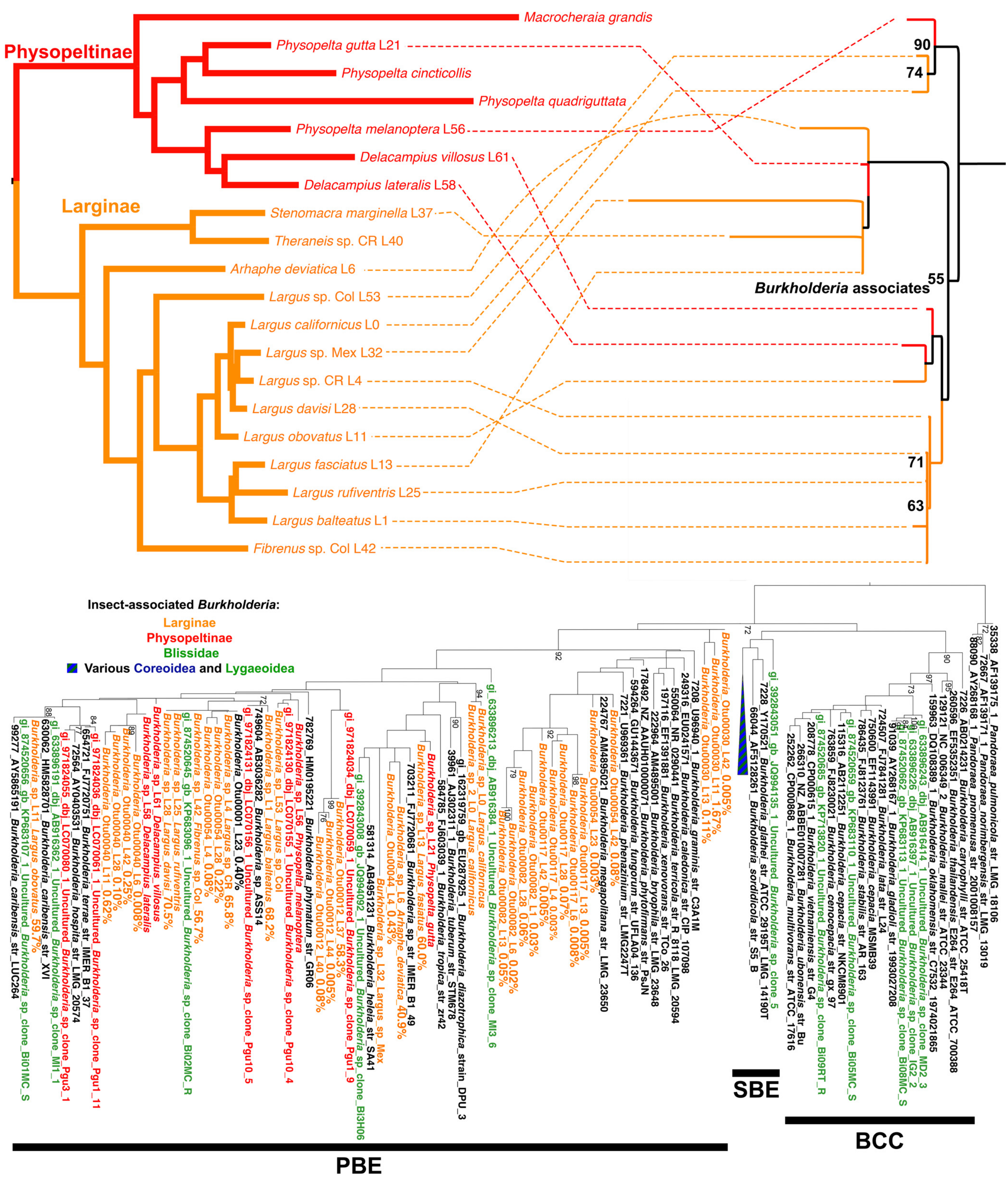

FIG 3 Phylogeny of *Burkholderia* symbionts. Bootstrap values of >70% are displayed on the bacterial phylogenies. (Top) Maximally concordant host (left) and symbiont (right) maximum likelihood phylogenies, produced by TreeMap 3, with the linkages shown. (Bottom) Phylogeny of newly obtained *Burkholderia* sequences with others from GenBank. Taxon names and branches are colored according to the host (orange, Larginae; red, Physopeltinae; green, Blissidae). OTUs from the Illumina data set (as well as full-length 16S rRNA sequences from dominant *Burkholderia* strains) are followed by the percentage of reads they represented, of the total for that sample. One node consisting only of lygaeoid- and coreoid-associated *Burkholderia* in the SBE clade was collapsed for easier visualization. BCC, *Burkholderia cepacia* complex.

of *L. californicus* reared in a laboratory died before molting, although eggs reared in the field and field-caught first instars were both viable to adulthood. This implies that symbionts are acquired during the first instar stage in *Largus* and potentially also in other Largidae species, in contrast to Alydidae, in which second instars acquire symbionts (13). Another major finding that suggests a horizontal transmission strategy in Largidae is the finding that, in all other cases of Heteroptera associated with *Burkholderia*, the symbiosis is acquired from the environment (although vertical transmission can occur up to ~30% of the time in Blissidae [15]).

Associated bacterial communities of Pyrrhocoroidea. Largidae species of six genera (16/17 species) were specifically associated with *Burkholderia* strains from a clade of nitrogen-fixing plant associates and plant symbionts. The exception, *Stenomacra tungurahuana* (L40), instead possessed a large number of reads from *Rickettsia*. A *Dysdercus* specimen (L23) also had an unusual microbiome, with a high concentration of Acetobacteraceae previously characterized from bees and a large number of reads identical to *Bartonella*, an intracellular pathogen. Both samples might be atypical due to infection with another bacterium or might represent samples that suffered from decomposition or degradation of DNA after inadequate preservation in ethanol, which was shown to have an effect on sequenced bacterial diversity in other insects (61, 62). However, these two samples did cluster with related members in PCoA plots based on distance matrices, except for the phylogenetically independent Bray-Curtis distances (see Fig. S3 in the supplemental material). Members of two largid genera that we were not able to sample, *Macrocheraia grandis* and *Iphita limbata*, were shown previously to possess ceca filled with bacteria of the same rod shape as *Burkholderia*, although cultured isolates displayed marked differences, being Gram positive or spore forming (41, 42).

We recovered a more restricted distribution of Pyrrhocoridae symbionts than that described previously. The most notable difference was the absence of a previously described *Coriobacterium* strain in all but the sampled *Dysdercus* species. Previously, *Coriobacterium* was shown to make up large proportions of bacterial communities in members of the genera *Scantius*, *Pyrrhocoris*, and *Dysdercus* (only the latter was sampled in this study) but represented <5% of reads in *Antilochus*, *Probergothius*, and *Dindymus* (35). Similarly, *Gordonibacter* has been shown to be present at low levels in Pyrrhocoridae genera including *Dindymus*, whereas we found either extremely low levels or no reads of *Gordonibacter* in our two sampled *Dindymus* species. In both of those predatory representatives, we found a microbiome dominated by a *Citrobacter*-type Enterobacteriaceae strain and a *Lactococcus* strain, both of which appear to be common insect gut inhabitants in Largidae and perhaps other insects. The previously described Pyrrhocoridae-associated *Clostridium* strain, which we also observed in all sampled Pyrrhocoridae species including predators, is surprising, as the evolution of a predatory life strategy in other Pentatomomorpha (such as the Asopinae [Pentatomidae] and Geocorinae [Geocoridae]) seems to negate dependence on any particular bacterium. This may reflect relatively recent evolution of this predatory trophic strategy. The observation of well-developed ceca in one ethanol-preserved female specimen (L54) of Pyrrhocoridae should be investigated with fresh or acetone-preserved specimens for verification. Although vestigial ceca have been noted in *Dysdercus*, *Antilochus*, *Probergothius*, and *Pyrrhocoris* (35), it is possible that some members of the well-supported clade containing *Melamphaus*, *Dermatinus*, *Ectatops*, and *Euscopus* retain functional ceca.

Evolutionary transitions of symbiont complexes. The exclusive association of Largidae with PBE clade *Burkholderia* is unlikely to be incidental. It is unlike other *Burkholderia* associations in Heteroptera, which are either specific associations with SBE clade *Burkholderia* or general associations with various clades of *Burkholderia*. The Pyrrhocoroidea lineage has now been shown to be one of the two earliest lineages of *Burkholderia*-associated Heteroptera and Largidae the earliest diverging single family that still retains this symbiosis (Fig. 4). It is likely that the ancestor of *Burkholderia*-associated Heteroptera was associated with *Burkholderia*, although it is not clear to which clade of *Burkholderia*, if any, it was allied. It is possible that this ancestor lacked specificity for *Burkholderia* as do extant members of Blissidae, which associate with *Burkholderia* in the three described clades. As lineages diverged, different mechanisms of specificity might have evolved, selecting for *Burkholderia* with increased lineage-specific fitness benefits. Nitrogen fixation could be the role of these nitrogen-fixing plant-associated symbiotic bacteria for their insect hosts, as suggested previously (36), and increased efficiency of nitrogen fixation (e.g., of elite commercial inoculants) could be the mechanism that selects for similar strains in geographically disparate Largidae.

Strict vertical transmission of symbionts, especially intracellular symbionts, can lead to rapid degradation of symbiont genomes, trapping the host and the symbiont in an "evolutionary rabbit hole" (63). While the ancestor of Heteroptera achieved independence from intracellular symbionts, some members developed novel associations with extracellular bacteria after reverting to herbivory. In the Pentatomoidea, these symbionts are primarily vertically transmitted and this mutualistic relationship is stable, due to shared selection acting on both the host and the symbiont due to partner fidelity (although a recent study suggests that vertically transmitted symbionts can be supplanted by suitable free-living bacteria [64]). For *Burkholderia*-associated Heteroptera, symbionts are primarily acquired selectively from the environment every generation, averting any transmission bottleneck that may lead to genome degradation (14) but requiring new host mechanisms for partner choice and, at least in similar systems, symbiont policing (65). Within Pyrrhocoridae, however, there has been a transition to vertically transmitted extracellular symbionts and, in several independent lineages of Lygaeoidea, reversions to vertically transmitted intracellular symbioses (Fig. 4). Transitions to vertically transmitted symbionts have been suggested to be driven by evolutionary pressures tied to specialization (35, 66), but many heteropteran plant specialists retain environmentally acquired symbioses (6, 67), suggesting a more nuanced evolutionary explanation. Comparative approaches focused on sister groups with different transmission strategies in the Heteroptera may yield useful biological correlations, although many additional phylogenetic and biological data will be required.

Many families of Heteroptera remain to be characterized (Fig. 4), especially using modern molecular methods, and examined representatives of other families demonstrate a potentially undersampled diversity of symbiotic complexes. In particular, the identity and clade of any symbiont present in members of the two families that are sisters to all other Coreoidea, namely, Hyocephalidae and Stenocephalidae (the former being associated with *Aca-*

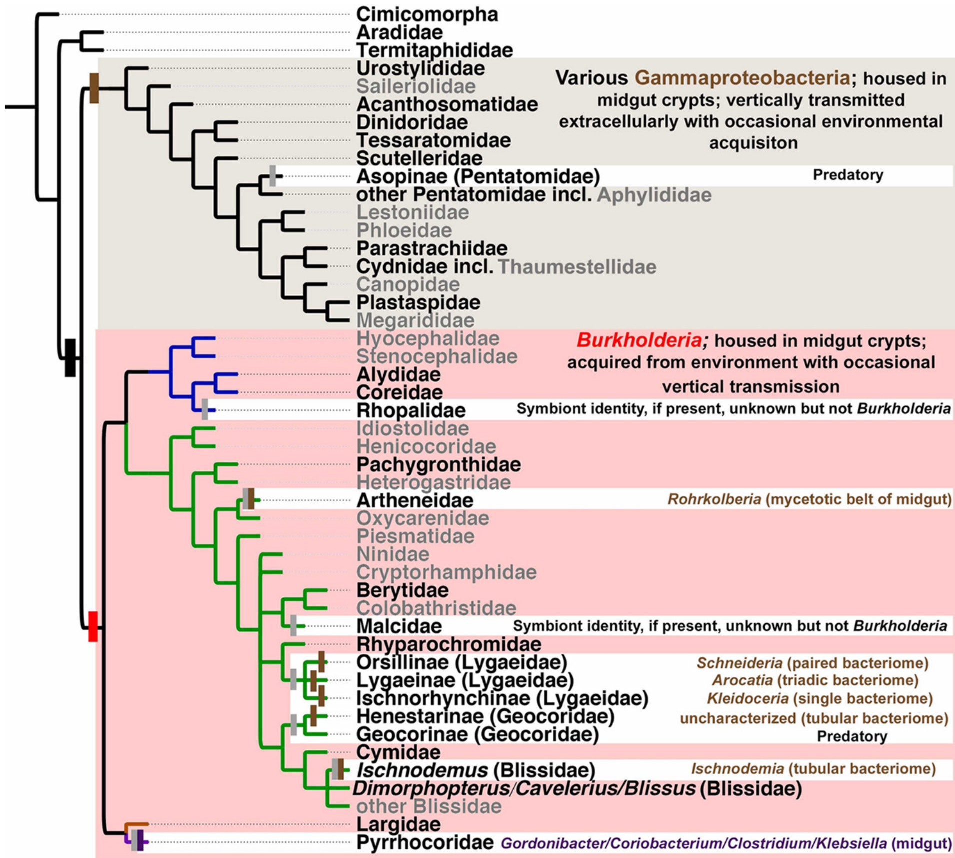

FIG 4 Transitions of symbiotic complexes in Pentatomomorpha. Various transitions of original partnerships with symbionts (large red bar for *Burkholderia* and large brown bars for various *Gammaproteobacteria*) after the evolution of ceca (large black bar) are indicated. Smaller bars indicate a loss of ceca (gray, vestigial in Pyrrhocoridae), the evolution of a new association with gammaproteobacteria (brown), or a consortium of bacteria including two *Actinobacteria* (purple). White boxes around taxa indicate a transition or absence of the ancestral symbiont complex of that clade (as indicated by gray boxes for *Gammaproteobacteria* and red boxes for *Burkholderia*) for taxa that are suspected or confirmed to retain that symbiosis. Taxa with names in gray have not yet been examined. The phylogeny is based on relationships recovered in this work (for Coreoidea) and well-supported relationships from Bayesian and likelihood analyses based on six Hox gene fragments (76). Families not represented in those analyses or without well-supported relationships were placed using successive weighting parsimony analyses based on morphology, as in reference 77 for Lygaeoidea and reference 78 for Pentatomoidea. References for symbiotic associates are as in the text (14, 21, 66, 79). Only one member of Lygaeinae (*Arocatus longiceps*) has so far been demonstrated to be associated with a symbiont (66). Oxycarenidae are purported to possess an unpaired bacteriome, but the identity of the bacterial inhabitant is unknown (80).

cia and *Eucalyptus* seeds and the latter being specialists on seeds of Euphorbiaceae [25]), may contribute to our understanding of how the evolution of strict associations with clade-specific *Burkholderia* arose. While the genome of a SBE clade *Burkholderia* strain isolated from Alydidae has been published (68), to date it has not provided much insight into nutritional supplementation. Unlike strict symbionts, these bacteria have large genomes; therefore, it is more difficult to discern function from the retention of genes alone. The sequencing of PBE clade *Burkholderia* symbionts from Largidae, which in at least one case are easily cultured, may provide insight into shared genes among symbiotic strains, as well as differences.

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