Evidence for Cascades of Perturbation and Adaptation in the Metabolic Genes of Higher Termite Gut Symbionts

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ABSTRACT **Termites and their gut microbes engage in fascinating dietary mutualisms. Less is known about how these complex symbioses have evolved after first emerging in an insect ancestor over 120 million years ago. Here we examined a bacterial gene, formate dehydrogenase (*fdhF*), that is key to the mutualism in 8 species of "higher" termite (members of the *Termitidae*, the youngest and most biomass-abundant and species-rich termite family). Patterns of *fdhF* diversity in the gut communities of higher termites contrasted strongly with patterns in less-derived (more-primitive) insect relatives (wood-feeding "lower" termites and roaches). We observed phylogenetic evidence for (i) the sweeping loss of several clades of *fdhF* that may reflect extinctions of symbiotic protozoa and, importantly, bacteria dependent on them in the last common ancestor of all higher termites and (ii) a radiation of genes from the (possibly) single allele that survived. Sweeping gene loss also resulted in (iii) the elimination of an entire clade of genes encoding selenium (Se)-independent enzymes from higher termite gut communities, perhaps reflecting behavioral or morphological innovations in higher termites that relaxed preexisting environmental limitations of Se, a dietary trace element. Curiously, several higher termite gut communities may have subsequently reencountered Se limitation, reinventing genes for Se-independent proteins via convergent evolution. Lastly, the presence of a novel *fdhF* lineage within litter-feeding and subterranean higher (but not other) termites may indicate recent gene "invasion" events. These results imply that cascades of perturbation and adaptation by distinct evolutionary mechanisms have impacted the evolution of complex microbial communities in a highly successful lineage of insects.**

IMPORTANCE **Since patterns of relatedness between termite hosts are broadly mirrored by the relatedness of their symbiotic gut microbiota, coevolution between hosts and gut symbionts is rightly considered an important force that has shaped dietary mutualism since its inception over 120 million years ago. Apart from that concerning lateral gene or symbiont transfer between termite gut communities (for which no evidence yet exists), there has been little discussion of alternative mechanisms impacting the evolution of mutualism. Here we provide strong gene-based evidence for past environmental perturbations creating significant upheavals that continue to reverberate throughout the gut communities of species comprising a single termite lineage. We suggest that symbiont extinction events, sweeping gene losses, evolutionary radiations, relaxation and reemergence of key nutritional pressures, convergent evolution of similar traits, and recent gene invasions have all shaped gene composition in the symbiotic gut microbial communities of higher termites, currently the most dominant and successful termite family on Earth.**

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Identifying factors associated with changing genetic diversity in natural microbial populations is crucial for understanding past and present ecology. Host-associated microbial populations have garnered much interest, as principles of evolution uncovered in the context of host-microbe interactions have wide-ranging applications (e.g., human health, animal development, agriculture) (1–3). In particular, studies of animal-microbe mutualism have revealed that microbial symbionts exert an important selective force for evolution in their eukaryotic hosts (3). Equally intriguing are the pressures that symbiosis imparts on the evolution of the hosts' microbial counterparts.

Many evolutionary studies on microbial symbiosis have explored the highly intimate mutualisms existing between insects and the microbial endosymbionts that live inside host insect cells (e.g., aphid [*Buchnera*] and tsetse fly [*Wigglesworthia*]) (4–6). These nutritional symbioses are obligate and characterized by low species complexity (typically, 1 symbiont species is present) (7–9). As such, they have been useful model systems for identifying the major evolutionary consequences of symbiosis in the microbial partners of mutualism: cospeciation with the host, genome reduction, low genomic GC content, and accelerated sequence evolution (10–14). However, such studies have limited potential for revealing the consequences of symbiosis between animal hosts and their extracellular symbionts, a category that includes gut tract microbes. As these symbioses can involve multiple microbial partners functioning as a "community bioreactor" to affect health

or disease in their host, determining how interactions between different symbiont species have evolved is necessary to understanding host-symbiont synergisms.

The obligate nutritional mutualism occurring between wood-feeding termites and their specific and complex hindgut microbiota offers an enticing subject for studies of evolutionary themes in polymicrobial, extracellular symbiont communities. Remarkably, mutualism, which underpins the insect's ability to access lignocellulose as a food source, predates the evolution of termites from their wood-feeding roach ancestors over 120 million years ago (15). Similar to endosymbioses, this long-term association has been facilitated by symbiont transfer between host generations (16), allowing coevolution between hosts and the symbiont community. Cospeciation as an evolutionary theme has indeed emerged over the past decade (e.g., 17–23); however, factors like diet, gut anatomy, and geography are also potential determinants of gene diversity in gut communities. Compared to endosymbionts, gut symbionts are exposed to considerably more environmental influence as food and environmental microbes continually pass through the gut tract. Under certain conditions, these microbes may establish as new symbionts or may horizontally transfer their genes to preexisting symbionts. Thus, termites and their rich diversity of gut microbial partners, which can number over 300 bacterial phylotypes (24, 25), provide exceptional opportunities for studying factors affecting evolution in symbiont communities.

Comparing symbiont genes in a wide range of termite host species is one way to learn about factors influencing symbiont evolution. Previously, we applied this comparative approach to symbiont metabolic genes encoding hydrogenase-linked formate dehydrogenase (*fdhF*) (37), important for CO_2 reductive acetogenesis, a key mutualistic process in lignocellulose degradation (26, 27). In all wood-feeding termites and roaches, CO_2 reductive acetogenesis is the major means of recycling the energy-rich H_2 derived from wood-polysaccharide fermentations into acetate, the insect's primary fuel source (28, 29). Study of *fdhF* in evolutionary less derived wood-feeding insects (the so-called primitive, "lower" termites and their wood-feeding roach relatives) implied that the trace element selenium has shaped the gene content of gut symbionts since termites diversified from roaches ~150 million years ago (15). More broadly, results suggested that, within related insect hosts that feature the same type of gut tract architecture and similar gut communities and diets, acetogenic symbionts as a metabolic subgroup of the greater gut community have maintained remarkably similar strategies to deal with changes in a key nutrient. But how have these symbionts (more precisely, their genes) responded to drastic changes in the termite gut, for example, to major alterations in gut tract architecture and to restructuring of the gut community itself?

To address this question, a comparison of *fdhF* genes present in extant insect species possessing gut communities representing "before" and "after" snapshots of drastic change is needed. The gut communities of lower termites represent the "before" condition. Gut communities in higher termites, which form the most recently evolved termite lineage (*Termitidae*), represent the "after" snapshot (30). Higher termites are known for highly segmented gut tracts and their lack of symbiotic gut protozoa (31), the primary sources of H_2 within the guts of earlier-evolved wood-feeding insects. Gut segmentation and the extinction of lignocellulose-fermenting protozoa are events that are thought to have occurred early during the emergence of this lineage, possibly in the late Cretaceous era (80 million years ago) to early Cenozoic era (50 million years ago) (15). Since then, higher termites with their gut symbionts have found ways to access polysaccharides bound in forms other than wood (e.g., dry grass, leaf litter, organic compounds in soil) and have become the most abundant and diverse termite group on Earth.

Here we investigated whether the dramatic shifts in termite biology and microbiology marking the diversification of higher termites into their extant forms have had lasting effects on the H_2-consuming symbiont community. To accomplish this, we analyzed the phylogeny of *fdhF* from the symbiotic gut communities of 8 higher termite species. The specimens belonged to the *Nasutiterminae* and *Termitinae* subfamilies, the two most numerically abundant and species-rich subfamilies within *Termitidae* (generally recognized as comprising four subfamilies [15, 32–34]), and were sampled from rainforest, beach, and desert environments in Central America and the southwestern United States. We compared higher termite *fdhF* sequences to each other and to previous data from less-derived wood-feeding insects. Our results indicate that the H_2-consuming bacterial community in higher termites experienced early evolutionary extinctions, possibly due to extinction of H_2-producing protozoa, followed by evolutionary radiation, convergent evolution, and even invasion. The latter three outcomes may have been influenced by trace element bioavailability. Together, the data provide a clear example of an extinction propagating through a chain of microbial mutualism, emphasizing the connectivity of symbiosis involving complex extracellular microbial communities such as those in the termite gut.

RESULTS AND DISCUSSION

To profile *fdhF* in the whole-gut microbial communities of higher termites with different species affiliations (see Fig. S1 in the supplemental material), habitats, and lifestyles, we constructed *fdhF* gene inventories (30 to 107 clones per species [total, 684]) from the whole-gut tracts of six higher termite species from Costa Rica and two higher termite species from California (Table 1). The broader taxonomic affiliations for higher termite specimens are indicated in a schematic termite cladogram (Fig. 1). Multiple genotypes (8 to 59) and phylotypes (4 to 12 operational taxonomic units, or OTUs, at a 97% protein sequence similarity cutoff) were recovered from the guts of each termite sample (see Table S1 in the supplemental material). This represents significantly more diversity than that discovered by metagenomic analysis of a wood-feeding higher termite's gut microbiota (35). In total, 62 novel FDH_H OTUs were documented in higher termites. To stringently estimate sampling completeness, we compared the number of observed OTUs to that predicted by the 95% higher confidence interval for mean Chao1 values (see Table S1 in the supplemental material), calculated using EstimateS (36). On average, 4.9 ± 4.5 (1 standard deviation [SD]) more OTUs were missing per inventory. However, by comparing the observed number of OTUs with mean Chao1 values, on average, less than 1 OTU remained undiscovered per inventory.

Broad-scale diversity of higher termite *fdhF*. Our previous studies of *fdhF* (37, 38) have shown that genes for hydrogenase-linked formate dehydrogenase enzymes (FDH_H [EC 1.2.1.2], encoded by enteric *Gammaproteobacteria*, *Spirochaetes*, and *Firmicutes*) are widespread in the guts of lower termites and wood roaches. Almost all *fdhF* recovered from these insects grouped

TABLE 1 Characteristics of higher termites examined in this study

Insect	Family (subfamily)[a]	Nest, location[b]	Ecosystem[c]	Food[d]	Soil[e]
Nasutitermes sp. Cost003	*Termitidae* (*Nasutitermitinae*)	Arboreal, forest (CR)	Premontane-wet rainforest transition	Wood	Low
Nasutitermes corniger Cost007	*Termitidae* (*Nasutitermitinae*)	Arboreal, forest-beach transition (CR)	Lowland moist forest	Palm	Low
Rhynchotermes sp. Cost004	*Termitidae* (*Nasutitermitinae*)	Arboreal, forest (CR)	Premontane-wet rainforest transition	Leaf litter	Medium
Microcerotermes sp. Cost006	*Termitidae* (*Termitinae*)	Arboreal, forest-beach transition (CR)	Lowland moist forest	Palm	Low
Microcerotermes sp. Cost008	*Termitidae* (*Termitinae*)	Arboreal, forest-beach transition (CR)	Lowland moist forest	Palm	Low
Amitermes sp. Cost010	*Termitidae* (*Termitinae*[f])	Subterranean, root zone (CR)	Premontane-wet forest	Roots/soil	High
Amitermes sp. JT2	*Termitidae* (*Termitinae*[f])	Subterranean galleries, desert (JT)	Warm temperate desert	Dry grass/soil	High
Gnathamitermes sp. JT5	*Termitidae* (*Termitinae*[f])	Subterranean galleries, desert (JT)	Warm temperate desert	Dry grass/Yucca/soil	High

[a] Termite family classifications are based on references 15 and 55.
[b] Data represent nest type, collection site. CR, Costa Rica; JT, Joshua Tree National Park, CA.
[c] Ecosystem terminology is based on the Holdridge life zone classification of land areas (58). Life zone categories for collection sites are based on maps in Holdridge et al. (58) and Enquist (59).
[d] Possible food source based on vegetation near collection location, insect trails, and/or laboratory feeding.
[e] Predicted level of soil exposure based on nest location (subterranean or above ground), food substrate, and foraging style.
[f] It is unclear whether *Amitermes* spp. affiliate within the subfamily *Termitinae* or rather constitute their own subfamily.

most closely with genes from the *Treponema primitia* termite gut acetogenic spirochete isolate, earning that phylogenetic clade the name "gut spirochete group." To determine the relationship between the sequence of higher termite *fdhF* and previously published sequences (and to guide detailed phylogenetic analyses), we constructed a phylogenetic "guide" tree (Fig. 2) based on 542 aligned amino acids in FDH_H, the catalytic subunit responsible for CO_2 reduction/formate oxidation in formate hydrogen lyase complexes (39).

Based on 3 tree construction methods (maximum likelihood, parsimony, and distance), higher termite sequences consistently clustered into 4 major clades (labeled A to D in Fig. 2). Sequences from higher termites with diets likely predominantly consisting of lignocellulosic substrates such as wood, palm, and dried grass (74% to 100%; Table 2) grouped into the Gut Spirochete Group (clade A; Fig. 2). Similar to other environmental sequences in the Gut Spirochete Group, higher termite sequences (37 phylotypes) most probably belonged to those of uncultured acetogenic *Spirochetes*. The presence of a diagnostic amino acid character uniquely shared among gut spirochete group sequences supported this phylogenetic inference (37). Interestingly, sequences from litter-feeding and subterranean higher termites (8% to 51%; Table 2) formed a novel cluster, designated the "AGR group" (for *Amitermes-Gnathamitermes-Rhynchotermes*) (clade B [17 phylotypes]; Fig. 2). Relatively few sequences (0% to 18%; Table 2)

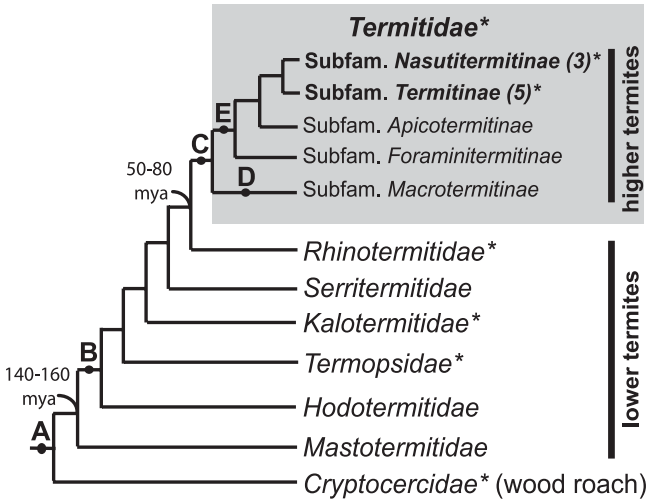

FIG 1 Schematic cladogram of major termite families and higher termite subfamilies showing major events in gut habitat evolution. Key events were (A) hindgut fermentation of lignocellulose; (B) loss of *Blattabacterium* fat body endosymbionts; (C) loss of mutualistic gut protists; (D) gain of mutualism with *Termitomyces* fungus; and (E) development of diverse feeding habits (e.g., soil feeding [15, 30]). Estimated timings (in millions of years ago [mya]) for the divergence of termites from wood roaches and of higher from lower termites are indicated. Family and subfamily associations of higher termites examined in this study are indicated in bold. Numbers of species examined are indicated in parentheses. This study analyzed gut community *fdhF* in the insect groups marked by asterisks.

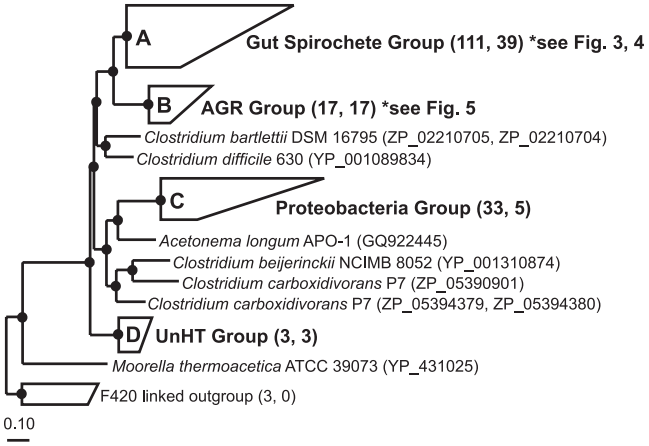

FIG 2 Protein phylogeny of hydrogenase-linked formate dehydrogenases (FDH_H). Sequences from the gut microbial communities of higher termites, lower termites, and wood-feeding roaches and from pure microbial cultures form four major clades (clades A, B, C, and D). Numbers in parentheses next to grouped clades denote the total number of sequences within a clade and the number of sequences recovered from higher termites. The tree was constructed with 542 ClustalW-aligned amino acids (61) and Phylip PROML (66) within the ARB software environment (65). A metagenomic sequence (IMG gene object identity no. 2004163507) from a *Nasutitermes* higher termite (31) was added by parsimony (253 amino acids); this fragment affiliates with the Gut Spirochete Group (clade A). Filled circles indicate nodes supported by maximum-likelihood, parsimony (PROTPARS; >60 of 100 bootstrap resamplings), and distance (Fitch) tree construction methods. The tree was outgrouped with F_{420}-linked FDH from methanogenic *Archaea* (GenBank accession no. P6119, CAF29694, and ABR54514). The scale bar indicates 0.1 amino acid changes per alignment position. Accession numbers for sequences comprising grouped clades are in Table S4 in the supplemental material.

TABLE 2 Distribution of higher termite inventory sequences among four major FDH_H clades (Fig. 2)

Source insect (number of clones)	No. of samples in indicated category			
	Gut spirochete	AGR	Proteobacteria	UnHT
Nasutitermes sp. Cost003[a] (104)	99	0	0	1
Nasutitermes corniger Cost007 (30)	86	0	7	7
Rhynchotermes sp. Cost004[a] (107)	47	51	2	0
Microcerotermes sp. Cost006 (74)	96	0	4	0
Microcerotermes sp. Cost008 (84)	100	0	0	0
Amitermes sp. Cost010[a](100)	85	13	0	2
Amitermes sp. JT2 (101)	92	8	0	0
Gnathamitermes sp. JT5 (84)	74	8	18	0

[a] Distribution based on sequences from combined L1 and L2 inventories. (See Tables S2 and S3 in the supplemental material for more information on L1 and L2.)

affiliated with clade C (as shown in Fig. 2) (5 phylotypes), a group defined by proteobacterial sequences and accordingly named the "*Proteobacteria* group." Clade D in Fig. 2 (3 phylotypes) was the least represented in inventories (0% to 7%; Table 2) and (like the AGR group) lacked pure culture representatives. This clade was named the "UnHT group" (for unclassified higher termite).

Genetic extinction and evolutionary radiation within higher termite gut communities. As higher termite sequences predominantly clustered into the gut spirochete group, we performed a phylogenetic analysis using 3 different treeing methods that focused on gut spirochete group sequences (Fig. 3). Unexpectedly, every higher termite sequence in this major clade group fell into a single subclade, the "higher termite spirochete" clade. This stood in striking contrast to our prior observations that *fdhF* genes from lower termites and wood roaches tend to form multiple, deeply branching but interspersed clades throughout the gut spirochete group. These results indicate that a large swath of *fdhF* diversity previously present in the guts of lower termite-like ancestors was lost from gut communities during higher termite evolution, consistent with a genetic bottleneck in the *fdhF* gene pool.

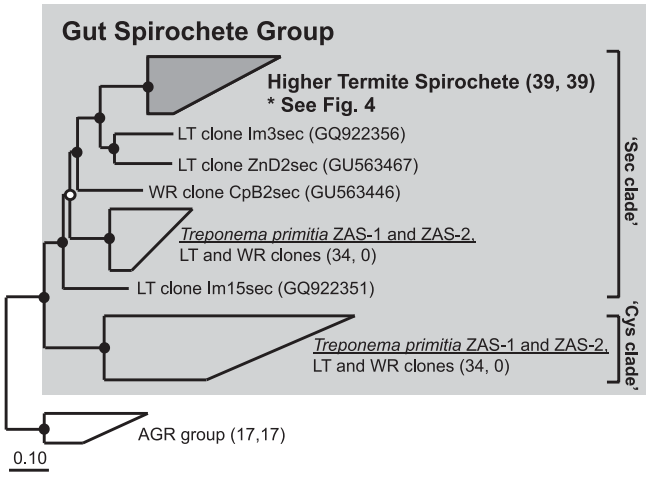

FIG 3 Protein phylogeny of Sec and Cys clade sequences within the Gut Spirochete Group of FDH_H (light gray [previously clade A], Fig. 2). Higher termite sequences grouped with a metagenomic sequence from *Nasutitermes* (IMG gene object identity no. 2004163507) to form the "Higher Termite Spirochete" group, highlighted in dark gray. "LT" and "WR" in sequence names denote clone sequences from lower termites and wood roaches, respectively. Insect sources are denoted in the sequence name by "Zn," "Im," and "Cp" (*Zootermopsis nevadensis*, *Incisitermes minor*, and *Cryptocercus punctulatus*, respectively). Numbers of sequences within grouped clades are indicated in parentheses as described for Fig. 2. The tree was constructed with 604 ClustalW-aligned amino acids and Phylip PROML. The metagenomic sequence was added in by parsimony methods. Filled circles indicate nodes supported by maximum-likelihood, parsimony (PROTPARS; >60 of 100 bootstrap resamplings), and distance (Fitch) tree construction methods. Unfilled circles indicate nodes supported by only 2 tree construction methods. The scale bar indicates 0.1 amino acid changes per alignment position. Accession numbers for sequences in grouped clades are in Table S4 in the supplemental material.

To substantiate phylogenetic observations of genetic bottlenecking and the accompanying hypothesis of gene extinction during higher termite gut community evolution, we assayed higher termite guts for the presence of "Cys" clade *fdhF*, a major phylogenetic group which comprised roughly half of all *fdhF* variants in the guts of lower termites and wood roaches (37). Using Cys clade-specific primers (Cys499F1b, 1045R; see Text S1 in the supplemental material), we screened the gut DNA of all higher termite samples, 3 lower termite species from Southern California representing 3 termite families, and a wood-feeding roach for Cys clade genes by PCR. No product (or correctly sized product) was detected from any higher termite templates after 30 cycles of PCR amplification (see Fig. S2 in the supplemental material). In contrast, all amplifications from lower termite and roach gut templates yielded robust products. Bearing in mind the inherent limitations of primer-based assays, the results independently corroborate inventory data which pointed to unique sweeping losses of *fdhF* diversity having occurred in higher termite gut communities.

To gain insight on when such drastic culling of *fdhF* gene diversity might have occurred, we analyzed the phylogeny of Higher Termite Spirochete clade sequences (Fig. 4). Sequences from the most closely related higher termites clustered into shallow clades, suggesting recent coevolution between host and *fdhF* encoded by gut symbionts. However, sequences from higher termite species representing different subfamilies formed deeply branching clades whose branching order could not be resolved. This type of "spoke" topology in phylogenetic trees, wherein clades radiate with no resolvable order like spokes from a wheel, is typical of adaptive (or "ecological") radiations (40). Based on uncertainties in branching order at the subfamily level, the radiation may have occurred sometime during the emergence of higher termite subfamilies. Since adaptive radiations commonly occur after massive extinctions, the major loss in *fdhF* diversity observed in both inventory and PCR data may have taken place earlier than subfamily diversification, possibly during the evolution of the last common ancestor (LCA) of all higher termites over 50 million years ago

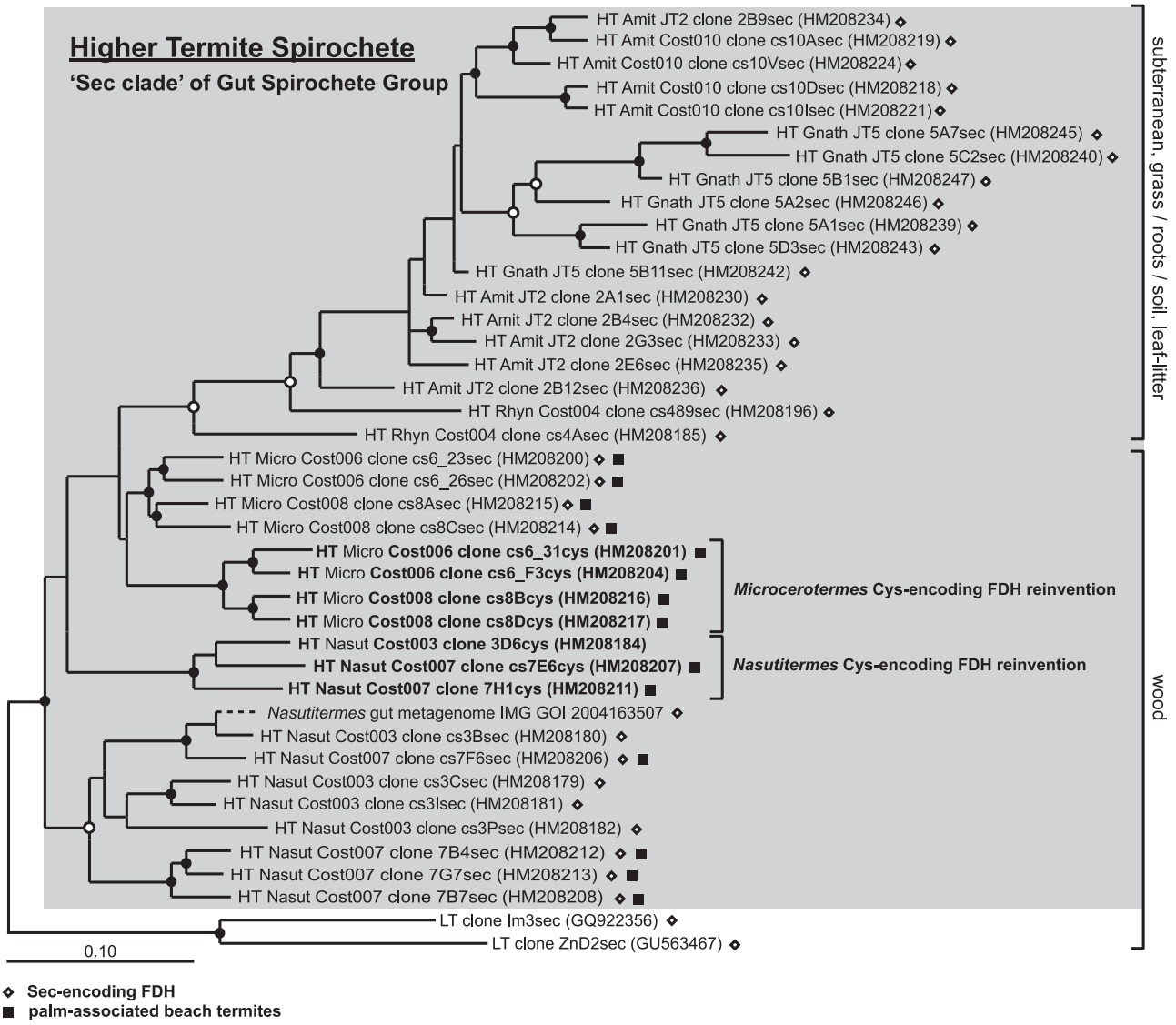

FIG 4 Protein phylogeny of Higher Termite Spirochete Group sequences (gray box [dark gray clade in Fig. 3]) within the Sec clade of the Gut Spirochete Group (light gray clade in Fig. 3). "HT," "LT," and "WR" in sequence names indicate the insect source (higher termite, lower termite, wood roach); "Cost" and "JT" refer to higher termite species from Costa Rica and California, respectively (see Table 1). Open diamonds next to sequences and "sec" in the sequence name denote selenocysteine FDH_H; "cys" in the sequence name denotes a cysteine-encoding variant. Filled squares indicate sequences derived from higher termites collected at Costa Rican beaches. Sequences predicted to result from evolutionary reinventions and the likely food substrates for insects are also indicated. The tree was constructed with 604 ClustalW-aligned amino acids and Phylip PROML. The branching position of a *Nasutitermes* metagenomic FDH_H fragment (added by parsimony using 250 amino acids) is indicated with a dashed line; thus, the phylogenetic distance represented by this dashed line is not comparable to that corresponding to any other sequence. Filled circles indicate nodes supported by PROML, parsimony (Phylip PROTPARS), and distance (Fitch) methods of tree construction. Unfilled circles indicate nodes supported by 2 of 3 tree construction methods. The scale bar corresponds to 0.1 amino acid changes per alignment position. GenBank accession numbers are in parentheses.

(15). The earliest evolved higher termites belong to the fungus-feeding subfamily *Macrotermitinae* (15, 33). Examining *fdhF* in a fungus-feeding termite should help clarify the timing of *fdhF* extinction.

"Chain of extinction" hypothesis: disappearance of H_2-producing protists and H_2-consuming dependents. Given that the *fdhF* gene extinction event (or events) occurred during time periods relevant to the LCA of higher termites, the most plausible cause of *fdhF* extinction would seem to relate to another extinction that transpired in the same time period: the extinction of H_2-producing gut protozoa. While these links are circumstantial, the link between *fdhF* and protozoa also makes functional sense. A dramatic extinction of primary H_2 producers (leading to a shift in niche occupancy) in the lower termite-like LCA would undoubtedly propagate down the microbial "food chain" to H_2-consuming symbionts such as H_2-consuming acetogens that possess *fdhF*. The result of this propagation could manifest itself in extant higher termites in one of two ways: (i) a dramatic shift in the abundance of H_2-consuming symbionts (and their *fdhF* genes) relative to that found in less-derived termites or (ii) a dramatic loss of diversity. Our results support the latter scenario, implying that the consequences of protozoon extinction for symbionts (and

their genes) lying "downstream" in H_2 metabolism were more wave-like than ripple-like.

Not all genes, however, went extinct. Those that survived waves of extinction underwent an explosive radiation to fill out previously occupied niches. We posit that their genetic descendants form the Higher Termite Spirochete Group.

The data also provide circumstantial support for a previous hypothesis on the nature of association between certain protozoa and ectosymbiotic *Spirochetes*. Leadbetter et al. (41) proposed an H_2-based symbiosis to explain the presence of *Spirochetes* attached to protozoon surfaces. The results described here strengthen the consequent implication that some ectosymbiotic *Spirochetes* may be acetogenic and also draw attention to unexplored metabolic dependencies between protists and free-living *Spirochetes* that may not require physical proximity.

Selenium dynamics: sweeping gene extinction followed later by occasional convergent evolution. Our previous study of Gut Spirochete Group FDH_H from the guts of less-derived wood-feeding insects revealed two functional enzyme variants differing in a key catalytic residue (Fig. 3) (37). "Sec" clade sequences have been predicted to encode enzymes that contain the trace element selenium in the form of selenocysteine at the active site. In contrast, Cys clade sequences generally encode selenium-independent variants containing cysteine, instead of selenocysteine, at the active site. Study of *T. primitia* (38) and other organisms (42–44) that possess dual-enzyme variants indicates that organisms switch to using their selenium-independent enzymes under conditions of selenium limitation.

In higher termites, the striking absence of Cys clade gene variants implied that some selective pressure related to selenium limitation was relaxed in higher termite gut communities, such that genes for selenium-independent enzymes were lost by genome reduction from symbiont genomes. An alternative explanation was that the characteristic absence of Cys clade genes in higher termites related to sampling differences between studies. To address this concern, we collected 2 lower termite species from the same habitats as a subset of higher termites (Costa Rican lower termite *Coptotermes* sp. Cost009 collected near *Microcerotermes* sp. Cost006 and Cost008; desert-adapted lower termite *Reticulitermes tibialis* JT1 collected near higher termite species *Amitermes* sp. JT1 and *Gnathamitermes* sp. JT5) and performed PCR screening on whole-gut community DNA with Cys clade-specific primers. Correctly sized PCR amplicons were observed for all lower termites, regardless of where they were collected (see Fig. S2 in the supplemental material), to independently support inventory data. Thus, the absence of Cys clade genes is a characteristic feature of higher termite gut microbial communities, rather than a result of sampling differences.

To further explore the dependence of higher termite FDH_H on selenium (a trace nutrient whose bioavailability varies with redox state [45, 46]), we inspected every higher termite Sec clade FDH_H sequence for the selenocysteine amino acid. We discovered that several sequences from Costa Rican higher termites actually include selenium-independent FDH_H (clones cs6_31cys, cs6_F3cys, cs8Bcys, cs8Dcys, 3D6cys, cs7E6cys, and 7H1cys). Since these cysteine-containing FDH_H variants were nested within the Sec clade, they must have originated from the duplication of a selenium-dependent FDH_H gene followed by mutational modification of the active site selenocysteine into cysteine. Also of note is the clustering of instances of *Microcerotermes* cysteine-containing FDH_H with each other (Fig. 4), to the exclusion of cysteine FDH_H from *Nasutitermes*. This result points to two independent gene duplication events, each of which has resulted in the "reinvention" (or convergent evolution) of a selenium-independent FDH_H gene from Sec clade FDH_H gene stock in termites. To our knowledge, these data provide the first examples of convergent evolution by symbiotic gut microbes in the termite gut.

The forces that have selected for convergent evolution are intriguing. One possibility is that the selenium content of the termite's diet may vary enough to affect selenium bioavailability in the gut tract and thus select for one or the other gene variant. This hypothesis stems from the observation that the majority of reinvented cysteine variants (Fig. 4) were identified in termites collected from palm trees in beach areas where plants are regularly submerged in seawater. Estimates of total selenium concentrations in the surface mixed layers of the ocean are 4 orders of magnitude lower than estimates of concentrations in surface soils (47). Thus, seawater may flush selenium from beach soil, reducing selenium levels in plants and consequently in the diet of termites. But even if dietary selenium levels drove convergent evolution, the larger question of why selenium-dependent FDH_H genes are favored over selenium-independent variants in higher termites remains unanswered. Perhaps a structural aspect of the gut tract in higher termites makes the same amount of selenium more easily bioavailable in higher termite than in lower termite guts. Perhaps behavioral innovations relating to, e.g., increased and more effective grazing for nutrients are at play. The continued study of selenium biology and chemistry in termite guts, and of the termites in which they reside, should provide further insight into such possibilities.

Recent gene invasion into subterranean and litter-feeding higher termite gut communities. Gut preparations from subterranean (*Amitermes* sp. Cost010, *Amitermes* sp. JT2, and *Gnathamitermes* sp. JT5) and litter-feeding (*Rhynchotermes* sp. Cost004) higher termites featured a novel clade of FDH_H absent in other termites (clade B in Fig. 2). Figure 5 shows the detailed phylogeny of AGR group sequences. Since we could not infer the identity of uncultured organisms encoding these sequences from phylogeny, we inspected the sequences for possible indel signatures. Indeed, AGR sequences contained an amino acid indel (Fig. 5, right panel) similar to that previously observed in Gut Spirochete Group sequences, weakly suggesting a spirochetal origin for AGR group genes (or for that indel).

We hypothesized that AGR group genes might be diagnostic markers for subterranean and litter-feeding higher termite diets and behaviors. To test this hypothesis, we designed AGR clade-specific primers (AGR193F, 1045R; see Text S1 in the supplemental material). We screened lower and higher termite gut DNA templates using nested PCR methods (see Fig. S3 in the supplemental material). Robust amplicons were consistently detected in every subterranean and litter-feeding higher termite but not in arboreal higher termites, lower termites, or *Cryptocercus punctulatus*, supporting the conjecture that AGR group alleles are characteristic of subterranean and litter-feeding higher termite gut communities.

To understand why AGR group sequences would be present in only a subset of higher termites, we compared the relative abundances of AGR sequences (Table 2) with the host's predicted diet (Table 1). AGR sequences represented the most abundant phylotype in leaf litter-feeding termites (51%; *Rhynchotermes* sp.

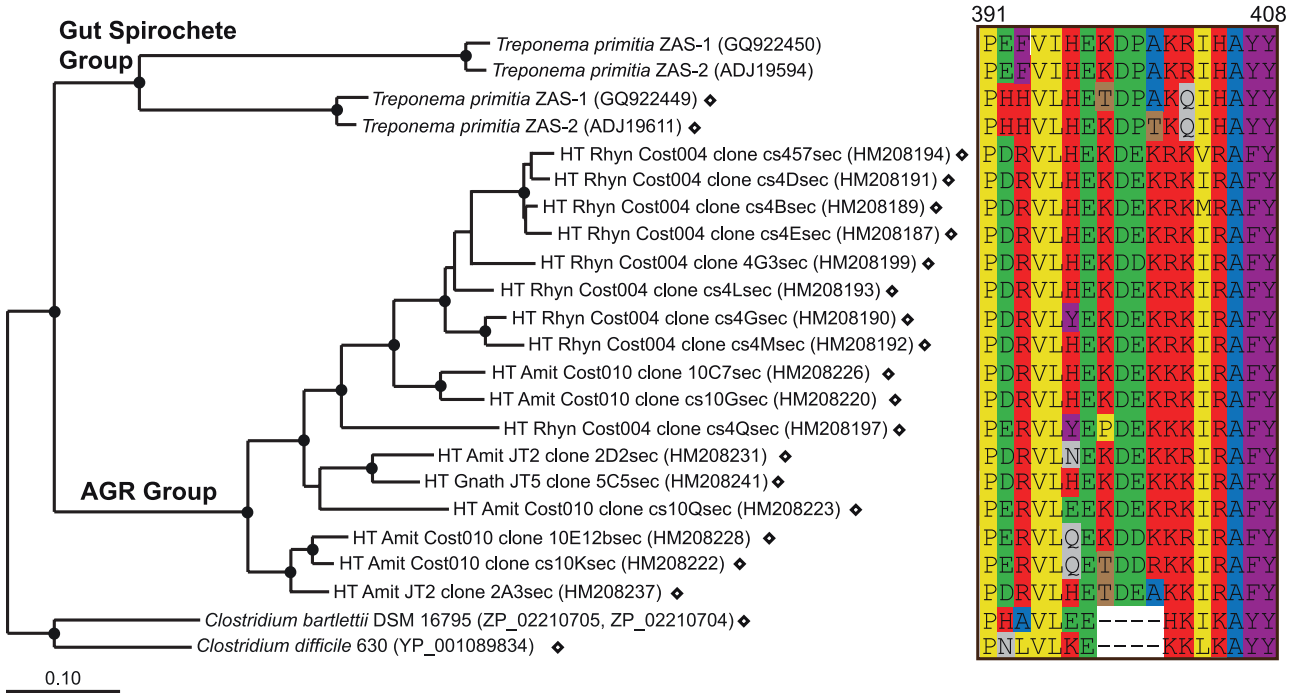

FIG 5 Protein phylogeny (left panel) and amino acid character (right panel) analysis of AGR group sequences (clade B, Fig. 2). In the left panel, sequences from *T. primitia* represent the gut spirochete group (clade A, Fig. 2). Open diamonds located next to sequences and clone names containing "sec" denote selenocysteine FDH_H; unmarked sequences denote cysteine-encoding FDH_H. The tree was constructed with 595 ClustalW-aligned amino acids using Phylip PROML. Filled circles indicate nodes supported by PROML, parsimony (Phylip PROTPARS), and distance (Fitch) methods of analyses. The scale bar indicates 0.1 amino acid changes per alignment position. In the right panel, numbers above the alignment refer to amino acid positions in the selenocysteine FDH_H of *T. primitia* sp. ZAS-2 (ADJ19611). Canonical amino acid one-letter coding applies. Amino acids are colored based on shared physical-chemical characteristics: yellow/hydrophobic, blue/small, brown/nucleophilic, purple/aromatic, green/acidic, red/basic, gray/amide.

Cost004) but appeared at lower frequencies (8% to 13%) in subterranean termites with diets containing monocots (such as sugarcane roots, grass, and *Yucca*) and were not recovered from termites feeding on wood. Based on tannin levels reported for bark, leaves, and wood (48), the presence of AGR group sequences may positively relate to dietary tannin levels and represent a novel marker for an as-yet-unappreciated group of uncultured acetogens, perhaps ones that exhibit greater tolerance to phenolic compounds such as tannin. Alternatively, they may represent a group of nonacetogenic, tannin-tolerant, heterotrophic bacteria that ferment residual sugars in decaying leaves and employ the enzyme in direction of formate oxidation, perhaps in concert with nonacetogenic formyl-tetrahydrofolate synthetase genes inventoried from the guts of litter and subterranean higher termites (49). In any case, the phylogenetic remoteness of the AGR group from other major FDH clades suggests that a niche that was previously small (or absent) in wood-feeding termites gained importance in higher termites that feed on decaying plant materials that have substantial contact with soil.

The basal location of subterranean termite sequences (Fig. 5) hints that the influx of AGR-type gene stock into gut communities occurred in a termite belonging to the *Termitinae* subfamily and that such genes may have been laterally transferred into the *Nasutitermitinae*. It remains to be determined whether the initial influx manifested itself as the lateral transfer of AGR genes from an organism passing through the gut to an established gut symbiont or as the invasive establishment of a novel group of gut symbionts. Indeed, complex phylogenetic relationships between spirochete rRNA genes and host termites (50, 51) which are not strictly cocladogenic imply that acquisition of gut symbionts has been ongoing during termite evolution, a concept outlined previously (52, 53). However, these events most likely occurred in the very distant past, as there is also strong evidence in support of the idea of broad levels of spirochete coevolution with lower termites (21). The select presence of AGR-type genes in litter and subterranean higher termite guts suggests a more recent acquisition in the evolutionary history of this successful lineage of termite hosts.

PCA: the past shapes most of the present. To quantify the importance of different factors associated with FDH_H phylogeny, we performed a principal component analysis (PCA) (Fig. 6) using UniFrac phylogeny statistics software (54). The first principal component (P1 [27.64% of total variance]; Fig. 6a and b) clearly separates lower termite and wood roach inventories from the higher termite inventories, a result consistent with P1 tracking the presence (lower termite, wood roach) or absence (higher termite) of flagellate protozoa in gut communities. This result supports our hypothesis that *fdhF* gene extinction results from protozoal extinctions. The second (Fig. 6a and c) and third (Fig. 6b and c) principal components accounted for similar levels of variance (15.46% and 14.25%). P2 clustered inventories containing *Protobacteria* clade sequences, whereas P3 grouped those containing AGR clade sequences. The latter grouping supports the idea that dietary variables (amount of soil and form of lignocellulose in diet) also play roles in shaping gut communities (55–57). PCA did not appear to cluster the data on the basis of geography, nest type,

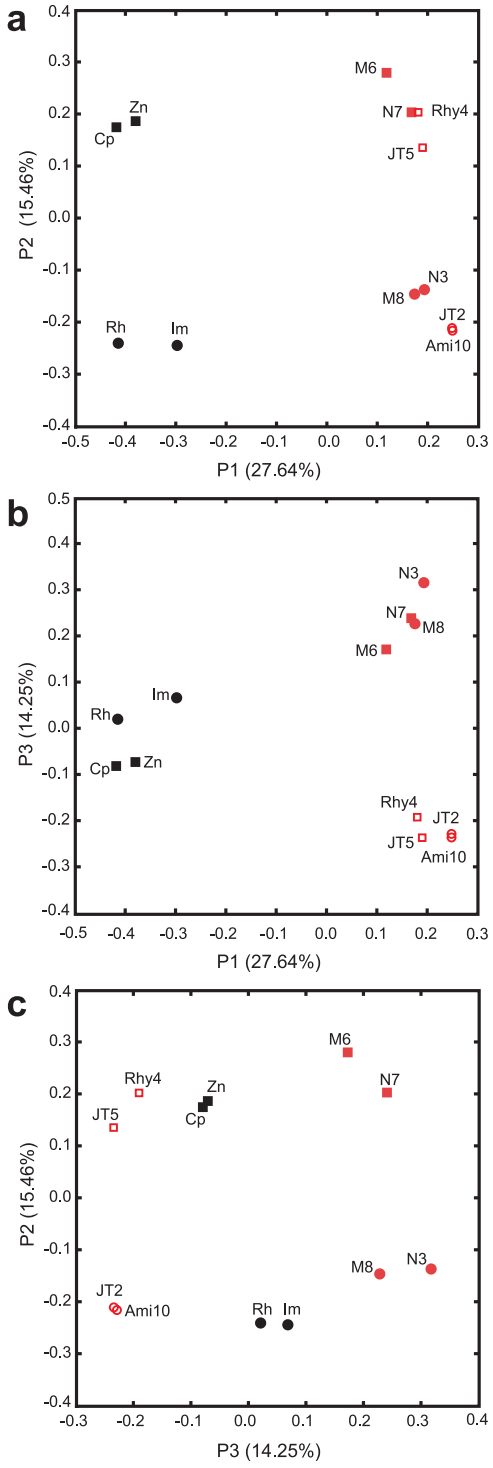

FIG 6 UniFrac principal component analysis of FDH_H phylogeny associated with the gut microbial communities of termites and related insects. For species of wood roach and lower termites, Cp represents *C. punctulatus*; Zn, *Z. nevadensis*; Rh, *R. hesperus*; and Im, *I. minor*. For species of higher termites, N3 represents *Nasutitermes* sp. Cost003; N7, *Nasutitermes corniger* Cost007; M6, *Microcerotermes* sp. Cost006; M8, *Microcerotermes* sp. Cost008; Rhy4, *Rhynchotermes* sp. Cost004; A10, *Amitermes* sp. Cost010; Jt2; *Amitermes* sp. JT2; and Jt5, *Gnathamitermes* sp. JT5. Black shapes represent lower termite and wood roach inventories; red shapes, higher termite inventories; squares, inventories containing *Proteobacteria* group sequences; open shapes, inventories containing AGR group sequences. The tree in Fig. 2 was analyzed with UniFrac (termite species as the environment variable; 100 permutations).

or habitat—the diversity of which was considerable in the sampled species. Based on these data, the transition from lower termite body and gut community plans to the higher termite forms seems to far outweigh in importance other variables for shaping *fdhF* diversity in higher termites. This is consistent with the notion that the signal imprinted long ago in *fdhF* sequence diversity in higher termites was the mass extinction of protozoa during their transition.

Model for *fdhF* evolution in wood-feeding dictyopteran insects. Based on our findings, we constructed a schematic modeling the evolutionary trajectory of *fdhF* in the guts of wood-feeding insects, beginning with *fdhF* in the LCA of termites and wood-feeding cockroaches and continuing to the present day (Fig. 7). The evolutionary sequence highlights the importance of past extinction events as key determinants of present diversity. Previous data (37) imply that a spirochete member of the gut community within the LCA of termites and roaches possessed an ancestral *fdhF* gene, which underwent duplication and mutational modification into selenocysteine- and cysteine-encoding forms. These two functional variants of *fdhF* then coradiated with gut communities and the host insects as wood-feeding insects were diversifying into termite and roach forms to create the "Sec" and "Cys" clades. Here we have documented a severe trimming of Sec clade diversity and complete loss of all Cys clade genes. We estimate that this occurred during the emergence of the higher termite line, when guts became segmented, foraging behaviors diversified, and cellulolytic protozoa went extinct from the gut community. It is unclear whether losses of Sec and Cys clade genes represent single or multiple extinction events. In any case, genes surviving extinction radiated to fill the newly emptied (or created) niches within higher termite guts. In particular, the convergent evolution of selenium-independent *fdhF* suggests an adaptive radiation into selenium-limited niches that have recently become available in a subset of higher termites. It also appears that some aspect of litter-feeding and subterranean lifestyles has allowed the more recent establishment of a novel clade of *fdhF*, possibly reflecting an invasion by non-gut-adapted species.

Conclusions. The overarching goal of this study was to understand how symbiont communities and their genes have been impacted by drastic change and other perturbations over evolutionary time scales. We accomplished this goal using the obligate nutritional mutualism between termites and their hindgut microbial communities as a "backdrop" for an evolutionary case study of symbiosis. Comparative analysis of a symbiont metabolic gene unveiled a striking implication for evolutionary biology in complex microbial communities wherein the metabolisms of community members form a network of dependent interactions: collapse of a functional population (or network node) within a symbiont community can have dramatic and long-lasting effects on the genes carried by symbionts occupying niches downstream in the chain of community metabolism.

Connectivity and adaptation are themes that have emerged from this study of symbiont communities. The challenge now is to understand the specific interactions on which connectivity was based in the distant past. Studying the genes and organisms involved in present-day interactions between specific microbes within termite gut communities should give us clues to how the past has shaped and continues to shape the present.

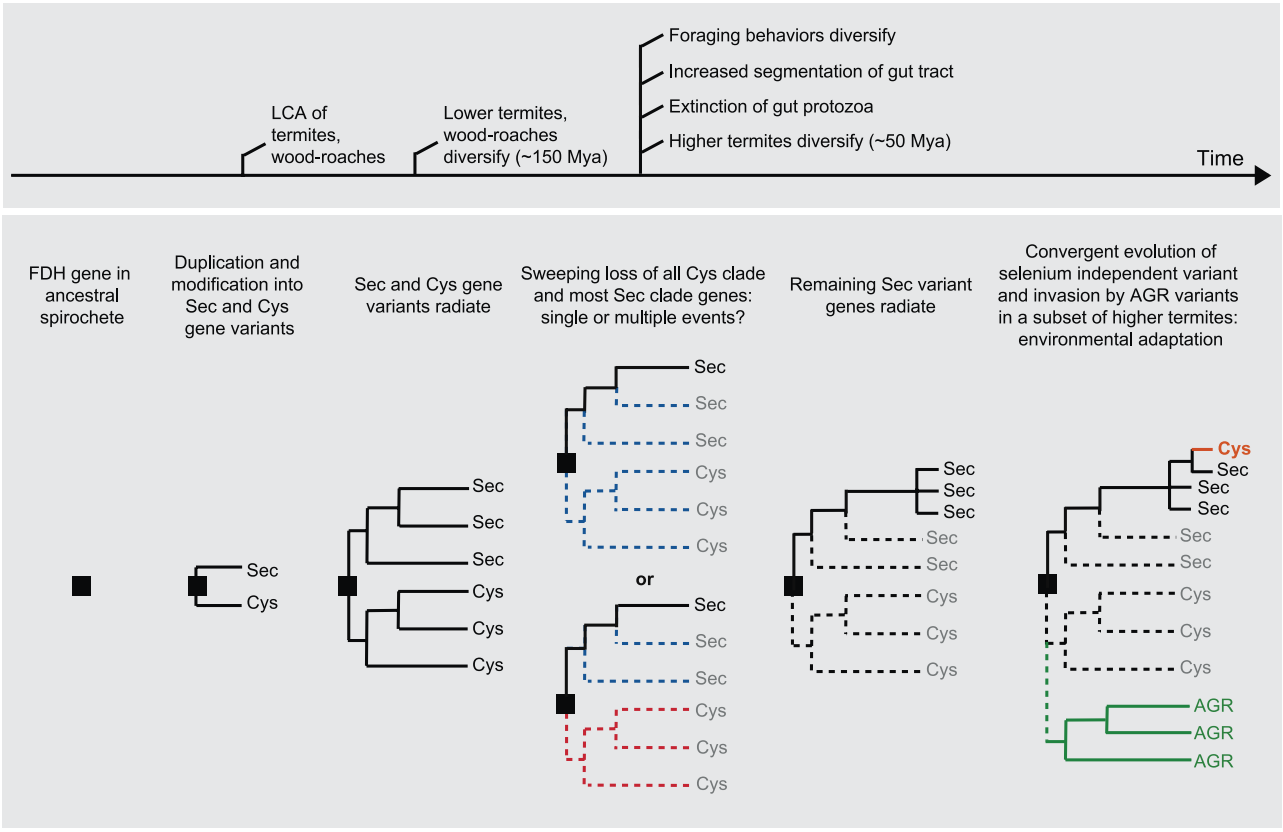

FIG 7 Inferred evolutionary history for *fdhF* in the symbiotic gut microbial communities of lignocellulose-feeding insects.

MATERIALS AND METHODS

Insect collection and classification. Details on insect collection can be found in Ottesen and Leadbetter (49). Briefly, termite species obtained by permit in Costa Rica were *Nasutitermes* sp. Cost003, collected from the National Biodiversity Institute of Costa Rica (INBio) forest; *Rhynchotermes* sp. Cost004, from leaf litter within INBio; *Amitermes* sp. Cost010, from soil-encrusted decayed sugar cane at a sugar cane plantation in Grecia; *Nasutitermes corniger* Cost007, *Microcerotermes* spp. Cost006 and Cost008, from unidentified species of palm growing at a beach in Cahuita National Park (CNP); and *Coptotermes* sp. Cost009 (lower termite, family *Rhinotermitidae*), from wood near CNP's Kelly Creek Ranger Station. Termites species obtained under a U.S. Park Service research permit from Joshua Tree National Park, CA, were *Amitermes* sp. JT2 and *Gnathamitermes* sp. JT5, collected from subterranean nests, and *Reticulitermes tibialis* JT1 (lower termite, family *Rhinotermitidae*), collected from a decayed log in a dry stream bed.

Termites were identified based on the gene sequence of mitochondrial cytochrome oxidase 2 (COII; see Fig. S1 in the supplemental material) and on morphology. In general, inadequate COII sequence data prevented taxonomic assignments past the genus level. Genus names for *Rhynchotermes* sp. Cost004 and *Gnathamitermes* sp. JT5 specimens were assigned based on head and mandible morphology and collection location. COII analyses confirmed that the 8 termite species examined in this study represented distinct lineages in the subfamilies *Nasutitermitinae* and *Termitinae*. Classification of termite habitats was based on the Holdridge life zone classification of land areas (58) and life zone maps in references 59 and 60.

DNA extraction. For each termite species, the entire hindguts of 20 worker termites were extracted within 48 h of collection, pooled into 500 μl 1× Tris-EDTA (TE) buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8), and stored at −20°C until DNA extraction. Whole-gut community DNA was obtained using the method described in reference 61.

***fdhF* amplification and cloning.** PCR mixtures containing universal *fdhF* primers (1 μM of each degenerate form) (37) were assembled with 1× FAILSAFE Premix D (Epicentre Biotechnologies, Madison, WI). Polymerase (EXPAND High Fidelity polymerase; Roche Applied Science, Indianapolis, IN) (0.07 to 0.14 U μl^{-1}) and gut DNA template (0.05 to 1 ng μl^{-1}) concentrations were adjusted so that reactions would yield similar amounts of PCR product. Thermocycling conditions for PCR performed using a Mastercycler Model 5331 thermocycler (Eppendorf, Westbury, NY) were 2 min at 94°C, 25 cycles of 94°C for 30 s, 51°C, 53.6°C, or 55°C for 1 min, and 68°C for 2 min 30 s, and 10 min at 68°C. Details on PCR are presented in Table S2 in the supplemental material. Annealing amplifications at 51°C yielded products of multiple sizes upon gel electrophoresis with 1.5% (wt/vol) agarose (Invitrogen, Carlsbad, CA). The correctly sized bands were excised and gel purified with a QIAquick gel extraction kit (Qiagen, Valencia, CA). To ensure product specificity, PCR was performed at higher annealing temperatures (53.6°C for *Microcerotermes* sp. Cost008 and *Amitermes* sp. Cost010 and 55°C for *Nasutitermes* sp. Cost003 and *Rhynchotermes* sp. Cost004). These reactions yielded single product bands upon electrophoresis. All PCR products were cloned using a TOPO-TA cloning kit (Invitrogen). Clones (30 to 107 per termite species) were screened for the presence of the correctly sized insert by PCR and gel electrophoresis. PCR mixtures contained T3 (1 μM) and T7 (1 μM) primers, 1× FailSafe PreMix D (Epicentre), 0.05 U μl^{-1} *Taq* polymerase (New England Biolabs, Beverly, MA), and 1 μl of cells in 1× TE buffer as the template. Thermocycling conditions were 2 min at 95°C, 30 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 2 min 30 s, and 10 min at 72°C. Primer design and PCR amplifications of Cys and AGR group *fdhF* genes are described in Text S1 in the supplemental material.

RFLP analysis, sequencing, and diversity assessment. Most inventories were subjected to restriction fragment length polymorphism (RFLP)

typing, wherein correct-sized products generated by screening PCRs were digested with the restriction enzyme RsaI (New England Biolabs) and electrophoresed on a 2.5% (wt/vol) agarose gel (Invitrogen). Plasmids from clones with unique RFLP patterns were purified using a QIAprep Spin Miniprep kit (Qiagen). For *Nasutitermes* sp. Cost003, *Rhynchotermes* sp. Cost004, and *Amitermes* sp. Cost010 inventories, generated at an annealing temperature of 51°C, plasmids from clones having the correct-sized products were purified for sequencing without RLFP typing. Plasmids were sequenced with T3 and T7 primers at Laragen, Inc. (Los Angeles, CA) using an Applied Biosystems Incorporated ABI3730 automated sequencer. Lasergene software (DNASTAR, Inc., Madison, WI) was used to assemble and edit sequences. Sequences were aligned with ClustalW (62), manually adjusted, and grouped into operational taxonomic units at a 97% protein similarity level based on distance calculations (Phylip Distance Matrix, Jones-Thornton-Taylor correction) and DOTUR (63). The program EstimateS v8.2.0 (36) was used to assess inventory diversity and completeness.

COII amplification for termite identification. Mitochondrial cytochrome oxidase subunit II (COII) gene fragments from Costa Rican termites were amplified from DNA containing both insect and gut community material using primers A-tLEU and B-tLYS and the protocol described by Miura et al. (64, 65). COII gene fragments from Californian termites were amplified using the supernatant of a mixture containing an individual termite head crushed in 1× TE buffer as the template. Primers and PCR conditions were identical to those employed for Costa Rican termite COII. PCR products were purified using a QIAquick PCR purification kit (Qiagen), sequenced, and analyzed to verify the identity of termite specimens.

Phylogenetic and principal component analysis. Phylogenetic analyses of protein and nucleotide sequences were performed with ARB version 09.08.29 (66). Details of tree construction can be found in the figure legends. In general, the trees show results from Phylip PROML analysis; node robustness was analyzed with PROTPARS and Fitch distance methods as well (67). The same filter and alignments were employed when additional tree algorithms were used to infer node robustness. All phylogenetic inference models were run while assuming a uniform rate of change for each nucleotide or amino acid position. Principal component analysis of FDH_H phylogeny and environment data was performed using Unifrac software (54).

Nucleotide sequence accession numbers. GenBank accession numbers for sequences determined during this study are listed in Table S4 in the supplemental material.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00223-12/-/DCSupplemental.

Text S1, DOCX file, 0.1 MB.
Figure S1, PDF file, 1.1 MB.
Figure S2, PDF file, 0.2 MB.
Figure S3, PDF file, 0.2 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.
Table S3, DOCX file, 0.1 MB.
Table S4, DOCX file, 0.1 MB.

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All authors participated in insect collection. X.Z. and J.R.L. conceived of the experiments. X.Z. performed the experiments and analyzed the data. X.Z. and J.R.L. wrote the manuscript.

The authors have no competing interests.

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