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INSIGHTS FROM INSECT–MICROBE SYMBIOSES

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For most of the nearly 4-billion-year history of life on Earth, microbial evolution was tied to the diversity of microniches in habitats that were part of the planet's geological history. Diversification of microorganisms was governed by the physical and chemical characteristics of their environment as well as by their interactions with one another. However, in the last 0.5 billion years or so, microbes have had increasing opportunities to establish interactions with plants and animals. As these so-called crown eukaryotes have diversified, they have added greatly to the habitats and lifestyles available for microbial life. On the zoological side, the best example of this is the insects, which include more species than all other animals combined (Wilson 1992). Insects have established a cornucopia of interactions with Bacteria and Archaea as well as

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with fungal and protistan members of the Eucarya (Buchner 1965; Anderson et al. 1984; Martin 1987; Schwemmler and Gassner 1989; Kane 1997).

In the macrocentric world of biological science, insects are often represented as the poster child for undiscovered numbers of species. Estimates of their biodiversity range from 2 to 100 million species, close to 900,000 of which have been described (Hammond 1994; Nielsen and Mound 1997). Naturally, as difficult as it has been to estimate the biodiversity of insects (all of which are visible to the naked eye), similar estimates of microbial biodiversity are subject to far greater uncertainty. For example, as Trüper (1992) noted, it would not be surprising to find that there is at least one new species of bacterium associated with every species of insect, although to date, <1% of described insect taxa have been examined for microbes of any sort. Any attempt to review the full extent of insect–microbe interactions, therefore, would be hopelessly incomplete. For that reason, we limit this chapter to insights that have been gained about microbial diversity through paradigmatic studies of their symbiotic interactions with insects. By symbiotic interaction, we mean a long-term association of two or more different taxa in close proximity to one another, a commonly used definition proffered by Smith and Douglas (1987).

Studies of insect–microbe symbioses offer unique opportunities to craft well-defined hypotheses about the patterns and processes that govern microbial evolution and diversification. Cultivation-independent studies of free living microbes often reveal such amazing diversity in the environment that it is difficult to define more specific questions about what has been observed. By contrast, comparative studies with uncultivated microbial symbionts of insects have often resulted in the development of a focused line of scientific inquiry. For example, does a microbial phylogeny indicate an ancient single colonization event, followed by millions of years of cospeciation between microbe and host in which their evolutionary trajectories have been tightly linked? Or does the phylogeny suggest a more tenuous association, in which the symbiont has frequently been transferred horizontally between insect taxa or repeatedly and independently acquired from the environment? How variable and complex are insect gut communities? Are they the results of stable associations over evolutionary time or is functional redundancy so pervasive that one microbial taxon can easily displace another?

While insect–microbe symbioses offer unique opportunities to examine these issues, the impact of the lessons learned extend to the microbial world writ large. In this chapter, we discuss a potpourri of symbiotic interactions between microbes and insects and explore what each of them can teach us about microbial biodiversity.

WOLBACHIA: HORIZONTAL TRANSFER BETWEEN A BROAD SPECTRUM OF INSECT TAXA

A variety of microbial symbionts have a significant effect on the population biology and evolution of insects (O'Neill et al. 1997). The most well studied

of these are bacterial symbionts in the genus *Wolbachia* that reside in the cytoplasm of cells in insect testes or ovaries and that have thus far been uncultivable. *W. pipientis* was originally identified as the causative agent of cytoplasmic incompatibility in the mosquito *Culex pipens* (Yen and Bar 1971). The incompatibility typically results when males harboring the symbiont are crossbred with symbiont-free females. In that case, no progeny are produced. By contrast, symbiont-free males cross-bred with symbiont-containing females and crosses in which both sexes either lack or contain symbionts are comparably productive. Also, crosses involving different strains of *Wolbachia* can produce incompatibilities. For successful crosses, the inheritance of *Wolbachia* cells by the host is strictly maternal (as it is for mitochondria). From the microbe's point of view, the net effect of these incompatibilities is the elimination of hosts that either do not carry *Wolbachia* or that carry a different strain of *Wolbachia*. Thus, when symbionts compete for hosts, a bacterium that induces mating incompatibilities will spread faster relative to competitors that do not induce similar incompatibilities.

Originally, the presence of *Wolbachia* was thought to be quite rare and restricted to a few distinct insect taxa, and its affiliation to cultured bacteria was unknown. Amplification and sequencing of its 16S rRNA genes first indicated that *Wolbachia* is an α-proteobacterium related to *Rickettsia*-like bacteria that cause arthropod-borne diseases in mammals, and that the symbiont is present in a wide variety of insect orders (O'Neil et al. 1992). Its host range is now known to include crustaceans, mites, and filarial nematodes. In addition to cytoplasmic incompatibility, *Wolbachia* can be the cause of parthenogenesis induction, feminization, and male killing, depending on the host (Stouthamer et al. 1999). The phylogeny of *Wolbachia* and its relatives has also been inferred using the genes *ftsZ*, *groEl*, and *wsp* (van Meer et al. 1999), and polymerase chain reaction–based surveys have shown that 15–20% of all insect species may harbor the symbiont (Werren and Windsor 2000).

The *Wolbachia* clade appears to be at least 100 million years old (Weisberg et al. 1989). This substantial age, combined with the fact that *Wolbachia* bacteria are inherited vertically from generation to generation, suggested that they may have co-speciated with their hosts during an extensive evolutionary history. However, this is clearly not the case, inasmuch as phylogenies of the symbionts and their hosts are not topologically congruent (O'Neill et al. 1992). In fact, *Wolbachia* have frequently been horizontally transferred between both distantly and closely related groups of insects and other arthropods. *Wolbachia* has been successful in the exploration of a varied and unique lifestyle as an insect symbiont. This is evidenced by the diversity of mechanisms with which it manipulates its hosts to enhance its transmission and its projected occurrence in hundreds of thousands of insect species around the world. Current research is exploring whether *Wolbachia* might, at times, confer a selective advantage on its hosts, whether *Wolbachia* can drive host speciation, and whether it can potentially be used as an insect-control agent (Stouthamer et al. 1999).

BUCHNERA: HOST-SYMBIONT CO-SPECIATION OVER A 200 MILLION YEAR PERIOD

Many insects harbor bacterial symbionts in specialized cells called bacteriocytes (formerly "mycetocytes") (Buchner 1965). In the last 10 years, a major research effort has focused on the primary endosymbiont *Buchnera aphidicola* found in bacteriocytes of aphids, a large and ancient group of insects that feed on plant sap. Considerable biochemical and molecular evidence support a physiological basis for the symbiotic interaction between *Buchnera* and aphids: The bacteria are able to synthesize several essential amino acids required by the host, including leucine and tryptophan (Douglas 1998; Baumann et al. 2000).

A series of recent advances has lead to a keen understanding of the evolutionary history of this association. These are summarized in greater detail elsewhere (Bauman et al. 2000; Moran and Bauman 2000) and are only briefly recounted here. Among the first findings was that, in direct contrast to the situation of *Wolbachia*, *Buchnera* (which are γ-proteobacteria) have undergone strict co-speciation with their aphid hosts (Figure 1). A morphology-based phylogeny of the aphids exhibited perfect congruence with a molecular phylgogeny of the bacteria based on 16S rDNA sequences, indicating that the symbiotic interaction was first established in the common ancestor of aphids and that it has undergone strict vertical transmission ever since.

Although microorganisms leave little trace in the fossil record, the situation is different for aphids. Comparisons with the aphid fossil record permitted Moran et al. (1993) to conclude that the symbiosis began with a single acquisition of the endosymbiont ca. 200 million years ago, and allowed them to assign an age to a variety of other speciation events in the aphid–*Buchnera* radiation (Figure 1). In turn, the authors were able to correlate these speciation events with fine-scale comparisons of the nucleotide substitution rate in *Buchnera*, calibrating a molecular clock for the symbionts. More recently, 24 loci were sequenced for four *Buchnera* strains that reside in each of two species pairs of aphids, for which similiar divergence dates were known (Clark et al. 1999). From this comparison, an overall synonymous (i.e., third position) substitution rate of 0.2/site/10^6 generations was estimated, a rate that is about twice as fast as that estimated for free-living bacteria.

Bacteriocyte symbionts have also co-speciated with their respective hosts in other insect groups, including cockroaches (Bandi et al. 1995), tsetse flies (Chen et al. 1999), and carpenter ants (Sauer et al. 2000). However, these symbionts do not share direct common ancestry with *Buchnera*. In fact, molecular phylogenetic evidence indicates each insect–microbe interaction evolved independently from a lineage of free-living bacteria. Nevertheless, correlating the bacterial molecular clock with insect host species divergence dates derived from the fossil record indicates that the bacteriocyte symbionts share in common a 16S rRNA sequence divergence rate of 1–2%/50 million years, a rate that, again, is about twice that of free-living bacteria (Ochman et al. 1999).

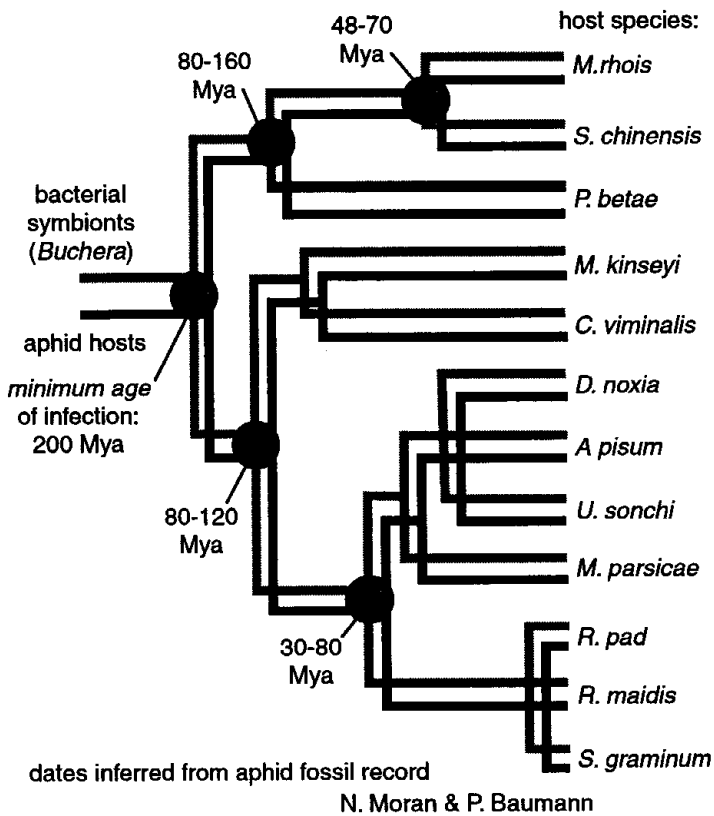

Figure 1. The phylogenies of aphids and *Buchnera* endosymbionts mirror one another, demonstrating co-evolution. Dates from the aphid fossil record (shown at various nodes in the phylogeny) were used to calibrate a molecular clock for genes of the endosymbiotic bacteria. Modified with permission from Moran et al. (1993).

Vertically transmitted symbionts such as these typically also have a low mol% G + C content (e.g., 28% in *Buchnera*). Both an increased mutation rate and base composition bias are predicted from the accumulation of mildly deleterious mutations in symbiotic bacteria whose life history prevents recombination and exposes them to a genetic bottleneck as they are transmitted vertically during each host's life cycle (Moran 1996). Increased mutation rate and A-T composition bias could complicate phylogenetic reconstructions through, respectively, long-branch attraction and convergent sequence evolution. As Moran and Baumann (2000) point out, naive phylogenetic placement of organisms that exhibit these characteristics, via a large database platform such as the Ribosomal Database Project (RDP) (Maidak et al. 2001), will be particularly prone to these sorts of errors.

Buchnera have relatively small genomes of ca. 650 Kilobases (Charles and Ishikawa 1999; Wernegreen et al. 2000). This situation will undoubtedly facilitate a comparative genomics approach to address questions about molecular mechanisms that govern the diversification of insect bacteriocyte symbionts,

providing models for similar studies of free-living microbes (Moran and Wernegreen 2000).

ATTINE ANT–FUNGUS SYMBIOSES: REPEAT DOMESTICATION, SYMBIONT ROTATION, AND CO-SPECIATION

Diversity of Attine Ant–Fungus Symbioses

Fungus-growing ants in the strictly neotropical tribe Attini currently comprise ca. 200 described species in 12 genera (Schultz and Meier 1995). All of these species obligately depend on the cultivation of fungi for food (Weber 1972; Schultz and Meier 1995), suggesting that fungiculture dates back to the origin of the attine clade 50–60 million years ago in Amazonian South America (Mueller et al., in press). All attines exhibit comparable suites of behavioral and physiological adaptations that testify to the functional integration between the ant and fungal partners of this symbiosis.

First, all attine ants appear to distribute enzymes of fungal origin throughout their fungal gardens by defecating in areas with active fungal growth (Martin 1984, 1987). These enzymes are ingested when the ants feed on the fungus, pass unharmed through the ants' guts, and help digest the substrate when applied by the ants with their feces, thereby accelerating fungal growth (Boyd and Martin 1975; Martin 1984, 1987). Second, all attines eliminate undesired microorganisms from the garden through a combination of constant, physical weeding (Weber 1954, 1972), secretion of antibiotics from various glands of the ants (Schildknecht and Koob 1971; Martin 1987; Jaffe et al. 1994; Knapp et al. 1994), or application of antibiotics derived from actinomycete bacteria that the ants are growing on specialized regions on their integument and that specifically target virulent garden parasites in the fungal genus *Escovopsis* (Currie et al. 1999a, 1999b). Third, all attines propagate their cultivars vegetatively within nests (i.e., as asexual clones), and asexual propagation also occurs between parent and offspring nests. Foundress queens obtain fungal inocula from the natal nest, carry it with them in a specialized pocket in their mouths during the mating flight, and then use it to start their own gardens (Ihering 1898; Wheeler 1907). This mode of propagation suggested the long-standing hypothesis that attine fungi are ancient clones that have strictly co-evolved with their hosts (Weber 1972). However, more recent molecular-ecological and phylogenetic work (Mueller et al. 1998) refuted this hypothesis of long-term co-evolution (see below).

In contrast to the above fungicultural features common to all attine ants, the substrates used by the ants and the cultivated fungi differ markedly among ant lineages. Two genera of so-called leafcutter ants with large colonies, *Acromyrmex* and *Atta*, culture their fungi on freshly cut foliage and flowers, making these ants major agricultural pests. Leafcutter ants are grouped with three other genera into the "higher attines" (Weber 1972; Schultz and Meier

1995), a monophyletic clade that comprises about one-half of the species diversity of the tribe. Ants in this monophyletic clade cultivate highly derived fungi that themsleves are monophyletic, suggesting specialization of the higher-attine ants on this group of fungi. Nevertheless, phylogenetic topologies within the two respective clades are clearly incongruent (Chapela et al. 1994; Rehner, personal communication), possibly because of frequent cultivar transfers among higher-attine ant lineages.

Ants in the remaining seven genera of "lower attines" are inconspicuous and behaviorally cryptic. They have small nests with one or a few walnut-size gardens in chambers constructed underground or under logs and stones. Lower attines also do not attack live plants but use a diversity of mostly dead vegetable matter (dry leaves, dry flower parts, seeds, or wood) that they collect on the gound as manure for their fungi. Compared to the economically important leafcutter ants, lower attines have received little attention; and their taxonomies, ecologies, and life histories are largely unknown (Weber 1972; Mueller and Wcislo 1998). This lack of knowledge is unfortunate, because it is exactly these basal lineages that are most likely to reveal ancestral forms of ant–fungus associations and that thus are most informative for elucidating the origin and diversification of the attine ant–fungus mutualism.

An anomaly among the lower attines is the monophyletic *Cyphomyrmex-rimosus* group. These ants cultivate their fungi as a yeast (single-celled growth form) rather than the mycelial form (hyphae) typical for all other attine ants. Yeast growing is a derived form of fungiculture that evolved from mycelial cultivation within the genus *Cyphomyrmex*, suggesting that the ants evolved the ability to manipulate fungal development, switch the fungus from a mycelial mode into a yeast mode, and maintain it permanently in the yeast state. The yeast fungus reverts to a mycelial form when cultured artificially in the laboratory. The selective advantages of yeast cultivation over mycelial cultivation are unclear. One hypothesis, suggested by studies on foraging behavior, is that the ants are ingesting sugary plant juices (floral or extrafloral nectar) to grow fungi in their crop (Murakami and Higashi 1997), and fungi cultured under these conditions may grow faster in a single-celled yeast state than when cultured in a filamentous, hyphal state.

The Diversity of Attine Fungi

The great majority of attine fungi belong to the tribe Leucocoprineae (family Lepiotaceae, order Agaricales, phylum Basidiomycotina) (Hervey et al. 1977; Chapela et al. 1994; Mueller et al. 1998; Johnson 1999), which is a large group of mushrooms that is particularly diverse in the tropics (Guzman and Guzman-Davalos 1992; Johnson 1999). Leucocoprineae are specialized plant-litter decomposers, and the attine and leucocoprineous ancestors probably first encountered each other in this microhabitat (Mueller et al., in press). The cultivation of tricholomataceous fungi by some ants in the genus *Apterostigma* is the only known exception to this ancestal association with leucocoprineous

fungi and indicates an evolutionarily unique switch to a fungus outside the Lepiotaceae (Chapela et al. 1994; Villesen et al., personal communication). Switching between cultivars and domesticating novel cultivars were probably original attributes of the attine symbiosis at a stage when the ants facultatively associated with fungi (Weber 1972; Mueller et al., in press). Indeed, repeated domestication events were implicated by phylogenetic analyses because (1) some attine cultivars are more closely related to free-living fungi than to other cultivars and (2) ant and cultivar phylogenies are topologically incongruent (Chapela et al. 1994; Mueller et al. 1998) (Figure 2). Both of these phylogenetic patterns are inconsistent with a scenario of a single domestication event followed by strict clonal propagation.

To assess the number of independent fungal domestications and the number of lateral cultivar transfers between ant lineages during the evolution of the attine ant–fungus symbiosis, Mueller et al. (1998) surveyed 309 free-living and 553 ant-cultivated fungi collected mostly from Central America (Panama, Costa Rica) and northern South America (Trinidad, Guyana). The survey focused on the seven genera of "lower" (phylogenetically basal) attine ants, those most likely to have retained the least modified forms of the ancestral farming behavior. Population genetic and phylogenetic analyses of these free-living and cultivated fungi revealed the following surprising results about the ant–fungal diversification during the 50-million-year history of this symbiosis.

First, phylogenetic patterns indicate a minimum number of five independent domestications by attine ants. This number likely underestimates the true number of domestication events that may have occurred during attine evolution, because most of the fungal diversity probably was missed, even in the extensive survey of hundreds of cultivated fungi (Mueller et al. 1998), and because many fungal lineages presumably were lost during the million-year evolutionary history of the ant–fungus symbiosis. Indeed, sequence identity in a fast-evolving gene (the internal transcribed spacer region of nuclear rDNA) between cultivated and free-living fungi suggests that some domestications may have occurred recently in evolutionary time and that acquisition of novel fungal strains from free-living sources may even be an ongoing process.

Second, while any single attine nest contains only a single cultivar, different nests of the same ant species may contain distantly related cultivars. Specifically, most attine species cultivate at least two fungal lineages; and, in one extreme case, a single attine ant species was found to cultivate eight distinct fungal lineages in only two tropical locations (Trinidad and Panama). The only exceptions are the yeast-cultivating *Cyphomyrmex* spp., which specialize on a derived monophyletic group of cultivars. Within this yeast group, however, the same ant species may cultivate a diverse set of fungi. Thus, while the yeast-cultivating *Cyphomyrmex* spp. are specialized on a narrow fungal clade, there is no apparent specialization within that clade. This same pattern—specialization on a defined clade of fungi but no correspondence between ant and fungal lineages within the fungal clade—appears to hold for the leafcutter ants

(Rehner, personal communication) and for the *Apterostigma* ant lineages that switched to a distantly related group of tricholomataceous fungi (Villesen et al., personal communication).

Third, the same or closely related fungi may be cultivated by distantly related ants. Such cultivar sharing generates topological incongruence across ant and fungal phylogenies and indicates that different ants acquired cultivars from the same free-living population or that they acquired cultivars from each other. The latter hypothesis was confirmed by AFLP fingerprint analyses, which revealed that cultivars grown by different ant species may be genetically identical. This suggests that cultivar clones are occasionally transferred laterally between ant species and that some of these transfers must have occurred so recently that the transferred cultivars in the recipient ant species have yet to diversify from the ancestral cultivars in the donor ant species. The ecological and evolutionary reasons for lateral cultivar transfers are unclear. Such transfers may occur fortuitously (e.g., gardens from two nests are simultaneously disturbed and mixed) or after accidental garden loss that forces ants to obtain a replacement from a neighboring colony (Mueller et al. 1998). Indeed, behavioral experiments simulating pathogen-driven loss of entire gardens indicate that garden-deprived colonies can regain fungal cultivars by joining a neighboring colony, by stealing part of a neighbor's garden, or by aggressive usurpation of a neighbor's garden (Adams et al. 2001).

Fourth, phylogeographic patterns of cultivar usage in different geographic locations revealed that cultivar communities are diverse at each location and that cultivar lineages are largely shared between locations, indicating that ants disperse and mix cultivar lineages across locations or that free-living populations from which cultivars are drawn are widespread. The absence of large-scale biogeographic patterns does not preclude the possible existence of

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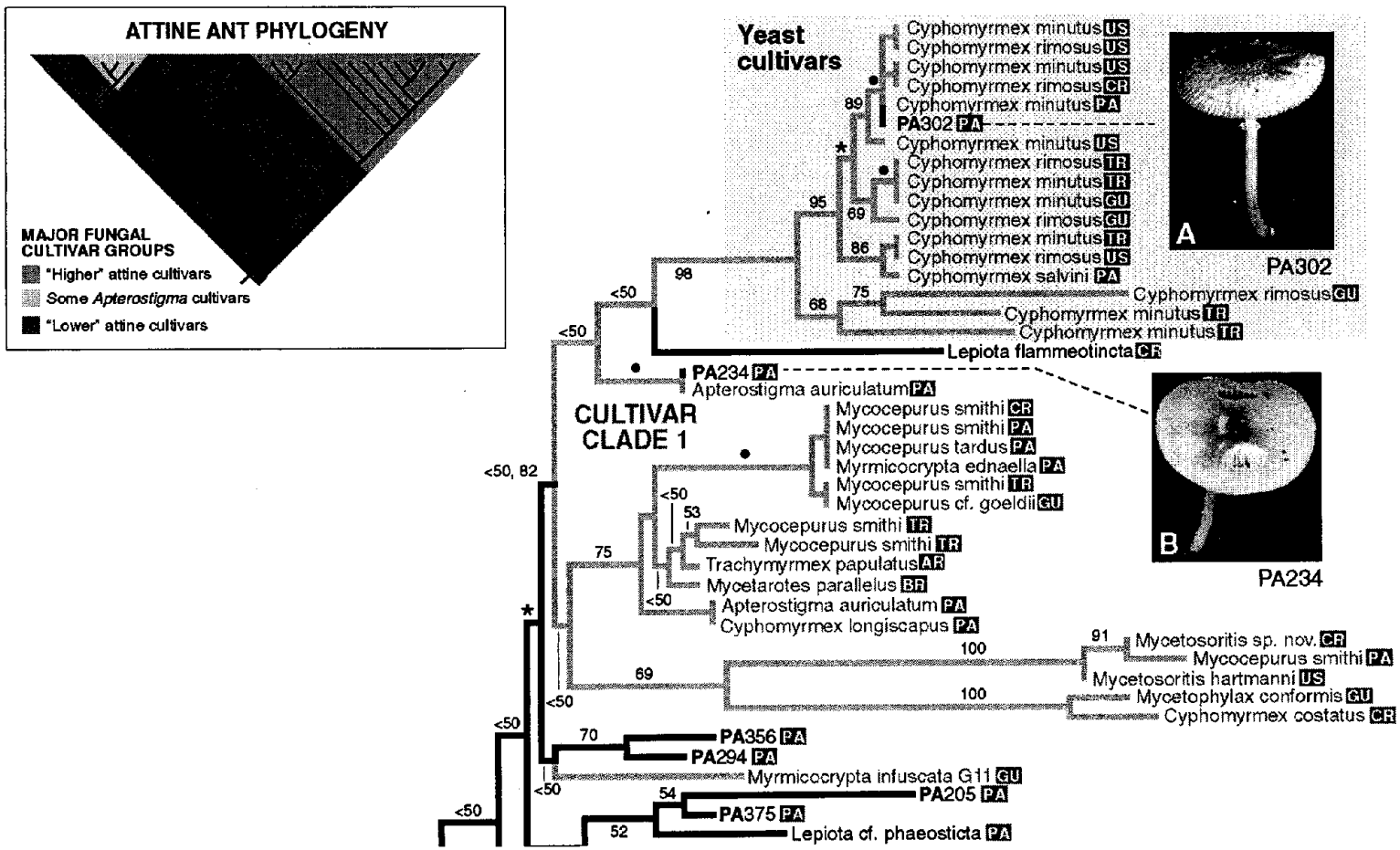
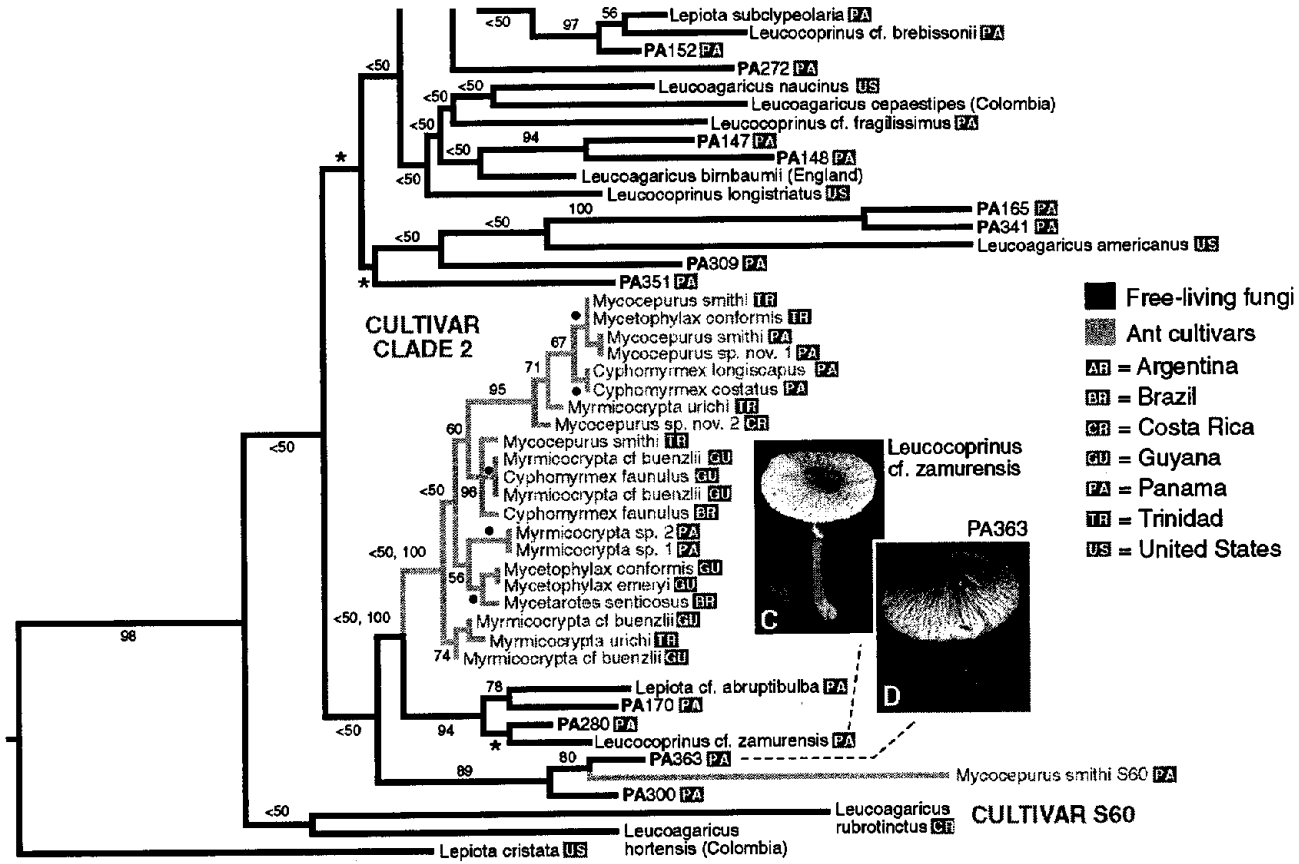
See Figure on pp. 298–299

Figure 2. See ftp site for color figure. Phylogeny for 57 attine cultivars and 36 free-living fungi in the tribe Leucocoprineae (Lepiotaceae, Basidiomycota). All cultivars belong to a group grown only by "lower attines," basal attine ant lineages that are most likely to have retained the ancestral states of ant-fungus associations that existed early in the history of this 50-million-year-old symbiosis. Cultivated fungi are marked in grey by their respective ant host species. Free-living fungi are marked in black; some of these are undescribed species and are denoted by their collection IDs. Two free-living counterparts of ant-cultivated fungi (labelled A & B) show identical sequences with those of two cultivated fungi, thus revealing two recent domestications of cultivars by ants (see text). Statistically distinct cultivar clades (Clade 1, Clade 2, *Mycocepurus smithi* S60) provide evidence for a minimum of three additional domestications. Almost all ant species cultivate several, phylogenetically distant cultivars. The tree shown is the strict consensus of five equally parsimonious trees obtained by parsimony analysis and successive approximations weighting of 1422bp sequence of two nuclear rDNA regions (ITS, 25S), and resembles in all important details the tree found using maximum likelihood. Numbers on branches are bootstrap values from 500 pseudoreplicates. Asterisks (*) indicate branches not present in the strict consensus of 180 equally parsimonious trees found in the analysis prior to successive weighting; bullets (•) indicate branches above which a single representative taxon was included in the analyses. Figure adapted from Mueller *et al.* (1998).


ATTINE ANT PHYLOGENY
MAJOR FUNGAL CULTIVAR GROUPS
"Higher" attine cultivars
Some Apterostigma cultivars
"Lower" attine cultivars
Yeast cultivars
Cyphomyrmex minutus US
Cyphomyrmex rimosus US
Cyphomyrmex minutus US
Cyphomyrmex rimosus CR
Cyphomyrmex minutus PA
PA302 PA
Cyphomyrmex minutus US
Cyphomyrmex rimosus TR
Cyphomyrmex minutus TR
Cyphomyrmex minutus GU
Cyphomyrmex rimosus GU
Cyphomyrmex minutus TR
Cyphomyrmex rimosus US
Cyphomyrmex salvini PA
Cyphomyrmex rimosus GU
Cyphomyrmex minutus TR
Cyphomyrmex minutus TR
Lepiota flammeotincta CR
PA234 PA
Apterostigma auriculatum PA
CULTIVAR CLADE 1
Mycocepurus smithi CR
Mycocepurus smithi PA
Mycocepurus tardus PA
Myrmicocrypta ednaella PA
Mycocepurus smithi TR
Mycocepurus cf. goeldii GU
Mycocepurus smithi TR
Mycocepurus smithi TR
Trachymyrmex papulatus AR
Mycetarotes parallelus BR
Apterostigma auriculatum PA
Cyphomyrmex longiscapus PA
Mycetosoritis sp. nov. CR
Mycocepurus smithi PA
Mycetosoritis hartmanni US
Mycetophylax conformis GU
Cyphomyrmex costatus CR
PA356 PA
PA294 PA
Myrmicocrypta infuscata G11 GU
PA205 PA
PA375 PA
Lepiota cf. phaeosticta PA
A
PA302
B
PA234



BRANCH LENGTH SCALE: ⊢——⊣ = 10 nucleotide changes

ecological zoning on a microecological scale (usage of different fungi in different habitats), but this hypothesis remains to be tested.

Microbial Diversity in Attine Gardens

In all attine species, gardens appear to support only a single dominant mutualistic fungus. However, specialized fungal parasites in the genus *Escovopsis* (Hypocreales: Ascomycotina) can infiltrate gardens and evade the weeding activity of the ants (Currie et al. 1999a, 1999b). Careful isolations from intact and apprently healthy attine gardens combined with experimental infection of gardens demonstrated that (1) *Escovopsis* fungi occur in gardens of ants from throughout the entire attine phylogeny, (2) *Escovopsis* is a specialist in attine gardens, (3) *Escovopsis* is highly virulent and is able to devastate gardens even in the presence of weeding ants, and (4) gardens of higher attines show more severe infestations with *Escovopsis* than gardens of lower attines. The latter point supports theoretical predictions of disease evolution under long-term clonality (the more anciently domesticated symbionts of higher attines), compared to the more recently acquired symbionts of the lower attines (see above).

As a defense against *Escovopsis* parasites, attine ants forged an association with a second group of symbionts, actinomycete bacteria (putatively identified as *Streptomyces* spp.) that the ants are cultivating on specialized regions of their cuticle (Currie et al. 1999, 1999b). These actinomycetes produce chemicals specifically inhibiting the growth of the *Escovopsis* parasites, and the ants are able to upregulate actinomycete production in nests heavily infested with *Escovopsis* and downregulate when infestations are under control (Currie, personal communication). The diversity of the attine ant–fungus symbiosis thus gains further complexities through the addition of these two types of microbial symbionts. It is at this point unknown how the respective phylogenies of the four players in this quadripartite symbiosis relate to each other.

Apart from these four symbionts, additional microbes, including bacteria and yeasts, have been described from attine gardens (Craven et al. 1970; Scheld 1971; Weber 1972; Tauk and Serzedello 1975; Bacci et al. 1995). Some of these microbes undoubtedly are pathogenic; but it is not unlikely that other microbes serve specific functions in the gardens or are beneficial to the overall garden community, thus adding to the microbial complexity and diversity of the attine ant–fungus symbiosis. Additional functions may include specific digestive capabilities, production of nutrients essential to either ants or cultivated fungi, chemical or antibiotic defenses, or any other control of undesirable microorganisms or pathogen vectors (e.g., mites, collembolans) that could invade gardens.

Diversity through Symbiosis Plasticity

Evolutionarily ancient, mutualistic symbioses are generally characterized by extreme codependency and fine-tuned functional integration between

mutualists (Douglas 1994; Thompson 1994), but they presumably evolved from plastic associations in which each symbiont was capable of independent existence and in which symbionts reassociated frequently (Herre et al. 1999). This ability to reassociate and to acquire partners de novo presumably affords greater flexibility or greater evolutionary potential (Maynard Smith and Szathmary 1999) and may have been retained for this reason in many mutualistic symbioses, including the primitive attine ants (Mueller et al. 1998) (Figure 2). Highly specific mutualistic symbioses, on the other hand, do not allow for this free reassociation of symbiotic partners, but the reduced flexibility is presumably offset by greater efficiency of specialized and highly integrated partners (Thompson 1994; Herre et al. 1999). As described above, this latter scenario appears to apply to the relationships that fungi have with several derived attine ant lineages (Mueller et al. 1998; Rehner et al., personal communication; Villesen et al., personal communication). Attine ants and their fungi as a whole, therefore, exhibit a rich diversity of mutualistic symbioses, with repeated transitions from plastic to specialized mutualisms that can be traced with phylogenetic techniques.

TERMITE–MICROBE SYMBIOSES: PATTERNS OF DIVERSITY IN COMPLEX MICROBIAL COMMUNITIES

The Diversity of Termite–Microbe Symbioses

Microorganisms have probably entered into a broader spectrum of symbiotic interactions with termites (Isoptera) than with any other insect order. Intracellular bacteriocyte symbionts and *Wolbachia* have both been detected in termites (Bandi and Sacchi 2000). Although the evolutionary history of the interaction has not yet been studied in detail, basidiomycete fungi of the genus *Termitomyces* are cultivated as gardens and consumed by members of the highly specialized termite subfamily Macrotermitinae in a symbiosis that shares many similarities with that of the attine ants (Martin 1987). However, the greatest challenge from a microbial diversity perspective lies in studying the symbiotic interactions between termites and their gut microbial communities, without which the insects cannot digest their food and survive. These communities can serve as a useful model for detecting patterns of microbial diversity, because they exhibit levels of complexity commonly seen in the microbial world; but their association with termites provides a more tractable framework for comparative study.

There are ca. 2200 described species of termites, which are classically divided into two phylogenetically distinct groups: the lower and higher termites. The lower termites consist of six families, all of which consume wood, and harbor anaerobic, cellulolytic protozoa and a diversity of prokaryotes, primarily in the hindgut region of their digestive tract. The higher termites, which are more nutritionally diverse, include 75% of all species grouped into the

single family Termitidae. Higher termites also have a complex community of prokaryotes in their hindgut, but they have not retained an association with the various cellulolytic protists found in the lower termites. For a few select termite species, much progress has been made in understanding the contribution of the microbiota to carbon and nitrogen metabolism in the gut and their role in host nutrition. This subject will not be covered here, except insofar as it relates to what termite–microbe symbioses reveal about microbial biodiversity. Information on the metabolic aspects of termite gut symbioses is available (Breznak and Brune 1994; Kane 1997; Radek 1999; Abe et al. 2000; Brune and Friedrich 2000).

Protozoa in the Guts of Lower Termites

Strictly anaerobic, cellulolytic, flagellate protozoa belonging to the orders Trichomonadida, Oxymonadida and Hypermastigida occur in the hindguts of lower termites (Honigberg 1970). Since Honigberg's 1970 review, most work on these protozoa has been concerned with the micro-anatomy and ultrastructure of individual species (Radek 1999), but these studies don't speak to the origin and evolutionary history of termite–protozoa symbioses. An extensive catalog of these fascinating protozoa was provided by Yamin (1979), who also was the first to successfully culture one (Yamin 1981). Yamin's catalog covered ca. 200 species of termites and lists the distribution of >400 species of protozoa in guts. Only a few of these have been cultured, however, and so their classification must be viewed with caution, complicating an assessment of protozoan diversification patterns. Nevertheless, a few general characteristics of termite–protozoa associations are evident: (1) some termite species contain only a few types of protozoa, whereas others can harbor ≥20 species; (2) the same or similar flagellate species can be found in different host taxa; and (3) population-level differences in flagellate composition can occur within a single species (Yamin 1979; Kitade and Matsumoto 1993). Based on this information, a hypothesis of strict co-evolution between termites and their protozoa can be rejected.

Some correspondance between flagellate community composition and termite taxonomy was suggested by a restricted survey of protist distribution patterns in 84 colonies of various species of *Reticulitermes* collected throughout Japan (Kitade and Matsumoto 1993). The distribution patterns of 15 protist species were significantly more similar between termites from colonies of a single species or subspecies than among termites of different species. Further surveys of this type are needed and must include molecular typing of gut protozoa (below) before a more general picture of the evolutionary history of lower termite–protozoa symbioses can emerge.

Since ca. 1997, molecular phylogenetic analyses of small subunit (SSU) rRNA genes (i.e., 18S rDNA) have provided some insights into the identity and evolutionary relationships of protozoa in termite guts. Trichomonad SSU rDNA sequences were recovered from *Mastotermes darwiniensis* hindguts and

assigned to cells of *Pentatrichomonoides scroa* and *Metadevescovina extranea* through the use of flourescent in situ hybridization (FISH) techniques (Berchtold and König 1995), and SSU rDNA seqeunces of the trichomonad *Pseudotrypanosma giganteum* were determined from cells that had been removed by micropipett from guts of *Porotermes adamsoni* (Keeling et al. 1998). Hypermastigote (*Trychonympha*) SSU rDNAs have been sequenced from *Reticulitermes speratus* (Ohkuma et al. 1998), *Zootermopsis angusticolis* (Dacks and Redfield 1998), and *P. adamsoni* (Keeling et al. 1998). In the latter study, genes representing SSU rDNAs from other (unknown) trichomonads and hypermastigotes were also amplified and sequenced from hindgut samples of *P. adamsoni*, *Cryptotermes brevis*, and *C. dudleyi*, and sequences representing two protozoan symbionts recovered from *Reticulitermes flavipes* hindguts, and originally identified as trichomonad-like sequences (Gunderson et al. 1995), were re-analyzed. The *R. flavipes* symbiont sequences were determined to be from a *Trichonympha*, in one case, and as likely coming from a hypermastigote in the family Spirotrichonymphidae, in the other (Keeling et al. 1998). Taken as a whole, the net effect of these molecular phylogenetic studies is that they have helped buttress the validity of protozoan taxonomic group concepts, but deeper nodes in the protist phylogenetic tree are still not well resolved (Inoue et al. 2000). Moreover, these efforts have been piecemeal, limited in scope, and fallen short of the comprehensive, comparative approach required to trace complex evolutionary interactions between symbionts and their hosts.

The Prokaryotic Community in Termite Guts

Microscopic examination of the gut communities of all termites examined to date reveals an impressive diversity of prokaryotic morphotypes that likely only hint at the true microbial diversity in these tiny habitats. Most cells have been observed in a highly concentrated culture in the hindgut region, although a few have been documented in the foregut, and midgut regions and in the mixed-segment found in certain higher termites (Abe et al. 2000). Prokaryotes that have been cultured from termite guts encompass aerobic, anaerobic, and facultative forms, including enteric and lactic acid bacteria, *Bacterioides*, actinomycetes, acetogens, sulfate reducers, methanogens, and spirochetes (below). Culturing of isolates has frequently resulted in the description of new and interesting species and genera and confirmed the presence of microorganisms capable of a metabolic activity believed to be important to the host, such as nitrogen fixation, uric acid degradation, fermentation, and acetogenesis or methanogenesis from $H_2 + CO_2$ (Breznak and Brune 1994; Kane 1997). However, studies with pure culture isolates have revealed little about the evolutionary foundation of these communities and their associations with host species.

Kane and Pierce (1994) first proposed that a culture-independent molecular phylogenetic approach be applied to the hindgut communities of termites.

A variety of higher and lower termites have now been examined using these methods. Through polymerase chain reaction (PCR) amplification and sequencing of 16S rRNA genes (sometimes coupled with FISH), these studies have identified molecular isolates affiliated with spirochetes in the genus *Treponema* (Berchtold et al. 1994; Paster et al. 1996; Berchtold and König 1996), enteric bacteria, sulfate-reducing bacteria, relatives of *Bacteroides* (Ohkuma and Kudo 1996), and methanogens (Shinzato et al. 1999). Not surprisingly, most of these sequences are not identical to any known prokaryotic taxa, confirming that termite guts are a large reservoir of uncharacterized microbial diversity. FISH was also used in combination with oxygen profile measurements on gut samples from *M. darwiniensis* in the first study to carefully quantify the location and composition of various prokaryote assemblages in different regions and compartments of a termite hindgut (Berchtold et al. 1999).

A related and intriguing line of inquiry has been followed by using amplification, sequencing, and phylogenetic analysis of the *nifH* gene from several lower and higher termite taxa. Okuma et al. (1999) described recovery of termite gut *nifH* sequences representing four major clades of *nifH* genes: proteocyano (α-, β-, and γ-proteobacteria), anaerobe (clostridia, δ-proteobacteria, low G-C gram-positive bacteria), *anf*-methano (methanogen alternative nitrogenase), and pseudo-*nif* (divergent, also derived from methanogens). Despite such a high diversity of *nifH* genes, a trend was suggested in which termite family relationships were correlated with *nifH* gene type. For example, pseudo-*nif* sequences were the most common sequence type recovered from three species of higher termites, whereas this sequence type was less common in six species of lower termites. By contrast, anaerobe seqeunces were the most common sequence type recovered from two species in the family Rhinotermitidae, and *anf*-methano sequences were the most common type recovered from two of three species of the family Kalotermitidae, although the later sequence type was completely absent from the Rhinotermitidae species. Nevertheless, an impressive diversity of sequence types was found in most of the termites examined, and the results are inconsistent with a scenario of parallel evolution between termites and nitrogen-fixing prokaryotic symbionts.

Patterns of Prokaryotic Diversity in Termite Guts

In the terminal steps of anaerobic decomposition in termite hindguts, two groups of H_2-oxidizing, CO_2-reducing prokaryotes consume reductant produced by fermentative activities of other gut microbes and are of specific interest from a microbial diversity perspective. One group, methanogens, reduces CO_2 to methane and belongs to a distinct lineage of the Archaea (Ferry 1993). The other group, acetogens, are bacteria that reduce CO_2 to acetate. They are a physiologically defined, polyphyletic guild of anaerobic microbes, most of

which are associated with the low GC-content, Gram-positive clade of the Bacteria, (Drake 1994).

The extent to which CO_2 is reduced to methane versus acetate in termite hindguts is correlated with host diet. Although most termite species appear to exhibit both metabolic activities, in guts of soil-feeding termites methanogenesis is the dominant process, whereas in wood-feeding feeding termites it's acetogenesis (Brauman et al. 1992). Lower termites are all wood feeders, which implies that the termite common ancestor was acetogenic. The number of independent origins of soil feeding in higher termites has been estimated to be as few as one to as many as six (Noirot 1992; Eggleton, personal communication). Accordingly, whether a switch to methanogenesis in guts is a true synapomorphy or due to ecological constraints of soil organic matter fermentation remains to be determined.

Current research is exploring patterns of diversity for some of the microorganisms implicated in methanogenesis and acetogenesis. For example, the relationship between host diet and gut prokaryotic community profiles in 24 taxonomically and nutritionally diverse termite species were explored by using probes targeting SSU rRNAs, including those of different groups of methanogens (Brauman et al. 2001). Differences in profiles were correlated with termite diet, as evidenced by higher abundances of archaeal SSU rRNAs in guts of soil- vs. wood-feeding Termitidae. Profiles also readily distinguished communities in guts of wood-feeding taxa of phylogenetically higher versus lower termites. Probes specific for different assemblages of methanogens detected members of only two of seven families—Methanobacteriaceae and Methanosarcinaceae—but no clear relationship was evident between methanogen profiles and termite diet or taxonomy.

The first acetogens isolated from various termites (*Sporomusa termitida*, *Acetonema longum*, and *Clostridium mayombei*) were all endospore formers associated with the low GC-content, gram-positive bacterial phylum (Breznak 1994). By contrast, the discovery of an acetogenic spirochete isolate from *Zootermopsis angusticolis* expanded acetogenesis into a new phylum and shed light on what is the most morphologically conspicuous group of prokaryotes in a vast majority of termite hindgut communities (Leadbetter et al. 1999).

Monophyly of the spirochete clade enabled Lilburn et al. (1999) to use spirochete-specific 16S rDNA primers for an in-depth molecular survey of their diversity in termite guts. While phylogenetic analysis indicated that all sequences recovered in the survey were affiliated with the genus *Treponema*, at least 21 new species were identified (based on a sequence similarity value of ≤97%) from the guts of *Reticulitermes flavipes* alone. Spirochete 16S rDNAs were also amplified and sequenced from guts of *Coptotermes formosanus* and *Zootermopsis angusticolis* with similar results. Spirochete sequences obtained by Lilburn et al. (1999) were combined with those of previous studies (Berchtold et al. 1994, 1996; Paster et al. 1996), as well as those from cultured treponemes, and used to infer a phylogenetic tree. This tree was then used as

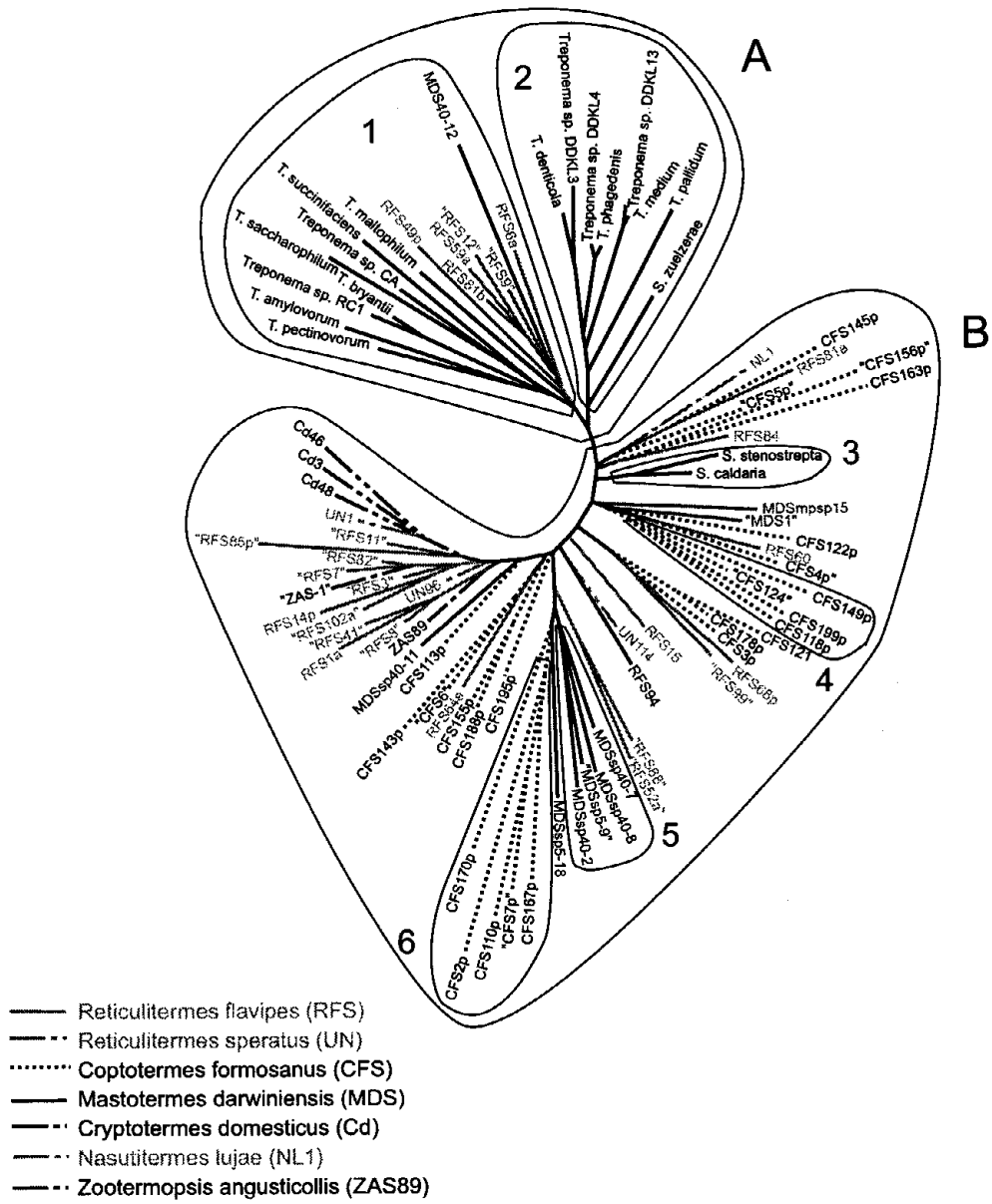
a framework on which additional partial spirochete 16S rDNA sequences from Lilburn et al. (1999) and from two other studies (Okhuma and Kudo 1996; Okhuma et al. 1998) were appended by using a parsimony criterion in the ARB program (Strunk and Ludwig 1997). The final tree depicting the phylogeny of all known spirochete sequences, taken from guts of 7 diverse termite species is shown in Figure 3. The vast majority (>60) of termite spirochete sequences formed a clade that contained only two cultured representatives, *Spirochaeta caldaria* and *S. stenostrepta* (spirochaetas whose phylogeny is associated with *Treponema* based on the 16S rDNA sequence). Another 7 termite gut sequences formed a clade derived from other cultured *Treponema.*

A variety of patterns are suggested in the treponeme phylogeny in Figure 3. First, the tree implies that there have been multiple independent origins of spirochetes in termite guts, although this is difficult to evaluate without statistical studies of the deeper nodes in the tree. Second, the tree does not provide support for a hypothesis of strict cospeciation between termites and spirochetes. Third, clades consisting of a collection of sequences from an individual species are evident (e.g., taxon groups 4 and 5), indicating that termite guts are a significant source of endemism for spirochetes. Conversely, individual sequences from one termite species were, in a few cases, most closely related to a group of sequences from another species (e.g., MDS40-12 in clade 1, or ZAS-1 in a clade of sequences from *R. flavipes*), suggesting that transfer of spirochetes between termite taxa occurs as well.

Despite the intensive nature of this spirochete survey, the results hint that even more intensive sampling of termite gut spirochetes is warranted. Moreover, guts from 30 or more individuals were pooled into single nucleic acid extraction for this study, so variation within a colony or species could not be evaluated. Although this study was prompted, in part, by the isolation of an acetogenic termite gut spirochete, it remains to be seen whether the other spirochete taxa detected in termite guts are also acetogenic.

CONCLUSION

The brief vignettes recounted in this chapter testify that there is much to learn about patterns of microbial diversification and the processes that underlie them by studying symbioses between insects and microbes. Molecular ecological/phylogenetic approaches have been used successfully to interrogate *Wolbachia* and *Buchnera*, intracellular insect symbionts with widely contrasting lifestyles. Similar approaches have now been expanded to study symbioses between attine ants and their fungal and bacterial partners, as well as complex interactions between termites and communities of symbionts in their guts. These studies suggest that the long-term survival of a nutritional-based symbiosis may rely on coordinate diversification between host and microbe as well as on the host's ability to switch to novel symbionts with novel properties or to find a suitable replacement if a partner is lost.



Reticulitermes flavipes (RFS)
Reticulitermes speratus (UN)
Coptotermes formosanus (CFS)
Mastotermes darwiniensis (MDS)
Cryptotermes domesticus (Cd)
Nasutitermes lujae (NL1)
Zootermopsis angusticollis (ZAS89)

Figure 3. See ftp site for color figure. Phylogenetic tree depicting diversity and relationships of 16S rRNA sequences of cultured treponemal spirochetes and those recovered from guts of seven different termite species. Branch pattern indicates host termite species. Reprinted with permission from Lilburn et al. (1999).

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