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Diversity within diversity: Molecular approaches to studying microbial interactions with insects

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Summary. DNA sequence information has greatly augmented the number of characters available for analysis in phylogenetic research. Nowhere is this more evident than in studies of microbial evolution. Ribosomal DNA sequence data has simultaneously permitted the distinction between individual species and the inference of their phylogenetic relationships in many cases where both were formerly impossible. These have contributed to our understanding of the ecology of particular microbe-host interactions and the history of these relationships over evolutionary time. We describe examples from two ends of the ecological spectrum in insect/bacterial associations: one in which bacteria mediate host cytoplasmic incompatibility and parthenogenesis, and the other in which mycetocyte bacteria augment host nutrition. In the former, the pattern of bacterial interaction is general, with the same or closely related strains of the genus *Wolbachia* associating with a wide range of insect taxa. In the latter, concordance between host and microbe phylogenies suggests cospeciation between bacteria and host, although it is as yet unclear whether this process has involved step-wise, reciprocal coevolution. We conclude with a discussion of how developments in molecular techniques may aid in analyzing more complex interactions between insects and microbes.

Introduction

Microbes can be as small as 0.2 μM in diameter, and frequently lack distinctive morphological characteristics that could be used in systematic analysis. Moreover, until recently, isolation and growth in pure culture was required for unambiguous identification, although this has been possible for less than ten percent of the observed microbial world (Bull and Hardman, 1991; Trüper, 1992). Even for those microbes that can be cultured, phylogenetic inference based on morphological and physiological properties has been difficult. As a result, studies of interactions between insects and microorganisms have lagged far behind those of insects and plants, despite the fact that microbes include the most ancient and diverse life forms on earth (Woese, 1987; Knoll, 1992), and interactions between insects and microorganisms are common (Buchner, 1965; Dasch et al., 1984; Anderson et al., 1984; Martin, 1987; Berenbaum, 1988; Schwemmler and Gassner, 1989; Barbosa et al., 1991; Douglas, 1992).

One of the greatest impacts of the application of molecular techniques to phylogenetic studies thus far has been the dramatic advance in systematics gained from comparisons of small subunit (16S or 18S)

ribosomal RNA (rRNA) sequences of bacteria (Woese, 1987, 1991; Ochman, this volume) and eukaryotic protists (Sogin et al., 1986; Sogin, 1991). Molecular phylogenetic tools based on these rRNA studies are being used to address formerly intractable ecological and evolutionary questions about microorganisms (Stahl et al., 1991; Stahl and Amann, 1991) including those that interact with insects.

This chapter reviews two general areas where molecular methods have illuminated ecological and evolutionary aspects of insect-microbe symbiosis. By symbiosis, we mean any association between different species. We use these examples as a basis to discuss changing views on the nature of interspecific interactions, and to suggest directions where techniques in molecular biology will be useful for future research. Although molecular methods are the emphasis of this volume, it is evident from what follows that the best approach to studying insect-microbe interactions is the integration of molecular techniques with other experimental studies.

Wolbachia and bacterial mediation of cytoplasmic incompatibility and parthenogenesis in insects

Bacterial 16S rRNA gene sequences (16S rDNAs) have shed light on two phenomena related to insect reproduction. These are caused by cytoplasmically inherited bacterial endosymbionts present in testes or ovaries. The first of these, cytoplasmic incompatibiltiy, was originally thought to be a relatively unusual phenomenon found in a few insect species. This incompatibility results from bacteria that interfere with sperm-derived chromosomes in fertilized eggs by preventing proper condensation (Breeuwer and Werren, 1990; O'Neill and Karr, 1990). A typical consequence is that symbiont-containing males crossed with symbiont-free females produce no viable progeny. However, the reciprocal cross (symbiont-free males with symbiont-containing females) produces normal numbers of progeny, as do crosses in which both parents contain symbionts.

The second case is when bacteria cause parthenogenesis by preventing segregation of chromosomes in unfertilized eggs (Stouthamer et al., 1993). In the parasitoid wasps, *Trichogramma deion* and *Muscidifurax uniraptor*, for example, this results in thelytoky, or the production of only female progeny (Stouthamer et al., 1990; Stouthamer and Luck, 1993). Until recently, we could only speculate about whether or not bacteria involved in each of the two processes were related, about how many different bacterial taxa were involved, and whether such symbionts are host-specific.

Although a review of these subjects is beyond the scope of this article, endosymbiont-mediated effects such as cytoplasmic incompatibility and parthenogenesis can have a strong impact on the population dynamics and evolution of insects (for example, see Hoffmann et al., 1990; Aeschilmann, 1990; Turelli and Hoffmann, 1991; Beard et al., 1993; Ebbert, 1993; Hurst, 1993; Luck et al., 1993; Stouthamer, 1993; Stouthamer and Luck, 1993).

The 16S rDNAs of bacteria responsible for cytoplasmic incompatibility in mosquitos, beetles, wasps, a moth, and a fruit fly have been sequenced (Tab. 1). Two important conclusions are evident from these studies. First, cytoplasmic incompatibility-causing bacteria surveyed to date are closely related, belonging to the α-subgroup of the proteobacteria (Tab. 1); they are all different species or strains of the genus *Wolbachia* (after Hertig, 1936). Second, the phylogenies of these symbionts are not congruent with those of their hosts (O'Neill et al., 1992).

Table 1. Diversity of insects containing *Wolbachia* that have been examined by comparative sequence analysis of endosymbiont rRNA genes

Insect order (group)	Host	Effect of *Wolbachia* on host[a]	References
Coleoptera			
(beetles)	*Attagenus unicolor*	?	O'Neill et al., 1992
	Diabrotica virgifera	?	O'Neill et al., 1992
	Hypera postica	CI	O'Neill et al., 1992
	Tribolium confusum	CI	O'Neill et al., 1992; Rousset et al., 1992
Diptera			
(mosquitos)	*Aedes albopictus*	CI	O'Neill et al., 1992
	Culex pipens	CI	O'Neill et al., 1992; Rousset et al., 1992
(fruit flies)	*Anastrepha suspensa*	?	O'Neill et al., 1992
	Drosophila simulans	CI	O'Neill et al., 1992; Rousset et al., 1992
(maggot flies)	*Rhagoletis mendax*	?	O'Neill et al., 1992
	R. pomonella	?	O'Neill et al., 1992
Homoptera			
(plant-hopper)	*Laodelphax striatellus*	CI	Rousset et al., 1992
Hymentoptera			
(wasps)	*Muscidifurax uniraptor*	P	Stouthamer et al., 1993
	Nasonia vitripennis	CI	Breeuwer et al., 1992
	N. giraulti	CI	Breeuwer et al., 1992
	N. longicornis	CI	Breeuwer et al., 1992
	Trichogramma deion	P	Stouthamer et al., 1993
	T. pretiosum	P	Stouthamer et al., 1993
	T. cordubensis	P	Rousset et al., 1992; Stouthamer et al., 1993
	T. oleae	P	Rousset et al., 1992
Lepidoptera			
(moths)	*Corcyra cephalonica*	?	O'Neill et al., 1992
	Ephestia cautella	CI	O'Neill et al., 1992
	E. kuehniella	?	Rousset et al., 1992
	Sitotroga cerealella	?	O'Neill et al., 1992

[a]CI, cytoplasmic incompatibility; P, parthenogenesis; ?, effect unknown.

Thus, 16S rDNA analyses indicate that *Wolbachia* has been able to transfer horizontally between hosts from a wide range of insect orders rather than becoming established in an ancestral insect and cospeciating with its host.

A similar approach has been applied to bacteria that cause parthenogenesis in the parasitic wasps *Muscidifurax uniraptor* and *Trichogramma* spp. Phylogenetic analysis of 16S rDNA sequences revealed that these bacteria are also part of the *Wolbachia* group (Stouthamer et al., 1993). Surprisingly, although members of *Wolbachia* form a monophyletic clade within the α-proteobacteria, parthenogenesis-causing symbionts from *M. uniraptor* and *Trichogramma* sp. do not form a clade within the *Wolbachia* group. However, *Wolbachia* from six parthenogenetic strains within the genus *Trichogramma* do form their own monophyletic clade (Stouthamer et al., 1993). Note that within the Hymenoptera, which are haplo/diploid, *Wolbachia*-like bacteria can cause cytoplasmic incompatibility in *Nasonia*, and thelytokyous parthenogenesis in *Trichogramma* sp. and *Muscidifurax uniraptor*.

That the *Wolbachia* group contains symbionts that cause either cytoplasmic incompatibility or parthenogenesis was confirmed by using phylogenetic analyses of partial 16S and 23S rRNA gene sequences of appropriate bacteria (Rousett et al., 1992). This study also concluded that *Wolbachia* is the causative agent of cytoplasmic incompatibility in a terrestrial isopod, *Porcellio dilatatus*, and feminization in another, *Armadillidium vulgare*. *Wolbachia*-like 16S rDNA sequences have been recovered from the ovaries of eight other insect taxa, despite the fact that neither cytoplasmic incompatibility nor parthenogenesis have been reported for these species (Tab. 1). It is not clear whether this means that *Wolbachia* may be present without altering reproduction, or that such effects are, as yet, undetected.

Molecular phylogenies of bacteria that mediate cytoplasmic incompatibility and parthenogenesis have demonstrated that what were once considered rare and distinct phenomena caused by host-specific symbionts are probably common and caused by closely related members of a single microbial group. *Wolbachia* bacteria thus appear to be relatively generalized with respect to their insect hosts. Whether one or a series of bacterial activities have somewhat different effects in the wide variety of taxa in which *Wolbachia* reside or whether closely related bacteria react differently in different hosts is the topic of current investigation (Boyle et al., 1993; Breeuwer and Werren, 1993a,b).

Mycetocyte bacteria

Members of at least six insect orders harbor intracellular microorganisms (usually bacteria, but sometimes yeasts) restricted to specialized

cells known as mycetocytes (Douglas, 1989a). These endosymbionts benefit their hosts inasmuch as hosts treated with antibiotics cease ordinary growth patterns and usually die without producing progeny. That the endosymbionts also benefit from the association is usually assumed because of their growth within the host, although, for reasons discussed below, few experimental studies have explored the costs and benefits of the association for the endosymbionts (Douglas and Smith, 1989).

The mycetocyte bacteria of many insect taxa are not well studied, and because the associations seem diverse in form and function, it is difficult to generalize about them. Nevertheless, much has been learned from aphids and other Homoptera that feed on plant sap (for a detailed review, see Douglas, 1989a). To summarize:

i) insect offspring do not acquire mycetocyte endosymbionts from the environment or from food; they usually inherit them maternally via transoviral transmission;

ii) apparently the main benefit conferred upon the host by endosymbionts is nutritional augmentation by mechanisms such as recycling uric acid N (e.g., in cockroaches; Cochran, 1985) or synthesizing host-essential amino acids (e.g., in aphids; Douglas, 1988, 1989a,b; Douglas and Prosser, 1992; Prosser and Douglas, 1992);

iii) these mycetocyte bacteria have resisted cultivation, despite many attempts, although they can be mechanically isolated, maintained and studied for a short time in a metabolically active state (e.g., Harrison et al., 1989; Ishakawa, 1982; Whitehead and Douglas, 1993).

Morphological differences between prokaryotic endosymbionts in mycetocytes from different insect taxa, or sometimes within a host, and differences in location of mycetocytes (e.g., in fat body, free in the hemocoel or associated with the digestive tract; Douglas, 1989a) suggested that prokaryotic mycetocyte endosymbionts are probably polyphyletic. Comparative sequence analysis of 16S rRNA genes revealed that, in contrast to the generalist pattern of association across taxa demonstrated by *Wolbachia*, mycetocyte bacteria appear to be specialized with respect to their hosts (Baumann et al., 1993). For example, aphids, whiteflies, and mealybugs (all sternorrhynchan homopterans) each harbor their own lineage of mycetocyte bacteria (Tab. 2). Those from aphids and whiteflies are members of the γ-subdivision of the proteobacteria, while those from mealybugs are affiliated with the β-subdivision, and each lineage is distinguished from other lineages within the proteobacteria (Munson et al., 1991a; Munson et al., 1992; Clark et al., 1992).

The most extensive work on mycetocyte bacteria has been done with aphids. Phylogenetic analysis of 16S rDNAs from symbionts of 11 species (representing four aphid families) indicated that they form a clade collectively described as belonging to the species *Buchnera*

Table 2. Diversity of homopteran mycetocyte bacteria that have been examined by comparative sequence analysis of their 16S rDNAs

Bacterial subgroup	Symbiont groups based on 16S rDNAs	Insect group Family (Tribe)	Host species	References
γ-Proteobacteria	Aphid symbionts[a]	Aphids		
		Aphidae		
	B. aphidicola – A_1	(Aphidini)	*Acyrthosiphon pisum*	Munson et al., 1991a,b;
			Diuraphis noxia	Baumann et al., 1993;
			Myzus persicae	Moran et al., 1993
			Uroleucon sonchi	
		– – –		
	B. aphidicola – A_2	(Macrosiphini)	*Rhopalosiphum maidis*	
			R. padi	
		– – –	*Schizaphis graminum*	
	B. aphidicola – B	Drepanosiphidae	*Chaitophorus viminalis*	
		Mindaridae	*Mindarus victoriae*	
		– – –		
	B. aphidicola – C	Pemphigidae	*Melaphis rhois*	
			Pemphigus betae	
			Schlectendalia chinensis	
	S-symbionts[b]	Aphid (S)	*Acyrthosiphon pisum*	Unterman et al., 1989;
	&	Whitefly (S)	*Bemisia tabaci*	Campbell et al., 1992;
	Weevil symbionts	Weevil	*Sitophilus oryzae*	Baumann et al., 1993
		Weevil	*S. zeamais*	
	Whitefly symbionts	Whiteflies	*Bemisia tabaci*	Clark et al., 1992
			Trialeurodes vaporariorum	
			Siphonius phillyrae	
β-Proteobacteria	Mealybug symbionts	Mealybugs	*Dysmicoccus neobrevipes*	Munson et al., 1992
			Pseudococcus longispinus	
			P. maritimus	

[a] *B. aphidicola* subgroups (A, B, C) correspond to major nodes in a phylogenetic tree inferred from comparative analysis of aphid mycetocyte endosymbiont 16S rDNAs (Moran et al., 1993).
[b] S, secondary symbiont (see text for details).

aphidicola (Munson et al., 1991a; Munson et al., 1991b). Moreover, the bacterial phylogeny is congruent with a morphology-based phylogeny of their aphid hosts. Moran et al. (1993) propose that 200 to 250 million years ago, a free-living bacterium associated with an aphid became an endosymbiont. Both partners continued to evolve, giving rise to extant species of aphids and endosymbionts, and cospeciating in the process.

In light of these results, it would be of considerable interest to examine the phylogenetic position of mycetocyte bacteria from other insect groups, such as representatives of those that feed on vertebrate blood (sucking lice, tsetse flies, etc.), to see whether specialized relationships similar to those of aphids and their symbionts are common in mycetocyte symbioses. A close association between cockroaches and their mycetocyte endosymbionts has been suggested based on DNA thermal stability studies (Wren et al., 1988), but this has yet to be followed up with comparative sequence analyses.

Moran and her colleagues (1993) compared the rate of base substitution in the symbiont 16S rRNA genes (relatively constant in different aphid-associated bacteria) with the aphid fossil record to provide an improved estimate of the prokaryotic "molecular clock" (see also Harvey and May, 1993). Since prokaryotes have little or no fossil record, this calibration of their divergence is an important offshoot of using a molecular approach to study interactions between microorganisms and insects. The rate thus obtained (0.01 to 0.02 base changes per site per 50 Myr) was in close agreement with previous studies, but should still be viewed with caution as it is not clear whether the 16S rRNA "clock" of free-living bacteria "ticks" at the same rate as it does in endosymbiotic lineages (c.f. Vawter and Brown, 1986).

Other interesting observations from 16S rDNA analyses include the discovery that the so-called secondary, or S-symbionts sometimes observed in nearby tissue are phylogenetically distinct from the primary endosymbionts of mycetocytes examined thus far (Baumann et al., 1993). The 16S rRNA genes of S-symbionts from the aphid, *Acrythosiphon pisum*, and the white fly, *Bemisia tabaci*, are closely related to one another, but they are associated with different lineage of the γ-Proteobacteria than are those of the primary endosymbionts of either insect species (Tab. 2).

Confusingly, 16S rDNA sequences obtained from two rhyncophorine weevils, *Sitophilus oryzae* and *S. zeamais*, were closely related to those of aphid and white fly S-symbionts (Campbell et al., 1992). By contrast, 16S rDNAs from the cleonine weevils, *Bangasternus orientalis* and *Rhinocyllus conicus*, were found to be closely related to *Wolbachia* sequences (Campbell et al., 1992). However, these studies were based on sequences obtained from homogenates of whole weevils. Since no effort was made to separate and identify bacteria from mycetocytes,

testes or ovaries, it is premature to conclude that the sequences thus obtained came from mycetocyte symbionts.

Ecological and coevolutionary perspectives

Molecular data were essential in revealing that the microbial examples described above illustrate familiar ecological and evolutionary themes (Futuyma and Moreno, 1988). In the case of *Wolbachia*, the bacteria seem to have ecologically general (eurytopic) life-history strategies with respect to their host resources: they inhabit a broad spectrum of insects, and can be transmitted horizontally across taxa (Boyle et al., 1993; Braig et al., 1994). By contrast, mycetocyte bacteria appear to have established ecologically specialized (stenotopic) associations, such as those found between strains of *Buchnera aphidicola* and various aphids. Although the aphid mycetocytes are only one example, their specialization appears to contradict theoretical arguments that partners of mutualistic endosymbiotic symbioses should show low host specificity (Law, 1985).

Rigorous testing of insect/host coevolutionary hypotheses has come from phylogenetic analyses of congruence between insect families or genera and their respective host plants (Mitter et al., 1988; Mitter et al., 1991). However, partners in ecological associations that demonstrate considerable concordance in their phylogenies are not necessarily co-evolved, and this would be especially true for insect/endosymbiont interactions in which the associated bacteria have ceased to be free-living. Vertical transmission of an endosymbiont is likely to result in congruent phylogenies between host and bacterium: when a host undergoes speciation, its endosymbiont would subsequently undergo cospeciation as a consequence of reproductive isolation. In this case, speciation of the endosymbiont need not be driven by an evolutionary response; it could simply be an accidental biproduct of host diversification. Concordant phylogenies would not be evidence for reciprocal, step-wise coevolution since they did not result from reciprocal adaptations and counteradaptations between host and bacteria. Farrell and Mitter (1993) point out that congruence could also arise without co-evolution if subsequent colonization of particular hosts is severely constrained by features related to the hosts' phylogeny.

An evaluation of insect/endosymbiont interactions requires special conceptual considerations compared with free-living organisms such as insects and plants. Both partners in an insect/endosymbiont association can be seen as engaging in an evolutionary race leading toward mutual metabolic exploitation, leaving in their wake a diversity of new taxa. But such mutualisms might eventually end in situations where the host gains a sufficient upper hand in the interaction such that its symbiont loses its ability to be free-living and ultimately becomes an organelle (Margulis, 1993), at which point simple descriptions of mutualism and/or parasitism begin to break down. The heuristically valuable (++, +−, −−) interactions of ecologists were designed with larger organisms in mind. Moreover, these concepts were built upon the notion of definite zero points from which net costs and benefits could be measured, whereas such set points are clearly difficult to determine in organisms that have already become dependent upon each other (Douglas and Smith, 1989).

Is there evidence that cospeciation between *B. aphidicola* and its hosts involves a reciprocal, coevolutionary process? Molecular evidence supports the role of *Buchnera* in augmenting the nutrition of its host: the detection of symbiont-associated tryptophan synthetase activity (Douglas and Prosser, 1992) was recently confirmed by cloning and sequencing genes for tryptophan biosynthesis from *B. aphidicola* (Munson and Baumann, 1993). By contrast, metabolic benefits to the endosymbiont are difficult to disentangle from metabolic dependence on its host (Douglas and Smith, 1989). Nevertheless, even if metabolic exploitation of its host leads to dependence, it is still a form of reciprocal evolutionary exchange.

We might assume that an obligate endosymbiont would incur benefit by turning over some of the metabolic costs of its former free-living lifestyle in subordination to its host. To address this, a variety of other genes have been characterized from the type strain of *B. aphidicola*, the endosymbiont of the aphid *Schizaphis graminum* (Baumann et al., 1993). However, unlike endosymbiotically derived organelles, evidence indicates that *B. aphidicola* has probably retained most of the genes that are required of free-living bacteria, such as those for ATP synthesis, DNA biosynthesis, transcription and translation. Perhaps the association between *B. aphidocola* and its hosts is too recent to provide much evidence for this sort of reciprocal interchange.

Interactions between termites and their symbiotic microbial communities

Insect-microbe interactions of the type described above focus on a one-on-one isolation of the relationship between host and symbiont or symbiont population. By contrast, microorganisms in nature often live in highly diverse and interactive communities. Classic examples of this are the microbial communities that reside in the hindguts of termites. The 2200 described termite species exhibit a wide variety of dietary habits such as wood-feeding, soil-feeding, grass-feeding and fungus-cultivating (Wood and Johnson, 1986), and have established symbiotic relationships with different combinations of fungi, protozoa and bacteria, interacting via complex biochemical food-webs to assist them in

their digestion and nutrition (Brauman et al., 1992; Breznak and Brune, 1994). Have the cooperative populations in these communities co-evolved in synchrony to work as a coherent functional unit with their host or, conversely, do gut populations consist primarily of microbial residents of wood or soil that are easily acquired as the animal forages?

It has been possible to isolate and describe some of the nutritionally relevant microbial taxa from termite guts (for example, H_2/CO_2-acetogenic bacteria; Breznak et al., 1988; Kane and Breznak, 1991; Kane et al., 1991), but the vast majority of microbes therein remain uncultured. A molecular approach based on rRNA gene sequences similar to that used for *Wolbachia* and *Bunchnera* could provide information about the phylogenetic identity (and thus aspects of the ecology and evolution) of many members of the termite hindgut community. This might begin to address to what extent the evolutionary histories of gut community members are linked to one another and to the evolution of their hosts' dietary diversification (Martin, 1991; Noirot, 1992). However, such an endeavor presents technical challenges not confronted in previous studies with insect symbionts. One must be able to identify relevant populations from a complex community in the gut of one termite species and then compare them with their counterparts in the guts of others.

Molecular methods to examine the symbiotic gut communities of termites as described below are diagrammed in Figure 1. Targeting the 16S rRNA genes of a few populations among many in a complex sample is made possible by taking advantage of the nature of sequence conservation. Certain regions are universally conserved, while others are conserved among members of a large or small phylogenetic group, while still other regions are relatively variable (and thus are genus-, species- or population-specific). This information can be used to design PCR primers that will selectively amplify the 16S rDNAs of a subset of the community. For example, two groups of archea in marine coastal waters were identified by using PCR primers biased towards the 16S rDNAs of archea (DeLong, 1992).

Another related approach involves the use of "phylogenetically-nested" oligonucleotide hybridization probes targeted to hierarchial assemblages of 16S rRNAs (Stahl and Kane, 1992). Probes have been designed to target highly conserved regions of 16S rRNAs shared by large groups (e.g., bacteria vs. archaea), or more variable regions shared only by members of a given genera, species or population (Stahl and Amann, 1991; Amann et al., 1992; Devereux et al., 1992). Probes can either be fluorescently labeled to distinguish microscopically targeted cells (DeLong et al., 1989), or radioactively labeled to quantify microbial groups or selected populations (Stahl et al., 1988; Stahl and Amann, 1991).

Selective PCR amplification and sequencing can be used in combination with oligonucleotide probe hybridization to focus on individual

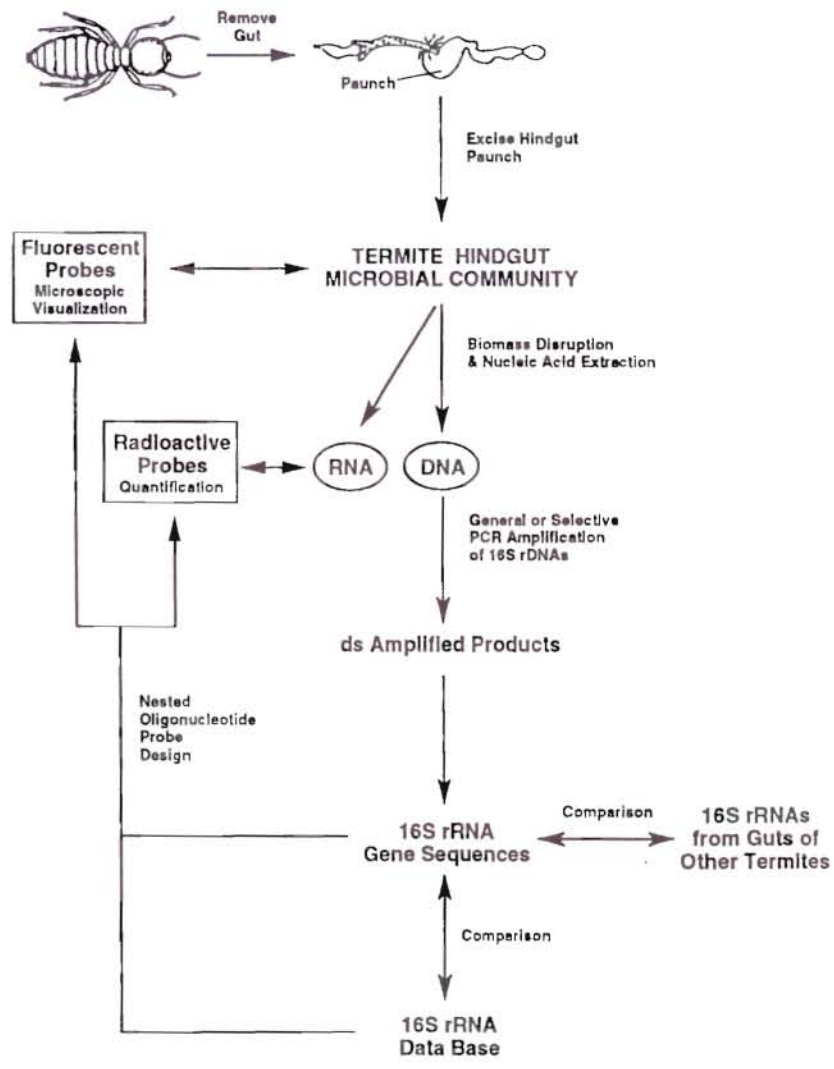

Figure 1. Molecular phylogenetic characterization of termite hindgut microbial communities by using 16S rDNA sequencing and rRNA-targeted oligonucleotide hybridization probes.

populations in complex communities such as those in termite hindguts (Fig. 1). For example, a probe that selectively targeted the 16S rRNAs of sulfate-reducing bacteria was used to visualize members of this group in multispecies anaerobic biofilms (Amann et al., 1992). A PCR primer targeting the same region of the 16S rRNA gene was then used to selectively recover sequences from the biofilms representing two distinct populations of sulfate-reducing bacteria. From these environmentally-

derived sequences, population-specific probes were designed, fluorescently labeled, and used to visualize the cells *in situ*, and to monitor their enrichment and isolation (Amann et al., 1992; Kane et al., 1993).

This same approach can be applied to the study of physiologically relevant and phylogenetically coherent groups of microbes that make up microbial communities in the guts of various termite species. Current research is aimed at recovering 16S rDNA sequences of termite gut protozoa and bacteria and to exploring the gut contents of termite species representing different feeding guilds. Molecular-phylogenetic approaches hold great promise in teasing apart ecological and evolutionary interactions between termites and their complex gut microbiota.

Conclusions

This chapter has emphasized the use of molecular-phylogenetic methods in investigating the relationships of several insect-microbe symbioses. Have molecular techniques taught us something new about the ecology and evolution of these relationships that we did not already know? Our answer to this is yes, for two reasons, one pragmatic and the other conceptual. On the pragmatic side, DNA sequence data have allowed for the identification of particular microorganisms and their placement in a phylogenetic context that provides information about the nature of their evolution with their insect hosts. Relationships of both a general and specialized nature have been documented. On the conceptual side, an understanding of these phenomena will enable us to evaluate the importance of particular species interactions in generating and maintaining diversity. We conclude with a note on the promise of new techniques: although they have brought us a long way toward understanding the ecology and evolution of particular microorganisms, the challenge remains of developing methods to characterize whole communities of microbes, such as those found in the guts of termites. The diversity of these communities is vast, and techniques of rapid identification and assessment will aid in understanding assembly rules governing the evolution of cooperative interactions between microbial populations.

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References

Aeschlimann, J.P. (1990) Simultaneous occurrence of thelytoky and bisexuality in Hymenoptera species, and its implications for biological control of pests. *Entomophaga* 35: 3–5.

Amann, R.I., Stromley, J., Devereux, R., Key, R. and Stahl, D.A. (1992) Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. *Appl. Environ. Microbiol.* 58: 614–623.

Anderson, A., Rayner, D.M. and Walton, D.W.H. (1984) *Invertebrate-Microbial Interactions*. Cambridge University Press, Cambridge, UK.

Barbosa, P., Krischik, V.A. and Jones, C.G. (1991) *Microbial Mediation of Plant-Herbivore Interactions*. John Wiley & Sons, Inc., New York.

Baumann, P., Munson, M.A., Lai, C.-Y., Clark, M.A., Baumann, L., Moran, N.A. and Campbell, B.C. (1993) Origin and properties of bacterial endosymbionts of aphids, whiteflies, and mealybugs. *ASM News* 59: 21–24.

Beard, C.B., O'Neill, S.L., Tesh, R.B., Richards, F.F. and Aksoy, S. (1993) Modification of arthropod vector competence via symbiotic bacteria. *Parasitology Today* 9: 179–186.

Berenbaum, M.R. (1988) Allelochemicals in insect-microbe-plant interactions; agents provocateurs in the coevolutionary arms race. *In:* P. Barbosa and D. Letourneau (eds): *Novel Aspects of Insect-Plant Interactions*. John Wiley & Sons, Inc., pp. 97–123.

Boyle, L., O'Neill, S.L., Robertson, H.M. and Karr, T.L. (1993) Interspecific and intraspecific horizontal transfer of *Wolbachia* in *Drosophila*. *Science* 260: 1796–1799.

Braig, H.R., Guzman, H., Tesh, R.H. and O'Neill, S.L. (1994) Replacement of the natural *Wolbachia* symbiont of *Drosophila simulans* with a mosquito counterpart. *Nature* 367: 453–455.

Brauman, A., Kane, M.D., Labat, M. and Breznak, J.A. (1992). Genesis of acetate and methane by gut bacteria of nutritionally diverse termites. *Science* 257: 1384–1386.

Breeuwer, J.A.J. and Werren, J.H. (1990) Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. *Nature* 346: 558–560.

Breeuwer, J.A.J., Stouthamer, R., Barnes, S.M., Pelletier, D.A., Weisburg, W.G. and Werren, J.H. (1992) Phylogeny of cytoplasmic incompatability microorganisms in the parasitoid wasp genus *Nasonia* (Hymenoptera: Pteromalidae) based on 16S ribosomal DNA sequences. *Insect Mol. Biol.* 1: 25–36.

Breeuwer, J.A.J. and Werren, J.H. (1993a) Effect of genotype on cytoplasmic incompatibility between two species of *Nasonia*. *Heredity* 70: 428–436.

Breeuwer, J.A.J. and Werren, J.H. (1993b) Cytoplasmic incompatibility and bacterial density in *Nasonia vitripennis*. *Genetics* 135: 565–574.

Breznak, J.A., Switzer, J.M. and Seitz, H.J. (1988) *Sporomusa termitida* sp. nov., an H_2/CO_2-utilizing acetogen isolated from termites. *Arch. Microbiol.* 150: 282–288.

Breznak, J.A. and Brune, A. (1994) Role of microorganisms in the digestion of lignocellulose by termites. *Ann. Rev. Entomol.* 39: 453–487.

Buchner, P. (1965) *Endosymbiosis of Animals with Plant Microorganisms*. Interscience, New York.

Bull, A.T. and Hardman, D.J. (1991) Microbial diversity. *Curr. Opin. Biotechnol.* 2: 4211–4218.

Campbell, B.C., Bragg, T.S. and Turner, C.E. (1992) Phylogeny of symbiotic bacteria of four weevil species (Coleoptera: Curculionidae) based on analysis of 16S ribosomal DNA. *Insect Biochem. Molec. Biol.* 22: 415–421.

Clark, M.A., Baumann, L., Munson, M.A., Baumann, P., Campbell, B.C., Duffus, J.E., Osborne, L.S. and Moran, N.A. (1992) The eubacterial endosymbionts of whiteflies (Homoptera: Aleyrodoidea) constitute a lineage distinct from the endosymbionts of aphids and mealybugs. *Curr. Microbiol.* 25: 119–123.

Cochran, D.G. (1985) Nitrogen excretion in cockroaches. *Ann. Rev. Entomol.* 30: 29–49.

Dasch, G.A., Weiss, E. and Chang, K.-P. (1984) Endosymbionts of insects. *In:* J.G. Holt and N.R. Krieg (eds): *Bergey's Manual of Systematic Bacteriology*, Vol. 1. Williams & Williams, Baltimore, pp. 811–833.

DeLong, E.F., Wickham, G.S. and Pace, N.R. (1989) Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. *Science* 243: 1360–1363.

DeLong, E.F. (1992) Archaea in coastal marine environments. *Proc. Natl. Acad. Sci. USA* 89: 5685–5689.

Devereux, R., Kane, M.D., Winfrey, J. and Stahl, D.A. (1992) Genus and group-specific hybridization probes for determinative and environmental studies of sulfate-reducing bacteria. *Syst. Appl. Microbiol.* 15: 601–609.

Douglas, A.E. (1988) Sulphate utilization in an aphid symbiosis. *Insect Biochem.* 18: 599–605.

Douglas, A.E. (1989a) Mycetocyte symbiosis in insects. *Biol. Rev.* 64: 409–434.
Douglas, A.E. (1989b) Nutritional interactions between *Myzus persicae* and its symbionts. *In:* R.K. Campbell and R.D. Eikenbary (eds): *Aphid Plant Genome Interactions*. Elsevier, Amsterdam, pp. 319–312.
Douglas, A.E. and Smith, D.C. (1989) Are endosymbioses mutualistic? *Trends Ecol. Evol.* 4: 350–352.
Douglas, A.E. (1992) Microbial brokers of insect-plant interactions. *In:* S.B.J. Menken, J.H. Visser and P. Harrewijn (eds): *Proc. 8th Inter. Symp. Insect Plant Relationships*. Kluwer Academic Publishing, Dordrecht, The Netherlands.
Douglas, A.E. and Prosser, W.A. (1992) Synthesis of the essential amino acid tryptophan in the pea aphid (*Acyrthosiphon pisum*) symbiosis. *J. Insect Physiol.* 38: 565–568.
Ebbert, M.A. (1993) Endosymbiotic sex ratio distorters in insects and mites. *In:* M.A. Ebbert and D.L. Wrensch (eds): *Evolution and Diversity of Sex Ratio in Insects and Mites*. Chapman and Hall, New York, pp. 150–191.
Farrell, B.D. and Mitter, C. (1993) Phylogenetic determinants of insect/plant community diversity. *In:* R. Ricklefs and D. Schluter (eds): *Species Diversity in Ecological Communities: Historical and Geographical Perspectives*. University of Chicago Press, pp. 253–266.
Futuyma, D.J. and Moreno, G. (1988) The evolution of ecological specialization. *Ann. Rev. Ecol. Syst.* 19: 207–233.
Harrison, C.P., Douglas, A.E. and Dixon, A.F.G. (1989) A rapid method to isolate symbiotic bacteria from aphids. *J. Insect Physiol.* 53: 427–428.
Harvey, P.H. and May, R.M. (1993) Bacterial tick-tock. *Nature* 365: 492.
Hertig, M. (1936) The rickettsia *Wolbachia pipentis* (gen. et sp. n.) and associated inclusions of the mosquito, *Culex pipens*. *Parasitology* 28: 435–486.
Hoffmann, A.A., Turelli, M. and Harsham, L.G. (1990) Factors affecting the distribution of cytoplasmic incompatibility in *Drosophila simulans*. *Genetics* 126: 933–948.
Hurst, L.D. (1993) The incidences, mechanisms and evolution of cytoplasmic sex ratio distorters in animals. *Biol. Rev.* 68: 121–193.
Ishikawa, H. (1982) Isolation of the intracellular symbionts and partial characterization of their RNA species of the elder aphid, *Acyrthosiphon magnoliae*. *Compar. Biochem. Physiol.* 72B: 239–247.
Kane, M.D. and Breznak, J.A. (1991) *Acetonema longum* gen. nov. sp. nov., an H_2/CO_2 acetogenic bacterium from the termite *Pterotermes occidentis*. *Arch. Microbiol.* 156: 91–98.
Kane, M.D., Brauman, A. and Breznak, J.A. (1991) *Clostridium mayombei* sp. nov. an H_2/CO_2 acetogenic bacterium from the gut of the African soil-feeding termite, *Cubitermes speciosus*. *Arch. Microbiol.* 156: 99–104.
Kane, M.D., Poulsen, L.K. and Stahl, D.A. (1993) Monitoring the enrichment and isolation of sulfate-reducing bacteria by using oligonucleotide hybridization probes designed from environmentally derived 16S rRNA sequences. *Appl. Environ. Microbiol.* 59: 682–686.
Knoll, A. (1992) The early evolution of eukaryotes: a geological perspective. *Science* 256: 622–627.
Law, R. (1985) Evolution in a mutualistic environment. *In:* D.H. Boucher (ed.): *The Biology of Mutualism*. Oxford University Press, New York; pp. 145–170.
Luck, R.F., Stouthamer, R. and Nunney, L.P. (1993) Sex determination and sex ratio patterns in parasitic hymenoptera. *In:* M.A. Ebbert and D.L. Wrensch (eds): *Evolution and Diversity of Sex Ratio in Insects and Mites*. Chapman and Hall, New York; pp. 442–476.
Margulis, L. (1993) *Symbiosis in Cell Evolution*, Second Edition. W. H. Freeman and Co., New York.
Martin, M.M. (1987) *Invertebrate-Microbial Interactions*. Cornell University Press, Ithaca, New York.
Martin, M.M. (1991) The evolution of cellulose digestion in insects. *Phil Trans. Roy. Soc. Lond.* B 333: 281–288.
Mitter, C., Farrell, B. and Wiegmann, B. (1988) The phylogenetic study of adaptive zones: has phytophagy promoted insect diversification? *Amer. Natur.* 132: 107–128.
Mitter, C., Farrell, B. and Futuyma, D.J. (1991) Phylogenetic studies of insect-plant interactions: insights into the genesis of diversity. *Trends Ecol. Evol.* 6: 290–293.
Moran, N.A., Munson, M.A., Baumann, P. and Ishikawa, H. (1993) A molecular clock in endosymbiotic bacteria is calibrated using the insect hosts. *Proc. Roy. Soc. Lond.* B 253: 167–171.
Munson, M.A., Baumann, P., Clark, M.A., Baumann, L., Moran, N.A., Voegtlin, D. J. and Campbell, B.C. (1991a) Evidence for the establishment of aphid-eubacterium endosymbiosis in an ancestor of four aphid families. *J. Bacteriol.* 173: 6321–6324.
Munson, M.A., Baumann; P. and Kinsey, M.G. (1991b) *Buchnera* gen. nov. and *Buchnera aphidicola* sp. nov., a taxon consisting of the mycetocyte-associated, primary endosymbionts of aphids. *Int. J. Syst. Bacteriol.* 41: 566–568.
Munson, M.A., Baumann, P. and Moran, N.A. (1992) Phylogenetic relationships of the endosymbionts of mealybugs (Homoptera: Pseudococcidae) based on 16S rDNA sequences. *Mol. Phylogen. Evol.* 1: 26–30.
Munson, M.A. and Baumann, P. (1993) Molecular cloning and nucleotide sequence of a putative *trpDC(F)BA* operon in *Buchnera aphidicola* (endosymbiont of the aphid *Schizaphis graminum*. *J. Bacteriol.* 175: 6426–6432.
Noirot, C. (1992) From wood- to humus-feeding: an important trend in termite evolution. *In:* J. Billen (ed.): *Biology and Evolution of Social Insects*. Leuven University Press, Leuven, Belgium, pp. 107–119.
O'Neill, S.L. and Karr, T.L. (1990) Bidirectional incompatibility between conspecific populations of *Drosophila simulans*. *Nature* 348: 178–180.
O'Neill, S.L., Giordano, R., Colbert, A.M.E., Karr, T.L. and Robertson, H.M. (1992) 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc. Natl. Acad. Sci. USA* 89: 2699–2702.
Prosser, W.A. and Douglas, A.E. (1992) A test of the hypothesis that nitrogen is upgraded and recycled in an aphid (*Acyrthosiphon pisum*) symbiosis. *J. Insect Physiol.* 38: 93–99.
Rousset, F., Bouchon, D., Pintureau, B., Juchault, J. and Solignac, M. (1992) *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. *Proc. Roy. Soc. Lond.* B. 250: 91–98.
Schwemmler, W. and Gassner, G. (1989) *Insect Endocytobiosis: Morphology, Physiology, Genetics, Evolution*. CRC Press, Inc. Boca Raton, Florida.
Sogin, M.L., Elwood, H.J. and Gunderson, H.J. (1986) Evolutionary diversity of the eukaryotic small subunit rRNA genes. *Proc. Natl. Acad. Sci. USA* 83: 1383–1387.
Sogin, M.L. (1991) The phylogenetic significance of sequence diversity and length variations in eukaryotic small subunit ribosomal RNA coding regions. *In:* L. Warren and H. Koprowski (eds): *Perspectives on Evolution*. Plenum Press, New York, pp. 175–188.
Stahl, D.A., Flesher, B., Mansfield, H.R. and Montgomery, L. (1988) Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. *Appl. Environ. Microbiol.* 54: 1079–1084.
Stahl, D.A. and Amann, R. (1991) Development and application of nucleic acid probes. *In:* E. Stackebrandt and M. Goodfellow (eds): *Nucleic Acid Techniques in Bacterial Systematics*. John Wiley & Sons, Ltd., New York, pp. 205–248.
Stahl, D.A., Krupp, G. and Stackebrandt, E. (1991) RNA sequencing. *In:* C.J. Howe and E.S. Ward (eds): *Nucleic Acids Sequencing: A Practical Approach*. IRL Press at Oxford University Press, Oxford, pp. 137–183.
Stahl, D.A. and Kane, M.D. (1992) Methods of microbial identification, tracking and monitoring of function. *Curr. Opin. Biotechnol.* 3: 244–252.
Stouthamer, R., Luck, R.F. and Hamilton, W.D. (1990) Antibiotics cause parthenogenetic *Trichogramma* (Hymenoptera/Trichogrammatidae) to revert to sex. *Proc. Natl. Acad. Sci. USA* 87: 2424–2427.
Stouthamer, R. (1993) The use of sexual versus asexual wasps in biological control. *Entomophaga* 38: 3–6.
Stouthamer, R. and Luck, R.F. (1993) Influence of microbe-associated parthenogenesis on the fecundity of *Trichogramma deion* and *T. pretiosum*. *Entomol. Exp. Appl.* 67: 183–192.
Stouthamer, R., Breeuwer, J.A.J., Luck, R.F. and Werren, J.H. (1993) Molecular identification of microorganisms associated with parthenogenesis. *Nature* 361: 66–68.
Turelli, M. and Hoffmann, A.A. (1991) Rapid spread of an inherited incompatibility factor in California *Drosophila*. *Nature* 353: 440–442.
Trüper, H.G. (1992) Prokaryotes: an overview with respect to biodiversity and environmental importance. *Biodiversity and Conservation* 1: 227–236.
Unterman, B.M., Baumann, P. and McLean, D.L. (1988) Pea aphid symbiont relationships established by analysis of 16S rRNAs. *J. Bacteriol.* 171: 2970–2947.
Vawter, L. and Brown, W.M. (1986) Nuclear and mitochondrial DNA comparisons reveal extreme rate variation in the molecular clock. *Science* 234: 194–196.

Whitehead, L.F. and A.E. Douglas (1993) A metabolic study of *Buchnera*, the intracellular bacterial symbionts of the pea aphid *Acyrithosiphon pisum*. *J. Gen. Microbiol.* 139: 821–826.
Woese, C.R. (1987) Bacterial evolution. *Microbiol. Rev.* 51: 221–271.
Woese, C.R. (1991) Prokaryotic systematics: the evolution of a science. *In:* A. Balows, H.G. Trüper, M. Dworkin, T.N. Harder and K.-H. Schleifer (eds): *The Prokaryotes*, Second Edition. Springer-Verlag, New York, pp. 3–18.
Wood, T.G. and Johnson, R.A. (1986) The biology, physiology and ecology of termites. *In:* S.B. Vinson (ed.): *Economic Impact and Control of Social Insects*. Praeger Press, pp. 1–68.
Wren, H.N., Johnson, J.L. and Cochran, D.G. (1988) Evolutionary inferences from a comparison of cockroach nuclear DNA and DNA from their fat-body and egg symbionts. *Evolution* 43: 276–281.