

**Use of DNA sequences to reconstruct the history of the association between members of the Sternorrhyncha (Homoptera) and their bacterial endosymbionts**

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**Abstract.** The suborder Sternorrhyncha (Insecta: Homoptera) includes aphids, whiteflies, psyllids and scale insects; these are all large diverse groups of herbivorous insects that feed on plant sap and that include many of the most damaging agricultural pests. All of these insects are dependent on obligately intracellular procaryotic symbionts for their survival. In this collaborative project between Nancy Moran and Carol von Dohlen, both insect biologists, and Paul Baumann, a bacteriologist, molecular phylogenetic methods have been used to explore the evolutionary history of these mutualistic interactions. Using PCR amplification with procaryote-specific primers, DNA sequences have been obtained for the 16S ribosomal gene of the endosymbionts from approximately 20 species of Sternorrhyncha, including 13 aphids. On the basis of these sequences as well as published 16S sequences from representative procaryotes, a phylogenetic tree has been constructed, using parsimony methods. Results indicate that the primary endosymbionts of aphids belong to a single distinctive clade that has descended from a single infection of a common ancestor of aphids; this conclusion is based on complete congruence between the phylogenetic tree for the endosymbionts and that for the aphid hosts. Based on fossils that give minimal ages for certain of the aphid lineages, the association is at least 80 million years old. The rate of change of the 16S sequence is roughly constant among lineages within the aphid primary endosymbiont clade. Again using fossil dating, we have estimated absolute rates of substitution, and estimates are consistent for three different aphid clades. These rates have been used to test the hypothesis based on a biogeographic argument that the divergence between the Asian and American members of the Melaphidina dates to the Eocene (approximately 50 MY ago): the molecular data are highly supportive of the hypothesis. The 16S genes of secondary endosymbionts of aphids and of endosymbionts of mealybugs and whiteflies have also been sequenced, and results indicate that, for each insect group, endosymbiotic bacteria are descendants of independent infections by other members of the  $\gamma$ -subdivision of the Proteobacteria for each insect group.

INTRODUCTION

Mutualistic, endosymbiotic bacteria are ubiquitous in members of the insect order Homoptera (Buchner, 1965). These bacteria inhabit specialized cells called mycetocytes and are maternally transmitted (Douglas, 1989a). Among the best known endosymbioses are those of aphids (e.g., Douglas, 1989b; Hinde, 1971; Houk, 1987; Houk & Griffiths, 1980) and mealybugs (Coccoidea: Pseudococcidae; e.g., Louis, 1965, 1967; Schrader, 1923), both in the suborder Sternorrhyncha. A variety of studies have established the vital role of

the symbionts in the lives of aphids and related insects (Douglas, 1989a, 1989b; Douglas & Prosser, 1992; Houk & Griffiths, 1980). Two of the most compelling facts indicating the dependence of the aphid on the symbionts are (i) the elaborate mechanisms that assure that the progeny are infected with the symbionts (Buchner, 1965; Hinde, 1971) and (ii) the numerous studies in which treatment with procaryote-specific antibiotics results in elimination of the symbionts with an accompanying decrease in insect growth, fecundity, and, often, lifespan (Douglas, 1989a, 1989b; Houk & Griffiths, 1980; Mittler, 1971). Aphids and other homopterans feed on plant saps, which are nutritionally deficient diets for eucaryotes (Klinghauf, 1987); their endosymbionts appear to provide limiting amino acids and possibly vitamins to their hosts (Douglas, 1989a, 1989b; Douglas & Prosser, 1992).

Until recently, some fundamental questions regarding the nature of these endosymbiotic associations and their evolutionary history were unapproachable. For example, what are the evolutionary affinities of endosymbionts? Do the endosymbionts in aphids fall within a single clade, all descendants of a single ancient infection, or do they descend from multiple infections? Do different infections all come from a single related group of free-living bacteria or from a variety of different procaryote types? To what extent do endosymbiotic bacteria and their insect hosts cospeciate and to what extent are bacteria transferred horizontally among different host lineages? How often are bacterial associates replaced by another bacterial lineage? How many infections have occurred within the aphids and within other groups of Homoptera? How old are these associations?

New methods in both molecular biology and phylogenetic analysis now allow us to determine the relationships of procaryotic symbionts to other bacteria and to reconstruct the relationships of interacting lineages of both symbionts and their hosts. The resulting phylogenetic knowledge can provide fundamental knowledge concerning the evolutionary history of these associations. We have been obtaining molecular sequence data for both endosymbionts and their aphid hosts, and we have been using these data to reconstruct the evolutionary history of these associations. Here, we summarize and interpret results from our studies so far, which are presented in more detail in the cited publications.

#### SUMMARY OF RESULTS FROM STUDIES OF THE MOLECULAR PHYLOGENETICS OF THE ENDOSYMBIONTS OF APHIDS AND RELATED INSECTS

In initial studies using DNA sequence data to characterize the endosymbiotic associations of aphids, analysis of the 16S ribosomal DNA sequences primary (P-) and secondary (S-) symbionts of the pea aphid, *Acyrtosiphon pisum*, indicated that these represented two different infections by members of the  $\gamma$ -3 subdivision of the Proteobacteria (Unterman et al., 1989; Unterman & Baumann, 1990). Those early studies have now been extended to the P-symbionts of 11 additional aphid species and also to three mealybug species and three whitefly species (Table 1). We have conducted parsimony analyses with the sequences obtained for these taxa together with published 16S sequences for procaryotes representing major subdivisions of eubacteria. By comparing the phylogenies of endosymbionts and their host insects, we have been able to make inferences regarding the evolutionary history of these mutualistic associations. Furthermore, based on evidence that the sequences of the P-symbionts evolve at roughly constant rates, we have been able to use these data to estimate dates for events in aphid and bacterial evolution. The results of

these studies permit the following preliminary conclusions concerning the evolutionary relations of these endosymbionts:

1) All P-symbionts are members of the  $\gamma$ -3 subdivision of the Proteobacteria (as defined by Stackebrandt et al., 1989) (Munson et al., 1991a; Moran et al., 1993). For the selected set of aphid species analyzed, the P-symbionts form a monophyletic group for which the sister clade includes *Proteus vulgaris* and *Escherichia coli*. The primary endosymbionts have been given the designation *Buchnera aphidicola* (Munson et al., 1991b).

2) The phylogeny derived from the 16S rDNA sequence of the P-symbionts of 11 aphid species is consistent with current aphid classification. In fact, the shortest tree is completely congruent with Heie's (1987) tentative phylogeny for the aphids (Munson et al., 1991a). These results are strong evidence for a single infection by a procaryote of an ancestor of the examined aphids. This procaryote was the common ancestor of all P-symbionts which have been inherited through vertical transmission within lineages of aphid hosts. Based on fossils assignable to several of the aphid families in 80-million-year-old amber (Heie, 1987), we can infer that the endosymbiotic association is at least that old. Following this ancient infection, host and symbionts cospeciated, resulting in the modern array of aphids and symbionts.

3) We have compared sequence differences among the 16S ribosomal genes of P-symbionts of aphid pairs representing different evolutionary distances. The 16S rDNAs of *E. coli* and *P. vulgaris*, distant relatives in the  $\gamma$ -3 subdivision, show 102 base differences. Comparable differences among P-symbionts of distantly related aphids are consistent with an ancient origin of the association. Furthermore, relative rates tests indicate that rates of substitution within the 16S rDNA are roughly constant among different lineages of the P-symbionts (Moran et al., 1993). Using fossils to estimate ages of ancestral aphid-endosymbiont pairs, the rate of substitution is approximately 1–2% per 50 million years.

4) The estimated molecular clock for the aphid P-symbionts appears to be useful for dating events in aphid evolution. For example, this rate, combined with the sequence divergence observed between an Asian and an American species of Melaphidina, confirms that the split between the ancestors of these species is very ancient (Moran et al., 1993) as hypothesized by a previous argument based on biogeography and host plant fossils (Moran, 1989). In addition, this molecular clock for the endosymbiont sequences would place the origin of the Aphidoidea (excluding Phylloxeridae and Adelgidae) at 160–280 million years ago; this age is consistent with previous estimates of the age of the superfamily.

5) As previously noted the S-symbiont of *A. pisum* is a member of the Enterobacteriaceae, also within the  $\gamma$ -3 subdivision of the Proteobacteria (Unterman et al., 1989).

6) Symbionts of the three examined members of the family Pseudococcidae (mealybugs) are related and are members of the  $\beta$ -subdivision of the Proteobacteria (Munson et al., 1992). These results indicate that an ancestor of these mealybugs was infected with another procaryote, distinct from that which infected ancestral aphids.

7) Symbionts of the three species of Aleyrodidae (whiteflies) belong to a distinct clade within the  $\gamma$ -subdivision of the Proteobacteria. They are closely related to one another and unrelated to the symbionts of aphids or mealybugs, indicating that they descend from a single ancestral infection.

8) Earlier work by PB established that the primary and secondary symbionts of *A. pisum* have a single copy of the rDNA operon (Unterman et al., 1989, 1990). A single copy has also been demonstrated in symbionts of 8 of the 10 additional aphid species that have been examined (Baumann et al., 1993).

9) The symbionts we have examined are distinct from such intracellular procaryotic parasites or commensals as *Rickettsia*, *Coxiella*, *Ehrlichia*, *Wolbachia*, and *Chlamydia* (Woese, 1987).

TABLE 1. Species of aphids and related insects for which the 16S ribosomal gene of the endosymbionts has been sequenced and used in phylogenetic analyses.

Aphidoidea (aphids)

<i>Pemphigus betae</i>	(Pemphigidae)
<i>Melaphis rhois</i>	(Pemphigidae)
<i>Schlechtendalia chinensis</i>	(Pemphigidae)
<i>Mindarus victoriae</i>	(Mindaridae)
<i>Chaitophorus viminalis</i>	(Drepanosiphidae)
<i>Rhopalosiphum maidis</i>	(Aphididae: Aphidini)
<i>Rhopalosiphum padi</i>	(Aphididae: Aphidini)
<i>Schizaphis graminum</i>	(Aphididae: Aphidini)
<i>Acyrtosiphon pisum</i>	(Aphididae: Macrosiphini)
<i>Diuraphis noxia</i>	(Aphididae: Macrosiphini)
<i>Uroleucon sonchi</i>	(Aphididae: Macrosiphini)
<i>Myzus persicae</i>	(Aphididae: Macrosiphini)

Coccoidea (scale insects)

<i>Pseudococcus longispinus</i>	(Pseudococcidae)
<i>Pseudococcus maritimus</i>	(Pseudococcidae)
<i>Dysmicoccus neobrevipes</i>	(Pseudococcidae)

Aleyrodoidea (whiteflies)

<i>Bemisia tabaci</i> (2 strains)	(Aleyrodidae)
<i>Trialeurodes vaporariorum</i>	(Aleyrodidae)
<i>Siphonius phillyreae</i>	(Aleyrodidae)

FUTURE WORK

Current studies involve the use of DNA sequences from the aphid nuclear genome for reconstruction of phylogenetic relationships of the aphid hosts (von Dohlen & Moran, in prep.). The primary objective of this work is to improve the reliability of phylogenetic hypotheses based on morphology, in particular, to add taxa for which morphology does not give a firm indication of relationships. In addition, studies in the PB laboratory are directed at characterization of the genome of the primary endosymbiont, with the aim of determining how it has diverged from free-living relatives (summary in Baumann et al., 1993).

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