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Short Communication

Evolution of male-killing *Spiroplasma* (Procaryotae: Mollicutes) inferred from ribosomal spacer sequences

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Many arthropods harbor symbiotic microorganisms that are vertically transmitted through eggs. Some of these maternally inherited symbionts have adopted a parasitic strategy, manipulating host reproduction to spread through and persist in host populations instead of providing a direct physiological benefit to their host. Reported cases of this behavior include cytoplasmic incompatibility, feminization of genetic males, induction of parthenogenesis, or the killing of male offspring during embryogenesis (male-killing). These cases are almost exclusively associated with bacteria of the genus *Wolbachia* (O'Neill et al., 1997). Male-killing is the main exception to this pattern, since it was found to be produced by at least nine different bacteria of four different taxonomic groups (Mollicutes, Enterobacteriaceae, Rickettsiaceae, Flavobacteria) (Hurst et al., 1997, 1999a, b, c). The observed taxonomic diversity suggests that the male-killing behavior arose several times independently.

We report on the evolution of male-killing within the genus *Spiroplasma* (Procaryotae: Mollicutes), using DNA sequence data. Spiroplasmas have been reported to produce male-killing in the *Drosophila willistoni* species group (Diptera: Drosophilidae) and in two beetles, *Adalia bipunctata* and *Harmonia axyridis* (Coleoptera: Coccinellidae) (Hurst et al., 1999c; Majerus et al., 1999; Williamson and Poulson, 1979). The phylogenetic analysis of 16S rDNA sequences showed the symbiont of *D. willistoni*, *Spiroplasma poulsonii* of *Spiroplasma* serogroup II, and the ladybird male-killers to fall into distantly related clades. The latter were here found to form a monophyletic clade with *Spiroplasma ixodetis* of *Spiroplasma* serogroup VI. However, 16S rDNA provided no sufficient resolution to determine the exact phylogenetic relationships within this clade (Majerus et al., 1999; for serogroup classification of *S. ixodetis* and *S. poulsonii*, see Tully et al., 1995; Williamson et al., 1999). Consequently, although male-killing apparently arose twice within the genus *Spiroplasma*, once in the lineage leading to *S. poulsonii* and once in the *S. ixodetis*-clade, it still remains unknown whether male-killing spiroplasmas of ladybirds evolved

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more than once. Furthermore, these spiroplasmas were shown to be present in extremely distant host populations from within the continuous geographic range of the two coccinellid species (Hurst et al., 1999c; Majerus et al., 1998). Data on the extent of gene flow between the host populations are not yet available. Therefore it cannot be excluded that spiroplasmas in these hosts are subject to geographic differentiation or that they have originated from independent infections. We employed DNA sequences of the ribosomal spacer and adjacent regions, 143 bp of the 3′ end of 16S rDNA and 44 bp of the 5′ end of 23S rDNA, to address these questions. This DNA region was chosen because it can easily be isolated via PCR, using primers in conserved regions of the adjacent 16S and 23S rRNA genes, and it is likely to be more variable and more phylogenetically informative than 16S rDNA because of its noncoding structure (e.g., Aakra et al., 1999; Harasawa, 1999).

Ladybird lineages infected with male-killing spiroplasmas were identified as described previously (Hurst et al., 1999c). Our study included two host specimens of *A. bipunctata* from Bielefeld and one each from Berlin, Bayreuth (all Germany), St. Petersburg, Moscow (both European part of Russia), and Tuva (Asian part of Russia). For *H. axyridis*, one host specimen from each, Novosibirsk (Siberia, Russia) and Sapporo (Japan), was studied. Samples of *S. ixodetis* (type strain Y32; accession number ATCC33835) (Tully et al., 1995) and *S. poulsonii* (type strain DW-1; accession number ATCC43153) (Williamson et al., 1999) were additionally analyzed. Molecular methods were as given in Hurst et al. (1999c). A PCR isolation of the ribosomal spacer region was performed with primers "Ha-In-1" (Hurst et al., 1999c) and "SP-ITS-N2" (5′ GGTAGTCACGTCCTTCATCG). Amplification products were cloned and subsequently sequenced for both strands and three clones per host specimen, using pUC/M13 primers and "SP-ITS-J1" (5′ TCTTGTACACACCGCCCGTC), which anneals with a conserved region at the 3′ end of 16S rDNA. Majority-rule consensus sequences were thereafter generated for each host specimen to guard against PCR errors (these sequences have been submitted to the EMBL data base with accession numbers AJ130952–AJ130955). An alignment of the sequences obtained and the previously published sequence of *Spiroplasma citri* (accession number X63781) was produced by using the program CLUSTAL W (Thompson et al., 1994). Homology could not be ascertained for all alignment positions because of length variability of the spacer, and ambiguous positions were excluded from the data set (positions 172–216, 251–272, 308–454; the complete alignment has been deposited in the EMBL alignment database under accession number DS38158). The alignment used for phylogenetic analysis thus consisted of 282 alignment sites, of which 54 were variable (19.15%). The maximum likelihood and unweighted maximum parsimony analyses (Swofford et al., 1996) were performed with the program PAUP* version 4.0b1, written by David L. Swofford, using a heuristic search with the branch-swapping nearest-neighbor interchanges algorithm. For maximum likelihood, we employed the HKY85 substitution model with rate heterogeneity across sites (four discrete gamma rate categories). The transition/transversion ratio and the shape of the gamma rate distribution were estimated from the data. Sequences were added randomly with 10 repetitions. Bootstrap analysis was based on maximum likelihood and unweighted maximum parsimony, using the same settings as above and 100 bootstrap replicates. The Kishino-Hasegawa test (Kishino and Hasegawa, 1989) was performed as implemented in PAUP*, using maximum likelihood.

The data obtained provided information on the following aspects:

First, in each of the two coccinellid species, male-killing spiroplasmas are not indicated to be subject to geographic differentiation or to have originated from independent invasion events. This is best documented for *A. bipunctata*, for which spiroplasma sequences were identical for all seven host specimens from six populations from almost the whole Eurasian continent (West Germany to the East Russian border with Mongolia). Male-killing spiroplasmas of *H. axyridis* from the two different populations similarly showed no nucleotide differences in the ribosomal spacer region.

Second, the results confirm that male-killing arose at least twice within the spiroplasmas. The ladybird male-killers and *S. ixodetis* form a well-supported monophyletic clade that is distantly related to other spiroplasmas, including the male-killer *S. poulsonii* (Fig. 1). Although the pronounced "distance" of this clade to the remaining taxa suggests that its inferred position within the *Spiroplasma* phylogeny may be affected by long-branch attraction (cf. Swofford et al., 1996; see also remote position of this clade in the 16S rDNA phylogenetic trees in Hurst et al., 1999c; Majerus et al., 1999;

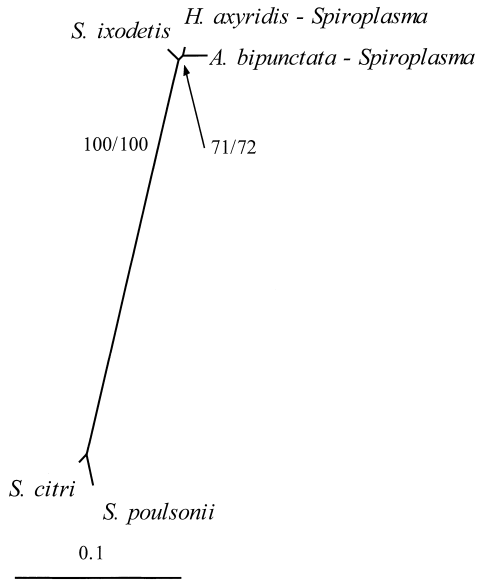

Fig. 1. Phylogenetic relationships between *Spiroplasma* bacteria inferred from the ribosomal spacer region.

Branches are drawn in proportion to the number of substitutions per site, as estimated with maximum likelihood. Values next to branches refer to the results of bootstrap analysis based on maximum likelihood (before slashes) and unweighted maximum parsimony (after slashes).

Weisburg et al., 1989), such an effect is unlikely to have biased monophyly of *S. ixodetis* and the ladybird male-killers or the relationships between these because they bear highly similar sequences and are related to each other along comparatively short branches (Fig. 1). It is thus worth noting that the close relationship of these taxa, isolated from hosts of different arthropod groups (Acari, Insecta), highlights the importance of horizontal transfer events in the evolution of these symbionts.

Third, male-killing spiroplasmas of ladybirds are likely to have evolved only once within the *S. ixodetis*-clade, although the hypothesis of a multiple origin cannot yet be rejected. Maximum likelihood and unweighted maximum parsimony produced identical tree topologies that uniformly show the spiroplasmas from *A. bipunctata* and *H. axyridis* to be monophyletic (Fig. 1). However, although the isolated ribosomal spacer region is more variable than the previously studied 16S rDNA (within the *S. ixodetis*-clade, 1.21 to 2.43% nucleotide differences for those parts of the spacer region that could be aligned without ambiguities for all taxa included, and 0.3 to 1.23% for complete 16S rDNA), tests on the robustness of the inferred topology indicate that this region may still not contain sufficient phylogenetic information. Bootstrap analysis did not provide very high support for the clade of male-killing spiroplasmas of ladybirds, regardless of the tree reconstruction method used (Fig. 1). This is corroborated by the results of the Kishino-Hasegawa test used to compare the three possible alternative phylogenetic relationships within this clade: Although the highest likelihood score was obtained for the tree topology that showed the ladybird male-killers to be monophyletic ($\ln L = -626.566$), neither of the alternative topologies was significantly worse ($p = 0.685$ for both alternative trees). The origin of male-killing within this clade therefore requires further investigation.

Fourth, besides the main purpose of our study, the results suggest that length and sequence variation of the spiroplasma ribosomal spacer region provide a powerful molecular tool for identification of spiroplasma infections. The spacer region had a length of 372–373 bp for the taxa of the *S. ixodetis*-clade, 459 bp for *S. poulsonii*, and 452 bp for *S. citri*. The nucleotide sequence divergence between the taxa included ranged from 1.21 to 18.88% for those parts of the spacer region that permitted unambiguous sequence alignment. Such variability should allow employment of these sequences as species-specific markers, as shown previously for ribosomal spacer regions of other bacterial groups (e.g., Daffonchio et al., 1998; Jensen et al., 1993). The availability of such a diagnostic marker is expected to be especially useful for rapid identification and the study of transmission routes of spiroplasmas that play a role in insect and plant diseases.

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