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The male-killing *Spiroplasmas* of *Drosophila nebulosa* and *Drosophila willistoni* have identical ITS sequences.

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Several species of the *Drosophila willistoni* group were first shown to carry male-killing bacteria during the 1950's. Certain females collected from the field produced only daughters, their sons being killed during embryogenesis. This trait was shown to be maternally inherited and associated with a *Spiroplasma* bacterium that killed male hosts only (reviewed in Williamson and Poulson, 1979). Male-killing bacteria have since been found in many insect species, and several bacteria have been shown to exhibit male-killing (Hurst and Jiggins, 2001). The *Spiroplasma* of *D. willistoni* has been cultured and formally described as *Spiroplasma poulsonii* (Williamson *et al.,* 1999).

The aim of this study was to investigate the closeness of the relationship between *Spiroplasma* strains from different *Drosophila* species. Do different species of fly have similar *Spiroplasma,* or are they different *Spiroplasma* altogether? If they are similar *Spiroplasma,* can we say whether they are similar because of shared ancestry or because of horizontal transmission of the bacterium between members of the group? Previous studies have shown all male-killing bacteria in these flies have similar morphology. However, the observation that mixing of infections from two different *willistoni* species leads to a 'clumping' reaction, in which all the *Spiroplasma* stick together, indicates they are not identical (Williamson and Poulson, 1979).

The relative ease of molecular phylogenetic analysis has made it possible for us to reassess the relationship between the different strains. The 16S-23S ribosomal spacer region (ITS; intergenic transcribed spacers) has been used in previous studies to investigate bacterial genetic relationships (Daffonchio *et al*., 1998; Jensen *et al.,* 1993). Because it is a non-coding region it is more variable and therefore more informative in differentiating between close relatives than the traditional 16S rDNA sequence (Aakra *et al.,* 1999). Schulenburg *et al.* (2000) used this region in their study of male-killing *Spiroplasma* relationships and concluded that the variability in this region of sequence would facilitate its use as a species-specific marker, evolving at double the rate of 16S rDNA.

The ITS region was sequenced for two recently collected *D. nebulosa* infections from Guadeloupe and also for a *Spiroplasma* infection originating from *D. nebulosa* and microinjected into *D. melanogaster* over forty years ago. Primers JO4 and N2 were used and the method in Schulenburg *et al.* (2000) followed, giving a sequence length of around 450bp. The new *Spiroplasma* sequences were all identical to that of *S. poulsonii* from *D. willistoni* (Schulenburg *et al.*, 2000, Accession no. AJ130995).

We conclude, therefore, that these *Spiroplasma* strains are closely related. What is unsure is whether they are similar because of common descent (the bacterium was present and maintained in these flies since their split), or whether they are similar because the bacterium has transmitted horizontally between host species. We can approach this issue by comparing the divergence of host and *Spiroplasma*.

The sequence divergence between different bacteria can be calculated by reference to the 16S 'clock' gene (Moran *et al.*, 1993) which has a lineage divergence of 1% per 50ma (2% sequence divergence). The ITS sequence evolves at double this rate, *i.e*., 1% per 25ma (2% sequence divergence) (Schulenburg *et al*., 2000). The date of the divergence between *D. nebulosa* and *D. willistoni* can be calculated by comparing COI sequences. Brower (1994) showed the rate of divergence of COI in *Drosophila* is 1.1% per million years per lineage at silent sites (2.2% sequence divergence). The COI sequences from *D. nebulosa* (U51605) and *D. willistoni* (U51589) (Gleason *et al*., 1998) were found to have 25 silent sites and 2 non silent sites over 471 bases. This gives an estimated divergence time around 7.2 million years ago (*i.e.,* ca. 5-10 million years ago) for the separation of *D. willistoni* and *D. nebulosa*. Unfortunately this means we cannot tell if horizontal transmission has occurred. During this timeframe, we would expect the ITS sequence of the *Spiroplasma*, to have accumulated 2-3 mutations, using the 'clock' above. Whilst absence of divergence in the ITS sequence is consistent with horizontal transmission, the low level of divergence expected with common ancestry forbids us from making the conclusion that their relatedness is because of a recent horizontal transmission event.

In conclusion, the *D. nebulosa Spiroplasma* strains are closely related to that of *S. poulsonii* in *D. willistoni*, and we can consider this a single instance of male-killing. However, the clumping reaction observed previously indicates subtle genetic differentiation, and we cannot formally distinguish between common ancestry and horizontal transmission as the cause of their close relationship.

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