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Molecular identification of a male-killing agent in the ladybird *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae)

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Introduction

Inherited bacteria that manipulate the reproduction of their hosts are common in invertebrates. Of four known manipulative strategies caused by bacteria in insects, three, namely cytoplasmic incompatibility, feminization and parthenogenesis induction, appear to be caused solely by members of the genus *Wolbachia*. In contrast, the fourth, male-killing, is associated with a variety of bacteria. Within the coccinellids, which are particularly prone to invasion by male-killing endosymbionts (Majerus & Hurst, 1997), bacteria of four groups, *Rickettsia* (Werren *et al*., 1994), *Spiroplasma* (Hurst *et al*., 1999a), Flavobacteria (Hurst *et al*., 1997) and *Wolbachia* (Hurst *et al*., 1999b) have been reported to be associated with the male-killing trait. That one species of coccinellid, *Adalia bipunctata*, harbours four different bacteria, a *Rickettsia*, a *Spiroplasma* and two *Wolbachia*, demonstrates the susceptibility of these beetles to male-killers.

Two questions arise from these observations. First, are there constraints on the evolution of a male-killing strategy within inherited bacteria, or can all such bacteria evolve this phenotype? Second, is invasion by more than one male-killer a general feature of those insect host species which are susceptible to male-killing?

The Asian ladybird *Harmonia axyridis* (Pallas) harbours a maternally inherited, female-biased sex ratio trait, taken to result from male-killing (Matsuka *et al*., 1975; Gotoh & Niijima, 1986; Majerus *et al*., 1998). This species shows all the features suggested by Majerus & Hurst (1997) to be necessary for the invasion and establishment of male-killers in a coccinellid: aphidophagy, laying eggs in tight batches, sibling egg cannibalism and significant levels of neonate larval mortality due to starvation. Previous studies have shown variation in the vertical transmission efficiencies between male-killing matrilines (Matsuka *et al*., 1975; Majerus *et al*., 1998), and both progressive sex ratio families (progenic sex ratio becomes progressively female biased) and revertant families (initial female bias in progenic sex ratio is lost) (Matsuka *et al*., 1975). Differences in the phenotypic expression of female-biased traits might be indicative of different causative agents of male-killing, making *H. axyridis* a suitable candidate in which to seek diversity in male-killers over geographical distance. Furthermore, establishment of the identity of the male-killer(s) in this species may help indicate which bacteria are most prone to evolving male-killing.

Results

Matrilines from four Japanese and one Mongolian population were designated as all female (SR), predominantly female (incomplete sex ratio – iSR) or normal (N), using phenotypic criteria (egg hatch rates and progenic sex ratios) defined by Majerus *et al*. (1998). Two hundred and five matrilines: sixty-five from Sapporo City, Hokkaido (31 SR, 1 iSR, 33 N), forty from Tsukuba City, Honshu (2 SR, 3 iSR, 35 N), forty-five from Fuchu City, Honshu (5 SR, 3 iSR, 37 N), six from Mt. Fugi (1 SR, 1 iSR, 4 N) and forty-six from Altai (1 SR, 45 N) were assayed.

To identify the agent(s) involved in male-killing in this species, molecular characterization of the 16S ribosomal DNA (rDNA) gene was carried out on SR lines from the most geographically distant populations. Identical 16S rDNA sequences of 1449 bp were obtained from SR individuals from Sapporo and Altai (EMBL accession no. AJ132412). When subjected to a BLAST search (Altschul *et al*., 1990), this sequence showed a high homology to 16S rDNA of Group VI spiroplasmas.

A *Spiroplasma*-specific polymerase chain reaction (PCR) was then used to test for *Spiroplasma* presence in SR,

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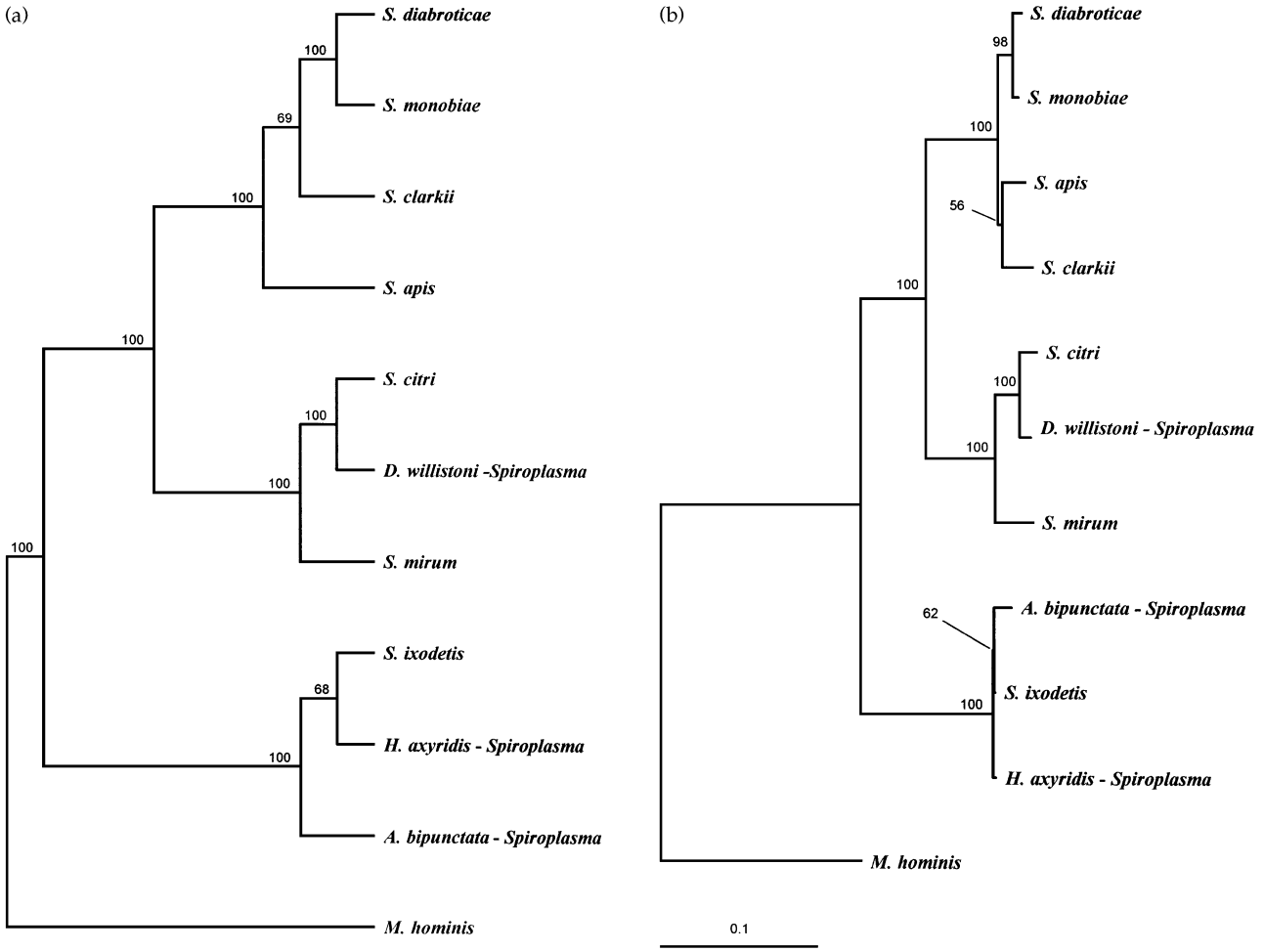

Figure 1. The phylogenetic position of the bacterium associated with male-killing in *H. axyridis*, as ascertained from analysis of 16S rDNA sequences from various members of the genus *Spiroplasma* and *Micoplasma hominis* (outgroup). Bootstrap values are given above branches. (a) Phylogenetic relationships were estimated through maximum parsimony, using the nearest-neighbour interchanges branch-swapping algorithm of PAUP v. 3.1. Bootstrap analysis with 1000 replicates was used to test the robustness of the tree obtained. (b) Phylogenetic relationships were estimated through maximum likelihood, using the programs PUZZLE 4.0 and DNAML of PHYLIP. Bootstrap analysis was performed with 100 replicates.

iSR and N lines from all populations. This gave a positive result for all individuals tested from SR and iSR matrilines. Tests of individuals from N families that had been artificially infected via microinjection and had subsequently produced a significantly female-biased progenic sex ratio also gave a positive result. All positives gave the same sized band, irrespective of phenotypic expression of the trait (SR or iSR), or geographical origin. Individuals from matrilines designated N and progeny of females produced subsequent to antibiotic treatment produced no such band. All samples tested positive with the insect/coleopteran COI PCR, validating the PCR tests. We conclude that presence of the PCR band correlates with presence of the male-killing trait.

To further corroborate the association between bacterial presence and male-killing, blood smears, from ladybirds of differing sex-ratio status, and stained with DAPI (Hurst *et al.*, 1996), were examined microscopically. Examination of smears showed the presence of bacteria in lymph and haemocytes of SR and iSR, but not N, individuals.

Results of phylogenetic analyses

Phylogenetic analysis using maximum parsimony and maximum likelihood showed the bacterium to be most closely related to *Spiroplasma ixodetis*, a *Spiroplasma* from ticks (Tully *et al.*, 1995), and the male-killing *Spiroplasma* from *A. bipunctata* (Hurst *et al.*, 1999a). These three form a monophyletic group (Fig. 1), with one species not known to cause male-killing, and the other two exhibiting this trait. However, 16S rDNA seems to provide insufficient phylogenetic information to resolve the relationships between members of this group with absolute certainty. Of the 1449 nucleotide positions in the alignment, 429 were variable over the complete data

set. However, only eighteen nucleotide positions showed variability between members of this clade. Low levels of variability here lead to inconsistent results from different tree reconstruction methods. Maximum parsimony analysis shows greatest similarity between the *H. axyridis* and tick symbionts (Fig. 1a), whereas those of *A. bipunctata* and the tick form a monophyletic group in the maximum likelihood tree (Fig. 1b). Bootstrap values for the branches separating the taxa of the clade were low, regardless of tree reconstruction method. Lack of sufficient phylogenetic information at this level was confirmed by the results of the Kishino–Hasegawa test. Using maximum likelihood, all three possible rearrangements of the branching order of the *S. ixodetis* clade were compared while keeping the rest of the tree topology constant. Although the tree shown in Fig. 1(b) had the highest log likelihood, none of the alternative tree topologies was significantly worse at 5% confidence limits (Kishino & Hasegawa, 1989).

Discussion

Here we have identified a single bacterial type associated with male-killing in *H. axyridis* from four different populations varying in longitude by 57° and in latitude by 18°. This strongly contrasts with studies of *A. bipunctata*, where four phylogenetically distinct bacteria have been found to cause male-killing in Europe and Asia, with several populations harbouring at least two bacteria at significant prevalences. The data obtained are insufficient to state with certainty that *H. axyridis* is host to just a single male-killer for three reasons. First, male-killers may be present in populations at low frequency, and low-prevalence male-killers may have been missed given the size of samples assayed. Second, only five populations of *H. axyridis* were assayed. Third, the phenotypic expression of the SR trait was relatively uniform in all the female-biased matrilines, with high vertical transmission and no cases of spontaneous reversion or the progressive sex ratio trait recorded by Matsuka *et al.* (1975) being noted. Although this observation may be a consequence of the similar treatment given to all samples in Cambridge, the existence of other male-killers in this species cannot be excluded.

Nevertheless, if the data is taken at face value, the contrast between the diversity of male-killers in *A. bipunctata* and the uniformity in *H. axyridis*, may be considered. Hurst *et al.* (1999a) note that the presence of two male-killers in *A. bipunctata* may be a transitional oddity as one male-killer replaces a less effective one. Alternatively, they argue that a selection–migration model could explain the data observed in *A. bipunctata*. The uniformity of the male-killing agent in *H. axyridis* may be the end result of a pattern of multiple invasion and replacement that is currently underway in *A. bipunctata.* Certainly, the high vertical transmission efficiency of the *H. axyridis Spiroplasma* may be indicative of a highly competitive male-killer. Studies of the costs of bearing this male-killer to female hosts would be valuable. It is of course possible that the *Spiroplasma* was the first male-killer to invade *H. axyridis*, and the competitiveness of this male-killer has precluded invasion by others that are less efficient. The variation in external environmental conditions to which the *H. axyridis* populations studied herein are exposed appear as great or greater than those of the different populations of *A. bipunctata* that have been shown to harbour male-killers. Consequently, the differences in male-killing between the species cannot be explained in these terms. Differences in the genetic environment provided by hosts may theoretically provide an explanation, but comment must await evidence of host nuclear genes which confer some degree of resistance to male-killers.

Finally, we note that the bacterium associated with male-killing in *H. axyridis* is closely related to a bacterium previously reported to exhibit this trait. This finding is not sufficient to allow appreciable comment on the range of endosymbiotic taxa that can cause male-killing. However, it is another datum in a growing set that should in time allow prediction, using an appropriate distribution, of the diversity of male-killers.

Experimental procedures

Ladybird samples

Samples of *H. axyridis* were collected from four Japanese populations, Sapporo City, Hokkaido; Tsukuba City, Fuchu City and Mt. Fugi, Honshu, and from the Altai Mountains, Mongolia. Ladybirds were transferred to Cambridge, England. Females were mated, fed *Acyrthosiphon pisum* (Harris) and allowed to oviposit. Egg hatch rates were assessed as described in Majerus *et al.* (1998). Larvae were reared to adulthood and progenic sex ratios recorded.

Molecular characterization of the 16S rDNA gene

Genomic DNA was extracted from whole ladybirds frozen in liquid nitrogen and ground up. Samples were incubated in 250 µl of digestion buffer (80 mM ethylenediaminetetra-acetic acid (EDTA), 1% sodium dodecyl sulphate (SDS), 160 mM sucrose, 100 mM Tris–HCl pH 8.0), containing 20 µg proteinase K, at 37 °C, overnight (or 65 °C for 1 h). An equal volume of 1 : 1 phenol/chloroform was added, the tubes shaken thoroughly and then spun for 10 min. The aqueous layer was transferred to a new tube and the previous step repeated using an equal volume of chloroform. The DNA was precipitated using 1/2 volume of 7.5 M NH_4-AC and 2 volumes 100% ethanol, washed with 70% ethanol and resuspended in 100 µl of sterile distilled water.

Each DNA sample was analysed for bacterial presence through a PCR reaction using general bacterial 16S rDNA primers (Weisberg *et al.*, 1991). Primers 27f (5′-GAGAGTTTGATCCTGGCTCAG-3′)

and 1495r (5′-CTACGGCTACCTTGTTACGA-3′) were used in 50-µl reactions containing 1 µl of genomic ladybird DNA, 5 µl NH_4 buffer, 5 µl dNTPs (2 mM with respect to each dNTP), 2.5 µl $MgCl_2$ (50 mM); 0.5 µl each primer (50 pmol/µl), 0.5 µl Taq polymerase and sterile distilled water to give a volume of 50 µl. Amplification controls were run for the PCR cocktail and an infected *A. bipunctata*. Cycle conditions on a Hybaid omnigene PCR machine were: one cycle of 2 min, 94 °C; thirty-five cycles of 15 s, 94 °C; 30 s, 55 °C; 3 min, 72 °C; one cycle 20 min, 72 °C. PCR products were visualized using UV light following electrophoresis on a 1% agarose gel containing ethidium bromide.

The agent in samples producing a positive 16S PCR was identified by purifying the PCR product using Prep-a-Gene (Biorad) and direct sequencing using dye-labelled terminators in a cycle-sequencing reaction, the products being visualized on an ABI 373 automated sequencing machine. Both strands of the whole unit were sequenced.

Samples that failed to produce a band with the general bacterial 16S rDNA PCR were further tested using primers which amplify the COI gene from insect mitochondrial DNA (mtDNA) (Simon *et al.*, 1994), to check that the DNA preparation did not contain factor(s) inhibiting PCR reaction.

Use of Spiroplasma*-specific PCR to assess correlation between* Spiroplasma *and SR trait*

Strength of correlation between *Spiroplasma* presence and male-killing was determined by screening with *Spiroplasma*-specific primers. The primers used were SP-ITS-JO4 (5′-GCCAGAAG-TCAGTGTCCTAACCG-3′) and SP-ITS-N55 (5′-ATTCCAAGGCA-TCCACCATACG-3′) which were designed to span the spacer region between the 16S and 23S rRNA genes (J. H. G. V. D. Schulenburg *et al*. unpublished). Individuals tested were from SR matrilines (Sapporo, eleven lines; Altai, one line; Fuchu, two lines; Tsukuba, two lines; Fugi, two lines), one iSR line (Fuchu), at least two N matrilines from each of the five populations, and males from both N and iSR matrilines. In addition, female and male progeny of SR females from Sapporo that had reverted to normal sex ratio following tetracycline treatment and female progeny from N matrilines that were successfully infected with the trait via microinjection (for method see Gotoh, 1982) were tested.

Phylogenetic analyses

To assess the phylogenetic position of the *H. axyridis* male-killer, the sequence obtained was aligned, by eye, with a previous alignment of 16S rDNA sequences from closely related species and an out-group (Hurst *et al.*, 1999a). We analysed the aligned sequences by applying the maximum parsimony method, using the program PAUP v. 3.1 (Swofford, 1993) and a heuristic search modus based on the nearest-neighbour interchanges branch-swapping algorithm. Bootstrap analysis (same settings as above) with 1000 replicates was used to test the tree robustness. Maximum likelihood analysis was also performed using PUZZLE 4.0 (Strimmer & von Haesler, 1996) and DNAML of PHYLIP (Felsenstein, 1995). As PUZZLE is based on a fast but only approximate search algorithm, the program was used to estimate transition/transversion ratios and rates and probabilities of eight gamma rate heterogeneity categories from the data set. Estimation of parameters used the Hasegawa–Kishino–Yano (HKY) model (Hasegawa *et al.*, 1985). Rate heterogeneity across sites was modelled using the discrete gamma distribution (Yang, 1994) with eight categories. The parameter estimates were then used in the program DNAML of PHYLIP. As the results from DNAML can show bias resulting from sequence input order, all DNAML analyses used five jumbles of random input order. Further, a maximum likelihood bootstrap analysis was performed using the same procedure as above and 100 bootstrap replicates. The significance of alternative tree topologies was evaluated using the Kishino–Hasegawa test (Kishino & Hasegawa, 1989).

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References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* **215**: 403–410.

Felsenstein, J. (1995) *PHYLIP: Phylogenetic inference package V. 3.57c*. Department of Genetics, University of Washington, Seattle.

Gotoh, T. (1982) Experimental transfer of abnormal 'Sex-Ratio' in the Lady-bird beetle, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae). *Appl Entomol Zool* **17**: 319–324.

Gotoh, T. and Niijima, K. (1986) Characteristics and agents of abnormal sex ratios (SR) in two aphidophagous coccinellid species. *Ecology of the Aphidophaga* (Hodek, I., ed.), pp. 545–550. Academia, Prague.

Hasegawa, M., Kishino, H. and Yano, K. (1985) Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J Mol Evol* **22**: 160–174.

Hurst, G.D.D., Hammarton, T.M., Bandi, C., Majerus, T.M.O., Bertrand, D. and Majerus, M.E.N. (1997) The diversity of inherited parasites of insects: the male-killing agent of the ladybird beetle *Coleomegilla maculata* is a member of the Flavobacteria. *Genet Res* **70**: 1–6.

Hurst, G.D.D., Jiggins, F.M., Schulenburg, J.H.G.V.D., Bertrand, D., West, S.A., Goriacheva, I.I., Zakharov, I.A., Werren, J.H., Stouthamer, R. and Majerus, M.E.N. (1999b) Male-killing *Wolbachia* in two species of insect. *Proc R Soc Lond B* **266**: 735–740.

Hurst, G.D.D., Schulenburg, J.H.G.V.D., Majerus, T.M.O., Bertrand, D., Zakarov, I.A., Baungaard, J., Völkl, W., Stouthamer, R. and Majerus, M.E.N. (1999a) Invasion of one insect species, *Adalia bipunctata*, by two different male-killing bacteria. *Insect Mol Biol* **8**: 133–139.

Hurst, G.D.D., Walker, L.E. and Majerus, M.E.N. (1996) Bacterial infection of hemocytes associated with the maternally inherited male-killing trait in British populations of the two spot ladybird, *Adalia bipunctata*. *J Invert Pathol* **68**: 286–292.

Kishino, H. and Hasegawa, M. (1989) Evaluation of the maximum

likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. *J Mol Evol* **29**: 170–179.

Majerus, M.E.N. and Hurst, G.D.D. (1997) Ladybirds as a model system for the study of male-killing symbionts. *Entomophaga* **42**: 13–20.

Majerus, T.M.O., Majerus, M.E.N., Knowles, B., Wheeler, J., Betrand, D., Kuznetzov, V.N., Ueno, H. and Hurst, G.D.D. (1998) Extreme variation in the prevalence of inherited male-killing microorganisms between three populations of *Harmonia axyridis* (Coleoptera: Coccinellidae). *Heredity* **81**: 683–691.

Matsuka, M., Hashi, H. and Okada, I. (1975) Abnormal sex ratio found in the lady beetle *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae). *Appl Entomol Zool* **10**: 84–89.

Simon, C., Frati, F., Beckenback, A., Crespi, B., Liu, H. and Flook, P. (1994) Evolution, weighting and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Ann Entomol Soc Am* **87**: 651–701.

Strimmer, K. and von Haesler, A. (1996) Quartet puzzling: a quartet maximum-likelihood method for reconstructing tree topologies. *Mol Biol Evol* **13**: 964–969.

Swofford, D.L. (1993) *PAUP: Phylogenetic analysis using parsimony V3.1.* Natural History Museum, University of Arizona.

Tully, J.G., Rose, D.L., Yunker, C.E., Carle, P., Bové, J.M., Williamson, D.L. and Whitcomb, R.F. (1995) *Spiroplasma ixodetis* sp. nov., a new species from *Ixodes pacificus* ticks collected in Oregon. *Int J Syst Bacteriol* **45**: 23–28.

Weisberg, W.G., Barns, S.M., Pelletier, D.A. and Lane, D.J. (1991) 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**: 697–703.

Werren, J.H., Hurst, G.D.D., Zhang, W., Breeuwer, J.A.J., Stouthamer, R. and Majerus, M.E.N. (1994) Rickettsial related male-killing in the ladybird beetle (*Adalia bipunctata*). *J Bacteriol* **176**: 388–394.

Yang, Z. (1994) Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: approximate methods. *J Mol Evol* **39**: 309–314.