History of Infection With Different Male-Killing Bacteria in the Two-Spot Ladybird Beetle *Adalia bipunctata* Revealed Through Mitochondrial DNA Sequence Analysis

J. Hinrich G. v. d. Schulenburg,*,[1] Gregory D. D. Hurst,† Dagmar Tetzlaff,* Gwendolen E. Booth,* Ilia A. Zakharov‡ and Michael E. N. Majerus*

**Department of Genetics, University of Cambridge, Cambridge CB2 3EH, United Kingdom, †Department of Biology, University College London, London NW1 2HE, United Kingdom and ‡Vavilov Institute of General Genetics, Russian Academy of Science, 117809 GSP-1 Moscow B-333, Russia*

Manuscript received June 13, 2001
Accepted for publication December 18, 2001

ABSTRACT

The two-spot ladybird beetle *Adalia bipunctata* (Coleoptera: Coccinellidae) is host to four different intracellular maternally inherited bacteria that kill male hosts during embryogenesis: one each of the genus Rickettsia (α-Proteobacteria) and Spiroplasma (Mollicutes) and two distinct strains of Wolbachia (α-Proteobacteria). The history of infection with these male-killers was explored using host mitochondrial DNA, which is linked with the bacteria due to joint maternal inheritance. Two variable regions, 610 bp of cytochrome oxidase subunit I and 563 bp of NADH dehydrogenase subunit 5, were isolated from 52 *A. bipunctata* with known infection status and different geographic origin from across Eurasia. Two outgroup taxa were also considered. DNA sequence analysis revealed that the distribution of mitochondrial haplotypes is not associated with geography. Rather, it correlates with infection status, confirming linkage disequilibrium between mitochondria and bacteria. The data strongly suggest that the Rickettsia male-killer invaded the host earlier than the other taxa. Further, the male-killing Spiroplasma is indicated to have undergone a recent and extensive spread through host populations. In general, male-killing in *A. bipunctata* seems to represent a highly dynamic system, which should prove useful in future studies on the evolutionary dynamics of this peculiar type of symbiont-host association.

MALE-KILLING is known from an increasing number of arthropod host species and seems to be particularly common in ladybird beetles. It refers to the killing of male embryos or larvae by intracellular, maternally inherited microorganisms. Male-killing is used by these symbionts to enhance their spread through host populations, since infected female hosts gain in fitness by the death of their brothers due to inbreeding avoidance, reduction of antagonistic sibling interactions, and/or the availability of additional resources (Hurst *et al.* 1997; Hurst and Jiggins 2000).

A unique situation was recently reported from the two-spot ladybird beetle, *Adalia bipunctata* L. (Coleoptera: Coccinellidae): It harbors at least four different male-killing bacteria, one each of the genus Rickettsia (α-Proteobacteria) and Spiroplasma (Mollicutes), and two distinct strains of Wolbachia (α-Proteobacteria;Werren *et al.* 1994; Hurst *et al.* 1999a,b). Two of these (Rickettsia, Spiroplasma) show a large overlap in their geographic distribution, whereas the Wolbachia could be isolated only from Central Russia. In Moscow (Russia), all four symbionts coexist (Hurst *et al.* 1999a,b; Majerus *et al.* 2000; Schulenburg *et al.* 2000b). The above finding is particularly unusual as minimal models of male-killing suggest the coexistence of several male-killers to be unstable at equilibrium (Hurst *et al.* 1997; Randerson *et al.* 2000). Consequently, either male-killing in *A. bipunctata* is currently not at equilibrium or there are other factors such as resistance genes in the host species or continuously fluctuating environmental conditions that permit establishment of a stable equilibrium (see also Hurst *et al.* 1999b; Majerus *et al.* 2000; Randerson *et al.* 2000).

More detailed information on the unusual coexistence of several male-killers is of great importance for our general understanding of male-killing, as it may permit detection of the factors that shape the evolutionary dynamics of this type of symbiont-host association. This study focuses on characterizing the history of infection with male-killing symbionts. Such historical information can most efficiently be extracted from molecular markers. However, bacterial DNA sequences are unsuitable for this approach. Previous analyses highlighted that none of the DNA regions currently available from the three symbiont taxa permits inference of relative diversification dates because they do not evolve with

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. AJ312060, AJ312061, and AJ313061–AJ313084.

[1]*Corresponding author:* Abteilung für Evolutionsbiologie, Institut für Evolution und Ökologie der Tiere, Westfälische Wilhelms-Universität Münster, Hüfferstr. 1, 48149 Münster, Germany.
E-mail: hschulen@uni-muenster.de

constant rates, thus rendering application of a molecular clock invalid (Schulenburg *et al.* 2000a,b). Therefore, we decided to use host mitochondrial DNA (mtDNA), which is in linkage disequilibrium with the intracellular bacteria, because both are maternally inherited. Consequently, mtDNA genealogies should permit reconstruction of symbiont evolution within the host.

An association between mitochondria and intracellular symbionts has already been documented for other symbiotic bacteria that manipulate host reproduction. The best-studied example refers to *Drosophila simulans* (Diptera), in which Wolbachia bacteria cause cytoplasmic incompatibility. Here, mtDNA variants were found to "hitchhike" with particular symbiont strains that spread through the host population, leading to congruence between mtDNA and Wolbachia gene genealogies (Nigro and Prout 1990; Turelli *et al.* 1992; Ballard *et al.* 1996; James and Ballard 2000). A similar relationship between the spread of Wolbachia symbionts and the evolution of the respective host mtDNA molecules was observed in the mosquito *Culex pipiens* (Diptera; Guillemaud *et al.* 1997), the woodlouse *Armadillidium vulgare* (Crustacea: Isopoda; Rigaud *et al.* 1999), the oak gallwasp *Biorhiza pallida* (Hymenoptera; Rokas *et al.* 2001), fire ants of the genus Solenopsis (Hymenoptera; Shoemaker *et al.* 2001), and the Asian rice gall midge *Orseolia oryzae* (Diptera; Behura *et al.* 2001).

For male-killing symbionts, the dynamics of such an association has so far been investigated only by computer simulation, based on population genetic models of mtDNA evolution and spread of a single male-killing symbiont (Johnstone and Hurst 1996). Intriguingly, the results obtained suggest that the evolution of host mtDNA should be severely affected in this case. As killing of male hosts confers a fitness advantage to the surviving siblings, maternally inherited genes from male-killer-bearing individuals will gain increased population frequencies. Hence, an increase in the abundance of daughters of infected broods will produce a concomitant increase in the frequency of a mitochondrial variant originally associated with the symbiont. This mitochondrial variant can even be predicted to reach an equilibrium frequency in the host population higher than that of the male-killing symbiont because mitochondria must show 100% vertical transmission, whereas transmission of the bacteria is usually imperfect (*e.g.*, Hurst *et al.* 1997). Consequently, the distribution of mitotypes in both the infected and the uninfected part of the host population provides information as to the history of infection with male-killing symbionts.

Here, we present an analysis of host mtDNA for a combined inference of the history of the different male-killing symbionts in *A. bipunctata*. For this purpose, mtDNA sequence data from two variable regions, part of the cytochrome oxidase subunit I (COI) and part of the NADH dehydrogenase subunit 5 gene (ND5), were isolated from 52 *A. bipunctata* specimens with known infection status and from different locations across Eurasia. For reliable outgroup comparisons, data were also obtained from two additional ladybird beetle species, *A. decempunctata* L. and *Coccinella septempunctata* L. Both phylogenetic and population genetic analyses were subsequently used to infer mtDNA genealogy and population dynamics as estimates of the demography of male-killing symbionts in *A. bipunctata*.

MATERIALS AND METHODS

Materials: Ladybird beetle material was either derived from previous studies (Hurst *et al.* 1993, 1999a,b; Werren *et al.* 1994; Majerus *et al.* 2000; Schulenburg *et al.* 2000b, 2001a) or obtained in the course of the current work, as indicated in Table 1. In the former case, infection status had been inferred using a combination of breeding experiments and molecular genetic techniques. In the latter case, ladybird specimens from different locations were first sexed using the method of Randall *et al.* (1992). Female beetles were then analyzed for the presence of the previously identified male-killing bacteria. For this purpose, we employed PCR assays specific for three bacterial groups (the *Spiroplasma ixodetis* clade, the genus Rickettsia, and A + B group Wolbachia) as described previously (Majerus *et al.* 2000; Schulenburg *et al.* 2001a). All infected specimens contained only one of the four different symbionts, consistent with previous studies that did not furnish evidence of double infections (Hurst *et al.* 1993; 1999a,b; Werren *et al.* 1994; Majerus *et al.* 2000; Schulenburg *et al.* 2000b, 2001a).

Strategy for choice of variable mtDNA regions: The entire cytochrome oxidase subunits I and II (COI + II) gene region and almost the complete NADH dehydrogenase subunit 5 (ND5) gene region were first isolated from two *A. bipunctata* specimens and a single *A. decempunctata* individual. Sequence analysis of these regions served to identify mtDNA fragments that were sufficiently variable for population genetic analysis. The COI + II and ND5 gene regions were chosen for such an analysis because they have previously been indicated to contain some of the most variable parts of the mitochondrial genome in insects (*e.g.*, Beard *et al.* 1993; Simon *et al.* 1994; Lunt *et al.* 1996; Ballard 2000). Analysis of noncoding DNA such as the A + T-rich region was not attempted because in insects, this region has in various cases been found to be less variable than some of the coding DNA (Zhang and Hewitt 1997). More importantly, interpretation of the variation observed in this region is hampered in this class of organisms by an extremely high preponderance of A and T nucleotides (usually >90%) and the abundance of indels and tandemly repeated elements, which all complicate reliable identification of homologies (Zhang and Hewitt 1997). The most variable parts within the COI + II and ND5 gene regions were then sequenced from various *A. bipunctata*.

Molecular methods: Genomic DNA was isolated from individual ladybirds, using the CTAB-based protocol outlined in Schulenburg *et al.* (2001b). The COI + II and ND5 gene regions were amplified via PCR from *A. bipunctata* from two different populations, Cambridge and Moscow (specimens BCA26 and MOS18, respectively), and *A. decempunctata* from Berlin. For this purpose, primers were designed in conserved regions adjacent to the genes, using a comparison with previously published complete mtDNA sequences from insects (Clary and Wolstenholme 1985; Beard *et al.* 1993; Crozier and Crozier 1993; Mitchell *et al.* 1993; Flook *et al.* 1995;

TABLE 1

Ladybird specimens, infection status, and mtDNA haplotype isolated

Specimen	Origin[a]	Infection[b]	COI[c]	ND5[c]
BCA26[d]	Cambridge (England)	U*	1	A
C51	Cambridge (England)	R	9	I
C61	Cambridge (England)	R	9	I
BBI10	Bielefeld (Germany)	U	1	A
BBI20	Bielefeld (Germany)	U	9	I
BBI21	Bielefeld (Germany)	R	9	I
BBI22	Bielefeld (Germany)	U	2	A
BBI23	Bielefeld (Germany)	U	2	A
BBI24	Bielefeld (Germany)	U	3	B
BBI31	Bielefeld (Germany)	S	2	A
BBI33	Bielefeld (Germany)	R	10	J
BBI42	Bielefeld (Germany)	U	1	F
BBI44	Bielefeld (Germany)	U	7	A
BBI50	Bielefeld (Germany)	U	1	A
BBI72	Bielefeld (Germany)	U	1	A
BBI127	Bielefeld (Germany)	U*	2	A
BBI128	Bielefeld (Germany)	U*	4	A
BBI147	Bielefeld (Germany)	R*	9	I
108	Bielefeld (Germany)	S	1	C
110	Berlin (Germany)	R	10	J
63	Bayreuth (Germany)	S	1	C
66	Bayreuth (Germany)	S	1	A
109	Ribe (Denmark)	R	9	I
125	Ribe (Denmark)	R	7	A
114	St. Petersburg (Russia)	S	1	A
BSP152	St. Petersburg (Russia)	R*	9	I
PBSP7	St. Petersburg (Russia)	R*	9	I
PBSP44	St. Petersburg (Russia)	R*	10	J
A2	Moscow (Russia)	S	1	A
146	Moscow (Russia)	WZ	1	B
192	Moscow (Russia)	WY	3	D
I22	Moscow (Russia)	WY	6	B
MOS3	Moscow (Russia)	R	10	J
MOS6	Moscow (Russia)	WZ	1	B
MOS9	Moscow (Russia)	S	1	A
MOS18[d]	Moscow (Russia)	WY	8	H
MOS33	Moscow (Russia)	S	1	A
BMO10	Moscow (Russia)	U	3	B
BMO13	Moscow (Russia)	U	3	B
BMO14	Moscow (Russia)	U	1	A
BMO15	Moscow (Russia)	U	1	C
BMO17	Moscow (Russia)	U	1	E
BMO19	Moscow (Russia)	U	3	D
BMO23	Moscow (Russia)	U	2	A
BMO31	Moscow (Russia)	U	1	C
BMO43	Moscow (Russia)	U	5	B
BMO45	Moscow (Russia)	U	1	G
PBMO39	Moscow (Russia)	R*	10	J
PBMO41	Moscow (Russia)	S*	1	B
PBMO57	Moscow (Russia)	U*	9	I
T17B	Tomsk (Russia)	WZ	1	B
TyN	Tuva (Russia)	S	1	B

[a] Locations are given from west to east.

[b] The hosts were either uninfected (U) or infected with Rickettsia (R), Spiroplasma (S), Wolbachia strain Y (WY), or Wolbachia strain Z (WZ); asterisk denotes specimens for which infection status had been confirmed only by bacteria-specific PCR tests; for the remaining specimens, it was inferred previously using both breeding experiments and molecular approaches (Hurst *et al.* 1993, 1999a,b; Werren *et al.* 1994; Majerus *et al.* 2000; Schulenburg *et al.* 2000b, 2001a).

[c] Numbers and letters indicate different COI and ND5 gene mitotypes, respectively.

[d] Specimens for which complete COI + II and ND5 gene region sequences were obtained.

TABLE 2

Primers used for amplification and sequencing of ladybird mtDNA

Name[a]	Oligonucleotide sequence (5′ → 3′)
COI + II	
TY-J-1462*	CCGTATCGCTTTAATTCAGCCAC
C1-N-1742	CGGATTAAGWCGWGGGAATGCTA
C1-J-1951*	GATCTCATCAATCTTAGGAGCTG
C1-J-2165	GGGGATCCAGTTTTATACCAACA
C1-N-2191	CCCGGTAAAATTAAAATATAAACTTC
C1-J-2630*	CTTTCTATAGGAGCTGTATTTGC
C1-N-2618*	CCCTATAATAGCAAATACAGCTCC
TL2-J-3042	GGCAGATTAGTGCATTGGATTTA
TL2-N-3014*	CCAATGCACTAATCTGCCATATTA
C2-J-3411	GCCCTGATACTGAAGTTATGAATA
C2-N-3389	GGCCATAACTTCAGTATCATTG
TK-N-3795*	GGGCTATAATATGGTTTAAGAGA
ND5	
N3-J-5945*	TATATCATGAATGAAATCAAGG
TN-J-6172*	GAGGTAAATCACTGTTAATGA
N5-J-6567	CCCTGTCCTCCTATTAATTCTGA
N5-N-6555	CAGAATTAATAGGAGGACAGGG
N5-J-7183*	CCCATATAACGAATATCTTGGCAA
N5-N-7312	GCTTTATCTACTTTAAGTCAA
N5-N-7789*	GGTTGAGATGGTTTAGGCTTA
N5-N-7849*	TGATTTGTGGTATCAATGATA

[a] Primer nomenclature follows Simon *et al.* (1994), using the complete mitochondrial DNA sequence of *D. yakuba* (Clary and Wolstenholme 1985) as the reference genome; primers marked with an asterisk were employed for both PCR and DNA sequencing; all others were used only for sequencing.

Lewis *et al.* 1995; see Table 2 for primer sequences). The COI + II gene region was isolated in two overlapping fragments, using primers TY-J-1462 × TL2-N-3014 and C1-J-2630 × TK-N-3795. The ND5 gene region was obtained in a continuous fragment from *A. bipunctata* with primers N3-J-5945 × N5-N-7849. These primers failed to amplify this region from *A. decempunctata*. On the basis of the sequences from *A. bipunctata* and published data, two additional primers, TN-J-6172 and N5-N-7789, were therefore constructed and subsequently employed for PCR isolation of the ND5 gene region from this ladybird species. Using PCR and partial sequencing of the amplification product, these two primers were also used on one of the *A. bipunctata* specimens (MOS18) to confirm that they produce results identical to those obtained with the more distal primers.

PCR was performed with the Expand High Fidelity PCR system (Boehringer Mannheim Ltd., Mannheim, Germany), following manufacturer's instructions. PCR cycling was controlled by the Progene thermal cycler (Techne Ltd.), using the following profile: 2 min at 94°, followed by 10 cycles of 15 sec at 94°, 1 min at 50°, 1 min and 30 sec at 70°, followed by 20 cycles of 15 sec at 94°, 1 min at 50°, and 1 min and 30 sec at 70° with an additional 10 sec for each cycle, and completed by a final extension step of 10 min at 70° (see instructions of the Expand High Fidelity PCR system; Boehringer Mannheim). PCR products were subsequently purified with Microcon-50 microconcentrators (Amicon, Beverly, MA) and directly sequenced for both strands, using a variety of internal sequencing primers (Table 2). DNA sequencing was performed with the ABI Prism BigDye Terminator cycle sequencing kit and the ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA). The resulting sequences were aligned by eye, taking into account the coding structure of the genes, and the variation between taxa and across sites was assessed.

Reliability of mtDNA data: DNA sequencing reactions on the PCR fragments produced, in each case, distinct unambiguous signals for the identified nucleotides. Therefore, they did not indicate presence of intraindividual heteroplasmy, which may hamper reliable analysis of mtDNA (*e.g.*, Rand 1993; Vogler and DeSalle 1993; Vogler *et al.* 1993). In addition, intraspecific variation between the protein-encoding genes was exclusively synonymous. The majority of differences between species were similarly due to silent substitutions. Since nuclear copies of mitochondrial genes lose their function and are thus expected to show a random distribution of substitutions (no bias toward synonymous substitutions; see Zhang and Hewitt 1996), the sequences isolated are unlikely to represent nuclear integrations, which would show nonmaternal inheritance and would thus forbid inference of symbiont evolution.

Isolation of variable mtDNA regions: On the basis of an analysis of variation across sites, the two most variable regions were chosen for demographic inferences: 610 bp in the middle of the COI gene and 563 bp toward the 5′ end of the ND5 gene. These regions were isolated from *A. bipunctata* specimens with known infection status from a variety of locations (Table 1). They were also studied for two additional *A. decempunctata* specimens from Cambridge and Moscow and one *C. septempunctata* specimen from Berlin, to obtain reliable data for outgroup comparisons. For all specimens, the complete COI gene and most of the ND5 gene were amplified via PCR as above (primers TY-J-1462 × TL2-N-3014 for COI and TN-J-6172 × N5-N-7789 for ND5). The variable regions were then directly sequenced from PCR products with internal primers (C1-J-1951 and C1-N-2618 for COI; N5-J-7183 and N5-N-7789 for ND5).

Phylogenetic analysis: DNA sequences obtained were aligned manually. The incongruence-length difference test ($P = 0.42$, Farris *et al.* 1995), as implemented in PAUP*, version 4.0b8 (Swofford 1998), and the pattern of variation between sequences suggested homogeneity of character partitions between the two gene regions. Analysis was thus performed on combined COI and ND5 gene region sequences. For this purpose, four data sets were constructed as outlined in Table 3.

Phylogenetic tree estimation was performed with maximum likelihood as implemented in PAUP*, 4.0b8 (Swofford 1998), using data set 1. Choice of substitution model was based on the approach of Huelsenbeck and Crandall (1997). Likelihood scores were thus obtained for a variety of substitution models and thereafter compared using the program MODELTEST (Posada and Crandall 1998). The general time reversible model with gamma-distributed rate heterogeneity across sites and a proportion of invariable positions (GTR-Γ-I; *e.g.*, Yang *et al.* 1994; Yang 1996) was found to provide the most realistic representation of sequence evolution for our data set. This substitution model was subsequently used for tree estimation and nonparametric bootstrapping (Felsenstein 1985). Both procedures were performed with the heuristic search modus using branch swapping by nearest-neighbor interchanges and random addition of sequences with 10 repetitions. Nonparametric bootstrapping was based on 100 replicate data sets.

Analysis of genetic differentiation: MtDNA genetic differentiation within *A. bipunctata* was assessed using differently structured data sets (Table 3). Data set 2 included all sequences isolated from uninfected *A. bipunctata* and was subdivided into those derived from Eastern (St. Petersburg, Moscow, Tomsk, Tuva) and Western ladybird populations (Cambridge, Bielefeld, Berlin, Bayreuth, Ribe). Data set 3 was similarly split up into Western and Eastern populations but contained all *A. bipunctata* mtDNA sequences obtained from the respective

TABLE 3

Composition of data sets used for mtDNA analysis

Data set[a]	N^b	Sequences included	Data set substructure
1	55	All sequences, including outgroup	None
2	23	All from uninfected *A. bipunctata*	Geographic origin
3	52	All from *A. bipunctata*	Geographic origin
4	52	All from *A. bipunctata*	Association with male-killers

[a] Data set number.
[b] Number of sequences included.

locations. Data set 4 was subdivided into sequences isolated from uninfected specimens or from ladybirds that harbored Rickettsia, Spiroplasma, or the two Wolbachia male-killers. Note that the two Wolbachia strains were taken together to increase sample size of this data subset, and no additional Wolbachia-host lineages are as yet available. Combination of these two strains is justified because both form an exclusive monophyletic group (Schulenburg *et al.* 2000a). The three structured data sets were then used to test whether mtDNA from *A. bipunctata* showed significant genetic differentiation in relation to bacterial infection or to geographic origin. In the former case, significant differentiation would indicate that bacteria were primarily maternally inherited and thus bias distribution of mitotypes among host specimens. In the latter case, geographic subdivision was analyzed for both uninfected and all studied specimens to assess whether the hypothesis of linkage disequilibrium between mitochondria and male-killing symbionts or the presence of additional bacteria in the Eastern populations (namely the Wolbachia symbionts) had any effect on the distribution of mitotypes between geographic regions. For each subset of data sets 2, 3, and 4, mitotype frequencies were recorded. In addition, the nucleotide diversity, π (Nei 1987), was calculated with the program ARLEQUIN, version 2.0 (Schneider *et al.* 2000), assuming the TN-Γ substitution model (Tamura and Nei 1993). Genetic differentiation was investigated by an analysis of variance framework, as initially defined by Cockerham (1969, 1973) and Weir and Cockerham (1984), using the analysis of molecular variance (AMOVA) and the related fixation indices (F_{ST}) as implemented in the program ARLEQUIN (Excoffier *et al.* 1992; Schneider *et al.* 2000). AMOVA and the F_{ST} measures allow consideration of both the frequency of haplotypes and also the evolutionary distances between these. In comparison to the original approach and most alternative methods, they therefore permit a more realistic assessment of the presence and extent of genetic differentiation (Excoffier *et al.* 1992; Schneider *et al.* 2000). The evolutionary distances were estimated from pairwise compared mitotypes assuming the TN-Γ model, which is the most sophisticated substitution model available in ARLEQUIN and thus most similar to the previously identified best-fitting model for this data set (GTR-Γ-I). The α-shape parameter for gamma-distributed rate heterogeneity across sites, as required for the TN-Γ model implementation in ARLEQUIN, was estimated *a priori* with PAUP*, version 4.0b8, using data set 1. In AMOVA, a significant deviation from a random distribution of haplotypes is inferred by permuting haplotypes among data subsets, using 1000 replications.

RESULTS

General information: COI and ND5 gene sequences were obtained from 52 *A. bipunctata* specimens from nine different populations. Of these, 13 were infected with Rickettsia, 10 with Spiroplasma, 3 each with Wolbachia strain Y and Z, and 23 were uninfected. The ladybirds included produced 10 different sequences for both COI and ND5. Linkage between COI and ND5 mitotypes differed between some specimens (Table 1). A combined gene data set therefore included 16 different mitochondrial variants. Data for 3 specimens of *A. decempunctata* from Cambridge, Berlin, and Moscow, and from a single ladybird of *C. septempunctata* were additionally considered for outgroup comparisons. As *A. decempunctata* from Cambridge and Bielefeld produced identical sequences in both regions, only one of them was included in subsequent analyses.The combined sequence alignment comprised 1173 positions (610 from the COI and 563 from the ND5 gene). Of these, 253 were variable between all sequences included (21.57% of the alignment sites) and 84 between those from *A. bipunctata* (7.16%). Pairwise compared sequences from this ladybird produced up to 68 nucleotide differences (5.80%), although the majority of unique sequences did not differ by more than six positions (0.51%). Variation between species was much higher, ranging between 115 and 185 differences between pairwise compared sequences (9.80–15.77%; Table 4).

Distribution of mitotypes among specimens: Nine of the 16 different mitotypes were isolated from at least two specimens. Five were found in at least five beetles, including mitotypes 1A, 1B, 2A, 9I, and 10J. Specimens from Eastern and Western populations yielded about the same number of different mitotypes, irrespective of infected ladybird beetles being excluded from the data set or not (in the former case, 9 Western *vs.* 13 Eastern mitotypes; in the latter case, 7 *vs.* 9; see data sets 2 and 3, Table 5). Three mitotypes were found exclusively in the West (1F, 4A, 7A) and 7 exclusively in the East, including 1 of the more frequent mitotypes (1B; the others were: 1E, 1G, 3D, 5B, 6B, 8H). Nevertheless, 5 mitotypes were present in both the Eastern and Western populations, including 4 of the most frequent mitotypes (1A, 2A, 9I, 10J). Nucleotide diversity estimates were almost identical for the different geographic regions (data sets 2 and 3, Table 5). Beetles with male-killing symbionts produced a total of 10 mitotypes. One of these was associated with more than one male-killer taxon: mitotype 1B was present in three specimens with

TABLE 4

Number and percentage of nucleotide differences between mtDNA sequences

	1A	1B	1C	1E	1F	1G	2A	3B	3D	4A	5B	6B	7A	8H	9I	10J	AD1	AD2	CS
1A		1	1	1	1	1	1	2	3	1	2	4	1	5	38	57	117	119	180
1B	0.09		2	2	2	2	2	1	2	2	1	3	2	4	38	56	117	119	179
1C	0.09	0.17		2	2	2	2	3	4	2	3	5	2	6	39	58	117	119	180
1E	0.09	0.17	0.17		2	2	2	3	4	2	3	5	2	6	39	56	116	118	179
1F	0.09	0.17	0.17	0.17		2	2	3	4	2	3	5	2	6	37	58	118	120	180
1G	0.09	0.17	0.17	0.17	0.17		2	3	4	2	3	5	2	4	37	56	116	118	179
2A	0.09	0.17	0.17	0.17	0.17	0.17		3	4	2	3	5	2	6	39	56	118	120	181
3B	0.17	0.09	0.26	0.26	0.26	0.26	0.26		1	3	2	2	3	3	39	57	116	118	180
3D	0.26	0.17	0.34	0.34	0.34	0.34	0.34	0.09		4	3	3	4	4	40	58	117	119	181
4A	0.09	0.17	0.17	0.17	0.17	0.17	0.17	0.26	0.34		3	5	2	6	39	58	118	120	180
5B	0.17	0.09	0.26	0.26	0.26	0.26	0.26	0.17	0.26	0.26		4	3	5	39	55	118	120	179
6B	0.34	0.26	0.43	0.43	0.43	0.43	0.43	0.17	0.26	0.43	0.34		5	5	40	57	117	119	179
7A	0.09	0.17	0.17	0.17	0.17	0.17	0.17	0.26	0.34	0.17	0.26	0.43		6	37	56	116	118	180
8H	0.43	0.34	0.51	0.51	0.51	0.34	0.51	0.26	0.34	0.51	0.43	0.43	0.51		38	56	115	117	181
9I	3.24	3.24	3.32	3.32	3.15	3.15	3.32	3.32	3.41	3.32	3.32	3.41	3.15	3.24		68	126	126	185
10J	4.86	4.77	4.94	4.77	4.94	4.77	4.77	4.86	4.94	4.94	4.69	4.86	4.77	4.77	5.80		120	122	184
AD1	9.97	9.97	9.97	9.89	10.16	9.89	10.16	9.89	9.97	10.06	10.06	9.97	9.89	9.80	10.74	10.23		2	175
AD2	10.14	10.14	10.14	10.06	10.23	10.06	10.23	10.06	10.14	10.23	10.23	10.14	10.06	9.97	10.74	10.40	0.17		177
CS	15.35	15.26	15.35	15.26	15.35	15.26	15.43	15.35	15.43	15.35	15.26	15.26	15.35	15.43	15.77	15.69	14.92	15.09	

The number and percentage of differences are given above and below the diagonal, respectively. Mitotypes are abbreviated as in Table 1. AD1 and AD2 denote the sequences from *A. decempunctata* from Cambridge/Berlin and Moscow, respectively. CS indicates sequences from *C. septempunctata.*

TABLE 5

Characteristics of data sets 2, 3, and 4

Data set[a]	Subsets[a]	π ± SD[b]	N_S[c]	N_M[d]	Mitotype distribution[e]															
					1A	1B	1C	1E	1F	1G	2A	3B	3D	4A	5B	6B	7A	8H	9I	10J
2	$West_{uninfected}$	0.8126 ± 0.4517	12	7	4				1		3	1		1			1		1	
	$East_{uninfected}$	0.9610 ± 0.5335	11	9	1		2	1		1	1	2	1		1				1	
3	$West_{all}$	2.8142 ± 1.4176	24	9	5		2		1		4	1		1			2		6	2
	$East_{all}$	2.3777 ± 1.1946	28	13	5	5	2	1		1	1	2	2		1	1		1	3	3
4	Uninfected	0.8536 ± 0.4517	23	12	5		2	1	1	1	4	3	1	1	1		1		2	
	Rickettsia	4.9963 ± 2.5969	13	3													1		7	5
	Spiroplasma	0.0790 ± 0.0669	10	4	5	2	2				1									
	Wolbachia	0.2266 ± 0.1616	6	4		3							1			1		1		

[a] Data sets and subset structures are as indicated in Tables 1 and 3; Uninfected refers to mitotypes from uninfected specimens, whereas Rickettsia, Spiroplasma, and Wolbachia refer to those from females that harbored the respective male-killers; $West_{uninfected}$ and $East_{uninfected}$ are the mitotypes from uninfected ladybirds from either Western (Cambridge, Bielefeld, Berlin, Bayreuth, Ribe) or Eastern (St. Petersburg, Moscow, Tomsk, Tuva) populations, respectively; $West_{all}$ and $East_{all}$ include all mitotypes from the respective populations; for further details, see Tables 1 and 3.

[b] Nucleotide diversity with its standard deviation, calculated as described in Nei (1987) using the TN-Γ substitution model as implemented in the program ARLEQUIN (Schneider *et al.* 2000); all values are multiplied by 100.

[c] Number of sequences included.

[d] Number of different mitotypes.

[e] List of the different mitotypes from *A. bipunctata* and their frequency in each of the subsets; nomenclature of mitotypes is as given in Table 1.

Wolbachia and two with Spiroplasma infections. The remaining mitotypes were never associated with more than one male-killing bacterium (data set 4, Table 5). In general, uninfected ladybird beetles and those that harbored Wolbachia symbionts produced a larger relative number of different mitotypes than did specimens with the other two male-killers. However, nucleotide diversity was clearly highest for the specimens infected with Rickettsia, followed by uninfected ladybirds, those with Wolbachia, and finally those with Spiroplasma male-killers (data set 4, Table 5). If the two Wolbachia strains were dealt with separately, then the three specimens with strain Y had 3 different mitotypes (3D, 6B, 8H). They produced a larger value for π ($\pi = 0.3509 \pm 0.2972$) than did those associated with strain Z, which all bore identical mitotypes (mitotype 1B; $\pi = 0$).

Tree estimation: Maximum likelihood tree estimation identified mitotype 10J to be the representative of the most basal lineage of the *A. bipunctata* clade. Mitotypes 9I and 7A were respectively found to belong to the next two early diversifying lineages. The inferred tree also indicated a monophyletic origin of mitotypes 3B, 3D, 6B, and 8H, and also of this clade and 1B and 5B (Figure 1). However, the majority of *A. bipunctata* mitotypes, all of which share a high degree of sequence similarity (mitotypes 1A–8H), were separated by only very short branches. This suggests that the data may not contain sufficient information for reliable phylogenetic inferences at this level. This is confirmed by nonparametric bootstrapping. With the exception of an early diversification of mitotypes 10J and 9I, no other clades within the *A. bipunctata* assemblage were supported by bootstrap values >50. Uninfected specimens are found in the clade of the highly similar mitotypes and also in the lineage leading to mitotype 9I, but not 10J. Spiroplasma and Wolbachia male-killers are associated only with the clade of highly similar mitotypes, whereas Rickettsia-bearing hosts are found additionally among the members of the early diversifying lineages (Figure 1).

Analysis of genetic differentiation: The results of AMOVA clearly showed absence of significant differentiation between mitotypes from the different geographic regions (data sets 2 and 3). The variance observed within data subsets was much higher than the variance between data subsets. This was reflected by a small value for the overall fixation index, which in this case was identical to the F_{ST} measure of the genetic distance between data subsets. This parameter was not significantly different from zero and, hence, supported genetic homogeneity between geographic regions (Table 6). Note that the value for Φ (equivalent to F_{ST}) had a negative prefix for data set 2. This was due to the fact that it was computed from the variance components, one of which was similarly negative (that inferred from the distribution of mitotypes among subsets). Usually, negative variance components are produced only if their nominal value is close to zero, which, in the above case, indicated lack of genetic structure between data subsets. Such negative prefixes should not have any effect on the inferred statistical significance of population differentiation (see discussion on this topic on the ARLEQUIN webpage; http://lgb.unige.ch/arlequin/software/2.000/doc/faq/faqlist.htm). The above results contrasted with those obtained from data set 4, in which an association between mitotypes and bacterial infections was tested. Here, variances observed among and within data subsets were almost identical. In addition, the overall fixation index, Φ, was higher than those previously inferred for data sets 2 and 3. Moreover, AMOVA clearly demonstrated that mitotypes cannot be assumed to be randomly distributed among data subsets (data set 4, Table 6). Therefore, homogeneity between these had to be rejected, suggesting that genetic differentiation of mtDNA is related to the presence of different male-killing symbionts. Interestingly, pairwise inferred F_{ST} values indicated significant genetic differentiation to be the result of differences between the Rickettsia and Spiroplasma, Wolbachia, or Uninfected subsets and also between those of Spiroplasma- and Wolbachia-bearing specimens. In contrast, the diversity of mitotypes from uninfected ladybirds did not appear to differ from that associated with either Wolbachia or Spiroplasma infections (Table 7).

DISCUSSION

Our study included ladybird beetles from across almost the whole Eurasian continents. The majority of specimens were derived from a German and a Central Russian population. Despite the enormous geographical distances between collection sites, mtDNA diversity was not found to correlate with geographic origin, suggesting considerable genetic exchange between ladybird beetle populations. Significant mtDNA differentiation was instead associated exclusively with the presence of the different male-killing bacteria. This supports the basic assumption used in this study that the distribution of mitotypes is, in general, linked with the distribution of the different male-killing symbionts. Bacteria thus appear to be primarily maternally inherited without significant levels of horizontal transmission. Consequently, host mtDNA should represent a suitable marker to reconstruct the evolutionary history of male-killing symbionts in relation to each other. On the basis of the results of phylogenetic and population genetic analyses and in consideration of the theoretical work by Johnstone and Hurst (1996), Rickettsia bacteria are inferred to have invaded the host species earlier than the other male-killing symbionts. Mitotypes associated with Rickettsia belonged to the three major lineages of the *A. bipunctata* clade, including the most basal branches, which clearly lack Spiroplasma- or Wolbachia-infected specimens. Uninfected ladybirds contained mitotypes that were identical or, at least, highly similar to those associated with the Rickettsia, in agreement with the model of John-

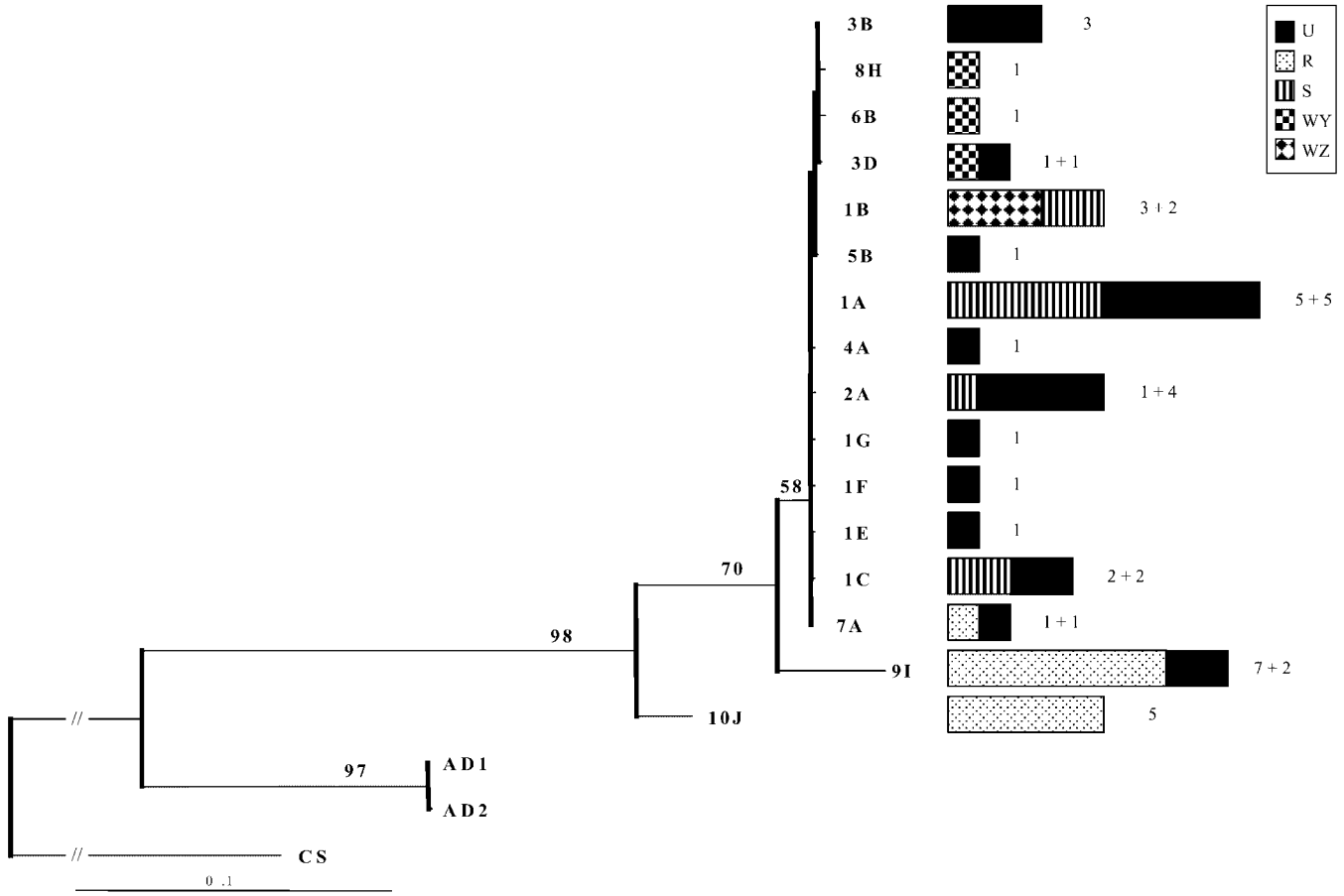

FIGURE 1.—Phylogeny of ladybird mtDNA sequences (left) and the number of specimens bearing one of the mitotypes from *A. bipunctata* (right). Tree topology and branch lengths were estimated with maximum likelihood, as implemented in PAUP*, version 4.0b8 (SWOFFORD 1998), using the general time reversible model with rate heterogeneity across sites and a proportion of invariable positions (*e.g.*, YANG *et al.* 1994; YANG 1996). Tree search was based on branch swapping by nearest-neighbor interchanges and random addition of sequences with 10 repetitions. The tree shown produced a likelihood of $\ln L = -3030.26$. Values above branches refer to the results of nonparametric bootstrapping (FELSENSTEIN 1985), based on the same methods as above and 100 replicate data sets. Only values >50 are shown. Branch lengths are given in proportion to the estimated number of substitutions per site (see bar in bottom left corner). The branches leading to the outgroup CS were shortened to permit increasing the resolution of the *A. bipunctata* clade (see double slash). Note that no resolution is obtained for the relationships between (i) 3B, 8H, 6B, and 3D; (ii) this clade, 1B, and 5B; (iii) the latter group, 1A, 4A, 2A, 1G, 1F, 1E, 1C, and 7A. Nomenclature of *A. bipunctata* mitotypes is as in Table 1. AD1 and AD2 denote the sequences from *A. decempunctata* from Cambridge/Berlin and Moscow, respectively. CS refers to that from *C. septempunctata*. On the right, horizontal bars are given in proportion to the number of specimens with one of the mitotypes from *A. bipunctata* as indicated. Infection status of these specimens is illustrated such that different patterns denote that host specimens were uninfected (U) or harbored male-killing Rickettsia (R), Spiroplasma (S), Wolbachia strain Y (WY) or Wolbachia strain Z (WZ; see legend in the far right corner; see also Tables 1 and 3 for details).

STONE and HURST (1996). Furthermore, the Rickettsia data subset produced the highest nucleotide diversity index π for data set 4 (data subdivided according to infection status). This is similarly consistent with an early association of Rickettsia male-killers and *A. bipunctata*. One peculiarity requires special consideration. Mitotype 10J shows considerable differences to the other mtDNA variants. It was found exclusively in specimens with Rickettsia but not in uninfected beetles or those with one of the other symbionts. There are four possible explanations. The finding of such a strict and apparently isolated association could be due to perfect vertical transmission efficiency of Rickettsia such that the associated mitotype cannot "escape" to the uninfected part of the host population. However, Rickettsia transmission rates were consistently found to be smaller than 100% in previous breeding experiments, which included host lineages associated with 10J (Rickettsia from *A. bipunctata* specimens BBI33, 110, and MOS3; HURST *et al.* 1993; MAJERUS *et al.* 2000). Another explanation is long-term coadaptation between Rickettsia and 10J mitochondria such that uninfected beetles with 10J mitotypes show reduced fitness. In this case, Rickettsia associated with 10J should clearly differ from those associated with other mitotypes. This is not confirmed by a previous analysis of Rickettsia gene sequences, where Rickettsia

TABLE 6

Results of AMOVA

Source of variation[a]	ν[b]	*SSD*[c]	Variance[d]	Φ[e]	*P*[f]
Data set 2 (uninfected, divided into geographic regions)					
Among subsets	1	1.356	−0.3332		
Within subsets	21	108.787	5.1803		
Total	22	110.143	4.8471	−0.0688	0.5191
Data set 3 (all, divided into geographic regions)					
Among subsets	1	15.182	0.0023		
Within subsets	51	756.165	15.1233		
Total	52	771.346	15.1256	0.0002	0.3744
Data set 4 (all, divided according to infection status)					
Among subsets	3	297.166	7.43934		
Within subsets	48	474.180	9.87875		
Total	51	771.346	17.31809	0.42957	<0.0001

[a] Nomenclature of data sets refers to Tables 3 and 5; all calculatiions were performed with AMOVA (Excoffier *et al.* 1992) as implemented in ARLEQUIN (Schneider *et al.* 2000).
[b] Degrees of freedom.
[c] Sum of squared deviations.
[d] Variance components.
[e] Overall fixation index.
[f] Probability of homogeneity between subsets, as calculated using 1000 permutations.

associated with different mitotypes (including 10J and 9I; host specimens BBI21, 110, MOS3) all bore identical sequences (Schulenburg *et al.* 2001a). The observed peculiarity could also have resulted from a specific event in a subset of the populations such as a recent population bottleneck. In this case, mitotype 10J is expected to be confined to a small geographic region. However, as it is present in different Western and Eastern populations, this hypothesis also seems unlikely. The apparent absence of mitotype 10J in uninfected ladybirds is thus most likely due to incomplete sampling, in combination with a recent spread of one of the other male-killers that is associated with a different mitotype (see below).

Mitotypes isolated from ladybirds with either Spiroplasma or Wolbachia symbionts were highly similar. They were inferred by ML tree estimation to belong to the same clade and were consistently shown to produce the smallest π values for data set 4. This clearly supports the above conclusion that Spiroplasma and Wolbachia male-killers have been present in *A. bipunctata* for a shorter period of time than the Rickettsia male-killer. In addition, almost all of the uninfected specimens bore mitotypes that were identical or highly similar to those associated with the Spiroplasma or Wolbachia male-killers. This indicates a high degree of genetic exchange between mitotype pools of Spiroplasma- or Wolbachia-bearing ladybird beetles and uninfected specimens. According to theoretical work (Johnstone and Hurst 1996), high prevalence of a particular class of mitochondrial variant among uninfected hosts should result from the recent spread of a male-killing symbiont through the host population. We here propose that it is most likely the Spiroplasma male-killer that has undergone a recent and extensive spread through host populations.

First, a recent spread of Rickettsia symbionts seems unlikely. If this were the case, then the Rickettsia-associated mitotypes 9I and 10J, which were clearly different from the remaining *A. bipunctata* mtDNA variants, would be expected to show a higher frequency among uninfected specimens.

Second, extensive screening of Eurasian host populations revealed that Wolbachia symbionts were present exclusively in Central Russia where they were found at comparatively low prevalence (Hurst *et al.* 1993, 1999a,b; Majerus *et al.* 2000; Zakharov *et al.* 1996, 2000; Schulenburg *et al.* 2000b, 2001a). Such a finding is clearly inconsistent with a recent spread of this symbiont through the host species.

Third, Spiroplasma bacteria did show a high prevalence in, at least, the Eastern host populations (Zakh-

TABLE 7

F_{ST} values inferred from pairwise compared subsets of data set 4

	Spiroplasma	Wolbachia	Uninfected
Rickettsia	0.4697	0.3910	0.4888
Spiroplasma		0.3771	−0.0099
Wolbachia			0.0289

[a] Pairwise F_{ST} values were calculated from variance components using the program ARLEQUIN (Excoffier *et al.* 1992; Schneider *et al.* 2000); data set substructure is as indicated in Tables 1, 3, and 5.

AROV *et al.* 1996; HURST *et al.* 1999b; MAJERUS *et al.* 2000; our unpublished data). In addition, they were found consistently in breeding experiments to show one of the highest transmission rates of the male-killers from *A. bipunctata* (ZAKHAROV *et al.* 1996; HURST *et al.* 1999b; MAJERUS *et al.* 2000). High transmission efficiency can be assumed to be associated with increased competitiveness (HURST *et al.* 1997; RANDERSON *et al.* 2000).

In conclusion, analysis of host mtDNA indicated that the unusual coexistence of several male-killing symbionts in *A. bipunctata* is associated with subsequent invasion events and spread of particular symbionts through host populations. Male-killing in *A. bipunctata* thus appears to be a highly dynamic system. It is therefore expected to prove extremely valuable in future studies on the evolutionary dynamics of male-killing since it may aid in identifying the factors that determine invasion, spread, maintenance, and also loss of male-killing bacteria.

The history of infection in this host species clearly warrants further investigation. In particular, information content of the data should be increased in the future by the inclusion of additional variable mtDNA regions in order to define more precisely the evolutionary relationships of the highly similar mitotypes. Similarly, sample size of data subsets should be increased, particularly in those cases where the number of host lineages was small, *e.g.*, the Wolbachia data subsets. This first requires isolation of additional Wolbachia-bearing host lineages since only six have been identified to date, all of which were included in this study. More detailed insights into symbiont demography in *A. bipunctata* would also be aided by an extension of the model of JOHNSTONE and HURST (1996) to explore the dynamics of mtDNA evolution in response to the spread of several male-killing symbionts.

We thank C. Maddren for technical assistance and J. W. O. Ballard, J. M. Koene, and two anonymous reviewers for comments on an earlier version of the manuscript. J. H. G. v. d. Schulenburg was funded by a Training and Mobility of Researchers (TMR) fellowship from the European Union and, for the collection of ladybird beetles, by travel grants from both Magdalene College (Cambridge, England) and the Cambridge Philosophical Society (Cambridge, England). G. D. D. Hurst was supported by a Biotechnology and Biological Sciences Research Council (BBSRC) D. Phillips fellowship and I. A. Zakharov was supported by grants of the Russian Foundation for Basic Research (grant nos. 99-04-48193 and 00-15-97777).

LITERATURE CITED

BALLARD, J. W. O., 2000 Comparative genomics of mitochondrial DNA in Drosophila simulans. J. Mol. Evol. **51:** 64–75.

BALLARD, J. W. O., J. HATZIDAKIS, T. L. KARR and M. KREITMAN, 1996 Reduced variation in Drosophila simulans mitochondrial DNA. Genetics **144:** 1519–1528.

BEARD, C. B., D. M. HAMM and F. H. COLLINS, 1993 The mitochondrial genome of the mosquito Anopheles gambiae: DNA sequence, genome organization, and comparisons with mitochondrial sequences of other insects. Insect Mol. Biol. **2:** 103–124.

BEHURA, S. K., S. C. SAHU, M. MOHAN and S. NAIR, 2001 Wolbachia in the Asian rice gall midge, Orseolia oryzae (Wood-Mason): correlation between host mitotypes and infection status. Insect Mol. Biol. **10:** 163–171.

CLARY, D. O., and D. R. WOLSTENHOLME, 1985 The mitochondrial DNA molecule of Drosophila yakuba—nucleotide sequence, gene organization, and genetic code. J. Mol. Evol. **22:** 252–271.

COCKERHAM, C. C., 1969 Variance of gene frequencies. Evolution **23:** 72–84.

COCKERHAM, C. C., 1973 Analyses of gene frequencies. Genetics **74:** 679–700.

CROZIER, R. H., and Y. C. CROZIER, 1993 The mitochondrial genome of the honeybee *Apis mellifera* —complete sequence and genome organization. Genetics **133:** 97–117.

EXCOFFIER, L., P. SMOUSE and J. QUATTRO, 1992 Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics **131:** 479–491.

FARRIS, J. S., M. KALLERSJO, A. G. KLUGE and C. BULT, 1995 Testing significance of incongruence. Cladistics **10:** 315–319.

FELSENSTEIN, J., 1985 Confidence limits on phylogenies—an approach using the bootstrap. Evolution **40:** 783–791.

FLOOK, P. K., C. H. F. ROWELL and G. GELLISSEN, 1995 The sequence, organization, and evolution of the *Locusta migratoria* mitochondrial genome. J. Mol. Evol. **41:** 928–941.

GUILLEMAUD, T., N. PASTEUR and F. ROUSSET, 1997 Contrasting levels of variability between cytoplasmic genomes and incompatibility types in the mosquito *Culex pipiens*. Proc. R. Soc. Lond. Ser. B **264:** 245–251.

HUELSENBECK, J. P., and K. A. CRANDALL, 1997 Phylogeny estimation and hypothesis testing using maximum likelihood. Annu. Rev. Ecol. Syst. **28:** 437–466.

HURST, G. D., and F. M. JIGGINS, 2000 Male-killing bacteria in insects: mechanisms, incidence, and implications. Emerg. Infect. Dis. **6:** 329–336.

HURST, G. D. D., M. E. N. MAJERUS and L. E. WALKER, 1993 The importance of cytoplasmic male killing elements in natural populations of the two spot ladybird, *Adalia bipunctata* (Linnaeus) (Coleoptera: Coccinellidae). Biol. J. Linn. Soc. **49:** 195–202.

HURST, G. D. D., L. D. HURST and M. E. N. MAJERUS, 1997 Cytoplasmic sex-ratio distorters, pp. 125–154 in *Influential Passengers*, edited by S. L. O'NEILL, A. A. HOFFMANN and J. H. WERREN. Oxford University Press, Oxford.

HURST, G. D. D., F. M. JIGGINS, J. H. G. v. d. SCHULENBURG, D. BERTRAND, S. A. WEST *et al.*, 1999a Male-killing *Wolbachia* in two species of insect. Proc. R. Soc. Lond. Ser. B **266:** 735–740.

HURST, G. D. D., J. H. G. v. d. SCHULENBURG, T. M. O. MAJERUS, D. BERTRAND, I. A. ZAKHAROV *et al.*, 1999b Invasion of one insect species, *Adalia bipunctata*, by two different male-killing bacteria. Insect Mol. Biol. **8:** 133–139.

JAMES, A. C., and J. W. O. BALLARD, 2000 The expression of cytoplasmic incompatibility and its impact on population frequencies and the distribution of *Wolbachia* strains in *Drosophila simulans*. Evolution **54:** 1661–1672.

JOHNSTONE, R. A., and G. D. D. HURST, 1996 Maternally inherited male-killing microorganisms may confound interpretation of mitochondrial DNA variability. Biol. J. Linn. Soc. **58:** 453–470.

LEWIS, D. L., C. L. FARR and L. S. KAGUNI, 1995 *Drosophila melanogaster* mitochondrial DNA—completion of the nucleotide sequence and evolutionary comparisons. Insect Mol. Biol. **4:** 263–278.

LUNT, D. H., D. X. ZHANG, J. M. SZYMURA and G. M. HEWITT, 1996 The insect cytochrome oxidase I gene: evolutionary patterns and conserved primers for phylogenetic studies. Insect Mol. Biol. **5:** 153–165.

MAJERUS, M. E. N., J. H. G. v. d. SCHULENBURG and I. A. ZAKHAROV, 2000 Multiple causes of male-killing in a single sample of the two-spot ladybird, *Adalia bipunctata* (Coleoptera: coccinellidae) from Moscow. Heredity **84:** 605–609.

MITCHELL, S. E., A. F. COCKBURN and J. A. SEAWRIGHT, 1993 The mitochondrial genome of *Anopheles quadrimaculatus* species a—complete nucleotide sequence and gene organization. Genome **36:** 1058–1073.

NEI, M., 1987 *Molecular Evolutionary Genetics*. Columbia University Press, New York.

NIGRO, L., and T. PROUT, 1990 Is there selection on RFLP differences in mitochondrial DNA? Genetics **125:** 551–555.

Posada, D., and K. A. Crandall, 1998 Modeltest: testing the model of DNA substitution. Bioinformatics **14:** 817–818.

Rand, D. M., 1993 Endotherms, Ectotherms, and mitochondrial genome size variation. J. Mol. Evol. **37:** 281–295.

Randall, K., M. E. N. Majerus and H. Forge, 1992 Characteristics for sex determination in British ladybirds (Coleoptera: Coccinellidae). Entomologist **111:** 109–122.

Randerson, J. P., N. G. C. Smith and L. D. Hurst, 2000 The evolutionary dynamics of male-killers and their hosts. Heredity **84:** 152–160.

Rigaud, T., D. Bouchon, C. Souty-Grosset and R. Raimond, 1999 Mitochondrial DNA polymorphism, sex ratio distorters and population genetics in the isopod *Armadillidium vulgare*. Genetics **152:** 1669–1677.

Rokas, A., R. J. Atkinson, G. S. Brown, S. A. West and G. N. Stone, 2001 Understanding patterns of genetic diversity in the oak gallwasp *Biorhiza pallida*: demographic history of a *Wolbachia* selective sweep? Heredity **87:** 294–304.

Schneider, S., D. Roessli and L. Excoffier, 2000 Arlequin: a software for population genetics data analysis. Version 2.0, Genetics and Biometry Lab, Dept. of Anthropology, University of Geneva, Geneva.

Schulenburg, J. H. G. v. d., G. D. D. Hurst, T. M. E. Huigens, M. M. M. van Meer, F. M. Jiggins *et al.*, 2000a Molecular evolution and phylogenetic utility of *Wolbachia ftsZ* and *wsp* gene sequences with special reference to the origin of male-killing. Mol. Biol. Evol. **17:** 584–600.

Schulenburg, J. H. G. v. d., T. M. O. Majerus, C. M. Dorzhu, I. A. Zakharov, G. D. D. Hurst *et al.*, 2000b Evolution of male-killing *Spiroplasma* (Procaryotae: Mollicutes) inferred from ribosomal spacer sequences. J. Gen. Appl. Microbiol. **46:** 95–98.

Schulenburg, J. H. G. v. d., M. Habig, J. J. Sloggett, K. M. Webberley, D. Bertrand *et al.*, 2001a Incidence of male-killing *Rickettsia* spp. (α-proteobacteria) in the ten-spot ladybird beetle *Adalia decempunctata* L. (Coleoptera: Coccinellidae). Appl. Environ. Microbiol. **67:** 270–277.

Schulenburg, J. H. G. v. d., J. M. Hancock, A. Pagnamenta, J. J. Sloggett, M. E. N. Majerus *et al.*, 2001b Extreme length and length variation in the first ribosomal internal transcribed spacer of ladybird beetles (Coleoptera: Coccinellidae). Mol. Biol. Evol. **18:** 648–660.

Shoemaker, D. D., K. G. Ross, L. Keller, E. L. Vargo and J. H. Werren, 2001 Wolbachia infections in native and introduced populations of fire ants (Solenopsis spp.). Insect Mol. Biol. **9:** 661–673.

Simon, C., F. Frati, A. Beckenbach, B. Crespi, H. Liu *et al.*, 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.

Swofford, D. L., 1998 *PAUP*—Phylogenetic Analysis Using Parsimony (* and Other Methods)*. Version 4. Sinauer Associates, Sunderland, MA.

Tamura, K., and M. Nei, 1993 Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. **10:** 512–526.

Turelli, M., A. A. Hoffmann and S. W. McKechnie, 1992 Dynamics of cytoplasmic incompatibility and mtDNA variation in natural *Drosophila simulans* populations. Genetics **132:** 713–723.

Vogler, A. P., and R. DeSalle, 1993 Phylogeographic patterns in coastal North American tiger beetles, *Cicindela dorsalis*, inferred from mitochondrial DNA sequences. Evolution **47:** 1192–1202.

Vogler, A. P., R. DeSalle, T. Assmann, C. B. Knisley and T. D. Schultz, 1993 Molecular population genetics of the endangered tiger beetle *Cicindela dorsalis* (Coleoptera: Cicindelidae). Ann. Entomol. Soc. Am. **86:** 142–152.

Weir, B. S., and C. C. Cockerham, 1984 Estimating F-statistics for the analysis of population structure. Evolution **38:** 1358–1370.

Werren, J. H., G. D. D. Hurst, W. Zhang, J. A. J. Breeuwer, R. Stouthamer *et al.*, 1994 Rickettsial relative associated with male killing in the ladybird beetle (*Adalia bipunctata*). J. Bacteriol. **176:** 388–394.

Yang, Z., 1996 Among-site variation and its impact on phylogenetic analyses. Trends Ecol. Evol. **11:** 367–371.

Yang, Z., N. Goldman and A. Friday, 1994 Comparison of models for nucleotide substitution used in maximum-likelihood phylogenetic estimation. Mol. Biol. Evol. **11:** 316–324.

Zakharov, I. A., I. I. Goryacheva, E. V. Shaikevich, J. H. G. v. d. Schulenburg and M. E. N. Majerus, 2000 *Wolbachia*, a new bacteria causing sex ratio bias in the two-spot ladybird *Adalia bipunctata* L. Russ. J. Genet. **36:** 385–388.

Zakharov, I. A., G. D. D. Hurst, N. E. Chernysheva and M. E. N. Majerus, 1996 Maternally inherited bacterium causing female bias in the St. Petersburg population of *Adalia bipunctata* does not belong to the genus *Rickettsia*. Russ. J. Genet. **32:** 1303–1306.

Zhang, D. X., and G. M. Hewitt, 1996 Nuclear integrations—challenges for mitochondrial DNA markers. Trends Ecol. Evol. **11:** 247–251.

Zhang, D. X., and G. M. Hewitt, 1997 Insect mitochondrial control region: a review of its structure, evolution and usefulness in evolutionary studies. Biochem. Syst. Ecol. **25:** 99–120.

Communicating editor: D. Charlesworth