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Comparative study of mtDNA in species of the genus *Adalia* (Coleoptera: Cocinellidae) and origin of ancient mitochondrial haplotypes in the gene pool of *Adalia bipunctata*

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Abstract. Fifteen different mitochondrial haplotypes of the mtDNA gene *COI* encoding cytochrome C oxidase subunit I were identified in the 127 individuals of *Adalia bipunctata* studied. Two mitochondrial haplotypes, H9 and H10, differed greatly from the others. The mitochondrial polymorphism in *A. bipunctata* is ancient, though its age remains to be evaluated. It is shown that mitochondrial haplotypes H9 and H10 and others coexisted in the original population of *A. bipunctata* before it spread throughout Eurasia from Western Europe to the Baikal Area, and before the differentiation of the subspecies *A. bipunctata fasciatopunctata*, which differs from the European form in its elytral pattern. In order to evaluate the possible origin of the ancient mitochondrial haplotypes in the gene pool of *A. bipunctata* sequences of the mtDNA gene *COI* and of the rRNA second internal transcribed spacer of the four species of *Adalia*: *A. bipunctata*, *A. decempunctata*, *A. frigida* and *A. tetraspilota*, were compared. It is suggested that infection with *Rickettsia* had an important role in the preservation of the mitochondrial haplotypes H9 and H10 during the evolution of *Adalia*.

INTRODUCTION

The ladybirds of the genus *Adalia* are a popular object of research in ecological and population genetics. The elytral and pronotum patterns of the species in this genus are very variable. The analyses of the variation in these morphological traits were mainly carried out on species of ladybird belonging to the genus *Adalia* (Lus, 1928; Majerus, 1994). As shown by genetic studies, the forms of *A. bipunctata* found in nature are not only phenotypically but also genotypically distinct; each colour phenotype corresponds to a particular genotype (Lus, 1928, 1932; Majerus, 1994). The first report of molecular polymorphism in *A. bipunctata* is that of Schulenburg et al. (2002). It is known that at least two species (*A. bipunctata* and *A. decempunctata*) are infected with symbiotic bacteria (Hurst et al., 1999; Von der Schulenburg et al., 2001). Under the term symbiosis are included any type of persistent biological interaction (ie mutualistic, commensalistic or parasitic). In ladybirds these bacteria kill male progeny. Since these male-killing bacteria are transferred transovarially bacterial infection is correlated with host mitochondrial haplotypes (Schulenburg et al., 2002). Studies of the mitochondrial polymorphism in populations of the two-spot ladybird beetle, *A. bipunctata*, by Schulenburg et al. (2002) revealed two peculiar mitochondrial haplotypes. These mitochondrial haplotypes, H9 and H10, were considerably different from the other. An attempt to evaluate the time of divergence of the mitochondrial haplotype H10 by Jiggins & Tinsley (2005) gave an age of 2–2.5 million years. Consequently, this mitochondrial polymorphism is referred to as ancient.

Which taxon of the genus *Adalia* was the source of the mitochondrial haplotypes H9 and H10? There are three species that are quite similar to *A. bipunctata* L.: *A. decempunctata* L., *A. tetraspilota* Hope and *A. frigida* Schneid. (Lusis, 1973). *A. decempunctata* occurs throughout the temperate zones in Europe and coexists there with *A. bipunctata*. These species can mate, but the eggs do not develop (Lusis, 1973). *A. tetraspilota* is an Indian species, which is found in the southern part of the former Soviet Union republics in Middle Asia. For example, in Tashkent (Uzbekistan) Lusis reports that these two species coexist, which we can confirm based on our observations. Lusis states that these two species are reproductively isolated (Lusis, 1973). Finally, *A. frigida* inhabits the northern part of Eurasia, from Scandinavia to Yakutia. Based on our observations, *A. bipunctata* and *A. frigida* coexist in Arkhangelsk. *A. frigida* occurring in North America (Wingo, 1952; Smith, 1953) and Russia (Lusis, 1973) has been studied. It is not clear to what extent these species are reproductively isolated.

In this work we compared the DNA sequences of the mtDNA gene *COI* and the rRNA second internal transcribed spacer (ITS2) of individuals of *A. bipunctata* with mitochondrial haplotypes H9 and H10 and also sequences common in this species with homologous DNA sequences from other species of the genus *Adalia*. We aimed to evaluate the possible origin of these ancient mitochondrial haplotypes in the gene pool of *Adalia bipunctata* and to analyze the role of male-killing bacteria in their spread.

TABLE 1. Estimates of the average evolutionary divergence recorded between sequence pairs in different species of *Adalia*.

	*A. bipunctata**	*A. frigida*	*A. tetraspilota*	*A. decempunctata*	H9**	H10***
*A. bipunctata**						
A. frigida	0.043					
A. tetraspilota	0.062	0.066				
A. decempunctata	0.120	0.138	0.129			
H9**	0.043	0.023	0.067	0.136		
H10***	0.067	0.070	0.069	0.135	0.067	

* *A. bipunctata* (includes all mitochondrial haplotypes except H9, H10); ** *A. bipunctata* mitochondrial haplotype H9; *** *A. bipunctata* mitochondrial haplotype H10.

MATERIAL AND METHODS

The DNA was isolated from adult individuals of the following species of *Adalia*: *A. bipunctata* (collected in Russia: in St. Petersburg, June 2009; Arkhangelsk, August 2010; Kem, August, 2010; in the eastern part of Russia in Ulan-Ude, Baikal Area, September 2010 and also in Uzbekistan, Tashkent, June 2011); *A. decempunctata* (collected in Sweden, Stockholm, Kungens Kurva area, 2001); *A. frigida* (collected in Arkhangelsk, August 2005); *A. tetraspilota* (collected in Uzbekistan, Tashkent, June 2011).

DNA was isolated from the ladybirds using the DIAtom™ DNA Prep Kit (Isogen, Moscow, Russia). Polymerase chain reaction (PCR) was run in thermocyclers GeneAmp^R PCR System 2700 (Applied Biosystems, Foster City, CA, USA) with amplification kits GenePak™ PCR Core (Isogene, Moscow, Russia), 1 mkg of extracted DNA and 5 pmol of each primer.

The mtDNA gene *COI* was amplified using primers LCO and HCO (Folmer et al., 1994). PCR conditions were: step 1, 94°, 1 min; step 2 with 5 cycles (94°C, 1 min, 45°C, 1.5 min, 72°C, 1.5 min); step 3 with 35 cycles (94°C, 1 min, 50°C, 1.5 min, 72°C, 1 min); final synthesis at 72°C, 5 min.

The primers C1-j-1951 and C1-N-2618 (Schulenburg et al., 2002) were used to amplify the most variable region in the middle part of the gene *COI*. The ITS2 region was amplified using the primers complementary to the 5,8S and 28S rRNA (Porter & Collins, 1991). PCR conditions for these two primers sets were: heating for 5 min at 94°C; then 35 cycles: denaturing at 94°C, 30 s, annealing at 55°C, 40 s, synthesis at 72°C, 40 s; final synthesis at 72°C, 10 min.

After electrophoresis on a 1% agarose gel (Sigma, St. Louis, MO, USA), the amplified DNA fragments were isolated from the gel using the JetQuiCk Gel Extraction Spin Kit (Genomed, Löhne, Germany) and then sequenced using both primers on an ABI 310 automated sequencer using the ABI PRISM BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). The newly identified sequences were submitted to GenBank, accession numbers of the gene *COI* JQ757048–JQ757053 and of the ITS2 JX459794–JX459830.

The sequences obtained were analyzed using software Chromas (http://www.technelysium.com.au) and the phylogenetic analysis in MEGA4 (Tamura et al., 2007). The evolutionary distances for *COI* were computed using five methods: (1) the p-distance method; (2) the Jukes-Cantor method commonly used to evaluate evolution of protein molecules; (3) the Tamura-Nei method commonly used to estimate the number of nucleotide substitutions in the control region of mitochondrial DNA; (4) the Kimura 2-parameter method, which is a simple way of estimating the evolutionary rate of base substitutions by comparing nucleotide sequences; and (5) the Tajima-Nei method in MEGA4 developed for estimating the evolutionary distance between nucleotide sequences (Tamura et al., 2007).

The values of divergence for the sequences of the *COI* gene obtained using all the above methods were practically identical (not shown). Table 1 presents the data obtained using the p-distance method. This method corresponds to the method used to evaluate the criteria for intra- and inter-species sequence divergence of mitochondrial genes in animals (Kartavtsev, 2011). The number of base differences per site averaged over all *COI* sequence pairs between groups is shown in Table 1. All results are based on the pairwise analysis of 20 sequences of *COI*. Among them 15 different mitochondrial haplotypes of *A. bipunctata* were studied. Mitochondrial haplotypes of *A. decempunctata* and *A. tetraspilota* were identified using two individuals of each species. Since we had the progeny of one individual of *A. frigida* the mtDNA of only one individual of this species (mother) was studied. Analyses were conducted in MEGA4 using the Maximum Composite Likelihood model. Codon positions included were 1st + 2nd + 3rd. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 970 positions of the *COI* gene in the final dataset. The analysis of nucleotide polymorphism from aligned DNA sequences of both *COI* and ITS2 data was done using the DNA Sequence Polymorphism (DnaSP) software (Librado & Rozas, 2009).

Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). The bootstrap consensus trees inferred using the Neighbour-Joining method was taken to represent the evolutionary history of *Adalia* based on *COI* and ITS2. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are expressed in terms of the number of base substitutions per site. Codon positions included were 1st + 2nd + 3rd. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option).

RESULTS

Mitochondrial DNA

We compared the sequences of the mtDNA gene *COI* of four species of *Adalia: A. bipunctata, A. decempunctata, A. frigida* and *A. tetraspilota*. Two *COI* gene fragments were analyzed: the 5'-terminal Barcode fragment, traditionally used in comparative analysis of species and the middle fragment, which is considered to be the most variable (Schulenburg et al., 2002). The size of the PCR product was about 700 bp in both cases. The amplified fragments were sequenced. The primers were selected to produce overlapping amplified sequences, thus allowing

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                                111111 1111111111 1222222222 2222222333 3333333333 3333333444 4444444444 4445555555]
[                 12333455 5666022445 5566678889 9023344456 6677889000 0111233335 5667789011 2334455578 9990011122]
[                3907349812 7036209140 3725670395 8181836752 4536284036 9289403691 7162879814 9584556976 2581703625]
#A.bipunctata H1   ATCAATTCAC TATCTACATA CCATTAGTGC ATATTCTTTG AAGATAATAT TTTAATGTTA TTCTTTATTT ATGTCCATTA TAATAAATAA
#A.bipunctata H2   .......... .......... .......... .......... .......... .......... .......... .......... ..........
#A.bipunctata H3   .......... .......... .......... .......... .......... .......... .......... .......... ..........
#A.bipunctata H4   .......... .......... .......... .......... .......... .......... .......... .........G ..........
#A.bipunctata H7   .......... .......... .......... .......... .......... .......... .......... .......... ..........
#A.bipunctata H11  .......... .......... .......... .......... .......... .......... .......... .......... ..........
#A.bipunctata H12  .......... .......... .......... .......... .......... .......... .......... .......... ..........
#A.bipunctata H13  .......... .......... .......... .......... .......... .......... .......... ....T..... ..........
#A.bipunctata H14  .......... .......... .......... .......... .......... .......... .......... .......... A.........
#A.bipunctata H15  .......... .......... .......... .......... .......... .......... .......... .........G ..........
#A.bipunctata H16  .......... .......... .......... .......... .......... .......... .......... .......... ..........
#A.bipunctata H17  .......... .......... .......... .......... .......... .......... .......... .......... ..........
#A.bipunctata H18  .......... .......... .......... .......... .......... .......... .......... .......... ..........
#A.bipunctata H10  .AT.GCAT.T .......G.. .T.GCGA.AT G..C.T.C.. ..A..G.CC. .....C...G .C.C...... .AT.T..... .T....GC..
#A.bipunctata H9   .AT...CT.. C.....AG.. .....GA.A. ...C....C. ..A......C ..C...... .......... ..ACT..... ....TGG...
#A.frigida 1       .A....CTG. C.....AG.. .....GA.A. ........C. ..AG.....C ..C...A... .......C.. T..CT..... ....T.G...
#A.tetraspilota 1  GA...CAT.. ...T.G.G.. TT.A..A... .C.CCTA... ..AG.G.C.C ...C..A... ...C...... ..TC....C. .T.A......
#A.tetraspilota 2  GA...CAT.. ...T.G.G.. TT.A..A.A. .C.CCTA... ..AG.G.C.C ...C..A... ...C...... ..TC....C. .T.A......
#A.decempunctata 1 TC.G..AT.T .TC.C...AT TTT...TA.T ..G..T...A TGAGATTCC. CA.TC.AAGT CCTACCT.CA ..T.T.CAA. .TT.T...GG
#A.decempunctata 2 TC.G..AT.T .TC.C...AT TTT...TA.T ..G..T...A TGAGATTCC. CA.TC.AAGT CCTACCT.CA ..T.T.CAA. .TT.T...GG

[                5555555555 5555666666 6666667777 7777777777 7777788888 8888888888 8889999999 9999999999 ]     1111]
[                3334667788 8999011233 5667790122 2234556667 7889900112 2334555688 9990011233 4445556667 ]   880456]
[                4789476925 8147628706 7395802403 6985060251 7395817092 5146258758 1273658106 5681243690 ]   897524]
#A.bipunctata H1   TATTAACTTA AGTACCTTTA AAATTAGTAC ATTTTTCAAT ATATTACCAT CTGTAAACAA ACTTATTTAC TTATCATGGC     VIMNTI
#A.bipunctata H2   .......... .......... .......... .......... .......... .......... ........G. ..........     ......
#A.bipunctata H3   .......... .......... .......... ..C....... .......... .......... .......... ..........     ......
#A.bipunctata H4   .......... .......... .......... .......... .......... .......... .......... ..........     ......
#A.bipunctata H7   .......... .......... ......A... .......... .......... .......... .......... ..........     ......
#A.bipunctata H11  ........G. .......... .......... .......... .......... .......... .......... ..........     ......
#A.bipunctata H12  .......... ...G...... .......... ..C....... .......... .......... .......... ..........     ......
#A.bipunctata H13  ........G. .......... ......A... ..C....... .......... .......... .......... ..........     ....M.
#A.bipunctata H14  .......... .......... .......... .......... .......... .......... .......... ..........     ......
#A.bipunctata H15  ........G. .......... .......... .......... .......... .......... .......... .....G....     ......
#A.bipunctata H16  .......... .......... .......... ..C....... .......... .......... .......... ........T.     ......
#A.bipunctata H17  .......... .......... .......... .......... .......... .......... .......... .....G....     ......
#A.bipunctata H18  .......... .......... .......... .......... .......A.. .......... .......... ..........     ......
#A.bipunctata H10  ..CC.GTC.. ..C......G ......A..T ....C.T..C .CG.CGTT.. ..A.GGT... ...C..CCGT ........T.     ...K..
#A.bipunctata H9   ..C....... GA...T.... ......A.G. G....CT... ........G. ..AC.TT... ...C...... ......CAA.     ......
#A.frigida 1       ..C.G.T... .....T...G ..T...AC.. ......T... ........G. ..AC..T... C..C...... .......AA.     ......
#A.tetraspilota 1  .........G .....T.CC. G.....T..T ......T..A .C...G.TG. .CA...TT.T .T.C..C..T ...CTG.A..     ..L...
#A.tetraspilota 2  .........G .....T.CC. G.....T..T ......T..A .C...G.TG. .CA...TT.T .T.C..C..T ...CTG.A..     ..L...
#A.decempunctata 1 CG.CT..... GA..TTC.A. .GTCCCA.GT .GCCCA.TCA G..AA.TA.C TCA....TT. .TC.GC...T CCC..T..CT     IVL...
#A.decempunctata 2 CG.CT..... GA..TTC.A. .GT.CCA.GT .GCCCA.TCA G..AA.TA.C TCA....TT. .TC.GC...T CCC..T..CT     IVL...
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Fig. 1. Alignment of the different nucleotide and amino acid sites of the gene *COI*. H1–H18 – mitochondrial haplotypes of *A. bipunctata*.

us to combine the sequences for further analysis. The 970-bp combined fragment was analyzed.

Mitochondrial divergence between species

Of all the 20 mitochondrial haplotypes recorded in four species, 170 nucleotide sites were variable and 144 of them parsimony-informative. Five nucleotide substitutions, including three at the first nucleotide position, result in an amino acid substitution (Fig. 1). Methionine (M) common to all *A. bipunctata* and *A. frigida* studied is replaced by Leucine (L) in *A. decempunctata* and *A. tetraspilota*. The *A. decempunctata* studied differed from the other species at position 88: Isoleicine (I) is replaced by Valine (V) and V by L at position 89 (Fig. 1). The minimum evolutionary divergence (nucleotide variations per site) recorded between *A. bipunctata* and *A. frigida* was 4.3%. Maximum differences of about 13% were recorded between *A. decempunctata* and other species (Table 1).

Mitochondrial polymorphism within *A. bipunctata*

Fifteen different mitochondrial haplotypes were identified in the 127 individuals of *A. bipunctata* studied. The numbering of the mitochondrial haplotypes follows Schulenburg et al. (2002) and continues on with the newly identified (H11–H18) mitochondrial haplotypes. The main part of the sample, 56 (44%) individuals were of mitochondrial haplotype H1, 31 (24.4%) of H10, 20 (16%) of H7, 5 (4%) of H9, 3 (2.4%) of H3, 2 (1.6%) of H2, 2 (1.6%) of H11 and one individual (0.8%) each of H4, H12–H18 Among the sequences of the *COI* gene from *A. bipunctata* 85 sites were found to be variable with most of the sequences differing in from 1–4 nucleotides. There are 40 variable sites in mitochondrial haplotypes H9 and H10. Most of the nucleotide substitutions are synonymous. H13 differs from H1 by four nucleotide substitutions, one of which results in the substitution of the amino-acid Methionine (M) for Threonine (T), H10 differs from H1 by 60 nucleotide substitutions, resulting in the substitution of Lysine (K) for Asparagine (N). No other nucleotide substitution results in an amino acid substitution (Fig. 1). Most of the nucleotide sequences of the gene *COI* of *A. bipunctata* studied varied by less than 0.3%. Exceptions are the DNA sequences of *A. bipunctata*, common for the mitochondrial haplotypes H9 and H10, which differ from the other *A. bipunctata* haplotypes by 4.3 and 6.7%, respectively.

Fig. 2. Map of distribution of the species of *Adalia* and of known mtDNA haplotypes of *A. bipunctata.* Circles denote *A. bipunctata* and the coloured sectors in the pie diagrams correspond to mitochondrial haplotypes H1 (in H1 we include H1 and all other mitochondrial haplotypes, except H9 and H10), H9 and H10. Haplotypes recorded for Moscow (Russia) and Bielefeld (Germany) (Schulenburg et al., 2002) and Cambridge (England) (Schulenburg et al., 2002; Jiggins & Tinsley, 2005). Triangles mark the sites where *A. frigida* was collected, squares – *A. tetraspilota*, diamonds – *A. decempunctata.*

Mitochondrial haplotype H9

The mitochondrial haplotype H9, reported in populations of *A. bipunctata* in the northern part of Europe (Schulenburg et al., 2002; Jiggins & Tinsley, 2005), was found by us in St. Petersburg and Arkhangelsk (Fig. 2). This mitochondrial haplotype differs from the groups "common" for *A. bipunctata* (all *A. bipunctata* studied except those with mytotypes H9 and H10) by 4.3%; this divergence is due to synonymous sites. H9 differs from *A. frigida*, *A. tetraspilota* and *A. decempunctata* mitochondrial haplotypes by 2.3%, 6.7% and 13.6%, respectively. As shown by DNA sequence polymorphism analysis comparing H9 and other mitochondrial haplotypes of the gene *COI* fragment studied, 22, 40–48, 62 and 117 sites differed between H9 and, respectively, *A. frigida*, *A. bipunctata*, *A. tetraspilota* and *A. decempunctata*. Minimum average number of nucleotide differences (Kt) and, consequently, minimum nucleotide diversity (PiT) were recorded between mitochondrial haplotype H9 and common mitochondrial haplotypes of *A. bipunctata* and between H9 and *A. frigida*. The differences between H9 and *A. tetraspilota* are about 4 times greater and *A. decempunctata* about 7 times greater (Table 2).

Mitochondrial haplotype H10

The mitochondrial haplotype H10 was present in populations in the European part of Russia, St. Petersburg, Arkhangelsk and Kem, and also in the eastern part of Russia in Ulan-Ude, Baikal Area (Fig. 2). The DNA sequence polymorphism analysis comparing H10 and other related sequences of the *COI* gene revealed 64 segregating sites for H10 compared with *A. frigida*, 66–68 with *A. bipunctata*, 64 with *A. tetraspilota* and 116 with *A. decempunctata*. The sequences of the type H10 differ from the group of mitochondrial haplotypes common for *A. bipunctata* by almost 7%, by the same percentage from *A. frigida* and *A. tetraspilota*, and by 13.5% from *A. decempunctata.* Evolutionary divergence between H10 and *A. bipunctata* is more, than between *A. bipunctata* and *A. frigida* and between *A. bipunctata* and *A. tetraspilota* (Table 1). The Kt and PiT values of the mitochondrial haplotype H10 and those common to *A.bipunctata* are two times lower than those of *A. frigida* and *A. tetraspilota*, and more than 3 times lower than those of *A. decempunctata* (Table 2).

The mitochondrial haplotype H10 is equally genetically distant from *A. bipunctata*, *A. frigida* and *A. tetraspilota*, and more distant from *A. decempunctata*. The mitochondrial haplotype H9 is genetically close to *A. frigida* and *A. bipunctata*, even closer than H10 to *A. bipunctata*. Genetic distances for H9 and either *A. tetraspilota* or *A. decempunctata* are practically the same as for H10 (Table 1, Fig. 3). It should be noted that the differences for H9 and H10 were similar for all species with the exception of

TABLE 2. Polymorphism in the sequences of mitochondrial haplotypes H9 and H10 compared with that in other mitochondrial haplotypes.

	mitochondrial haplotype H9		mitochondrial haplotype H10	
	Kt	Pit	Kt	Pit
A. bipunctata (-H9, H10)	11.876	0.012	19.333	0.019
A. frigida	14.667	0.015	42.667	0.044
A. tetraspilota	41.167	0.042	42.500	0.044
A. decempunctata	77.833	0.080	77.167	0.079

Kt – average number of nucleotide differences, PiT – nucleotide diversity (DnaSP v5. Librado & Rozas, 2009).

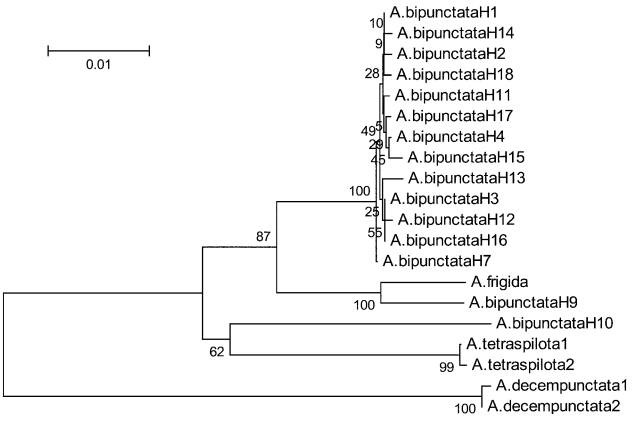

Fig. 3. Phylogeny based on sequences of the *COI* gene. Evolutionary relationships based on 20 sequences of species of *Adalia* inferred using the Neighbour-Joining method. H1–H18 – different mitochondrial haplotypes of *A. bipunctata*. The optimal tree with the sum of branch lengths = 0.17308113 is shown (967 positions in the final dataset).

A. frigida, which showed about a 3 times greater similarity with H9 than H10.

ITS2 in the ribosomal gene cluster

As a control, we studied the region of the second transcribed spacer (ITS2) in the ribosomal gene cluster of *Adalia*. The amplified fragment spanned 900 bp. The following individuals were studied: 11 *A. bipunctata* of mitochondrial haplotype H1, 4 of H9, 8 of H10 and 3 *A. frigida*, 8 *A. tetraspilota* and 1 *A. decempunctata*. Unlike mtDNA, the fragment of ITS2 studied was absolutely conserved within a species. The sequences of the ITS2 of all the *A. bipunctata* studied, independent of their mitochondrial haplotypes, were practically identical. The average number of base substitutions per site based on the analysis of 25 *A. bipunctata* sequences (within species DNA polymorphism) is 0.001. The ITS2 in other species is also monomorphic (Fig. 4). Divergences recorded between species of *Adalia* are: 1.8% between *A. bipunctata* and *A. frigida*, 41.2% between *A. bipunctata* and *A. decempunctata*, 57.8% between *A. bipunctata* and *A. tetraspilota*, 41.6% between *A. decempunctata* and *A. frigida*; *A. tetraspilota* differs from *A. frigida* and *A. decempunctata* by 58.5% and 57.7%, respectively. The intergroup genetic diversity, Gst, between the four species of *Adalia* studied is almost four times lower for mtDNA (Gst: 0.259) than for ITS2 rRNA (Gst: 0.824), but the Haplotype diversity, Hd of mtDNA (Hd 0.988) is higher than that of ITS2 (Hd 0.595).

DISCUSSION

Earlier (Shaikevich et al., 2012) we studied mitochondrial polymorphism in populations of *A. bipunctata* in relation to their infection with symbiotic bacteria. In this work we compared the variants of *A. bipunctata* mtDNA with that recorded in three closely related species: *A. decempunctata*, *A. frigida* and *A. tetraspilota.* All four of the species studied are also characterized by a nuclear

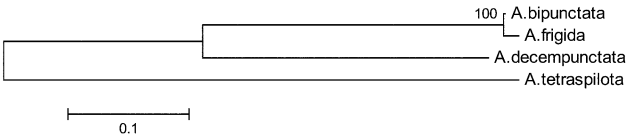

Fig. 4. Evolutionary relationships based on sequences of ITS2. Evolutionary relationships of 4 species of *Adalia*. The evolutionary history was inferred using the Neighbour-Joining method. The optimal tree with the sum of branch lengths = 1.08817898 is shown (673 positions in the final dataset).

marker, ITS2 rRNA. This is the first molecular genetics study of *A. frigida* and *A. tetraspilota*.

Among the four species of *Adalia* studied the most closely related are *A. bipunctata* and *A. frigida*. The phylogenetic relationships of these species have not been previously studied. Our study has revealed a 4.3% divergence in *COI* and 1.8% in ITS2 between *A. bipunctata* and *A. frigida*. These differences are greater than the values of evolutionary divergence common for subspecies. Independent of the mitochondrial haplotype the ITS2 rRNA sequence of *A. bipunctata* is not polymorphic. ITS2 sequences of *A. frigida* are different from those of *A. bipunctata* and each are monomorphic. Variations in mtDNA, rRNA and the data in the literature on *A. frigida* (Lusis, 1976) allow us to suggest that *A. frigida* is a separate species. However, this species needs further evaluation, and the individuals from geographically remote habitats should be studied.

Based on the gene *COI* mtDNA, *A. decempunctata* is the most distant species, whereas based on nucleotide composition of ITS2 *A. tetraspilota* is evolutionarily most distant from the other species studied. Mutation rate is higher in the rRNA spacer, but the absence of recombination results in a better preservation of rare mutations in the mitochondrial genome; consequently, there is greater diversity of mtDNA haplotypes in species of *Adalia* and in the related intergroup genetic diversity in rRNA.

Schulenburg et al. (2002) report 10 variable mitochondrial haplotypes in *A. bipunctata*. We found 8 more new mitochondrial haplotypes based on the polymorphism recorded in mtDNA of individuals of *A. bipunctata* from various populations. The most frequent mitochondrial haplotype of the gene *COI* in *A. bipunctata* was named H1. Fifteen other mitochondrial haplotypes differed from H1 by 1–4 substitutions in the most variable 532-bp fragment from the middle part of the gene. Two more mitochondrial haplotypes are also notably different from these 16: H9 and H10. These mitochondrial haplotypes were recorded in populations of *A. bipunctata* from practically throughout the whole area of the distribution of this species (Fig. 2).

Jiggins & Tinsley (2005) estimated the divergence time of the mitochondrial haplotype H10 and other mitochondrial haplotypes as between 2.1–2.5 million years ago. If we assume that the frequency of mutation in the mtDNA of *Adalia* is equal to that of *Drosophila*, 6.2×10^{-8} (Haag-Liautard et al., 2008), then assuming one generation per year the divergence time is 1.13 million years. For an

average of 1.5 generations per year the divergence time is 750 thousand years.

We attempted to estimate the divergence between the mitochondrial haplotypes H1, H9, and H10 by comparing 571-bp sequences of the 5' part of the *COI* gene. These sequences are considered standard and are widely used for genome barcoding. The difference between mitochondrial haplotypes H1 and H10 was 7.00%, and between H1 and H9 4.55%. As shown by Kartavtsev (2011), based on a meta-analysis of the barcoding of thousands of species of animal, insects included, mean intra-populational variation in p-distances (%) is 0.89, mean variation between subspecies and twin species is 3.78 and between morphologically distinct species of one genus is 11.06. Therefore, the difference between the mitochondrial haplotypes H1 and H10 are close to the level of variation for "distinct" species. Comparison of longer, 970 bp, sequences of the *COI* gene resulted in an evolutionary divergence of 4.3% between the H9 and the other mitochondrial haplotypes, and 6.7% between H10 and other mitochondrial haplotypes of *A. bipunctata*. This confirms the above conclusions.

It is important to show that the individuals with mitochondrial haplotypes H9 and H10 and other mitochondrial haplotypes occur in the same population. Jiggins & Tinsley (2005) compared samples of individuals carrying mitochondrial haplotypes H10 and H7 for the alleles of the nuclear gene *g6pd*. In this work we compared the sequences of the second internal transcribed spacer of the ribosomal locus from samples of individuals with mitochondrial haplotypes H9, H10 and H1. Among *A. bipunctata*, independent of the mitochondrial haplotype, no polymorphism was found in the ITS2 rRNA sequence. Both studies revealed no difference between individuals with different mitochondrial haplotypes.

The oldest age for coexistence of mitochondrial haplotypes H1 and H10 in the *A. bipunctata* gene pool is confirmed by the similarity in the mtDNA polymorphism recorded in European populations and in those from Ulan-Ude, Baikal Area (Fig. 2). However, the population in the Baikal area belongs to a separate subspecies described by Lusis as *A. bipunctata fasciatopunctata* Fald. (Lusis, 1973). Consequently, mitochondrial haplotypes H10 and other mitochondrial haplotypes must have coexisted in the original population of *A. bipunctata* before it spread through Eurasia, from western Europe to the Baikal Area and the differentiation of the subspecies *A. bipunctata fasciatopunctata*, which differs from the European form in its elytral pattern. Therefore, the mitochondrial polymorphism in *A. bipunctata* is in fact ancient, though its precise age remains to be evaluated.

The similarity between the sequence of mitochondrial haplotype H9 and the respective DNA sequence of *A. frigida* is notable (Fig. 3). Probably, mitochondrial haplotype H9 originated from an ancestral species of *A. frigida*. It is known that *A. frigida* inhabits a vast area extending from Scandinavia to Yakutia. We had only one female (and her progeny) of this species from Arkhangelsk. Probably, a mitochondrial haplotype close to H9 is still present in the *A. frigida* gene pool. Thus, it appears promising to study the mtDNA of individuals of *A. frigida* collected from various geographical locations. The mitochondrial haplotype H10 is very distinct from the common haplotypes of *A. bipunctata* and the sequences of other species (Fig. 3). That it is considerably different from the other haplotypes indicates that this haplotype was incorporated into the *A. bipunctata* gene pool as a result of interspecies hybridization. Studies of the extant species of the genus *Adalia* failed to find the ancestral species for the H10 mitotype. Probably, the species from which *A. bipunctata* got mitochondrial haplotype H10 is now extinct.

In order to understand the factors determining the preservation of the mitochondrial haplotypes H9 and H10 in the *A. bipunctata* gene pool, it is important to take into consideration that only individuals carrying mitochondrial haplotypes H9 and H10 are mostly infected with the cytoplasmic symbiotic bacterium *Rickettsia AB* (Schulenburg et al., 2002; Jiggins & Tinsley, 2005; Shaikevich et al., 2012), which kills ladybird males at an early stage in their embryonic development. We found no individuals of *A. bipunctata* carrying the other mitochondrial haplotypes infected with *Rickettsia*, however other studies report cases of infection of individuals with the mitochondrial haplotype H7 (Schulenburg et al., 2002; Jiggins & Tinsley, 2005). Infected females produce all-female progeny, however, as pointed out long ago by Lus (1947) they have an advantage over non-infected females: the emerging larvae consume non-developing eggs with male embryos and thus obtain sufficient food to complete their initial stages of development. Cytoplasmic bacteria, like mitochondria, are transferred to the progeny transovarially, i.e. through the female line, demonstrating a peculiar effect of linkage disequilibrium with mtDNA. Therefore, the females that produce non-male progeny have a biological advantage, which might account for the continued survival of infected lines and maintenance of the mitochondrial haplotypes "linked" with the infection in the population.

The following scenario may be suggested for the origin of the modern *A. bipunctata* gene pool. Some time ago three similar species coexisted: *A. bipunctata*, *Adalia Y* and *Adalia Z*, the two latter carrying mtDNA of type H9 and H10, respectively. The individuals of *Adalia Y* and *Adalia Z* were infected with *Rickettsia*, which resulted in a sharp decrease in the number of males in these species. The lack of males favoured those *Adalia Y* and *Adalia Z* females that mated with males of *A. bipunctata*. The hybrid females were again inseminated by males of *A. bipunctata*. Then the *Adalia Y* and *Adalia Z* species became extinct and their mtDNA was incorporated into the gene pool of *A. bipunctata*. In the extension of the range of *A. bipunctata* in Eurasia and its differentiation into subspecies, the mitochondrial haplotypes H9 and H10, linked with *Rickettsia*, were preserved in most populations of *A. bipunctata*.

A situation similar to what we describe here for *Adalia* is recorded for *Drosophila*. In populations of *D. quinaria*

there is a peculiar mtDNA variant that differs from the standard at the species-level. Comparison of this mtDNA variant with the mtDNA of known related *Drosophila* species failed to find the donor species for this haplotype. The flies carrying the peculiar haplotype are infected with the symbiotic bacterium *Wolbachia*. The authors (Dyer et al., 2011) suggest that the mitochondrial haplotype was introduced into the gene pool by interspecies hybridization with a now extinct species and preserved in the gene pool of *D. quinaria* together with symbiotic bacteria. Wide occurrence of symbiotic bacterial infection in insects suggests that other similar cases will be reported.

Two mitochondrial haplotypes identified earlier in a geographically isolated population of *Coccinella septempunctata* in Japan, show a 4% difference from the common haplotypes (Marin et al., 2010). Symbiotic bacteria have not been found in European populations of *C. septempunctata* (M.E.N. Majerus, pers. commun.). However, it would be interesting to know if this is the case in Japan.

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