Spiroplasma infection in Harmonia axyridis - Diversity and multiple infection

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Abstract

The heritable endosymbiotic bacterium Spiroplasma is found in the harlequin ladybird Harmonia axyridis. The proportion of beetles infected with Spiroplasma in different native H. axyridis populations varies from 2% to 49%. We investigated the polymorphism of Spiroplasma strains in samples from individual beetles from Kyoto, Vladivostok, Troitsa Bay, Novosibirsk, and Gorno-Altaisk. To identify Spiroplasma strains, we analyzed nucleotide polymorphisms of the 16S rRNA gene and the ribosomal internal transcribed spacer (ITS1). The majority of infected beetles were infected with two or more Spiroplasma strains. We measured Spiroplasma density in beetles with different infection status using quantitative PCR. The abundance of Spiroplasma in samples with a single infection is an order of magnitude lower than in samples with multiple infections. Density dependent biological effects of Spiroplasma are discussed.

Introduction

Many insect species are infected with intracellular symbiotic bacteria, which are inherited maternally and affect host reproduction. Among such bacteria, Wolbachia, Spiroplasma, Rickettsia, and Cardinium are widely known. Spiroplasma is one of the most prevalent and well characterized facultative insect endosymbionts, and it is estimated to infect 5–10% of all insect species [1, 2]. This bacterium is an endosymbiont of some members of all the main insect orders: Coleoptera [3–8], Diptera [1, 9–15], Hemiptera [16–18], Homoptera [19–22], Lepidoptera [23, 24], and Odonata [25]. Spiroplasma is found in the midgut, filter chamber, malpighian tubules, hindgut, fat tissues, hemocytes, muscle, trachea, salivary glands, reproductive tissues and eggs [23, 26, 27]. Phylogenetic reconstruction of the genus Spiroplasma using the 16S rRNA gene sequences revealed that the genus comprises four distinct clades: the Ixodetis clade, the Citri-Chrysopica-Mirum clade, the Apis clade sensu lato, and the Mycoides-Entomoplasmaeae clade [28, 29].

In insects, the effects of symbiosis with Spiroplasma range from mutualistic to parasitic. In some insects, Spiroplasma determines male-killing at an embryogenesis stage [5, 8, 30], resulting in strongly female-biased offspring. The male-killing spiroplasmas are known in Drosophila [31, 32], ladybird beetles: Adalia bipunctata, Anisosticta novemdecimpunctata, Harmonia axyridis, Menochilus sexmaculatus [3, 5, 6, 8, 33]; in lepidopterans: Danaus chrysippus, Ostrinia...
The authors have declared that no competing interests exist.

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Ethical approval is not required for this study because we use only DNA preparations and do not experiment in any way with living insects.

**Amplification**

All DNA samples from beetles were tested before use. To validate the prepared templates, the mitochondrial cytochrome oxidase 1 (COI) gene region of *H. axyridis* was amplified by PCR primers LCO1490 and HCO2198 [43]. To detect *Spiroplasma* infection and to study *Spiroplasma* diversity, we used *Spiroplasma*-specific primers that amplify the 301-bp DNA fragment including ITS1 complete sequence [6], and *Spiroplasma*-specific primers that amplify the 1028 bp fragment of 16S rRNA gene [18] (Table 1). The amplification reaction was conducted under the following conditions: initial denaturation (one cycle 4 min at 95˚C), followed by 38 cycles that include denaturation (30 s at 95˚C), annealing (40 s at 59˚C for ITS1 and 40 s at 53˚C for 16S rRNA), and polymerization 40 s at 72˚C. This was followed by a cycle of final polymerization (5 min at 72˚C). For a positive control, we used a DNA sample of *H. axyridis*, infected by *Spiroplasma* from the DNA collection at the Vavilov Institute of General Genetics RAS. For a negative control, we used DNA samples from uninfected *H. axyridis* imago from the same collection.

**qPCR**

Using a NanoDrop8000 (Termo Scientific, Germany), DNA concentrations were measured. Samples were then diluted as necessary so that each were of the same DNA concentration for qPCR. Real-time qPCR reactions were carried out in the ANK-32 real-time PCR system (Synsyntol, Russia). Quantitative PCR was performed in 25-μL reactions containing Lightcycler 480 SYBR Green I (Invitrogen) and 0.5 μM of each of the primers. The following thermal cycling protocol was applied: 95˚C for 5 min followed by 35 cycles at 95˚C for 10 s, 60˚C for 10 s, then 72˚C for 30 sec. Three technical replicates per biological sample were performed for each set of primers, SP-ITS-JO4 and SP-ITS-N55 for the internal transcribed spacer (ITS1) of *Spiroplasma* and CPS-F and CPS-R for the carbamoyl phosphate synthetase (CPS) gene of *H. axyridis* (Table 1). Melting curves were examined to confirm the specificity of amplified products. Cycle threshold (Ct) values were obtained using the ANK-32 real-time PCR system at default threshold settings. The efficiency of each primer pair was predetermined in separate experiments using serial 10-fold dilutions of the DNA samples. The amplification efficiency of CFP (E = 1.94) is different from that of the *Spiroplasma* ITS1 sequence (E = 1.72). Therefore, qPCR from CFP was used to confirm *H. axyridis* total DNA quantity (Table 2). To estimate

| PCR fragment name                        | Fragment length (bp) | Primer name   | Primers sequence                          | Primers melting temperature (Tm) | Reference |
|------------------------------------------|----------------------|---------------|-------------------------------------------|---------------------------------|-----------|
| Fragments of the rRNA repeat of *Spiroplasma* sp |                      |               |                                           |                                 |           |
| ITS1                                     | 301                  | SP-ITS-JO4    | 5’-GCCAGAAGTCAGTTCCCTACCG-3’               | Tm = 59˚C                       | [6]       |
|                                           |                      | SP-ITS-N55    | 5’-ATCCCAAGCCCATCCACATACG-3’               | Tm = 59˚C                       |           |
| 16S rRNA                                 | 1028                 | spi_f1        | 5’-GGGTGGAACACGCTTCTCTC-3’                 | Tm = 53˚C                       | [17]      |
|                                           |                      | spi_r3        | 5’-CCCTCTCTCCTCCTTACACTA-3’               | Tm = 53˚C                       |           |
| Fragments of the nuclear gene of *H. axyridis* |                      |               |                                           |                                 |           |
| carbamoylphosphate synthetase (CPS)      | 711                  | CPS-F         | 5’-TGCCAGTAACATTTTCTCTC-3’                 | Tm = 60˚C                       | This paper |
|                                           |                      | CPS-R         | 5’-CCATCAGTTGGCTCTCACCA-3’                 | Tm = 59˚C                       |           |

https://doi.org/10.1371/journal.pone.0198190.t001
differences in Spiroplasma DNA quantities between samples, ratios of Spiroplasma DNA quantities between samples were calculated as: \( \text{No/Mo} = e^{\Delta Ct} \), where (No) is the initial concentration in sample N, (Mo) is the initial concentration in sample M, and \( \Delta Ct \) is the difference in the number of control cycles between samples N and M. The quantity of Spiroplasma ITS1 DNA in sample N19 was used as a proxy for Spiroplasma DNA titers in other samples. To determine the 95% confidence interval of the mean Ct, the one-sample Student t test was used. To compare relative quantifications of Spiroplasma DNA in samples with single and multiple infections, the Mann-Whitney test was used.

Electrophoresis, elution, cloning, and sequencing

PCR products were run on an 1.5% agarose gel, then extracted from the gel and purified with an elution kit (Zymoclean™ Gel DNA Recovery Kit, Zymo Research, USA), according to the manufacturer’s instructions. PCR product cloning was performed using the pGEM®-T Easy Vector System, according to standard protocols (Fermentas InsTAcClone™ PCR Cloning Kit). We use random sampling of the clones for sequencing. Sequencing of the amplification products was conducted with both primers on an ABI PRISM 3500 instrument using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, United States), according to the manufacturer’s instructions.

Multiple sequence alignment and phylogenetic analysis

Sequences were aligned using the Clustal W algorithm in the MEGA 4.00 program package [44]. As a reference sequence for the rRNA gene cluster, we used Spiroplasma sequence from the closely related \( H. axyridis \) species of ladybirds \( Anisosticta novemdecimpunctata \) (GenBank ID: AM087471). The Median-Joining network of the Spiroplasma rRNA genes from different strains was constructed in the PopART program [45] using the TCS algorithm [46].
Results

*Spiroplasma* ITS1 and the fragment of 16S rRNA gene nucleotide variability are presented in Tables 3 and 4. *Spiroplasma* sequences from females G9, K13, N19, and N1 showed no evidence of ambiguous nucleotide positions. This allowed us to conclude that these females were infected with a single *Spiroplasma* strain. The ITS1 sequences from G9 and K13 females were identical to the sequence of the *Spiroplasma* strain HARFUKU2 that was previously found in *H. axyridis* from Japan (GenBank ID: AB127933) [37]. The ITS1 sequences in samples N1 and N19 were previously recorded as the Sib1 strain (GenBank ID: KR363169) and Sib19 strain (GenBank ID: KR363170), respectively.

However, in most *Spiroplasma*-infected *H. axyridis* females from Gorno-Altaisk (G16), Birobidzhan (Bi16, Bi29), Troitsa Bay (T5, T21), Vladivostok (V37, V42), and Kyoto (K11, K15), some nucleotides were ambiguously read at phylogenetically informative sites of both ITS1 and in the fragment of 16S rRNA gene. Because the emergence of ambiguous sites could indicate multiple infections in females, we cloned PCR fragments and then a set of individual clones were sequenced. We cloned the ITS1 fragment with ambiguous sites of all samples and the 16S gene fragment of three samples: G16, V37 and Tr21.

*Spiroplasma* strain identification of individual clones is presented in Tables 5 and 6. Newly obtained sequences of the *Spiroplasma* 16S rRNA gene and ITS1 were deposited into GenBank (GenBank ID: KR363166-KR363168, MF543310-MF543312). Multiple infections with *Spiroplasma* strains were detected in *H. axyridis* females from five studied populations in both (western and eastern) parts of the native range. HARFUKU1 and HARFUKU2 *Spiroplasma* strains co-occur in most females with multiple infections. HARFUKU1 strain is absent only in one female from Vladivostok (V42, Table 5). Rare *Spiroplasma* strains were detected in multiply infected females from Birobidzhan, Vladivostok and Troitsa Bay. One beetle from Japan (K13) and one beetle from the Gorno-Altaisk (G9) are infected only by HARFUKU2. The sequences of the Sib19 strain and Bi22 strain found in females from Troitsa Bay and Birobidzhan were identical.

### Table 3. *Spiroplasma* ITS1 nucleotide variability. Nucleotides of phylogenetically informative polymorphic positions are indicated.

| Names of *H. axyridis* infected females | Names of *Spiroplasma* strains | Nucleotide positions ** |
|----------------------------------------|-------------------------------|------------------------|
|                                        | HARFUKU1*                     | T G A G T G A T C C T G C C |
|                                        | HARFUKU2*                     | C G A G T G A T C C T G C C |
|                                        | G9; K13                       | C A A T T |
|                                        | N19                           | C A A T T |
|                                        | N1                            | C A A T T |
|                                        | G16; Bi16, Bi29; T5, T21; V37, V42; K11, K15 | Y R M Y |

*according to [37]

** As a reference sequence we used ribosomal genes cluster of *Spiroplasma* from *A. novemdecimpunctata* (GenBank ID: AM087471). The letters indicate the collection site: Bi–Birobidzhan, G–Gorno-Altaisk, K–Kyoto, N–Novosibirsk, T–Troitsa Bay, V–Vladivostok. The characters indicate the sample numbers.

https://doi.org/10.1371/journal.pone.0198190.1003
Because HARFUKU1 and HARFUKU2 have the same length and differ only by few point nucleotide substitutions that do not alter the structure of the PCR fragments ends, we assume that possible differences in the amplification efficiency of these fragments in one reaction and possible differences in the cloning efficiency are negligible. Therefore, we approximate the quantitative relationship of Spiroplasma strains in the case of multiple infection from the number of plasmid clones of different types in a random sampling of clones (Tables 5 and 6). In a random sampling of clones, we found a total of 26 HARFUKU1 and 87 HARFUKU2 strains. Based on this data, we propose that the density of HARFUKU2 may be more than twice the density of HARFUKU1 in beetles with double infection. Analysis of the 16S rRNA gene polymorphism confirms the presence of multiple infections in females. Overall, we identified 45 clones of this fragment: 27 clones corresponding to the Ha-2 strain and 18 clones corresponding to the Ha-1 strain. One beetle from Japan (K13) and one beetle from the Altai (G9) are infected only by Ha-2 strain. Since it is known that the ITS1 sequence is physically linked to the 16S rRNA sequence in the ribosomal gene cluster and based on the infection of the beetles K13 and G9 with only one Spiroplasma strain it can be assumed that Ha2 16S rRNA fragment is physically linked to HARFUKU2 fragment of ITS1. Consequently Ha1 16S rRNA fragment is linked to HARFUKU1 fragment of ITS1. Quantitative data of the number of clones of different types supports this assumption. Obviously, the 3 sequence of the 16S rRNA fragment found in the V37 female, is linked with the Bi24 ITS1 strain and Ha4 and Ha5 sequences of the 16S rRNA fragment found in females from Novosibirsk infected with a single Spiroplasma strain, are linked with Sib1 and Sib19 fragments of ITS1 respectively.

Phylogenetic analysis

The results of the phylogenetic analysis of ITS1 and the 16S rRNA gene fragment of Spiroplasma from H. axyridis are presented in Figs 1 and 2. All identified Spiroplasma strains belong to the Ixodetis clade. The nucleotide divergence of the ITS1 fragment is π = 0.0091. There are 17 variable sites, and of these, there are 16 phylogenetically informative sites. The nucleotide divergence of the 16S rRNA fragment is π = 0.002. There are 19 variable sites, four of them are phylogenetically informative.

Survey for abundance of Spiroplasma in H. axyridis

The Spiroplasma relative densities were measured in 13 DNA samples from H. axyridis. The results are shown in Table 7. To verify the data we divided our sampling into two groups.

** https://doi.org/10.1371/journal.pone.0198190.t004

Table 4. Nucleotide variability of 16S rRNA fragments of Spiroplasma of H. axyridis. Nucleotides of phylogenetically informative polymorphic positions are indicated.

| Names of H. axyridis infected females | Nucleotide positions** |
|---------------------------------------|------------------------|
|                                       | 1 2 3 4 5 6 7 8 9 10 | 1 2 3 4 5 6 7 8 9 10 |
| Reference strain -Spiroplasma from A. novemdecimpunctata | G A G N A T C G G A T C C G G C G G |
| N19                                   | G G G G G G G G G G G |
| N11                                   | A A G G C T T T T C G T T A A |
| G9; K13; G17; A16; A16; B12; T15; T21; V36; V42; K11; K15 | R K K K K K K K K K |
|                                       | ** The numbers of the variable nucleotides are given by the sequence of the ribosomal genes cluster of A. novemdecimpunctata (GenBank ID: AM087471), which was used as a reference sequence.
The first group included four beetles (G9, G13, N1, N19) infected with only one *Spiroplasma* strain. The second group included beetles with multiple infections. Comparison of the groups based on the value of *Spiroplasma* relative densities was performed using the Mann-Whitney test. The value of Mann–Whitney U test (U-empirical = 0) is less than U-critical = 3 for p ≤ 0.01) confirms that this groups are different. We may conclude that the relative density of *Spiroplasma* in samples with a single infection is lower than in samples with multiple infections. All samples with high *Spiroplasma* density are infected simultaneously with strains HARFUKU1 and HARFUKU2. This is likely to be one of the necessary conditions to achieve high *Spiroplasma* density.

Table 5. ITS1 based identification of *Spiroplasma* strains from individual females of *H. axyridis*.

| Female number of *H. axyridis* | Strain name (number of clones in parenthesis) | GenBank ID: |
|-------------------------------|------------------------------------------------|-------------|
| **Western populations**       |                                                |             |
| G16                           | (1)–HARFUKU1                                  | AB127932    |
|                               | (5)–HARFUKU2                                  | AB127933    |
| G9                            | HARFUKU2                                      | AB127933    |
| N1                            | Sib1                                          | KR363169    |
| N19                           | Sib19                                         | KR363170    |
| **Eastern populations**       |                                                |             |
| Bi16                          | (2)–HARFUKU1                                  | AB127932    |
|                               | (14)–HARFUKU2                                 | AB127933    |
|                               | (2)–Bi24                                       | KR363168    |
|                               | (1)–Bi10                                       | KR363166    |
| Bi29                          | (3)–HARFUKU1                                  | AB127932    |
|                               | (11)–HARFUKU2                                 | AB127933    |
|                               | (1)–Bi24                                       | KR363168    |
|                               | (2)–Bi22                                       | KR363167    |
| V37                           | (7)–HARFUKU1                                  | AB127932    |
|                               | (6)–HARFUKU2                                  | AB127933    |
|                               | (1)–Bi24                                       | KR363168    |
| V42                           | (14)–HARFUKU2                                 | AB127933    |
|                               | (1)–Bi10                                       | KR363166    |
| T5                            | (6)–HARFUKU1                                  | AB127932    |
|                               | (6)–HARFUKU2                                  | AB127933    |
|                               | (4)–Bi22                                       | KR363167    |
|                               | (4)–Tr54                                       | MF543310    |
|                               | (2)–Tr55                                       | MF543311    |
| T21                           | (6)–HARFUKU1                                  | AB127932    |
|                               | (10)–HARFUKU2                                 | AB127933    |
|                               | (3)–Tr21                                       | MF543312    |
| K11                           | (2)–HARFUKU1                                  | AB127932    |
|                               | (12)–HARFUKU2                                 | AB127933    |
| K15                           | (5)–HARFUKU1                                  | AB127932    |
|                               | (9)–HARFUKU2                                  | AB127933    |
| K13                           | HARFUKU2                                      | AB127933    |

Note: the numbers in parentheses indicate the number of plasmid clones of this type in a random sample set of clones.

https://doi.org/10.1371/journal.pone.0198190.t005
We detected nine *Spiroplasma* strains within populations of the *H. axyridis* native range. The two most common strains—HARFUKU1 and HARFUKU2—as well as two minor strains—Sib1 and Sib19—have been previously noted in Japanese and Novosibirsk populations, respectively [42, 37]; the rest of the strains described here are recorded for the first time. Our study is the first to note the diversity of *Spiroplasma* from a single host. Previously, the diversity of *Spiroplasma* strains was investigated in the genus *Drosophila*. In Drosophilidae, the infected species has a single *Spiroplasma* strain [15]. However, a diversity of strains that infect one host species have been repeatedly discussed for other reproductive endosymbiotic bacteria. In particular, the diversity of *Wolbachia* strains was found in *Drosophila simulans*, *Drosophila melanogaster*, *Culex pipiens* [47, 48, 49]; a diversity of *Rickettsia* and *Arsenophonus* strains were found in *Bemisia tabaci* invasive biotype [50]. Interpretation of the data of *Rickettsia* and *Arsenophonus* diversity in *B. tabaci* requires some caution; it is known that *B. tabaci* is a cryptic species complex comprising at least 24 morphologically indistinguishable species [51].

All *Spiroplasma* strains previously detected in *H. axyridis* belong to the Ixodetis clade. The Sib1 strain belongs to the *Spiroplasma* cluster whose members infect arachnids; three other strains are phylogenetically close to the male-killing *Spiroplasma* from *A. bipunctata* [42]. Five new *Spiroplasma* lines (Fig 2), which differ from previously identified strains by single nucleotide substitutions, also belong to the Ixodetis clade and are phylogenetically close to *Spiroplasma* from the ladybird beetle *Anistotica novemdecempunctata*. The position on the median network (Fig 1) of the Bi22 strain, which is closely related to the *Spiroplasma* strain from *Ixodes ricinus*, suggests that this strain is ancestral to the remaining *Spiroplasma* strains of *H. axyridis*, with the exception of the Sib1 strain. The remaining strains can be considered to be derivatives of the primary ones via the accumulation of point mutations. However, on the other hand, the HARFUKU2 strain could be acquired independently by *H. axyridis*. In this case, the Bi10 and Bi24 strains could arise as a result of recombination between HARFUKU1 and HARFUKU2, which is possible when the strains coexist in one individual. Such coexistence has been demonstrated in our study. The formation of new lines via the recombination

### Table 6. 16S rRNA based identification of *Spiroplasma* strains from individual females of *H. axyridis*.

| Female number of *H. axyridis* and geographical locations | Strain name (number of clones in parenthesis) | GenBank ID: |
|-----------------------------------------------------------|-------------------------------------------------|-------------|
| Western populations                                       |                                                 |             |
| G16                                                       | (8) Ha1                                         | MF538703    |
|                                                           | (7) Ha2                                         | MF538704    |
| G9                                                        | Ha2                                            | MF538704    |
| N1                                                        | Ha4                                            | MG672513    |
| N19                                                       | Ha5                                            | MG672514    |
| Eastern populations                                       |                                                 |             |
| V37                                                       | (8) Ha1                                         | MF538703    |
|                                                           | (7) Ha2                                         | MF538704    |
|                                                           | (2) Ha3                                         | MF538705    |
| Tr21                                                      | (2) Ha1                                         | MF538703    |
|                                                           | (13) Ha2                                        | MF538704    |
| K13                                                       | Ha2                                            | MF538704    |

Note: the numbers in parentheses indicate the number of plasmid clones of this type in a random sample set of clones.

https://doi.org/10.1371/journal.pone.0198190.t006

### Discussion

We detected nine *Spiroplasma* strains within populations of the *H. axyridis* native range. The two most common strains—HARFUKU1 and HARFUKU2—as well as two minor strains—Sib1 and Sib19—have been previously noted in Japanese and Novosibirsk populations, respectively [42, 37]; the rest of the strains described here are recorded for the first time. Our study is the first to note the diversity of *Spiroplasma* from a single host. Previously, the diversity of *Spiroplasma* strains was investigated in the genus *Drosophila*. In Drosophilidae, the infected species has a single *Spiroplasma* strain [15]. However, a diversity of strains that infect one host species have been repeatedly discussed for other reproductive endosymbiotic bacteria. In particular, the diversity of *Wolbachia* strains was found in *Drosophila simulans*, *Drosophila melanogaster*, *Culex pipiens* [47, 48, 49]; a diversity of *Rickettsia* and *Arsenophonus* strains were found in *Bemisia tabaci* invasive biotype [50]. Interpretation of the data of *Rickettsia* and *Arsenophonus* diversity in *B. tabaci* requires some caution; it is known that *B. tabaci* is a cryptic species complex comprising at least 24 morphologically indistinguishable species [51].
of the original lines coexisting in the same organism was previously noted in Wolbachia [52–57]. However, evidence of Spiroplasma strain recombination requires an analysis of a longer genome fragment than we had in our possession. The Sib1 strain, found only in one female from the Novosibirsk population, likely infected H. axyridis independently. The uniqueness of the Spiroplasma Sib1 strain infecting female N1 from Novosiborsk is supported by data pertaining to the variability of both ITS1 and 16S rDNA (Figs 1 and 2).

The Spiroplasma strains detected in this study exist in various combinations and quantitative ratios in different females and in different populations of the native range of H. axyridis. The proportion of HARFUKU2 (Ha2) (Tables 5 and 6) in the total strain pool is more than 62%. This strain is found in females with both multiple and single infections (Table 5). The HARFUKU1 strain is also widespread in the H. axyridis area, where it is detected at a frequency of nearly 22%. Although we found this strain only in multiply infected females in combination with the strain HARFUKU2, in Japanese populations HARFUKU1 occurs in females with a single infection [42]. The Bi22 strain, which is identical to the Sib19 strain (5% in the total strain pool) was detected in H. axyridis as a single infection only in Novosibirsk (as Sib19), and as a multiple infection in females from the eastern part of the range (Table 5). Minor strains of Tr21, Tr55 and Tr54 were found only in two females with multiple infections in the Troitsa Bay population. The Bi10 and Bi24 strains were detected in four females from two different populations of the eastern part of the range.

Single-strain Spiroplasma female infections were detected only in Novosibirsk (Sib1, Sib19), Gorno-Altaisk (HARFUKU2), and Kyoto (HARFUKU2) populations, which are all located on the periphery of the native range. In the same edge populations (Gorno-Altaisk and Kyoto), the diversity of Spiroplasma strains was reduced in multiply infected females, which carry only two Spiroplasma strains (HARFUKU1 and HARFUKU2). The number of Spiroplasma strains ranged from 3–5 in multiply infected females from other populations (Table 5). We hypothesize that a decrease in the overall diversity of Spiroplasma strains from the center to the periphery of the range, both in one host individual and in populations as a whole, reflects the well-known pattern of the microevolutionary process, which consists of reductions in diversity in edge populations [58–60]. The possibility cannot be excluded that Spiroplasma reduces the fitness of infected beetles in conditions of ecological pessimum at the border of the H. axyridis native range (Novosibirsk, Gorno-Altaisk). In this case, a mono infection and a decrease in bacterial abundance may be considered to be a stage of Spiroplasma elimination from the populations of H. axyridis. The assumption of the negative Spiroplasma effect on the viability of H. axyridis is indirectly supported by the absence (or very low occurrence) of Spiroplasma in invasive populations of H. axyridis [61].

The diversity of Spiroplasma strains in H. axyridis is the result of at least two events of infection of the host. The existence of repeated infections of H. axyridis was previously shown for its symbiotic bacteria Rickettsia [61] and Wolbachia [62]. Multiple infection events are known for other symbiotic pairs. D. simulans is infected by five strains of Wolbachia that span across both supergroup A and B, including three supergroup A strains and two supergroup B strains [47, 63]. These strains attained a different density in the host cells and, accordingly, determined different levels of cytoplasmic incompatibility, as well as different levels of protection against pathogens.
Spiroplasma infection in Harmonia axyridis
In most of the samples studied, we detected infection with two or more Spiroplasma strains. The same type of infection, more than a single strain in one host–can be assumed in ticks. At least in the study of the symbiotic community structure in Zygiella x-notata Clerck, 1757 (Araneae: Araneidae) within the Spiroplasma, there was evidence for several “heterozygous nucleotide positions”. Individual Spiroplasma ITS sequences contained up to eight such sites [64], which we suggest is the result of multiple infections. Multiple infections are also known for Wolbachia [65–67]. Because multiple infections are widespread among Spiroplasma infected individuals (88%), it is possible that such co-infections are functionally significant for H. axyridis and favored by selection.

The effects of symbiotic bacteria are density-dependent. Male-killing correlates with Spiroplasma density in species of the genus Drosophila [35]. The level of cytoplasmic incompatibility and the level of antiviral protection in Drosophila positively correlate with Wolbachia density [48, 68–71]. Until now, the mechanisms of density formation for Spiroplasma remain unknown. Mechanisms of density formation were studied for Wolbachia, and these mechanisms depend both on the characteristics of the host and bacterial genomes. In most cases of multiple Wolbachia infections, the density of each strain is under independent host control. The strains do not compete with each other, except for the only known case today, when one Wolbachia strain was shown to be suppressed by another strain during co-infection [65, 67, 68, 72–76]. Bacterial density may be under the control of bacterial genes. The density of

**Fig 2. 16S rRNA median network of Spiroplasma strains from H. axyridis.** This reconstruction is based on the analysis of 16S rRNA gene fragment polymorphisms. Characteristics of the strains are presented in Table 6. Mutations are indicated by dashes. The size of the circles is proportional to the number of sequences in each group. Spiroplasma from A. novemdecimpunctata, a closely related ladybird species, was used as a control.

https://doi.org/10.1371/journal.pone.0198190.g002

| Beatle number | Identified Spiroplasma strains | DNA concentration (ng/ul) | Ct FAM (ITS1 Spiroplasma) of three technical PCR replicates | Mean number of Ct FAM (ITS1 Spiroplasma) with 95% confidence interval | Spiroplasma density |
|---------------|--------------------------------|--------------------------|-------------------------------------------------------------|---------------------------------------------------------------------|-------------------|
| 1 G9          | HARFUKU2                        | 1.2                      | 32.44; 31.90; 31.27                                          | 31.87 ± 1.45                                                       | 1.53              |
| 2 G13         | HARFUKU2                        | 1.2                      | 31.39; 31.90; 32.50                                          | 31.93 ± 1.39                                                       | 1.49              |
| 3 N1          | Sib1                           | 1.2                      | 33.05; 32.40; 31.00                                          | 32.15 ± 2.60                                                       | 2.27              |
| 4 N19         | Sib19                          | 1.2                      | 31.95; 33.00; 33.03                                          | 32.66 ± 1.53                                                       | 1.00              |
| 5 G 16        | HARFUKU1; HARFUKU2              | 1.2                      | 24.92; 24.60; 24.49                                          | 24.67 ± 0.55                                                      | 76.18             |
| 6 V37         | HARFUKU1; HARFUKU2; Bi24       | 1.2                      | 25.22; 25.12; 23.79                                          | 24.71 ± 1.98                                                      | 74.55             |
| 7 V42         | HARFUKU2; Bi10                 | 1.2                      | 26.22; 26.33; 26.47                                          | 26.34 ± 0.31                                                      | 30.80             |
| 8 T5          | HARFUKU1; HARFUKU2; Bi22; Tr54; Tr55 | 1.2  | 25.02; 24.57; 23.91                                          | 24.50 ± 1.39                                                      | 83.54             |
| 9 21          | HARFUKU1; HARFUKU2; Ty21      | 1.2                      | 23.61; 25.34; 23.05                                          | 24.00 ± 2.97                                                      | 109.56            |
| 10 Bi16       | HARFUKU1; HARFUKU2; Bi24; Bi10 | 1.2                      | 23.07; 23.89; 25.40                                          | 24.12 ± 2.94                                                      | 102.66            |
| 11 Bi29       | HARFUKU1; HARFUKU2; Bi24; Bi22 | 1.2                      | 23.22; 24.43; 24.11                                          | 23.92 ± 1.56                                                      | 114.42            |
| 12 K11        | HARFUKU1; HARFUKU2              | 1.2                      | 24.22; 23.92; 23.26                                          | 23.80 ± 1.22                                                      | 122.12            |
| 13 K15        | HARFUKU1; HARFUKU2              | 1.2                      | 24.22; 24.11; 23.04                                          | 23.79 ± 1.62                                                      | 122.78            |

* The Spiroplasma density in sample N19 was used as a proxy for Spiroplasma titers.

Ct FAM—The value of the reference cycle (fluorescent dye: SYBR Green I).

https://doi.org/10.1371/journal.pone.0198190.t007
Wolbachia strain wMelPop depends on the number of copies of a special operon named Octomom [48, 77]. Ambient temperature and the host diet also influence the bacterial density [75, 78, 79]. The density of symbiotic bacteria is consistently increased during embryonic and larval development [35]. In this study, we showed that two parameters—infections type (mono- or multiple infection) and Spiroplasma density are correlated. Molecular mechanisms of genetic control of the Spiroplasma density, as well as the biological consequences of single and multiple infections, remain unclear. It can be assumed that the increased density of Spiroplasma in beetles infected with several strains can increase the stability of the infection, reducing the likelihood of spontaneous loss of Spiroplasma, which explains the wide spread of multiple infection in H. axyridis. Quite possible Spiroplasma strains can interact according to the principle of complementarity. Our data can be the basis for further experimental study of the genetic control of Spiroplasma density in H. axyridis.

The diversity of strains seems to provide Spiroplasma with a variety of manipulations with the reproductive strategy of the host. Cytological studies of Spiroplasma revealed two scenarios of interactions with H. axyridis embryos. In the first case, embryo development stops at the stage of yellow eggs, and there are four critical development points, after which embryos die. In the second scenario, embryos died at the stage of gray eggs immediately before hatching [80]. We suggest that such differences can be the result of competition between two Spiroplasma strains with different and density dependent effects. Therefore, it is of considerable interest to investigate associations between the type of infection (single or multiple), and the degree of manifestation of male-killing and probably cytoplasmic incompatibility, which has not been shown in H. axyridis.

Supporting information
S1 Fig. Map of H.axyridis sampling locations.
(TIF)

Acknowledgments
The authors are grateful to M. Ya. Orlova-Ben’kovskaya, L. V. Frisman, V. P. Koralev, and A.V. Zimenko for providing them with the H. axyridis collections. We used the materials of the Coccinellidae Collection of the Koltzov Institute of Developmental Biology of RAS. The Collection is supported by the contract 0108-2017-0010. We also used the materials of the Genetic Collection of Insects Models for biomedical and population-genetic studies of the Vavilov Institute of General Genetics RAS. The study of Spiroplasma in western group of populations of H.axyridis was supported by the Russian Foundation for Basic Research (Project No. 15-04-07466_A). The study of Spiroplasma in eastern group of populations of H.axyridis—the source of the global invasion, was supported by the Russian Science Foundation (Project No. 16-16-00079.

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