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Signs of Neutralization in a Redundant Gene Involved in Homologous Recombination in Wolbachia Endosymbionts

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Abstract

Genomic reduction in bacterial endosymbionts occurs through large genomic deletions and long-term accumulation of mutations. The latter process involves successive steps including gene neutralization, pseudogenization, and gradual erosion until complete loss. Although many examples of pseudogenes at various levels of degradation have been reported, neutralization cases are scarce because of the transient nature of the process. Gene neutralization may occur due to relaxation of selection in nonessential genes, for example, those involved in redundant functions. Here, we report an example of gene neutralization in the homologous recombination (HR) pathway of Wolbachia, a bacterial endosymbiont of arthropods and nematodes. The HR pathway is often depleted in endosymbiont genomes, but it is apparently intact in some Wolbachia strains. Analysis of 12 major HR genes showed that they have been globally under strong purifying selection during the evolution of Wolbachia strains hosted by arthropods, supporting the evolutionary importance of the HR pathway for these Wolbachia genomes. However, we detected signs of recent neutralization of the ruvA gene in a subset of Wolbachia strains, which might be related to an ancestral, clade-specific amino acid change that impaired DNA-binding activity. Strikingly, RuvA is part of the RuvAB complex involved in branch migration, whose function overlaps with the RecG helicase. Although ruvA is experiencing neutralization, recG is under strong purifying selection. Thus, our high phylogenetic resolution suggests that we identified a rare example of targeted neutralization of a gene involved in a redundant function in an endosymbiont genome.

Key words: endosymbiosis, gene loss, molecular evolution, selection relaxation, genomic reduction, nonorthologous gene displacement.

Introduction

Host-restricted intracellular bacteria, either as parasites, commensals or mutualistic symbionts, exhibit multiple distinguishing genomic features in comparison with their free-living relatives. Hence, obligate bacterial endosymbionts (that live and replicate exclusively in the cytoplasm of the host cells) are characterized by reduced genomes, accelerated DNA sequence evolution, and strong A+T nucleotide compositional bias (Wernegreen 2005; Moran et al. 2008; Moya et al. 2008; McCutcheon and Moran 2012; Van Leuven and McCutcheon 2012). These features are the consequences of the process of genomic reduction, which is triggered by enhanced genetic drift and relaxation of selection because of effective population size reduction and stable environmental conditions (Moran 1996; Nilsson et al. 2005; Toft and Andersson 2010). The initial stages are characterized by large genomic deletions mediated by recombination between proliferating mobile genetic elements (Moran and Plague 2004; Plague et al. 2008; Walker and Langridge 2008; Cerveau, Leclercq, Bouchon, et al. 2011). On the long term, relaxed purifying selection leads to the accumulation of slightly deleterious mutations and the inactivation of nonessential genes or genes with redundant functions (Ohta and Gillespie 1996; Silva et al. 2001; Tamas et al. 2002; Daqan et al. 2006; Moran et al. 2009) (fig. 1). Coupled with a deletion bias, newly formed pseudogenes are ultimately lost along with mobile genetic elements (Mira et al. 2001; Moran and Mira 2001; Ogata et al. 2001; Gómez-Valero et al. 2004; Fuxelius et al. 2008) (fig. 1). In addition, gene loss can involve genes carrying essential functions such as DNA repair, which further increases the rate of gene
loss (Dale et al. 2003; Rocha et al. 2005). In particular, genes involved in the homologous recombination (HR) pathway are often depleted in these genomes, implicating fewer recombination events (Dale et al. 2003; Rocha et al. 2005; Moran et al. 2008). This is consistent with the long-term genomic stability observed in various ancient endosymbiont genomes (Tamas et al. 2002; Silva et al. 2003). Although the later stages of gene loss in endosymbionts (from gene inactivation to complete loss) have been well-documented (fig. 1 and references therein), few studies have described examples of the initial step of gene loss, that is, neutralization preceding inactivation. This is because most analyzed genomes are from ancient endosymbionts with highly reduced genomes, in which the process of gene loss is already at an advanced stage.

Various mechanisms can lead to gene neutralization in endosymbiont genomes, either by altering all genes of the genome in the same manner or by targeting specific genes. In the first case, enhanced genetic drift and less efficient purifying selection, coupled with a Muller’s ratchet effect due to reduced opportunities for recombination, lead to the global accumulation of slightly deleterious mutations in many genes. This scenario explains the general acceleration in DNA sequence evolution observed in endosymbiont genomes (Moran 1996; Ohta and Gillespie 1996; Wernegreen and Moran 1999; Itoh et al. 2002; Woolfit and Bromham 2003; Wernegreen and Funk 2004; Fry and Wernegreen 2005; Blanc et al. 2007; McCutcheon and Moran 2012). Gene neutralization can also occur due to targeted relaxation of selection on a gene that became superfluous for the endosymbiont, as in the case in which a gene is involved in a redundant function with another gene (Moran et al. 2008; Moya et al. 2008). This is well illustrated by functional complementation, which may happen when multiple endosymbionts co-occurring within a single host can fulfill the same function. This has been demonstrated for the endosymbionts Buchnera aphidicola BCc and Serratia symbiotica in the insect Cinara cedri, in which B. aphidicola is undergoing genome degradation and functional replacement by the coexisting S. symbiotica (Pérez-Brocal et al. 2006). Alternatively, functional redundancy may occur within a single endosymbiont genome. However, to our knowledge, there has been no report clearly demonstrating targeted relaxation of selective pressures in such functionally redundant genes leading to neutralization. This is because gene neutralization is a transient stage preceding actual pseudogenization, whose detection requires a high phylogenetic resolution and, thus, investigation of a large set of closely related endosymbiont strains.

In this study, we report an example of likely gene neutralization in a redundant portion of the HR pathway within the bacterial endosymbiont Wolbachia. Wolbachia are maternally inherited microorganisms that have been associated with arthropod and nematode hosts for greater than 100 Myr and are able to manipulate arthropod host reproduction to increase their own transmission (Bandi et al. 1998; Werren et al. 2008; Cordaux et al. 2011). Wolbachia endosymbionts present a large genetic diversity, with multiple phylogenetic supergroups defined with capital letters (Lo et al. 2007). In particular, Wolbachia strains from supergroups A and B are found in arthropods and Wolbachia strains from supergroups C and D are found in nematodes. Multiple Wolbachia genomes have been sequenced; they show typical features of long-term obligate endosymbionts, such as reduced genome size, accelerated DNA sequence evolution, and A+T nucleotide bias (Wu et al. 2004; Foster et al. 2005; Klasson et al. 2008; Klasson et al. 2009; Darby et al. 2012). Yet, despite their ancient association with invertebrates, many Wolbachia genomes contain recently active mobile genetic elements (Cordaux et al. 2008; Kent and Bordenstein 2010; Cerveau, Leclercq, Leroy, et al. 2011; Leclercq et al. 2011). In addition, several Wolbachia strains experience recombination (Werren and Bartos 2001; Ellegaard et al. 2013) and gene conversion (Cordaux 2009). Strikingly, the HR pathway, which is commonly depleted in long-term endosymbionts (Akman et al. 2002; Tamas et al. 2002; Dale et al. 2003; Gil et al. 2003; Rocha et al. 2005), is apparently intact in some (Wu et al. 2004; Foster et al. 2005), but not all (Darby et al. 2012), Wolbachia genomes.

The HR pathway is involved in DNA repair of single- and double-strand breaks and is responsible for large-scale genomic rearrangements and incorporation of homologous foreign DNA (Aravind et al. 2000; Kowalczykowski 2000; Zuñiga-Castillo et al. 2004) (fig. 2). Interestingly, a crucial step of the HR pathway (branch migration) may be fulfilled by genes with overlapping functions, that is, the RuvAB complex or the RecG helicase, although efficiency is reduced when only one of the two possibilities is active (Meddows et al. 2004). In the context of genomic reduction undergone by Wolbachia, such functional redundancy is predicted to be dispensable (Mendonça et al. 2011). These observations raise the question whether the HR pathway in general and functional redundancy in this pathway in particular are maintained by purifying
identification of HR genes

We selected 12 genes encoding the major proteins predicted to be involved in the HR pathway in Wolbachia (Aravind et al. 2000; Kowalczykowski 2000; Zuniga-Castillo et al. 2004; Cromie 2009) (fig. 2). The genes are: recJ, recF, recO and recR (which initiate the repair of single-strand DNA breaks), addA and addB (which initiate the repair of double-strand DNA breaks), recA (which catalyzes strand exchange), ruvA and ruvB (which perform branch migration), ruvC (which resolves Holliday junction intermediates), and priA (which initiates replication during double-strand break repair). In addition, we selected recG whose protein function overlaps with that of the RuvAB complex (Meddows et al. 2004).

We analyzed these 12 genes in a diverse set of Wolbachia strains encompassing the four major supergroups A–D of Wolbachia diversity. We obtained sequences of HR genes by similarity searches using BLASTp (Altschul et al. 1990) against 20 sequenced Wolbachia genomes from supergroups A–D, as described in table 1. We also performed targeted resequencing of 14 additional Wolbachia strains from supergroups A and B (supplementary table S1, Supplementary Material online). Total DNA extraction, polymerase chain reaction (PCR), and sequencing were performed as previously described (Cordaux et al. 2008). In brief, DNA was extracted using a standard phenol–chloroform protocol, PCR was performed using the PCR primers and conditions provided in supplementary table S2, Supplementary Material online, and purified PCR products were directly sequenced on an ABI Prism 3130 Genetic Analyzer. The nucleotide sequences generated in this study have been deposited in GenBank under accession numbers KM066817–KM0066942.

Sequence Analyses of Wolbachia Supergroups A and B

Nucleotide sequences of HR genes from 29 Wolbachia strains were aligned together by codons using the Muscle algorithm implemented in MEGA5 software (Tamura et al. 2011). We removed seven palindromic regions because they were difficult to align with confidence. These regions, resembling Wolbachia palindromic elements (WPE) (Ogata et al. 2005), were located in addA (nucleotide positions [np] 289–459, 907–1146, and 3076–3387), addB (np 778–1023 and np 2452–2655), and priA (np 523–1365 and 2587–2790). As these WPE-like sequences are inserted in frame and do not generate premature stop codons, they apparently do not inactivate the HR genes. The only exception is addA in the six Wolbachia strains from supergroup B hosted by isopods, in which a premature stop codon was generated at np 3100–3102, resulting in the deletion of the entire nuclease domain. This deletion may have limited functional consequences, as it has been shown that the loss of the nuclease domain significantly reduces efficiency of the exonuclease activity of the AddAB complex, but repair can still operate (Amundsen et al. 2009). Nevertheless, we conservatively split addA into two parts for our evolutionary analyses: addA_1 encompasses np 1–3099 and addA_2 encompasses np 3103–3837.

To avoid biased evolutionary analyses due to poor resolution, we removed all but one representative for all groups in which Wolbachia strains showed less than 0.2% pairwise nucleotide divergence across the 12 HR genes. This filter resulted in the removal of ten Wolbachia strains from analyses.
| Subgroup | Strain | Host | Accession Number | addA | addB | priA | recA | recF | recG | recJ | recO | recR | ruvA | ruvB | ruvC | Reference |
|----------|--------|------|------------------|------|------|------|------|------|------|------|------|------|------|------|------|---------|
| A        | wMel*  | Drosophila melanogaster | AE017196 | WD_0359 | WD_0912 | WD_1200 | WD_1050 | WD_0824 | WD_0312 | WD_0219 | WD_1180 | WD_1113 | WD_1112 | WD_0142 | Wu et al. 2004 |
| wRi*     | Drosophila simulans | CPO01391 | WRI_004270 | WRI_008710 | WRI_011780 | WRI_010830 | WRI_013130 | WRI_007900 | WRI_004620 | WRI_002090 | WRI_011550 | WRI_012870 | WRI_012860 | WRI_01110 | Klason et al. 2009 |
| wHa*     | Drosophila simulans | CPO03884 | wHa_04850 | wHa_07710 | wHa_10020 | wHa_08760 | wHa_06107 | wHa_06960 | wHa_01890 | wHa_09660 | wHa_09320 | wHa_09310 | wHa_06060 | Ellegaard et al. 2013 |
| wUni     | Malaria/fluxus unikapit | ACP00000000 | M | F | F | F | F | F | M | F | F | F | F | F | Klasson et al. 2009 |
| wWlu     | Drosophila sylvesteri | AAGP00000000 | F | F | F | F | F | F | F | F | F | F | F | F | Remington et al. unpublished |
| wSuzi    | Drosophila suzuki | CADU00000000 | F | F | F | F | F | F | F | F | F | F | F | F | Klasson et al. 2008 |
| B        | wPip-P*  | Culex | AM998887 | WP0175 | WP0219 | WP0766 | WP0932 | WP0901 | WP0482 | WP0630 | WP0359 | WP0769 | WP0988 | WP0987 | WP1146 | Klasson et al. 2008 |
| wAlbB    | Aedes albopictus | CAGB00000000 | F | F | F | F | F | F | F | F | F | F | F | F | F | Mavingui et al. 2012 |
| wDi      | Diaphorina citri | AMZ00000000 | F | F | F | F | F | F | F | F | F | F | F | F | F | Ellegaard et al. 2013 |
| wNo*     | Drosophila simulans | CPO03883 | wNo_09810 | wNo_11150 | wNo_04260 | wNo_05950 | wNo_05210 | wNo_03260 | wNo_00850 | wNo_04290 | wNo_06840 | wNo_06830 | wNo_0860 | Ellegaard et al. 2013 |
| wVfB     | Nasonia vitriannca | AERW00000000 | F | F | F | F | F | F | F | F | F | F | F | F | F | Kent et al. 2011 |
| wBoI     | Hypohiponos bolina | CACH00000000 | F | F | F | F | F | F | F | F | F | F | F | F | F | Duploy et al. 2013 |
| wPip-JB  | Culex | ABZA00000000 | F | F | F | F | F | F | F | F | F | F | F | F | F | Salzberg et al. 2009 |
| wPip-Mol | Culex piipes molestus | CACK00000000 | F | F | F | F | F | F | F | F | F | F | F | F | F | Sikkens et al. unpublished |
| C        | wOo*     | Onchocerca ochengi | HE660029 | wOo_08780 | wOo_07360 | wOo_05130 | P | P | P | P | P | wOo_01160 | P | P | P | P | P | Darby et al. 2012 |
| wDir     | Dirofilaria immitis | dirofilaria. | F | F | P | P | P | P | P | P | P | P | P | P | P | Godel et al. 2012 |
| D        | wBm*     | Brugia malayi | AE017321 | Wbm0172 | Wbm0272 | Wbm0247 | Wbm0126 | Wbm0635 | Wbm0288 | Wbm0674 | Wbm0251 | Wbm0252 | Wbm0697 | Foster et al. 2005 |
| wBmB     | Wucheria bancrofti | ADHD00000000 | F | M | Partial | F | M | Partial | F | M | M | M | M | M | M | Dejardins et al. 2013 |
| wVol     | Onchocerca volvulus | ADHE00000000 | Partial | M | M | M | M | M | M | M | M | M | M | M | M | Dejardins et al. 2013 |
| wLs      | Lutomosoides sigmodonite | lutomosoides. | F | F | F | Partial | M | Partial | M | Partial | M | M | M | M | M | Comandatore et al. 2013 |

Notes.—F, gene found (detected by BLASTp with start and stop codons); Partial, partial gene found (detected by BLASTp but lacking start or stop codon); P, pseudogene (only detected by BLASTn); M, missing gene (detected neither by BLASTp nor BLASTn). Locus tags are provided for the seven completed genomes (identified by *). The 14 other genomes are incomplete; therefore missing genes may be truly absent or may just be absent from the genome assembly.
Selection Analyses

Selection analyses were performed by calculating the ratios of the rates of nonsynonymous (Ka) to synonymous (Ks) nucleotide substitutions per site on each HR gene as implemented in codeml PAML4 software (Yang 2007). Ka/Ks ratios >1, $<1$, and =1 are indicative of positive selection, purifying selection, and neutral evolution, respectively. Pairs of models were compared using likelihood ratio tests (LRTs), as described in Yang (1998). When the $P$ value of an LRT was significant at the 5% level, the model with the highest likelihood value was considered as the best-fit model. Otherwise, the two models were not significantly different, which means that adding complexity to the model does not improve its likelihood. As a result, the simplest model was considered as the best-fit model. We performed four successive types of comparisons.

First, we investigated global patterns of selection by comparing two models: 1) The neutral model with the Ka/Ks ratio forced to 1 in all branches of the phylogenetic tree (the simplest model in the comparison), and 2) a single Ka/Ks ratio model with the same Ka/Ks ratio in all branches.

Second, for HR genes in which the single Ka/Ks ratio model was the best-fit model, we searched for selection heterogeneity by comparing two models: 1) The single Ka/Ks ratio model from the previous analysis (the simplest model in the comparison), and 2) a 3-Ka/Ks ratio model that implements a different Ka/Ks ratio for each of three monophyletic clusters (supergroup A, supergroup B hosted by insects, and supergroup B hosted by isopods).

Third, for HR genes in which the 3-Ka/Ks ratio model was the best-fit model, we searched for neutralization signal in the cluster with the highest Ka/Ks ratio in the 3-Ka/Ks ratio model by comparing two models: 1) the 3-Ka/Ks ratio model with the Ka/Ks ratio forced to 1 in the cluster with the highest Ka/Ks ratio in the 3-Ka/Ks ratio model (the simplest model in the comparison), and 2) the 3-Ka/Ks ratio model from the previous analysis.

Fourth, for the HR gene showing signs of neutralization, we searched for positive selection on codons (which may have inflated the Ka/Ks ratio) by using the branch-site model (Yang et al. 2005; Zhang et al. 2005).

To evaluate the robustness of our analyses, we used multiple phylogenetic frameworks based on a resampling strategy. Specifically, following a jackknife procedure, we removed each gene in turn from the concatenated alignment of the 12 HR genes and recalculated a phylogenetic tree based on the remaining genes. For each of the 12 alignments, we used MEGA5 to identify the best substitution model (GTR+G with $\gamma = 5$ in all cases). Next, each alignment was used to build a maximum-likelihood tree with 1,000 bootstrap replicates (85% partial deletion), using MEGA5. Hence, we obtained 12 phylogenetic trees, each of which was used as a phylogenetic hypothesis for selection analyses. The 12 trees corresponded to five different topologies exhibiting minor variations in terminal branching patterns (supplementary fig. S1, Supplementary Material online). This enabled us to calculate confidence intervals for LRTs.

**Results and Discussion**

**Distribution of HR Genes in Wolbachia Strains**

Analysis of the distribution of the 12 HR genes in 20 sequenced Wolbachia genomes from superfamilies A–D (table 1) revealed that most of the HR genes from supergroup C Wolbachia
Table 2
Summary of Selection Analysis for 12 HR Genes in 18 Wolbachia Strains

| Gene  | Best-Fit Model | P Value (Range) | Ka/Ks Values (Range) |
|-------|----------------|-----------------|----------------------|
| addA_1| 3 Ka/Ks        | <0.02           | A: 0.214–0.221;      |
|       |                |                 | B insects: 0.277–0.291; |
|       |                |                 | B isopods: 0.175–0.193 |
| addA_1| 1 Ka/Ks        | <0.001          | 0.187–0.199          |
| addB  | 3 Ka/Ks        | <0.001          | A: 0.324–0.343;      |
|       |                |                 | B insects: 0.431–0.447; |
|       |                |                 | B isopods: 0.382–0.395 |
| priA  | 1 Ka/Ks        | <0.001          | 0.143–0.147          |
| recA  | 1 Ka/Ks        | <0.001          | 0.049–0.050          |
| recF  | 1 Ka/Ks        | <0.001          | 0.225–0.239          |
| recG  | 1 Ka/Ks        | <0.001          | 0.128–0.136          |
| recJ  | 1 Ka/Ks        | <0.001          | 0.112–0.113          |
| recO  | 3 Ka/Ks        | <0.02           | A: 0.294–0.307;      |
|       |                |                 | B insects: 0.158–0.182;  |
|       |                |                 | B isopods: 0.283–0.289 |
| recR  | 1 Ka/Ks        | <0.001          | 0.075–0.081          |
| ruvA  | Neutal foreground| 0.066–0.094    | A: 0.299–0.316;      |
|       |                |                 | B insects: 0.211–0.216;  |
|       |                |                 | B isopods: 1          |
| ruvB  | 1 Ka/Ks        | <0.001          | 0.072–0.074          |
| ruvC  | 1 Ka/Ks        | <0.001          | 0.218–0.221          |

Note.—Detailed results are provided in supplementary tables S4–S6, Supplementary Material online.

strains were pseudogenized (Darby et al. 2012; Godel et al. 2012), indicating that the HR pathway has been inactivated in these strains. In addition, most of the HR genes were missing in all supergroup D strains except wBm, which has an apparently intact HR pathway (Foster et al. 2005). Because wBm is the only finished supergroup D genome (Foster et al. 2005), we cannot conclude whether some HR genes have not been sequenced or the HR pathway has been inactivated in other supergroup D genomes. In contrast, the HR pathway is apparently intact in Wolbachia strains from supergroups A and B (Wu et al. 2004; Klasson et al. 2008). The difference in completeness of the HR pathway or lack thereof is not really surprising given the contrast in host range (arthropods vs. nematodes for supergroups A/B and C/D, respectively) and lifestyle (reproductive parasite vs. mutualist for supergroups A/B and C/D, respectively) of these supergroups. Based on these observations, we focused our selection analyses on supergroups A and B. To obtain a more extensive sampling of HR genes in supergroups A and B, we performed targeted resequencing of 14 additional Wolbachia strains. After removal of strains showing less than 0.2% pairwise nucleotide divergence across the 12 HR genes (see Materials and Methods), our final data set consisted of 18 supergroups A and B Wolbachia strains and wBm (supergroup D) as an outgroup.

Selective Pressures on HR Genes
To measure the selective pressures acting on HR genes in supergroups A and B Wolbachia strains, we compared the Ka/Ks ratios of the 12 genes by using LRTs as implemented in PAML 4 (Yang 2007). To evaluate the robustness of our results, we performed analyses using multiple phylogenetic frameworks, based on a jackknife resampling strategy (see Materials and Methods). Assuming a single Ka/Ks ratio in each HR gene tree, the best-fit to the data was obtained for Ka/Ks ratios ranging from 0.05 (for recA) to 0.35 (for addB), which are all significantly less than 1 (all P < 0.001; table 2 and supplementary table S4, Supplementary Material online). This is indicative of purifying selection globally acting on all 12 HR genes. This strong conservation suggests that the HR pathway is evolutionarily important for Wolbachia strains from supergroups A and B. HR, together with repeats, creates high levels of genomic variation in prokaryotes (Rocha et al. 2005; Treangen et al. 2009). Strikingly, Wolbachia genomes from supergroups A and B bear an unusually high proportion of repeats and experience recombination (Werren and Bartos 2001; Kent and Bordenstein 2010; Cerveau, Leclercq, Leroy, et al. 2011; Leclercq et al. 2011; Ellegaard et al. 2013). Genome plasticity (and the factors favoring this plasticity) may represent an appreciable evolutionary advantage for Wolbachia, perhaps in relation with its ability to horizontally transfer between various arthropod host species (Cordaux et al. 2001) and with the fact that most of these strains are involved in an evolutionary arms race with their hosts (Cordaux et al. 2011). For example, it has been shown that recombination caused by repeats and HR genes enables some parasites to respond specifically to the adaptive immune system of the host (Finlay and Falkow 1997; Mehr and Seifert 1998).

Heterogeneity and Relaxation of Selection Pressures
To investigate whether purifying selection patterns have been stably maintained throughout Wolbachia evolution, we tested for heterogeneity in Ka/Ks ratios in each HR gene by comparing the above results to a model in which different Ka/Ks ratios were allowed in the three monophyletic clusters of the ingroup, each consisting of six Wolbachia strains: supergroup A, supergroup B hosted by insects, and supergroup B hosted by isopods (fig. 3). In this clustering scheme, average nucleotide divergence across the 12 HR genes ranged from 1% to 2% within groups and 6–10% between groups (fig. 3).

The model with three Ka/Ks ratios was significantly better than the model with a single Ka/Ks ratio under all 12 jackknifed phylogenetic hypotheses for four HR genes: addA_1, addB, recO, and ruvA (all P < 0.05; table 2 and supplementary table S5, Supplementary Material online). The 3-Ka/Ks ratio model was also better for recF and ruvC albeit for only a subset of jackknifed phylogenetic hypotheses. Given the limited robustness of the results for recF and ruvC, we conservatively rejected heterogeneity in Ka/Ks ratios for these two genes. In contrast, the results are robust for the four other genes addA_1, addB, recO, and ruvA, as all jackknifed trees consistently support heterogeneity in Ka/Ks ratios.
Heterogenous selection patterns have already been observed among different endosymbiont strains and were found to result from the effect of strong genetic drift or enhanced mutation rate (Wernegreen and Moran 1999; Itoh et al. 2002; Woolfit and Bromham 2003; Wernegreen and Funk 2004; Fry and Wernegreen 2005; Blanc et al. 2007), as expected in the context of genomic reduction undergone by Wolbachia.

To investigate whether heterogeneity in Ka/Ks ratios in these four HR genes may reflect relaxation of selective pressures leading to neutralization in a subset of the Wolbachia strains, we tested whether the highest Ka/Ks ratio in the 3-Ka/Ks model was different from 1. We predicted that the elevated Ka/Ks ratio should be significantly different from 1 under relaxed selection without neutralization, and not significantly different from 1 under neutral evolution. For addA, addB, and recO, we found that the 3-Ka/Ks ratios model was significantly better than the model assuming neutralization in a subset of Wolbachia strains, under all 12 jackknifed phylogenetic hypotheses (P < 0.01; table 2 and supplementary table S6, Supplementary Material online). In contrast, for ruvA, the two models were not significantly different from each other, implying that the highest Ka/Ks ratio (0.44) in the 3-Ka/Ks ratio model is not significantly different from 1. This result was robust, as it was supported by all 12 jackknifed phylogenetic hypotheses (P ranging from 0.066 to 0.094). In other words, these results indicate relaxed selection leading to neutralization in ruvA in supergroup B Wolbachia strains from isopods.

To substantiate these results, we further analyzed ruvA molecular evolution in Wolbachia strains from isopods. Average genetic distance between isopod Wolbachia strains for ruvA was 1.2% (range 0.2–2.0%) (supplementary fig. S1, Supplementary Material online). There was also no obvious sign of pseudogenization in ruvA, as it does not contain any inactivating mutation (i.e., frameshift or premature stop codon) or homopolymeric tract of poly(A) (>9 bp), which could induce slippage of RNA polymerase (Tamas et al. 2008). In addition, to test whether positive selection, rather than neutral evolution, may have inflated the Ka/Ks ratio, we used the branch-site model of PAML to search for positively selected codons in ruvA (Yang et al. 2005; Zhang et al. 2005). No codon was found to be under positive selection (P = 0.71). Therefore, there is no evidence to support an effect of positive selection in our results.

| Substitution Type | Nucleotide Substitution | Functional Domain* | Amino Acid Substitution | Amino Acid Property Change |
|-------------------|-------------------------|--------------------|-------------------------|----------------------------|
| NS                |                         |                    |                         |                            |
| G70A              | I                       | V24I               | Neutral > positive      |                            |
| G125A             | I                       | S42N               |                         |                            |
| T353C             | II                      | L118P              | Aliphatic > nonaliphatic|                            |
| G493A             | III                     | D165N              | Negative > neutral      |                            |
| T497C             | III                     | T166M              | Nonhydrophobic > hydrophobic|                            |
| C524A             | III                     | P175Q              | Hydrophobic > nonhydrophobic|                            |
| A539G             | III                     | K180R              |                         |                            |
| T87C              | I                       |                    |                         |                            |
| T280C             | II                      |                    |                         |                            |
| A357G             | II                      |                    |                         |                            |
| T457C             | III                     |                    |                         |                            |
| A498G             | III                     |                    |                         |                            |
| A513G             | III                     |                    |                         |                            |

*Domains I and II are implicated in tetramORIZATION of the protein and junction-DNA binding; domain III is implicated in branch migration through heteroduplex contact with RuvB.

To further investigate the evolutionary history of ruvA in supergroup B Wolbachia strains from isopods, we searched for potential ancestral substitutions specific to isopod Wolbachia strains that might have contributed to impair RuvA functional efficiency and, possibly, triggered gene neutralization. Inspection of the ruvA sequence alignment revealed 14 nucleotide substitutions exclusively shared by isopod Wolbachia strains, all located in functional domains, eight of which being nonsynonymous substitutions (table 3). Interestingly, five of these amino acid changes modify the physicochemical properties of the RuvA protein, in terms of charge, hydrophobicity or aliphatic property. Unfortunately, Wolbachia endosymbionts are unculturable bacteria. Therefore, the actual consequences of these five amino acid changes on RuvA functionality cannot be directly tested. Nevertheless, to hint at the potential functional consequences of these amino acid changes, we reviewed the literature and collated a list of 51 amino acid sites in the approximately 200 amino acid-long RuvA protein that are considered important for proper folding, multimerization or DNA binding, based on crystallography (Rafferty et al. 1996; Nishino et al. 1998; 2000; Roe et al. 1998; Ariyoshi et al. 2000; Yamada et al. 2002; Prabu et al. 2006, 2009) or mutagenesis studies (Nishino et al. 1998; Privzentez et al. 2005; Baharouglu et al. 2008; Fujisawa et al. 2008; Le Masson et al. 2008; Mayanagi et al. 2008; Bradley et al. 2011). We found that one amino acid change inducing a physicochemical
Neutralization of Redundant Gene in Wolbachia

modification in isopod Wolbachia RuvA falls in this list of 51 important amino acids (Lys-118). In Escherichia coli, Lys-118 plays an important role in DNA binding through nonpolar interaction with its aliphatic chain (Aryoshii et al. 2000). In all supergroup A and supergroup B Wolbachia strains from insects, Lys is replaced by Leu, but this change is not expected to impair function as Leu is also an aliphatic amino acid. In contrast, in the ancestor of all supergroup B Wolbachia strains from isopods, the aliphatic Leu was replaced by the nonlinear aliphatic Pro, which may have altered RuvA DNA-binding activity. Strikingly, in the wAse isopod Wolbachia strain, an additional nonsynonymous substitution resulted in the replacement of Pro by Ser (which is a nonaliphatic amino acid). We speculate that this substitution was not eliminated by purifying selection because the ancestral Pro had already contributed to impair RuvA function in isopod Wolbachia strains. If so, Leu-118-Pro might have played a key role in ruvA neutralization in these Wolbachia strains.

Although we cannot formally ascertain which (if any) of the five amino acid changes may have been primordial in the initiation of the ruvA neutralization process in isopod Wolbachia strains, a plausible evolutionary scenario is that a nonsynonymous substitution occurred in the ancestral ruvA gene of isopod Wolbachia that significantly affected RuvA functionality (possibly the one leading to Leu-118-Pro). RuvA is part of the RuvAB complex, which performs branch migration during the HR process. Interestingly, RuvAB and RecG functions overlap (Meddows et al. 2004) and our results indicate that RecG has been consistently evolving under strong purifying selection throughout supergroups A and B Wolbachia evolution (Ka/Ks = 0.13; P < 0.001). Therefore, the impaired RuvA function may have resulted in a decreased efficiency of the RuvAB complex, which may have been compensated by RecG in the isopod Wolbachia ancestor, as part of a process of nonorthologous gene displacement (Koonin et al. 1996). As soon as RuvA function was impaired, gene sequence could start undergoing independent neutral evolution in each lineage derived from this ancestor (table 3), thereby reinforcing functional impairment and gene neutralization. As a result, a pair of genes with redundant functions is now experiencing contrasting selective pressures in Wolbachia strains from isopods, with recG experiencing strong purifying selection, whereas ruvA has been neutralized. In support to our inferences, it is noteworthy that nonorthologous gene displacement has already been documented for recombination functions, as exemplified by the substitution of rucC by recU or recBCD by addAB (Ayora et al. 2004; Rocha et al. 2005; Cromie 2009).

**Conclusion**

In the context of genomic reduction undergone by Wolbachia genomes, redundancy is expected to be lost through the process of pseudogenization (Ogata et al. 2001; Silva et al. 2001; Moya et al. 2008). Numerous reports have focused on global pseudogenization events affecting the overall repertoire of genes of a genome due to enhanced genetic drift and less effective purifying selection (Moran 1996; Itoh et al. 2002; Woolfit and Bromham 2003; Fuxellius et al. 2008; McCutcheon and Moran 2012). In contrast, studies reporting relaxation of selection targeting redundant genes are scarce. This has been reported in the case of the functional replacement of B. aphidicola BCC by S. symbiotes in the host C. cedri (Pérez-Brocal et al. 2006). However, in this example, redundancy occurs between two different but closely interacting genomes, whereas we report a case of probable gene neutralization in the context of functional redundancy within a single genome.

Our results provide empirical evidence to support that relaxation of selection on specific genes and genetic drift act in synergy with the process of Muller’s ratchet (Moran 1996; McCutcheon and Moran 2012). It is even plausible that genetic drift triggers specific relaxation of selection while slightly and mildly deleterious mutations are accumulating. In the case of redundant genes, these mutations would render the gene less “efficient” in its function in comparison with the analogous gene. As a result, the analogous gene would be favored to fulfill the function relative to the gene affected by slightly deleterious mutations and/or some compensatory measures would be set up. On the long-term, this would render the gene nonessential and thus initiate the process of specific neutralization, ultimately leading to pseudogenization and complete loss. In any event, targeted neutralization of RuvA indicates that genomic reduction is an ongoing process in Wolbachia endosymbionts.

**Supplementary Material**

Supplementary figure S1 and tables S1–S6 are available at Genome Biology and Evolution online (http://www.gbe.oxfordjournals.org/).

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