Phylogenomic analysis of *Wolbachia* strains reveals patterns of genome evolution and recombination

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Significance Statement

For the intracellular symbiont *Wolbachia*, evolutionary analysis and supergroup classification were previously based on the multilocus sequence typing (MLST) genes. We performed phylogenomic analyses of genome sequenced *Wolbachia* strains from six supergroups. 210 single-copy protein coding genes were identified in these strains, and our interclade recombination screening method discovered 14 inter-supergroup recombination events, including A-E events which were not described before. We conclude that recombination between supergroups occurred in at least 2.9% of the core genes. We also observed almost perfect correlation in evolutionary divergence between genome sequences and MSLT genes, suggesting that MLST remains a useful tool in strain identification and evolutionary relationship analysis, before speedy and affordable genome sequencing and assembly approaches are readily available for single arthropods.

Abstract

Wolbachia are widespread intracellular bacteria that mediate many important biological processes in arthropod species. In this study, we identified 210 conserved single-copy genes in 33 genome-sequenced *Wolbachia* strains in the A, B, C, D, E and F supergroups. Phylogenomic analyses with these core genes indicate that all 33 *Wolbachia* strains maintain the supergroup relationship, which was classified previously based on the multilocus sequence typing (MLST) genes. Using an interclade recombination screening method, 14 inter-supergroup recombination events were discovered in six genes (2.9%) among 210 single copy orthologs. This finding suggests a relatively low frequency of intergroup recombination. Interestingly, they have occurred not only between A and B supergroups (9 events), but also between A and E supergroups (5 events). Maintenance of such transfers suggests possible roles in *Wolbachia* infection related functions. Comparisons of strain divergence using the five genes of the MLST system show a high correlation (Pearson correlation coefficient r = 0.98) between MLST and whole genome divergences, indicating that MLST is a reliable method for identifying related strains when whole genome data are not available. The phylogenomic analysis and the identified core gene set in our study will serve as a valuable foundation for strain identification and the investigation of recombination and genome evolution in *Wolbachia*.

Background

The obligate intracellular bacteria *Wolbachia* commonly infect arthropods and filarial nematodes (Werren 1997; Fenn and Blaxter 2006; Werren, et al. 2008). In particular, more than half of the arthropod species are infected by *Wolbachia* (Hilgenboecker, et al. 2008; Zug and Hammerstein 2012), possibly representing a dynamic equilibrium between gain and loss on a global scale (Werren and Windsor 2000; Bailly-Bechet, et al. 2017; Klopfstein, et al. 2018). The *Wolbachia*-host interaction generally spans a range from reproductive parasitism to mutualism. *Wolbachia* can alter the host reproduction to enhance their own transmission in different ways, such as feminization of genetic males, male-killing, parthenogenetic induction, and cytoplasmic incompatibility (Stouthamer, et al. 1999; Werren, et al. 2008). Other effects of *Wolbachia* can include viral suppression (Hedges, et al. 2008), and nutritional mutualism (Hosokawa, et al. 2010). In nematodes, *Wolbachia* appear to have evolved a long-standing mutualistic relationship (Fenn and Blaxter 2006). *Wolbachia* strains have been found to move between species by horizontal (infectious) transmission, even between distantly related hosts, and by hybrid introgression between closely related species. (Werren and Wan 1995; Heath, et al. 1999; Raychoudhury, et al. 2009). *Wolbachia pipientis* have been divided into supergroups (A-H) based on 16S ribosomal RNA sequences and other sequence information, including six supergroups (A,B and E-H) primarily identified in arthropods and two supergroups (C and D) commonly found in filarial nematodes (Werren, et al. 2008). However, it has been proposed that supergroup G be decommissioned, as it is based primarily on recombinant *wsp* sequences and cluster with A supergroup based on five multi-locus strain typing genes (Baldo, Dunning Hotopp, et al. 2006; Baldo and Werren 2007), eight supergroups (A- H) are still widely used in the research community. A multi-locus strain typing (MLST) system based on five house-

keeping genes, (*coxA*, *gatB*, *hcpA*, *ftsZ* and *fbpA*) has been developed for *Wolbachia* (Baldo, Dunning Hotopp, et al. 2006), and is widely used for strain typing and to characterize strain variation within *Wolbachia*. However, the increasing number of genome sequences for *Wolbachia* allows for more detailed characterization of their diversity, including inter-strain recombination events.

Genomic studies of *Wolbachia* started with the first complete genome of the A-*Wolbachia* parasite of *Drosophila melanogaster* (*w*Mel) published in 2004 (Wu, et al. 2004), and followed by the complete genome of D-*Wolbachia* (*w*Bm) in nematode *Brugia malayi* in 2005 (Foster, et al. 2005). Many more genomes have been published in the last decade, and a list of sequenced whole genomes of *Wolbachia* is summarized in Supplemental Table S1.

Because of its endosymbiotic nature, multiple different *Wolbachia* strains can be present in the same host cells, allowing the potential for homologous recombination between strains (Jiggins, et al. 2001; Jiggins 2002). Studies have observed recombination across strains and supergroups (Werren and Bartos 2001; Baldo, et al. 2005; Duron, et al. 2005), which may be mediated by bacteriophages and lead to mosaic genomes in *Wolbachia* (Klasson, et al. 2009; Kent, et al. 2011; Duplouy, et al. 2013). Although co-infection of different strains exist in the same arthropod host, with recombination particularly in associated phage (Chafee, et al. 2009), the supergroups may still remain genetically distinct clades (Ellegaard, et al. 2013). Recombination events in *Wolbachia* have been discovered in *Wsp* (Werren and Bartos 2001) and other genes in Crustaceans (Verne, et al. 2007), mites (Ros, et al. 2012) and various arthropod species (Werren and Bartos 2001; Reuter and Keller 2003; Baldo, et al. 2005; Baldo, Bordenstein, et al. 2006; Ilinsky and Kosterin 2017). No inter-strain recombination has been reported in the filarial nematode *Wolbachia* strains (Foster, et al. 2011).

Most of the previous research on recombination has focused on five MLST genes, *Wolbachia* surface protein (*wsp*), and 16S rRNA, or for a subset of genomes from the A-D and F supergroups (Lindsey, et al. 2016). Therefore, whole-genome analyses in a large number of *Wolbachia* strains of all supergroups are needed to identify additional homologous recombination events among *Wolbachia* across the different supergroups. In this study, we performed phylogenomic analyses on 33 annotated *Wolbachia* genomes, and analyzed the individual gene trees to identify potential recombination events across the supergroups. Relatively low frequencies of inter-supergroup recombination events were found, indicating a general genetic cohesiveness of supergroups. However, between supergroup recombination is still evident, and could play a role in *Wolbachia* adaptation.

Results

Phylogenomic analysis of annotated *Wolbachia* genomes

To identify a core gene set for phylogenomic analysis of *Wolbachia* strains, we initially compared 34 publicly available and annotated *Wolbachia* genomes as of November 2019, which include sixteen A-group, twelve B-group, two C-group, two D-group and one for E and F-group strains from diverse host species (Supplemental Table S1). Single-gene ortholog clusters were generated using the procedure described in the Methods. A total of 210 single-gene ortholog clusters (listed in Supplemental Data S1) were identified that are shared among the 34 *Wolbachia* genomes. This is a smaller set than the 496 *Wolbachia* gene orthologs detected in (Lindsey, et al. 2016) for 16 *Wolbachia* strains, but ours included a larger strain set (34 *Wolbachia* strains), and we restricted our analysis to single-copy orthologs across all of the genomes.

Based on the concatenated coding nucleotide and protein sequences of this core gene set, Maximum Likelihood (ML) phylogenetic trees of 34 *Wolbachia* genomes confirmed the separation of different supergroups A (*w*Suzi, *w*Spc, *w*Ri, *w*Ha, *w*Au, *w*Mel, *w*MelPop, *w*Gmm, *w*Uni, *w*DacA, *w*Nfe, *w*Npa, *w*Nfla, *w*Nleu, *w*VitA, *w*OneA1), B (*w*AlbB, *w*Stri, *w*Di, *w*No, *w*Tpre, *w*DacB, *w*VitB, Ob_Wba, *w*Bol1, *w*Pip_Mol, *w*Pip), C (*w*Oo, *w*Ov), D (*w*Bm, *w*Wb), E (*w*Fol) and F (*w*Cle) with 100% bootstrap support (Supplemental Figure S1, Data S2 and S3). One of the B-*Wolbachia*, *w*Con, appeared to be phylogenetically distant from other B strains (Supplemental Figure S1). However, its genome size is 2.11Mb, almost double the B-*Wolbachia* average (1.288 Mb). Further examination of the genome assembly suggested *w*Con is potentially a mixed assembly of one A and one B *Wolbachia* genomes. Therefore, we excluded *w*Con and reconstructed the ML tree using the rest 33 *Wolbachia* nucleotide sequences (Figure 1,

Supplemental Data S2 and S3). For comparisons of nucleotide and protein phylogenies, we also constructed an ML phylogenetic tree of concatenated protein sequences from these core genes using RAxML (Stamatakis 2014). The protein ML phylogenetic tree (Supplemental Figure S2, Data S4 and S5) matched well with the nucleotide coding sequence ML tree, having the same 100% bootstrap for the same clades in A and D supergroups, and a very few variations in bootstrap values but same clustering patterns in other supergroups. (Figure 1).

As expected, our genomic analyses support extensive horizontal movement of *Wolbachia* strains between divergent host species. For example, *w*OneA1, which is an A-supergroup bacterium in the parasitoid *Nasonia oneida* (Wang, et al. 2019) is more closely related to a subset of A-*Wolbachia* found in *Drosophila* (*w*Ha, *w*Ri, *w*Spc and *w*Suzi) than to *w*VitA and *w*Uni in closely related parasitoid wasps. This pattern was previously observed using MLST genes in *Wolbachia* (Raychoudhury, et al. 2009), but is now supported by a much larger data set. The B-supergroup mosquito *Wolbachia w*Alb in *Aedes albopictus* gives another example of obvious major host shift (Figure 1 and Figure S3).

Identification of inter-supergroup recombination events

Our focus in this study is to evaluate between supergroup recombination in *Wolbachia*. We therefore developed a prescreening method to detect candidate between supergroup recombination events, and applied it to the 210 single-copy ortholog gene set. For each *Wolbachia* strain on an individual gene nucleotide tree, we computed the branch length distance to all other strains. We defined recombination candidates if their the nearest neighboring strain belongs to a different supergroup based on the concatenated gene tree (see Methods). The interclade recombination score (IR score) can range from 0 to 100. This method works for detecting recombinants within supergroups containing more than one genome sequenced strain

(see Methods). Five genes with IR>65 were chosen as the cutoff for further investigation. We identified recombination events between A and B, and A and E supergroups. We also examined all 210 RAxML gene trees with both the corresponding protein and nucleotide sequence alignments. Both tree topologies and bootstrap values support the recombination events detected by the screening method (Supplemental Data S3 and S5). One additional recombination event was found for an A group strain that contains an E group version *dnaK* gene (Table 1).

A total of 5 genes (2.4%) with 9 recombination events were identified between A and B supergroups, including B-supergroup genes *FtsH* (ATP-dependent metalloprotease) and *rplU* (50S ribosomal protein L21) in A-supergroup strains *w*Au (Figure 2A) and *w*DacA (Figure 2B) respectively (B-in-A events in Table 1), and 7 A-in-B recombination events in *coxB* (cytochrome c oxidase subunit II), *WONE_04820* (hypothetical protein) and *argS* (arginine-tRNA ligase) (Table 1 and Supplemental Table S2). GARD algorithm (Kosakovsky Pond, et al. 2006) was used to detect intragenic recombination in these events and identify recombination breakpoints if intragenic recombination is involved. Two breakpoint positions among the identified genes were detected by GARD, including one breakpoint position at 816 bp in *ftsH* gene with a *P-value* of 0.0002, another breakpoint position at 561 bp in *argS* gene with a *P-value* of 0.0006.

Here we describe the recombination events in more detail. There are two cases of A-*Wolbachia* strains that contain a B-*Wolbachia* gene transfer. For *ftsH*, the A-*Wolbachia w*Au strain gene clusters with B-*Wolbachia* strains with an IR score of 99.9, and this recombination event is supported in the nucleotide tree with a bootstrap value of 100 (Figure 2A, Supplemental Data S3 and S5). As a universally conserved gene in bacteria, *ftsH* is known to be crucial for the proteolytic degradation of specific integral membrane proteins and cytoplasmic proteins, and it

also targets soluble signaling factors like heat-shock sigma factor σ32 and transcriptional activator λ-CII (Wolfgang 1999). A second B into A recombination event involves a B-group *rplU* gene that has inserted into the A-*Wolbachia* *w*DacA (IR = 71), which is also supported with a bootstrap value of 100 in the corresponding nucleotide tree (Figure 2B, Supplemental Data S3 and S5). Less is known about the function of *rplU*, except for its interaction with 23S rRNA (Vladimirov, et al. 2000).

Three additional genes reveal recombination events of individual A-*Wolbachia* genes into B-*Wolbachia* strains. The *coxB* gene from an A-*Wolbachia* was transferred to B-*Wolbachia* *w*AlbB (IR =92), supported by the corresponding nucleotide and trees with a bootstrap value of 99 (Figure 3A, Supplemental Data S3 and S5). The *coxB* protein is a component of the electron transport chain which drives oxidative phosphorylation. The second case of an A to B transfer involves the hypothetical protein *WONE_04820* gene. An A-*Wolbachia* gene is present in three B-*Wolbachia* strains *w*Di, *w*AlbB and *w*Tpre (IR = 91, 83 and 67, respectively). The corresponding nucleotide tree supports the general pattern with a bootstrap value of 74 (Figure 3B, Supplemental Data S3 and S5). Based on the concatenated tree topology, it is difficult to resolve whether these indicate a single or independent transfer events, given that the three strains are not monophyletic within the B supergroup (Figure 1). The function of this gene is currently unknown.

In each case for the above examples, the complete gene was recombined into a different supergroup. However, recombination events can also occur within genes, as has been documented for the highly recombinogenic *wsp* gene (Baldo et al 2005). For *argS*, we found evidence for intragenic recombination (Figure 4A and 4B, Supplemental Data S3 and S5), with significantly different topologies between the 5' region (positions 1-561 bp) compared to the rest

of the gene (positions 562-1707 bp). Intragenic recombination is supported by GARD, which identified the breakpoint at 561 (*P*-value = 0.0006). As a member of the class I aminoacyl-tRNA synthetase family, expression of *argS* is reported to increase the aminoacyl-tRNA synthetase activity in bacteria (Oguiza, et al. 1993). In addition, there is also an apparent A-B recombinant event in the *coxB* gene of *w*DacA based on a stretch of 5 A-B diagnostic SNPs (position 151, 194, 226, 245 and 285 in Figure 3C).

For the E supergroup there is only one released genome (*w*Fol). Nevertheless, we also found some evidence for recombination events between A and this single representative of the E supergroup. For instance, *w*Fol genes cluster with A-*Wolbachia* in *coxB*, *WONE_04820* and *argS* (Figure 3A, 3B and 4A, Supplemental Data S3 and S5). Given the high similarity among most sequenced A-*Wolbachia*, it is not possible to confidently identify which is the likely source. In addition, there appear to be two E-group genes that have transferred into the A-*Wolbachia* strain *w*DacA, *argS* and *DnaK* (Figure 4B and 5; Supplemental Data S3 and S5). A better understanding of the evolutionary history of these transfers will be gained with additional E supergroup genome sequences.

Taken together, 97% of the single copy orthologs agree with the supergroup classification in *Wolbachia***,** with a few cases of likely recombination events between *Wolbachia* strains of different supergroups. The recombination between A and B supergroups in gene *coxB* was reported by a previous study of 6 *Wolbachia* strains (Ellegaard, et al. 2013), and the remaining identified inter-supergroup recombination events are novel findings in our study. The finding also indicates that these recombination events involve relatively small regions, rather than large recombination events involving many genes. The frequent gene order rearrangements observed

in *Wolbachia* may make larger recombination tracks between supergroups less successful, as they are more likely to involve vital gene losses due to lack of synteny.

Concordance of MLST genes and whole genome divergence

The MLST system (Baldo, Dunning Hotopp, et al. 2006) has been variously used for strain typing of *Wolbachia*, identification of related strains, recombination within genes (e.g. the *wsp* locus) and for phylogenetic inferences among strains. Recently, reliability of the MLST system has been criticized (Bleidorn and Gerth 2018), with whole genome sequencing stated to be preferred. Although whole genome data sets would always be desirable, the number of *Wolbachia* whole genome sequences is small compared to the many hundreds of MLST sequences currently available for comparative analyses. We therefore undertook to compare genetic divergence based the MLST to our set of 211 genes in 34 different *Wolbachia* strains.

The MLST performed very well in both identifying closely related strains and in genetic divergence among strains compared to the genome wide data set. The Pearson correlation coefficient of estimated evolutionary divergence with core gene set and *gatB*, *fbpA*, *hcpA*, *coxA* and *ftsZ* is 0.96, 0.9, 0.97, 0.92 and 0.97, respectively with *P*-value < 2.2×10^{-16} (Table 1, Supplemental Data S6). The Pearson correlation coefficient of estimated evolutionary divergence with core gene set and the concatenated MLST set is 0.98 with *P*-value < 2.2×10^{-16} (Figure 6). Therefore, MLST is a reliable method for strain identification and close relationships among strains, even when similar strains occur in very different hosts, such as *w*DacB in a hemipteran (a true bug) and *w*VitB in a hymenopteran (a parasitoid wasp), or *w*Bol1 in a lepidopteran (butterfly) and *w*Pip in a dipteran (a mosquito) (Figure 1 and Figure S3). Eventually, whole genome data sets will supplant the MLST system. However, with over 1900 isolates in the *Wolbachia* MLST database, this will likely take some time, and until then, MLST remains a

reliable method for identifying closely related *Wolbachia* strains and their host associations. Furthermore, closely related *Wolbachia* strains identified by MLST, that differ in host type of phenotypic effects on hosts (e.g. cytoplasmic incompatibility, feminization, male-killing, parthenogenesis, viral suppression), can be used for targeted whole genome sequencing to reveal possible mechanisms involved in host and phenotypic shifts.

Discussion

The phylogenomic analysis of 33 annotated *Wolbachia* genomes in our study is the most comprehensive phylogenomic and evolutionary analysis conducted in *Wolbachia* strains to date. By including almost all available *Wolbachia* genomes in NCBI, we confirmed at the genome level that these *Wolbachia* strains group into distinct clusters (A, B, C, D, E, F supergroups) and different *Wolbachia* co-infected in the same host kept strain boundaries (Ellegaard, et al. 2013). 204 of the 210 single gene trees are consistent with the strain tree. Six gene trees have major rearrangements among *Wolbachia* groups (Figures 2-5), indicating potential recombination events between strains. We estimated that recombination events between supergroups occurred in at least 2.9% of the core genes in the *Wolbachia* genomes, and recombination may be one of the evolutionary forces shaping the *Wolbachia* genomes.

In total, there are a total of 14 recombination events detected in six genes. Nine of these involve A-B recombination in five genes. The five genes with distinct tree structure differences from the consensus *Wolbachia* tree include *ftsH*, *rpIU*, *coxB*, *hypothetical protein WONE_04820* and *argS*. In addition, five events were detected between the A and E supergroups. Most recombination events involved the entire gene, whereas a single intragenic event was found in *argS*. A second intragenic event may also be present in *coxB* (Figure 3C) although it was not detected by the Interclade Recombination or GARD methods. We conclude that inter-supergroup recombination is uncommon among the set of 210 core single ortholog genes used in this study. Recombination may be more frequent in other genes, and clearly is so in phage associated genes (Bordenstein and Bordenstein 2016; Wang, et al. 2016) and the surface protein *wsp* (Baldo, Bordenstein, et al. 2006). Furthermore, within supergroup recombination is also

likely to be more common, although also more difficult to quantify due to the greater similarity within these groups.

Among the 14 recombination events observed, *argS* (5 events) and *WONE_04820* (4 events) appear to be particularly prone to inter-supergroup recombination (Table 1). *argS* is a class I aminoacyl-tRNA synthetases which catalyzes the ligation of arginine to its transfer RNA, while the function of *WONE_04820* is not clear. *WONE_04820* is conserved in *Wolbachia* and no known functional domains could be identified. In addition, two bacterial strains appear to be more prone to inter-supergroup recombination (*w*DacA and wDi). Notably, both are found in hemipterans. More sequencing of *Wolbachia* from different insect orders is needed, as the current set are predominantly from Diptera and Hymenoptera.

Recombination events among A and B *Wolbachia* supergroups have been documented in previous studies, and we identified addition cases through the phylogenomic analysis among 33 sequenced genomes. Interestingly, we also discovered recombination events between A and E supergroups, which was not known previously. The E group *Wolbachia* is found in springtails (Vandekerckhove, et al. 1999; Czarnetzki and Tebbe 2004; Fountain and Hopkin 2005). A recent study characterized the *Wolbachia* in 11 collembolan species by MLST, and found that nearly all are E group *Wolbachia* that are monophyletic, based on phylogenetic reconstruction using MLST genes (Ma, et al. 2017). Our genome analysis of the single collembolan *Wolbachia* genome reveals a number of candidate recombination events, including intergroup recombination between A and E in *coxB*, *dnaK*, *WONE_04820* and *argS*. Targeted sequencing of these genes in the additional collembolan species or additional genome sequencing will help reveal the origins and directions of these events. We further speculate that selective maintenance of such transfers could suggest a possible role in E *Wolbachia* function, such as parthenogenesis induction found

in this springtail (Ma, et al. 2017). The focus of this study has been on recombination between supergroups, where the phylogenetic signal to noise ratio is much stronger. However, although more difficult to document, intra-supergroup recombination is likely to be more extensive than between supergroups, and is a topic worthy of future study.

It has been recently argued that MLST genotyping has little utility in phylogenetic analyses, and should be supplanted by genomic studies (Bleidorn and Gerth 2018). When the MLST system was developed, it was pointed out by the authors that the system would be most useful for identifying relatively closely related *Wolbachia*, due to potential recombination among more divergent strains (Baldo and Werren 2007). However, our comparison on genome sequence indicates that MLST typing is largely valid, both for supergroup identification and detection of closely related strains. Related *Wolbachia* based on MLST results are also closely related in the genome-wide analysis. This suggests that, until *Wolbachia* genome sequencing becomes much less expensive and can be readily performed on single arthropods, that MLST will remain a useful tool for identification of strains, their relationships, and host affinities. Nevertheless, caution should be exercised due to some documented recombination events within MLST genes and among them (Raychoudhury, et al. 2009). Therefore, topologies should be compared among genes for evidence of discordance, rather than simply relying of phylogenetic reconstructions of concatenated sequences.

Methods

Phylogenomic analysis of annotated *Wolbachia* genomes

To examine the phylogeny of *Wolbachia* at the genome level, we conducted phylogenomic analysis using 34 annotated *Wolbachia* genomes (GenBank accession numbers and reference papers listed in Table S1). Homologous genes and ortholog clusters among all 34 *Wolbachia* genomes were determined by using OrthoFinder v1.1.8 (Emms and Kelly 2015) with default settings. 210 single-copy ortholog groups were identified, and gene IDs in each of the ortholog groups were used to extract the corresponding nucleotide and protein sequences from 34 *Wolbachia* genomes. The 210 core single-copy genes in all 34 *Wolbachia* genomes were aligned with MAFFT (Katoh and Standley 2014) at the protein sequence level. PAL2NAL (Suyama, et al. 2006) was used to check the consistency between the nucleotide and protein sequences, and all inconsistent nucleotide sequences downloaded from GenBank were manually corrected. 210 core single-copy genes were identified for the subsequent analysis, their accession numbers are listed in Supplemental Data S1. These single-gene alignments were concatenated into one alignment to use in the subsequent phylogenetic analysis. A Maximum Likelihood (ML) tree was constructed with the GTRGAMMA model and 1,000 bootstrap replicates by RAxMLv8.2 (Stamatakis 2014) using the concatenated nucleotide sequence alignment of the core gene set. For phylogenetic analysis of protein sequences from the core gene set, the best-fit model of protein evolution was searched by ProtTest 3 (Darriba, et al. 2011). The final ML phylogenetic tree was inferred by using RAxML v8.2 (Stamatakis 2014) with the FLU protein model (best-fit model identified by ProtTest 3) and 1,000 rapid bootstrap replicates.

The single gene ML trees for all 210 core genes were constructed with their corresponding nucleotide sequence alignments using the GTRGAMMA model and 1,000

bootstrap replicates by RAxML v8.2 (Stamatakis 2014). We also constructed protein trees for these identified genes with their corresponding protein sequence alignments using the best fit protein model detected by ProtTest 3 (Darriba, et al. 2011) and 1,000 rapid bootstrap replicates by RAxML v8.2 (Stamatakis 2014). The gene trees and protein trees were visualized using FigTree v1.4.4 (Rambaut 2018). For better viewing of short branches, transformation and rerooting were performed in FigTree to generate the main figures. The original gene trees were shown in Supplemental Figure S4.

Identification of individual gene trees with intergroup recombination events

To search for interclade recombination events, we developed a prescreening tool for the identification of specific gene/protein recombinants that move a particular gene/protein outside its respective supergroup. Based on the concatenated strain phylogeny, we assign a supergroup identity for each strain. For every gene, we calculate the branch length between all strain combinations, and then determine the nearest neighbor based on shortest branch length. Candidate recombination events are then identified as those for which the nearest neighbor is in a different supergroup. Because some supergroups only have a single representative, the method is most effective at finding candidate recombination to or from A, B, C, and D supergroups. Next, an Interclade Recombination (IR) score was used to quantify the degree of divergence of the gene from its strains' supergroup. The distance from the candidate gene to its nearest neighbor (Nn) is compared to the average interclade distance (IC) distance of the recombination candidate gene to other members of its strain's supergroup (based on the concatenated phylogeny) using the IR metric below.

$$\textbf{IR score} = \left(1 - \frac{Nn}{IC}\right) \times 100$$

An IR score can range from 0 to 100, with a larger score indicating a recombination between supergroups.

We further manually compared gene trees in both nucleotide and protein level as follows: 1) the nucleotide ML trees were compared to the concatenated ML tree to manually confirm the recombination events; 2) nucleotide sequence alignments were further inspected for informative SNPs that separate different supergroups; 3) the single-gene protein trees were also compared to the concatenated protein tree to check for consistency of supergroup classification. Inference of intragenic recombination events and the breakpoints was conducted on nucleotide sequence alignments using the GARD algorithm (Kosakovsky Pond, et al. 2006) with default parameters using the datamonkey web server (http://www.datamonkey.org/). The individual trees with potential recombination events were defined as trees with IR score larger than 65 for A/B recombination. Three additional candidate genes are all less than 60. We inspected the trees and found they are not interclade recombination events. The A/E recombination was identified by manual evaluations as only one species is available in E supergroup.

Phylogenetic analysis of *Wolbachia* in *Nasonia* using MLST genes.

The five MLST (Multi Locus Sequence Typing) genes (Baldo, Dunning Hotopp, et al. 2006; Jolley and Maiden 2010) were examined to further characterize the phylogenetic relationships of *Wolbachia* strains in *Nasonia*. These genes include *gatB* (aspartyl/glutamyl-tRNA (Gln) amidotransferase, subunit B), *coxA* (cytochrome c oxidase subunit I), *hcpA* (conserved hypothetical protein), *ftsZ* (cell division protein) and *fbpA* (fructose-bisphosphate aldolase). The pairwise evolutionary divergence distances between 33 *Wolbachia* species were estimated with both the core gene set identified in this study, five MLST genes and the concatenated sequence of these five MLST genes in 33 *Wolbachia* species by using the

Maximum Composite Likelihood model (Tamura, et al. 2004) in MEGA7 (Kumar, et al. 2016). Estimates of evolutionary divergence using the *ftsZ* gene were only conducted among 31 *Wolbachia* species, excluding *w*Bm and *w*Wb, because of the inability to correctly annotate *ftsZ* in these species. The Pearson correlation coefficient of estimated evolutionary divergences with the core gene set and the MLST gene set (each MLST gene and the concatenated sequence of five MLST genes) was calculated with Hmisc package (Harrell Jr and Harrell Jr 2019) in R.

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Data Availability Statement

The data underlying this article are available in the Dryad Digital Repository, at https://doi.org/10.5061/dryad.kg87554.

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Table legends

Table 1. List of 6 *Wolbachia* genes with interclade recombination events.

Table 2. Correlation of evolutionary divergence estimates between *Wolbachia* species using 210 core gene set and five MLST genes.

Figure legends

Figure 1. Phylogenomic relationships of 33 *Wolbachia* strains.
The phylogenetic tree was constructed using Maximum Likelihood method from a concatenated nucleotide sequence alignment of 210 single-copy orthologous genes among 33 genome-sequenced *Wolbachia* strains. Numbers on the branches represent the support from 1,000 bootstrap replicates. Branch transformation and rerooting were performed in FigTree 1.4.4. The assembly names were color-coded based on supergroup identity (A-F). Host taxonomic classifications and species common names were labeled.

Figure 2. Inter-supergroup recombination events of B supergroup genes *fstH* and *rplU* in A-*Wolbachia* strains.

(***A-B***) Nucleotide ML trees reveal interclade recombination events, in which genes from an A-*Wolbachia* clusters with B supergroup. The supergroup identities are labeled using the same color code as in Figure 1. Bootstrap values greater than 50 are shown in the figure. (***C-D***) Super group informative SNP positions are plotted for all strains (green: A; blue: C; yellow: G; pink: T). These SNPs showed the general pattern of recombination, whether entire genes between clades or between clade recombination within genes.

Figure 3. Inter-supergroup recombination events of A supergroup genes *coxB* and *WONE_04820* in B-*Wolbachia* and E-*Wolbachia* strains.

(***A***) Nucleotide ML trees reveal interclade recombination events, in which *coxB* genes from *w*AlbB (B-*Wolbachia*) and *w*Fol (E-*Wolbachia*) cluster with A supergroup, with a bootstrap support of 99. (***B***) Nucleotide ML trees reveal interclade recombination events, in which hypothetical protein *WONE_04820* from *w*AlbB, *w*Di, *w*Tpre, (B-*Wolbachia*) and *w*Fol (E-*Wolbachia*) clusters with A supergroup, with a bootstrap support of 74. (***C-D***) Super group informative SNP positions are plotted for all strains (green: A; blue: C; yellow: G; pink: T). Bootstrap values greater than 50 are shown in the figure.

Figure 4. Intragenic recombination event between supergroups in *argS* gene.

Intragenic recombination event was detected by the GARD method in *argS* (*P*-value = 0.0006), and the inferred breakpoint is at 561 bp position in this gene. (***A-B***) Nucleotide ML trees for the 5' region (1-561 bp) and 3' region (positions 562-1707 bp), respectively. *argS* genes from *w*Di, *w*No, (B-*Wolbachia*) and *w*Fol (E-*Wolbachia*) cluster with A supergroup (***A***).

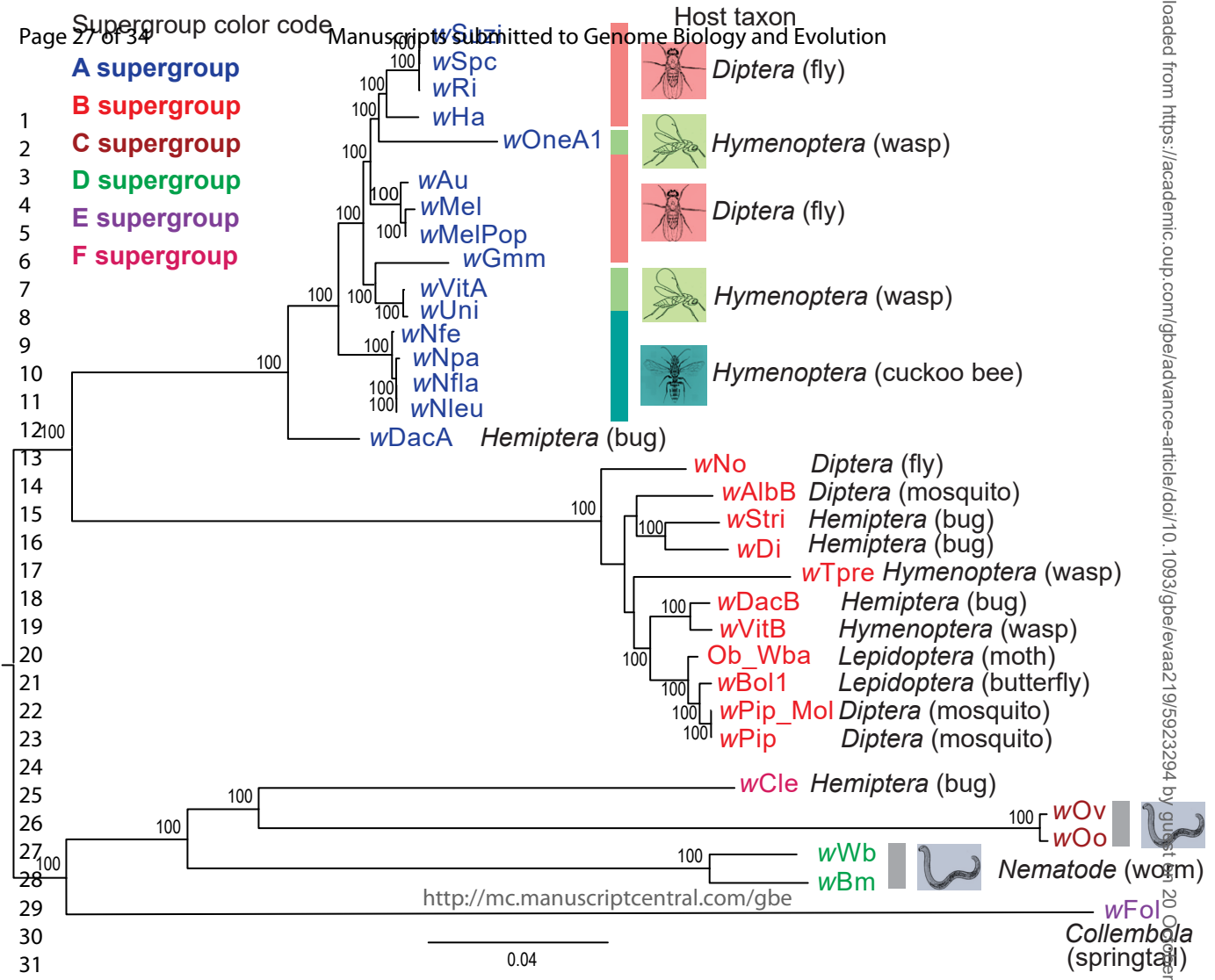
The supergroup classifications follow the color code in previous figures. Bootstrap values above 50 are shown in the figure. (A) *argS* from starting site to 561 bp, B-*Wolbachia* *w*Di, *w*No and E-*Wolbachia* *w*Fol cluster with A supergroup with 19 bootstrap support; whereas (B) *argS* from 562 bp to stop site, *w*Di (B-*Wolbachia*) clusters with A supergroup with 79 bootstrap support, and *w*DacA (A-*Wolbachia*) clusters with E-*Wolbachia* with 35 bootstrap support, indicating intragenic recombination events; (C) Nucleotides at selected positions (1-561 bp in *argS*) support the tree topology in (A); (D) Nucleotides at selected positions (562-1707 bp in *argS*) supported the tree topology in (B).

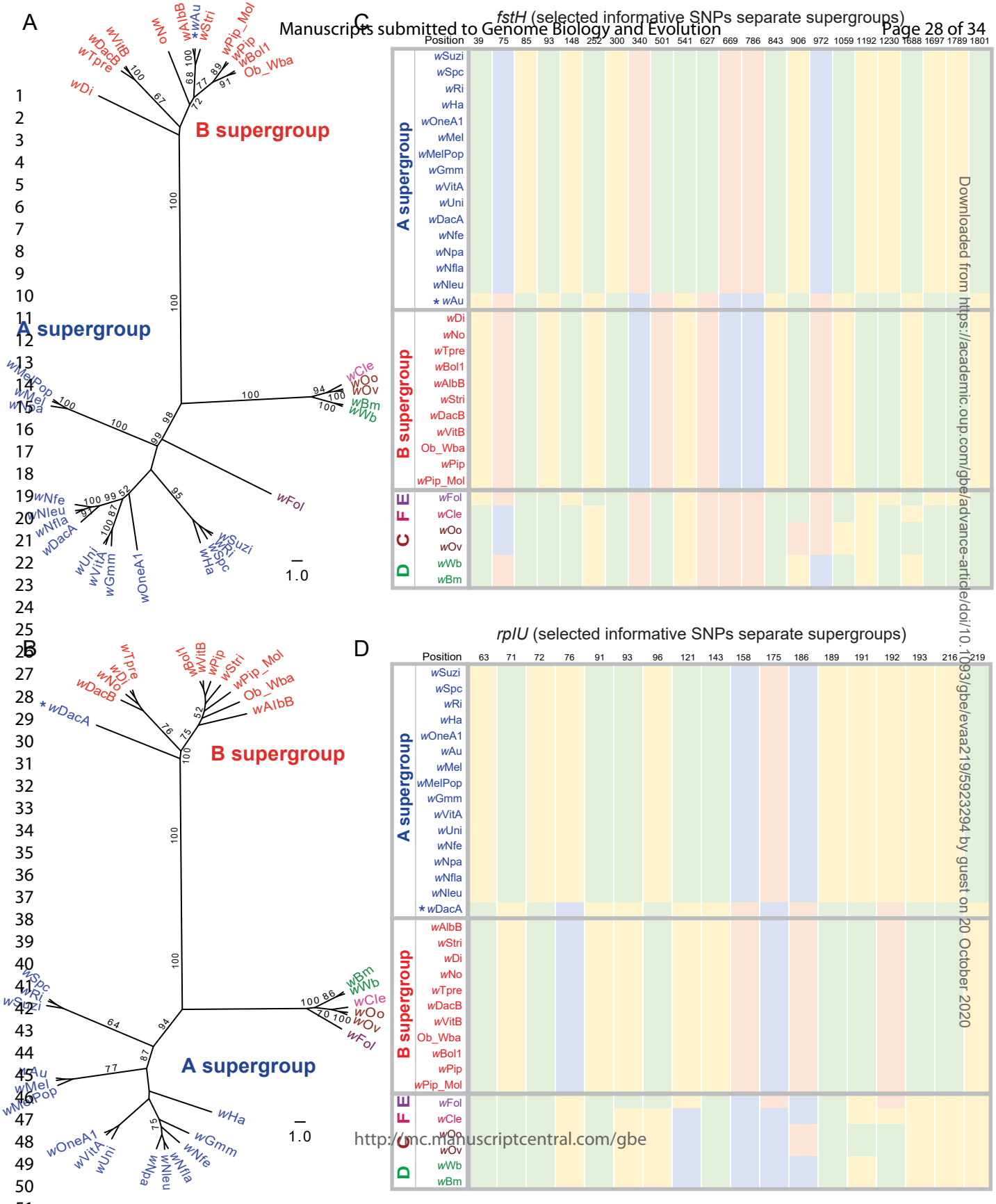
Figure 5. The nucleotide ML tree reveals recombination event where A-*Wolbachia* cluster with E-*Wolbachia* in *dnaK* gene.

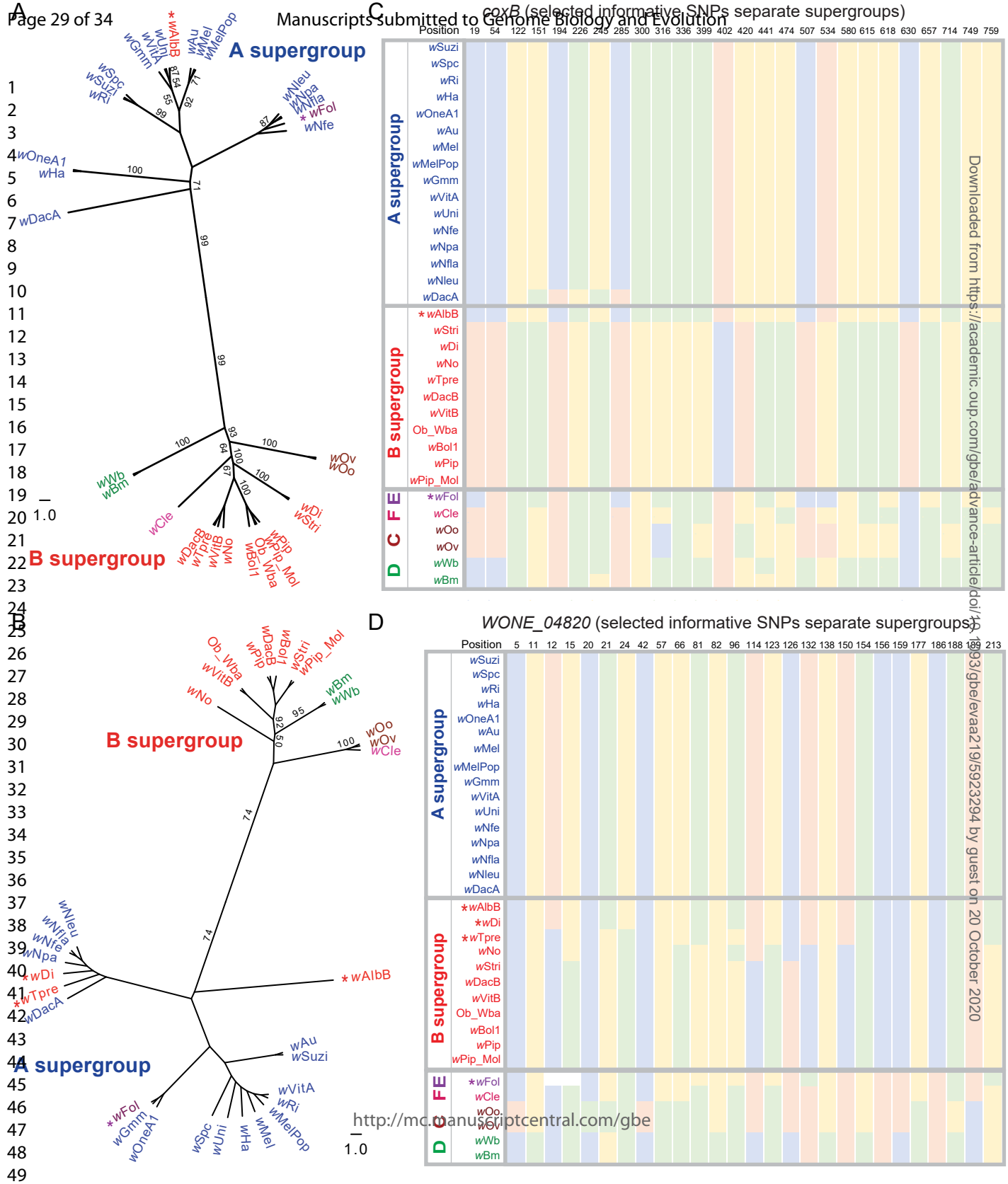
The supergroup classifications follow the color code in earlier figures. Bootstrap values above 50 are shown in the figure. Nucleotides at selected positions are shown in the right panels. *w*DacA (A-*Wolbachia*) clusters with *w*Fol (E supergroup) with 90 bootstrap support.

Figure 6. Correlation of evolutionary divergence estimated by core gene set and the five concatenated MLST genes.

Pearson correlation coefficient = 0.98, *P*-value < 2.2 x 10^{-16}.



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B supergroup
wAlbB
wStri
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wBol1
wPip
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wBm
wWb
wOo
wOv
wCle
wAu
wSuzi
wSpc
wRi
wMel
wMelPop
wGmm
wVitA
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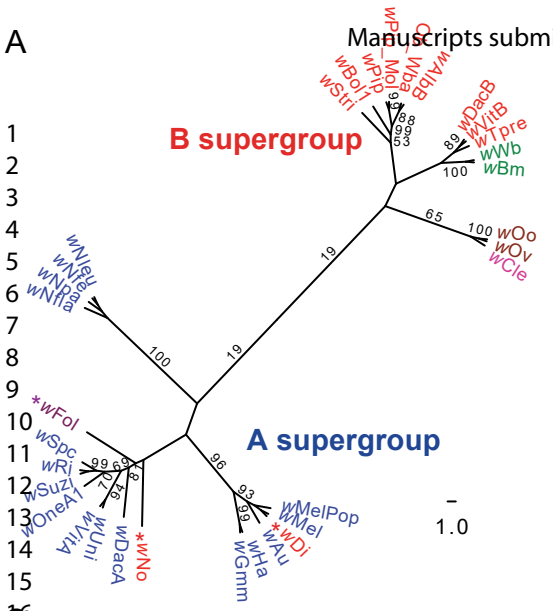

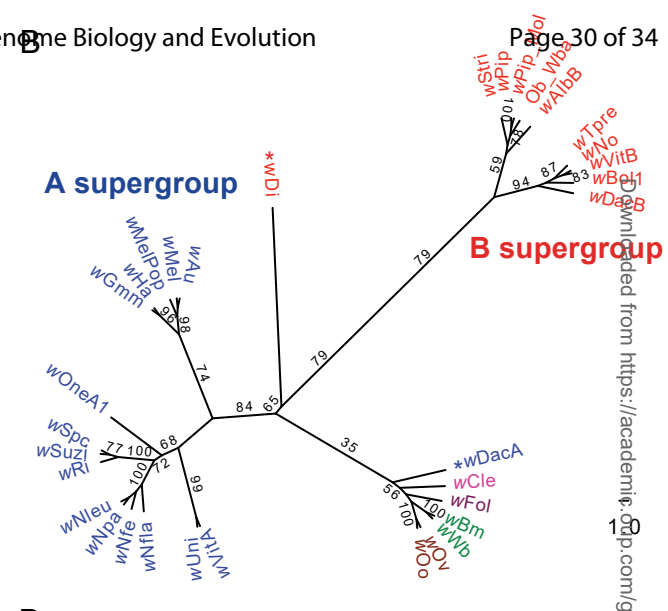

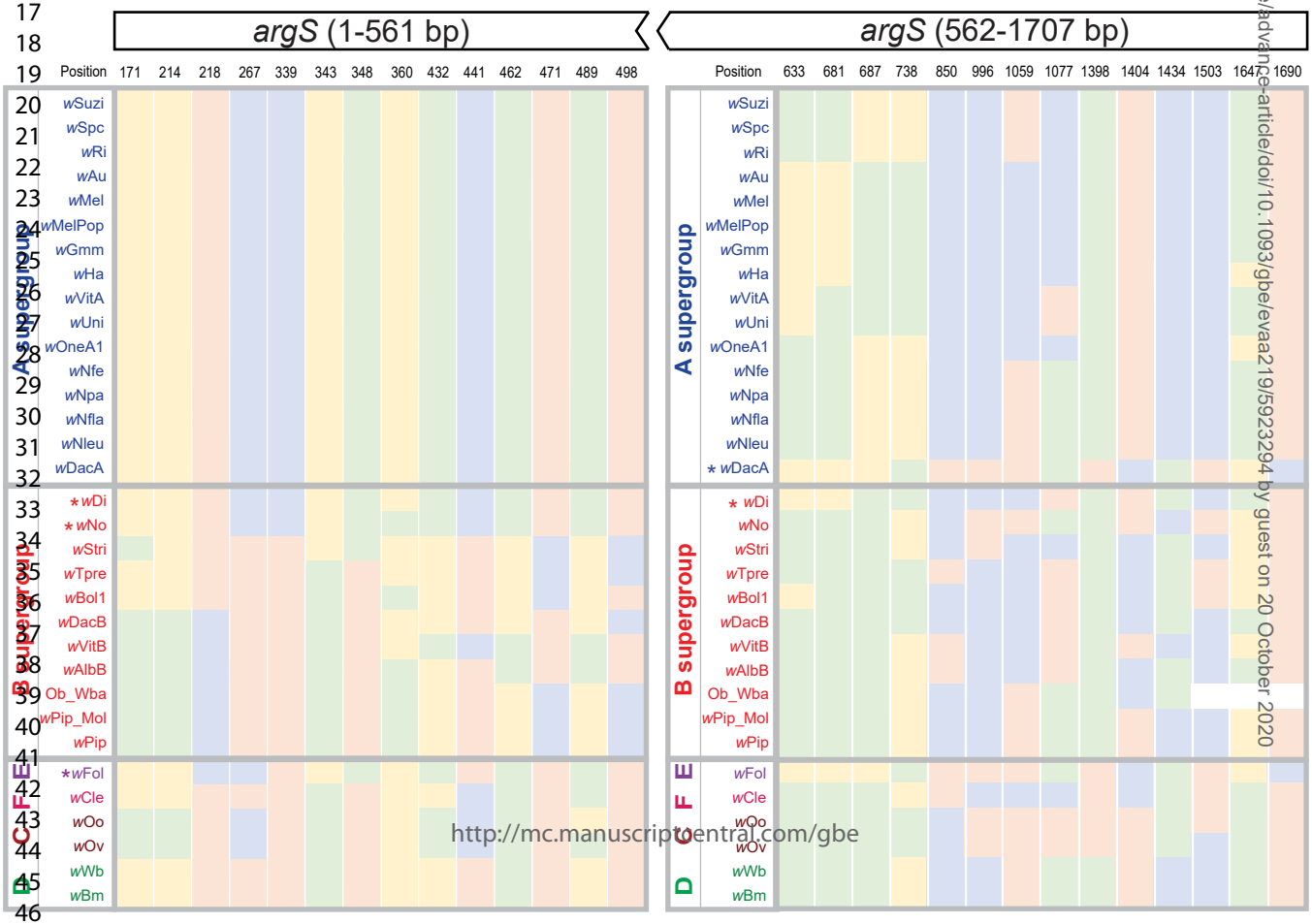

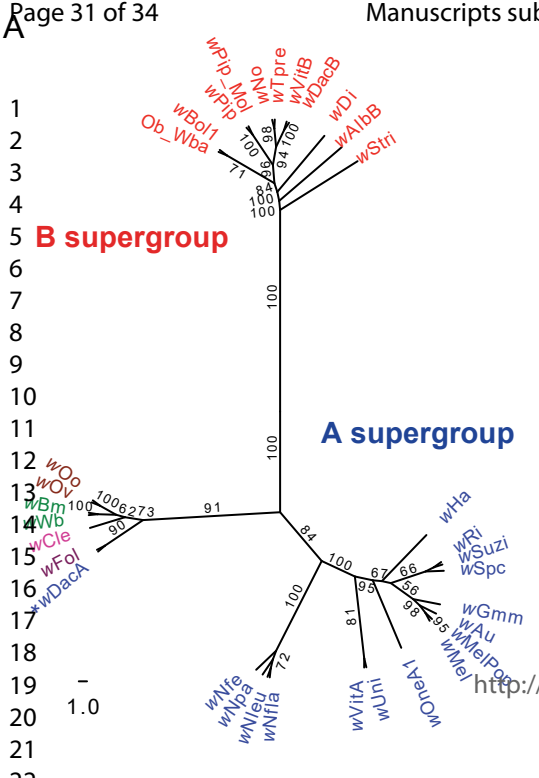

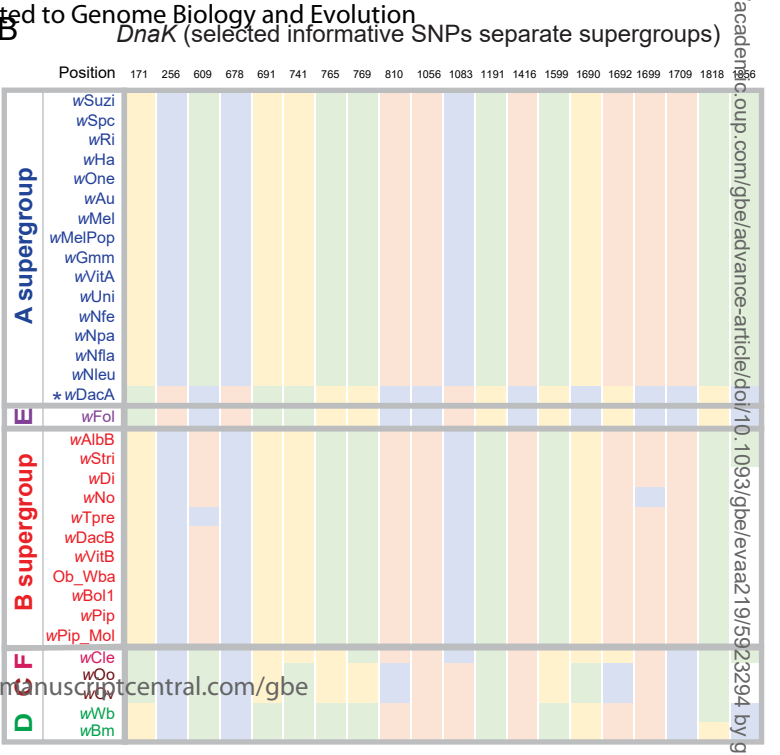

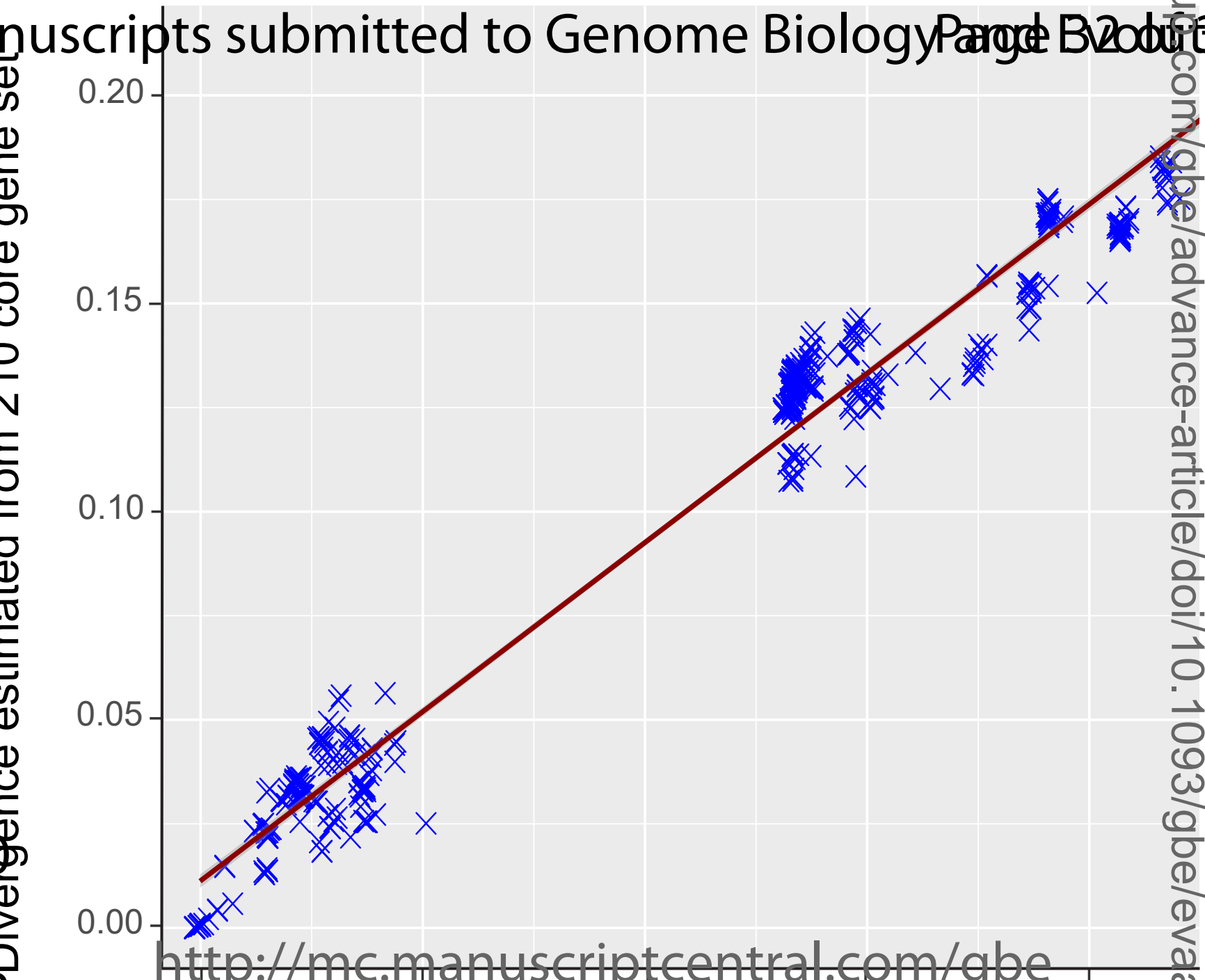

Table 1. List of 6 *Wolbachia* genes with interclade recombination events.

Gene name	Gene description	Intragenic recombination breakpoint	Species with interclade recombination	Interclade recombination score	Nucleotide tree shown in
ftsH	ATP-dependent metalloprotease FtsH	816 bp, *P*=0.002	*w*Au (B-in-A)	99.9	Figure 2A
rplU	50S ribosomal protein L21	None	*w*DacA (B-in-A)	71.0	Figure 2B
coxB	cytochrome c oxidase subunit II	None	*w*AlbB (A-in-B) *w*Fol (A-in-E)	92.3 NA	Figure 3A
WONE_04820	hypothetical protein	None	*w*Di (A-in-B) *w*AlbB (A-in-B) *w*Tpre (A-in-B) *w*Fol (A-in-E)	91.3 82.6 67.1 NA	Figure 3B
argS	arginine-tRNA ligase	1-561 bp	*w*Di (A-in-B) *w*No (A-in-B) *w*Fol (A-in-E)	99.9 43.9 NA	Figure 4A
		562-1707 bp	*w*Di (A-in-B) *w*DacA (E-in-A)	23.3 NA	Figure 4B
dnaK	Chaperone protein DnaK	None	*w*DacA (E-in-A)	NA	Figure 5

Table 2. Correlation of evolutionary divergence estimates between *Wolbachia* species using the 210 core gene set and five MLST genes.

Correlation coefficient (rho)	core gene set	gatB	fbpA	hcpA	coxA	ftsZ*
core gene set	1	0.96	0.90	0.97	0.92	0.97
gatB		1	0.86	0.91	0.90	0.94
fbpA			1	0.87	0.84	0.92
hcpA				1	0.89	0.96
coxA					1	0.92
ftsZ*						1

*Estimates of evolutionary divergence using *ftsZ* gene were only conducted among 31 *Wolbachia* species excluding *w*Bm, *w*Wb and *w*Con, because of the inability to correctly annotate *ftsZ* in these 3 species.