Article

Insights into the evolution of *Wolbachia* supergroup through the lens of genomic variation, phylogenetic and recombination analysis

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Abstract

Wolbachia is an alpha-proteobacteria and endosymbiont, causing infection in arthropods as parasites and in nematodes as mutualists. Unraveling the evolution of *Wolbachia* supergroups is the most fascinating topic among the scientific community. In this quest, we analyzed 30 *Wolbachia* genomes that belong to 11 supergroups (A, B, C, D, E, F, J, L, M, S, and T). We also performed average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH) analysis to understand the supergroup demarcation. Furthermore, we carried out multi-locusphylogenetic analysis using 189 single-copy orthologs followed by recombination analysis. We found that ANI values for each strain belonging to the same supergroups are supported by a threshold value of ≥95% except for two strains *wCtub* and *wDcau* (ANI value is 82%). dDDH analysis finds that most supergroups follow species boundary thereshold except supergroup J. Further, a phylogenomic tree was reconstructed for supergroup analysis and found that the strains *wCtub* and *wDcau* were monophyletic and belong to the same supergroup J. Further, strains from supergroup A and supergroup B were monophyletic. Supergroups J and C were monophyletic, and supergroup S was an outgroup to them. Supergroup T was an outgroup to supergroups C, D, F, J, and S. Supergroups E, L and M were at the base of other supergroups radiation (i.e., supergroups A, B, C, D, F, J, S, and T). Recombination analysis finds 8 genes (out of 189 genes) showed genetic recombination, which infers the role of recombination has minimal effect in *Wolbachia* supergroup evolution. Overall, this study concludes that besides the 16S rRNA-based phylogeny, ANI analysis, and dDDH test, phylogenomic study indispensable for unraveling the evolution of *Wolbachia* supergroups.

Keywords: *Wolbachia*; evolution; phylogenomics; average nucleotide identity; dDDH; recombination.

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1 Introduction

Wolbachia is an endosymbiotic alpha-proteobacteria from Rickettsia that infect many arthropods and nematodes. (Sironi et al., 1995; Werren et al., 1995). These bacteria are gram-negative and obligately intracellular (Harris et al., 2010). Their genomes have been analyzed to determine the type and nature of symbiosis they perform in their host (Hoerauf et al., 2003; Duron et al., 2007; Hosokawa et al., 2010; Lindsey et al., 2016; Badawi et al., 2018). Their nature of relationships in the hosts is a reproductive parasite in arthropods, nutritional mutualists in bed bugs, and obligate mutualism in filarial nematodes (Bouchon et al., 1998; Kageyama et al., 2002). All reproductive manipulation in the host—mostly arthropods and some nematodes—was mediated by *Wolbachia*, using parthenogenesis (P), feminization (F), male-killing (MK), inducing cytoplasmic incompatibility (CI), and nutritional supplementation (Sironi et al., 1995; Werren et al., 2008; Cordaux et al., 2011; Miyata et al., 2017). According to estimates by Hilgenboecker et al. (2008), Zug and Hammerstein (2012), and Kajtoch and Kotásková (2018), up to 40–76% of insects may be infected with *Wolbachia*.

Supergroups, which first appeared in 1998, are different monophyletic lineages into which *Wolbachia* have been divided (Zhou et al., 1998). This idea was later made famous by Lo et al. (2002). Initially, the majority of *Wolbachia* strains' molecular characterizations were based on either a single gene or a multilocus gene (Lo et al., 2002; Casiraghi, 2005; Baldo et al., 2006; Lo et al., 2007; Bordenstein et al., 2009; Ferri et al., 2011; Glowska et al., 2015; Konecka et al., 2015; Lefoulon et al., 2016; Ma et al., 2017; Khoo et al., 2020), and the *Wolbachia* surface protein genes, often known as wsp genes, were used to delineate supergroups by setting a threshold of 2.5% divergence value (Zhou et al., 1998). In addition, it was evident that wsp genes might recombine amongst *Wolbachia* strains (Baldo et al., 2005). As a result, Baldo et al. (2006) introduced the multilocus sequence typing (MLST) technique, which later emerged as the frequently used method for supergroup classification. Supergroups A and B comprised most of the supergroups' genomes sequenced when this MLST approach was established (Baldo et al., 2006). Bleidorn and Gerth (2018) recently conducted a study to review the MLST paradigm. There, they assessed the *Wolbachia* MLST markers' characteristics and contrasted them with 252 additional single-copy loci found in the genomes of different *Wolbachia* strains. According to them, MLST loci outperform but do not reflect the properties of a *Wolbachia* strain very well because they are highly conserved and slow-evolving genes. Therefore, they suggested using whole genome typing methods and criticized using MLST markers.

Till now, *Wolbachia* has been divided into 21 supergroups, namely A–F, H–Q, and S-W (Lefoulon et al., 2020; Laidoudi et al., 2020; Konecka, 2021; Baimai et al., 2021; Sharma and Som, 2023). Two supergroups, G and R, had been described as invalid (Baldo et al., 2007; Gerth et al., 2016). *Wolbachia* supergroups A and B are the most primitive, and more research has been done on these two supergroups, and hence reported that these are diverse groups (Ishmael et al., 2009). Among all the *Wolbachia* supergroups, the parasitic supergroups are A, B, E, H, I, K, M, N, O, P, Q, S, and U, which are found in arthropods (Casiraghi, 2004; Fenn et al., 2006; Comandatore et al., 2013: Lefoulon et al., 2020b: Baimai et al. 2021). Supergroups which are restricted to filarial nematodes belong to C, D, and J (Bandi et al., 1998; Casiraghi, 2004; Haegeman et al., 2009; Lefoulon et al., 2016), whereas supergroup L is found only in plant-parasitic nematodes (Haegeman et al., 2009; Brown et al., 2016). Supergroup F is, so far, the only known clade comprising symbionts of filarial nematodes (wMhie as mutualistic nature) as well as arthropods (wCle as nutrition action mutualism as well as a parasite in nature) (Lo et al., 2002; Hosokawa et al., 2010; Ferri et al., 2011; Lefoulon et al., 2012). A new supergroup, T, is found in the host *Cimex hemipterus* (Bed bug), showing nutritional mutualism with the host (Laidoudi et al., 2020). Additionally, supergroups V and W found coinfecting cat fleas (*Ctenocephalides felis*) shows parasitic and mutualistic life-style respectively (Driscoll et al., 2020; Sharma and Som, 2023).

As *Wolbachia* strains show an endosymbiotic nature, there is a prevalence of the possibility of the presence of multiple different strains in the same host. Due to the co-existence of multiple strains in the same host cell, there develops a high chance of homologous recombination (Jiggins et al., 2001; Jiggins et al., 2002). Previous studies reported recombination between the strains (Wang et al., 2020). No recombination has been discovered in filarial nematodes (Foster et al., 2011). Wang et al. (2020) did a comprehensive study on identifying recombination with 33 *Wolbachia* strains and six supergroups. They reported only six genes (2.9%) for recombination. This also suggests that the role of homologous recombination between inter-supergroups is very minute for shaping the *Wolbachia* genomes.

This article focused on complete genomesof *Wolbachia*from 11 supergroups. The objective is to find evolutionary relationships among all supergroups and the role of homologous recombination in inter-supergroup genome evolution. To complete these objectives, we performed comparative genomic analysis, ANI test, dDDH test, multilocus phylogenetic, and recombination analyses on 189 single-copy genes from 30 *Wolbachia* strains belonging to 11 supergroups.

2 Materials and Methods

2.1 Data collection

To find the supergroup relationships of the *Wolbachia* genomes, we first took 16S rRNA sequences for phylogenetic analysis because it is a highly conserved gene and can show species delineation. For this, we use strains of all the previously identified 11 supergroups, consisting of 30 *Wolbachia* strains in the study. Then we use complete genomes of selected 30 *Wolbachia* strains from 11 supergroups (i.e., A-F, J, L, M, S, and T). Details of the 30 genomes are given in (Table 1). Furthermore, we took 189 coding DNA sequences (single-copy genes) and their amino acid sequences for each *Wolbachia* genome. Therefore, this study included 12,096 sequences and all the sequences were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/).

Table 1 Genomic details of the *Wolbachia* strains used in the study.

S.No.	Strains	NCBI Id	Supergroups	Length (in Mbp)	GC%	Proteins	Pseudogenes	Host
1	wMel	NC_002978.6	A	1.27	35.2	1143	103	Arthropods
2	wNpa	PRJNA322628	A	1.34	35.2	1,217	138	Arthropods
3	wNfla	PRJNA322628	A	1.33	35.2	1203	140	Arthropods
4	wHa	NC_021089.1	A	1.30	35.1	1123	91	Arthropods
5	wInc	CP011148.1	A	1.27	35.8	953	242	Arthropods
6	wCauA	CP041215.1	A	1.45	35	1258	123	Arthropods
7	wRi	NC_012416.1	A	1.44	35.2	1237	95	Arthropods
8	wNo	NC_021084.1	B	1.30	34.0	1071	105	Arthropods
9	wPip_Mol	PRJEB4607	B	1.44	35.0	1122	87	Arthropods
10	wVitB	PRJNA61407	B	1.11	34.0	900	116	Arthropods
11	wAlbB	PRJNA508212	B	1.49	34.5	1173	193	Arthropods
12	wMeg	CP021120.1	B	1.37	34.0	1116	111	Arthropods
13	wMau	CP034335.1	B	1.27	34.0	1110	99	Arthropods
14	wBtab	CP016430.1	B	1.31	35.1	979	222	Arthropods
15	wOo	NC_018267.1	C	0.96	32.1	646	47	Nematodes

16	wOvul	NZ_HG810405.1	C	0.96	32.1	650	45	Nematodes
17	Dimm	CP046578	C	0.92	32.7	660	24	Nematodes
18	wBm	NC_006833.1	D	1.08	34.2	832	128	Nematodes
19	wWb	PRJNA388334	D	1.06	34.3	811	124	Nematodes
20	wBpah	NZ_CP050521.1	D	1.07	34.2	857	130	Nematodes
21	wLsig	CP046577	D	1.04	32.1	714	74	Nematodes
22	wFol	NZ_CP015510.2	E	1.80	34.4	1540	84	Arthropods
23	wCle	NZ_AP013028.1	F	1.25	36.3	1012	174	Arthropods
24	wMhie	PRJNA593581	F	1.02	36.1	960	145	Nematodes
25	wCtub	CP046579	J	0.86	32.3	623	29	Nematodes
26	wDcau	CP046580	J	0.86	28.0	607	12	Nematodes
27	wPpe	PRJNA343941	L	0.97	32.1	851	76	Nematodes
28	wApol	PRJNA593570	S	1.44	35.5	1121	468	Arthropods
29	wPni	PRJNA628023	M	1.46	34.1	1261	227	Arthropods
30	wChem PL13	NZ_CP061738.1	T	1.29	35.4	1075	125	Arthropods

2.2 Average Nucleotide Identity (ANI) and digital DNA-DNA Hybridization (dDDH) measures

The supergroups are sub-species level, and their genomes are close enough to each other, so genomic divergence analysis is required for supergroup identification. Accordingly, we performed an ANI analysis using OrthoANIu tool (Yoon et al., 2017). ANI measures nucleotide-level genomic similarity between the coding regions of two genomes, and here we attempt to find the divergence of genomes to check whether two genomes are from the same supergroup or belong to different supergroups based on their genomic content similarity. We also compared both genomes with other genomes belonging to different supergroups to check for their genomic similarity and divergence.

We also used dDDH to calculate *in-silico* genome-to-genome comparison using the GGDC tool (Meier-Kolthof et al., 2013). The dDDH analysis emerged as an alternative to the wet-lab DNA–DNA hybridization of species delineation. In GGDC, we used the genome blast distance phylogeny approach to calculate the probability that an inter-genomic distance yielded a dDDH larger than 70%, representing a novel species-delimitation threshold (Auch et al., 2010).

2.3 Phylogenetic analysis

In the phylogenetic analysis, we reconstructed the 16S rRNA phylogeny of 30 strains from 11 supergroups. Further, we used coding DNA and amino acid sequences for the phylogenomic analysis. We prepared a set of ortholog protein sequences by performing an All-vs-All BLAST similarity search on the whole proteome datasets for every 30 genomes (https://blast.ncbi.nlm.nih.gov/Blast.cgi). We selected the sequences with the best reciprocal BLAST hits. The parameters used are top BLAST score results having >70% query coverage and E-value $<10^{-5}$. We used the orthoven2 package (Xu et al., 2019). Further, we aligned all the ortholog sequences by MUSCLE (Edgar et al., 2004) and removed poorly aligned sites by TRIMAL (Capella-Gutierrez et al., 2009). Then, we concatenated the aligned protein sequences using MEGAX (Kumar et al., 2018). Then, the phylogenetic tree was reconstructed by using the IQTREE package (Trifinopoulos et al., 2016). We also performed the model test to find the best suitable model using ModelFinder (Kalyaanamoorthy et al., 2017). This entire protocol is also used for coding DNA sequences and reconstructing nucleotide trees using the IQTREE package. We have visualized and edited the trees using iTOL (Letunic et al., 2021).

2.4 Recombination

After the phylogenomic analyses, we performed a recombination test on all orthologs used for phylogenomic tree reconstruction. We did a recombination test to find the traces of homologous recombination in *Wolbachia* supergroup evolution and check whether there is any recombination in the novel strains. We used RDP4 (Recombination Detection Programme 4) package to check gene-wise recombination between the *Wolbachia* strains (Martin et al., 2015). Here we used seven methods to detect recombination events for a gene between the *Wolbachia* strains. These methods were RDP, GENECONV, Bootscan, MaxChi, Chimera, SisScan and 3Seq. A recombination event is detected in a gene when at least five methods support it with the E-value <10-05.

3 Results

3.1 ANI analysis

The ANI is a similarity index between a given pair of genomes routinely used to define Archaea and Bacteria species boundaries, and a cut-off score of >95% indicates that they belong to the same species (Figueras et al., 2014). ANI between the other 30 strains was calculated by using the OrthoANIu tool (Yoon et al., 2017). We compare all strains with other supergroups to check whether they belong to the same or any other supergroup. There was an apparent distinction in the overall ANI distribution between intra- and interspecies relationships at around 95-96% ANI (Kim et al., 2014). We found that ANI values for each strain belonging to the same supergroups are supported by a threshold value barrier (i.e., ≥95%). There is an exception for two strains wCtub and wDcau; the ANI value is 82%. These two strains belong to supergroup J (Lefoulon et al., 2020). Detailed ANI values for individual genomes are given in (Fig. 1).

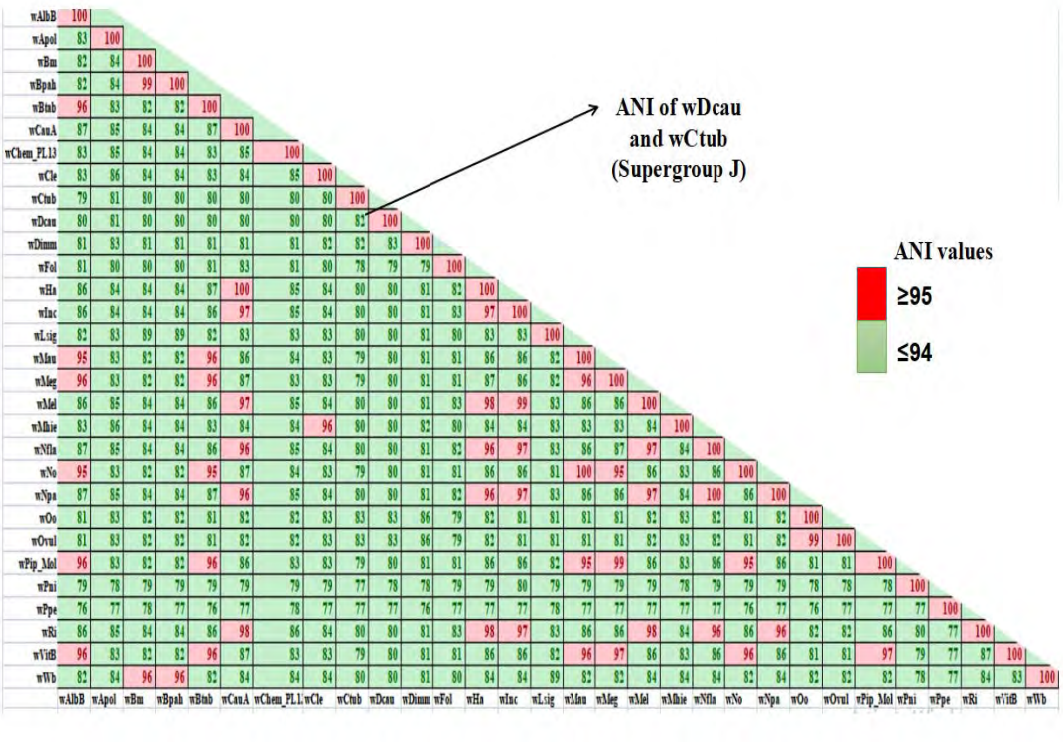

Fig. 1 Average nucleotide identity (ANI) values for 30 genomes. Red color boxes indicate an ANI value ≥95%, and green color indicates an ANI value ≤94%.

3.2 dDDH analysis

We compared dDDH values for all 30 strains of *Wolbachia* genomes from 11 supergroups (Fig. 2). We found that most genomes follow species boundary distinction except supergroup J. The species belonging to the same supergroup must have a dDDH value ≥70% (Kim et al., 2014).

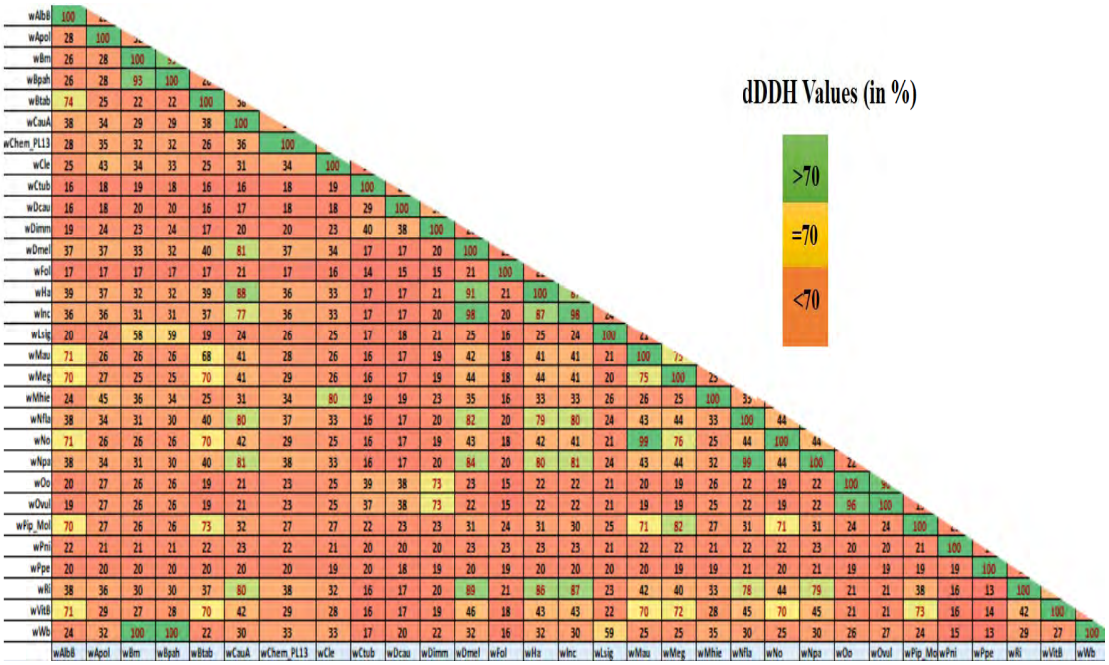

Fig. 2 dDDH analysis of 30 *Wolbachia* genomes. The yellow colour indicates the dDDH value =70%. Yellow to green indicates dDDH values 70 to 100, and yellow to orange indicates dDDH values below 70.

3.3 Phylogenomic analysis

3.3.1 16S rRNA phylogeny

In this study, first, we performed a 16S rRNA phylogenetic analysis using 30 strains from 11 supergroups. The tree was reconstructed with the ML method and the HKY+F+I+G4 model given in (Fig. 3). Here, we found that all the strains cluster together according to their supergroup boundary (for example, strains from supergroup A cluster together and strains from supergroup B cluster together and both the clusters differentiated separately). Supergroup E at the base of supergroup A, Supergroup C, J, F and S forms cluster together, Supergroup D and T cluster together, and Supergroup M and L are at the base of all other supergroups.

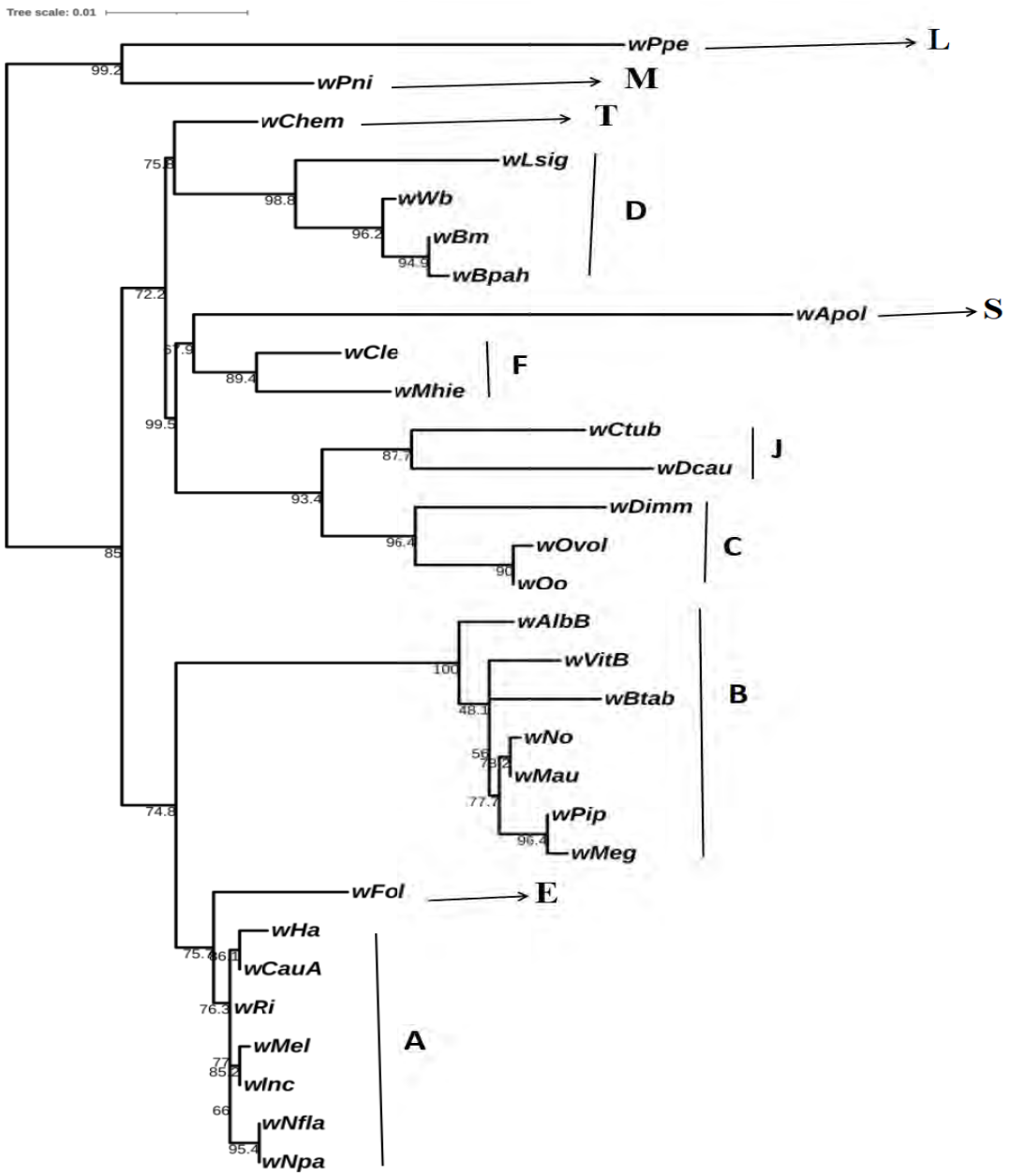

Fig. 3 *Wolbachia* 16S rRNA gene phylogeny. The phylogenetic tree includes 30 strains of *Wolbachia* belongingto 11 supergroups. Tree reconstructed using the ML method, HKY+F+I+G4 model of the nucleotide of evolution and 1000 bootstrap replicates.

3.3.2 RbMLST phylogenetic analysis

Then we performed phylogenetic analysis using ribosomal DNA sequences, frequently called ribosomal multi-locus sequence typing (RbMLST) (Fig. 4). Here we founddifferent scenario of evolutionary relationships. All the strains cluster together according to their supergroup boundary (for example, strains from supergroup A cluster together and strains from supergroup B cluster together and both the clusters differentiated separately). Supergroup E at the base of supergroup A, B, C, D, F, J, S and T, and Supergroup M and L are at the base of all other supergroups.

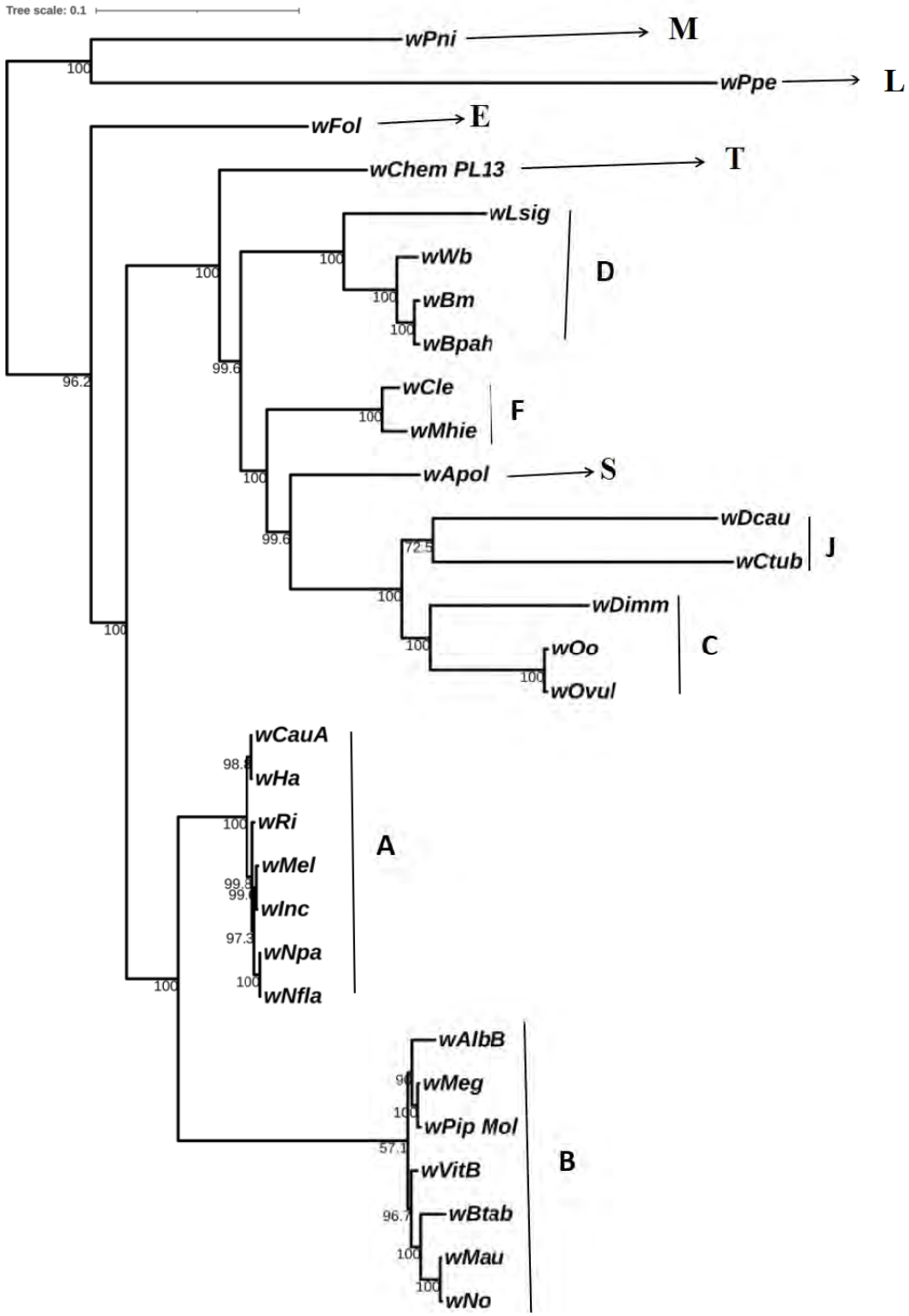

Fig. 4 RbMLST gene phylogeny of *Wolbachia*. Tree consists of 32 ribosomal genes from 30 strains, and a total of 13,805bp used. The tree was reconstructed using the maximum likelihood method with the GTR+G+I model of nucleotide evolution used with 1000 bootstraps.

3.3.3 Phylogenomic analysis

Further, we did a comprehensive phylogenetic analysis using a set of 189 single-copy orthologs genes identified by all-vs-all BLAST hits using the OrthoVenn2 package. Hence, we performed Maximum Likelihood (ML) phylogenetic analyses based on 189 single-copy ortholog proteins using coding nucleotide sequences of the respective amino acid sequences.

Here we hypothesize that each strain differentiated according to its supergroup boundaries. To validate our hypothesis, we reconstructed ML trees by concatenating the aligned 189-coding DNA sequences. Phylogenetic tree depicts that all the strains cluster in the clade-wise manner (Fig. 5). Strain wPni from supergroup M and strain wPpe from supergroup L are monophyletic, and wFol (supergroup E) evolved as an outgroup to them. This relationship between the strains wPpe, wPni and wFol is also reported in previous studies (Gerth et al., 2014; Brown et al., 2017; Lefoulon et al., 2020).

Other strains of the supergroup F clade (consisting of two strains wCle and wMhei), are monophyletic. ANI results supported this result, which reported that the strains wCle and wMhei (Supergroup F). Thus, both ANI and phylogenetic studies indicate that these strains share a close ancestry after being the hybrid supergroup (i.e., supergroup F, which consists of parasitic and nematode symbionts). But there were some contradictionsalso prevails, for example in the case of Supergroup J, strain wCtub and strain wDcau Both ANI and dDDH did not support them to be in the same group but phylogenetic analysis supports.

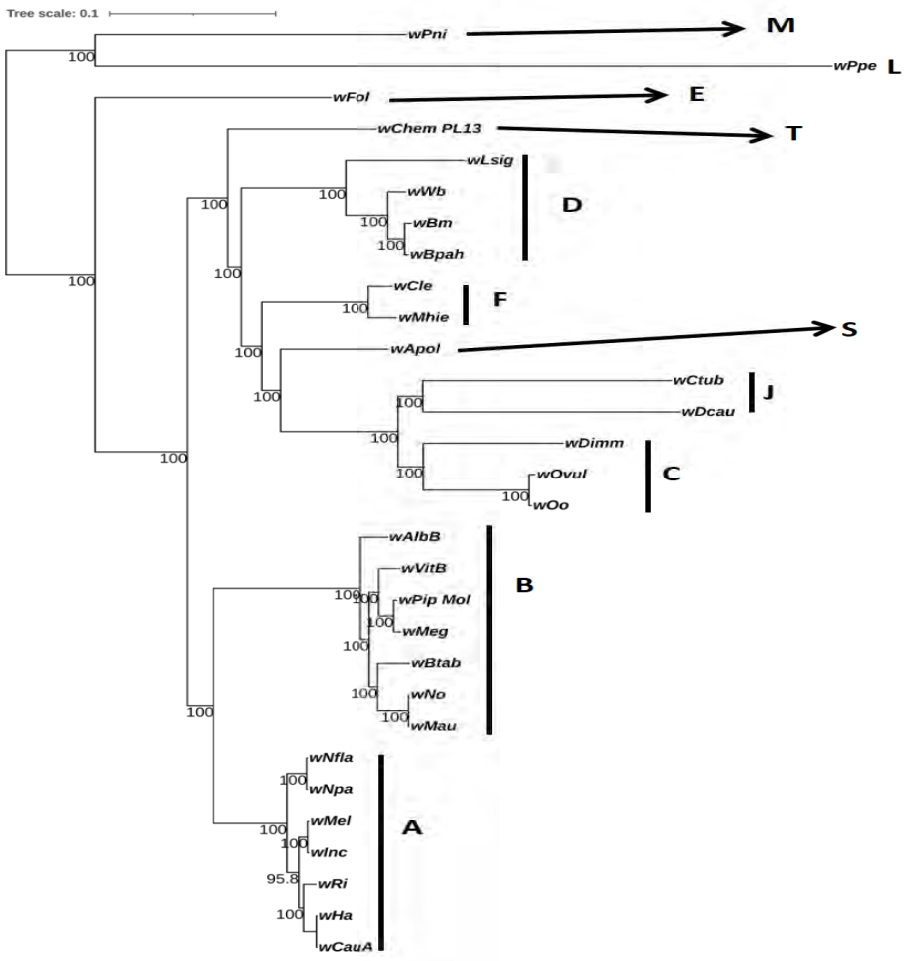

Fig. 5 *Wolbachia* supergroup phylogeny. Tree consists of 189 orthologs from 30 strains, and a total of 1,51,165bp used. The tree was reconstructed using the maximum likelihood method with the GTR+G+I model of nucleotide evolution used with 1000 bootstraps.

3.4 Recombination

Recombination is essential to bacterial evolution (Didelot et al., 2012). Thus, we intended to investigate putative/homologous recombination events between the strains. Accordingly, we performed the recombination test on all 189 orthologous genes set using the RDP4 package. We identified eight genes (4.2% of 189 genes) that showed inter-supergroup recombination, seven of which were from supergroups A and B. A detailed description of the recombination analysis is given in Table 2.

Table 2 List of *Wolbachia* genes shows inter-supergroup recombination events.

Genes	Recombination breakpoint			Sequences (supergroup)			Detection Methods						
	No. Events	Begin	End	Recombinant	Major Parent	Minor Parent	RDP	GENE CONV	Bootscan	Maxchi	Chimaera	SiSscan	3Seq
FtsH	1	1253	1647	wNfla (A)	wNpa (A)	wNo (B)	2.86E-20	7.61E-18	2.00E-19	6.01E-16	4.17E-14	7.48E-12	2.13E-21
glpX	1	91	399	wBtab (B)	wAlbB (B)	wRi (A)	1.18E-10	1.03E-12	4.62E-12	6.85E-09	1.95E-09	NS	1.48E-13
gyrA	1	1526	2295	wVitB (B)	wNo (B)	wNpa (A)	5.54E-13	5.64E-08	1.53E-12	5.96E-16	5.71E-15	7.33E-10	5.41E-18
	2	2090	2295	wMeg (B)	wNo (B)	wNpa (A)	1.93E-10	7.23E-08	1.62E-10	3.19E-07	6.63E-07	3.02E-08	1.21E-09
leuS	1	765	847	wAlbB (B)	wVitB (B)	wCle (F)	4.69E-14	2.78E-12	3.29E-14	1.16E-05	6.96E-05	4.78E-07	7.15E-12
mgtE	1	1070	1253	wVitB (B)	wPip_Mol(B)	wCauA (A)	3.12E-12	7.04E-11	6.00E-13	1.22E-07	2.42E-07	8.85E-11	6.84E-13
purL	1	1777	2032	wMeg (B)	wVitB (B)	wNpa (A)	7.17E-12	3.36E-09	6.15E-12	5.30E-10	8.57E-07	1.33E-08	2.02E-12
ppdk	1	311	803	wVitB (B)	wPip_Mol(B)	wRi (A)	1.49E-15	8.20E-14	1.35E-15	1.98E-10	2.76E-08	8.90E-12	2.36E-04
sucC	1	834	1161	wAlbB (B)	wNo (B)	wInc (A)	8.30E-14	2.40E-13	2.03E-15	3.55E-11	2.15E-11	5.07E-12	1.89E-21

4 Discussion

4.1 Revisiting the *Wolbachia* supergroup phylogenies: An assessment

Evolutionary biologists have been interested in understanding the underlying evolutionary mechanism of *Wolbachia* and its supergroups classification since 1992 when O'Neill reported that *Wolbachia pipientis* belong to the alpha-subdivision of the Proteobacteria (O'Neill et al., 1992). After that, in the last 30 years, a vast number of research articles have been published on various evolutionary aspects of *Wolbachia*, such as supergroup identification, inter/intra group recombination, HGT etc. and made significant progress towards understanding *Wolbachia* diversity and lifestyle using a single gene to few hundred genes (Baldo et al., 2005; Bordenstein et al., 2009; Ellegaard et al., 2013). Initially, due to limited data, most of the phylogenetic inferences were based on single to few genes with limited supergroups (Lo et al., 2002; Casiraghi, 2005; Bordenstein et al., 2009; Ferri et al., 2011; Glowska et al., 2015). In the last decade, because of the advancement of high throughput sequencing technology, *Wolbachia*'s evolution has been studied using upto few hundred single-copy orthologs (Ellegaard et al., 2013; Gerth et al., 2014; Comandatore et al., 2015; Wang et al., 2020; Lefoulon et al., 2020) and made substantial progress on the *Wolbachia* evolution and diversity including discovery/classification of 20 supergroups. Unfortunately, despite substantial efforts, there is no

univocal *Wolbachia* supergroup phylogeny. Instead, there is a ubiquitous discordance among the different phylogenies inferred using up to a few hundred loci. This is probably because of *Wolbachia*'s inherent genomic complexity. The problem is further extrapolated because the concatenation of aligned sequences called the supermatrix approach, suffered from various biological factors such as HGT, gene loss/gain, recombination and heterotactic etc (Seo et al., 2005). Details of the negative factors and their causes and consequences have been thoroughly discussed in the review by Som (2015), which is beyond the scope of the article. Therefore, the dream of a fully resolved *Wolbachia* supergroup phylogeny can be archived by overcoming the aforesaid biological factors by correct loci selection, incorporating a large no of loci, appropriate model and method of tree reconstruction which can deal with the heterotactic problem (i.e., within site-specific rate variation) caused by the concatenation of the fast and slowly evolving genes (Lopaze, 2002; Rokas and Carroll, 2005; Philippe, 2005; Heath et al., 2008; Som et al., 2009; Som, 2013; Som, 2015).

4.2 Recombination in *Wolbachia* genomes

Our study identified eight genes showing recombination (i.e., about 4.2% of the total 189 orthologs). Most show inter-supergroup recombination (mostly between two supergroups, A-B). We did not find any putative homologous recombination between *Wolbachia* showing mutualistic life style, it might be because they somehow able to established mutual understanding between their host to get energy requirements by establishing healthy protein-protein between nematode host as in the case of supergroups C, D and J (Sharma et al., 2023). A similar study was done by Wang et al. (2020), where they got six genes showing inter-clade recombination. This might be due to taxon selection, as we selected 30 genomes from 11 supergroups, and they used 33 genomes with six supergroups. These recombination results indicate that in the evolution and diversification of *Wolbachia* supergroups, the role of homologous recombination is diminishing.

5 Conclusions

In our analysis, we found that both ANI and dDDH values were not ≥ 95% and 70% respectively for all strains that belong to the same supergroup, suggesting that both ANI and dDDH tests are required for species delimitation analysis. Further, phylogenetic analyses including 16S rRNA, RbMLST and Phylogenomic study based on orthologous genes from whole genomes were also contradictory, as 16S rRNA-based phylogeny suggest that supergroups E is closer to supergroup A, however RbMLST and phylogenomic tree suggest that supergroup E has highly diverged and present at the base of *Wolbachia* radiation. This study also reports that the role of recombination has a minimal effect in supergroup evolution. Thus our analyses recommend that along with ANI and dDDH tests, phylogenetic studies (i.e., 16S rRNA-based phylogeny, RbMLST and phylogenomic analysis) are required to reveal *Wolbachia* supergroup delimitation. The lack of full genomic data limits our analysis, so more *Wolbachia* genomes are required for further insights into *Wolbachia* evolution and diversity.

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