

## SUPPLEMENTAL INFORMATION

### **Distinct *Wolbachia* localization patterns in oocytes of diverse host species reveal multiple strategies of maternal transmission**

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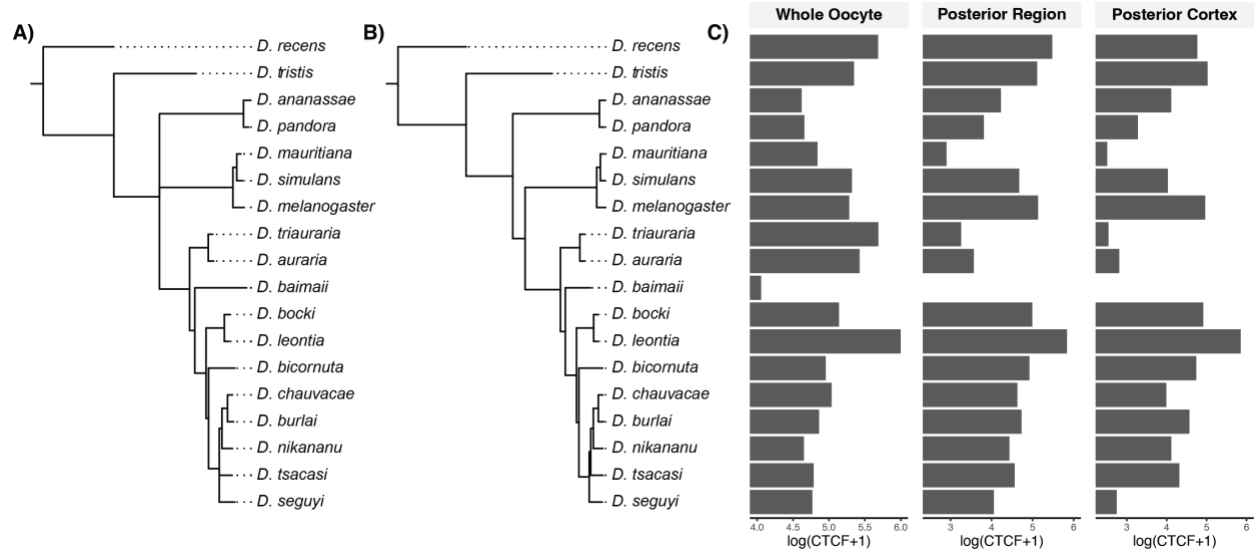
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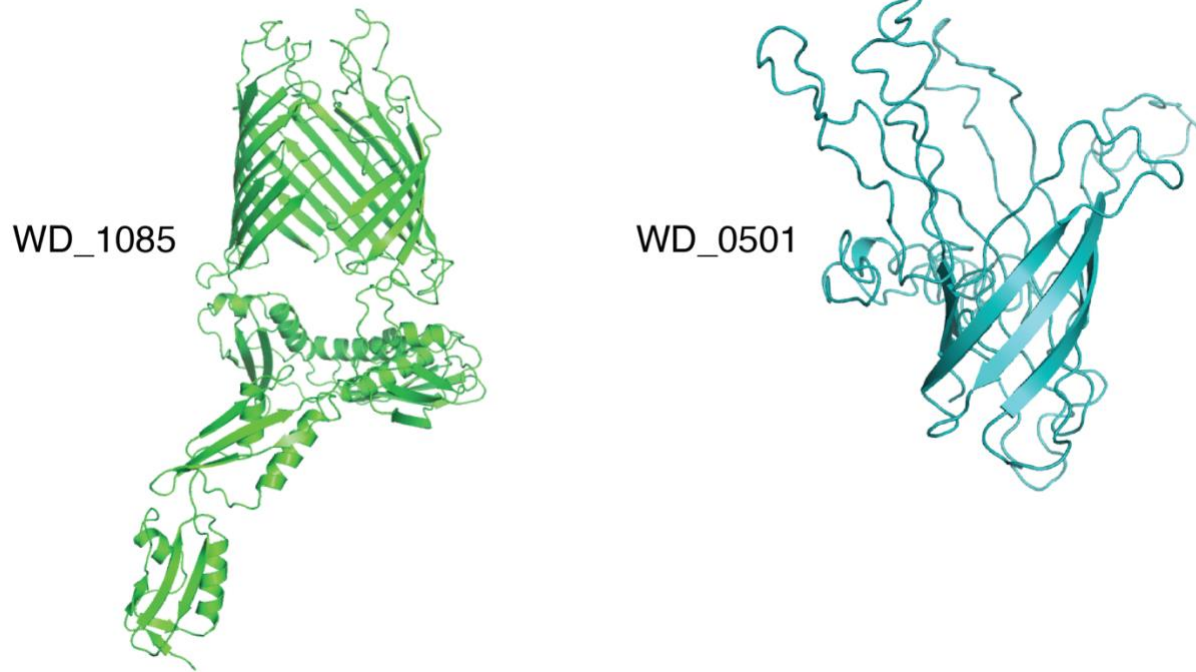
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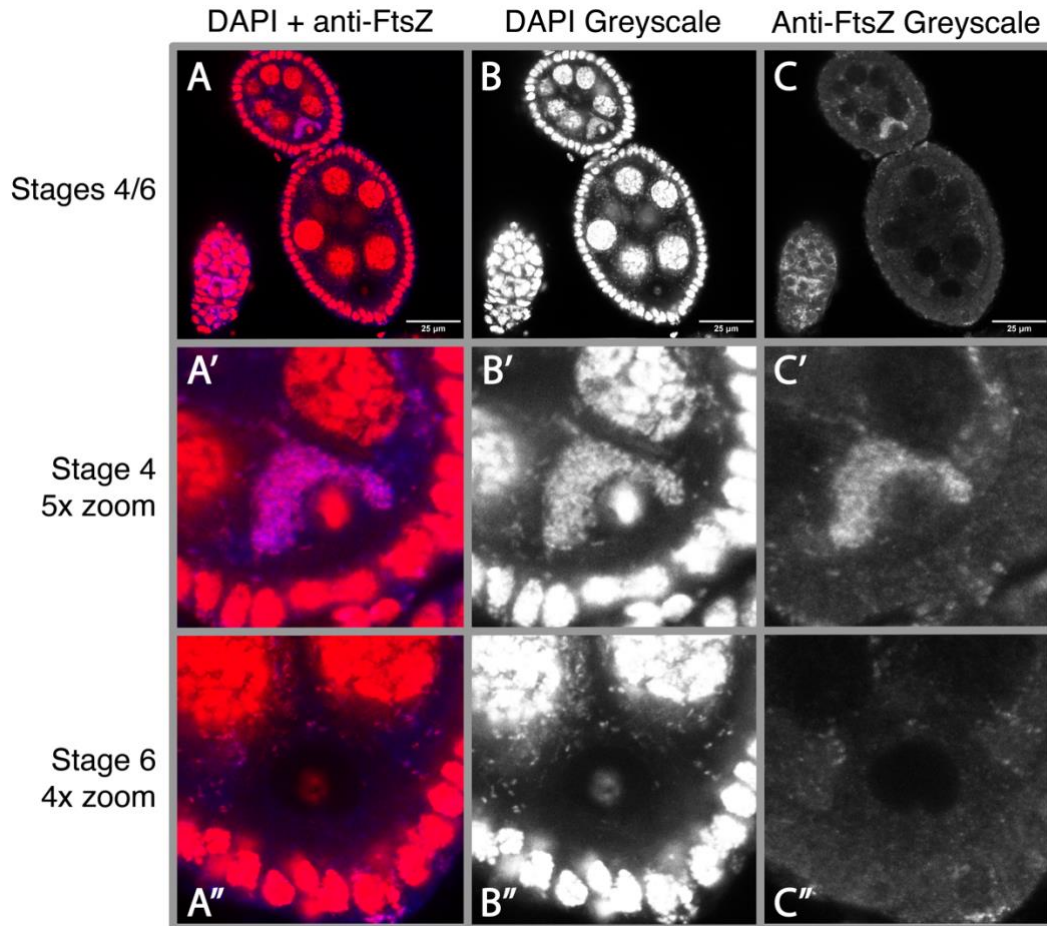
**Supplemental Figure 1.** (A) Estimated Bayesian phylogram of host species based on 20 nuclear loci. All nodes have posterior probabilities >0.95. (B) Bayesian phylogram estimated using similar methods, but with a constrained topology to account for previously resolved relationships among subgroups within the *melanogaster* species group ((*montium*, *melanogaster*) *ananassae*) based on Turelli et al., (2018) and Suvorov et al., (2022). (C) Mean estimates of *Wolbachia* abundance (log-transformed CTCF) in the whole oocyte, posterior region, and the posterior cortex. For *D. simulans*, the mean values include both wRi- and wHa-infected individuals.



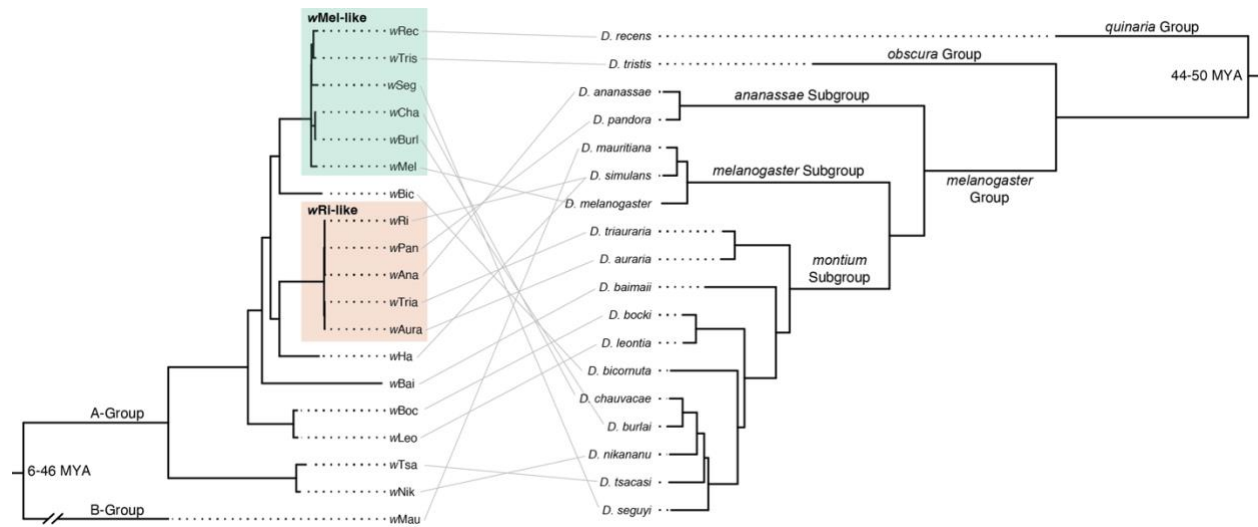
**Supplemental Figure 2.** Illustrations of the homology models for the *Wolbachia* surface proteins WD\_1085 and WD\_0501.



**Supplemental Figure 3.** (A) Representative image of the reduction of *w*Mel *Wolbachia* titer between stages 4/5 and 6 in *D. melanogaster* oogenesis. *Wolbachia* stained with anti-FtsZ shown in pink. Scale bars set at 25  $\mu$ M. (B) Greyscale DAPI channel. (C) Greyscale of anti-FtsZ channel. (A'-C') 5X zoom around the nucleus of the oocyte during stage 4 shown for each channel. (A''-C'') 4X zoom around the nucleus during stage 6 for each respective channel seen above. At least 10 oocytes across 3 slides were imaged with Z-stacks for a visual description of this trend.

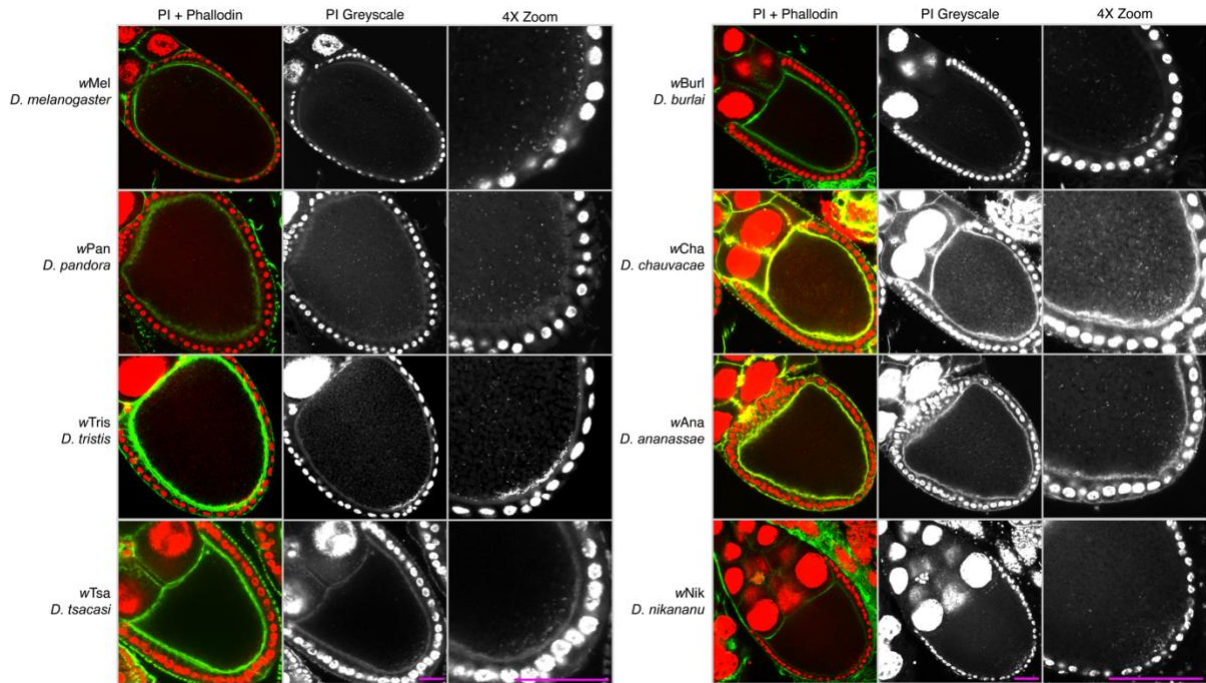


**Supplemental Figure 4.** Bayesian phylograms depicting the evolutionary relationships among *Wolbachia* strains diverged up to 46 million years (left) and *Drosophila* host species diverged up to 50 million years (right). *wMel*- and *wRi*-like clades of closely related *Wolbachia* are labeled on the left. Grey lines pair *Wolbachia* strains with their *Drosophila* host species, which highlights patterns of topological discordance due to introgressive and horizontal transfer of *Wolbachia* among host species. The *Wolbachia* phylogram was estimated using 66 full-length and single-copy genes (43,275 bp) of equal length. All nodes have posterior probabilities >0.95. The host phylogram was estimated using 20 conserved single-copy genes (see Materials and Methods regarding host tree topology). *Wolbachia* and host divergence times in millions of years (MYA) are reproduced from Meany et al. (2019) and Suvorov et al. (2022), respectively.



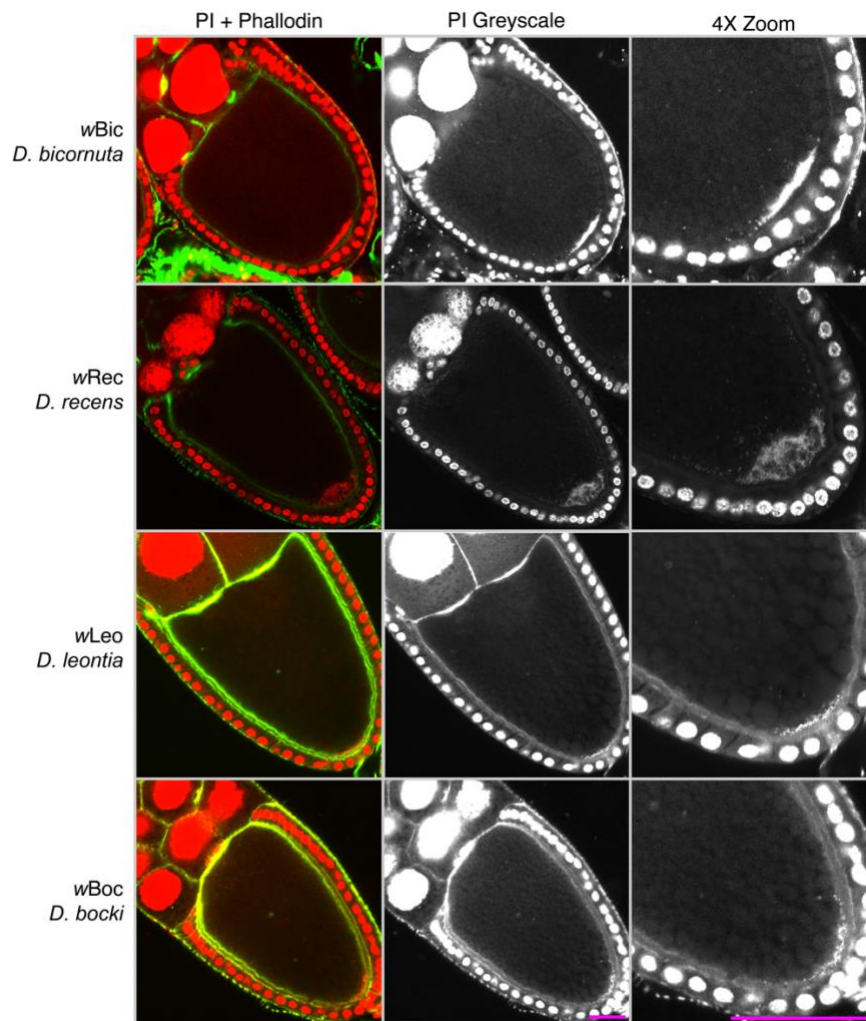
**Supplemental Figure 5.** Representative images of *Wolbachia* posterior localization patterns. Confocal micrographs of *Drosophila* oocytes DNA-stained with PI (red) and actin-stained with phalloidin (green) show representative examples of infected *Drosophila* species with different *Wolbachia* localization classes. Second columns depict single greyscale channel images of PI staining. Third columns depict an enlarged PI-stained image of the posterior region of each oocyte. Panels A, B and C are grouped into *Wolbachia* strains that exhibit a Posteriorly Localized, Posteriorly Clumped, or Dispersed localization pattern respectively. Scale bar set at 25  $\mu$ M.

**A**

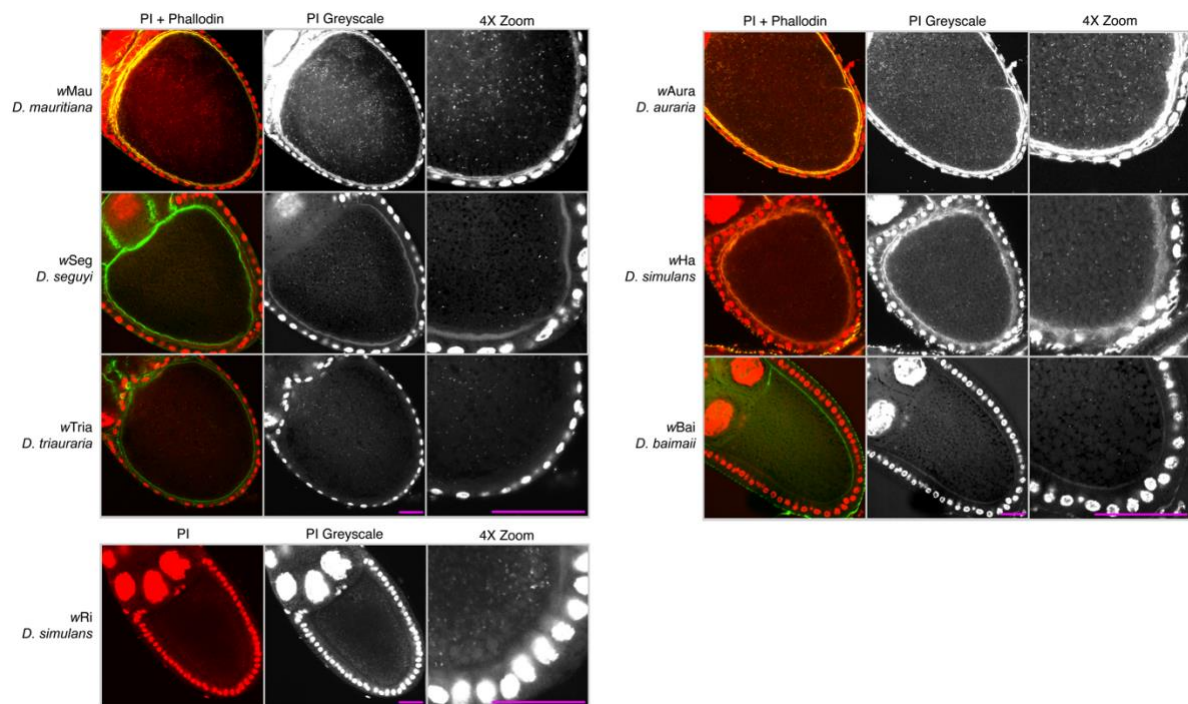




**B**

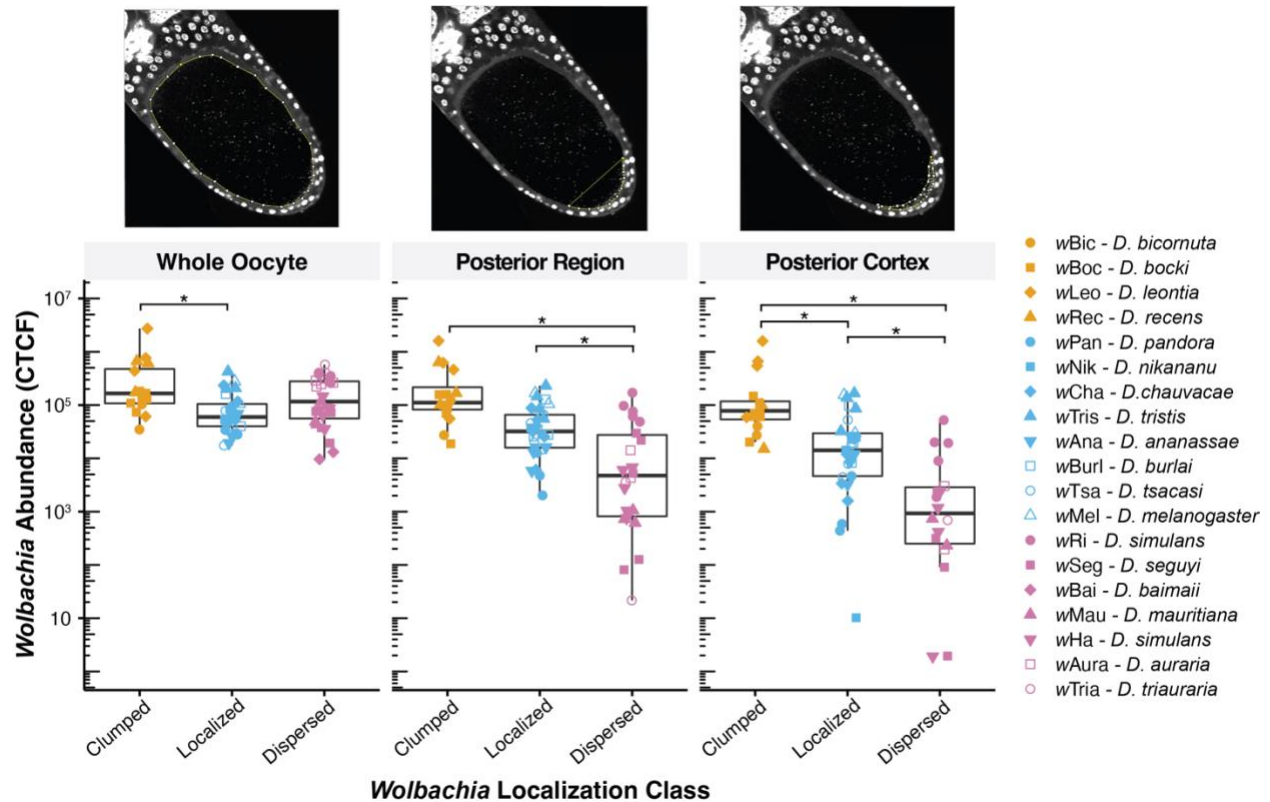


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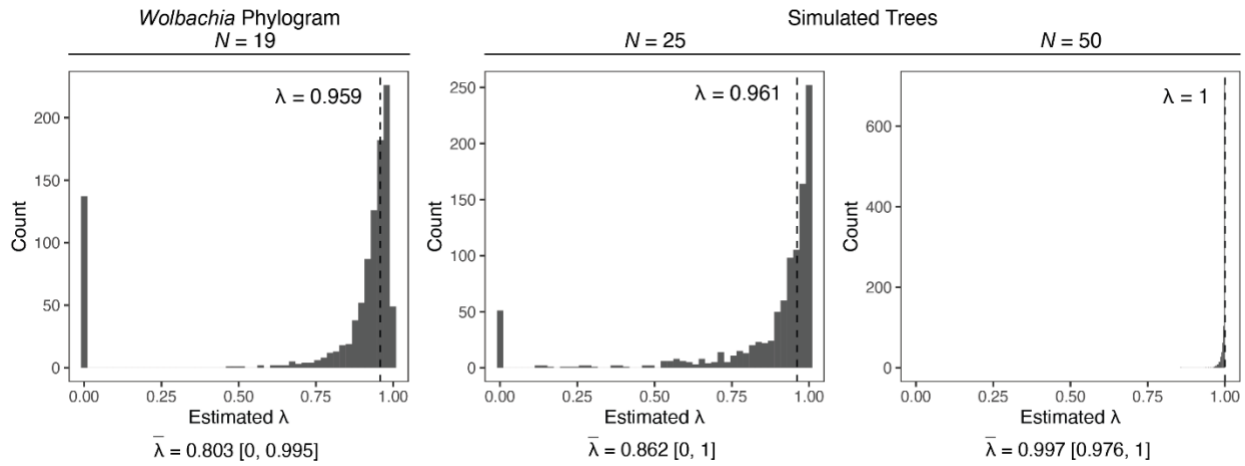




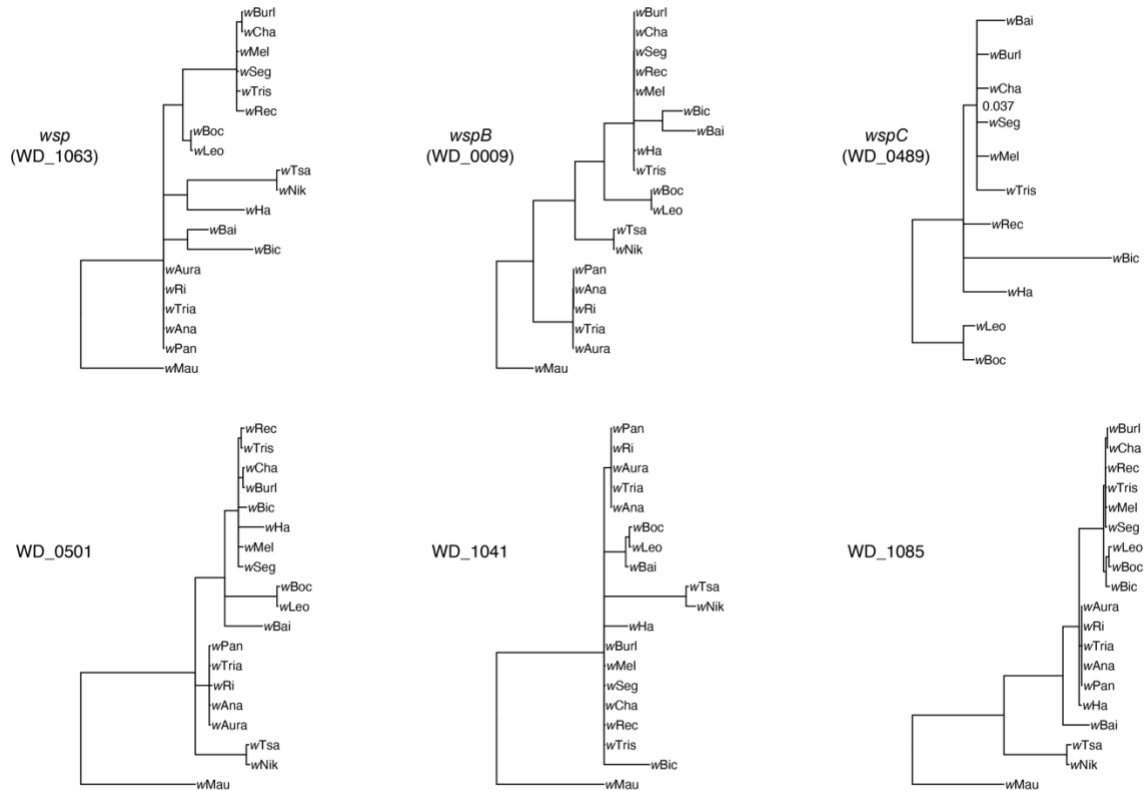
**Supplemental Figure 6.** Cellular *Wolbachia* abundance in stage 10 oocytes measured as *Wolbachia* fluorescence due to propidium iodide (CTCF). Points are color coded by *Wolbachia* localization class (Posteriorly Clumped, Posteriorly Localized, Dispersed), with unique shapes indicating each *Wolbachia* strain and host species. Asterisks indicate significant differences among groups based on one-way ANOVAs and Tukey comparisons using  $P$ -values  $<0.05$  adjusted for multiple comparisons. Above, a schematic is shown for fluorescent quantification of different regions of the oocyte using the PI greyscale channel: the whole oocyte, the posterior region, and the posterior cortex.



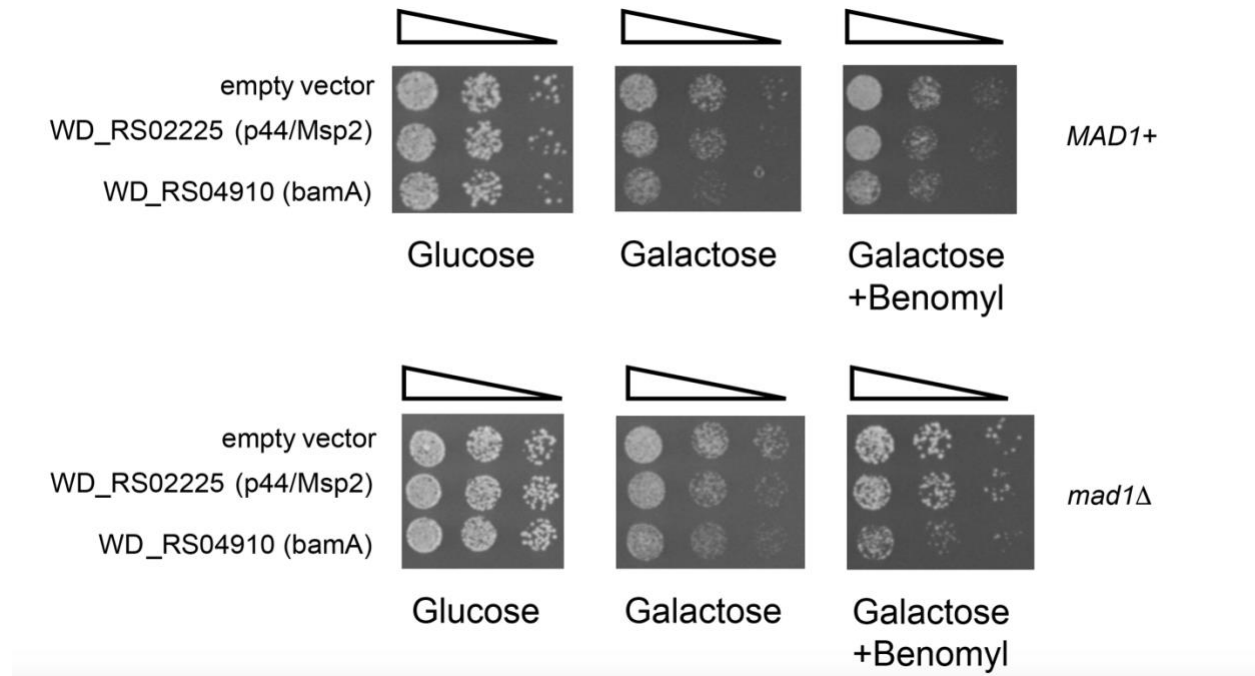
**Supplemental Figure 7.** Distribution of maximum likelihood estimates of  $\lambda$  from 1,000 bootstrap replicates based on the *Wolbachia* phylogram and *Wolbachia* abundance at the oocyte posterior cortex (log-transformed CTCF). The bootstrap analysis of our *Wolbachia* phylogram is shown to the left ( $N = 19$  *Wolbachia* strains). To the right are simulated phylogenies with an increasing number of *Wolbachia* strains included ( $N = 25, 50$ ). For simulated trees, character evolution was simulated with our  $\lambda$  estimate of 0.959 using the “sim.bdtree” and “sim.char” functions in the *Geiger* R package (Harmon et al. 2008). For each graph, fitted  $\lambda$  values for the original phylogeny are shown above with a vertical dashed line. Note that fitted  $\lambda$  values for the simulated phylogenies differ slightly from  $\lambda = 0.959$ , because “sim.char” uses a Brownian-motion model to simulate character evolution along the phylogeny. Below each graph, the mean estimate of  $\lambda$  from the 1,000 replicates ( $\bar{\lambda}$ ) is shown with associated 95% confidence intervals. Small phylogenies (e.g.,  $N = 19$ ) are likely to generate many near-zero  $\lambda$  values by chance, not necessarily because the phylogeny is unimportant for trait evolution (Boettiger et al. 2012). As the number of strains in our analysis increases ( $N = 25, 50$ ), bootstrapped estimates of  $\lambda$  cluster around the true  $\lambda$  value fitted to the original phylogeny.



**Supplemental Figure 8.** Gene trees of *Wolbachia* surface proteins. All nodes have Bayesian posterior probabilities of >0.95 unless otherwise noted (see only *wspC*).



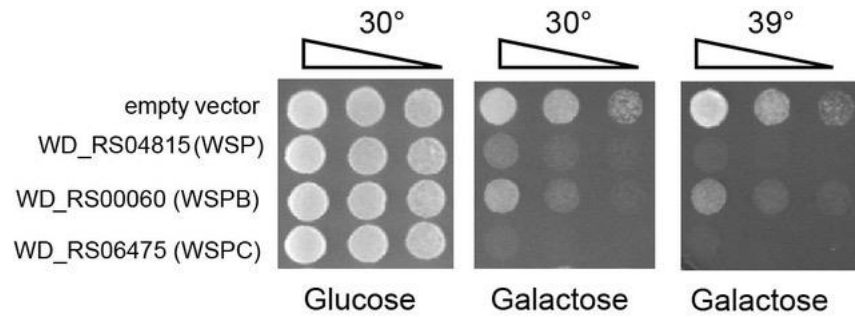
**Supplemental Figure 9.** To gain insight into WD\_0501 (RS0225/p44/Msp2) and WD\_1085 (RS04910/bamA) cellular function, we ectopically expressed these genes in yeast using a Galactose inducible promoter. Under normal growth conditions, ectopic expression of both genes inhibited growth, with WD\_1085 (RS04910/bamA) exhibiting a more pronounced inhibition. Growth inhibition of WD\_1085 was dramatically increased when the integrity of microtubules was compromised with the yeast placed in the *mad1* spindle assembly checkpoint background or the microtubules were compromised directly through the addition of benomyl. These results suggest that WD\_1085 may directly or indirectly interact with host microtubules.



**Supplemental Figure 10.** Full amino acid alignment of *wspB* (WD\_0009) homologs in *wMel* and the *Wolbachia* strains with a putatively pseudogenized version of the surface protein. Stop codons are indicated by asterisks and insertions of varying length are indicated by “XXX”.

<u>wMel reference</u>	MISKKTLAVTAFALLLSQQSFASETEGFYFGSGYYGQYLNNTSVLKTSTTGKLNLSINDR	60
<u>wHa</u>	MISKKTLAVTAFALLLSQQSFASETEGFYFGSRYYGQYLNNTSVLKTSTTGKLNLSINDR	60
<u>wBai</u>	MISKKTLAVTALALLLSQQSFASETEGFYFGSGYYGQFFNGMGELKTSTTDIKNLSINDR	60
<u>wBoc</u>	MISKKTLAVTALALLLSQQSFASETEGFYFGSGYYGQFFNGMGELKTSTTDIKNLSINDR	60
<u>wLeo</u>	MISKKTLAVTALALLLSQQSFASETEGFYFGSGYYGQFFNGMGELKTSTTDIKNLSINDR	60
<u>wBic</u>	MISKKTLAVTAFALLLSQQSFASETEGFYFGSGYYGQYLNNTSVLKTSTTGKLNLSINDR	60
<u>wMel reference</u>	GAQNTGQSLSEYKGDYNPPFAANVAFGYTGELGNNSYRAELEGMYSSVKVD---NIGLT	117
<u>wHa</u>	GAQNTGQSLSEYKGDYNPLLQMWHLVTQENWVTTAIGLNWKGCIIL*KWI---ILV*Q	117
<u>wBai</u>	DAQNTKGQSLSEYKGDYNPPL-----VIQGNWVTTAIGLNWKGCIIL*KWI---ILV*Q	111
<u>wBoc</u>	GAQNTKGQSLSKYKGDYNPPFAANVALGYTGELGNNSCRAELEGMYSSVKVDXXXILV*Q	120
<u>wLeo</u>	GAQNTKGQSLSKYKGDYNPPFAANVALGYTGELGNNSCRAELEGMYSSVKVDXXXILV*Q	120
<u>wBic</u>	GAQNTGQSLSEYKGDYNPPFAANVAFGYTGELGNNSYRAELEGMYSSVKVD---NIGLT	117
<u>wMel reference</u>	SSQITVSYLKETGEDDPDKETYLYSAAVSHDQIENISVMANVYHHWKSDFSFSPYVGIGI	177
<u>wHa</u>	VAK*LFHT*RRLVRILIKKLISIVLQLVMTKLRTYL*WQMFIIIGKVTVSLFLLTLVLGS	177
<u>wBai</u>	VAK*LFHI*RTLVRMLIKKLISIVLQLIMTKLRTYL*WQMFIIIGKVTVSLFLLTLVLES	171
<u>wBoc</u>	VVK*LFHT*RTLVRMLVKKLVSIVLQLIMTKLKTCL*WQMFIIIGRVTVSLFLLMLGLES	180
<u>wLeo</u>	VVK*LFHT*RTLVRMLVKKLVSIVLQLIMTKLKTCL*WQMFIIIGRVTVSLFLLMLGLES	180
<u>wBic</u>	SSQITVSYLKETGEDDPNKETYLYSAAVSHDQIENISVMANVYHNWKSDFSFSPYVGIGI	177
<u>wMel reference</u>	GATRMTMFEKPSIRPAGQLKAGFDYRINEDVN---MHIGYRGFGAIGSDIKLTAKRLGQV	234
<u>wHa</u>	VQQE*RCLKNRQ*DPQVN*KLALTIA*TKM*I---CISDIEVLVLLVAILSLQQKG*DKW	234
<u>wBai</u>	VQQE*RCLKNRQ*DPQVK*KLALTIT*TKM*I---CISDIEVLVLLVAILSLQQKS*DKW	228
<u>wBoc</u>	VQQE*QCLKNRQ*DPQVN*KLALIIT*TKM*I---CISDIEVLVLLVLLVMVLNLQRM	237
<u>wLeo</u>	VQQE*QCLKNRQ*DPQVN*KLALIIT*TKM*I---CISDIEVLVLLVLLVMVLNLQRM	237
<u>wBic</u>	GATRMTMFEKPSIRPAGQLKAGFDYRINEDV*XXXCISDIEVLVLLVAILSLQQKG*DKW	237
<u>wMel reference</u>	VDDPNNDKKKK-----LNPSSGSKVTEEINIGNQLFHTHGIEAGLTFHFASKA	282
<u>wHa</u>	*TTLIMIKKRS-----LILAQVAK*LRK*I*VINYFIHTV*RLVLLSILPAKL	282
<u>wBai</u>	*TTLIMIKKRS-----LILAQVAKYSISHNSPNRS*EGRINSTTKGFE*RVN-	275
<u>wBoc</u>	LEKL*MILITGIKRSMLIRVNQQMQQKKI*I*VIHRFTHTV*RLALLSTLPAKL	291
<u>wLeo</u>	LEKL*MILITGIKRSMLIRVNQQMQQKKI*I*VIHRFTHTV*RLALLSTLPAKL	291
<u>wBic</u>	*TTLIMIKKRS-----LILAQVAKYSTSRNSPNRIRYVIMAMVEQ*HIMICSD	285

**Supplemental Figure 11.** Predicted *Wolbachia* surface proteins WD\_1063 (*wsp*/RS04815), WD\_0009 (*wspB*/RS00060), and WD\_0489 (*wspC*/RS06475) were assayed for their effect on eukaryotic cellular processes through ectopic expression in yeast. Note that galactose induction of gene expression of *wspC* kills the cells, while expression of *wsp* and *wspB* inhibits growth.





**Supplemental Table 1.** Scaffold count, N50, and total assembly size of each new *Wolbachia* assembly.

Host species	<i>Wolbachia</i>	Scaffold Count	N50	Total Assembly Size
<i>D. baimaii</i>	wBai	209	9004	1135209
<i>D. bicornuta</i>	wBic	140	16267	1192611
<i>D. bocki</i>	wBoc	227	7115	1090928
<i>D. burlai</i>	wBurl	128	20905	1248003
<i>D. chauvacae</i>	wCha	406	4802	1300201
<i>D. tsacasi</i>	wTsa	59	29597	1132109
<i>D. nikananu</i>	wNik	83	22663	1257108
<i>D. seguyi</i>	wSeg	201	8880	1174927
<i>D. tristis</i>	wTris	114	20171	1264866
<i>D. leontia</i>	wLeo	186	9149	1096851

**Supplemental Table 2.** Summary of domain annotation results from HHPred for WD\_1085 and WD\_0501.

Query name	Number	Hit	Name	Prob	E-value	Aligned cols	Target length	Query residues	Domain length
WD_1085	1	COG4775	BamA; Outer membrane protein assembly factor BamA [Cell wall/membrane/envelope biogenesis]	100	3.10E-63	733	766	25-778	753
WD_1085	2	COG0729	TamA; Outer membrane translocation and assembly module TamA [Cell wall/membrane/envelope biogenesis]	100	2.00E-42	505	594	227-778	551
WD_1085	3	COG2831	FhaC; Hemolysin activation/secretion protein [Intracellular trafficking, secretion, and vesicular transport]	100	2.90E-39	490	554	237-778	541
WD_1085	4	KOG2602	Predicted cell surface protein homologous to bacterial outer membrane proteins [General function prediction only]	100	1.00E-31	433	457	315-778	463
WD_1085	5	PF19143.3	Omp85_2 ; OMP85 superfamily	99.94	2.60E-23	308	360	419-778	359
WD_1085	6	COG4775	BamA; Outer membrane protein assembly factor BamA [Cell wall/membrane/envelope biogenesis]	99.93	6.20E-21	569	766	25-637	612
WD_1085	7	COG4775	BamA; Outer membrane protein assembly factor BamA [Cell wall/membrane/envelope biogenesis]	99.92	2.80E-22	310	766	93-433	340
WD_1085	8	PF01103.26	Omp85 ; Omp85 superfamily domain	99.92	5.40E-22	306	424	454-778	324
WD_1085	9	PF03865.16	ShlB ; Haemolysin secretion/activation protein ShlB/FhaC/HecB	99.87	2.10E-19	285	310	416-742	326
WD_1085	10	COG4775	BamA; Outer membrane protein assembly factor BamA [Cell wall/membrane/envelope biogenesis]	99.61	2.50E-13	232	766	24-275	251
WD_0501	1	PF05736.14	OprF ; OprF membrane domain	99.78	3.00E-16	134	186	159-303	144
WD_0501	2	COG3637	LomR; Opacity protein and related surface antigens [Cell wall/membrane/envelope biogenesis]	99.75	1.20E-15	135	199	166-303	137
WD_0501	3	COG3047	OmpW; Outer membrane protein W [Cell wall/membrane/envelope biogenesis]	99.74	1.80E-15	144	213	159-303	144
WD_0501	4	PF06316.14	Ail_Lom ; Enterobacterial Ail/Lom protein	99.7	1.20E-14	179	199	3-303	300
WD_0501	5	PF19573.2	DUF6089 ; Domain of unknown function (DUF6089)	99.67	3.60E-14	186	226	4-303	299
WD_0501	6	PF13505.9	OMP_b-brl ; Outer membrane protein beta-barrel domain	99.65	8.40E-14	175	209	7-303	296
WD_0501	7	SCOP_d1p4ta_	f.4.1.1 (A:) Outer membrane protein NspA {Neisseria meningitidis [TaxId: 487]}   CLASS: Membrane and cell surface protei	99.64	2.10E-13	154	155	95-303	208
WD_0501	8	PF01389.20	OmpA_membrane ; OmpA-like transmembrane domain	99.62	2.60E-13	173	202	95-303	208
WD_0501	9	SCOP_d1qjpa_	f.4.1.1 (A:) Outer membrane protein A (OMPA) transmembrane domain {Escherichia coli [TaxId: 562]}   CLASS: Membrane and	99.61	4.60E-13	167	171	94-303	209
WD_0501	10	PF03922.17	OmpW ; OmpW family	99.6	3.40E-13	164	201	96-303	207

**Supplemental Table 3.** Summary of results for *Wolbachia* surface proteins.

Gene	Description	Significant Phylogenetic Signal	Ectopic Expression
WD_1063 (wsp/RS04815)	surface antigen Wsp	None	Growth inhibition
WD_0009 (wspB/RS00060)	surface antigen Wsp paralog	None	Growth inhibition
WD_0489 (wspC/RS06475)	surface antigen, Wsp paralog	None	Lethality
WD_1085 (RS04910)	outer membrane protein assembly factor BamA	Oocyte posterior, posterior cortex	Growth inhibition and sensitive to microtubule checkpoints/inhibitors
WD_0501 (RS0225)	P44/Msp2 family outer membrane protein	Oocyte posterior	Slight growth inhibition
WD_1041 (RS04710)	peptidase M2	None	Not tested

**Supplemental Table 4.** Estimates of phylogenetic signal (Pagel's  $\lambda$ ) with 95% confidence intervals (CI) for *Wolbachia* surface proteins.

Locus	Whole Oocyte				Posterior				Posterior Cortex			
	$\lambda$	Lower CI	Upper CI	<i>P</i>	$\lambda$	Lower CI	Upper CI	<i>P</i>	$\lambda$	Lower CI	Upper CI	<i>P</i>
WD_1063 ( <i>wsp</i> )	0.000	0.000	0.578	1.000	0.945	0.000	0.993	0.071	0.000	0.000	0.633	1.000
WD_0009 ( <i>wspB</i> )	0.000	0.000	0.401	1.000	0.000	0.000	0.374	1.000	0.279	0.000	0.720	0.655
WD_0489 ( <i>wspC</i> )	0.210	0.000	0.947	0.659	0.000	0.000	0.845	1.000	0.000	0.000	0.811	1.000
WD_0501	0.000	0.000	0.597	1.000	0.992	0.521	1.000	0.022	0.000	0.000	0.536	1.000
WD_1041	0.000	0.000	0.692	1.000	0.000	0.000	0.555	1.000	0.000	0.000	0.484	1.000
WD_1085	0.000	0.000	0.553	1.000	0.974	0.000	0.998	0.001	0.942	0.000	0.994	0.028