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Determination of *Wolbachia* Diversity in Butterflies from Western Ghats, India, by a Multigene Approach

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Members of the genus *Wolbachia* are intracellular bacteria that are widespread in arthropods and establish diverse symbiotic associations with their hosts, ranging from mutualism to parasitism. Here we present the first detailed analyses of *Wolbachia* in butterflies from India with screening of 56 species. Twenty-nine species (52%) representing five families were positive for *Wolbachia*. This is the first report of *Wolbachia* infection in 27 of the 29 species; the other two were reported previously. This study also provides the first evidence of infection in the family Papilionidae. A striking diversity was observed among *Wolbachia* strains in butterfly hosts based on five multilocus sequence typing (MLST) genes, with 15 different sequence types (STs). Thirteen STs are new to the MLST database, whereas ST41 and ST125 were reported earlier. Some of the same host species from this study carried distinctly different *Wolbachia* strains, whereas the same or different butterfly hosts also harbored closely related *Wolbachia* strains. Butterfly-associated STs in the Indian sample originated by recombination and point mutation, further supporting the role of both processes in generating *Wolbachia* diversity. Recombination was detected only among the STs in this study and not in those from the MLST database. Most of the strains were remarkably similar in their *wsp* genotype, despite divergence in MLST. Only two *wsp* alleles were found among 25 individuals with complete hypervariable region (HVR) peptide profiles. Although both *wsp* and MLST show variability, MLST gives better separation between the strains. Completely different STs were characterized for the individuals sharing the same *wsp* alleles.

Wolbachia species are intracellular obligatory symbionts belonging to the family *Anaplasmataceae* that infect a large variety of arthropods and filarial nematodes (6, 50). These bacteria establish diverse symbiotic associations with their hosts ranging from mutualism to parasitism (51). They are known to manipulate biology of their host by inducing male killing, feminization, parthenogenesis, cytoplasmic incompatibility, and speciation through reproductive isolation (46, 50, 51). Their main strategy of transfer is vertical cytoplasmic inheritance; however, horizontal transfer across different hosts also occurs (5, 21) and accounts for the widespread distribution of these bacteria, which infect around 16% to 66% of insect species (14, 17). A remarkable genetic diversity exists in *Wolbachia*, and gene phylogenies show the existence of 11 supergroups (A to K) (7, 11, 30, 38, 39). Studies of *Wolbachia* using multilocus sequence typing (MLST) have demonstrated discriminatory power of these approaches in accurately characterizing and identifying various *Wolbachia* strains (2, 3, 36, 41, 43, 54, 55). *Wolbachia* infections have been reported in various Lepidoptera families such as Lycaenidae, Pieridae, Nymphalidae, Hesperiidae, Pyralidae, Noctuidae, and Lasiocampidae (14, 18, 23, 41, 48).

Butterflies are mainly day-flying insects of the order Lepidoptera, comprising the true butterflies (superfamily Papilionoidea), the skippers (superfamily Hesperioidea), and the moth-butterflies (superfamily Hedyloidea). They exhibit genetic polymorphisms, mimicry, and aposematism. Some butterflies have evolved symbiotic and parasitic relationships with social insects, such as ants (15, 37). Butterflies serve as important plant pollinators and help to pollinate more than 50 economically important crop plants (8). Some species in their larval stages are pests and damage domestic crops or trees (10, 13).

The considerable ecological, biological, and behavioral diversity of butterflies suggests the need for further characterization of *Wolbachia* to understand the impact of infection on their reproduction, evolution, and speciation. Data on the molecular biology and phenotypic effects of *Wolbachia* from some butterfly species show the presence of supergroup A and B *Wolbachia* strains (12, 14, 18, 23, 41, 48). *Wolbachia* strains in butterflies have been implicated in basic biological processes such as sex ratio distortion, sex determination, sperm-egg compatibility, and speciation (33, 42, 47). However, the distribution of *Wolbachia* strains among butterfly species is largely unknown.

India's diverse fauna includes a rich variety of butterflies, comprising 1,501 species, which accounts for one-fifth of the known butterfly species in the world (16, 26). Western Ghats harbor 330 known species (16) belonging to 166 genera and 5 families (Lycaenidae, Pieridae, Nymphalidae, Papilionidae, and Hesperiidae [27]) and including 37 endemic species and another 23 shared only with Sri Lanka (16). Curiously, this tropical group from India and particularly Western Ghats, which is a biodiversity hot spot, has not yet been explored for *Wolbachia* infection. In the present

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report we show (i) the presence of *Wolbachia* among a sample of butterflies from Western Ghats belonging to five families; (ii) the diversity of *Wolbachia* strains within these butterflies, determined by using MLST and *wsp* genes; (iii) the phylogenetic relatedness of butterfly *Wolbachia* strains; and (iv) the role of recombination and point mutation in generating new sequence types (STs) in *Wolbachia*.

MATERIALS AND METHODS

Insects and DNA extraction. Butterflies used in this study were collected during 2006 to 2008 from different regions of Western Ghats, India (Table 1). Legs of the butterflies were removed and preserved in absolute ethanol at −20°C until DNA extraction. Legs were used for screening, and the rest of the specimen was preserved for identification. Use of legs in screening for *Wolbachia* is a common practice (19, 24, 29, 31, 33). DNA was extracted from tissue using a QIAamp DNA minikit (Qiagen) following the manufacturer's instructions. In cases where specimens were small, abdomens were used. The specimens of all the butterflies under study were morphologically identified at the specimen collection and preservation center of the Department of Zoology, Modern College, Pune, India.

***Wolbachia* DNA amplification and sequencing.** The quality of DNA extracted from samples was checked by PCR targeting butterfly DNA using arthropod-specific 28S primers, amplified as described by Werren et al. (53), and samples with weak or no amplification were extracted again. All the specimens were screened initially for *Wolbachia* infection by PCR for the *wsp* (9) and *ftsZ* (5) genes using primers and previously described protocols. Primer details and PCR protocols for amplification of the five reported *Wolbachia* MLST genes (*ftsZ*, *coxA*, *fbpA*, *hcpA*, and *gatB*) and *wsp* genes are described elsewhere (2). The sequence data were analyzed against the *Wolbachia* MLST database (http://pubmlst.org/wolbachia/). All PCR products were purified using the polyethylene glycol (PEG)-NaCl method (44). The successfully amplified products of the five MLST genes and the *wsp* gene were sequenced bidirectionally with the respective primers using a BigDye terminator cycle sequencing kit, version 3.1 (Applied Biosystems). Sequences were obtained using an automatic DNA sequencer (3730 DNA analyzer; ABI).

At least one HVR for *wsp* gene was sequenced for 38 individuals. *wsp* typing assigned the *wsp* allele to 25 individuals with complete profiles for four HVRs. Alleles for 13 individuals could not be assigned due to incomplete HVR profiles. However, HVR peptide numbers were assigned to these individuals. Four strains could not be amplified with the *wsp* gene.

***Wolbachia* genetic diversity.** Estimates of genetic diversity (*Pi*), number of variable sites (VI), and ratios of synonymous substitutions per synonymous site to nonsynonymous substitutions per nonsynonymous site (K_a/K_s) were performed by DNAsp, version 4.10.2 (40).

Recombination and pairwise genetic distance analysis. MaxChi (45) and GENECONV (35) programs in the RDP3 package (32) were used to perform the recombination analysis of the concatenated MLST gene alignment from butterfly STs. A Bonferroni correction was applied, and 100 permutations were generated. The highest acceptable *P* value cutoff was set at 0.05. The pairwise genetic distance of different *Wolbachia* strains was tested by using the Kimura 2-parameter method in MEGA4 (25, 49). Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) program, version 17.0. Comparisons between the supergroups were performed using the Mann-Whitney U test for independent pairwise comparisons. A percent similarity matrix was calculated in Phylip 3.69. A concatenated data set for all MLST genes was considered for pairwise comparisons. The percent differences in pairwise comparisons between supergroups were evaluated by a Wilcoxon signed-rank test. A *P* value of less than 0.05 was considered statistically significant.

Phylogenetic analysis. We retrieved STs and *wsp* alleles currently available in the MLST database for all members of supergroup B and Lepidoptera representatives of supergroup A. *Wolbachia* gene sequences (MLST and *wsp*) generated in this study were aligned with homologous sequences deposited in the *Wolbachia* MLST database using ClustalX, version 2.0.9 (28). All sequences were manually edited using MEGA4 (49). Unrooted phylogenetic trees were constructed using Bayesian inference and the neighbor-joining method for a concatenated data set for the five MLST genes and a separate data set for the *wsp* gene. For Bayesian inference of phylogeny, the program MrBayes 3.1.2 was used (20). The analysis for each gene consisted of 3,000,000 generations with sampling every 100 generations. The first 12,000 trees (40%) were discarded as "burn-in." Before the probabilistic phylogenetic analyses were carried out, appropriate models of sequence evolution for each data set were chosen via the Akaike information criterion (AIC) using the program MrModeltest 2.2 (34). The selected model of nucleotide substitution was GTR+I+G for concatenated MLST gene sequences and the *wsp* gene. The final alignments consisted of 2,079 bp for concatenated MLST gene sequences and 495 bp for *wsp* gene fragments. Only the strains with full STs (complete five MLST alleles) were selected to construct the phylogenetic tree for the concatenated data set. The strains with incomplete allelic profiles were therefore omitted from the concatenated analysis. Similarly, strains with at least three complete HVRs were selected to construct the *wsp* phylogenetic tree. Three independent runs were performed for each data set. In phylogenetic trees, levels of confidence for each node are shown in the form of Bayesian posterior probabilities (BPP). BPP below 0.50 are not shown. NJ trees were constructed using MEGA 4.1 with 1,000 bootstrap replicates and the Kimura 2-parameter method as a model of nucleotide substitution.

Nucleotide sequence accession numbers. Sequences obtained in this study were deposited in the *Wolbachia* MLST and GenBank databases with the alleles and accession numbers, respectively, noted in Table 2.

RESULTS

A total of 118 individuals representing 56 species belonging to five families of Lepidoptera were screened for *Wolbachia* by PCR assay using *Wolbachia*-specific *wsp* and *ftsZ* gene primers. The infection status of each species and the number of individuals screened are listed in Table 1. Twenty-nine species representing all five families were positive for *Wolbachia* (Table 1). In total, 44 of the 118 individuals were positive.

At least one MLST gene was amplified and sequenced for all 44 individuals. Two individuals exhibited the presence of multiple *Wolbachia* infections, with double peaks in the chromatograms. These strains were removed from the analysis. Complete MLST profiles were generated for 26 *Wolbachia* strains, whereas repeated failures to PCR amplify particular *Wolbachia* genes (MLST and *wsp*) resulted in 16 incomplete profiles (Table 2).

Sequence typing was performed on the 26 complete MLST strains using the *Wolbachia* MLST database (http://pubmlst.org/wolbachia/) (Table 2). Characterization of allelic profiles indicated the presence of 15 STs in butterflies from this study. Of these, the allelic profiles and STs for 13 strains were new to the MLST database, whereas two STs (ST41 and ST125) were previously known (Table 2).

Divergence among the 26 STs accounted for 97 variable sites (VI) out of 2,073 sites (4.679%) with concatenated alignment of all five *Wolbachia* MLST genes (Table 3). The gene *coxA* showed the highest nucleotide divergence, with 36 variable sites out of 402 (8.955%), followed by *gatB*, with 28 variable sites out of 369 (7.588%) (Table 3). The average K_a/K_s per gene was found to be <1 (average K_a/K_s across genes is 0.16877), which indicates strain evolution mainly by synonymous substitutions. This is in line with a scenario of strong purifying selection.

Occurrence of recombination and point mutations. The recombination analysis based on the MLST concatenated data indicated that recombination events occurred in three STs, ST151

TABLE 1 Screening of *Wolbachia* in butterflies from different families

Family	No. of specimens	Butterfly	Collection location[a]	No. of specimens		Body part used for DNA isolation
				Total	Positive	
Papilionidae	8	*Graphium agamemnon*	F	2	0	Leg
		Papilio demoleus[b]	D, F	2	1	Leg
		Papilio polytes	F	4	0	Leg
Nymphalidae	53	*Junonia arithya*	F	1	0	Leg
		Acraea violae	F	2	0	Leg
		Ariadne merione[b]	B, F, G	3	1	Leg
		Byblia ilithyia	B	1	0	Leg
		Charaxes dolon	F	1	0	Leg
		Danaus chrysippus[b]	E, F	3	1	Leg
		Danaus genutia	F	1	0	Leg
		Euploea core	F, H	2	0	Leg
		Euthalia lubentina	F	1	0	Leg
		Euthalia nais	F	1	0	Leg
		Hypolimnas bolina	C, F	7	3	Leg
		Hypolimnas misippus	F	4	0	Leg
		Junonia almana	F	1	0	Leg
		Junonia hierta	F	1	0	Leg
		Junonia lemonias[b]	C, F	3	1	Leg
		Lethe europa	F	1	0	Leg
		Melanitis leda[b]	D, F	2	1	Leg
		Moduza procris	F	1	0	Leg
		Neptis hylas[b]	F	2	1	Leg
		Parantica aglea[b]	E, H	2	1	Leg
		Polyura athamas	F	1	0	Leg
		Precis iphita[b]	E, H	2	1	Leg
		Tirumala limniace[b]	F	2	1	Leg
		Tirumala septentrionis	H	1	0	Leg
		Vanessa cardui	F	1	0	Leg
		Ypthima asterope[b]	C, E	5	1	Abdomen
		Ypthima sp.	C	1	0	Abdomen
Pieridae	36	*Anaphaeis aurota*	F	1	0	Abdomen
		Catopsilia pomona[b]	E, F	10	2	Leg
		Cepora nerissa[b]	H	1	1	Leg
		Colotis amata[b]	A	2	2	Abdomen
		Delias eucharis[b]	F	1	1	Leg
		Eurema brigitta	F	2	0	Abdomen
		Eurema hecabe	C, D, E, F, G, H	11	5	Abdomen
		Eurema laeta[b]	C, F	2	1	Abdomen
		Ixias marianne	F	2	0	Leg
		Ixias pyrene[b]	F	1	1	Leg
		Leptosia nina[b]	H	1	1	Abdomen
		Pareronia valeria[b]	F, H	2	2	Leg
Lycaaenidae	16	*Caleta caleta*[b]	E	1	1	Abdomen
		Castalius rosimon[b]	C, F	2	2	Abdomen
		Curetis thetis	F	1	0	Abdomen
		Jalmenus evagoras[b]	E	2	2	Abdomen
		Jamides bochus	F	1	0	Abdomen
		Pseudozizeeria maha[b]	F	2	2	Abdomen
		Talicada nyseus[b]	F	3	3	Abdomen
		Tarucus nara[b]	C	1	1	Abdomen
		Zizeeria knysna[b]	F	2	2	Abdomen
		Euchrysops cnejus	C	1	0	Abdomen
Hesperiidae	5	*Telicota ancilla*	H	1	0	Abdomen
		Borbo cinnara	B	1	0	Abdomen
		Taractrocera ceramus[b]	E	2	1	Abdomen
		Udaspes folus[b]	H	1	1	Abdomen

[a] A, Ahemadnagar; B, Alandi; C, Junnar; D, Khanapur; E, Mulshi; F, Pune city; G, Satara; and H, Thane.
[b] Butterfly found to harbor *Wolbachia* for the first time.

TABLE 2 Complete and partial MLST and WSP profiles of *Wolbachia* isolates

ID	Collection reference no.	Host		Collection location[a]	Strain	*wsp* allele (accession no.)	*wsp* profile				Allele (accession no.)					ST
		Species	Family				HVR1	HVR2	HVR3	HVR4	*gatB*	*coxA*	*hcpA*	*ftsZ*	*fbpA*	
208	B4	*Ariadne merione*	Nymphalidae	F	Amer_B_wMer	64 (JN236173)	35	35	38	44	96 (JN236109)	14 (JN236015)	40 (JN236146)	73 (JN236075)	4 (JN236043)	153
209	B21	*Colotis amata*	Pieridae	A	Cama_B_wAma1	(JN236208)		8	10		4 (JN236103)	14 (JN236010)	40 (JN236141)	7 (JN236070)	4 (JN236038)	147
210	B22	*Colotis amata*	Pieridae	A	Cama_B_wAma2	10 (JN236195)	10	8	10	8	16 (JN236104)	14 (JN236011)	40 (JN236142)	36 (JN236071)	4 (JN236039)	150
211	F37	*Caleta caleta*	Lycaenidae	E	Ccal_B_wCal	(JN236206)	10	8	10		9 (JN236128)		40 (JN236152)			
212	F48	*Cepora nerissa*	Pieridae	H	Cner_B_wNer	10 (JN236181)	10	8	10	8	4 (JN236127)	14 (JN236021)	3 (JN236154)	36 (JN236092)	4 (JN236058)	145
213	F22	*Catopsilia pomona*	Pieridae	F	Cpom_B_wPom	10 (JN236202)	10	8	10	8	39 (JN236116)	14 (JN236026)		36 (JN236085)		
214	F3	*Catopsilia pomona*	Pieridae	F	Cpom_B_wPom	(JN236178)		8	10	8	9 (JN236118)					
215	F14	*Castalius rosimon*	Lycaenidae	C	Cros_B_wRos	10 (JN236182)	10	8	10	8	9 (JN236112)		40 (JN236150)			
216	B5	*Castalius rosimon*	Lycaenidae	F	Cros_B_wRos										4 (JN236044)	
217	F36	*Danaus chysippus*	Nymphalidae	E	Dchr_B_wChr	(JN236174)	2	142	131		39 (JN236120)	11 (JN236028)	101 (JN236163)	36 (JN236081)	4 (JN236052)	151
218	B28	*Delias eucharis*	Pieridae	F	Deuc_B_wEuc	10 (JN236197)	10	8	10	8	39 (JN236107)	14 (JN236014)	40 (JN236145)	36 (JN236074)	4 (JN236042)	41
219	F78	*Eurema hecabe*	Pieridae	F	Ehec_B_wHec3	10 (JN236184)	10	8	10	8	102 (JN236137)	14 (JN236034)	29 (JN236170)	36 (JN236099)	42 (JN236065)	157
220	B7	*Eurema hecabe*	Pieridae	F	Ehec_B_wHec1	10 (JN236199)	10	8	10	8	39 (JN236108)	14 (JN236016)	40 (JN236147)	36 (JN236078)	4 (JN236046)	41
221	F47	*Eurema hecabe*	Pieridae	E	Ehec_B_wHec1	10 (JN236188)	10	8	10	8	39 (JN236126)	14 (JN236020)	40 (JN236153)	36 (JN236091)	4 (JN236057)	41
222	F53	*Eurema hecabe*	Pieridae	H	Ehec_B_wHec1	10 (JN236189)	10	8	10	8	39 (JN236132)	14 (JN236030)	40 (JN236164)	36 (JN236095)	4 (JN236061)	41
223	F7	*Eurema hecabe*	Pieridae	D	Ehec_B_wHec2	(JN236171)	19	17	24		102 (JN236136)	14 (JN236033)	100 (JN236167)	36 (JN236098)	4 (JN236064)	156
224	F9	*Eurema laeta*	Pieridae	C	Elae_B_wLae	(JN236172)	19	17	24		9 (JN236138)	80 (JN236035)	100 (JN236169)	8 (JN236100)	156 (JN236066)	149
225	B11	*Hypolimnas bolina*	Nymphalidae	F	Hbol_B_wBol	10 (JN236200)	10	8	10	8	9 (JN236102)	14 (JN236008)	40 (JN236140)	73 (JN236068)	4 (JN236037)	148
226	F15	*Hypolimnas bolina*	Nymphalidae	C	Hbol_B_wBol	(JN236191)		8			4 (JN236113)		40 (JN236151)	36 (JN236079)		
227	B14	*Hypolimnas bolina*	Nymphalidae	F	Hbol_B_wBol	10 (JN236180)	10	8	10	8		14 (JN236009)		36 (JN236069)		
228	F38	*Junonia iphita*	Nymphalidae	E	Jiph_B_wIph	10 (JN236183)	10	8	10	8	9 (JN236121)		100 (JN236158)	73 (JN236080)	4 (JN236053)	
229	F20	*Junonia lemonias*	Nymphalidae	C	Jlem_B_wLem	10 (JN236198)	10	8	10	8	4 (JN236114)	14 (JN236024)	40 (JN236161)	36 (JN236088)	4 (JN236050)	146
230	F21	*Ixias pyrene*	Pieridae	F	Ipyr_B_wPyr	10 (JN236203)	10	8	10	8	39 (JN236115)	14 (JN236025)	40 (JN236159)	36 (JN236084)	4 (JN236048)	41
231	F45	*Jalmenus evagoras*	Lycaenidae	E	Jeva_B_wEva1	(JN236176)	18	16	23		101 (JN236124)	79 (JN236029)	99 (JN236156)	73 (JN236089)	155 (JN236055)	155
232	F46	*Jalmenus evagoras*	Lycaenidae	E	Jeva_B_wEva2	(JN236175)	18	16			101 (JN236125)	79 (JN236019)	40 (JN236155)	73 (JN236090)	4 (JN236056)	154
233	F49	*Udaspes folus*	Hesperidae	H	Ufol_B_wFol	10 (JN236179)	10	8	10	8	39 (JN236129)			36 (JN236093)	4 (JN236059)	
234	F65	*Zizeeria knysna*	Lycaenidae	F	Zkny_B_wKny	10 (JN236194)	10	8	10	8	39 (JN236135)	14 (JN236032)	40 (JN236166)	36 (JN236097)	4 (JN236063)	41
235	F51	*Leptosia nina*	Pieridae	H	Lnin_B_wNin	10 (JN236177)	10	8	10	8	39 (JN236131)	14 (JN236023)	40 (JN236162)	7 (JN236094)	4 (JN236062)	152
236	F5	*Melanitis leda*	Nymphalidae	D	Melanitis leda						39 (JN236130)	9 (JN236022)				
237	B26	*Neptis hylas*	Nymphalidae	F	Nhyl_B_wHyl	(JN236186)	10	8			39 (JN236106)	14 (JN236013)	40 (JN236144)	36 (JN236073)	4 (JN236041)	41
238	F39	*Parantica aglea*	Nymphalidae	E	Pagl_B_wAgl						39 (JN236122)				4 (JN236054)	
239	F6	*Papilio demoleus*	Papilionidae	D	Pdem_B_wDem	10 (JN236193)	10	8	10	8	39 (JN236134)					
240	B8	*Pseudozizeeria maha*	Lycaenidae	F	Pmah_B_wMah	10 (JN236201)	10	8	10	8	39 (JN236110)	14 (JN236017)	40 (JN236148)	36 (JN236077)	4 (JN236047)	41
241	F29	*Pseudozizeeria maha*	Lycaenidae	F	Pmah_B_wMah	10 (JN236205)	10	8	10	8	39 (JN236117)	14 (JN236027)	40 (JN236160)	36 (JN236083)	4 (JN236051)	41
242	B25	*Pareronia valeria*	Pieridae	F	Pval_B_wVal	(JN236185)	10	8	10		39 (JN236105)	14 (JN236012)	40 (JN236143)	36 (JN236072)	4 (JN236040)	41
243	F54	*Pareronia valeria*	Pieridae	H	Pval_B_wVal	(JN236207)	10	8	10		39 (JN236133)	14 (JN236031)	40 (JN236165)	36 (JN236096)	4 (JN236060)	41
244	F34	*Teractrocera ceramus*	Hesperidae	E	Tcer_B_wCer	(JN236187)	10	8						36 (JN236082)		
245	F16	*Tarucus nara*	Lycaenidae	C	Tnar_B_wNar									36 (JN236087)	4 (JN236049)	
246	RP3	*Telicada nyseus*	Lycaenidae	F	Tnys_B_wNys1	10 (JN236190)	10	8	10	8	4 (JN236139)	14 (JN236036)	40 (JN236168)	36 (JN236101)	4 (JN236067)	146
247	B9	*Telicada nyseus*	Lycaenidae	F	Tnys_B_wNys2	10 (JN236196)	10	8	10	8	4 (JN236111)	14 (JN236018)	40 (JN236149)	73 (JN236076)	4 (JN236045)	125
248	F4	*Telicada nyseus*	Lycaenidae	F	Tnys_B_wNys	10 (JN236204)	10	8	10	8	4 (JN236123)		40 (JN236157)			
249	F35	*Ypthima asterope*	Nymphalidae	E	Yast_B_wAst	10 (JN236192)	10	8	10	8	39 (JN236119)			36 (JN236086)		

[a] A, Ahemadnagar; B, Alandi; C, Junnar; D, Khanapur; E, Mulshi; F, Pune city; G, Satara; and H, Thane.

TABLE 3 Genetic features at five MLST and *wsp* loci

Locus	Pi_s	Pi_a	K_a/K_s	% VI
gatB	0.04352	0.00494	0.113511	7.588
coaX	0.06432	0.00534	0.083022	8.955
hcpA	0.02250	0.00510	0.226666	5.630
fbpA	0.00523	0.00220	0.420650	3.782
ftsZ	0.01270	0.0000	0.00000	1.379
MLST concatenated	0.04038	0.00575	0.142397	4.679
wsp	0.06589	0.03153	0.478524	29.580

(beginning breakpoint; 1234, ending breakpoint, 9) and ST154 and ST155 (beginning breakpoint; 773, ending breakpoint, 9). The events revealed that this localized divergence was the result of recombination involving the major parent ST149 for all three STs, the minor parent ST145 for ST151, and the minor parent ST153 for ST154 and ST155 (Table 4). Even if ST151, ST154, and ST155 are recombinant, the phylogeny inference based on MLST placed these three STs in supergroup B.

ST154 and ST155 were observed in *Jalmenus evagoras* specimens which were collected from the same location (Mulshi, Pune, India). These STs are results of the point mutations in *hcpA* (base 1004) and *fbpA* (base 1890). (All the base positions given here are based on the concatenated MLST gene data set.) ST145 (*Cepora nerissa*, Pieridae) and ST146 (*Junonia lemonias*, Nymphalidae, and *Talicada nyseus*, Lycaenidae) are very closely related and arose as a result of the point mutation at base 1181 in *hcpA*. Surprisingly, these two STs were found in distinctly different hosts from three localities and belonging to three different families. *Hypolimnas bolina* from the family Nymphalidae (this study) and *Colias erate poliographus* from the family Pieridae (from Japan) were found to harbor very closely related *Wolbachia* (ST148 and ST141, respectively). These STs were also formed as a result of point mutations in gene gatB at bases 315 and 359. ST125 (*Hypolimnas bolina* and *Talicada nyseus*) and ST147 were formed as a result of point mutations at bases 1503 (*ftsZ*) and 1709 (*fbpA*). ST41 was observed among a wide range of butterfly hosts, and it showed point mutations in *gatB* at positions 120 and 359 relative to ST150, which was found in *Colotis amata*. These results suggest that along with the recombination, point mutation events also play a crucial role in the genesis of new STs in *Wolbachia*.

Genetic distance among STs. To detect whether the genetic variation (percent similarity) was statistically significant, a nonparametric Mann-Whitney U test was independently conducted between *Wolbachia* representatives of supergroup A from the MLST database, all supergroup B strains, and the samples from this study. Three different tests were conducted for the three data sets described above (Table 5). The data reveal that the genetic difference among *Wolbachia* strains in this study and the genetic difference among the rest of the supergroup B *Wolbachia* strains

TABLE 4 Occurrence of recombination in the concatenated data set

Recombinant sequence	Major parent	Minor parent	Average *P* value by:	
			MaxChi	GENECONV
ST151	ST149	ST145	2.720×10^{-2}	1.201×10^{-5}
ST154	ST149	ST153	2.298×10^{-3}	1.729×10^{-4}
ST155	ST149	ST153	2.298×10^{-3}	1.729×10^{-4}

TABLE 5 Comparison of genetic variation (% similarity) in *Wolbachia* isolates

Groups	Mann-Whitney U test value	*P* value
Supergroups A and B	2,086.500	0.038
Supergroup B and this study	4,565.500	0.00
Supergroup A and this study	360.000	0.001

are not significantly different (U = 4565.5, $P = 0.00$). The genetic difference between supergroup A and supergroup B *Wolbachia* strains is significantly higher (U = 2086.5, $P = 0.038$) than that between supergroup A strains and butterfly symbiont strains in this study (U = 360.0, $P = 0.001$). This suggests that butterfly *Wolbachia* isolates from this study are very closely related to the rest of the supergroup B *Wolbachia* isolates in the MLST database.

Diversity of *Wolbachia* strains in butterflies. Phylogenetic reconstructions for all genes by Bayesian inference and neighbor-joining methods showed similar results. Phylogenetic reconstructions based on concatenated alignment of *hcpA*, *gatB*, *coxA*, *ftsZ*, and *fbpA* indicated a strong clustering of all butterfly *Wolbachia* isolates from this study within supergroup B; none of the strains belonged to supergroup A (Fig. 1). *Wolbachia* strains from Western Ghats Lepidoptera were classified in three major clusters. A large cluster with strong support (0.88) includes 11 STs that are newly described in this study, two STs found in this study but previously known to occur in other Lepidoptera, four STs previously found in other Lepidoptera, and one ST from *Culex* mosquitoes. A second clade contains three lepidopteran *Wolbachia* isolates from other regions of the world and ST149 from Western Ghats along with one *Wolbachia* isolate from the Hymenoptera. A third clade contains *Wolbachia* isolates from diverse insect taxa, as well as isolates identified from our sample in *Danaus chrysippus* (ST151).

Most of the strains were remarkably akin in terms of their *wsp* genotypes. HVR1, HVR2, and HVR3 showed same variability, with the presence of five alleles in the sequenced data set. HVR4 showed the presence of two alleles, though this region was not sequenced for most of the individuals. Only two *wsp* alleles were found among 25 individuals with complete HVR peptide profiles. However, unique partial HVR peptide profiles indicated the possibility of the presence of three additional *wsp* alleles for (i) *Danaus chrysippus* (collection reference number F36), (ii) *Eurema hecabe* (F7) and *Eurema laeta* (F9), and (iii) *Jalmenus evagoras* (F45 and F46). Analysis of the relationships in phylogeny among butterfly strains supports five main clusters (Fig. 2). The most prevalent allele, *wsp-10*, was shared by 16 butterfly species from our study and six insect species representing Lepidoptera (French Polynesia, Ghana, Japan, Malaysia, and Taiwan) and Diptera (United States) from the MLST database (Fig. 2). The strains from *Ariadne merione* (B4) harbored *wsp-64*, which was shared with *Colias erate poliographus* (Japan), *Eurema hecabe* (Japan), and *Surendra vivarna* (Malaysia) (Fig. 2).

DISCUSSION

This is the first detailed analysis of *Wolbachia* in butterflies from India. For 27 butterfly species, this was also the first detection of infection by *Wolbachia*, though its occurrence in *Hypolimnas bolina* and *Eurema hecabe* was reported previously (14, 48). The

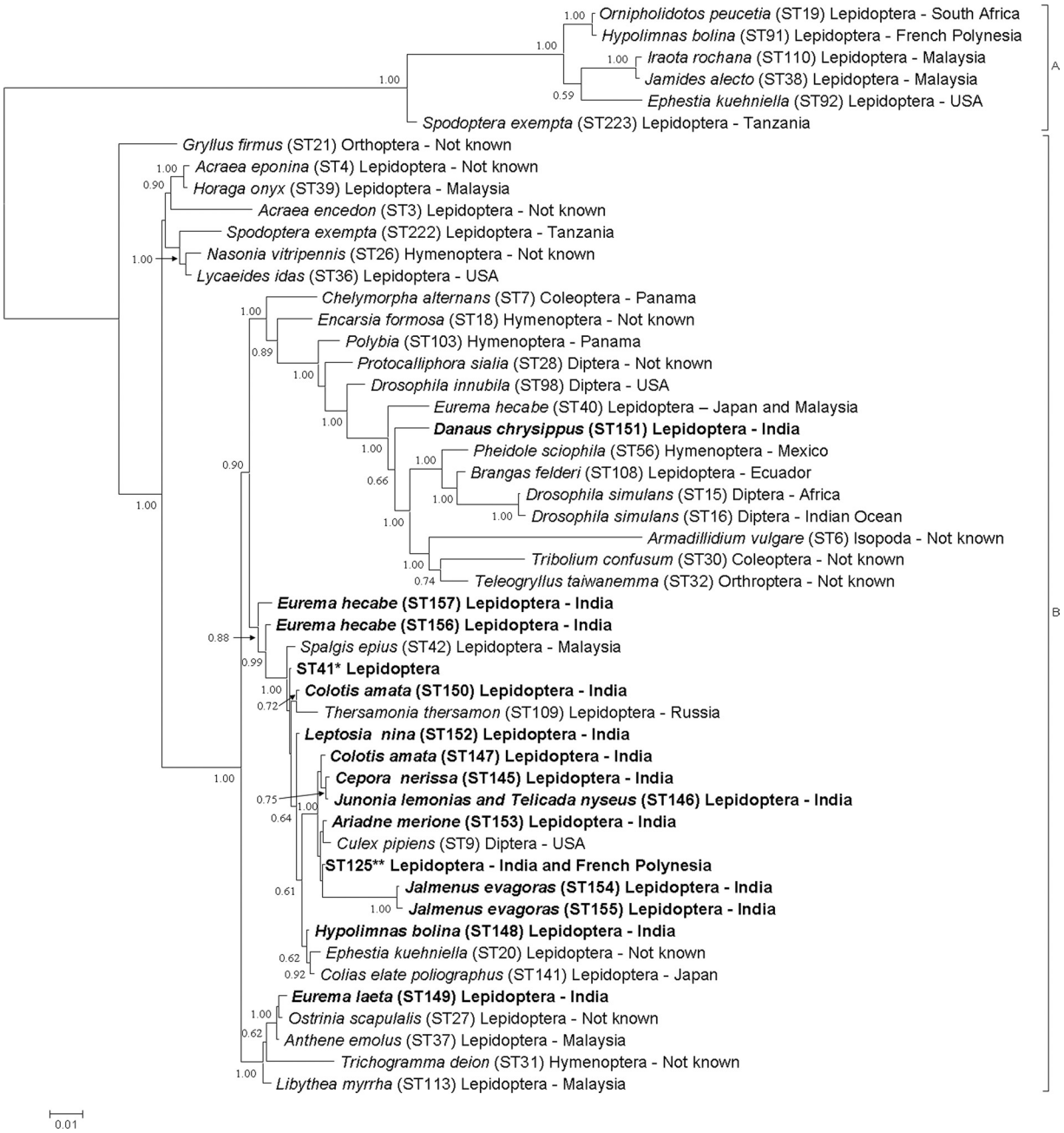

FIG 1 Unrooted phylogenetic relationships between *Wolbachia* strains from butterflies (bold) and those infecting other organisms, representing two supergroups (50 *Wolbachia* isolates), based on concatenated alignment of MLST loci (2,079 bp). *Wolbachia* supergroups are shown to the right side of the host species names. The bar shows substitutions per site. *, ST shared by samples from *Pareronia valeria* (B25 and F54), *Neptis hylas* (B26), *Delias eucharis* (B28), *Eurema hecabe* (B7, F47, and F53), *Pseudozizeeria maha* (B8 and F29), *Ixias pyrene* (F21), *Zizeeria knysna* (F65), *Nacaduba angusta* (Malaysia), *Azanus mirza* (Ghana), *Celastrina argiolus* (United States), *Eurema mandarina* (Japan) and *Eurema hecabe* (Japan) from MLST database. **, ST shared by *Talicada nyseus* (B9) from our study and *Hypolimnas bolina* (French Polynesia) and *Spodoptera exempta* (Tanzania) from the MLST database; STs and allele numbers are shown after each species name in parentheses.

butterfly *Wolbachia* strains belonged to supergroup B. Good-quality chromatograms were obtained for 42 *Wolbachia* strains, suggesting amplification of a single *Wolbachia* strain during the reaction. Mixed signals were observed in the chromatograms of samples from *Eurema hecabe* (F43) and *Zizeeria knysna* (B6), indicating the presence of more than one *Wolbachia* strain. Hence, these sequences were omitted from further analyses. This indicated the utility of MLST primers to detect multiple infections, as investigated by Baldo et al. (2).

It is interesting to note the existence of a number of new STs present in Indian butterflies that had not previously been found in butterflies or other arthropods. The phylogenetic tree constructed using the shared region of *wsp* nucleotides also demonstrated the affiliation of butterfly *Wolbachia* from this study with supergroup

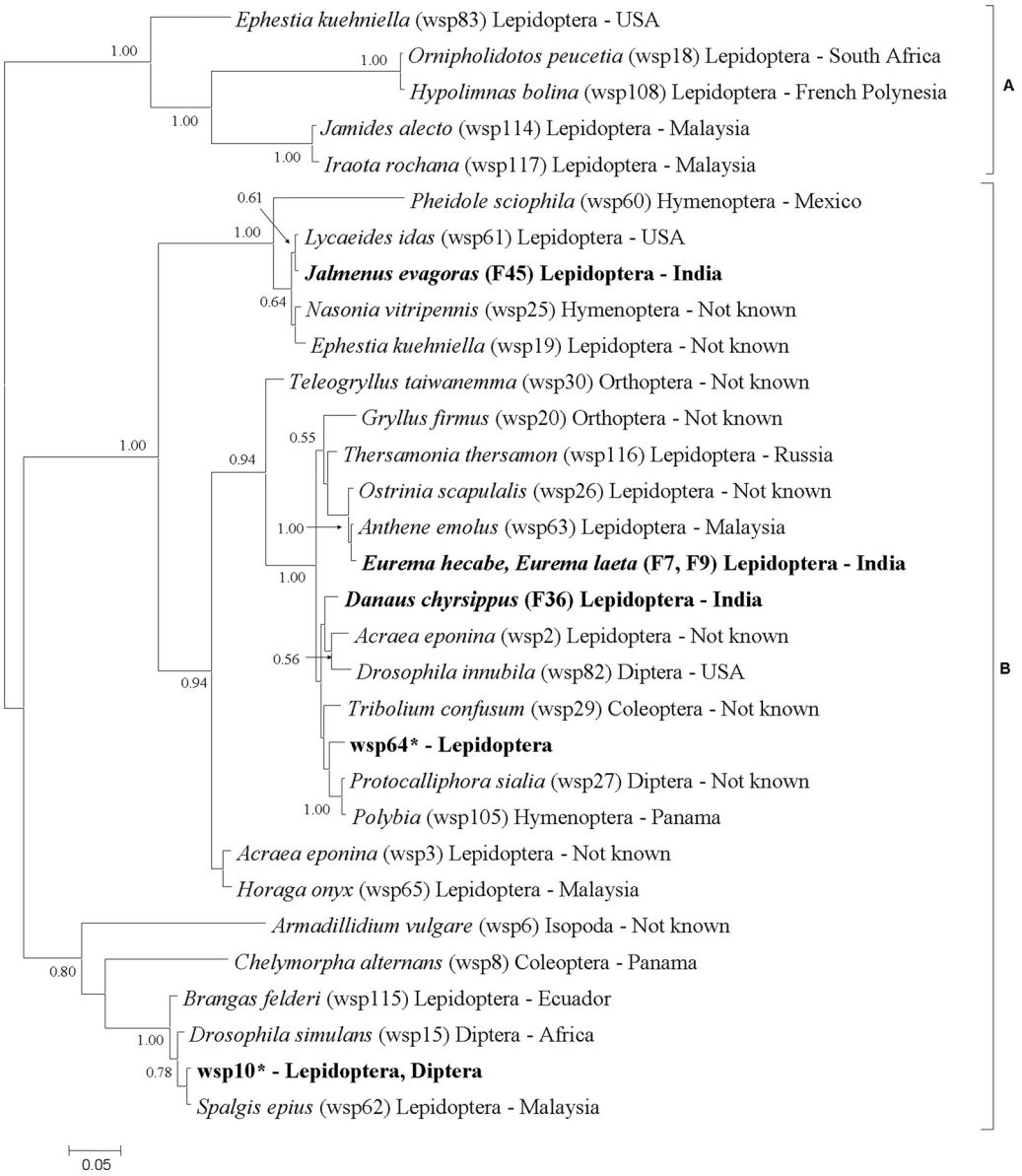

FIG 2 Unrooted phylogenetic relationships between *Wolbachia* from butterflies (bold) and those infecting other organisms representing two supergroups (31 *Wolbachia* isolates), based on *wsp* loci (495 bp). The *wsp* alleles are shown after each species name in parentheses. *Wolbachia* supergroups are shown to the right side of the host species names. The bar shows substitutions per site. *, *wsp-10* shared by *Hypolimnas bolina* (French Polynesia and India), *Azanus mirza* (Ghana), *Castalius rosimon* (India), *Catopsilia pomona* (India), *Celastrina argiolus* (United States), *Cepora nerissa* (India), *Colotis amata* (India), *Culex pipiens* (United States), *Delias eucharis* (India), *Eurema hecabe* (India), *Eurema mandarina* (Japan and Taiwan), *Ixias pyrene* (India), *Junonia iphita* (India), *Junonia lemonias* (India), *Leptosia nina* (India), *Nacaduba angusta* (Malaysia), *Papilio demoleus* (India), *Pseudozizeeria maha* (India), *Talicada nyseus* (India), *Ypthima asterope* (India), *Zizeeria knysna* (India), and *Udaspes folus* (India). **, *wsp-64* shared by *Ariadne merione* (India), *Colias erate poliographus* (Japan), *Eurema hecabe* (Japan), and *Surendra vivarna* (Malaysia).

B. As has been observed for other *Wolbachia* strains (2, 4), the *wsp* phylogeny is not concordant with the MLST gene phylogeny. These results are consistent with the high rates of recombination between *wsp* and other MLST genes and within the *wsp* locus (2, 4). In our study, legs were used to screen for *Wolbachia*. It is possible that some infection types were missed by this method, although it likely also facilitates our analysis by reducing the complications of multiple infections in evaluations MLST types. Legs are commonly used in some *Wolbachia* screens (19, 24, 29, 31, 33), in part because the remainder of the specimen is then preserved for identification.

The MLST system works better than *wsp* typing in butterflies. Both *wsp* typing (% VI, 29.580; K_a/K_s, 0.478524) and MLST (% VI, 4.679; K_a/K_s, 0.142397) were found to give variability among the strains. All the strains from this study consistently belonged to supergroup B according to both the typing methods. Some strains sharing common STs also shared *wsp* alleles. ST41 and *wsp-10* were shared among five Western Ghats strains and five butterfly species from other geographic regions in the MLST database.

In contrast, many species differed in their STs and *wsp* alleles. Eight completely different STs were characterized for the individ-

uals sharing same *wsp* allele 10 (Table 2). Two individual *Talicada nyseus* specimens (B9 and RP3) shared a *wsp* allele 10 but harbored two distinctly different STs (ST125 and ST146). Four different *Eurema hecabe* individuals shared a *wsp* allele (*wsp-10*), but MLST typing characterized two different strains in these four samples, with ST157 in one (F78) and ST41 in the other three (B7, F47, and F53). Strains grouped closely on the basis of *wsp* were found to differ in their phylogenetic affiliation as determined by MLST (Fig. 1 and 2). Phylogenetic reconstruction using *wsp* showed the same affiliation for *Eurema hecabe* (F7) and *Eurema laeta* symbionts (F9) (Fig. 1), while they had two distinctly different STs (ST156 and ST149) and were positioned at different places in MLST phylogeny (Fig. 1). The *Ariadne merione* symbiont (ST153) shared a *wsp* allele (*wsp-64*) with isolates from *Colias erate poliographus* (ST141), *Eurema hecabe* (ST40), and *Surendra vivarna* (ST40) but formed a separate clade in MLST phylogeny (Fig. 1 and 2). The *Ariadne merione* isolate (ST153) clustered with *Culex pipiens* (ST9) but had a distinctly different *wsp* allele (*wsp-64* and *wsp-10*, respectively). The isolate from *Jalmenus evagoras* (ST155) was positioned at different places in both phylogenies (Fig. 1 and 2). In the *wsp* phylogeny, it formed a clade with *Lycaeides idas* (*wsp-61*), while in the MLST phylogeny, it formed a clade with a cluster including *Drosophila simulans* (ST15) and *Teleogryllus taiwanemma* (ST32) (Fig. 1 and 2). *Danaus chrysippus* (F36) harbored a unique strain that formed separate clades within supergroup B *Wolbachia* in both the phylogenetic reconstructions (Fig. 1 and 2). The MLST tree was found to show better separation among the strains (Fig. 2).

Extensive recombination in *wsp* (4) and throughout the genome at large was observed (5, 22, 52). The MLST system developed by Baldo and colleagues provides a standardized and rigorous framework for studies of *Wolbachia* strains (2). Combined with extensive sampling from related hosts, this MLST approach has successfully been applied to *Wolbachia* strains from the spider genus *Agelenopsis* (1), the spider *Hylyphantes graminicola* (54), the scorpion genus *Opistophthalmus* (3), the termite genera *Odontotermes* and *Coptotermes* (43), and the lone star tick, *Amblyomma americanum* (55). This study provides another example of *Wolbachia* strain diversity, specifically, that in a community of butterfly hosts.

Role of point mutation and recombination in forming new STs in *Wolbachia*. The recombination was detected only within the butterflies in this study and not in existing STs from the MLST database (Table 4). Within supergroup B, three distinct clades, one comprising lepidopteran species with the exception of the Hymenoptera (*Trichogramma*), the second comprising lepidoptera with the exception of the Diptera (*Culex*), and the third including insects from the Hymenoptera, Coleoptera, Orthoptera, Diptera, and Lepidoptera, were observed. ST151, ST154, and ST155, which were revealed to be recombinant, fall within the two different clusters. Though all the strains were not monophyletic, all populations were identified as strains belonging to supergroup B (Fig. 1).

Point mutations were detected not only in *Wolbachia* isolates in closely related hosts but also in *Wolbachia* isolates found in butterflies from different families and localities. The isolates from *Cepora nerissa* (ST145), *Junonia lemonias* (ST146), and *Talicada nyseus* (ST146) show only one base change. These hosts belong to three different families and were collected from three different locations (Table 2). Similarly, *Hypolimnas bolina* (ST148 from India, in this study) and *Colias erate poliographus* (ST141 from Japan) were found to harbor very closely related *Wolbachia* isolates. These STs were also formed as a result of point mutations in *gatB* at bases 315 and 359. Two new STs (ST154 and ST155) were observed in *Jalmenus evagoras* specimens which were collected from the same location (Table 2). These STs are results of point mutations in gene *hcpA* (base 1004) and *fbpA* (base 1890). Interestingly, none of these hosts showed multiple *Wolbachia* infection. This suggests that same butterfly hosts within same locality harbor different *Wolbachia* strains. *Hypolimnas bolina* (ST125) and *Talicada nyseus* (ST147) showed mutations at only two positions (base 1503 of *ftsZ* and base 1709 of *fbpA*). Surprisingly, the *Hypolimnas bolina* host of *Wolbachia* (ST125) was collected from French Polynesia, while the later one, *Talicada nyseus* (ST147), was collected from India. Although these two places are geographically completely separated, the two distinct butterfly host species harbor very closely related *Wolbachia*. ST41 was observed among a wide range of butterfly hosts (42.31% of the infected specimens) from three different families and different localities. This ST showed point mutations in *gatB* at positions 120 and 359 relative to ST150 from *Colotis amata*. From the data presented in this study, it can be inferred that point mutations within MLST genes play a crucial role in generating new *Wolbachia* STs.

Butterfly host-*Wolbachia* relationships. Butterfly *Wolbachia* phylogenies revealed a very distinct pattern of distribution. *Wolbachia* strains from same or different butterfly hosts from this study were closely related to each other and to representatives of lepidopteran symbionts reported in the MLST database (Table 2; Fig. 1). At the same time, the same host species, *Eurema hecabe* (ST40, ST41, ST156, and ST157), *Colotis amata* (ST147 and ST150), *Hypolimnas bolina* (ST91, ST125, and ST148), *Talicada nyseus* (ST125 and ST146), and *Jalmenus evagoras* (ST154 and ST155) carried distinctly different *Wolbachia* isolates (Table 2 and Fig. 1). A strict geographical congruence between the *Wolbachia* from butterfly species was not observed (Fig. 1). In terms of geography, *Wolbachia* strains have been recovered from lepidopteran host species in Ecuador, French Polynesia, Ghana, Japan, Malaysia, Russia, South Africa, Taiwan, Tanzania, and the United States. Country-wise relatedness was not observed for butterfly *Wolbachia* isolates, since distantly related hosts from different countries shared closely related strains (Fig. 1).

There are different possibilities for scenarios describing the evolution of the distribution and transfer of butterfly *Wolbachia* isolates. As butterflies share *Wolbachia* variants with divergent host species, the scenario of long-term cocladogenesis of *Wolbachia* and butterfly as in the case of clade C and D *Wolbachia* strains and filarial nematodes looks unfeasible. Alternatively, a scenario of *Wolbachia* invasion before differentiation of butterfly host species could be possible. In such scenario, the common ancestor of the butterfly host complex could have been originally infected with multiple *Wolbachia* strains, and loss and or acquisition of *Wolbachia* might have occurred during species differentiation. Horizontal transfer of divergent *Wolbachia* from outside the butterfly host genus in already genetically differentiated species might be other possibility. Strict association of one *Wolbachia* strain with one butterfly species appears to be an impractical explanation, as similar strains are shared by different host species. Point mutation and recombination within *Wolbachia* strains after the acquisition of *Wolbachia* in host species could be the other possibility, as observed in this study.

Phylogenetically diverse types of *Wolbachia* (supergroups B and A) have been reported from butterfly hosts in studies carried out so far (12, 14, 18, 23, 41, 48). Currently, the MLST database has a record for *Wolbachia* strains from the families Nymphalidae, Lycaenidae, and Pieridae. *Wolbachia* strains from supergroups A and B have been found in members of the Nymphalidae and Lycaenidae, while supergroup B strains have been found in the Pieridae. Russell et al. (41) reported the presence of supergroup A and B *Wolbachia* isolates in the Lycaenidae. The survey carried out by Tagami and Miura (48) reported the presence of supergroups A and B in the family Nymphalidae and supergroup B in the rest of the butterfly families except the family Papilionidae, in which they did not find *Wolbachia* infection. The present study suggests the presence of only supergroup B *Wolbachia* in the sampled populations of India of five different families with first detection in family Papilionidae. It is worthwhile adding here that different *Wolbachia* strains infecting the same or closely related butterfly species share close genetic relatedness with strains infecting other lepidopteran or insects. This advocates possibility of horizontal movement of *Wolbachia* to species of the complex, or to their last common ancestor.

The prevalence of the *Wolbachia* was high in some of the butterfly populations in this study. When the butterfly populations with a sample size of more than three are considered, *Wolbachia* prevalences are 100% in *Talicada nyseus*, 45% in *Eurema hecabe*, and 43% in *Hypolimnas bolina*. The prevalence and distribution of the symbionts in these species give an indication of the impact of *Wolbachia* on butterfly populations and merit further study. Although the *Wolbachia* phenotype in some of the butterflies is currently known, our study lays the groundwork for further biological investigations of the effects of *Wolbachia* on Indian butterfly populations and the relevance of *Wolbachia* in the evolutionary process of their butterfly hosts.

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