zones of Indian Ocean over the last sixty years. *Global Planet. Change*, 2014; doi:10.1016/j.gloplacha.2014.02.001.
5. Unnikrishnan, A. S. and Shankar, D., Are sea-level-rise trends along the north Indian Ocean coasts consistent with global estimates? *Global Planet. Change*, 2007, **57**, 301–307.
6. Nandy, S. and Bandyopadhyay, S., Trends of sea level change in the Hugli estuary, India. *Indian J. Geo-Mar. Sci.*, 2011, **40**(6), 802–812.
7. Vinogradov, S. V. and Ponte, R. M., Low frequency variability in coastal sea level from tide gauges and altimetry. *J. Geophys. Res.*, 2011, **116**, C07006; doi:10.1029/2011JC007034.
8. Woodworth, P. L. and Player, R., The permanent service for mean sea level: an update to the 21st century. *J. Coast. Res*., 2003, **19**, 287–295.
9. Peltier, W. R., Global isostacy and the surface of the ice-age Earth: the ICE-5G (VM2) model and GRACE. *Annu. Rev. Earth Planet. Sci.*, 2004, **32**, 111–149.
10. CLS/CNES, France, CalVal *in situ* altimetry/tide gauges. Validation of altimeter data by comparison with tide gauges measurements for TOPEX/Poseidon, Jason-1, Jason-2 and Envisat. CLS DOS/NT/12-016. SALP-RP-MA-EA-22046_CLS, May 2012.
11. Shankar, D. *et al.*, Minima of interannual sea-level variability in the Indian Ocean. *Prog. Oceanogr.*, 2010, **84**(3–4), 225–241.
12. Aparna, S. G., McCreary, J. P., Shankar, D. and Vinayachandran, P. N., Signatures of Indian Ocean Dipole and El Nino–Southern Oscillation events in sea level variations in the Bay of Bengal. *J. Geophys. Res. C*, 2012, **117**, C10012, 13.
13. Nidheesh, A. G., Lengaigne, M., Vialard, J., Unnikrishnan, A. S. and Dayan, H., Decadal and long-term sea level variability in the tropical Indo-Pacific Ocean. *Climate Dyn.*, 2013, **41**(2), 381–402.
14. Masters, D., Nerem, R. S., Choe, C., Leuliette, E., Beckley, B., White, N. and Ablain, M., Comparison of global mean sea level time series from TOPEX/Poseidon, Jason-1 and Jason-2. *Mar. Geodesy*, 2012, **35**, 20–41.
15. Han, W. *et al*., Patterns of Indian Ocean sea-level change in a warming climate. *Nature Geoscience*, 2010, **3**, 546–550; DOI: 10.1338/NGEO 901.
16. Kulkarni, A. V. and Karyakarte, Y., Observed changes in Himalayan glaciers. *Curr. Sci.*, 2014, **106**(2), 237–243.
17. IPCC, Summary for policymakers. In *Climate Change 2013: The Physical Science Basis*, Contribution of Working Group I to the Fifth Assessment of the Intergovernmental Panel on Climate Change (eds Stocker, T. F. *et al.*), Cambridge University Press, Cambridge, UK, 2013, p. 27.
18. Bala, G., Why the hiatus in global warming in the last decade? *Curr. Sci.*, 2013, **105**(8), 1031–1032.

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Distribution and molecular characterization of *Wolbachia* endosymbionts in odonata (insecta) from Central India by multigene approach

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Wolbachia **are maternally inherited bacterial endosymbionts of arthropods distributed among a wide range of hosts. It is now well known that they induce reproductive manipulations in their arthropod hosts by various phenotypic effects. The objective of the present study was to investigate *Wolbachia* infection among the insect order Odonata comprising 16 species from 5 families. Fifteen odonate species representing five families were found to harbour *Wolbachia* with the overall infection rate of 70%, out of which fourteen species are reported for the first time. According to multilocus sequence typing (MLST) data and phylogenetic analysis, all odonate *Wolbachia* species belong to supergroup F, except *Trithemis pallindinervis*, which belongs to supergroup B. MLST data reveal 20 new, highly similar STs (99.32 ± 0.34). We found a high rate of *Wolbachia* infection in Odonata of India, which indicates importance of this association. The characterization of these *Wolbachia* strains promises to lead to a deeper insight into this interaction, which is essential for further studies based on their phenotypic effects. The study suggests that all the characterized *Wolbachia* STs are totally new and arise as a result of point mutation.**

Keywords: Multilocus sequence typing, phenotypic effects, point mutations.

THE Alphaproteobacteria *Wolbachia* are intracellular and maternally inherited bacterial symbionts found in many arthropod and filarial nematodes. Along with vertical cytoplasmic inheritance, *Wolbachia* are also known to transfer horizontally across different hosts[1]. Along with symbiotic associations like mutualism and parasitism, *Wolbachia* can influence the host population by different

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reproductive alterations such as parthenogenesis, male killing, cytoplasmic incompatibility, speciation and feminization[2,3]. According to various reports, the infection of *Wolbachia* was observed in 15–25% of insect species[2,4,5], which was reworked to 40% according to a meta-analysis by Zug and Hammerstein[6]. A notable genetic diversity of *Wolbachia* divided them in 14 different supergroups (A–N)[7]. Two multilocus sequence typing (MLST) systems, one developed by Baldo *et al.*[8] and other by Paraskevopoulos *et al.*[9] are now well known for accurately characterizing and identifying various *Wolbachia* strains[4,9,10].

Odonata, an order of class Insecta, encompasses both dragonflies (Anisoptera) and damselflies (Zygoptera). Odonates are hemimetabolous, in which adults are terrestrial and larvae aquatic. Adult odonates usually feed on mosquitoes, blackflies and other insects and serve as a biocontrol agent. India has diverse fauna and so far, 475 species of odonates belonging to 142 genera and 19 families have been identified. Among these, about 200 species are found in peninsular India. About 67 species of peninsular Indian odonates are endemic and most of them are found in riverine ecosystem[11].

Odonates are highly diverse (5740 extant species in 33 families and just over 600 genera)[12]. They play a significant role in ecology as indicators of aquatic ecosystem health and being top predators maintain the balance at tropic levels of the food chain[13], making them important order to further study *Wolbachia* infection. Although molecular data for these odonate *Wolbachia* have been reported[14,15], no attempt has been made to determine strain diversity by multigene approach. In addition, there is no report available on *Wolbachia* infection in odonates from India so far. Besides, Odonata are basal group of insects belonging to Palaeoptera along with mayflies (Ephemeroptera). Hence studying *Wolbachia* infection in evolutionary, ancient, basal insect group may provide deep insight into the evolutionary history of insect–*Wolbachia* interaction.

In the present study, we show: (i) presence of *Wolbachia* among a sample of odonates from Nagpur region, Maharashtra, India belonging to five families; (ii) diversity of *Wolbachia* within these odonates using MLST genes, and (iii) phylogenetic affiliation of odonate *Wolbachia*.

All odonate samples were collected from the surrounding regions of Telenkhedi Lake and Ambazari Lake, Nagpur, and were preserved in absolute ethanol at –20°C until further processing. Odonate collection information and sample size are listed in Table 1. All adult odonate samples were collected during 2010. DNeasy Blood and Tissue Kit (QIAGEN®) at National Centre for Cell Science, Pune was used to carry out DNA extraction from the tip of the abdomen. Quality of DNA was checked by polymerase chain reaction (PCR) performed using arthropod-specific 28S rRNA gene primers as reported by Werren *et al.*[5]. All the specimens were preserved and morphologically identified at the specimen Collection Centre of Department of Zoology, Hislop College, Nagpur. Except two, the gender of all specimen, was identified.

Sixty odonates were screened initially for *Wolbachia* infection by PCR for the *Wolbachia*-specific 16S rRNA gene using primers and reported protocols[16]. Primer details and PCR protocols as described by Baldo *et al.*[8] were used for amplification of the five reported *Wolbachia* MLST genes (*ftsZ*, *coxA*, *fbpA*, *hcpA* and *gatB*). All PCR products were purified using PEG–NaCl method[17]. The successfully amplified products of the five MLST genes were sequenced bidirectionally with the respective primers using BigDye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, at National Centre for Cell Science, Pune). Sequences were obtained

Table 1. Screening of *Wolbachia* in dragonflies, damselflies and parasitic mites

Type	Family	*N*	Scientific name of odonate sample	Collection site*	No. of specimens	Positive specimens
Dragonflies	Aeshnidae	1	*Anax guttatus*	B	1	1
	Macromiidae	2	*Epophthalmia vittata*	B	2	1
	Gomphidae	2	*Ictinogomphus rapax*	B	2	2
	Libellulidae	52	*Acisoma panerpoides panerpoides*	A	4	4
			Brachythemis contaminata†	A and B	12	6
			Crocothemis servilia	A and B	6	3
			Diplocodes trivialis	A	4	4
			Neurothemis tulia†	B	2	2
			Orthetrum sabina	A and B	10	8
			Orthetrum glaucum	B	2	1
			Pantala flavescens	A and B	6	4
			Rhyothemis variegeta	A and B	2	0
			Trithemis pallindinervis	A and B	4	3
Damselflies	Coenagrionidae	3	*Aciagrion pallidum*	A	1	1
			Ceriogrion coromandelianum	A	1	1
			Ischnura senegalensis	B	1	1

*A, Telenkhedi Lake; B, Ambazari Lake. †Indicates the odonates which are already reported for *Wolbachia* infection.

using an automatic DNA sequencer (3730xl DNA analyzer, ABI) and were deposited in the *Wolbachia* MLST and GenBank databases with alleles and accession numbers respectively (Table 2). The sequence data were analysed using *Wolbachia* MLST database (http://pubmlst.org/*Wolbachia*/).

Estimates of genetic diversity (P_i), variable sites (VI) and ratio of synonymous substitutions per synonymous site over non-synonymous substitutions per non-synonymous site (K_a/K_s) were made using DNAsp[18] (version 4.10.2). MaxChi[19] and GENECONV[20] methods in the RDP2 package[21] were used to perform recombination analyses of the concatenated gene alignments. Parameters were set as follows; triplets were scanned using different values of fraction of variable sites per window. A Bonferroni correction was applied and 100 permutations were generated. The highest acceptable *P* value cut-off was set to 0.05. The pairwise genetic distance of different *Wolbachia* strains was tested using the Kimura 2-parameter method in MEGA5 (refs 22 and 23).

We retrieved all the strain types (STs) available publically in the MLST database till the last analysis for F supergroup ($n = 12$) and representatives for supergroup A ($n = 4$), B ($n = 4$), D ($n = 1$) and H ($n = 1$). *Wolbachia* MLST gene sequences generated in the present study were aligned with homologous sequences deposited in *Wolbachia* MLST database using ClustalX (version 2.0.9)[24]. All sequences were manually edited using MEGA5 (ref. 23). Unrooted phylogenetic trees were constructed using Bayesian inference and Neighbor joining method for concatenated dataset as well as for individual MLST genes. Bayesian inference of phylogeny was performed as discussed earlier[4]. The selected model of nucleotide substitution was 'GTR + I + G' for concatenated MLST genes and concatenated dataset of *fpbA* and *ftsZ* genes. The final alignments consisted of 2079 and 864 respectively. Three independent runs were performed for each dataset. 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. STs and allele number are shown after each species name in parenthesis. *Wolbachia* supergroups are shown to the right side of the host species names. Scale bar represents substitutions per site. NJ trees were constructed using MEGA 5 with 1000 bootstrap replication. The selected models with the lowest Bayesian Information Criterion (BIC) scores are T92 + G + I for concatenated MLST genes, HKY + I for *coxA* gene and T92+G for the remaining genes.

A total of 60 odonates were screened initially for *Wolbachia* infection using PCR assay targeting *Wolbachia*-specific 16S rRNA gene. These odonates represent five different families with domination of Libellulidae ($n = 52$; Table 1). From this study, 15 odonate species (93.75%) were found to be positive for *Wolbachia* infection, with the exception of *Rhyothemis variegeta* (Table 1). Among these 15 species, 13 are reported first time for *Wolbachia* infection. In total, 42 out of 60 odonates specimens were infected with *Wolbachia* (Table 1). All the *Wolbachia* under this study belong to F supergroup except strain from *Trithemis pallindinervis* which belongs to B supergroup. This is the first detailed report of characterization of 20 new *Wolbachia* ST from the odonates using MLST.

Few attempts were made to check infection of *Wolbachia* in odonates. Thipaksorn *et al.*[15], extensively surveyed the prevalence of *Wolbachia* among the tropical odonates from Thailand. Out of the 19 genera and 33 species ($n = 427$) screened, *Wolbachia* infection was found in four species (*Agriocnemis f. femina, Pseudagrion pruinosum*, *Brachythemis contaminata* and *Neurothemis t. tullia*). Gossum *et al.*[25] discussed the presence of *Wolbachia* in several species of *Nesobasis* from unpublished data of Charlat *et al.* Jeyaprakash and Hoy[14] reported *Wolbachia* infection in a dragonfly species *Perithemis tenera*, but did not report the infection frequency. In the present study, infection rate of *Wolbachia* was found to be 70% in odonate hosts collected from Nagpur region. The meta-analyses by Zug and Hammerstein[6] suggested that *Wolbachia* infects at least 40% of all insect species. However, our findings at least in odonates show significantly higher infection rate. Considering the minimum sample size of four, the prevalence of *Wolbachia* infection ranges from 50% (*Crocothemis servilia*, *Brachythemis contaminata*) to 100% (*Diplocodes trivialis*, *Acisoma panerpoides panerpoides*; Table 1).

Genotyping of the odonate *Wolbachia* using MLST showed significant strain diversity. At least one MLST gene was amplified and sequences were obtained for all 43 specimens. Complete MLST profiles were obtained for 27 *Wolbachia* species, while repeated failures to PCR amplify particular *Wolbachia* MLST genes resulted in incomplete profiles for the rest (Table 2). The odonate species, *Orthetrum sabina* showed presence of the same *Wolbachia* ST242 in seven specimens (four males and three females). Therefore, one representative for each of the males and females has been included in Table 2. Sequence typing was performed on all the complete MLST profiles using *Wolbachia* MLST database (http://pubmlst.org/*Wolbachia*/) (Table 2). Characterization of allelic profile indicated presence of 20 new *Wolbachia* STs (Table 2). Phylogenetic analysis using concatenated MLST dataset showed their affiliation to F supergroup (Figure 1), except *T. pallindinervis* which belongs to supergroup B *Wolbachia* (Table 2). Phylogenetic analysis illustrated a monophyletic group of odonate *Wolbachia* within supergroup F (Figure 1).

Divergence among all the STs considered for phylogenetic analysis accounted for 621 variable sites (VI) out of 2079 sites (29.87%) in the concatenated dataset for five MLST genes (see supplementary material, Table S1 online). The gene *hcpA* showed highest nucleotide divergence with 161 variable sites out of 444 (36.26%),

Table 2. Complete and partial multilocus sequence typing (MLST) profiles of *Wolbachia* in odonate samples collected from India

ID	host_genus	host_species	Sex	*gatB*	*coxA*	*hcpA*	*ftsZ*	*fbpA*	Strain type	Strain	Supergroup
358	*Diplocodes*	*trivialis*	Male	168 (KC915339)	146 (KC915243)	173 (KC915369)	134 (KC915307)	126 (KC915273)	237	Dtri_F_Odo1	F
359	*Anax*	*guttatus*	Male	168 (KC915353)	146 (KC915256)	174 (KC915382)	134 (KC915322)	126 (KC915285)	238	Agut_F_Odo2	F
360	*Brachythemis*	*contaminata*	Female	168 (KC915346)	147 (KC915250)	173 (KC915376)	132 (KC915314)	226 (KC915280)	239	Bcon_F_Odo3	F
361	*Pantala*	*flavescens*	Male	168 (KC915338)	147 (KC915242)	173 (KC915368)	134 (KC915306)	226 (KC915272)	240	Pfla_F_Odo4	F
362	*Trithemis*	*pallindinervis*	Female	168 (KC915332)	147 (KC915237)	174 (KC915363)	134 (KC915298)	126 (KC915267)	241	Tpal_F_Odo5	F
363	*Orthetrum*	*sabina*	Male	168 (KC915343)	147 (KC915247)	175 (KC915373)	132 (KC915311)	226 (KC915277)	242	Osab_F_Odo6	F
364	*Orthetrum*	*sabina*	Female	168 (KC915344)	147 (KC915248)	175 (KC915374)	132 (KC915312)	226 (KC915278)	242	Osab_F_Odo6	F
366	*Orthetrum*	*sabina*	Female	168 (KC915334)	147 (KC915239)	177 (KC915365)	132 (KC915300)	226 (KC915269)	243	Osab_F_Odo7	F
368	*Diplocodes*	*trivialis*	Female	169 (KC915341)	146 (KC915245)	173 (KC915371)	134 (KC915309)	126 (KC915275)	245	Dtri_F_Odo9	F
369	*Diplocodes*	*trivialis*	Female	169 (KC915342)	146 (KC915246)	173 (KC915372)	134 (KC915310)	226 (KC915276)	246	Dtri_F_Odo10	F
370	*Neurothemis*	*tulia*	Male	169 (KC915350)	146 (KC915254)	174 (KC915380)	132 (KC915318)	126 (KC915283)	247	Ntul_F_Odo11	F
371	*Acisoma*	*panerpoides panerpoides*	Male	169 (KC915329)	146 (KC915235)	174 (KC915361)	134 (KC915295)	126 (KC915264)	248	Apan_F_Odo12	F
372	*Aciagrion*	*pallidum*	Female	169 (KC915333)	146 (KC915238)	175 (KC915364)	132 (KC915299)	125 (KC915268)	249	Apal_F_Odo13	F
373	*Acisoma*	*panerpoides panerpoides*	Female	169 (KC915327)	146 (KC915234)	175 (KC915359)	134 (KC915293)	126 (KC915262)	250	Apan_F_Odo14	F
374	*Orthetrum*	*glaucum*	Male	169 (KC915354)	147 (KC915257)	173 (KC915383)	132 (KC915323)	226 (KC915286)	251	Ogla_F_Odo15	F
375	*Pantala*	*flavescens*	Male	169 (KC915337)	147 (KC915241)	173 (KC915367)	134 (KC915305)	125 (KC915271)	252	Pfla_F_Odo16	F
376	*Diplocodes*	*trivialis*	Male	169 (KC915340)	147 (KC915244)	173 (KC915370)	134 (KC915308)	126 (KC915274)	253	Dtri_F_Odo17	F
377	*Epophthalmia*	*vittata*	Male	169 (KC915352)	147 (KC915255)	174 (KC915381)	134 (KC915321)	125 (KC915284)	254	Evit_F_Odo18	F
378	*Acisoma*	*panerpoides panerpoides*	Female	169 (KC915330)	147 (KC915236)	178 (KC915362)	134 (KC915296)	126 (KC915265)	255	Apan_F_Odo19	F
379	*Brachythemis*	*contaminata*	Male	170 (KC915326)	147 (KC915233)	178 (KC915358)	133 (KC915291)	125 (KC915261)	256	Bcon_F_Odo20	F
380	*Ictinogomphus*	*rapax*	Male	82 (KC915347)	147 (KC915251)	174 (KC915377)	132 (KC915315)	125 (KC915281)	257	Irap_F_Odo21	F
381	*Ictinogomphus*	*rapax*	Female	82 (KC915348)	147 (KC915252)	174 (KC915378)	132 (KC915316)	125 (KC915282)	257	Irap_F_Odo21	F

(*Contd*)

Table 2. (*Contd*)

ID	host_genus	host_species	Sex	*gatB*	*coxA*	*hcpA*	*ftsZ*	*fbpA*	Strain type	Strain	Supergroup
382	*Neurothemis*	*tulia*	Female	169 (KC915349)	147 (KC915253)	175 (KC915379)	132 (KC915317)			Ntul_F_Odo22	F
383	*Acisoma*	*panorpoides panorpoides*	Female	169 (KC915328)		176 (KC915360)	133 (KC915294)	226 (KC915263)		Apan_F_Odo23	F
367	*Ischnura*	*senegalensis*		169 (KC915325)		178 (KC915355)		226 (KC915258)		Isen_F_Odo8	F
384	*Crocothemis*	*servilia*	Male	82 (KC915324)	146 (KC915231)		132 (KC915287)			Cser_F_Odo24	F
385	*Brachythemis*	*contaminata*	Female			173 (KC915356)	132 (KC915290)	226 (KC915260)		Bcon_F_Odo25	F
386	*Ceriogrion*	*coromandelianum*	Male	169 (KC915331)			132 (KC915297)	126 (KC915266)		Ccor_F_Odo26	F
387	*Crocothemis*	*servilia*	Female	169 (KC915336)			132 (KC915302)	126 (KC915270)		Cser_F_Odo27	F
388	*Trithemis*	*pallindinervis*					36 (KC915288)	4 (KC915259)		Tpal_B_Odo28	B
389	*Brachythemis*	*contaminata*	Male		147 (KC915232)		132 (KC915289)			Bcon_F_Odo29	F
390	*Brachythems*	*contaninata*	Male	168 (KC915335)			132 (KC915301)			Bcon_F_Odo30	F
391	*Pantala*	*flavescens*	Female		146 (KC915240)		132 (KC915303)			Pfla_F_Odo31	F
392	*Pantala*	*flavescens*	Female			173 (KC915366)	132 (KC915304)			Pfla_F_Odo32	F
393	*Trithemis*	*pallindinervis*	Male	168 (KC915351)			132 (KC915320)			Tpal_F_Odo33	F
394	*Crocothemis*	*servilia*	Female				132 (KC915319)			Cser_F_Odo34	F
395	*Brachythemis*	*contaminata*	Male				132 (KC915292)			Bcon_F_Odo35	F

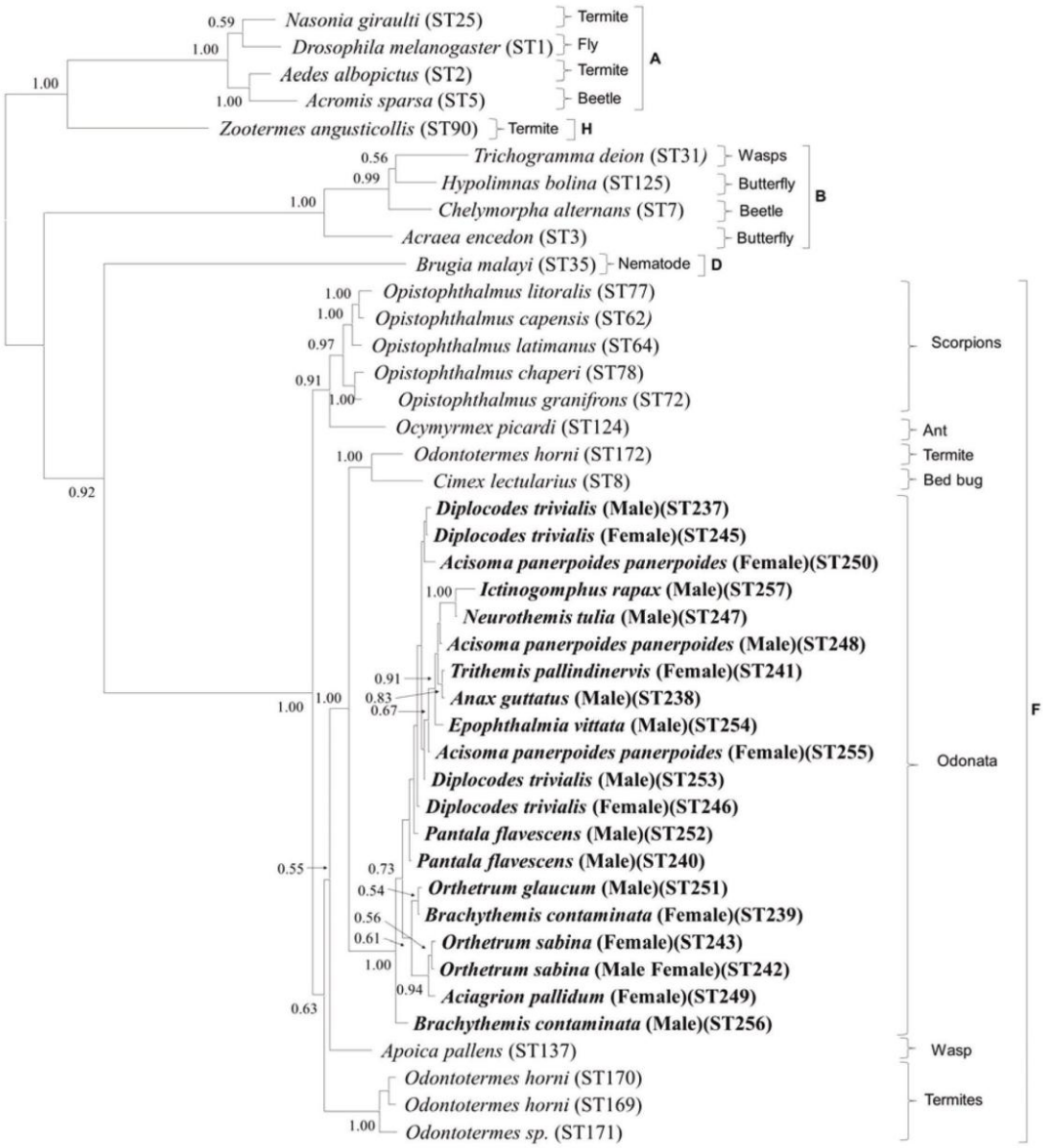

Figure 1. Unrooted phylogenetic relationship between *Wolbachia* from odonates (bold) and those infecting other organisms representing five supergroups (42 *Wolbachia*), based on concatenated alignment of MLST loci (2079 bp).

followed by *fbpA* with 134 variable sites out of 429 (31.24%; Table S1). Average K_a/K_s per gene was found to be ⊥1 (average K_a/K_s across genes is 0.08698), which indicates strain evolution mainly by synonymous substitutions. This suggests the case of strong purifying selection. All odonate *Wolbachia* strains in this study resemble each other remarkably (99.32% ± 0.34) compared to remaining F supergroup representatives (97.21% ± 1.08; see supplementary material, Table S2 online). Since we used tip of the abdomen for DNA extraction, detection of endosymbionts from its prey like mosquitoes, blackflies and other blood-sucking insects that remained undigested in the gut of odonates may be possible. Perhaps, this possibility can be ruled out because all STs are remarkably similar to each other and super infection was not observed for any of the specimens.

The recombination analysis using MLST concatenated dataset showed that none of the odonate *Wolbachia* strains obtained in this study is recombinant. Point mutation seems to play a crucial role in the formation of new strains, irrespective of whether *Wolbachia* hosts are closely related with each other or not. Different specimens of same host from same locality were found to harbour different STs which are formed as a result of point mutations e.g. *Diplocodes trivialis* (ST245, 253 and 237). In some specimens we observed the same *Wolbachia* strain in the same host collected from different localities, e.g. *Orthetrum sabina* (ST242 and 243). Surprisingly,

Wolbachia strains from two distinct host, *Anax guttatus* (ST238) and *Trithemis pallindinervis* (ST241) belonging to different families and also from different localities were found to be similar, and resulted from point mutation in gene *coxA*. These results support the role of point mutation in evolving new *Wolbachia* STs in butterflies[4].

A high mean, pairwise genetic distance (0.0232) was observed in 32 STs representing the entire F supergroup *Wolbachia* from the database and those under the present study. The mean pairwise genetic distance within the 20 odonate *Wolbachia* STs from this study was significantly lower (0.0069).

Phylogenetic reconstructions for all genes by both Bayesian inference and neighbour-joining methods showed similar topology and therefore only Bayesian phylogenetic trees are shown here. Phylogenetic reconstructions based on concatenated alignment of *hcpA*, *gatB*, *coxA*, *ftsZ* and *fbpA* genes (Figure 1) and those for individual MLST genes (data not shown) indicate a strong clustering of odonate *Wolbachia* within supergroup F except for a specimen of *Trithemis pallindinervis* which belongs to supergroup B (Table 2; see supplementary material; Figure S1 online). Within supergroup F cluster, all the odonate *Wolbachia* strains show a separate clade alongside the clade of *Cimex lectularius* (ST8), and termite genus *Odontotermes* (ST172; Figure 1).

Within a cluster of odonate *Wolbachia*, no specific clustering was observed at family level. Out of two *Brachythemis contaminata*, ST256 was found to be the most divergent in the odonates clade. Many odonate *Wolbachia* strains from same host do not show host-specific clustering. However, this was observed for some odonate *Wolbachia* like *Pantala flavescens* and *Orthetrum sabina* (Figure 1). Gender-specific clustering was observed neither within the same host species nor in overall odonate clade (Figure 1).

According to a meta-analysis by Zug and Hammerstein[6], around 40% of arthropod species are infected with *Wolbachia*. Such ubiquitous infection is not only because of its strength of vertical transmission within the host, but also due to occurrence of multiple horizontal transmissions between different hosts[3]. Higher number of STs (n = 20) was observed in the odonate *Wolbachia*, but they are remarkably similar to each other. There are two possibilities of having closely related *Wolbachia* strains in odonates: (i) hybridization in odonate hosts leads to introgression and (ii) horizontal transfer of *Wolbachia* strains.

For the first possibility, natural hybridization is not a common process in odonates, because their morphological incompatibility plays a crucial role in reproductive isolation[26,27]. Few reports describe the intrageneric hybridization present in genus *Ischnura*[28] and *Mnais*[29] of damselflies. Some reports have also showed intrageneric introgression in the dragonfly. However, intergeneric hybridization in odonates is not reported to the best of our knowledge. Hence, we might rule out the first possibility. Even if hetero-specific copulation with insemination can take place (in dragonflies), post-copulatory reproductive mechanism (e.g. chromosomal incompatibility or zygote mortality) could still prevent hybridization. Evidently, hybrid odonates do exist, but are rare[30].

The second observation hypothesized that the common ancestor of the odonate species might have been initially uninfected and *Wolbachia* might be acquired at different stages of the speciation. Horizontal acquisition of *Wolbachia* to already differentiated odonate species from another host could have been a possibility. We have observed *Wolbachia* infection from supergroup F and B in same host species *T. pallindinervis* (Table 2). As most of the *Wolbachia* strains from odonates belong to F supergroup, there are chances that *T. pallindinervis* acquired B supergroup *Wolbachia* by horizontal transmission.

F supergroup *Wolbachia* has been reported in different arthropod[10,31,32] and nematode hosts[33]. According to investigations carried out so far, parasitic behaviour for F supergoup *Wolbachia* has not been observed. In fact, mutualistic association of F supergoup *Wolbachia* with nematode host has been studied recently[34] and the possibility of obligate nutritional mutualism with the bedbug *Cimex lectularius* has been proposed as *Wolbachia* was found to reside in a bacteriome[35]. This raises interesting possibility that *Wolbachia* in the F supergoup are nutritional mutualists to other arthropod hosts. Separate investigations will be needed to understand association of F supergoup *Wolbachia* and their different hosts[10,31]. Recombination was not observed in F supergroup *Wolbachia*, including in the present study, which is contrast with the A and B supergroup *Wolbachia*, that are mostly parasitic. Moreover, no recombination was reported from C and D supergroups, which are mutualistic. Beside this, little nucleotide variation and a wide host range from arthropods to nematodes in F supergroup, the possibility of horizontal transfer is also likely[36]. According to our observations and available data, there is a possibility of both horizontal and vertical transfer in many supergroup F *Wolbachia* hosts. The recombination may not have contributed in the evolution of F supergroup *Wolbachia*. More thorough experimentation will be required to understand the evolution of *Wolbachia* in their host species and in the less studied F supergroup.

We have inferred the association of *Wolbachia*–odonate based on a phylogenetic tree that consisted of 20 new *Wolbachia* strains from Indian odonates. High frequency of *Wolbachia* infection in Indian odonates gives an indication of the importance of this association and also provides a roadmap for further studies with higher resolution based on their phenotypic effects.

1. Huigens, M. E., de Almeida, R. P., Boons, P. A., Luck R. F. and Stouthamer, R., Natural interspecific and intraspecific horizontal

transfer of parthenogenesis-inducing *Wolbachia* in *Trichogramma* wasps. *Proc. Biol. Sci*., 2004, **271**, 509–515.
2. Salunkhe, R. C., Narkhede, K. P. and Shouche, Y. S., Distribution and evolutionary impact of *Wolbachia* on butterfly hosts. *Indian J. Microbiol*., 2014, **54**, 249–254.
3. Werren, J. H., Baldo, L. and Clark, M. E, *Wolbachia*: master manipulators of invertebrate biology. *Nature Rev. Microbiol*., 2008, **6**, 741–751.
4. Salunke, B. K. *et al*., Determination of *Wolbachia* diversity in butterflies from Western Ghats, India, by a multigene approach. *Appl. Environ. Microbiol*., 2012, **78**, 4458–4467.
5. Werren, J. H., Windsor, D. M. and Guo, L., Distribution of *Wolbachia* among Neotropical arthropods. *Proc. R. Soc. London, Ser. B*, 1995, **262**, 197–204.
6. Zug, R. and Hammerstein, P., Still a host of hosts for *Wolbachia*: analysis of recent data suggests that 40% of terrestrial arthropod species are infected. *PLoS ONE*, 2012, **7**(6), e38544.
7. Augustinos, A. A. *et al.*, Detection and characterization of *Wolbachia* infections in natural populations of aphids: is the hidden diversity fully unraveled? *PLoS ONE*, 2011, **6**(12), e28695.
8. Baldo, L. *et al.*, Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis*. *Appl. Environ. Microbiol*., 2006, **72**, 7098–7110.
9. Paraskevopoulos, C., Bordenstein, S. R., Wernegreen, J. J., Werren, J. H. and Bourtzis, K., Toward a *Wolbachia* multilocus sequence typing system: discrimination of *Wolbachia* strains present in *Drosophila* species. *Curr. Microbiol*., 2006, **53**, 388–395.
10. Salunke, B. K. *et al.*, Diversity of *Wolbachia* in *Odontotermes* spp. (Termitidae) and *Coptotermes heimi* (Rhinotermitidae) using the multigene approach. *FEMS Microbiol. Lett*., 2010, **307**, 55–64.
11. Subramanian, K. A., *Dragonflies of India – A Field Guide*, Vigyan Prasar, Department of Science and Technology, Government of India, 2009.
12. Kalkman, V. J., Clausnitzer, V., Dijkstra, K. D. B., Orr, G. A., Paulson, D. R. and Tol, J. V., Global diversity of dragonflies (Odonata) in freshwater. *Hydrobiologia*, 2008, **198**, 351–363.
13. Mitra, T. R., Geographic distribution of Odonata (Insecta) of eastern India. *Mem. Zool. Sur. India*, 2002, **19**, 1–208.
14. Jeyaprakash, A. and Hoy M. A., Long PCR improves *Wolbachia* DNA amplification: wsp sequences found in 76% of sixty-three arthropod species. *Insect Mol. Biol*., 2000, **9**, 393–405.
15. Thipaksorn, A., Jamnongluk, W. and Kittayapong, P., Molecular evidence of *Wolbachia* infection in natural populations of tropical odonates. *Curr. Microbiol*., 2003, **47**, 314–318.
16. O'Neill, S. L., Giordano, R., Colbert, A. M. E., Karr, T. L. and Robertson H. M., 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc. Natl. Acad. Sci. USA*, 1992, **89**, 2699–2702.
17. Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA, 2nd edn, 1989.
18. Rozas, J. and Rozas, R., DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics*, 1999, **15**, 174–175.
19. Maynard, S. J., Analyzing the mosaic structure of genes. *J. Mol. Evol*., 1992, **34**, 126–129.
20. Padidam, M., Sawyer, S. and Fauquet, C. M., Possible emergence of new geminiviruses by frequent recombination. *Virology*, 1999, **265**, 218–225.
21. Martin, D. P., Williamson, C. and Posada, D., RDP2: recombination detection and analysis from sequence alignments. *Bioinformatics*, 2005, **21**, 260–262.
22. Kimura, M., A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol*., 1980, **16**, 111–120.
23. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S., MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. *Mol. Biol. Evol*., 2011, **28**, 2731–2739.
24. Larkin, M. A. *et al.*, Clustal W and Clustal X version 2.0. *Bioinformatics*, 2007, **23**, 2947–2948.
25. Gossum, H. V. *et al.*, Male rarity and putative sex-role reversal in Fijian damselflies (Odonata). *J. Trop. Ecol*., 2007, **23**, 591–598.
26. Miller, P. L., The structure and function of the genitalia in the Libellulidae (Odonata). *Zool. J. Linn. Soc*., 1991, **102**, 43–73.
27. Tennessen, K. J., Review of reproductive isolating barriers in Odonata. *Adv. Odonatol.*, 1882, **1**, 251–265.
28. Liliana, M., Rosa, A., Sanchez, G. and Adolfo, C. R., Hybridization between *Ischnura graellsii* (Vander Linder) and *I. elegans* (Rambur) (Odonata: Coenagrionidae): are they different species? *Biol. J. Linn. Soc. London*, 2002, **76**, 225–235.
29. Hayashi, F., Dobata, S. and Futahashi, R., Disturbed population genetics: suspected introgressive hybridization between two *Mnais* damselfly species (Odonata). *Zool. Sci.*, 2005, **22**, 869–881.
30. Corbet, P. S., *Dragonflies: Behaviour and Ecology of Odonata*, Harlet Books (B.H. Harley and Harley Ltd), Great Horkesley, England, 1999.
31. Salunke, B. K., Salunkhe, R. C., Patole, M. S. and Shouche, Y. S., *Wolbachia* and termite association: present status and future implications. *J. Biosci*., 2010, **35**, 171–175.
32. Vaishampayan, P. A., Dhotre, D. P., Gupta, R. P., Lalwani, P., Ghate, H., Patole, M. S. and Shouche, Y. S., Molecular evidence and phylogenetic affiliations of *Wolbachia* in cockroaches. *Mol. Phylogenet. Evol*., 2007, **44**, 1346–1351.
33. Ferri, E. *et al.*, New insights into the evolution of *Wolbachia* infections in filarial nematodes inferred from a large range of screened species. *PLoS ONE*, 2011, **6**(6), e20843.
34. Taylor, M. J., Voronin, D., Johnston, K. L. and Ford, L., *Wolbachia* filarial interactions. *Cell Microbiol*., 2013, **15**, 520–526.
35. Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X. Y. and Fukatsu, T., *Wolbachia* as a bacteriocyte-associated nutritional mutualist. *Proc. Natl. Acad. Sci*., *USA*, 2010, **107**, 769–774.
36. Panaram, K. and Marshall, J. L., F supergroup *Wolbachia* in bush crickets: what do patterns of sequence variation reveal about this supergroup and horizontal transfer between nematodes and arthropods? *Genetica*, 2007, **130**, 53–60.

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