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Wolbachia Population in Vectors and Non-vectors: A Sustainable Approach Towards Dengue Control

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Abstract

Wolbachia is gram negative obligate endosymbiont known for reproductive manipulation in the host. It is important to study the presence of natural *Wolbachia* in mosquitoes which can later help in understanding the effect of transfected strain on indigenous strain. With this view, the present study is undertaken to focus on the prevalence, diversity, infection frequencies, phylogeny and density of indigenous *Wolbachia* strains in wild mosquito species of Odisha. Our study confirms *Wolbachia* presence in *Ae. albopictus, Cx. quinquefasciatus, Cx. vishnui, Cx. gelidus, Ar. subalbatus, Mn. uniformis,* and *Mn. indiana. Wolbachia* in the above mosquitoes were separated into two supergroups (A and B). *Ae. albopictus*, the major vector of dengue and chikungungunya had both super-infection and mono-infection. The ovaries of *Ae. albopictus* were highest in density of *Wolbachia* as compared to midguts or salivary glands. *w*AlBA and *w*AlbB density were variable in mosquitoes of F1 generation for both the sex and at different age. We also found that *Wolbachia* super-infection in females tends to increase whereas *w*AlbA density reduced completely as compared to *w*AlbB in males when they grew old. Giemsa stained squashed ovaries revealed pink pleomorphic *Wolbachia* cells with different shapes and forms. This study is unique in its kind covering the major aspects of the endosymbiont *Wolbachia* and focusing on its potential as a biocontrol agent in arboviral outbreaks. Knowledge on potential of the indigenous strain and interactions between *Wolbachia* and viruses can be utilized further to reduce the global burden of vector borne diseases.

Introduction

Mosquitoes are vectors gaining medical importance throughout the globe due to transmission of numerous diseases like malaria, filaria, dengue, chikungunya, Japanese encephalitis, etc. A brief update on their population structure and distribution prototype is required to evaluate their roles in pathogen transmission and development of their control strategies. Vector borne diseases are expanding its horizon to newer geographical regions due to progressive urbanization, globalization and climatic changes. Vector control remains the only choice to reduce the disease burden. Emergence of insecticide resistance and efforts in developing effective vaccines has urged scientists to develop alternate tools to combat vector borne diseases [20, 23]. In this scenario, sterile male technique, *Wolbachia*-infected replacement, [17, 22] and radiation are needed to be implemented to combat the dengue and other vector populations in endemic areas [13, 51].

Wolbachia, a gram negative, obligate, endosymbiont resides mainly in reproductive tissues of insects. They are known for both maternal and horizontal transmission [32, 34, 45]. They are known to infect 40–75% of insect species [55]. To facilitate their spread in the host providing them fitness benefit they cause reproductive manipulations including feminization, parthenogenisis, male killing and cytoplasmic induction (CI) [35, 46]. The main theme behind the use of *Wolbachia* as a biological control is its ubiquity in insect vectors, widespread distribution, reproductive parasitism, antiviral protection which has been explored by researchers in the world, and thus successful outcomes have also been demonstrated in field trials [12, 18, 24, 30]. 16Sr

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RNA sequences revealed seven (A–H) supergroups with *Wolbachia* in arthropods belonging to A and B supergroups [25, 46]. Multiple or single strain of *Wolbachia* may infect single or multiple hosts which may provide a complement or interference for transfected strains [27, 41].

It is highly crucial to identify natural occurrence of *Wolbachia* in mosquitoes for purposes of medical importance. For this, a critical assessment on the incidence of *Wolbachia* in natural populations of mosquitoes is essential that may aid in a thorough understanding on the effect of transfection of new *Wolbachia* strains to mosquitoes already inhabited by indigenous *Wolbachia* strain within.

There have been a few studies in India till date to detect the presence of *Wolbachia* in wild populations of mosquitoes [29] and *Drosophila* [28]. India is a hub for various vector borne diseases claiming health and lives in lacs. The present study will focus on the prevalence, diversity, and infection frequencies of indigenous *Wolbachia* strains in wild mosquito species collected from different locations of an Indian state, Odisha through the surface protein gene, *wsp*, a quick evolving gene. An investigation on phylogeny of *wsp* sequences for *Wolbachia* strains similarities and variations was also done with an attempt to discuss the existing subgroups. The density distribution study in organs and sex was done with the aim to further analyze the potential of indigenous *Wolbachia* in viral inhibition. This study will provide a base for controlling arboviral diseases in many parts of Odisha. We used standard and quantitative PCR based techniques along with staining protocol to detect *Wolbachia* differential distribution in somatic tissues and germlines.

Materials and Methods

Selection of Study Area

The districts of Odisha with repeated outbreaks of vector borne diseases were selected for the study. Samples were collected from four distinct physiographical regions; coastal plains (Jagatsinghpur and Kendrapara), central tableland (Angul), northern plateau (Keonjhar and Mayurbhanj), and eastern ghats (Kalahandi) (Fig. 1). Aquatic stages were collected from urban and rural areas of each district.

Sampling and Rearing

Adult samples were collected from the above mentioned districts through mechanical aspirator. Larvae and pupae collection was made through dip method or by Pasteur pipette [47] from peri-domestic water collections (used tires, discarded small and large wastes, tree holes etc.) and domestic containers (cement tanks, earthen pots, plastic buckets), labeled and brought to laboratory for eclosion. Larvae and pupae were reared to adults at room temperature (29–30 °C) and relative humidity 70 ± 5% with 16:8 light:dark cycle. Larvae were fed on yeast powder and adults on 10% sucrose and rabbit blood. The eclosed adults were identified based on the standard keys described by Barraud, Christophers, and Harbach [3, 5, 11]. The identified samples were immediately processed or subjected to storage at − 80 °C for future use.

Primer Designing

Strain specific primers (new) were designed from sequences obtained from Genbank, NCBI for *Ae. albopictus*. The designing was carried out using PRIMER 3 software. The designed primers are *wsp*FN/*w*AlbAN for strain A and *wsp*FN/*w*AlbBN for strain B (*wsp*FN-5′-GAAGATATG CCTATCACTCC-3′; *w*AlBAN-5′GTATGTCAGCACTCC TTT-3′; *w*AlBBN-5′-CCCAGAAATCAAGCTTTATGC-3′).

Molecular Analysis

DNA was extracted using HiPura™ insect DNA purification kit (Himedia) from adults of *Aedes, Culex, Armigeres, Anopheles,* and *Drosophila* species (as positive control). DNA was also extracted from ovaries, salivary glands and midguts of female mosquitoes every alternate day till day 30 of survival. Along with this DNA extraction was done for individual male *Ae albopictus* on each day till day 20 of their survival. Extracted DNA was stored at − 20 °C for future use.

Standardization, Optimization, and Evaluation of PCR

Screening for *Wolbachia* in mosquito and *Drosophila* were done by *wsp* gene (81F/691R) specific PCR. Samples positive for *wsp* primers were genotyped using newly designed strain specific primers *wsp*FN/*w*AlbAN *wsp*FN/*w*AlbBN (for *Ae. albopictus*). *Wolbachia* supergroup and subgroup primers were later used for further confirmation [53]. The reaction mixture for PCR included 0.25 mM dNTPs, 2.5 mM primers and 1 U Taq DNA polymerase following cycling conditions: 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 56 °C for 1 min and 72 °C for 2 min, followed by 72 °C for 10 min. 10 µl of the PCR products were run 1.5% agarose gel.

Sequencing and Phylogenetic Analysis

1.5% Agarose gel was used for PCR product visualization under UV transilluminator and subjected to purification by HiPura™ PCR product purification kit (Himedia). EDTA/ethanol was used for the precipitation of PCR product after which 10 µl of Hi-Di-formamide was used for pellet

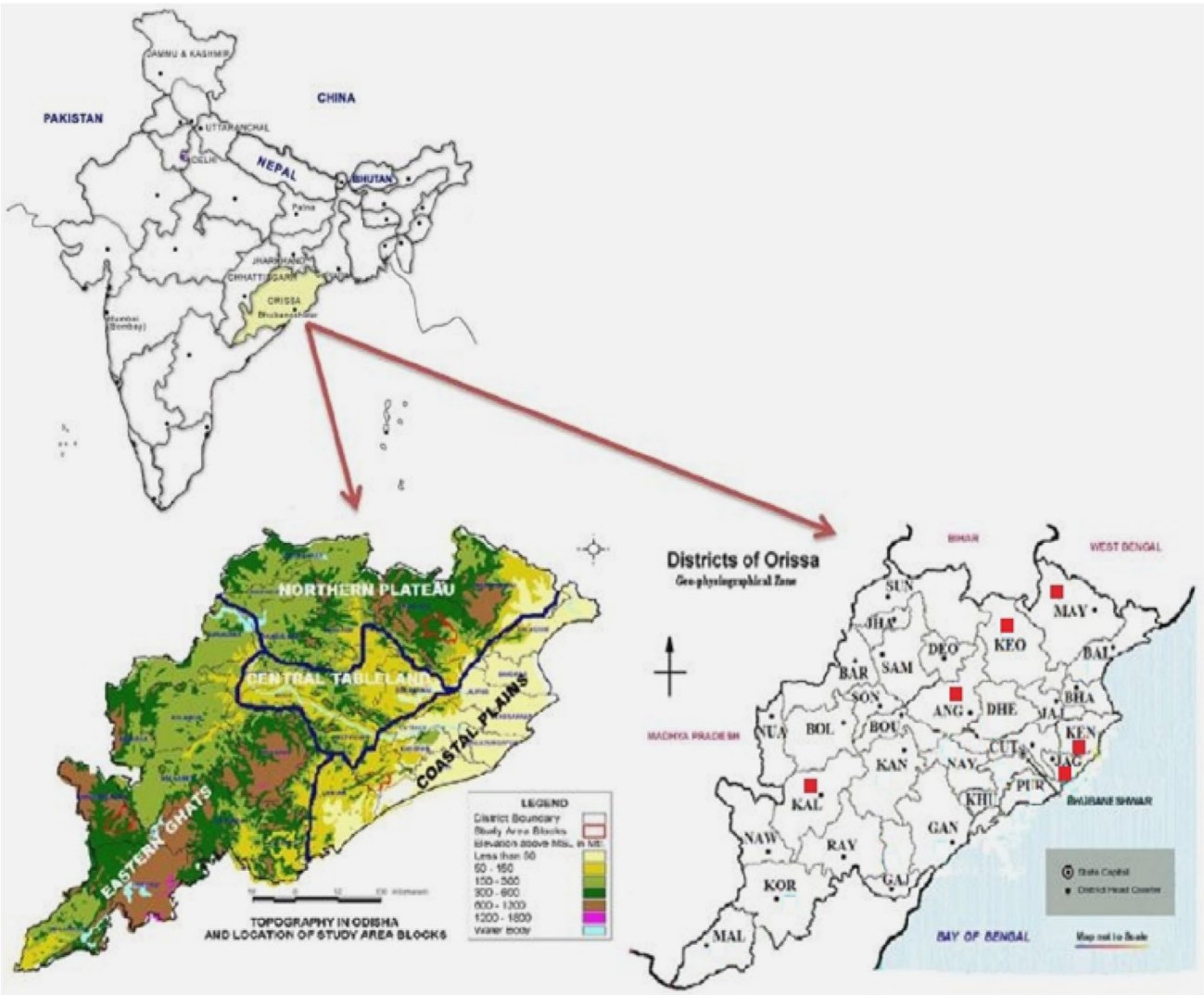

Fig. 1 Map showing four physiographical region of Odisha and red squares show collection areas

resuspension. The suspension was then subjected to direct sequencing following the manufacturer's instructions in a 16 capillary (90 cm) automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). The sequencing mixture contained 2.5 × sequencing buffer, 5 × big dye terminator, 20 mM of either forward or reverse primers of *wsp* genes and 50 ng of purified PCR product. The cycling parameters used were as follows: 96 °C for 1 min followed by 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. The sequences obtained were later edited and analyzed through the Sequencing analysis software (Applied Biosystems, Foster City, CA, USA).

The *wsp* gene sequences generated from the study were deposited in GenBank. Multiple sequence alignment was done using Clustal Omega and phylogenetic analysis were performed using partial *wsp* gene sequences of the mosquito isolates obtained from Odisha and other regions of Asia through MEGA (Version 7.0.26) (Arizona State University, Tempe, AZ, USA) using the maximum-likelihood method [36]. 500 bootstrap replications under the nearest-neighbor-interchange procedure were used to confirm the robustness of each node.

Wolbachia q-PCR

The densities of *Wolbachia* were quantified in whole body of wild strain of *Ae. albopictus* at day 0, 5, 18 and 30 post eclosion. Further densities were also quantified in wild male and female of *Ae. albopictus*. Along with quantifying *Wolbachia* from whole body of mosquitoes, *Wolbachia* was also quantified from salivary gland, midgut and ovary. The primers exclusively concentrated on *Wolbachia* surface protein (*wsp*) of *w*AlbA and *w*AlbB. The *Wolbachia* genome copy number was normalized using the mosquito *actin* gene. *Ae.*

albopictus actin gene was amplified with the forward primer actAlb-dir (GCAAACGTGGTATCCTGAC) and reverse primer actAlb-rev (GTCAGGAGA ACTGGGTGCT). A standard curve mentioned by Tortosa et al. [38] was used as the reference. q-PCR reactions were performed in a 20 µl total volume containing 2 ng of genomic DNA, 0.5 µM of each primer and 3 µl of FastStart Universal SYBR Green Master (Roche). Cycling was performed using a Light-Cycler480 Instrument (Roche) for 45 amplification cycles of 94 °C for 4 s, 65 °C for 15 s and 72 °C for 25 s.

Microscopic Analysis

Commercially prepared Giemsa stain (SRL-45881) was used to stain ovaries of wild female *Ae. albopictus* and *Cx. quinquefasciatus*. A standardized staining condition (taking into account varying staining time, dilution, and buffer pH) as mentioned by Wright [50] was used to visualize mosquito *Wolbachia* in ovary smears.

Statistical Analyses

Wolbachia positivity in frequency between ovaries and midgut/salivary gland in *Ae. albopictus, Ar. subalbatus* and *Cx. quinquefasciatus* was calculated using one-way ANOVA. The density of *Wolbachia* between ovaries, midgut and salivary gland of *Ae. albopictus* was also calculated using one-way ANOVA. A comparison between male and female infection status from *Ae. albopictus* collected from four physiographical regions was calculated using Fisher's exact test.

GenBank Submissions MF805773, MF805774, MF805775, MF805776, MF805777, MH753508, MH753510, MH765637, MH765639, MH765641, MH765643, MH765645, MH765647, MH765649, MH765651.

Results

Distribution of Mosquito Species in Odisha

A total of 1634 mosquitoes were collected during the period May 2017 to Feb 2018. Following the standard keys of identification the mosquitoes included the species like *Anopheles culicifacies, Anopheles stephensi, Anopheles barbirostris, Anopheles subpictus, Anopheles vagus, Anopheles minimus, Anopheles fluviatilis, Aedes aegypti, Aedes albopictus, Aedes vittatus, Culex quinquefasciatus, Culex vishnui, Culex gelidus, Armigeres subalbatus, Armigeres theobaldi, Mansonia indiana, Mansonia uniformis*, and *Mansonia annulifera*. Along with mosquitoes 27 *Drosophila simulans* were also collected for reference as positive control.

Wolbachia Distribution in Mosquito Species

Wolbachia was detected in 7 of 18 species of mosquitoes collected. The details are mentioned in Table 1. *Anopheles* sp, *Ae. aegypti, Ae. vittatus, Ar. theobaldi*, and *Mn. annulifera* were completely devoid of *Wolbachia*. A standard PCR was performed using *Wolbachia* specific primer (81F/691R) that amplified at 610 bp. A semi nested PCR using the amplified product from standard PCR produced bands at 577 bp and 449 bp for supergroups A and B respectively. Further A and B sub grouping primers amplified around 379 bp and 501 bp respectively. Newly designed strain specific primers for *Ae. albopictus* amplified at 65 bp for subgroup A and 164 bp for subgroup B as depicted in Fig. 2.

On screening for subgroups, it was found that 80% of *Ae. albopictus* were super-infected (*w*AlbA + *w*AlbB), 5% were mono-infected with *w*AlbA and 10% were mono-infected with *w*AlbB. Along with this, 58.3% of *Mn. uniformis* and 38.8% of *Mn. indiana* were found to be superinfected; 85% of *Cx. quinquefasciatus* and 68.4% of *Cx. vishnui* were found to be mono-infected with *w*AlbB; 38.4% of *Cx. gelidus* and 65% of *Ar. subalbatus* were found to be mono-infected with *w*AlbA. *D. simulans* taken as control was found to be 100% mono-infected with wAlbA.

Sub-grouping and Phylogenetic Analysis

Sequences of *wsp* gene generated from the study were subjected to alignment using Clustal Omega. This included 15 sequences from this study and 14 sequences derived from NCBI. The phylogenetic analysis revealed separate clusters A and B belonging to two supergroups A and B of *Wolbachia*.

Maximum-Likelihood Method based on Tamura–Nei model [36] with a bootstrap value of 500 was employed to study the evolutionary history. *Wsp* gene sequences of *Ae. albopictus* are found to be present in both the groups A and B. *Cx. quinquefasciatus, Cx vishnui. Mn indiana, Mn. uniformis,* and *D. simulans* are clustered under supergroup B whereas *Ar. subalbatus, Cx. gelidus, and D. simulans* are clustered under supergroup A. *D. simulans* (positive control) had A infection. Stars indicate sequences with accessions generated from studies in different parts of Asia.

Three *wsp* sequences from *Ae. albopictus* and two sequences from *Ar. subalbatus* were found in a clade where *Wolbachia* AlbA sequences of *Ae. albopictus* (AF020058, AF397411 and JX476002) and *Cx. gelidus* (HM007831) were clustered together along with positive *D. simulans* (AF020067). Other sequences from *Ae. albopictus, Cx. quinquefasciatus* belonged to the clade where *Wolbachia w*AlbB (Pip) sequences from *Ae. albopictus* (HM007829, AF397412), *Cx. quinquefasciatus* (AF020060), *Cx. vishnui* (HM007825, KY71015), *Mn. uniformis* (KY523674),

Table 1 Entomological collection with *Wolbachia* presence determined by *wsp* gene in vectors and non-vectors of Odisha

Sl no	Species	Numbers screened		*Wolbachia infection status*					
				Both A and B		Only A		Only B	
		Male	Female	Male	Female	Male	Female	Male	Female
1	*Anopheles culicifacies*	3	10	0	0	0	0	0	0
2	*Anopheles stephensi*	0	4	0	0	0	0	0	0
3	*Anopheles barbirostris*	3	10	0	0	0	0	0	0
4	*Anopheles subpictus*	1	10	0	0	0	0	0	0
5	*Anopheles vagus*	8	10	0	0	0	0	0	0
6	*Anopheles minimus*	3	10	0	0	0	0	0	0
7	*Anopheles fluviatilis*	0	10	0	0	0	0	0	0
8	*Aedes aegypti*	10	10	0	0	0	0	0	0
9	*Aedes albopictus*	10	10	7	9	1	0	1	1
10	*Aedes vittatus*	7	10	0	0	0	0	0	0
11	*Culex quinquefasciatus*	10	10	0	0	0	0	7	10
12	*Culex vishnui*	9	10	0	0	0	0	4	9
13	*Culex gelidus*	3	10	0	0	1	4	0	0
14	*Armigeres subalbatus*	10	10	0	0	6	7	0	0
15	*Armigeres theobaldi*	8	10	0	0	0	0	0	0
16	*Mansonia indiana*	8	10	3	4	0	0	0	0
17	*Mansonia uniformis*	2	10	2	5	0	0	0	0
18	*Mansonia annulifera*	5	10	0	0	0	0	0	0
19	*Drosophila simulans*	10	10	0	0	10	10	0	0

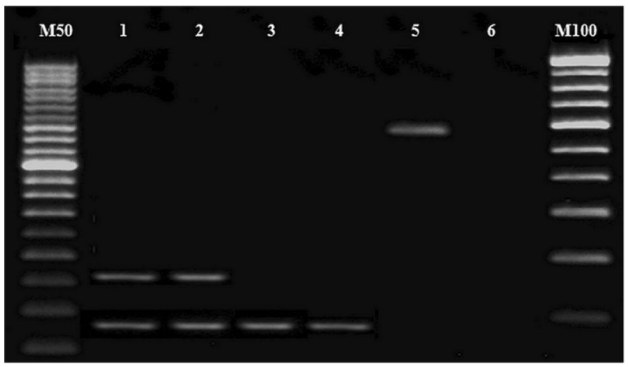

Fig. 2 Gel picture amplifying *w*AlbA and *w*AlbB from *Wolbachia* positive *Ae. albopictus*. Lane 1 and 2 represents super-infection in females (65 bp for wAlbA and 164 bp for wAlbB). Lane 3 and 4 represents mono-infection in males (65 bp for wAlbA). Lane 5 is positive control *Wolbachia* DNA (610 bp; primers of Zhou et al. [53]) and lane 6 is negative control containing reaction mixture without *Wolbachia* DNA

and *Mn. indiana* (AF317492) are clustered. The details of the sequences generated from the study are mentioned in Table 2.

Thus, a monophyletic group with representative members of *Wolbachia* from arthropods were clearly distinguished under groups A and B (Fig. 3).

Table 2 Details of sequences generated from the study

Host species	*Wolbachia* supergroup	*Wolbachia* subgroup	GenBank Accession Numbers
Ae. albopictus	A	AlbA	MF805774
			MF805776
			MF805777
Ar. subalbatus	A	AlbA	MH753508
			MH753510
D. simulans	A	Mel	MH765637
			MH765639
			MH765641
			MH765643
Ae. albopictus	B	Pip	MF805773
			MF805775
Cx. quinquefasciatus	B	Pip	MH765645
			MH765647
			MH765649
			MH765651

Organ Specific *Wolbachia* Screening in Mosquito Species

On screening *Wolbachia* in different organs of female mosquitoes of *Ae. albopictus, Cx. quinquefasciatus* and *Ar.*

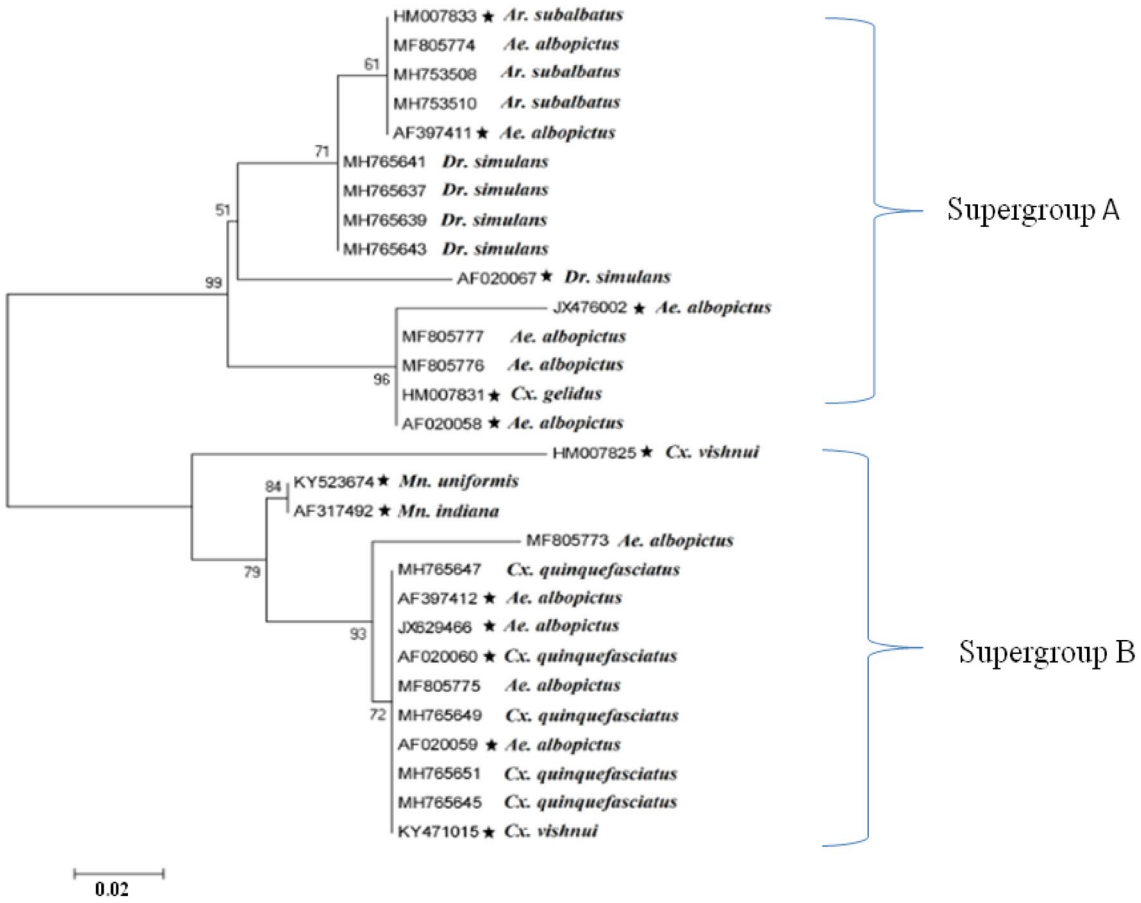

Fig. 3 Phylogenetic tree based on *wsp* sequences of *Wolbachia* (Maximum-Likelihood Method)

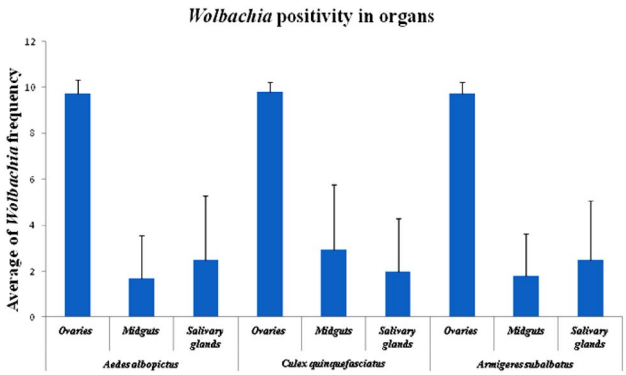

Fig. 4 *Wolbachia* positivity (in frequency) in different organs of *Ae. alhopictus, Cx. quinquefasciatus* and *Ar. subalbatus*

subalbatus, it was found that ovaries were the highest to be infected followed by salivary glands and midguts (Fig. 4). One-way ANOVA revealed that there was a significant difference in *Wolbachia* positivity between ovaries and midguts/salivary glands in all the above mentioned species at $P < 0.05$. However, there was no significant difference in *Wolbachia* positivity between respective organs of three species studied at $P < 0.05$.

Based on these results, we tried to quantify *Wolbachia* in ovaries, midguts and salivary glands of *Ae. albopictus*. It was found that the number of *wsp* gene per host actin gene was highest in ovaries followed by salivary glands and midguts ($n = 5$ for each organ). Further one-way ANOVA suggested a significant difference between densities of all the tested organs at $P < 0.05$ (Fig. 5).

Sex-Specific *Wolbachia* Infection Pattern in *Ae. albopictus*

The density of *Wolbachia* in wild male and female *Ae. albopictus* was compared. There was 1.8 and 18.1 copies of wsp A and wsp B per host actin gene in male as different against 7.8 and 13 copies in female. The standard PCR in male *Ae. albopictus*, revealed no wAlbA mono-infection at day 3 of their survival suggesting the complete clearance of this strain by day 3 of survival. The prevalence of *w*AlbA and *w*AlbA + *w*AlbB differed significantly between

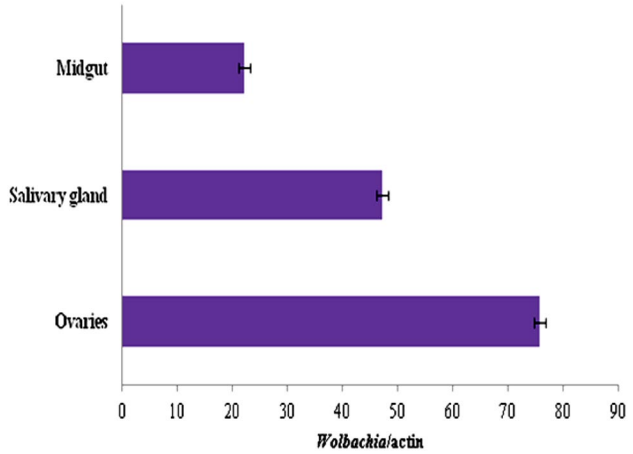

Fig. 5 *Wolbachia* density in organs of Fl reared *Ae. albopictus*

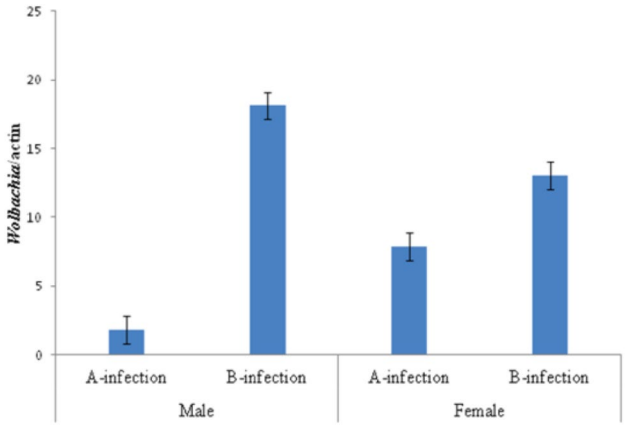

Fig. 6 *Wolbachia* density in wild male and female *Ae. albopictus*

males and females for four physiographical regions of Odisha (Fisher's exact test, $P < 0.05$) (Fig. 6).

On quantifying *Wolbachia* at day 0, 5, 15, and 30 post eclosion, it was found that *Wolbachia* super-infection in females tend to increase whereas wAlbA density reduced completely as compared to wAlbB in males when they grew old (Fig. 7).

Microscopic Identification

Giemsa stained microscopic images of squashed ovary of wild caught *Ae. albopictus* and *Cx. quinquefasciatus* showed the presence of pink pleomorphic cells of *Wolbachia* with shapes ranging from cocci, comma to bacillus and chain forms. *Wolbachia* could not be observed in the ovaries of *Ae. aegypti* (Fig. 8).

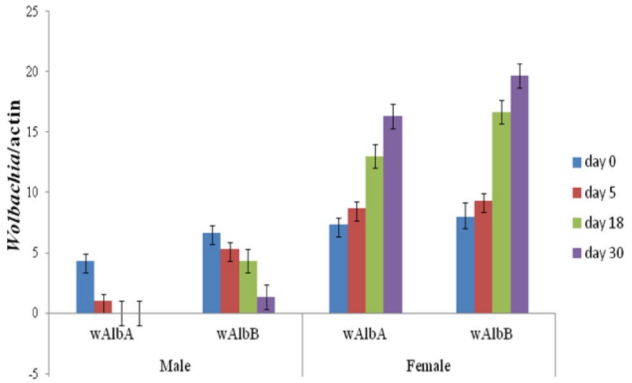

Fig. 7 *Wolbachia* density in Fl reared male and female *Ae. albopictus* at 0, 5, 18, and 30 days post eclosion

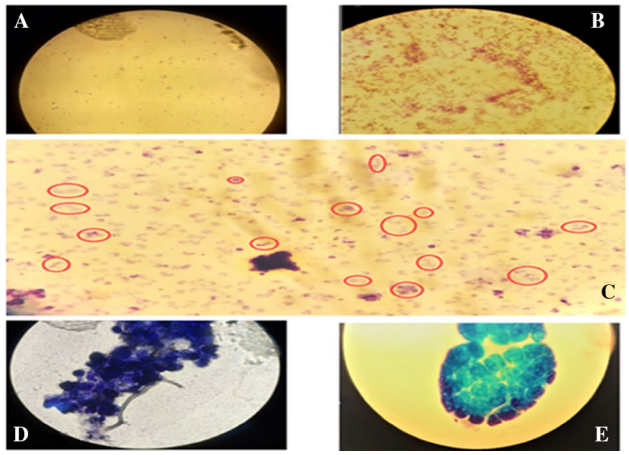

Fig. 8 **a** Giemsa stained smear of squashed ovary of *Ae. aegypti* without *Wolbachia*. **b** Giemsa stained smear of squashed ovary of *Ae. albopictus* with *Wolbachia* colonies. **c** Giemsa stained smears showing pleomorphic forms (red rounds) of *Wolbachia* in *Cx. quinquefasciatus*. **d** Giemsa stained ovary of *Ae. aegypti* without *Wolbachia*. **e** Purple stained *Wolbachia* on the follicle cells of ovary in *Ae. albopictus*

Discussion

Ever since the discovery of *Wolbachia* in *Culex pipiens* in 1936 [14], the importance of this endosymbiont has reached its peak. The common arthropods with natural infection of *Wolbachia* include *D. melanogaster, Cnaphalocrocis medinalis, Lutzomyia* sp. and *Ae. albopictus* [4, 6, 33, 40]. The general and strain differentiating primers [53] amplified *wsp* gene from *Wolbachia*-infected insects thus making *wsp* helpful in determining the evolutionary relationships between strains. On account of variability of *wsp*, more studies on biological aspects of *Wolbachia* can be done in future.

In this study a survey on vectors and non-vectors in Odisha was done to determine the extent of indigenous

Wolbachia infections. The result indicates the presence of indigenous *Wolbachia* in *Ae. albopictus, Ar. subalbatus, Mn. uniformis, Mn. indiana, Cx. quinquefasciatus, Cx. vishnui*, and *Cx. gelidus. D. simulans* was taken as a positive control for *Wolbachia*. None of the field collected *Anopheles* species, *Ae. aegypti* and *Mn. annulifera* had *Wolbachia* in them. This is in concordance with the results of Hughes et al. [16], Kittayapong et al. [21], de Oliveira et al. [7], Wiwatanaratanabutr [48], and Van den Berg et al. [42]. Molecular analysis revealed that *Cx. quinquefasciatus, Cx vishnui. Mn indiana, Mn. uniformis* and *D. simulans* belong to supergroup B (wPip-group B) whereas *Ar. subalbatus, Cx. gelidus, D. simulans*, and *D. melanogaster* belong to supergroup A (*w*AlbA-group A).

Ae. albopictus, an efficient vector of dengue and chikungunya, had either super-infection (*w*AlbA-group A and *w*Pip-group B) or mono-infection with wPip-group B. Kittyapong et al. [21] reported the same results. Both *Mn. uniformis* and *Mn. indiana* were also found to be superinfected. This shows that vector and non-vector species are natural niche to strain A and strain B of *Wolbachia*. Our results also confirm *D. simulans* to be infected with strain A of *Wolbachia*. Many studies have shown *D. melanogaster* to be naturally infected with *w*Mel strain of *Wolbachia*. The induction of cytoplasmic incompatibility in females can be attributed to multiple *Wolbachia* strain infections [45]. The results from PCR screening were further co-evaluated/co-confirmed with phylogenetic analysis of the *wsp* sequences. *Wolbachia* sub-grouping is of utmost importance in tracing the evolutionary significance between the host and the symbiont [44].

The phylogenetic analysis also established the co-infection of *w*AlbA and *w*AlbB in *Ae. albopictus* mosquitoes collected from different regions. As observed, *Wolbachia* strains are distributed in both group A and group B, similar to those reported by Zhou et al. [53], Van Meer et al. [43], and Ruang-Areerate et al. [31]. High homology between *w*AlbA and *w*AlbB strains of *Ae. albopictus* indicates the stability of these two strains. Moreover, the high homology of *Wolbachia* from *Ae. albopictus, Cx. quinquefasciatus* and *Ar. subalbatus* may predict its origin from the same ancestral bacterial strain. A further study on comparison between *Wolbachia* strains can estimate a vector's capacity to acquire, replicate and transmit a novel pathogen.

Wolbachia quantitative study also indicated that ovaries of tested mosquitoes were infected from day 0 of emergence while midgut and salivary glands acquired *Wolbachia* infection at 8 and 10 days post eclosion respectively. This might indicate the gradual spread of *Wolbachia* within organs when a female mosquito grows old. The high density of *Wolbachia* in the ovaries in the present study justifies its transmission during oogenesis as evident from Giemsa staining in the ovarian cells. This affirms its presence in germ cell cytoplasm, follicular cells, nurse cells, and future oocytes. Since *Wolbachia* is transmitted transovarially from infected mother to offspring, the early stages of oogenesis or embryogenesis concentrates itself mainly in the germ-line of arthropods and nematodes [26, 37, 45]. Quantitative studies in other organs indicated that the density of *Wolbachia* is lower in salivary gland and lowest in the midgut which is similar to other reports where *Wolbachia* presence was observed in salivary glands, midguts, and ovaries of *Ae. albopictus* [40, 54]. *Wolbachia* infection in midgut and salivary glands might explain the reduction in replication and transmission of dengue virus in *Ae. albopictus*. Many authors described *Wolbachia* presence in various somatic tissues along with germ-line tissues in hosts making *Wolbachia* a potential organism incorporating disease resistant gene products [8].

Studies on density variance of individual *Wolbachia* strains showed a decrease in *w*AlbA density and increase in *w*AlbB density when males grow old; but super-infected females had an overall increase in density for both *w*AlbA and *w*AlbB. The study was conducted on laboratory reared F1 generation. *w*AlBA and *w*AlbB density were variable in mosquitoes of F1 generation for both the sex and at different age. This was further confirmed by standard PCR wherein there was no *w*AlBA infection in males of 3 day old; thereby confirming very low density of *w*AlbA that cannot be detected by standard PCR. The sensitivity of q-PCR made its detection even at 5 day of post eclosion. This explains the fact of only *w*AlbB infection existence in males. Females are thus considered to be a repertoire of *Wolbachia* prevalence in field population because of vertical transmission. This phenomenon has been clarified by studies of Tortosa et al. [39] which states that males receive super-infection at birth from their mothers but lose wAlbB when they grow old showing immunity to cytoplasmic incompatibility induced embryonic mortality. Here females show a virtual fixation of super-infection. Our study confirms a difference in total density of *Wolbachia* in wild male and female. Females tend to have a higher density than males. Studies have shown that density vary from region to region. Heterologous hosts encounter higher *Wolbachia* densities than native hosts, rendering more robustness for antiviral effects than native ones [10]. A decrease in density of *Wolbachia* occurred at high temperature and low nutrition [49].

We also performed microscopic studies on distribution of *Wolbachia* in ovaries of *Ae. albopictus* and later confirmed that PCR to be more sensitive and specific. Though microscopic method using Giemsa staining protocol helped in readily identifying morphology of *Wolbachia* present in *Ae. albopictus* and *Cx. quinquefasciatus* in laboratory, it could not confirm the distinction between two strains i.e., *w*AlbA and *w*AlbB inhabiting the mosquitoes. However, the PCR technique helped in strain typing of *Wolbachia* that may assist in further determining the competitive strain that can

in future help in transfection to vectors, thereby reducing the transmission of diseases.

The study provides evidences on potential vectors of dengue, chikungunya *(Ae. aegypti)* and malaria (*An. culicifacies* and *An. fluviatilis)* that are inefficient to harbor *Wolbachia* and they hardly encounter *Wolbachia* horizontal transmission. So, further studies in Odisha can be made by transfecting a foreign strain into dengue and malaria vectors to analyze their pathogen replication capabilities. Future studies can help identify the interaction between host, virus and *Wolbachia*. Many studies reported that *Wolbachia* diminishes viral replication [1, 2] and reduces the life span of the host [19]. This fact makes *Wolbachia* an alternate choice for vector control leading to a massive reduction of the disease transmission. Since *Wolbachia* is not naturally present in wild *Ae. aegypti*, transfected *Ae. aegypti* has been successfully incorporated in field trials to maximize their spread in wild populations [9, 15, 24, 52], thereby reducing vector competence. The mass releases of *w*Mel transfected *Ae. aegypti* in Australia has attempted success in reducing dengue burden in the released site with no adverse effects. With this, WHO along with various public health entities have advocated the use of *Wolbachia*-based strategies to control spread of arboviral diseases. Ample data on indigenous *Wolbachia* in mosquito may serve as a gateway for *Wolbachia*-based vector control approaches. It is compulsive to screen mosquito species for indigenous *Wolbachia* strains for its selection and future application in biocontrol strategies when conventional vector control strategies are less valuable. The current study is a preliminary effort to collect basic information regarding the extent of indigenous infections of *Wolbachia* in vectors and non-vectors of Odisha, India. Since Odisha is endemic for dengue and chikungunya, the study may be helpful to initiate vector control programs making a potential use of *Wolbachia*.

Wolbachia has been used as a driver for spread of disease resistant gene in mosquito being implemented worldwide. Our study provides a brief insight on the indigenous strain of *Wolbachia* circulating in mosquito species of Odisha. This study is unique in its kind covering the major aspects of the endosymbiont *Wolbachia* and focusing on its potential as a biocontrol agent in arboviral outbreaks. A robust knowledge on potential of the indigenous strain and further experiments including interactions between *Wolbachia* and viruses can be utilized further to reduce the global burden of vector borne diseases.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflicts of interest.

Ethical Approval All procedures performed in the study involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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