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Wsp gene based detection and characterization of *Wolbachia* in indigenous *Drosophila*

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Abstract

Wolbachia is a genus of intracellular symbiotic bacteria, vertically transmitted in more than 60% insects. *Wolbachia* interferes with host physiology and induces various alterations like cytoplasmic incompatibilty, parthenogenesis, feminization, male killing and inhibition of parasite and pathogen propagation. In last decade, it has been used as transinfected bioagent in the control strategies of vector-borne diseases. Current study involves the detection of *Wolbachia* from *Drosophila melanogaster* and *D. simulans* for the first time in Pakistan. The fruit flies collected were screened for the presence of *Wolbachia* using *Wolbachia* surface protein (*wsp*) gene specific primers by conventional polymerase chain reaction (PCR). *D. melanogaster* and *D. simulans* represented 90.6% (96/106) and 89.6% (69/77) *Wolbachia* infection respectively. Nucleotide BLAST reslut of partial *wsp* gene sequence from detected strain of *D. melanogaster* indicated 100% homology with *Walachia* endosymbiont of *D. melanogaster* reported from Australia and detected *Wolbachia* of *D. simulans* represented 100% homology with *Wolbachia* endosymbiont *w*Ri strain of *D. simulans* from Sweden. Both of the detected strains belonged to supergroup A and were named as *w*Mel (*D. melanogaster*) and *w*Riv (*D. simulans*) on the basis of host and *wsp* similarity.

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Introduction

Wolbachia variants, widely distributed in insects, have gained considerable attention due to the efficient maternal transmission and induction of reproductive modifications in their host. Cytoplasmic incompatibility (CI), parthenogenesis, feminization, male killing and pathogen inhibition are valuable features associated with *Wolbachia* (Hornett *et al.,* 2008).

Unidirectional or bidirectional cytoplasmic incompatibility has been observed among different strains of *D. simulans* fruit fly (Charlat *et al.,* 2002, James and Ballard 2000) and various other insects (Vavre *et al.,* 2000) including *D. melanogaster* (Riegler *et al.,* 2005). Bidirectional CI occurs when two partners are bearing dissimilar strains of *Wolbachia* and this cross is always mortal in both directions. *Wolbachia* induced CI between a sperm and an egg, results in destruction of zygote in diploid species or production of males in haplodiploid species. Transmission of bacteria occurs through eggs but not via sperms. *Wolbachia* is transmitted to next generations by females only, via their egg cytoplasm. Therefore, it is subjected to diverse selective pressures from nuclear genes: its own reproductive success is not affected in males, because they are 'dead ends' through which no bacterial transmission occurs (Huigens *et al.,* 2000).

Wolbachia may induce some additional variations in host genome. So, there are greater chances of divergence and reproductive isolation among populations of various infections. Other than this, *Wolbachia* induced CI can directly influence the gene flow between different populations. Thus, it can be regarded as a chief cause of speciation in such populations. Gene flow can be reduced by unidirectional incompatibility that occurs when infected males and uninfected females mate and this is thought to be the key reason of divergence between closely related species of *Drosophila* (Jaenike *et al.,* 2006).

Wolbachia may directly or indirectly influence the development of other pathogens in insect vector by interfering with its genetic mechanisms (Hughes *et al.,* 2011, Lu *et al.,* 2012, Raychoudhury *et al.,* 2010).

Wolbachia potential to inhibit parasite propagation and to induce CI has led the idea to use this endosymbiont as biological agent for the control of insect borne diseases. Moreover, *Wolbachia* can act as a gene vector to distribute preferred genotypes in natural population of disease vectors.

Exact estimates of *Wolbachia* occurrence are difficult to attain, and both under and overestimation may occur. Underestimation may be caused by incomplete sampling or by the fact that both infected and uninfected hosts normally coexist in the same population (Hilgenboecker *et al.,* 2008, Zug and Hammerstein 2012). *Wolbachia* detection or its mapping in host tissues is usually accomplished using different techniques such as; fluorescence-based methods (Albertson *et al.,* 2013, Casper-Lindley *et al.,* 2011), PCR assays, targeting 16S rRNA, *gatB*, *coxA*, *hcpA*, *groEL*, *ftsZ* and *wsp* genes (Baldo *et al.,* 2006, Chai *et al.,* 2011, Marcon *et al.,* 2011, Serbus *et al.,* 2012). 16S rRNA gene is regarded as conserved gene in contrast to *ftsZ* which is variable. Surface protein genes of bacteria are usually considered as suitable in detection and phylogenetic studies (Baldo *et al.,* 2005). *Wsp* gene comes under moderately variable genes. The *wsp* gene has been extensively used in detection and phylogenetic studies of *Wolbachia* in a wide range of insects.

First *Wolbachia,* whose complete genome was studied was isolated from *Drosophila melanogaster* (Wu *et al.,* 2004). Transfection of *Wolbachia* from *Ae. albopictus, Cx. Quinquefasciatus*, *D. melanogaster* and *D. simulans* has been established in mosquito vectors including *Ae. aegypti* (Walker *et al.,* 2011, Xi *et al.,* 2005), *Ae. albopictus* (Blagrove *et al.,* 2012, Braig *et al.,* 1994), in different countries. *In vitro* cultivation of *Wolbachia* and genetic manipulations are challenging as it is an intracellular obligate symbiont (Casper-Lindley *et al.,* 2011). There is great scope of further research on exploration of new genetic variants of *Wolbachia* in natural insect populations which will lead to use *Wolbachia* as biocontrol agent for vectors of many deadly diseases in Pakistan.

The objective of current study involved *wsp* gene based screening and characterization of *Wolbachia pipientis* in natural population of *D. melanogaster* and *D. simulans* in Punjab, Pakistan.

Materıals and methods

Drosophila Sampling

Two species of fruit flies, *D. melanogaster* (n=106) and *D. simulans* (n=77), were collected from two different geographical locations using mechaincal aspirator (Table 1). Insect species were identified on the basis of morphological criteria. Pure cultures were extended from each isofemale in insectary, Department of Zoology, GC University Lahore, Pakistan under standard conditions (25°C temperature, 80% humidity and light/ dark cycle of 12 hours).

Dissection of reproductive organs

The collected flies were paralysed using freeze shock method by keeping them at 4°C for 5-10 minutes. Each adult was dipped 3 times in sterile water, surface disinfected 2 times by rinsing in 70% ethanol for 5 min, and then transferred to successive sterile water baths 5 times, and once in sterilized NaCl 0.8% (Zouache *et al.,* 2009). The ovaries or testes were dissected under dissecting microscope using sterile pair of forceps. A single pair of ovaries or testes was considered as one sample. The tissues were homogenized using a sterile polypropylene pestle.

Genomic DNA Extraction

Total 400µL lysis buffer (100mM NaCl, 10mM Tris-HCL, 10mM EDTA, pH 8.0 and 0.5% SDS) along with 0.5mg/mL proteinase K was added to the homogenates and mixed. The samples in micro centrifuge tubes (1.5mL) were incubated overnight at 60°C. The tissues were shaken well and spun for 2 seconds to force condensation into bottom of the tube. In the fume hood 500 µL of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added to each sample and vortexed for 30 sec to achieve a milky emulsion in the tube. The tube was centrifuged at 14,000rpm for 5min. Denatured proteins collected at the interface was left as such and very clear supernatant (aqueous layer) was transferred carefully to upper chamber of an Amicon® ultra-0.5mL 100K centrifugal filter unit (Millipore corporation, Ireland; Cat# UFC510096) and lid was placed on the concentrator and spun at 500 ×*g* for 10min using Benchtop centrifuge (Hettich Mikro 200R, Tuttlingen, Germany). The liquid was passed through membrane to filtrate cup. Spin cap was removed and filtered material was discarded.

Then 300µL of 1X TE buffer (10mM Tris-HCl, pH 8.0; 0.1mM EDTA) was added to concentrator for washing and centrifuged at 500 ×*g* for 5 min. This step was repeated again. The concentrator was removed from the filtrate cup and carefully inverted onto a labelled retentate cup (reverse spin). The assembly was centrifuged at 500 ×*g* for 5 min. The concentrator cup was discarded. The retentate cup was capped which contains the DNA.

Quantification of DNA

The quality and quantity of the isolated DNA sample was estimated by the help of Nano Drop Lite Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). The 260/280 nm ratio was calculated to evaluate DNA purity. The ratio was <1.8 and DNA concentration was ranging from 450 to 1800ng/µL.

PCR amplification of wsp gene

The exponential amplification of extracted DNA (template) was carried out with PCR thermal cycler (Techne Progene, UK) in a total volume of 50µL contained 1X *Taq* buffer (without $MgCl_2$), 1.5mM $MgCl_2$, 0.2 µM dNTPs, 0.4µM each primer, 1 U *Taq* DNA polymerase (Invitrogen), 50ng of total genomic DNA. The sequence of primers pair for screening of wsp of *Wolbachia* were: 5'- GATCCTGTTGGT CCAATAAGTG -3' (forward) and 5'- AACGCTACTCC AGCTTCTGC -3' (reverse) according to (Albertson *et al.,* 2013). Thermal cycling conditions were as follows: one cycle of 94°C for 5min (initial denaturation) followed by 35 cycles of 94°C for 1min (denaturation), 55°C for 1min (annealing), 72°C for 1min (extension) and one cycle at 72°C for 10min (final extension).

The thermocycler was programmed to hold the product at 4°C after completion of cycles automatically.

Agarose Gel Electrophoresis

The extracted genomic DNA from different insect tissues underwent 1% agarose gel electrophoresis (in 1X TAE buffer), stained with ethidium bromide (EtBr) (as 0.5µg/mL) and the band was visualized and photographed by a UV trans-illuminator and Bio Doc-It® TS imaging system (Ultra-Violet Products Limited, Cambridge, UK). The DNA samples were kept at –20°C till PCR analysis.

Sequencing of wsp amplicons and data analyses

The selected amplified PCR products were sequenced from 1st Base Laboratories Sdn Bhd, Kembangan 43300, Selangor, Malaysia. Sequences were analysed for quality using Chromas software and subjected to BLAST search against the National Center for Biotechnology Information (NCBI) public sequence database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide sequences generated in current study have been deposited in Gen Bank data base and accession numbers were obtained. The Phylogenetic tree was traced by Maximum Likelihood statistical method rooted to the Tamura-Nei model. Evolutionary tree was constructed using MEGA6.

Results and discussion

Current research is the first report of *Wolbachia* detection in two sister species of fruit flies in Pakistan. *D. melanogaster* (n=106) collected from Faisalabad and *D. simulans* (n=77) from Lahore, Pakistan represented 90.6% and 89.6% *Wolbachia* infection respectively on the basis of *wsp* gene. *Wolbachia* infection level in both host species (*D. melanogaster* and *D. simulans*) has been reported as 100% previously (Casper-Lindley *et al.,* 2011). In current study, one of the main reasons of lower *Wolbachia* infection is that the *Drosophila* were collected from the field in natural conditions whereas the previosuly reported results involved laboratory reared *Drosophila*. Age and ecology of the host including geographical and seasonal variations are responsible for low infection rate in natural population. Numerous genes for the identification of *Wolbachia* strains has been identified including 16S rRNA, *ftsZ* and *wsp* genes (Rasgon and Scott, 2004). One individual (host) can have multiple *Wolbachia* infections (more that one strains). Moreover, different individuals of one host species can have different *Wolbachia* strains. Current study reports single *Wolbachia* infection in one host population. Both species have different *Wolbachia* strains.

The *wsp* gene sequence of *Wolbachia* from *D. Melanogaster* were submitted to Gen Bank under accession numbers KX650072 and that from *D. simulans* under KX650074 (Table 1). Both of the sequences were analysed through CLUSTAL W multiple sequence alignment tool and were represented (Fig. 1) using Bio Edit software. Almost 88% identities with complete query coverage were traced in both of the sequences indicating that detected *Wolbachia* strains were distantly related in evolutionary view point. Fig. 2 represents two relatively conserved regions in *wsp* partial sequence with no variation. These two regions (67 nucleotides from 140-206 position and 116 nucleotides from 399-514) were not only similar in both (*w*Mel and *w*Riv) detected strains but also similar in a large number of already reported sequences. In addition, three regions of *wsp* gene in both strains; 73 nucleotides (67-139), 192 nucleotides (207-398) and 73 nucleotides (515-587) were found with maximum variation. These hypervariable regions leads to diverse variety of *Wolbachia* strains in various insects. *wsp* was determined as moderately variable gene and sequence from this gene provides much more information about evolutionary relationship among *Wolbachia* strains (Zhou *et al.*, 1998). The recombination in surface protein gene of endobacteria also have strong impact on host intracelluar environment. The *wsp* gene had relatively more phylogenetic divergence rate as compared to most common 16S rRNA gene (Bazzocchi *et al.*, 2000). The *wsp* gene has been extensively used to infer phylogenetic and evolutionary interactions among *Wolbachia* of various hosts (Baldo *et al.,* 2005).

Table 1. Collection of fruit flies (*Drosophila*) for screening of *Wolbachia* from Faisalabad and Lahore, Pakistan.

Insect species	Latitude, Longitude*	Area	Collection	*Wolbachia* strain	Accession No.
D. melanogaster	N 31°12'59.3", E 72°59'57.2"	Faisalabad	March 2015	*w*Mel	KX650072
D. simulans	N 31°34'11.7", E 74°18'25.5"	Lahore	December 2014	*w*Riv	KX650074

*Geographical coordinates were collected (as degrees, minutes, seconds) using GPS Garmin GPSMAP® Olathe, Kansas, USA; 76CSx Model with the accuracy of ±5 m.

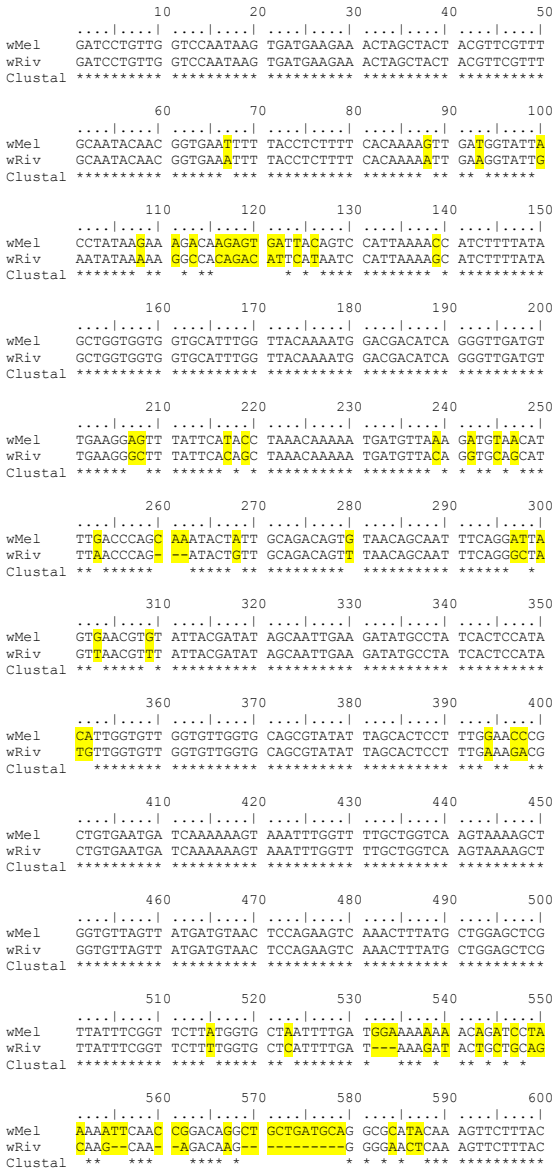

Fig. 1. Comparison of *wsp* partial sequences of *Wolbachia* endosymbionts detected from *D. melanogaster* and *D. simulans* Pakistan using CLUSTAL W.

Nucleotide BLAST reslut of *wsp* gene sequence from detected strain of *D. melanogaster* indicated 100% homology of with *Wolbachia* endosymbiont of *D. melanogaster* reported from Australia with Gen Bank accession AE017196 (Wu *et al.,* 2004). Moreover, the query has maximum (98%) homology with *Wolbachia* of same host from Australia and China with total score ranging from 1168 to 1153.

In contrast detected *Wolbachia* of *D. simulans* represented 100% homology with *Wolbachia* endosymbiont *w*Ri strain of *D. simulans* from Sweden having accession number CP001391. Likewise, the query also has 100% homology with *D. simulans* of United States. Both of the detected strains belonged to supergroup A and were named as wMel (*D. melanogaster*) and wRiv (*D. simulans*) on the basis of host and *wsp* gene similarity.

The phylogenetic analysis of detected *Wolbachia* strains is presented in Fig. 2. The *wsp* gene based hierarchy of both strains (wMel and wRiv) was constructed with the maximum log likelihood (-1432.5781). The primary ranking for the heuristic method was obtained automatically by applying Neighbor-Join and Bio NJ algorithms to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) methodology, and then selecting the topology with superior log likelihood value. The analysis involved 34 coding sequences including 475 positions. Codon positions encompassed were made with all possible frames excluding gaps and missing data in data set.

The dendogram represented that the *Wolbachia* hosts *D. melanogaster* was almost totally separated in one group from *D. simulans* indicating that both hosts harboring different *Wolbachia pipientis* including *w*Mel and *w*Riv strains . However, both strains come under supergroup A as they are sharing >85% homology.

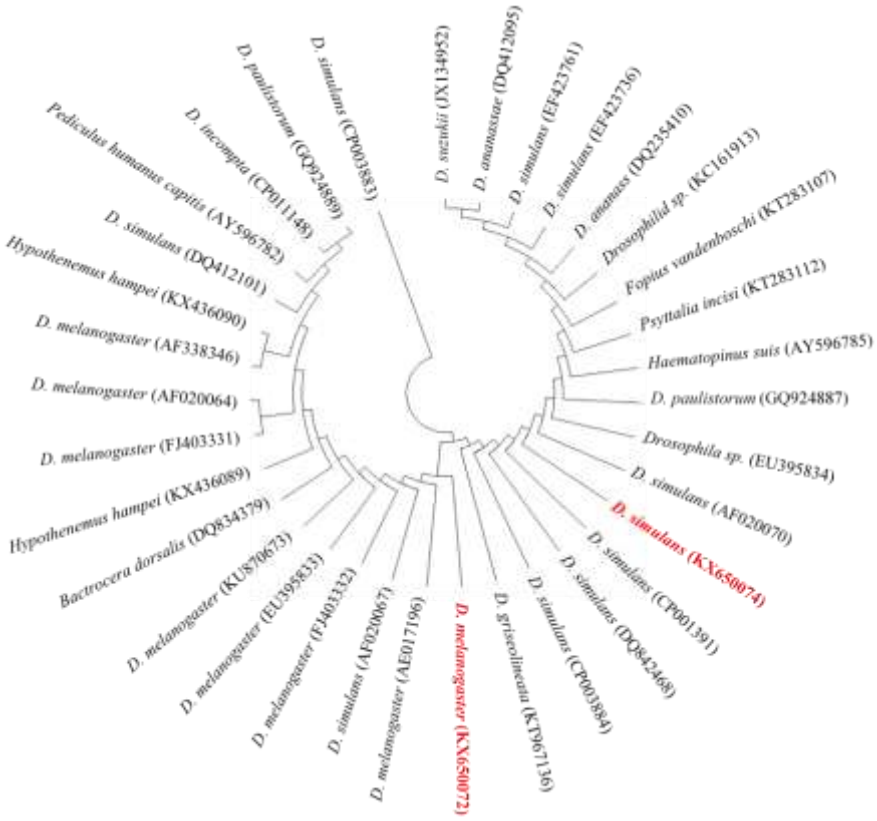

Fig. 2. Phylogenetic tree of *Wolbachia pipientis* found in Pakistani *D. melanogaster* and *D. simulans* on the basis of *wsp* gene using maximum likelihood method.

*Query in bold and red color represents *Wolbachia* host species (Gene Bank accession number).

Wolbachia induced reduction of life span and inhibition of parasite propagation in its host (Correa and Ballard, 2016; Joshi *et al.*, 2014) along with its diversity in more than 60% insects has gained much attention to use this endosymbiont in biological control of different vector borne diseases. *Wolbachia* also influences the host population by causing the cytoplasmic incompatibility when infected male mate with uninfected female or female having different strain of *Wolbachia* (Werren *et al.*, 2008). Trans-infection of *w*Mel *Wolbachia* from *D. melanogaster* has been successfully established in *Ae. aegypti* (dengue vector) population for the control of dengue disease in Australia (Hoffmann *et al.,* 2011). The knowledge about *Wolbachia* infections in several kinds of arthropods found in Pakistan is still inadequate. Current study reports high infestation of *Wolbachia* endosymbionts named *w*Mel and *w*Riv in local *D. melanogaster* and *D. simulans* respectively which will provide basis for determining the ways and strategies for vector control.

Future studies should investigate the life shortening and parasite inhibition potential of local *Wolbachia* strains in biocontrol control of disease vectors.

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