

Full Length Research Paper

# Molecular diagnosis of *Wolbachia* endosymbiont from Iranian scorpion *Hemiscorpius lepturus* using polymerase chain reaction (PCR) amplification of 16S rDNA gene

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There are several reports concerning the intracellular bacteria, *Wolbachia*, which infect an extensive range of invertebrates and promote a diverse array of reproductive alteration which encourage infection spread among its host population. This study reports the identification of *Wolbachia* in the Iranian scorpion named *Hemiscorpius lepturus* (*H. lepturus*) collected from the southwestern province of Khuzestan using partial polymerase chain reaction (PCR) amplification of *Wolbachia* 16S rDNA gene. PCR product was directly sequenced and the alignment of the sequence with similar sequences in GenBank showed high similarity with 16S rDNA gene of *Wolbachia* endosymbiont of *Drosophila melanogaster*.

**Key words:** *Wolbachia*, Iranian scorpion, 16S rDNA gene, *Hemiscorpius lepturus*.

## INTRODUCTION

*Wolbachia* is a gram negative, obligate, intracellular symbiont (Stephen et al., 2002) that is classified in  $\alpha$ -proteobacteria (Inaki et al., 2011). *Wolbachia* is probably the most common endosymbiont in the biosphere, and up to 66% of arthropod species are reported to be infected by it (Hilgenboecker, et al., 2008). The first description was done by Wolbach in 1924. The initial observation was reported in the ovaries of *Culex pipiens* mosquito. Hence, this unknown gram negative bacterium was classified as an unnamed Rickettsia (Hertig and Wolbach, 1924), and was later named *Wolbachia* to honor Wolbach.

*Wolbachia* is vertically transmitted through the egg cytoplasm and they manipulate their hosts' reproduction in various ways (Ravikumar et al., 2011). These ways are feminization of genetic males (Rousset et al., 1992),

parthenogenetic induction which results in development of unfertilized eggs (Stouthamer et al., 1993), the killing of male progeny from infected females (Hurst et al., 1999), and sperm-egg incompatibility (referred to as cytoplasmic incompatibility) (Bourtzis et al., 1996; Bram et al., 2011).

This intracellular bacterium is observed in a broad range of hosts. The different genome sequencing projects were carried out in order to understand *Wolbachia* distribution, evolution and the genetic basis of the phenotypes it induces (Inaki et al., 2011). The *Wolbachia*-host interaction ranges from mutualism to commensalism and parasitism (Xie et al., 2011; Gavotte et al., 2010). One of the fitness benefits of *Wolbachia* on its hosts is increasing the host resistance to infection with a range of pathogens (Bian et al., 2010; Moreira et al., 2009). *Wolbachia* cannot be cultured outside the host cells (O'Neill et al., 1992), and its potential application to the biocontrol of insect pests (Ravikumar et al., 2011) and vector-borne diseases such as dengue fever (Inaki et al., 2011), malaria and filariasis (Chaoyang et al., 2009)

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**Figure 1.** A photographic illustration of the male (upper figure) and female (lower figure) *Hemiscorpius lepturus*. Note the very short sting size and longer tail in the male.

have been studied.

On the basis of the molecular studies and phylogenetic analysis of 16S rDNA genes, groESL and wsp genes, *Wolbachia* was placed in family Anaplasmataceae with genera *Ehrlichia*, *Neorickettsia* and *Anaplasma* (Florence et al., 2003). A phylogenetic analysis of 16S rDNA sequences showed a 2% sequence divergence in *Wolbachia* strains from different arthropod species. This diversity resulted into a division of *Wolbachia* into two separate groups (A and B) (Jeyaprakash and Hoy, 2000). Subsequently, this division was subdivided into 11 subgroups (A to K). Phylogenetic analysis of other *Wolbachia* genes, including ftsZ and wsp, showed higher diversity (Werren et al., 1995; Zhou et al., 1998; Van et al., 1999).

The different surveys of arthropods by polymerase chain reaction (PCR) for *Wolbachia* infection suggested that it would be interesting to search for *Wolbachia* endosymbiont in arthropod species, such as scorpion, in order to estimate the presence and distribution of the infection with *Wolbachia* (O'Neill et al., 1992; Catharina et al., 2011). The initial report for the presence of *Wolbachia* in scorpion is related to southern African genus, *opisththalmus* (Laura et al., 2007). Therefore, the main purpose of this study is to search for *Wolbachia* 16S rDNA gene in the genomic DNA preparation made from the dangerous scorpion *H. lepturus* (*Hemiscorpiidae*). This scorpion has been responsible for stinging a great number of people annually in the southwestern dry and hot province of Khuzestan (Dehghani et al., 2007). Among different types of available scorpions in Khuzestan, *H. lepturus* is the most lethal one with major concern (Jalali et al., 2011). Furthermore, the use of the

naturally existing strains of *Wolbachia* to control the most dangerous scorpion of Iran is introduced as a topic of research.

## MATERIALS AND METHODS

### Scorpion samples

Iranian scorpions *H. lepturus* were collected from Khuzestan province. All scorpions were identified by trained health-caring personnel of the laboratory reference of Razi Institute in Ahvaz (Figure 1). The milking was carried out to allow the toxin-producing cells of the venom glands to enter into the secretory phase. They were killed five days after milking, kept in 95% ethanol and frozen at -20°C. 20 separated venom glands (ten males and ten females) were randomly used for total DNA extraction. DNA extraction from *Wolbachia*-infected scorpions were collected and stored at -80°C.

### Total DNA extraction

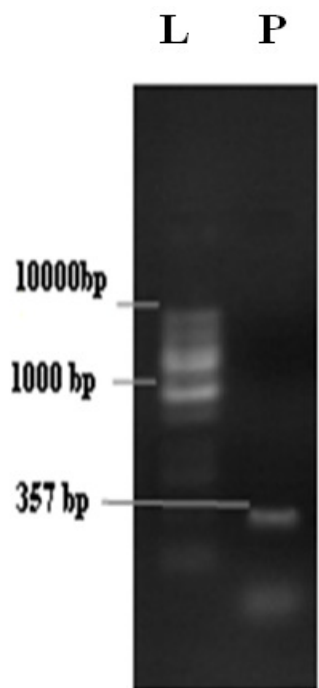
Total DNA was extracted from the venom glands of scorpions using the phenol-chloroform extraction method (Werren et al., 1995; Werren et al., 2000). The extracted DNA was dissolved in DEPC-ddH<sub>2</sub>O solution and kept until it was used for rDNA amplification by PCR.

### 16S rDNA amplification by PCR

The PCR amplification reactions were carried out by Bio-Rad thermocycler, in 25 µl reaction mixtures consisting of 0.6 mM of dNTP, 0.4 mM of each primer, 1 µl of the crude DNA extract, 1 Mm MgCl<sub>2</sub>, 2.5 µl of 10 x PCR buffer, 0.3 U of Taq polymerase and 1 µl of dimethyl sulfoxide (DMSO) 16S rDNA. Specific primers used in this work were WrDNA-R (5'-AGCTTCGAGTGAAACCAATTC) and WrDNA-F (5'-CATACCTATTCTGAAGGGATAG)(24). These amplify a

**Table 1.** PCR program used for amplification of 16S rDNA gene from *Wolbachia* endosymbiont of scorpion *H. lepturus*.

Cycle number	Step name	Duration time	Degree (°C)
1	Initial denaturation	5 min	95
35	Denaturation	40 s	94
	Annealing	90 s	51
	Extension	90 s	72
1	Final extension	10 s	72

**Figure 2.** PCR amplification of 16S rDNA gene from *Wolbachia* endosymbiont of scorpion *H. lepturus* using specific primers. Lane L: DNA ladder, Lane P: production of amplification of 16S rDNA gene.

357 bp fragment. These are designed from the moderately variable regions of 16S rDNA and are relatively conserved among *Wolbachia*. This gene is used routinely for phylogenetic studies as it is highly conserved between different species of bacteria and archaea (Coenye and Vandamme, 2003).

The PCR conditions for initial denaturation were carried out for 5 min at 95°C, followed by 30 cycles consisting of 94°C for 40 s, 51°C for 1 min and 72°C for 1 min, and a final extension for 10 min at 72°C as described in Table 1.

10 µl of the amplified product with a 100 bp DNA ladder were separated by 1% agarose gel electrophoresis including ethidium bromide and visualized by UV transilluminator to determine the presence and size of the amplified DNA.

#### DNA sequencing

The main band was identified and purified from agarose gel by QIA quick agarose Gel Extraction kit (Cat no: 28104) and then sent to Kawsar Biotech Company (Iran-Tehran) for nucleotide sequencing.

#### Sequence analysis

Sequence was compared with GenBank database using the BLAST software from NCBI site (<http://www.ncbi.nlm.nih.gov>), whereas sequence alignment was done using the CLC Main Workbench program.

#### RESULTS AND DISCUSSION

A total of 20 scorpions (ten males and ten females) *H. lepturus* were screened for *Wolbachia*. *Wolbachia* were found in ten of them. Figure 2 shows the PCR amplification of 16S rDNA of *Wolbachia* endosymbiont. As shown in the figure, PCR reaction amplified a fragment in the range of about 360 bp. According to the sequencing results, the length of the nucleotide sequence of the amplified fragment was 357 bp (Figure 3). Comparison of the 16S rDNA fragment with the GenBank database revealed that the nucleotide sequence of 16S rDNA of *Wolbachia* endosymbiont of *H. lepturus* was highly similar to that of *Drosophila melanogaster* (Figure 4).

In addition to insects, *Wolbachia* are also known to infect filarial nematodes, terrestrial crustaceans (isopods), mites, scorpions and spiders (Sether et al., 2010); though the *Wolbachia* infection in Iranian scorpions has not been previously determined. In this study, we found *Wolbachia* infection in scorpion *H. lepturus*. As described, 50% of the total collected scorpions were infected with *Wolbachia*.

Phylogenetic analysis of different strains of *Wolbachia* infecting arthropods using 16S rDNA gene did not show high level of divergences (Vaishampayan et al., 2007). This finding is similar with that of this study. However, a comparison of the 16S rDNA nucleotide sequence of *Wolbachia* endosymbiont of scorpion *H. lepturus* (from arachnidae) was highly similar (99%) with that of *Drosophila melanogaster* (from insect).

Rapid evolving bacteria cell-cycle gene *ftsZ* and 16S rDNA gene has shown that there are two major groups of *Wolbachia* (A and B) in arthropods (Fenn et al., 2006; Holden et al., 1993). These groups have been divided into 11 subgroups (A to K) as described by Zhou et al. (1998). Further, two additional groups (C and D) were also reported in filarial nematodes (Van et al., 1999); so we suggest that the determined *Wolbachia* in this study belong to group A or B.

GGTCGGTTCGTCGGGTTTCACACAGGTGTTGCATGGCTGTCGTCAGCTCGTGTCTGTG  
 AGATGTTGGGTTAAGTCCCAGCAACGAGCGCAACCCTCATCCTTAGTTACCATCAGGT  
 AATGCTGGGGACTTTAAGGAAACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGAT  
 GTCAAGTCATCATGGCCCTTATGGAGTGGGCTACACACGTGCTACAATGGTGGCTAC  
 AATGGGCTGCAAAGTCGCGAGGCTAAGCTAATCCCTTAAAAANCCATCTCAGTTCGGA  
 TTGTA CTCTGCAACTCGAGTGCATGAAGTTGGAATCGCTAGTAATCGTGGATCAGCA  
 CGCCACGGTGAATA

Figure 3. Nucleotide sequence of 16S rDNA gene from *Wolbachia* endosymbiont of scorpion *H. lepturus*.

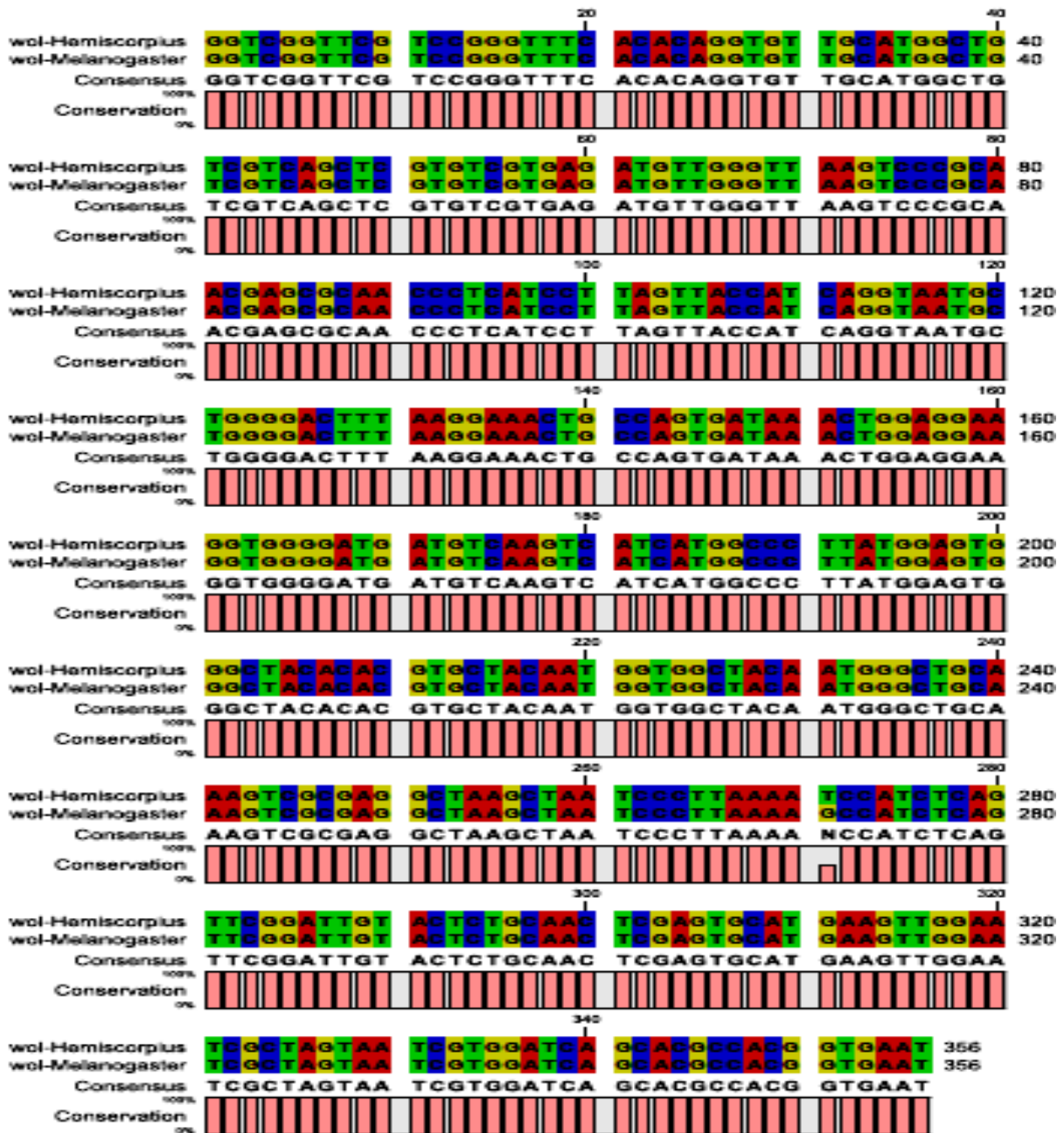


Figure 4. Sequence alignment of *Wolbachia* 16S rDNA gene from *H. lepturus* with 16S rDNA gene from *Drosophila melanogaster*. Wol-Hemiscorpius, *Wolbachia* 16S rDNA gene endosymbiont of *H. lepturus*; Wol-melanogaster, *Wolbachia* 16S rDNA gene endosymbiont of *D. melanogaster*.



ting how this bacterium infect a wide range of arthropods including scorpion. With the report of *Wolbachia* endosymbiont infection of *H. lepturus* scorpion, more evidences were provided that the bacteria of the *Wolbachia* group are more diverse than was previously pointed.

As described, the presence of *H. lepturus* is restricted to the southwestern part of Iran, Khuzestan with hot and dry climate conditions. This province has long and warm summers beside short, moderate winters. Summertime temperatures normally exceed 50°C. This finding may approve growing of *Wolbachia* in all geographic regions and the hottest temperatures conditions.

*Wolbachia* induce reproductive manipulation in arthropod. So, it is usual to conclude that treatment of *Wolbachia* can reduce the population of their hosts (Ravikumar et al., 2011). Therefore, if infection of arthropods is proved, we can apply this bacterium for biological control of arthropod pests. Therefore, this kind of descriptive information helps to propose experimental strategies by exploiting a *Wolbachia*-cytoplasmic incompatibility based mechanism to control medically important scorpions.

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