Co-occurrence of pederin-producing and *Wolbachia* endobacteria in *Paederus fuscipes* Curtis, 1840 (Coleoptera: Staphilinidae) and its evolutionary consequences

Naseh Maleki-Ravasan¹ | Niloofar Akhavan¹,² | Abbasali Raz¹ | Mahmood Jafari³ | Sedigheh Zakeri¹ | Navid Dinparast Djadid¹

¹Malaria and Vector Research Group (MVRG), Biotechnology Research Center (BRC), Pasteur Institute of Iran, Tehran, Iran
²Department of Biotechnology, Faculty of Advanced Sciences and Technology, Pharmaceutical Sciences Branch, Islamic Azad University, Tehran, Iran
³Department of Geology, Faculty of Sciences, Tarbiat Modares University, Tehran, Iran

Correspondence
Navid Dinparast Djadid, Malaria and Vector Research Group (MVRG), Biotechnology Research Center (BRC), Pasteur Institute of Iran, Tehran, Iran.
Email: navidmvrg@gmail.com

Abstract
The dual occurrence of *Pseudomonas*-like and *Wolbachia* endobacteria has not been investigated in the *Pederus* beetles yet. We investigated pederin-producing bacteria (PPB) infection in *Paederus fuscipes* specimens from the southern margins of the Caspian Sea by designed genus-specific (*OprF*) and species-specific (16S rRNA) primers. *Wolbachia* infection was studied through a nested-PCR assay of *Wolbachia* surface protein (*wsp*) gene. Of the 125 analyzed beetles, 42 females (82.35%) and 15 males (20.27%) were positive to PPB infection; this is the first study reporting male *P. fuscipes* infection to PPB. *Wolbachia* infection was found in 45 female (88.23%) and 50 male (67.57%) analyzed beetles. Surprisingly, a number of 36 females (70.59%) and 13 males (17.57%) were found to be infected with both PPB and *Wolbachia* endosymbionts. In general, population infection rates to PPB and *Wolbachia* were determined to be 45.6% and 76%, respectively. The infection rates of female beetles to PPB and PPB-*Wolbachia* were significantly higher than males. In *Paederus* species, only female beetles shelter PPB and the discovery of this bacterium in adult males may reflect their cannibalistic behavior on the contaminated stages. Phylogenetic analysis showed that the sequences of *OprF* gene were unique among *Pseudomonas* spp.; however, sequences of 16S rRNA gene were related to the PPB of *Pederus* species. The co-occurrence and random distribution of these endobacteria may imply putative tripartite interactions among PPB, *Wolbachia*, and *Paederus*. In order to elucidate these possible tripartite interactions, further studies are required even at gender level.

KEYWORDS
16S rRNA, *OprF*, *Paederus*, pederin, *Pseudomonas*-like, *wsp*
Rove beetles of Staphylinidae are the largest family of beetles and are distributed in a wide range of habitats. They include more than 63,000 known species arranged into thousands of genera and 32 subfamilies worldwide (Grebeníkův & Newton, 2009; Thayer, 2005). The genus *Paederus* Fabricius, 1775, which is classified into the tribe Paederini and subfamily Paederinae, currently comprises ~490 species (Nikbakhtzadeh, Naderi, & Safa, 2012; Vieira, Ribeiro-Costa, & Caron, 2014).

Fourteen species and subspecies of the *Paederus*, including five subgenera occur in Iran. Among them, six species *P. balachowskyi*, *P. balcanicus*, *P. fuscipes* *fusipes*, *P. littoralis iliae*, and *P. riparius* are present in three Southern Caspian Provinces, Gilan, Mazandaran, and Golestan (Nikbakhtzadeh et al., 2012).

In natural ecosystems (predominantly moist environments), staphylinid species are associated with various arthropods, higher plants, fungi, decomposing materials, mollusks, and vertebrates. Most of the rove beetles are predators of arthropods, and some of them are associated with social insects, while others are scavengers on decaying plant matter or live in nests of rodents (Frank & Thomas, 2016). Some species of rove beetles are important in terms of the biological control of insects of agricultural, medical, and veterinary importance (Echegaray & Cloyd, 2013).

*Paederus* species and its relatives are the agents of human dermatitis as well. They are active during daylight hours and can cause linear dermatitis on human skin and severe damage to human eyes (Echegaray & Cloyd, 2013). The genus *Paederus* Fabricius, 1775, which is classified into the tribe Paederini and subfamily Paederinae, currently comprises ~490 species (Nikbakhtzadeh, Naderi, & Safa, 2012; Vieira, Ribeiro-Costa, & Caron, 2014).

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*Paederus* species and its relatives are the agents of human dermatitis as well. They are active during daylight hours and can cause linear dermatitis on human skin and severe damage to human eyes (Nairobi eye). These beetles neither bite nor sting but release their hemolymph containing pederin, a potent vesicant toxin (C₂₅H₂₅NO₈; MM: 503.63; LD₅₀: 0.14 mg/kg rat i.p.), when they injured or crushed on human skin (Dettner, 2011; Iserson & Walton, 2012). This contact dermatitis is a distinctive stimulus form that can be distinguished by the rapid onset of erythematobullous lesions on the exposed areas (Mammino, 2011). If erythemas continue longer, other symptoms such as fever, edema, neuralgia, arthralgia, and vomiting may be observed as well (Rahman, 2006). It is supposed that pederin has antitumor and antiviral properties (Narquizian & Kocienski, 2000), presumably through the inhibition of DNA replication and protein synthesis (Dettner, 2011).

Pederin and its derivatives, namely, pseudephedrine and pederone, are synthesized by uncultured *Pseudomonas*-like endosymbionts located in the female accessory glands, restored in the hemolymph and transferred to the developmental stages through the contaminated eggs (Kellner, 1998, 2001; Kellner & Dettner, 1995). Studies based on 16S rRNA gene have shown that only female beetles contain the pederin-producing bacteria (PPB; Kellner, 2002). These bacteria are distributed in the rove beetle populations, through the transovarial transmission (Kador, Horn, & Dettner, 2011).

Naturally, pederin is used by *Paederus* species as a defensive compound against insect and arachnid predators (Kellner & Dettner, 1996). The immature stages of *P. fuscipes* and *P. riparius*, which were pederin positive, were repulsive for spiders of the families Lycosidae and Salticidae but not for insect predators (Kellner & Dettner, 1996).

*Wolbachia*, obligate endosymbionts, are estimated to infect 40% of terrestrial arthropod species (Zug & Hammerstein, 2012) and many parasitic filarial nematodes (Taylor, Bandi, & Hoerauf, 2005). They manipulate reproduction properties of the hosts through the induction of cytoplasmic incompatibility, parthenogenesis, feminization, and male killing (Hughes, Pamilo, & Kathirithamby, 2004; Li et al., 2016; Li, Wang, Bourguet, & He, 2013; Vavre, Fleury, Lepeit, Fouillet, & Boulétreau, 1999; Werren, 1997; Yun, Peng, Liu, & Lei, 2011).

*Wolbachia* strains and their role in arthropod host fitness have been reviewed recently (Zug & Hammerstein, 2015). It has been indicated that a *Wolbachia* strain could protect alfalfa weevil, *Hypera postica*, against the parasitic wasp, *Microtonus aethiopoides* (Hsiao, 1996). Recently, it has been shown that *Wolbachia* can protect *Culex pipiens* mosquitoes against *Plasmodium relictum*-induced mortality (Zele, Nicot, Duron, & Rivero, 2012). In addition, a new strain of *Wolbachia* has been reported in *Cimex lectularius* that appears to display an important role in bedbug fitness through provisioning of B vitamins (Nikoh et al., 2014). More recently, some strains of *Wolbachia* have been introduced as a weapon in the war against vector-borne pathogens (Hughes & Rasgon, 2014; Kambris, Cook, Phuc, & Sinkins, 2009). Therefore, a variety of *Wolbachia* strains can have either mutualistic or parasitic outcomes in the insect/pathogens/parasitoids assemblages (van Nouhuys, Kohonen, & Duplouy, 2016), which should be studied in details when their properties are exploited.

Initially, insect’s isolates of *Wolbachia pipientis* has been classified into two supergroups (A and B) and 12 groups based on the sequences of the major *Wolbachia* surface protein (wsp) gene (Zhou, Rousset, & O’Neill, 1998). Today, all invertebrate isolates of *Wolbachia* have been divided sequentially into 16 supergroups (A to Q) using the multilocus sequence typing (MLST) technique (Baldon et al., 2006; Glowksa, Dragun-Damian, Dabet, & Gerth, 2015).

Despite many advances in the study on *Wolbachia* infection in insects, our knowledge on the *Wolbachia* strain diversity/dispersion, or their effects on the beetle hosts is very limited. According to the findings of a review study (Kajtoch & Kotásková, 2018), *Wolbachia* infection was detected in 204 coleopteran species with average prevalence of 38.3%. The most intensively studied families have been herbivorous beetles of Curculionidae and Chrysomelidae. Coleoptera-infecting *Wolbachia* strains belonged to three supergroups of A, B, and F with single, double, or multiple infections in the studied species. *Wolbachia* has had a lot of effects on its beetle hosts ranging from selective sweep with host mtDNA and cytoplasmic incompatibility to other changes related to the reproductive or developmental phenotypes (Kajtoch & Kotásková, 2018).

Survival and reproduction of many insects rely on the endosymbiotic bacteria (Eleftherianos, Atri, Accetta, & Castillo, 2013; Ratzka, Gross, & Feldhaar, 2012). Therefore, PPB as defensive (Oliver & Moran, 2009) and *Wolbachia* as reproductive (Kajtoch & Kotásková, 2018) symbionts may play an important role in evolution and adaptation of *Paederus* species. As a matter of fact, PPB seems to affect
the capacity of the Paederus beetles to be causative agent of human linear dermatitis. It is also necessary to study the distribution of Wolbachia in the rove beetles to determine its function in host biology. Infection of Paederus species by PPB and Wolbachia has separately been investigated in a very few studies (Kador et al., 2011; Yun et al., 2011); however, dual occurring of these endobacteria has not been investigated yet. Hence, we studied co-occurrence of PPB and Wolbachia in P. fuscipes. The achieved results can contribute to pave the way to address interesting open queries on the evolutionary consequences of the interactions between these inherited bacteria and their host biology with further experiments.

2 | EXPERIMENTAL PROCEDURES

2.1 | Study areas

The specimens were collected from nine locations of two provinces of southern coast of Caspian Sea in Iran, Gilan (Bijar Boneh \( n = 6 \)), Vashmeh Sara \( n = 38 \), Kochesfahan \( n = 10 \), Chini Jan \( n = 8 \), Kalachai \( n = 1 \), and Tajan Gukeh \( n = 40 \) and Mazandaran (Rayan \( n = 6 \), Shirud \( n = 5 \), and Amol \( n = 11 \)). Live adult beetles were gathered from humid areas, principally from rice fields, using hand collection method. The specimens were kept in 70% ethanol in 4°C refrigerator until experiments.

2.2 | Morphological studies

The specimens were morphologically identified using available identification keys generated by Blackwelder (1957), Arnett and Thomas (2001), and Borror and DeLong’s (Triplehorn & Johnson, 2005).

2.3 | DNA extraction

Prior to molecular survey, to surface sterilize, the specimens were immersed twice in freshly prepared 70% ethanol for 2 min and rinsed vigorously with 0.9% normal saline. The whole bodies of adult beetles were homogenized in the DNA lysis buffer using sterile pestles. Genomic DNA of rove beetles was extracted using Collins DNA extraction method (Collins et al., 1987).

2.4 | Detection of PPB infection

2.4.1 | OprF primer designing and amplification

The major outer membrane protein of *Pseudomonas*, OprF, has been found only in *Pseudomonas* genus and considered as a diagnostic protein in *Pseudomonas* sensu stricto (Bodilis & Barray, 2006; Bouffartigues et al., 2011). A total of 44 sequences of OprF gene related to *Pseudomonas* isolates were extracted from the GenBank and aligned using Mega 5.0 software. The conserved regions among all *Pseudomonas* isolates were targeted to design genus-specific primers. Two primers, OPFF: 5’-GGGAGACTCAGGAAATGCTGTCATG-3’ and OPRFR: 5’-CAACGCTACGGGCGATGAT-3’, were designed based on the OprF-specific sequences to amplify 327bp of *Pseudomonas* spp. and PPB in the rove beetles. PCRs were done in a volume of 20 µl containing 5 pmol of each designed primer (Macrogen, Korea), 0.5 nmol dNTPs (Fermentas, USA), 1 U Taq DNA polymerase (CinnaGen, Iran), 2.5 µl buffer 10×, and 1–5 µl (~0.1 µg) of the extracted DNA from samples. The PCR thermal profile used with these primers was an initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 66°C for 30 s, 72°C for 25 s, and a final extension step at 72°C for 10 min. All specimens were firstly screened with OprF gene, and then positive ones were examined via 16S rRNA gene.

2.4.2 | 16S rRNA primer designing and amplification

Five available 16S rRNA sequences of PPB in rove beetles (*P. fuscipes* [AJ316016], *P. riparius* [AJ316018], *P. melanurus* [AJ316017], *P. ruficollis* [AJ316019], and *P. sabaeus* [AJ295331]) and five representative 16S rRNA sequences of other bacteria (*Pseudomonas aeruginosa* [AE004844], *Escherichia fergusonii* [NR_074902], *Salmonella enteric* [NR_119108], *Klebsiella pneumoniae* [NR_117686], and *Proteus mirabilis* [NR_114419]) were retrieved from the GenBank and subjected to PPB species-specific primer designing. After alignment, 16S-PPBF: 5’-ACCCGCATACGTCTAAGGGGAC-3’ and 16S-PPBR: 5’- CTCCTTGCGTTAGCCAAG-3’ primers were designed based on the 16S rRNA-specific sequences of PPB in rove beetles to amplify a 1,265-bp fragment of this gene. PCRs were the same as those performed for OprF primers. After an initial denaturation step of 5 min at 94°C, 35 cycles were carried out (denaturation for 30 s at 94°C, annealing for 30 s at 59°C, and elongation for 40 s at 72°C), followed by 10 min at 72°C.

2.5 | Detection of Wolbachia infection

Wolbachia infection was detected in rove beetles on the basis of Zhou et al.’s, (1998) introduced primers and through a nested-PCR assay recruited by Karami et al., (2016). Originally, primers of 81F: 5’-TGGTCAATAAGTGATGAAGAAAC-3’ and 69R: 5’- AAAATTAAACGCTACTCCA-3’ were designed to amplify a 632-bp fragment of the wsp gene. The PCR product of the first step was employed as a template for the second step. In this step, the primers of 691R and 183F: 5’-AGGAAACCGAATATTCG-3’ were used to amplify a 301-bp fragment. The PCR was performed in a total volume of 20 µl containing 5 µl (~0.5 µg) of genomic DNA for the first step, and 2.5 µl of PCR product for the second step of nested-PCR, one-time PCR buffer, 2.5 U Taq polymerase (CinnaGen, Iran), 1 µl of each primer (20 mM, Macrogen, Korea), 200 µM of each dATP, dTTP, dCTP, and dGTP (Fermentas, USA) and 1.5 mM of MgCl2 in an automated Thermocycler (Analytik Jena FlexCycler, Canada). The PCR conditions were set as an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min.
2.6 | Sequencing and phylogenetic analysis

All the PCR products from 16s rRNA, OprF, and wsp genes were analyzed by 1% agarose gel electrophoresis, followed by Green Viewer staining and visualization using a UV transilluminator. Amplicons of the representative specimens were extracted from the gel, and after purification was sequenced bidirectionally via the same amplification primers (Macrogen Company, Korea).

The raw sequences were initially edited by the Chromas 2.6.5 software through trimming of right and left cut-off regions that may contain poor qualities. The consensus of confident sequences was analyzed using NCBI (nucleotide collection) database. Multiple alignments of the studied sequences were generated by the Clustal Omega package (Sievers et al., 2011). BLOSUM62 and Kimura-2-Parameter models were used, respectively, to score the pairs of aligned OprF/wsp amino acids and 16S rRNA nucleotides. Phylogenetic trees were constructed using the maximum likelihood method embedded in Mega 5 software (Tamura et al., 2011). Confidence of internal nodes was tested by Bootstrap test with 1,000 replications.

The phylogeny of various Pseudomonas spp., including PPB, was evaluated based on the OprF gene sequences. The relationships between 16S rRNA gene sequences of PPB in Paederus specimens and their close relative, Pseudomonas aeruginosa, was investigated through the phylogenetic tree construction.

3 | RESULTS

3.1 | Morphological study

In this research, a total of 125 adult rove beetles, including 74 males and 51 females, were studied. All the collected specimens were taxonomically identified as Paederus fuscipes Curtis, 1840 (Coleoptera: Staphilinidae) by using the morphological keys mentioned in the Experimental Procedures.

3.2 | Detection of PPB and Wolbachia infection in

Prior to practical procedures, the specificity of designed primers was tested in silico. Performing BLAST searches showed that OprF primers were able to find cultured and uncultured Pseudomonas spp., which is in accordance with the desired specificity we expect for our study to identify Pseudomonas and Pseudomonas-like species, but not the other genera. Also, the 16S rRNA primers could amplify only PPB endosymbiotic of P. fuscipes, and it did not even reproduce symbionts which were present in the GenBank other than P. fuscipes.

In practice, both Pseudomonas-specific (OprF) and PPB-specific (16S rRNA) primers resulted in amplicon sizes of 327 and 1,265 bp, respectively, as expected. Applying the nested-PCR assay could easily detect the wsp, a single-copy gene. The PCR products of the first and the second steps of nested-PCR assay were roughly 650 and 500 bp, respectively.

3.3 | PPB and Wollbachia infection rates in P. fuscipes

The designed OprF primers could amplify all Pseudomonas species, including Pseudomonas-like PPB and P. aeruginosa (Figure 1).

![Figure 1](image-url)
However, the species-specific 16S rRNA primers that we designed could identify only Pseudomonas-like PPB (Figure 2). In total, of the 125 (51 female and 74 male) analyzed beetles, 42 females (82.35%) and 15 males (20.27%) were positive to OprF primers and the same rates (82.35% and 20.27%) were also positive to the PPB-specific 16S rRNA primers. PPB was detected not only in female beetles (as reported by Kellner, 2002) but also in male beetles. This is the first study reporting male P. fuscipes infection to PPB. Also, Wolbachia infection was found in 45 female (88.23%) and 50 male (67.57%) analyzed beetles. Surprisingly, a number of 36 females (70.59%) and 13 males (17.57%) were detected to be infected with both PPB and Wolbachia endosymbionts. Individual analysis of bacteria showed that six females (11.76%) and two males (2.7%) were PPB positive and nine females (17.65%) and 37 males (50%) were positive for wsp gene. The Chi-squared test showed no significant difference ($\chi^2 = 0.13$) of Wolbachia infection among male and female beetles, either alone or in combination with Pseudomonas. The infection rates of females to PPB and PPB-Wolbachia were significantly higher than males in both alone and combined analyses ($\chi^2 > 0.05$). Combined analysis showed that Wolbachia infection rate in females was more than males; however, this difference was not significant ($\chi^2 = 0.015$). Overall, our results pointed out that 45.6% and 76% of all the specimens were positive to PPB and Wolbachia endosymbionts, respectively. The infection results in both alone and combined analyses are depicted in Table 1.

### 3.4 | Sequence and phylogenetic analyses

Sequence analysis of OprF gene revealed the presence of two phylogenetically diverse groups in both male and female rove beetles; the first group of PPB sequences had 78% similarity to P. jinjuensis and P. citronellolis, and the second group of sequences was 100% identical to P. aeroginosa (Figure 1). Phylogenetic analysis showed that the sequences of OprF gene are unique among Pseudomonas spp.; however, the sequences of 16S rRNA gene were related to the PPB of Paederus species.

Comparative 16S rRNA gene sequence analysis showed that some specimens from Gilan (KY568938 & KY568939) and Mazandaran (KY568940) Provinces were 100% identical to each other. Nevertheless, there were minor differences between the samples from Gilan Province (KY568941 with 4 and KY568937 with 2 substitutions). In general, phylogenetic analysis of 16S rRNA gene from P. fuscipes specimens indicated that along with a sequence of the same species from Germany, the sequences of this study made a monophyletic clade were located as the sister clade of the sequences from P. ruficollis (France) and P. sabaues (Cameroon; Figure 2).

The wsp gene sequence analysis displayed that all Wolbachia strains, obtained from the collected P. fuscipes in the study areas, were 100% identical to each other. In addition, the results of the BLAST search indicated that these strains were fully similar to the wsp sequence of Aedes albopictus [AF020059], Drosophila simulans [AF020069 and AF020074], Culex pipiens [AF020061], and Lasioderma serricorne [AB469359], the members of the Pip group of supergroup B.

### 3.5 | Nucleotide sequence accession numbers

The nucleotide sequences determined in this study have been deposited into the GenBank database under the following accession numbers: OprF: KY568928-KY568936, 16S rRNA: KY568937-KY568941, and wsp gene: KY555600-KY555603. The representatives of each sequences group were applied to phylogenetic analysis (Figures 1 and 2).

### 4 | DISCUSSION

We studied dual occurrence of PPB and Wolbachia endobacteria in P. fuscipes rove beetles. The overall population infection rates to PPB and Wolbachia endosymbionts were revealed to be 45.6% and 76%, respectively. The PPB infection has previously been reported only in adult females (Kellner, 2002); however, here, we report the infection not only in females but also in male specimens. Detection of PPB in male beetles does not necessarily mean the existence of pederin substance in the male beetles. The PPB infection in females was found to be four times that of males (Table 1). These results are rational because the female Paederus have to transmit PPB to offspring and protect them against both conspecific and other natural
predators. Finding the PPB infection in adult males may be reflecting the cannibalistic behavior of the rove beetles, in part.

In this study, for the first time, PPB was detected at the genus and species levels, respectively, by OprF and 16S rRNA primers. The outcome results from both genus and species levels were in agreement with the detection of PPB. Initially, the specimens were screened with OprF gene (copy numbers ≃ 200,000 per bacterial genome [Hancock, Siehnel, & Martin, 1990]), and then positive specimens were examined via 16S rRNA gene with copy numbers 1–15 per bacterial genome (Rainey, Ward-Rainey, Janssen, & Hippe, 1996). The OprF is a protein that not only has widely been studied in vaccine researches (Rawling, Martin, & Hancock, 1995) but also considered as a diagnostic protein for Pseudomonas spp. (Bouffartigues et al., 2011). Our designed OprF primers could amplify both Pseudomonas-like PPB and P. aeruginosa (Figure 1). The P. aeruginosa is extensively distributed in the environment and can be both opportunistic and pathogenic microbial agent of plants, animals, and humans (Balcht & Smith, 1994). It has frequently been isolated from medical and non-medical insects (Bulla, Rhodes, & St. Julian, G., 1975; Maleki-Ravasan et al., 2015; Mitscherlich & Marth, 1984). Pseudomonas strains found in insects have been shown to protect host against toxic compounds in some cases (e.g., Ceja-Navarro et al., 2015); however, they display pathogenic characteristics in general (Vega & Kaya, 2012). The role of P. fuscipes originating Pseudomonas strains needs to be disclosed in future studies. Our designed species-specific 16S rRNA primers could identify only Pseudomonas-like PPB (Figure 2), an advantage that will be useful for the determination of PPB circulation pattern in the life cycle of Paederus beetles.

To raise the sensitivity and specificity of Wolbachia DNA amplification, we used a nested-PCR assay (Karami et al., 2016). Generally, in many specimens, PCR products of the first step were positive; however, in a few cases, the density of Wolbachia indeed was so low (as indicated by Arthofer, Riegler, Avtizs, & Stauffer, 2009) that we have to perform the second step. The use of other techniques, including high-quality polymerases, amplicon detection via DNA probes (Arthofer, Riegler, Schneider, et al., 2009) or high-throughput sequencing methods (NGS), is recommended. The frequency of Wolbachia in 128 species of beetles belonging to seven families of Buprestidae, Hydraenidae, Dytiscidae, Hydrophilidae, Gyrinidae, Haliliidae, and Noteridae showed to be 31% (Sontowski, Bernhard, Bleidorn, Schlegel, & Gerth, 2015). Oliveira et al. (2015) used three markers (16S rRNA, wsp, and ftsZ) to screen a broad range of Brazilian insect species and found Wolbachia infection in 13% (n = 25) of the studied coleopterans (Oliveira et al., 2015). Infection of P. fuscipes by Wolbachia strains was originally reported by Yun et al., (2011). They did not track the prevalence of Wolbachia infection in the rove beetles but provided evidence for indirect horizontal transmission of Wolbachia between predators and preys (Yun et al., 2011). In the present study, Wolbachia (combined) infection rate in female and male specimens was 88.23% and 67.57%, respectively (χ², p = 0.015). This difference is remarkable as the infection rates are in accordance with other studied insects including mosquitoes (Karami et al., 2016), and the fact is that no study has already been compared Wolbachia infection rates in the male and female beetles.

Herein, the phylogeny of P. fuscipes-infesting Wolbachia was not investigated; nonetheless, they were previously classified in the supergroup B, based on the 16S rRNA and wsp markers (Yun et al., 2011). MLST data are needed to determine their exact position among 16 supergroups.

Surprisingly, the coinfection rates of both PPB and Wolbachia were 70.59% in females and 17.57% in males. The frequency of both bacteria in females was four times that of males (χ², p < 0.0001). This co-occurrence may imply putative interactions among these endosymbionts.

Our results highlighted the coexistence of PPB (as defensive) and Wolbachia (as reproductive) secondary endosymbionts not only in females but also in males of P. fuscipes. These bacteria will potentially interact with the host beetle and with each other as well. As defined in defensive symbiosis, the symbionts protect their host against hostile agents, including pathogens, parasites, parasitoids, or predators by the production of diverse metabolites, antimicrobial compounds, or toxins (Flórez, Biedermann, Engl, & Kaltenpoph, 2015). Defensive compounds such as pederin, piericidin, streptochlorin, and diaphorin have been characterized from bacterial symbionts of diverse insects (Beemelmans, Gio, Rischer, & Poulsen, 2016). Although pederin can protect Paederus species from predation by natural enemies (Kellner & Dettner, 1995, 1996), its protective role against parasitoid wasps or entomopathogenic nematodes has not been inspected (Oliver & Moran, 2009). Also, the effects of Wolbachia infection on the life history of Paederus spp. are unclear. The reproductive phenotypes caused by Wolbachia in the P. fuscipes will need to be determined in the future surveys.

Given the transovarial transmission of Wolbachia as well as its relation to the reproductive phenotypes, the attention of researchers on Wolbachia infections should be drawn to the reproductive tissues. Dobson et al. (1999) have conversely demonstrated that

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**TABLE 1** Prevalence of PPB and Wolbachia infection in the Paederus fuscipes specimens collected from nine locations of two Northern provinces of Iran during 2016

| Endosymbiont beetle gender | Alone          | Wolbachia (%) | PPB-Wolbachia (%) | Combined       | PPB (%) | Wolbachia (%) |
|----------------------------|---------------|---------------|-------------------|----------------|---------|---------------|
| Male                       | PPB (%)       | Wolbachia (%) |                   | PPB (%)        | Wolbachia (%) |
| Male                       | 2 (2.7)       | 37 (50)       | 13 (17.57)        | 15 (20.27)     | 50 (67.57) |
| Female                     | 6 (11.76)     | 9 (17.65)     | 36 (70.59)        | 42 (82.35)     | 45 (88.23) |
| Total                      | 8 (6.4)       | 46 (36.8)     | 49 (39.2)         | 57 (45.6)      | 95 (76)   |
Wolbachia infections not only are distributed in germ line but also are present throughout insect somatic tissues. They have also reported that the distribution of Wolbachia in somatic tissues is varied between different Wolbachia/host associations (Dobson et al., 1999). Distribution of Wolbachia in the somatic and reproductive tissues of Paederus species needs to be determined in future.

The interaction between the PPB and Wolbachia has not been studied in any case. However, the asymmetrical interaction of Wolbachia and Spiroplasma endosymbionts had been indicated in the Drosophila melanogaster by Goto, Anbutsu, and Fukatsu (2006) who showed that Wolbachia could not affect the population of Spiroplasma, while Spiroplasma could negatively restrict the population of Wolbachia. Remarkably, they could not detect Wolbachia from the fly hemolymph, the principal location of Spiroplasma (Goto et al., 2006). Insect hemolymph is an operational area for innate immune responses where the phenol oxidase cascade factors, antimicrobial peptides, phagocytosis, and encapsulation of exotic agents are produced by hemocytes (Lavine & Strand, 2002; Naitza & Ligoxygakis, 2004; Theopold, Li, Fabbr, Scherfer, & Schmidt, 2002). In Paederus beetles, the addition of pederin toxin to the hostile environment of the hemolymph may render the condition more difficult for dwelling microorganisms, requiring further investigation.

Our results reported more frequency of both bacteria in females than that of males (χ², p < 0.0001). This observation may indicate tripartite interactions among Paederus, Wolbachia, and PPB. Recently, it has been proposed that the nature of the interaction between the insect host and Wolbachia bacterium is parasitic or mutualistic, and the induction/inhibition of reactive oxygen species would be an essential player in the new and native hosts (Zug & Hammerstein, 2015). The nature of Paederus–Wolbachia interaction is not known and requires being determined in upcoming studies. Moreover, it has previously been reported that antimicrobial peptides keep the insect’s endosymbionts under governor (Login et al., 2011). It is unclear whether the PPB regulates the population of Wolbachia via pederin or not. Hence, co-occurrence of Wolbachia and PPB in rove beetles may infer that Wolbachia is adapted to cope with adverse conditions triggered by PPB. Numerous Wolbachia strains have already been found in beetle’s eggs containing antimicrobially active components (Pankewitz, Zollmer, Hilker, & Graser, 2007). Thus, it seems that these kinds of adaptations are common features among the Wolbachia strains. As a conclusion, on the side of symbiosis, PPB and Wolbachia may interact with each other and Paederus beetles, while on the side of insect host, Paederus beetles exploit these defensive and reproductive symbionts to warrant their fitness in the environment. Details and nature of these interactions (even at gender level) call for further investigation and testing.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTION

NDD, NMR, and AAR conceived and designed the experiments. NMR and MJ collected the samples. NA performed the molecular experiments. NMR wrote the paper. NMR and AAR went through bioinformatics analyses. NMR and NDD analyzed and interpreted total data. NMR, NDD, SZ, and AAR involved in critical revision of manuscript. NDD and SZ financially supported the research. All authors read, discussed the results, and contributed to the final version of manuscript.

ETHICS STATEMENT

None required.

DATA ACCESSIBILITY

All data are included in the main manuscript. Sequences were also have deposited at the NCBI GenBank under accession number of KY568928–KY568936, KY568937–KY568941, and KY555600–KY555603.

ORCID

Naseh Maleki-Ravasan https://orcid.org/0000-0001-7209-5908
Navid Dinparast Djadid https://orcid.org/0000-0002-2277-4504

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