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Article

Sex-ratio deviation and sex-ratio distorting bacteria detected by multiplex PCR in the predatory mite *Amblyseius swirskii* (Acari: Phytoseiidae)

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ABSTRACT

The symbiotic relationship between microorganisms and arthropods is very common in nature. Some of the symbiotic microorganisms alter the normal sex-determining mechanisms in arthropods, causing sex-ratio deviation, killing males, and eventually leading to asexual reproduction and the possibility of species extinction. In the biocontrol of agricultural pests, predatory mites of the family Phytoseiidae are of great importance. Here, regarding the observance of sex-ratio deviation in *Amblyseius swirskii* (Acari: Phytoseiidae), the presence of three reproductive manipulator bacteria, i.e. *Wolbachia*, *Rickettsia*, and *Cardinium* were investigated in adult mites. Firstly, investigation of the female: male ratio in commercial mites showed that the ratio was about 42:58. Thus, there was an obvious deviation from the normal sex ratio (50:50). Therefore, to determine the presence of sex-determining bacteria, the whole DNA was extracted from *A. swirski* mites, or alternatively the adult mites were used. Using specific primers of the above-mentioned bacteria for *WSP*, *gtl*A, and *16S rDNA* genes, multiplex PCR was applied. The PCR products were sequenced, and the sequences were processed using MEGA 6 software, blasted, and checked within the NCBI gene bank. The results indicated the simultaneous presence of *Wolbachia* sp. and *Rickettsia* sp. in *A. swirskii* in all individuals tested, which meant 100% colonization. Therefore, the sex-ratio deviation in this species could be explained by the presence of sex-ratio distorting symbionts, although more studies are needed to prove this concept. Moreover, using individual adult mites directly in PCR tubes as the sources of raw DNA saved time and reduced the costs and labors in detecting the bacteria. Multiplex PCR also decreased the need for reagents, enzymes, DNA templates, etc., and saved a significant amount of time.

KEYWORDS: Endosymbiosis, maternally inherited bacteria, multiple infections, reproductive parasitism, *Rickettsia*, *Wolbachia*.

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INTRODUCTION

Molecular genetics has revolutionized the understanding of the structure, regulation, and development of arthropods (Hoy 2013). One of the biggest surprises was the discovery of the fact that the genome is very dynamic during evolution. Research has also shown that microbial symbiotic organisms play a vital role in the speciation and evolution of some mites (Hoffman 2014). The characteristics and details of the relationship between the host and the symbionts are often unknown, but with the help of molecular methods, they can be quickly discovered.

The importance of the arthropod symbionts can be seen in the fate of their hosts (Caldera *et al.* 2009). Indeed, symbiosis includes a continuum from mutualism (beneficial) to commensalism

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(neutral) or parasitism (parasitic) (Baumann 2005; Haine 2008). This can be in the form of tolerating environmental fluctuations, increasing the adaptability and fitness of the host, and maintaining or improving the host's immune system (Weiss and Aksoy 2011).

Phytoseiid predator mites are one of the biological control agents for some plant pests. They are the main natural enemies of spider mites of the family Tetranychidae and some other herbivorous mites. They also kill some small insects such as whiteflies, thrips and weevils. Thus, these predators are used commercially for pest control. Mites of the family Phytoseiidae have assumed a very effective role in establishing ecological balance in natural environments as an effective factor in the mass control of some plant pests. *Amblyseius swirskii* is one of the most important and famous predatory mites of the family Phytoseiidae. It can be seen on various crops such as apples, apricots, grapes, citrus fruits, vegetables, and cotton. This mite is omnivorous and can control pests such as thrips, whiteflies, eriophyid mites, and other greenhouse vegetable pests. Hence, *A. swirskii* has been used as a biological control agent (Arthurs *et al*. 2009; Kade *et al*. 2011; EPPO 2013; Cedola and Polack 2017).

Symbiotic microorganisms have fascinated scientists due to their wide distribution and their effects on the reproduction of their hosts. It seems that they are one of the effective forces in evolution and speciation, which are influential in determining the gender of arthropods with different mechanisms (Moran *et al*. 2008). Indeed, cytoplasmic sex ratio distorters are able to manipulate the sex ratio of their host and increase their self-emission (Werren *et al*. 2008; Engelstadter and Hurst 2009). They are often shown to decrease the compatibility of hosts and can cause their populations to die out. In infected populations, such bacteria can alter the sex ratio in favor of females, which theoretically allocates more resources to the female sex and increases bacterial transmission. In most symbioses, *Wolbachia* interferes with the reproduction of the host by providing a suitable ground for its reproduction and distribution within the host population (Stouthamer *et al*. 1999).

Multiplex PCR is a thrifty method that saves a significant amount of time, while decreasing the need for reagents, enzymes, DNA templates, etc. In this research, first, the sex ratio deviation in the predatory mite *A. swirskii* (Acari: Phytoseiidae) was investigated. Second, the presences of three sexual symbiotic bacteria, i.e. *Wolbachia*, *Rickettsia*, and *Cardinium*, in *A. swirskii* were explored using multiplex PCR with bacterial-specific primers, and sanger sequencing.

MATERIALS AND METHODS

Mite rearing and sampling to investigate the sex ratio

Amblyseius swirskii was reared at the Acarology Laboratory, Plant Protection Department of Bu-Ali Sina University, Hamedan, Iran, according to Swirski *et al*. (1967). Random samplings were performed at three times, and 50, 60 and 90 numbers of single mites were collected. The sample mites were first placed at −4 °C for 15 minutes and then female and male mites were counted. Mites were separated under a stereomicroscope (Olympus, Japan) with 10× magnification. Male mite bodies were smaller than females, and the end parts of female's bodies were wider than males. To count and separate mites, a three-zero brush, which is very thin and was moistened with 90% alcohol, was used to get mites more easily.

Molecular identification of the target bacteria

Sampling the mites

A total number of 60 single mite samples from males and females was collected randomly and transferred to PCR tubes separately.

DNA samples and primers

Three genera of sexual symbiotic bacteria, i.e. *Wolbachia*, *Rickettsia*, and *Cardinium* were investigated in *A. swirskii*. Mite's DNAs were extracted using CTAB protocol (Masoomi-Aladizgeh *et al*. 2016), or alternatively, single sterilized adult mites as the raw material was used for PCR. For investigating the presence/absence and the sequencing of *Wolbachia WSP*, *Rickettsia gtl*A, and *Cardinium 16S rDNA* genes, specific primers were used in the multiplex PCR assay (Table 1) (Zélé *et al*. 2018).

Table 1. Specific primers used in this study for the detection of the target bacteria in *A. swirskii* (Zélé *et al*. 2018).

Target gene	Primer name	Sequence (5' - 3')	Concentration (µM)	Product size (bp)
Wolbachia	WSPTG-F	GTTGGTGTTGGTGCAGCGTATGTAAGC	2	222
WSP	WSPTG-R	AGTGCTGTAAAGAACTTTGATTCCGCCATC	2	
Rickettsia	RICTG –F	AGGCTAATGGGCTTTGGTCATCGTGTAT	2	293
gtlA	RICTG R	TGTGCCATCCAGCCTACTGTTCTTGC	2	
Cardinium	CARDTG-F	GGCGGCTTATTAAGTCAGTTGTGAAATCCT	3	152
16S rDNA	CARDTG-R	GCTGCCTACGCTATTGGTATTCCTTATGAT	3	

Multiplex PCR, DNA sequencing, and analyses

Multiplex PCR technique was applied simultaneously to identify *Wsp*, *gltA*, and *16srDNA* genes belonging to *Wolbachia*, *Rickettsia*, and *Cardinium* bacteria, respectively (Zélé *et al*. 2018). In a total volume of 30 µL, 15 µL of Master Mix, 6 µL of specific primers (1 µL of each), 1 µL of DNA (or alternatively a single mite), and 8 µL of autoclaved double distilled water were added. Thermocycler (Techne TC-512, UK) was programmed for one cycle of 15 min. at 95 °C, followed by 35 cycles of 30 sec at 94 °C, 1 min. at 58 °C, 1 min. at 72 °C, and a final extension at 72 °C for 10 min.

The PCR products were sequenced using the same primers (Biomagic Gene Co., Iran), and the gene sequences were processed using MEGA 6 software and blasted (nucleotide blast) and checked in the NCBI gene bank. The most similar sequences with the lowest E-values were selected and analyzed.

RESULTS

Female-male ratio in A. swirskii

The ratio of 58.7:41.3 female:male was observed, instead of a normal sex ratio of 50:50 female: male (Table 2) . Therefore, there was an obvious deviation in the sex ratio of *A. swirskii* adult mites.

Table 2. Sax ratio in adult mites of *A. swirskii* in this research.

Random sampling stages	Total number of samples	Female numbers (%)	Male numbers (%)
1st	50	30 (60%)	20 (40%)
2nd	60	35 (58.3%)	25 (41.7%)
3rd	90	52 (57.8%)	38 (42.2%)
Average	200	117 (58.7%)	83 (41.3%)

Multiplex PCR results

Adult mites were investigated for their reproductive parasites using the specific primers and multiplex PCR assays. The results showed that all samples produced the expected bands of the target genes in *Wolbachia* (222 bp) and *Rickettsia* (293 bp) (Fig. 1). However, no expected bands for the *Cardinium* were amplified.

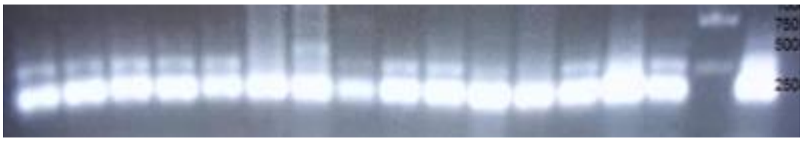

Figure 1. Gel electrophoresis of multiplex PCR products from individual mites, obtained from the target genes of the target sex-ratio distorting bacteria. The lower bands (222 bp) represent *Wolbachia*, the upper bands (293 bp) represent *Ricketssia*.

Sequence analyses

According to the data, the identities of two symbiotic bacteria were detected as *Wolbachia* sp. (96.9% identity) and *Rickettsia bellii* (100% identity), as the endosymbionts of *A. swirskii*.

DISCUSSION

Plant-associated arthropods usually host a number of endosymbiont cytoplasmic bacterial microorganisms which are vertically inherited from mother to offspring via the cytoplasm of the eggs (Moran *et al*. 2008). Research over the last decade has revealed the dominance of *Wolbachia*, *Rickettsia*, *Cardinium*, *Arsenophonus*, and *Spiroplasma* bacteria in arthropod species (Duron *et al*. 2008; Weinert *et al*. 2015). The most dominant bacterial genus in most groups is *Wolbachia*, after which *Rickettsia* and *Cardinium* are placed (Weeks *et al*. 2003; Zchori-Fein and Perlman 2004; Weinert *et al*. 2015). Moreover, these three bacterial genera are widespread in chelicerates, especially in mites, ticks, and spiders (Duron *et al*. 2008, 2017; Weinert *et al*. 2015; Zhang *et al*. 2016). The maternal inheritance of such sexual bacteria induces several phenotypic changes in their hosts, such as cytoplasmic incompatibility (CI, i.e., embryonic lethality where infected males mate uninfected females), feminization (i.e., the development of genetic males into females), male killing (i.e., male specific death during embryogenesis or late larval instars), or thelytokous parthenogenesis (i.e., the production of diploid daughters from unfertilized eggs) which manipulate the hosts' reproduction and allow the spread of the bacteria within host populations (Werren *et al*. 2008; Engelstadter and Hurst 2009).

Studies in herbivorous spider mites, e.g. the members of Tetranychidae, have shown that they are mostly infected with *Wolbachia* and *Cardinium* (Gotoh *et al*. 2003, 2007a, b; Liu *et al*. 2006; Xie *et al*. 2011; Ros *et al*. 2012; Suh *et al*. 2015; Zhang *et al*. 2016) and sometimes with *Spiroplasma* (Zhang *et al*. 2016; Staudacher *et al*. 2017) and *Rickettsia* (Hoy and Jeyaprakash 2005; Zhang *et al*. 2016). It is reported that *Wolbachia* and *Cardinium* may cause cytoplasmic incompatibility in spider mites (Gotoh *et al*. 2003, 2007a, b; Ros and Breeuwer 2009; Xie *et al*. 2011; Zhu *et al*. 2012; Suh *et al*. 2015; Xie *et al*. 2016). Moreover, these bacteria could be found simultaneously in the same individual host (Ros *et al*. 2012; Zhu *et al*. 2012; Zhao *et al*. 2013a, b; Xie *et al*. 2016). Furthermore, incidence of *Wolbachia*, *Cardinium* and *Spiroplasma* in predatory phytoseiid mites are reported. For example, *Cardinium* infection in *Euseius finlandicus* (Enigl and Schausberger 2007), and *Wolbachia*, *Cardinium*, and *Spiroplasma* in *Neoseiulus californicus* (Famah Sourassou *et al*. 2014) are seen.

In this study, we showed that the predatory mite *A. swirskii* had a significant deviation in its sex-ratio, which favored feminization. This was in accordance with sex ratio deviations in the mite populations that occurred by their bacterial endosymbionts (Werren *et al*. 2008; Engelstadter and Hurst 2009). This led us to investigate the dominant reproductive manipulator bacteria in *A. swirskii* population. Using specific primers for detecting *Wolbachia*, *Rickettsia*, and *Cardinium* by a novel multiplex PCR protocol, we showed for the first time the simultaneous presence of *Wolbachia* and *Rickettsia* endosymbionts in all tested individuals, which meant a 100% colonization. However, *Cardinum* was not detected in our assays. Since endosymbiont-induced incompatibility can be treated by some antibiotics, these findings might be of high value in mass production of the mite, and thereby in biocontrol programs, which need further investigations.

Moreover, in this research, besides using the CTAB protocol to extract DNA, which was time-consuming, laboring, and costly, we applied the individual adult mites directly in PCR tubes as the sources of raw DNA. This was very successful in detecting the endosymbionts in the shortest time, as well as cutting the costs, and labor. Moreover, our results indicate that contamination from proteins, polysacharides, etc., didn't interfere with the PCR. However, since the single mite used in the PCR tube couldn't be retrieved, the procedure was not reproducible. Furthermore, multiplex PCR decreased the need for reagents, enzymes, DNA templates, etc., and saved a significant amount of time. So, we suggest that the primers and the multiplex PCR protocol developed by Zélé *et al*. (2018) is an efficient and reliable protocol for detecting the simultaneous presence of bacterial endosymbionts in *A. swirskii*.

In conclusion, the assumptions of this research were the disruption of sex ratios and the possibility of the presence of sexual bacteria *Wolbachia*, *Cardinium*, and *Rickettsia* in the predatory mite *A. swirskii*, which was approved. Therefore, endosymbiotic bacteria can be considered one of the disturbing factors of sex ratios in *A. swirskii*. A metagenomic approach might discover the plethora of such bacteria in *A. swirskii*. Further, genetic crosses between males and females can determine the cellular and molecular mechanisms involved in disrupting the sexual ratio.

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بررسی انحراف نسبت جنسی و باکتری‌های برهم‌زننده نسبت جنسی در کنهٔ شکارگر *Amblyseius swirskii* (Acari: Phytoseiidae) با استفاده از روش Multiplex PCR

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چکیده

رابطه همزیستی میکروارگانیسم‌ها و بندپایان در طبیعت بسیار رایج است. برخی از میکروارگانیسم‌های همزیست بندپایان سازوکارهای تعیین جنسیت را تغییر داده و باعث انحراف نسبت جنسی، کشتن نرها و در نهایت تولیدمثل غیر جنسی و احتمال انقراض گونه‌ها می‌شوند. در مهار زیستی آفات کشاورزی، کنه‌های شکارگر خانوادهٔ Phytoseiidae از اهمیت زیادی برخوردارند. در این پژوهش، با توجه به مشاهده انحراف نسبت جنسی در *Amblyseius swirskii* (Acari: Phytoseiidae) حضور سه جنس باکتری درون‌زیست مؤثر در تعیین جنسیت، یعنی *Cardinium*، *Rickettsia* و *Wolbachia* در کنه‌های بالغ تجاری بررسی شد. در مرحله نخست، بررسی نسبت نر و ماده در کنه‌ها نشان داد که این نسبت در حدود ٥٨:٤٢ (حدود ٢: ٣) است. بنابراین، از نسبت جنسی طبیعی (٥٠: ٥٠) انحراف معنی‌داری وجود داشت. بر این اساس، برای بررسی وجود باکتری‌های نامبرده، کل DNA از کنه‌های *A. swirskii* استخراج شد و با استفاده از پرایمرهای اختصاصی برای توالی‌های ژن‌های ١٦S rDNA، *gtl*A و *WSP* از روش Multiplex PCR استفاده شد. افزون بر این، کنه‌های سالم بالغ نیز به عنوان مادهٔ اولیه PCR استفاده شدند. محصولات PCR توالی‌یابی شده و توالی‌ها با استفاده از نرم افزار مگا-٦ پردازش و در بانک ژن NCBI بلاست و شناسایی شدند. نتایج، حاکی از حضور صد در صدی *Wolbachia* و *Rickettsia* به عنوان باکتری‌های درون‌زیست جنسی در تمام کنه‌های مورد بررسی *A. swirskii* بود. بنابراین، انحراف نسبت جنسی در این کنه احتمالا می‌تواند به حضور باکتری‌های جنسی مذکور نسبت داده شود که این مورد نیاز به بررسی‌های بیشتری دارد. همچنین، استفاده از کنه‌های سالم بالغ به عنوان منبع DNA در انجام PCR، هزینه، زمان و زحمات را کاهش داد. روش Multiplex PCR هم نیاز به بافرها، آنزیم‌ها، DNA هدف، و مواد دیگر را کاهش داده و باعث صرفه جویی در وقت و هزینه‌ها شد.

واژگان کلیدی : باکتری درون‌زیست، باکتری‌های منتقل شونده مادری، آلودگی‌های چندگانه، پارازیتیسم جنسی، *Rickettsia*، *Wolbachia*.

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