



Endosymbionts and Phage WO Infections in Korean ant Species (Hymenoptera: Formicidae)

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ABSTRACT

Bacterial symbionts are common across insects, including ants (Hymenoptera: Formicidae). Reproduction-manipulating endosymbionts, such as *Wolbachia*, *Spiroplasma*, *Rickettsia*, and *Cardinium*, are closely associated with many aspects of host-insect life. In addition, phage WO plays an essential role in the phenotypic effects of *Wolbachia*. Although endosymbionts are possible biological control agents, there is a lack of knowledge of their rate of infection of ants in Korea. We tested a range of Korean ant species for the presence of *Wolbachia*, *Spiroplasma*, *Rickettsia*, *Cardinium*, and phage WO by extracting DNA from the ants and using specific primer sets to test the status of infections. In addition, the mitochondrial cytochrome c oxidase I (*COI*) gene of the host ants was amplified to confirm the molecular identification and phylogenetic relationship between the hosts. We found that infection with *Wolbachia* (29.6% of species) is relatively common when compared with that of other endosymbionts. Only one species was infected with *Spiroplasma*. Infection with *Rickettsia* and *Cardinium* was not detected in the examined ants. Most *Wolbachia* in ants were infected with phage WO. Although the phenotypic effects of endosymbionts in ants are still unknown, this first survey of endosymbionts in Korea is the first step toward the use of reproduction-manipulating endosymbionts.

Keywords: Ants, Bacteriophages, Infections, Symbiosis, *Wolbachia*

Introduction

Symbiotic bacteria interact with their host and affect the life of their host (Duron *et al.*, 2008). In particular, vertically transmitted endosymbionts coevolve with their host over a long evolutionary period. *Wolbachia*, which is the most prevalent endosymbiont, is an alpha-proteobacteria found in arthropods and nematodes, such as insects, isopods, mites, and spiders (Stouthamer *et al.*, 1999;

Werren *et al.*, 2008). *Wolbachia* infection rates have been inferred as 66% in insects (Hilgenboecker *et al.*, 2008), and the high rates of spread are thought to be the result of four effective phenotypic consequences that disturb the host's sex ratio: cytoplasmic incompatibility, feminization, male-killing, and parthenogenesis (Stouthamer *et al.*, 1999; Werren *et al.*, 2008). Three other genera, *Spiroplasma*, *Cardinium*, and *Rickettsia*, which are not as prevalent as *Wolbachia*, are also endosymbionts that disrupt the sex ratio of the host and have been found in various arthropods groups (Duron *et al.*, 2008). The *Wolbachia*-infecting phage WO was first detected by Masui *et al.* (2000) and was found in ~89% of *Wolbachia* (Bordenstein & Wernegreen 2004). In particular, genes derived from phage WO improve the phenotypic effect of cytoplasmic

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incompatibility (LePage *et al.*, 2017).

Ants are one of the most successful animals in terms of species richness and abundance, and they are distributed from arctic to tropical areas, including other extreme habitats, such as deserts (Hölldobler & Wilson, 1990). Recent studies have shown that ants have a variety of symbiont bacteria, and some of these play an important role in nutrition (Feldhaar *et al.*, 2007; Russell *et al.*, 2009; Stoll *et al.*, 2007). In addition to ants having these ecologically relevant microorganisms, they also have endosymbionts that alternate host sex ratios and provide an opportunity to study this mechanism. Because ant workers are functionally sterile, they are a dead end in terms of endosymbionts, such as *Wolbachia*. Nevertheless, *Wolbachia* infection is also found in ant workers (Russell, 2012). Consequently, studies on the interaction between *Wolbachia* and ants have been conducted, but there is minimal information on endosymbionts, including *Wolbachia* and ant species in Korea.

Biological control using reproduction-manipulating endosymbionts has emerged as a self-sustaining mechanism to reduce damage from invasive species and pests that are spreading as a result of climate change and trade. In many countries, the invasive species *Solenopsis invicta* is damaging crops and local diversity (Morrison *et al.*, 2004; Wojcik *et al.*, 2001). In Korea, *S. invicta* was first reported in 2017 (Lyu & Lee, 2017), and under conditions of future climate change, this species may reproduce and spread more successfully (Sung *et al.*, 2018). *Solenopsis invicta* harbors *Wolbachia*, and the evolutionary history of these two species has been reported to be very complex. Studies related to endosymbionts, including *Wolbachia*, are needed in ant species inhabiting Korea to inform the utilization of endosymbionts. Therefore, this study inves-

tigated the infection of four endosymbionts, *Wolbachia*, *Spiroplasma*, *Cardinium*, and *Rickettsia*, and phage WO in 27 species of ants in Korea and deduced the maternal lineage of the infected host.

Materials and Methods

Sample collection and DNA extraction

Seventy-five individuals representing 27 ant species were collected between 2014 and 2015 in Korea. Samples were stored in 100% ethanol at -20°C before genome DNA extraction. Genomic DNA was extracted using Blood and Tissue DNeasy kits (Qiagen, Hilden, Germany) following the manufacturer's instructions and stored at -20°C .

Polymerase chain reaction and phylogenetic and statistical analyses

The mitochondrial cytochrome c oxidase I (*COI*) gene of the host ants was amplified to confirm the molecular identification and phylogenetic relationship between the hosts. In addition, diagnostic polymerase chain reaction (PCR) was conducted using the different endosymbionts, *Wolbachia*, *Spiroplasma*, *Cardinium*, and *Rickettsia*, and phage WO specific primer sets to test the status of infections (Table 1, Kageyama *et al.*, 2006; Masui *et al.*, 2000; Werren & Windsor, 2000). The PCR protocol involved an initial denaturation at 94°C for 3 minutes followed by 35 cycles: 1 minute at 94°C , 1 minute at each annealing temperature (Table 1), 1 minute at 72°C , and a final extension at 72°C for 5 minutes. Maxime PCR PreMix Kits (iNtRON Biotechnology, Seongnam, Korea) were used for amplification with 16 μL of distilled water, 1 μL of each primer (10 pmol) and 2 μL of template DNA. The PCR products were visualized using a 1% agarose gel dyed

Table 1. Endosymbionts and phage-specific screening primers used in this study

Target	Primer	Sequence (5'-3')	Annealing temperature
<i>COI</i>	LCO1490	GGTCAACAAATCATAAAGATATTGG	48°C
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	
<i>Wolbachia</i>	Wol specF	CATACCTATTCTGAAGGGATAG	55°C
	Wol specR	AGATTCGAGTGAAACCAATTC	
<i>Spiroplasma</i>	SpoulF	GCTTAACTCCAGTTCGCC	60°C
	SpoulR	CCTGTCTCAATGTAAACCTC	
<i>Cardinium</i>	Ch-F	TACTGTAAGAATAAGCACCGGC	60°C
	Ch-R	GTGGATCACTTAACGCTTTTCG	
<i>Rickettsia</i>	Rb-F	GCTCAGAACGAACGCTATC	60°C
	Rb-R	GAAGGAAAGCATCTCTGC	
WO phage	WOorf7f	CCCACATGAGCCAATGACGTCTG	57°C 30 s, 65°C 1 min
	WOorf7r	CGTTCGCTCTGCAAGTACTCCATTAATAAC	

with TopGreen Nucleic Acid Gel Stain to confirm the infection of endosymbionts and phage WO (Genomic Base, Seoul, Korea).

Sequences were aligned and analyzed using Clustal W in MEGA ver. 7 (Kumar *et al.*, 2016). The sequences were submitted to the GenBank database under the following accession numbers: MT800204–MT800278. Phylogenetic relationships for host ants of the *COI* gene (611 bp) were inferred using the maximum likelihood (ML) method after selecting the substitution model in MEGA ver. 7. The selected model for the ML method was the general time-reversible model (GTR+G+1; Nei & Kumar, 2000). Phylogenetic tree support was evaluated using bootstrapping with 1,000 replications.

Results and Discussion

The infection status of four endosymbionts and phages WO was investigated in 27 ants ($n=75$ individuals) living in Korea. Species were considered to be infected with endosymbionts when at least one individual of the species was infected. The study found that eight species were infected with *Wolbachia* (29.6%), while only one species was infected with *Spiroplasma* (3.7%). *Rickettsia* and *Cardinium* infections were not detected (Table 2).

Previous studies on *Wolbachia* infection in Formicidae show infection rates of 34.1% (Russell, 2012) and 45.6% (Treanor & Hughes, 2019). In addition, according to Kautz

et al. (2013), *Spiroplasma* showed a higher infection rate than *Wolbachia* (28.4%) across the ants studied, which is contrary to the results of this study in which *Spiroplasma* infection was very rare. These different results are presumed to be because the ant species surveyed in this and the previous study are different, and the habitats of the surveyed species are different, even when the same species was investigated. This suggestion is in agreement with a previous study that shows that there can be a high variation in infection rate between different genera (Russell, 2012). In addition, Martins *et al.* (2012) confirmed that the infection rates of *Solenopsis spp.* populations in different regions are different. Therefore, the infection rate of ants inhabiting Korea cannot be inferred from overseas cases, and investigations are required on ants inhabiting Korea.

There are cases in which the host has been co-infected with different endosymbionts (Goodacre *et al.*, 2006), or *Spiroplasma* and *Wolbachia* infections have been associated (Jaenike *et al.*, 2010). However, unlike these cases, our study did not confirm an association between endosymbionts or infections of different endosymbionts. In addition, *Cardinium* infection was commonly found in spiders (Martin & Goodacre, 2009), but no infection was found in ants living in Korea, even though infection in *Formica* has been confirmed previously (Sirviö & Pamilo, 2010). Our results do not apply to all ant species inhabiting Korea, so there are limitations to our analysis.

Table 2. *Wolbachia*, *Spiroplasma*, *Cardinium*, *Rickettsia*, and phage WO identified using polymerase chain reaction in a range of ants

Subfamily	Genus	No. of species	Total no. of individuals	No. of infected species (no. of individuals)				
				<i>Wolbahica</i>	<i>Spiroplasma</i>	<i>Cardinium</i>	<i>Rickettsia</i>	WO phage
Dolichoderinae	<i>Dolichoderus</i>	1	1	0	0	0	0	
Formicinae	<i>Camponotus</i>	4	13	1 (1)	0	0	0	1 (1)
	<i>Formica</i>	5	17	4 (16)	0	0	0	4 (16)
	<i>Lasius</i>	4	8	0	0	0	0	
	<i>Paratrechina</i>	2	9	1 (3)	0	0	0	0
	<i>Polyrhachis</i>	1	2	0	0	0	0	
Myrmicinae	<i>Aphaenogaster</i>	1	2	0	0	0	0	
	<i>Crematogaster</i>	1	1	0	0	0	0	
	<i>Myrmica</i>	1	1	0	1 (1)	0	0	
	<i>Pheidole</i>	1	5	1 (5)	0	0	0	1 (4)
	<i>Pristomyrmex</i>	1	7	0	0	0	0	
	<i>Strumigenys</i>	1	1	0	0	0	0	
	<i>Tetramorium</i>	1	3	0	0	0	0	
	<i>Vollenhovia</i>	1	1	1 (1)	0	0	0	0
	Ponerinae	<i>Cryptopone</i>	1	1	0	0	0	0
<i>Pachycondyla</i>		1	3	0	0	0	0	

As a result of inferring the phylogenetic relationship to the mitochondrial gene (*COI*) of the host, there is no correlation between the endosymbiont infection and the phylogenetic relationship (Fig. 1). This is because a species that is not infected (*Formica sanguinea*) has been identified within the monophyletic genera (Fig. 1). In addition, *Wolbachia* infection rates differed between genera. All of the collected *Formica japonica*, *Pheidole fervida*, *Formica yessensis*, and *Paratrechina sakurae* individuals were infected with *Wolbachia* (Fig. 1). However, only one individual harbored *Wolbachia* in *Camponotus japonicus*. The infected *C. japonicus* individual was collected in a different region to the uninfected individuals. Therefore, this species is likely to show substantial variation in infec-

tion rates from region to region, which is in agreement with the proposal of different infection rates for different regions in ants (Martins *et al.*, 2012). In addition, infection loss and frequent horizontal transmission have been proposed (Frost *et al.*, 2010).

There is little known on the status of phage WO infection in Formicidae. According to a study of fig wasps in Hymenoptera to which Formicidae belongs, the phage WO infection rate of *Wolbachia*-infected species was 47.4% (9/19) (Wang *et al.*, 2016). Although belonging to the same order, this is different from the case of ants, which account for 75% of species infected with phage WO in *Wolbachia*. However, the results for ants showed infection rates similar to those estimated by Bordenstein

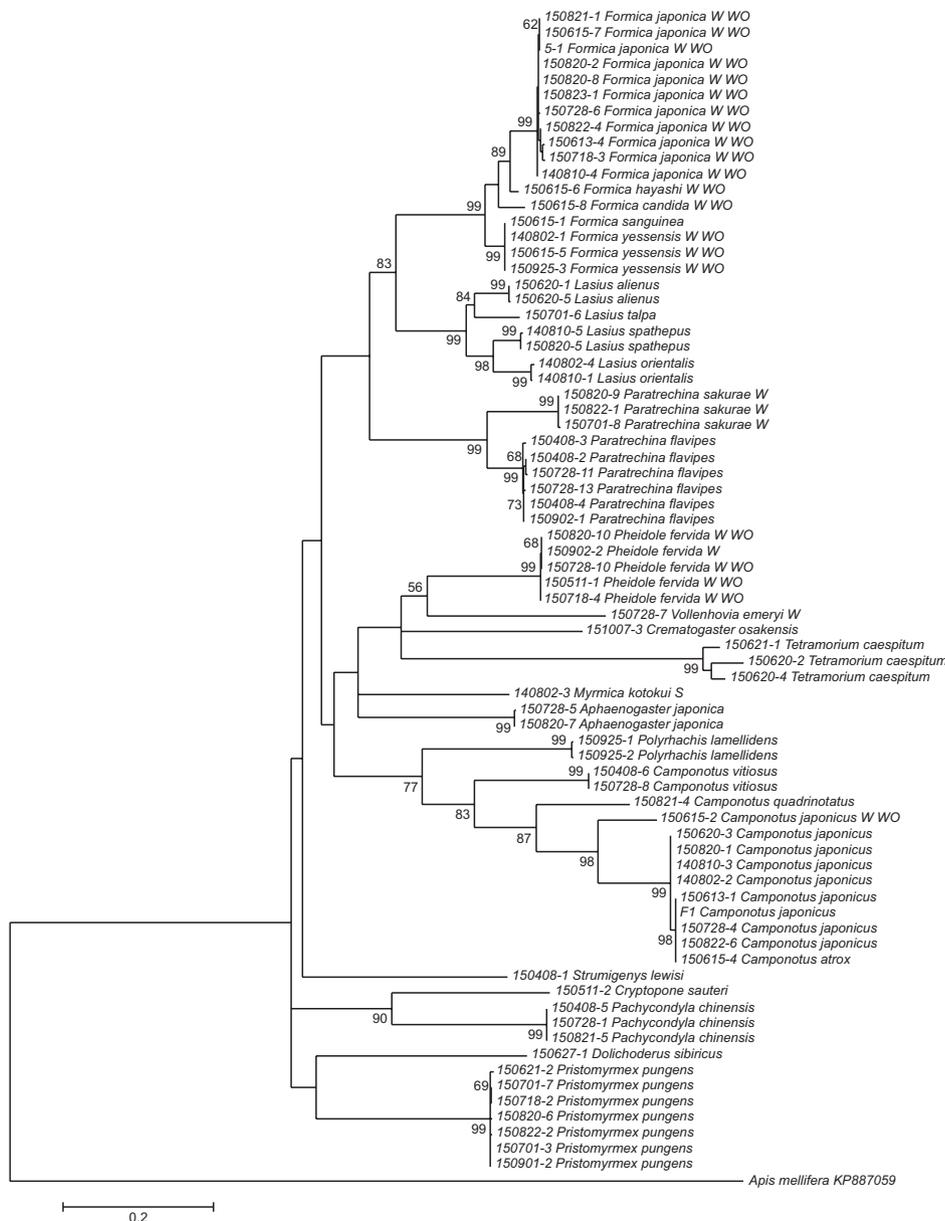


Fig. 1. Maximum likelihood phylogenetic tree based on the *COI* gene from ants in Korea. *Wolbachia*, *Spiroplasma*, and phage WO are indicated by W, S, and WO, respectively. The outgroup (*Apis mellifera*) sequence was obtained from the GenBank database. The GenBank accession number is shown after the species name. Numbers above branches refer to the bootstrap support (Bootstrap value=1,000).

and Wernegreen (2004). Considering the close relationship between phage WO and *Wolbachia*, this may be due to different *Wolbachia* species depending on the host, but more research on phage WO is required to clarify the differences in our results and those of previous studies.

As only one individual was examined in one species, a sampling bias may have occurred due to the small sample size. Therefore, although it is difficult to confirm that some of the species investigated in this study are endosymbiont-free, this result is meaningful as the first survey data for ants living in Korea. In addition, as this study contains ant species that have not been investigated in previous studies, it is a significant starting point for the use of reproduction-manipulating endosymbionts for applications, such as biological control. Based on this study, further studies considering ecological characteristics, such as habitat sharing with other species and colony founding methods, will be useful to provide fundamental data for studying interactions between ants and endosymbionts.

Conflict of Interest

The authors declare that they have no competing interests.

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