Evolutionary history of a global invasive ant, *Paratrechina longicornis*

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Chapter 1: General introduction

Biological invasions are one of the major threats to biodiversity and economy. The longhorn crazy ant, *Paratrechina longicornis* (Hymenoptera: Formicidae; Latreille, 1802), has been considered as a successful invasive species, as evident by its presence in most parts of the world. The longhorn crazy ant is highly adapted to disturbed and artificial environments, polygynous, and the mating can occur within the nest without a mating flight. This species is one of few ant species that can be cultured for multi-generations in the laboratory. All these characteristics make this ant an excellent model system to test and answer invasive biology-related questions. In this thesis, I focus on the invasion history and biology of longhorn crazy ant and its symbionts, to address questions through the lens of bioinvasion and evolutionary genetics.

1.1 Invasion history of *Paratrechina longicornis* is largely unknown

One of successful management strategies to mitigate the negative impacts of invasive species relies on reconstructing the invasion history, which traces patterns of ongoing invasion pathways and accordingly prioritizes quarantine resources to those of high invasion risk. Although *P. longicornis* has been found worldwide for more than a century, the origin and invasion history of this species remain controversial. By comparing the location and historical records of *P. longicornis*, scientists attempted to deduce the geographic origins of this ant and never reached a consensus. Wasmann (1905) concluded that *P. longicornis* probably originated in the Indian region because *P. longicornis* only found from coastal areas along major trade routes in other areas (Wasmann, 1905). In contrast, some other research groups regarded that *P. longicornis* originated in Southeast Asian or African (Wetterer, 2008; LaPolla et al., 2010; LaPolla et al., 2013; LaPolla and Fisher, 2014). Wetterer (2008) analyzed a large number of recent and historical records, and the origin of this ant remains ambiguous and highly

debatable. However, the limitation of traditional methods can be overcome by adding DNA-based molecular tools as numerous studies have applied genetic tools to identify source populations and potential invasive pathways of alien species (Corin et al., 2007; Ugelvig et al., 2008; Valade et al., 2009; Ascunce et al., 2011; Yang et al., 2012). To date, the global genetic structure of *P. longicornis* has not yet been extensively studied, partially because of the limited number of genetic markers currently available. Therefore, in Chapter 2, I developed 36 polymorphic microsatellite markers as a practical tool to assess the population genetics of *P. longicornis*. In Chapter 3 and 5, I used mtDNA and the new microsatellite markers to assess the global genetic structure of *P. longicornis*.

1.2 *Wolbachia* infection in *Paratrechina longicornis*

Wolbachia are probably the most successful symbionts of arthropods worldwide (Jeyaprakash and Hoy, 2000; Werren and Windsor, 2000). One of the possible explanations of this success can be attributed to manipulation of host reproductive system that enhances the spread of infections across generations. *Wolbachia* spread within host species by increasing the relative fitness of infected cytoplasmic lineages, either by conferring direct fitness benefits (Vavre et al., 1999) or by manipulating host reproduction via mechanisms such as cytoplasmic incompatibility (CI), male-killing, feminization of genetic males or thelytoky parthenogenesis (Werren et al., 2008; Saridaki and Bourtzis, 2010)

The Formicidae family exhibited a high *Wolbachia* infection rate of 34.1%, ranking 22nd out of 64 families (Russell, 2012). Although *Wolbachia* infection is a ubiquitous phenomenon across the ants, several core issues remain unclear for most ant species (Russell, 2012). For examples, we know little about the phenotypic effect of *Wolbachia* on ants the natural routes of horizontal transfer for the ants (Russell, 2012).

The paucity of relevant studies may result from the difficulty of rearing, breeding and maintaining stable ant colony in the laboratory conditions. The unique mating system of *P. longicornis*, however, makes it possible and feasible. *Paratrechina longicornis* virgin queens could directly mate with males within the nest without mating flight, which provides us a rare opportunity to manipulate the reproduction of *P. longicornis*, and test several hypotheses regarding *Wolbachia* infection in the ants. In Chapter 3, I characterized two types of *Wolbachia* strains, *w*LonA and *w*LonF, by multilocus sequence typing (MLST) in *P. longicornis*. The evolutionary histories of these two strains differ; *w*LonA appears to be primarily transmitted maternally, and patterns of mtDNA and nDNA variation and *w*LonA infection status are consistent with a relatively recent *Wolbachia*-induced selective sweep. On the other hand, the history of *w*LonF infections in *P. longicornis* appears to be characterized by frequent gains and losses over time. In Chapter 4, I attempted to elucidate sources of *w*LonF by surveying *Wolbachia* infections in various ant guests. I found *P. longicornis* and a specialist ant cricket *Myrmecophilus americanus* shared an identical *Wolbachia* strain (*w*LonF = *w*Mame1), impling the occurrence of *Wolbachia* horizontal transmission most likely through intimate ecological associations.

1.3 Unique reproduction mode in *Paratrechina longicornis*

Pearcy et al., (2011) demonstrated the occurrence of an extraordinary, double-clonal reproduction system in a population of *P. longicornis* from Thailand. In this population, queens are produced clonally from their mothers, males are produced clonally from their fathers, and workers are produced sexually (Fig. 1.1). Under this double-clonal system, workers are offspring of queen and male lineages that are genetically divergent from each other and are hence characterized by an excess of heterozygosity.

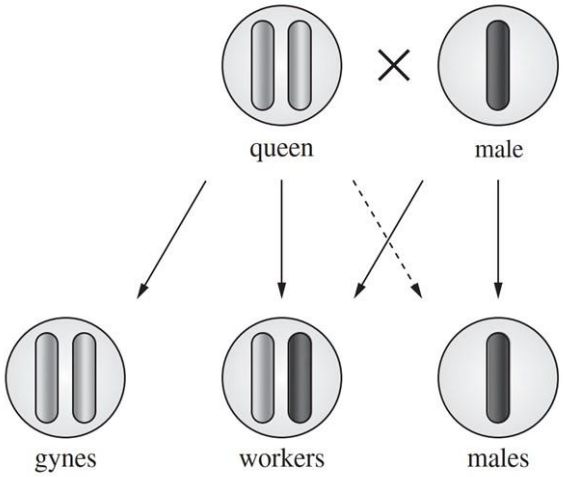

Figure 1.1 Clonal reproduction in queens and males of *P. longicornis*. This figure was adopted from Pearcy et al. (2011). Maternal (light) and paternal (dark) chromosomes are displayed. Contribution to the genome of the offspring is indicated by arrows (dashed arrow represents the mother laying haploid eggs with no actual contribution to the genome)

This clonal reproductive system has been reported in three other ant species, namely *Wasmannia auropunctata* (Fournier et al., 2005)*, Vollenhovia emeryi* (Ohkawara et al., 2006; Kobayashi et al., 2008) and *Cardiocondyla kagutsuchi* (Okita and Tsuchida, 2015), and these three species are either tramp or widespread exotic. Like other double-clonal ants, *P. longicornis* are born to be a successful invader as such reproduction system may contribute to avoid the risk of inbreeding associated with the early stage of colonization, high heterozygosity is maintained in the workers, and clonal queens can mate with their clonal brothers without any negative fitness consequence (Okamoto and Ohkawara, 2010a; Pearcy et al., 2011). In Chapter 5, I used the novel microsatellite markers to assess generality of this reproductive mode across the populations worldwide and the genetic lineage composition of male and queen founders of *P. longicornis*.

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Chapter 2 Novel microsatellite markers for the *Paratrechina longicornis*: a new set of molecular tool for inferring the invasion history of this globally distributed invasive ant

2.1 Introduction

Biological invasions are a major threat to biodiversity and economic activity (Schmitz and Simberloff, 1997; Pimentel et al., 2000; Occhipinti-Ambrogi and Savini, 2003). While a considerable amount of effort has been devoted to prevent, control, and eradicate invasive species worldwide, management strategies designed to mitigate their negative impacts partially rely on reconstructions of invasion routes; these enable the immediate source to be identified and thus facilitate the design of strategies for controlling (e.g., biocontrol agent) or preventing invasions (Hulme, 2009; Wilson et al., 2009; Estoup and Guillemaud, 2010). Reconstructing routes of invasion based on historical observation data is challenging since the data are often sparse and incomplete (Estoup and Guillemaud, 2010); however, this limitation can now be overcome by implementing DNA-based molecular tools (e.g., molecular markers) that give rise to much higher resolution when inferring potential invasive pathways. Molecular markers such as microsatellite markers and mitochondrial DNA (mtDNA) sequences have been widely applied to identify migration pathways, quantify gene flow among populations across spatial scales, estimate admixture between populations from different origins, and can be further used to reconstruct the invasive routs of an alien species (Lawson Handley et al., 2011). For example, the global invasion histories of the red imported fire ant, *Solenopsis invicta*, and the tropical fire ant, *S. geminata*, were both revealed by using mtDNA and an extensive number of microsatellite markers (Ascunce et al., 2011; Gotzek et al., 2015). Recent developments in sequencing technologies have allowed the quick and economic development of a large number of molecular markers for non-model species (Yang et al., 2015). Microsatellites have emerged as the markers of choice for high-resolution population analysis because of the advantages of high variability, easy access, and low cost (Guichoux et al., 2011).

The longhorn crazy ant, *Paratrechina longicornis* (Latreille, 1802), is regarded as a significant invasive species due to its ecological impacts (Wetterer, 2008). The native range of this invasive species and its invasion history, however, remain controversial (Wetterer, 2008; LaPolla and Fisher, 2014). A previous study reported that colonies of *P. longicornis* from Bangkok, Thailand display a remarkable genetic system, whereby workers are produced by sexual reproduction, whereas queens are clones of their mothers and males are clones of their fathers (Pearcy et al., 2011). Under this system, workers carry high levels of heterozygosity as they are produced from divergent queen and male clones. As a consequence, the spatial pattern

of genetic variation may be biased if analyzing the worker genotype alone due to the strong sex-associated structure between their male- and female-derived genomes. To overcome this, sexuals (i.e. male, queen and/or daughter queen) should be used instead (e.g., Foucaud et al., 2005; Kuhn et al., 2017) or, where sexuals are not available, male and queen lineages can be inferred based on worker genotype (e.g., Darras et al., 2014). *Paratrechina longicornis* sexuals are, however, difficult to find in the field, being located deep within the nest in cavities of concrete objects with narrow crevices, and produced only during the warm, rainy months (Trager, 1984; Tseng, personal observation). This limitation, therefore, highlights the need to develop microsatellite markers that can infer male and queen lineages from the worker genotype. We note that of the 15 previously published microsatellite markers (Molecular Ecology Resources Primer Development Consortium et al., 2011), 11 display the potential to distinguish queen and male alleles from worker genotypes (Pearcy et al., 2011). To increase the power and resolution of inference the invasive history of *P. longicornis*, we developed a new set of polymorphic microsatellite makers and characterized the novel markers using worker and sexual samples from three geographical regions in East and Southeast Asia.

2.2 Material and methods

Development of microsatellite markers

DNA libraries were prepared from genomic DNA of two *P. longicornis* queens from Thailand using a TruSeq Nano DNA Library Preparation Kit (Illumina, San Diego, CA, USA) and sequencing on an Illumina HiSeq 2000. De novo genome assembling was carried out by Kuora Co., Ltd (Taipei, Taiwan) following the procedure described below. Trimming and error-correction were performed with Trimmomatic (Bolger, 2014) and BBMap software (Bushnell, 2015). Error-corrected reads were assembled using Platanus (Kajitani et al., 2014). The draft contigs were then screened for microsatellite loci containing 10 or more dinucleotide repeats using MSATCOMMANDER (Faircloth, 2008). A total of 65 potential microsatellite loci were selected, and primer pairs were designed using the Primer3 program (Rozen and Skaletsky, 1999) embedded in MSATCOMMANDER with default settings (product sizes ranging from 150 to 350 bp).

The 65 potential microsatellite loci were screened for positive PCR amplification using agarose gel electrophoresis. PCR reactions contained a total volume of 20 µl, composed of 10 µl of EmeraldAmp® MAX PCR Master Mix (TaKaRa, Otsu, Shiga, Japan), 1 µl of 10 µM

primer pairs, 8 µl of ddH_2O and 1 µl of genomic DNA from one adult male (50 to 100 ng). The PCR conditions were as follows: initial denaturation at 94°C (3 min) followed by 35 cycles of 94°C (30 s), 55°C (30 s) and 72°C (40 s), with a final extension phase at 72°C (7 min). All obtained PCR products underwent gel electrophoresis; those that yielded a single band with the expected size were sequenced by Genomics BioSci and Tech Corp. (Taipei, Taiwan) using an ABI-3730 autosequencer to confirm if they corresponded to the expected microsatellite loci. The nucleotide sequences of confirmed microsatellites were deposited in NCBI GenBank (accession nos. KY912037–KY912074) (Table 2.1).

Sampling, DNA extraction, and microsatellite genotyping

Paratrechina longicornis samples were collected between 2012 and 2015 from 74 colonies in three geographical regions, namely Taiwan (Taiwan island, 27 colonies), Thailand (central Thailand, 29 colonies), and Okinawa (Okinawa island, Japan, 18 colonies). The distance between each sampled colony was at least 100 m. One worker per colony was used for subsequent population genetic analyses. In addition, 8 queens (Taiwan, 2 colonies; Thailand, 2 colonies) and 5 males (Taiwan, 3 colonies) were sampled and used in genetic analyses. Genomic DNA was extracted using the Gentra Puregene cell and tissue kit (Qiagen, Maryland, USA) according to the manufacturer's instructions and stored at -20 °C until use.

In order to genotype all individual ants in an economic manner, we performed multiplex PCR reactions with fluorescently labeled universal primers following the strategy described in Blacket et al., (2012). Four fluorescent labeled universal primers and modified locus-specific primers with a 5' universal primer sequence tail were used. Five to six loci were amplified per multiplex reaction. PCR reactions contained a total volume of 20 µl, composed of 10 µl of EmeraldAmp® MAX PCR Master Mix (TaKaRa, Otsu, Shiga, Japan), 1.5 µl of 10 µM primer pairs, 7.5 µl of ddH_2O and 1 µl of genomic DNA from the ant sample (50 to 100 ng). The PCR conditions were as follows: initial denaturation at 94°C (3 min) followed by 35 cycles of 94°C (30 s), 55°C (30 s) and 72°C (30 s), with a final extension phase at 72°C (30 min). The resulting PCR products were analyzed on an ABI-3730 Genetic Analyzer (Applied Biosystems) by Genomics BioSci and Tech Co., Ltd (Taipei, Taiwan). GeneMarker program (version 2.4.0, SoftGenetics LLC) was used to visualize and score alleles.

Characterization of microsatellite loci

Summary statistics of novel microsatellite markers including the number of alleles (Na), Shannon's information index (I), and observed heterozygosity (Ho) were calculated using GenAlEx 6.5 software (Peakall et al., 2006). Regional genetic differentiation, as expressed by Wright's F_{ST} (F_{ST}), Jost's estimate of differentiation (Dest), and Hedrick's standardized G_{ST} for small number of populations (G''_{ST}), was estimated using GenAlEx 6.5 software (Peakall et al., 2006).

2.3 Results

Among 65 primer sets tested, 36 succeeded in amplification and showed polymorphisms (Table 2.1). A total of 305 alleles were amplified from the 36 loci based on the 74 genotyped worker individuals. The number of alleles per locus ranged from 3 to 18, averaging 8.5 alleles per locus for the worker dataset (Table 2.1). The presence of null alleles was unlikely as the vast majority of workers were heterozygous (Table 2.1). All 36 loci were successfully amplified from queen and male samples, and the number of alleles per locus ranged from 1 to 6 in queens and 1 to 3 in males (Table 2.1). Among the 36 loci, queens and males had non-overlapping allele size ranges at 18 loci (Table 2.1, highlighted in bold).

From the 305 alleles observed in the worker dataset, 40, 45, and 2 private alleles (i.e., the number of alleles unique to a single population) were found in Taiwan, Thailand, and Okinawa populations, respectively. The frequencies of private alleles were generally low, with average frequencies of 0.026, 0.033, and 0.083 for *P. longicornis* in Taiwan, Thailand, and Okinawa, respectively. The number of alleles (Na), Shannon's information index (I), observed heterozygosity (Ho), and expected heterozygosity (He) for each region are listed in Table 2.2. The microsatellite polymorphism and genetic diversity, as expressed by the average number of alleles per locus and average Shannon's information index, were generally higher in *P. longicornis* in Taiwan and Thailand than in Okinawa (Table 2.2). *Paratrechina longicornis* in all regions displayed remarkably high levels of observed heterozygosity, with average values of 0.897, 0.901, and 0.880 for ants in Taiwan, Thailand and Okinawa, respectively (Table 2.2). Pairwise F_{ST} values were very low in all pairwise population comparisons, ranging from 0.016 to 0.020 (Table 2.3). Similar results were found for Dest and G''_{ST} yet genetic differentiation between regions were significant (Dest = 0.032–0.048; G''_{ST} = 0.048–0.070; Table 3). Overall, our data reveal a high degree of genetic variability,

and thus highlight the substantial potential of these newly developed markers on population genetic studies.

Table 2.1 Summary of general information for the 36 polymorphic microsatellite loci isolated from *Paratrechina longicornis*. (Na-W: number of alleles in workers; Na-Q: number of alleles in queens; Na-M: number of alleles in males; Ta: annealing temperature). The loci which queens and males have non-overlapping allele size ranges are highlighted in bold.

Locus	Repeat motif	Primer sequences (5'−3')	Na-W	Na-Q	Na-M	Ta (°C)	Size range (bp)	Accession No.
Prl102	(CT)^12	F: TCCAACTGACCCGGAAGAC	5	2	2	58	159-171	KY912037
		R: CGTACGGAATCGTGCGAAG						
Prl104	(AG)^15	F: GAGAGGGAACCCTGCTTCG	13	5	2	58	263-291	KY912038
		R: TCTGCCTGGTTTAGCCCTC						
Prl106	(AT)^17	F: CTCATCGACCCTTTGACGG	13	4	3	58	286-320	KY912039
		R: ACTGGTAAGTCCACTCCGC						
Prl107	(AT)^10	F: TCTCTGCAGCTGTGTCAGG	8	1	3	58	294-330	KY912040
		R: CGCAATTAGCGTCTCCGC						
Prl109	(CT)^12	F: CAGTCGCAACAATGGCGG	5	2	2	58	178-186	KY912041
		R: TGACGAAAGCACCCGTAGG						
Prl110	(CT)^15	F: CGTTATCCGTTCGTCACCG	14	1	3	58	179-231	KY912042
		R: GTGTCCGATGCAAATCCCG						
Prl111	(AG)^13	F: AGCTGTCTGATTTCGTCGC	14	4	3	58	277-317	KY912043
		R: AACGCCTTTAATCCGTCGC						
Prl113	(AT)^10	F: ATACACATTAGTGCATCCAACC	6	2	2	58	296-310	KY912044
		R: TTCGGCGTTCGTGAACAAG						
Prl118	(AG)^16	F: ACAGGAAGTCGCGGAGATG	8	2	2	58	255-279	KY912045
		R: AATGCGGTGGTCAAAGTGC						
Prl119	(AT)^13	F: ACAACTAATCGCCCGTAGC	5	1	3	58	288-306	KY912046
		R: TGGATCGTGAGATTTCCGTTTAG						
Prl120	(AG)^17	F: CGCATGTGAATGTAAACGATGG	18	6	1	58	297-341	KY912047
		R: CAGCTTGCGGTTCAAGGTC						
Prl121	(CT)^10	F: TAGTGCTGGATGCAGGGTG	5	2	1	58	307-315	KY912048
		R: ACGGCGTAGTACCTTCTGC						
Prl123	(AG)^12	F: ACCGCAGCGTTAATTGC	6	1	2	58	209-225	KY912049
		R: GTCTCCGGACCCATTCTCG						
Prl125	(CT)^10	F: AACACGGATGATTGCATGTC	7	4	1	58	281-301	KY912050
		R: GCCGTGATACGAACTTCCAC						
Prl126	(AT)^11	F: AAGAACTGCAAGAGTGCGG	6	2	2	58	301-317	KY912051
		R: GCACGTCCCGAGAAACATC						
Prl127	(AG)^12	F: AGCTTCCCGTACTTACACG	4	2	1	58	315-325	KY912052
		R: TGCAGAAAGTATGTCGCGATG						

Prl128	(AT)^15	F: AAATTCGTCATGTTCCAGATCC R: CAGCTGGCAAGGCATGAAC	10	3	2	58	312-340	KY912053
Prl130	(CT)^11	F: GCACGCGGAAGCAATTAAC R: GGACGCGTTGGAAAGTTCG	3	1	2	58	221-225	KY912054
Prl132	(CT)^14	F: GATGGCGGAAATACCGGAG R: TCGTTGACTTTACGTGTCGC	5	1	2	58	283-291	KY912055
Prl136	(AT)^14	F: TTGACACAGAAGGCATTTCG R: AGACGGGAGGAAATATCACGG	9	1	3	58	212-244	KY912056
Prl137	(AG)^20	F: CTTTACGTCCGCCGTTTCC R: CATACCTCGCATGGTACGC	11	2	2	58	215-243	KY912057
Prl138	(AG)^17	F: TAGACGGATTCTCCACGGC R: TCTTCGACGGAGGTTCGTG	8	1	2	58	206-230	KY912058
Prl139	(CT)^20	F: TCGATTGACCCGAATCCCG R: TTGTCAAGCCACGAGCATC	12	3	2	58	282-306	KY912059
Prl141	(AT)^18	F: CTGCGCAAATTGTTCTGCC R: TCCATCGTAGGAAGTCGGTC	11	2	3	58	313-353	KY912060
Prl143	(AG)^10	F: GGCTCGGAATAGCTTCCAC R: GTCCCGAGCGCAGTTTATG	6	2	2	58	220-234	KY912061
Prl144	(CT)^10	F: GACGGGTATCGGAACTTTGC R: ACCGCGTTATTTCCGGTTG	10	3	1	58	232-276	KY912062
Prl149	(AG)^12	F: AGACCATGGATCACTCCGC R: TCCGTACATTAATATTCTGCAGTTG	7	2	3	58	345-363	KY912063
Prl150	(AG)^11	F: TCAACCGTAGCATGTGTCTTC R: TCGACATTCTTCCAATTTCGTG	4	1	1	58	240-246	KY912064
Prl152	(GT)^16	F: TCACTATGCGACATCAACTATCG R: CGCGTAAATAAACACGCTTCC	8	2	2	58	249-273	KY912066
Prl155	(GT)^10	F: ATCAGCCAAAGGAATTAGCAC R: ACACCTCACATCTCTTGAATGG	3	1	1	58	347-359	KY912068
Prl156	(AT)^17	F: CTCAGCAGCGAGTTGTTCG R: TGCGGCTTTATATCGGAGC	12	2	3	58	346-376	KY912069
Prl158	(GT)^11	F: CTGCTTGTTCACATGTTCGC R: CGTGCTCGCATGTATGATTTC	6	2	1	58	266-294	KY912070
Prl161	(AG)^12	F: CCCAATGGCGCAGATAACG R: ACAGATTTAAAGCCAGCGCC	7	1	2	58	355-385	KY912071
Prl162	(AT)^13	F: GCGCGTAATCGACCAACTC R: GTTTCAAGGGCTCCTTCGC	11	2	3	58	347-379	KY912072
Prl165	(AG)^18	F: GATTGCTTCCTCGCGCTAC R: TTCTCTGTGCTGCGAAACG	8	2	2	58	283-299	KY912073
Prl166	(AG)^16	F: ACGTGGAATTCGTTTCGGC R: GAAGCCCATTCGCCCATTC	17	4	3	58	283-331	KY912074

Table 2.2 Genetic diversity across the 36 polymorphic microsatellite loci in Paratrechina longicornis workers from Thailand, Taiwan and Okinawa (N: sample size; Na: number of alleles; I: Shannon's information index; Ho: observed heterozygosity; He: expected heterozygosity)

Locus	Taiwan					Thailand					Okinawa				
	N	Na	I	Ho	He	N	Na	I	Ho	He	N	Na	I	Ho	He
Prl102	27	4	1.212	1.000**	0.675	29	4	1.087	0.931*	0.616	18	3	0.781	0.778	0.508
Prl104	26	8	1.849	1.000**	0.821	29	12	2.036	1.000***	0.829	17	7	1.600	1.000***	0.763
Prl106	27	12	2.090	0.889	0.832	29	12	2.234	1.000	0.872	18	8	1.842	0.944	0.818
Prl107	25	6	1.263	1.000	0.650	28	5	1.208	1.000**	0.641	17	3	0.966	1.000**	0.590
Prl109	27	5	1.304	0.815	0.685	29	5	1.304	0.862	0.688	18	5	1.200	0.778	0.644
Prl110	27	10	1.690	0.963	0.711	29	9	1.317	1.000	0.636	18	3	0.958	1.000***	0.586
Prl111	26	8	1.802	1.000	0.811	29	11	1.842	1.000*	0.778	18	7	1.604	1.000*	0.755
Prl113	27	5	1.329	1.000**	0.676	29	6	1.414	1.000**	0.691	17	4	1.212	1.000*	0.657
Prl118	27	8	1.505	1.000	0.697	29	6	1.391	1.000*	0.677	18	5	1.307	1.000	0.671
Prl119	24	5	1.052	1.000*	0.592	29	4	1.102	1.000***	0.618	16	3	1.040	1.000**	0.625
Prl120	27	12	2.174	1.000**	0.862	29	14	2.099	1.000**	0.816	18	7	1.483	1.000	0.701
Prl121	27	5	1.017	0.741	0.541	29	5	0.893	0.517	0.445	18	4	0.877	0.667	0.489
Prl123	27	6	1.289	0.926	0.650	26	5	1.172	0.962	0.649	16	3	0.769	0.750	0.490
Prl125	27	7	1.705	0.926	0.790	28	7	1.660	0.964**	0.783	18	7	1.760	0.889	0.809
Prl126	27	6	1.363	0.889	0.694	29	5	1.092	0.931*	0.606	18	2	0.692	0.944***	0.498
Prl127	27	4	0.979	1.000***	0.578	29	4	0.892	1.000***	0.548	18	3	0.800	1.000***	0.526
Prl128	27	10	1.858	1.000	0.809	29	8	1.703	1.000	0.795	17	6	1.501	1.000	0.730
Prl130	27	2	0.386	0.259	0.226	29	3	0.379	0.207	0.188	18	2	0.349	0.222	0.198
Prl132	27	4	1.039	0.889*	0.592	29	4	1.074	0.931**	0.613	18	3	0.949	0.944**	0.579
Prl136	27	6	1.269	1.000	0.647	29	7	1.375	1.000	0.666	18	4	1.211	1.000*	0.656
Prl137	27	8	1.647	1.000***	0.732	29	10	1.484	1.000	0.673	18	6	1.388	1.000***	0.688
Prl138	27	7	1.182	0.704	0.547	29	7	1.383	0.966	0.668	18	5	1.286	1.000	0.665
Prl139	27	9	1.981	0.926*	0.844	29	11	2.051	1.000**	0.841	18	8	1.771	0.889	0.772
Prl141	27	9	1.475	1.000***	0.683	29	9	1.605	1.000*	0.711	18	6	1.393	1.000***	0.691
Prl143	27	6	1.109	0.556*	0.524	29	5	0.825	0.379***	0.383	18	4	0.693	0.389***	0.366
Prl144	27	9	1.854	0.963	0.806	29	8	1.719	1.000***	0.777	17	6	1.558	0.941	0.758
Prl149	25	7	1.723	1.000	0.782	29	6	1.623	0.931***	0.776	18	6	1.563	0.944**	0.755
Prl150	27	3	0.184	0.074	0.072	29	3	0.546	0.345	0.295	18	3	0.411	0.222	0.202
Prl152	27	8	1.598	0.889	0.737	29	5	1.258	0.793	0.671	17	6	1.589	0.941	0.775
Prl155	26	3	1.034	1.000***	0.622	27	3	1.031	1.000***	0.621	18	3	1.037	1.000***	0.623
Prl156	27	10	1.661	0.926	0.706	29	10	1.808	0.862	0.762	18	7	1.353	0.778	0.620
Prl158	27	5	1.087	1.000**	0.608	28	5	1.117	1.000**	0.610	18	4	0.906	1.000*	0.551
Prl161	26	6	1.265	0.962	0.652	29	6	1.295	0.862	0.659	18	4	0.932	0.667	0.505
Prl162	26	8	1.669	1.000***	0.743	29	10	1.623	1.000***	0.714	18	6	1.415	1.000***	0.698
Prl165	27	6	1.392	1.000**	0.709	28	8	1.551	1.000**	0.714	17	4	1.151	1.000**	0.652

Prl166	27	13	2.322	1.000	0.885	29	13	2.060	1.000**	0.820	18	11	2.183	1.000**	0.870

2.4 Discussion

In the present study, we developed a set of 36 microsatellite markers for the longhorn crazy ant *P. longicornis*. Descriptive statistics of the 36 microsatellite loci across all studied regions indicate that these loci are sufficiently polymorphic to conduct population genetic studies on this invasive ant. Among the 36 loci, queens and males had non-overlapping allele size ranges at 18 loci, implying these loci could be an ideal tool to infer genetic lineages of queens and males from worker data.

Table 2.3 Pairwise genetic differentiation among the three studied *Paratrechina longicornis* populations

Population pair	F_{ST}	Dest	G''_{ST}
Thailand vs. Taiwan	0.018**	0.048**	0.070**
Taiwan vs. Okinawa	0.020**	0.047**	0.069**
Thailand vs. Okinawa	0.016*	0.032*	0.048*

Significance was tested using 999 permutations for pairwise F_{ST}, Dest, and G''_{ST} (* $p < 0.05$; ** $p < 0.01$)

Our genetic analyses reveal a low yet significant level of genetic differentiation in *P. longicornis* among the three studied regions. One explanation for this pattern is that these three regions were colonized by genetically similar source populations and the time since introduction may have not been sufficient for drift or local adaptation to produce a measurable level of genetic differentiation. It is also likely that ongoing gene flow associated with extensive international commerce activities may have erased the signature of genetic differentiation among the three regions, given that *P. longicornis* is one of the most common ants found or intercepted on human-associated means of transport (Weber, 1939; Lester, 2005; Wetterer, 2008).

The levels of heterozygosity across the 36 loci were extremely high in workers of all studied regions. The high heterozygosity most likely results from the unusual reproductive mode of this species whereby workers are produced from hybrid mating from divergent queen and male clones. Our study suggests that this system is likely widespread in Asia and might be linked to the invasion success of *P. longicornis* as it acts as an adaptive trait to relax the costs associated with inbreeding (Pearcy et al., 2011).

In conclusion, we have developed 36 high-quality microsatellite markers for *P. longicornis*. These novel markers, combined with 15 previously published microsatellite markers (Molecular Ecology Resources Primer Development Consortium et al., 2011), potentially allow us to have higher resolving power in inferring the routes of introduction of *P. longicornis* and examining the population structure of this ant at a variety of geographical scales. These data, if obtained, would serve as baseline information for developing an effective control scheme or formulating appropriate quarantine procedures on *P. longicornis*.

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Chapter 3 Genetic diversity and *Wolbachia* infection patterns in *Paratrechina longicornis*

3.1 Introduction

Globalized human commerce has facilitated and intensified the spread of alien species, and the number of invasive species threatening native biodiversity, natural resources, and the economy continues to increase (Pimentel et al., 2000; Leppc et al., 2002; Occhipinti-Ambrogi and Savini, 2003; Meyerson and Mooney, 2007). Knowledge of the invasion histories, routes, and subsequent spread of invasive species provides important information for developing practical management strategies (Estoup and Guillemaud, 2010). Population genetic analyses *on invasive species may* provide insights into the introduction pathways and help us understand the mechanisms underlying the invasion success. Such analyses also may help define management objectives and assist policy makers in developing management, prevention, and restoration strategies (Abdelkrim et al., 2005; Le Roux and Wieczorek, 2009; Chadès et al., 2011; Cristescu, 2015).

The longhorn crazy ant, *Paratrechina longicornis* (Latreille, 1802) (Hymenoptera: Formicidae), is a widespread agricultural and household pest found throughout much of the tropics and subtropics in both the Old World and New World (Wetterer, 2008). A previous study demonstrated the occurrence of an extraordinary, double-clonal reproduction system in a population of *P. longicornis* from Thailand. In this population, queens are produced clonally from their mother, males are produced clonally from their fathers, and workers are produced sexually and characterized by an excess of heterozygosity (Pearcy et al., 2011). High heterozygosity of workers, close association with humans, and high adaptability in disturbed environments of this species may help explain to some extent how this ant spread rapidly around the world even prior to the 20th century (Weber, 1939; Harris and Berry, 2005; Lester, 2005; Wetterer, 2008; Pearcy et al., 2011). While the precise native range of this ant has been a source of debate and remains uncertain, distribution records of *P. longicornis* and its closest relatives suggest either a Southeast Asian or African origin (Wetterer, 2008; LaPolla et al., 2010, 2013; LaPolla and Fisher, 2014). A comprehensive phylogeographic study of *P. longicornis* is needed to help identify more precisely where the species originated as well as its subsequent dispersal routes around the globe.

Researchers routinely analyze both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) data to address questions in molecular ecology and invasion biology. Typically, low genetic variation within a focal population is interpreted as resulting from one or more

population bottlenecks after colonization. However, low mtDNA variation also can result from a recent "selective sweep" of a single, highly successful mtDNA variant, a process which may have no discernable effect on nDNA variation (Nei et al., 1975; Aquadro, 1997). This pattern also can stem from indirect selection associated with a selectively-favored, maternally-inherited symbiont (Hurst and Jiggins, 2005). Such symbionts are common in many insect populations and play a major role in shaping host mtDNA evolutionary history (Hurst and Jiggins, 2005; Moran et al., 2008; Charlat et al., 2009; Feldhaar, 2011; Richardson et al., 2012; Bennett and Moran, 2015; Schuler et al., 2016). If a maternally-inherited symbiont confers a sufficient selective advantage to spread within and among host populations, the mtDNA variant originally associated with this symbiont may spread with it, and result in a skewed frequency distribution of mtDNA alleles during the process (Caspari and Watson, 1959; Kambhampati et al., 1992; Turelli et al., 1992; Narita et al., 2006; Atyame et al., 2011; Schuler et al., 2016). Several genera of such bacterial symbionts are found in insects, including *Wolbachia*, *Cardinium*, *Rickettsia*, *Spiroplasma*, and *Arsenophonus* (Duron et al., 2008; Engelstädter and Hurst, 2009). Among these, *Wolbachia* appears to be the most widespread maternally-transmitted symbiont in insects (Zug and Hammerstein, 2012; Weinert et al., 2015). *Wolbachia* variants typically spread within host species by increasing the relative fitness of infected females, either by conferring direct fitness benefits, such as increased fecundity (Vavre et al., 1999; Weeks et al., 2007; Zélé et al., 2018) or providing nutrients (Hosokawa et al., 2010; Nikoh et al., 2014), or by manipulating host reproduction via cytoplasmic incompatibility (CI), male-killing, feminization of genetic males, or thelytokous parthenogenesis (Werren et al., 2008; Saridaki and Bourtzis, 2010; Ma and Schwander, 2017). A relatively high proportion of ant species harbor *Wolbachia* infections (34%; Russell, 2012; Russell et al., 2012). Thus, possible symbiont effects on mtDNA variation in ants cannot be ignored. Incorporation of data from nuclear genes is essential to verify results obtained for mtDNA data because *Wolbachia* selective sweeps often, but not always, have little to no effects on nuclear variation (Rokas et al., 2001).

This study constitutes the first attempt to understand worldwide genetic variation and prevalence of reproductive parasites in *P. longicornis*. We also assessed the geographic patterns of mtDNA variation in *P. longicornis*, to see if phylogeographic structure can help track the routes of dispersal of this invasive ant species. Our combined results allow us to test whether *Wolbachia* have exerted some selective pressure on mtDNA variation in *P. longicornis*. Also, patterns of mtDNA and nDNA variation were compared for incongruence, which would be

predicted if mtDNA variation has been affected by co-evolving reproductive parasite(s). Lastly, because mtDNA genomes and endosymbionts are maternally co-inherited, analyses of mtDNA structure and variation can shed light on historical transmission patterns (e.g., potential source and spread) of endosymbionts in *P. longicornis*.

3.2 Materials and methods

mtDNA sequencing and phylogenetic analyses

We obtained *P. longicornis* workers from field collections and from other researchers (Appendix 1). A total of 248 ant colonies were sampled across the current geographic distribution of *P. longicornis*, including 22 colonies from Northeast Asia, 81 colonies from East Asia, 71 colonies from South Asia, 9 colonies from Indian Subcontinent, 17 colonies from Oceania, 9 colonies from Polynesia, 9 colonies from North America, 2 colonies from South America, 19 colonies from Caribbean, 2 colonies from Arabia, 2 colonies from Southeastern Europe, 4 colonies from West Africa, and 1 colony from South Africa. To generate statistically unbiased samples, only a single worker ant was used from each colony for subsequent genetic analyses. DNA was extracted from individual *P. longicornis* workers using the Gentra Puregene cell and tissue kit (Qiagen, USA) following the manufacturer's instructions, and stored at -20 °C. Portions of the cytochrome oxidase subunit I (COI, 1,203 bp), an intergenic spacer (106 to 127 bp), tRNA–Leu (70 to 77 bp), and the cytochrome oxidase subunit II (COII, 547 bp) genes were amplified via polymerase chain reaction (PCR). PCR was performed using the primer pair C1-J-1745M-F/PLCOII-R2 for partial COI and PLCOII-F1/ C2-N-3661R for CO1-tRNA-COII region follow the PCR conditions described below (Degnan et al., 2004; Appendix 2). PCR mixtures contained 1-2 μL of template DNA, 0.2 μM of each primer, Takara EmeraldAmp Max PCR Master Mix (Takara, Japan) and water (20 μL reactions). PCR conditions included an initial denaturation step at 98°C (3 min) followed by 35 cycles of 94°C (30 s), 52°C (30 s), 72°C (2 min), and a final extension phase at 72°C (7 min). All PCR products were sequenced in both directions by Genomics BioSci and Tech Corp. (Taipei, Taiwan) using an ABI3730 sequencer. Sequence data were assembled using Sequencher 4.9 (GeneCodes).

Sequences were aligned using MUSCLE as implemented in MEGA 6 with default settings (Tamura et al., 2013). The intergenic spacer and tRNA–Leu region were excluded from phylogenetic analyses due to its ambiguous alignment. We performed phylogenetic analyses using two mtDNA datasets, one including all 248 *P. longicornis* workers and a second

containing only a single representative sequence for each of the 43 mitochondrial haplotypes and plus two outgroup taxa, *P. zanjensis* and *P. ankarana* (45 OTUs). PartitionFinder 1.0.1 software (Lanfear et al., 2012) was used to determine the best fit substitution model and partitioning scheme based on Akaike information criterion (AIC) scores. PartitionFinder for our full dataset indicated the best scheme had four partitions: first position of COI and COII, second position of COI, third position of COI and COII, and second position of COII. The preferred evolutionary model for these four partitions were GTR + G, HKY + I, GTR + G, and F81, respectively (GTR = General Time Reversible; G = gamma distribution; HKY = Hasegawa-Kishino-Yano; I = proportion of invariable sites; F81 = Felsenstein 1981). For the singleton haplotype dataset (45 OTU), PartitionFinder suggested the best scheme had five partitions: 1) first position of COI, 2) second position of COI, 3) third position of COI and COII, 4) first position of COII, and 5) second position of COII. The preferred evolutionary model for these five partitions was GTR + G, HKY + I, GTR + G, HKY + G, and F81, respectively. These best schemes were used as priors for Bayesian phylogeny inference. A Bayesian phylogeny was reconstructed using MrBayes 3.2.1 (Ronquist et al., 2012). Two independent runs of 10^7 generations with 4 MCMC (Markov Chain Monte Carlo) chains were conducted simultaneously, starting from random trees and resampling each tree every 1,000 generations. Posterior probabilities were obtained from the 50% majority-rule consensus of trees sampled after discarding the first 25% of sampled trees.

Network analysis and neutrality tests

A median joining mtDNA haplotype network was constructed using POPART (Leigh and Bryant, 2015; software available at: www.popart.otago.ac.nz) to infer relationships among haplotypes. Net genetic divergence between and within groups (p-distance) was estimated using MEGA 6 (Tamura et al., 2013). Population genetic parameters, including number of segregating sites *S* (Watterson, 1975), number of haplotypes *h*, haplotype diversity *Hd* (Nei, 1987), and nucleotide diversity π/bp (Nei, 1987), were estimated using DNASP v5.10 (Librado and Rozas, 2009). This software also was used to perform neutrality tests including Tajima's *D* (Tajima, 1989), Fu and Li's *D** and *F** tests (Fu and Li, 1993) and McDonald and Kreitman test (McDonald and Kreitman, 1991). A mtDNA sequence from *P. zanjensis* was used as the outgroup for neutrality tests. Negative values of Tajima's *D*, Fu and Li's *D** and F* may reflect a recent population expansion, purifying selection, or genetic hitchhiking, whereas positive values generally reflect a population bottleneck, genetic structure and/or balancing selection.

The McDonald and Kreitman test (M-K test) compares the ratio of fixed and polymorphic synonymous and nonsynonymous changes (McDonald and Kreitman, 1991). Additionally, the DHEW test (Zeng et al., 2007b) was performed to detect the signatures of positive selection and hitchhiking on host mtDNA as implemented in the DH program (Zeng et al., 2007a, 2007b). The DHEW test we used was a compound test of Tajima's *D* (Tajima, 1989), Fay and Wu's *Hn* (Fay and Wu, 2000), and Ewens-Watterson test (Watterson, 1978), and is thought to be more powerful in detecting positive selection and more robust to historical demographic changes. *P*-values of the DHEW test were estimated using 100,000 replications of coalescent simulation using DH package (available online: http://zeng-lab.group.shef.ac.uk/wordpress/?page_id=28). Normalized Fay and Wu's *Hn* (Fay and Wu, 2000; Zeng et al., 2006) was also calculated using the same package.

Screening for Wolbachia infection and MLST sequencing

We screened the DNA samples for *Wolbachia* using three primer pairs that amplified part of the *Wolbachia* surface protein gene (*wsp*), 16S rRNA gene, and cell division protein (*ftsZ*) (Appendix 3). PCR primers are published elsewhere and listed in Appendix 3. Three workers per colony were used to determine the infection status for each colony. Our preliminary results indicated a high intra-colony infection rate of *Wolbachia* in both workers and queens (approximately 0.96-0.97, Tseng et al., unpublished data). All PCRs were performed with at least one appropriate positive (DNA extracted from a *Wolbachia*-infected sample) and blank (ddH_2O). All PCR amplicons that yielded a single band on agarose gels were sequenced by Genomics BioSci and Tech Corp. (Taipei, Taiwan) using an ABI3730 sequencer. Some workers appeared to be infected with multiple *Wolbachia* (see Results for more details), and, in these cases, sequence data for individual *Wolbachia* was obtained by PCR using group- or strain-specific primers (Appendix 4).

Although *Wolbachia* surface protein (*wsp*) gene sequence data have been used for phylogenetic analyses in numerous studies, the phylogenetic relationships inferred using data based on a single gene may not be robust due to a high level of recombination among *Wolbachia* strains (Baldo and Werren, 2007). Therefore, we employed a multilocus sequence typing (MLST) approach developed by Baldo et al. (2006) in which a total of five MLST genes (*gatB*, *coxA*, *hcpA*, *ftsZ*, and *fbpA*) were sequenced following the methods of Baldo et al. (2006). *Wolbachia* strains were characterized by comparisons with other sequences in the *Wolbachia*

MLST database (http://pubmlst.org/*Wolbachia*/) and NCBI Genbank database (https://www.ncbi.nlm.nih.gov/genbank/). ClonalFrame version 1.1 was used to construct a *Wolbachia* MLST genealogy (Didelot and Falush, 2007). ClonalFrame accounts for both substitutions and recombination events, providing more reliable clonal relationships based on multilocus data (Didelot and Falush, 2007). Two independent runs were performed, each with 1,000,000 MCMC burn-in iterations and 1,000,000 as sampling period and a sampling frequency of 1,000. A 50% majority rule consensus tree was built from combined data from the two independent runs.

Reconstruction of ancestral states of Wolbachia infection status

The program BayesTraits was utilized to reconstruct the ancestral states of *Wolbachia* infections in *P. longicornis* mtDNA lineages (Pagel et al., 2004; Mark and Andrew, 2006). Two *Wolbachia* strains, *w*LonA and *w*LonF, were found in some of our *P. longicornis* samples (see Results for details). We tested for a correlation between the occurrence/absence of *w*LonA and the occurrence/absence of *w*LonF by performing BayesTrait analyses using both dependent (i.e., the infection history of *w*LonA was correlated with *w*LonF) and independent models. The difference between the two models was assessed by Bayes Factor (BF) based on the final harmonic mean of the likelihoods model. A log BF value greater than two was interpreted as supporting the dependent model (i.e., correlated patterns of infections). Prior to the MCMC runs, maximum likelihood analyses were performed using the consensus tree obtained from MrBayes, and the derived results were used to set the priors for MCMC analyses. Considering the results of the likelihood analysis, all MCMC priors were set as uniform distribution for all rates, with different ranges used for each parameter. A total of 7,500 trees were generated by MrBayes (full dataset with 248 OTUs, discarded first 25 % trees as burn-in) and used in the MCMC inferences to account for phylogenetic uncertainty. These input trees did not include an outgroup species because we focused only on infection histories of the two *Wolbachia* strains in *P. longicornis*. Terminal taxa were coded for presence (1) or absence (0) of *Wolbachia* infection. The rate deviation parameter was tuned automatically to achieve an average acceptance rate between 20% and 40% and ancestral states were reconstructed using the command "addnode". The MCMC chains were run for 10^9 iterations, sampled every 10^5 iterations with a burn-in of 10^8 iterations.

We tested for associations between mitochondrial lineages and *Wolbachia* infection status

by using the BaTS program (Bayesian tip-association significance testing) to compute the parsimony score statistic of clustering strength (PS), the association index statistic (AI), and the exclusive single-state clade size statistic (MC) (Parker et al., 2008). PS represents the most parsimonious number of character changes in the phylogeny. AI is an estimate of the frequency of the most common branch tip trait subtended by internal nodes. MC measures the size of the maximum monophyletic clade in which all tips share the same trait. Thus, a significantly lower value of PS, lower AI and higher MC would indicate a strong phylogeny-trait association. The association between mitochondrial lineage and *Wolbachia* infection is predicted to be strong if *Wolbachia* infections are transmitted vertically only, while frequent horizontal transfers of *Wolbachia* infections would erode this association. In BaTS analyses, 7,500 trees were generated by MrBayes as input and tested each parameter by generating a null distribution from 1,000 replicates.

nDNA analyses

We genotyped a subset of *P. longicornis* workers from three well-sampled regions (41 colonies from East Asia, 21 colonies from Northeast Asia, and 71 colonies from South Asia) at 20 microsatellite loci (Appendix 5) to test for congruence (or incongruence) of mtDNA and nuclear DNA variation patterns. DNA of the same worker (one worker per colony) was used for both mtDNA and microsatellite analyses. Microsatellite loci were amplified by using a multiplex PCR method following procedures described by Blacket et al. (2012). The purified PCR products were analyzed on an ABI-3730 Genetic Analyzer (Applied Biosystems) by Genomics BioSci and Tech Co., Ltd (Taipei, Taiwan). GeneMarker (version 2.4.0, Softgenetics LLC) was employed to visualize and score alleles. Genetic variation at each microsatellite locus was characterized in terms of number of alleles (*Na*), effective number of alleles (*Ne*), observed (*Ho*) and expected (*He*) heterozygosity, Shannon's information index (*I*), fixation index (*F*), and Hedrick's standardized G_{st} for small number of populations (G''_{ST}), using the program GENALEX 6.502 (Peakall and Smouse, 2006).

Genetic structure was assessed using the Bayesian model-based clustering software STRUCTURE 2.3.4 (Hubisz, Falush, Stephens, and Pritchard, 2009; Pritchard, Stephens, and Donnelly, 2000). Five independent STRUCTURE runs were executed for each of $K = 1–10$ (K, the number of assumed genetic clusters) under the admixture model and allele frequencies correlated with 1,000,000 MCMC iterations and an initial burn-in of 100,000 generations. The

optimal number of genetic clusters within the data was estimated by Evanno et al. (2005) in STRUCTURE HARVESTER v. 0.9.94 (Earl and vonHoldt, 2012) (available online: http://taylor0.biology.ucla.edu/structureHarvester/). STRUCTURE results were visualized using CLUMPAK server (Jakobsson and Rosenberg, 2007) (available online: http://clumpak.tau.ac.il/). Genetic relationships among populations were examined by applying a discriminant analysis of principal components (DAPC) (Jombart et al., 2010) available in the R package adegenet (Jombart, 2008) on all microsatellite data. Population labels were input as the prior cluster information in DAPC. The first 20 principal components (PCs) accounted for 80% of the total microsatellite genetic variation and were retained from the analysis.

3.3 Results

mtDNA analyses

We sequenced mtDNA of 248 *P. longicornis* workers (one per colony) from 13 geographic regions. A total of 43 different mtDNA haplotypes were found with 172 polymorphic sites present over the entire 1,750bp COI-COII region (Appendix 6; collection site information in Appendix 1). Nucleotide diversity was highest in samples from the Indian Subcontinent (0.041)

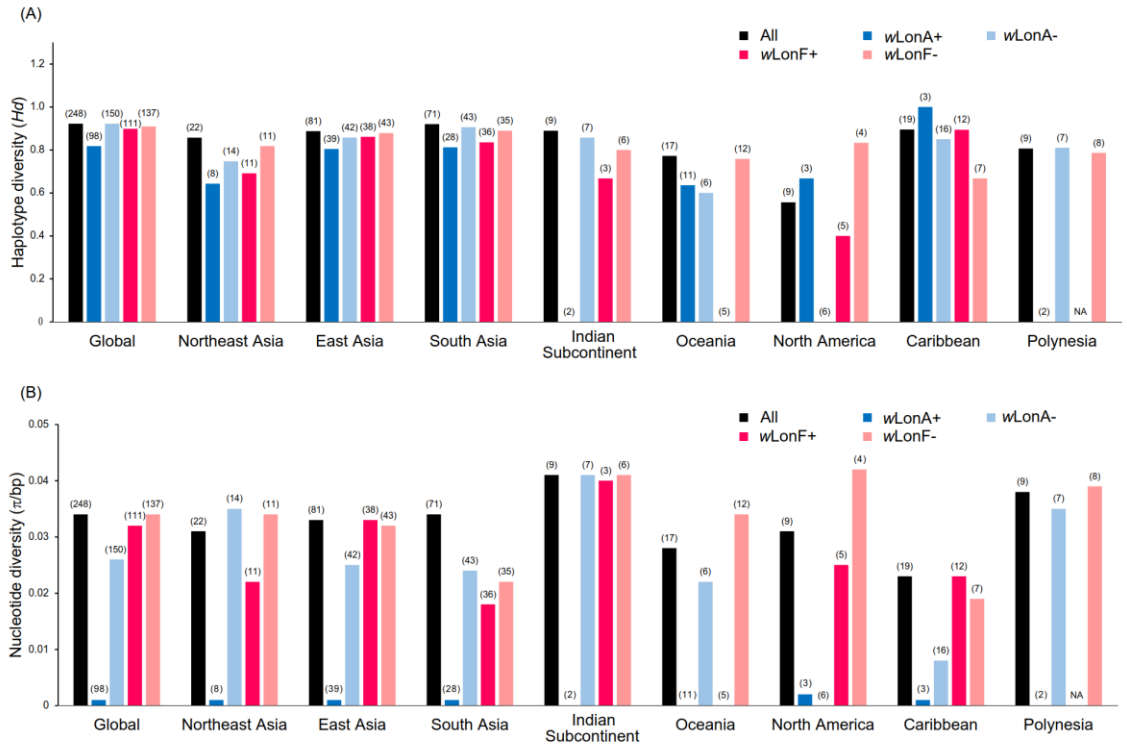

Figure 3.1 Regional mitochondrial genetic diversity of *Paratrechina longicornis* as expressed by (A) haplotype diversity and (B) nucleotide diversity with respect to their *Wolbachia* infection status. *w*LonA+, *w*LonA-, *w*LonF+, and *w*LonF- denote *w*LonA-infected, *w*LonA-uninfected, *w*LonF-infected, and *w*LonF-uninfected ants in a given region, respectively. Sample size of each region is indicated in parentheses.

(Fig. 3.1, Appendix 6; genetic diversity values from Arabia, Southeastern Europe, West Africa, and South America are likely biased due to low sample size). Nevertheless, the populations across Old World regions exhibit similar levels of genetic diversity. Bayesian phylogenetic analyses indicated the presence of two mtDNA clades (Clade I and II), one of which (Clade II) was divided into three subclades (Clade II-1, -2 and -3) (Fig. 3.2). The average genetic distance between Clades I and II was 0.057, suggesting deep divergence between the two clades. The average pairwise genetic distance among haplotypes was higher within Clade II (0.010) compared with Clade I (0.002). Average genetic distances among workers within the three subclades each had a mean value of 0.001. MtDNA variation was not strongly correlated with

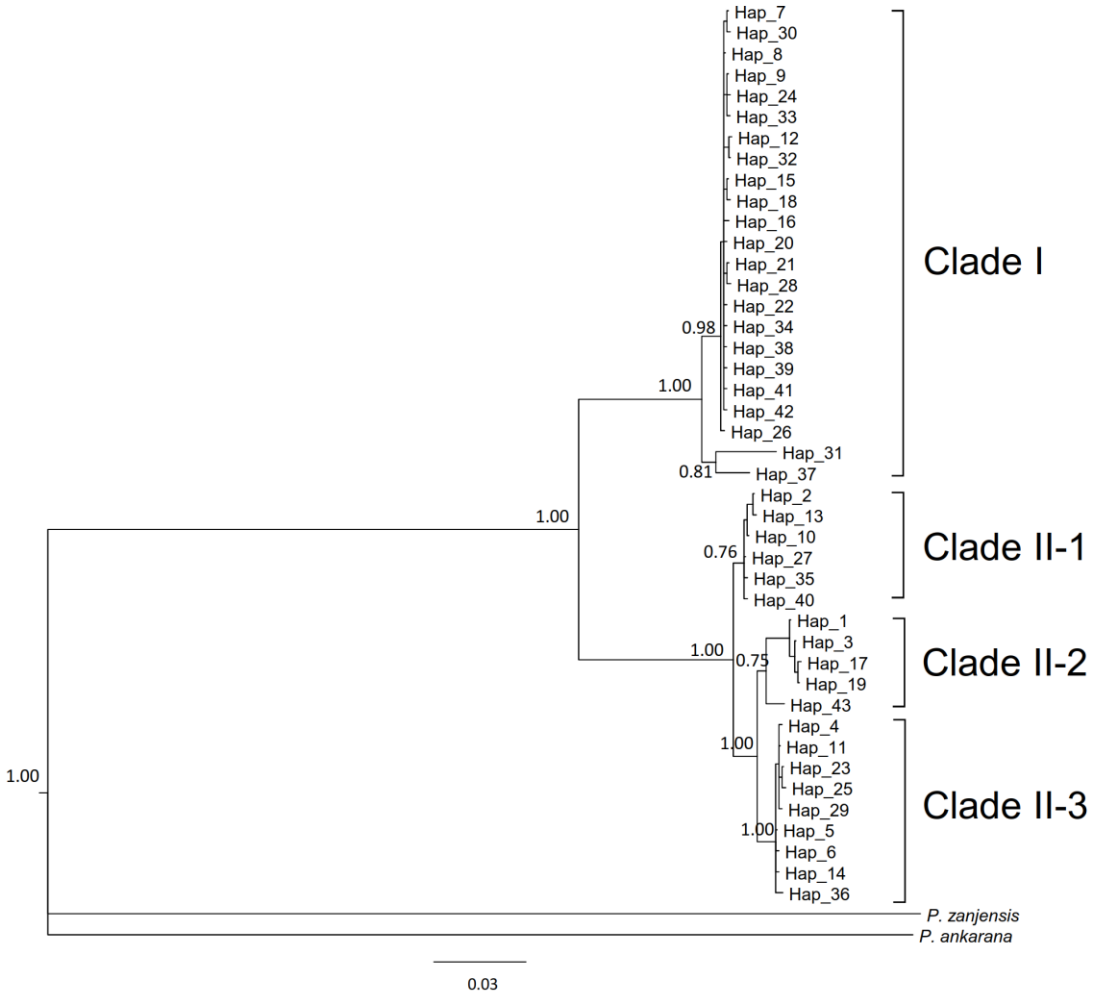

Figure 3.2 The 50% majority rule consensus tree for all sampled *Paratrechina longicornis*, inferred by Bayesian analysis. Numbers above branches indicate Bayesian posterior probability calculated by MrBayes. Refer to Table S1 for respective geographic information of each haplotype.

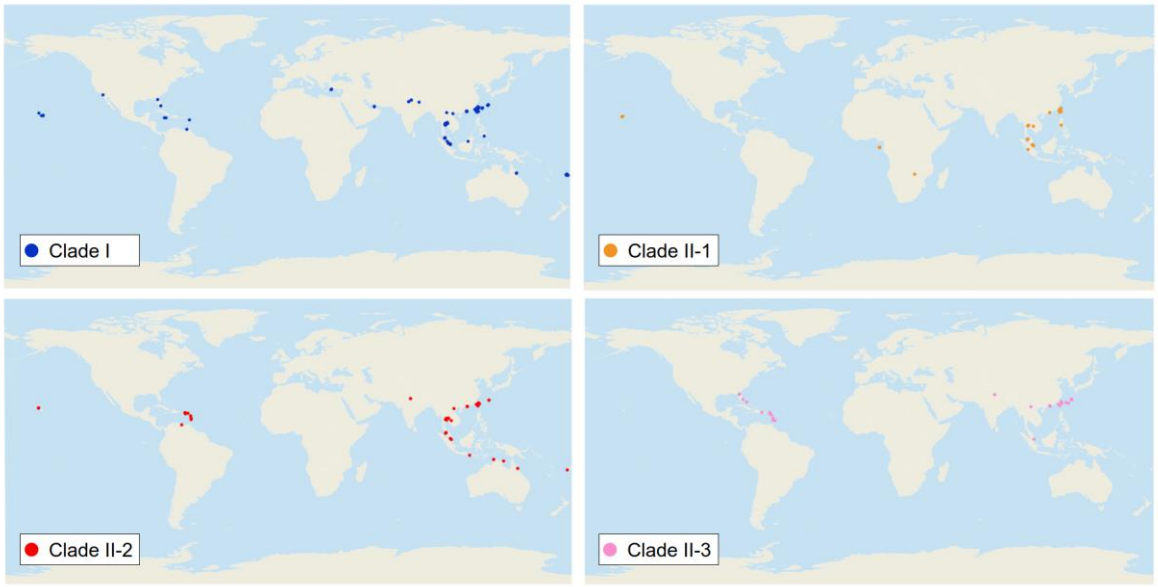

Figure 3.3 Distribution of all mitochondrial haplogroups in the study regions. Haplogroups are denoted by colors: Clade I (blue), Clade II-1 (orange), Clade II-2 (red), and Clade II-3 (pink).

geographic location (Fig. 3.3). Workers belonging to Clades I and II were found at 11 of the 13 sampled geographic regions (all except South and West Africa; Fig. 3.3). Workers with haplotypes belonging to subclade II-1 were found in the Old World, but not in the New World (Fig. 3.3). The median-joining network constructed for all 43 unique mtDNA haplotypes further revealed no clear spatial clustering of haplotypes from Clade I (Fig. 3.4). In particular, haplotype Hap08 (Clade I) was common across the sampled ranges and was connected to several tip haplotypes with low frequency, implying that this haplotype may represent a putative ancestral haplotype within Clade I from which the latter are derived. Similar to Clade I, the haplotype network revealed negligible spatial clustering in Clade II (Fig. 3.4), with approximately half of all haplotypes in this clade present in more than one geographic region.

Wolbachia infections in *P. longicornis*

Our assays for the presence of five putative reproductive parasites in all sampled *P. longicornis* populations detected only *Wolbachia*. Both sequence data and phylogenetic analyses of concatenated MLST data suggest that two *Wolbachia* strains, *w*LonA and *w*LonF, occur in *P. longicornis*, with the former belonging to supergroup A and the latter to supergroup F (Fig. 3.5). Forty-two of the 248 *P. longicornis* workers were infected with *w*LonA only (17 %), 55 workers were infected with *w*LonF only (22 %), 56 workers were co-infected with *w*LonA and *w*LonF (23 %), and 95 workers were uninfected (38 %) (Table 3.1). *Wolbachia* infection status was strongly associated with mtDNA variation. Specifically, the majority of ants belonging to Clade

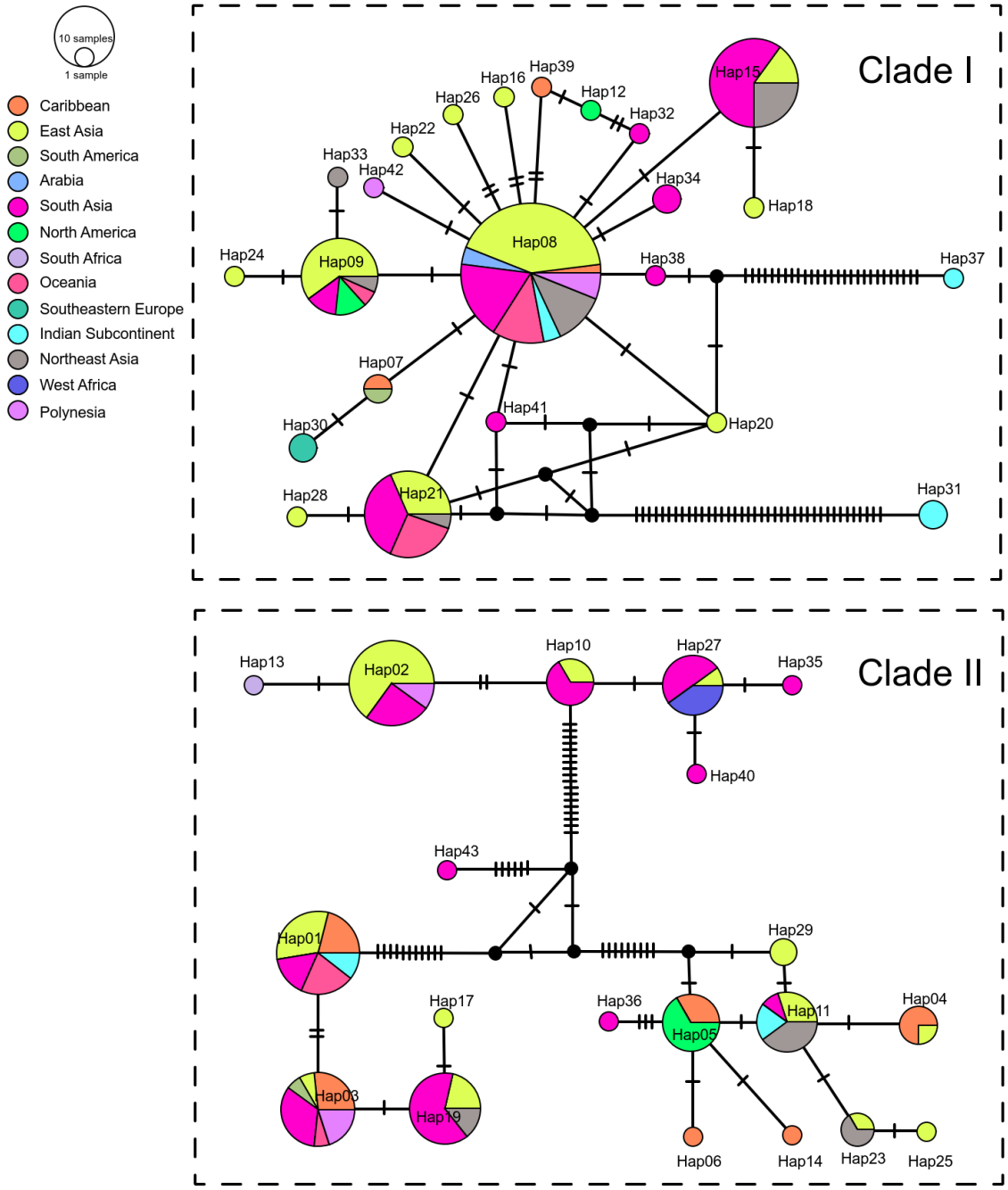

Figure 3.4 Haplotype networks of the mitochondrial genes. Circle sizes are proportional to the number of sequences per haplotype. Colors correspond to geographic regions.

I were either infected with *w*LonA only (33 %) or co-infected with *w*LonA and *w*LonF (44 %), whereas none of workers belonging to Clade II was infected with *w*LonA (Table 3.1). We did not observe a significant association between *Wolbachia* infection status and host geographic range (Fig. 3.6).

Table 3.1 Prevalence of *Wolbachia* *w*LonA and *w*LonF infections in *Paratrechina longicornis*.

No. nests (percentage) †	*w*LonA	*w*LonAF	*w*LonF	Uninfected
Clade I	42 (33 %)	56 (44%)	8 (6%)	21 (17%)
Clade II	0 (0%)	0 (0%)	47 (39%)	74 (61%)
Total	42 (17 %)	56 (23 %)	55 (22 %)	95 (38 %)

† Three workers from each nest were used to screen for *Wolbachia* infections

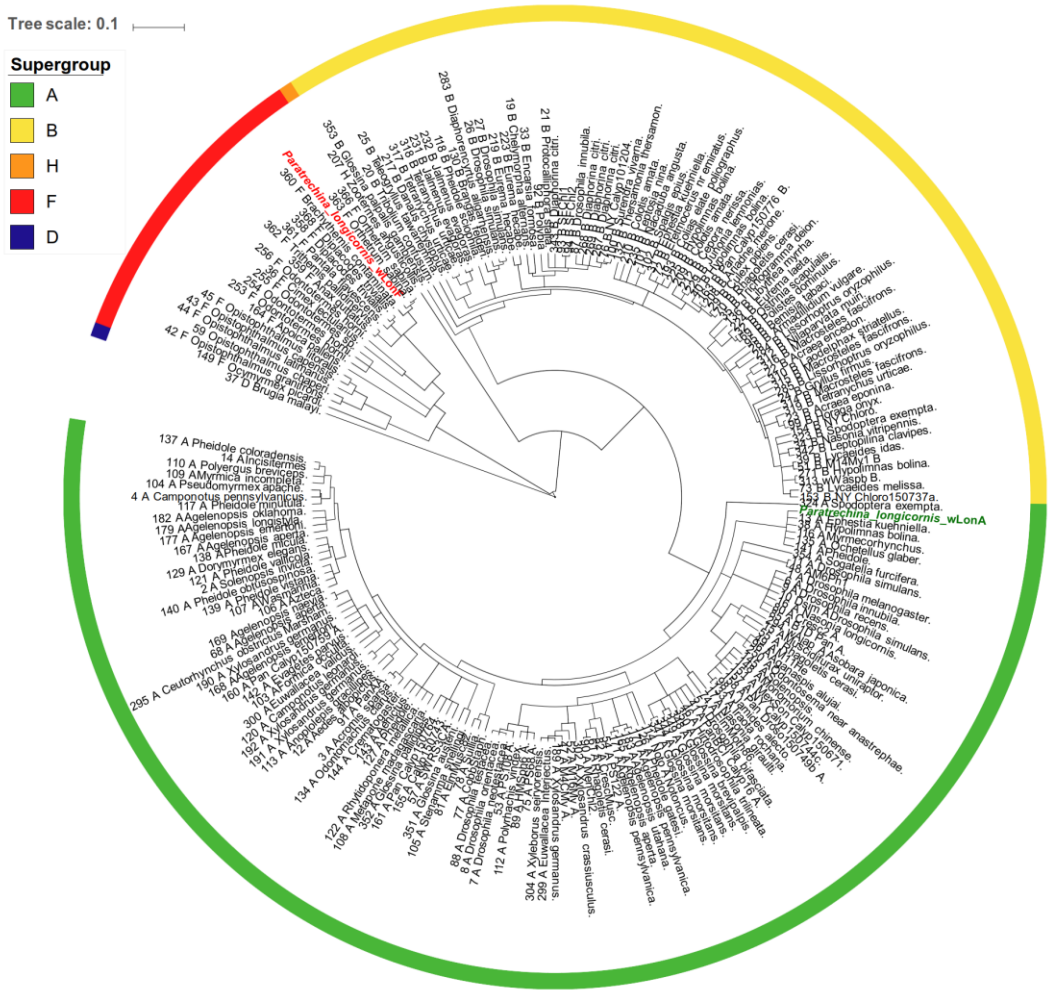

Figure 3.5 ClonalFrame genealogy of 5-locus MLST data in GenBank for *Wolbachia*. The two *Wolbachia* strains detected in this study are marked in green (*w*LonA) and red (*w*LonF). Information regarding host and *Wolbachia* supergroup is obtained from PubMLST database.

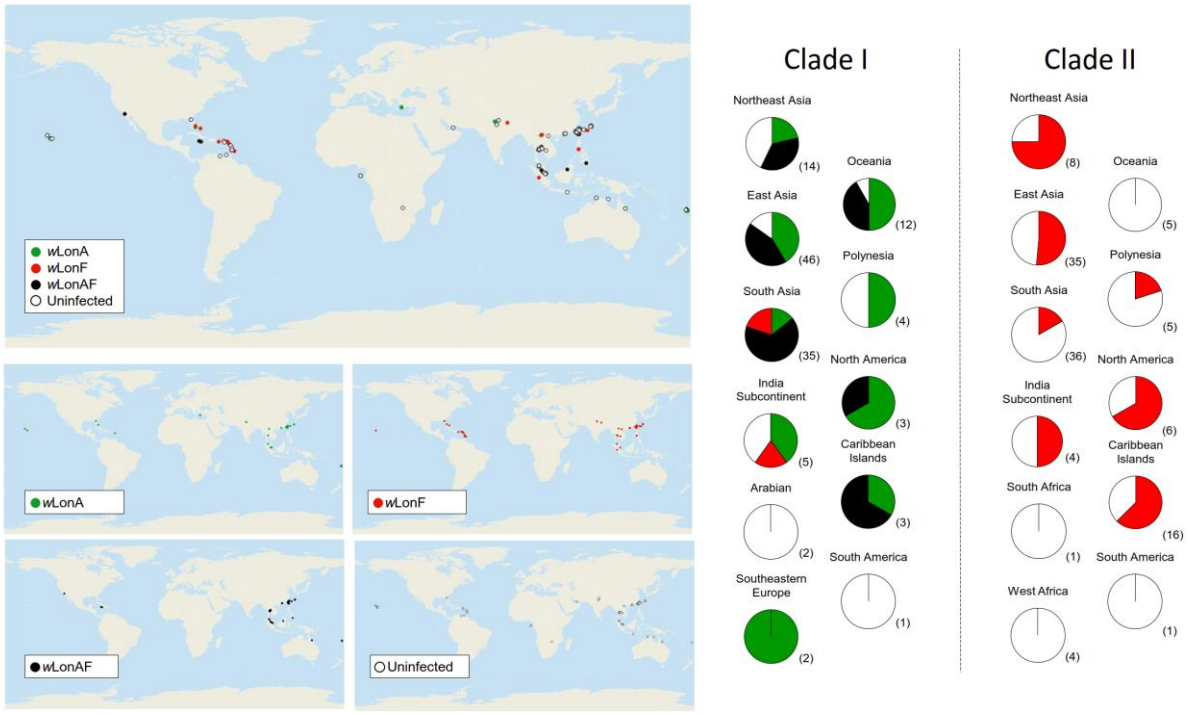

Figure 3.6 Geographic distribution of *Wolbachia* infection of *Paratrechina longicornis* in the study regions. Different colors represent different infection status: *w*LonA infected (green), *w*LonF infected (red), *w*LonA and *w*LonF co-infection (black), and uninfection (white) individuals. Pie charts show the prevelance of each *Wolbachia* strain in each geographic region. Sample sizes are shown in parentheses

The MLST allelic profile for *w*LonA was identical to a sequence type in the *Wolbachia* MLST database, whereas *w*LonF represented a new sequence type that has not been reported in the database. *w*LonA allelic profiles for *gatB*, *coxA*, *hcpA*, *ftsZ*, *fbpA* and *wsp* were 7, 6, 7, 3, 8 and 18, respectively (Appendix 7). *w*LonA belongs to sequence type 19 (ST-19), and is similar to *Wolbachia* variants detected in a moth (*Ephestia kuehniella*), several ants (*Technomyrmex albipes*, *Leptomyrmex* sp., *Pheidole plagiara*, *P. sauberi*, and *Leptogenys* sp.) and two butterflies (*Ornipholidotos peucetia* and *Aricia artaxerxes*) (Appendix 7). *w*LonA shared an identical sequence type with *Wolbachia* Ekue_A (ID 13) detected from *Ephestia kuehniella*, a transinfected A group *Wolbachia* from *Cadra cautella* (*w*CauA) to *E. kuehniella*. *Wolbachia* *w*CauA has been reported to cause cytoplasmic incompatibility (CI) in *C. cautella*, and male killing in *E. kuehniella* (Sasaki, Kubo, and Ishikawa, 2002; Sasaki, Massaki, and Kubo, 2005). *w*LonF allelic profiles for *gatB*, *coxA*, *hcpA*, *ftsZ*, *fbpA* and *wsp* were 168, 147, 262, 132, 226 and 708, respectively (Appendix 7), and were unique to consider *w*LonF a new sequence type (denoted as ST-471). Similar sequence types included ST-239, ST-242, and ST-243, all of which were detected from two dragonflies (*Brachythemis contaminata* and *Orthetrum sabina*).

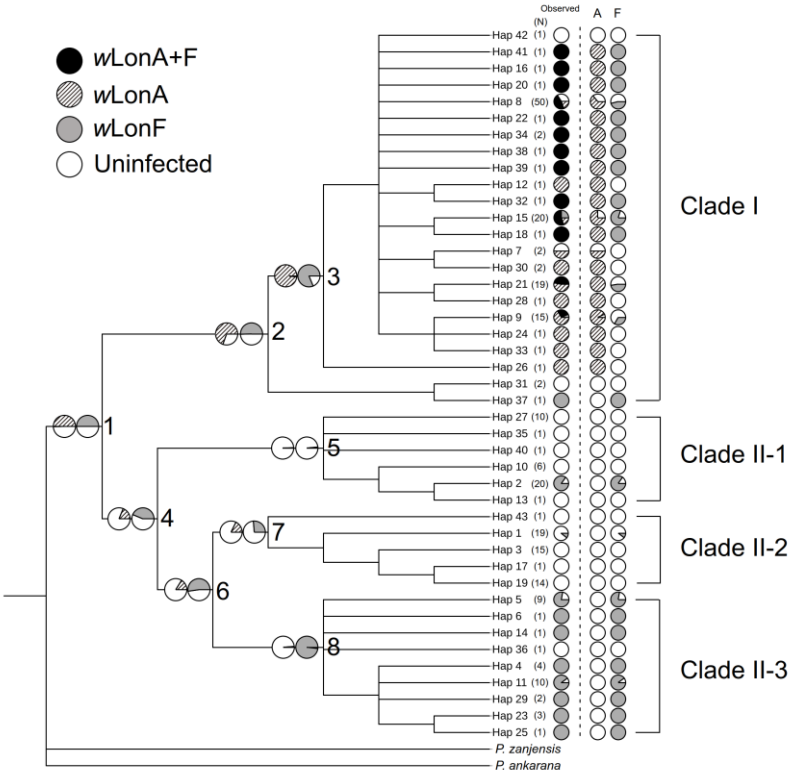

Figure 3.7 Simulated ancestral states of *Wolbachia* infection in *Paratrechina longicornis* inferred by BayesTraits. For each haplotype, pie charts following the haplotype name indicate observed *Wolbachia* infection status combined, *w*LonA only and *w*LonF only (*w*LonA+F co-infection: black; *w*LonA infected: upward diagonal; *w*LonF infected: grey; lack of infection: white). Pie charts on branches indicate simulated probabilities of *Wolbachia* infected status (left: *w*LonA; right: *w*LonF) for each numbered node.

The most similar *wsp* sequence to *w*LonF in GenBank was from a *Wolbachia* variant infecting bat flies *Cyclopodia dubia* (KT751165; 99 % similarity) (Wilkinson et al., 2016).

Wolbachia *infection history in* P. longicornis

The BayesTraits analyses indicated the dependent model was not significantly better than the independent model (log Bayes factors = 0.707), suggesting no correlation between *w*LonA infection status and *w*LonF infection status. Therefore, infection history of *w*LonA and *w*LonF was inferred separately (Fig. 3.7). BayesTraits analyses suggested a single ancestral *w*LonA infection (on the common ancestor of node 2 or node 3, Fig. 3.7) occurred in *P. longicornis* that subsequently has been characterized by vertical *Wolbachia* transmission in the populations of Clade I with only occasional losses of infections (Fig. 3.7). On the other hand, the history *w*LonF infections in *P. longicornis* appears to be characterized by frequent gains of *w*LonF through horizontal transmission as well as frequent losses of *w*LonF over time.

Table 3.2 Significance of correlations between *Paratrechina longicornis* mtDNA phylogeny and *Wolbachia* infection status as identified by BaTS. Association index statistic (AI) and parsimony score (PS) statistic of clustering strength, and exclusive single-state clade size (MC) statistic.

Statistics	Observed mean (95 % CI)	Null mean (95 % CI)	*P*-value
*w*LonA			
AI	3.10 (2.03, 4.19)	10.46 (9.82, 11.06)	< 0.0001
PS	24.29 (22.00, 27.00)	77.69 (74.31, 80.41)	< 0.0001
MC (Uninfected)	121.00 (121.00, 121.00)	5.63 (5.03, 6.50)	0.001
MC (Infected)	19.89 (14.00, 24.00)	3.46 (3.17, 4.26)	0.001
*w*LonF			
AI	6.37 (4.87, 7.89)	10.82 (10.17, 11.47)	< 0.0001
PS	49.67 (45.00, 54.00)	81.54 (77.87, 84.61)	< 0.0001
MC (Uninfected)	33.40 (30.00, 42.00)	4.97 (4.45, 5.71)	0.001
MC (Infected)	10.56 (5.00, 18.00)	3.90 (3.55, 4.57)	0.001

Although the association between *w*LonF infection status and host mtDNA phylogeny was weaker than that of *w*LonA (both AI and PS values of *w*LonF were higher than those of *w*LonA), the BaTS results indicated that both *w*LonA and *w*LonF are significantly associated with the host mtDNA phylogeny (Table 3.2). These results suggest that *w*LonF infection within *P. longicornis* has been shaped by both horizontal and vertical transmission. For example, individuals bearing haplotype 2 (Hap 2) likely obtained *w*LonF via horizontal transmissions whereas the high prevalence of *w*LonF in Clade II-3 is consistent with vertical transmission of *w*LonF over time (Fig. 3.7).

Similar trends were found for estimates of Tajima's *D*, Fu and Li's *D**, and Fu and Li's *F**statistic for all groups harboring *w*LonA (i.e., generally less than zero). However, the normalized *Hn* statistics of Fay and Wu test and results of DHEW varied among regions (Appendix 8). We obtained a negative yet significant estimate of *Hn* only for *w*LonA-infected workers from East Asia, and the results of DHEW tests for *w*LonA-infected groups from East Asia and South Asia regions were significant. The NI estimates from M-K test were larger than one for all groups harboring *w*LonA, and were significant in groups from Northeast and East Asia.

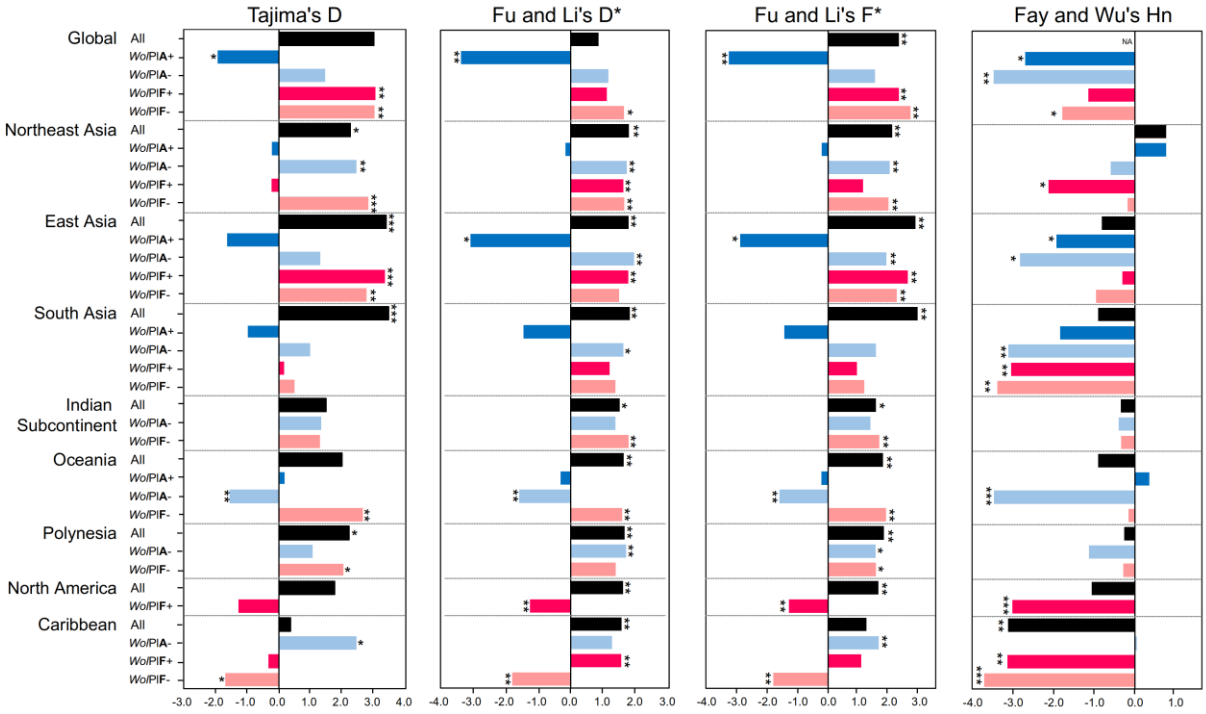

Figure 3.8 Tests for departure from neutrality for mtDNA sequence variation in *Paratrechina longicornis*. *w*LonA+, *w*LonA-, *w*LonF+, and *w*LonF- denote *w*LonA-infected, *w*LonA-uninfected, *w*LonF-infected, and *w*LonF-uninfected ants in a given region, respectively. *P < 0.05; **P < 0.01; ***P < 0.0001; statistics significantly deviated from expectations under neutrality.

Tajima's *D*, Fu and Li's *D**, Fu and Li's *F** generally were positive for *w*LonA-uninfected, *w*LonF-infected and *w*LonF-uninfected groups for each region with few exceptions. The significant negative estimates of *D*, *D**, *F**, *Hn* and significant results of DHEW test were observed in three groups, *w*LonA-uninfected workers from Oceania, *w*LonF-infected workers from North America, and *w*LonF-uninfected workers from Caribbean.

Patterns of mtDNA variation within Wolbachia*-infected and -uninfected ants*

Analyses of mtDNA variation revealed that nucleotide diversity and numbers of segregating sites are much lower in *w*LonA-infected workers than those in *w*LonA-uninfected workers within all sampled regions, except North America (Fig. 3.1, Appendix 6). Estimates of nucleotide diversity were more than 8-fold lower for *w*LonA-infected workers than in *w*LonA-uninfected workers despite limited differences in mtDNA variation between *w*LonF-infected workers and *w*LonF-uninfected workers.

Statistical tests of departures from neutral expectations are presented in Appendix 8 and Fig. 3.8. Estimates of Tajima's *D*, Fu and Li's *D**, Fu and Li's *F**, and Fay and Wu's *Hn* were negative and statistically significant when all *w*LonA-infected workers were combined (Global

group; Appendix 8, Fig. 3.8). The result of the DHEW test on the combined *w*LonA-infected workers supported the hypothesis of selection influencing mtDNA variation (Appendix 8). The neutrality index (NI) of the McDonald-Kreitman (M-K) test was greater than one and deviated from neutral expectations (Appendix 8). In contrast, Tajima's *D*, Fu and Li's *D**, Fu and Li's *F** tests performed on combined samples in other three groups (*w*LonA-uninfected, *w*LonF-infected and *w*LonF-uninfected workers) were all positive, but only five of these estimates were significant (Appendix 8, Fig. 3.8). The estimates of Fay and Wu's *Hn* in *w*LonA-uninfected and *w*LonF-uninfected workers were negative and significantly less than zero. However, the results of DHEW tests failed to support the presence of positive selection of these two groups. The results of M-K tests indicated that the NI of these three groups were not significantly different from one.

Nuclear DNA variation and population genetic structure

We compared the extent of mtDNA and nuclear (microsatellite) differentiation in the three selected Asia regions. A total of 191 alleles were observed across all loci for the 134 sampled workers. The average number of alleles in each sampled region ranged from 4.85 to 8.10 (Appendix 9). Shannon's information index was used to assess gene diversity (Fig. 3.9; Appendix 9), and, when incorporating infection status into analysis, genetic diversities among *Wolbachia*-infected workers are similar to those for uninfected workers across the three selected regions.

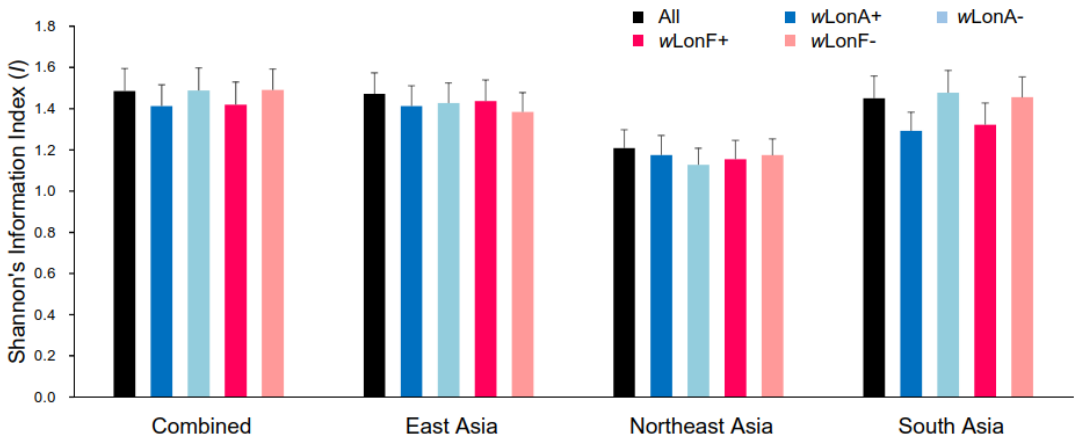

Figure 3.9 Genetic diversity, as expressed by Shannon's information index, of *Paratrechina longicornis* in selected regions based on 20 microsatellite markers. *w*LonA+, *w*LonA-, *w*LonF+, and *w*LonF- denote *w*LonA-infected, *w*LonA-uninfected, *w*LonF-infected, and *w*LonF-uninfected ants in a given region, respectively. Error bars indicate standard errors.

Bayesian cluster analysis performed using STRUCTURE revealed six distinct genetic clusters for the entire data set (K = 3 based on ΔK statistic; Appendix 10). Most workers were admixed (i.e., had membership in more than one cluster; Fig. 3.10), and genetic differentiation among geographic regions or mtDNA clades was not observed. DAPC analysis indicated the lack of differentiation among groups in each of the three selected regions as well as between the two mtDNA clades (Fig. 3.11). The estimate of G''_{ST} between the two mtDNA clades was

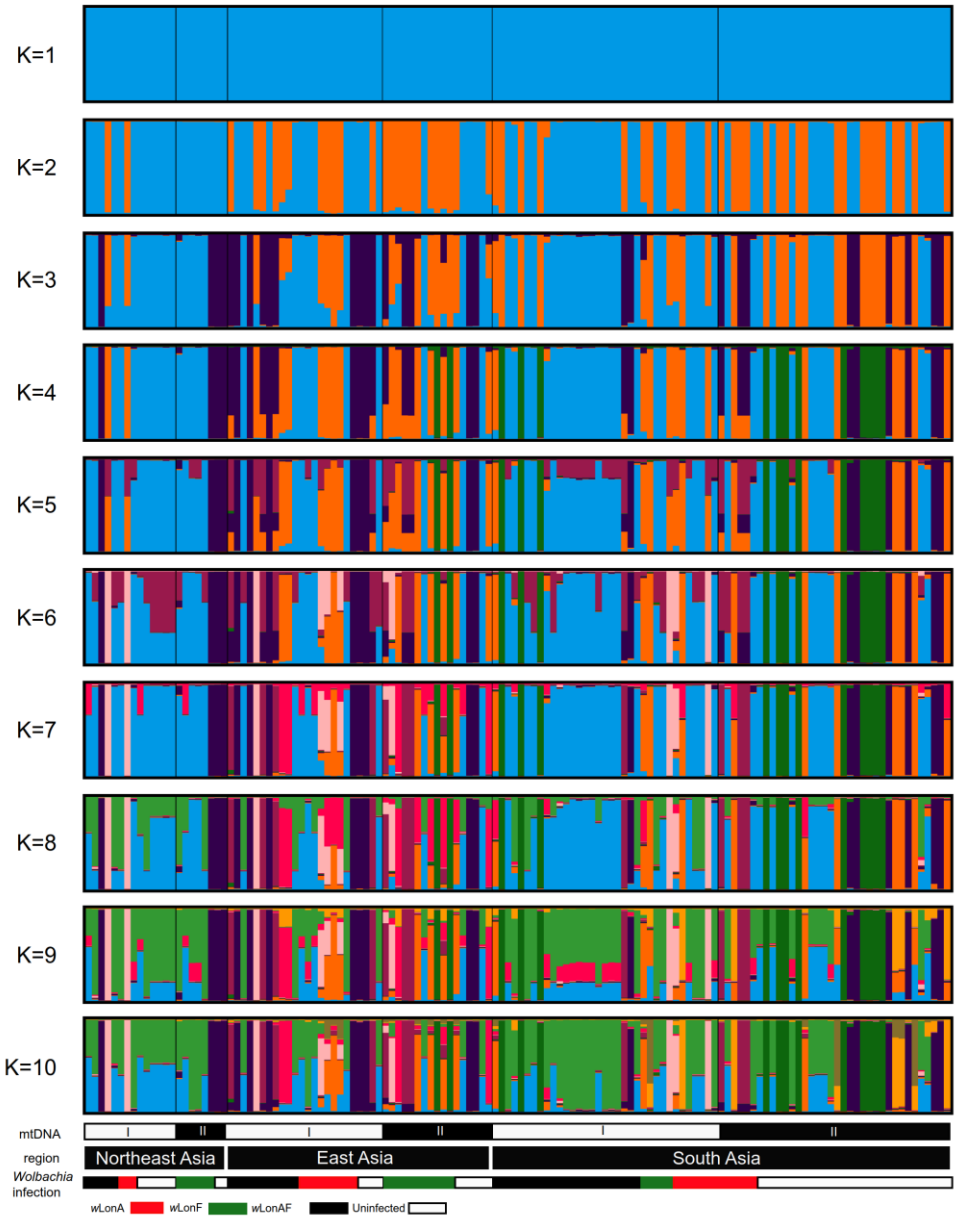

Figure 3.10 *Paratrechina longicornis* population clustering analyses from Structure based on 20 microsatellite loci. Results from K=1 to K=10 are shown with the major mode generated by CLUMPAK with the highest mean posterior probability. Samples are organized by mtDNA clade, geographic regions and *Wolbachia* infection status.

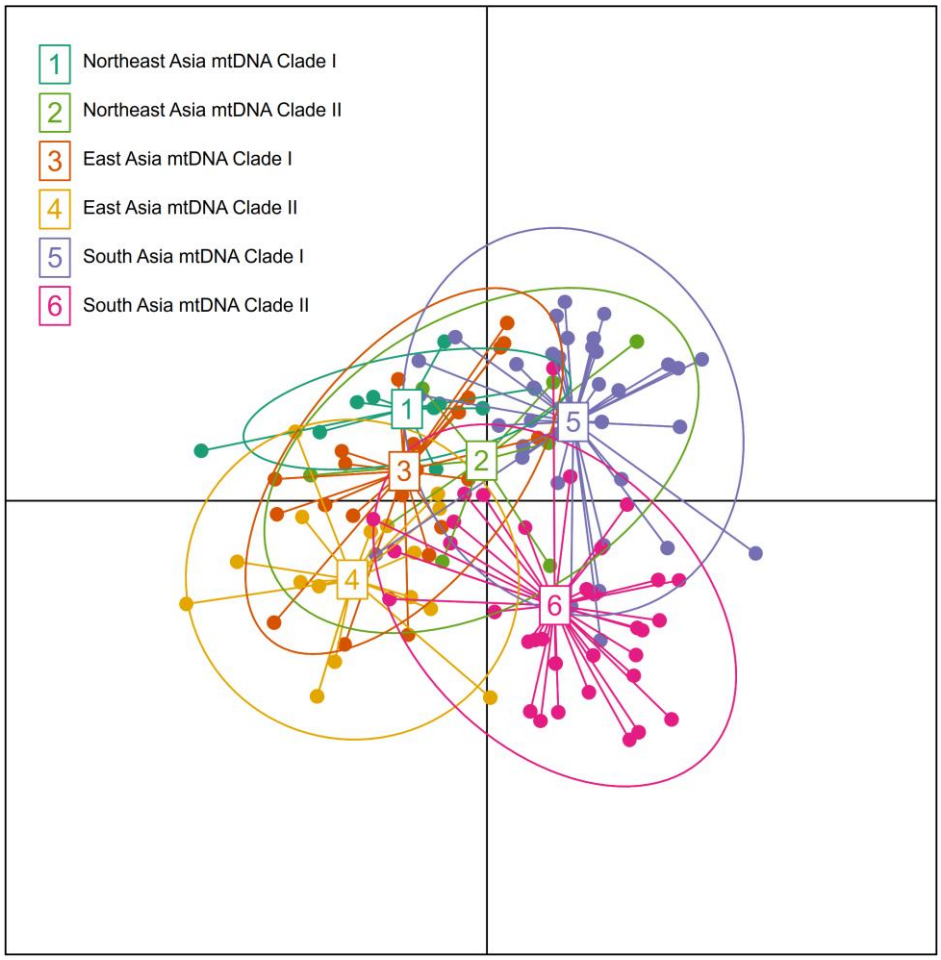

Figure 3.11 Discriminant analysis of principal components (DAPC) of nuclear DNA variation for *Paratrechina longicornis* populations from selected Asia regions. Area identities are labelled in the center of the dispersion, while the large open circle indicates the 90% inertia ellipses for each group.

0.049 (P = 0.001), suggesting a low level of nuclear differentiation between workers from the two mtDNA clades.

3.4 Discussion

Our results showed that global patterns of mtDNA and nDNA variation among populations of *P. longicornis* were discordant, characterized by two highly divergent mtDNA clades with no parallel pattern of nuclear genetic divergence (based on microsatellite loci) between workers from the two mtDNA clades. Several evolutionary scenarios possibly explaining such mitochondrial-nuclear discordance include sex-biased dispersal, local adaptation, historical demography, incomplete lineage sorting, and endosymbiont-driven hitchhiking effects (reviewed in Toews and Brelsford, 2012). Male-biased dispersal (López-Uribe et al., 2014) and local adaptation of mtDNA haplotypes (Cheviron and Brumfield, 2009; Ribeiro et al., 2011;

Spottiswoode et al., 2011) are unlikely for at least two reasons: 1) the geographical distributions of ants from the two mtDNA clades overlap considerably and coexist in virtually all geographic regions we sampled and 2) all ant samples were collected from human-modified habitats (e.g., roadsides, parks or near buildings), suggesting negligible habitat preference between ants from the two clades. The strong association between *Wolbachia* infection status and host mtDNA lineage, as well as reduced mtDNA diversity associated with *w*LonA in *P. longicornis*, are consistent with *Wolbachia* influencing patterns of host mtDNA structure and variation. Levels of nuclear variation were nearly identical for ants from the *w*LonA-infected workers (mtDNA Clade I) and uninfected groups (mtDNA Clade II), which is consistent with the prediction that *Wolbachia* endosymbionts have minimal or no effects on nuclear genetic variation and divergence (assuming host reproduces sexually) due to biparental inheritance (Rokas et al., 2001).

Results from additional analyses also were largely consistent with the predicted effects of *Wolbachia* on mtDNA variation. Tajima's D, Fu and Li's D*, and Fu and Li's F* tests for departures from neutral evolution were negative for all groups harboring *w*LonA and for global datasets. Moreover, the results of Fay and Wu's *Hn* and DHEW were also consistent with expected patterns for a relatively recent *Wolbachia*-driven selective sweep occurring in some, but not all, geographic regions, such as East and South Asia, despite the fact that mtDNA variation is low in almost all populations harboring *w*LonA in every geographic region. One potential explanation for these inconsistencies is that selective sweeps of *Wolbachia* in some of these populations may have occurred far enough in the distant past such that any signature of selection on the mtDNA may have been eroded. The results of M-K tests also imply mtDNA substitution patterns may have been influenced by *w*LonA, but surprisingly that a signature of purifying selection is registered. The NI values of M-K test for groups harboring *w*LonA ranged between 5.074 and 12.888, and were significantly larger than values for groups from Northeast and East Asia. One possible explanation is that hitchhiking events associated with *w*LonA resulted in accumulation of slightly deleterious mutations (Shoemaker et al., 2004; Fay, 2011) followed by negative selection as *Wolbachia*-driven haplotype replacements cease (Bazykin and Kondrashov, 2011).

The virtual absence of genetic structure across a large geographic area in *P. longicornis* and the co-occurrence of divergent mtDNA haplotypes in almost every geographic region suggest that human-mediated, long-distance movement of this species is common. The time of

divergence between any random pair of *w*LonA-uninfected groups (i.e., mtDNA subclades II-1, 2, and 3) is roughly 34,000 years ago (estimated based on average of pairwise genetic distances assuming a substitution rate of 1.455% per site per million years, the estimated rate for the COI gene of ants; Resende et al., 2010), which apparently predates potential human dispersal. However, this divergence, along with the deep divergence between Clades I and II, may stem from accelerated mtDNA substitution rates due to recurrent *Wolbachia* sweeps (Shoemaker et al., 2004), with the assumption that the former had infected ancestor at some point in time in the past. A likely explanation for the specific genetic patterns observed in *P. longicornis* is the ant has experienced multiple human-mediated dispersal events from genetically distinct source populations followed by global dispersal (i.e., high propagule pressure as a result of rampant migration/movement). The genetic patterns we describe are similar to those found in several other globally distributed insects, especially those that are common in human-modified landscapes (e.g., German cockroach [*Blattella germanica*], American cockroach [*Periplaneta americana*]; Vargo et al., 2014; von Beeren et al., 2015), further highlighting the role of human-mediated dispersal in shaping population structure of insect species closely associated with humans.

Reduction in genetic variation as a result of a population bottleneck is a common feature observed in the introduced ranges of numerous invasive species (Nei et al., 1975; Allendorf and Lundquist, 2003; Dlugosch and Parker, 2008). The general consensus is that *P. longicornis* originated in the Old World tropics, but to narrow this down further to a specific sub-region has been somewhat controversial (Wetterer, 2008). We did not find evidence for reduced mtDNA diversity in any sampled subregions of the Old World. Also, mtDNA structure within Clade II appears to be less associated with *Wolbachia* infections, and inferring the origin of this ant using mtDNA patterns of variation in Clade II remains challenging primarily due to insufficient sampling in certain areas (Appendix 11). Identification of the native range of *P. longicornis* on a finer geographic scale is further obscured by presumed frequent human-mediated dispersals, a multi-century old invasion history, and the potential effects of *Wolbachia* infections on mtDNA variation. However, it is interesting to note that haplotypes from the northern part of India and Nepal (Himalayan region) (Hap31 and Hap37) are divergent from other haplotypes in Clade I and form a clade sister to all other haplotypes in this clade (Fig. 3.2), implying the populations in Himalayan region might be the source of invasive populations of Clade I. More comprehensive sampling and additional nuclear data from queens and males may help efforts

to identify the likely origin and to reconstruct with more confidence the invasion history of this ant.

The loss of *Wolbachia* in invasive ranges is common, but not universal, in invasive insects possibly due to founder effects or altered selection pressures in the new habitats (Shoemaker et al., 2000; Tsutsui et al., 2003; Reute et al., 2004; Yang et al., 2010; Nguyen et al., 2016). For example, both the red imported fire ant, Solenopsis invicta, and the Argentine ant, Linepithema humile, had higher Wolbachia infection prevalences in their native populations compared with introduced populations where the symbionts are nearly absent (Shoemaker et al., 2000; Tsutsui et al., 2003; Reuter et al., 2004; Yang et al., 2010). However, we did not observe a similar phenomenon in P. longicornis. Both *wLonA* and *wLonF* are found throughout geographic range of *P. longicornis*, including known invasive areas such as North America and Caribbean (Fig. 3.6). Nevertheless, a few individuals in clade I (*w*LonA lost) and subclade II-3 (*w*LonF lost) have lost *Wolbachia* and this loss appears to be stochastic. The loss of *Wolbachia* likely is attributable to imperfect maternal transmission or natural curing events (Stevens and Wicklow, 1992; Hoffmann and Turelli 1997; Clancy and Hoffmann, 1998).

Our simulation results suggest *wLonA* was acquired by the common ancestor of Clade I, and the fitness advantage associated with harboring *wLonA* infections compared with uninfected ants may have facilitated the spread of *Wolbachia* and the associated mtDNA haplotype. One possible fitness advantage of harboring wLonA infections could be Wolbachia-induced cytoplasmic incompatibility (CI). Although spread of *Wolbachia* inducing CI in haplodiploid species appears to be less efficient than in diploid species (Vavre et al., 2000), limited movement of *P. lonigicornis* (Trager, 1984; Harris and Berry, 2005) could have enabled *Wolbachia* to increase in frequency within small local populations through genetic drift, allowing the bacterium to exceed the threshold frequency for spread in a host population *(*Vavre et al., *2003)*. While this possibility remains to be tested, a survey of *Wolbachia* prevalence across numerous ant species appears supportive that *Wolbachia* infections generally are more prevalent in ant species have limited mobility (i.e. reproducing by budding or fusion) (Wenseleers et al., 1998).

In contrast, *w*LonF appears to have been gained and lost multiple times in *P. longicornis* over evolutionary time and has had little or no significant effect on host mtDNA variation. One possible explanation for this pattern is that *w*LonF is simply a passive passenger in longhorn

crazy ant (e.g., having negligible fitness effects), and persists because the rate of *w*LonF loss occurs roughly at the same rate as horizontal transmission (Hoffmann, Clancy, and Duncan, 1996; Charlat, 2004; Bouwma and Shoemaker, 2011). Invasive species may acquire new *Wolbachia* in their new environments (Rocha et al., 2005; Himler et al. 2011; Schuler et al., 2013). For example, the North American fruit fly, *Rhagoletis cingulata* (Diptera: Tephritidae) acquired a new *Wolbachia* strain through interspecific horizontal transmission from the Eurasian endemic *R. cerasi* (Schuler et al., 2013). However, supergroup F *Wolbachia*, to the best of our knowledge, has only been discovered in a single ant species, *Ocymyrmex picardi* (Russell et al., 2009), suggesting that the prevalence of this variant is very low in ants, and that acquisition of this variant from other sympatric ant species in the introduced range of *P. longicornis* appears unlikely. Effective and efficient horizontal transmission of *Wolbachia* depends on intimate ecological associations that provide opportunities to bring *Wolbachia* into close contact with novel hosts (Sintupachee et al., 2006; Stahlhut et al., 2010; Boivin et al., 2014). Host-parasitoid associations have been commonly suggested as a route of the *Wolbachia* horizontal transmission, and evidence this has occurred includes between host and parasitoid (Heath et al., 1999), between hosts (Ahmed et al., 2015), and between parasitoids sharing the same host (Huigens et al., 2000; Huigens et al., 2004). Other putative ecological associations for successful *Wolbachia* horizontal transmission are prey-predator and parasite-host associations (Le Clec'h et al., 2013; Brown and Vett, 2015). One well-known case for the latter involves an inquiline social parasite (*Solenopsis daguerrei*) and its ant host (*S. invicta*) (Dedeine et al., 2005). While no social parasites have been reported in *P. longicornis* to date, colonies of this ant often host a variety of arthropods, such as the ant cricket *Myrmecophilus americanus*, the beetle *Coluocera maderae* and the ant mite *Macrodinychus multispinosus* (Wollaston, 1854; Wetterer, 2008; Wetterer and Hugel, 2008; Lachaud et al., 2016). These arthropods, termed "myrmecophiles", represent good candidates for *Wolbachia* transfer because they all have intimate ecological associations and interactions with their ant hosts (Kronauer and Pierce, 2011). A future *study of Wolbachia in the organisms* ecologically associated with *P. longicornis* may uncover the routes and mechanisms underlying *Wolbachia* horizontal transmission in this ant.

3.5 References

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Chapter 4 Phylogenetic evidence for horizontal transmission of *Wolbachia* among ants and ant crickets

4.1 Introduction

Wolbachia are widespread maternally-transmitted intracellular bacteria, present in approximately half of all arthropod species (Zug et al., 2012; Weinert et al., 2015). Discordant phylogenies between *Wolbachia* and their hosts suggest that *Wolbachia* infections also include horizontal transmission (HT) between species (Baldo et al., 2006, 2008; Raychoudhury et al., 2009; Ahmed et al., 2016), which is analogous to an epidemiological process driven by the ability of a pathogen to invade and maintain in novel host populations (Bailly-bechet et al., 2017). *Wolbachia* must pass three main filters before successfully colonizing a new host species (Vavre et al., 2003). First, *Wolbachia* must come into physical contact with the potential host (encounter filter), then evade the host's immune system and replicate in the new host (compatibility filter). Whether *Wolbachia* infection can reach a certain threshold to ensure its persistence in the population represents the third filter (invasion filter). The community composition may affect filter stringency and thus shape the epidemiological patterns of *Wolbachia* in a community. Communities composed of generalist or specialist species will affect both encounter and compatibility filters (Stahlhut et al., 2010; Boivin et al., 2014). For example, the intimate interspecific interactions by specialists are predicted to favor *Wolbachia* transmission. However, these interactions may also restrict the transmission sources to a few species (Boivin et al., 2014).

An ecological interaction between an infected and uninfected species is considered necessary for interspecific *Wolbachia* transmission (Hurst et al., 1992; Stahlhut et al., 2010; Kittayapong et al., 2003). For example, *Wolbachia* HT between ant hosts and their inquiline ants most likely occurs through intimate contact (VanBorm et al., 2003; Dedeine et al., 2005; Tolley et al., 2019). Ant nests are often utilized by other non-ant invertebrates (Kronauer and Pierce, 2011), offering an excellent opportunity to test if *Wolbachia* HT remains feasible among distantly related species. Kleptoparasitic ant crickets (Orthoptera: Myrmecophilidae) are an intriguing group to test for *Wolbachia* HT at the inter-ordinal level. Since different ant cricket species display differential host specificity and integration levels, rendering them suitable for examining how host specificity/integration shapes *Wolbachia* infection patterns. The host-specialist ant cricket *Myrmecophilus albicinctus* engages in intimate behavioral interactions including trophallaxis with the yellow crazy ant (*Anoplolepis gracilipes*), and possesses low survivorship in the absence of the ants (Komatsu et al., 2009). In contrast, host-generalist *M. quadrispina* feeds independently, often escapes ant attack by swift movements, and is capable

of surviving without ants (Komatsu et al., 2009). There are also some single-host ant cricket species that show no signs of intimacy toward their host and are termed non-integrated host-specialists (Komatsu et al., 2013).

In the present study, we hypothesize that integration level toward host ant and/or host specificity of ant crickets are crucial in governing *Wolbachia* HT between ant and ant crickets. We conducted an extensive *Wolbachia* survey in ant crickets, attempted to reconstruct history of *Wolbachia* HT, and examined relationships between ant crickets of different host specificity/integration levels and *Wolbachia* infection patterns. We hypothesize that 1) inter-ordinal transfer of *Wolbachia* occurs frequently among ant crickets and ants; 2) integrated host-specialist ant crickets most likely share the same (or similar) *Wolbachia* with their ant hosts; 3) generalist ant crickets likely have a higher *Wolbachia* diversity than specialists due to a higher chance of interacting with ants harboring different types of *Wolbachia*.

4.2 Materials and methods

Seven ant cricket species were used in this study, including two integrated specialist species [*M. albicinctus* (n = 38) and *M. americanus* (n = 40)], three non-integrated specialist species [*M. antilucanus* (n = 26), *M. dubius* (n = 23), *M. hebardi* (n = 26)], and two generalist species [*M. quadrispina* (n = 31) and *Myrmophilellus pilipes* (n = 23)]. Most of the ant cricket species were collected from Asia (Appendix 12).

Myrmecophilus albicinctus, *M. antilucanus*, *M. dubius*, and *M. hebardi* are specialists associated with *A. gracilipes*; *M. americanus* is associated with the longhorn crazy ant, *Paratrechina longicornis* (Hsu et al., 2019; Wetterer and Hugel, 2008). The two host-generalist species were reported in nests of more than ten ant species each (Komatsu et al., 2009; Komatsu and Maruyama, 2016; Hsu et al., 2019), and our generalist samples were collected from ant colonies of six ant species (Appendix 12).

Genomic DNA was extracted from legs of ant cricket using the Gentra Puregene cell and tissue kit (Qiagen, USA). To detect *Wolbachia* infection, polymerase chain reaction (PCR) was employed to amplify partial *Wolbachia* surface protein gene (*wsp*) [primers were listed in Table 4.1, and PCR conditions follow (Tseng et al., 2019)], with inclusion of positive control and blank (ddH_2O). The multi-locus sequence typing (MLST) gene sequences of *Wolbachia* (*hcpA*, *ftsZ*, *gatB*, *coxA* and *fbpA*; 2,565 bp) from the ant crickets with single infection were amplified following Baldo et al. (2006). Primers and PCR conditions followed the description on

PubMLST (https://pubmlst.org/wolbachia/info/protocols.shtml). When necessary, to distinguish multiple sequences from individual crickets with multiple *Wolbachia* infections, the amplified products of *wsp* gene were cloned using the TOPO TA cloning kit (Invitrogen, United States). Twenty colonies were selected from each PCR reaction and sequenced. To further confirm the infection status of individuals with multiple infections, an additional four specific primer sets were designed based on the sequencing results of the cloning experiment (Table 4.1), and each amplicon was also sequenced.

Table 4.1 Primer sequences and PCR conditions used in this study

Wolbachia type	name	Primer sequences for *wsp* gene (5'-3')	Ta (°C)	Size (bp)	Reference
Wolbachia universal	81F	TGGTCCAATAAGTGATGAAGAAAC	50	610	(Zhou et al., 1998)
	691R	AAAAATTAAACGCTACTCCA			
*w*Msp4	M4F	GGACACAGACATTCATAATCCA	54	308	This study
	M4R	TATAGGTTTGACCATCCACG			
*w*Msp5	M5F	AAAGCTTTTGATCCTTTCA	54	408	This study
	M4R	GCTAGCACCATAAGARCCA			
*w*Mame1	A1F	AAGGTGATAAAGATCAAGATCCTT	54	439	This study
	A1R	TACCATCACCCTTAGTTGTTGCAT			
*w*Mame2	A2F	AGATAATAAAGACCAAGACCT	54	285	This study
	A2R	GGACTCTTTAAAGGATTGCTA			

To rule out the possibility that detected *Wolbachia* were derived from parasitic filarial nematodes frequently found inside insects (Fox, 2018), we screened for the presence of filarial nematodes using two polymerase chain reaction assays involving two nematode universal primer pairs [5.8s-1/KK-28S-22 (Barrière and Félix, 2006); SSU18/SSU26R (Floyd et al., 2002)] that amplify the ITS2 region and 18S RNA gene of nematodes, respectively. A PCR mixture was set up in a reaction volume of 25 μl using Takara EmeraldAmp Max PCR Master Mix (Takara, Japan). PCRs were carried out following the procedures described in main text with slight modifications (56 °C as annealing temperature for ITS2 region; 50 °C for 18S RNA gene). We detected no sign of filarial nematodes in any ant cricket samples.

The phylogenetic status of identified *Wolbachia* strains and co-phylogenetic patterns of *Wolbachia* and the cricket hosts were examined based on three datasets: (1) *wsp* gene (2) MLST dataset, and (3) partial mtDNA *cytb* gene of the crickets. *Wolbachia* strains were characterized by comparisons against sequences available in the GenBank and PubMLST databases. We estimated the *wsp* and ant cricket phylogenies using a maximum-likelihood (ML) method with

RAxML Blackbox web-servers (Kozlov et al., 2019), and phylogenies of *Wolbachia* MLST were inferred using both ML and Bayesian method with RAxML Blackbox and ClonalFrame 1.1 (Didelot and Falush, 2007). Alignment of *wsp* dataset was constructed on the GUIDANCE2 Server (Penn et al., 2010) based on codons using the MAFFT algorithm (Katoh and Standley, 2013), and ambiguous alignments with the confidence score below 0.7 were excluded (417 bp were remained). The nucleotide substitution models and the best partitioning schemes were estimated with PartitionFinder version 2.1.1 (Lanfear et al., 2012) using the Akaike information criterion and a heuristic search algorithm. The best partitioning scheme selected by PartitionFinder for *wsp* gene was data partitioned by codon positions under the GTR+I+G model of rate substitution. We estimated the *wsp* phylogeny using a maximum-likelihood (ML) method with RAxML Blackbox web-servers (Kozlov et al., 2019) implementing the optimal substitution model and partitions estimated in PartitionFinder.

Wolbachia MLST loci were concatenated and aligned using MUSCLE as implemented in MEGA 6 with default settings (Tamura et al., 2013). ML phylogeny was inferred using RAxML Blackbox under the GTR+I+G model of rate substitution, and data partitioned by both gene and codon position (7 partitions) as suggested by the PartitionFinder version 2.1.1 (Lanfear et al., 2012). Bayesian analyses were conducted using ClonalFrame 1.1 (Didelot and Falush, 2007). Two independent runs were performed with 1,000,000 generations each, a sampling frequency of 1,000, and a burn-in of 50%. A 50% majority rule consensus tree was built from combined data from the two independent runs.

The sequence of partial mtDNA *cytb* gene for ant crickets were obtained from a previous study (Hsu et al., 2019)(GenBank accession number: MN064914-MN065077), and aligned using MUSCLE as implemented in MEGA 6 with default settings. ML phylogeny was inferred using RAxML Blackbox under the GTR+I+G model of rate substitution, with data partitioned by codon positions as suggested by the PartitionFinder version 2.1.1 (Lanfear et al., 2012).

The *Wolbachia* MLST gene sequences of *M. americanus* were characterized using genome sequences generated by the high-throughput sequencing method. DNA libraries were prepared from genomic DNA of three *M. americanus* collected from Taiwan using the Truseq Nano DNA HT Sample Prep Kit (Illumina, USA) for 350 bp inserts, and each DNA library was sequenced on the Illumina Hiseq 4000 platform by Genomics BioSci and Tech Corp (Taipei, Taiwan), generating 150 bp paired-end reads. Trimmomatic 0.36 (Bolger et al., 2014) was employed to

remove adaptor sequences and trim bases with quality lower than 20 (QV20). The *wsp* sequences in *M. americanus* were identical to *Wolbachia* strains *w*Msp4 and *w*Msp5 from ant crickets, and *w*LonF from longhorn crazy ant, *Paratrechina longicornis* (see Results for more details). To confirm *Wolbachia* strain identity detected in *M. americanus*, we mapped the sequencing reads of *M. americanus* onto the MLST reference sequences from *w*Msp4, *w*Msp5 and *w*LonF using bowtie2 v2.3.3 (Langmead and Salzberg, 2012) in the local alignment mode. Integrative Genomics Viewer (IGV version 2.5.3) was used to visualize the mapping results (Robinson et al., 2017). Multiple reads matched with the entire reference sequences perfectly, with one exception: the reference sequence *fbpA* of *w*LonF was only partially aligned (Appendix 13). Therefore, a primer pair, [FbpwLonF-F (5'- GCTCCAATTCTTTGCATTCAA-3') and FbpwLonF-R (5'- CCAATTCGTTTGGATAACGAT-3')], was designed to amplify the *fbpA* sequence unique to *w*LonF in *M. americanus* samples. The PCR conditions included an initial denaturation step at 95°C (3 min) followed by 35 cycles of 94°C (30 s), 55°C (30 s), 72°C (1 min) and a final extension phase at 72°C (7 min). The PCR amplicons were purified and sequenced. A total of five *M. americanus* infected with *w*Mame1 were sequenced in both directions by using the specific primers. The results indicated that the sequences obtained from *M. americanus* were identical to the *fbpA* sequence of *w*LonF. Summing up, we conclude that the *Wolbachia* in *M. americanus* were identical to *w*Msp4, *w*Msp5 and *w*LonF at both *wsp* and MLST loci, assuming no sequence recombination among strains.

We evaluated the influence of ant cricket type on the prevalence of *Wolbachia* by generalized linear mixed model (GLMM) using the '*lme4*' package (Bates et al., 2014). We treated the presence or absence of *Wolbachia* infection as the response variable, the type of ant crickets was a fixed effect, and the species was included as a random factor nested within type of ant crickets. We conducted post-hoc analyses for pairwise comparisons with Tukey's HSD, using the glht function in the '*multcomp*' package (Hothorn et al., 2008).

4.3 Results

We found *Wolbachia* infection frequency varied across different ant cricket species (Fig. 4.1A). We identified ten *Wolbachia* strains from the studied ant crickets based on the *wsp* gene sequence: *w*Mame1, *w*Mame2, and *w*Msp1-*w*Msp8. Three of which (*w*Mame1, *w*Msp4, and *w*Msp6) had *wsp* sequences identical to strains previously reported from ants (Table 4.2). Three others (*w*Msp2, *w*Msp3, and *w*Msp7) had sequences that differed from known ant-infecting

strains by less than 1% (Table 4.2).

We excluded *w*Mame1 and *w*Mame2 from the MLST analysis because individual crickets bearing one of the two strains were always found to be infected with other closely related strains, while the remaining eight strains, *w*Msp1-*w*Msp8, are represented by six unique MLST (Table 4.3). These strains were identified as members in either *Wolbachia* supergroup A or F (Fig. 4.2). Most ant crickets were infected with supergroup A *Wolbachia*, and *w*Msp4 was among the most widespread strain, which was shared among four species (Fig. 4.1A). Supergroup F *Wolbachia* was found in two phylogenetically distant species, *M. americanus* and *M. quadrispina* (Fig. 4.1A). Comparison of phylogenetic trees of host and *Wolbachia* indicated no evidence of cricket-*Wolbachia* co-divergence (Fig. 4.1A). *Myrmecophilus americanus* harbored the highest *Wolbachia* prevalence and diversity. Most infected *M. americanus* had more than two *Wolbachia* strains (triple infection: 43%; quadruple infection: 55%), while single or double infections were common in other species (Appendix 12). The *Wolbachia* prevalence was higher for integrated specialists than the other two types (Fig. 4.1B), and the differences were statistically supported (GLMM, Tukey contrast test, $P < 0.05$; Table 4.4).

Phylogeny analysis of the *wsp* gene revealed *Wolbachia* strains isolated from ant crickets were scattered across the phylogenetic tree (Fig. 4.3A). Supergroup A *Wolbachia* from ant crickets were often clustered with *Wolbachia* of ant origin. For instance, the *wsp* sequences of *w*Msp4 and *w*Msp6 were most similar to those from ants (Fig. 4.3B, Table 4.2). *w*Msp4 and *w*Msp7 are virtually identical to *Wolbachia* ST-57 (ant origin, host: *Camponotus leonardi*)(Fig. 4.3C, Table 4.2). The *wsp* sequence of wMsp6 was identical to those isolated from ants, weevils, and lepidoptera (Table 4.2). The MLST type of *w*Msp6 was identical to a sequence type previously identified in the MLST database, ST-19, which was found in ants, lepidoptera, beetles, and wasps (Fig 4.3C).

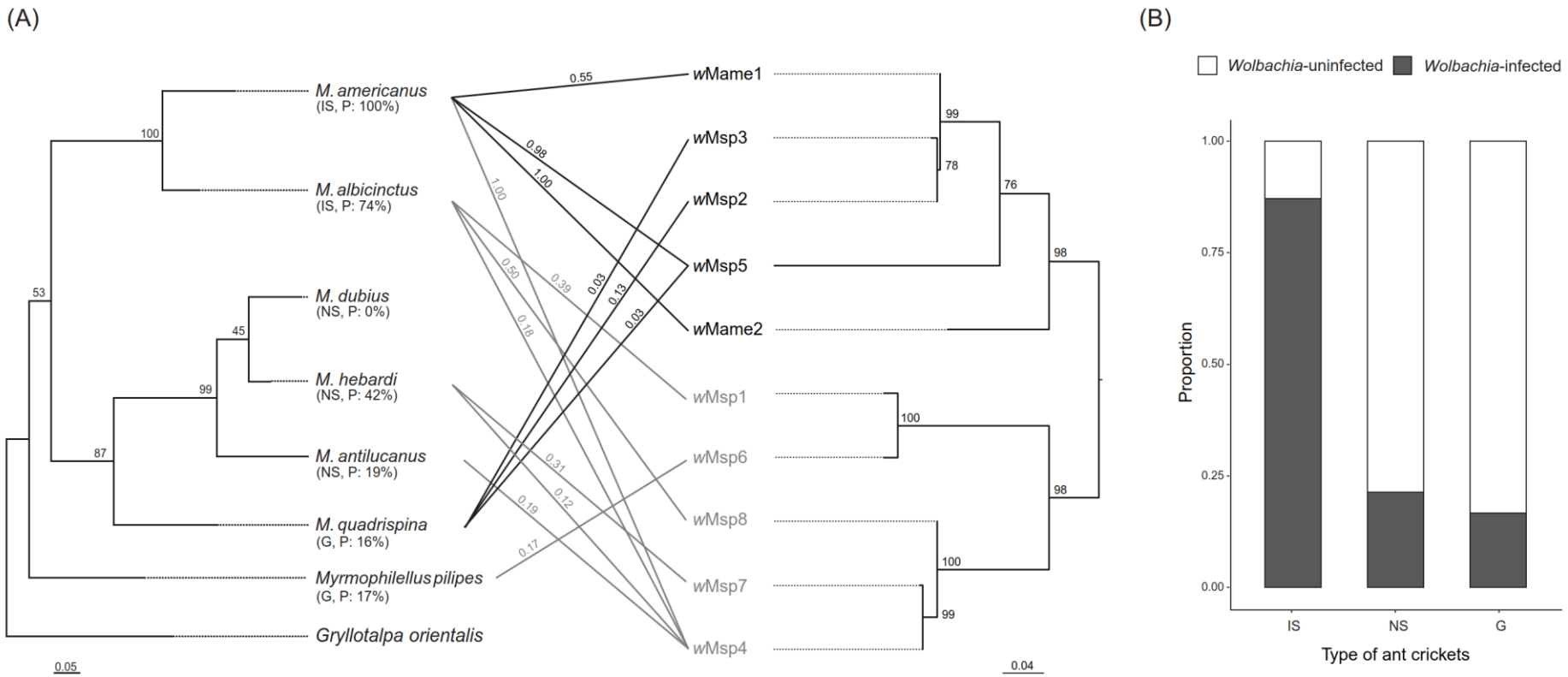

Figure 4.1 (A) Phylogenetic patterns of ant crickets (left) and corresponding *Wolbachia* strains (right). IS, NS, and G denote integrated specialist, non-integrated specialist, and generalist, respectively. P denotes *Wolbachia* prevalence in each ant cricket species. Host-*Wolbachia* associations are indicated by lines (black: supergroup A; gray: supergroup F), and the number above the line indicates infection rate of each *Wolbachia* strain. (B) *Wolbachia* infection rate in three types of ant crickets.

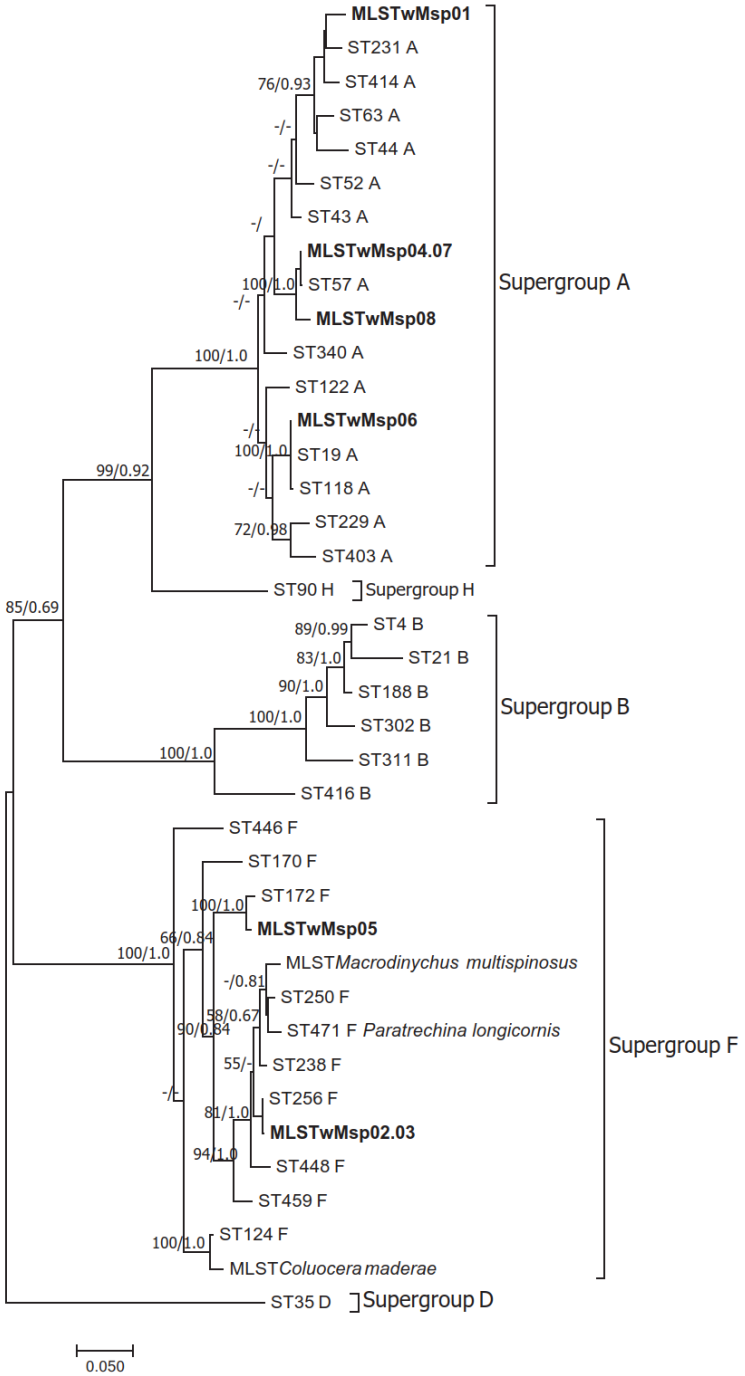

Figure 4.2 Maximum Likelihood (ML) phylogeny of *Wolbachia* based on the concatenated MLST data. The topology resulted from the Bayesian inference was similar to that inferred with ML methods. The *Wolbachia* strains obtained from ant crickets are indicated in bold. ML bootstrap values (left) and Bayesian posterior probability (right) are given (only values > 50% are shown).

Table 4.2 Sequence comparisons of *wsp* between *Wolbachia* from tested ant crickets and those found in GenBank and PubMLST databases

Strain	GenBank accession no./ PubMLST id	Sequence similarity	Host	Common name
wMame1	KU527459	100%	*Paratrechina longicornis*	longhorn crazy ant
	id: #1828	100%	*Paratrechina longicornis*	longhorn crazy ant
wMame2	KC161941	97.22%	*Tachinid* sp.	tachinid fly
	KC161936	97.22%	*Pyralidid* sp.	pyralidid moth
wMsp1	MG797608	99.63%	*Loxoblemmus equestris*	hard-headed cricket
wMsp2	KU527459	99.62%	*Paratrechina longicornis*	longhorn crazy ant
wMsp3	KU527459	99.23%	*Paratrechina longicornis*	longhorn crazy ant
wMsp4	KU527484	100%	*Tetramorium lanuginosum*	wooly ant
	KC137165	100%	*Odontomachus* sp.	trap jaw ant
	GU236978	100%	*Aulacophora nigripennis*	leaf beetle
	MG551859	100%	*Octodonta nipae*	nipa palm hispid beetle
	id: #120	100%	*Camponotus leonardi*	carpenter ant
wMsp5	KM078883	94.81%	*Chorthippus parallelus*	meadow grasshopper
	JN701984	94.02%	*Chorthippus parallelus*	meadow grasshopper
wMsp6	KU527480	100%	*Tapinoma sessile*	odorous house ant
	KU527478	100%	*Tapinoma melanocephalum*	ghost ant
	HQ602874	100%	*Ceutorhynchus neglectus*	weevil
	AB024571	100%	*Ephestia cautella*	almond moth
	id: #111	100%	*Technomyrmex albipes*	white-footed ant
	id: #115	100%	*Leptomyrmex* sp.	spider ant
	id: #141	100%	*Pheidole* sp.	big-headed ant
	id: #146	100%	*Leptogenys* sp.	razorjaw ant
	id: #116	100%	*Myrmecorhynchus* sp.	ant
	id: #124	100%	*Pheidole plagiara*	big-headed ant
	id: #125	100%	*Pheidole sauberi*	big-headed ant
	id: #1827	100%	*Paratrechina longicornis*	longhorn crazy ant
	id: #135	100%	*Ochetellus glaber*	black household ant
	id: #13	100%	*Ephestia kuehniella*	mediterranean flour moth
	id: #123	100%	*Ornipholidotos peucetia*	glasswings
	id: #451	100%	*Aricia artaxerxes*	northern brown argus
wMsp7	KU527484	99.81%	*Tetramorium lanuginosum*	wooly ant
	KC137165	99.81%	*Odontomachus* sp.	trap jaw ant
	GU236978	99.81%	*Aulacophora nigripennis*	leaf beetles
	MG551859	99.81%	*Octodonta nipae*	the nipa palm hispid beetle
	id: #120	99.81%	*Camponotus leonardi*	carpenter ants
wMsp8	EF219194	95.10%	*Ixodes ricinus*	castor bean tick

Table 4.3 MLST allelic profiles of the *Wolbachia* strains recovered from the tested ant crickets

Strain	id	*gatB*	*coxA*	*hcpA*	*ftsZ*	*fbpA*
*w*Msp01	1926	294	291	331	251	459
*w*Msp02	1927	170	147	178	252	125
*w*Msp03	1928	170	147	178	252	125
*w*Msp04	1929	49	44	297	42	49
*w*Msp05	1930	295	94	332	85	460
*w*Msp06	1931	7	6	7	3	8
*w*Msp07	1932	49	44	297	42	49
*w*Msp08	1933	49	44	333	253	49

Supergroup F *Wolbachia* in ant crickets were frequently clustered with those in phylogenetically distant hosts. *w*Msp5 was clustered with *Wolbachia* from scale insects, grasshoppers, and scorpions based on *wsp* gene (Fig. 4.3B), and its MLST type was most similar to *Wolbachia* from a termite (ST172) (Fig. 4.3D). The *wsp* sequences of *w*Mame2 differed from all known *Wolbachia* strains. Three *Wolbachia* strains from ant crickets, namely *w*Msp2, *w*Msp3 and *w*Mame1, were closely related to each other (pairwise identity > 99.3 %) and clustered with other *Wolbachia* strains (*w*Mul and *w*LonF) isolated from their ecologically associated hosts (Fig. 4.3B). *w*LonF was detected in *P. longicornis* (Tseng et al., 2019), while *w*Msp2-3, *w*Mame1 and *w*Mul were detected from ant guests associated with *P. longicornis*: generalist ant cricket *M. quadrispina* (*w*Msp2-3), integrated specialist ant cricket *M. americanus* (*w*Mame1), and parasitoid mite *Macrodinychus multispinosus* (*w*Mmul) (Fig 4.3B, Table 4.2). These strains also were highly similar to each other at MLST loci (pairwise identity > 98.7 %). We, however, failed to recover the corresponding MLST gene sequences of *w*Mame1 because co-infection of *w*Msp4-5 and *w*Mame2 was invariably found in *w*Mame1-infected individuals (Appendix 12). We argue that the *w*Mame1 was identical with *w*LonF at both *wsp* gene (Table 4.2) and MLST loci, given the finding that all the five MLST alleles of *w*LonF were detected in the genome sequence of *M. americanus* (Appendix 13).

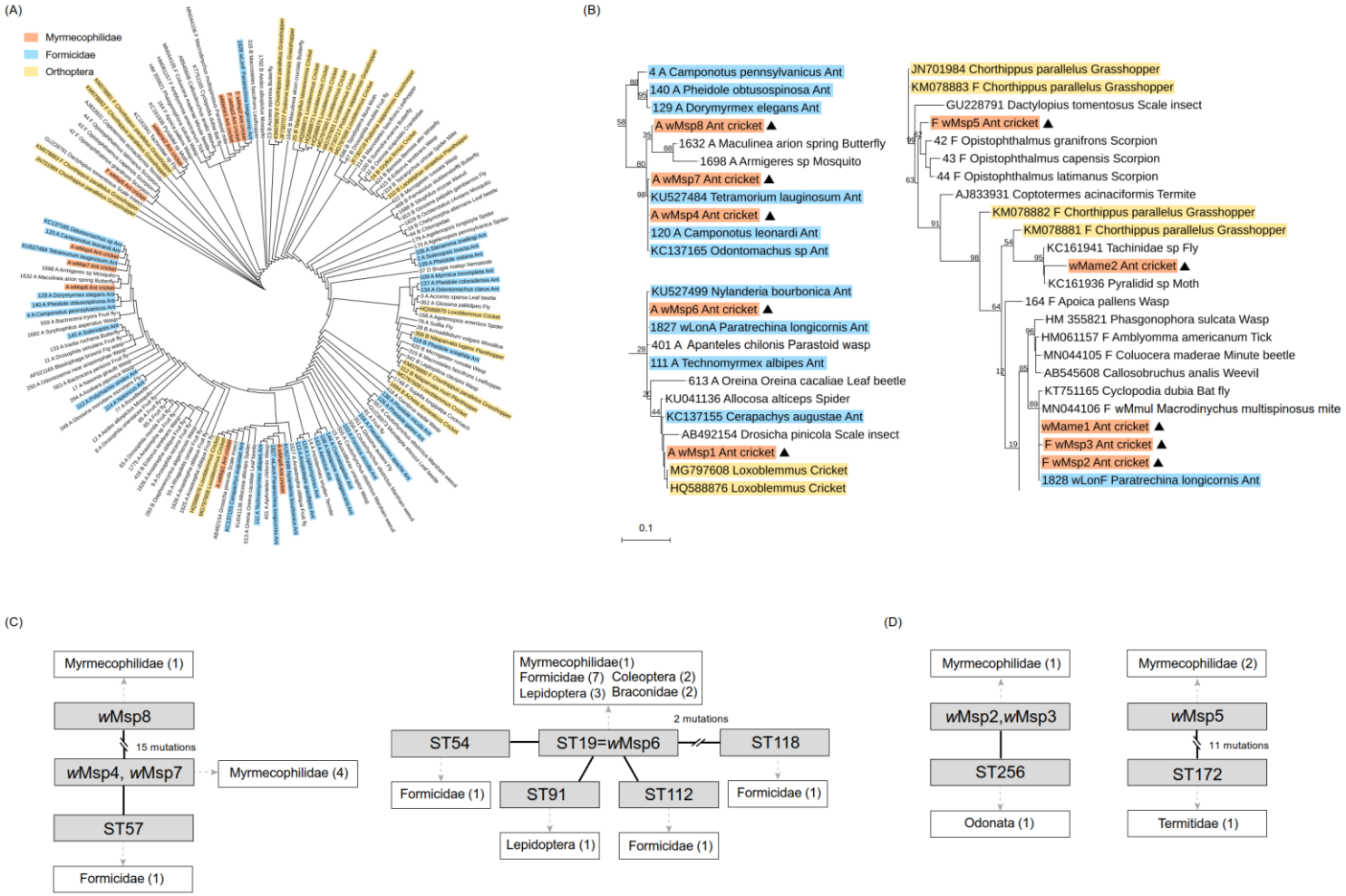

Figure 4.3 Genealogical relationships of *Wolbachia* strains. (A) Phylogenetic tree and (B) subtrees for *Wolbachia* strains based on the *wsp* gene. Strains are represented by the infected arthropod host species with which they are associated. *Wolbachia* from ant crickets, ants, and orthoptera are colored orange, blue and yellow, respectively. Sequences generated in the current study are indicated by triangles. Relationships among supergroup A *Wolbachia* (C) and supergroup F *Wolbachia* (D) strains based on MLST. Strains that differ in a single mutation are connected with a solid line. Host information was provided in white box and the number of host species is indicated in parentheses.

Table 4.4 Results of GLMMs on the effect of type of ant crickets on the presence of *Wolbachia*. GLMM includes 'type' of ant crickets (IS: integrated specialist; NS: non-integrated specialist, G: generalist) as a fixed effect and 'species' as a random effect nested within 'type'. Pairwise comparison between types are based on Tukey's post-hoc tests applied to generalized linear mixed models

Response variable	Fixed effect	Estimate	SE	Z-value	P-value
Wolbachia infection (presence/absence)	(Intercept)	-1.6802	0.93427	-1.798	0.07211
	type (IS)	4.28317	1.40371	3.051	0.00228**
	type (NS)	-0.09594	1.24002	-0.077	0.93833
Tukey's post-hoc tests	Contrast	Estimate	SE	Z-value	P-value
	IS-G == 0	4.28317	1.40371	3.051	0.00654**
	NS-G == 0	-0.09594	1.24002	-0.077	0.9967
	NS-IS == 0	-4.37911	1.37476	-3.185	0.00419**

4.4 Discussion

One major finding of this study is the extensive sharing of *Wolbachia* strains among ants and ant crickets, including three cases where ants and ant crickets share identical or nearly identical *Wolbachia* strains (*w*Mame1, *w*Msp4, and *w*Msp6). The *w*Mame1 *Wolbachia* strain is only known from *M. americanus* and its exclusive host, *P. longicornis*. The *w*Msp4 *Wolbachia* that we found in four specialist ant crickets has been previously found in ants from three subfamilies (*Myrmicinae*, *Ponerinae*, and *Formicinae*) (Table 4.2). Surprisingly, we failed to detect *w*Msp4 *Wolbachia* from the ant-crickets' hosts (i.e., *P. longicornis* and *A. gracilipes*). The *w*Msp6 (ST-19) strain was detected in the host-generalist ant cricket *Myrmophilellus pilipes*, and some *w*Msp6 (ST-19)-harboring ant species such as *Pheidole* sp. are reported as hosts of *Myrmophilellus pilipes* (Komatsu and Maruyama, 2016). Given that predation often serves a route for *Wolbachia* HT (Kittayapong et al., 2003; Hoy and Jeyaprakash, 2005; LeClec'h et al., 2013), ant-crickets may acquire novel *Wolbachia* strains through preying on host ants or stealing ant food (Henderson and Akre, 1986; Hölldobler and Wilson, 1990; Komatsu et al., 2009; Komatsu and Maruyama, 2016). Conversely, ants may acquire *Wolbachia* through preying on ant crickets.

Identical *Wolbachia* (*w*Mame1) shared between *M. americanus* and its ant host *P. longicornis* suggests the occurrence of HT. Integrated host-specialists possess high degrees of host dependence, and acquire food exclusively via trophallaxis with ants (Komatsu et al., 2009; Komatsu et al., 2010; Komatsu et al., 2017), readily providing opportunities for transferring *Wolbachia* between the two interacting parties. This pattern is consistent with the prediction in

which social interactions may facilitate *Wolbachia* HT between cohabiting species (VanBorm et al., 2003; Dedeine et al., 2005; Tolley et al., 2019). Nevertheless, the absence of shared *Wolbachia* between host-specialist *M. albicinctus* and its ant host *A. gracilipes* suggests cohabitation may not always result into successful *Wolbachia* HT.

In this study, we proposed that generalist species potentially have a higher likelihood of transmitting and/or acquiring *Wolbachia* than specialists. We found that the integrated host-specialist harbors a higher *Wolbachia* prevalence and diversity among three types of ant cricket species. Two mechanisms may explain the observed patterns: (1) a higher rate of *Wolbachia* acquisition, and/or (2) a lower rate of *Wolbachia* loss. While our study design precludes us from testing which factor is preferred, the high prevalence of *w*Msp4 in *M. americanus* partially supports the latter. *w*Msp4 is maintained with a high frequency only in *M. americanus* (Fig. 4.1A), but not other *w*Msp4-infected ant cricket species, suggesting *M. americanus* is particularly prone to *Wolbachia* infection. The reasoning behind high diversity and prevalence of *Wolbachia* in integrated host-specialists remains unclear. One speculative hypothesis is that the degree of host dependence may interact with *Wolbachia* persistence within host populations due to different selection forces operating on hosts, or factors related to lifestyle (e.g. limited dispersal) can be beneficial for the establishment of *Wolbachia* (Treanor and Hughes, 2019). Support for this observation is available in fire ants and their social parasites in which an unexpectedly high *Wolbachia* diversity was found in the social parasites (up to eight strains), but the free-living hosts rarely harbor more than one *Wolbachia* strain (Shoemaker et al., 2000; Dedeine et al., 2005).

In conclusion, we demonstrated that HT represents a prevailing path for *Wolbachia* transmission and that host life-history traits may have shaped *Wolbachia* prevalence and diversity in host populations. Further *Wolbachia* surveys on species with similar kleptoparasitic nature may uncover the generality of this phenomenon and underlying mechanisms.

4.5 References

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Chapter 5 Reproductive system and patterns of spread of *Paratrechina longicornis*

5.1 Introduction

Invasive ants are among the most damaging pests and have become a worldwide problem (Lowe et al., 2000, Holway et al., 2002, Lach and Hooper-Bùi, 2010). Understanding the dispersal dynamics of invasive species has become crucial, as it assists in predicting the rate and pathway of the spread, as well as in implementing effective control or quarantine measures if necessary (Sakai et al., 2001, Abdelkrim et al., 2005, Lawson Handley et al., 2011). Many tramp ant species share a suite of characteristics that facilitate their establishment in new environments, including polygyny, unicoloniality, and propagation by budding without a nuptial flight (Passera, 1994). For ant species that propagate through colony budding, natural dispersal range is expected to be limited due to the lack of effective dispersal by winged reproductives, and may result in a detectable pattern of isolation by distance (IBD), an increase of genetic differentiation with geographical distances, and/or spatial genetic structure of geographic populations (Wright 1943, Malécot 1955, Kimura and Weiss 1964). Such a genetic pattern has been observed in several ant species with a restricted dispersal ability, such as *Cataglyphis cursor* (Fonscolombe), *Myrmica rubra* (L.) and *M. ruginodis* Nylander (Seppä and Pamilo, 1995, Clémencet et al., 2005). Nevertheless, anthropogenic forces such as human-mediated dispersal may contribute to erase the signature of regional genetic structure, resulting into genetic homogenization and thus the absence of spatial genetic structure. This is particularly the case for many invasive ant species in the introduced ranges because they frequently travel with humans as stowaway and establish in human-disturbed areas (Tsutsui et al., 2001, Zheng et al., 2018).

In addition to dispersal mode, reproduction mode may also influence invasiveness and genetic structure of an invasive species (Sakai et al., 2001, Barrett, 2011; Rabeling and Kronauer, 2013). In most ant species, fertilized eggs become females, while unfertilized eggs develop parthenogenetically into males (arrhenotoky). Several species have, however, evolved alternative reproductive strategies such as female production through thelytokous parthenogenesis, male production through androgenesis or social hybridogenesis (reviewed in Rabeling and Kronauer, 2013; Goudie and Oldroyd 2018). Thelytoky is particularly common among invasive ant species and seems to possess evolutionary advantage to overcome the challenges associated with low population densities during colonization (Rabeling and Kronauer, 2013). Some ants have evolved a mixed mode of reproduction, where queens produce new queens asexually but workers sexually, thereby enabling them equipped with the

advantages stemmed from both sexual and asexual reproductive systems (reviewed in Wenseleers and Van Oystaeyen, 2011; Rabeling and Kronauer, 2013).

The longhorn crazy ant, *Paratrechina longicornis* (Latreille, 1802) is regarded as a significant invasive species due to its ecological impacts (Wetterer 2008). Yet, population studies of this species are surprisingly scarce despite this species arguably being the world's most widespread ant species (Wetterer 2008). A previous study demonstrated the occurrence of an extraordinary, double-clonal reproduction system in a population of *P. longicornis* from Thailand. In this population, queens are produced clonally from their mother, males are produced clonally from their fathers, and workers are produced sexually (Pearcy et al., 2011). Under this double-clonal system, workers are offspring of queen and male lineages that are genetically divergent from each other and are characterized by an excess of heterozygosity. As consequence, spatial pattern of genetic variation may be biased if analyzing worker genotype alone due to strong sex-associated structure between male- and female-derived genomes within the workers and thus requires either sexuals (male, queen and daughter queen) as alternatives or inference of male and queen lineages based on worker genotype (e.g. Darras et al., 2014).

In this study, I used the novel set of microsatellite marker (Chapter 2) to assess spatial genetic structure in worldwide populations of *P. longicornis*. One hypothesis we proposed to test in this study is that while colonies of *P. longicornis* are believed to reproduce predominantly through budding (Trager 1984, Harris and Berry 2005), close association with humans and high adaptability in urbanized habitat should allow this species to disperse as an accidental hitchhiker with anthropogenic activities, especially in the introduced ranges (Wetterer 2008). We hypothesize that if human-assisted transport plays a key role in shaping the dispersal pattern of *P. longicornis*, no genetic structuring should be observed in *P. longicornis*. As *P. longicornis* possesses an unusual genetic system whereby queens, males and workers carry different genetic make-up, parental alleles were inferred from worker genotypes using parent-offspring analyses and utilized for the spatial genetic analyses. The results are expected to lead to a better understanding of dispersal pattern of this invasive ant and to shed light on the extent in which the dispersal is due to human activities (e.g. jump dispersal), and how local spatial genetic structure is influenced by the double clonal reproductive system.

5.2 Material and methods

Sampling, DNA extraction, microsatellite genotyping and characterization

We obtained *P. longicornis* workers from field collections and from other researchers (Appendix 1). A total of 248 ant colonies were sampled across the current geographic distribution of *P. longicornis*, including 22 colonies from Northeast Asia, 81 colonies from East Asia, 71 colonies from South Asia, 9 colonies from Indian Subcontinent, 17 colonies from Oceania, 9 colonies from Polynesia, 9 colonies from North America, 2 colonies from South America, 19 colonies from Caribbean, 2 colonies from Arabia, 2 colonies from Southeastern Europe, 4 colonies from West Africa, and 1 colony from South Africa. Genomic DNA was extracted using the Gentra Puregene cell and tissue kit (Qiagen, USA) according to the manufacturer's instructions and stored at -20 °C upon usage.

To genotype all individual ants in an economic manner, we performed multiplex PCR reactions with fluorescently labeled universal primers following the strategy described in Blacket et al., (2012). Four fluorescent labeled universal primers and modified locus-specific primers with a 5' universal primer sequence tail were used. Five to six loci were amplified per multiplex reaction. Multiplex PCR reactions were conducted in a 15 µl volume containing 7.5 µl of EmeraldAmp® MAX PCR Master Mix (TaKaRa), 0.2 µM each primer, and 2-4 ng of genomic DNA. The PCR conditions include an initial denaturation step at 94°C (3 min) followed by 35 cycles of 94°C (30 s), 55°C (30 s), 72°C (30 s) and a final extension phase at 72°C (30 min). The resulting PCR products were analyzed on an ABI-3730 Genetic Analyzer (Applied Biosystems) by Genomics BioSci and Tech Co., Ltd (Taipei, Taiwan). GeneMarker program (version 2.4.0, SoftGenetics LLC) was used to visualize and score alleles. Summary statistics of novel microsatellite markers including the number of alleles (Na), Shannon's information index (I), observed heterozygosity (Ho), expected heterozygosity (He), and deviation from Hardy–Weinberg equilibrium were calculated using GenAlEx 6.5 software.

Identification of clonal multilocus lineage

In a double-clonal population, workers are offspring of queen and male lineages that are genetically divergent from each other and are characterized by an excess of heterozygosity. Our results suggested that this system is rather widespread in Asian populations of *P. longicornis* and that the queens and males always belonged to separate gene pools (See Results for more details). Therefore, population genetic analyses were conducted independently for worker,

queen and male data, and only reproductive genotypes were used for the spatial analyses. In localities where reproductive individuals were sampled with workers, parental alleles were inferred using parent-offspring analyses, while in others the paternal and maternal alleles were deduced from worker genotypes using observed allele frequencies of queen and male whenever possible.

The allele size ranges of the queen and male gene pools were determined for each locus using available reproductive genotypes as references, and were used to infer the parental alleles of workers. For example, at locus *Prl136*, worker genotypes were always combinations of a small allele 212 bp and a large allele ranging from 218 to 244 bp. The 212 bp allele was observed in queens, whereas 218 bp, 220 bp and 222 bp alleles were found in males only (Fig. 5.1). We therefore concluded that the allele 212 bp of worker was of maternal origin, while the large alleles (218-244 bp) were of paternal origin. In total, we were able to infer with confidence paternal and maternal alleles from workers for a total of 14 loci at which queens and males had non-overlapping allele size ranges (see Results and Fig. 5.1). Subsequent genetic analyses were based on the 14 loci.

In order to identify queen and male clonal lineage in our samples, pairwise genetic distances were calculated with the R package 'poppr' (Kamvar et al., 2014) using the Bruvo's distance which is based on a stepwise mutation model for microsatellites. Neighbor-Joining trees were produced based on Bruvo's distances for the queen and male datasets. Individuals with similar allelic combinations were grouped into clonal multilocus lineage (MLL, at least three individuals) based on Bruvo's distance with a cutoff of 0.1. The geographic distribution of MLLs were then visualized and mapped using Quantum GIS 3.4.2 (Quantum GIS Development Team, 2018).

Evaluation of spatial genetic structure

Spatial genetic structure was assessed using spatial principal component analysis (sPCA) implemented in the R-package adegenet (Jombart, 2008). The method sPCA summarizes genetic variation and its spatial pattern while taking both allele frequencies and the spatial autocorrelation between individuals into account (Jombart et al., 2008). The Monte-Carlo randomization tests (999 permutations) were performed to statistically test the presence of global and local spatial structure, where global structure (e.g. patches and clines) indicates a positive spatial autocorrelation that genotypes of neighboring sites tend to be similar, whereas

local structure indicates a negative spatial autocorrelation that neighboring sites tend to be dissimilar.

5.3 Results and Discussion

Genotyping data has been obtained from 78 workers, 9 queens and 5 males collected in three geographic regions, Thailand (central Thailand, 30 localities), Taiwan (Taiwan Island, 30 localities), and Japan (Okinawa Island, 18 localities). Our results revealed large differences among queen, male and worker genotypes (Fig. 5.1, Appendix 14). Remarkably high levels of heterozygosity was observed in workers in each region (Fig. 5.2). In contrast to workers, queens were confirmed to be homozygous at most of the loci. Thirty of 36 loci were homozygous in all queens (Taiwan, 7 queens; Thailand, 2 queens; 2 localities each; Fig. 5.2). When multiple queens were genotyped in a locality (2 colonies; 3 and 4 queens, respectively), all had identical multilocus genotypes as expected under thelytokous parthenogenesis.

Queens and males appeared to have distinct allele size ranges at 14 of 36 loci, while workers carried both queen and male alleles at these 14 loci (Fig. 5.1). Workers were almost invariably heterozygous for these 14 loci (except at *Prl110* where one worker was homozygous). These 'bimodal' allele size distributions suggested that workers are products of interbreeding between two divergent gene pools in the three regions. The most likely explanation for this pattern was a complete segregation of the male and female gene pools across the population. The paternal and maternal alleles of 78 workers were successfully recovered at the 14 of 36 loci based on observed genotypes of queens and males. Note that four individuals were treated as missing data due to ambiguous assignment of paternal types (one at locus *Prl110*, two at locus *Prl137*, one at locus *Prl141*, with each belonging to four different workers).

To compare genetic variation in paternal and maternal lineages, we combined the inferred parental genotypes data and observed reproductive genotypes. After removing redundant clonal genotypes within localities, we were left with 79 paternal genotypes (4 observed haplotypes and 74 inferred haplotypes) and 78 maternal genotypes (4 observed diploid genotypes and 74 inferred haplotypes). The paternal lineages displayed 44 % to 133 % more alleles than maternal lineages depending on the region. In total, 85 alleles were found in paternal lineages across the 14 loci, while only 37 alleles were recovered in maternal lineages.

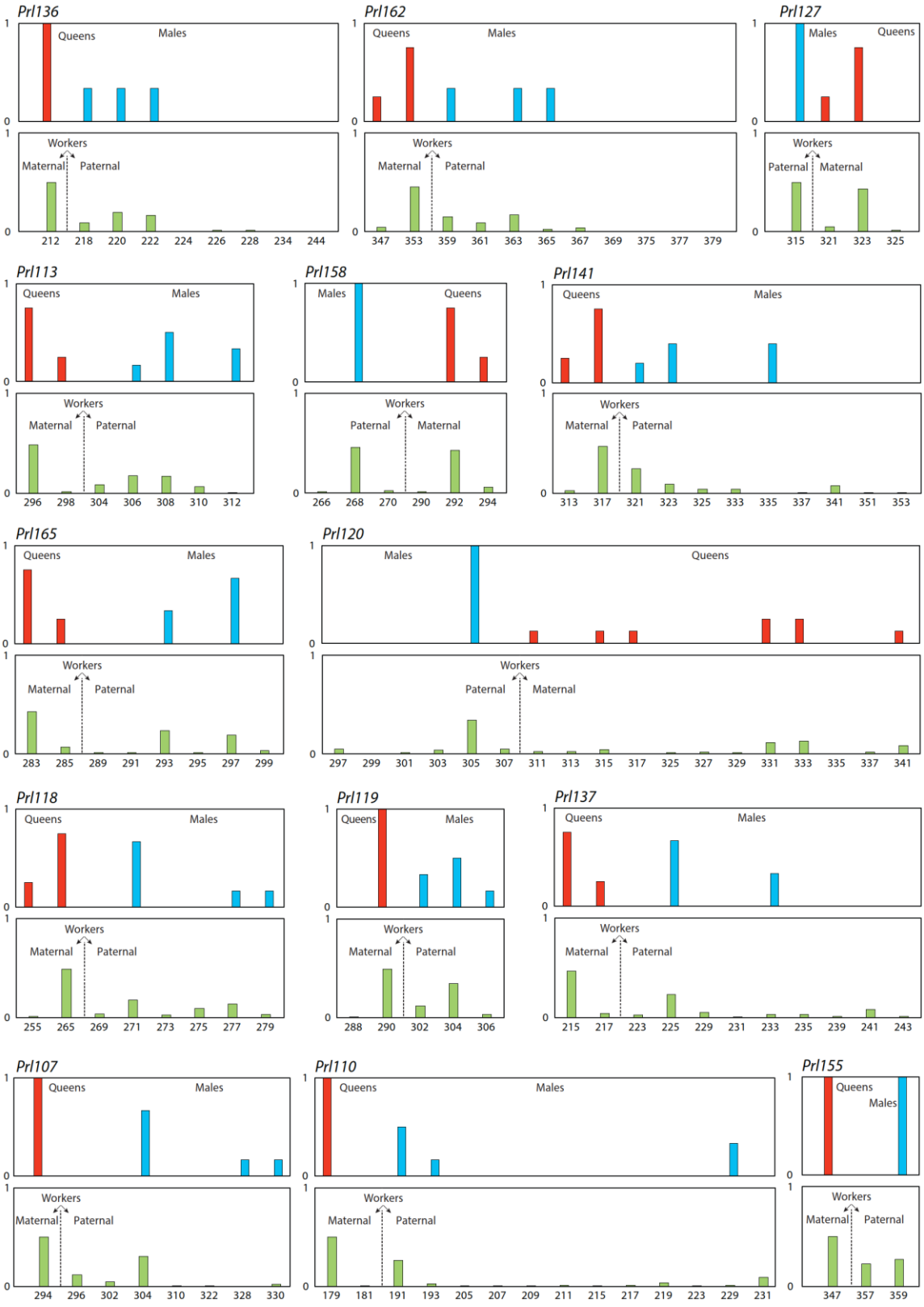

Figure 5.1 Allele frequencies in *Paratrechina longicornis* males (blue), queens (red), and workers (green) sampled from the three studied regions. Frequencies were inferred from 5 males, 9 queens, and 78 worker genotypes. This figure only presents the allele frequencies at 14 loci which were used in spatial genetic analysis. The allele frequencies at the other 22 loci were available in Appendix 14).

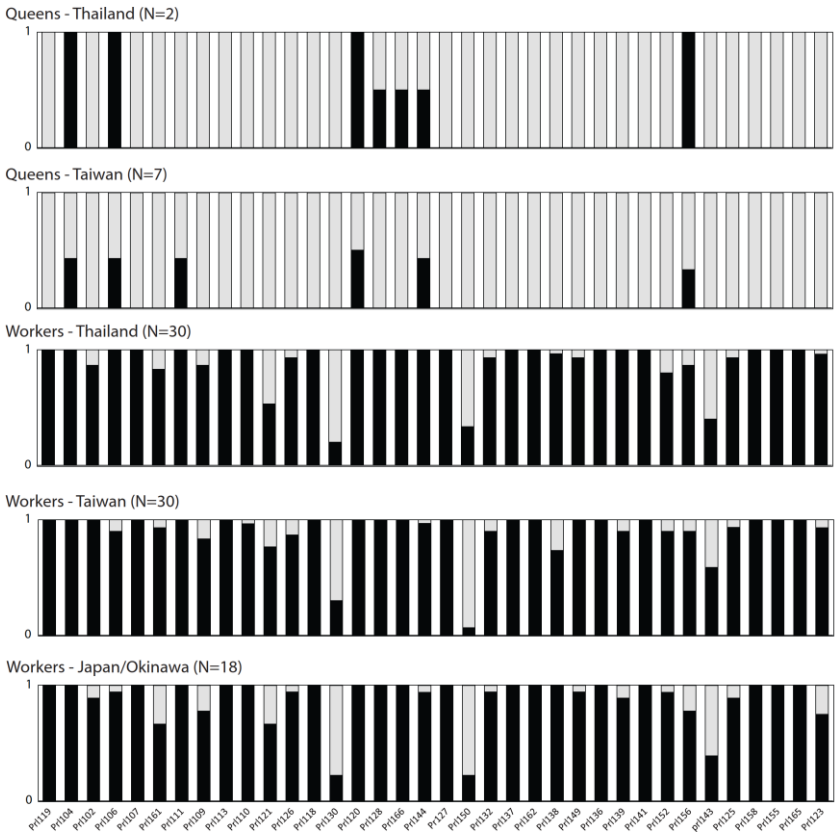

Figure 5.2 Proportion of heterozygous (black) and homozygous (gray) loci in *Paratrechina longicornis* workers and queens across the three studied regions.

A high diversity of multilocus genotype was observed in paternal lineages. Among the 79 male genotypes, 44 different multilocus genotypes were grouped into eight multilocus lineages (MLLs). Four multilocus lineages were found in all regions (MLL1, MLL2, MLL3, and MLL6), and the other two were found in two of the sampled regions (MLL4 and MLL8) (Fig. 5.3A). No clear differentiation between regions was detected among male lineages (Fig. 5.3A).

Maternal lineages exhibited comparatively low allelic richness at 13 of 14 loci. Among these, seven loci were either monomorphic or only had a single rare allele (e.g., allele frequency < 0.05), while six loci had two or three common alleles. These 13 loci were always homozygous in the nine genotyped queens. The remaining loci (*Prl120*) showed 12 different alleles and appeared heterozygous in most genotyped queens (Fig. 5.1, 5.2). This highly heterozygous locus was excluded when constructing Neighbor-Joining tree and analyzing spatial genetic pattern, as only one of two alleles was available for most of the queens. The analysis of the 13

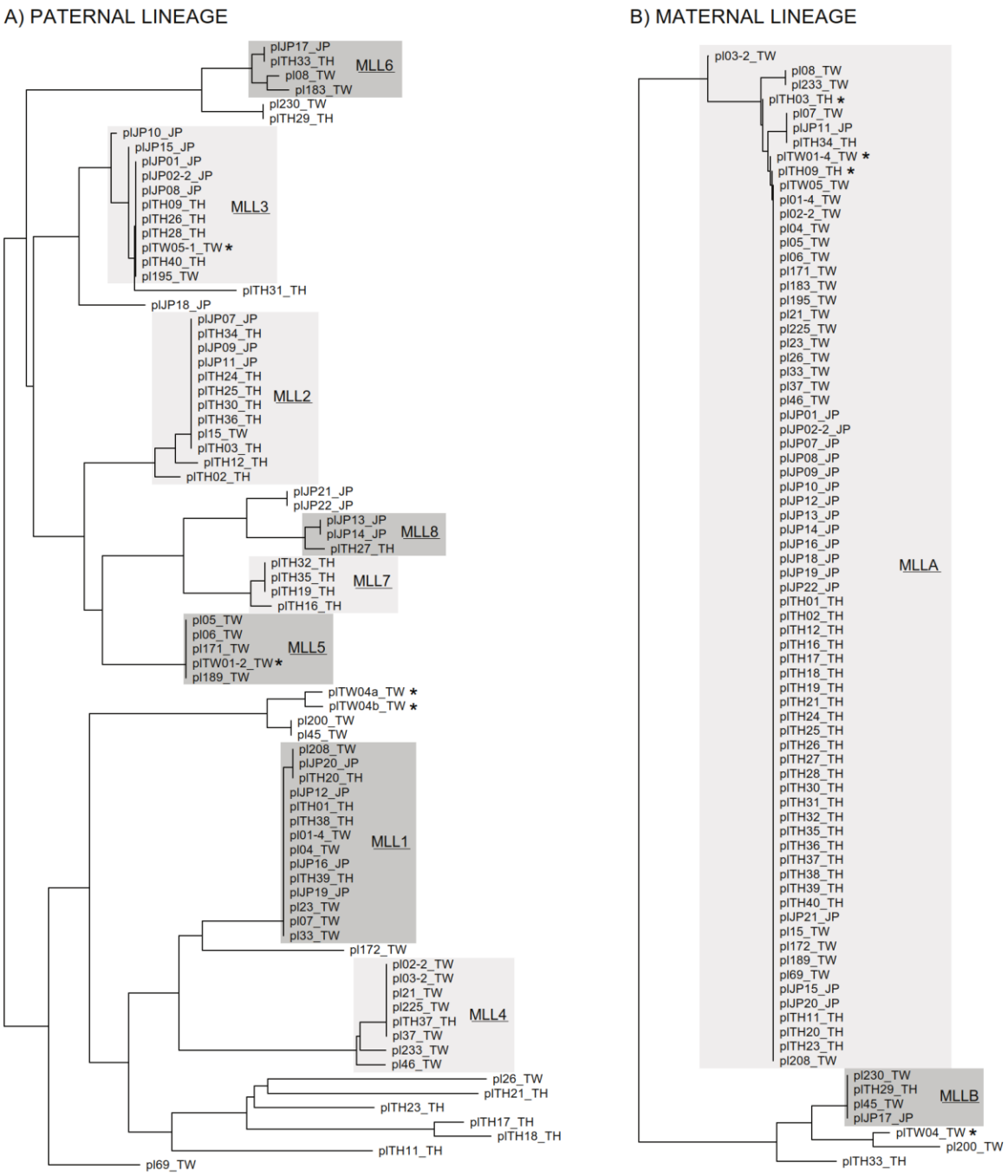

Figure 5.3 Neighbor-joining trees of (A) paternal and (B) maternal lineage of *Paratrechina longicornis* from the three studied regions. The capital letters after underline symbol denote the sampling region (TH: Thailand, TW: Taiwan, JP: Japan/Okinawa). All paternal and maternal genotypes were deduced from workers except those directly from queens and males as indicated by a star symbol.

remaining loci revealed the presence of two major maternal MLLs, namely MLLA (71 localities) and MLLB (4 localities). The two maternal MLLs were not correlated with their corresponding geographic regions, both, however, were found in Thailand, Taiwan and Japan/Okinawa (Fig. 5.3B).

Our population genetic analyses revealed dramatic differences in allelic patterns among queens, males and workers of *P. longicornis* in Thailand, Taiwan and Japan/Okinawa. The fixed heterozygosity observed in workers indicated that they were all hybrids of two divergent lineages. The high proportion of homozygous loci observed in queens supported the hypothesis that they were produced through automictic parthenogenesis (Pearcy et al., 2006; Rabeling and Kronauer, 2013). Besides, that males carry different alleles than queens was consistent with males being androgenetic clones. Altogether, our data suggested that all colonies from the three studied regions follow a double-clonal reproduction system, whereby queens are clones of their mothers, males are clones of their fathers and workers are produced by sexual reproduction (Pearcy et al., 2011). This unusual reproductive strategy has been reported in three other ant species, namely *Wasmannia auropunctata* (Fournier et al., 2005), *Vollenhovia emeryi* (Ohkawara et al., 2006; Kobayashi et al., 2008) and *Cardiocondyla kagutsuchi* (Okita and Tsuchida, 2015). These double-clonal ants are born to be successful invaders as they appear to avoid the risk of inbreeding associated with the early stage of colonization; high heterozygosity is maintained in the workers, and clonal queens can mate with their clonal brothers without any negative fitness consequence (Okamoto and Ohkawara, 2010; Pearcy et al., 2011). The fact that all these ants as well as *P. longicornis* are tramp/invasive species thriving in at least some part of their distribution is consistent to such hypothesis.

The little fire ant, *W. auropunctata* is by far the most studied double-clonal ant. In this species, queens are occasionally produced by sexual reproduction and males can be produced by arrhenotokous parthenogenesis. These recombination events reshuffle gene pools and lead to the formation of new derived clonal pairs (Foucaud et al., 2006, 2009, 2010; Mikheyev et al., 2009). By contrast, our results suggested that queens and males of *P. longicornis* belong to two separate evolutionary units that remain divergent over time. The absence of recombination between queens and males is expected to have major effects in shaping evolution of genomes in the two sexes (Sykes and West, 2005). Interestingly, our analyses revealed that males of *P. longicornis* exhibit much higher allelic richness and clonal diversity than queens. This discrepancy possibly stems from differences in mutation rates between sexes. In many organisms, the number of germline cell divisions during gametogenesis is higher in males than in females, thus leading to a male mutation bias (Sayres et al., 2011). Ploidy differences between sexes may also influence mutation rates. Recent research on yeast indeed showed that small-scale mutations are more frequent in haploids than in diploids (Sharp et al., 2018). Additional

explanations, such as variance in sex-specific difference in reproductive success (Heyer et al., 2012) or clonal sweeps on female genome cannot be ruled out.

As both reproductive castes of *P. longicornis* are produced parthenogenetically, a single colony fragment consisting both sexuals and possibly workers should be able to establish a new population (Foucaud et al., 2006; Mikheyev et al., 2009). The finding of divergent lineages within each studied region, however, suggests that populations of *P. longicornis* may have resulted from multiple introductions involving genetically distinct propagules. The clonal diversity observed within each region unlikely stems from post-introduction genetic drift as some queen and male lineages co-existed in all three regions studied. *Paratrechina longicornis* represents one of the most frequently intercepted ant species at the US, New Zealand and Taiwan borders (Ward et al., 2006; Bertelsmeier et al., 2018; Lee et al., unpublished data), and is generally intercepted in commodities from different regions. Such interception patterns not only indicate a high degree of propagule pressure for this species, but also serve as empirical support for the possibility of multiple, repeated introductions of this ant from genetically divergent populations into a single region.

Like many other invasive ant species, *P. longicornis* is believed to disperse primarily by budding (Trager, 1984; Harris and Berry, 2005), and thus a spatial pattern of genetic variation should be registered among colonies within a region. Nevertheless, our results reveal no spatial pattern in both paternal and maternal genetic lineages in the three studied regions (Fig. 5.3, Table 3). Genetically distinct MLLs, instead of forming discrete clonal patches, were found to co-exist in a relatively smaller geographic scale, suggesting that the spread of this species is mainly associated with anthropogenic dispersal (human-assisted long distance dispersal) rather than natural colony budding. This study, along with others involving various tramp ant species (e.g. *Tapinoma melanocephalum*; Zheng et al., 2018), demonstrates that the high level of tolerance/affinity to humanized habitats serves as an adaptive trait that allows these ants not only to travel around the globe as a commensal hitchhiker (primary spread), but also facilitates the secondary spread within each region (Wetterer 2008, 2009, 2010, 2012; Bertelsmeier et al., 2017). Moreover, the presence of multiple male MLLs at a fine spatial scale may be an important component of adaptation for this species, especially in its invasive range. Since the allele richness of queen in this study is low, male-mediated gene flow (or migration of male clone among colonies) appears to be crucial force in maintaining worker genetic diversity within both colonies and populations. Further studies on colony structure of *P. longicornis* are

warranted to shed light on potential effects of male gene flow on genetic diversity at both colony and population levels.

In summary, genetic patterns of *P. longicornis* in the three studied regions likely reflect footprint left by frequent dispersal events associated with human-mediated transport at among- and within-region levels. The findings also suggest that human-mediated dispersal rather than colony budding is primarily responsible for the spread of this species in the three studied regions and possibly other introduced populations. Studies on average dispersal distance, frequency and potential human-associated "vector" are underway and would provide insightful information towards efficient management of this invasive species.

5.4 References

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Chapter 6 Conclusions

In the present study, we developed 36 polymorphic microsatellite markers for the longhorn crazy ant *P. longicornis*, examined worldwide mtDNA and nDNA (microsatellite) variations in *P. longicornis* and its associated *Wolbachia* bacterial symbionts. Analyses of mtDNA sequences of 13 geographic regions reveal two highly diverged mtDNA clades that co-occur in most of the geographic regions. These two mtDNA clades are associated with different *Wolbachia* infection patterns, but are not congruent with patterns of nDNA variation. Two *Wolbachia* strains, *w*LonA and *w*LonF, occur: *w*LonA appears to be primarily transmitted maternally, and its infection status is consistent with a relatively recent *Wolbachia*-induced selective sweep. On the other hand, the history of *w*LonF infections in *P. longicornis* appears to be characterized by frequent gains and losses over time. Identical *Wolbachia* strain shared between specialist ant cricket *Myrmecophilus americanus* and *P. longicornis* implies the occurrence of *Wolbachia* horizontal transmission possibly through intimate ecological associations. The estimation of nDNA variation in worldwide populations reveals an extremely high level of heterozygosity, a possible genetic consequence derived from its unique reproductive mode where workers are produced from hybridization of divergent queen and male clones. Our study show that this system is widespread across our studied populations of *P. longicornis* and might represent an adaptive trait linked to the invasion success of this species as it potentially relaxes the costs associated with inbreeding.

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Appendix

Appendix 1 Profiles for *Paratrechina longicornis* specimens used in Chapter 3 and Chapter 5.

ID	mt Clade	Haplotype	Infection type	Latitude	Longitude	Region	Country
plJP01	I	Hap15	A	26.21891	127.68644	Northeast Asia	Japan
plJP02-2	II-3	Hap11	F	26.1784797	127.7994308	Northeast Asia	Japan
plJP03	II-3	Hap23	F	24.35555	124.24238	Northeast Asia	Japan
plJP04	I	Hap15	AF	24.39036333	124.2460911	Northeast Asia	Japan
plJP05	I	Hap33	A	24.31386	123.90633	Northeast Asia	Japan
plJP06	I	Hap8	N	24.33107972	123.9090897	Northeast Asia	Japan
plJP07	II-3	Hap23	F	26.398756	127.758078	Northeast Asia	Japan
plJP08	I	Hap8	N	26.333547	127.787008	Northeast Asia	Japan
plJP09	II-3	Hap11	F	26.167364	127.828897	Northeast Asia	Japan
plJP10	I	Hap15	AF	26.14476	127.66473	Northeast Asia	Japan
plJP11	I	Hap15	A	26.0957	127.6828	Northeast Asia	Japan
plJP12	I	Hap21	AF	26.096	127.7218	Northeast Asia	Japan
plJP13	I	Hap8	N	26.14186	127.74891	Northeast Asia	Japan
plJP14	I	Hap8	N	26.13774	127.72902	Northeast Asia	Japan
plJP15	II-3	Hap11	F	26.6777	127.8912	Northeast Asia	Japan
plJP16	II-3	Hap11	F	26.33846	127.84567	Northeast Asia	Japan
plJP17	I	Hap9	AF	26.36461	127.85358	Northeast Asia	Japan
plJP18	I	Hap15	AF	26.23266	127.68115	Northeast Asia	Japan
plJP19	II-2	Hap19	N	26.23752	127.67418	Northeast Asia	Japan

plJP20	II-2	Hap19	N	26.24146	127.6792	Northeast Asia	Japan
plJP21	I	Hap8	N	26.43612	127.79304	Northeast Asia	Japan
plJP22	I	Hap8	N	26.20983	127.65172	Northeast Asia	Japan
pl01-4	I	Hap8	N	25.157405	121.401672	East Asia	Taiwan
pl02-2	I	Hap15	AF	25.056328	121.224689	East Asia	Taiwan
pl03-2	II-1	Hap2	F	24.828056	121.071289	East Asia	Taiwan
pl04	I	Hap8	N	24.81848	121.12813	East Asia	Taiwan
pl05	I	Hap8	A	24.81684	121.11055	East Asia	Taiwan
pl06	II-2	Hap1	N	24.49518	120.82783	East Asia	Taiwan
pl07	I	Hap21	AF	24.06126	120.43017	East Asia	Taiwan
pl08	II-1	Hap2	F	24.98925	121.46495	East Asia	Taiwan
pl09	II-3	Hap25	F	25.05575	121.1943	East Asia	Taiwan
pl10	I	Hap8	A	23.96461	120.57375	East Asia	Taiwan
pl11	I	Hap9	A	24.08009	120.55845	East Asia	Taiwan
pl114	I	Hap9	AF	22.66592	120.31297	East Asia	Taiwan
pl115	II-1	Hap10	N	22.62255	120.28884	East Asia	Taiwan
pl12	I	Hap9	A	24.08269	120.55847	East Asia	Taiwan
pl13	I	Hap9	A	24.0808	120.5587	East Asia	Taiwan
pl14	II-2	Hap1	N	23.97484	120.68483	East Asia	Taiwan
pl15	I	Hap15	A	24.07217	120.87296	East Asia	Taiwan
pl17	I	Hap8	A	23.75804	120.6715	East Asia	Taiwan

pl171	I	Hap8	A	22.6258	120.341	East Asia	Taiwan
pl172	II-1	Hap2	F	22.62573	120.36373	East Asia	Taiwan
pl18	I	Hap15	AF	23.80967	120.72036	East Asia	Taiwan
pl180	I	Hap16	AF	22.75782	121.10272	East Asia	Taiwan
pl182	II-1	Hap2	F	22.77547	121.1477	East Asia	Taiwan
pl183	I	Hap8	A	22.53192	120.96728	East Asia	Taiwan
pl185	II-2	Hap17	N	23.56713	119.56514	East Asia	Taiwan
pl186	I	Hap8	N	23.5676	119.5621	East Asia	Taiwan
pl187	I	Hap9	A	23.55607	119.6021	East Asia	Taiwan
pl189	II-3	Hap11	F	23.66052	119.56007	East Asia	Taiwan
pl19	I	Hap8	AF	23.91944	120.67461	East Asia	Taiwan
pl195	I	Hap18	AF	22.06374	121.56617	East Asia	Taiwan
pl200	I	Hap9	A	23.56369	119.48947	East Asia	Taiwan
pl208	I	Hap8	AF	24.22642	120.8794	East Asia	Taiwan
pl21	I	Hap8	N	23.76022	120.61775	East Asia	Taiwan
pl22	II-1	Hap2	F	23.48456	120.468	East Asia	Taiwan
pl225	II-1	Hap2	F	22.91977	121.13971	East Asia	Taiwan
pl23	II-2	Hap19	N	23.11964	120.36213	East Asia	Taiwan
pl230	I	Hap9	AF	21.93197	120.82416	East Asia	Taiwan
pl233	I	Hap20	AF	22.0025	120.7456	East Asia	Taiwan
pl24	I	Hap9	A	23.14064	120.32557	East Asia	Taiwan

pl26	I	Hap21	A	23.13657	120.30029	East Asia	Taiwan
pl27	II-1	Hap2	F	23.29165	120.39574	East Asia	Taiwan
pl29	II-1	Hap10	N	22.65576	120.29132	East Asia	Taiwan
pl30	II-1	Hap2	N	22.67611	120.3119	East Asia	Taiwan
pl31	II-2	Hap1	N	22.64306	120.6106	East Asia	Taiwan
pl32	I	Hap21	A	22.59472	120.61087	East Asia	Taiwan
pl33	I	Hap8	AF	24.9022	121.8621	East Asia	Taiwan
pl35	I	Hap21	A	23.78891	120.47762	East Asia	Taiwan
pl36	I	Hap22	AF	23.8253	120.4559	East Asia	Taiwan
pl37	II-1	Hap2	F	23.79779	120.46528	East Asia	Taiwan
pl38	II-1	Hap2	F	23.79232	120.44801	East Asia	Taiwan
pl39	I	Hap8	AF	23.77125	120.41276	East Asia	Taiwan
pl40	II-1	Hap2	N	23.76207	120.38989	East Asia	Taiwan
pl41	I	Hap8	N	23.76186	120.359	East Asia	Taiwan
pl42	I	Hap8	AF	23.65795	120.31242	East Asia	Taiwan
pl43	I	Hap8	N	23.61172	120.30735	East Asia	Taiwan
pl44	II-1	Hap2	F	23.552	120.3471	East Asia	Taiwan
pl45	I	Hap9	A	23.45888	120.3325	East Asia	Taiwan
pl46	I	Hap21	AF	23.46512	120.24691	East Asia	Taiwan
pl47	II-2	Hap19	N	23.41133	120.30818	East Asia	Taiwan
pl48	I	Hap8	AF	23.4287	120.3979	East Asia	Taiwan

pl49	I	Hap8	AF	22.99459	120.23331	East Asia	Taiwan
pl50	II-2	Hap1	N	22.99847	120.19744	East Asia	Taiwan
pl51	II-2	Hap1	N	22.99391	120.20748	East Asia	Taiwan
pl58	II-3	Hap11	F	22.52346	120.46428	East Asia	Taiwan
pl69	II-3	Hap23	F	23.899	121.5503	East Asia	Taiwan
pl74	II-2	Hap1	N	23.96713	121.60876	East Asia	Taiwan
pl76	I	Hap24	A	24.02891	121.62731	East Asia	Taiwan
plCN01	I	Hap26	A	21.39219	101.31512	East Asia	China
plCN02	I	Hap21	AF	22.29695007	114.1742821	East Asia	China
plCN04.2	I	Hap8	AF	22.25664636	113.9027274	East Asia	China
plCN07	II-1	Hap27	N	22.27944	114.1579	East Asia	China
plCN09	I	Hap28	A	22.26393	114.23711	East Asia	China
plCN10	II-3	Hap11	N	22.29715	114.27222	East Asia	China
plCN11	II-2	Hap19	N	22.19617	113.54118	East Asia	China
plCN12	I	Hap8	N	22.50213	114.12656	East Asia	China
plCN13	I	Hap8	AF	22.41307	114.21012	East Asia	China
plCN14	II-1	Hap2	F	22.31048	114.1581	East Asia	China
plCN15	II-2	Hap3	N	22.28171	114.18877	East Asia	China
plCN16	II-3	Hap29	F	22.53706	114.05406	East Asia	China
plCN17	II-3	Hap4	F	22.54667	114.12677	East Asia	China
plCN18	II-3	Hap29	F	22.54469	114.08518	East Asia	China

pl12.348	II-3	Hap11	F	21.683	102.1	South Asia	Laos
plID01	II-1	Hap2	F	-0.828076	100.53021	South Asia	Indonesia
plID02	II-2	Hap19	N	-8.50957	115.261704	South Asia	Indonesia
plKH02	II-1	Hap2	F	13.412693	103.867024	South Asia	Cambodia
plKH03	II-2	Hap1	N	13.369017	103.864459	South Asia	Cambodia
plMY01	I	Hap8	AF	3.21726	101.72442	South Asia	Malaysia
plMY02	I	Hap34	AF	5.661726	100.508539	South Asia	Malaysia
plMY03	I	Hap8	AF	5.612921	100.486052	South Asia	Malaysia
plMY04	I	Hap34	AF	5.41792	100.337	South Asia	Malaysia
plMY05	II-2	Hap19	N	5.325918	100.287427	South Asia	Malaysia
plMY07	II-2	Hap19	N	5.277888	100.27023	South Asia	Malaysia
plMY08	I	Hap21	AF	5.372663	100.237777	South Asia	Malaysia
plMY09	II-1	Hap27	N	5.406769	100.280058	South Asia	Malaysia
plMY10	II-1	Hap2	F	5.440082	100.287224	South Asia	Malaysia
plMY101	I	Hap21	AF	3.42173	115.152667	South Asia	Malaysia
plMY11	I	Hap21	AF	5.354718	100.300785	South Asia	Malaysia
plMY13	I	Hap8	AF	1.863265	102.966965	South Asia	Malaysia
plMY14	II-1	Hap10	N	1.841309	102.955261	South Asia	Malaysia
plMY15	I	Hap21	A	1.842982	102.93743	South Asia	Malaysia
plMY16	II-2	Hap19	N	1.858027	102.940938	South Asia	Malaysia
plMY17	II-2	Hap19	N	5.661996	100.502915	South Asia	Malaysia

plMY18	II-1	Hap27	N	5.674444	100.508889	South Asia	Malaysia
plMY19	II-2	Hap3	N	5.62605	100.46523	South Asia	Malaysia
plMY20	I	Hap8	A	1.486775	103.930877	South Asia	Malaysia
plMY21	I	Hap15	A	1.487547	103.929632	South Asia	Malaysia
plMY22	II-1	Hap10	N	1.492245	103.927948	South Asia	Malaysia
plMY23	I	Hap8	AF	1.494658	103.922013	South Asia	Malaysia
plMY24	I	Hap21	AF	2.137032	102.505825	South Asia	Malaysia
plMY25	II-1	Hap10	N	1.871323	102.996996	South Asia	Malaysia
plMY26	II-2	Hap19	N	5.601889	100.480544	South Asia	Malaysia
plMY27	I	Hap21	A	5.369258	100.248221	South Asia	Malaysia
plMY28	I	Hap15	F	1.411972	103.845186	South Asia	Singapore
plMY29	II-1	Hap35	N	1.275823	103.624204	South Asia	Singapore
plMY30	II-3	Hap36	N	1.312363	103.939681	South Asia	Singapore
plMY31	II-1	Hap10	N	1.335399	103.745502	South Asia	Singapore
plMY32	I	Hap21	AF	1.294067	103.85398	South Asia	Singapore
plMY34	II-2	Hap3	N	1.338513	103.743131	South Asia	Singapore
plPH03	I	Hap15	AF	6.692472	125.350278	South Asia	Philippines
plPH04-1	II-1	Hap2	F	14.156441	121.233857	South Asia	Philippines
plPH04-2	II-1	Hap2	F	14.156441	121.233857	South Asia	Philippines
plTH01	II-2	Hap19	N	13.8421	100.573	South Asia	Thailand
plTH02	I	Hap38	AF	13.84194	100.57374	South Asia	Thailand

plTH03	I	Hap39	AF	13.72999	100.53828	South Asia	Thailand
plTH11	II-1	Hap27	N	13.80283	100.55336	South Asia	Thailand
plTH12	I	Hap15	AF	13.80625	100.55512	South Asia	Thailand
plTH16	II-1	Hap27	N	13.77629	100.45629	South Asia	Thailand
plTH17	II-2	Hap3	N	13.80057	100.18766	South Asia	Thailand
plTH18	II-2	Hap19	N	13.80889	100.16084	South Asia	Thailand
plTH19	II-1	Hap40	N	13.96531	100.08373	South Asia	Thailand
plTH20	II-2	Hap19	N	14.45797	100.53773	South Asia	Thailand
plTH21	I	Hap15	F	14.593	100.3782	South Asia	Thailand
plTH23	II-2	Hap1	N	14.59216	100.37907	South Asia	Thailand
plTH24	I	Hap8	F	14.54583	100.50112	South Asia	Thailand
plTH25	I	Hap8	AF	14.34815	100.5806	South Asia	Thailand
plTH26	I	Hap15	F	14.34539	100.59335	South Asia	Thailand
plTH27	I	Hap15	AF	14.35998	100.59255	South Asia	Thailand
plTH28	I	Hap15	F	14.3501	100.5424	South Asia	Thailand
plTH29	I	Hap9	A	14.35295	100.53183	South Asia	Thailand
plTH30	I	Hap15	AF	13.68233	100.65976	South Asia	Thailand
plTH31	II-2	Hap1	N	13.68005	100.66018	South Asia	Thailand
plTH32	II-1	Hap27	N	14.22814	100.70685	South Asia	Thailand
plTH33	I	Hap9	F	14.58983	101.02333	South Asia	Thailand
plTH34	I	Hap41	AF	14.83454	101.54985	South Asia	Thailand

plTH35	II-2	Hap3	N	14.8746	101.7244	South Asia	Thailand
plTH36	I	Hap15	AF	15.21134	101.76631	South Asia	Thailand
plTH37	I	Hap8	AF	15.29943	101.73737	South Asia	Thailand
plTH38	I	Hap8	AF	14.56549	101.97845	South Asia	Thailand
plTH39	II-2	Hap3	N	14.51466	101.95918	South Asia	Thailand
plTH40	I	Hap15	AF	14.46594	101.90431	South Asia	Thailand
plVN01	I	Hap15	F	20.99266	105.49518	South Asia	Vietnam
plVN02-1	II-2	Hap43	N	20.99266	105.49518	South Asia	Vietnam
pl12.101	I	Hap8	N	25.327	55.391	Arabia	Arabia
pl12.99	I	Hap8	N	25.276	55.3	Arabia	Arabia
pl05.246	II-2	Hap3	N	18.357	-65.027	Caribbean	USA-Virgin Islands
pl05.324	II-2	Hap1	F	18.338	-64.666	Caribbean	USA-Virgin Islands
pl06.134	II-2	Hap1	F	18.011	-63.043	Caribbean	France-St Martin
pl06.264	II-3	Hap4	F	18.083	-67.939	Caribbean	USA-Puerto Rico
pl06.647	II-3	Hap5	F	13.364	-61.136	Caribbean	St. Vincent & The Grenadines
pl06.816	II-2	Hap1	N	13.761	-60.932	Caribbean	Saint Lucia
pl07.163	II-3	Hap4	F	17.128	-62.612	Caribbean	St Kitts & Nevis
pl07.382	II-3	Hap4	F	18.043	-63.117	Caribbean	France-St Martin
pl07.561	II-3	Hap6	F	16.79	-62.211	Caribbean	UK-Montserrat
pl07.681	I	Hap7	A	16.772	-62.219	Caribbean	UK-Montserrat
pl10.62	II-3	Hap5	F	24.981	-77.46	Caribbean	Bahamas

pl11.24	II-2	Hap3	N	16.225	-61.531	Caribbean	France-Guadeloupe
pl13.382	II-2	Hap3	N	17.702	-64.785	Caribbean	USA-Virgin Islands
pl14.136	II-3	Hap14	F	13.117	-59.6	Caribbean	Barbados
plHug087	II-2	Hap1	N	14.75777	-60.922011	Caribbean	France-Martinique
plHug101	II-2	Hap3	N	14.683965	-60.940304	Caribbean	France-Martinique
plHug88	II-3	Hap5	F	14.75547	-60.910702	Caribbean	France-Martinique
plJM02-3	I	Hap32	AF	18.32025	-78.09965	Caribbean	Jamaica
plJM03-3	I	Hap8	AF	18.237484	-77.050172	Caribbean	Jamaica
plIN01-1	II-3	Hap11	F	29.386128	79.110206	Indian Subcontinent	India
plIN01-2	II-3	Hap11	F	29.386128	79.110206	Indian Subcontinent	India
plIN02-1	I	Hap8	A	28.5411067	77.2107594	Indian Subcontinent	India
plIN02-2	I	Hap8	A	28.5411067	77.2107594	Indian Subcontinent	India
plIN04-1	II-2	Hap1	N	27.174121	78.041145	Indian Subcontinent	India
plIN04-2	II-2	Hap1	N	27.174121	78.041145	Indian Subcontinent	India
plIN05-1	I	Hap31	N	29.384286	79.106085	Indian Subcontinent	India
plIN05-2	I	Hap31	N	29.384286	79.106085	Indian Subcontinent	India
plNP01	I	Hap37	F	28.201085	83.945061	Indian Subcontinent	Nepal
pl12.349	II-3	Hap5	F	26.529	-80.056	North America	USA-Florida
pl12.357	I	Hap12	A	25.7454	-80.1763	North America	USA-Florida
plUS08	II-3	Hap5	F	29.6524	-82.312	North America	USA-Florida
plUS09	II-3	Hap5	F	29.6455	-82.308	North America	USA-Florida

plUS10	II-3	Hap5	N	29.6453	-82.31	North America	USA-Florida
plUS11	II-3	Hap5	N	29.6417	-82.3106	North America	USA-Florida
plUS12	I	Hap9	A	29.6511	-82.3737	North America	USA-Florida
plUS13	II-3	Hap5	F	29.65072	-82.3741	North America	USA-Florida
plUS19	I	Hap9	AF	32.81001	-116.94625	North America	USA-CA
plAU01	I	Hap8	AF	-16.87701	145.75383	Oceania	Australia
plAU02	II-2	Hap1	N	-12.170968	136.76458	Oceania	Australia
plAU03	II-2	Hap1	N	-11.385879	130.426509	Oceania	Australia
plAU04	II-2	Hap1	N	-11.385879	130.426509	Oceania	Australia
plAU12	I	Hap8	AF	-16.9301307	145.7720749	Oceania	Australia
plAU17	I	Hap8	N	-16.9133044	145.7700533	Oceania	Australia
plAU19	II-2	Hap1	N	-16.8733555	145.7563153	Oceania	Australia
plFJ01	I	Hap8	AF	-17.772803	177.367195	Oceania	Fiji
plFJ02	I	Hap21	A	-18.105823	178.39531	Oceania	Fiji
plFJ03	I	Hap21	A	-18.113325	178.473873	Oceania	Fiji
plFJ04	I	Hap21	A	-18.145858	178.447527	Oceania	Fiji
plFJ05-1	I	Hap8	AF	-17.441404	177.861391	Oceania	Fiji
plFJ05-2	I	Hap8	AF	-17.441404	177.861391	Oceania	Fiji
plFJ06	I	Hap9	A	-17.449162	177.983061	Oceania	Fiji
plFJ07	II-2	Hap3	N	-17.79612	177.398636	Oceania	Fiji
plFJ08	I	Hap21	A	-18.21001	177.711677	Oceania	Fiji

plFJ09	I	Hap21	A	-18.08804	177.552228	Oceania	Fiji
plUS01	I	Hap8	A	21.307922	-157.816293	Polynesia	USA-Hawaii
plUS02	II-2	Hap3	N	21.270779	-157.697124	Polynesia	USA-Hawaii
plUS03	II-1	Hap2	N	19.410158	-155.893298	Polynesia	USA-Hawaii
plUS04	I	Hap8	A	19.724453	-155.084908	Polynesia	USA-Hawaii
plUS05-2	I	Hap8	N	19.662305	-155.006883	Polynesia	USA-Hawaii
plUS14	I	Hap42	N	19.64074	-155.99731	Polynesia	USA-Hawaii
plUS15	II-1	Hap2	F	19.71444	-155.03995	Polynesia	USA-Hawaii
plUS16	II-2	Hap3	N	21.27906	-157.82815	Polynesia	USA-Hawaii
plUS17	II-2	Hap3	N	21.27898	-157.83346	Polynesia	USA-Hawaii
pl12.67	II-1	Hap13	N	-16.534	28.803	South Africa	Zimbabwe
pl10.201	II-2	Hap3	N	10.597	-67.007	South America	Venezuela
pl10.289	I	Hap7	N	10.998	-63.867	South America	Venezuela
plGR01	I	Hap30	A	36.44634	28.2257	Southeastern Europe	Greece
plGR03	I	Hap30	A	36.091819	28.088709	Southeastern Europe	Greece
plST01-1	II-1	Hap27	N	0.367658	6.712249	West Africa	São Tomé and Príncipe
plST01-2	II-1	Hap27	N	0.367658	6.712249	West Africa	São Tomé and Príncipe
plST01-3	II-1	Hap27	N	0.367658	6.712249	West Africa	São Tomé and Príncipe
plST01-4	II-1	Hap27	N	0.367658	6.712249	West Africa	São Tomé and Príncipe

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Appendix 2 Sequences of polymerase chain reaction (PCR) primers used for amplification of mtDNA gene.

Name	Primer sequences (5' – 3')	Primer position	Ta (°C)	Reference
C1-J-1745M-F†	CCTCGAATAAATAATATAAGATTTTGAC	COI	52	Modified from (Degnan et al., 2004)
PLCOII-R2	TTAGATTGCAGGAATTTCGTTATATCT	COI	52	This study
PLCOII-F1†	ACCACGTCGTTATTCTGACTATC	COI	52	This study
C2-N-3661R	CCACAAATTTCTGAACATTGACCA	COII	52	(Degnan et al., 2004)

†Primer pair C1-J-1745M-F/PLCOII-R2 amplified partial COI region (approximately 1151 bp), and PLCOII-F1/ C2-N-3661R amplified partial COI-tRNA-COII (approximately 917-928 bp) region

Appendix 3 Sequences of polymerase chain reaction (PCR) primers used for detection of the reproductive parasites in Chapter 3.

Species	Name	Primer sequences (5' – 3')	Ta (°C)	Size (bp)	Reference
Wolbachia sp.	81F	TGGTCCAATAAGTGATGAAGAAAC	50	610	(Zhou et al., 1998)
	691R	AAAAATTAAACGCTACTCCA			
	16SwolF†	TTGTAGCCTGCTATGGTATAACT	54	896	(O'Neill et al., 1992)
	16SwolR†	GAATAGGTATGATTTTCATGT			
	fts-F†	GTATGCCGATTGCAGAGCTTG	55	769	(Kondo et al., 1999)
	fts-R†	GCCATGAGTATTCACTTGGCT			

† Primers were used to double-check the infection status of *Wolbachia* when the result of *Wolbachia* detection by using primer 81F and 691R was negative.

Appendix 4 Specific primers used for detection and amplification of *Wolbachia* in Chapter 3.

DNA target	Name	Primer sequences (5' – 3')	Ta (°C)	Size (bp)	Reference
Wolbachia A supergroup	136F	TGAAATTTTACCTCTTTTC	50	556	(Zhou et al., 1998)
	691R	AAAAATTAAACGCTACTCCA			
Wolbachia B supergroup	81F	TGGTCCAATAAGTGATGAAGAAAC	50	442	(Zhou et al., 1998)
	522R	ACCAGCTTTTGCTTGATA			
*Wolbachia w*LonA	WspSpePlA-F	GTTCGTTTGCAATACAACGGTG	54	430	This study
	WspSpePlA-R	TGTCATAGCTGACACCAGCTCTTGC			
*Wolbachia w*LonF	WspSpePlF-F	AAGGTGATAAAGATCAAGATCCTT	54	439	This study
	WspSpePlF-R	TACCATCACCCTTAGTTGTTGCAT			

Appendix 5 Sequences of polymerase chain reaction (PCR) primers used for amplification of microsatellite loci.

Name	Repeat motif		Primer sequences (5' – 3')	Ta (°C)
Prl102	(CT)^12	F:	TCCAACTGACCCGGAAGAC	58
		R:	CGTACGGAATCGTGCGAAG	
Prl104	(AG)^15	F:	GAGAGGGAACCCTGCTTCG	58
		R:	TCTGCCTGGTTTAGCCCTC	
Prl106	(AT)^17	F:	CTCATCGACCCTTTGACGG	58
		R:	ACTGGTAAGTCCACTCCGC	
Prl107	(AT)^10	F:	TCTCTGCAGCTGTGTCAGG	58
		R:	CGCAATTAGCGTCTCCGC	
Prl109	(CT)^12	F:	CAGTCGCAACAATGGCGG	58
		R:	TGACGAAAGCACCCGTAGG	
Prl110	(CT)^15	F:	CGTTATCCGTTCGTCACCG	58
		R:	GTGTCCGATGCAAATCCCG	
Prl111	(AG)^13	F:	AGCTGTCTGATTTCGTCGC	58
		R:	AACGCCTTTAATCCGTCGC	
Prl113	(AT)^10	F:	ATACACATTAGTGCATCCAACC	58
		R:	TTCGGCGTTCGTGAACAAG	
Prl118	(AG)^16	F:	ACAGGAAGTCGCGGAGATG	58
		R:	AATGCGGTGGTCAAAGTGC	
Prl119	(AT)^13	F:	ACAACTAATCGCCCGTAGC	58
		R:	TGGATCGTGAGATTTCCGTTTAG	
Prl120	(AG)^17	F:	CGCATGTGAATGTAAACGATGG	58
		R:	CAGCTTGCGGTTCAAGGTC	
Prl121	(CT)^10	F:	TAGTGCTGGATGCAGGGTG	58
		R:	ACGGCGTAGTACCTTCTGC	
Prl123	(AG)^12	F:	ACCGCAGCGTTAATTGC	58
		R:	GTCTCCGGACCCATTCTCG	
Prl125	(CT)^10	F:	AACACGGATGATTGCATGTC	58
		R:	GCCGTGATACGAACTTCCAC	
Prl126	(AT)^11	F:	AAGAACTGCAAGAGTGCGG	58
		R:	GCACGTCCCGAGAAACATC	
Prl127	(AG)^12	F:	AGCTTCCCGTACTTACACG	58
		R:	TGCAGAAAGTATGTCGCGATG	
Prl128	(AT)^15	F:	AAATTCGTCATGTTCCAGATCC	58
		R:	CAGCTGGCAAGGCATGAAC	
Prl130	(CT)^11	F:	GCACGCGGAAGCAATTAAC	58
		R:	GGACGCGTTGGAAAGTTCG	
Prl132	(CT)^14	F:	GATGGCGGAAATACCGGAG	58

		R:	TCGTTGACTTTACGTGTCGC	
Prl136	(AT)^14	F:	TTGACACAGAAGGCATTTCG	58
		R:	AGACGGGAGGAAATATCACGG	

Appendix 6 Regional genetic diversity of *Paratrechina longicornis*. Number of individuals sampled (N), number of segregating sites (*S*), number of haplotypes (*h*), haplotype diversity (*Hd*), and nucleotide diversity (π/bp). *w*LonA+, *w*LonA-, *w*LonF+, and *w*LonF- denote *w*LonA-infected, *w*LonA-uninfected, *w*LonF-infected, and *w*LonF-uninfected ants in a given region, respectively. Note that the *w*LonA+ group includes *w*LonA and F co-infected ants and *w*LonA single infected ants, and *w*LonA- group includes uninfected ants and *w*LonF single infected ants.

Geographic regions †	N	*S*	*h*	*Hd*	π/bp
All	248	172	43	0.922	0.034
*w*LonA+	98	21	20	0.818	0.001
*w*LonA-	150	164	27	0.922	0.026
*w*LonF+	111	146	24	0.898	0.032
*w*LonF-	137	161	27	0.909	0.034
Northeast Asia	22	125	8	0.857	0.031
*w*LonA+	8	4	4	0.643	0.001
*w*LonA-	14	122	4	0.747	0.035
*w*LonF+	11	117	4	0.691	0.022
*w*LonF-	11	110	5	0.818	0.034
East Asia	81	137	23	0.887	0.033
*w*LonA+	39	12	11	0.804	0.001
*w*LonA-	42	130	13	0.858	0.025
*w*LonF+	38	124	14	0.861	0.033
*w*LonF-	43	133	15	0.878	0.032
South Asia	71	136	19	0.920	0.034
*w*LonA+	28	7	8	0.812	0.001
*w*LonA-	43	135	14	0.906	0.024
*w*LonF+	36	121	10	0.835	0.018
*w*LonF-	35	133	13	0.889	0.022
Indian Subcontinent	9	145	5	0.889	0.041
*w*LonA+	2	0	1	0.000	0.000
*w*LonA-	7	137	4	0.857	0.041
*w*LonF+	3	106	2	0.667	0.040
*w*LonF-	6	131	3	0.800	0.041
Oceania	17	115	5	0.772	0.028
*w*LonA+	11	2	3	0.636	0.000
*w*LonA-	6	114	3	0.600	0.022
*w*LonF+	5	0	1	0.000	0.000
*w*LonF-	12	115	5	0.758	0.034
Polynesia	9	124	4	0.806	0.038
*w*LonA+	2	0	1	0.000	0.000
*w*LonA-	7	124	4	0.810	0.035

*w*LonF-	8	124	4	0.786	0.039
North America	9	110	3	0.556	0.031
*w*LonA+	3	4	2	0.667	0.002
*w*LonA-	6	0	1	0.000	0.000
*w*LonF+	5	108	2	0.400	0.025
*w*LonF-	4	110	3	0.833	0.042
Caribbean	19	128	9	0.895	0.023
*w*LonA+	3	3	3	1.000	0.001
*w*LonA-	16	28	6	0.850	0.008
*w*LonF+	12	125	7	0.894	0.023
*w*LonF-	7	115	3	0.667	0.019
Arabia	2	0	1	0.000	0.000
Southeastern Europe	2	0	1	0.000	0.000
West Africa	4	0	1	0.000	0.000
South America	2	115	2	1.000	0.066

† South Africa excluded from analysis due to sample size of one.

Appendix 7 Allelic profiles, sequence types (ST), and wsp allele numbers of the two *Wolbachia* strains in *Paratrechina longicornis*. The most similar allelic profiles in PubMLST database are also displayed.

Host	Strain	ID	Supergroup	ST	*gatB*	*coxA*	*hcpA*	*ftsZ*	*fbpA*	*wsp*
Ephestia kuehniella	Ekue_A	13	A	19	7	6	7	3	8	18
Technomyrmex albipes	Talb_A	111	A	19	7	6	7	3	8	18
Leptomyrmex sp.	Lept_A	115	A	19	7	6	7	3	8	18
Ornipholidotos peucetia	Opeu_A	123	A	19	7	6	7	3	8	18
Pheidole plagiara	Ppla_A_20-05	124	A	19	7	6	7	3	8	18
Pheidole sauberi	Psau_A	125	A	19	7	6	7	3	8	18
Leptogenys sp.	Lepg_A_06-03	146	A	19	7	6	7	3	8	18
Aricia artaxerxes	Aart_A	451	A	19	7	6	7	3	8	18
P. longicornis	***w*LonA**	**1827**	**A**	**19**	**7**	**6**	**7**	**3**	**8**	**18**
Brachythemis contaminata	Bcon_F_Odo3	360	F	239	168	147	173	132	226	NA
Orthetrum sabina	Osab_F_Odo6	363	F	242	168	147	175	132	226	NA
Orthetrum sabina	Osab_F_Odo7	366	F	243	168	147	177	132	226	NA
P. longicornis	***w*LonF**	**1828**	**F**	**471**	**168**	**147**	**262**	**132**	**226**	**708**

NA, Not applicable

Appendix 8 Tests for departure from neutrality for mtDNA sequence variation in *Paratrechina longicornis*. Tajima's *D*, Fu and Li's *D**, Fu and Li's *F**, normalized Fay and Wu's *Hn*, DHEW test *P*-value, and Neutrality index from McDonald–Kreitman test (M-K test). *w*LonA+, *w*LonA-, *w*LonF+, and *w*LonF- denote *w*LonA-infected, *w*LonA-uninfected, *w*LonF-infected, and *w*LonF-uninfected ants in a given region, respectively.

Geographic regions	N	Tajima's *D*	Fu and Li's *D**	Fu and Li's *F**	Fay and Wu's *Hn*	DHEW test *P*-value	Neutrality index
All	248	2.991	0.839	**2.328****	NA	NA	1.612
*w*LonA+	98	**-1.927***	**-3.377****	**-3.252****	**-2.689***	**<0.001**	**12.888*****
*w*LonA-	150	1.445	1.135	1.550	**-3.465****	0.863	1.014
*w*LonF+	111	**3.037****	1.087	**2.337****	-1.137	0.999	1.317
*w*LonF-	137	**3.006****	**1.615***	**2.715****	**-1.771***	0.997	1.481
Northeast Asia	22	**2.249***	**1.766****	**2.111****	0.78	0.709	0.947
*w*LonA+	8	-0.222	-0.176	-0.189	0.780	0.709	**12.737***
*w*LonA-	14	**2.437****	**1.699****	**2.033****	-0.587	0.353	0.746
*w*LonF+	11	-0.234	**1.589****	1.155	**-2.115***	**0.037***	0.945
*w*LonF-	11	**2.798*****	**1.623****	**1.987****	-0.168	0.193	0.883
East Asia	81	**3.380*****	**1.751****	**2.875****	-0.801	1.000	1.131
*w*LonA+	39	-1.629	**-3.082***	**-2.877***	**-1.920***	**0.024***	**9.603****
*w*LonA-	42	1.302	**1.925****	**1.915****	**-2.82***	0.823	0.775
*w*LonF+	38	**3.342*****	**1.742****	**2.630****	-0.294	0.998	0.947
*w*LonF-	43	**2.759****	1.451	**2.257****	-0.948	0.998	1.061
South Asia	71	**3.454*****	**1.791****	**2.943****	-0.894	1.000	1.105
*w*LonA+	28	-0.998	-1.462	-1.428	-1.828	**0.021***	5.074
*w*LonA-	43	0.98	**1.591***	1.574	**-3.100****	0.707	1.013
*w*LonF+	36	0.16	1.163	0.954	**-3.035****	0.293	0.971
*w*LonF-	35	0.481	1.348	1.195	**-3.374****	0.460	0.933
Indian Subcontinent	9	1.483	**1.488***	**1.574***	-0.335	1.000	0.683
*w*LonA-	7	1.33	1.347	1.404	-0.386	1.000	0.518

*w*LonF-	6	1.294	**1.759****	**1.691****	-0.323	1.000	0.687
Oceania	17	1.984	**1.597****	**1.810****	-0.888	0.544	0.839
*w*LonA+	11	0.199	-0.330	-0.205	0.362	0.323	12.789
*w*LonA-	6	**-1.546****	**-1.587****	**-1.584****	**-3.454*****	**0.001****	0.719
*w*LonF-	12	**2.648****	**1.553****	**1.910****	-0.143	0.245	0.839
Polynesia	9	**2.209***	**1.627****	**1.833****	-0.248	0.089	0.849
*w*LonA-	7	1.062	**1.667****	**1.561***	-1.119	**0.028***	0.849
*w*LonF-	8	**2.051***	1.357	**1.574***	-0.275	0.059	0.849
North America	9	1.756	**1.585****	**1.660****	-1.051	0.095	0.947
*w*LonF+	5	-1.267	**-1.267****	**-1.267****	**-3.005*****	**0.006****	0.768
Caribbean	19	0.365	**1.540****	1.264	**-3.107****	0.267	1.241
*w*LonA-	16	**2.439***	1.255	**1.673****	0.053	0.947	1.640
*w*LonF+	12	-0.313	**1.523****	1.084	**-3.133****	0.112	1.153
*w*LonF-	7	**-1.683***	**-1.804****	**-1.793****	**-3.703*****	**0.019***	0.839

*$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$; statistics significantly deviated from expectations under neutrality
Groups with samples size smaller than 5 were excluded from the analyses
NA; Not applicable

Appendix 9 Genetic diversity of *Paratrechina longicornis* in Asia regions based on 20 microsatellite markers. Sample size (N), average number of alleles (*Na*), average number of effective alleles (*Ne*), average of observed heterozygosity (*Ho*), average of expected heterozygosity (*He*), and average of Shannon's information index (*I*). *w*LonA+, *w*LonA-, *w*LonF+, and *w*LonF- denote *w*LonA-infected, *w*LonA-uninfected, *w*LonF-infected, and *w*LonF-uninfected ants in a given region, respectively.

Geographic regions	N	*Na*	*Ne*	*Ho*	*He*	*I*
Asia	134	9.55	3.80	0.91	0.68	1.49
*w*LonA+	56	7.80	3.53	0.90	0.66	1.41
*w*LonA-	78	8.55	3.87	0.91	0.69	1.49
*w*LonF+	69	8.25	3.60	0.90	0.66	1.42
*w*LonF-	65	8.50	3.87	0.91	0.69	1.49
Northeast Asia	22	4.85	3.08	0.90	0.63	1.21
*w*LonA+	8	4.30	3.09	0.89	0.62	1.18
*w*LonA-	14	4.00	2.91	0.91	0.62	1.13
*w*LonF+	11	4.35	2.96	0.91	0.62	1.16
*w*LonF-	11	4.50	2.95	0.89	0.63	1.17
East Asia	41	7.90	3.76	0.91	0.68	1.47
*w*LonA+	20	6.40	3.67	0.92	0.68	1.41
*w*LonA-	21	6.65	3.63	0.90	0.68	1.43
*w*LonF+	22	6.75	3.74	0.90	0.68	1.44
*w*LonF-	19	6.25	3.55	0.92	0.67	1.38
South Asia	71	8.10	3.73	0.90	0.68	1.45
*w*LonA+	28	6.25	3.16	0.90	0.64	1.29
*w*LonA-	43	7.45	3.91	0.91	0.69	1.48
*w*LonF+	36	6.55	3.29	0.90	0.64	1.32
*w*LonF-	35	6.95	3.87	0.91	0.69	1.46

Appendix 10 The estimated mean log probability of the data *LnP*(*K*), *standard deviation* of *LnP*(*K*) and *ΔK* for *Paratrechina longicornis* inferred by STRUCTURE

K	Reps	Mean *LnP*(*K*)	Stdev *LnP*(*K*)	*Ln'(K)*	|*Ln*"(*K*)|	*ΔK*
1	5	-7987.60	0.54	--	--	--
2	5	-7454.86	69.40	532.74	136.50	1.97
3	5	-7058.62	11.19	396.24	96.40	8.62
4	5	-6758.78	39.63	299.84	88.36	2.23
5	5	-6547.30	43.26	211.48	95.02	2.20
6	5	-6430.84	30.03	116.46	17.94	0.60
7	5	-6332.32	90.78	98.52	103.60	1.14
8	5	-6337.40	263.17	-5.08	96.60	0.37
9	5	-6245.88	243.52	91.52	1956.90	8.04
10	5	-8111.26	3376.97	-1865.38	--	--

Appendix 11 Genetic diversity of *Paratrechina longicornis* for ants belonging to Clade II among various geographic regions. Number of individuals sampled (N), number of segregating sites (*S*), number of haplotypes (*h*), haplotype diversity (*Hd*), nucleotide diversity (π/bp)

Geographic regions	N	*S*	*h*	*Hd*	π/*bp*
Northeast Asia	8	27	3	0.714	0.007
East Asia	35	44	12	0.830	0.010
South Asia	36	49	11	0.881	0.009
Indian Subcontinent	4	23	2	0.667	0.009
West Africa	4	0	1	0.000	0.000
Oceania	5	2	2	0.400	0.001
Polynesia	5	28	2	0.600	0.010
North America	6	0	1	0.000	0.000
Caribbean	16	28	6	0.850	0.008

Appendix 12 Profile information of the ant cricket samples used in Chapter 4

Sample	Species	*Wolbachia* strain	Locality		Host
AnoTH04C04	*Myrmecophilus albicinctus*	wMsp4, wMsp8	Thailand	Nong Sarai, Pak Chong District	*Anoplolepis gracilipes*
AnoBOT01C03	*Myrmecophilus albicinctus*	wMsp1	Malaysia	George Town, Pulau Pinang	*Anoplolepis gracilipes*
AnoKIC03	*Myrmecophilus albicinctus*	wMsp1	Malaysia	George Town, Pulau Pinang	*Anoplolepis gracilipes*
Anomy35C01	*Myrmecophilus albicinctus*	wMsp1	Malaysia	Gelugor, Pulau Pinang	*Anoplolepis gracilipes*
Anomy36C02	*Myrmecophilus albicinctus*	wMsp1	Malaysia	Gelugor, Pulau Pinang	*Anoplolepis gracilipes*
Anomy36C03	*Myrmecophilus albicinctus*	wMsp1	Malaysia	Gelugor, Pulau Pinang	*Anoplolepis gracilipes*
Ano84.C01	*Myrmecophilus albicinctus*	wMsp1	Taiwan	Daxi Dist., Taoyuan City	*Anoplolepis gracilipes*
Ano84.C02	*Myrmecophilus albicinctus*	wMsp1	Taiwan	Daxi Dist., Taoyuan City	*Anoplolepis gracilipes*
Ano84.C03	*Myrmecophilus albicinctus*	wMsp1	Taiwan	Daxi Dist., Taoyuan City	*Anoplolepis gracilipes*
Ano84.C04	*Myrmecophilus albicinctus*	wMsp1	Taiwan	Daxi Dist., Taoyuan City	*Anoplolepis gracilipes*
Ano125C02	*Myrmecophilus albicinctus*	wMsp1, wMsp8	Taiwan	Taitung Dawu Township	*Anoplolepis gracilipes*
Ano125C04	*Myrmecophilus albicinctus*	wMsp1, wMsp8	Taiwan	Taitung Dawu Township	*Anoplolepis gracilipes*
Ano125C05	*Myrmecophilus albicinctus*	wMsp1, wMsp8	Taiwan	Taitung Dawu Township	*Anoplolepis gracilipes*
Ano125C06	*Myrmecophilus albicinctus*	wMsp1, wMsp8	Taiwan	Taitung Dawu Township	*Anoplolepis gracilipes*
Ano125C08	*Myrmecophilus albicinctus*	wMsp1, wMsp8	Taiwan	Taitung Dawu Township	*Anoplolepis gracilipes*
Ano125C09	*Myrmecophilus albicinctus*	wMsp1, wMsp8	Taiwan	Taitung Dawu Township	*Anoplolepis gracilipes*
AgrJP18.4C1	*Myrmecophilus albicinctus*	wMsp4, wMsp8	Japan	Nakagami District, Okinawa	*Anoplolepis gracilipes*
AnoJP46C02	*Myrmecophilus albicinctus*	wMsp4, wMsp8	Japan	Onna-son, Kunigami-gun, Okinawa	*Anoplolepis gracilipes*
AnoJP47C04	*Myrmecophilus albicinctus*	wMsp4, wMsp8	Japan	Onna-son, Kunigami-gun, Okinawa	*Anoplolepis gracilipes*
AnoJP48C01	*Myrmecophilus albicinctus*	wMsp4, wMsp8	Japan	Onna-son, Kunigami-gun, Okinawa	*Anoplolepis gracilipes*
AnoJP49C02	*Myrmecophilus albicinctus*	wMsp4, wMsp8	Japan	Onna-son, Kunigami-gun, Okinawa	*Anoplolepis gracilipes*
AnoTH04C02	*Myrmecophilus albicinctus*	wMsp4, wMsp8	Thailand	Nong Sarai, Pak Chong District	*Anoplolepis gracilipes*

AnoNT01c02	*Myrmecophilus albicinctus*	wMsp8	Taiwan	Chushan Township, Nantou County	*Anoplolepis gracilipes*
AnoNT01c04	*Myrmecophilus albicinctus*	wMsp8	Taiwan	Chushan Township, Nantou County	*Anoplolepis gracilipes*
AnoNT01c06	*Myrmecophilus albicinctus*	wMsp8	Taiwan	Chushan Township, Nantou County	*Anoplolepis gracilipes*
AnoNT01c09	*Myrmecophilus albicinctus*	wMsp8	Taiwan	Chushan Township, Nantou County	*Anoplolepis gracilipes*
AnoNT01c11	*Myrmecophilus albicinctus*	wMsp8	Taiwan	Chushan Township, Nantou County	*Anoplolepis gracilipes*
AnoNT01c12	*Myrmecophilus albicinctus*	wMsp8	Taiwan	Chushan Township, Nantou County	*Anoplolepis gracilipes*
AnoBOT01C01	*Myrmecophilus albicinctus*	Uninfected	Malaysia	George Town, Pulau Pinang	*Anoplolepis gracilipes*
AnoKIC04	*Myrmecophilus albicinctus*	Uninfected	Malaysia	George Town, Pulau Pinang	*Anoplolepis gracilipes*
AnoKIC06	*Myrmecophilus albicinctus*	Uninfected	Malaysia	George Town, Pulau Pinang	*Anoplolepis gracilipes*
Anomy35C09	*Myrmecophilus albicinctus*	Uninfected	Malaysia	Gelugor, Pulau Pinang	*Anoplolepis gracilipes*
Anomy35C10	*Myrmecophilus albicinctus*	Uninfected	Malaysia	Gelugor, Pulau Pinang	*Anoplolepis gracilipes*
Anomy35C11	*Myrmecophilus albicinctus*	Uninfected	Malaysia	Gelugor, Pulau Pinang	*Anoplolepis gracilipes*
Anomy35C12	*Myrmecophilus albicinctus*	Uninfected	Malaysia	Gelugor, Pulau Pinang	*Anoplolepis gracilipes*
mal20	*Myrmecophilus albicinctus*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
mal80	*Myrmecophilus albicinctus*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
Ano84.C05	*Myrmecophilus albicinctus*	Uninfected	Taiwan	Daxi Dist., Taoyuan City	*Anoplolepis gracilipes*
07.323-1	*Myrmecophilus americanus*	wMsp4, wMame2	Antigua	Darkwood Beach	*Paratrechina longicornis*
14.489-1	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame1, wMame2	Singapore	City Hall	*Paratrechina longicornis*
12.358-10	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame1, wMame2	USA	Virginia Key, FL	*Paratrechina longicornis*
12.358-11	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame1, wMame2	USA	Virginia Key, FL	*Paratrechina longicornis*
08.818-1	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame1, wMame2	USA	Big Pine Key, FL	*Paratrechina longicornis*
08.709-3	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame1, wMame2	Bonaire	Kralendijk	*Paratrechina longicornis*
08.745-1	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame1, wMame2	Bonaire	Belnem	*Paratrechina longicornis*
11.543-1	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame1, wMame2	Curaçao	Juan Domingo	*Paratrechina longicornis*
11.368-1	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame1, wMame2	Curaçao	Playa Forti	*Paratrechina longicornis*

11.507-2	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame1, wMame2	Curaçao	Koredor	*Paratrechina longicornis*
11.24-1	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame1, wMame2	Guadeloupe	Carénage	*Paratrechina longicornis*
10.454-1	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame1, wMame2	Jamaica	Negril	*Paratrechina longicornis*
plmy89Mame01	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame1, wMame2	Malaysia	Pulau Pinang	*Paratrechina longicornis*
plmy89Mame02	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame1, wMame2	Malaysia	Pulau Pinang	*Paratrechina longicornis*
plmy89Mame03	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame1, wMame2	Malaysia	Pulau Pinang	*Paratrechina longicornis*
11.268-1	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame1, wMame2	Martinique	Le Marin	*Paratrechina longicornis*
hug88	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame1, wMame2	Martinique	Spoutourne	*Paratrechina longicornis*
07.642-1	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame1, wMame2	Montserrat	Brades	*Paratrechina longicornis*
07.561-1	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame1, wMame2	Montserrat	Brades	*Paratrechina longicornis*
plTH22Mame1	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame1, wMame2	Thailand	Wiset Chai Chan Dist, Ang Thong Province	*Paratrechina longicornis*
plTH29Mame1	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame1, wMame2	Thailand	Ban Pom, Phra Nakhon Si Ayutthaya District	*Paratrechina longicornis*
12.356-1	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame1, wMame2	USA	Rickenbacker Causeway, Miami, FL	*Paratrechina longicornis*
12.391-1	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame1, wMame2	USA	Lake Worth, FL	*Paratrechina longicornis*
14.376-1	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame2	Singapore	City Hall	*Paratrechina longicornis*
08.813-1	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame2	USA	Key West, FL	*Paratrechina longicornis*
07.359-1	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame2	Antigua	Boons Bay	*Paratrechina longicornis*
07.507-2	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame2	Antigua	Long Bay	*Paratrechina longicornis*
07.815-1	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame2	Aruba	Cas di Paloma	*Paratrechina longicornis*
10.62-1	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame2	Bahamas	New Providence, Coral Harbour	*Paratrechina longicornis*
08.694-1	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame2	Bonaire	Bezu	*Paratrechina longicornis*
08.709-4	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame2	Bonaire	Kralendijk	*Paratrechina longicornis*
10.303-1	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame2	Jamaica	Montego Bay	*Paratrechina longicornis*
hug087	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame2	Martinique	Tartane	*Paratrechina longicornis*
hug101	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame2	Martinique	Le Robert	*Paratrechina longicornis*

07.382-1	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame2	St. Martin	Airport Road	*Paratrechina longicornis*
MameTw01-1	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame2	Taiwan	Dacun Township, Changhua County	*Paratrechina longicornis*
MameTw01-2	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame2	Taiwan	Dacun Township, Changhua County	*Paratrechina longicornis*
MameTw01-3	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame2	Taiwan	Dacun Township, Changhua County	*Paratrechina longicornis*
MameMyn01	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame2	Taiwan	Taichung	*Paratrechina longicornis*
MameMyn02	*Myrmecophilus americanus*	wMsp4, wMsp5, wMame2	Taiwan	Taichung	*Paratrechina longicornis*
Ano85.C01	*Myrmecophilus antilucanus*	wMsp4	Taiwan	Da'an Dist., Taipei City	*Anoplolepis gracilipes*
Ano97C01	*Myrmecophilus antilucanus*	wMsp4	Taiwan	Sanwan Township, Miaoli County	*Anoplolepis gracilipes*
Ano97C02	*Myrmecophilus antilucanus*	wMsp4	Taiwan	Sanwan Township, Miaoli County	*Anoplolepis gracilipes*
Ano105C01	*Myrmecophilus antilucanus*	wMsp4	Taiwan	Daan Dist. Taipei	*Anoplolepis gracilipes*
Ano105C02	*Myrmecophilus antilucanus*	wMsp4	Taiwan	Daan Dist. Taipei	*Anoplolepis gracilipes*
Ano36.2C01	*Myrmecophilus antilucanus*	Uninfected	Malaysia	Gelugor, Pulau Pinang	*Anoplolepis gracilipes*
Ano36.2C04	*Myrmecophilus antilucanus*	Uninfected	Malaysia	Gelugor, Pulau Pinang	*Anoplolepis gracilipes*
Ano36.2C05	*Myrmecophilus antilucanus*	Uninfected	Malaysia	Gelugor, Pulau Pinang	*Anoplolepis gracilipes*
Ano36.2C06	*Myrmecophilus antilucanus*	Uninfected	Malaysia	Gelugor, Pulau Pinang	*Anoplolepis gracilipes*
Ano36.2C10	*Myrmecophilus antilucanus*	Uninfected	Malaysia	Gelugor, Pulau Pinang	*Anoplolepis gracilipes*
mun1-r10	*Myrmecophilus antilucanus*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
mun1-r11	*Myrmecophilus antilucanus*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
mun1-r14	*Myrmecophilus antilucanus*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
mun1-r3	*Myrmecophilus antilucanus*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
mun1-r6	*Myrmecophilus antilucanus*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
mun1-r7	*Myrmecophilus antilucanus*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
mun1-r8	*Myrmecophilus antilucanus*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
mun1-r9	*Myrmecophilus antilucanus*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
UnknowC01	*Myrmecophilus antilucanus*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*

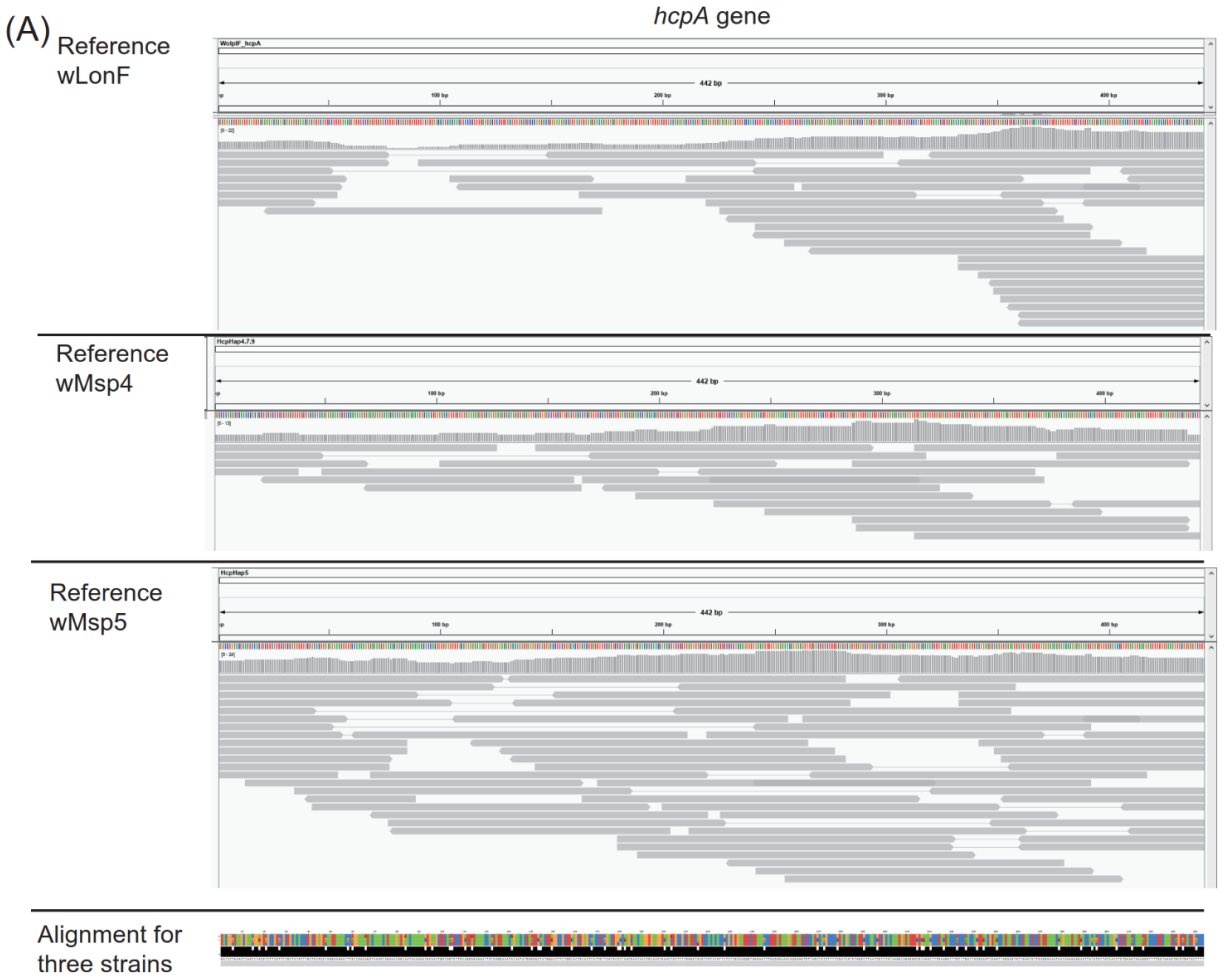
Ano95C01	*Myrmecophilus antilucanus*	Uninfected	Taiwan	Hsinpu town,Hsinchu County	*Anoplolepis gracilipes*
Ano95C02	*Myrmecophilus antilucanus*	Uninfected	Taiwan	Hsinpu town,Hsinchu County	*Anoplolepis gracilipes*
Ano95C03	*Myrmecophilus antilucanus*	Uninfected	Taiwan	Hsinpu town,Hsinchu County	*Anoplolepis gracilipes*
Ano95C04	*Myrmecophilus antilucanus*	Uninfected	Taiwan	Hsinpu town,Hsinchu County	*Anoplolepis gracilipes*
Ano95C05	*Myrmecophilus antilucanus*	Uninfected	Taiwan	Hsinpu town,Hsinchu County	*Anoplolepis gracilipes*
Ano95C06	*Myrmecophilus antilucanus*	Uninfected	Taiwan	Hsinpu town,Hsinchu County	*Anoplolepis gracilipes*
AnoTH04C03	*Myrmecophilus antilucanus*	Uninfected	Thailand	Nong Sarai, Pak Chong District	*Anoplolepis gracilipes*
AnoBOT01C02	*Myrmecophilus dubius*	Uninfected	Malaysia	George Town, Pulau Pinang	*Anoplolepis gracilipes*
AnoBOT01C04	*Myrmecophilus dubius*	Uninfected	Malaysia	George Town, Pulau Pinang	*Anoplolepis gracilipes*
AnoBOT01C05	*Myrmecophilus dubius*	Uninfected	Malaysia	George Town, Pulau Pinang	*Anoplolepis gracilipes*
AnoBTG01	*Myrmecophilus dubius*	Uninfected	Malaysia	George Town, Pulau Pinang	*Anoplolepis gracilipes*
AnoBTG02	*Myrmecophilus dubius*	Uninfected	Malaysia	George Town, Pulau Pinang	*Anoplolepis gracilipes*
AnoBTG03	*Myrmecophilus dubius*	Uninfected	Malaysia	George Town, Pulau Pinang	*Anoplolepis gracilipes*
AnoBTG04	*Myrmecophilus dubius*	Uninfected	Malaysia	George Town, Pulau Pinang	*Anoplolepis gracilipes*
AnoBTG05	*Myrmecophilus dubius*	Uninfected	Malaysia	George Town, Pulau Pinang	*Anoplolepis gracilipes*
AnoBTG06	*Myrmecophilus dubius*	Uninfected	Malaysia	George Town, Pulau Pinang	*Anoplolepis gracilipes*
AnoKIC01	*Myrmecophilus dubius*	Uninfected	Malaysia	George Town, Pulau Pinang	*Anoplolepis gracilipes*
AnoKIC02	*Myrmecophilus dubius*	Uninfected	Malaysia	George Town, Pulau Pinang	*Anoplolepis gracilipes*
AnoKIC05	*Myrmecophilus dubius*	Uninfected	Malaysia	George Town, Pulau Pinang	*Anoplolepis gracilipes*
mpv-r1	*Myrmecophilus dubius*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
mpv-r10	*Myrmecophilus dubius*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
mpv-r11	*Myrmecophilus dubius*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
mpv-r2	*Myrmecophilus dubius*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
mpv-r5	*Myrmecophilus dubius*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
mpv-r6	*Myrmecophilus dubius*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*

mpv-r8	*Myrmecophilus dubius*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
mpv-r9	*Myrmecophilus dubius*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
NP01	*Myrmecophilus dubius*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
NP02	*Myrmecophilus dubius*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
UnknowC03	*Myrmecophilus dubius*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
mp-r12	*Myrmecophilus hebardi*	wMsp4	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
mp-r3	*Myrmecophilus hebardi*	wMsp4	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
mp-r5	*Myrmecophilus hebardi*	wMsp4	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
Anomy35C02	*Myrmecophilus hebardi*	wMsp7	Malaysia	Gelugor, Pulau Pinang	*Anoplolepis gracilipes*
Anomy35C03	*Myrmecophilus hebardi*	wMsp7	Malaysia	Gelugor, Pulau Pinang	*Anoplolepis gracilipes*
Anomy35C04	*Myrmecophilus hebardi*	wMsp7	Malaysia	Gelugor, Pulau Pinang	*Anoplolepis gracilipes*
Anomy35C05	*Myrmecophilus hebardi*	wMsp7	Malaysia	Gelugor, Pulau Pinang	*Anoplolepis gracilipes*
Anomy35C06	*Myrmecophilus hebardi*	wMsp7	Malaysia	Gelugor, Pulau Pinang	*Anoplolepis gracilipes*
Anomy36C01	*Myrmecophilus hebardi*	wMsp7	Malaysia	Gelugor, Pulau Pinang	*Anoplolepis gracilipes*
Cam01C05	*Myrmecophilus hebardi*	wMsp7	Malaysia	Lebuh Relau, Pulau Pinang	*Camponotus* sp.
mp-r8	*Myrmecophilus hebardi*	wMsp7	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
Ano17.09C01	*Myrmecophilus hebardi*	Uninfected	Taiwan	Hengchun Township, Pingtung County	*Anoplolepis gracilipes*
Ano17.09C02	*Myrmecophilus hebardi*	Uninfected	Taiwan	Hengchun Township, Pingtung County	*Anoplolepis gracilipes*
Ano17.10C03	*Myrmecophilus hebardi*	Uninfected	Taiwan	Hengchun Township, Pingtung County	*Anoplolepis gracilipes*
Ano17.12C09	*Myrmecophilus hebardi*	Uninfected	Taiwan	Hengchun Township, Pingtung County	*Anoplolepis gracilipes*
Ano40.C01	*Myrmecophilus hebardi*	Uninfected	Taiwan	Maolin Dist., Kaohsiung City	*Anoplolepis gracilipes*
Anodyu01C01	*Myrmecophilus hebardi*	Uninfected	Taiwan	Dacun Township, Changhua County	*Anoplolepis gracilipes*
Anodyu01C02	*Myrmecophilus hebardi*	Uninfected	Taiwan	Dacun Township, Changhua County	*Anoplolepis gracilipes*
Anodyu01C03	*Myrmecophilus hebardi*	Uninfected	Taiwan	Dacun Township, Changhua County	*Anoplolepis gracilipes*
Anodyu01C04	*Myrmecophilus hebardi*	Uninfected	Taiwan	Dacun Township, Changhua County	*Anoplolepis gracilipes*

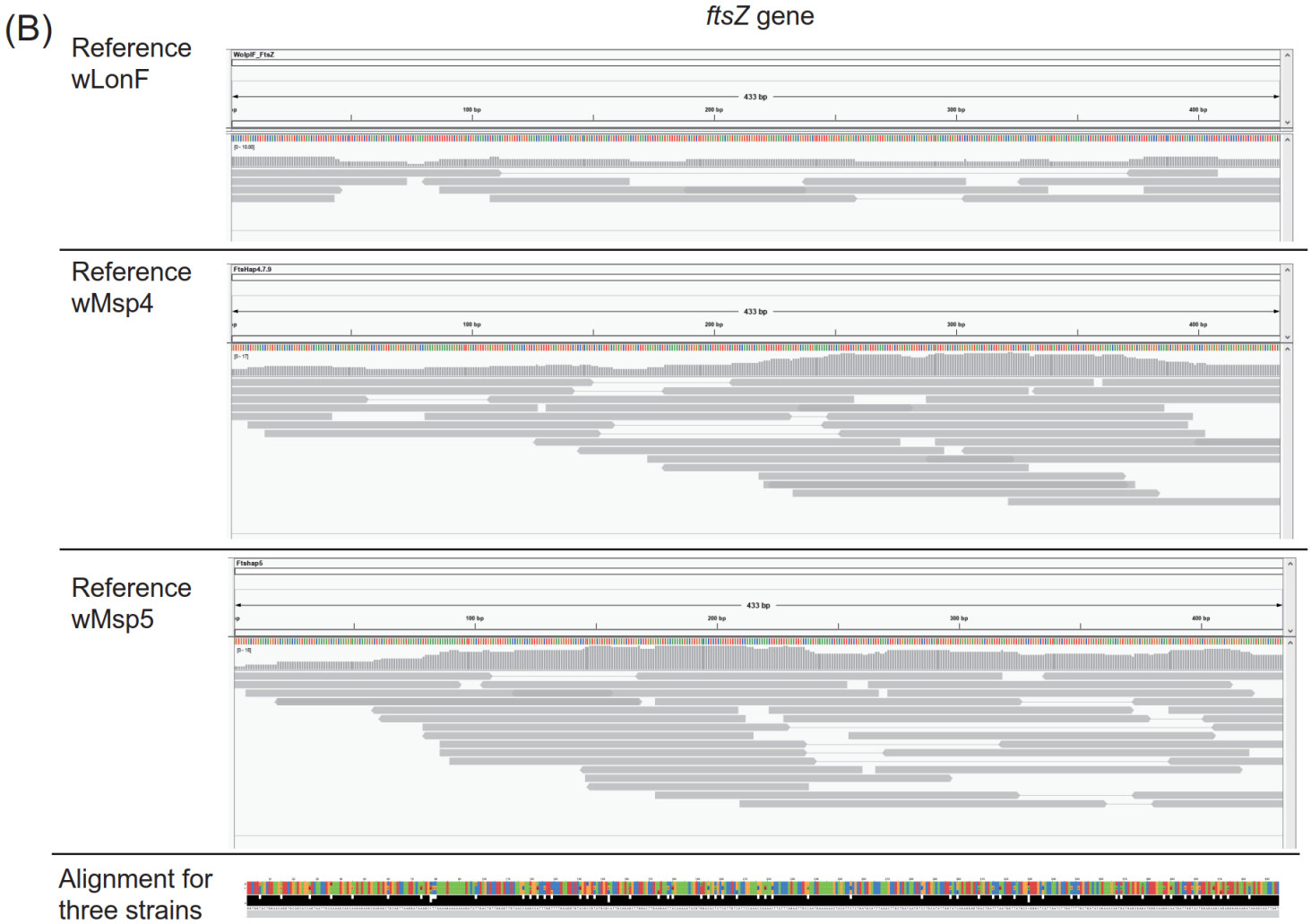
AnoNT01c03	*Myrmecophilus hebardi*	Uninfected	Taiwan	Chushan Township, Nantou County	*Anoplolepis gracilipes*
AnoNT01c05	*Myrmecophilus hebardi*	Uninfected	Taiwan	Chushan Township, Nantou County	*Anoplolepis gracilipes*
AnoNT01c07	*Myrmecophilus hebardi*	Uninfected	Taiwan	Chushan Township, Nantou County	*Anoplolepis gracilipes*
AnoTH04C01	*Myrmecophilus hebardi*	Uninfected	Thailand	Nong Sarai, Pak Chong District	*Anoplolepis gracilipes*
AnoTH04C05	*Myrmecophilus hebardi*	Uninfected	Thailand	Nong Sarai, Pak Chong District	*Anoplolepis gracilipes*
AnoTH04C06	*Myrmecophilus hebardi*	Uninfected	Taiwan	Daan Dist. Taipei	*Anoplolepis gracilipes*
pheton01.C01	*Myrmecophilus quadrispina*	wMsp2	Taiwan	Shalu Dist., Taichung City	*Carebara* sp. (Pheidologeton)
pheton01.C02	*Myrmecophilus quadrispina*	wMsp2	Taiwan	Shalu Dist., Taichung City	*Carebara* sp. (Pheidologeton)
pheton01.C04	*Myrmecophilus quadrispina*	wMsp2	Taiwan	Shalu Dist., Taichung City	*Carebara* sp. (Pheidologeton)
Pl330.C01	*Myrmecophilus quadrispina*	wMsp2, wMsp3	Taiwan	Taiping Dist., Taichung City	*Paratrechina longicornis*
Ano95C20	*Myrmecophilus quadrispina*	wMsp5	Taiwan	Hsinpu town,Hsinchu County	*Anoplolepis gracilipes*
Agr18.1C1	*Myrmecophilus quadrispina*	Uninfected	Taiwan	New Taipei City	*Anoplolepis gracilipes*
Agr18.1C2	*Myrmecophilus quadrispina*	Uninfected	Taiwan	New Taipei City	*Anoplolepis gracilipes*
Agr18.1C3	*Myrmecophilus quadrispina*	Uninfected	Taiwan	New Taipei City	*Anoplolepis gracilipes*
Agr18.1C4	*Myrmecophilus quadrispina*	Uninfected	Taiwan	New Taipei City	*Anoplolepis gracilipes*
Agr18.1C5	*Myrmecophilus quadrispina*	Uninfected	Taiwan	New Taipei City	*Anoplolepis gracilipes*
Agr18.1C6	*Myrmecophilus quadrispina*	Uninfected	Taiwan	New Taipei City	*Anoplolepis gracilipes*
Agr18.2C1	*Myrmecophilus quadrispina*	Uninfected	Taiwan	New Taipei City	*Anoplolepis gracilipes*
Ano17.06C06	*Myrmecophilus quadrispina*	Uninfected	Taiwan	Hengchun Township, Pingtung County	*Anoplolepis gracilipes*
Ano68.C01	*Myrmecophilus quadrispina*	Uninfected	Taiwan	Wulai Dist., New Taipei City	*Anoplolepis gracilipes*
Ano68.C02	*Myrmecophilus quadrispina*	Uninfected	Taiwan	Wulai Dist., New Taipei City	*Anoplolepis gracilipes*
Ano68.C03	*Myrmecophilus quadrispina*	Uninfected	Taiwan	Wulai Dist., New Taipei City	*Anoplolepis gracilipes*
Ano68.C04	*Myrmecophilus quadrispina*	Uninfected	Taiwan	Wulai Dist., New Taipei City	*Anoplolepis gracilipes*
Ano71.C01	*Myrmecophilus quadrispina*	Uninfected	Taiwan	Xiulin Township, Hualien County	*Anoplolepis gracilipes*
Ano71.C02	*Myrmecophilus quadrispina*	Uninfected	Taiwan	Xiulin Township, Hualien County	*Anoplolepis gracilipes*

Ano95C08	*Myrmecophilus quadrispina*	Uninfected	Taiwan	Hsinpu town,Hsinchu County	*Anoplolepis gracilipes*
Ano97C03	*Myrmecophilus quadrispina*	Uninfected	Taiwan	Sanwan Township, Miaoli County	*Anoplolepis gracilipes*
Ano97C04	*Myrmecophilus quadrispina*	Uninfected	Taiwan	Sanwan Township, Miaoli County	*Anoplolepis gracilipes*
Phe17.01C03	*Myrmecophilus quadrispina*	Uninfected	Taiwan	Renai Township, Nantou County	*Pheidole* sp.
Phe17.01C05	*Myrmecophilus quadrispina*	Uninfected	Taiwan	Renai Township, Nantou County	*Pheidole* sp.
Phe17.01C06	*Myrmecophilus quadrispina*	Uninfected	Taiwan	Renai Township, Nantou County	*Pheidole* sp.
pheton01.C03	*Myrmecophilus quadrispina*	Uninfected	Taiwan	Shalu Dist., Taichung City	*Carebara* sp. (Pheidologeton)
pl349C01	*Myrmecophilus quadrispina*	Uninfected	Taiwan	Kinmen	*Paratrechina longicornis*
pl349C02	*Myrmecophilus quadrispina*	Uninfected	Taiwan	Kinmen	*Paratrechina longicornis*
pl367C01	*Myrmecophilus quadrispina*	Uninfected	Taiwan	huisun NantouCounty	*Paratrechina longicornis*
pl367C02	*Myrmecophilus quadrispina*	Uninfected	Taiwan	Renai Township, Nantou County	*Paratrechina longicornis*
pl368C01	*Myrmecophilus quadrispina*	Uninfected	Taiwan	Renai Township, Nantou County	*Paratrechina longicornis*
Cam01C08	*Myrmophilellus pilipes*	wMsp6	Malaysia	Lebuh Relau, Pulau Pinang	*Camponotus* sp.
Dia01C02	*Myrmophilellus pilipes*	wMsp6	Malaysia	Lebuh Relau, Pulau Pinang	*Diacamma* sp.
mun2-r3-1	*Myrmophilellus pilipes*	wMsp6	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
Plmy100C01	*Myrmophilellus pilipes*	wMsp6	Taiwan	Air Hitam, Pulau Pinang	*Anoplolepis gracilipes*
Cam01C01	*Myrmophilellus pilipes*	Uninfected	Malaysia	Lebuh Relau, Pulau Pinang	*Camponotus* sp.
Cam01C02	*Myrmophilellus pilipes*	Uninfected	Malaysia	Lebuh Relau, Pulau Pinang	*Camponotus* sp.
Cam01C03	*Myrmophilellus pilipes*	Uninfected	Malaysia	Lebuh Relau, Pulau Pinang	*Camponotus* sp.
Cam01C04	*Myrmophilellus pilipes*	Uninfected	Malaysia	Lebuh Relau, Pulau Pinang	*Camponotus* sp.
Cam01C06	*Myrmophilellus pilipes*	Uninfected	Malaysia	Lebuh Relau, Pulau Pinang	*Camponotus* sp.
Cam01C07	*Myrmophilellus pilipes*	Uninfected	Malaysia	Lebuh Relau, Pulau Pinang	*Camponotus* sp.
Dia01C01	*Myrmophilellus pilipes*	Uninfected	Malaysia	Lebuh Relau, Pulau Pinang	*Diacamma* sp.
mun2-r1-1	*Myrmophilellus pilipes*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
mun2-r1-2	*Myrmophilellus pilipes*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*

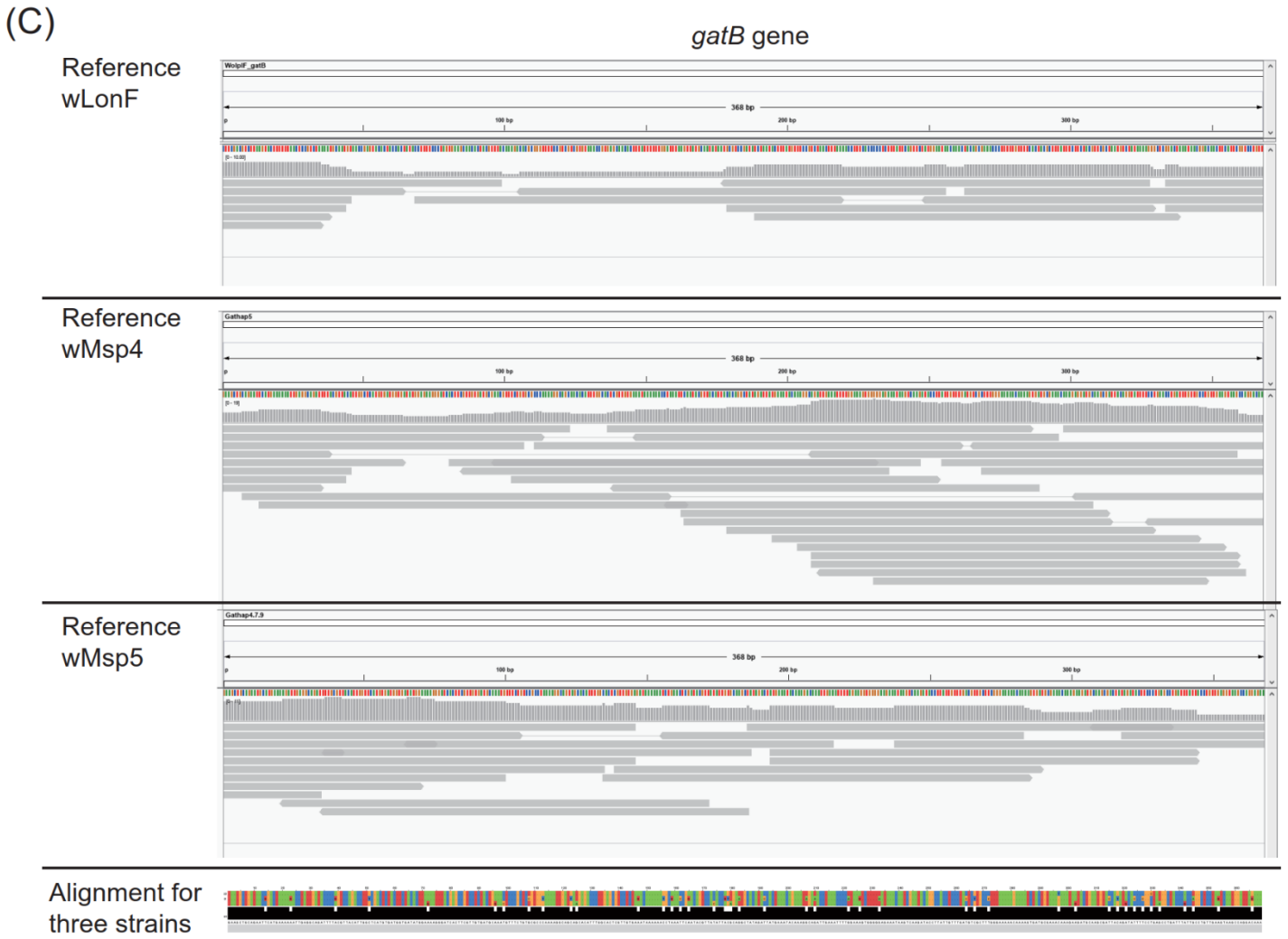
mun2-r2-1	*Myrmophilellus pilipes*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
mun2-r3-2	*Myrmophilellus pilipes*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
mun2-r4-1	*Myrmophilellus pilipes*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
mun2-r4-2	*Myrmophilellus pilipes*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
mun2-r4-3	*Myrmophilellus pilipes*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
plMYBPC01	*Myrmophilellus pilipes*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
plMYUSMC01	*Myrmophilellus pilipes*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
plMYUSMC02	*Myrmophilellus pilipes*	Uninfected	Malaysia	Pulau Pinang	*Anoplolepis gracilipes*
PlSG04C01	*Myrmophilellus pilipes*	Uninfected	Singapore	Mount Faber Park	*Anoplolepis gracilipes*
plmy100C02	*Myrmophilellus pilipes*	Uninfected	Malaysia	Air Hitam, Pulau Pinang	*Anoplolepis gracilipes*



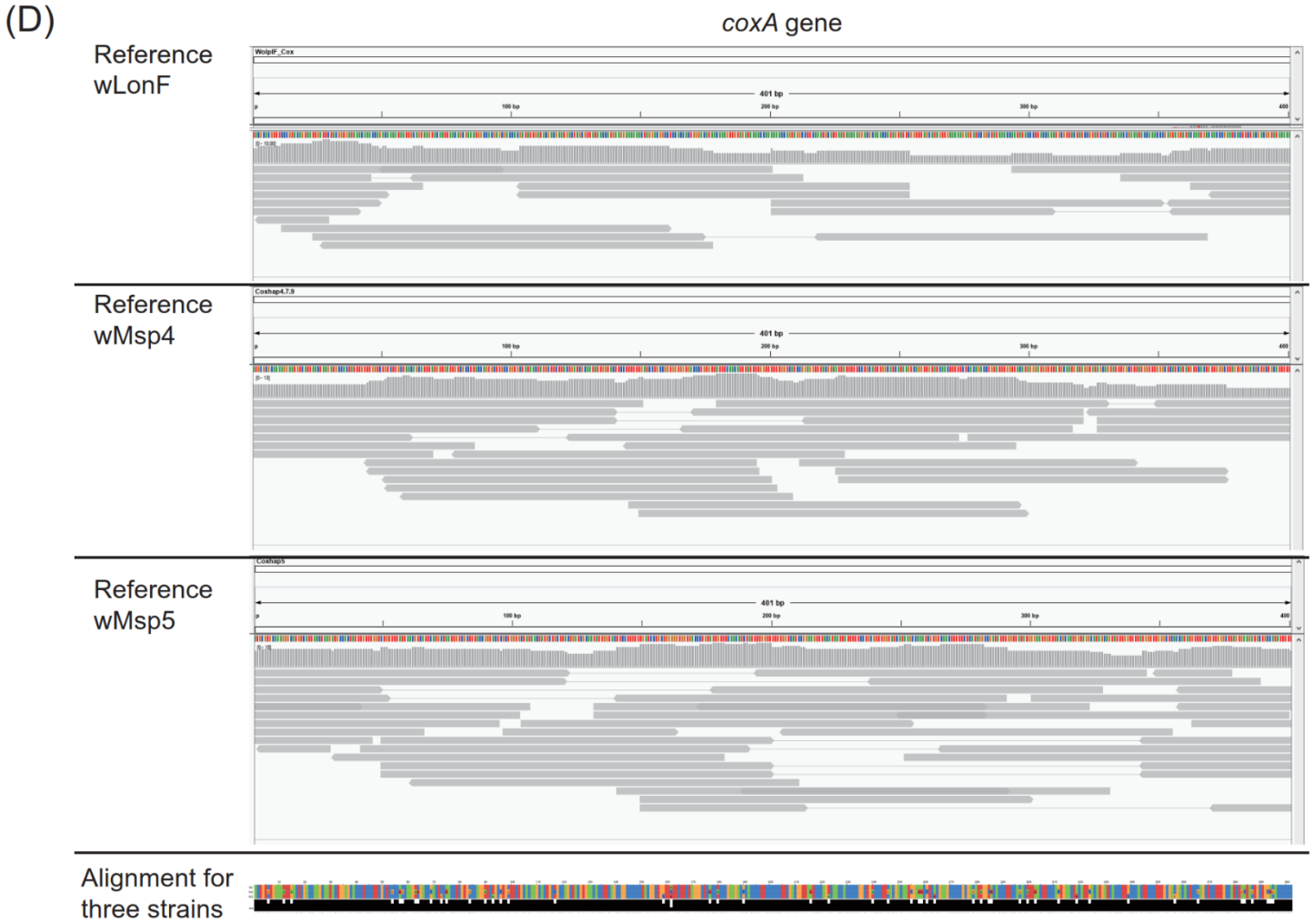
Appendix 13 Alignment of Illumina paired-end sequence reads to the reference MLST sequences (A) *hcpA*, (B) *ftsZ*, (C) *gatB*, (D) *coxA* and (E) *fbpA* of *w*LonF (top), *w*Msp4 (middle), and *w*Msp5 (bottom). Note only those reads perfectly matching the reference sequences were shown.



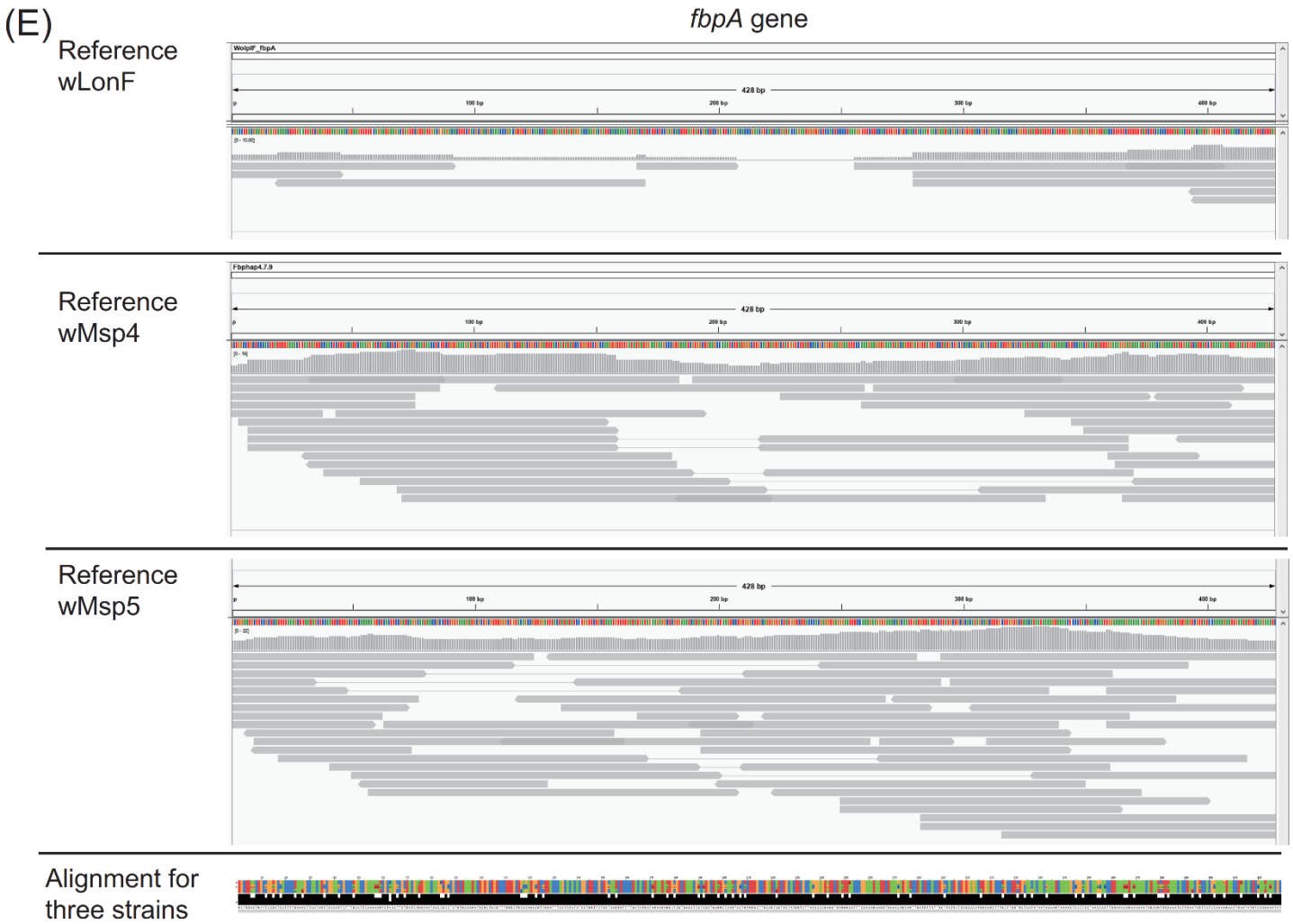
Appendix 13 (Continued)



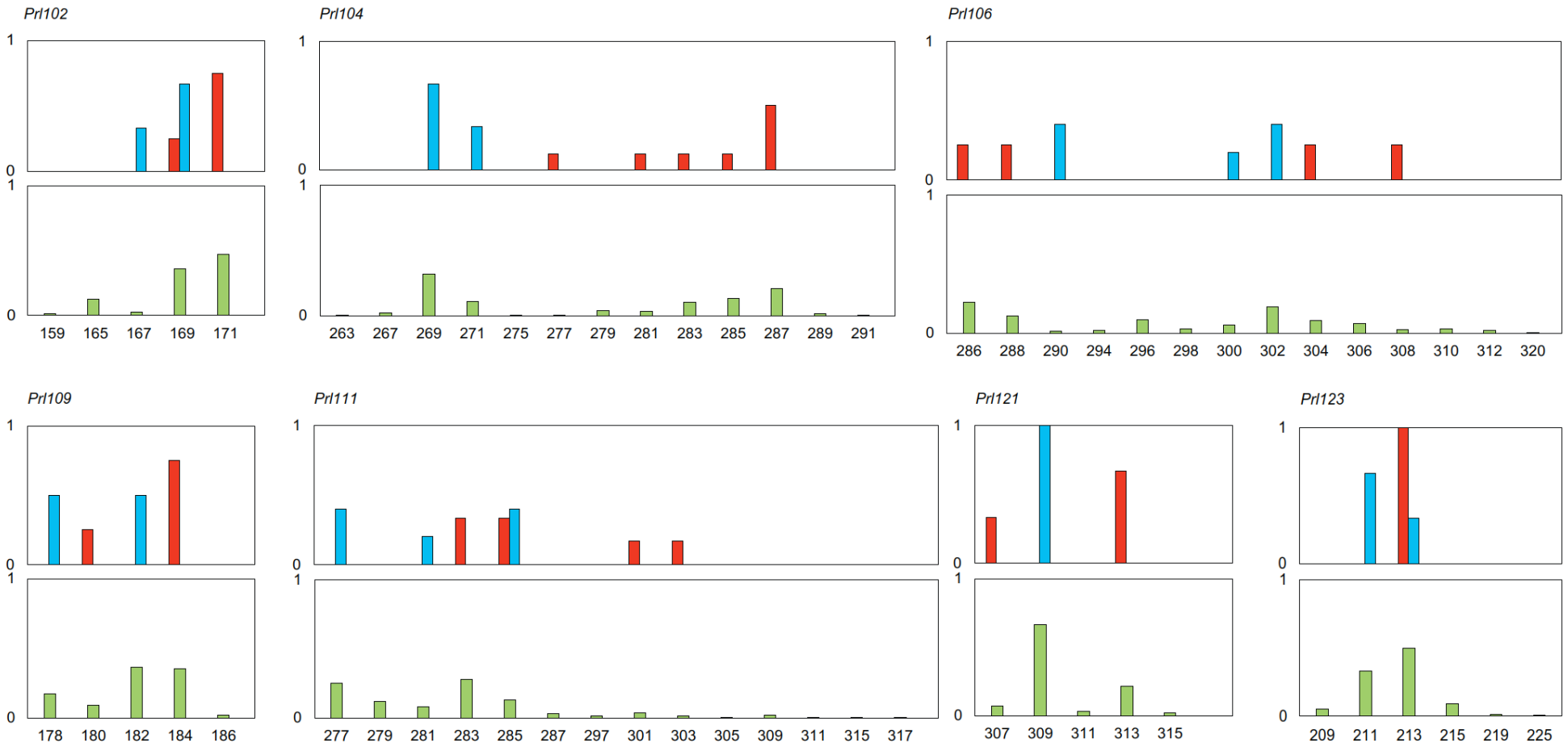
Appendix 13 (Continued)



Appendix 13 (Continued)



Appendix 13 (Continued)



Appendix 14 Allele frequencies in *Paratrechina longicornis* males (blue), queens (red), and workers (green) sampled from the three studied regions. Frequencies were inferred from 5 males, 9 queens, and 78 worker genotypes.