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Diversity and Regional Variation of Endosymbionts in the Green Peach Aphid, *Myzus persicae* (Sulzer)

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Abstract: The green peach aphid, *Myzus persicae*, is globally distributed and an important pest of many economically valuable food crops, largely due to its ability to transmit plant viruses. Almost all aphids, including *M. persicae*, carry the obligate symbiont *Buchnera aphidicola*, which provides essential amino acids that aphids cannot obtain from the phloem of plants themselves. Many aphids also harbor facultative (secondary) endosymbionts, which provide benefits under specific ecological conditions. In this study, we screened for secondary endosymbionts in *M. persicae*, with a particular focus on Australian populations where this species is growing in status as a major agricultural pest. We compared 37 Australian *M. persicae* populations with other populations, including 21 field populations from China and 15 clones from the UK, France, Italy, Greece, USA, Spain, South Korea, Chile, Japan and Zimbabwe. No secondary endosymbionts were identified in *M. persicae* samples outside of China, despite samples covering a wide geographic range and being collected from several host plant families. We detected two secondary endosymbionts (*Rickettsia*, *Spiroplasma*) in Chinese samples, although diversity appeared lower than detected in a recent study. We also found very high clonal diversity in Chinese samples based on DNA microsatellite markers in comparison with lower clonal diversity from Australia. These patterns may indicate a higher diversity of secondary endosymbionts (and clonal diversity) in the native range of *M. persicae* when compared to its invasive range.

Keywords: regional variation; primary endosymbiont; secondary endosymbiont; *Myzus persicae*

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1. Introduction

Bacterial symbionts are widespread in insects, and their symbiotic associations range from obligate mutualism to facultative parasitism [1]. In aphids, the primary (also called obligate) symbiont *Buchnera aphidicola* provides essential amino acids that aphids cannot obtain from the phloem of host plants themselves [2,3]. Secondary endosymbionts (also called facultative) can influence ecologically important traits in aphids, including resistance to parasitoid wasps [4], tolerance to heat stress [5], and host plant utilisation patterns [6]. An association between endosymbiont persistence and colonization of new plant species has been described by Henry et al. [7] in the pea aphid, *Acyrthosiphon pisum* (Harris) (Homoptera: Aphididae). Endosymbionts are spread within populations through vertical transmission, whereby the bacteria are passed from the mother directly to her offspring (which is the main pathway) and through horizontal transmission, for example through host feeding and via parasitoids (e.g., [8]).

The green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), is globally distributed and one of the most economically important aphid crop pests [9]. It has a host

range of more than 400 plant species [10], multiple methods of causing plant damage [11], and widespread resistance to insecticides [12,13]. In addition, *M. persicae* is an important vector of many plant viruses, that can cause considerable harm to host plants [14]. *Myzus persicae* is thought to be of Chinese origin, similar to its primary host, the peach, *Prunus persica*, and has invaded many countries on every continent, except Antarctica [15,16]. Studies of genetic variation within Chinese *M. persicae* populations collected from their primary host plant *P. persica* using DNA microsatellite markers have shown that the genetic structure of these populations involved a split into a southern group and a northern group divided by the Yangtse River. However, the historical demography of *M. persicae* in China remains unknown [17]. The subsequent invasion of this species has included Australia, where *M. persicae* was first detected in 1893 [18], and where it is now found in every state and territory. Singh et al. [15] recently used a high-quality chromosome-scale genome assembly with resequenced genomes of 127 globally sampled *M. persicae* to provide evidence of migration/gene flow between Australia and some populations in Europe and Asia, suggesting multiple incursions.

Artificial endosymbiont infections in aphids can be generated through microinjection of hemolymph across aphid strains or species [19,20], and there is increasing interest in the use of such endosymbiont transfections in pest control. There may be opportunities to release aphid strains generated through transinfection with favourable traits and with endosymbionts able to spread these through wild populations. When applying endosymbiont technology, it is important to understand the status of natural infections in populations of the target aphid, particularly when secondary endosymbionts can vary in incidence between geographic locations as demonstrated in the pea aphid [21]. In *M. persicae*, a few studies have reported that secondary endosymbiont infections may be very rare [22,23], but Xu et al. [24] observed a high diversity of secondary endosymbionts in several Chinese *M. persicae* populations.

In order to better understand the diversity and regional variation of endosymbionts in *M. persicae* and to investigate the ecological and evolutionary factors that might influence the ability of endosymbionts to invade local populations, we characterized the secondary endosymbiont diversity in *M. persicae* populations and some laboratory clones using 16S metabarcoding and quantitative PCR. We undertook broad-scale sampling of aphids from different host plants throughout Australia, China and several other countries to provide baseline data for the exploitation of endosymbiont technology in *M. persicae* control. Our work suggests variation in the incidence of secondary endosymbionts between *M. persicae* within China and elsewhere.

2. Methods

2.1. Aphids

Thirty-two *M. persicae* samples were collected by direct searching for aphids from a variety of host plants from around Australia between 2019 and 2021, while several historical samples, which were collected from the field and had been placed in culture, were also included (Table 1). Ten individuals from each sample were stored in 100% ethanol and frozen at −20 °C for later molecular analysis. The samples in culture were maintained as asexual lines established from a single female in the laboratory on single bok choy (*Brassica napus* subsp. *chinensis*) leaves that were placed in 60 mm petri dishes containing agar (1%). Petri dishes were kept in a controlled temperature cabinet at 11 °C, with a 16L:8D h photoperiod. Twenty-one aphid samples were collected from China (n = 2 to 25) from eight different plant hosts between 2016 and 2021. An additional 15 clones from Europe, the USA, Chile, Zimbabwe, South Korea and Japan (N = 5) were included in this study. These clones were established from aphids collected as part of a global study investigating the evolution of resistance in *M. persicae* [15], with these aphids being maintained as asexual lineages on individual wombok (*Brassica rapa* var. pekinensis) leaves in small plastic cups at 20 °C, with a 16L:8D h photoperiod prior to this study.

Table 1. Summary of aphid samples tested. Collection country and host plant are given, along with numbers tested and microsatellite-defined clonal types where available.

M. persicae Sample	Latitude	Longitude	Host Plant	Date Collected	Aphids Tested	Method	Type of Sample	Microsatellite Defined Clones
AUS_Alloway171	−24.955	152.394	*Celosia* sp.	5 April 2020	10	16S	Field sample	171
AUS_Boggabilla209	−28.718	150.033	*Brassica napus*	16 September 2020	10	16S	Field sample	209
AUS_Bowen158	−20.010	148.188	*Solanum melongena*	16 August 2021	10	16S	Field sample	158
AUS_BrunswickEast_1	−37.776	144.975	*Capsicum* sp.	13 May 2022	10	16S	Field sample	
AUS_BrunswickEast_2	−37.776	144.975	*Solanum lycopersicum*	29 September 2021	10	16S	Field sample	
AUS_Colevale171	−19.505	147.328	*Capsicum chinense*	27 August 2021	10	16S	Field sample	171
AUS_Conara	−41.833	147.464	*Brassica napus*	23 October 2019	10	16S	Field sample	4, 36, 78, 157, 209
AUS_Curyo	−35.848	142.780	*Brassica napus*	21 September 2019	10	16S	Field sample	
AUS_Dookie	−36.344	145.654	*Brassica napus*	23 September 2013	10	16S	Field sample	209, 211
AUS_Elliott158	−24.983	152.304	*Capsicum annum*	4 October 2017	10	16S	Field sample	158
AUS_Hurstbridge	−37.642	145.198	*Solanum betaceum*	16 May 2021	10	16S	Field sample	
AUS_Kendenup98	−34.480	117.404	*Brassica napus*	17 September 2019	10	16S	Field sample	98
AUS_Lab	−36.723	142.175	*Trifolium* sp.	7 October 2019	10	16S	Laboratory colony	
AUS_Lockier209	−29.155	115.360	*Brassica napus*	22 September 2020	10	16S	Field sample	209
AUS_Melbourne	−37.817	144.965	*Helianthus annuus*	26 March 2021	10	16S	Field sample	
AUS_Morangarell209	−34.220	147.714	*Brassica napus*	9 September 2019	10	16S	Field sample	209
AUS_MtKelly171	−19.696	147.319	*Cucurbita* sp.	10 August 2021	10	16S	Field sample	171
AUS_Munglinup209_2	−33.681	120.820	*Brassica napus*	29 August 2018	10	16S	Field sample	209
AUS_NorthMelbourne [1]	−37.795	144.949	*Plantago* sp.	15 April 2020	10	16S	Field sample	
AUS_Osborne_1 [2]	−19.706	147.361	*Capsicum frutescens*	26 August 2020	10	16S	Field sample	158, 171
AUS_Osborne_2 [2]	−19.706	147.361	*Capsicum frutescens*	26 August 2020	10	16S	Laboratory colony	158
AUS_Osborne_3 [2]	−19.706	147.361	*Capsicum frutescens*	26 August 2020	10	16S	Laboratory colony	171
AUS_Osborne_4 [3]	−19.706	147.361	*Capsicum frutescens*	26 August 2020	10	16S	Field sample	158, 171
AUS_Osborne_5 [3]	−19.706	147.361	*Capsicum frutescens*	26 August 2020	10	16S	Laboratory colony	158
AUS_Osborne_6 [3]	−19.706	147.361	*Capsicum frutescens*	26 August 2020	10	16S	Laboratory colony	171

Table 1. *Cont.*

M. persicae Sample	Latitude	Longitude	Host Plant	Date Collected	Aphids Tested	Method	Type of Sample	Microsatellite Defined Clones
AUS_Osborne171	−19.706	147.361	*Capsicum frutescens*	26 August 2020	10	16S	Field sample	171
AUS_Osborne158	−19.706	147.361	*Capsicum frutescens*	26 August 2020	10	16S	Field sample	158
AUS_SouthGreenough158	−29.028	114.832	*Capsicum* sp.	30 October 2019	10	16S	Field sample	158
AUS_StLucia209	−27.496	153.009	*Brassica oleracea*	16 November 2021	10	16S	Field sample	209
AUS_StRonans209	−31.909	116.703	*Brassica napus*	28 September 2021	10	16S	Field sample	209
AUS_Parkville	−37.780	144.940	*Brassica oleracea*	12 March 2021	10	16S	Field sample	
AUS_PascoeValeSouth	−37.738	144.935	*Capsicum* sp.	11 March 2021	10	16S	Field sample	
AUS_Penfield188	−34.687	138.633	*Solanum melongena*	10 October 2014	10	16S	Field sample	188
AUS_Preston	−37.742	145.000	*Prunus persica*	10 October 2020	10	16S	Field sample	
AUS_Preston158_1 [4]	−37.733	145.009	*Brassica oleracea*	22 April 2020	10	16S	Field sample	158
AUS_Preston158_2 [5]	−37.733	145.009	*Brassica oleracea*	22 April 2020	10	16S	Field sample	158
AUS_Preston209	−37.742	145.000	*Solanum betaceum*	23 November 2021	10	16S	Field sample	209
China_Beijing_Haidian	39.944	116.288	*Capsicum* sp.	22 October 2021	25	16S	Field sample	
China_Beijing_Daxing	39.733	116.349	*Capsicum* sp.	21 March 2021	10	16S	Field sample	
China_Beijing_1	39.903	116.401	*Arabidopsis thaliana*	2016	10	qPCR	Field sample	253, 254, 256, 257, 258, 259
China_Beijing_2	39.903	116.401	*Amygdalus persica*	2016	5	qPCR	Field sample	103, 244, 307, 315
China_Beijing_3	39.903	116.401	*Amygdalus persica*	2016	5	qPCR	Field sample	237, 244, 304, 313
China_Fujian_Ningde	26.160	119.767	*Brassica napus*	2005	5	qPCR	Clone_laboratory	
China_Gansu_Lanzhou_1	36.062	103.832	*Nicotiana tabacum*	2016	6	qPCR	Field sample	110, 260
China_Gansu_Lanzhou_2	36.061	103.832	*Nicotiana tabacum*	2016	6	qPCR	Field sample	223, 249, 291, 309
China_Guangxi_Hezhou	24.468	111.130	*Solanum melongena*	21 June 2018	10	16S	Field sample	
China_Hebei_Tangshan	39.958	117.967	*Prunus persica*	2016	5	qPCR	Clone_laboratory	
China_Jiangsu_Nanjing	32.060	118.791	*Raphanus sativus*	2016	12	qPCR	Field sample	241, 245, 247, 256, 302, 303, 310, 316
China_Shanxi_Jinzhong	37.421	112.545	*Brassica oleracea*	2016	12	qPCR	Field sample	231, 255, 256
China_Shandong_Qindao	36.066	120.378	*Amygdalus persica*	2016	4	qPCR	Field sample	105, 140, 266, 314

Table 1. *Cont.*

M. persicae Sample	Latitude	Longitude	Host Plant	Date Collected	Aphids Tested	Method	Type of Sample	Microsatellite Defined Clones
China_Sichuan_Deyang	38.142	104.417	*Brassica napus*	12 March 2021	10	16S	Field sample	
China_Xinjiang_Tulufan_1	42.941	89.183	*Prunus persica*	2016	4	qPCR	Field sample	6, 59, 121, 292
China_Xinjiang_Tulufan_2	42.941	89.183	*Prunus persica*	2016	8	qPCR	Field sample	10, 86, 120, 141, 143, 228, 308, 317
China_Yunnan_Kunming_1	25.009	102.825	*Brassica oleracea*	19 July 2020	10	16S	Field sample	
China_Yunnan_Yuxi	24.094	101.910	*Nicotiana tabacum*	20 July 2020	10	16S	Field sample	
China_Yunnan_Kunming_2	24.883	102.832	*Nicotiana tabacum*	2016	5	qPCR	Field sample	117, 290
China_YunnanKunming_3	24.883	102.832	*Nicotiana tabacum*	2016	5	qPCR	Field sample	117, 261, 290
China_Y_Kunming_4	24.883	102.832	*Nicotiana tabacum*	2016	2	qPCR	Field sample	117
Chile_Duao	35.558	71.588	*Prunus persica*	2018	5	qPCR	Clone_laboratory	
France	Unknown	Unknown	*Prunus persica*	2009	5	qPCR	Clone_laboratory	
Greece_ Tyrnavos	39.759	22.286	*Prunus persica*	2018	5	qPCR	Clone_laboratory	
Greece_ Neo Keramidi	40.286	22.463	*Nicotiana tabacum*	2018	5	qPCR	Clone_laboratory	
Italy_Salvo	42.048	14.734	*Prunus persica*	2012	5	qPCR	Clone_laboratory	
Italy_Benevento	41.130	14.783	*Nicotiana tabacum*	1999	5	qPCR	Clone_laboratory	
Japan	Unknown	Unknown	*Solanum melongena*	1983	5	qPCR	Clone_laboratory	
South Korea_ North Gyeongsang	35.848	129.202	*Brassica oleracea*	Unknown	5	qPCR	Clone_laboratory	
Spain	37.755	1.103	*Capsicum* sp.	Unknown	5	qPCR	Clone_laboratory	
UK_ Worcestershire	52.255	2.267	*Chrysanthemum*	1982	5	qPCR	Clone_laboratory	
UK_1	Unknown	Unknown	*Brassica oleracea*	2004	5	qPCR	Clone_laboratory	
UK_2	Unknown	Unknown	*Beta vulgaris*	1974	5	qPCR	Clone_laboratory	
UK_3	Unknown	Unknown	*Solanum tuberosum*	2007	5	qPCR	Clone_laboratory	
USA_ North Carolina	35.883	77.665	*Nicotiana tabacum*	2015	5	qPCR	Clone_laboratory	
Zimbabwe	Unknown	Unknown	*Nicotiana tabacum*	2010	5	qPCR	Clone_laboratory	

[1] Orange color morph from AUS_NorthMelbourne. [2] Green color morph from samples preserved in 100% ethanol immediately after field collection or from lab colony established from AUS_Osborne. [3] Red color morph from AUS_Osborne. [4] Green color morph from AUS_Preston158. [5] Pink color morph from AUS_Preston158.

A map displaying the location of all *M. persicae* samples used here is provided in Figure 1.

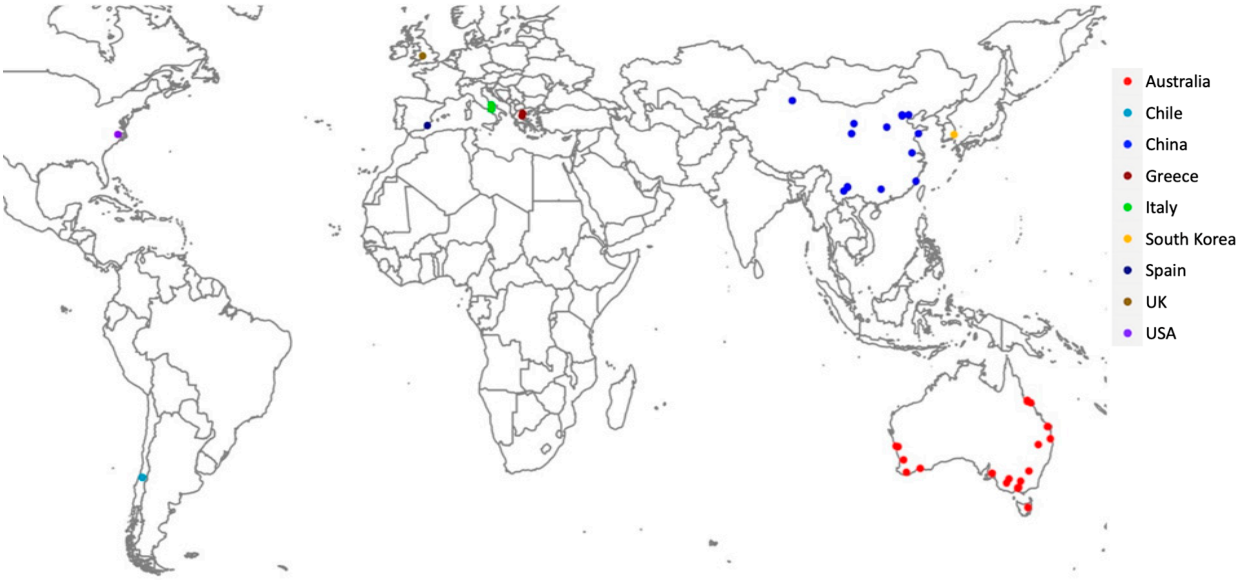

Figure 1. Geographic origin of *M. persicae* samples used in this study.

2.2. Clonal Assignment of M. persicae

Aphids from 27 Australian samples and 13 Chinese samples were genotyped to determine the clonal make-up of each sample. Two to 12 aphids from each population were genotyped using 10 previously described DNA microsatellite loci: M35, M37, M40, M49, M55, M63, M86, myz2, myz9 and myz25 [25,26]. DNA was extracted by homogenising individual aphids in a 200 µL solution containing 5% Chelex 100 resin (Bio-Rad Laboratories, Hercules, CA, USA) according to methods described previously [24]. Samples were centrifuged for 2 min at 20,800 g (Eppendorf Centrifuge 5417 C, Hamburg, Germany) and 2 µL of the supernatant was used as template in polymerase chain reactions. Loci were pooled into three groups, labelled with unique fluorophores (FAM, NED, VIC, and PET) and coamplified by multiplex PCR using a Qiagen multiplex kit and an Eppendorf Mastercycler S gradient PCR machine. Genotyping was subsequently performed using a 3730 capillary analyzer (Applied Biosystems, Melbourne, Australia) and product lengths were scored manually using GeneMapper version 4.0 (Applied Biosystems).

2.3. 16S rRNA Gene Metabarcoding and Quantitative PCR

We used DNA metabarcoding to characterise the microbiome of some *M. persicae* samples listed in Table 1 (all Australian populations and a few Chinese populations). For these samples, individuals were pooled to provide sufficient DNA for next generation sequencing. Two replicate DNA extractions, each containing a pool of 5 individuals, were performed for each sample using a DNeasy® Blood & Tissue kit (Qiagen, Hilden, Germany). Metabarcoding targeted the hypervariable V3-V4 region of the bacterial 16S rRNA gene and was carried out by Novogene (Novogene (HK) Co. Ltd., Hong Kong, China), using the universal primers 341F and 806R. Sequence analysis was performed using a standard QIIME2 pipeline [27]. Firstly, primer sequences were trimmed from reads with the cutadapt plugin. Sequence quality filtering and error correction, assembly of paired-end reads, and chimera removal were performed with the DADA2 plugin. DADA2 was then used to group reads into amplicon sequence variants (ASVs), which are analogous to Operational Taxonomic Units (OTUs) clustered at a 100% identity threshold. Background filtering was performed on a sample-wise basis. ASVs that made up less than 0.1% of the reads in a sample were removed from that sample. Taxonomic identity was assigned to ASVs with the classify-sklearn plugin, using a naïve Bayes classifier that had been trained against

the SILVA 16S rRNA database [28] (release 132; 99% identity criterion). The identity of endosymbiont ASVs was further investigated with blastn searches (nr/nt database). LFN (low-frequency noise) filters were used to discard variants with low read counts and sequencing contamination (Yang et al., unpublished). An average of 283,084 reads per sample were retained after each of the quality filtering and assembly steps from our 16S metabarcoding data.

Quantitative PCR (qPCR) assays were used to screen for secondary endosymbionts in *M. persicae* samples which comprised a limited number of individuals and/or had insufficient DNA amounts for metabarcoding. DNA was extracted from individual aphids (variable numbers per population; Table 1) using 150 µL of 5% Chelex 100 resin as described previously, with PCRs undertaken using a LightCycler® 480 High Resolution Melting Master (HRMM) kit (Roche Diagnostics Australia Pty. Ltd., Castle Hill, Australia) and IMMOLASETM DNA polymerase (5 U/µL) (Bioline; Cat. No. BIO-21047). The PCR conditions for DNA amplification began with a 10-min pre-incubation at 95 °C (Ramp Rate = 4.8 °C/s), followed by 40 cycles of 95 °C for 5 s (Ramp Rate = 4.8 °C/s), 58 °C for 15 s (Ramp Rate = 2.5 °C/s), and 72 °C for 30 s (Ramp Rate = 4.8 °C/s). Two primer sets were applied to amplify markers to confirm the quality of aphid DNA (β-actin as reference gene) [29] and the presence or absence of the target endosymbiont infection (Table 2). Crossing point (Cp) values of three consistent replicate runs were averaged. Differences in Cp values between the actin and the target endosymbiont markers were transformed by 2n to produce relative endosymbiont density measures.

Table 2. Primers used for the detection of endosymbionts in this study.

Target Endosymbiont	Primer Name	Primer Sequence	Reference
Arsenophonus	Arsen_yaeT_F	AATATGCCTGTTCGGGTAGG	[30]
	Arsen_yaeT_R	GTTGGCCGCTCTTTTACTTG	
Hamiltonella defensa	Ham_16Sl_F1	AGGAGGAAGCGATAAATGC	This study
	Ham_16Sl_R1	CCCTCTAGAAAACTCTAGCGAC	
Regiella insecticola	U99F	ATCGGGGAGTAGCTTGCTAC	[31]
	16SB4	CTAGAGATCGTCGCCTAGGTA	
Rickettsia	Rickettsia_16S_F1	GTGCGTAGGCGGTTTAGTA	This study
	Rickettsia_16S_R1	TTGTAGCCCAGATGACCG	
Rickettsiella viridis	RCL16S-211F	GGGCCTTGCGCTCTAGGT	[31]
	RCL16S-470R	TGGGTACCGTCACAGTAATCGA	
Serratia symbiotica	Serr_16S_F1	TTGTTGCCAGCGATAAAG	This study
	Serr_16S_R1	CCATTGTAGCACGTGTGT	
Wolbachia	Wol_16S_F	CCAGCAGCCGCGGTAAT	[32]
	Wol_16S_R	CGCCCTTTACGCCCAAT	
	Wol_probe	CGGAGAGGGCTAGCGTTATTCGGAATT	
Reference gene	actin_aphid_F1	GTGATGGTGTATCTCACACTGTC	This study
	actin_aphid_R1	AGCAGTGGTGGTGAAACTG	

Presence of the endosymbiont amplicon was confirmed by running a standard PCR using the same program and primers with qPCR, running 10 µL of PCR product on a 2% molecular biology grade agarose gel (Scientifix, Springvale, Australia) and observing a clear band with appropriate size for each primer pair. PCR products were then sent for Sanger sequencing (Macrogen, Inc., Geumcheongu, Seoul, South Korea). Sequencing chromatograms were examined and processed with Geneious 9.18 software (Biomatters,

Inc., Auckland, New Zealand). The 16S rRNA gene sequencing data were analysed with the program Geneious. A phylogenetic tree was constructed with a neighbour-joining model applied to a genetic distance matrix with the Tamura-Nei2 model implemented with 1000 bootstrap replications in Geneious.

3. Results and Discussion

In this study, we examined endosymbiont diversity in 73 *M. persicae* samples from 18 different host plants, focusing on Australia but also including samples collected more widely. Importantly, this included samples from China, which is believed to represent the region where this species originated [17]. Genotyping across 10 microsatellite DNA loci identified 10 distinct clones among the Australian *M. persicae* populations sampled here and 47 clones among the Chinese populations sampled (Table 1). No secondary endosymbionts were detected within the 37 Australian samples (Table 3, Figure 2), even though individuals from Australian samples represented multiple clones as characterized by microsatellites, were sampled over a broad geographic range, collected from multiple plant families and included multiple color morphs. These patterns are consistent with the lack of secondary endosymbionts in the 15 clones from the UK, France, Italy, Greece, the USA, Spain, South Korea, Chile, Japan and Zimbabwe, and also from other studies of *M. persicae* outside of China [22,23,33].

Table 3. Infections by endosymbionts detected in *M. persicae* samples tested in this study and in Xu et al. (2019) [24].

Endosymbiont	No. Aphids Infected/Sample Size			
	This Study			Xu et al. (2019) [24]
	China	Australia	Other Countries	China
Buchnera aphidicola	21/21	37/37	15/15	92/92
Serratia symbiotica	0/21	0/37	0/15	15/92
Rickettsiella viridis	0/21	0/37	0/15	NA
Hamiltonella defensa	0/21	0/37	0/15	4/92
Rickettsia	4/21	0/37	0/15	15/92
Regiella insecticola	0/21	0/37	0/15	12/92
Wolbachia	0/21	0/37	0/15	53/92
Arsenophonus	0/21	0/37	0/15	15/92
Spiroplasma	1/21	0/37	0/15	3/92

We then screened 21 *M. persicae* populations from China using qPCR, the putative native range, in order to provide a comparison to invaded populations. *Rickettsia* was detected in four out of 21 samples, each of which was collected from tobacco, *Nicotiana tabacum*. We also detected *Spiroplasma* from a single population collected on radish, *Raphanus sativus* (Figure 3, Table 3). The incidence of endosymbiont detection in the Chinese samples (5/21) differs from the incidence of detection in Australian samples (0/37) (Fisher's exact test, $p = 0.004$, IBM Statistics SPSS version 26), even though the sample size of clones from China was smaller (Table 1). A previous screen of diversity in secondary endosymbionts from Chinese populations also identified *Rickettsia* and *Spiroplasma* from samples of *M. persicae*. In addition, another five endosymbionts were detected including *Wolbachia*, which was abundant across the populations (Table 3) [24]. Although the sample sizes of field

collections were not specified in this paper, it is unclear why we failed to detect these other endosymbionts and particularly *Wolbachia* in our field samples from China.

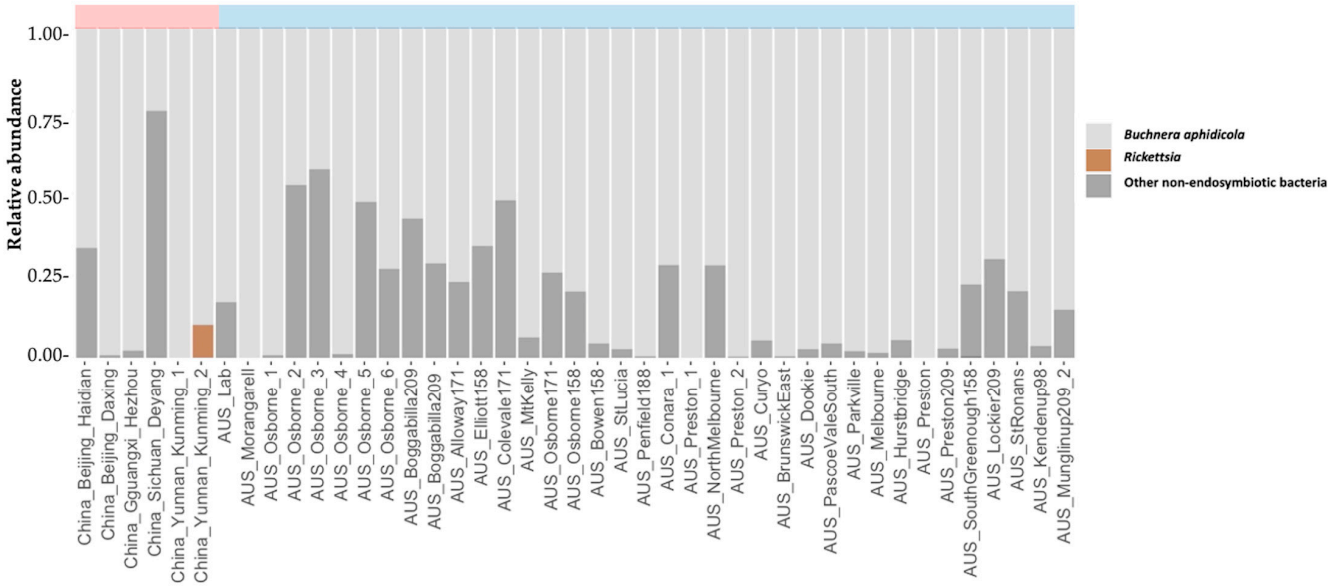

Figure 2. Secondary endosymbionts in *M. persicae* populations tested via 16S metabarcoding. Mean relative abundances are shown for each sample included within the study (calculated from 2 technical replicates). Known secondary endosymbionts are shown in color, while the primary endosymbiont, *Buchnera aphidcola*, and non-endosymbiotic bacteria are shown in grey. The color label on the top of the bar chart represents China, and blue represents Australia. Note that sample codes can be found in Table 1. The group of 'other non-endosymbionts' includes all the bacterial detected except for *Buchnera* and 8 secondary endosymbionts recorded in aphids (*Serratia*, *Hamiltonella*, *Regiella*, *Rickettsia*, *Rickettsiella*, *Spiroplasma*, *Wolbachia and Arsenophonus*).

It is possible that some detections in previously published work represents contaminants. Note that we set a sensitive cut-off at 0.1% to remove ASVs with low relative abundance and validated all our positive detections through PCR and Sanger sequencing, whereas previous work has relied on metabarcoding data and set a much lower cut off at 0.005%, with the exception of qPCR screening for *Rickettsia*. It is also possible that there is geographic or seasonal variation in the distribution of endosymbionts. Although we had multiple sampling points in China, there was only one that overlapped with the samples scored as *Wolbachia* infected by Xu et al. (M37103, *Amygdalus persica*, Beijing) [24]. Seasonal changes in *Wolbachia* infection have been documented in some insects [34] and may occur for other endosymbionts.

Nevertheless, the results from our survey combined with the data from Xu et al. [24] suggest that secondary endosymbionts are more common in the native range of this species than the introduced range, which is also consistent with our failure to detect secondary endosymbionts in a sample of the global clonal collection from Singh et al. [15]. Note that Singh et al. [15] also failed to detect any secondary endosymbionts in whole genome sequence data from >110 fully sequenced globally sampled clonal lines of *M. persicae*, although this method is less sensitive than metabarcoding of the microbiome.

The phylogenetic analyses of endosymbionts based on sequences obtained from both the 16S data and the Sanger data indicates that the *Rickettsia* we detected is not closely linked to *Rickettsia* from other aphids, but instead connects to other arthropod groups including weevils (e.g., *Sitona obsoletusand* and *Liophloeus* sp.) and green lacewings (*Pseudomallada ventralis*) (Figure S1, Supplementary Data). However, the phylogenic analysis of the *Spiroplasma* sequences indicated that the secondary endosymbiont we identified was closely connected to an aphid-specific group, which was separate from the *Spiroplasma* sequences from *Drosophila* and several other insects, including the mealybug *Antonina crawii* (Figure S2,

Supplementary Data). Perhaps *Rickettsia* has a high rate of horizontal transmission which may explain its similarity across disparate taxonomic groups, but at this stage we are unaware of any literature on horizontal transmission in this group and additional molecular analyses based on more comprehensive sequence data are required.

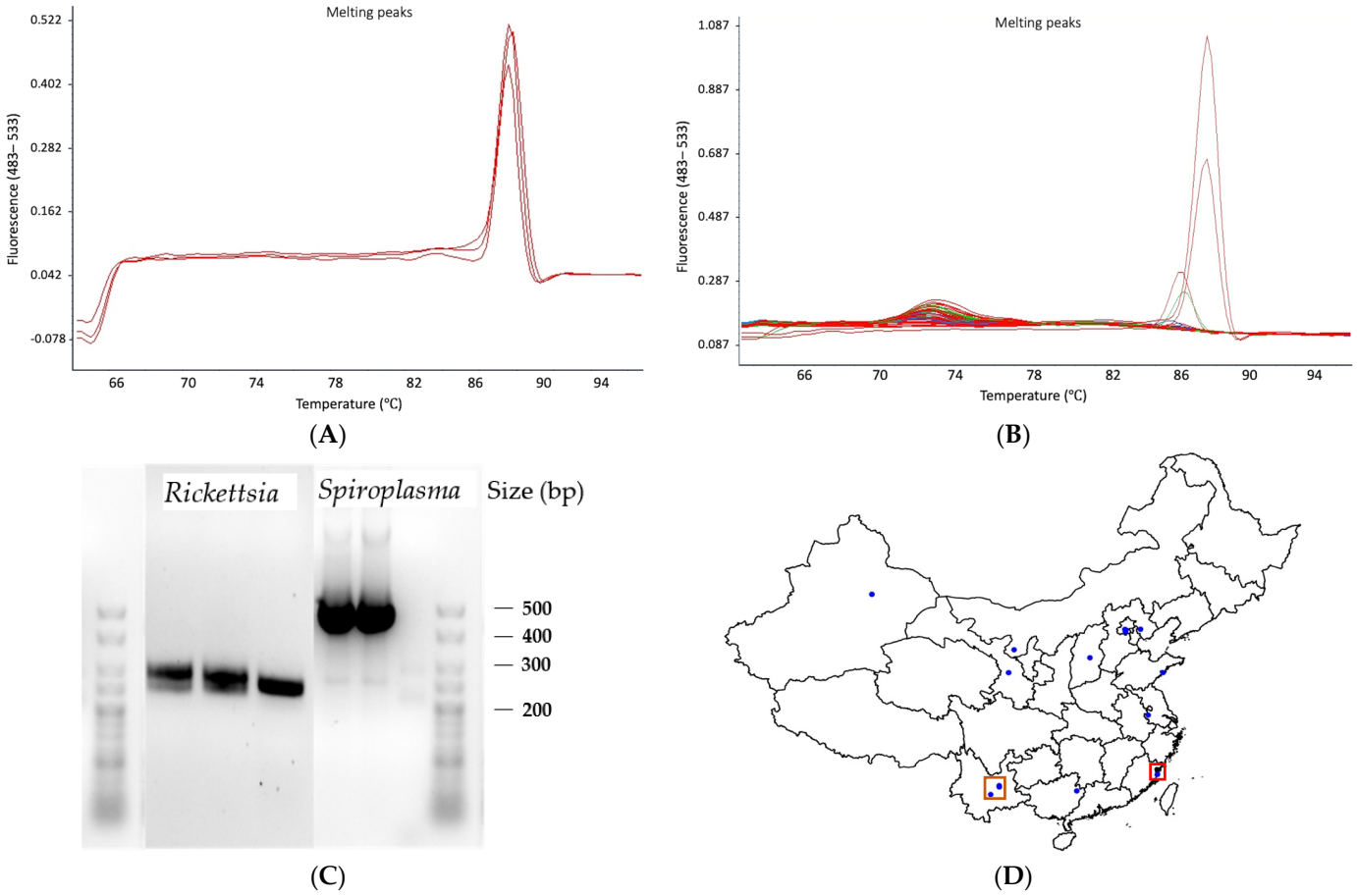

Figure 3. qPCR and PCR amplification of *Rickettsia* and *Spiroplasma*. (**A**). Melting curve of *Rickettsia* in qPCR showing distinct Tm value of 87 degrees. (**B**). Melting curve of *Spiroplasma* in qPCR. (**C**). Agarose gel images of *Rickettsia* and *Spiroplasma* amplicons in traditional PCR. (**D**) Map showing the 21 sampling points of *M. persicae* in China (blue dots) with *Rickettsia* points (brown square) and *Spiroplasma* points (red square) indicated.

Several reasons might explain the absence of secondary endosymbionts outside of China. Firstly, it may be the case that the colonization process of invasive *M. persicae* involved a low number of individuals and that by chance these lacked secondary endosymbionts. If this happened, we might expect lower nuclear genetic diversity in colonized regions (or lower clonal diversity in cases where there is no sex). Singh et al. [15] found that both host plant and geography play a significant role in partitioning genetic variation in *M. persicae* global populations. However, genetic divergence between Chinese *M. persicae* populations was not higher than divergence detected between Australian *M. persicae* populations. This may reflect limited sampling of Chinese clones by Singh et al. [15] given that they characterized only 9 opportunistically collected clones from China. Based on microsatellite data, we detected much higher clonal diversity in Chinese populations, with 47 clones detected from 53 individuals, compared to only 10 clones from 270 individuals detected in Australia (binomial test comparing two proportions, $z = 14.6$, $p < 0.001$).

Secondly, it may be that secondary endosymbionts have been lost in colonized regions. This might occur if there is imperfect maternal transmission and no selective advantage of individual aphids with these endosymbionts in newly colonized environments. Numerous

phenotypes have been associated with aphid endosymbionts that could contribute to selective differences between host aphid lineages, including resistance to parasitoid wasps [4], tolerance to heat stress [5], and host plant utilisation patterns [6], although very few of these have been examined in *M. persicae*. One exception is the secondary endosymbiont *Regiella*, which has been associated with parasitoid resistance in *M. persicae* [35]. Interestingly the natural *Regiella* strain used in that study was collected from Bacchus Marsh, Australia on wild mustard, *Hirschfeldia incana*, in 2003 [36]. Clearly this was a serendipitous finding given that our much more extensive collection indicated an absence of *Regiella* in all 32 *M. persicae* field populations tested. We have also failed to collect this endosymbiont from aphids from this host plant in subsequent work (Yang et al., unpublished data). Perhaps the *Regiella* strain was a recent introduction into Australia which has been lost because of a selective disadvantage and/or transmission leakage. Or perhaps the abundance of secondary endosymbiont seasonally changes. In China, there was no association between the presence of secondary endosymbionts and host plant, but *Regiella* was commonly detected [24].

It is not yet clear if the loss of endosymbionts from the invaded range of a species is a general finding or specific to *M. persicae*. Perhaps the most extensively investigated comparison of native and invasive ranges involves endosymbionts in the mosquito *Aedes albopictus*. In this species, Yang et al. [37] showed that two *Wolbachia* strains were widely distributed across both its native and introduced ranges, with only minor population differences in endosymbiont frequency. In other species, variation in the distribution of endosymbionts has been found across populations, such as the pea aphid *A. pisum* [21], the spider mite *Tetranychus truncates* [38], and various plant parasitic nematodes [39]. These comparisons do not provide a contrast between invaded versus native range populations, but there are other instances (for instance in fire ants [40] and in thrips [41]) where there has been a loss of symbionts in the invasive range.

These results have implications for the introduction of secondary endosymbionts for pest control. This is an area of increasing interest, particularly from the perspective of blocking plant viral transmission [42,43] and changing pesticide susceptibility [44]. Based on the current work, a lack of endosymbionts in the invasive range of this species means that any secondary endosymbionts artificially introduced into *M. persicae* populations from this range will not have to compete with other secondary endosymbionts already present in field populations. Interactions among endosymbionts are known from other work [45] and could otherwise lead to a suppression of newly introduced endosymbionts in a native host. We have recently introduced two endosymbionts from other aphid species into *M. persicae* (Gu et al., under review) and into the oat aphid, *Rhapalosiphum padi* [46] from their invasive range. These have proven to be stable introductions, and we are currently exploring if they have favorable characteristics (such as blocking plant virus transmission or decreasing host fitness) that may make them suitable for introductions into pest aphid populations in Australia and elsewhere.

Endosymbionts have also been implicated in color variation among aphids [47], including in a recent study where the endosymbiont *Rickettsiella viridis* was transferred to *M. persicae* which modified the aphid body color from light to dark green (Gu et al. under review). Although color morphs were not specifically considered in this study, we did test individuals from Australia that varied considerably in color, ranging from light green, to orange, pink and red. Since we did not find secondary endosymbionts associated with these color morphs, it appears that environmental factors generate color variation in Australian *M. persicae*.

4. Conclusions

In summary, there appear to be differences in the diversity (number and variety) of secondary endosymbionts found in *M. persicae* from invaded and native range (China) populations. Secondary endosymbionts in colonized regions may have been lost due to imperfect maternal transmission, founder events and/or selection associated with different

conditions in the invaded region. The lack of secondary endosymbionts in the invaded regions open up the possibility of manipulating populations in these regions through the introduction of novel endosymbionts.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15020206/s1, Figure S1: Phylogenetic analysis of *Rickettsia* based on 16S rRNA gene variation; Figure S2: Phylogenetic analysis of *Spiroplasma* based on 16S rRNA gene variation; Supplementary Data: Chromatogram from Sanger sequencing.

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