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Genetic Diversity of *Tamarixia radiata* Populations and Their Associated Endosymbiont *Wolbachia* Species from China

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Abstract: *Tamarixia radiata* is one of the established biocontrol pests against the major Asian citrus psyllid, *Diaphorina citri*, a vector of *Candidatus* Liberibacter that is a causal agent of citrus Huanglongbing (HLB) disease. Updated information and regional exploration on biocontrol pests are important elements for effective disease management strategies. In this study, the diversity and parasitism rate of *T. radiata* populations were evaluated. Due to the importance of the host–parasitoid relationship, the presence of *Wolbachia* as an endosymbiont was also investigated. The parasitism rate of various *T. radiata* populations from Ecuador and China ranged between 57.27% and 66.32%, respectively, with a non-significant emergence rate and a statistically similar sex ratio. Sequence analysis of ITS and *COI* from *T. radiata* populations was consistent with the morphological hypothesis that the collections represent a single species, whereas phylogeny of the *wsp* gene confirmed the presence of *Wolbachia pipientis* as an endosymbiont within *T. radiata* populations. Based on partial *COI* sequences, the maximum genetic diversity such as total haplotype diversity (Hd = 0.788), nucleotide, diversity (π = 0.2439), and average nucleotide difference (k = 171.844) was also estimated for different *T. radiata* populations. Furthermore, neutrality tests based on *COI* sequences indicated an overall contraction in *T. radiata* populations, whereas an expansion trend was observed in associated *W. pipientis* strains. This study clearly demonstrated the presence of genetically diverse *T. radiata* populations that were able to parasitize *D. citri* effectively, and these can be further explored as promising biocontrol candidates in integrated pest management strategies to solve citriculture economic loss caused by *D. citri*.

Keywords: biocontrol agent; *Tamarixia radiata*; parasitism rate; *Wolbachia pipientis*; COI; wsp; *Diaphorina citri*

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1. Introduction

Parasitoids constitute a species-rich group containing more than 20% of the world's insect species [1], divided into six families (Aphelinidae, Encyrtidae, Eulophidae, Eupelmidae, Pteromalidae, and Signiphoridae) within the order Hymenoptera. According to the parasitoids' taxonomic position, Eulophidae contains many species associated with insect

hosts [2]. These parasitoids use their hosts for nourishment through direct feeding and gradually kill them [3,4]. Consequently, they effectively reduce their host populations in natural ecosystems and act as biological control agents in the agro-ecosystems. *Tamarixia* species are parasitoids of psyllids and widely distributed as a classical biological control agent for the management of *D. citri* in many regions, including Taiwan [5], Reunion Island, Guadeloupe [6] Florida, California, and Texas [7–9], due to their high parasitism capability and field adaptation. *Tamarixia radiata* Waterston (Hymenoptera: Eulophidae) was first reported by Tang (1988) in Fujian province, China, as a nymph parasitoid of the Asiatic citrus psyllid (ACP) *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), a primary pest of citrus species/hybrids (Rutaceae: Aurantioideae: Aurantieae) and a disseminating source of the bacteria *Candidatus* Liberibacter asiaticus (CLas), which causes the citrus greening disease known as Huanglongbing (HLB) [10–12]. The infestation of *D. citri* has been observed in various provinces of China, including Fujian, Guangxi, Guangdong, Guizhou, Hunan, Jiangxi, Sichuan, Taiwan, Yunnan, and Zhejiang [13–16], as well as Hainan, Henan, Hong Kong, and Macau [17]. At present, the primary method for the management of *D. citri* is mainly based on the adhesive use of chemical pesticides that caused the resistance of insect pests and their harmful effects on non-target organisms [17]. Thus, due to the adverse effects of insecticides on the fauna and flora, there is a crucial need to introduce an environmentally friendly alternative measure such as biological control agents, natural insect enemies, and entomopathogenic fungi to eradicate *D. citri* [18–20].

The literature shows that *T. radiata* can significantly parasitize populations of *D. citri*; however, parasitism percentages are highly variable due to the seasonal period and various ecological sites [8,9] and found 20% and 56% parasitism rates of *T. radiata* in Florida. In Puerto Rico, the apparent parasitism of *T. radiata* to older fifth instar of *D. citri* nymphs at some locations averaged 70–100% parasitism [21]. Previous studies indicated that temperature, host stage, and geographical location significantly affect parasitism as the highest parasitism percentage (78%) was observed in *T. radiata* populations from southern China [22]. Several variable factors have significant effects on survival, parasitism, emergence, and fecundity of parasitoids. Environmental factors such as temperature and geographical location are the most important among these variables as they directly influence the fitness of parasitoids [23].

Formerly, the genetic diversity of *T. radiata* from several countries has also been studied using DNA marker sequencing. These molecular approaches are rapid characterization tools for identifying genetic diversities or similarities among geographically isolated *T. radiata* populations [24–27]. Molecular markers consisting of the mitochondrial gene (*COI*) and internal transcribed spacer (ITS1 and ITS2) regions have been extensively used to investigate the genetic diversity and phylogenetic relationships between the insect–host and parasitoids [28–30].

Endosymbionts also influence fundamental biological processes in their host, including physiology, evolution, nutrition, reproduction, metabolism, immune homeostasis, defense mechanism, and other fitness attributes [31–40]. Several endosymbionts associated with *D. citri* have been identified, such as *Candidatus* Carsonella rudii, *Candidatus* Profftella armatura, *Candidatus* Liberibacter asiaticus, and *Wolbachia* [31,41,42]. Furthermore, various *Wolbachia* strains have been reported from many insect parasitoids associated with disease transmission and manipulation of their host *Wolbachia* resident strains [43]. Thus, the characterization of *Wolbachia* in *T. radiata* will be a helpful biological control against *D. citri*. Despite the biological importance of *T. radiata*, its population's genetic variation has not been studied from climatically diverse zones in China. Therefore, the current study examines the genetic diversity of *T. radiata* from various geographic locations and identifies their associated *Wolbachia* populations using the *wsp* gene.

2. Materials and Methods

2.1. Collection of Insects and Rearing

A total of twelve *T. radiata*, populations, four each from Fujian (Fuzhou), Guangdong (Zhaoqing), and Jiangxi (Ganzhou) Figure S1, were collected from May to July in 2020. We also used one population from Guayaquil city, Guayas, Ecuador. In the previous study, the life table of Ecuador population of *T. radiata* has already been reported without any comparative molecular data [44]. Therefore, we decided to include Ecuador population of *T. radiata* (which is already maintained in our lab) for a preliminary comparative molecular analysis with other *T. radiata* populations collected from China. The different populations of *T. radiata* were maintained on *D. citri* nymphs and adults collected from citrus orchards in Fujian in May 2020. The stock populations were reared on orange jasmine (*Murraya paniculata*) (Sapindales: Rutaceae) and kept in mesh cages (0.60 m wide; 0.50 m deep; 0.50 m high) under control conditions (25 ± 2 °C; 70 ± 5% RH; a photoperiod of 14 h light and 10 h dark) in the laboratory of Insect Ecology and Biological Control, Fujian Agricultural and Forestry University, Fuzhou, China [44]. This study was conducted in three phases: biological parameters, molecular analysis, and endosymbiont characterization.

2.2. Biological Aspects (Parasitism, Emergence, and Sex Ratio) of Different Populations of Tamarixia radiata

Each population of *T. radiata* was reared and maintained in 5 plastic rearing bottles (0.2 m in height and 0.08 m in diameter). Each bottle contained 20 nymphs of *D. citri* (third–fifth instar) placed on a 15 cm tall *M. paniculata* shoot. To keep the shoot fresh for several days, it was implanted in a water-soaked cotton plug in a polypropylene deli container (25 mL) (Figure S2). Previously matted females *T. radiata* (24 h old) were introduced into each bottle containing nymphs for 48 h and then removed. Subsequently, potentially parasitized nymphs in rearing bottles were kept at 27 ± 1 °C, RH 70 ± 5% with L: D = 14:10 photoperiod. The experimental setup was observed for each population and replicated five times, which means there was a total of 100 *D. citri* nymphs investigated per population. (All experiments were run at the same time.)

2.3. DNA Extraction and Isolation

Before DNA extraction, all the *T. radiata* populations were surface-sterilized in 75% ethanol for 90 s, followed by three rinses in sterilized distilled water. For DNA extraction, each population's specimens were crushed separately using a DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's guidelines. The quality of DNA was assessed by electrophoresis on 1% agarose gel and quantified using Nanodrop 1000 spectrophotometer (Thermo-Scientific, Waltham, MA, USA). DNA samples were stored at −20 °C for further use.

2.4. Amplification of DNA and Sequencing

The internal transcribed spacer (ITS1 and ITS2) regions and cytochrome oxidase subunit I (*COI*) was amplified using specific primers for the identification of *T. radiata* populations, while endosymbiont *Wolbachia* was confirmed using *wsp* gene-specific primers (Table 1). Each PCR reaction was carried out in a 25 uL reaction mixture containing 2 uL DNA template (100 ng/uL), 2 uL (10 mM) of each forward and reverse primers, and 12.5 uL of 2XTaq PCR Master mix (Tiangen Biotechnology, Beijing, China). The amplification protocol, annealing temperatures, and expected product size (bp) are summarized in Table 1 according to the literature [45–47]. The PCR products were visualized by 1% agarose gel electrophoresis and purified using purification kit QIAQuick (QIAGEN, Hilden, Germany). Sequencing of amplified DNA fragments was performed at BioSune Biotechnology Co., Ltd. (Shanghai, China).

Table 1. Primers and annealing temperature used for PCR.

Primer	Primer Sequence (5′-3′)	(Annealing Temp °C)	Expected Product Size (bp)
ITS1- 18s F	GTGAACCTGCGAAGGA		
ITS1- 5.8s R	GTTCATGTCCTGCAGTTCACA	55 °C for 30 sec	503–515
ITS2- 5.8s F	TGTGAACTGCAGGACACATGAAC		
ITS2- 28s R	ATGCTTAAATTTAGGGGGTA	50 °C for 30 sec	489–490
C1-J-1718 F	GGAGGATTTGGAAATTGATTAGTTCC		
C1-*n*-2191R	CCCGGTAAAATTAAAATATAAACTTC	50 °C for 20 sec	518–574
wsp 81F	TGGTCCAATAAGTGATGAAGAAAC		
wsp 691R	AAAAATTAAACGCTACTCCA	55 °C for 60 sec	567

2.5. Sequence Composition and Phylogenetic Analysis

Obtained sequences of ITS1 and ITS2 regions, COI, and *wsp* genes were first assembled with DNAMAN v5 (Lynnon BioSoft, Vaudreuil-Dorion, Quebec, QC, Canada). Assembled sequences were subjected to BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 1 May 2021) to determine their identity with those deposited in GenBank [46]. The sequences showing the highest similarity index were retrieved and aligned using the Clustal W program BioEdit v7 [48]. For COI and *wsp* genes, nucleotide sequences were translated to amino acid sequences with ExPASY tools [49]. Our ITS1, ITS2, *COI*, and *wsp* sequences of all *T. radiata* populations were deposited in the NCBI GenBank database and their accession numbers are shown in Table S1.

The phylogenetic analysis of ITS1, ITS2, *COI*, and *wsp* sequences of different populations of *T. radiata* was conducted using MEGA-X [50]. For additional support and diversity, already reported ITS1 sequences of *T. radiata* populations from Bhutan, Mexico, Vietnam, and USA (Florida and Texas regions) had been used [25,27]. Meanwhile, sequences from Bhutan, Finland, India, Puerto Rico, Vietnam, and USA (Florida and Texas regions) were employed for ITS2 analysis of *T. radiata populations* [25,27]. For phylogenetic tree constructions, the General Time Reversible under the initial tree option of maximum parsimony method was used for the maximum likelihood phylogeny [51], while the Maximum Composite Likelihood method was considered for Neighbor-Joining topology [52]. The heuristic search trees were obtained with 1000 bootstrap support [50]. Furthermore, MEGA-X software implemented the Maximum Likelihood model to calculate the genetic distances of the aligned sequences for province-wise and overall populations of *T. radiata* from China [50].

2.6. Genetic Diversity and Distribution Analysis

The nucleotide polymorphism evaluation parameters, including haplotype number (h), haplotype diversity (Hd), nucleotide diversity (π), and the average number of nucleotide differences (k) were calculated using DnaSP 5 within and overall populations of *T. radiata* collected from China [53]. The neutrality test indices, i.e., Tajima's D, Fu, and Li's D* statistical test values for deviating neutral evolution pattern, were calculated within and overall *T. radiata* populations. Further, population analysis was performed with the Raggedness indices model [53].

2.7. Data Analysis

The parasitism percentage was determined on the fifth day based on the number of nymphs parasitized. The percentage of parasitism was calculated as the ratio of the number of emerged *T. radiata* to the combined total number of emerged *D. citri* and *T. radiata*. The number of emerged *T. radiata* evaluated the emergence percentage divided by the number of parasitized *T. radiata.* The sex ratio is based on the number of females (*T. radiata*) divided by the total number of emerged *T. radiata* for each population. The differences in the parasitism, emergence, and sex ratio among different populations of *T. radiata* were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test with a significant difference at $p < 0.05$.

3. Results

In this study, nymphs of ACP were found to be parasitized with *T. radiata* on *M. paniculata* plants were collected in Fujian, Guangdong, and Jiangxi provinces in China, while a previously collected population of *T. radiata* from Guayas, Ecuador, maintained in insectary since 2018, was also included in analysis. All the *T. radiata* populations were kept in separate cages under controlled conditions and reared successfully for the present investigation. The study of *T. radiata* populations determines biological aspects, including parasitism rate, emergence rate, and sex ratio from each population by parasitizing third–fifth instar nymphs of *D. citri*. The obtained sequences of ITS1, ITS2, COI, and *wsp* were blasted in the NCBI database to identify populations of *T. radiata* and their associated *Wolbachia* strains. The blast results showed more than 97–99% sequence identity with their respective species or strains deposited in the NCBI database and represented in the phylogenetic trees.

3.1. Efficacy of Parasitism, Emergence, and Sex Ratio on a Different Population of Tamarixia radiata

The parasitism rate of different *T. radiata* populations after parasitization of *D. citri* third–fifth instar nymphs are provided in Table 2. The results showed a statistical parasitism percentage difference between China and Ecuador populations as well as within *T. radiata* populations from China ($F_{3.39}$ = 15.57, $p < 0.001$). The parasitism percentage of the Fujian population was significantly higher (68.20%) than the Jiangxi population (63.54%), whereas there was no significant difference observed between populations from Guangdong and Fujian provinces. In addition, the minimum parasitism (57.28%) was observed by the Guayas population of *T. radiata.* After the potential parasitism occurred post-*T. radiata*-release, the highest emergence rate (86.61%) was observed in the Fujian population. However, no statistical dissimilarities were observed in the emergence percentage among and in between China and Ecuador populations. The trends in the sex ratio of different *T. radiata* populations were substantial and similar to the emergence rate. Furthermore, the female proportion results showed that the F1 generation of Guayas' population was lower (58.40%), whereas there was a statistical similarity in the overall population of China and Ecuador (Table 2).

Table 2. Means and SE of the parasitism, emergence, and sex ratio of different populations of *Tamarixia radiata* in third–fifth instar nymphs of *Diaphorina citri*.

Population	Parasitism (%)	Emergence (%)	Sex ratio
Fujian	68.20 ± 3.24a	86.61 ± 2.29a	62.81%
Guangdong	64.26 ± 2.86a	85.74 ± 2.16a	61.60%
Jiangxi	63.54 ± 2.94b	85.33 ± 2.43a	62.14%
Guayas	57.28 ± 5.00c	82.95 ± 3.07a	58.40%

Means in the same column with the same letter are not significantly different (Tukey's test: $p < 0.05$).

3.2. Sequence and Phylogenetic Analysis of the ITS1 Region

The length of the ITS1 sequences from all populations of *T. radiata* ranged from 503 to 515 bp. The alignment results showed maximum sequence similarity among Fujian-1, Guangdong-2, Guayas-1, and Jiangxi-1 populations with a difference of one nucleotide at their respective positions of 18, 22, 45, and 47. The phylogenetic analysis showed that *T. radiata* individuals collected from Fujian, Guangdong, Jiangxi, and Guayas formed a separate clade with the Bhutan and Vietnam populations of *T. radiata* (Figure 1A,B). However, the Fujian-1 population (MW537748) is grouped with the population reported from Vietnam, while *Tamarixia triozae* Burks is grouped into a distinct clade. The pairwise distance divergence ranged between 0.0020% and 0.0048% among the populations obtained from Fujian, Guangdong, and Jiangxi (Table 3). The 34 nucleotide sequences involved 563 positions in the final dataset at pairwise deletion and gamma distribution options, including 1000 bootstrap values. Furthermore, the Maximum Likelihood method presented

86% similarity among all population sequences (Figure 1A). In the Neighbor-Joining tree, all populations showed a resemblance of 96% with all *T. radiata* populations (Figure 1B).

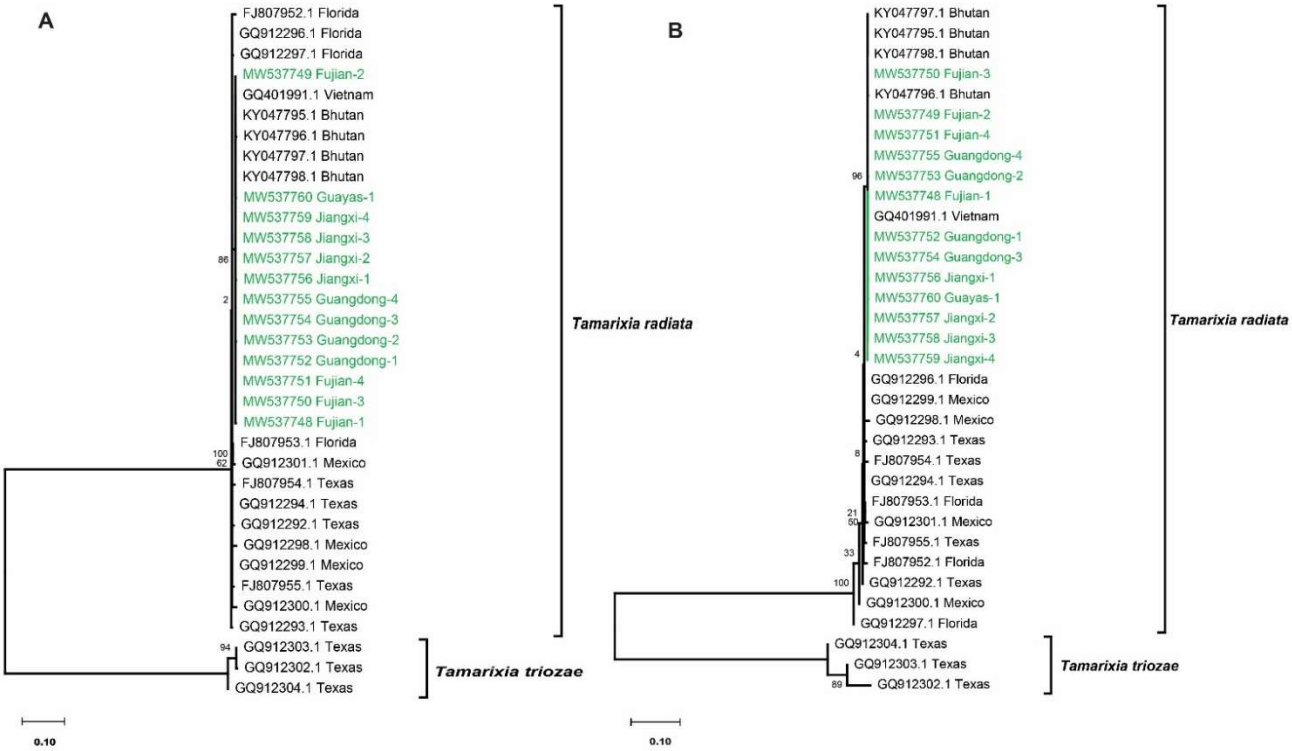

Figure 1. Phylogenetic analysis based upon ITS1 region of thirteen different sequences of *Tamarixia radiata* populations from different geographical locations. (**A**) Tree was constructed using the Maximum Likelihood (ML) method through the General Time-Reversible model under the maximum parsimony, while (**B**) tree was constructed through the Neighbor-Joining method Maximum Composite Likelihood model. All *Tamarixia radiata* sequences obtained in the study are highlighted in green lines and font.

Table 3. Distance divergence (%) within the provinces and overall populations of *Tamarixia radiata*.

	Population	Samples (*n*)	Min. Dist.	Max. Dist.
ITS1	Fujian	4	0.0020	0.0028
	Jiangxi	4	0.0020	0.0028
	Guangdong	4	0.0020	0.0028
	Overall	12	0.0020	0.0048
ITS2	Fujian	4	0.0020	0.0047
	Jiangxi	4	0.0021	0.0067
	Guangdong	4	0.0020	0.0043
	Overall	12	0.0020	0.0243
COI	Fujian	4	0.0001	0.0053
	Jiangxi	4	0.0001	0.0040
	Guangdong	4	0.0020	0.0040
	Overall	12	0.0021	3.0552
wsp	Fujian	4	0.0022	0.0072
	Jiangxi	4	0.0022	0.0046
	Guangdong	4	0.0011	0.0038
	Overall	12	0.0022	0.0099

3.3. Sequence and Phylogenetic Analysis of the ITS2 Region

Obtained sequences of the ITS2 region of parasitoid *T. radiata* ranged from 489 to 490 bp. A difference of nine nucleotides was observed among nucleotide sequences of Fujian-1, Guangdong-2, and Jiangxi-1. However, minimum nucleotide difference was

observed within populations of Fujian, Guangdong, Jiangxi, and Guayas. All the collected *T. radiata* populations (Fujian, Guangdong, Jiangxi, and Guayas) are grouped into a single clade with other *T. radiata* populations collected from *D. citri* host insect insects from several geographical locations. Similarly, the other species of *Tamarixia* (*Tamarixia drukyulensis* Yefremova and Yegorenkova) clustered into a separate clade concerning their host insect (*Diaphorina communis* Mathur) (Figure 2A,B). According to the sequenced database, the maximum distance divergence ranged between 0.0043% and 0.0243% in Fujian, Guangdong, and Jiangxi populations, while minimum variation was observed within each group (Table 3). This analysis involved 44 nucleotide sequences with 561 positions in the final dataset at pairwise deletion and gamma distribution option, including 1000 bootstrap values. Moreover, according to the Maximum Likelihood method, all populations showed 100% resemblance with the entire group (Figure 2A). The Neighbor-Joining tree presented up to 94% similarity among all group sequences (Figure 2B).

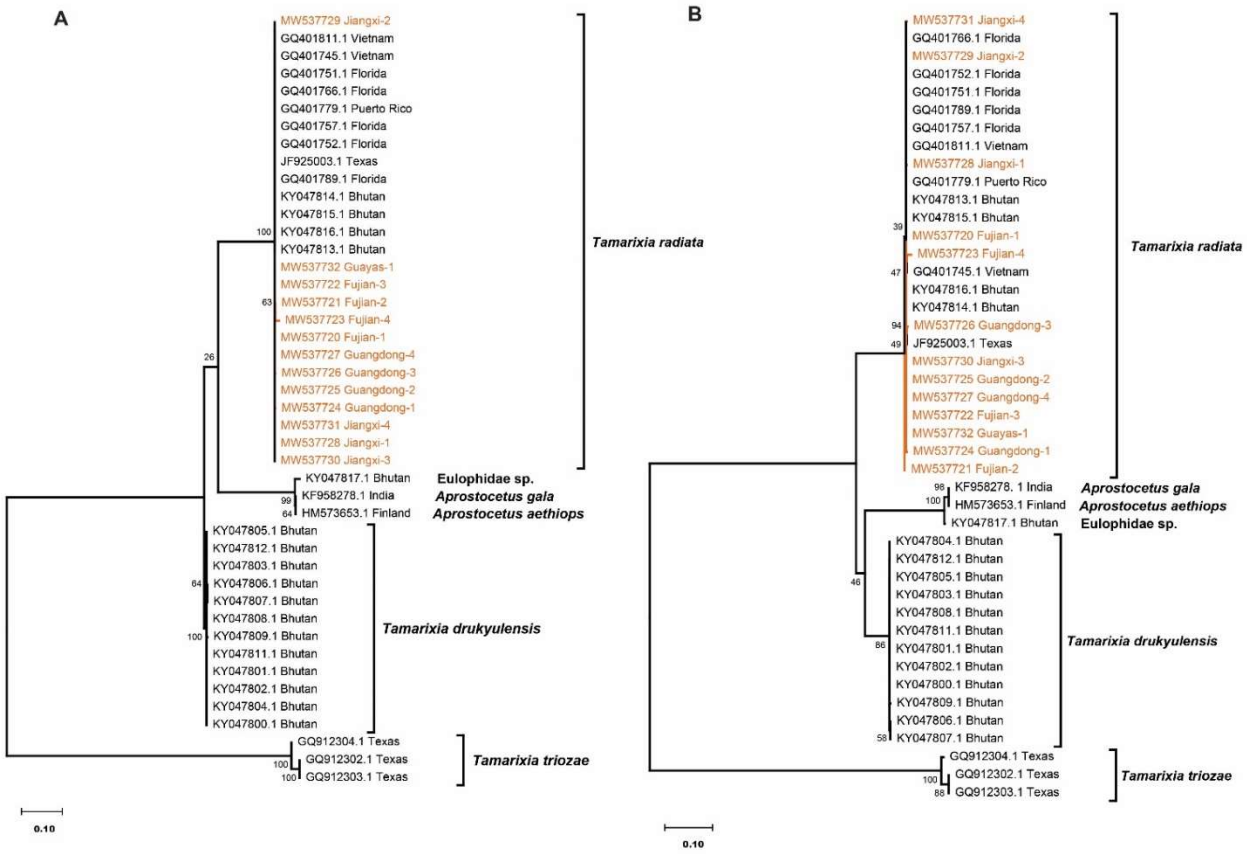

Figure 2. Phylogenetic analysis ITS2 region of *Tamarixia radiata* populations from different geographical locations. (**A**)The phylogenetic tree was constructed through the Maximum Likelihood method through the General Time-Reversible model under maximum parsimony, and (**B**) the phylogenetic tree was constructed through the Neighbor-Joining method Maximum Composite Likelihood model. All *Tamarixia radiata* sequences obtained in the study are highlighted in orange lines and font.

3.4. Sequence and Phylogenetic Analysis of COI

The obtained sequence length of *COI* was 488–565 bp from each of *T. radiata* populations collected in Fujian, Guangdong, Jiangxi, and Guayas. Phylogenetic analysis showed that two variable sites corresponding to transitions and transversions were identified in the *COI* sequences of *T. radiata* populations clustered into two distinct clades based on the haplotype diversity (H1 and H2). In the tree topology, the first clade showed H-2 from the Fujian population grouped with the Mexican population of *T. radiata*. The second clade represents H-1, which is frequently encountered from China (Guangdong and Jiangxi) to Ecuador (Guayas) grouped with the Mexican and USA (Florida and Taxes) popula-

tions of *T. radiata* (Figure 3A,B). However, the distance divergence was calculated using the Maximum Composite Likelihood model, and variation ranged between 0.0021% and 3.05% (Table 3). The 32 nucleotide sequences with 628 positions were involved in the final dataset at pairwise deletion and gamma distribution options, including 1000 bootstrap values. Moreover, according to the Maximum Likelihood and Neighbor-Joining methods, all populations showed 100% resemblance with the entire group of *T. radiata* populations (Figure 3A,B).

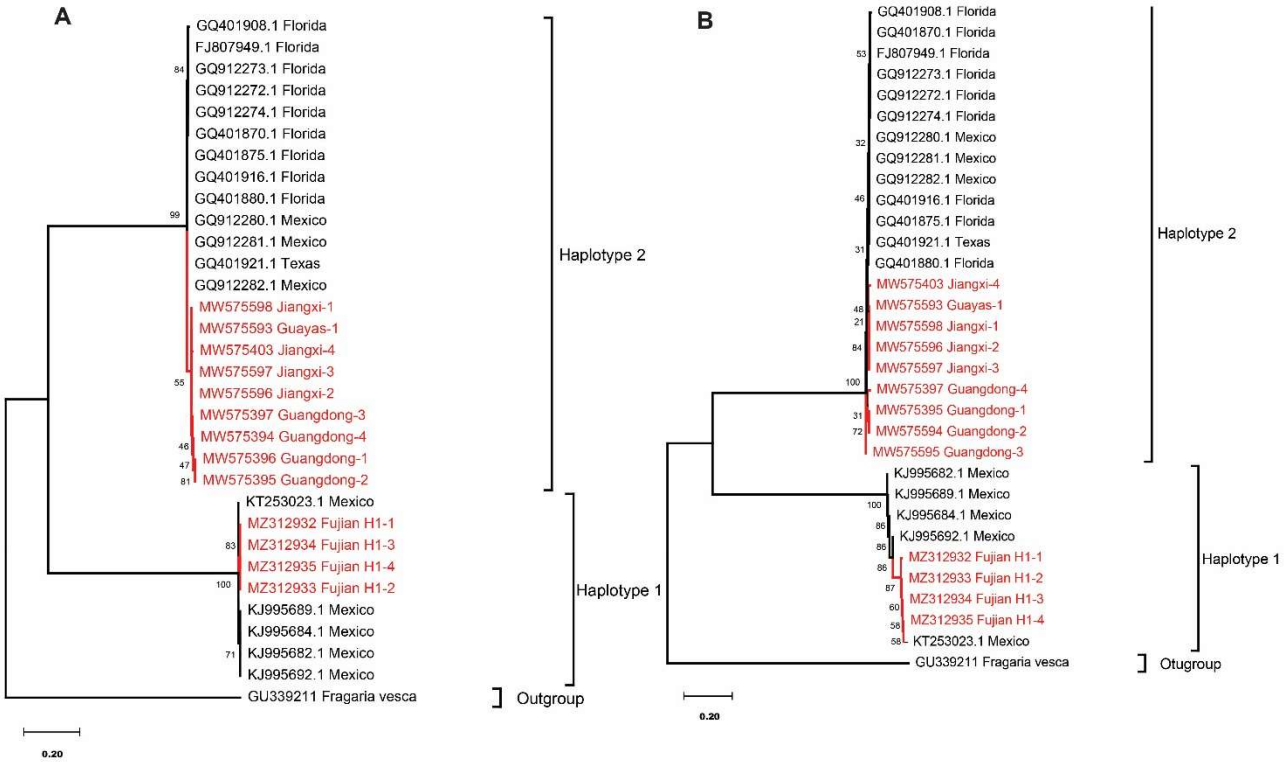

Figure 3. Phylogenetic analysis based upon an alignment of *COI* sequences of 13 different populations of *Tamarixia radiata*. (**A**) Phylogenetic tree based on the Maximum Likelihood method through the General Time-Reversible model under the initial tree option of maximum parsimony, while (**B**) shows a phylogenetic tree constructed through the Neighbor-Joining method by the Maximum Composite Likelihood model. All *COI* sequences of *Tamarixia radiata* obtained in the study are highlighted in red lines and font.

3.5. Phylogenetic Analysis of Wolbachia Based on the wsp Gene

The presence of *Wolbachia* as endosymbionts is well-known in the previous host-parasitoid relationship of *D. citri* and its endoparasitoid (*Diaphorencyrtus algerihnses* Shafee, Alam and Agarwal) [54]. However, the presence of *Wolbachia* in *T. radiata* has been confirmed using the *wsp* gene in the present study. Phylogenetic analysis showed that *Wolbachia* sequences obtained in this study clustered in a single sub-clade with already reported *Wolbachia pipientis* from *Drosophila melanogaster* and *Aedes albopictus* with maximum bootstrap support. Based on sequence alignment and phylogenetic analysis, we could identify *Wolbachia* endosymbiont as *W. pipientis* from different populations of *T. radiata.* Previous research describes at least two *Wolbachia* strains (A and B groups) based on host–parasitoids relationships [55]. To evaluate the phylogenetic position and diversity of *Wolbachia* within *T. radiata* populations, we performed a detailed analysis using the *wsp* sequence data. Our study contained four 16s RNA used as an outgroup for *Wolbachia* sequenced from different hosts (*D. algerihnses* (EF433794), *D. citri* (EF433793, AB038370), *Culex pipiens* (U23709) according to Meyer and Hoy (2008)). Based on the Maximum Likelihood and Neighbor-Joining method, the phylogenetic tree clustered the entire *Wolbachia* sequences obtained

from *T. radiata* populations into Subdivision-B [55], which showed a strong relationship with previously reported *Wolbachia* strains. Analysis results also showed that previous *Wolbachia* sequences (data from the literature) in *D. citri* also belong to Subdivision-B, as observed in *T. radiata* populations. *Wolbachia* sequences found in *T. radiata* and *D. citri* are closely related but not identical and therefore positioned into two separate subclades. Accordingly, the horizontal transfer might have occurred between host and parasitoid, but if so, it was not a recent one. Pairwise sequence divergence of 0.0035%–0.0117% was observed within the *Wolbachia* strains (Table 3). This analysis involved 77 nucleotide sequences with 670 positions in the final dataset at pairwise deletion and gamma distribution options, including 1000 bootstrap values. Moreover, in the Maximum Likelihood and Neighbor-Joining method, all populations assembled in both trees showed a resemblance of 97% and 99% within the group, respectively (Figure 4A,B).

3.6. Population Genetic Diversity Analysis

All the populations of *T. radiata* were collected from different geographical regions and showed some genetic variation within and overall populations. The genetic diversity was calculated based on haplotype diversity (Hd), nucleotide diversity (π), and the average number of nucleotide differences (k) ranges from (0.500–0.833), (0.00193–0.00252), and (1.0000–1.50000) for the COI gene, respectively (Table 4). Likewise, the COI gene showed maximum nucleotide variations up to 243 mutations overall among the Chinese populations based on the Eta value and revealed a high genetic diversity (Table 4).

Table 4. Genetic diversity of different populations of *Tamarixia radiata* calculated via nucleotides polymorphism data analysis.

Gene	Nucleotide Diversity								Neutrality Tests			
	Population	***n***	**S**	**k**	**Eta**	**Hd**	**Θ**	**π**	**Fu and Li' s D***	***p*-Value**	**Tajima' s D**	***p*-Value**
COI	Fujian	4	3	1.50000	3	0.500	0.00290	0.00265	−0.7544	0.41	−0.7544	0.10
	Jiangxi	4	2	1.00000	2	0.500	0.00211	0.00193	−0.7099	0.10	−0.7099	0.10
	Guangdong	4	2	1.16667	2	0.833	0.00211	0.00255	0.5915	0.10	0.5915	0.10
	Overall	12	243	117.848	243	0.788	0.16625	0.24349	1.6893	0.02 *	2.1857	0.05 *
wsp	Fujian	4	3	1.50000	3	0.833	0.00353	0.00324	−0.7544	0.10	−0.7544	0.10
	Jiangxi	4	2	1.16667	2	0.833	0.00236	0.00252	0.59158	0.10	0.5915	0.10
	Guangdong	4	3	1.50000	3	0.833	0.00353	0.00324	−0.7544	0.10	−0.7544	0.10
	Overall	12	6	1.37879	6	0.833	0.00429	0.00298	−1.1084	0.10	−1.1671	0.10

Abbreviations: Eta = total number of mutations, *n* = number of samples, k = average number of nucleotide differences, S = number of segregating sites, Θ = nucleotide substitution rate, π = nucleotide diversity, Hd = haplotype diversity, Fu and Li's D* = variation among different haplotypes in the population, and Tajima's D is the sequence comparison for segregating sites as well as the mean pairwise variation * $p \leq 0.05$.

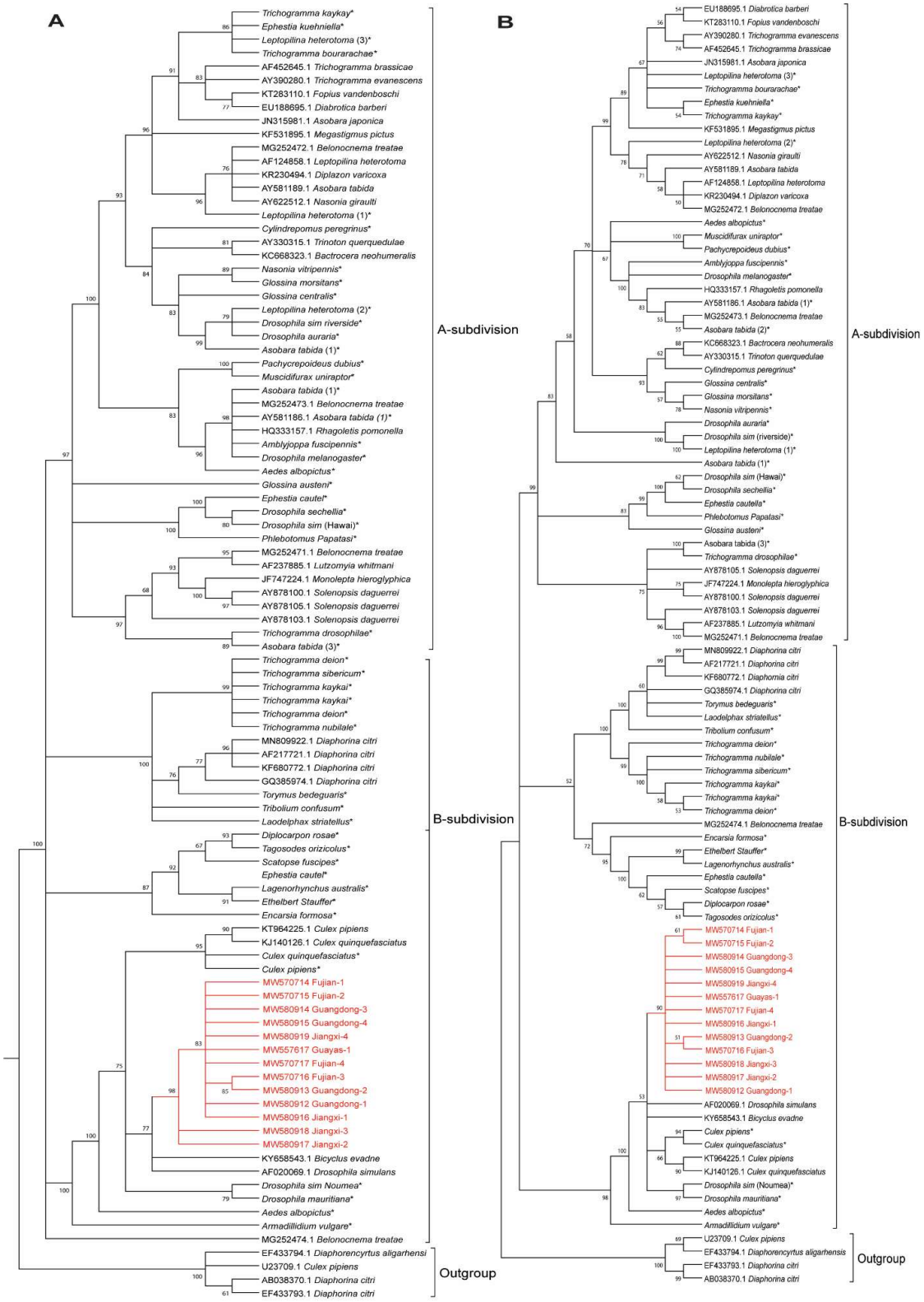

Figure 4. Phylogeny of *wsp* gene from *Wolbachia* associated with different insect species, including *Tamarixia radiata* from this study. (**A**) The phylogenetic tree was constructed through the Maximum Likelihood method. (**B**) The phylogenetic tree was constructed through the Neighbour-Joining method by the Maximum Composite Likelihood model. All *wsp* sequences of *Wolbachia* obtained in the study are highlighted in red lines and font. For tree construction, *wsp* gene sequences were collected from the NCBI database for tree construction, representing different insect species and host–parasitoid relationships. The four sequences of 16s RNA were chosen as an outgroup. For tree construction, the bootstrap value was replicated 1000 times. The sequence represented by * is taken from Vavre et al., 1999.

3.7. Demographic Analysis

The neutrality tests were performed to confirm the significance of genetic diversity within and overall populations of *T. radiata* and its associated endosymbiont *W. pipientis.* The results of the *COI* gene from both neutrality indices showed a significant positive *p*-value of Tajima's D (1.6893) and Fu and Li's D* test and (2.1857), respectively (Table 4). A positive value of these tests is evidence for a deficiency of alleles and an expected decrease in population size. The neutrality tests showed a non-significant negative value of neutrality indices analysis for the *wsp* gene (Table 4). These negative *p*-values indicate an excess of rare mutations that favor population expansion. These results may suggest an excess number of low-frequency alleles typical of positive selection or recent population expansion. DNA sequences from different genes were analyzed for population size changes to enrich the genetic diversity results among different *T. radiata* populations and their associated *Wolbachia* strains to observe pairwise nucleotide differences (mismatch distribution). The pairwise nucleotide differences graph showed a bimodal curve in the *COI* gene under a sudden contraction model that expected the population bottleneck (Figure 5A). In contrast, the pairwise mismatch distributions plot, which comprises the *W. pipientis* strain of the *wsp* gene, was smooth and unimodal, which may indicate that a strong population subdivision confers a stable population size (Figure 5B).

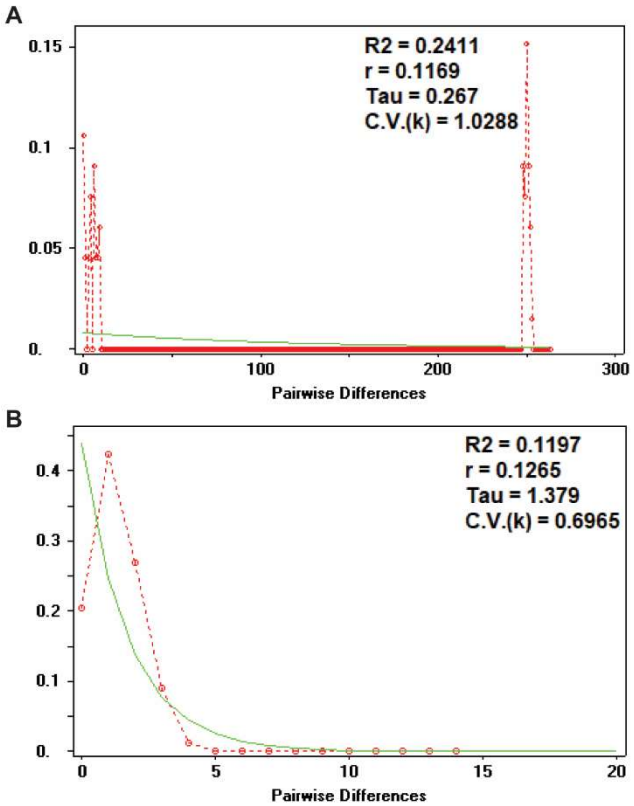

Figure 5. Pairwise nucleotides differences for *Tamarixia radiata* of *COI* (**A**) and *wsp* (**B**) gene sequences for the mismatch distributions; the solid lines show expected frequency distribution, while dotted lines show the distribution observed under the sudden-expansion model. *R2* = Ramos-Onsins and Rozas statistics; r = Raggedness statistic; Tau = date of the growth; C.V.= coefficient of variation.

4. Discussion

The ACP is the primary vector of the gravest disease of the citrus industry, known as a citrus greening disease [56], and *T. radiata* is one of the recommended biological controls for sustainable management of *D. citri* in different geographic areas worldwide. It has been reported that *T. radiata* has a very high reproductive rate with a short life span and is frequently reared in control conditions [57]; nevertheless, little work has been completed

in insectary to assess the parasitism rates and genetic diversity of different geographical populations of *T. radiata* from China. The objective of this study was first to examine the biological parameters of different populations of *T. radiata*, their genetic diversity, and the presence of their associated *Wolbachia* species and its diversity in the various populations. *T. radiata* is a solitary, arrhenotokous ectoparasitoid of third, fourth, or fifth instars of *D. citri* nymphs as it can kill approximately 500 nymphs by oviposition and feeding [6,58,59]. The environmental factors may affect the parasitism percentage of *T. radiata* and directly influence the foraging behavior of parasitic wasps. Among these factors, temperature, hostage, and geographical locations may directly affect the parasitism and emergence of *T. radiata* [23]. The previous studies revealed that the *T. radiata* preferred older nymphs of *D. citri* (fourth to fifth) at a temperature range from 25 to 30 °C and reported the highest parasitism percentage (85.50%) and emergence rate (88.30%) [22]. The parasitism of older instars psyllid nymphs in orange jasmine ranging from 48% to 70% was recorded during spring and summer at Río Piedras and San Juan, respectively [21]. According to Wang et al. 1999, temperatures ranging from 20 to 30 °C were favorable for parasitism and reproduction of *T. radiata*. However, *T. radiata* may feed and kill all instars of *D. citri* nymphs but mostly preferred fifth instar host because the progeny sex ratio of parasitoids mainly influences the host instar stage and oviposition [60]. In the present study, the different populations of parasitoids performance (parasitism rate, emergence rate, and sex ratio) may provide insights into the behavior and interactions of the parasitoid concerning its geographical location. The current findings show that *T. radiata* preferred parasitized (third–fifth instar) nymphs of *D. citri* on *M. paniculate* plants under control conditions (27.5 ± 1 °C, 70 ± 5% RH) and the maximum percentage of parasitism was observed from *T. radiata* populations from Fujian (68.20%) and minimum from Guayas (57.28%). Similarly, the rate of emergence may range from 86.61% to 82.95% for third–fifth instar psyllid nymphs. Likewise, results from the life table of *T. radiata* have reported a higher parasitism rate (77.24%) at a temperature of 26.3 °C. [23]. Previous research revealed that a series of complex environmental factors had significant effects on survival, parasitism, and reproduction of *T. radiata*. Accordingly, the highest parasitism percentage was observed in summer and the lowest in winter in Sao Paulo [61].

In the molecular study, the ITS (ITS1, ITS2) regions and *COI* were used to determine nucleotide diversity between and within populations of *T. radiata* sampled across China (Fujian, Guangdong, and Jiangxi) and one population from Ecuador (Guayas). We also amplified the *wsp* gene to confirm the identification and variability of *Wolbachia* strains as an associated endosymbiont in *T. radiata* populations. Molecular analysis of *T. radiata* is primarily related to genetic diversity within the species using conserved ITS regions and the *COI* gene [24,27,62]. Among the conserved regions, the ITS loci are useful diagnostic markers due to their high divergence levels between species but low levels of variation within a species [33,63]. The ITS regions have been used to study phylogenetic relationships and resolve taxonomic controversies in various insect species, including *T. triozae*, *T. mercet*, and *T. drukyulensis* [25,27]. In the current study, the ITS regions have been used to construct the phylogenetic tree with twenty-one (ITS1) and thirty-one (ITS2) reference sequences of *Tamarixia* reported from the different agro-ecological zones, including *T. radiata* sequence from China populations and one from Ecuador (Guayas). Sequence analysis of ITS1 and ITS2 showed no variability among the populations of *T. radiata* from China and Ecuador. The phylogenetic tree's topology based on ITS regions represented strong intermingled relationships between and within all the populations of *T. radiata* (Figure 1A,B and Figure 2A,B). However, sequence analysis of ITS regions showed minor distance divergence for province and overall *T. radiata* populations from Fujian, Guangdong, and Jiangxi, ranging from 0.0028% to 0.0048% (ITS1) and 0.0043% to 0.0243% (ITS2), respectively (Table 3). The minimum divergence in conserved regions in the *T. radiata* populations supports the that these different populations represent a single species.

In previous studies, mitochondrial genes have been used to analyze the genetic diversity and genetic differentiation of the *Tamarixia* species [24,25,27]. Proper assimilation

and understanding of the arthropods' genetic variability found it essential to mitigate and improve its monitoring, which further facilitates the implementation of need-based management strategies. In this study, *T. radiata* populations' maximum nucleotide diversity was observed based on Eta mutations in the total *COI* sequences, as observed in the Chinese population (Table 4). The previous studies also revealed two haplotypes (Hap-1 and Hap-2) for *T. radiata* in the Sonora region of Mexico and America (Florida, Texas) [24,27]. The previous analysis also revealed that H1 and H2 have been present in reported populations from Mexico, Texas, Florida, but the distribution and frequency bias by H2 was greater than that of H1. Moreover, haplotype H2 was found in all samples obtained from 11 states of Mexico, and H1 was only found in Yucatan [64]. *COI* gene analyses have also been widely used for the phylogeny of population and genetic inferences. Based on the *COI* phylogeographic analysis, it was observed that *T. radiata* populations from China showed two types of haplotype diversity (H1, H2). However, *T. radiata* populations from Fujian fall into separates clades based on the H1 type of haplotype (Figure 3A,B). In contrast, the population of *T. radiata* from Jiangxi Guangdong and Guayas showed H2 haplotype diversity and is similar to the previously reported populations of *T. radiata* from Mexico and Florida. Furthermore, the previous work showed a high number of haplotypes H2 samples obtained from the northeast states of Nuevo León, and Tamaulipas and the western states of Colima and Michoacán, and H1 was only obtained from the Tamaulipas state of Mexico. These findings suggest gene flow due to the release and introduction of *T. radiata* between countries [61]. The high haplotype diversity and nucleotide diversity (Hd > 0.78, $\pi > 0.24$) observed in overall populations from China represent stable populations with a long evolutionary history [65], and these populations are the contributors to the nucleotide diversity of *T. radiata*. Likewise, the averaged maximum genetic distance deviation among all *T. radiata* populations was observed at 0.0053% and 3.0552% (Table 3). The neutrality indices of Tajima's D tests' positive values represent a lack of rare alleles, which may be due to balancing selection or population contraction. In contrast, the negative values indicate an excess of low-frequency alleles and represent a recent selective sweep or population expansion [66,67]. However, *p*-values of neutrality tests indicated a positive significant genetic diversity for total *T. radiata* populations, which may support population contraction, and the mismatch distributions could represent the bottleneck growth. The significant positive values of Tajima's D (2.1857) indicated that the population was undergoing balanced selection and had no further rapid expansion in the future. Additionally, Fu and Li's D* (1.6893) positive significance indicated that background selection had a significant effect on haplotypes of the total populations from China.

The facultative endosymbiont *Wolbachia* was previously reported in bacteriocytes and multiple somatic and reproductive tissues in various insect hosts and parasitoids [68]. *Wolbachia* has been found in multiple tissues, bacteriocytes, and somatic and other reproductive tissues in various insect pests [33,69]. The association of *Wolbachia* can affect its host insect's behavior and biology by manipulating their resident strains [70]. Therefore, the characterization of *Wolbachia* in *T. radiata* is of particular interest due to its impact on host biology and its potential to control *D. citri*. By analyzing *Wolbachia* infection status using the *wsp* gene within *T. radiata* populations and its distribution across the three provinces from China and one province from Ecuador, we found the presence of *W. pipientis* in association with *T. radiata*. Our results showed that *W. pipientis* obtained from *T. radiata* formed a clade with different insects, including different species of *Drosophila, Aedes, Armadillidium,* and *Culex.* These results revealed that the previous literature also showed that the *W. pipientis* host range is broader than initially thought [71]. However, we did not find any evidence of an association between *Wolbachia* and its host's geography. The phylogenetic analysis showed that the strains of *W. pipientis* found in *T. radiata* related to supergroup B (Figure 4A,B) but distinct from *D. citri* positioned into a separate subclade. None of these results is surprising since the phylogeny of *Wolbachia* is known to be incongruent with that of its arthropod hosts, precisely because horizontal transfers between species are not uncommon at a large phylogenetic scale. However, it has been proposed that supergroup

G be decommissioned, as it is based primarily on recombinant *wsp* sequences and clusters with A supergroup based on five multilocus sequence typing genes [72,73], and eight supergroups (A–H) are still widely used in the research community. An MLST system based on five house-keeping genes (coxA, gatB, hcpA, ftsZ, and fbpA) has been developed for *Wolbachia* [72] and is widely used for strain typing and to characterize strain variation within *Wolbachia* [74]. With the differentiation of *Wolbachia* from *T. radiata* and *D. citri* within the B subdivision, the idea of recent and rapid expansion of the B-clade *Wolbachia* could result from more frequent transfers of *Wolbachia* between host and parasitoid. In previous studies, rapid expansion has also been observed in *Wolbachia* strains associated with *Drosophila* species and their associated parasitoids [55,75]. However, the genetic diversity was calculated through the particular mutations in the *wsp* gene. The neutrality tests (Tajima's D, −1.1671 and Fu and Li's D*−1.1084) and sequence mismatch distributions analysis of total populations of *Wolbachia* strains were negative and not significant, indicating that the expansion of these populations was limited [66,76].

5. Conclusions

This study investigates the population genetic diversity of twelve populations of *T. radiata* from different provinces in China and one population from Ecuador. The results showed that different populations of *T. radiata* were distinguished into two clades of haplotypes (H1–H2). The populations of *T. radiata* from Fujian fall into H-1, and Jiangxi, Guangdong, and Guayas were H-2, the most frequent population clade. The total genetic diversity of *T. radiata* was maximum as indicated by the total haplotype diversity (Hd = 0.788), nucleotide diversity (π = 0.243), and average nucleotide difference (k = 1177). Tajima's *D* and Fu and Li's *D** analyses indicated that these populations of *T. radiata* had population contraction and were limited by local area. The presence of *Wolbachia* strains from all *T. radiata* populations had a strong interaction with previously reported *wsp* gene strains. The negative values of the neutrality test may indicate long-term balancing selection or population expansion. The perceptive study of different *T. radiata* populations can be used to design any independent strategy to carry out integrated pest management of *D. citri.*

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11102018/s1. The supplementary material associated with this article is mentioned as follows: Figure S1. Sampling site locations of *Tamarixia radiata* on the orange jasmine plants in the different provinces of China indicated with different colors (source: https://github.com/huangbuyi/svg-china-map/blob/master/china_provinces_map.svg, accessed on 30 June 2021). Figure S2. Experimental unit for the rearing of *Tamarixia radiata* populations. Table S1. Sequences of *Tamarixia radiata* populations used for molecular analysis and their GenBank accession numbers.

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Data Availability Statement: The ITS1, ITS2, *COI*, and *wsp* sequences of all *Tamarixia radiata* populations were deposited in the NCBI GenBank database with accession numbers are presented in Table S1.

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