Molecular Evidence Suggests That Populations of the Asian Citrus Psyllid Parasitoid *Tamarixia radiata* (Hymenoptera: Eulophidae) From Texas, Florida, and Mexico Represent a Single Species

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ABSTRACT We genetically characterized *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) populations from Texas, Florida, and Mexico and the sister taxon *Tamarixia triozae* (Burks) by two molecular methods. *T. radiata* is an ectoparasitoid of Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), nymphs. The populations were submitted to intersimple sequence repeat-polymerase chain reaction DNA fingerprinting with two primers. No fixed banding pattern differences were uncovered among the populations of *T. radiata* with either primer, whereas different patterns were observed in *T. triozae*, suggesting that there is no genetic differentiation among the populations. Support for these results was obtained by sequence analyses of the internal transcribed spacer region one and the mitochondrial cytochrome oxidase subunit one (COI) gene. In both genes, the intrapopulational variation range (percentage divergence, %D) fell within the interpopulational variation range. The %D at the COI gene between *T. radiata* and *T. triozae* was 9.0–10.3%. However, haplotype structure was uncovered among the populations. No haplotypes were shared between Florida and Texas/Mexico, whereas sharing was observed between Texas and Mexico. Two population-specific nucleotides were identified that allowed the discrimination of the Florida and Texas/Mexico individuals. A neighbor-joining and a parsimonious tree clustered the populations into two distinct clades. The Florida population clustered into one clade, whereas the Texas/Mexico populations clustered into another clade. The COI phylogeographic analysis suggests that the population of *T. radiata* in Texas did not come from Florida. The data also suggest that the population in Texas came from Mexico, or vice versa, because the Mexico population showed less haplotype diversity.

KEY WORDS natural enemy, ectoparasitoid, Asian citrus psyllid, biological control, DNA fingerprinting

Tamarixia radiata (Waterston) (Hymenoptera: Eulophidae) is a natural enemy of the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) (Husain and Nath 1927, Aubert 1987). This ectoparasitic eulophid originated in northeastern India (Punjab) (Waterston 1922) and was imported into Réunion Island where it was reported to have successfully suppressed populations of *D. citri* (Aubert and Quilici 1983, 1984; Étienne et al. 2001). Because of this high efficiency and success at controlling *D. citri* populations, *T. radiata* was imported from Réunion Island to several other countries, such as Taiwan (Chien et al. 1989), Mauritius (Joomaye and Aubert 1987, an internal report), and Guadaloupe (Étienne et al. 2001). *T.*

radiata was imported from Taiwan and Vietnam into Florida for classical biological control of *D. citri* (Hoy et al. 1999, Hoy and Nguyen 2001, McFarland and Hoy 2001). Good levels of suppression, but lower than in Réunion Island, were achieved in Mauritius (Joomaye and Aubert 1987, an internal report) and Guadaloupe (Étienne et al. 2001) with *T. radiata.* However, in countries such as Taiwan, the Philippines, Nepal, and Indonesia, *T. radiata* apparently did not have much success (da Graça 1991, Gottwald et al. 2007), including Florida where *T. radiata* efficiency is reported to be low (≈20%) (Michaud 2002a,b, 2004; Halbert and Manjunath 2004; Qureshi et al. 2008). In much of South and Southeast Asia, including India, the impact of *T. radiata* can be reduced by the presence of hyperparasites. Réunion Island is free of hyperparasites and this may, in part, explain the success of *T. radiata* in this country (Aubert 1987). Hyperparasites have not been reported from Florida (Michaud 2004), so environmental or ecological factors also may play a role in the success of *T. radiata.* For example, in the island of Puerto Rico, where *T. radiata* was inadvertently introduced, it was reported to produce good levels of

suppression of *D. citri* populations (Pluke et al. 2008). However, the influence of environmental or ecological factors requires further investigation.

D. citri is widespread throughout Southeast Asia (Aubert 1987). It has been recorded in South America (Brazil) for the past 60 yr (da Costa Lima 1942, Catling 1970, Hodkinson and White 1981), and recently *D. citri* has expanded its range into North America (Halbert and Nuñez 2004), including Florida (Knapp et al. 1998), Texas (French et al. 2001), and more recently California and the southeastern states Alabama, Georgia, Louisiana, and Mississippi (Gomes 2008a,b,c). It is interesting to note that *T. radiata* seems to be moving along with the expansion of *D. citri* in America, because it has been inadvertently introduced into many areas, such as, Brazil, Venezuela, Argentina, Puerto Rico, and Texas (Cáceres and Aguirre 2005, Torres et al. 2006, Lizondo et al. 2007, Hall 2008, Pluke at al. 2008). In Texas, for example, it is believed that both the psyllid and its natural enemy originated from Florida on imported potted plant material [jasmine orange, *Murraya paniculata* (L.)] (Michaud 2004). However, there are no experimental data to confirm this conclusion. *T. radiata* is routinely found parasitizing *D. citri* nymphs in Texas, but its incidence on its host population is fairly low (1–10%) (M.S., unpublished data). The development of any biological control program of *D. citri* in Texas (specifically with imported *T. radiata*) will require a proper establishment of the identity of *T. radiata* already present in the state.

The objective of the current study was to genetically characterize *T. radiata* populations from Texas, Florida, and Mexico and the closely related sister taxon *Tamarixia triozae* (Burks) by using two molecular methods: inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) DNA fingerprinting and a phylogeographic approach by sequencing standard partial genes (internal transcribed spacer region 1 [ITS1] and mitochondrial cytochrome oxidase subunit 1 gene [COI]). We have used the ISSR-PCR method extensively because of its increased power to resolve genetic relationships. The sensitivity of the method is due to the fact that it targets random simple sequence repeats or microsatellites within the entire genome, therefore providing information from many loci within the nuclear DNA (Zietkiewicz et al. 1994; Karp and Edwards 1997; Witsenboer and Michelmore 1997; Wolfe and Liston 1998; de León and Jones 2004, 2005; de León et al. 2004a,b, 2006b, 2008a; Triapitsyn et al. 2008). Phylogenetics is also a widespread approach for delineating morphologically similar species; in addition, it has been used in identifying geographic origins of invasive species (Avise 2000; Roderick and Navajas 2003; Brown 2004; Roderick 2004; de León et al. 2006b, 2008a; Triapitsyn et al. 2008).

Methods and Materials

Insect Collection. Surveys of *D. citri* natural enemies were conducted between spring and summer 2006 in south Texas. Developing *D. citri* nymphs on new flush shoots of sweet orange (*Citrus sinensis* L.), grapefruit (*Citrus paradisi* Macfayden), and orange jasmine were excised and put in aquatubes containing a hydroponic solution. These flush shoots were individually put in petri dishes and kept at room temperature ($\approx 25°C$) in the laboratory. They were checked daily, and the parasitoids that emerged were collected and stored in 95% alcohol. Parasitoid specimens were first examined and identified with an SMZ 10 stereomicroscope (Olympus, Tokyo, Japan) and later sent for confirmation to the USDA–APHIS–PPQ Systematic Entomology Laboratory in Beltsville, MD. Additional adult parasitoids were stored at $-80°C$ for subsequent molecular testing. *T. radiata* adults also were obtained from Florida. These parasitoids emerged from parasitized *D. citri* nymphs collected from a grapefruit orchard at the University of Florida Citrus Research and Education Center in Lake Alfred, FL. *T. radiata* adult specimens that emerged from psyllid nymphs collected in Huatabampo, Sonora, Mexico, from orange jasmine were obtained through our collaboration with colleagues at the University of Nuevo León in Monterrey, Mexico. The sister taxon or outgroup *T. triozae* was collected in Weslaco, TX (North American origin) on the potato/tomato psyllid *Bactericerca cockerelli* (Sulc) (Hemiptera: Psyllidae), and a colony was reared on tomato (*Solanum lycopersicum* L.) and potato (*Solanum tuberosum* L.) (Solanales: Solanaceae). The species was kindly provided by Tong-Xian (T-X) Liu of Texas AgriLIFE Research (Weslaco, TX). Voucher specimens of *T. radiata* and *T. triozae* in the current study are deposited at the Entomological Laboratory of Texas A&M-Kingsville Citrus Center in Weslaco, TX.

Genomic DNA Isolation and ISSR-PCR DNA Fingerprinting. Total genomic DNA extraction per individual was performed as described in de León and Jones (2004), de León et al. (2004a,b, 2006b, 2008a), and Triapitsyn et al. (2008). ISSR-PCR assays were performed with the 5′-anchored primer HVH(TG)$_7$T (Zietkiewicz et al. 1994), where H = A/T/C and V = G/C/A, and the compound primer T(GT)$_7$(AT)$_2$ (Witsenboer et al. 1997) as described previously (de León and Jones 2004, 2005; de León et al. 2004a,b, 2006b, 2008a; Triapitsyn et al. 2008). The ISSR-PCR assays were performed in a final volume of 20 μl with the following components: 1× PCR buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4, 1.5 mM MgCl$_2$, and 0.01% gelatin), 0.25 mM deoxynucleotide triphosphates, 0.25 μM ISSR primer, 1.0 μl of stock genomic DNA, and 2 U of *Taq*DNA Polymerase (New England Biolabs, Ipswich, MA). The cycling parameters were as follows: one cycle at 94°C for 2 min followed by 45 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min. Negative control reactions were performed in the absence of genomic DNA. To confirm for the presence of genomic DNA, reactions were performed with 1 μl of stock DNA and insect 28S primers (forward, 5′-CCCTGTTGAGCTTGACTCTAGTCTGG-C-3′ and reverse, 5′-AAGAGCCGACATCGAAG-GATC-3′) (Werren et al. 1995; de León et al. 2004a, 2006a; de León and Jones 2005) at a T$_m$ of 65°C with 1.5 mM MgCl$_2$ and the assay conditions described above.

Amplification and Sequencing of the ITS1 and ITS2 and the Partial COI. Primers ITS5 (forward, 5′-GGAAGTAAAAGTCGTAACAAGG-3′) (White et al.

Table 1. Mitochondrial COI diagnostic or population-specific nucleotides[a]

COI	Diagnostic Nucleotide no.		GenBank no.	ITS1	GenBank no.
	88	444			
T. radiata				*T. radiata*	
Clade 1					
Florida #1 [H1]	C	T	FJ807948	Florida #1 [v1]	FJ807952
Florida #2 [H2]	C	T	FJ807949	Florida #2 [v2]	FJ807953
Florida #3 [H2]	C	T	GQ912272	Florida #3 [v3]	GQ912295
Florida #4 [H2]	C	T	GQ912273	Florida #4 [v4]	GQ912296
Florida #5 [H2]	C	T	GQ912274	Florida #5 [v5]	GQ912297
Florida #6 [H3]	C	T	GQ912275		
Clade 2					
Texas #1 [H4]	T	G	FJ807950	Texas #1 [v6]	FJ807954
Texas #2 [H5]	T	G	FJ807951	Texas #2 [v7]	FJ807955
Texas #3 [H6]	T	G	GQ912276	Texas #3 [v8]	GQ912292
Texas #4 [H7]	T	G	GQ912277	Texas $4 [v9]	GQ912293
Texas #5 [H7]	T	G	GQ912278	Texas #5 [v10]	GQ912294
Texas #6 [H8]	T	G	GQ912279		
Mexico #1 [H7]	T	G	GQ912280	Mexico #1 [v11]	GQ912298
Mexico #2 [H7]	T	G	GQ912281	Mexico #2 [v4]	GQ912299
Mexico #3 [H7]	T	G	GQ912282	Mexico #3 [v12]	GQ912300
Mexico #4 [H7]	T	G	GQ912283	Mexico #4 [v13]	GQ912301
Mexico #5 [H9]	T	G	GQ912284		
Mexico #6 [H7]	T	G	GQ912285		
Outgroup				Outgroup	
T. triozae #1 [H1]			GQ912286	*T. triozae* #1 [v1]	GQ912302
T. triozae #2 [H2]			GQ912287	*T. triozae* #2 [v2]	GQ912303
T. triozae #3 [H3]			GQ912288	*T. triozae* #3 [v3]	GQ912304
T. triozae #4 [H2]			GQ912289		
T. triozae #5 [H4]			GQ912290		
T. triozae #6 [H5]			GQ912291		

[a] Also included are the GenBank accession numbers for COI and ITS1 DNA sequences from *T. radiata* and the sister taxon *T. triozae.* The mitochondrial haplotype number [H] and the ITS1 rDNA fragment variant number [v] are listed after each individual. Specific details about the haplotypes are not shown here.

1990) and RNA2 (reverse, 5′-CACGAGCCGAGT-GATCCACCGCTAAGAGT-3′) (Chang et al. 2001) were used to amplified the ITS1 rDNA gene fragment as described previously (de León et al. 2004a, Triapitsyn et al. 2008). The amplification reactions were performed with ThermoPol reaction buffer [1×: 20 mM Tris-HCl (pH 8.8 at 25°C), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100] (New England Biolabs), and 2.0 mM $MgCl_2$] with the following cycling parameters: one cycle at 94°C for 3 min followed by 30 cycles at 94°C for 20 s, 55°C for 20 s, and 72°C for 40 s. Primers 5.8S-F (forward, 5′-TGTGAACTGCAGGACACATGAAC-3′) (Porter and Collins 1991) and ITSd (reverse, 5′-TCCTCCGCT-TATTGATATGC-3′) (White et al. 1990) were used to amplify the ITS2 rDNA gene fragment. The cycling parameters were the same as described above except that the T_m was 60°C and the last cycle at 72°C was for 30 s. To amplify the COI partial gene the following primers C1-J-1718 (forward, 5′-GGAGGATTTG-GAAATTGATTAGTTCC-3′) and C1-N-2191 (reverse, 5′-CCCGGTAAAATTAAAATATAAACTTC-3′) of Simon et al. (1994) were used as described previously (de León et al. 2006a,b, 2008a). ThermoPol reaction buffer and 2.0 mM $MgCl_2$ were used and the cycling parameters were as follows: one cycle for 3 min at 94°C followed by 40 cycles at 94°C for 20 s, 48°C for 20 s, and 72°C for 40 s.

For sequencing, the amplification products were subcloned with the TOPO cloning kit (Invitrogen, Carlsbad, CA), plasmid minipreps were prepared by the QIAprep spin miniprep kit (QIAGEN, Valencia, CA), and sequencing was performed by Davis Sequencing (Davis, CA) as described previously (de León et al. 2006a,b, 2008a; Triapitsyn et al. 2008). GenBank accession numbers for both gene fragments (COI and ITS1) are listed in Table 1.

DNA Sequence Analysis. The DNA sequencing software program Sequencher (Gene Codes Corp., Ann Arbor, MI) was used to process the raw sequences. DNA sequences were aligned with the program DNAStar (DNAStar, Madison, WI) using the ClustalW algorithm (Higgins et al. 1994), which was used to calculate percentage divergence (%D), as measured as a function of genetic distance and phylograms (neighbor-joining and maximun parsimony) were reconstructed as described in de León et al. (2006b, 2008a). Mitochondrial COI sequences were translated into amino acid sequences by using the invertebrate mitochondrial code with the computer program EMBOSS Transeq (http://www.ebi.ac.uk/emboss/transeq/index.html).

Results

Presence of *T. radiata* in Texas. Of the ≈ 1,000 *D. citri* nymphs collected and incubated in the laboratory between April and July 2006, 73 were found to be parasitized with *T. radiata*. The identity of this parasitoid was confirmed by Dr. Gregory Evans (USDA–
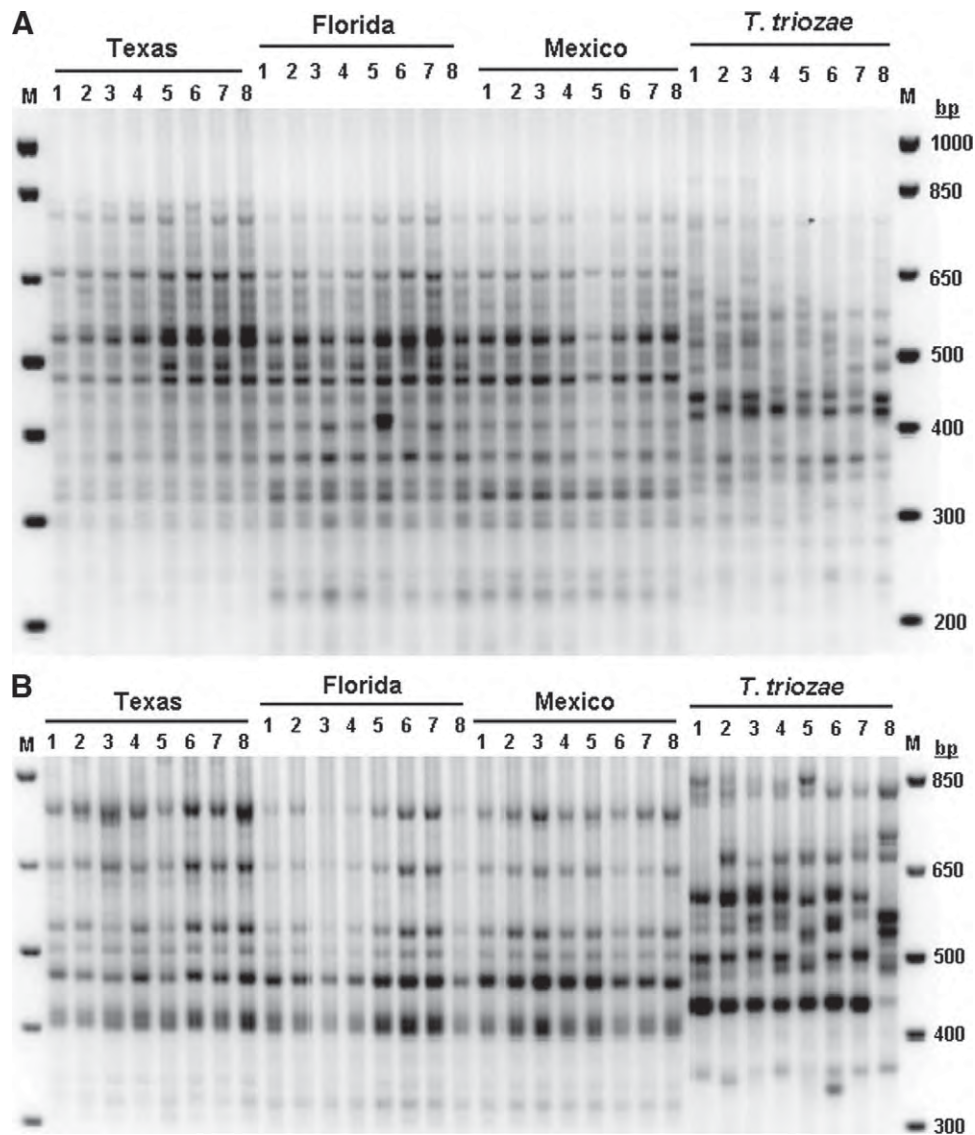
Fig. 1. ISSR-PCR DNA fingerprinting. Total genomic DNA from eight individuals each from populations of *T. radiata* from Texas, Florida, and Mexico was used to perform DNA fingerprinting assays with both (A) a 5′-anchored ISSR primer and (B) a compound ISSR primer. M, 1.0-kb Plus DNA ladder.

APHIS–PPQ Systematic Entomology Laboratory, Beltsville, MD).

Morphological comparisons of *T. radiata* collected in Texas showed this species is essentially identical to *T. radiata* from Florida. Inasmuch as the parasitoid has not been previously intentionally imported into Texas, it is quite possible that it may have been introduced along with its *D. citri* host on orange jasmine plants. The importation of potted orange jasmine plants into Texas from Florida had been until recently a common practice by many Texas nursery personnel.

ISSR-PCR DNA Fingerprinting. The results of these experiments are shown in Fig. 1 with the two different ISSR primers. The 5′-anchored primer generated ≈10 consistent bands ranging from below the 850 bp marker to above the 300 bp marker (Fig. 1A). The bands below the 300 bp marker were not consistent. The *T. radiata* populations from Texas, Florida, and Mexico basically showed the same ISSR-PCR DNA banding patterns, indicating that there is no genetic differentiation among the three populations based on this primer. However, the sister taxon *T. triozae* showed a different ISSR banding pattern with little band sharing with *T. radiata;* however, variation was observed in this species. No genetic differentiation was observed with the ISSR compound primer either as the consistent bands seemed equally among the three populations (Fig. 1B). This primer generated about seven consistent bands ranging from below the 850-bp marker to above the 400-bp marker. *T. triozae*
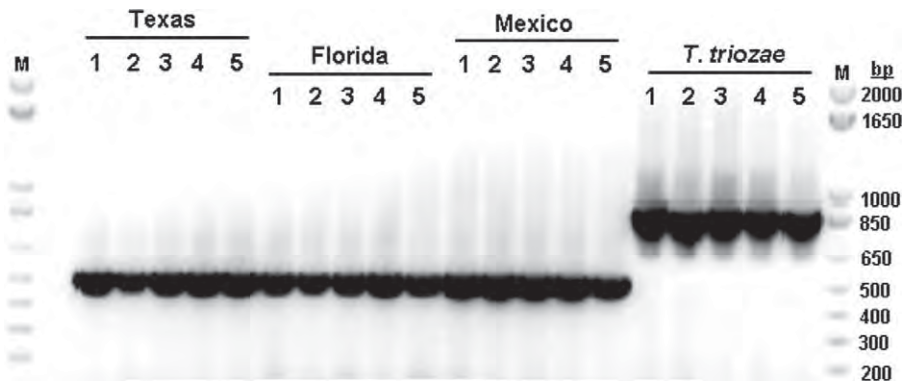
Fig. 2. Amplification of the ribosomal ITS1. The ITS1 rDNA gene fragment was amplified with standard ITS1-specific primers with genomic DNA from five individuals each from Texas, Florida, and Mexico. The sister taxon *T. triozae* is included for comparison. M, 1.0-kb Plus DNA ladder.

was observed with a completely different banding pattern with the compound primer compared with *T. radiata.* Banding pattern variation was again observed in *T. triozae* with the ISSR compound primer.

Amplification of ITS1 and ITS2. Because ITS rDNA fragment size differences can be an indication of species level divergence (Collins and Paskewitz 1996; Stouthamer et al. 1999; de León et al. 2004a, 2006b), we amplified the ITS1 and ITS2 gene fragments from the *T. radiata* populations with standard primers. Differences in DNA fragment size were not observed for either of the ITS fragments among the populations from Texas, Florida, and Mexico. The size of the ITS1 rDNA fragments was 520–521 bp (Fig. 2) and the size of the ITS2 rDNA fragments was ≈600 bp (data not shown). The size of the ITS1 gene fragment in *T. triozae* varied from *T. radiata* in being ≈866–867 bp (Fig. 2), making this gene fragment a simple diagnostic tool for discriminating the two closely related species. No size difference in the ITS2 gene fragments was observed between the two species.

DNA Sequence Analyses of the ITS1 and COI Gene Fragments. To confirm the results of the electrophoretic methods described above, we sequenced a nuclear (ITS1) and a mitochondrial (COI) gene from individuals from Texas, Florida, and Mexico. Sequencing of the ITS1 gene fragment produced 13 variants (v1-v13) (Table 1) out of 14 individuals based on nucleotide differences, demonstrating extensive genetic variation. The ITS1 gene fragments ranged in size from 520 to 521 bp. The ITS1 rDNA variants were not geographic-specific and varied among each other by only five to nine nucleotides. This type of natural variation is expected for ITS regions (de León et al. 2006b). The levels of genetic divergence in the ITS1 gene fragment were determined by calculating the pairwise estimates for genetic distance. The intrapopulational variation for Texas, Florida, and Mexico was 0.6–1.0, 0.2–1.0, and 0.6–2.0%, respectively, demonstrating overlapping results. The variance for all populations combined was 0.0–2.0%. Alignment of the *T. triozae* ITS1 fragments was not attempted due to the size differences (345–347 bp) observed

between the two species. Attempts to align fragments that differ by this many nucleotides could lead to erroneous and inaccurate results. Genetic variation, however, was observed within the ITS1 fragment of *T. triozae* (Table 1).

Sequencing of the COI partial gene generated fragments of 518 bp across all populations of *T. radiata* (18 total individuals). The same size fragment was also observed in *T. triozae* (six individuals). Overall, one to six nucleotide differences were observed among all individuals of the three populations. Haplotype variation was observed within the COI gene of *T. radiata* as nine haplotypes (H1–H9) out of 18 individuals were uncovered (Table 1). No haplotypes were shared between the Florida and the Texas/Mexico populations, indicating haplotype structure. Florida was observed with three haplotypes (H1–H3), Texas with five haplotypes (H4–H8), and Mexico with two haplotypes (H7 and H9). Haplotypes 1–3 were unique to Florida, H4–6 and H8 were unique to Texas, and H9 was unique to Mexico. Haplotype 7 was shared by both the Texas and Mexico populations, demonstrating their genetic similarly at the COI gene. The intrapopulational variation was 0.0–0.4, 0.0–1.4, and 0.0–0.2% for Florida, Texas, and Mexico, respectively. The overall populational variation (0.0–1.4%) fell within the intrapopulational range (Table 2). Because several specimens per population were analyzed, the COI sequence data uncover two diagnostic or clade-specific nucleotides in the Florida and Texas/Mexico populations. At nucleotide #88, for example, all individuals from Florida were observed with a cytosine (C),

Table 2. Pairwise DNA sequence distances (percentage divergence, range) of COI gene fragments from populations of *T. radiata* from Florida (FL), Texas (TX), and Mexico (MX), including the outgroup *T. triozae*

	FL	TX	MX
FL	0.0–0.4		
TX	0.4–1.4	0.0–1.4	
MX	0.4–0.8	0.0–1.0	0.0–0.2
T. triozae	9.0–9.9	9.0–10.3	9.0–10.3

whereas all individuals from Texas and Mexico were observed with a thymine (T) (Table 1). These nucleotide differences allowed the discrimination of the *T. radiata* populations by COI sequence analysis. None of the nucleotide differences led to amino acid substitutions or changes in enzyme restriction sites. The percentage divergence between *T. radiata* and the sister taxon *T. triozae* ranged from 9.0 to 10.3%, highly suggesting that the populations of *T. radiata* from Florida, Texas, and Mexico represent a single species. Comparison of the COI sequence data demonstrated that *T. triozae* varied from *T. radiata* by 42 nucleotides that led to four amino acid substitutions ($T^{35}M$, $L^{94}I$, $L^{101}M$, and $S/P^{146}A$).

The phylogeographic analysis of the COI sequence data from the three populations of *T. radiata* is shown in Fig. 3. A neighbor-joining distance phylogram clustered the Florida population into a distinct clade (clade 1), whereas the Texas and Mexico populations both fell into a separate clade (clade 2) (Fig. 3A). Strong bootstrap support was seen in the *T. radiata* major clade (100%) and in the Florida subclade (clade 2) (87%). A single most parsimonious tree demonstrated the same tree topology as the neighbor-joining tree with support values of 100 and 67% for the *T. radiata* major clade and clade 1 (Florida), respectively (Fig. 3B).

Discussion

To our knowledge, the current report represents the first molecular study of *T. radiata* populations using the ISSR-PCR DNA fingerprinting method. The advantage of this procedure is that it is very sensitive and requires no prior knowledge of DNA sequence information. In addition, ISSR-PCR can be used across various species, even those belonging to different kingdoms using the same primer, an advantage that sequence-specific microsatellites do not have (Zietkiewicz et al. 1994, Karp and Edwards 1997, Witsenboer and Michelmore 1997, Wolfe and Liston 1998, de León and Jones 2004). In our laboratory, we have seen, in the majority of cases, a very good correlation between ISSR-PCR banding pattern differences and species level divergence; for example, in species such as *Gonatocerus morrilli* (Howard) and *Gonatocerus walkerjonesi* S. Triapitsyn (Hymenoptera: Mymaridae). Fixed ISSR-PCR banding pattern differences were identified in the two species. These species are very closely related and were once considered a single species (Huber 1988, Triapitsyn 2006); both are primary egg parasitoids of the glassy-winged sharpshooter, *Homalodisca vitripennis* (Germar) (Hemiptera: Cicadellidae) (de León et al. 2004b, 2006b; Triapitsyn 2006; de León and Morgan 2007). Another example is the *Gonatocerus tuberculifemur* (Ogloblin) species complex that includes *Gonatocerus deleoni* Triapitsyn, Logarzo, & Virla (de León et al. 2008a, Triapitsyn et al. 2008). This species complex was also once considered to be a single species (Ogloblin 1957). In this particular case, DNA sequence data inferred from COI, ITS1, and ITS2 gene fragments could not dis-

criminate all five strains or species within the complex, whereas ISSR-PCR had the capacity to discriminate all five species within the complex. The differentiation of the *G. tuberculifemur* species complex by the ISSR-PCR method was confirmed by cross-mating studies of most of the species as reproductive incompatibility was demonstrated (Triapitsyn et al. 2008). However, lack of fixed ISSR-PCR banding pattern differences in geographic populations of *Gonatocerus ashmeadi* Girault (de León and Jones 2005) demonstrated that they were all a single species as the geographic populations were reproductively compatible (Vickerman et al. 2004). However, ISSR-PCR was sensitive enough to detect geographic variation in *G. ashmeadi* populations (de León and Jones 2005).

Likewise, in the current study, ISSR-PCR, using two sensitive primers—a 5′-anchored and a compound primer—did not uncover any fixed banding pattern differences in the populations of *T. radiata* from Texas, Florida, and Mexico (Fig. 1). However, ISSR-PCR banding pattern differences were clearly observed between the two closely related species *T. radiata* and *T. triozae*. The data highly suggest that these three populations of *T. radiata* represent a single species, as genetic differentiation was not detected. Lending support to this interpretation were the results from the DNA sequence data inferred from the mitochondrial COI gene and the nuclear ITS1 gene fragment. In both cases, the intrapopulational variation range fell within the interpopulational variation range. The %D among all populations of *T. radiata* at the COI gene was 0.0–1.4%, whereas the %D between *T. radiata* and its sister taxon *T. triozae* was 9.0–10.3% (Table 2). In addition, size differences in the amplification products of either gene fragment were not observed between or within the populations (Fig. 2). For ITS rDNA gene fragments, size differences can serve as a rapid diagnostic tool to discriminate very closely related species (Collins and Paskewitz 1996; de León et al. 2004a, 2006b; de León and Morgan 2007). Size-wise, the very conserved protein-coding genes, such as the COI and COII genes, are very stable (Simon et al. 1994) and are not expected to vary in size within populations of the same species or within closely related species, such as egg parasitoids belonging to the genus *Gonatocerus* Nees (e.g., de León et al. 2008a). Any size differences in these conserved genes are usually an indication of separate species. For example, a 2-bp difference in the size of the COII gene was observed in two closely related species, *G. walkerjonesi* and *G. morrilli* (de León et al. 2006b). Size differences at the COI gene, however, are more common among highly diverged species. For example, the size fragments of the COI gene of two closely related endoparasitoids, *Encarsia diaspidicola* Silvestri and *Encarsia berlesei* Howard (Hymenoptera: Aphelinidae), was 518 bp, whereas that of a scale insect, *Colobopyga pritchardiae* (Stickney) (Hemiptera: Halimoccidae), was 524 bp (J.H.L., unpublished data). Of course, lack of size differences in gene amplification products does not mean that species are not genetically diverged.
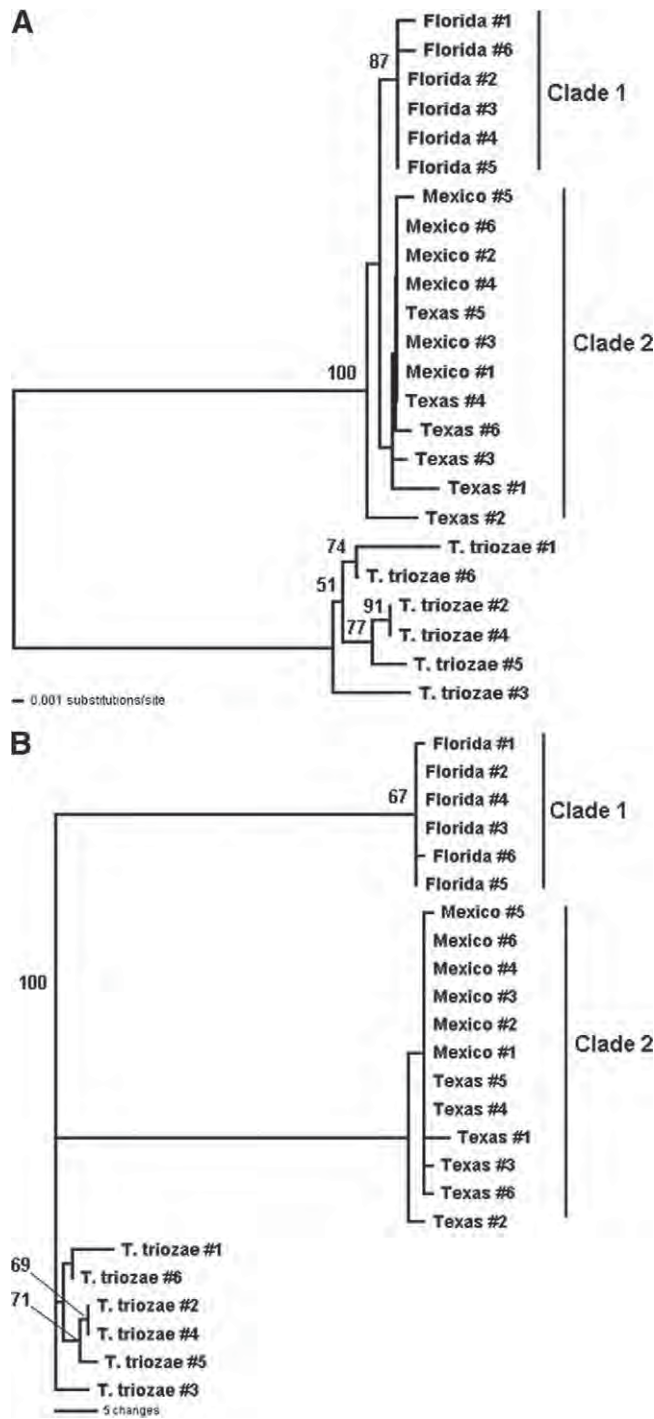
Fig. 3. Phylograms inferred from the mitochondrial COI partial gene from geographic populations of *T. radiata*. (A) Neighbor-joining distance tree and (B) Parsimonious tree: bootstrap 50% majority-rule consenses tree based on 49 informative characters. Tree length = 73 steps; consistency index (CI) = 0.932; and retention index (RI) = 0.979. *T. triozae* individuals (6) were included as an outgroup. The trees display bootstrap values as percentage of 1,000 replications. To account for intra and interpopulational variation, several individuals (six) were included.

Caution, however, must be exercised when analyzing ITS regions because there are many examples in the literature where neither the size of the ITS fragment nor the nucleotides sequences vary in closely related species. Triapitsyn et al. (2008) have shown that both the ITS1 and ITS2 fragments do not vary

among the nucleotide sequences in two closely related species, *G. tuberculifemur* and *G. deleoni.* These species were discriminated by COI sequence data, by ISSR-PCR analysis, and by cross-mating studies. In the genus *Anagrus*, COI sequence analyses discriminated three closely related species, *Anagrus atomus* (L.), *Anagrus ustulatus* Haliday, and *Anagrus erythroneurae* Trjapitzin et Chiappini, but the ITS2 fragment could not discriminate *A. atomus* from *A. ustulatus* (de León et al. 2008b). Another example was demonstrated by Stouthamer et al. (2000) with cryptic species (reproductively incompatible) of *Trichogramma*, *Trichogramma minutum* Riley and *Trichogramma platneri* Nagarkatti. As with other closely related species, the ITS2 fragment could not be used to discriminate these cryptic species. These examples, among others in the literature, including several with fungi (e.g., Baayen et al. 2002), demonstrate that even if organisms share the same ITS regions, this does not necessarily mean that they represent a single species. Thus, the ITS regions alone cannot be used to determine species status without other supporting data, such as, cross-mating studies, morphological analyses, and other molecular methods.

Based on the results from the COI phylogeographic analysis, however, haplotype or geographic structure was identified. There was no haplotype sharing between the Florida population and the Texas/Mexico populations, whereas sharing was observed between the Texas and Mexico populations. These results indicate that gene flow is occurring between the Texas and Mexico populations of *T. radiata* but not between the Texas/Mexico and the Florida population. Therefore, the data suggest that the population of *T. radiata* from Texas probably did not come from Florida. This is demonstrated on the phylograms on Fig. 3 showing that the Florida population clustered into its own clade (clade 1) with strong bootstrap support. Lending support to this observation is the fact that the Texas and Mexico populations shared the same two diagnostic nucleotides that allowed them to be distinguished from the Florida population (Table 1). As mentioned, the thought in the literature was that *T. radiata* was inadvertently introduced into Texas from Florida (Michaud 2004), as it is well known that Texas nurserymen traditionally imported orange jasmine plants from Florida. The current experimental data now shed light on a different scenario and suggest that the population of *T. radiata* from Texas came from Mexico, or vice versa, because the Mexico population was observed with less haplotype diversity. This could be a possibility because based on the theories of founding events, founding populations will experience a genetic bottleneck that will lead to a severe decrease in genetic diversity (Narang et al. 1993, Unruh and Woolley 1999, Roderick and Navajas 2003, Roderick 2004). However, more experimental data will be required to confirm this possibility.

The present results raise new questions about *T. radiata.* For example, 1) Could *T. radiata* be native to Texas or the American continent? Hoy et al. (1999) imported *T. radiata* from Taiwan into Florida; yet, our data suggest that the Texas and Mexico populations did not come from Florida. *T. radiata* has not been deliberately released into any other country of the American continent but has been inadvertently introduced into several countries (Cáceres and Aguirre 2005, Torres et al. 2006, Lizondo et al. 2007, Hall 2008, Pluke at al. 2008). Another possibility is that *T. radiata* was inadvertently introduced into the American continent (excluding Florida) at different ports of entries via movement of plant material from various Asian countries, so 2) Can genetic structure be identified in *T. radiata* found within each Texas, Mexico, or South American with more extensive sampling? Also, 3) Where did the populations of *T. radiata* in Texas and Mexico come from? Could they have come from Puerto Rico, South America, somewhere in Asia, or were they already present? The process of collecting more geographic populations of *T. radiata* is in motion to begin to answer theses questions. Cross-mating and morphological studies also would be required in populations that show genetic differentiation to confirm a species level divergence.

In conclusion, invasive pests usually do not bring their natural enemies with them into a new environment; therefore, the pests are allowed to multiply to levels that can cause great damage to crops of interest. In classical biological control, natural enemies are usually collected from the pest's area of origin and released into the new environment (Narang et al. 1993, Roderick and Navajas 2003. However, before this approach can be implemented, an extensive survey of the landscape should be performed for the presence of natural enemies that could be the same species as the ones being imported. Molecular genetic, cross-mating, and morphological studies are critical to accurately identify both the native and the candidate biological control agent. If hybridization occurs between the two strains, it could make it impossible to track and monitor the establishment of the imported natural enemy, as discussed by Rugman-Jones et al. (2009). We therefore, recommend that no releases of *T. radiata* be made in Texas for the Asian citrus psyllid until the situation with this species is resolved with more extensive experimental data.

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