Overview of worldwide diversity of *Diaphorina citri* Kuwayama mitochondrial cytochrome oxidase 1 haplotypes: two Old World lineages and a New World invasion

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Abstract

Relationships among worldwide collections of *Diaphorina citri* (Asian citrus psyllid) were analyzed using mitochondrial cytochrome oxidase I (mtCOI) haplotypes from novel primers. Sequences were produced from PCR amplicons of an 821 bp portion of the mtCOI gene using *D. citri* specific primers, derived from an existing EST library. An alignment was constructed using 612 bps of this fragment and consisted of 212 individuals from 52 collections representing 15 countries. There were a total of eight polymorphic sites that separated the sequences into eight different haplotypes (Dcit-1 through Dcit-8). Phylogenetic network analysis using the statistical parsimony software, TCS, suggests two major haplotype groups with preliminary geographic bias between southwestern Asia (SWA) and southeastern Asia (SEA). The recent (within the last 15 to 25 years) invasion into the New World originated from only the SWA group in the northern hemisphere (USA and Mexico) and from both the SEA and SWA groups in the southern hemisphere (Brazil). In only one case, Reunion Island, did haplotypes from both the SEA and SWA group appear in the same location. In Brazil, both groups were present, but in separate locations. The Dcit-1 SWA haplotype was the most frequently encountered, including ∼50% of the countries sampled and 87% of the total sequences obtained from India, Pakistan and Saudi Arabia. The second most frequently encountered haplotype, Dcit-2, the basis of the SEA group, represented ∼50% of the countries and contained most of the sequences from Southeast Asia and China. Interestingly, only the Caribbean collections (Puerto Rico and Guadeloupe) represented a unique haplotype not found in other countries, indicating no relationship between the USA (Florida) and Caribbean introductions. There is no evidence for cryptic speciation for *D. citri* based on the COI region included in this study.

Keywords: genetic structure, statistical parsimony, Asian citrus psyllid, citrus, citrus greening disease, Huanglongbing, novel COI primers

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Introduction

The Asian citrus psyllid, *Diaphorina citri* Kuwayama, Hemiptera: Psyllidae, is a phloem feeding insect native to Asia and the Far East with a host range of only *Citrus* spp. and its near *Rutaceae* relatives (Halbert & Núñez, 2004). *Diaphorina citri* causes little direct feeding damage, but it vectors the phloem limited bacterial citrus pathogens, 'Candidatus Liberibacter asiaticus', 'C. L. africanus', and 'C. L. americanus', which cause 'citrus greening disease' or Huanglongbing (HLB), a disease which is considered one of the most damaging to citrus (Capoor *et al.*, 1967; Martinez & Wallace, 1967; Teixeira *et al.*, 2005). The impact of this disease on world citrus production and concern over its continued spread have lead to research on the vector's plant host range, interactions with parasitoids and host-vector-pathogen interactions (Bové, 2006; Halbert & Manjunath, 2004; Hall *et al.*, 2008). Despite these endeavors, there is virtually no information available regarding *D. citri* genetic diversity and the implications this may hold for these and other studies.

The Asian citrus psyllid's current distribution extends from the Arabian Peninsula (Saudi Arabia and Yemen) and from Afghanistan through to the Indian Subcontinent, Southeast Asia and the Ryukyu Archipelago and Kyushu in Japan, Taiwan, Hong Kong and the coastal provinces of Guangxi, Guangdong, Fujian and Zhejiang in China, the Philippine Islands and through the Indonesian archipelago to northeastern Papua New Guinea; in the Pacific Islands of Hawaii and Guam; the continental USA (Alabama, California, Florida, Georgia, Louisiana, Mississippi, South Carolina and Texas), as well as the Caribbean (Bahamas, Cayman Islands, Cuba, Jamaica, Dominican Republic, Guadeloupe and the Commonwealth of Puerto Rico), Central (Honduras and Mexico) and South America (Argentina, Brazil, Paraguay, Uruguay and Venezuela) and the Indian Ocean islands of Mauritius and Réunion (Costa Lima, 1942; French *et al.*, 2001; Halbert & Manjunath, 2004; Halbert & Núñez, 2004; Mead, 1977; Pluke *et al.*, 2008; Tsai *et al.*, 2000). Because of the lack of global phylogenetics for this species, hypotheses describing its origin include either southeastern Asia or southwestern Asia (India/Pakistan) (Mead, 1977). Anthropogenic spread via the pathway of infested plant material has been the principal means by which this pest has spread. This was responsible for the New World invasions that have seen its distribution expand to include countries in North, Central and South America and the Caribbean (Núñez, 2004). *Diaphorina citri* was first reported from the continental USA (Florida) in 1998 (Bové, 2006) and now occurs from Florida to California.

Understanding the genetic diversity underlying an invasive pest is a key element that supports the development of effective management strategies (Rosen, 1978; Schauff *et al.*, 1998). Complicating the analysis of genetic diversity among world populations of many invasive insects is that many contain a cryptically diverse collection of populations that cannot be easily categorized by morphological characteristics (Scheffer, 2000; Clarke *et al.*, 2005). Diversity can be represented as distinct biotypes and may extend to reproductive isolation, suggesting the existence of multiple cryptic species (Bickford *et al.*, 2007; De Barro *et al.*, 2011). The emergence of molecular systematic approaches has allowed the more precise and rapid characterization of diversity among populations of invasive species beyond what can be ascertained from morphological evaluations. This information can be used to identify the geographic origin of the invaders, if not directly, then at least from an evolutionary perspective (Xavier *et al.*, 2009; De Barro & Ahmed, 2011). Such knowledge is invaluable when looking for effective biological control agents, and understanding basic biology and ecology.

The purpose of this study was to use mitochondrial cytochrome oxidase I (mtCO1) to assess the genetic relationships between different collections of *D. citri* from around the world. The questions we set out to answer were: (i) Can mtCOI haplotype analysis provide an insight into phylogeographic relationships of Old World *D. citri* populations? If so, (ii) is there population structure that can be used to monitor global movement of specific genetic types? And, (iii) is there evidence of cryptic species based on relative genetic relationships of individuals from the different collections?

Methods

Collection of global D. citri *collections and outgroup selection*

Individuals were collected from Bali, Brazil, China, USA (Florida and Texas), Guadeloupe, India, Java, Mauritius Island, Mexico, Pakistan, Puerto Rico, Réunion Island, Saudia Arabia, Taiwan, Texas-USA, Thailand and Vietnam (table 1). All material was stored in 70% ethanol until DNA was extracted. In most cases, multiple individuals were collected in each location. Truncated sequences (sequences less than 550 base pairs) were deleted to leave a final aligned data set of 212 individual sequences. These sequences were then screened using a similarity matrix, and eight unique haplotypes were identified. An outgroup mtCOI sequence was from *Pachypsylla venusta*, the hackberry petiole gall psyllid (NC_006157). This was chosen because it was the closest psyllid relative for which the entire 612 bp mtCOI sequence was available and was used to aid in alignment.

Identification of novel COI primers specific for D. citri

Amplification of mtCOI was not successful using the universal primers C1-J-2195 and TL2-N-3014 (Simon *et al.*, 1994). New mtCOI primers for *D. citri* were designed from an EST library generated at the USDA (Hunter *et al.*, 2008) and available in GenBank (http://www.ncbi.nlm.nih.gov/). The primers were: DCITRI COI-L 5'- AGG AGG TGG AGA CCC AAT CT and DCITRI COI-R 5'- TCA ATT GGG GGA GAG TTT TG. These amplify an 821 base pair fragment of the mtCOI coding region.

DNA extraction, amplification and sequencing

Total DNA was extracted using Cartagen's (http://www.cartagen.com) rapid homogenization kit for plant leaf DNA amplification (catalog number 20700-500, lot number 08180400134). The 30 μl PCR reactions were heated at 94°C for two minutes followed by 35 cycles of 30 s at 94°C denaturation, 30 s at 53°C annealing, 1 min at 72°C extension and a final extension of 72°C for 10 min in a MJ Research PTC-200 Peltier thermal cycler. The PCR reactions were composed of 27 μl Platinum PCR SuperMix (Invitrogen, catalog number 11306-016), 1 μl forward primer (10 ρmol), 1 μl reverse primer (10 ρmol), and 1 μl DNA template. Prior to sequencing, the amplified products were cleaned using the montage PCR filter units from Millipore (catalog number UFC7PCR50). All sequencing was performed using the amplification primers and BigDye® Terminator Cycle Sequencing Kits. Sequence

Table 1. Accession numbers for each of the 212 sequences from 52 populations of *Diaphorina citri* used to generate fig. 1.

Location	Locality	Genebank Code	Accession Number	Haplotype Assignment*
USA, Florida	St. Lucie, Co. (32)	psy1-1-dcitrico1	FJ190167	Dcit-1
		psy1-2-dcitrico1	FJ190168	Dcit-1
		psy1-3-dcitrico1	FJ190169	Dcit-1
		psy1-4-dcitrico1	FJ190170	Dcit-1
		psy1-5-dcitrico1	FJ190171	Dcit-1
		psy9-1-dcitrico1	FJ190192	Dcit-1
		psy9-2-dcitrico1	FJ190193	Dcit-1
		psy9-3-dcitrico1	FJ190194	Dcit-1
		psy9-4-dcitrico1	FJ190195	Dcit-1
		psy9-5-dcitrico1	FJ190196	Dcit-1
		psy10-1-dcitrico1	FJ190197	Dcit-1
		psy10-2-dcitrico1	FJ190198	Dcit-1
		psy10-3-dcitrico1	FJ190199	Dcit-1
		psy10-4-dcitrico1	FJ190200	Dcit-1
		psy10-5-dcitrico1	FJ190201	Dcit-1
		psy11-1-dcitrico1	FJ190202	Dcit-1
		psy11-2-dcitrico1	FJ190203	Dcit-1
		psy11-3-dcitrico1	FJ190204	Dcit-1
		psy11-4-dcitrico1	FJ190205	Dcit-1
		psy11-5-dcitrico1	FJ190206	Dcit-1
		psy18-1-dcitrico1	FJ190232	Dcit-1
		psy18-2-dcitrico1	FJ190233	Dcit-1
		psy18-3-dcitrico1	FJ190234	Dcit-1
		psy18-4-dcitrico1	FJ190235	Dcit-1
		psy18-5-dcitrico1	FJ190236	Dcit-1
		psy30-1-dcitrico1	FJ190277	Dcit-1
		psy30-3-dcitrico1	FJ190278	Dcit-1
		psy21-1-dcitrico1	FJ190246	Dcit-1
		psy21-2-dcitrico1	FJ190247	Dcit-1
		psy21-3-dcitrico1	FJ190248	Dcit-7
		psy21-4-dcitrico1	FJ190249	Dcit-1
		psy21-5-dcitrico1	FJ190250	Dcit-1
	Palm Beach, Co. (16)	psy2-1-dcitrico1	FJ190172	Dcit-1
		psy2-2-dcitrico1	FJ190173	Dcit-1
		psy2-3-dcitrico1	FJ190174	Dcit-1
		psy2-4-dcitrico1	FJ190175	Dcit-1
		psy2-5-dcitrico1	FJ190176	Dcit-1
		psy4-1-dcitrico1	FJ190182	Dcit-1
		psy4-2-dcitrico1	FJ190183	Dcit-1
		psy4-3-dcitrico1	FJ190184	Dcit-1
		psy4-4-dcitrico1	FJ190185	Dcit-1
		psy4-5-dcitrico1	FJ190186	Dcit-1
		psy16-1-dcitrico1	FJ190226	Dcit-1
		psy16-5-dcitrico1	FJ190227	Dcit-1
		psy20-1-dcitrico1	FJ190242	Dcit-1
		psy20-2-dcitrico1	FJ190243	Dcit-1
		psy20-4-dcitrico1	FJ190244	Dcit-1
		psy20-5-dcitrico1	FJ190245	Dcit-1
	Brevard, Co. (10)	psy13-1-dcitrico1	FJ190211	Dcit-1
		psy13-2-dcitrico1	FJ190212	Dcit-1
		psy13-3-dcitrico1	FJ190213	Dcit-1
		psy13-4-dcitrico1	FJ190214	Dcit-1
		psy13-5-dcitrico1	FJ190215	Dcit-1
		psy19-1-dcitrico1	FJ190237	Dcit-1
		psy19-2-dcitrico1	FJ190238	Dcit-1
		psy19-3-dcitrico1	FJ190239	Dcit-1
		psy19-4-dcitrico1	FJ190240	Dcit-1
		psy19-5-dcitrico1	FJ190241	Dcit-1
	Miami-Dade, Co. (4)	psy23-1-dcitrico1	FJ190256	Dcit-1
		psy23-3-dcitrico1	FJ190257	Dcit-1
		psy23-4-dcitrico1	FJ190258	Dcit-1
		psy23-5-dcitrico1	FJ190259	Dcit-1
	Broward, Co. (2)	psy39-2-dcitrico1	FJ190310	Dcit-1
		psy39-5-dcitrico1	FJ190311	Dcit-1

Table 1. (*Cont.*)

Location	Locality	Genebank Code	Accession Number	Haplotype Assignment*
	Indian River, Co. (10)	psy8-1-dcitrico1	FJ190187	Dcit-1
		psy8-2-dcitrico1	FJ190188	Dcit-1
		psy8-3-dcitrico1	FJ190189	Dcit-1
		psy8-4-dcitrico1	FJ190190	Dcit-1
		psy9-5-dcitrico1	FJ190191	Dcit-1
		psy14-1-dcitrico1	FJ190216	Dcit-1
		psy14-2-dcitrico1	FJ190217	Dcit-1
		psy14-3-dcitrico1	FJ190218	Dcit-1
		psy14-4-dcitrico1	FJ190219	Dcit-1
		psy14-5-dcitrico1	FJ190220	Dcit-1
	Okeechobee, Co. (9)	psy12-1-dcitrico1	FJ190207	Dcit-1
		psy12-2-dcitrico1	FJ190208	Dcit-1
		psy12-4-dcitrico1	FJ190209	Dcit-1
		psy12-5-dcitrico1	FJ190210	Dcit-1
		psy22-1-dcitrico1	FJ190251	Dcit-1
		psy22-2-dcitrico1	FJ190252	Dcit-1
		psy22-3-dcitrico1	FJ190183	Dcit-1
		psy22-4-dcitrico1	FJ190254	Dcit-1
		psy22-5-dcitrico1	FJ190255	Dcit-1
	Martin, Co. (5)	psy15-1-dcitrico1	FJ190221	Dcit-1
		psy15-2-dcitrico1	FJ190222	Dcit-1
		psy15-3-dcitrico1	FJ190223	Dcit-1
		psy15-4-dcitrico1	FJ190224	Dcit-1
		psy15-5-dcitrico1	FJ190225	Dcit-1
	Collier, Co. (6)	psy55-4-dcitrico1	FJ190372	Dcit-1
		psy55-5-dcitrico1	FJ190373	Dcit-1
		psy56-1-dcitrico1	FJ190374	Dcit-1
		psy56-2-dcitrico1	FJ190375	Dcit-1
		psy56-3-dcitrico1	FJ190376	Dcit-1
		psy56-5-dcitrico1	FJ190377	Dcit-1
USA, Texas	Hidalgo, Co. (5)	psy3-1-dcitrico1	FJ190177	Dcit-1
		psy3-2-dcitrico1	FJ190178	Dcit-1
		psy3-3-dcitrico1	FJ190179	Dcit-1
		psy3-4-dcitrico1	FJ190180	Dcit-1
		psy3-5-dcitrico1	FJ190181	Dcit-1
Brazil	Sao Paulo (17)	psy17-1-dcitrico1	FJ190228	Dcit-2
		psy17-2-dcitrico1	FJ190229	Dcit-2
		psy17-3-dcitrico1	FJ190230	Dcit-2
		psy17-4-dcitrico1	FJ190231	Dcit-2
		psy42-2-dcitrico1	FJ190321	Dcit-1
		psy42-3-dcitrico1	FJ190322	Dcit-1
		psy42-4-dcitrico1	FJ190323	Dcit-1
		psy42-5-dcitrico1	FJ190324	Dcit-1
		psy43-1-dcitrico1	FJ190325	Dcit-2
		psy43-2-dcitrico1	FJ190326	Dcit-2
		psy43-3-dcitrico1	FJ190327	Dcit-2
		psy43-5-dcitrico1	FJ190328	Dcit-2
		psy44-1-dcitrico1	FJ190329	Dcit-2
		psy44-2-dcitrico1	FJ190330	Dcit-2
		psy44-3-dcitrico1	FJ190331	Dcit-2
		psy44-4-dcitrico1	FJ190332	Dcit-2
		psy44-5-dcitrico1	FJ190333	Dcit-2
Puerto Rico	Univ. of PR (3)	psy24-3-dcitrico1	FJ190260	Dcit-3
		psy24-4-dcitrico1	FJ190261	Dcit-3
		psy24-5-dcitrico1	FJ190262	Dcit-3
Indonesia	Java (10)	psy26-2-dcitrico1	FJ190263	Dcit-2
		psy26-4-dcitrico1	FJ190264	Dcit-2
		psy26-5-dcitrico1	FJ190265	Dcit-2
		psy31-1-dcitrico1	FJ190279	Dcit-2
		psy31-2-dcitrico1	FJ190280	Dcit-2
		psy31-3-dcitrico1	FJ190281	Dcit-2
		psy31-5-dcitrico1	FJ190282	Dcit-2
		psy45-1-dcitrico1	FJ190279	Dcit-1
		psy45-2-dcitrico1	FJ190280	Dcit-1
		psy45-3-dcitrico1	FJ190336	Dcit-1

Table 1. (*Cont.*)

Location	Locality	Genebank Code	Accession Number	Haplotype Assignment*
	Bali (6)	psy27-2-dcitrico1	FJ190266	Dcit-2
		psy28-1-dcitrico1	FJ190267	Dcit-2
		psy28-2-dcitrico1	FJ190268	Dcit-2
		psy28-3-dcitrico1	FJ190269	Dcit-2
		psy28-4-dcitrico1	FJ190270	Dcit-2
		psy28-5-dcitrico1	FJ190271	Dcit-2
Vietnam	Hanoi (10)	psy29-1-dcitrico1	FJ190272	Dcit-2
		psy29-2-dcitrico1	FJ190273	Dcit-2
		psy29-3-dcitrico1	FJ190274	Dcit-2
		psy29-4-dcitrico1	FJ190275	Dcit-2
		psy29-5-dcitrico1	FJ190276	Dcit-2
		psy57-1-dcitrico1	FJ190378	Dcit-2
		psy57-2-dcitrico1	FJ190379	Dcit-2
		psy57-3-dcitrico1	FJ190380	Dcit-2
		psy57-4-dcitrico1	FJ190381	Dcit-2
		psy57-5-dcitrico1	FJ190382	Dcit-2
Taiwan	Taipei (5)	psy32-1-dcitrico1	FJ190283	Dcit-2
		psy32-2-dcitrico1	FJ190284	Dcit-2
		psy32-3-dcitrico1	FJ190285	Dcit-2
		psy32-4-dcitrico1	FJ190286	Dcit-2
		psy32-5-dcitrico1	FJ190287	Dcit-2
Pakistan	Punjab (5)	psy33-1-dcitrico1	FJ190288	Dcit-6
		psy33-2-dcitrico1	FJ190289	Dcit-6
		psy33-3-dcitrico1	FJ190290	Dcit-6
		psy33-4-dcitrico1	FJ190291	Dcit-6
		psy33-5-dcitrico1	FJ190292	Dcit-6
Thailand	Hat Yai (4)	psy34-2-dcitrico1	FJ190293	Dcit-2
		psy34-3-dcitrico1	FJ190294	Dcit-2
		psy34-4-dcitrico1	FJ190295	Dcit-2
		psy34-5-dcitrico1	FJ190296	Dcit-2
China	Zhejaing (3)	psy35-2-dcitrico1	FJ190297	Dcit-5
		psy35-4-dcitrico1	FJ190298	Dcit-5
		psy35-5-dcitrico1	FJ190299	Dcit-5
	Fuzhou (9)	psy51-1-dcitrico1	FJ190357	Dcit-2
		psy51-2-dcitrico1	FJ190358	Dcit-2
		psy51-3-dcitrico1	FJ190359	Dcit-2
		psy51-4-dcitrico1	FJ190360	Dcit-2
		psy51-5-dcitrico1	FJ190361	Dcit-2
		psy52-1-dcitrico1	FJ190362	Dcit-2
		psy52-2-dcitrico1	FJ190363	Dcit-2
		psy52-3-dcitrico1	FJ190364	Dcit-2
		psy52-4-dcitrico1	FJ190365	Dcit-2
	Gangzhou (4)	psy54-1-dcitrico1	FJ190366	Dcit-2
		psy54-2-dcitrico1	FJ190367	Dcit-8
		psy54-4-dcitrico1	FJ190368	Dcit-2
		psy54-5-dcitrico1	FJ190369	Dcit-2
Mexico	Akil, Yucatan (6)	psy36-1-dcitrico1	FJ190300	Dcit-4
		psy36-2-dcitrico1	FJ190301	Dcit-4
		psy36-3-dcitrico1	FJ190302	Dcit-4
		psy37-1-dcitrico1	FJ190303	Dcit-4
		psy37-2-dcitrico1	FJ190304	Dcit-4
		psy37-3-dcitrico1	FJ190305	Dcit-4
	Nuevo Leon (4)	psy38-1-dcitrico1	FJ190306	Dcit-1
		psy38-3-dcitrico1	FJ190307	Dcit-1
		psy38-4-dcitrico1	FJ190308	Dcit-1
		psy38-5-dcitrico1	FJ190309	Dcit-1
Mauritius	(5)	psy40-1-dcitrico1	FJ190312	Dcit-2
		psy40-2-dcitrico1	FJ190313	Dcit-2
		psy40-3-dcitrico1	FJ190314	Dcit-2
		psy40-4-dcitrico1	FJ190315	Dcit-2
		psy40-5-dcitrico1	FJ190316	Dcit-2
Reunion	(4)	psy41-1-dcitrico1	FJ190317	Dcit-2
		psy41-2-dcitrico1	FJ190318	Dcit-1
		psy41-3-dcitrico1	FJ190319	Dcit-2
		psy41-4-dcitrico1	FJ190320	Dcit-2

Table 1. (*Cont.*)

Location	Locality	Genebank Code	Accession Number	Haplotype Assignment*
Saudi Arabia	(5)	psy46-1-dcitrico1	FJ190337	Dcit-1
		psy46-2-dcitrico1	FJ190338	Dcit-1
		psy46-3-dcitrico1	FJ190339	Dcit-1
		psy46-4-dcitrico1	FJ190340	Dcit-1
		psy46-5-dcitrico1	FJ190341	Dcit-1
India	(4)	psy47-1-dcitrico1	FJ190342	Dcit-1
		psy47-2-dcitrico1	FJ190343	Dcit-1
		psy47-4-dcitrico1	FJ190344	Dcit-1
		psy47-5-dcitrico1	FJ190345	Dcit-1
Guadeloupe	(11)	psy48-1-dcitrico1	FJ190346	Dcit-3
		psy48-2-dcitrico1	FJ190347	Dcit-3
		psy48-3-dcitrico1	FJ190348	Dcit-3
		psy48-4-dcitrico1	FJ190349	Dcit-3
		psy49-1-dcitrico1	FJ190350	Dcit-3
		psy49-3-dcitrico1	FJ190351	Dcit-3
		psy49-4-dcitrico1	FJ190352	Dcit-3
		psy49-5-dcitrico1	FJ190353	Dcit-3
		psy50-1-dcitrico1	FJ190354	Dcit-3
		psy50-2-dcitrico1	FJ190355	Dcit-3
		psy50-5-dcitrico1	FJ190356	Dcit-3

* Haplotype assignments are as defined in table 2.

analysis was conducted on an Applied Biosystems 3730×1 DNA Analyzer. Sequence fragments were assembled with Sequencher ® version 4.2 (Gene Codes Corporation, 2004) and aligned using ClustalX (Thompson *et al.*, 1997). Minor alignment issues were corrected using Se-Al (Rambaut, 2000). Sequences generated at the United States Horticultural Research Laboratory (USHRL) were deposited in GenBank (table 1), and voucher specimens are deposited in the USHRL collection in Fort Pierce, Florida.

Haplotype network construction

A haplotype network was constructed using the statistical parsimony program TCS (Clement *et al.*, 2000). TCS estimates phylogenetics networks from DNA sequences or nucleotide distances using the statistical parsimony algorithm which estimates the 95% parsimony limits and outgroup weights (Templeton *et al.*, 1992). Computation was performed using an alignment of each of the eight haplotypes produced in ClustalX. The program was used to construct a statistical parsimony cladogram from parsimonious connections calculated at $P_j \geq 0.95$ and a matrix of absolute pairwise differences was calculated. Haplotype frequencies were not used to calculate outgroup probabilities, as there was minimal sharing of haplotypes among locations and our sampling effort was not uniform (e.g. there was more sampling within Florida than elsewhere).

Results and discussion

Identification of D. citri *mtCOI* haplotypes

Alignment of the mtCOI sequences showed the presence of eight polymorphic sites within the 612 bps. Four of the substitutions corresponded to synonymous sites and four to non-synonymous (amino-acid altering) sites (table 2). Six of the eight polymorphisms were the result of transitions while two were from transversions. The eight haplotypes were

highly similar with shared identities >99% (table 2). Two of these haplotypes (Dcit-1 and Dcit-2) were the most frequent and represented 87% of the sequences (table 3). Of the remaining six haplotypes, two (Dcit-7 and Dcit-8) were rare variants found among populations predominated by Dcit-1 or Dcit-2. The other four haplotypes (Dcit-3, Dcit-4, Dcit-5 and Dcit-6), without the benefit of larger sample sizes, appear to be fixed in their respective populations.

The rare haplotypes Dcit-7 and Dcit-8 were each recovered from a single individual in Florida and China, respectively. Moreover, each of these rare variants differed from all other sequences by a single non-synonymous (amino acid altering) substitution at an otherwise invariant site. Inspection of sequence chromatograms shows clean sequence reads for both variants, indicating that these were not related to sequence read errors.

Global relationships of D. citri

Due to the minimal sequence diversity in this intraspecific haplotype comparison, and the resulting poor support for the phylogenetic inference using standard tree construction methods (data not shown), it was determined that a haplotype network analysis would be the most appropriate way to consider the relationship between haplotypes (Hart & Sunday, 2007; Chen *et al.*, 2010; De Barro & Ahmed, 2011). The analysis using TCS is graphically represented in fig. 1.

By considering the two most frequently encountered haplotypes and their singleton derivatives as separate 'groups', Dcit-1 groups with Dcit-4, Dcit-6 and Dcit-7 to form what we refer to as the SWA group. Dcit-1 was the most frequently encountered haplotype in this group. The other haplotype group was composed of Dcit-2 and Dcit-8 and represents the SEA group. Placement of Dcit-3 (all Caribbean collections) and Dcit-5 (a single Chinese collection) was ambiguous, possibly because of the small numbers collected from those areas and because of the greater number

Table 2. Unique haplotypes and polymorphism locations observed from the alignment of the 212 *Diaphorina citri* mtCOI sequences.

Haplotype	Base location relative to start of the FJ190167 accession							
	130	138	217	447	500	533	612	645
Dcit-1	G	A	G	G	G	A	A	A
Dcit-2	A	A	G	G	G	A	A	G
Dcit-3	G	G	G	G	G	A	T	G
Dcit-4	G	A	G	G	G	C	A	A
Dcit-5	A	A	G	G	G	C	A	A
Dcit-6	G	A	G	A	G	A	A	A
Dcit-7	G	A	G	G	A	A	A	A
Dcit-8	A	A	A	G	G	A	A	G
Sub. Type*	Ns	S	Ns	S	Ns	Ns	S	S

* Substitution type is indicated as (S) synonymous or (Ns) nonsynonymous.

Table 3. Geographic distribution and abundance of *Diaphorina citri* haplotypes.

Location	Number of individuals (collections)	Abundance by haplotype							
		Dcit-01	Dcit-02	Dcit-03	Dcit-04	Dcit-05	Dcit-06	Dcit-07	Dcit-08
Florida, USA	94 (22)	93						1	
Texas, USA	5 (1)	5							
Mexico	10 (3)	4			6				
Puerto Rico	3 (1)			3					
Guadeloupe Island	11 (3)			11					
Brazil	17 (4)	4	13						
Saudi Arabia	5 (1)	5							
Pakistan	5 (1)						5		
India	4 (1)	4							
China	16 (4)		12			3			1
Taiwan	5 (1)		5						
Vietnam	10 (2)		10						
Thailand	4 (1)		4						
Indonesia	16 (5)	3	17						
Mauritius Island	5 (1)		5						
Reunion Island	4 (1)	1	3						
Total	212 (52)	122	69	14	6	3	5	1	1

of nucleotide substitutions in relation to the most common sequences.

This network analysis shows a diverged haplotype, Dcit-3, distributed in the Caribbean yet absent from all other samples (fig. 1). This result was surprising since the Caribbean was thought to be the source of *D. citri* that entered Florida (Halbert & Núñez, 2004). Since Dcit-3 is not found in Florida, either the Florida *D. citri* have come from some location other than the Caribbean or there is greater diversity in the Caribbean than we observed in the four collections (Psy-24, -48, -49, -50) and 13 individuals obtained from Puerto Rico and Guadaloupe (table 1). The relatively distant relationship between the Florida and Caribbean haplotypes (three substitutions, see fig. 1) indicate that the invasion of Florida did not come from the established Caribbean (Guadeloupe and Puerto Rico) populations, but more sampling is needed to verify these findings. Further, because *D. citri* is hypothesized to have entered the Caribbean as recently as the 1990s (Hall, 2008) and the Caribbean samples have a mtCOI genotype that was not found anywhere else in the old world, we are left to draw the conclusion that there is more worldwide diversity than what was captured in our sampling.

Within the Old World, few haplotypes predominate. However, the distribution of these haplotypes suggests a

phylogeographic break. Dcit-2 occurs in >90% of all the Southeast Asia individuals and 75% of all Chinese samples. Despite its common occurrence in SE Asia and China, it appears to be absent from the Middle East and the Indian subcontinent. Conversely, Dcit-1 and Dcit-6, just one synonymous mutation away, are the only haplotypes observed in both the Middle East and Indian subcontinent. Considering all the haplotypes present in Old World collections, Dcit-1 and Dcit-6 displayed a southwestern Asia (SWA) bias and Dcit-2, -5 and -8 a southeastern Asia bias.

In the New World, where *D. citri* was introduced within the last 25 years (Halbert & Núñez, 2004), a geographical bias to haplotype distribution was also observed. With the exception of one individual carrying haplotype Dcit-7, all 23 USA collections (99 individuals) contained Dcit-1. The individual carrying Dcit-7, which differs by a single non-synonymous substitution, was collected from St Lucie County, Florida alongside Dcit-1. In the four Brazilian collections, both Dcit-1 and Dcit-2 haplotypes were observed, but not in the same collections. In Mexico, three collections were analyzed, one from northern Mexico near the Texas, USA border and the other two from the Yucatan Peninsula. The collection close to Texas contained the same haplotype found in Texas (Dcit-1), but the Yucatan Peninsula collections
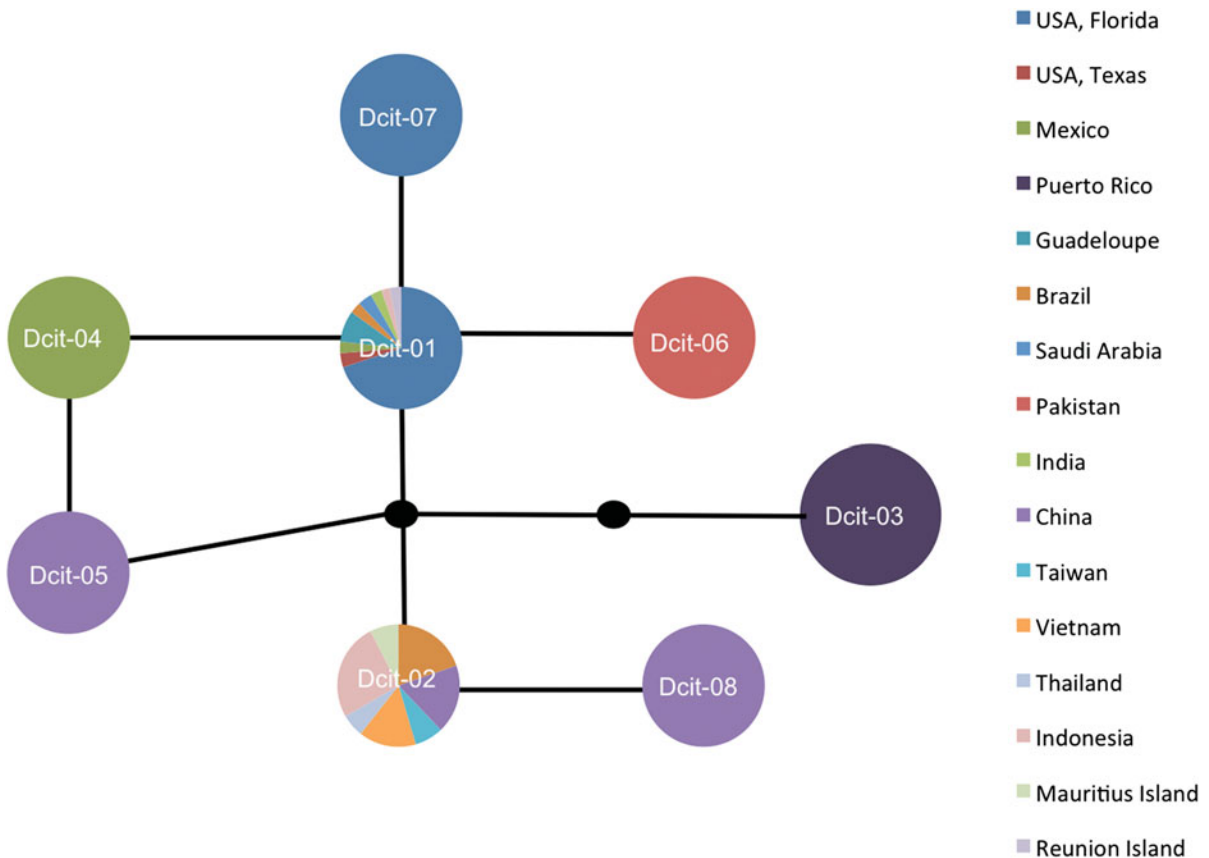
Fig. 1. Haplotype network generated with statistical parsimony analysis of *D. citri* mtCOI DNA sequence. Multicolored markers indicate the presence and identity of multiple haplotypes found within a region. The block dots represent hypothetical haplotypes. See table 3 to evaluate haplotype frequencies.

were fixed for a unique haplotype Dcit-4, which differed from Dcit-1 by a single nucleotide. Although *D. citri* is a pest that has spread worldwide, unlike other pests such as the whitefly *Bemisia tabaci* (Boykin *et al.*, 2007b; Dinsdale *et al.*, 2010), the low level of sequence diversity indicates that it is most likely a single species rather than a species complex.

A major aim of this study was to show how *D. citri* in Florida are related to *D. citri* elsewhere. We found that the Florida *D. citri* were most closely related to those from Mexico, Pakistan, Brazil, Texas, Saudi Arabia, India, Indonesia and Réunion Island, and includes the SWA sequences. New World populations show three distinct separations based on phylo-geographic interpretation of the haplotype network (fig. 1). North American continental populations (USA: Florida and Texas; and Mexico) belong to the SWA group, whereas individuals from locations in South America were either SWA or SEA in origin; SWA and SEA did not co-occur in any of the collections, suggesting two separate introductions. The only case where a collection from a single location contained individuals from both SWA and SEA was in the Indian Ocean island of Réunion. It is interesting to consider here whether there is some factor that limits the capacity of these two groups to co-occur. The only other New World locations sampled were the two Caribbean islands of Puerto Rico and Guadeloupe, where both contained the same haplotype Dcit-3.

Geographic origins of D. citri

Network-based genealogies can offer insight into ancestral sequences, as these sequences are expected to be common, widely distributed and highly connected (Chen *et al.*, 2010). While these predictions hold in theory, application is often difficult. Insufficient or non-uniform sampling schemes can bias the frequencies of observed haplotypes. Anthropogenic activity can alter geographical distributions at a scale and rate well beyond the ability of genetic systems to equilibrate. In our study, >60% of our samples are from the New World where *D. citri* is invasive. Using frequency of occurrence or geographic distribution as a metric for predicting ancestry would bias predictions by frequency of invasion and invasion success.

While both haplotype frequency and geographical distribution are likely not reflective of equilibrium evolutionary processes, patterns of haplotype connectivity may be more robust. Using this criterion, the mitochondrial haplotype network for *D. citri* suggests a basal and thus ancestral position for Dcit-1 (fig. 1). Even a conservative analysis, excluding all New World haplotypes that were singleton branches from the main Dcit-1 and Dcit-2 groups leaving only old world haplotypes, predicts Dcit-1 as ancestral based on connectivity.

Although early work suggested a far eastern Asia (Asia region to the east of India) origin of *D. citri* (Mead, 1977), more

recent reconstructions based on plant host origins and historical information suggest a southwestern Asia origin, i.e. India (Hall, 2008). Therefore, ancestral placement of the SWA group supports the more contemporary view of *D. citri* evolution. However, because of limited sampling in the Old World, we do not have a clear phylogeographic picture of the complete distribution of the SWA and SEA groups. The phylogeographic interpretations of our data identify two geographical redistributions of *D. citri*. First, some apparent geographical separation between the SWA and SEA groups that requires more intense sampling in the Old World to provide a true characterization of this relationship, and then a more recent radiation of the SWA and SEA groups into the New World most likely within the last 25 years (Halbert & Núñez, 2004). There is one earlier report of *D. citri* from Brazil in the early 1940s (Costa Lima, 1942); however, no one can substantiate this record, and it is certain that established populations were not reported until much more recently.

Conclusion

Our data indicate that with respect to the mCOI haplotypes and within the limits of our global sampling, there is very little genetic diversity among the populations worldwide. Despite this limited diversity, enough information was gained to partially answer the three questions on which this research was based.

(i) Can mtCOI haplotype analysis provide an insight into phylogeographic relationships of Old World *D. citri* populations? Limited sampling became a factor in this interpretation; however, two mtCOI haplotypes predominate, Dcit-1 and Dcit-2. These differed from each other by just two nucleotides and formed the basis of Dcit-1 group individuals (Dcit -1,-4, -6 and -7) and Dcit-2 group individuals (Dcit-2 and -8), as defined by those individuals whose mtCOI sequence diverges from the most common sequence (either Dcit-1 or Dcit-2) by only one nucleotide. Distribution of these two haplotype groups displayed some apparent Old World geographical bias. The Dcit-1 group was more abundant in SWA samples, and the Dcit-2 group was more abundant in SEA samples. Clearly, more sampling was done in SEA (Indonesia, Vietnam, Taiwan, Thailand and China) with 12 different collections, than in SWA (Pakistan, Saudia Arabia and India) with only three collections. However, if collections from Mauritius and Reunion are excluded as anthropogenic introductions because they are not considered a center of *D. citri* diversity and are isolated islands where both Dcit-1 and-2 are present, 11 of the 12 SEA samples contained individuals belonging to the Dcit-2 group, while all SWA samples contained only Dcit-1 group individuals. Based on these results, there is an appearance of geographically distinct populations; however, due to the limited sequence divergence between the observed groups, more in-depth sampling is needed for this to be verified. The authors suggest that further sampling from these Middle East and Asian countries needs to be conducted to better survey diversity and also utilize the other genetic markers such as microsatellites (Boykin *et al.*, 2007a).

(ii) Is there population structure that can be used to monitor global movement of specific genetic types? Analysis of the New World collections showed that invasions into North America came from the SWA group only, whereas those involving South America came from both the SWA and SEA groups. There was extensive sampling in the USA (22 collections within Florida) and only minimal sampling in South America (four collections), so it is unlikely that SEA populations were missed in the USA. Despite this observed difference in haplotype distribution in the New World, our limited Old World sampling and the small amount of global mtCOI sequence variation precludes the ability to provide a concise determination of origin. However, knowledge from this research may be useful in searching for effective biological control agents and may help explain differences in effectiveness of *Tamarixia radiata*, a parasitoid introduced in the New World to control invasive populations of *D. citri* (Hoy & Nguyen, 2001; Hoy *et al.*, 2004; Michaund, 2004; Pluke *et al.*, 2008; Barr *et al.*, 2009). Although, one needs to be cautious here, as ecological factors may be as or more important than genetic variability within the parasitoid and should not be overlooked.

(iii) Is there evidence of cryptic species based on relative genetic relationships of collections? All individuals are closely related, and there is no evidence for complex cryptic species structure within these populations. This may be expected as genetic variation is expected to increase with time since divergence (globalization) and increased host variability (local adaptation). *D. citri* has a very limited host range (only within the Rutaceae) and has only recently (within the last 15 to 25 years) spread to the New World.

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