Phylogeographic Structure in *Anastrepha ludens* (Diptera: Tephritidae) Populations Inferred With mtDNA Sequencing

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J. Econ. Entomol. 108(3): 1324–1336 (2015); DOI: 10.1093/jee/tov082

ABSTRACT *Anastrepha ludens* (Loew) (Diptera: Tephritidae), the Mexican fruit fly, is a major pest of citrus and mango. It has a wide distribution in Mexico and Central America, with infestations occurring in Texas, California, and Florida with origins believed to have been centered in northeastern Mexico. This research evaluates the utility of a sequence-based approach for two mitochondrial (COI and ND6) gene regions. We use these markers to examine genetic diversity, estimate population structure, and identify diagnostic information for *A. ludens* populations. We analyzed 543 individuals from 67 geographic collections and found one predominant haplotype occurring in the majority of specimens. We observed 68 haplotypes in all and see differences among haplotypes belonging to northern and southern collections. Mexico haplotypes differ by few bases possibly as a result of a recent bottleneck event. In contrast to the hypothesis suggesting northeastern Mexico as the origin of this species, we see that specimens from two southern collections show high genetic variability delineating three mitochondrial groups. These data suggest that Central America is the origin for *A. ludens*. We show that COI and ND6 are useful for phylogeographic studies of *A. ludens.*

KEY WORDS *Anastrepha ludens,* mitochondrial DNA, Mexican fruit fly, sequencing, COI

The Mexican fruit fly (mexfly), *Anastrepha ludens* (Loew) (Diptera: Tephritidae), a major pest of citrus and mango (White and Elson-Harris 1992), is distributed from the southern tier states of the United States through Central America (Stone 1942, Enkerlin et al. 1989, Foote et al. 1993, Hernandez-Ortiz and Aluja 1993). This species is of great interest for quarantine purposes because it is frequently recovered in citrus production areas in the United States, including the Lower Rio Grande Valley of Texas, Arizona, and California. In fact, a portion of those occasional finds in Texas and California have led to the establishment of a quarantine status in some state counties as reported by APHIS-PPQ and can be accessed through the North American Plant Protection Organization's (NAPPO) Phytosanitary Alert System (www.pestalert.org). Mexican fruit fly has also been intercepted in Florida but with less frequency (Steck 1998).

The major commercial hosts of Mexican fruit fly, citrus (*Citrus* spp.: Rutaceae) and mango (*Mangifera indica*: Anacardiaceae), were introduced to the New World about 400 yr ago, the latter fruit by Christopher Columbus (Mukherjee 1972). Baker et al. (1944) noted that species of the native genus *Casimiroa* (Rutaceae) are heavily infested by *A. ludens* in northeastern Mexico, and perhaps even more significantly, attacked by a wide array of parasitoids there, suggesting that *Casimiroa* usage by *A. ludens* is an ancient, or at least pre-Columbian, host–plant relationship. On that basis, Baker et al. (1944) suggested that northeastern Mexico was the ancestral range of this species. Baker et al. (1944) also assembled anecdotal evidence that Mexican fruit fly infestation of citrus and mango was a relatively modern occurrence, perhaps in the 20th century. The first report of *A. ludens* as a pest was that of Riley (1887) who noted that while it was a pest of citrus in the Mexican state of Morelos (in south-central Mexico), it had not been found in oranges imported to the US from the gulf coast of Mexico.

The present range of *A. ludens* is from Texas south to Panama (Stone 1942). Also, while a continual presence of mexfly in southern Mexico (including Guatemala and Honduras) has been reported by several authors (Stone 1942, Baker et al. 1944, Hernandez-Ortiz 1992, White and Elson-Harris 1992, Hernandez-Ortiz and Aluja 1993), the presence of this pest in isthmian Central America (Costa Rica and Panama) has a complicated historical record and may be recent. Stone (1942) cited *A. ludens* specimens collected in Costa Rica in 1936 and in Panama in 1904. However, subsequent pest surveys in Costa Rica (Jiron and Zeledon 1979, Jiron and Hedstrom 1988) did not

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recover this species. Additional collections by Jiron and Hedstrom (1988) yielded no Mexican fruit fly among the 15,000 specimens recovered during the 1985–1986 seasons. A comprehensive review of museum specimens combined with trapping and rearing of infested host material (Jirón et al. 1988) identified seven Mexican fruit fly specimens; three of these were specimens referenced by Stone (1942) that were gathered in 1936 and an additional four recovered in 1980. All were reared from white sapote, *Casimiroa edulis* Llave & Lex, a plant cultivated but not native to Costa Rica. Citrus and mango have been cultivated in the region since the 18th century (Reuther et al. 1967, Mukherjee 1972), Central American populations of *A. ludens*, at least until recently, were not reported to cause economic damage to any of these hosts. All evidence suggests that Mexican fruit fly has been, at best, uncommon in the southern portion of its current range. For example, in a survey of insect pests of coffee plantations in Costa Rica, Fischel (1982, unpublished thesis) stated that the only fruit fly found in oranges was the medfly (*Ceratitis capitata*). As late as 1988, Jiron and Hedstrom (1988) reported that *A. ludens* was not present in a 3-yr trapping survey of Costa Rica that included 11,000 adults. In a separate paper in which they surveyed infested fruits for larvae, Jirón et al. (1988) reared specimens of *A. ludens* only from *C. edulis*. The latter plant is cultivated in, but not native to, Costa Rica.

Due to the importance of this species as a pest of commercial fruit, its frequent human-mediated dispersal and evidence of a recent range expansion into Costa Rica and Panama (Jirón et al. 1988, Foote et al. 1993), an understanding of Mexican fruit fly population structure could contribute to improved management strategies. We evaluate the genetic diversity and population structure of the Mexican fruit fly throughout its geographic range based on DNA sequences of two segments of the fly's mitochondrial genome. Mitochondrial DNA has been a marker of choice in many phylogeographic studies (Avise 2004). Previous studies utilizing molecular methods have addressed interspecific (Smith-Caldas et al. 2001, Barr et al. 2005, Barr and McPheron 2006) and intraspecific (Steck 1991, Boykin et al. 2006, 2010, Alberti et al. 2008, Bomfim et al. 2011, Ruiz-Arce et al. 2012) relationships among and within members of the genus *Anastrepha*. This is the first phylogeographic study that examines the genetic diversity of Mexican fruit fly across its full geographic range in order to evaluate the data for evidence of reproductive isolation among regions. Our study also tests the hypothesis of an origin of the species in northeastern Mexico (Baker et al. 1944) and subsequent spread throughout the region. Based on population genetic structure, the usefulness of the mitochondrial markers for tracking introduction pathways is considered.

Materials and Methods

Sample Collection. *Anastrepha ludens* specimens used in this study were collected in traps (adults) or from host fruit material (larvae) between 1998 and 2006. The 67 sampling sites and host association of samples are described in Table 1, and the sites are depicted graphically in Fig. 1. Individuals were collected from citrus and mango in Costa Rica, where the only previously reported infested host was white sapote, *C. edulis* (Jiron and Hedstrom 1988). Also included in our analyses are samples from three sites in Panama, the southernmost edge of Mexican fruit fly distribution. Specimens were identified using morphological characters by trained taxonomists (D. B. Thomas), either fresh frozen or placed in alcohol, and shipped to the USDA Center for Plant Health Science and Technology, Mission Lab in Edinburg, TX, or to Penn State University, Department of Entomology, University Park, PA. Specimens were maintained at −80°C at both locations prior to analysis.

DNA Extraction, PCR Amplification, and Sequencing. DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) following standard DNeasy guidelines for animal tissues. Extractions were stored at −20°C for the duration of the study. Whenever possible, DNA from one specimen per collection was isolated via a nondestructive method (Barr et al. 2006) to serve as a voucher. Samples were amplified for a c. 1,100 bp fragment of the mitochondrial cytochrome oxidase subunit I (COI) using the primers tRNA-cys2 5′-ACTCCTTTAGAATTGCAGTCTAAT-3′ and COId-r 5′-GGGCTCATACAATAAATCCTAAT-3′ (Ruiz-Arce et al. 2012). A c. 800 bp fragment of the mitochondrial NADH dehydrogenase subunit 6 (ND6) gene was amplified using the primers TT-J-9886 5′-TAAAAACATTGGTCTTGTAA-3′ (Barr et al. 2006) and ND6r 5′-TTATGATCCAAAATTTCATCA-3′ (Ruiz-Arce et al. 2012). PCR reactions were performed according to the method described by Ruiz-Arce et al. (2012). PCR products were stained with Sybr Green (Invitrogen, Carlsbad, CA, USA) fluorescent dye at 2/10,000× and loaded on 1.2% electrophoresis agarose gels. Documentation of these gels was via a Gel Doc (Bio-Rad Laboratories, Hercules, CA, USA) imaging system using Quantity One software. Amplification products were purified with ExoSAP-IT (Affymetrix USB, Santa Clara, CA, USA) prior to sequencing. PCR products were sequenced asymmetrically using 3′ BigDye-labeled dideoxynucleotide triphosphates (v 3.1 dye terminators, Applied Biosystems, Foster City, CA, USA) and run on an ABI 3730XL DNA Analyzer with the ABI Data Collection Program (v 2.0) at the Huck Institute's Nucleic Acid Facility at Penn State University.

Data Analysis. Sequences were edited with Sequencher 4.8 (Gene Codes Corp., Ann Arbor, MI, USA). All sequences were aligned with Mega4 (Tamura et al. 2007) using ClustalW and trimmed to a length of 1,090 and 736 bp for COI and ND6, respectively. Sequences were concatenated using DnaSP 4.10 (Rozas et al. 2003) and subsequent analyses were carried out on the 1,826 bp fragment.

Haplotypes were identified from these combined sequences with DnaSP and Mega4. The relative

Table 1. Geographical origins and information on collections of *Anastrepha ludens* included in this study.

Site#	Population				*n*	Latitude	Longitude	Elev (ft)	Year coll.	Host
	Country	State	Locality	Loc code						
1	Belize	Cayo Dist	Benque Viejo	BEL1	3	18.266667 N	−88.45 W	20	1999	Grapefruit
2	Belize	Corozal Dist	Corozal	BEL2	10	18.22255 N	−88.32295 W	59	1999	Grapefruit
3	Belize	Stann Creek Dist	Dangriga	BEL3	8	17.183333 N	−88.58333 W	554	1999	Grapefruit
4	Belize	Cayo Dist	Georgeville	BEL4	7	17.183 N	−88.966 W	505	1999	Grapefruit
5	Belize	Stann Creek Dist	Pomona	BEL5	6	16.983 N	−88.366 W	135	1999	Grapefruit
6	Costa Rica	Alajuela Prov	Naranjo	CR1	5	10.09 N	−84.4 W	3796	2000	Mango
7	Costa Rica	Puntarenas Prov	Puntarenas	CR2	8	9.2 N	−84.006 W	0	2005	Sour orange
8	Costa Rica	San Jose Prov	Puriscal	CR3	10	9.8 N	−84.3 W	3304	2000	
9	Costa Rica	San Jose Prov	Acosta	CR4	7	9.805 N	−84.14 W	4429	2000	Orange
10	Costa Rica	San Jose Prov	Rosario	CR5	9	9.68 N	−84.06 W	3310	2000	Orange
11	Costa Rica	San Jose Prov	San Isidro	CR6	7	9.677 N	−84.074 W	5410	2000	Grapefruit
12	Guatemala	Escuintla	Palin	GT1	8	14.298 N	−90.638 W	2848	1998/2001	Grapefruit
13	Guatemala	Guatemala	Sacatepequez	GT2	10	14.576465 N	−90.72872 W	5138	1999	Sapote
14	Honduras	Atlantida	Jutiapa	HON1	4	15.770928 N	−86.51461 W	36	2001	Grapefruit
15	Honduras	Atlantida	Tela	HON2	9	15.779946 N	−87.45791 W	30	2001	Orange
16	Honduras	Cortes	Lago Yojoa	HON3	5	14.943928 N	−88.015 W	2169	2001	Orange
17	Honduras	Yoro	Guaymitas	HON4	6	15.502526 N	−87.71162 W	154	2001	Orange
18	Honduras	F. Morazan	Zamorano	HON5	8	14.7309 N	−87.27327 W	2044	2004	Sour orange
19	Mexico	Chiapas	Cacahoatan	MX1	20	14.959421 N	−92.16796 W	1240	1999	Sour orange
20	Mexico	Chiapas	Huehuetan	MX2	7	15.03195 N	−92.38451 W	407	2003	Sour orange
21	Mexico	Chiapas	Tapachula	MX3	18	14.90675 N	−92.26068 W	594		Sour orange
22	Mexico	Chiapas	Tapanatepec	MX4	11	16.3722 N	−94.217 W	266	2000	Mango
23	Mexico	Chiapas	Union Juarez	MX5	18	15.065 N	−92.081 W	4469		Sour orange
24	Mexico	Colima	Colima	MX6	9	19.245226 N	−103.7335 W	1598	2006	Sour orange
25	Mexico	Colima	Tecoman	MX7	9	19.237 N	−103.704 W	1680	2006	Mango
26	Mexico	Guerrero	Acahuizotla	MX8	2	17.405868 N	−99.44621 W	3655	2005	Mango
27	Mexico	Hidalgo	Chalcocotipa	MX9	10	20.497 N	−98.924 W	6745	2004/2006	Orange
28	Mexico	Hidalgo	Chalcocotipa	MX10	7	20.497 N	−98.924 W	6745	2004/2006	Orange
29	Mexico	Jalisco	Cd Guzman	MX11	10	19.701 N	−103.466 W	5020	2006	Sour orange
30	Mexico	Jalisco	Cofrida	MX12	10	20.038333 N	−103.9668 W	3763	2006	Sour orange
31	Mexico	Jalisco	Mezquitlan	MX13	9	19.817 N	−104.34 W	3176	2006	Orange
32	Mexico	Jalisco	La Sauceda	MX14	8	20.42046 N	−105.5679 W	1693	2000	Sour orange
33	Mexico	Jalisco	La Sauceda	MX15	10	20.42046 N	−105.5679 W	1693	2006	Mango
34	Mexico	Jalisco	Tamazulita	MX16	7	19.684428 N	−103.2495 W	3757	1999	Orange
35	Mexico	Mexico	Zacazonapan	MX17	10	19.574 N	−99.063 W	7717	2006	Sour orange
36	Mexico	Michoacan	Apatzingan	MX18	4	19.0786 N	−102.351 W	1053	2000	Grapefruit
37	Mexico	Michoacan	El Salitre	MX19	4	20.654 N	−100.378 W	6667	2005	Grapefruit
38	Mexico	Michoacan	nr. Nueva Italia	MX20	9	19.73 N	−101.206 W	6260	2006	Mango
39	Mexico	Michoacan	Patzcuaro	MX21	10	19.517 N	−101.61 W	7057	2006	Grapefruit
40	Mexico	Michoacan	San J de Lima	MX22	9	18.566 N	−103.633 W	361	2005	Orange
41	Mexico	Morelos	Mazatepec	MX23	11	18.7255 N	−99.363 W	3222	1999	Sour orange
42	Mexico	Nayarit	San Blas	MX24	9	21.541 N	−105.284 W	26	2003	Mango
43	Mexico	Nayarit	Jala	MX25	10	21.102 N	−104.5157 W	4974	2005	Sour orange
44	Mexico	Nayarit	Jala	MX26	8	21.10856 N	−104.4452 W	23	2006	Sour orange
45	Mexico	Nuevo Leon	Allende	MX27	7	25.28466 N	−100.0404 W	1578	2003	Sour orange
46	Mexico	Nuevo Leon	Cola de Caballo	MX28	8	25.31364 N	−100.1336 W	3858	1999	Sapote
47	Mexico	Nuevo Leon	Linares	MX29	9	24.85512 N	−99.56573 W	1158	2000	Grapefruit
48	Mexico	Nuevo Leon	St Rosa Canyon	MX30	18	26.03975 N	−100.0409 W	1929	1999	Sapote
49	Mexico	Puebla	La Ceiba	MX31	2	19.1 N	−98.22 W	7093	1999	Grapefruit
50	Mexico	Queretaro	Ahuacatlan	MX32	7	20.384 N	−99.9576 W	6535	2005	Sour orange
51	Mexico	Queretaro	Jalpan	MX33	3	20.578 N	−100.3874 W	6010	2005	Sour orange
52	Mexico	Quintana Roo	Andres	MX34	2	19.290705 N	−88.22398 W	85	2001	Orange
53	Mexico	Quintana Roo	Bacalar	MX35	3	18.676757 N	−88.38782 W	0	2001	Grapefruit
54	Mexico	Quintana Roo	Senor	MX36	3	19.811 N	−88.113 W	72	2001	
55	Mexico	San Luis Potosi	Huichihuayan	MX37	9	21.4999 N	−98.95 W	1631	2000	Orange
56	Mexico	San Luis Potosi	Picholco	MX38	1	21.379 N	−98.867 W	374	2004	Orange
57	Mexico	Tabasco	Chontalpa	MX39	3	17.665 N	−93.48 W	171	2006	Orange
58	Mexico	Tabasco	Macuspana	MX40	6	17.698071 N	−92.62833 W	49	2001	Grapefruit
59	Mexico	Tamaulipas	Canon Caballero	MX41	13	24.613 N	−98.559 W	968	2000	Sapote
60	Mexico	Tamaulipas	Ocampo	MX42	11	22.87722 N	−99.25183 W	676	2000	Orange
61	Mexico	Tamaulipas	Santa Engracia	MX43	8	24.029 N	−99.276 W	807	1999	Grapefruit
62	Mexico	Veracruz	Alameda	MX44	10	19.803 N	−96.143 W	0	2005	
63	Mexico	Veracruz	Nautla	MX45	15	20.202049 N	−96.77031 W	7	2001	Orange
64	Mexico	Veracruz	Teocelo	MX46	10	19.387409 N	−96.95669 W	3766	2005	Sapote
65	Panama	Panama Prov	Altos de Pacora	PAN1	3	9.081758 N	−79.29082 W	52	2006	Trap
66	Panama	Chiriqui Prov	Boquete	PAN2	3	8.774567 N	−82.432 W	3543	2006	Trap
67	Panama	Chiriqui Prov	Chiriqui	PAN3	10	8.19187 N	−82.26255 W	0	2006	Grapefruit

n, number of individual specimens analyzed; feet above sea level. nr., near locality. Blank fields indicate missing information.

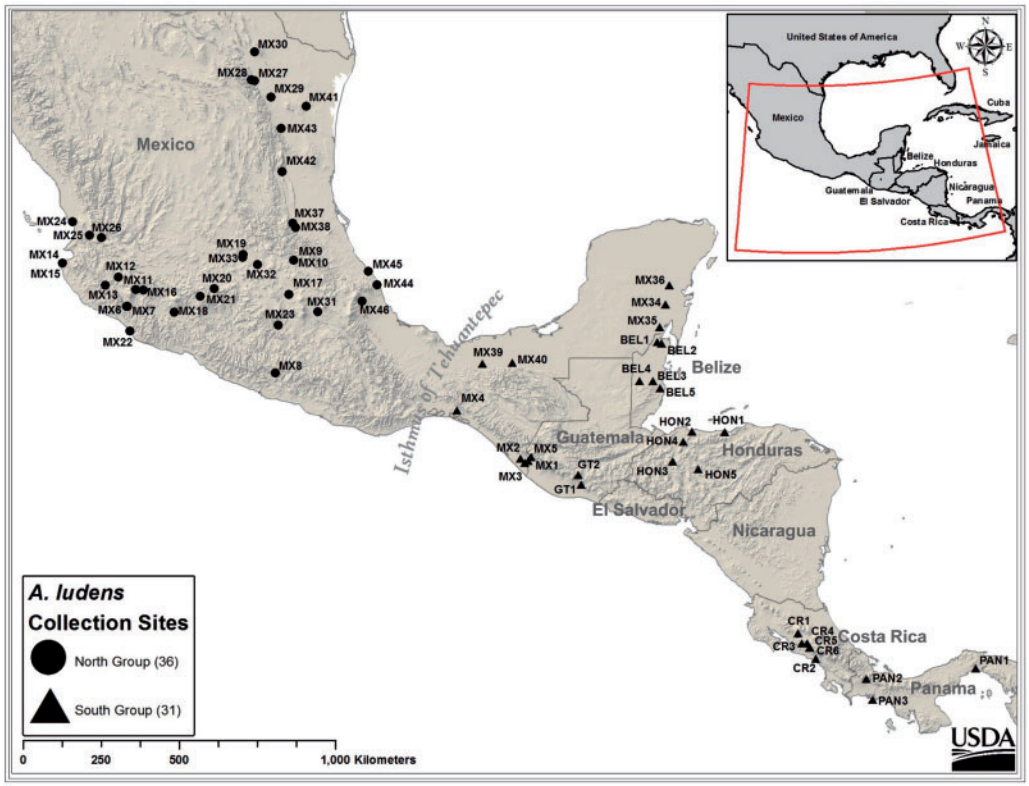

Fig. 1. Map showing sites for northern and southern collections of *A. ludens* collections used for the study. Sites show coding corresponding to locality in Table 1.

haplotype frequencies within populations were calculated using ARLEQUIN 3.5.1.3 (Excoffier and Lischer 2010). Diversity estimates, the demographic parameters Tajima's D (Tajima 1989), Fu and Li's D and F (Fu and Li 1993), and Fu's Fs (Fu 1997), were implemented to test for deviations from neutrality as a means for identifying population bottlenecks and population expansion. Mismatch distributions were estimated with DnaSP as an indicator of recent population growth, where a unimodal distribution could suggest an expanding population and multiple modes an established population.

The Sierra Madre Oriental and Occidental as well as the Isthmus of Tehuantepec (Fig. 1) are considered to be major biogeographic barriers. Both mountain ranges as well as distinct biotas on the opposite sides of the Isthmus (Halffter 1987, Peterson et al. 1999) may present formidable challenges to gene flow. These barriers permit the delineation of collections into 1) four geographic groups when all three barriers are considered and 2) two groups when collections are separated into north and south by the Isthmus. AMOVA was used to examine for population structure patterns that may be due to geography for these two hypotheses. Measures to estimate the source of variation among groups, among populations within groups, and within populations using analysis of molecular variance (AMOVA) using a permutation method with 10,000 replications was applied using ARLEQUIN. Once the most appropriate delineation was identified, demographic parameters were then estimated. The 95% confidence intervals used to estimate Fu's Fs, Tajima's D, Fu and Li's F, and Fu and Li's D were assessed by 10,000 coalescent simulations using DnaSP to test the hypothesis that all mutations are selectively neutral. Our collection records revealed that individuals from near Nueva Italia, Michoacan (MX20), were collected from a packing house and represent multiple geographic sites and not from one individual site. The sites are within close proximity to the MX20. However as a caution, these individuals were excluded from demographic analyses. ARLEQUIN was used to calculate: pairwise population sample differentiation (*F*st) using Weir and Cockerham's (1984) estimates of Wright's *F*-statistics (1951). The relationship between mtDNA divergence and geographic distance (isolation by distance) for populations was tested using Mantel tests (Mantel 1967) with 1,000 replications in isolation by distance (IBD) version 1.52 (Bohonak 2002).

A maximum parsimony haplotype network was constructed according to the algorithm described in Templeton et al. (1992) and implemented in the program TCS 1.18 (Clement et al. 2000). TCS was used to generate schematics representative of parsimonious relationships between and among collections according to geographic association.

Phylogenetic tree reconstruction was estimated using maximum likelihood (ML) and neighbor-joining methods (NJ). The appropriate model of sequence evolution was determined using Mega5 (Tamura et al. 2007). The T92+G+I substitution model with a gamma distribution (Γ) 0.77 and with 0.81 invariant sites. This model was selected based on Bayesian Information Criterion scores and bootstrap analyses were conducted with 1,000 replicates (Felsenstein 1985). Confidence in phylogenetic analysis was assessed according to Hillis and Bull (1993) for trees produced via both ML and NJ methods. We rooted these trees with sequences from one individual of *Anastrepha pseudoparallela.* This species was chosen based on its phylogenetic relationship to *A. ludens* (McPheron et al. 1999).

Results

Mitochondrial Variability in *A. ludens*. A total of 68 composite (COI + ND6) haplotypes were observed in the 543 individuals of *A. ludens* sequenced. There were 105 (5.7%) polymorphic sites, with no stop codons observed (Suppl Tables 1 and 3–8 [online only]). Our analyses supported the delineation of collections into North and South groups (explained below). The most abundant haplotype (AL03) was found in 351 (65%) individuals sequenced and was observed over a widespread geographic range (Table 2). We see that the second most common haplotype (AL04) occurred in 34 (6%) specimens sequenced, all of which were found only in southern collection sites. The mean number of haplotypes per collection site was 2.7, ranging between 1 and 8 per population. The presence of an extremely abundant haplotype and many low-frequency, closely related haplotypes is reflected in both the haplotype (0.58) and nucleotide (0.00184) diversity of the overall sample (Table 3).

When analyzed as northern and southern collections (Fig. 1) we see that the majority (74%) of flies in the study have haplotypes present in both regions (Table 2). The northern collections are represented by 306 flies and 35 haplotypes. Of these northern flies, 251 (82%) have haplotypes also present in the southern collection. Southern collections include 237 flies and 42 haplotypes. Of these southern flies, 148 (62%) have a haplotype present in the northern region. Only 9 (13%) of the 68 haplotypes sampled in the study are present in both regions yet these comprise the largest number of flies.

The majority (87%) of haplotypes in the study were private: 26 haplotypes to northern collections and 33 haplotypes to southern collections. Of these haplotypes, 39 (66%) were singletons (sampled from a single individual), 9 (15%) were sampled from only two flies each, and the others were sampled from three to 10 flies each. In addition to differences based on private haplotypes, the two regions had different levels of genetic diversity. The northern collection showed haplotype and nucleotide diversities of 0.47 and 0.00061, respectively (Table 3). In comparison, the southern collection had relatively higher values of 0.69 and 0.00327, for these respective estimates.

Among the six collections from Costa Rica, 46 of the specimens sequenced successfully. A total of five haplotypes (AL01-05) were recovered from these samples resulting in a higher haplotype/nucleotide diversity estimate (0.75/0.00426) as compared with all (including Costa Rica) southern collections (0.69/0.00327) and the entire dataset average (0.58/0.00184). These haplotypes included the AL03 haplotype, common across the entire range, as well as haplotypes AL01 and AL05 which were seen to be restricted to Costa Rica collections. Haplotypes AL02 and AL04 were also recovered from Costa Rica samples and have been seen in flies from southern collections in southern Mexico, Belize, Guatemala, Honduras, and Panama. These five haplotypes differed by an average of 12 bases or 0.65%.

For Panama, 16 flies showed four haplotypes. Haplotype AL03, common among many collections across the entire range, was recovered from 9/16 flies. Haplotype AL04, restricted to southern collections, was seen in 5/16 flies. And finally, haplotypes AL54 and AL68 which were considered private to Chiriqui (PAN3) and Boquete (PAN2), respectively, were seen in one fly from each collection. These four haplotypes differed by an average of 6.5 bases or 0.36%. The haplotype and nucleotide diversities in Panama were 0.62 and 0.00107, respectively, which reflect lower diversities than Costa Rica and may be explained because Panama is at the extreme distribution range for this fly and represents an area that has experienced a more recent invasion by *A. ludens.* The low diversity may also be due to the reduced sampling in Panama that was conducted for this study. It may be that additional collections in Panama will result in a different diversity estimate.

These data show that collections from Costa Rica and Panama, considered at the extreme edge of this fly's range, yielded seven haplotypes showing higher haplotype (0.75) and nucleotide (0.00426) diversities than the entire southern collection.

Population Structure, Neutrality Tests, and Demographic Inferences. We examined the source of variation by AMOVA analysis for collections. Overall, an AMOVA of the sequence variation with populations nested within groups revealed that grouping according to North and South collections provided the highest amount of variation as compared with collections grouped when considering the Sierra Madre Oriental and Occidental as barriers. Grouping collections according to North and South provided 69.71% of the variation was from within populations, 23.95% from among populations within groups, and 6.35% from between groups (Table 4).

Approximately 31% of the pairwise comparisons yielded significant *F*st estimates ranging from 0.0526 to 0.9123 (Suppl Table 2 [online only]). These represent collections showing significant differentiation consistent with population structure and included comparisons between many of the North and South collections. We report negative values in Suppl Table 2 (online only) as represented by 0 for estimates of *F*st. The relationship between mtDNA divergence and geographic distance (isolation by distance) for populations was tested using

Table 2. The geographic distribution of 68 haplotypes observed among the *A. ludens* collections examined. Data are sorted by collection (Coll.) where N refers to northern and S to southern collections as shown in haplotypes distribution map (Fig. 1). Collection site codes (e.g., MX37) correspond to Table 1. GenBank accession numbers to COI and ND6 sequences that composed the 68 concatenated haplotypes of *A. ludens* are shown. The haplotype cluster is also referenced (Fig. 4)

Haplotype	COI GenBank no.	ND6 GenBank no.	Coll.	Cluster	Origin of population (n = no. of individuals)
AL06	HM538293	HM538338	N	I	MX37(1)
AL07	HM538294	HM538338	N	I	MX15(4), MX26(2), MX37(1)
AL12	HM538299	HM538338	N	I	MX30(1), MX43(1), MX44(1), MX45(1)
AL23	HM538307	HM538348	N	I	MX23(1)
AL29	HM538313	HM538338	N	I	MX7(6), MX45(1)
AL30	HM538292	HM538349	N	I	MX42(1)
AL31	HM538314	HM538338	N	I	MX30(1)
AL32	HM538292	HM538346	N	I	MX20(1), MX30(1)
AL39	HM538292	HM538351	N	I	MX30(1)
AL40	HM538320	HM538338	N	I	MX15(1), MX25(1), MX28(1)
AL45	HM538325	HM538341	N	II	MX10(2)
AL46	HM538292	HM538341	N	I	MX10(1)
AL50	HM538292	HM538355	N	I	MX27(1)
AL51	HM538328	HM538338	N	I	MX6(2), MX11(7), MX24(1)
AL52	HM538292	HM538356	N	I	MX24(1)
AL55	HM538331	HM538338	N	I	MX21(2)
AL58	HM538332	HM538338	N	II	MX26(1)
AL59	HM538292	HM538359	N	I	MX26(1)
AL60	HM538333	HM538338	N	I	MX26(1)
AL61	HM538292	HM538360	N	I	MX26(1)
AL62	HM538292	HM538361	N	III	MX20(1)
AL63	HM538334	HM538338	N	I	MX20(1)
AL64	HM538292	HM538362	N	I	MX46(1)
AL65	HM538292	HM538363	N	I	MX6(1)
AL66	HM538335	HM538338	N	I	MX44(1)
AL67	HM538336	HM538338	N	I	MX17(1)
AL11	HM538298	HM538341	N, S	II	BEL4(1), GT1(1), HON5(3), MX10(2), MX35(2)
AL13	HM538300	HM538338	N, S	I	MX2(1), MX3(2), MX4(1), MX12(2), MX14(1), MX17(1), MX20(1), MX23(1), MX24(1), MX43(1), MX45(2), MX46(1)
AL14	HM538301	HM538338	N, S	I	MX3(1), MX45(2)
AL18	HM538292	HM538343	N, S	III	BEL5(2), HON4(1), MX20(1)
AL28	HM538312	HM538338	N, S	I	MX1(1), MX45(2)
AL03	HM538292	HM538338	N, S	I	BEL1(2), BEL2(8), BEL3(7), BEL4(2), BEL5(4), CR2(1), CR3(6), CR4(6), CR5(3), GT1(1), GT2(3), HON1(2), HON2(6), HON3(3), HON4(2), MX1(11), MX2(4), MX3(10), MX4(6), MX5(18), MX6(6), MX7(3), MX8(2), MX9(10), MX10(2), MX11(3), MX12(8), MX13(9), MX14(7), MX15(5), MX16(7), MX17(8), MX18(4), MX19(4), MX20(4), MX21(7), MX22(9), MX23(9), MX24(6), MX25(9), MX26(2), MX27(6), MX28(7), MX29(9), MX30(14), MX31(2), MX32(7), MX33(3), MX34(2), MX36(3), MX37(7), MX38(1), MX39(3), MX40(6), MX41(11), MX42(10), MX44(8), MX45(7), MX46(7), PAN1(1), PAN3(8)
AL36	HM538318	HM538338	N, S	I	BEL2(1), MX21(1)
AL41	HM538321	HM538338	N, S	I	MX1(1), MX43(6), MX46(1)
AL08	HM538295	HM538338	N, S	I	MX41(2)
AL01	HM538290	HM538337	S	II	CR5(3), CR6(2)
AL02	HM538291	HM538337	S	II	BEL1(1), CR3(3), CR5(3)
AL04	HM538292	HM538339	S	I	CR1(1), CR2(7), CR3(1), CR4(1), CR6(5), GT1(2), GT2(6), HON3(1), MX1(1), MX3(4), PAN1(2), PAN2(2), PAN3(1)
AL05	HM538291	HM538340	S	II	CR1(4)
AL09	HM538296	HM538337	S	II	HON(2)
AL10	HM538297	HM538337	S	II	MX35(1)
AL15	HM538302	HM538342	S	II	GT2(1)
AL16	HM538298	HM538338	S	II	BEL4(1)
AL17	HM538303	HM538338	S	I	BEL4(2)
AL19	HM538304	HM538344	S	II	HON2(1)
AL20	HM538305	HM538345	S	II	HON2(1)
AL21	HM538300	HM538346	S	I	HON2(1), HON5(1)
AL22	HM538306	HM538347	S	III	HON3(1)
AL24	HM538308	HM538341	S	II	BEL2(1)
AL25	HM538309	HM538341	S	II	HON4(1)
AL26	HM538310	HM538338	S	I	HON4(1)
AL27	HM538311	HM538338	S	II	HON4(1)
AL33	HM538315	HM538338	S	I	MX1(1)
AL34	HM538316	HM538338	S	I	MX1(2)
AL35	HM538317	HM538338	S	I	MX4(2)

(*continued*)

Table 2. (Continued)

Haplotype	COI GenBank no.	ND6 GenBank no.	Coll.	Cluster	Origin of population (n = no. of individuals)
AL37	HM538319	HM538350	S	II	BEL3(1)
AL38	HM538298	HM538343	S	III	BEL4(1)
AL42	HM538322	HM538337	S	II	MX1(1)
AL43	HM538323	HM538338	S	I	MX1(2)
AL44	HM538324	HM538352	S	I	MX3(1)
AL47	HM538326	HM538353	S	II	GT1(1)
AL48	HM538304	HM538354	S	II	GT1(2), HON5(2)
AL49	HM538327	HM538341	S	II	GT1(1)
AL53	HM538329	HM538338	S	I	MX2(2)
AL54	HM538330	HM538357	S	II	PAN3(1)
AL56	HM538304	HM538343	S	III	HON5(1)
AL57	HM538300	HM538358	S	III	HON5(1)
AL68	HM538292	HM538364	S	I	PAN2(1)

Table 3. Diversity estimates for north and south collections of *A. ludens* examined in this study. The values in the columns correspond to sample size (n), segregating sites (S), nucleotide diversity (π), number of haplotypes (H), and haplotype diversity (Hd), average number of nucleotide differences (k). One northern population (MX20), $n = 9$, was not included in the estimation of Fu's Fs (Fs), Tajima's D (D_1), Fu and Li's F (F), and Fu and Li's D (D_2).

Region	n	S	π	H	Hd	K	Fs	D_1	F	D_2
North	306	61	0.00061	35	0.466	1.117	-39.609^{*}	-2.58198^{**}	$-4.46603^{\dagger}$	$-4.72188^{\dagger}$
South	237	89	0.00327	42	0.686	5.972	-11.493^{*}	$-1.83577^{\dagger\dagger}$	$-3.17914^{\dagger}$	$-3.38012^{\dagger}$
All	543	105	0.00184	68	0.576	3.363	-34.369^{*}	-2.28020^{**}	$-4.41829^{\dagger}$	$-5.18456^{\dagger}$

$^{*}P < 0.000$, $^{**}P < 0.01$, $^{\dagger}P < 0.02$, $^{\dagger\dagger}P < 0.05$.

Table 4. Partitioning of DNA variance at three hierarchical levels as revealed by AMOVA for *A. ludens* mitochondrial DNA sequences. Regions tested are discussed in the text

			Among populations			
	Within populations		Within groups		Among groups	
Group division	% var	F_{ST}	% var	F_{SC}	% var	F_{CT}
1. Regions ($n = 2$)	69.71	$0.30293^{\dagger}$	24.95	$0.25569^{\dagger}$	6.35	0.06347^{*}
2. Regions ($n = 4$)	71.23	$0.28769^{\dagger}$	25.04	$0.26013^{\dagger}$	3.72	0.03724^{**}

$^{\dagger}P = 0.0000$, $^{*}P = 0.0001$, $^{**}P = 0.0052$; One population (MX38) consisting of a single individual was not considered in the analysis.

Mantel tests. For the entire dataset, the Mantel test showed values that were positively correlated. These values were shallow but significant ($r = 0.1767$, $P = 0.0020$) indicating isolation by distance. However, when analyzed separately, this pattern disappears both among northern and southern collections which showed a non-significant correlation.

Populations within both northern and southern geographic collections reveal negative values that deviate significantly from expectations under neutrality using Tajima's D, Fu's Fs, and Fu and Li's D and F (Table 3). This deviation can be explained by either demographic or selection factors. The combined dataset shows a multimodal curve (Fig. 2A) with mismatch distribution analyses. A constant-sized population is projected to show multi-modality while an expanding population shows a smooth, unimodal distribution (Slatkin and Hudson 1991). The dataset was then partitioned to test northern and southern geographic collections separately. The mismatch distribution for northern populations (Fig. 2B) showed a smooth curve typifying a recently expanding population and indicated that the observed differences followed the distribution of expected differences under an expanding population model. Southern populations (Fig. 2C) showed multiple peaks observed for the pooled dataset and suggestive of a constant-sized population (Slatkin and Hudson 1991).

In order to further explore the association among populations and respective haplotypes, we constructed a haplotype network. The haplotype network (Fig. 3) showed a star-like arrangement in which most of the unique haplotypes were closely related to the common central haplotype (AL03). Five loops were observed in the haplotype network. There is evidence of some grouping in the network that shows association to geography. The clearest pattern is the grouping of 40 haplotypes with the predominant AL03 haplotype which we call Haplogroup I. These haplotypes are not more than two mutational steps from AL03. The other haplotypes connected to Haplogroup 1 by five or more mutational steps. Although diversity separating these other haplotypes can be high (e.g., 23 steps), we observe two branches connected to Haplogroup I. These are

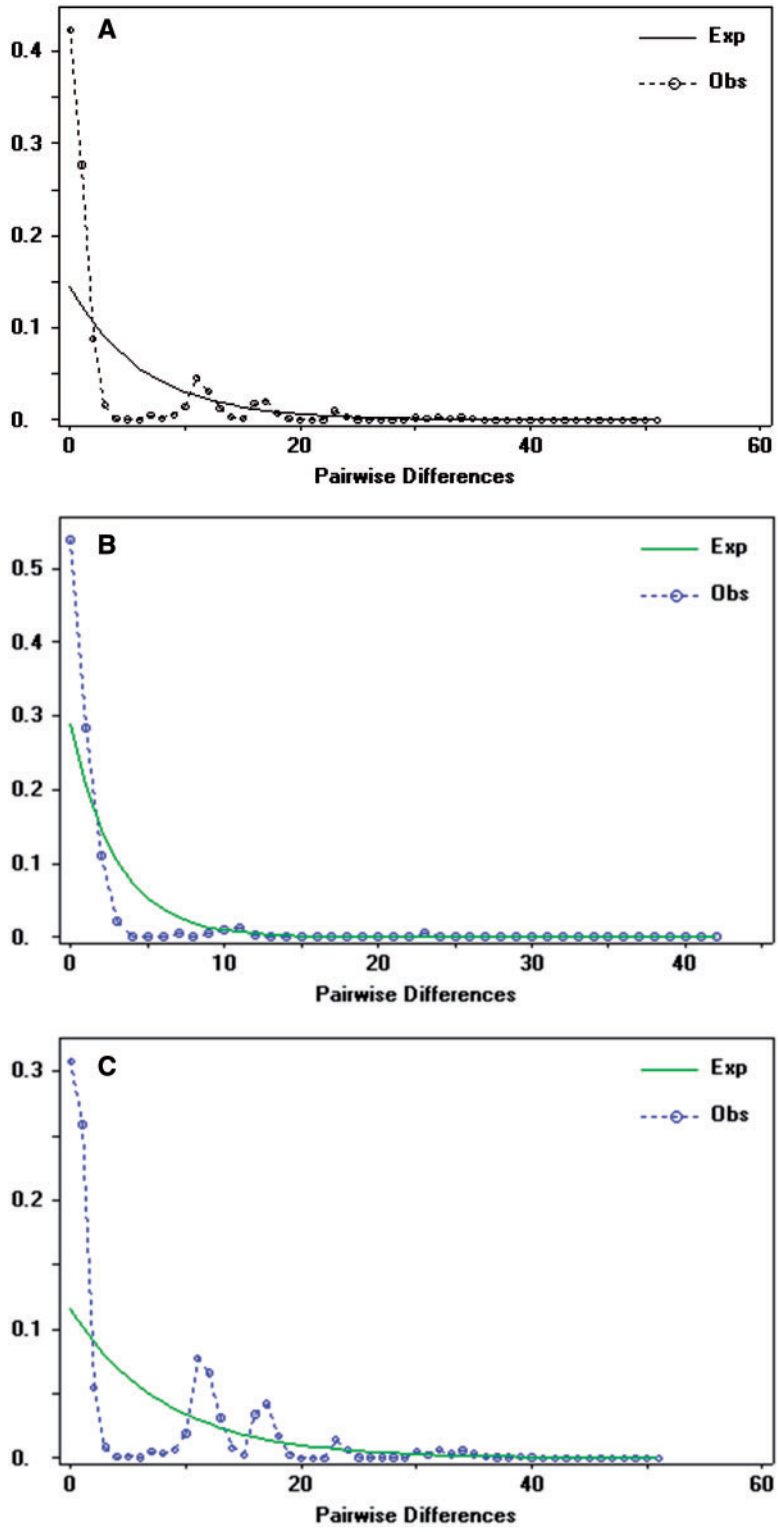

Fig. 2. Mismatch distributions for mtDNA haplotypes of A. *ludens*. The distributions for the combined dataset (A), as well as northern (B), and southern (C) collections are shown.

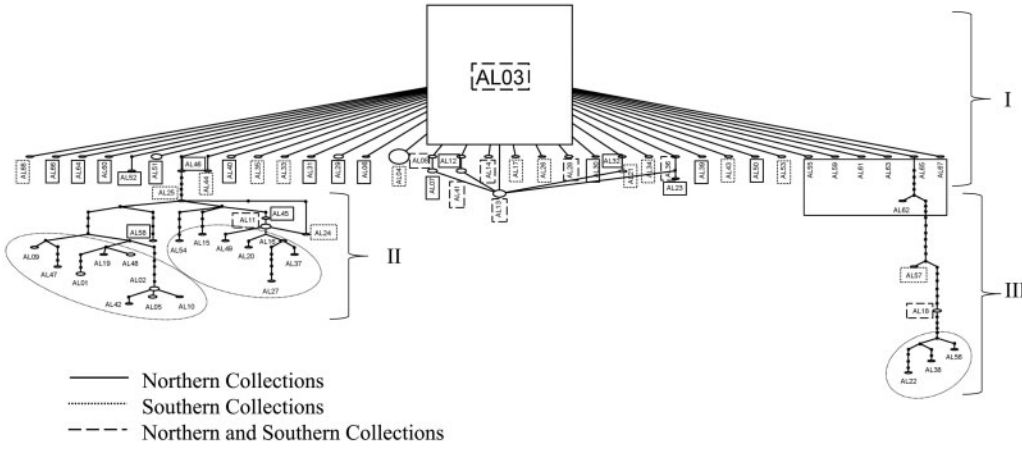

Fig. 3. Statistical parsimony network for *A. ludens*. Numbers correspond to 68 haplotypes with mutational steps noted as filled ovals while open ovals represent haplotypes. Haplogroup I, II, and III, discussed in the text, are also shown.

referred to as Haplogroup II (connecting to AL46) and III (connecting to AL65). Mean nucleotide diversity among haplotypes in Haplogroups I, II, and III are 0.2, 0.4, and 0.8%, respectively.

Based on our network, Haplogroup I included haplotypes present in northern, southern, or both collections. These 40 haplotypes represent 487 (91%) of the 537 flies studied. In contrast the other haplogroups represented fewer flies and had evidence of geographic association. The Haplogroup II haplotypes were sampled from 47 flies of which 42 (89%) were from southern collections. Similarly, seven (78%) of the nine flies with Haplogroup III haplotypes were from southern collections. The two flies collected in the north with Haplogroup III haplotypes were both from a packing house (MX20, site 38 in Table 1) and may not represent that location. For the Haplogroup II and III flies, 87.5% were associated with southern collections.

Phylogenetic analyses of the haplotype dataset resulted in patterns similar to the network results (Figs. 3 and 4, and Suppl Figs. 1–3 [online only]). Many of the 68 haplotypes had very short branches separating them from AL03 (i.e., Cluster I). The haplotypes in Cluster II form a clade in the ML and NJ trees each with bootstrap support >70% (Figs. 4 and Suppl Fig. 1 [online only]). The Cluster I and II haplotypes also formed a clade in the ML and NJ trees with bootstrap support of 84% in the ML tree. The Cluster III haplotypes were separated by longer branches in comparison to the other haplotypes. In the ML and NJ trees the AL65 haplotype was separated from the other Cluster III haplotypes by bootstrap values of 88–89%.

Discussion

Genetic Diversity of *A. ludens*. We have successfully sequenced fragments of the ND6 and COI genes from 543 mexfly samples. A total of 68 haplotypes were sampled from the collection and haplotype and nucleotide diversities for the dataset were estimated to be 0.58 and 0.00184, respectively. This is low compared with mitochondrial diversity estimates reported for other fruit fly pests such as *A. obliqua*, where haplotype (0.65) and nucleotide (0.00465) diversities were considerably higher using the same molecular loci (Ruiz-Arce et al. 2012). No study has estimated genetic diversity of *A. ludens* across its entire range before. Our data do not suggest a high level of diversity that is structured among collections and therefore, are not consistent with a cryptic species complex. This is important because the species is the target of sterile insect technique programs and cryptic species could negatively impact efficacy. Relatively low genetic diversity for the species makes the COI gene amendable to DNA barcode projects.

Population Structure Within *A. ludens*. The presence of one abundant haplotype (AL03, 65%) distributed throughout the range of the species with a large number of closely-related, low-frequency haplotypes scattered over the geographic distribution demonstrates a high degree of homogeneity among populations. Yet, the distribution of low-frequency haplotypes fell in a pattern which supported population structure between northern and southern collections using AMOVA. These data showed the existence of structure among groups and populations. We observed numerous unique haplotypes private to various northern and southern collections further suggesting differentiation and restricted gene flow among these two groups. The Mantel test showed a pattern of IBD among northern and southern localities collectively, however the analysis of the northern and southern collections analyzed individually failed to indicate such.

Ancestral Range and Expansion of *A. ludens*. We found that it was not possible to determine the ancestral haplotypes (terminal nodes) using the topology of a phylogenetic tree (Krell and Cranston 2004) but our branch lengths do support the genetic diversity and network estimates in that southern collections are more

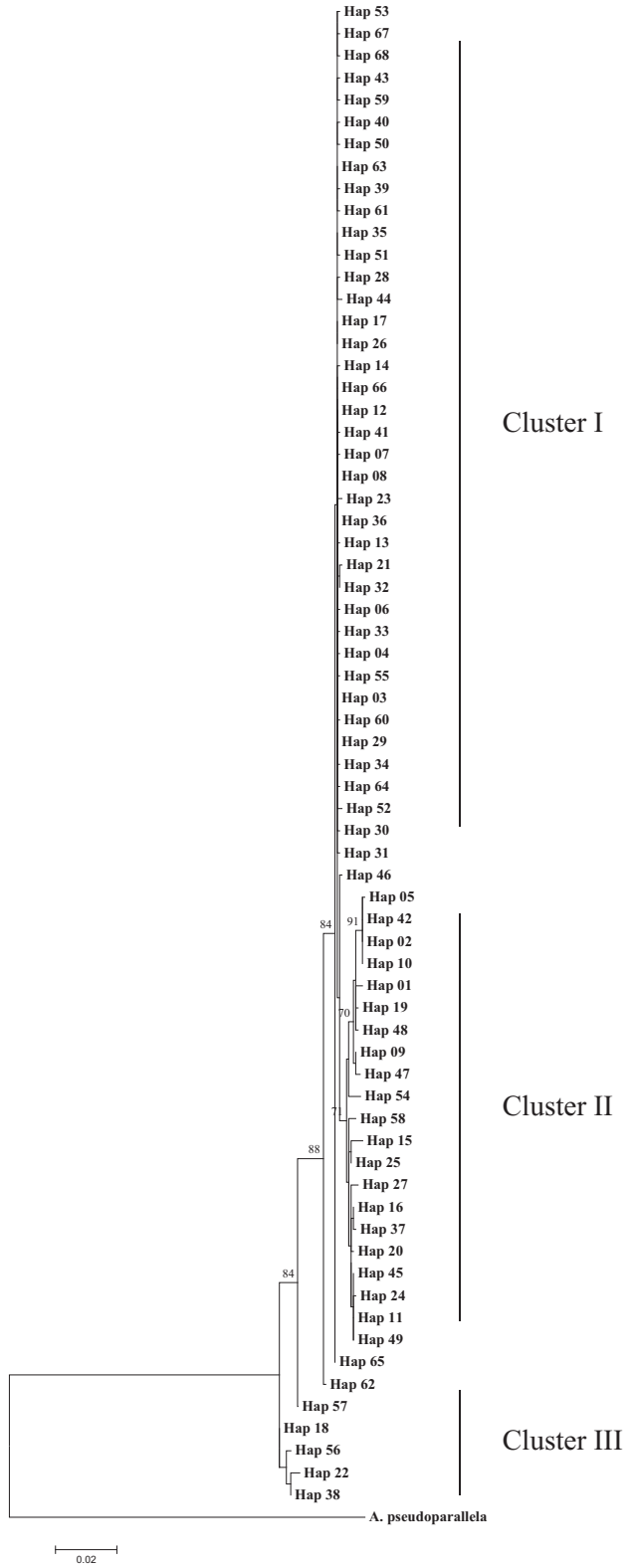

Fig. 4. ML tree showing the phylogeny of 68 *A. ludens* COI + ND6 haplotypes AL01–AL68. Numbers above branches show bootstrap support for ML (1,000 replicates). Missing bootstrap values designate unsupported branches with respective method or methods according to Hillis and Bull (1993). Tree was rooted with one *A. pseudoparallela*.

diverse. The reconstruction of intra-specific variation using trees is not always recommended (Smouse 1998, Huson and Bryant 2006) but the topology, branch lengths, and bootstrap support values of our searches support structure in the dataset.

These analyses suggest a potential origin of this species in Central America, in contrast to the assessment by Baker et al. (1944) suggesting that origin is the Sierra Madre Oriental in northeastern Mexico. The Michoacán (MX20) samples with haplotypes in the relatively diverse Cluster III (AL18, AL62) could represent introductions from southern regions under this scenario.

We found no evidence to support the hypothesis of northern Mexico as the ancestral origin of the species. Based on haplotype and nucleotide diversity estimates, the southern collections were more diverse than the northern collections. The high haplotype diversity observed among southern collections suggests that this is the source of origin for this fruit fly species. Anecdotal evidence using RFLP analysis also suggested that collections from southern Mexico and Central America showed a higher diversity index (J Gomes da Silva-Miller, unpublished). Although political barriers and trade restrictions among these regions could have contributed to the observed genetic patterns, available information supports a southern origin.

There is also evidence of historical population expansion for the northern population. Low nucleotide and haplotype diversity, in a star-shaped haplotype network with an abundance of low-frequency haplotypes, support the notion that populations occurring in the northern range of the species experienced a recent bottleneck event, perhaps due to expansion that followed a population crash or due to a recent introduction (founder effect). Mismatch distribution views the northern collection of haplotypes as undergoing a range expansion and southern collections as stable populations. Tests of selection (e.g., Tajima's D) generated negative values that are consistent with population expansion, the alternative hypothesis of a recent bottleneck would generate significantly positive values. Differences among these two groups could be attributed to cultural practices and trade restrictions across these regions that might also limit gene flow. The ecological reasons underlying this distinction remain unclear.

We hypothesize that the fly evolved in southern Mexico or Central America and then moved into northern Mexico. This introduction involved one or more founder events followed by a range expansion on available commercial hosts. This resulted in an increase in the common haplotype. As a result of increased cultivation, transportation and trade of fruits these populations moved back into the southern region and became the dominant haplotype. This hypothesis is consistent with our observations on regional genetic diversity, network structure, mismatch distributions, and tests of selection.

The high genetic diversity we observed for collections of Costa Rica and Panama is interesting. Since most of these haplotypes are restricted to southern collections, it is very likely that they may have been present for some time in these areas, perhaps at low densities. It is also likely that this area experience a recent introduction from neighboring countries. Additional sampling is needed to address these hypotheses.

Diagnostic Utility for Pathway Studies. The haplotypes assorted into three phylogenetic clusters (Fig. 4). Cluster I: containing a widespread but homogenous genotype (AL03) which is dominant throughout Mexico; II: diverse genotypes found scattered from Panama to northern Mexico; and III: diverse genotypes centered in nuclear Central America with relictual populations in Mexico. The areas where *A. ludens* is reported to be a pest are dominated by haplotypes recovered from populations assignable to Cluster I.

The presence of one common haplotype among northern and southern collections presents some challenges to the use of our data for diagnosing geographic source of captured flies. Preferably, a pathway tool should provide sufficient resolution so that the analysis of a captured individual provides evidence that can exclude a good portion of the geographic range for that species. Unfortunately, it would be very difficult to arrive at a limited number of geographic sites for a capture that showed the very common AL03 haplotype. The list of likely sources for AL03 includes 61 geographic sites in Mexico and Central America, none of which can be excluded using this method alone.

In fact, the presence of many rare Cluster I haplotypes in northern and southern collections suggest that this cluster may not be a reliable diagnostic for predicting a geographic source. For example, a fly with the AL06 haplotype could not be reliably identified as a 'northern' fly because this rare haplotype may also be in the south where we did not happen to sample for our study, simply by chance. Those rare haplotypes may increase in frequency if additional sampling were to be conducted. Additional sampling might also reveal novel haplotypes. While all this may provide better insight into the genetic diversity for this pest, it is unlikely that this information would improve the diagnostic capacity for these methods.

In contrast, the haplotypes in Clusters II and III do have a geographic association with collection sites when grouped as northern and southern. Even when the packing house samples (MX20) are included, 87.5% of the flies in these clusters are from southern collections. The utility of this approach to diagnosis does have several important practical limitations. Flies with these haplotypes are not commonly sampled based on our study. If our sampling estimates are indicative of fly capture genetic diversity, then we would only expect to find diagnosable haplotypes 10% (56/537) of the time. All other captures would not provide any resolution: the fly could be from any source. Without targeting pathways known to originate in the southern range of our study, this success rate could be much lower if proximity, infestation rates, and production levels are not even among the regions.

Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

Acknowledgments

We are grateful to Roxanne E. Farris, Rosita Deleon, Juan D. Vasquez, Lisa A. Ledezma, and Oscar Obregon at the USDA-APHIS-CPHST Mission Laboratory in Edinburg, TX, for providing DNA extractions, and to Samuel Gum and Oralia Ruiz for their technical assistance at Penn State University, University Park, PA. In addition, we thank Dr David W. Bartels at the USDA-APHIS-CPHST Mission Laboratory for map construction. We also thank Dr Deborah S. Grove (Director of the Genomics Core Facility), Ashley Price, and Sheila Plock for their assistance at the Pennsylvania State University-Huck Institute Nucleic Acid Facility. The use or mention of a trademark or proprietary product does not constitute an endorsement, guarantee, or warranty of the product and does not imply its approval to the exclusion of other suitable products by the U.S. Department of Agriculture, an equal opportunity employer. This work was supported by the United States Department of Agriculture-Animal Plant Health Inspection Service-Center for Plant Health Science & Technology Project Code#M6M02, USDA/Penn State cooperative agreement 07-8100-1051-CA.

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Received 18 April 2014; accepted 6 March 2015.