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Population genetics and recent colonization history of the invasive drosophilid *Zaprionus indianus* in Mexico and Central America

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Abstract *Zaprionus indianus*, also known as the African fig fly, is an invasive pest of a variety of commercial and native fruit. The species was first reported in Brazil in 1999, but has established itself in much of the New World within the last 10–15 years. We used nucleotide sequences from a segment of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene to examine haplotype relationships, population structure, and infer the colonization history of *Z. indianus* in Mexico and Panama. Construction of a haplotype network showed that six COI haplotypes, obtained from flies collected at six localities in Mexico and one in Panama, clustered into three distinct clades. Clade composition was generally consistent in flies from Panama to northwestern Mexico, and analysis of molecular variance indicated no significant structure among populations. Three of the six haplotypes from Mexico and Panama were identical to previously reported haplotypes from Brazil. None of the six haplotypes, however, were shared with previously reported haplotypes from potential source populations in the Old World. The results of our genetic analysis suggest that the invasion of *Z. indianus* into Central America and Mexico most probably includes a northward migration of individuals from Brazil, with the possibility of at least one additional introduction of *Z. indianus* to the New World. Additional sequence data from potential source populations in the Old World will be required to confidently determine the number of introductions of *Z. indianus* into the New World, and to identify the geographic source.

Keywords Cytochrome *c* oxidase subunit I (COI) · Dispersal · Haplotype network · Population structure

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Introduction

Within the last 15 years, *Zaprionus indianus* Gupta (Diptera: Drosophilidae) has undergone a rapid and widespread invasion of the Western Hemisphere from

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its native range in sub-Saharan Africa (van der Linde et al. 2006; Commar et al. 2012). First recorded in Brazil in 1999, it is hypothesized that *Z. indianus* was introduced in a contaminated commercial fruit shipment from Africa (Vilela 1999). After its arrival, *Z. indianus* rapidly spread throughout Brazil and other South American countries (van der Linde et al. 2006; Soto et al. 2006), and became an important pest of figs, oranges and peaches (Santos et al. 2003). Allozyme evidence suggested that the rapid dispersal of *Z. indianus* in Brazil was aided by commercial transportation of fruit along major highways (Galego and Carareto 2010). Although the precise geographic source of the propagule that invaded Brazil is unknown, various lines of evidence, including allozymes (Mattos-Machado et al. 2005; Galego and Carareto 2007), chromosome inversions (Ananina et al. 2007) and morphological traits (David et al. 2006), suggest that it was a single large propagule from western Africa that contained much of the genetic diversity of the source population.

Commonly referred to as the fig fly, or African fig fly, *Z. indianus* poses a serious potential threat as an agricultural pest throughout the Americas owing to its ability to rapidly colonize new habitats (da Mata et al. 2010) and utilize more than seventy species of both commercial and native fruit as hosts for feeding and development (Santos et al. 2003; van der Linde et al. 2006; Lavagnino et al. 2008; Commar et al. 2012). Shortly after the initial Brazilian record, reports of *Z. indianus* from North America began to appear, first in the state of Chiapas in southern Mexico in 2002 (Castrezena 2007) and then in Panama in 2003 (van der Linde et al. 2006). By 2005 *Z. indianus* had been reported in Florida, USA (van der Linde et al. 2006). In 2006 it was recorded from San Carlos, Sonora in northwestern Mexico, and in southwestern USA (California and Arizona) (Castrezena 2007). It was found in the Cape Region of Baja California Sur in 2010 (Castrezana 2011). Recently (September 2012) *Z. indianus* was found in Virginia, USA where crop damage was reported in wine grapes (D. Pfeiffer, personal communication). [Updated distribution maps of *Z. indianus* can be found at http://www.kimvdlinde.com/professional/Zaprionus%20indianus.html (accessed 27 January 2014) (van der Linde 2010)].

The flies now invading North America have been assumed to represent progeny from the initial propagule of *Z. indianus* that colonized South America (Yassin et al. 2009; Commar et al. 2012). It is also possible, however, that multiple invasions of *Z. indianus* have occurred by commercial air and/or maritime transport from the Old World, both in North and South America. Furthermore, it is unclear if invasive populations in the New World regions are genetically differentiated. This could result from limited dispersal among populations that were genetically distinct due to separate introductions from different source populations, or from natural selection on populations arising from a single introduction that colonized varying habitat types. Previous studies on insects have shown that mitochondrial DNA (mtDNA) markers have proven useful in monitoring population genetics of both natural biological invasions (Yassin et al. 2009) and planned introductions of biological control insects (Franks et al. 2011). In the present study we used mtDNA sequences from a segment of the COI gene, commonly referred to as the barcode segment (Ratnasingham and Hebert 2007), to test these hypotheses. In an attempt to identify potential source populations of *Z. indianus*, we analyzed COI haplotype relationships of newly sampled invasive populations from Mexico and Panama with new COI sequences obtained from flies from Africa (Cameroon and Malawi), together with selected GenBank sequences from other Old World populations. We also tested for structure between populations from Mexico and Panama to determine whether samples from these widespread localities are genetically isolated.

Materials and methods

Samples

Samples of *Z. indianus* ($N = 99$) were collected during 2011–2013 from six widely-separated localities in Mexico and one in Gamboa, Panama. The Mexico localities included a peninsular site near La Paz, Baja California Sur (El Carrizal), Isla Isabel, a small isolated Pacific island located about 28 km off the coast of the state of Nayarit, and four mainland localities [Alamos, Sonora; Guanajuato, Guanajuato; the northern outskirts of Querétaro, Querétaro (Juriquilla); and Oaxaca, Oaxaca] (Fig. 1). The La Paz site is located in the southernmost part of the Sonoran Desert. The Alamos and Isla Isabel sites are located in

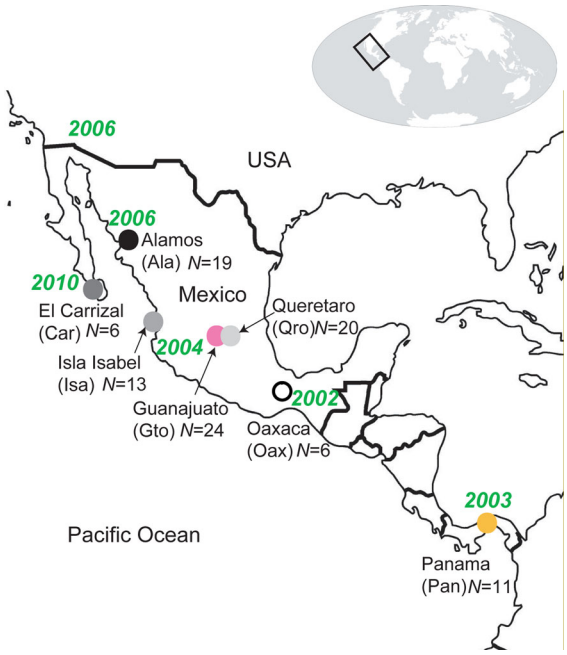

Fig. 1 Map showing collecting localities and sample sizes for *Zaprionus indianus* in Mexico and Panama, with locality abbreviations shown in *parentheses*. The colors of the spots identifying each locality correspond to the color-coded localities in Fig. 2. Years shown in *green* represent the approximate progression of the invasion of *Z. indianus* in the study area based on observations given in Castrezana (2007, 2011) and van der Linde et al. (2006)

a tropical deciduous forest biome. Isla Isabel is a national park and wildlife reserve, with remnants of previous human perturbation. The Guanajuato, Querétaro and Oaxaca sites were in urban or semi-urban areas. The site in Panama was tropical rain forest. Flies were collected from baits consisting of a variety of rotting fruits, including bananas, oranges, and mangoes. Some flies from Juriquilla were caught from fallen rotting citrus fruits. Flies from Africa were collected at Yokadouma, Cameroon during 2005 ($N = 9$) and at Lujeri, Malawi during 2009 ($N = 1$).

Molecular protocol and data analysis

Total genomic DNA was usually extracted from individual flies using the DNeasy^{TM} (QIAGEN Inc., Valencia, CA) protocol. For flies collected at Isla Isabel, and for several of those from Querétero, however, a crude homogenate was used in the polymerase chain reaction (PCR). Individual flies were homogenized in 50 µl 10 mM Tris–HCl (pH 8.2), 1.0 mM EDTA, 25 mM NaCl, and treated with 200 µg × ml^{-1} proteinase K for 30 min at 37 °C. The homogenate was then heated to 95 °C for 2 min, placed on ice, and 5 µl used for the PCR. A 658 bp segment of the mitochondrial COI gene was amplified by PCR using primers LCO1490f and HCO2198r (Folmer et al. 1994). This segment corresponds to nucleotide positions 1,515–2,172 in the complete mitochondrial genome of *Drosophila yakuba* (GenBank accession no. NC001322). Details of PCR and sequencing reactions are found in Pfeiler et al. (2010). Sequences were proofread and aligned in either ClustalX 1.81 (Thompson et al. 1997) or Sequencher 4.1 (GeneCodes Corp., Ann Arbor, MI) followed by manual editing. All alignments were straightforward. Translation of sequences in MEGA version 5.0.5 (Tamura et al. 2011) revealed no frameshifts or stop codons. Mean CG content was 33.3 %. Sequences of COI haplotypes of *Z. indianus* from Mexico, Panama, Cameroon and Malawi are deposited in GenBank (Accessions numbers KF736180–KF736195).

Calculations of uncorrected *p*-distance and Kimura (1980) 2-parameter (K2P) genetic distances (*d*) were carried out in MEGA. Calculations of genetic diversity indices were performed in DnaSP version 5.10.01 (Librado and Rozas 2009). Neutrality tests [Tajima's (1989) *D* and Fu's (1997) F_S] were carried out in ARLEQUIN version 3.5.1.3 (Excoffier and Lischer 2010). Analysis of molecular variance (AMOVA, Excoffier et al. 1992) performed in ARLEQUIN was used to test for structure among the seven populations of *Z. indianus* from Mexico and Panama. The calculation of significance ($\alpha = 0.05$) of pairwise comparisons of the fixation index Φ_{ST} was based on 10,000 permutations of the data matrix.

A haplotype network of COI sequences for *Z. indianus* was constructed using statistical parsimony implemented in TCS version 1.21 (Clement et al. 2000). The connection limit among haplotypes was set to the default value of 95 %. In addition to the 109 new COI sequences of *Z. indianus* from Mexico, Panama, Cameroon and Malawi, we also incorporated 24 GenBank sequences (Accession numbers EF632353–EF632372; KC994623, −28, −29 and −31) for *Z. indianus* from southeastern USA ($N = 1$), South America ($N = 7$), Africa ($N = 9$), the Middle East ($N = 5$) and India ($N = 2$) encompassing fourteen additional countries.

Results

Genetic diversity indices and results of neutrality tests (Tajima's D and Fu's F_S) for COI in *Z. indianus* from each sampling locality in Mexico and Panama, and in the combined data set, are shown in Table 1. Corresponding values for the Afrotropical Cameroon population are shown for comparison. Individual and overall values for haplotype and nucleotide diversity from all Mexico/Panama localities ($N = 99$) were relatively low (overall values of 0.579 and 0.00801, respectively) compared with the Cameroon population (0.972 and 0.01856, respectively). Haplotype diversity of flies from each of the Mexico/Panama localities ranged from 0.453 to 0.867; nucleotide diversity ranged 0.00722 to 0.01094. Overall, there were fifteen variable sites and six haplotypes in the 658 bp gene segment of the Mexico/Panama data set. Values for Tajima's D and Fu's F_S were not significant. A value of 0.8 % was found for both mean uncorrected p-distance and mean K2P distance among sequences. Because p-distances and K2P distances were identical, or nearly identical, in all analyses, below we report only p-distances.

There was no evidence for structure among populations of *Z. indianus* in Mexico and Panama. The AMOVA (Table 2) showed that 100 % of the genetic variation in COI was distributed within populations (overall $\Phi_{ST} = -0.027$; $P = 0.78$). None of the pairwise comparisons of Φ_{ST} among the seven localities was significant (Table 3).

Results from the TCS program (Fig. 2) showed that COI haplotypes of *Z. indianus* collected worldwide resolved in a single network at a 95 % connection limit, indicating a close relationship among all haplotypes, as expected for a single species. The six haplotypes seen in flies from Mexico and Panama partitioned into three distinct clades (A, B and C) within the network. These six haplotypes, however, did not correspond to any COI haplotypes of *Z. indianus* from potential source populations in the Old World available in GenBank, or to any of our new sequences from Cameroon and Malawi.

Pairwise comparisons of mean p-distance among the three Mexico/Panama clades ranged from 1.3 to 1.7 %. The mean p-distance among the combined samples from the New and Old Worlds ($N = 133$) was 1.1 % (range 0.0–3.8 %). The most abundant clade seen in Mexico and Panama (clade A), comprising 63 of the 99 individuals analyzed, was present at all localities sampled and contained one predominant haplotype ($N = 60$) and two minor haplotypes (Fig. 2). The common haplotype of clade A was also found in a single individual from Brazil (GenBank KC994623). The second most abundant clade (clade B; $N = 22$), also found at all localities in Mexico and Panama, consisted of a single haplotype. Finally, clade C ($N = 14$) was present at all sites, except Oaxaca, and consisted of two haplotypes of equal frequency. Both haplotypes of clade C were also present in two flies from Brazil (KC994628 and KC994629). The two haplotypes in clade C differed by a single mutation.

Table 1 Summary of genetic diversity indices and results of neutrality tests (Tajima's D and Fu's F_S) in the 658 bp mitochondrial COI gene segment of *Zaprionus indianus* from Mexico, Panama and Cameroon

Locality[a]	N	k	K	h (±SD)	π (±SD)	Tajima's D	Fu's F_S
Mexico							
Ala	19	14	5	0.591 ± 0.118	0.00747 ± 0.00166	0.84	3.71
Car	6	14	4	0.867 ± 0.129	0.01094 ± 0.00203	1.07	1.89
Isa	13	14	3	0.513 ± 0.144	0.00764 ± 0.00210	0.47	5.95
Gto	24	14	3	0.453 ± 0.095	0.00722 ± 0.00147	0.94	8.15
Qro	20	15	5	0.695 ± 0.081	0.00932 ± 0.00111	1.66	5.02
Oax	6	11	2	0.600 ± 0.129	0.01003 ± 0.00216	2.24	6.46
Panama	11	14	4	0.600 ± 0.154	0.00790 ± 0.00195	0.39	3.47
Total	99	15	6	0.579 ± 0.046	0.00801 ± 0.00062	2.26	9.20
Cameroon	9	29	8	0.972 ± 0.064	0.01856 ± 0.00182	0.37	–0.69

[a] See Fig. 1 for Mexico locality abbreviations. N, number of sequences; k, number of variable sites; K, number of haplotypes; h, haplotype diversity; π, nucleotide diversity; none of the values for D or F_S were significant at the 0.05 level

Table 2 AMOVA of 99 COI sequences of *Zaprionus indianus* from Mexico and Panama

Source of variation	*df*	Sum of squares	Variance components	Percentage of variation	Φ_{ST} (*P* value)
Among pops	6	10.381	−0.07062 Va	−2.69	−0.027 (0.781)
Within pops	92	247.781	2.69327 Vb	102.69	

Table 3 Pairwise comparisons of Φ_{ST} for populations of *Zaprionus indianus* from Mexico and Panama based on COI sequences

	Ala (19)	Car (6)	Isa (13)	Gto (24)	Qro (20)	Oax (6)	Pan (11)
Ala	–						
Car	−0.025	–					
Isa	−0.066	−0.039	–				
Gto	−0.029	−0.016	−0.043	–			
Qro	−0.027	−0.095	−0.037	−0.008	–		
Oax	0.057	−0.150	0.039	0.013	−0.031	–	
Pan	−0.060	−0.037	−0.073	−0.002	−0.041	0.077	–

None of the pairwise Φ_{ST} values were significant at the 0.05 level. Number of individuals from each locality is shown in parentheses. See Fig. 1 for locality abbreviations

Discussion

Analysis of nucleotide sequences from the COI barcode segment revealed that *Z. indianus*, a recently introduced drosophilid in the Americas, showed a lack of population structure over a wide geographic area from Panama to northwestern Mexico, a distance of approximately 3,800 km. Allozyme studies of *Z. indianus* in Brazil also suggested little population structure among widely separated populations, as well as a single introduction from tropical Africa followed by rapid expansion (Mattos-Machado et al. 2005). To our knowledge, the only other mtDNA population genetic study on *Z. indianus* was conducted on invasive populations in the Nile Delta region of northern Egypt in which genetic variability in COII was analyzed (Yassin et al. 2009). Although sample sizes were low in the eight populations analyzed in that study (4 or 5 individuals per population), a very low level of genetic variability, attributed to a population bottleneck, and significant population structure along a southeastern–northwestern cline were found. It was suggested that two independent invasions had occurred about 10 years apart, the first arriving from the south and the second arriving to the northwestern region of the Nile Delta from Asia. Interestingly, when the two northwestern populations containing the genetic signature of the Asian invasion were omitted, no significant population structure was found among the remaining populations (Yassin et al. 2009). Overall, these studies suggest a model in which an expansion wave of a single introduction of the invading *Z. indianus* will occur without producing population structure, assuming that selection is not acting on individual genotypes.

The reproductive biology of *Z. indianus* suggests it is capable of rapid invasion and gene flow, which is consistent with the lack of population structure revealed in our analysis. First, it is able to utilize multiple food types to feed and breed (Commar et al. 2012), increasing the number of suitable resources available in a variety of localities. Second, female *Z. indianus* appear to be sperm limited in nature (G. Hanna and T.A. Markow, unpublished results). Wild caught females only produce an average of 51 progeny and in the laboratory females remate daily, receiving enough sperm from a given copulation to produce only 40 progeny. Rapid remating coupled with dispersal to new habitats suggests that females are capable of transporting multiple genetic variants when they disperse, potentially contributing to the panmixia observed in New World populations of this species. A similar situation is seen in several other drosophilids, such as *D. nigrospiracula*, in which females receive few sperm on any given copulation, remate rapidly, and show no population structure across

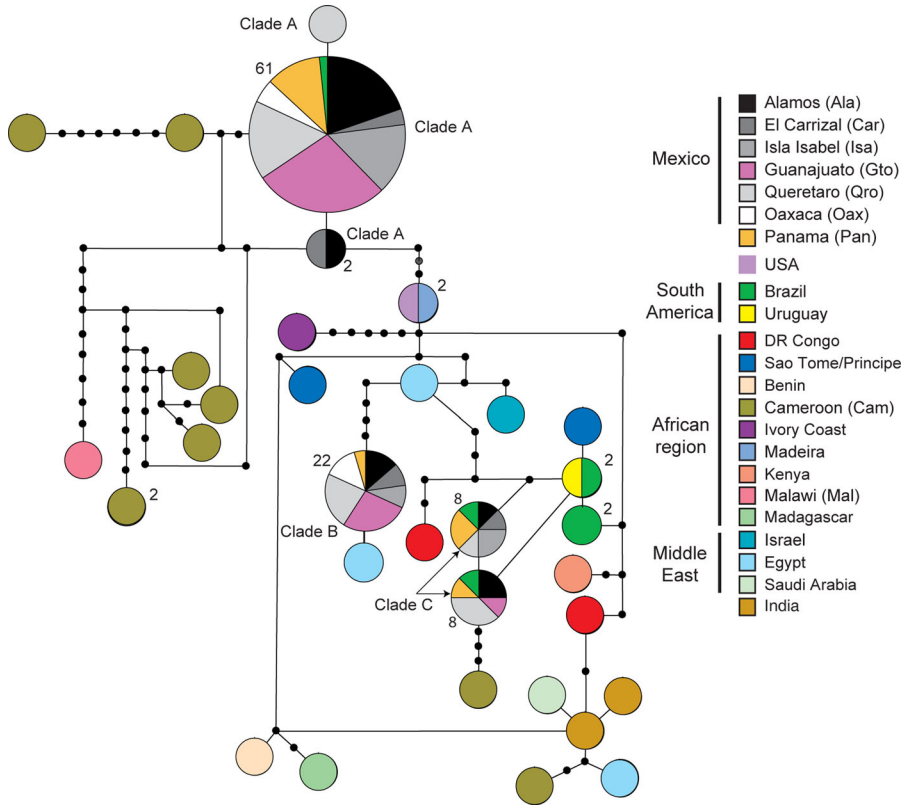

Fig. 2 TCS haplotype network for new COI sequences for *Zaprionus indianus* from Mexico ($N = 88$), Panama ($N = 11$), Cameroon ($N = 9$) and Malawi ($N = 1$), and GenBank sequences ($N = 24$) from specimens collected in the USA (Florida), South America, Africa, the Middle East and India, based on a 95 % connection limit. Locality abbreviations for the new sequences are shown in *parentheses*. *Each line* segment between haplotypes represents a single mutational step. Inferred intermediate haplotypes not sampled are shown as *small black dots* on the line segments. Size of the *circles* is proportional to haplotype frequency. *Numbers* next to the *circles* represent number of individuals with that haplotype, if greater than one. Geographical localities are color-coded. The three clades of *Z. indianus* from Mexico are labeled with *letters* [A (three haplotypes; $N = 63$), B (one haplotype; $N = 22$) and C (two haplotypes; $N = 14$)]

distances similar to those sampled here for *Z. indianus* (Pfeiler et al. 2005).

The COI haplotype network (Fig. 2) showed that the composition of the three clades (A, B and C) of *Z. indianus* from Mexico and Panama was remarkably consistent throughout the region. Individuals from each clade were found at all localities, except Oaxaca where clade C was absent. Although the absence of clade C at Oaxaca might be explained by the low sample size at that locality ($N = 6$), all three clades were present on the Baja California peninsula at El Carrizal where sample size was also $N = 6$. Invasive populations from Central America and Mexico were collected in vastly different habitats that included desert and tropical rainforest, as well as mainland, insular and peninsular localities. Despite this variation, the haplotype consistency among all localities suggests selection is presently not acting on the different haplotypes of the three clades of *Z. indianus*. However, we cannot rule out that dispersal among populations may be masking a detectable signal of selection.

Although the initial propagule of *Z. indianus* arriving to Brazil in 1999 was thought to have been large (Mattos-Machado et al. 2005; David et al. 2006; Ananina et al. 2007), overall COI genetic diversity of this propagule is unknown. The low genetic diversity found in Mexico and Panama flies compared with the Cameroon population, however, is consistent with the findings of Yassin et al. (2009) in Egypt, and suggests that a reduction of genetic diversity (i.e. bottleneck) occurred during the initial invasion of South America.

Although this suggestion cannot be confirmed until we identify the geographic source of the propagule, it seems unlikely that the propagule would have contained all the genetic diversity of the source population.

Three of the six COI haplotypes in *Z. indianus* from Mexico and Panama, including the most common haplotype of clade A and both haplotypes of clade C, also have been reported in flies from Brazil. In addition, two additional COI haplotypes found in three individuals from Brazil and one from Uruguay (Yassin et al. 2008) were very closely related to the two clade C haplotypes, being separated by only 1–3 mutational steps (Fig. 2). These findings are consistent with a scenario of a northward migration out of Brazil of *Z. indianus* presently colonizing Mexico and Central America. The haplotype corresponding to clade B, however, did not correspond to available haplotypes from South America or the Old World, although it was separated by only a single mutational step from the haplotype of a fly from Alexandria, Egypt (KC994631) (Fig. 2). This observation suggests two alternative explanations. The first is that there have been two or more independent introductions of *Z. indianus* in the New World. Separate introductions of *Z. indianus* could have occurred at the Panama Canal, Mexico City, or other major ports in central and southern Mexico. Consistent with this scenario, none of the COI haplotypes from Mexico, Panama, or South America correspond to the haplotype found in *Z. indianus* sampled from Florida, USA and Madeira (an archipelago off the west coast of North Africa) (Yassin et al. 2008; also see Fig. 2). The second possibility is that an expansion wave from a single introduction to South America is occurring and that the haplotype from clade B, as well as the haplotype from Florida, are present in South America, but have not been detected. The lack of population structure and similarities in clade composition of Mexico and Panama flies are consistent with this second scenario.

Several factors complicate our efforts to precisely identify the geographic source and invasion dynamics of *Z. indianus* in Mexico and Panama, and the New World in general. The most critical of these is the low number of *Z. indianus* COI sequences from the Old World available for comparisons. Only 29 COI haplotypes are presently available for *Z. indianus* worldwide, including those from Cameroon and Malawi (present study), and none of these correspond to the six haplotypes of *Z. indianus* found in Mexico and Panama (Fig. 2). One haplotype from Cameroon, however, is separated from the common haplotype of clade A by only three mutational steps (Fig. 2), consistent with the hypothesis of a western Africa origin of the New World invasion, although clade B may represent the independent arrival of a Middle East propagule as suggested above. Another potential problem is that the sequences reported by Yassin et al. (2008) were obtained from laboratory cultures started from one or a few females, thus inbreeding and genetic drift may have occurred and resulted in haplotypes not representative of the common haplotype at each of the reported localities. More data from field collected flies from the Old World and South America will be required to assess genetic diversity and population structure in flies from different localities and to confidently identify source populations (Muirhead et al. 2008).

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