GENETIC DIFFERENCES BETWEEN *Anastrepha ludens* (Loew) POPULATIONS STEMMING FROM A NATIVE AND AN EXOTIC HOST IN NE MEXICO[*]

DIFERENCIAS GENÉTICAS ENTRE POBLACIONES DE *Anastrepha ludens* (Loew) DE HOSPEDEROS NATIVOS Y EXÓTICOS EN EL NE MÉXICO

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ABSTRACT

The objective of this study was to determine the genetic structure of populations of the mexican fruit fly *Anastrepha ludens* (Loew) in NE citrus growing regions of Mexico. The work was conducted during 2005 at the Center of Genomic Biotechnology in Reynosa, Tamaulipas, Mexico. AFLP markers using four different pairs of initiators were used on specimens collected from the native, ancestral host, yellow chapote *(Casimiroa greggii* S. Wats) and the exotic host, citrus (*Citrus sinensis* (L.) Osbeck cv Valencia). The four combinations of initiators amplified an average of 95 AFLP fragments. A total of 382 products were obtained and 259 (67%) of them were polymorphous. The overall index of genetic diversity was 28%, with 3% difference between the genotypes collected from yellow chapote as compared to the genotypes collected from sweet orange. Cluster analysis and principal components showed a close genetic relation among *A. ludens* specimens regardless of the origin. Although, the dendrogram showed two main groups, the genetic distance varied from 0.0 to 10%, suggesting that despite the reproductive isolation, host groups are not evolving. These results are discussed in the context of evolution and conclude on practical implications of the ongoing eradication program through sterile insect technique and the geographical origin of this species.

Key words: AFLP markers, genetic diversity, host fidelity.

RESUMEN

El objetivo de este trabajo fue determinar la estructura genética de poblaciones de mosca mexicana de la fruta *Anastrepha ludens* (Loew) en las regiones citrícolas del noreste de México. La caracterización molecular se realizó durante 2005 en Centro de Biotecnología Genómica del Instituto Politécnico Nacional en Reynosa, Tamaulipas. Se utilizaron marcadores AFLP con cuatro diferentes pares de iniciadores para analizar individuos de *A. ludens* colectados del hospedero nativo chapote amarillo (*Casimiroa greggii* S. Wats) y del hospedero exótico naranja dulce (*Citrus sinensis* L. Osbeck cv Valencia). Las cuatro combinaciones de iniciadores amplificaron en promedio 95 fragmentos de AFLP. Se obtuvieron 382 productos de los cuales, 259 (67%) fueron polimórficos. El índice general de diversidad genética fue de 28% con 3% de diferencia entre los genotipos colectados de chapote amarillo y los de naranja dulce. El análisis de conglomerados y de componentes principales mostró estrecha relación genética entre individuos de *A.*

ludens independientemente de su origen. No obstante de que el dendrograma divide las poblaciones de mosca mexicana de la fruta en dos grandes grupos, la distancia genética varió de 0.0 a 10%, lo que sugiere que no obstante el aislamiento reproductivo entre los grupos, estos no están en evolución. Estos resultados se discuten en el contexto de evolución (historia de vida) y se concluye sobre las implicaciones que tienen sobre la campaña en curso para la erradicación mediante la técnica de insectos estériles y para determinar el origen geográfico de estas especies.

Palabras clave: diversidad genética, fidelidad al hospedero, marcadores AFLP.

INTRODUCTION

The genetic structure of *A. ludens* populations can be an important component for a successful region-wide pest management (Via, 1990; Krafsur, 2005) and it is fundamental to establish the origin and identity of the invasive species into agricultural regions (Roderick, 1996; Armstrong and Ball, 2005). The mexican fruit fly complex is different enough from others that renaming it has been long overdue (Baker *et al*., 1944; Aluja *et al*., 2003; Hernández-Ortíz *et al*., 2004). In the *A. fraterculus* sibling complex, some sibling species appear to have different geographic range, but others do not. Sibling species exhibit differences in biology, host diversity (Aluja *et al*., 2000a, 2003) and consistent morphological characteristics (Hernandez-Ortíz *et al*., 2004). Mating compatibility tests among different populations of South America *A. fraterculus* have provided evidence of prezygotic reproductive isolation due in part to asynchronies in the daily patterns of sexual activity (Vera *et al*., 2006). The latter finding has important management implications, particularly on the sterile insect technique (SIT). *A. ludens* is the most devastating pest of citrus in Mexico and Central America (Aluja, 1993; Thomas, 2003); however, its genetic structure is almost unknown. Up to date the only published report indicate that it is difficult to genetically differentiate *A. ludens* populations, because it's high intra-population variability (Mangan and Moreno, 2003). Currently, this species are subject to an area-wide management program based on the sterile insect technique that has resulted in the partial/temporary declaration of several fruit fly free areas in NW Mexico (Reyes *et al*., 2000). Introduced *A. ludens* populations have been eradicated through SIT from the San Diego area in the US. (Dowell *et al*., 2000) and have been a recurrent problem in the Rio Grande valley of Texas (Thomas, 2003).

In NE Mexico, the widespread abundance of naturally occurring ancestral hosts of *A. ludens* such as the yellow chapote (*Casimiroa greggi* S. Wats), hinders the official declaration of low pest prevalence area; therefore, a stiff regulation on citrus fruit transport has been impose. It is assumed that there is a permanent massive flow of mexican fruit flies from their native hosts (yellow chapote and white zapote [*Casimiroa edulis* Llave *et* Lex]) into citrus groves (Enkerlin, 1987), that complicates an effective pest control. However, there is no solid evidence based on the genetic structure of the populations that demonstrates that there is a flow of flies from the native habitats to crop areas. Studies on mobility and longevity of sterile mexican fruit flies revealed that adults can fly up to 9 km; however, 98% were recaptured within 100 m from the release site (Enkerlin 1987, Thomas & Loera-Gallardo, 1998). The latter study determined a mean mobility distance of 240 m (using a regression model), which suggest that mexican fruit flies in citrus, reproduce within the orchard or at nearby neglected plantations. Nevertheless, studies on the reproductive phenology of *A. ludens* in NE Mexico (Enkerlin, 1987; Thomas, 2003) suggest that the fly is essentially bivoltine in the area and may exploit yellow chapote fruits during spring and citrus at fall. A recent study compared the reaction of wild and lab-reared *A. ludens* strains to host fruits, the results showed that wild flies were incapable of recognize introduced grapefruit as host, while laboratory flies did. Such reaction is the effect of selective pressures to which the mexican fruit flies have been submitted at the laboratory (Robacker and Fraser, 2004) and a manifestation of the inherent plasticity of this species. Also, when wild and lab-reared flies had previous contact with yellow chapote, the attraction towards this host increased.

The objective of this study was to determine the genetic structure of *A. ludens* populations from two of the most important citrus producing states of Mexico: Tamaulipas and Nuevo Leon. Molecular characterization of *A. ludens* populations is important for the successful application of the SIT in pest management programs. If *A. ludens*, as is the case with *A. fraterculus*, is found to enclose a complex of cryptic species, then the strain currently being release on SIT programs in Mexico would be useless at certain areas due to reproductive isolation. Furthermore, molecular characterization of *A. ludens* populations will be an aid to confirm and identify the origin of recurrent putative introductions into citrus growing areas of California, Texas and Florida.

MATERIALS AND METHODS

DNA collection and extraction

A total of 25 specimens (adult and larvae) of the mexican fruit fly, *A. ludens* were directly collected from infested *C. greggii* (yellow chapote) and *Citrus sinensis* cv. Valencia (citrus), in different geographical regions within NE Mexico during 2005. (Table 1). The specimens were preserved in ethanol (90%), then washed with sterilized water (to remove alcohol) and stored at- 80 ºC. The specimens were ground to a fine powder with a mortar and pestle. The protocol for DNA extraction was according to Corsini *et al*., 1999, with extraction solution (50 mM Tris HCl, pH 8.0, 50 mM, EDTA, 3% w/v SDS and 0.1 M 2-mercaptoetanol) and 25 µl proteinase K (10 mg ml).

Table 1. Host of origin and location where *Anastrepha ludens* specimens were collected.

Num. of collection	Host	Location /Coordinates
CHATAM1, 2, 3, 4 and 5	*Casimiroa greggii*	Tamaulipas, Mexico N 23º 45' W 99º 03'
CHATAM6, 7, 8, 9 and 10	*Casimiroa greggii*	Tamaulipas, Mexico N 23º 59' W 99º 06'
CHANL1, 2, 3 and 4	*Casimiroa greggii*	Nuevo Leon, Mexico N 24º 52' W 99º 43'
CITAM 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10	*Citrus sinensis*	Tamaulipas, Mexico N 23º 45' W 98º 59'
CITSLP (C)	*Citrus sinensis*	San Luis Potosi, Mexico N 21º 55' W 99º 59'

Numbers refer to individuals labeled in Figures 1 and 2.

AFLP analysis

The AFLP protocol utilized was that of Vos *et al*. (1995). It consists in the genomic DNA digestion and ligation of specific adaptors, followed by two PCR selective amplifications. For DNA digestion a reaction mixture of 30 µL containing 3 µL of DNA (150 ng µL^{-1}), 1.5 µL of *Eco*RI (Roche®) of 10 U µL^{-1}, 1.5 µL of *Tru*9I (Roche®) of 10 U µL^{-1} and 3 µL of 10X RL was used. The samples were incubated for 4 h at 37°C, followed by heating the reaction mixture to 70°C for 15 min to inactivate the restriction enzymes. The specific adapters were united to the sites of restriction. The mixture consisted of 1 µL of *Eco*RI adapter (5 pmol), 1 µL of *Mse*I adapter (50 pmol), 1.2 µL of 10 mM ATP, 1 µL of 10X RL buffer and 1 µL of T4 DNA ligase (Roche®) of 1 U µL^{-1} in a total volume of 10 µL. The preamp included 5 µL of the ligation mixture plus the addition of a mixture of oligonucleotides specific AFLP (50 ng µL^{-1}), 2 µL of 10 mM dNTPs in a total volume of 25 µL. The reaction mixture consisted of 0.2 µL addition of Taq DNA polymerase (Roche®) (5 U µL^{-1}) and 5 µL of 10X PCR buffer in a final volume of 20 µL. The total volume of the reaction mixture was 50 µL. The mixture was subjected to PCR amplification in a GeneAmp 9 700 thermocycler (Applied Biosystems®). The following PCR program was used: 94 ºC for 30 s, 56 ºC for 60 s and 72 ºC for 60 s for 20 cycles. Subsequently, 2.5 µL of pre-amplified DNA was added with 0.5 µL of the oligonucleotide *Eco*RI + 3 (*Eco*RI + AAA), 0.6 µL of oligonucleotide *Mse*I + 3 (AAA, AAC, ATG and AGG), both with a concentration of 50 ng µL^{-1}, and 0.4 µL of 10 mM dNTPs mix, for a total volume of 5 µL, plus 0.2 µL of Taq DNA polymerase 5 U µL^{-1} and 1.4 µL of 10 X PCR buffer, for a total volume of 11 µL.

The reaction mixture was subjected to the following PCR program: 94 ºC for 30 s, 65 ºC for 30 s and 72 ºC for 60 s; this for 11 cycles where the temperature was reduced by alignment of 0.7 ºC per cycle and then continued with the following PCR program: 94 ºC for 30 s, 56 ºC for 30 s and 72 ºC for 60 s for 23 cycles. Amplified products were separated in acrylamide gel at 6%. Electrophoresis was performed at 2 000 V for 3 h. For detection of amplified products using the Silver Sequence Staining Reagents kit Promega®. The AFLP analysis was conducted at the laboratory of plant biotechnology of the Genomic Biotechnology Center of the National Polytechnic Institute in Reynosa, Tamaulipas, Mexico.

Data analysis

All bands that showed molecular weight over 250 bp were included in the AFLP analysis. It was assumed that bands of the same molecular weight in different individuals were identical. All gels were scored visually for both polymorphic and monomorphic bands. Band presence was indicated by a (1) and absence by a (0). Analysis of molecular variance was used to estimate variance components among and within groups, following Excoffier *et al.* (1992). Wright's (1951) F statistics were used to estimate the amount of genetic differentiation among populations and subdivisions of populations. Genetic relationship was calculated by the method of Nei and Li (1979), using the S-Plus Version 4.0 (S-Plus, 1997). Principal components (CPs) and cluster analysis were performed. Cluster analysis was performed on the relationship matrices using the unweighted pair group method arithmetic average (UPGMA), (Avise, 1994) and the relationships were graphically presented as dendrograms. Finally, the diversity index was calculated as $DI = 1 - p_i^2$, where p_i is the frequency of the ith allele, each allele is considered as a unique locus and at the same time, an amplification fragment (Powell *et al.*, 1996).

RESULTS AND DISCUSSION

The four-primer combinations amplified among 91 and 99 bands, with a mean of 95 for each pair of primers. A total of 382 products were obtained and 259 of them (67%) were polymorphic (Table 2). A slight difference among hosts was noticed, specimens from yellow chapote showed 3% difference with respect to specimens from citrus (Table 3).

Table 2. Primer pairs used, and number and percentages of polymorphic products obtained in each assay.

AFLP Primer pairs *Eco* RI / *Mse*I	Monomorphic	Amplified Products		Total	Polymorphisms (%)
		Polymorphic			
AAA / AAA	26				
		65		91	71
AAA / AAC	23	72		95	75
AAA / ATG	39	60		99	60
AAA / AGG	35	62		97	63
Total	123	259		382	
Mean	31	65		95	67

Table 3. Index of genetic diversity in the mexican fruit fly (*Anastrepha ludens*) according to Powell *et al.* (1996).

A. ludens population	Index
Casimiroa greggii	0.29
Citrus sinensis	0.26
Nuevo Leon, Mexico	0.28
Tamaulipas, Mexico	0.28
C. greggii, Tamaulipas	0.29
C. greggii, Nuevo Leon	0.28
C. sinensis, Tamaulipas	0.26

The analysis of molecular variance (AMOVA) showed significant differences (*p*<0.001) within *A. ludens* populations (among specimens), while no population differences in regard to host or region were observed. The fixation index (F_{ST}) for the three analyzed levels (population, host and region), showed a close relationship among *A. ludens* specimens in regard to host and region as evidenced by negligible genetic differences among subpopulations. Similarly, cluster analysis and principal components analysis confirmed that there is a strong genetic relationship among specimens of *A. ludens* (Table 4).

Table 4. Analysis of molecular variance of different *Anastrepha ludens* populations according to AFLP information.

Source of variation	d. f.	Sum of squares	Variance components	Percentage variation	P
Among hosts	1	58	2.06	5.66	ns
Among regions	1	47.1	2.15	5.91	ns
Within populations (specimens)	21	675.9	32.19	88.43	<0.001
Total	23	781	36.04		
Fixation Index					
F_{ST} (hosts)			0.062		
F_{ST} (regions)			0.058		

P= probability; ns= no significative.

The dendrogram based on the cluster analysis of AFLP data (Figure 1) showed one main group that includes 88% of the specimens, with differences varying from 0.0 to 7.6%. In this main group, a defined grouping pattern was observed: first, genotypes of flies collected from yellow chapote, followed by genotypes from citrus, including the control CITSLP; however, two specimens from yellow chapote (CHANL1 and CHANL2) were mixed with specimens collected from citrus, and two genotypes from yellow chapote (CHATAM6 and CHANL4) clearly differ from the rest.
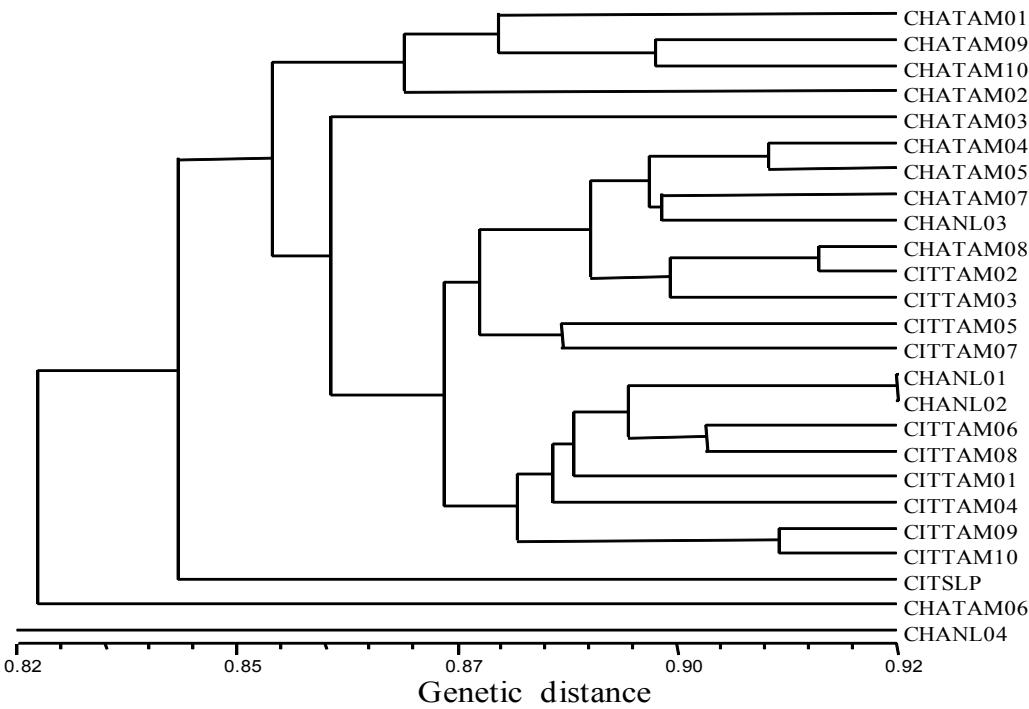

Figure 1. Dendrogram based on 382 AFLPs (obtained by the UPGMA method) showing genetic associations among *A. ludens* specimens from different hosts and geographical areas.

Due to the observed trend of grouping by host, which suggests the possibility of a marker linked to a fruit fly-host relation, a second cluster analysis was performed, using specimens collected from Tamaulipas and San Luis Potosi (citrus) as control. One thousand bootstrap replicates were obtained from the original data and a distance matrix was

calculated for each replicate. In this new analysis, two main genotype groups were observed; the first one, included yellow chapote and citrus specimens from Tamaulipas and the other the citrus specimen from San Luis Potosi (control). The dendrogram showed that all specimens from yellow chapote are closely related, followed by citrus specimens collectedin Tamaulipas (Figure 2).

This suggests the existence of a DNA marker linked to the fruit fly-host relation. Moreover, in the matrices of AFLP, it was observed that the Eco/Mse AAA/AAA pair of primers amplify a DNA band which was present in the whole set of specimens collected in citrus; meanwhile, in the yellow chapote specimens this DNA band was absent, except for one individual.
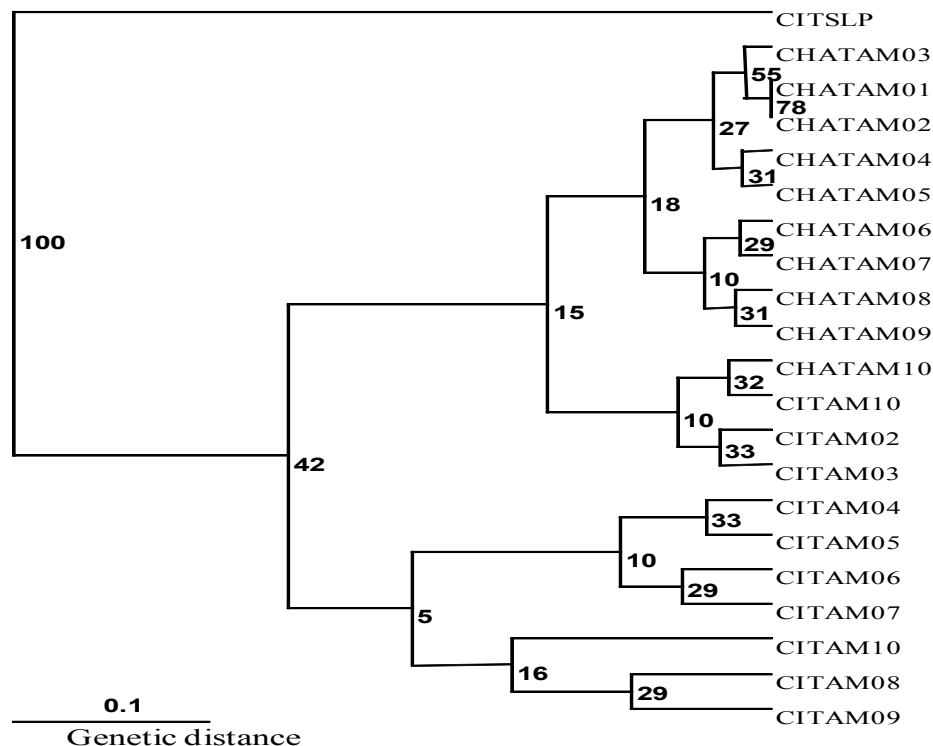

Figure 2. Dendrogram based on 382 AFLPs (obtained by the UPGMA method) showing genetic associations among *A. ludens* specimens from different hosts in Tamaulipas, Mexico. Numbers above the branches represent bootstrap support values for 1 000 replicates.

The AFLP analysis showed a high level of polymorphism (67%), with a mean index of diversity of 28%. The technique was sensitive enough to detect differences among individuals. A slight trend toward greater genetic diversity of *A. ludens* populations stemming from native yellow chapote was observed, which is possibly due to the fact that these populations have been less perturbed (Robacker and Fraser, 2004) and subjected to a lesser selection than citrus populations subjected to frecuent insecticide sprays.

A weak grouping trend related to host origin was observed; particularly in the specimens from Tamaulipas (Figure 2). The analyses showed a close relation

among specimens from the same host with genotypes collected from yellow chapote separated from those collected from citrus.

Phenotypic plasticity has been defined as the ability of a single genotype to produce more than one alternative form in response to environmental conditions (West-Eberhard, 1989). It has been recognized that plasticity itself is a trait subject to natural selection and evolutionary change (Williams, 1966). The results of this research indicate that *A. ludens* exploiting different hosts have a slightly different genetic composition, probably owing to the fact that citrus colonization by yellow chapote flies implies going through

a series of bottlenecks as described by Alberti *et al*. (1999) for *A. fraterculus* in Argentina. Nevertheless, the detected differences were not significant, suggesting that reproductive isolation is not evolving among the studied populations. It is important to note that *A. ludens* is polyphagous and multivoltine (Aluja, 1994; Thomas, 2003). Multivoltine fruit flies have to cope with long periods when host fruit is not available without undergoing diapuase. There are a few possible life strategies to overcome this hurdle. The first one would be through evolution of adult longevity, which has occurred in some *Anastrepha* species (Aluja *et al*., 2000a) but not in the case of *A. ludens*; the second would be by exploiting hosts with long fruiting periods, as it is the papaya fruit fly, *Toxotrypana curvicauda* Gerstaecker. Multivoltine fruit flies can bridge the fruiting gaps of their main host by exploiting alternate hosts (Aluja and Mangan, 2008). This survival strategy might select for genetic plasticity but also restricts genetic differentiation of host plant races. A likely scenario would explain the observed results, where an *A. ludens* population switching from chapote to citrus undergoes a genetic bottleneck that can change allele frequency, followed by a return to the ancestral host that may result in homogenizing the gene pool. This life strategy and the phenotypic plasticity observed may help to explain the findings of Robacker and Fraser (2004) showing that mexican fruit flies with previous contact with yellow chapote will prefer this host over other alternatives regardless of their own origin. It also provides support to the idea that chapote populations move back and forth from chapote to citrus as documented by Enkerlin (1987) and more recently by Thomas (2003).

Practical implications of these findings suggest that at a regional scale, the sterile insect technique can be applied for *A. ludens* without the need to colonize different strains stemming from different hosts. AFLP markers proved to be a useful tool to differentiate among *A. ludens* specimens allowing to detect a high level of polymorphism, measure genetic diversity and to detect genetic differences in host relation. It is important to mention that this is the first step of several more to follow attempting to characterize *A. ludens* populations within their geographical range. Molecular scrutiny of these populations at a wider scale is essential for a thorough understanding of the evolution of *A. ludens* life strategies. Wide range sampling is also critical to rule out geographical effects (Atlantic-Pacific, altiplano-coastal plains) on *A. ludens* population's genetic composition and to get evidence that these factors may be leading to the evolution of inter population reproductive isolation. That attempt will also contribute to determine the origin of recurrent introductions of *A. ludens* into citrus growing regions of the U. S., whether occur by natural dispersal (the case of Texas and Nuevo Leon), or human transport. It is also necessary to widen the scope of sampling of host-associated populations considering that both chapote and citrus belong to Rutaceae and *A. ludens* has been reported infesting plants in very different families: Solanaceae (Thomas, 2004), Anacardaceae, and Rosaceae (Aluja *et al*., 2000b; Norrbom, 2003). Based on the above, the next step to genetically characterize the *A. ludens* populations in Mexico, will be to extend the sampling to other regions and other host plants and to incorporate other molecular tools as microsatellites.

CONCLUSIONS

Despite there is a high level of polymorphism (67%) in *A. ludens* in NE Mexico, the genetic diversity of this species is low (ID 28%).

The AFLP technique was sensitive enough to detect differences among individuals, a silght 3% difference was observed among specimens collected from yellow chapote as compared to those collected from sweet orange.

Cluster analysis and principal components showed a tight genetic relation among *A. ludens* individual regardless of origin.

Although the dendrogram divided divide the mexican fruit fly populations into two main groups, the genetic distance (0.0 to 10%) suggest that despite the reproductive isolation, host groups are not evolving.

ACKNOWLEDGEMENTS

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