

Reproductive compatibility among Mexican populations of *Anastrepha obliqua*: theoretical and management implications

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Abstract

The *fraterculus* species group, composed of 34 species in the genus *Anastrepha* (Diptera: Tephritidae), includes the *fraterculus* cryptic species complex formed by eight reproductively isolated morphotypes. A previous study revealed six genetic mitochondrial types of *Anastrepha obliqua*, suggesting the existence of a second cryptic species complex. However, marked discrepancies between nuclear and mitochondrial loci rather suggest incomplete lineage sorting or introgression between *A. obliqua* and *A. fraterculus*. Such hybridization could nevertheless result in reproductive isolation, an outcome that could affect efficacy of area-wide management for the most important pest of mangos in America. Two mitochondrial types occur in Mexico, and the limits of a third one, encompassing Central American populations, have not been clearly established. Here, we tested reproductive compatibility among three *A. obliqua* populations from the Pacific and a population from the Gulf of Mexico. No evidence of pre-zygotic isolation was found. Flies from the Atlantic mated randomly for equal duration with individuals from three Pacific populations. Homotypic and heterotypic crosses produced similar numbers of eggs, with heterotypic crosses of Pacific males and Atlantic females hatching in lower proportions. Larvae of all cross types developed equally in mangos and exhibited no sex ratio distortion of hybrid F1. The three mitochondrial types identified in Mexico and Central America do not appear to be cryptic species and can be managed using one single strain for the sterile insect technique.

KEYWORDS

area-wide management, cryptic species, *fraterculus* species group, reproductive isolation

1 | INTRODUCTION

Cryptic species are two or more distinct, but morphologically similar, species that have been classified as a single species (Pfenninger & Schwenk, 2007). Although the existence of cryptic species has been acknowledged for more than 300 years, the advent of molecular tools for biological scrutiny has facilitated their detection (Bickford et al., 2007), and greatly contributed to disclosing hidden diversity (Adams, Raadik, Burrige, & Georges, 2014; Hebert, Penton, Burns, Janzen, & Hallwachs, 2004). The widespread occurrence of cryptic species across insect taxa (Pfenninger & Schwenk, 2007) may not

only lead to revisions of global species diversity (Scheffers, Joppa, Pimm, & Laurance, 2012), but has important implications for conservation, identification of invasive species, pest management, public health and biological control (Bickford et al., 2007; Heraty, 2009; Liu, Colvin, & De Barro, 2012; Pavan, Rivas, Dias, & Gurgel-Gonçalves, 2015; Perring, 2001).

Tephritid fruit flies are currently composed of approximately 4,500 species, among which several (ca. 200) are of economic importance (Norrbom, 2004). In addition to their status as pests, Tephritidae includes several species that have played an important role in evolutionary biology for the understanding of rapid

divergence (Berlocher & Feder, 2002; Clarke et al., 2005). Various tephritid genera include species groups with large numbers of sibling, sister, cryptic species and host races (Berlocher, 2000; Clarke et al., 2005; Condon & Steck, 1997; Craig, Horner, & Itami, 2001; Steck, 1991; Virgilio, Backeljau, Barr, & De Meyer, 2008). In the subtropical genus *Anastrepha*, the *fraterculus* species group is currently composed of 34 species, four of which are of economic importance (Norrbom et al., 2012). Nevertheless, one of these species, *Anastrepha fraterculus* (Wiedemann), is actually a complex of at least eight cryptic species (Hernández-Ortiz, Bartolucci, Morales-Valles, Frías, & Selivon, 2012; Hernández-Ortiz, Canal, Salas, Ruiz-Hurtado, & Dzul-Cauich, 2015). Cryptic species of *A. fraterculus* exhibit slight differences in ovipositor and wing shape (Hernández-Ortiz, Gómez-Anaya, Sánchez, McPherson, & Aluja, 2004; Hernández-Ortiz et al., 2012, 2015), host plant affiliation (Aluja et al., 2003), pheromone and cuticular hydrocarbon composition (Břízová et al., 2013; Vaničková, Břízová, Mendonça, Pompeiano, & Do Nascimento, 2015), mating behaviour (Cáceres et al., 2009; Selivon, Perondini, & Morgante, 2005; Vaničková, Hernández-Ortiz et al., 2015; Vera et al., 2006) and genetic structure (Manni et al., 2015; Selivon et al., 2005; Silva & Barr, 2008; Steck, 1991; Vaničková, Hernández-Ortiz et al., 2015).

Delimitation of cryptic species complexes may require the application of comprehensive integrative approaches (Gebiola, Gómez-Zurita, Monti, Navone, & Bernardo, 2012; Katz, Giordano, & Soto-Adames, 2015; Riedel, Sagata, Suhardjono, Tänzler, & Balke, 2013). Such approaches have been successfully applied both, to separate (Cáceres et al., 2009; Dias et al., 2016; Selivon, Vretos, Fontes, & Perondini, 2004; Selivon et al., 2005) or, to cluster (Krosch et al., 2013; Schutze et al., 2012) morphologically cryptic species groups of fruit flies. Among other methods, studying reproductive compatibility, both at pre-zygotic and post-zygotic levels, has contributed to identifying species boundaries and ongoing divergence among cryptic fruit fly taxa (Devescovi et al., 2014; Roriz, Japyassú, & Joachim-Bravo, 2017; Rull, Aluja, & Feder, 2010; Rull et al., 2013; Selivon, Perondini, & Morgante, 1999; Tadeo et al., 2015).

The West Indian fruit fly, *Anastrepha obliqua* Macquart, is a member of the *fraterculus* species group and the most important pest of mangos, *Mangifera indica* L., in Latin America and Mexico (Aluja et al., 2014). Mangos are an important export commodity for several tropical and Latin American countries (Litz, 2009). As part of the Mexican Fruit Fly National Campaign, a programme based on the sterile insect technique (SIT) was launched in 2001 against *A. obliqua* in western Mexico (Artiaga-López, Hernández, Dominguez, Moreno, & Orozco-Dávila, 2004). Mass production of *A. obliqua* is currently undertaken in the southern state of Chiapas where pupae are irradiated and shipped to packing stations in the North of Mexico for periodic release (Orozco-Dávila et al., 2017; Rull, Birke, Ortega, Montoya, & López, 2012). Ruiz-Arce, Barr, Owen, Thomas, and McPherson (2012) examined mitochondrial genetic diversity of *A. obliqua* among 54 geographic populations in Mexico, Central America, the Caribbean and South America. Six populations were identified in the analysis, two of which, the Mesoamerican and western Mexican groups, occur in Mexico. The degree of differences among the genetic types

identified by Ruiz-Arce et al. (2012) suggested that a second cryptic species complex in the *fraterculus* species group could be formed by nominal *A. obliqua* across its distributional range. Nevertheless, more recently Scally et al. (2016), using a multilocus data set comprising nuclear and mitochondrial loci, suggested that *A. obliqua* is a monophyletic group. They attributed prior results to possible introgression and interbreeding between different *Anastrepha* species, which has been shown to be possible in the laboratory (Rull et al., 2017; Santos, Uramoto, & Matioli, 2001).

In Mexico, populations from the Pacific Coast and the Gulf of Mexico exhibit slight differences in host plant affiliation. Specifically, flies from Chiapas State in the South infest guavas (*Psidium guajava* L.) (Aluja et al., 1987), whereas flies from Veracruz State along the Gulf coast do not (Birke & Aluja, 2011). These populations are separated by ~500 km and a mountainous chain that only connects the two regions with habitat suitable for *A. obliqua* hosts, through the narrow Isthmus of Tehuantepec. Ruiz-Arce et al. (2012) classified a population of *A. obliqua* from Oaxaca (Zacatepec) as Mesoamerican, yet they did not analyse populations from the Pacific Coast of Mexico south of this sampling point. In fact, Southern Oaxaca and Chiapas populations could belong to the Central American type by being a continuation of the Guatemalan population (Yepocapa). No important geographical barrier exists among these populations, and the distance that separates Oaxaca (Zacatepec) and the Oaxacan-Chiapas border (~260 km) is not much greater than the one that separates it from Yepocapa (Guatemala) (~380 km), which is only ~150 km away from the Mango producing area of Southern Chiapas (Soconusco).

In the case of populations that Ruiz-Arce et al. (2012) classified as "Western México," the Mexican Trans-Volcanic Belt could represent an important geographical barrier isolating these populations from those of south-western Mexico (potentially "Central American") and the Gulf of México ("Mesoamerica").

If mitochondrial types actually represent cryptic species, it could be expected that some form of reproductive isolation has evolved between these taxa. Alternatively, if mitochondrial DNA structural patterns are explained by introgression, this could result in potential changes in mating behaviour and unexpected genetic variation introduced through hybridization with other species (Scally et al., 2016). If any type of reproductive isolation exists, this will have important practical implications, as different mass rearing strains would be needed for effective area-wide SIT control.

Here, we examined pre-zygotic (mating discrimination and mating duration) and post-zygotic isolation (hybrid fertility, larval development and F1 sex ratio) among flies from a population from the Gulf of Mexico (Veracruz, "Mesoamerican") and three populations from the Pacific Coast of Mexico, belonging to south Oaxaca ("Mesoamerican-Central American"), south Chiapas ("Central American") and Central Sinaloa ("Western Mexican"). Our goals were to characterize Mexican populations, contribute to defining the geographical range of genetic types of *A. obliqua*, establish the existence or absence of an *A. obliqua* cryptic species complex within the *fraterculus* species group, or identify potential mating incompatibilities

arising from hybridization with closely related species in the *fraterculus* species complex.

2 | MATERIALS AND METHODS

2.1 | Biological material

Anastrepha obliqua flies from the Pacific were recovered as larvae from infested *Spondias purpurea* L. fruits in Ejido los Corazones, in the south of Oaxaca State (14°49'46.68"N; 92°17'08.69"W), within the distributional range of either Mesoamerican or Central American mitochondrial type, in June 2015. Another collection was from infested mango fruits (*M. indica* L. cv Ataulfo type niño) near Tapachula, in the south of Chiapas State (14°49'46.68"N; 92°17'08.69"W), within the range of the Central American type, in July in 2016. Flies from Sinaloa, within the range of the Western Mexican type, were collected as larvae from infested *S. purpurea* in the locality of Palmito de Verde (22°39'35.24"N; 105°46'14.10"W) in September 2016. Flies from the Atlantic coast of Mexico were collected from *S. purpurea* and mango, *M. indica* var criollo., near Jalcomulco, Veracruz State (within the range of the Mesoamerican

type) (Coord. 19°19'37.54"N; 96°45'25.91"W) between June and September in both 2015 and 2016. Collection sites and separation distance (Km) from the Veracruz population are shown in Figure 1. Collected fruits from Veracruz were taken to the laboratory at the Instituto de Ecología A.C. in Xalapa Veracruz, fruits from Oaxaca and Chiapas were taken to the Moscafrut facility in Metapa in Chiapas, and fruits from Sinaloa were taken to the Comité Estatal de Sanidad Vegetal del Estado de Sinaloa and processed following the methodology described by Rull, Aluja, Feder, and Berlocher (2006). Recovered pupae in Oaxaca, Chiapas and Sinaloa were mixed with vermiculite and sent by airplane to the Instituto de Ecología A.C. in 1-L containers 2 days before adult emergence. Pupae from different collection sites were placed over a thin layer of vermiculite in 200-ml plastic containers and moistened with 0.2% (wt/vol) sodium benzoate solution to allow adult emergence. Fly populations were maintained at $26 \pm 1^\circ\text{C}$ temperature, a 13-hr:11-hr light: dark photoperiod and 65% relative humidity. At emergence, adults were counted and sorted by location, sex and age in 3-L plastic cages covered with mosquito mesh on the upper side. No more than 30 flies were placed in a single cage to reduce wing damage. Flies were fed ad libitum with a 3:1 mixture of sugar and yeast hydrolyzate enzymatic (MP Biomedicals,



FIGURE 1 Collection sites and distance (km) from the Veracruz population for three Mexican populations of *Anastrepha obliqua*

Fisher scientific, UK) and provided with water until sexually mature (20 days).

2.2 | Isolation tests

Anastrepha obliqua isolation tests were carried out in two laboratories at the Instituto de Ecología AC in 2015 and 2016. Isolation tests between populations of Veracruz and Oaxaca were carried out in 2015, and tests between Veracruz and south Chiapas and between Veracruz and Sinaloa were carried out in 2016. Laboratory conditions were maintained as described above. All tests were done following a similar methodology.

2.3 | Pre-zygotic isolation tests

Pre-zygotic isolation tests were carried out in six PVC pipe-framed experimental cages of $0.6 \times 0.6 \times 1$ m covered with white nylon gauze of 1-mm mesh. While such cages are smaller than those recommended to test mating performance of mass-reared sterile males (FAO/IAEA/USDA, 2003), at densities of up to 30 couples, they provide ample space for female rejection to occur. Small enclosures ($30 \times 30 \times 30$ cm) tend to favour mass-reared males, while large cages (3 m in diameter) favour wild individuals (Rull, Brunel, & Mendez, 2005). Nevertheless, in our experiments all flies were directly collected from the wild and therefore shared the same handicap. Using smaller cages allowed higher replication and environmental control, which was key for synchronization of sexual receptivity of adults from geographically disjunct populations. Our cages had been successfully used to test reproductive isolation between *A. fraterculus* and *A. obliqua* (Rull et al., 2017), and we feel confident that results obtained using such cages reflect existing patterns among populations. A white light lamp (960 lm) was hung in the centre top of each

cage, at a distance of 15 cm above the cage ceiling. Each cage contained one 0.9-m high mango sapling, *M. indica* L. var Manila, with 10–12 leaves to serve as fly resting and mating arena during the experiment. The evening before tests, flies were marked on the dorsal part of the thorax with a small spot of vinyl acrylic paint (Vinci de México, S.A. de C.V., Mexico City, Mexico) to distinguish populations during the experiment. Colours were assigned at random. Previous studies indicated that this type of mark does not interfere with fly sexual activity or behaviour (Rull et al., 2005). Ten pairs of *A. obliqua* from each test population (20 pairs in total) were released inside each cage. Adult flies were used from 20 to 30 days of age for populations from Veracruz and Oaxaca in 2015. In this case, flies from spanning from 20 to 30 days of age had to be used to reach adequate sample size. For populations from Veracruz, Chiapas and Sinaloa in 2016, tested flies were between 15 and 17 days of age given that enough adults emerged in synchrony. Regardless, adult *A. obliqua* from 15 to 30 days can be considered in all cases sexually mature. Flies were introduced into cages from 7:30 to 7:50 a.m., several minutes before observations. Fly observation started at 8:00 hr and ended at 13:00 hr. The variables recorded during observation were the choice of partner (male–female) for mating according to population of origin and the duration of copulation in minutes. When a copulation began, the pair was gently extracted from the experimental cage by means of a 35-ml glass vial where it was maintained until its end. After that, couples of a similar male–female combination were transferred to a transparent 3-L plastic cage and maintained under laboratory conditions as described above for post-zygotic isolation tests. Overall, the experiment involved two replicates each comprising six cages (Figure 2) with a total of 12 replicates for Veracruz \times Oaxaca in 2015; and three replicates each comprising 3–4 cages with a total of 11 replicates for Veracruz \times Chiapas and 10 replicates for Veracruz \times Sinaloa, in 2016.



FIGURE 2 Caged mango saplings where mating compatibility tests between an Atlantic (Veracruz) and three Pacific (Chiapas, Oaxaca, and Sinaloa) *Anastrepha obliqua* populations were conducted. Top left, an *Anastrepha obliqua* mating couple

2.4 | Post-zygotic isolation tests

Randomly selected couples from pre-zygotic isolation tests, separated according to each of four homotypic and heterotypic male–female combinations, were maintained under similar laboratory conditions in groups of four couples per cage in 2015 and five couples per cage in 2016. Cages were provided with water and food. Two days after pre-zygotic isolation observations, an artificial spherical device made with a mixture of agar and diet (3.19% agar, 96.32% water, 0.30% yeast protein, 0.13% sugar, 0.024% nipagin, 0.01% sodium benzoate, 0.03% green food colouring) was introduced into each cage to serve as an egg laying substrate. The spheres were replaced with new ones every other day during 6 days in 2015 and every day during 5 days in 2016, giving a total of three or five agar spheres per cage, respectively. Eggs were carefully extracted from spheres using a scalpel under a binocular microscope, counted and placed on a black cloth over moist cotton in a 90 × 15 mm Petri dish. After 5 days, eggs were inspected and the number of hatched eggs recorded. Five replicates (each comprising a cage with four couples) were performed for each male–female mating combination in 2015 and between seven and eight replicates in tests of 2016. After flies were exposed to spherical agar fruits, a mango fruit was exposed in cages every other day to give a total of three exposed mango fruits per cage. During the first exposure, a mango fruit cv Tommy Atkins was used, followed by two subsequent exposures of the Manila variety. Two days after exposure, mangos were removed and individually placed into 0.8-L plastic cups lined with vermiculite and covered with a nylon mesh. Three weeks later, fruits were inspected and the total number of pupae recovered from each fruit was counted. Pupae were then placed into 35-ml plastic cups with vermiculite awaiting for adult emergence. Pupae were moistened regularly, as described above. Finally, the number and sex of emerged adults was recorded.

2.5 | Statistical analysis

Mating partner choice, the frequency of choice for each male–female combinations between the Atlantic and each one of the three Pacific populations and the duration of copula for each mating combination were analysed by means of a generalized linear model (GLZ) with a Poisson error distribution specified (GLZs are generalized linear models [GLMs] to fit data that follow probability distributions other than the normal distribution). The overall degree of sexual isolation was established by calculating the Ipsi index (Rolán-Alvarez & Caballero, 2000), which ranges from –1 to +1, with 0 indicating random mating, using Jmating v1.0.8 software (Carvajal-Rodríguez & Rolán-Alvarez, 2006). The total number of eggs recovered in artificial fruit from each cage and the percentage of hatched eggs were analysed by one-way analysis of variance (ANOVA) and GLZ with a Poisson distribution error. The total number of pupae recovered from mangos exposed to different male–female combinations was analysed by one-way ANOVA and GLZ with a Poisson distribution error. A two-way ANOVA was also performed to evaluate the mean numbers of adults emerged according to sex. All analyses were

performed using Statistica v7 (Stat Soft Inc.) and graphed using SigmaPlot v10.0 (Jandel Scientific, 1992).

3 | RESULTS

3.1 | Veracruz vs. Oaxaca

3.1.1 | Pre-zygotic tests

A total of 108 copulation events were recorded between *A. obliqua* populations in the six cages over two replicates. No significant differences in the frequency of mate choice were observed among different male–female combinations of *A. obliqua* populations ($\chi^2 = 5.766$; $df = 3$; $p = .124$) (Figure 3a). No significant difference was observed in the duration of copulations ($\chi^2 = 3.043$; $df = 3$; $p = .385$) (Table 1).

The overall measure of sexual isolation (Ipsi index), considering the frequency of copula for all possible combinations, revealed no sexual isolation between adults from different populations (Ipsi = 0.0709; $SD = 0.0995$; $p = .4798$). These results suggest the absence of geographically based mating preferences within either the Veracruz or the Oaxaca population.

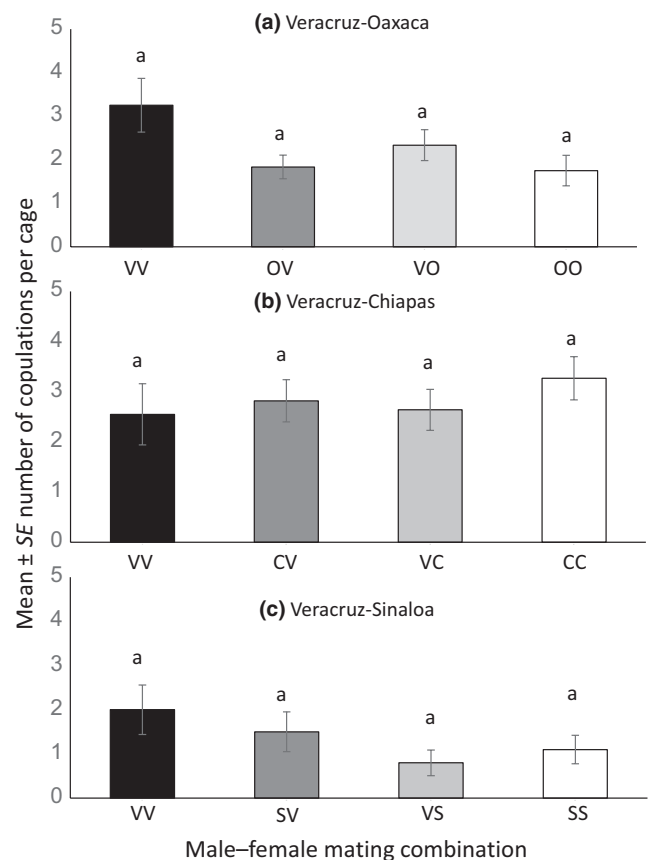


FIGURE 3 Mean ($\pm SE$) number of copulations per observational period and experimental cage (replicate) according to male–female mating combinations between sexually mature adult *Anastrepha obliqua* from (a) Veracruz and Oaxaca, (b) Veracruz and Chiapas and (c) Veracruz and Sinaloa

TABLE 1 Mating duration, number of eggs laid in agar spheres, percentage of emerged adults in mango fruits, number of emerged adults and sexual proportion to different male–female combinations for according to different male–female combinations for two *Anastrepha obliqua* populations

Variable	Veracruz-Oaxaca populations	
	Mating combinations	Mean ± SE
Duration of copula (min)	♂Veracruz-♀Veracruz	42.5 ± 3.9 a
	♂ Oaxaca -♀Veracruz	52.9 ± 4.6 a
	♂Veracruz-♀ Oaxaca	54.8 ± 6.4 a
	♂ Oaxaca -♀ Oaxaca	47.2 ± 4.5 a
No. of eggs in agar spheres (4 couples)	♂Veracruz-♀Veracruz	93.8 ± 8.7 a
	♂ Oaxaca -♀Veracruz	28.5 ± 4.8 c
	♂Veracruz-♀ Oaxaca	59.9 ± 9.2 b
	♂ Oaxaca -♀ Oaxaca	48.4 ± 9.0 bc
Number of F1 pupae in mango fruits	♂Veracruz-♀Veracruz	126.4 ± 11.3 ab
	♂ Oaxaca -♀Veracruz	210.2.8 ± 47.3 a
	♂Veracruz-♀ Oaxaca	70.6 ± 7.1 ab
	♂ Oaxaca -♀ Oaxaca	53.2 ± 22.3 b
Number of adults emerged	♂Veracruz-♀Veracruz	12.7 ± 4.19 a
	♂ Oaxaca -♀Veracruz	14.6 ± 5.0 a
	♂Veracruz-♀ Oaxaca	1.8 ± 0.5 b
	♂ Oaxaca -♀ Oaxaca	3.8 ± 1.6 b
Adult emergence by sex (male–female)	♂Veracruz-♀Veracruz	(M) 13.0 ± 6.2
		(F) 12.4 ± 6.6
	♂Oaxaca-♀Veracruz	(M) 15.8 ± 8.8
		(F) 13.4 ± 5.9
	♂Veracruz-♀Oaxaca	(M) 1.8 ± 0.8
		(F) 1.8 ± 0.8
	♂Oaxaca-♀Oaxaca	(M) 4.2 ± 2.6
		(F) 3.4 ± 2.0

Means followed by a similar letter are not significantly different after comparison among treatments (ANOVA, Tukey's HSD, $p > .05$).

3.1.2 | Post-zygotic tests

The fertility analysis conducted among different male–female mating combinations of *A. obliqua* from Veracruz and Oaxaca showed significant differences in the total number of eggs laid by four couples in cages ($F = 6.129$, $df = 3, 16$; $p = .007$) (Table 1). However, no significant differences were observed among male–female combinations in relation to the percentage of hatched eggs ($F = 0.853$; $df = 3, 16$; $p = .485$), with a hatching rate that varied between 16% and 23%, as is indicated in bars in Figure 4a.

Overall, a total of 2,302 pupae were recovered from fruit exposed to different male–female combinations. Significant differences were found in the total number of pupae recovered per fruit among treatments ($F = 10.06$; $df = 3, 16$; $p < .001$) (Table 1).

With regard to the percentage of adults that emerged from pupae, no significant differences were observed among treatments

($\chi^2 = 1.553$; $df = 3$; $p = .670$) with an emergence range that varied between 67% and 75% (Table 1).

A total of 1,572 adults emerged out of 2,302 pupae collected from fruit. Overall, 49% of adults were males and 51% females. When total adults and sex were analysed with a two-way ANOVA, significant differences were found in the total number of emerged adults among treatments ($\chi^2 = 435.30$; $df = 3$; $p < .001$), but not in sexual proportion ($\chi^2 = 0.247$; $df = 1$; $p = .619$). No significant interaction was detected between factors ($\chi^2 = 7.039$; $df = 3$; $p = .071$) (Table 1).

3.2 | Veracruz vs. Chiapas

3.2.1 | Pre-zygotic tests

A total of 124 copulation events were recorded between *A. obliqua* populations over all replicates. No significant difference in the frequency of mate choice was observed among different male–female combinations of *A. obliqua* populations ($\chi^2 = 1.221$; $df = 3$; $p = .075$) (Figure 3b). No significant difference was observed in the duration of copulations ($\chi^2 = 2.121$; $df = 3$; $p = .548$) (Table 2). The overall measure of sexual isolation (Ipsi index), considering the frequency of copula for all possible combinations, revealed no sexual isolation between adults from different populations (Ipsi = 0.0294, $SD = 0.0916$, $p = .764$). These results also suggest the absence of geographically based mating preferences within either the Veracruz or a Chiapas population.

3.2.2 | Post-zygotic tests

The fertility analysis conducted among different male–female mating combinations of *A. obliqua* showed no significant differences in the total number of eggs laid by five couples in cages ($F = 0.967$, $df = 3, 24$; $p = .425$) (Table 2). However, significant differences were observed among male–female combinations in relation to the percentage of hatched eggs ($F = 4.26$; $df = 3, 24$; $p = .015$), with a hatching rate that varied between 11% and 38%, as is indicated in Figure 4b.

Overall, a total of 1,428 pupae were recovered from fruit exposed to different male–female combinations. No significant differences were found in the total number of pupae recovered per fruit among treatments ($F = 3.24$; $df = 2.47$; $p = .087$) (Table 2).

With regard to the number of adults that emerged from pupae, significant differences were observed among treatments ($\chi^2 = 185.71$; $df = 3$; $p < .001$) with greater numbers of adults emerging from heterotypic combinations involving females from Veracruz. From a total of 988 adults that emerged out of 1,428 pupae collected from fruit, overall 505 (51.12%) were males and 483 (48.88%) females. When total adults and sex were analysed, no significant differences were found in the percentage of emerged adults among treatments ($\chi^2 = 185.78$; $df = 3$; $p < .001$), but not in the sexual proportion ($\chi^2 = 0.48980$; $df = 1$; $p = .484$) (Table 2).

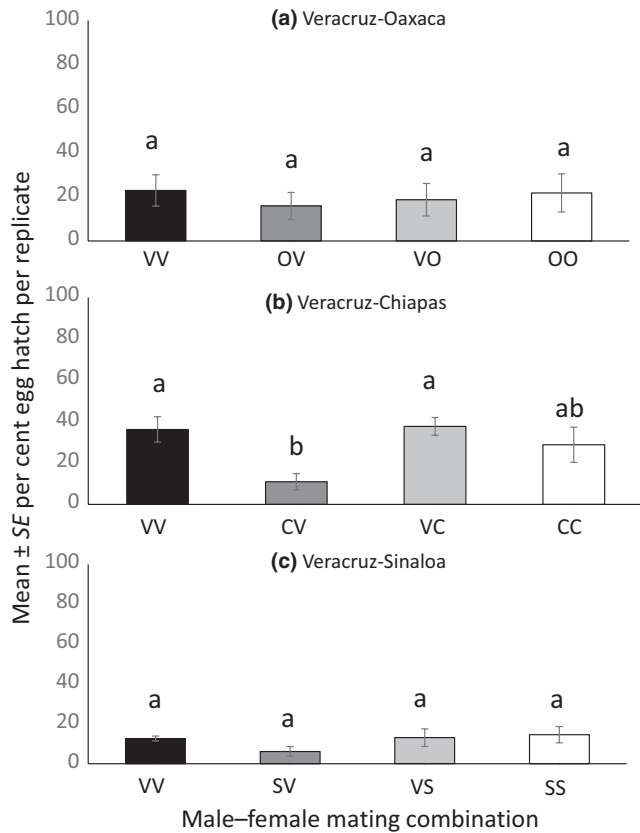


FIGURE 4 Mean (±SE) per cent egg hatch of eggs recovered from 3-L Plexiglass cages with four and five couples of all possible male–female mating combinations of sexually mature adult *Anastrepha obliqua* from (a) Veracruz and Oaxaca, (b) Veracruz and Chiapas and (c) Veracruz and Sinaloa

3.3 | Veracruz vs. Sinaloa

3.3.1 | Pre-zygotic tests

An overall of 55 copulation events were recorded between *A. obliqua* populations. Despite this lower number of copulations observed when compared with previous experiments, no significant difference in the frequency of mate choice was observed among different male–female combinations ($\chi^2 = 5.741$; $df = 3$; $p = .125$) (Figure 3c). No significant difference was observed in the duration of copulations ($\chi^2 = 4.227$; $df = 3$; $p = .238$) (Table 2), but it is noteworthy that even when no statistical differences were found, a longer duration was observed in the homotypic matings of the Veracruz population. The overall measure of sexual isolation (Ipsi index), considering the frequency of copula for all possible combinations, revealed no sexual isolation between adults from different populations (Ipsi = 0.151; $SD = 0.145$; $p = .317$). These results suggest the absence of geographically based mating preferences within either the Veracruz or the Sinaloa population.

3.3.2 | Post-zygotic tests

The fertility analysis conducted with agar spheres showed no significant differences in the total number of eggs laid by five couples in

cages ($F = 0.451$, $df = 3, 28$; $p = .718$) (Table 2) nor in the percentage of hatched eggs ($\chi^2 = 2.540$; $df = 3$; $p = .468$) (Figure 4c).

A total of 600 pupae were recovered from fruit exposed to different male–female combinations. No significant differences were found in the total number of pupae recovered per fruit among treatments ($X^2 = 1.77$; $df = 3$; $p = .622$).

With regard to the percentage of adults that emerged from pupae, no significant differences were observed among treatments ($\chi^2 = 0.760$; $df = 3$; $p = .859$). A total of 571 adults emerged out of 600 pupae collected from fruit, with an overall 287 (50.26%) males and 284 (49.74%) females. No significant differences were found in the total number of emerged adults among treatments ($\chi^2 = 1.800$; $df = 3$; $p = .615$), nor in sexual proportion ($\chi^2 = 0.016$; $df = 1$; $p = .901$) (Table 2).

4 | DISCUSSION

Mating discrimination and hybrid viability tests can be a powerful tool for evolutionary studies and species delimitation. Previous reviews of data on 119 pairs of closely related *Drosophila* with known genetic distances concluded that mating discrimination and the unviability or sterility of hybrids evolve gradually over time (Coyne & Orr, 1989, 1997). Data from crossing experiments and behavioural observations among 14 of 28 genetically distinct *Bemisia tabaci* types revealed a consistent pattern of reproductive isolation among putative species (Liu et al., 2012). A similar outcome has been obtained for cryptic species of the *fraterculus* species complex where all tested morphologically and genetically distinct type pairs (putative species) have shown some form of reproductive isolation, both at the pre-zygotic and post-zygotic levels (Cáceres et al., 2009; Devescovi et al., 2014; Roriz et al., 2017; Rull et al., 2013; Selivon et al., 1999; Vaničková, Hernández-Ortiz et al., 2015; Vera et al., 2006). Conversely, three of the four described species in the *Bactrocera dorsalis* complex, a serious economic problem for fruit production in Asia and Africa, were fused in a single taxon following review of taxonomic, genetic and reproductive compatibility data (Schutze et al., 2015). Also, morphologically and genetically similar populations of *A. fraterculus* in Northern Argentina and Southern Brazil, separated by more than 450 km, were found to be fully compatible (Rull, Abraham et al., 2012). Our results suggest that the Chiapas, Oaxaca and Sinaloa populations are the same biological entity as populations from Veracruz. The Jalcomulco population of our study was collected very near the Actopan site in Veracruz, reported by Ruiz-Arce et al. (2012) as the Mesoamerican mitochondrial type, our Sinaloa population is well within the area where the Western Mexican type was identified, while the Chiapas populations could belong to the Central American type. In any case, the degree of reproductive compatibility among these types is much greater than the one exhibited by several morphotypes of *A. fraterculus* (Cáceres et al., 2009; Devescovi et al., 2014; Roriz et al., 2017; Rull et al., 2013; Selivon et al., 2005), suggesting, in agreement with Scally et al. (2016), that at least in Mexico, these types do not represent cryptic species.

TABLE 2 Mating duration, number of eggs laid in agar spheres, percentage of emerged adults in mango fruits, number of adults emerged and sexual proportion to different male–female combinations for two *Anastrepha obliqua* populations from Veracruz and south Chiapas (a) and Veracruz and Sinaloa (b)

Variable	(a) Veracruz-Chiapas south populations		(b) Veracruz-Sinaloa populations	
	Mating combinations	Mean ± SE	Mating combination	Mean ± SE
Duration of copula (min)	♂Veracruz-♀Veracruz	42.5 ± 3.6 a	♂Veracruz-♀Veracruz	48.6 ± 4.8 a
	♂Chiapas-♀Veracruz	38.2 ± 3.7 a	♂Sinaloa-♀Veracruz	38.9 ± 6.9 a
	♂Veracruz-♀Chiapas	35.8 ± 2.9 a	♂Veracruz-♀Sinaloa	31.3 ± 3.5 a
	♂Chiapas-♀Chiapas	36.3 ± 3.6 a	♂Sinaloa-♀Sinaloa	41.0 ± 6.7 a
No. of eggs in agar spheres (5 couples)	♂Veracruz-♀Veracruz	147.6 ± 12.6 a	♂Veracruz-♀Veracruz	200.0 ± 39.2 a
	♂Chiapas-♀Veracruz	138.1 ± 15.8 a	♂Sinaloa-♀Veracruz	163.4 ± 34.9 a
	♂Veracruz-♀Chiapas	128.6 ± 14.2 a	♂Veracruz-♀Sinaloa	146.4 ± 35.5 a
	♂Chiapas-♀Chiapas	116.0 ± 11.9 a	♂Sinaloa-♀Sinaloa	190.6 ± 37.1 a
	♂Veracruz-♀Veracruz	71.0 ± 17.1 a	♂Veracruz-♀Veracruz	14.0 ± 7.0 a
	♂Chiapas-♀Veracruz	66.9 ± 17.5 a	♂Sinaloa-♀Veracruz	17.6 ± 7.6 a
	♂Veracruz-♀Chiapas	32.3 ± 7.3 a	♂Veracruz-♀Sinaloa	14.1 ± 6.7 a
	♂Chiapas-♀Chiapas	33.9 ± 6.9 a	♂Sinaloa-♀Sinaloa	29.1 ± 15.0 a
Number of F1 pupae in mango fruits	♂Veracruz-♀Veracruz	27.4 ± 4.9 a	♂Veracruz-♀Veracruz	77.5 ± 12.9 a
	♂Chiapas-♀Veracruz	23.5 ± 5.3 ab	♂Sinaloa-♀Veracruz	60.4 ± 17.7 a
	♂Veracruz-♀Chiapas	9.3 ± 2.2 b	♂Veracruz-♀Sinaloa	61.82 ± 18.1 a
	♂Chiapas-♀Chiapas	10.7 ± 1.9 b	♂Sinaloa-♀Sinaloa	59.36 ± 17.4 a
Sexual proportion/total treatment (male–female)	♂Veracruz-♀Veracruz	(M) 27.7 ± 7.8 a	♂Veracruz-♀Veracruz	(M) 6.1 ± 3.2 a
	♂Chiapas-♀Veracruz	(F) 27.1 ± 6.8 a	♂Sinaloa-♀Veracruz	(F) 6.6 ± 3.7 a
	♂Chiapas-♀Veracruz	(M) 26.7 ± 10.0 a	♂Sinaloa-♀Veracruz	(M) 9.5 ± 3.9 a
	♂Chiapas-♀Chiapas	(F) 20.3 ± 4.3 a	♂Sinaloa-♀Sinaloa	(F) 7.4 ± 3.3 a

Means followed by a similar letter are not significantly different after comparison among treatments (ANOVA, Tukey's HSD, $p > .05$).

In order to establish or dispel the existence of an *A. obliqua* cryptic species complex, examining reproductive isolation among South American populations, in particular from Peru, could yield conclusive results. Peruvian *A. obliqua* appears to be consistently different from samples of other North, Central and South American regions both at the morphological (Castañeda, Selivon, Hernández-Ortiz, Soto, & Canal, 2015) and the molecular levels (Ruiz-Arce et al., 2012; Scally et al., 2016). An analysis of reproductive compatibility of the Peruvian type against the Western Mexican and/or Eastern Brazilian types should greatly contribute to clarify the integrity of *A. obliqua* as a widespread Neotropical species or as a complex potentially composed of two or three cryptic species.

Our results leave some open questions. The first one is how the Oaxaca population maintains its integrity in the face of gene flow from the southern Chiapas and Central American type populations. There is no apparent barrier to movement between members of these two demes, and in our opinion no obvious environmental differences between the habitats they occupy. It is also difficult to understand why and how these two populations diverged. In fact, the Mexican morphotype of the *A. fraterculus* species complex extends from northern Mexico to Panama (Hernández-Ortiz et al., 2004, 2012), and Mexican fruit fly (*Anastrepha ludens*) populations, from Mexico to Central America appear to be relatively homogeneous

(Ruiz-Arce, Owen, Thomas, Barr, & McPherson, 2015). Perhaps, as suggested by Scally et al. (2016), introgression, particularly between *A. obliqua* and *A. fraterculus*, may be one explanation for the discrepancy and the high mitochondrial diversity reported for *A. obliqua* could be the result of incomplete lineage sorting. These two species have been found to mate and interbreed under artificial conditions (Rull et al., 2017; Santos et al., 2001) and could potentially hybridize in nature.

Another question is raised by the low levels of egg hatch recorded among *A. obliqua* populations and the asymmetric reduction in fertility between the Veracruz-Chiapas hybrids. These patterns suggest the existence of one or several *Wolbachia* strains harboured by different *A. obliqua* populations. Similar post-zygotic isolation patterns have been found between cryptic species in the *A. fraterculus* complex (Devescovi et al., 2014), which harbour different strains of *Wolbachia* (Prezotto, Perondini, Hernández-Ortiz, Marino, & Selivon, 2017; Selivon et al., 2002, 2005). Exploring the existence of the bacteria and characterizing its different strains among *A. obliqua* populations are important since it could facilitate colonization, strain refreshment, efficient mass rearing for application of the SIT, and eventually lead to the development of the incompatible insect technique (IIT) (Zhang, Lees, Xi, Bourtzis, & Gilles, 2016).

Sporadic failures of the screwworm eradication programme in Mexico were attributed to the existence of cryptic species and mating types exhibiting pre-zygotic isolation (Richardson & Averhoff, 1982). In some cases, however, eradication of Western Mexican populations was achieved using a southern strain because the only reproductive barrier between the two populations was a reduction in hybrid male viability uncoupled from differences in mating behaviour (Mangan, 1986). A similar case could be occurring within *A. obliqua*. However, the lack of systematic studies on screwworm "ecotype" mating compatibility generated a controversial debate that is currently unresolved (Klassen & Curtis, 2005). From a management perspective, our study implies that SIT can be applied in Mexico over a large area encompassing coastal plains and mid-elevation areas facing the Gulf of Mexico, the entire Yucatan peninsula and the Pacific coastal plain from Guatemalan border to Sinaloa using a single mass rearing strain. These regions include all important mango-growing areas in the country, Actopan in Veracruz, Tapanatepec-Chahuities in Oaxaca, the Soconusco in Chiapas and all regions of Guerrero, Michoacán, Nayarit and Sinaloa.

Despite the advent and development of accurate and cheap molecular tools, conventional simple crossing experiments can be very informative and contribute to delimiting species boundaries, the evolution of reproductive isolation, environmental factors that select for it, and the geographical range of distribution of diverging taxa. From a management perspective, it is important to establish if *A. obliqua* is a cryptic species complex across its entire Neotropical range and perform a comprehensive study of its genetic structure in Mexico. Overall, the *fraterculus* species group appears to be another excellent candidate group within Tephritidae for the study of speciation.

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AUTHOR CONTRIBUTION

Study design and drafting of the article were done by JR, RL and ET. Obtention of biological material, data collection and analysis were done by ET, RL, FDF, JA and MA, and funding was secured by JR, RL and MA. Critical revision of the study and of the article was done by all authors.

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