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Original article

Experimental hybridization and reproductive isolation between two sympatric species of tephritid fruit flies in the *Anastrepha fraterculus* species group

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Abstract Among tephritid fruit flies, hybridization has been found to produce local adaptation and speciation, and in the case of pest species, induce behavioral and ecological alterations that can adversely impact efficient pest management. The *fraterculus* species group within *Anastrepha* (Diptera: Tephritidae), is a rapidly radiating aggregate, which includes cryptic species complexes, numerous sister species, and several pest species. Molecular studies have highlighted the possibility of introgression between *A. fraterculus* and *A. obliqua*. Reproductive isolation has been studied among morphotypes of the *A. fraterculus* species

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complex as a tool for species delimitation. Here we examined the existence and strength of prezygotic and postzygotic isolation between sympatric populations of two closely related species within the highly derived *fraterculus* group (*A. fraterculus* and *A. obliqua*), coexisting in nature. Although adults of both species showed a strong tendency for assortative mating, a small proportion of hybrid pairings in both directions were observed. We also observed asymmetric postzygotic isolation, with one hybrid cross displaying a strong reduction in fecundity and F1 egg fertility. Survival was greater for the progeny of homotypic and hybrid crosses in the maternal host. There was a marked female biased sex ratio distortion for both F1 hybrid adults. Hybridization between *A. fraterculus* and *A. obliqua* in nature may be difficult but possible; these two species display stronger reproductive isolation than all pairs of species previously examined in the *A. fraterculus* species complex. Asymmetric postzygotic isolation is suggestive of *Wolbachia* mediated cytoplasmic incompatibilities that may be exploited in area-wide pest management.

Key words *Anastrepha obliqua*; *Anastrepha fraterculus*; prezygotic isolation; postzygotic isolation; Haldane's rule; introgression

Introduction

Tephritid fruit flies in the temperate genus *Rhagoletis* have been a model system for the study of speciation and supported a long and heated debate on the plausibility and frequency of sympatric speciation as a mode of divergence among phytophagous insects (Berlocher & Feder, 2002). With the advent of molecular techniques, research on fruit flies advanced our understanding of mixed models of speciation, in particular those involving speciation with gene flow (Feder *et al.*, 2003). Experimental hybridization studies and molecular data revealed that several *Rhagoletis* species in the *pomonella*, *suavis*, and *cingulata* species

groups potentially interbreed, leading in some cases to speciation and adaptive introgression (Schwarz & McPheron, 2007; Rull *et al.*, 2010; 2012; Tadeo *et al.*, 2015; Arcella *et al.*, 2015). In general, many more animal species are likely exchanging genes than has been previously appreciated, with some groups exhibiting greater frequency of introgression than others (Mallet *et al.*, 2016). This could represent an overlooked factor in speciation and rapid radiation that merits further scrutiny.

The *fraterculus* species group, the most derived within the genus *Anastrepha*, composed of at least 29 species, is a subtropical group where cryptic species and several sister species have been identified (Norrbom *et al.*, 2000; Zucchi, 2000). Both *A. fraterculus* and *A. obliqua* belong in the *fraterculus* group. Molecular studies on the *fraterculus* species group have faced difficulties in definition and placement of some species (Smith-Caldas *et al.*, 2001). In the particular case of *A. fraterculus*, integrative multidisciplinary approaches have allowed identification of up to eight cryptic species, among which, the Mexican form ranges from southern US to Panama (Hernández-Ortiz *et al.*, 2012; Rull *et al.*, 2013; Dias *et al.*, 2016). Based on the results of mitochondrial sequences, Ruiz-Arce *et al.* (2012) identified six different genetic *A. obliqua* types across America that raised the possibility that more than one cryptic species complex exists in the *frateculus* group. However, a recent study using nuclear and mitochondrial multilocus data suggests that gene flow and introgression between species (*A. fraterculus* × *A. obliqua*) may better explain the high mitochondrial diversity previously detected across *A. obliqua* populations (Scally *et al.*, 2016). In Mexico, *A. obliqua* and *A. fraterculus* widely overlap in distribution across the Sierra Madre Occidental and the Sierra Madre Oriental, in areas at mid elevation (600 – 1000 m altitude) along both the Pacific and the Atlantic (Gulf of Mexico) coasts where their respective main host plants (*Spondias* spp. and Mango in the case of *A. obliqua*, and Guava in the case of *A. fraterculus*) also overlap (Sivinski *et al.*, 2000). These two species share at least 13 components of their

complex sexual pheromones that consist of as many as 40 compounds (Fig. 1 and Table S1). Therefore, females of both species could be attracted to lekking sites, where males of one species are calling in areas where populations overlap.

Although interbreeding can result in homogenization among species and the disappearance of species boundaries, in the case of the *fraterculus* species group Based on pheromone composition and behavioral responses of hybrids between two morphotypes of the *fraterculus* cryptic species complex, hybridization has been postulated as a possible mode of divergence among species in the *fraterculus* species group (Cáceres *et al.*, 2009; Segura *et al.*, 2011). By combining previously isolated gene pools, interspecific hybridization can result in the origin of new genotypes and rapid long-lasting changes among interbreeding species (Schwenk *et al.*, 2008). Interspecific hybridization could not only contribute in explaining rapid divergence in radiating species groups, but in the case of tephritid fruit flies, which are of major economic importance, it could have practical implications on the application of management tools, such as the sterile insect technique. Interspecific hybridization can alter behavior, pheromone composition, host plant utilization and adaptation to novel environments (Cáceres *et al.*, 2009; Segura *et al.*, 2011; Oroño *et al.*, 2013; Arcella *et al.*, 2015), all of which may impact the efficiency of pest management programs.

Reproductive isolation among different morphotypes of the *fraterculus* cryptic species complex has been examined in some detail due to the implications for potential application of the sterile insect technique and regulation concerning international trade in agricultural products, particularly fruits and vegetables (Hendrichs *et al.*, 2015). Aluja *et al.* (2009) found full mating compatibility among geographically distant and ecologically distinct populations of *A. ludens* from Mexico, while Dos Santos *et al.* (2001) reported postzygotic isolation and sex ratio distortion among *A. fraterculus*, *A. sororcula* and *A. obliqua*. Overall,

populations of the same species appear to freely interbreed, cryptic species exhibit strong prezygotic isolation and asymmetric postzygotic isolation, and sister species forced to mate under laboratory conditions show reduced hybrid fitness and sex ratio distortion. In all cases, hybridization appears possible, although no formal test on prezygotic isolation has been performed between *A. fraterculus* and any closely related sister species.

Experiments testing reproductive compatibility among species with different taxonomical status in the *fraterculus* species group may allow estimates of potential and extent of interspecific introgression. Additionally, correlating genetic distance with the strength of reproductive isolation has been used as an approach to analyze the time course of speciation in *Drosophila* (Coyne & Orr, 1997). As such, building a molecular and behavioral data-base for the *fraterculus* species group could facilitate a similar analytical approach. Finally, a comparison of the strength of reproductive isolation among pairs differing in the degree of differentiation such as populations, cryptic species, sister species and non- cryptic species in the group could serve as a tool for resolving the status and delimiting the extent of distributional ranges of cryptic species complexes of economic importance.

Here, we test for potential hybridization between sympatric Mexican populations of *A. fraterculus* and *A. obliqua*, two morphologically similar (males cannot be distinguished without examining the aedagus) and closely related species in the *fraterculus* species group. To build a comparative database we adopt a methodological approach previously used (see Rull *et al.*, 2013) to estimate the degree of prezygotic and postzygotic isolation between populations and cryptic species and populations within the *fraterculus* species group.

Materials and methods

Biological Material

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For prezygotic isolation tests, adults of *A. obliqua* were recovered during August 2015 from infested *Spondias mombin* L. near the locality of Jalcomulco, Veracruz ($19^{\circ}19'37.54''$ N; $96^{\circ}45'25.91''$ W, 365 m altitude), whereas adults of *A. fraterculus* were recovered from infested *Psidium guajava* L. at las Animas in Xalapa, Veracruz ($19^{\circ}31'56.06''$ N; $96^{\circ}53'31.46''$ W, 1322 m altitude). For postzygotic isolation tests, adults of *A. obliqua* were recovered during the first week of November 2015 from *Spondias purpurea* L. fruit near Tuzamapan, Veracruz ($19^{\circ}24'50.80''$ N; $96^{\circ}52'05.64''$ W, 938 m altitude), whereas *A. fraterculus* was recovered from *P. guajava* L. near San Marcos de León, Veracruz ($19^{\circ}25'07.42''$ N; $96^{\circ}58'30.36''$ W, 1183 m altitude).

Collected fruit were taken to the laboratory at the Instituto de Ecología A.C. in Xalapa, Veracruz, and processed following methods described in Rull *et al.* (2006). Recovered pupae were placed over a thin layer of vermiculite in 200 mL plastic containers and were regularly moistened with a 0.2% sodium benzoate solution to allow adult emergence. Flies were maintained at a 26 ± 1 °C temperature, a 13 : 11 light : dark photoperiod and 65% relative humidity. At emergence, adults were counted and sorted according to species, sex and age in plastic cages (3 L volume) covered with mosquito mesh on the upper side. No more than 30 flies were kept in a single cage to reduce wing damage to adults. Flies were fed with a mixture (3:1) of sugar and hydrolyzed yeast protein and provided with water until sexually mature (20 days).

Prezygotic isolation tests

Prezygotic isolation tests were run simultaneously in six experimental cages of $0.6 \times 0.6 \times 1$ m covered with white tulle cloth of 1 mm mesh (Fig. 2). While such cages are

smaller than field cages recommended by USDA/IAEA (2003) for SIT mating compatibility tests, in our experience, when 20–30 fly couples of each species/population/strain are observed, they offer ample space for female rejection to occur without restriction. In the center top of each cage, a white light lamp (960 lm) was hung at a distance of 150 mm above the cage ceiling. Each cage contained one 0.9 m tall mango sapling, *Mangifera indica* L., with 10–12 leaves to provide opportunity for fly resting and mating during the experiment . The tests were run indoors in order to ensure a temperature of at least 26 °C under which flies are less active. The evening before tests, flies were marked on the back of the thorax with a small spot of acrylic paint (Vinci de México, S.A. de C.V., Mexico City, Mexico) to easily distinguish species during the experiment. Colors were initially assigned at random and then alternated between species during subsequent replicates. Previous studies indicated that this type of mark does not interfere with fly sexual activity or behavior (Rull *et al.*, 2013). Ten pairs of sexually mature adults from 20 to 30 days of age of both species were released inside each cage. Flies were introduced in cages from 07:30 – 07:50 h, several minutes before observations began. Based on Aluja *et al.* (2000a), fly observations started at 08:00 h and ended at 13:00 h, a period that covered the morning calling period of *A. obliqua* (Aluja & Birke, 1993) and the peak calling period of the Mexican form of *A. fraterculus* (Rull *et al.*, 2013). The variables recorded during observation were the choice of partner for mating according to species and the duration of copulation in minutes. When flies began to copulate, the pair was gently removed, from the experimental cage by means of a 35 ml glass vial where they were maintained until the end of the copulation. Couples were removed to prevent other flies from disturbing them. Six cages were observed over each of four days, totaling 24 cages.

Postzygotic isolation tests

Five sexually mature virgin males and females were placed in plastic cages (3 L volume, as described above) to include all homotypic and heterotypic male-female combinations (*A. fraterculus* ♂ × *A. fraterculus* ♀; *A. fraterculus* ♂ × *A. obliqua* ♀; *A. obliqua* ♂ × *A. fraterculus* ♀; *A. obliqua* ♂ × *A. obliqua* ♀). Cages were maintained under similar laboratory conditions, and flies were provided with water and food. Two days after the introduction of couples in the cages, an artificial spherical device made with a mixture of agar and diet (Freeman & Carey, 1990) (3.19% agar, 96.32 % water, 0.30% yeast protein, 0.13% sugar, 0.024% Nipagin and 0.01% sodium benzoate, 0.03% green food coloring) was introduced in each cage to serve as an oviposition substrate. Agar spheres with diet were used, because previous efforts to recover hybrids using standard agar spheres for egg recovery and carrot diet for larval rearing (see Rull *et al.*, 2013) had yielded poor results. Agar spheres with diet were found to enhance egg laying by wild females of both species during preliminary tests. Spheres with diet were replaced every other day (three times) so that a total of three spheres were exposed over a week in each cage. All eggs were carefully extracted using a scalpel under a binocular microscope, counted and aligned over a black cloth placed over a moist piece of cotton in a 90 × 15 mm Petri dish. After five days, eggs were observed and the number of hatched eggs recorded. Eight replicates were run for each male-female mating combination.

After exposure to agar spheres, a mango and four guavas (roughly equivalent in weight) were introduced into cages to recover F1 progeny.. Fruit was left in cages for six days, removed and placed individually in plastic cylindrical containers (70 mm high × 150 mm in diameter with a capacity of 800 mL) lined with a fine layer of vermiculite according to the mating combination of its cage of origin. Three weeks later, fruit were inspected to verify the absence of slow developing larvae and the total pupae recovered per fruit were counted

and placed into 35 mL plastic cups with vermiculite to wait for adult emergence. Pupae were moistened regularly as described above. The number and sex of emerged adults according to mating combination per fruit type were recorded and when possible wing length of the F1 progeny was measured (with the exception of *A. obliqua* × *A. fraterculus* males that could not be recovered for measurements).

Statistical analysis

To analyze mating partner choice, the frequency of choice for each male-female combination between species was analyzed by fitting a GLZ with a Poisson distribution error. The copula duration for each mating combination was analyzed using a one-way ANOVA with a similar error distribution. Overall degree of sexual isolation was established by calculating the Ipsi index defined by Rolán-Alvarez & Caballero (2000), which ranges from -1 to +1 with 0 = random mating, using Jmating v1.0.8 software (Carvajal-Rodríguez & Rolán-Alvarez, 2006). The total number of eggs recovered in artificial fruit per cage and the percentage of hatched eggs were analyzed using one-way ANOVA. The total number of pupae recovered from fruit exposed to different male-female combinations was analyzed by fitting a GLZ with a Poisson distribution error. A three-way ANOVA was run to evaluate the mean number of adults emerged according to sex and fruit. Adult F1 wing size was compared according to fruit origin and sex among the progeny of homotypic crosses and *A. fraterculus* × *A. obliqua* by fitting a GLZ with a Poisson distribution error. Post-Hoc tests were performed using Tukey HSD, transforming the data into ranges for the GLZs. All analyses were performed using Statistica v7 (Stat Soft Inc.) and graphed using SigmaPlot v10.0 (Jandel Scientific, 1992) the alpha value considered for significance was 0.05.

Results

Prezygotic isolation tests

A total of 105 copulations were observed in 24 cages over four days. Of these, 36 where between *A. obliqua* × *A. obliqua* , 14 between *A. obliqua* males × *A. fraterculus* females, one between an *A. fraterculus* male × and *A. obliqua* female , and 54 between *A. fraterculus* × *A. fraterculus* . There were significant differences in mating frequencies

according to mating combination ($\chi^2(3) = 33.215, P < 0.05$) with the cross of *A. fraterculus* males and *A. obliqua* females occurring at lower frequencies than both homotypic mating combinations (Table. 1). There were no significant differences in mating duration according to mating combination ($\chi^2(3) = 1.070, P = 0.784$) (Table 1). The Ipsi value was 0.7634 which was indicative of assortative mating between species (SD = 0.0514, $P < 0.05$), indices of asymmetry (IApsi: aa/bb 1.37, SD = 0.22, $P = 0.066$ and ab/ba 0.12, SD = 0.12, $P < 0.05$).

Postzygotic isolation tests

There were significant differences in fecundity in cages according to mating combination ($F_{3,28} = 8.7987, P < 0.05$). The homotypic cross of *A. fraterculus* × *A. fraterculus* yielded significantly more eggs per cage than any other mating combination (Fig. 3A). With respect to fertility, there were also significant differences in percent egg hatch according to mating combination ($\chi^2(3) = 13.574, P < 0.05$). A significantly higher proportion of eggs hatched in the homotypic cross of *A. obliqua* × *A. obliqua* than from any other mating combination, whereas the lowest prevalence of egg hatching was observed in the heterotypic cross of *A. fraterculus* × *A. obliqua* (Fig. 3B).

There were also significant differences in the number of F1 adults recovered from fruit exposed in cages according to mating combination ($\chi^2(3) = 10.753, P < 0.05$). The homotypic cross of *A. obliqua* yielded the greatest number of adults followed by the homotypic cross of *A. fraterculus* and both heterotypic crosses (Fig. 4). In addition, there were significant differences in the number of adults recovered from different adult mating combinations according to fruit ($\chi^2(3) = 32.109, P < 0.05$). Mating combinations involving *A. obliqua* females yielded significantly more adults in mango while those involving *A. fraterculus* females performed better in guava. For heterotypic mating combinations the

number of emerged males was significantly lower than for homotypic combinations when considering the total number of emerged flies ($\chi^2(9) = 19.551, P = 0.0209$) (Fig. 5).

Finally, there were significant differences in sex ratios among different mating combinations ($\chi^2(9) = 19.551, P < 0.05$) with heterotypic crosses yielding highly female biased progeny (Fig. 6). The mean (\pm S.E.) wing length of hybrid F1 progeny of the *A. fraterculus* \times *A. obliqua* (6.51 ± 0.07 mm) and *A. obliqua* \times *A. obliqua* (6.64 ± 0.1 mm) crosses were significantly longer than homotypic *A. fraterculus* (6.24 ± 0.07 mm) ($F_{2,96} = 5.653, P < 0.05$). Adults reared from mango (6.94 ± 0.07 mm) were larger than those reared from guava (5.98 ± 0.06 mm) ($F_{1,96} = 88.21, P < 0.05$). There was no significant effect of sex on wing length ($F_{1,96} = 0.11, P = 0.74$) the only significant interaction was recorded between sex and fruit with females developing in guava being slightly smaller than males and those reared from mango larger than males ($F_{1,96} = 4.252, P < 0.05$).

Discussion

Anastrepha fraterculus and *Anastrepha obliqua* exhibited a strong tendency towards assortative mating in large cages in the laboratory. However, hybrid matings in both directions were observed, suggesting that interspecific matings between these two species may occur in nature. In general, *A. obliqua* females mated less frequently than *A. fraterculus* females and there were no differences in mating duration among hybrid and homotypic pairs. Despite the fact that prezygotic isolation between these two species is strong, it is not complete. In small enclosures, all mating combinations produced eggs. Nevertheless, the hybrid cross of *A. fraterculus* males \times *A. obliqua* females produced fewer eggs that hatched in lower proportions than any other mating combination. Larvae derived from mating

combinations involving *A. obliqua* females developed better in mango, while those involving *A. fraterculus* females developed better in guava. Both hybrid mating combinations resulted in markedly female biased F1 progeny, while the progeny of both homotypic crosses was close to a 1:1 sex ratio. In sum, hybridization between *A. fraterculus* and *A. obliqua* in nature is likely to be rare, yet is not impossible.

All studied tephritid fruit fly species in the genus *Anastrepha* exhibit a lek-based mating system in which males aggregate in mating arenas and release a sex pheromone that attracts females (Aluja *et al.*, 2000a). Within leks, males perform an elaborate courtship ritual that involves wing displays and the production of acoustic signals (Webb *et al.*, 1984). We did not test for cross responses of females and males to homotypic or heterotypic male produced pheromone, which could represent an additional prezygotic mating barrier to gene flow, yet in close proximity, our results indicate that hybridization between *A. fraterculus* and *A. obliqua* is uncommon but not impossible. As shown in Figure 1, there are 13 common compounds in the sexual pheromone of these two species based on literature reports. It is therefore possible that females of the other species could be attracted to leks in areas where populations of both species coexist, particularly if the “leking tree” is also used for resting or is close to hosts or feeding sites as reported for *A. obliqua* by Aluja & Birke (1993). Prezygotic isolation was stronger for *A. fraterculus* males × *A. obliqua* females than for *A. obliqua* males × *A. fraterculus* females, perhaps because *A. fraterculus* males × *A. obliqua* females were less likely than *A. obliqua* males × *A. fraterculus* females to produce eggs and those eggs were less fertile, implying that there is stronger selection for mating discrimination to evolve among *A. obliqua* females than among *A. fraterculus*.

Fewer eggs were recovered from agar spheres in cages with *A. obliqua* females and *A. fraterculus* males than in cages with homotypic pairs of *A. obliqua*. The injection of

accessory gland fluids (without sperm) can induce egg laying in homotypic virgin females of some insect species including *Ceratitis capitata* (Larson *et al.*, 2012; Jang *et al.*, 1995). Egg laying induction, however, fails in heterotypic females, because many of these proteins are highly divergent between species (Larson *et al.*, 2012). Perhaps *A. fraterculus* ejaculates induced less egg laying in *A. obliqua* females. Alternatively, our results could imply that fewer females were successfully inseminated in hybrid than in homotypic cages and therefore did not engage in egg laying. Dissection of females mated with heterotypic and homotypic males and comparison of the numbers of mature eggs in the ovaries could resolve this issue. Such studies could be coupled with behavioral observations comparing mated female egg laying propensity across mating combinations.

In any case, egg hatch was lower for the heterotypic *A. fraterculus* × *A. obliqua* cross than for any other crosses, which represents strong asymmetric postzygotic isolation. By contrast, eggs from the *A. obliqua* × *A. fraterculus* cross hatched in greater proportions than the homotypic *A. fraterculus* × *A. fraterculus*. Asymmetric postzygotic isolation is common among other members of the *fraterculus* species group (Cáceres *et al.*, 2009; Rull *et al.*, 2013; Devescovi *et al.*, 2014). Such patterns could be explained by cytoplasmic incompatibility driven by the presence/absence of different strains of *Wolbachia*. In the *fraterculus* species group, *A. ludens* does not carry the bacterial symbiont (Martinez *et al.*, 2012), *A. suspensa* (Jeyaprakash & Hoy, 2000) and *A. obliqua* (Coscrato *et al.*, 2009) do, whereas cryptic species of *A. fraterculus* can display single and double strain infections (Selivon *et al.*, 2002; Cáceres *et al.*, 2009; Coscrato *et al.*, 2009; Marcon *et al.*, 2011). Novel strategies for area-wide pest management take advantage of *Wolbachia* induction of cytoplasmic incompatibility for the development of the incompatible insect technique (IIT) (Sadiraki & Bourtzis, 2010). Identifying *Wolbachia* strains in the populations involved in

this study could therefore contribute not only to explaining observed patterns of reproductive incompatibility, but has potential application for IIT-mediated area-wide pest management.

Another interesting finding of our study was the recovery of F1 adults from guavas exposed in cages with *A. obliqua* males × *A. fraterculus* females. This finding implies that because hybrid F1 larvae performed better in “novel” fruit than one of their homotypic parental species, host plant utilization could have a maternal heritable component. Under such a scenario, interspecific introgression could alter host use patterns and expand the host range of tephritid pest species. Such events could explain, for example, the ability of Mexican fruit flies (*A. ludens*), a species that primarily exploits native and introduced plants in the family Rutaceae, to exploit introduced plants in the Anacardiaceae, such as mango. More closely related to the topic addressed here, it could explain the rare infestation by *A. obliqua* of guavas under totally natural conditions in a clearly circumscribed region in Central Veracruz, Mexico (“La Víbora”, Tlalixcoyan [35 m above sea level]) where in single fruit, two *Anastrepha* species were recovered: *A. striata* and *A. obliqua* (Birke & Aluja, 2011). But in the same locality and neighboring ones, guavas are also infested by *A. fraterculus*, a species preferring highland habitats, over 800 m above sea level (Sivinski *et al.*, 2004). In another study, three-way infestations (*A. bahiensis*, *A. fraterculus* and *A. obliqua*) were detected in single *Myrciaria floribunda* (Myrtaceae) fruit, a key-stone species bridging periods of low host fruit prevalence for polyphagous/oligophagous *Anastrepha* species (Aluja *et al.*, 2000b). Thus, there is evidence that *A. obliqua* and *A. fraterculus* adults coincide in the field and may exhibit similar host use patterns.

There was a profound distortion of the sex ratio in the F1 progeny from hybrid matings in both directions. Hybrid progeny was strongly female biased, adhering to Haldane’s rule, “When in the F1 offspring of two different animal races one sex is absent, rare, or sterile, that sex is the heterozygous sex” (Haldane, 1922). Male F1 hybrids of two

different morphotypes of the *A. fraterculus* species complex (Peruvian x Argentinian morphotypes) produce a different blend of pheromone volatiles than either of the parental males (Cáceres *et al.*, 2009), and hybrid females appear to preferentially respond to hybrid pheromone blends (Segura *et al.*, 2011). These features were proposed to constitute a potential hybridization driven speciation mechanism in the *fraterculus* species group (Segura *et al.*, 2011). In the case of *A. obliqua* and *A. fraterculus*, such a mechanism is unlikely to function, given the scarcity of hybrid males. Additionally, for comparative purposes, we can conclude that postzygotic isolation is stronger between *A. obliqua* and *A. fraterculus* than among several morphotypes of the *A. fraterculus* cryptic species complex, among which sex ratio distortion has not evolved (Rull *et al.*, 2013; Devescovi *et al.*, 2014).

In conclusion we found that *A. fraterculus* and *A. obliqua* can potentially hybridize. Interspecific introgression could impact on pest management practices by altering sexual behavior and/or host plant use. Additionally, some of the observed patterns of postzygotic isolation suggest an interaction with *Wolbachia* symbionts that could be exploited for development of area-wide pest control strategies. Overall, reproductive isolation between *A. fraterculus* and *A. obliqua* was stronger than that documented for cryptic species of *A. fraterculus* using similar methodologies. This suggests that recent divergence in the *A. fraterculus* group is occurring within the *A. fraterculus* cryptic species complex with increasing strength of reproductive isolation evolving among more distant species. This conclusion could be verified or strengthened by further examination of reproductive isolation between species pairs in the *A. fraterculus* species group that vary in genetic distance.

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Disclosure

The authors declare that they have no any conflict of interest.

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Table 1. Total number, copulation frequency per replicate and mean duration (\pm S.E.) in minutes of copulations according to ♂♀ homotypic and heterotypic mating combinations among ten pairs of *Anastrepha obliqua* and ten pairs of *A. fraterculus* in experimental cages (N= 24 cages).

| ♂♀ Mating combination | <i>A. obliqua</i> × <i>A. obliqua</i> | <i>A. obliqua</i> × <i>A. fraterculus</i> | <i>A. fraterculus</i> × <i>A. obliqua</i> | <i>A. fraterculus</i> × <i>A. fraterculus</i> |
|---|---|--|--|---|
| Total number copulations | 36 | 14 | 1 | 54 |
| Frequencies of copulation. (Mean±S.E.) | 2.06 ± 0.50 ac | 0.82 ± 0.26 bc | 0.06 ± 0.60 b | 3.12 ± 0.57 a |
| Duration in minutes (Mean±S.E.) | 44.36 ± 3.30 a | 49.86 ± 11.42 a | 59.0 a | 43.72 ± 2.85 a |

Figure legends

Figure. 1 Sexual pheromone components shared between *Anastrepha obliqua* and *A. fraterculus* based on literature reports (Lima *et al.*, 2001; Santos, 2003; Brizová *et al.*, 2013; Bachman *et al.*, 2015; Milet-Pinheiro *et al.*, 2015; Ibañez-López and Cruz-López, 2001; López-Guillén *et al.*, 2008; López-Guillén *et al.*, 2011; Goncalves *et al.*, 2013). For further details, see Supplemental Table 1.

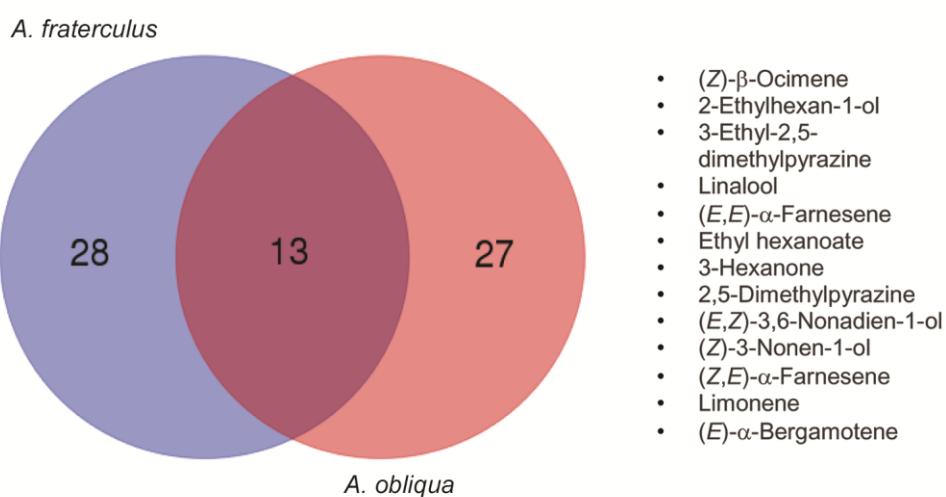


Figure. 2 Experimental $0.6 \times 0.6 \times 1$ m cages covered with white tulle cloth and used for prezygotic isolation tests among 10 pairs of sexually mature *Anastrepha obliqua* and *A. fraterculus*.



Figure. 3 A) Mean (\pm S.E.) number of eggs laid on three agar spheres over six days for 5 pairs of sexually mature flies according to mating combination (*A. fraterculus* ♂ \times *A. fraterculus* ♀; *A. fraterculus* ♂ \times *A. obliqua* ♀; *A. obliqua* ♂ \times *A. fraterculus* ♀; *A. obliqua* ♂ \times *A. obliqua* ♀); **B)** Mean percentage (\pm S.E.) of egg hatch for 5 pairs of sexually mature flies according to mating combination ($n = 8$).

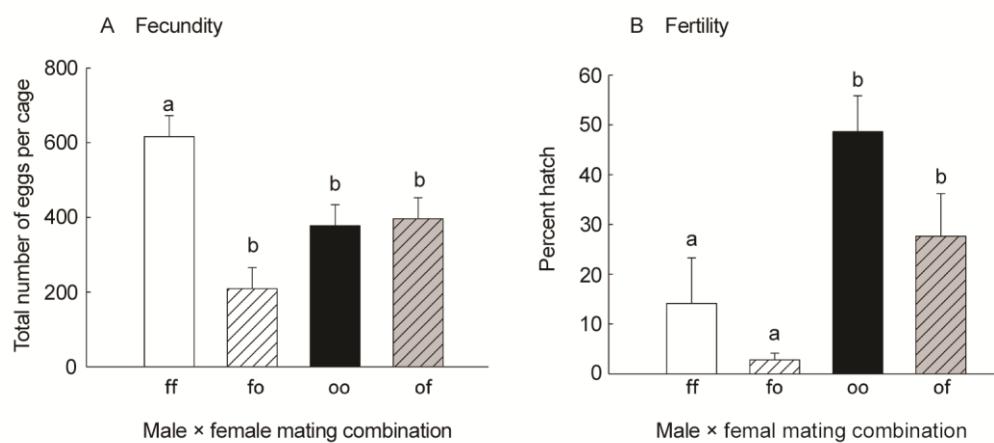


Figure. 4 Mean (\pm S.E.) number of eclosed F1 adults per cage from fruit (one mango and four guavas) exposed to 5 pairs of sexually mature flies according to mating combination (*A. fraterculus* ♂ \times *A. fraterculus* ♀; *A. fraterculus* ♂ \times *A. obliqua* ♀; *A. obliqua* ♂ \times *A. fraterculus* ♀; *A. obliqua* ♂ \times *A. obliqua* ♀) ($n = 8$).

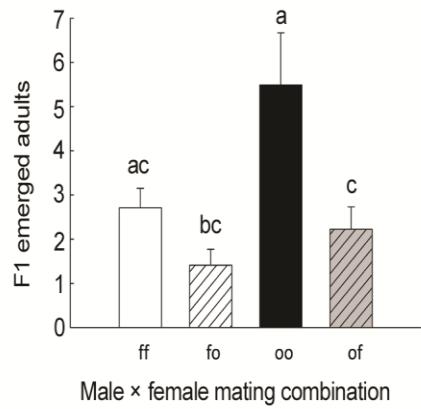


Figure. 5 Mean (\pm S.E.) number of eclosed F1 adults from fruit (one mango and four guavas) exposed to 5 pairs of sexually mature flies per cage according to mating combination and fruit type (guava or mango) (*A. fraterculus* ♂ \times *A. fraterculus* ♀; *A. fraterculus* ♂ \times *A. obliqua* ♀; *A. obliqua* ♂ \times *A. fraterculus* ♀; *A. obliqua* ♂ \times *A. obliqua* ♀) ($n = 8$).

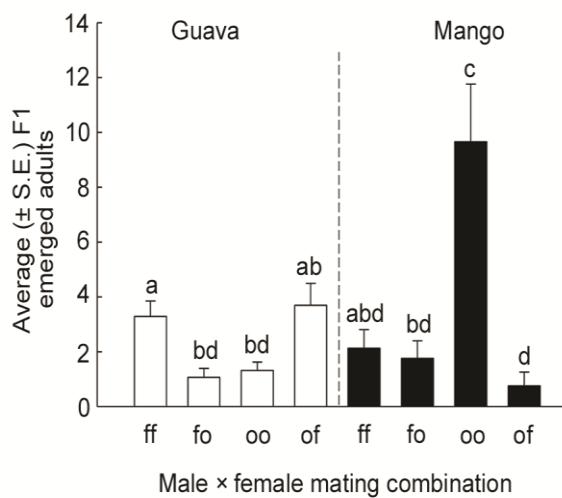


Figure. 6 Mean (\pm S.E.) number of eclosed male, female, and deformed F1 adults and average (\pm S.E.) number of recovered pupae per cage from fruit (one mango and four guavas) exposed to 5 pairs of sexually mature flies according to mating combination ($n = 8$).

