Original article

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

Juan Rull	extsuperscript{1}, Eduardo Tadeo	extsuperscript{2}, Rodrigo Lasa	extsuperscript{2}, Christian L. Rodríguez	extsuperscript{2}, Alma Altuzar-Molina	extsuperscript{2}, and Martín Aluja	extsuperscript{2}

	extsuperscript{1}PROIMI Biotecnología-CONICET, LIEMEN-División Control Biológico de Plagas, Av. Belgrano y Pje. Caseros, T4001MVB San Miguel de Tucumán, Tucumán, Argentina. 

	extsuperscript{2}Red de Manejo Biorracional de Plagas y Vectores, Instituto de Ecología, A.C., Xalapa, Veracruz 91070, México.

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...
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 *fraterculus* 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**

This article is protected by copyright. All rights reserved.
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°19'37.54" N; 96°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°31'56.06" N; 96°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°24'50.80" N; 96°52'05.64" W, 938 m altitude), whereas *A. fraterculus* was recovered from *P. guajava* L. near San Marcos de León, Veracruz (19°25'07.42" N; 96°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 × 0.6 × 1 m covered with white tulle cloth of 1 mm mesh (Fig. 2). While such cages are

This article is protected by copyright. All rights reserved.
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

This article is protected by copyright. All rights reserved.
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 \((A. \text{fraterculus} \, \text{♂} \times A. \text{fraterculus} \, \text{♀}; A. \text{fraterculus} \, \text{♂} \times A. \text{obliqua} \, \text{♀}; A. \text{obliqua} \, \text{♂} \times A. \text{fraterculus} \, \text{♀}; A. \text{obliqua} \, \text{♂} \times A. \text{obliqua} \, \text{♀})\). 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 (IApis: 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 (χ²(9) = 19,551, P = 0.0209) (Fig. 5).

Finally, there were significant differences in sex ratios among different mating combinations (χ²(9) = 19.551, P < 0.05) with heterotypic crosses yielding highly female biased progeny (Fig. 6). The mean (± S.E.) wing length of hybrid F1 progeny of the A. fraterculus × A. obliqua (6.51 ± 0.07 mm) and A. obliqua × A. obliqua (6.64 ± 0.1 mm) crosses were significantly longer than homotypic A. fraterculus (6.24 ± 0.07 mm) (F₂,₉₆ = 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) (F₁,₉₆ = 88.21, P < 0.05). There was no significant effect of sex on wing length (F₁,₉₆ = 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 (F₁,₉₆ = 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 × A. obliqua females produced fewer eggs that hatched in lower proportions than any other mating combination. Larvae derived from mating

This article is protected by copyright. All rights reserved.
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 “lekking 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 Vibora”, 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 × 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.

**Acknowledgments**
This work was partly funded by the Mexican Campaña Nacional Contra Moscas de la Fruta (Secretaría de Agricultura, Ganadería, Desarrollo Rural y Pesca-Consejo Nacional Consultivo Fitosanitario) grant to Martín Aluja. Additional funds were provided the Instituto de Ecología, A.C.

Disclosure

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

References

Aluja, M. and Birke, A. (1993) Habitat use by Anastrepha obliqua (Diptera: Tephritidae) in a mixed mango and tropical plum orchard. Annals of the Entomological Society of America, 86, 799–812.

Aluja, M., Piñero, J., Jácome, I., Díaz-Fleischer, F. and Sivinski, J. (2000a) Behaviour of flies in the genus Anastrepha (Tryptinae: Toxotrypanini). Fruit Flies (Tephritidae): Phylogeny and Evolution of Behavior. (Eds. M. Aluja & A. L. Norrbom) pp. 375–408. Boca Raton, USA, CRC Press.

Aluja, M., Piñero, J., López, M., Ruíz, C., Zúñiga, A., Piedra, E. et al. (2000b) New host plant and distribution records in Mexico for Anastrepha spp., Toxotrypana curvicauda Gerstacker, Rhagoletis zoqui Bush, Rhagoletis sp., and Hexachaeta sp. (Diptera: Tephritidae). Proceedings of the Entomological Society of Washington, 102, 802–815.

Aluja, M., Rull, J., Pérez-Staples, D., Díaz-Fleischer, F. and Sivinski, J. (2009) Random mating among Anastrepha ludens (Diptera: Tephritidae) adults of geographically distant
and ecologically distinct populations in Mexico. *Bulletin of Entomological Research*, 99, 207–214.

Arcella, T., Hood, G.R., Powell, T.H., Sim, S.B., Yee, W.L., Schwarz, D. *et al.* (2015) Hybridization and the spread of the apple maggot fly, *Rhagoletis pomonella* (Diptera: Tephritidae), in the northwestern United States. *Evolutionary Applications*, 8, 834–846.

Bachmann, G.E., Segura, D.F., Devescovi, F., Juárez, M.L., Ruiz, M.J., Vera, M. T. *et al.* (2015) Male sexual behavior and pheromone emission is enhanced by exposure to guava fruit volatiles in *Anastrepha fraterculus*. *PLoS ONE*, 10, e0124250.

Berlocher, S.H. and Feder, J.L. (2002) Sympatric speciation in phytophagous insects: moving beyond controversy? *Annual Review of Entomology*, 47, 773–815.

Birke, A. and Aluja, M. (2011) *Anastrepha ludens* and *A. serpentina* (Diptera: Tephritidae) do not infest *Psidium guajava* (Myrtaceae), but *A. obliqua* occasionally shares this resource with *A. striata* in nature. *Journal of Economic Entomology*, 104, 1204–1211.

Břízová, R., Mendonça, A.L., Vaníčková, L., Lima-Mendonça, A., da Silva, C.E., Tomčala, A. *et al.* (2013) Pheromone analyses of the *Anastrepha fraterculus* (Diptera: Tephritidae) cryptic species complex. *Florida Entomologist*, 96, 1107–1115.

Caceres, C., Segura, D.F., Vera, M.T., Wornoayporn, V., Cladera, J.L., Teal, P. *et al.* (2009) Incipient speciation revealed in *Anastrepha fraterculus* (Diptera: Tephritidae) by studies on mating compatibility, sex pheromones, hybridisation and cytology. *Biological Journal of the Linnean Society*, 97, 152–165.

Carvajal-Rodriguez, A. and Rolan-Alvarez, E. (2006) JMATING: a software for the analysis of sexual selection and sexual isolation effects from mating frequency data. *BMC Evolutionary Biology*, 6, 1.

This article is protected by copyright. All rights reserved.
Coscrato, V.E., Braz, A.S., Perondini, A.L., Selivon, D. and Marino, C.L. (2009) Wolbachia in *Anastrepha* fruit flies (Diptera: Tephritidae). *Current Microbiology*, 59, 295–301.

Coyne, J.A. and Orr, H.A. (1997) “Patterns of speciation in *Drosophila*” revisited. *Evolution*, 51, 295–303.

Devescovi, F., Abraham, S., Roriz, A.K.P., Nolazco, N., Castañeda, R., Tadeo, E. et al. (2014) Ongoing speciation within the *Anastrepha fraterculus* (Diptera: Tephritidae) cryptic species complex: the case of the Andean morphotype. *Entomologia Experimentalis et Applicata*, 152, 238–247.

Dias, V.S., Silva, J.G., Lima, K.M., Petitinga, C.S., Hernández-Ortiz, V., Laumann, R. A. et al. (2016) An integrative multidisciplinary approach to understanding cryptic divergence in Brazilian species of the *Anastrepha fraterculus* complex (Diptera: Tephritidae). *Biological Journal of the Linnean Society*, 117, 725–746.

Dos Santos, P., Uramoto, K. and Matioli, S.R. (2001) Experimental hybridization among *Anastrepha* species (Diptera: Tephritidae): production and morphological characterization of F1 hybrids. *Annals of the Entomological Society of America*, 94, 717–725.

Feder, J.L., Berlocher, S.H., Roethele, J.B., Dambroski, H., Smith, J.J., Perry, W.L. et al. (2003) Allopatric genetic origins for sympatric host-plant shifts and race formation in *Rhagoletis*. *Proceedings of the National Academy of Sciences USA*, 100, 10314–10319.

Freeman, R. and Carey, J.R. (1990) Interaction of host stimuli in the ovipositional response of the Mediterranean fruit fly (Diptera: Tephritidae). *Environmental Entomology*, 19, 1075–1080.
Gonçalves, G.B., Silva, C.E., Mendonça, A.D.L., Vaníčková, L., Tomčala, A. and Do Nascimento, R.R. (2013) Pheromone communication in Anastrepha obliqua (Diptera: Tephritidae): a comparison of the volatiles and salivary gland extracts of two wild populations. Florida Entomologist, 96, 1365–1374.

Haldane, J.B. (1922) Sex ratio and unisexual sterility in hybrid animals. Journal of Genetics, 12, 101–109.

Hendrichs, J., Vera, M.T., De Meyer, M. and Clarke, A.R. (2015) Resolving cryptic species complexes of major tephritid pests. ZooKeys, 540, 5.

Hernández-Ortiz, V., Bartolucci, A.F., Morales-Valles, P., Frías, D. and Selivon, D. (2012) Cryptic species of the Anastrepha fraterculus complex (Diptera: Tephritidae): a multivariate approach for the recognition of South American morphotypes. Annals of the Entomological Society of America, 105, 305–318.

Ibañez, L.A. and Cruz, L.L. (2001) Glándulas salivales de Anastrepha obliqua (Macquart) (Diptera: Tephritidae): análisis químico y morfológico y actividad biológica de los componentes volátiles. Folia Entomologica Mexicana, 40, 221–231.

Jang, E.B. (1995) Effects of mating and accessory gland injections on olfactory-mediated behavior in the female Mediterranean fruit fly, Ceratitis capitata. Journal of Insect Physiology, 41, 705–710.

Jeyaprakash, A. and Hoy, M.A. (2000) Long PCR improves Wolbachia DNA amplification: wsp sequences found in 76% of sixty-three arthropod species. Insect Molecular Biology, 9, 393–405.

Larson, E.L., Andrés, J.A. and Harrison, R.G. (2012) Influence of the male ejaculate on post-mating prezygotic barriers in field crickets. PLoS ONE, 7, e46202.

This article is protected by copyright. All rights reserved.
Lima, I.S., House, P.E. and Nascimento, R.R.D. (2001) Volatile substances from male *Anastrepha fraterculus* Wied. (Diptera: Tephritidae): identification and behavioural activity. *Journal of the Brazilian Chemical Society*, 12, 196–201.

López-Guillén, G., Cruz-López, L., Malo, E.A., González-Hernández, H., Cazares, C.L., López-Collado, J. *et al.* (2008) Factors influencing the release of volatiles in *Anastrepha obliqua* males (Diptera: Tephritidae). *Environmental Entomology*, 37, 876–882.

López-Guillén, G., López, L.C., Malo, E.A. and Rojas, J.C. (2011) Olfactory responses of *Anastrepha obliqua* (Diptera: Tephritidae) to volatiles emitted by calling males. *Florida Entomologist*, 94, 874–881.

Mallet, J., Besansky, N. and Hahn, M. W. (2016) How reticulated are species? *BioEssays*, 38, 140–149.

Marcon, H.S., Coscrato, V.E., Selivon, D., Perondini, A.L.P. and Marino, C.L. (2011) Variations in the sensitivity of different primers for detecting *Wolbachia* in *Anastrepha* (Diptera: Tephritidae). *Brazilian Journal of Microbiology*, 42, 778–785.

Martín, H., Toledo, J., Liedo, P. and Mateos, M. (2012) Survey of heritable endosymbionts in southern Mexico populations of the fruit fly species *Anastrepha striata* and *A. ludens*. *Current Microbiology*, 65, 711–718.

Milet-Pinheiro, P., Navarro, D.M.A., De Aquino, N.C., Ferreira, L.L., Tavares, R.F., Da Silva, R.C.C. *et al.* (2015) Identification of male-borne attractants in *Anastrepha fraterculus* (Diptera: Tephritidae). *Chemoecology*, 25, 115–122.

Norrbom, A.L., Zucchi, R.A. and Hernández-Ortiz, V. (2000) Phylogeny of the genera *Anastrepha* and *Toxotrypana* (Trypetinae: Toxotrypanini) based on morphology. *Fruit
flies (Tephritidae): Phylogeny and Evolution of Behavior. (eds. M. Aluja & A. L. Norrbom), p. 299–342. Boca Raton, Florida, CRC Press, 944p.

Oroño, L., Paulin, L., Alberti, A.C., Hilal, M., Ovruski, S., Vilardi, J. et al. (2013) Effect of host plant chemistry on genetic differentiation and reduction of gene flow among Anastrepha fraterculus (Diptera: Tephritidae) populations exploiting sympatric, synchronic hosts. Environmental Entomology, 42, 790–798.

Rolán-Alvarez, E. and Caballero, A. (2000) Estimating sexual selection and sexual isolation effects from mating frequencies. Evolution, 54, 30–36.

Ruiz–Arce, R., Barr, N.B., Owen, C.L., Thomas, D.B. and McPheron, B.A. (2012) Phylogeography of Anastrepha obliqua inferred with mtDNA sequencing. Journal of Economic Entomology, 105, 2147–2160.

Rull, J., Aluja, M., Feder, J.L. and Berlocher, S.H. (2006) The distribution and host range of hawthorn-infesting Rhagoletis (Diptera: Tephritidae) in Mexico. Annals of the Entomological Society of America, 99, 662–672.

Rull, J., Aluja, M. and Feder, J.L. (2010) Evolution of intrinsic reproductive isolation among four North American populations of R. pomonella. Biological Journal of the Linnean Society, 100, 213–223.

Rull, J, Tadeo, E. Aluja, M., Guillen, L., Egan, S.P. and Feder, J.L. (2012) Hybridization and sequential components of reproductive isolation between parapatric walnut-infesting sister species Rhagoletis completa and R. zoqui. Biological Journal of the Linnean Society, 107, 886–898.

Rull, J., Abraham, S., Kovaleski, A., Segura, D.F., Mendoza, M., Liendo, M.C. and Vera, M.T. (2013) Relative importance of prezygotic and postzygotic barriers to gene flow among three cryptic species within the Anastrepha fraterculus complex. Entomologia Experimentalis et Applicata, 148, 213–222.

This article is protected by copyright. All rights reserved.
Santos, J.C.G.S. (2003) Estudo da preferência alimentar de machos de *Anastrepha* spp. e dos constotuintes voláteis liberados por estes machos e por seu hospedeiro principal, *Psidium guajava* L. PhD thesis, Universidade Federal de Alagoas, Maceio, Brazil.

Saridaki, A. and Bourtzis, K. (2010) *Wolbachia*: more than just a bug in insect genitals. *Current Opinion in Microbiology*, 13, 67–72.

Scally, M., Into, F., Thomas, D.B., Ruiz-Arce, R., Barr, N.B. and Schuenzel, E.L. (2016) Resolution of inter and intra-species relationships of the West Indian fruit fly *Anastrepha obliqua*. *Molecular Phylogenetics and Evolution*, 101, 286–293.

Schwarz, D. and McPheron, B.A. (2007) When ecological isolation breaks down: sexual isolation is an incomplete barrier to hybridization between *Rhagoletis* species. *Evolutionary Ecology Research*, 9, 829–841.

Schwenk, K., Brede, N. and Streit, B. (2008) Extent, processes and evolutionary impact of interspecific hybridization in animals. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 363, 2805–2811.

Segura, D.F., Vera, M.T., Rull, J., Wornoayporn, V., Islam, A. and Robinson, A.S. (2011) Assortative mating among *Anastrepha fraterculus* (Diptera: Tephritidae) hybrids from two distinct populations as a possible route to radiation of the *fraterculus* cryptic species group. *Biological Journal of the Linnean Society*, 102, 346–354.

Selivon, D., Perondini, A.P., Ribeiro, A.F., Marino, C.L., Lima, M.M. and Coscrato, V.E. (2002) *Wolbachia* endosymbiont in a species of the *Anastrepha fraterculus* complex (Diptera: Tephritidae). *Invertebrate Reproduction & Development*, 42, 121–127.

Sivinski, J., Pinero, J. and Aluja, M. (2000) The distributions of parasitoids (Hymenoptera) of *Anastrepha* fruit flies (Diptera: Tephritidae) along an altitudinal gradient in Veracruz, Mexico. *Biological Control*, 18, 258–269.

This article is protected by copyright. All rights reserved.
Sivinski, J., Aluja, M., Piñero, J. and Ojeda, M. (2004) Novel analysis of spatial and temporal patterns of resource use in a group of tephritid flies of the genus *Anastrepha*. *Annals of the Entomological Society of America*, 97, 504–512.

Smith-Caldas, M.R., McPheron, B.A., Silva, J.G. and Zucchi, R.A. (2001) Phylogenetic relationships among species of the *fraterculus* group (*Anastrepha*: Diptera: Tephritidae) inferred from DNA sequences of mitochondrial cytochrome oxidase I. *Neotropical Entomology*, 30, 565–573.

Tadeo, E., Feder, J.L., Egan, S.P., Schuler, H., Aluja, M. and Rull, J. (2015) Divergence and evolution of reproductive barriers among three allopatric populations of *Rhagoletis cingulata* across eastern North America and México. *Entomologia Experimentalis et Applicata*, 156, 301–311.

Webb, J.C., Sivinski, J. and Litzkow, C. (1984) Acoustical behavior and sexual success in the Caribbean fruit fly, *Anastrepha suspensa* (Loew) (Diptera: Tephritidae). *Environmental Entomology*, 13, 650–656.

Zucchi, R.A. (2000) Lista das espécies de *Anastrepha*, sinonímias, plantas hospedeiras e parasitóides. *Moscas-das-frutas de Importância Econômica no Brasil* (eds. A. Malavasi & R.A. Zucchi) pp. 41–48., Ribeirão Preto, Holos.

Manuscript received November 24, 2016

Final version received April 18, 2017

Accepted May 02, 2017

**Table 1.** Total number, copulation frequency per replicate and mean duration (± 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).

This article is protected by copyright. All rights reserved.
♂♀ Mating combination | A. obliqua × A. obliqua | A. obliqua × A. fraterculus | A. fraterculus × A. obliqua | A. fraterculus × A. fraterculus
---|---|---|---|---
Total number of 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 × 0.6 × 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 (± 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* ♂ × *A. fraterculus* ♀; *A. fraterculus* ♂ × *A. obliqua* ♀; *A. obliqua* ♂ × *A. fraterculus* ♀; *A. obliqua* ♂ × *A. obliqua* ♀); B) Mean percentage (± S.E.) of egg hatch for 5 pairs of sexually mature flies according to mating combination (*n* = 8).
Figure. 4 Mean (± 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 ♀× A. fraterculus ♀; A. fraterculus ♂× A. obliqua ♀; A. obliqua ♂× A. fraterculus ♀; A. obliqua ♂× A. obliqua ♀) (n = 8).

Figure. 5 Mean (± 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 ♂× A. fraterculus ♀; A. fraterculus ♂× A. obliqua ♀; A. obliqua ♂× A. fraterculus ♀; A. obliqua ♂× A. obliqua ♀) (n = 8).
Figure. 6 Mean (± S.E.) number of eclosed male, female, and deformed F1 adults and average (± 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 ($n = 8$).