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# Evolution of pre-zygotic and post-zygotic barriers to gene flow among three cryptic species within the *Anastrepha fraterculus* complex

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## Abstract

Tropical tephritids are ideally suited for studies on population divergence and speciation because they include species groups undergoing rapid radiation, in which morphologically cryptic species and sister species are abundant. The *fraterculus* species group in the Neotropical genus *Anastrepha* is a case in point, as it is composed of a complex of up to seven *A. fraterculus* morphotypes proposed to be cryptic species. Here, we document pre- and post-zygotic barriers to gene flow among adults of the Mexican *A. fraterculus* morphotype and three populations (Argentina, Brazil, and Peru) belonging to two separate morphotypes (Brazilian 1 and Peruvian). We unveiled three forms of pre-zygotic reproductive isolation resulting in strong assortative mating. In field cages, free-ranging male and female *A. fraterculus* displayed a strong tendency to form couples with members of the opposite sex belonging to their own morphotype, suggesting that male pheromone emission, courtship displays, or both intervene in shaping female choice before actual contact and coupling. In addition, males and females of the Peruvian morphotype became receptive and mated significantly later than adults of the Mexican and Brazilian 1 morphotypes. After contact, Mexican females exhibited greater mating discrimination than males when facing adults of the opposite sex belonging to either the Peruvian or the Brazilian 1 morphotype as evidenced by vigorous resistance to penetration once they had been forcefully mounted by heterotypic males. Forced copulations resulted in production of F1 hybrids that were either less viable (and partially fertile) than parental crosses or even sterile. Our results suggest that the Mexican morphotype is a distinct biological entity and that pre-zygotic reproductive isolation through divergence in courtship or male-produced pheromone and other mechanisms appear to evolve faster than post-zygotic isolation in the *fraterculus* species group.

## Introduction

The early stage of speciation in animals is often characterized by the appearance of pre-zygotic and post-zygotic reproductive isolation (Moehring et al., 2004). However, the relative importance of different isolating barriers to speciation remains a topic of considerable debate (Coyne & Orr, 2004), particularly because of difficulties in distinguishing barriers that trigger speciation from those that accumulate after interruption of gene flow. The study of individual and relative contributions of all potential barriers among species that have recently achieved species status has been proposed to distinguish reproductive barriers that have actually contributed to speciation from those that accumulate after speciation is complete (Sobel et al., 2009; Nosil & Schluter, 2011; Marie Curie SPECIATION Network, 2012).

Tephritid fruit flies have often been used as model systems for the study of speciation (Bush, 1969; Feder

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et al., 1988; Craig et al., 1997; Berlocher & Feder, 2002), perhaps because this diverse family includes many groups undergoing recent and rapid radiation (Berlocher, 2000; Clarke et al., 2005; Condon et al., 2008; Virgilio et al., 2008). One of those groups is the *fraterculus* species group within the genus *Anastrepha*, a taxon of tropical and subtropical fruit flies comprised of at least 29 species, among which several are cryptic and sibling species that are morphologically and genetically similar (Smith-Caldas et al., 2001).

Within the *fraterculus* species group, *A. fraterculus,* originally thought to be a wide-ranging highly polyphagous species distributed from Argentina to southern USA (Stone, 1942), was found to be in fact a group of cryptic species (Steck, 1991). Further study of *A. fraterculus* revealed subtle morphological differences among populations of different geographical origin, among which the shape of the ovipositor is the most relevant diagnostic feature (Hernández-Ortiz et al., 2004). Different morphotypes of *A. fraterculus* are also genetically distinct (Morgante et al., 1980; Steck & Sheppard, 1993; Smith-Caldas et al., 2001; Selivon & Perondini, 2007; Ludeña et al., 2010) and, in some cases, have been found to exhibit different forms of pre- and post-zygotic isolation (Selivon et al., 1999; Vera et al., 2006; Cáceres et al., 2009). Recently, a comprehensive morphometric study encompassing 32 *A. fraterculus* populations across the Americas allowed the identification of seven different morphotypes (Hernández-Ortiz et al., 2012), which are in all likelihood a complex of cryptic species.

At the northern end of the *A. fraterculus* range, the Mexican morphotype has been found to exhibit host affiliations (Baker, 1945; Aluja et al., 2003), morphological features (Stone, 1942; Hernández-Ortiz et al., 2004), and a karyotype (Bush, 1962) that have distinguished its members from South American forms of *A. fraterculus*. The Mexican morphotype extends from Mexico to Panama and has often been hypothesized to be a distinct species (Baker, 1945; Bush, 1962; Aluja et al., 2003; Hernández-Ortiz et al., 2004, 2012). If so, it should be expected that isolating barriers prevent gene flow between this morphotype and southern morphotypes. The nature of these barriers and their relative strength can shed some light into speciation mechanisms explaining rapid radiation and the great diversity of tropical taxa of fruit flies, which are far less understood than those explaining speciation of some temperate tephritid genera (Segura et al., 2011).

Resolving part or the entire *fraterculus* species complex is also of practical importance due to the fact that its members are agricultural pests, which could be potentially managed under an areawide, environmentally friendly scheme through the application of the sterile insect technique (SIT) (Rull et al., 2012). The SIT involves mass rearing, sterilization, and the release of insects as an autocidal, species-specific pest control method. Under SIT programs, sterile males mate with wild fertile females, inducing sterility in the wild population (Knipling, 1959; Dyck et al., 2005). Therefore, in cases involving cryptic species complexes, it is necessary to delimit the geographical range of sexually compatible morphotypes to avoid releases of sterile males of a laboratory strain, which may be incompatible with the local morphotype and could result in failure to induce sterility in the wild population.

Here, aiming at contributing to species delimitation within the *fraterculus* complex, we evaluated the existence and strength of pre- and post-zygotic reproductive isolation among a population of the Mexican morphotype and populations from Argentina, Brazil, and Peru.

## Materials and methods

Experiments were carried out at the FAO/IAEA Agriculture and Biotechnology Laboratories in Seibersdorf, Austria.

### Biological material

Individuals of the Mexican *A. fraterculus* morphotype were recovered from several collections of naturally infested peach [*Prunus persica* L. (Rosaceae)] during the month of June 2010 in the vicinity of Xalapa, Veracruz (19°32′N, 96°55′W). The Argentinean population was obtained from a laboratory colony kept at the FAO/IAEA Laboratory. This strain was derived from a strain reared at the Estación Experimental Agroindustrial Obispo Colombres since 1997 and originally recovered from naturally infested guavas [*Psidium guajava* L. (Myrtaceae)] collected at the vicinity of Tafí Viejo, Tucumán (26°48′5″S, 65°9′50″W). Southern Brazilian populations were recovered from naturally infested guavas near the locality of Vacaria (28°27′52″S, 50°59′0″W) in April 2010 and araça [*Eugenia stipitata* Mc. Vaugh (Myrtaceae)] from the locality of Pelotas. They were taken to the FAO/IAEA Laboratories and reared for one generation on an artificial carrot diet (Tanaka et al., 1970) prior to experiments. Populations from Argentina and Brazil belong to the same morphotype and are not sexually isolated from each other (Rull et al., 2012). The Peruvian population stemmed from a laboratory strain artificially held at the IAEA laboratories since 2006 and originally collected from infested cherimoya *Annona cherimola* Miller (Annonaceae), near la Molina, Peru (12°00′03″S, 76°57′00″W) (Vera et al., 2006), and belongs to the Peruvian morphotype, which is partially isolated from the Brazilian 1 morphotype (Cáceres et al., 2009).

#### Pre-zygotic reproductive isolation tests

To establish the degree of pre-zygotic isolation between adults of different populations/morphotypes and adults of the Mexican morphotype, we performed a series of mating compatibility tests. For each population or strain, 200 pupae were placed in 15-cm-diameter × 45-cm-high cylindrical Plexiglass cages before adult emergence. Cages were covered at one end with a mesh and at the other with a long sleeve (also made with mesh) that could be tied and untied in a knot to facilitate fly transfer to and from the cage. At emergence, adults were sorted by sex and placed in similar cages with ad libitum access to water and a mixture of wheat germ, hydrolysed yeast, and sugar at a 1:1:3 ratio. One to 2 days before reaching sexual maturity (10–18 days depending on the strain), males and females of each population were marked on the back of the thorax with a small dot of distinctive acrylic paint. Twenty-five marked males and 25 marked females of each population were placed in smaller 11 × 11 × 17 cm square cages with water and food. The following day, at 08:00 hours (time at which lights were turned on in the laboratory where adults were kept), marked flies of both sexes and from two different populations were released inside a 2.0 × 1.6 × 1.9-m cage. In each cage, a 2-m-high potted *Citrus sinensis* Osbeck (L.) (Rutaceae) tree with a canopy of ca. 1.1 m in diameter provided the flies an arena for resting, calling, and mating. Cages were installed inside a greenhouse in which temperature and light could be manipulated. The greenhouse could be heated when necessary, and flies were released once the temperature reached 23 °C. Simultaneous releases were done in four adjacent cages. One observer in each cage recovered mating couples from the tree, cage walls, and ceiling and recorded the colour (origin) of male and female and time at which copulation initiated. Shortly after detection of a mating pair, the couple was gently captured in a 3.7-cm-diameter × 4-cm-high plastic cup that was capped and placed on a plastic tray to record the time at which copulation ended. Mating latency was calculated as time (in min) from the start of the experiment to each copulation. Flies were observed for ca. 3–7 h, a time lapse that covered the period of sexual activity for all populations. After this time, mated couples and remaining unmated adults were taken back to the laboratory. Six replicates each were performed between Mexican and Argentinean flies, and Mexican and southern Brazilian flies, whereas four replicates were carried out between Mexican and Peruvian adults.

#### Post-zygotic reproductive isolation tests

To identify existing post-zygotic barriers between morphotypes, up to 10 mated couples of each possible combination were taken from the field cage and placed in 45 × 15 cm cylindrical Plexiglass cages (as previously described) and were assessed for fertility (% egg hatch). To recover eggs, the bottom of a Petri dish (13.9 cm in diameter) was removed and replaced with a piece of cloth previously lined with a thin layer of black silicone (Sanitarsiliko; Murexin, Vienna, Austria). The oviposition device was placed over the top of the cylindrical Plexiglass cage and filled with tap water. With the aid of a Pasteur pipette, eggs were recovered every other day and placed on a piece of black filter paper.

Because some mating combinations (e.g., heterotypic crosses) yielded few mating couples (particularly those involving Mexican females and males from other populations), mated couples from such combinations were held individually in a 2.5 × 2.5-cm cage of 10 cm in height with an opening on top where a cylinder made of agar (as described in Abraham et al., 2011)) was placed to recover eggs. Agar cylinders were dissected under a microscope every 48 h and eggs aligned on a moist black filter paper. In addition, in an attempt to increase sample size, 10 sexually mature virgin Mexican females were placed in 45 × 15 cm Plexiglass cages with males of different populations (either Argentina, Brazil, or Peru) at a 3:1 male/female ratio. Mated couples were held individually as described above to recover eggs. Unmated females were separated from males and re-exposed the following day. Exposure was carried out from 08:00 to 12:00 h, and mated females were replaced with sexually mature, virgin females.

Because transferring eggs into diet right after collection resulted in no hatch, collected eggs were first transferred to a black filter paper and then placed in a Petri dish bottom that contained a thin piece of moistened sponge. The Petri dish was then covered and incubated at 27 °C and 65% r.h. for 48 h. When eggs began hatching, the filter paper was transferred to a 9-cm Petri dish filled with carrot larval diet. After an additional 48-h period, the number of hatched eggs was counted and recorded, and the filter paper was removed from the diet to prevent fungal growth. The Petri dishes were then covered and placed in a 250-ml cylindrical container covered with mesh, with a thin layer of vermiculite as pupation substrate. Plastic containers with Petri dishes were kept under a dark cloth at 27 °C and 65% r.h., and after 3 days, the tops of the Petri dishes were removed. When larvae completed development (attempting to leave the diet to pupate), diet was examined, and pupae and late instars were counted, placed on the vermiculite layer, and incubated at 27 °C and 65% r.h. for ca. 8–10 days, when adults began to emerge. At emergence, F1 adults were transferred to cylindrical Plexiglass cages

with water and food. F1 adults were left in cages for 15 days, and when couples began mating, an oviposition device (as described above) was placed on top of cages, eggs were recovered, and F2 egg hatch was recorded following the procedures described for F1 egg hatch estimation.

#### Data analysis

The number of copulations, mating duration in minutes, and latency to mate in minutes among homotypic and heterotypic male–female combinations within population pairs were compared by means of a Kruskal–Wallis test, followed by two-tailed multiple comparisons. Fertility (% egg hatch) of eggs recovered from homotypic and heterotypic crosses within population pairs and from the self-cross of resulting F1 adults was also compared with Kruskal–Wallis tests followed by two-tailed multiple comparisons. All analyses were carried out using STATISTICA, version 7 (Statsoft, Tulsa, OK, USA).

## Results

#### Pre-zygotic reproductive isolation

Mating compatibility tests revealed the existence of strong mating incompatibility between adults of the Mexican morphotype and adults of all tested South American populations of *A. fraterculus*. A Kruskal–Wallis test followed by two-tailed comparisons of means revealed significantly more homotypic than heterotypic matings for the Mexico–Argentina ($H_{3,24}$ = 18.65, P<0.001), Mexico–Brazil ($H_{3,24}$ = 18.16; P<0.001), and Mexico–Peru ($H_{3,24}$ = 12.79, P = 0.005) mating trials (Figure 1). Mating isolation indexes (Cayol et al., 1999) were on average 0.82 ± 0.06 for Mexico–Argentina, 0.89 ± 0.02 for Mexico–Brazil, and 0.74 ± 0.03 for Mexico–Peru (where ISI values of 1 represent absolute assortative mating, whereas 0 represents random mating). A Kruskal–Wallis test revealed no significant differences in latency to mate among different mating combinations for the Mexico–Argentina trials ($H_{3,98}$ = 3.72, P = 0.29) or the Mexico–southern Brazil mating trials ($H_{3,162}$ = 4.76, P = 0.19). However, Peru females mated with Peru males exhibited significantly longer mating latency than all other mating combinations within the Mexico–Peru trials ($F_{3,134}$ = 48.57, P<0.001; Table 1). There were significant differences in duration of copulations during the Mexico–Argentina trials ($H_{3,161}$ = 32.16, P<0.001). Argentinean females mated longer than Mexican females, irrespective of the male origin. Brazilian females and males mated longer than Mexican females and males ($H_{3,170}$ = 38.32, P<0.001). In contrast, there were no significant differences in mating duration among different mating combinations

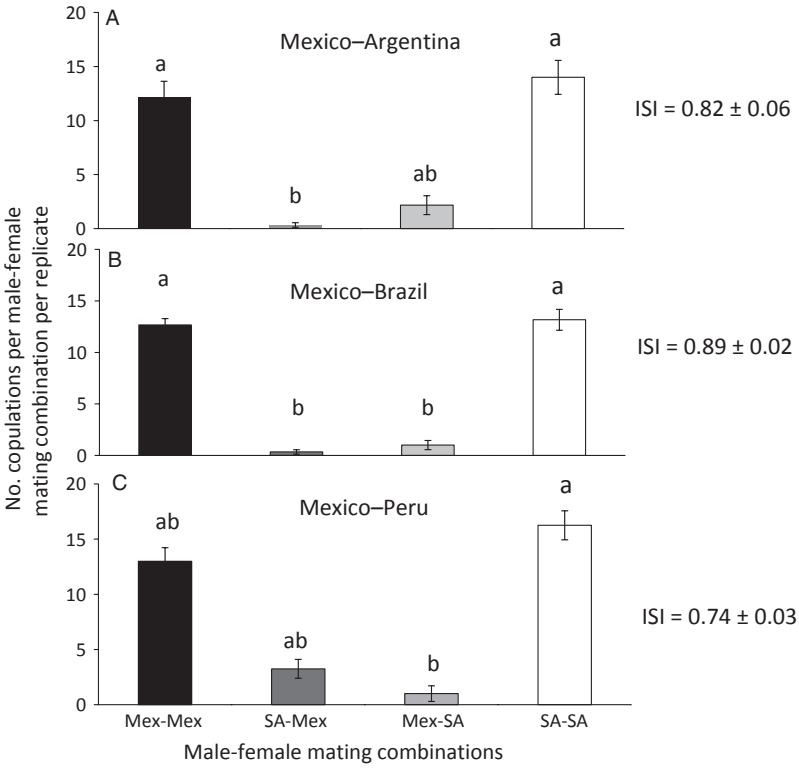


**Figure 1** Average number (± SE) of copulations per replicate, per male–female combination in field cage crossing experiments between adult *Anastrepha fraterculus* of the Mexican (MEX) morphotype and adults of three populations of South American (SA) *A. fraterculus* of the Brazilian 1 [(A) Argentina and (B) Brazil] or Peruvian [(C) Peru] morphotypes. Means topped with different letters are significantly different (Kruskal–Wallis: P<0.05).

during the Mexico–Peru trials ($H_{3,146}$ = 2.05, P = 0.56) (Table 2).

#### Post-zygotic reproductive isolation

*Homotypic and heterotypic cross-fertility.* Strong mating incompatibility resulted in recovery of few mated couples for some particular crosses. Of 172 matings in the Mexico–Argentina field cage trials, only two occurred between an Argentinean male and a Mexican female. Our attempts to force copulations in small enclosures in the laboratory yielded low numbers of heterotypic crosses as well. Although males repeatedly attempted copulation, females vigorously rejected mounting males and consequently prevented penetration. In all, five mated females did not oviposit in agar discs over the course of 3 weeks. Nevertheless, there were significant differences in per cent of eclosion of F1 eggs among homotypic crosses and Mexican males crossed with Argentinean females ($H_{2,23}$ = 13.417, P = 0.001; Figure 2A). In case of Mexican and southern Brazilian populations, although we only recovered two couples of Brazilian males and Mexican females in field cages (of a total of 163) and four more out of forced copulations in the laboratory, three females laid a total of 108 eggs. Although there were numerical differences in egg hatch between the Mexican homotypic cross and the cross of Brazilian males with Mexican females, all mating combinations produced similar levels of egg hatch ($H_{2,25}$ = 5.624, P = 0.13; Figure 2B). Finally, for the Mexico–Peru combination, we were also unable to recover eggs from the cross of Peruvian males and Mexican females, and the mean egg hatch for the Mexican homotypic crosses was numerically but not significantly higher than that found for the cross of Mexican males and Peruvian females ($H_{2,23}$ = 10.55, P = 0.051; Figure 2C).

*F1 sterility.* Fertility for the self-crosses of F1 progeny resulting from the cross of Mexican males and Argentinean females was significantly lower than that of F1 self-crosses of homotypic Mexican parentals ($H_{2,12}$ = 8.116, P = 0.017; Figure 3A). In case of the Mexican–Brazilian combination, the cross of three Brazilian males and three Mexican females produced 10 F1 females and 14 F1 males, of which after self-crossing, five females produced 292 eggs, but none hatched. Egg hatch from the Mexican homotypic F1 self-cross was greater than that of the F1 self-cross of Mexican males and Brazilian females ($H_{3,17}$ = 13.914, P = 0.003; Figure 3B). Finally, there were no statistical differences in F2 egg hatch among homotypic F1 self-crosses and the F1 self-cross of Mexican males and Peruvian females ($H_{2,12}$ = 4.903, P = 0.086; Figure 3C).

## Discussion

We found extremely strong pre-zygotic reproductive isolation among adults of the Mexican morphotype and adults of three South American populations. Pre-zygotic isolation in field cages was manifested in all cases through assortative mating and in case of trials involving Peruvian flies also through temporal isolation due to marked differences in latency to mate. In small enclosures, Mexican females were much more reluctant to accept copulations of mounting heterotypic males than Mexican males with heterotypic females of any origin. Egg hatch of heterotypic crosses involving Mexican males was lower than that of homotypic Mexican crosses in most cases, and the only instance in which F1 hybrids involving Mexican females could be obtained, they appeared to be sterile.

According to the biological species concept (BSC), species are groups of actually or potentially interbreeding populations that are reproductively isolated from other such groups (Mayr, 1963). The BSC has been criticized among other things because the degree of reproductive isolation between species is seldom discrete and can lead to arbitrary taxonomic decisions when attempting to assign species status to unresolved taxa (Sokal & Crovello, 1970). Additionally, the advent of modern genetics has allowed extensive documentation of gene flow among animal species (Mallet, 2005, 2007, 2008). Nevertheless, even

**Table 1** Average (± SE) latency to mate, measured as the time (min) elapsed from the beginning of observations to the beginning of copulations, for all formed couples in all possible male–female combinations between adults of the Mexican *Anastrepha fraterculus* morphotype and adults of three South American (SA) populations during field cage mating compatibility trials. Sample sizes are given in parentheses

| South American population | Mex♂ – Mex♀ | SA♂ – Mex♀ | Mex♂ – SA♀ | SA♂ – SA♀ |
|---|---|---|---|---|
| Argentina | 28.90 ± 4.79a (41) | 97a (1) | 31.30 ± 6.74a (10) | 30.32 ± 4.38a (46) |
| Brazil | 45.52 ± 6.23a (75) | 56.41 ± 41.0a (2) | 53.83 ± 18.54a (6) | 62.83 ± 6.17a (79) |
| Peru | 50.03 ± 7.51a (56) | 111.5 ± 31.3ab (12) | 49.5 ± 17.39a (6) | 194.5 ± 14.3b (62) |

Means within rows followed by different letters are significantly different (Kruskal–Wallis: P<0.05).

**Table 2** Average (± SE) copula duration in (min) for all possible male-female combinations between adults of the Mexican *Anastrepha fraterculus* morphotype and adults of three South American (SA) populations.

| South American population | Mex♂ – Mex♀ | SA♂ – Mex♀ | Mex♂ – SA♀ | SA♂ – SA♀ |
|---|---|---|---|---|
| Argentina | 41.89 ± 3.18a | 31.58 ± 7.88a | 69.66 ± 7.72b | 66.37 ± 3.96b |
| Brazil | 42.38 ± 3.17a | 40.0 ± 13.42ab | 66.08 ± 11.36ab | 77.36 ± 4.69b |
| Peru | 40.19 ± 3.18a | 35.41 ± 4.79a | 32.81 ± 5.84a | 43.85 ± 4.26a |

Means within rows followed by different letters are significantly different (Kruskal–Wallis: $P<0.05$).

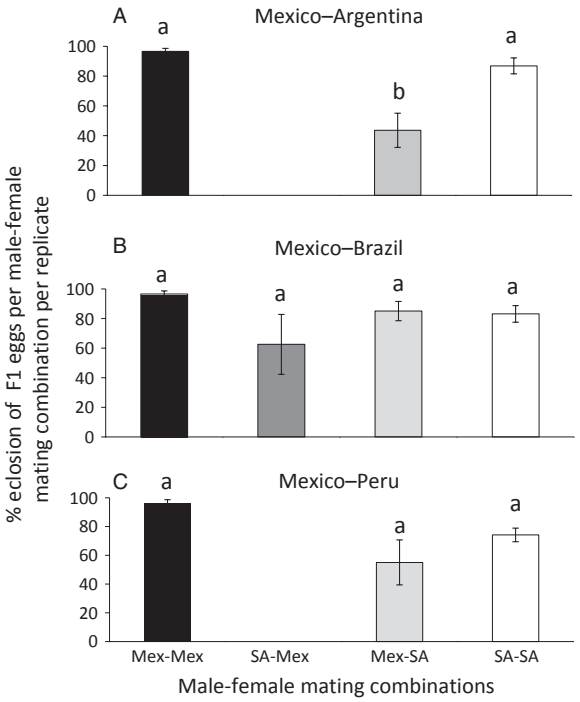


**Figure 2** Average percentage eclosion (± SE) of F1 eggs per replicate, per male–female combination from laboratory crosses between adult *Anastrepha fraterculus* of the Mexican (Mex) morphotype and adults of three populations of South American (SA) *A. fraterculus* of the Brazilian 1 [(A) Argentina and (B) Brazil] or Peruvian [(C) Peru] morphotypes. Means topped with different letters are significantly different (Kruskal–Wallis: $P<0.05$).

the most ardent detractors of the usefulness of BSC recognize that reproductive barriers are fundamental in the study of divergence (Sokal & Crovello, 1970). Moreover, examination of pre- and post-zygotic reproductive isolation between species or population pairs has proven to be an extremely useful tool in experiments attempting to understand speciation among some animal groups and in particular among the Diptera (Dobzhansky & Koller, 1938; Coyne & Orr, 1989, 1997; Cáceres et al., 2009; Rull et al., 2010). Here, the examination of interpopulation reproductive barriers allowed us to distinctly separate the Mexican morphotype from three South American populations of *A. fraterculus* belonging to two different morphotypes.

Pre-zygotic reproductive isolation was found to be extremely strong among adults of the Mexican morphotype and those of the three South American populations. The Argentinean population and the southern Brazilian population, which belong to the *A. fraterculus* Brazilian 1 morphotype (Hernández-Ortiz et al., 2012), had been found to be a single biological entity displaying no form of pre- or post-zygotic reproductive isolation in earlier studies (Rull et al., 2012), while the Peruvian population, classified as a member of the Peruvian morphotype, had been found to be isolated from *A. fraterculus* Brazilian 1 morphotype (Vera et al., 2006; Cáceres et al., 2009). Altogether, it appears that morphometric differentiation in the *A. fraterculus* cryptic species complex is a good proxy to distinguish specific biological entities and that these entities, as far as we know, are reproductively isolated from each other.

Pre-zygotic reproductive isolation among cryptic species of *A. fraterculus* may be linked to differences in male sexual pheromones (Cáceres et al., 2009) and also through female preferences attracted to lekking arenas by pheromones produced by calling males of their own morphotype (Segura et al., 2011). While long-distance attraction to calling males is perhaps the most important component of pre-zygotic isolation in *A. fraterculus*, other factors may also be at play. The great difficulties we faced when attempting to force heterotypic copulations between South American males and Mexican females suggest that close-range signals may also be playing a role in species recognition. These signals could include visual or chemical tactile stimuli. Regarding the latter, it has been proposed for *Drosophila* that some cuticular hydrocarbons can stimulate, whereas others can inhibit sexual behaviour and that major and minor components of the bouquet may be used both for intra- and interspecific communication to influence mate choice and reinforce sexual isolation among species (Ferveur, 2005).

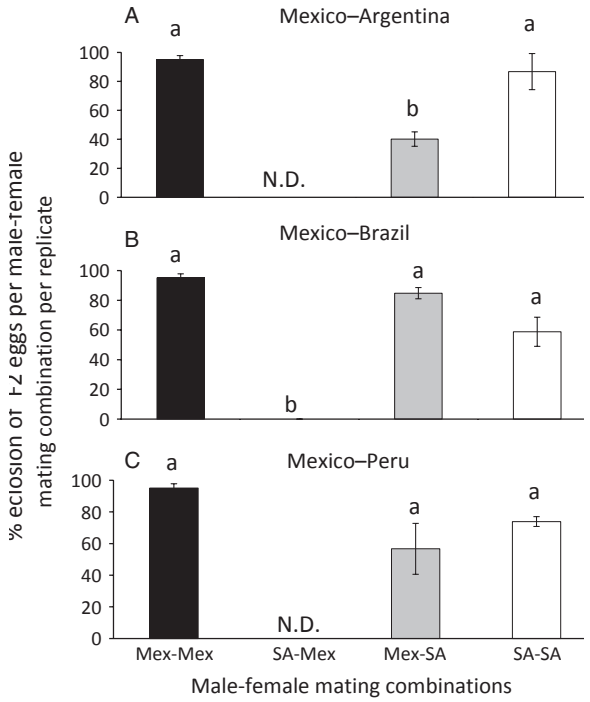


**Figure 3** Average percentage eclosion (± SE) of F2 eggs per replicate of male–female self-crosses between homotypic and heterotypic F1 adults resulting from crosses of Mexican (Mex) and (A) Argentinean, (B) Brazilian, or (C) Peruvian *Anastrepha fraterculus*. Means topped with different letters are significantly different (Kruskal–Wallis: $P<0.05$). When no eggs could be recovered for a particular cross, N.D. indicates no data.

The third form of pre-zygotic reproductive isolation that surfaced during our study was manifested through temporal partition of mating activity between adults of the Mexican morphotype and Peruvian flies. According to reinforcement theory, such a mechanism is more likely to evolve between species pairs that are or have been sympatric or parapatric after a period of isolation, through selection against maladaptive hybridization during secondary contact (Butlin, 1987; Hoskin et al., 2005). This additional pre-zygotic barrier to gene flow seems redundant for allopatric Mexican flies, which are already almost completely isolated from other *A. fraterculus* morphotypes (Brazilian 1), exhibiting identical temporal mating patterns. Understanding the evolution of different mating times will require further study of Peruvian populations, the identification of their geographical boundaries, and the assessment of isolation with neighbouring populations and closely related sympatric species, which in some cases have been found to be capable of hybridization with *A. fraterculus* (Santos et al., 2001). Alternatively, such behaviours could be the result of optimal conditions for mating (luminosity, temperature, and precipitation patterns) gradually changing with elevation or latitude, in which case pre-zygotic isolation, or at least temporal isolation, would be an indirect effect of local adaptation.

When analysing post-zygotic reproductive isolation in the *fraterculus* cryptic species complex, Selivon et al. (1999) found reduced egg hatch for hybrids in both directions and sex ratio distortion in laboratory crosses between Brazilian morphotypes 1 and 2 (from southern Brazil and San Salvador de Bahia area, respectively). Similarly, Cáceres et al. (2009) found a significant reduction in F1 egg hatch for the cross of Peruvian males and Argentinean females when compared to fertility levels of homotypic crosses and that of Argentinean males and Peruvian females. However, the cross of F1 hybrids was interfertile, suggesting that barring pre-zygotic barriers, gene flow between the Brazilian 1 and Peruvian morphotypes is still possible. In case of the Mexican morphotype, we also found a reduction in F1 fertility for the cross of Mexican males and Argentinean females. However, in the only case where we were able to obtain eggs from the cross of South American males and Mexican females, we found that heterotypic crosses in one direction produced sterile hybrids. In this case, the Mexican morphotype appears to exhibit stronger reproductive isolation than that found between the Peruvian and Brazilian 1 morphotypes; a finding that adds to the wealth of collective evidence showing that it is a distinct species (Stone, 1942; Baker, 1945; Bush, 1962; Aluja et al., 2003; Hernández-Ortiz et al., 2004, 2012).

From an analysis of reproductive isolation between pairs of 119 closely related species of *Drosophila,* Coyne & Orr (1989, 1997) concluded that among these flies, pre-zygotic isolation is stronger than post-zygotic isolation, and while for allopatric species, mating discrimination and post-zygotic isolation evolve at similar rates, in case of sympatric species, mating discrimination evolves well before severe hybrid sterility of inviability. Collective evidence indicates that among species of the *A. fraterculus* group, pre-zygotic isolation in the form of mating discrimination is also stronger than any form of post-zygotic barriers to gene flow. Laboratory experiments involving forced copulations to overcome naturally prevailing pre-zygotic isolating mechanisms result in production of hybrids between distinct morphotypes (Selivon et al., 2005; Cáceres et al., 2009), sister species (Santos et al., 2001), and distinct species (Santos et al., 2001). Furthermore, such hybrids, although less viable in the first generation, are interfertile and recover high levels of fertility after self- and back crosses with parentals (Santos et al., 2001; Cáceres et al., 2009).

Production of novel pheromone blends through hybridization and female preference for such blends has been

suggested as a fast sympatric mechanism for radiation of tropical fruit flies in the genus *Anastrepha* (Cáceres et al., 2009; Segura et al., 2011). It can be proposed that differences in host affiliation among some morphotypes of *A. fraterculus* (Baker et al., 1944; Baker, 1945; Aluja et al., 2003) could additionally influence the production of different pheromone blends (Symonds & Elgar, 2008) as has been found for closely related species in the tropical tephritid genus *Bactrocera* (Symonds et al., 2009), and interrupt gene flow between sympatric or parapatric host-associated populations. As has been proven for some species of *Drosophila* that develop on different larval diets (Rundle et al., 2005), *A. fraterculus* exploiting different hosts could produce males with distinct cuticular hydrocarbon profiles and females with preferences for such profiles and aversion for others. It has been proposed for herbivorous insects in general that specialization could be fostered by local adaptations to specific host food plants and reinforced by kairomones and sex pheromones (Loxdale et al., 2011). Our observations allow us to propose that cuticular hydrocarbons and other factors influencing female mating discrimination at close range can further reinforce isolation. Radiation in the tropical genus *Anastrepha* and probably other tropical genera in Tephritidae seems to be the result of ecological adaptation promoting reproductive isolation and speciation (sensu Funk et al., 2006). Studying the natural history and the evolution of reproductive barriers among cryptic and sister species of the *fraterculus* group is a viable experimental approach to gather empirical support for this hypothesis.

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## References

Abraham S, Goane L, Rull J, Cladera J, Willink E & Vera MT (2011) Multiple mating in *Anastrepha fraterculus* (Diptera: Tephritidae) females and its relationship with fecundity and fertility. Entomologia Experimentalis et Applicata 141: 15–24.

Aluja M, Pérez-Staples D, Macias-Ordoñez R, Piñero J, McPherson B & Hernández-Ortiz V (2003) Non host status of *Citrus sinensis* cultivar Valencia and *C. paradisi* cultivar Ruby Red to Mexican *Anastrepha fraterculus* (Diptera: Tephritidae). Journal of Economic Entomology 96: 1693–1703.

Baker AC (1945) Studies on the Mexican fruit fly known as *Anastrepha fraterculus*. Journal of Economic Entomology 35: 95–100.

Baker AC, Stone WE, Plummer CC & McPhail MA (1944) A review of studies of the Mexican fruit fly and related Mexican species. US Department of Agriculture Miscellaneous Publications 351: 1–155.

Berlocher SH (2000) Radiation and divergence in the *Rhagoletis pomonella* species group: inferences from allozymes. Evolution 54: 543–557.

Berlocher SH & Feder JL (2002) Sympatric speciation in phytophagous insects: moving beyond controversy? Annual Review of Entomology 47: 773–815.

Bush GL (1962) The cytotaxonomy of the larvae of some Mexican fruit flies in the genus *Anastrepha* (Tephritidae: Diptera). Psyche 69: 87–101.

Bush GL (1969) Sympatric host race formation and speciation in frugivorous flies of the genus *Rhagoletis* (Diptera: Tephritidae). Evolution 23: 237–251.

Butlin RK (1987) Species, speciation, and reinforcement. American Naturalist 130: 461–464.

Cáceres C, Segura DF, Vera MT, Wornoayporn V, Cladera JL 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.

Cayol JP, Vilardi JC, Rial E & Vera MT (1999) New indices and methods to measure the sexual compatibility and mating performance of medfly (Diptera: Tephritidae) laboratory reared strains under field cage conditions. Journal of Economic Entomology 92: 140–145.

Clarke AR, Armstrong KF, Carmichael AE, Milne JR, Raghu S et al. (2005) Invasive phytophagous pests arising through recent tropical evolutionary radiation: the *Bactrocera dorsalis* complex of fruit flies. Annual Review of Entomology 50: 293–319.

Condon M, Adams DC, Bann D, Flaherty K, Gammons J et al. (2008) Uncovering tropical diversity: six sympatric cryptic species of *Blepharoneura* (Diptera: Tephritidae) in flowers of *Gurania spinulosa* (Cucurbitaceae) in eastern Ecuador. Biological Journal of the Linnean Society 93: 779–797.

Coyne JA & Orr HA (1989) Patterns of speciation in *Drosophila*. Evolution 43: 362–381.

Coyne JA & Orr HA (1997) "Patterns of speciation in *Drosophila*" revisited. Evolution 51: 295–303.

Coyne JA & Orr HA (2004) Speciation. Sinauer Associates, Sunderland, MA, USA.

Craig TP, Horner JD & Itami JK (1997) Hybridization studies on the host races of *Eurosta solidaginis*: implications for host shifts and speciation. Evolution 55: 773–782.

Dobzhansky T & Koller PC (1938) An experimental study of sexual isolation in *Drosophila*. Biologisches Zentralblatt 58: 589–607.

Dyck VA, Hendrichs J & Robinson AS(Eds.) (2005) Sterile insect technique. Principles and Practice in Area-Wide Integrated Pest Management. Springer, Dordrecht, the Netherlands.

Feder JL, Chilcote CA & Bush GL (1988) Genetic differentiation between sympatric host races of *Rhagoletis pomonella*. Nature 336: 61–64.

Ferveur JF (2005) Cuticular hydrocarbons: their evolution and roles in *Drosophila* pheromonal communication. Behavioral Genetics 35: 279–295.

Funk DJ, Nosil P & Etges WJ (2006) Ecological divergence exhibits consistently positive associations with reproductive isolation across disparate taxa. Proceedings of the National Academy of Sciences of the USA 103: 3209–3212.

Hernández-Ortiz V, Gómez-Anaya JA, Sánchez A, McPheron BA & Aluja M (2004) Morphometric analysis of Mexican and South American populations of the *Anastrepha fraterculus* complex (Diptera: Tephritidae) and recognition of a distinct Mexican morphotype. Bulletin of Entomological Research 94: 487–499.

Hernández-Ortiz V, Bartolluci AF, Morales-Valle P, Frías D & 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.

Hoskin CJ, Higgie M, McDonald KR & Moritz C (2005) Reinforcement drives rapid allopatric speciation. Nature 437: 1353–1356.

Knipling EF (1959) Screwworm eradication: concepts and research leading to the sterile male method. Smithsonian Institution Publication 4365: 409–418.

Loxdale HD, Lushai G & Harvey JA (2011) The evolutionary improbability of 'generalism' in nature, with special reference to insects. Biological Journal of the Linnean Society 103: 1–18.

Ludeña B, Bayas R & Pintaud JC (2010) Phylogenetic relationships of Andean-Ecuadorian populations of *Anastrepha fraterculus* (Wiedemann 1830) (Diptera: Tephritidae) inferred from COI and COII gene sequences. Annales de la Societé Entomologique de France 46: 344–350.

Mallet J (2005) Hybridization as an invasion of the genome. Trends in Ecology and Evolution 20: 229–237.

Mallet J (2007) Hybrid speciation. Nature 446: 279–283.

Mallet J (2008) Hybridization, ecological races and the nature of species: empirical evidence for the ease of speciation. Philosophical Transactions of the Royal Society B 363: 2971–2986.

Marie Curie SPECIATION Network (2012) What do we need to know about speciation? Trends in Ecology and Evolution 27: 27–39.

Mayr E (1963) Animal Species and Evolution. Harvard University Press, Cambridge, MA, USA.

Moehring AJ, Li J, Schug MD, Smith SG, de Angelis M et al. (2004) Quantitative trait loci for sexual isolation between *Drosophila simulans* and *D. mauritania*. Genetics 167: 1265–1274.

Morgante JS, Malavasi A & Bush GL (1980) Biochemical systematics and evolutionary relationships of neotropical *Anastrepha*. Annals of the Entomological Society of America 73: 622–630.

Nosil P & Schluter D (2011) The genes underlying the process of speciation. Trends in Ecology and Evolution 26: 160–167.

Rull J, Aluja M & Feder JL (2010) Evolution of intrinsic reproductive isolation among four North American populations of *Rhagoletis pomonella*. Biological Journal of the Linnean Society 100: 213–233.

Rull J, Abraham S, Kovaleski A, Segura DF, Islam A et al. (2012) Argentinean and Southern Brazilian populations of *Anastrepha fraterculus* (Diptera: Tephritidae). Bulletin of Entomological Research 102: 435–443.

Rundle HD, Chenoweth SF, Doughty P & Blows MW (2005) Divergent selection and the evolution of signal traits and mating preferences. PloS Biology 3: e368.

Santos P, Uramoto K & Matioli SR (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.

Segura DF, Vera MT, Rull J, Wornoayporn V, Islam A & Robinson AS (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 ALP (2007) Especies crípticas del complejo *Anastrepha fraterculus* en Brazil. Moscas de la Fruta en Latinoamerica (Diptera: Tephritidae): Diversidad Biológica y Manejo (ed. by V Hernández-Ortiz), pp. 101–118. Sy G Editores, Distrito Federal, Mexico.

Selivon D, Perondini ALP & Morgante JS (1999) Haldane's rule and other aspects of reproductive isolation observed in the *Anastrepha fraterculus* complex (Diptera: Tephritidae). Genetics and Molecular Biology 22: 507–510.

Selivon D, Perondini ALP & Morgante JS (2005) A genetic-morphological characterization of two cryptic species of the *Anastrepha fraterculus* complex (Diptera: Tephritidae). Annals of the Entomological Society of America 98: 367–381.

Smith-Caldas MRB, McPheron BA, Silva JG & Zucchi RA (2001) Phylogenetic relationships among species of the fraterculus group (Anastrepha: Diptera: Tephritidae) inferred from DNA sequences of mitochondrial cytochrome oxidase 1. Neotropical Entomology 30: 565–573.

Sobel JM, Chen GF, Watt LR & Schemske DW (2009) The biology of speciation. Evolution 64: 295–315.

Sokal RR & Crovello TJ (1970) The biological species concept: a critical evaluation. American Naturalist 104: 127–193.

Steck GJ (1991) Biochemical systematics and population genetic structure of *Anastrepha fraterculus* and related species (Diptera:

Tephritidae). Annals of the Entomological Society of America 84: 10–28.

Steck GJ & Sheppard WS (1993) Mitochondrial DNA variation in *Anastrepha fraterculus*. Fruit Flies, Biology and Management (ed. by M Aluja & P Liedo), pp. 9–14. Springer, New York, NY, USA.

Stone A (1942) The fruit flies of the genus *Anastrepha*. US Department of Agriculture Miscellaneous publications 439, Washington, DC: 1–112.

Symonds MRE & Elgar MA (2008) The evolution of pheromone diversity. Trends in Ecology and Evolution 23: 220–228.

Symonds MRE, Moussalli A & Elgar MA (2009) The evolution of sex pheromones in an ecologically diverse genus of flies. Biological Journal of the Linnean Society 97: 594–603.

Tanaka N, Okamoto R & Chambers DL (1970) Methods of mass rearing the Mediterranean fruit fly currently used by the United States Department of Agriculture. Proceedings on the Sterile Male Techniques for Control of Fruit Flies, pp. 19–23. International Atomic Energy Agency, 1–5 September 1969, Vienna, Austria.

Vera MT, Cáceres C, Wornoayporn V, Islam A, Robinson AS et al. (2006) Mating incompatibility among populations of the South American fruit fly *Anastrepha fraterculus* (Diptera: Tephritidae). Annals of the Entomological Society of America 99: 387–397.

Virgilio M, Backeljau T, Barr N & De Meyer M (2008) Molecular evaluation of nominal species in the *Ceratitis fasciventris, C. anonae, C. rosa* complex (Diptera: Tephritidae). Molecular Phylogenetics and Evolution 48: 270–280.