Entomologia
Experimentalis et Applicata

DOI: 10.1111/eea.12219

# Ongoing speciation within the *Anastrepha fraterculus* cryptic species complex: the case of the Andean morphotype

Francisco Devescovi[1], Solana Abraham[2], Alzira K.P. Roriz[3], Norma Nolazco[4], Rosario Castañeda[5], Eduardo Tadeo[6], Carlos Caceres[7], Diego F. Segura[1], M. Teresa Vera[8], Iara Joachim-Bravo[3], Nelson Canal[5] & Juan Rull[6]*

[1]*Instituto de Genética 'E.A. Favret', INTA Castelar, El Ñandú y Aristizabal s/n, Hurlingham (1686), Buenos Aires, Argentina,* [2]*Laboratorio de Investigaciones Ecoetológicas de Moscas de la Fruta y sus Enemigos Naturales (LIEMEN), PROIMI, Av. Belgrano y Pasaje Caseros s/n (T4001MVB), San Miguel de Tucumán, Tucumán, Argentina,* [3]*Depto. Biologia Geral, Instituto de Biologia, Universidade Federal da Bahia – UFBA, Rua Barão do Geremoabo s/n, 40170-290, Campus Universitário de Ondina, Salvador, Bahia, Brazil,* [4]*Laboratorios de Sanidad Vegetal - SENASA, Av. La Molina 1915, Lima 12, La Molina, Lima, Perú,* [5]*Grupo De Investigación en Moscas De Las Frutas "GIMFRUT", Facultad de Ingeniería Agronómica, Universidad del Tolima, Barrio Santa Helena Parte Alta, A.A. 546, Ibague, Colombia,* [6]*Pest Management, Instituto de Ecología, Congregación el Haya, A.C., Carretera Antigua a Coatepec no. 351, Xalapa, Veracruz, C.P. 91070, México,* [7]*Insect Pest Control Laboratory, Entomology Unit, FAO/IAEA Agriculture and Biotechnology Laboratories, A-24444, Seibersdorf, Austria, and* [8]*Cátedra de Terapéutica Vegetal, Departamento de Sanidad Vegetal, Facultad de Agronomía y Zootecnia, UNT, Avenida Roca 1900 – (4000) San Miguel de Tucumán, Tucumán, Argentina*

Accepted: 16 June 2014

*Key words*: pre-zygotic isolation, post-zygotic isolation, neotropical, sterile insect technique, SIT, Diptera, Tephritidae

**Abstract**

The *Anastrepha fraterculus* (Wiedemann) (Diptera: Tephritidae) cryptic species complex is currently composed of seven taxonomically recognized morphotypes. Both, pre- and post-zygotic isolation has been documented among four of these morphotypes, revealing that in fact they appear to be distinct biological entities. In order to progress in the full delimitation of species within the complex, we examined reproductive isolation between a Colombian population of the Andean morphotype and populations belonging to four other morphotypes spanning from Mexico to Argentina. Flies from the Andean morphotype exhibited strong pre-zygotic mating isolation through temporal partitioning of mating activity. Post-zygotic isolation was observed for crosses of males of all morphotypes and Andean morphotype females, yet most of the F1 hybrid ♂ × F1 hybrid ♀ self-crosses showed normal levels of fertility, a finding suggesting a nuclear–cytoplasmic interaction according to previous studies. Overall, the Andean morphotype within the complex also appears to be a distinct biological entity. We discuss the implications of these findings for the understanding of speciation mechanisms in the Neotropical genus *Anastrepha*.

## Introduction

The biological species concept (BSC) defines a species as a group of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups (Mayr, 1942). Studying speciation of taxa with sexual reproduction can therefore be approached through the study of the evolution of reproductive isolation (Coyne & Orr, 2004). It has been proposed that pre-zygotic isolation tends to evolve faster than post-zygotic reproductive isolation among sympatric than among allopatric species (Coyne & Orr, 1989, 1997), therefore examining the strength of different isolating barriers can shed some light on speciation modes of a particular animal group.

The South American fruit fly *Anastrepha fraterculus* (Wiedemann) (Diptera: Tephritidae) has been found to be a complex of at least seven cryptic species exhibiting subtle

*Correspondence: Juan Rull, Pest Management, Instituto de Ecología A.C., Km 2.5 Carretera Antigua a Coatepec, Xalapa, Veracruz, C.P. 91070, Mexico. E-mail: juan.rull@inecol.mx

morphological and biological differences (Selivon et al., 2005; Vera et al., 2006; Hernández-Ortiz et al., 2012; Rull et al., 2013). There has been recent interest in delimitating reproductive boundaries among morphotypes of the complex, both for applied and fundamental scientific reasons. From an applied perspective, identifying reproductively isolated morphotypes is critical for an efficient application of the sterile insect technique (SIT) (Cayol et al., 2002; Rull et al., 2012) and, because different morphotypes exhibit different host affiliations (Aluja et al., 2003), it is also important when establishing scientifically based trade restrictions and quarantine treatments for fresh fruit imports (Aluja & Mangan, 2008). From a theoretical perspective, examining reproductive isolation among recently evolved morphotypes can contribute in particular to explain tropical fruit fly diversity (Segura et al., 2011; Oroño et al., 2013; Rull et al., 2013) and in general to provide empirical support for establishing the relative importance of pre- and post-zygotic barriers during speciation (Coyne & Orr, 2004).

There have been previous efforts to explore the existence and strength of reproductive barriers among morphotypes of the *A. fraterculus* species complex. A reduction in egg hatch and F1 sex ratio distortion for heterotypic crosses between adults of the Brazil-1 and Brazil-2 morphotypes was observed by Selivon et al. (1999) and later corroborated in studies examining other biological differences (Selivon et al., 2005). In contrast, no pre- or post-zygotic (Petit-Marty et al., 2004a,b) isolating barriers were found among populations of *A. fraterculus* within Argentina, which all belong to the Brazil-1 morphotype (Alberti et al., 2002). These findings were extended to include Brazilian populations south of Sao Paulo (Rull et al., 2012), allowing delimitation of this morphotype's range and corroboration of reproductive compatibility among populations from central Argentina to southern Brazil. In a study encompassing several populations, Vera et al. (2006) found mating discrimination and differences in the timing of mating activities among flies of the Andean, Brazil-1, and Peruvian morphotypes. Cáceres et al. (2009) examined in detail pre- and post-zygotic isolation between the Brazil-1 (from Argentina) and Peruvian morphotypes confirming Vera et al. (2006) findings and also documenting low egg hatch for the cross of Peruvian males and Brazil-1 females. More recently, Rull et al. (2013) found strong pre- and post-zygotic isolation among Mexican, Brazil-1, and Peruvian morphotypes. Collectively, all of the previously identified morphotypes of the *A. fraterculus* species complex that have been examined (four out of seven) display some degree of reproductive isolation among themselves.

Pre-zygotic isolation has been found to occur through either temporal partitioning of mating activity (flies of different morphotypes mate at different times of the day) or strong mating discrimination resulting in marked (and sometimes complete) assortative mating (Vera et al., 2006; Cáceres et al., 2009; Rull et al., 2013). Mating discrimination is mediated from a distance by differences in male pheromone composition (Cáceres et al., 2009; Brízová et al., 2013) and also perhaps, after male–female contact is established, through differences in cuticular hydrocarbon composition that result in female resistance to copulation after mounting (Rull et al., 2013). Although there are differences in mating duration among morphotypes (Vera et al., 2006; Rull et al., 2013), such differences are not influenced by male origin, do not result in differential sperm transfer, and do not influence female remating propensity or the duration of the refractory period (Abraham et al., 2014).

All of the examined morphotypes display some degree of post-zygotic (postcopulatory) isolation. In general, heterotypic crosses in one male–female direction result in a reduction of F1 egg hatch, which vary in degree (Selivon et al., 1999, 2005; Cáceres et al., 2009; Rull et al., 2013) and some morphotypes have been found to carry *Wolbachia* infections (Selivon et al., 2002; Cáceres et al., 2009; Coscrato et al., 2009; Marcon et al., 2011). Also, hybrid crosses in one direction between adults of some morphotypes have been found to produce an F1 female-biased progeny (Selivon et al., 1999). Hybrids between morphotypes produce different and unique blends of male sex pheromone (Cáceres et al., 2009) to which hybrid females preferentially respond (Segura et al., 2011) and, finally, per cent egg hatch of hybrid self-crosses appears to be restored to levels similar to those recorded for homotypic parental crosses (Cáceres et al., 2009).

Overall, pre-zygotic isolation has been found to be stronger than post-zygotic isolation among morphotypes of the *A. fraterculus* species complex. Even when some heterotypic crosses are very infrequent, F1 hybrids are viable and can be backcrossed with parentals, suggesting that hybridization could be a component, along with host plant chemistry, of a speciation mode explaining radiation of the group (Segura et al., 2011; Oroño et al., 2013). This pattern may indicate, according to findings for *Drosophila* (Coyne & Orr, 1989, 1997), that the *A. fraterculus* species group is undergoing rapid radiation and that this process may have been triggered in sympatry (pre-zygotic isolation through reinforcement evolves faster among sympatric taxa).

Here, we explore pre- and post-zygotic isolation between *A. fraterculus* of the Andean morphotype (Colombia) and adults belonging to four distinct morphotypes: Brazil-1 (Argentina), Brazil-3 (Parnamirim), Mexican, and Peruvian. According to patterns unveiled during previous studies, we expected to find reproductive isolation for all mating combinations involving the Andean morphotype, with strong pre-zygotic isolation and some degree of post-zygotic incompatibility.

## Materials and methods

### Biological material

Flies from the Andean morphotype were obtained from a laboratory colony originally established from infested coffee berries (*Coffea arabica* L.) and reared for research purposes at the Laboratorio de Entomología in the Universidad del Tolima in Ibagué, Colombia, since 2005. The colony is refreshed with field-collected flies on an annual basis. Flies from the Mexican morphotype were obtained from naturally infested peaches [*Prunus persica* (L.) Batsch] in the vicinity of Xalapa, Veracruz, Mexico (19°32′N, 96°55′W), in July 2012. Flies from the Brazil-3 morphotype were obtained from infested guava (*Psidium guajava* L.) in the locality of Parnamirim, Rio Grande do Norte, Brazil (05°54′57″ S, 35°15′46″ W), in March 2012. Flies from the Brazil-1 morphotype were obtained from a laboratory colony originally established in 1997 from infested guavas in the locality of Yerba Buena, Tucumán, Argentina (26°48′5″S, 65°9′50″W). And flies from the Peruvian morphotype were obtained from a colony held at La Molina mass rearing facility since 2002, originally collected from infested cherimoyas (*Annona cherimola* Miller) in La Molina, south-eastern Lima, Peru (12°00′03″ S, 76°57′00″W). Collection sites are shown in Figure 1. All flies were shipped as pupae to the Entomology Unit, FAO/IAEA Agriculture and Biotechnology Laboratory, in Seibersdorf, Austria, and maintained according to methods described by Rull et al. (2012) until experiments. Before testing, flies were sexed upon adult emergence and held in separation until sexual maturity (males: 10–25 days old; females: 15–25 days old) in cylindrical Plexiglas cages with free access to water and a diet consisting of wheat germ, hydrolysed yeast, and sugar at a 1:1:3 ratio.

### Pre-zygotic isolation

For pre-zygotic isolation tests, 20 sexually mature virgin males and 20 sexually mature virgin females of the Brazil-1,


**Figure 1** Collection sites of *Anastrepha fraterculus* flies of the Mexican (Xalapa, Mexico), Andean (Ibagué, Colombia), Brazil-3 (Parnamirim, Brazil), Peruvian (La Molina, Peru), and Brazil-1 (Yerba Buena, Tucumán, Argentina) morphotypes. Collection sites were highlighted on a map showing major biogeographic areas according to Morrone (2006) and modified by Hernández-Ortiz et al. (2012).

Andean, Mexican, and Peruvian morphotypes were marked on the notothorax with a distinctive dot of vinyl paint according to fly origin. No adults of the Brazil-3 morphotype were available for these tests. Flies were held in a dark room at 20 °C overnight and taken into a greenhouse at 26 °C, 65% r.h., around 08:30 hours. Within the greenhouse, 20 marked males of the Andean morphotype and 20 marked males of either the Brazil-1, Mexican, or Peruvian morphotype were released inside a cylindrical (2 m high, 3 m diameter) field cage containing a potted citrus tree (2 m high, 1.2 m canopy diameter) in its centre. After 15 min, 20 females from the Brazil-1, Mexican, or Peruvian morphotypes and 20 females of the Andean morphotype were released in the cages. Thereafter, mating couples were gently placed in a plastic transparent vial and immediately labelled with a permanent marker. The time at which the mating pair was detected as well as the colour mark of each sex were recorded. The vials containing couples were continuously inspected until flies disengaged and the copulation ending time was noted. Observations were carried out from 08:30 to 19:00 hours. Five replicates were performed for the Brazil-1–Andean combination, four for the Mexican–Andean combination, and two for the Peruvian–Andean combination.

#### Post-zygotic isolation

To assess the degree of post-zygotic isolation between the Andean and the Brazil-1, Brazil-3, Mexican, and Peruvian morphotypes, 10 sexually mature males and 10 sexually mature females of the four possible mating combinations [two pure parental crosses and hybrid crosses in both directions (reciprocal)] were placed inside cylindrical Plexiglas cages (45 cm high, 20 cm diameter) and were provided with water and adult diet. Flies were left in cages for 2 days and allowed to copulate (copulations were observed in all cages). Three cages were set up for every possible homotypic and Andean heterotypic combination (this resulted in 39 cages considering that only three cages of the Andean–Andean combination were set up).

An artificial egg-laying device, consisting of the bottom of a 10-cm-diameter Petri dish in which a ca. 9-cm-diameter hole was cut and covered with a piece of cloth that had been previously lined with a thin layer of black silicone, was placed on top of each cage. The device was filled with water to prevent dehydration of eggs. Eggs were recovered every 48 h using a Pasteur pipette, lined over a piece of black filter paper which was later placed over moist cotton inside a clean Petri dish. The eggs laid were counted and incubated under a dark cloth. After 2 days, the filter paper was transferred to a Petri dish with larval diet and left for a total of 6 days when egg hatch was recorded. For every cage, four egg collections of at least 30 eggs were made.

In the case of crosses involving Andean females, which had been selected to lay eggs on red wax domes placed on the bottom of laboratory cages, such devices had to be used. Red wax domes were also exposed for 48 h, after which period eggs were removed from domes with a paint brush aligned over a Petri dish and treated in an identical manner as eggs recovered from upper-cage egg-laying devices.

#### F1 sex ratio and viability

Each Petri dish containing diet with F1 larvae was left inside a plastic container with a tight sealing lid using vermiculite as pupation substrate. Larvae refraining from leaving the diet were gently transferred in the vermiculite to force pupation. One hundred pupae of every possible combination were placed in a Plexiglas cage to record adult emergence and calculate sex ratios. Emerging adults were allowed to mature sexually and mate with each other (i.e., F1♂ × F1♀), and egg-laying devices were placed on top of cages to verify viability (fertility/egg hatch) of the F1 progeny using similar methods as those described above for homo- and heterotypic crosses of parental morphotypes.

#### Statistical analysis

*Pre-zygotic isolation.* The total number of copulations in field cages was compared among male–female mating combinations using Kruskal–Wallis tests. In the case of the Andean–Peruvian combination total number of copulations per replicate per mating combination was compared to a uniform distribution of male–female mating combinations using a $\chi^2$ test (only two replicates could be performed). Latency to mate and duration of copulations were compared between Brazil-1 and Andean morphotypes using a Mann–Whitney U-test (there were no heterotypic matings). In the case of the Mexican–Andean and Peruvian–Andean combinations, latency to mate and duration of copulations were compared using Kruskal–Wallis tests. All analyses were carried out using STATISTICA, version 7 (Statsoft, Tulsa, OK, USA).

*Post-zygotic isolation.* Mean per cent egg hatch was compared among all possible male–female mating combinations between the Andean and either the Brazil-1, Mexican, Peruvian, or Brazil-3 morphotypes using one-way ANOVAs on arcsin$\sqrt{x}$-transformed proportions. Sex ratio of F1 adults was compared to a 1:1 ratio using a $\chi^2$ homogeneity test. Finally F1 × F1 egg hatch of all male–female mating combinations was contrasted against a uniform distribution of egg hatch with a $\chi^2$ homogeneity test.

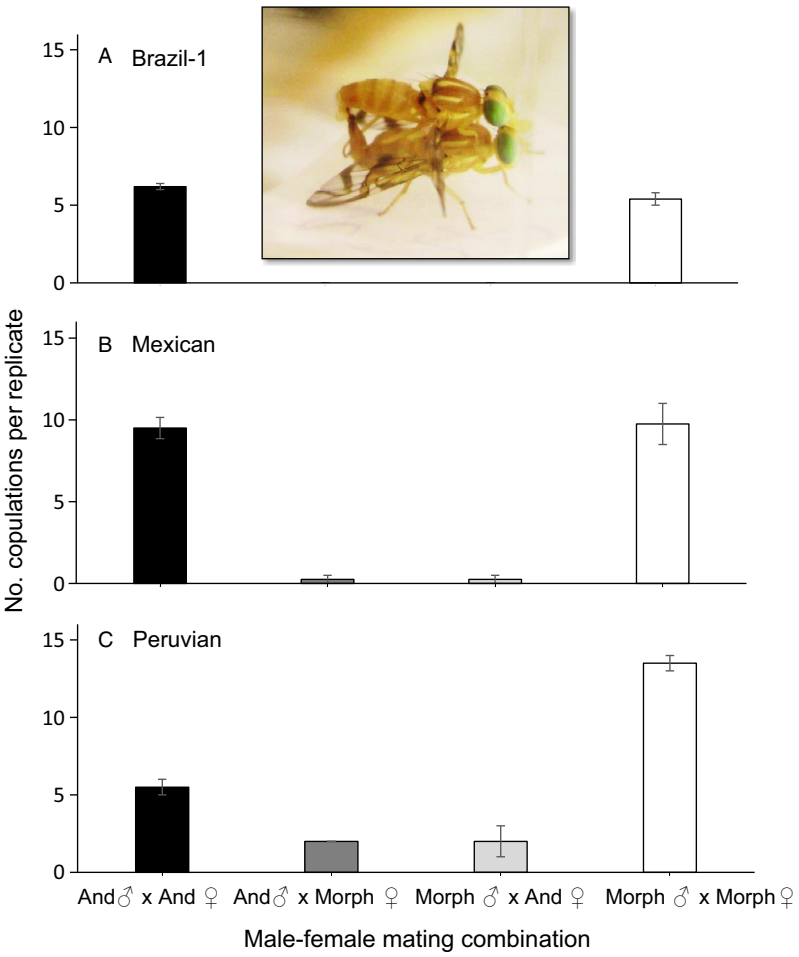


**Figure 2** Mean (± SE) number of copulations per replicate per male–female mating combination for adult *Anastrepha fraterculus* flies of the Andean (And) and (A) Brazil-1, (B) Mexican, and (C) Peruvian morphotypes (Morph).

## Results

### Pre-zygotic isolation

*Mating compatibility.* There were significant differences in total number of copulations among the four possible male–female combinations of Brazil-1 and Andean morphotypes (H = 17.45, d.f. = 3, P<0.01; n = 20); in fact there were no heterotypic copulations recorded for this morphotype combination (Figure 2A). For the Mexican–Andean combination, there was also a significantly higher number of homotypic than heterotypic copulations (H = 11.96, d.f. = 3, P<0.01; n = 16) (Figure 2B). In the case of the Peruvian–Andean morphotype combination, the $\chi^2$ test revealed significant deviations from random mating (homogeneous total copulations for all four possible male–female combinations) ($\chi^2$ = 30.69, d.f. = 3, P<0.01), with more homotypic than heterotypic copulations (Figure 2C).

**Table 1** Mean (± SE) latency to mate (min) for all possible male–female mating combinations between flies of the *Anastrepha fraterculus* Andean morphotype (And) and flies of the Brazil-1, Mexican, or Peruvian morphotype (Morph). Sample sizes in parenthesis

| | Mating combination | | | |
|---|---|---|---|---|
| Morphotype | And♂ × And♀ | Morph♂ × And♀ | And♂ × Morph♀ | Morph♂ × Morph♀ |
| Brazil-1 | 489.7 ± 6.01a (30) | – | – | 21.7 ± 4.04b (27) |
| Mexican | 457.71 ± 10.2a (39) | 207b (1) | 165b (1) | 82.2 ± 13.1b (38) |
| Peruvian | 476.36 ± 20.11a (11) | 481.5 ± 27.7a (4) | 315 ± 82ab (4) | 162.6 ± 25.2b (27) |

Means within a row followed by different letters are significantly different [Mann–Whitney (Brazil-1) and Kruskal–Wallis test: P<0.05].

*Latency to mate.* Flies from the Andean morphotype mated significantly later than flies from the Brazil-1 morphotype ($Z = 6.47$, $P<0.001$; Table 1). For the Mexican–Andean combination significant differences in latency to mate among the four male–female mating combinations were detected ($H = 59.03$, d.f. = 3, $P<0.001$; $n = 79$), with flies from the Andean morphotype mating later than Mexican flies (Table 1). For the Peruvian and Andean combinations, there were also significant differences in latency to mate among the four possible male–female mating combinations ($H = 27.41$, d.f. = 3, $P<0.001$; $n = 46$) with flies from the Andean morphotype mating substantially later than Peruvian flies (Table 1).

*Duration of copulation.* On average, Andean homotypic matings lasted shorter than homotypic Brazil-1 matings ($Z = 4.51$, $P<0.001$; Table 2). For the Mexican–Andean morphotypes no differences were found in mating duration among male–female mating combinations ($H = 3.74$, d.f. = 2, $P = 0.15$; $n = 73$) (Table 2). Similarly, there were no differences in mating duration among male–female combinations of Andean and Peruvian flies ($H = 4.33$, d.f. = 3, $P = 0.23$; $n = 45$) (Table 2).

#### Post-zygotic isolation

A one-way ANOVA on arcsin√x-transformed proportions revealed no significant differences in egg hatch among the four Brazil-1–Andean male–female mating combinations ($F_{3,8} = 2.72$, $P = 0.11$; Table 3). The cross of Mexican males and Andean females yielded significantly lower per cent of egg hatch than the Mexican homotypic cross ($F_{3,6} = 8.19$, $P = 0.015$; Table 3). The Peruvian male × Andean female cross produced significantly lower percentages of egg hatch than both homotypic crosses ($F_{3,8} = 7.48$, $P = 0.010$; Table 3). Similarly, Brazil-3 males × Andean females produced significantly lower percentages of egg hatch than the homotypic cross of Brazil-3 flies ($F_{3,8} = 7.32$, $P = 0.011$; Table 3).

#### F1 sex ratio and viability

There was no significant deviation from a 1:1 sex ratio for any of the F1 × F1 self-crosses ($\chi^2 = 0.83$, d.f. = 1, $P = 0.99$) and although all of them were viable (adults were interfertile), there were significant differences in mean per cent egg hatch ($\chi^2 = 68.54$, d.f. = 11, $P<0.01$; Figure 3). Specifically, the Andean × Andean, Brazil-1 × Andean, and Brazil-3 × Andean mating combinations had lower egg hatch than average, whereas the Andean × Peruvian and Andean × Brazil-3 had higher egg hatch than average.

## Discussion

Reproductive isolation between flies from the Andean morphotype and four other morphotypes within the *A. fraterculus* complex was found acting both at the pre- and post-zygotic level. Pre-zygotic isolation was strong (and sometimes complete) and this was mainly the result of differences in the timing of mating activity: adults of the Brazil-1, Mexican, and Peruvian morphotypes became sexually active in the morning/noon, whereas those of the Andean morphotype did so late in the afternoon. When forced to copulate at high densities, all mating combinations produced fertile hybrids; however, egg hatch was reduced in the case of Andean females that mated with males from other morphotypes. There was no distortion of sex ratio for hybrid progeny and F1 adults were viable and frequently displayed higher fertility levels than the parental hybrid cross.

Marked differences in the timing of mating activities of Andean *A. fraterculus* compared to common mating patterns within the *A. fraterculus* complex (early morning or mid-day) could have evolved in response to selection against maladaptive hybridization in sympatry (Butlin, 1987). However, it is unlikely that selection pressure for differences in diel rhythm emerged from coexistence with the tested morphotypes of *A. fraterculus*, which are all allopatric to the Andean morphotype (Hernández-Ortiz et al., 2012). In fact, differences in the timing of mating activity

**Table 2** Mean (± SE) duration of copulation (min) between flies of the *Anastrepha fraterculus* Andean (Colombia; And) morphotype and flies of the Brazil-1 (Argentina), Mexican, or Peruvian morphotype (Morph). Sample sizes in parenthesis

| | Mating combination | | | |
|---|---|---|---|---|
| Morphotype | And♂ × And♀ | Morph♂ × And♀ | And♂ × Morph♀ | Morph♂ × Morph♀ |
| Brazil-1 | 25.3 ± 2.8a (30) | – | – | 56.4 ± 5.5b (27) |
| Mexican | 50.8 ± 5.7a (39) | 5a (1) | – | 58.2 ± 4.8a (38) |
| Peruvian | 38.8 ± 6.5a (11) | 38.2 ± 5.1a (4) | 19.8 ± 4.6a (4) | 35.07 ± 3.6a (27) |

Means within a row followed by different letters are significantly different [Mann–Whitney (Brazil-1) and Kruskal–Wallis test: $P<0.05$].

**Table 3** Mean (± SE) egg hatch (%) for all possible male–female crosses between the *Anastrepha fraterculus* Andean morphotype (And) and flies of the Brazil-1, Brazil-3, Mexican, or Peruvian morphotypes (Morph)

| | Mating combination | | | |
|---|---|---|---|---|
| Morphotype | And♂ × And♀ | Morph♂ × And♀ | And♂ × Morph♀ | Morph♂ × Morph♀ |
| Brazil-1 | 0.59 ± 0.04a | 0.47 ± 0.10a | 0.51 ± 0.09a | 0.75 ± 0.04a |
| Mexican | 0.59 ± 0.04ab | 0.25 ± 0.12b | 0.49 ± 0.10ab | 0.76 ± 0.04a |
| Peruvian | 0.59 ± 0.04a | 0.08 ± 0.05b | 0.39 ± 0.18ab | 0.73 ± 0.07a |
| Brazil-3 | 0.59 ± 0.04ab | 0.30 ± 0.03b | 0.62 ± 0.13ab | 0.81 ± 0.04a |

Means within a row followed by different letters are significantly different (ANOVA: $P<0.05$).

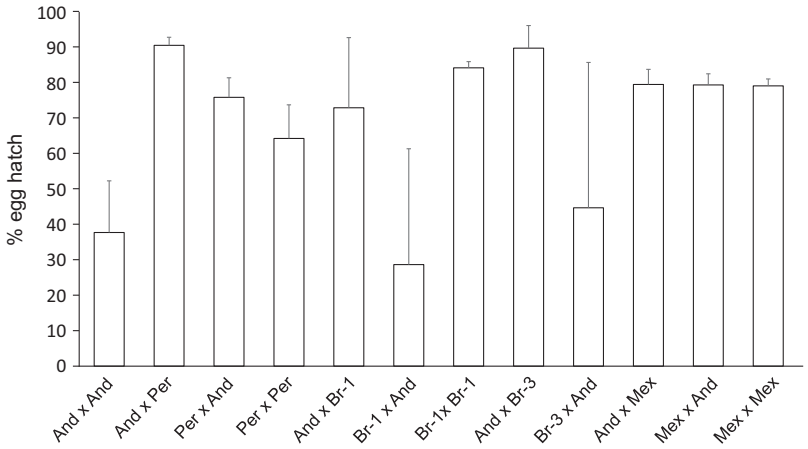


**Figure 3** Mean (+ SE) egg hatch (%) for F1♂ × F1♀ crosses of parental male–female homotypic and heterotypic mating combinations between *Anastrepha fraterculus* flies of the Andean (And) morphotype and either the Brazil-1 (Br-1), Mexican (Mex), Peruvian (Per), or Brazil-3 (Br-3) morphotypes.

among sympatric species in the *A. fraterculus* species group, such of *Anastrepha zenildae* Zucchi, *Anastrepha sororcula* Zucchi, *Anastrepha obliqua* Macquart, and *A. fraterculus* have been recently documented in Brazil (De Almeida et al., 2011). Similarly, *Anastrepha schultzi* Blanchard and *A. fraterculus* morphotype Brazil-1 coexist in northern Argentina, both exploiting native walnuts and guavas (Schliserman et al., 2004) and exhibiting marked differences in the time of mating activity (J Rull, S Ovruski & P Schliserman, unpubl.).

The Andean morphotype of *A. fraterculus* in Colombia is sympatric with *Anastrepha distincta* Greene, *A. sororcula*, and *A. obliqua* (Castañeda et al., 2010), three closely related species of the *A. fraterculus* group (Santos et al., 2001; Smith-Caldas et al., 2001). Among these sister species, it is known that *A. obliqua* has a peak of mating activity early in the morning (Aluja et al., 2000) whereas there is no published information on mating behaviour of the other species. Reproductive isolation through temporal partitioning of mating activity among allopatric *A. fraterculus* morphotypes may therefore be the by-product of selection against maladaptive hybridization on adults of the Andean morphotype and local guilds of sympatric sister species with spatial overlap.

Smith-Caldas et al. (2001) reported strong molecular divergence between geographically connected *A. fraterculus* populations at low (Caracas, Venezuela) and high (Merida, Venezuela) elevations. Flies from the highlands of Colombia and Venezuela belonging to the Andean morphotype exploit, among other hosts, large extensions of coffee (Nuñez et al., 2004; Castañeda et al., 2010), whereas flies from the Venezuelan morphotype (one of the least studied morphotypes in the complex)—which probably extend to the Caribbean lowlands of both countries and appear to be parapatric with the Andean morphotype—exploit other hosts such as tropical almonds and guava (Hernández-Ortiz & Morales-Valles, 2004). Under such a scenario, differences in host plant chemistry could contribute to morphotype divergence (Oroño et al., 2013), and reproductive isolation could have been reinforced in a contact zone at mid elevation. Comparing the degree of reproductive isolation in the contact zone and at the extremes of their distribution, as it has been done between allopatric and sympatric populations of sister species of *Drosophila* (Noor & Ortíz-Barrientos, 2006), could reveal whether or not divergence of these two morphotypes is the result of reinforcement. Studying host affiliation, diel rhythms, and mating isolation among parapatric

morphotypes and among sympatric sister species across elevation gradients in Colombia could contribute to our understanding of how mating patterns evolve under selection against maladaptive hybridization.

Finally, differences in the timing of mating activities could be the result of inhabiting environments that differ in physical attributes such as humidity, light intensity, temperature, vegetation structure, and guild of predators, which have all been shown to influence fruit fly mating behaviour (Hendrichs & Hendrichs, 1990; Hendrichs et al., 1994; Aluja et al., 2000). Therefore, the timing of mating activity of the Andean morphotype could have evolved as a response to local environmental conditions, given that the coolest part of the day in the Colombian highlands occurs early in the morning and late in the afternoon.

In the case of post-zygotic isolation, the cross of Peruvian, Mexican, and Brazil-3 males with Andean females resulted in lower egg hatch than homotypic mating combinations. However, the few obtained F1 adults were viable and laid eggs that hatched normally. Cáceres et al. (2009) reported similar findings for the cross of Brazil-1 females and Peruvian males, concluding that low egg hatch in the parental cross may be the result of major karyotypic differences between morphotypes that translated into large asynaptic areas in the hybrids. They further conclude that a nuclear–cytoplasmic interaction may be at play because the cross of identical genotypes (F1 × F1) did not result in reduced egg hatch. We detected very similar patterns between the Andean morphotype and the four other *A. fraterculus* morphotypes (Mexican, Brazil-1, Brazil-3, Peruvian). Some morphotypes of the *A. fraterculus* species complex have been found to carry single and even double infections of *Wolbachia* (Selivon et al., 2002; Cáceres et al., 2009; Coscrato et al., 2009; Marcon et al., 2011), although several morphotypes have not been examined yet. Post-zygotic isolation patterns in our study could be suggestive of cytoplasmic incompatibility due to *Wolbachia*, a hypothesis that could be corroborated by screening flies from the morphotypes evaluated here. Altogether, our results suggest that the *A. fraterculus* Andean morphotype is a distinct biological entity.

It is important to highlight the fact that our methodology, largely based on the BSC, is a useful tool for cryptic species delimitation within the *A. fraterculus* species group. Indeed, geographically isolated populations belonging to the same morphotype display similar mating patterns, random mating, and reproductive compatibility (Petit-Marty et al., 2004a,b; Rull et al., 2012), whereas populations belonging to different morphotypes show partial, and even complete, pre-zygotic reproductive isolation (Vera et al., 2006; Cáceres et al., 2009; Rull et al., 2013), asymmetrical post-zygotic isolation (Selivon et al.,1999,2005; Cáceres et al., 2009; Rull et al., 2013), and sex ratio distortion (Selivon et al., 1999; Cáceres et al., 2009). Collective evidence indicates that all of the morphotypes identified with taxonomic techniques (Hernández-Ortiz et al., 2012) tested so far are distinct biological species. Comparatively, similar methods have been applied to delimit putative cryptic species in the *Bactrocera dorsalis* (Hendel) complex, but yielded opposite results, with two cryptic species displaying full pre- and post-zygotic compatibility (Schutze et al., 2012, 2013).

Santos et al. (2001) examined post-zygotic isolation among three distinct species in the *A. fraterculus* group (*A. sororcula*, *A. obliqua*, and *A. fraterculus*) and found that some interspecific crosses did not yield offspring at all and others produced marked distortions in sex ratio (only females). These results, coupled with our findings, suggest that post-zygotic isolation among cryptic species in the *A. fraterculus* group is at an early evolutionary state and should become stronger over time and also that pre-zygotic isolation evolved faster within the complex than post-zygotic isolation. In fact, in our study the highest level of pre-zygotic isolation was recorded between flies from the Andean and Brazil-1 morphotypes. However, when females from the Andean morphotype were forced to copulate with males from the Brazil-1 morphotype, the percentage of egg hatch was comparable to that of homotypic crosses. According to Coyne & Orr (1989, 1997) such a pattern is suggestive of sympatric speciation events. Further, exploration of reproductive isolation between sister species pairs in the *A. fraterculus* species group may aid in understanding speciation mechanisms in tropical *Anastrepha* spp.

From an applied perspective, our results stress the importance to delimit the extent of the geographical range of different *A. fraterculus* morphotypes, given that they exhibit differences in host plant affiliations that have strong implications on commercial exchange of fresh fruit (Aluja et al., 2003). Additionally, efficient application of area-wide management schemes such as the SIT require that mass-reared strains released in a particular area belong to the same morphotype as wild populations causing economic damage to fruit production (Rull et al., 2012), a factor that becomes highly relevant considering the magnitude of pre-zygotic isolation among morphotypes revealed here.

So far, only the Venezuelan and Brazil-2 morphotypes remain to be tested for reproductive isolation. However, the *A. fraterculus* group as a whole may still contain substantial hidden diversity. For example, Ludueña et al. (2010) reported a distinct clade in the Ecuatorian Andes,

whereas Ruiz-Arce et al. (2012) revealed the existence of six genetically distinct populations of *A. obliqua* among which the magnitude of differences warrants taxonomic revision. This recent and rapid differentiation renders the *A. fraterculus* species group a potential model for the understanding of divergence in tropical frugivorous insects but also renders management of pest species a highly complex task.

## Acknowledgements

We are grateful to Thilakasiri Dammalage, Ulysses Santo Tomas, and Sohel Ahmad for technical assistance. Special thanks to Jesús Reyes for arranging fly supply and technical visits and to Jorge Hendrichs, Marc Vreysen, and Rui Pereira for their support during the entire experimental process at the FAO/IAEA Agriculture and Biotechnology Laboratories in Seibersdorf, Austria. We also thank John Jaenike and an anonymous reviewer for their constructive input. Funding was provided through a Research Contract (16038) as part of the FAO/IAEA Coordinated Research Project on Resolution of cryptic species complexes of tephritid pests to overcome constraints to SIT and International trade as well as a FAO/IAEA consultancy to JR and a COLCIENCIAS project (1105-489-25567) to NC and RC.

## References

Abraham S, Rull J, Mendoza M, Liendo MC, Devescovi F et al. (2014) Differences in sperm storage and remating propensity between adult females of two morphotypes of the *Anastrepha fraterculus* (Diptera: Tephritidae) cryptic species complex. Bulletin of Entomological Research 104: 376–382.

Alberti AC, Rodriguero MS, Cendra PG, Saidman BO & Vilardi JC (2002) Evidence indicating that Argentine populations of *Anastrepha fraterculus* (Diptera: Tephritidae) belong to a single biological species. Annals of the Entomological Society of America 95: 505–512.

Aluja M & Mangan RL (2008) Fruit fly (Diptera: Tephritidae) host status determination: critical conceptual, methodological, and regulatory considerations. Annual Review of Entomology 53: 473–502.

Aluja M, Pinero J, Jácome I, Diaz-Fleischer F & Sivinski J (2000) Behaviour of flies in the genus *Anastrepha* (Trypetinae: Toxotrypanini). Fruit Flies (Tephritidae): Phylogeny and Evolution of Behavior (ed. by M Aluja & AL Norrbom), pp. 375–406. CRC Press, Boca Raton, FL, USA.

Aluja M, Pérez-Staples D, Macias-Ordoñez R, Piñero J, McPheron 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.

Brízová R, Mendonça AL, Vanícková L, Mendonça AL, Da Silva CE et al. (2013) Pheromone analyses of the *Anastrepha fraterculus* (Diptera: Tephritidae) cryptic species complex. Florida Entomologist 96: 1107–1115.

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.

Castañeda MR, Osorio FA, Canal NA & Galeano PE (2010) Especies, distribución y hospederos del género *Anastrepha* Schiner en el departamento de Tolima Colombia. Agronomía Colombiana 28: 265–271.

Cayol JP, Coronado P & Taher M (2002) Sexual compatibility in medfly (Diptera: Tephritidae) from different origins. Florida Entomologist 85: 51–57.

Coscrato VE, Braz AS, Perondini AL, Selivon D & Marino CL (2009) *Wolbachia* in *Anastrepha* fruit flies (Diptera: Tephritidae). Current Microbiology 59: 295–301.

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.

De Almeida LM, Araújo A, Mendes NHD, de Souza JMGA & Menezes AAL (2011) The temporal pattern of mating behavior of the fruit fly, *Anastrepha zenildae* in the laboratory. Journal of Insect Science 11: 1–9.

Hendrichs J & Hendrichs MA (1990) Mediterranean fruit fly (Diptera: Tephritidae) in nature: location and diel pattern of feeding and other activities on fruiting and non fruiting hosts and non hosts. Annals of the Entomological Society of America 83: 632–641.

Hendrichs J, Katsoyannos BI, Wornoayporn V & Hendrichs MA (1994) Odour-mediated foraging by yellow jacket wasps (Hymenoptera: Vespidae): predation on leks of pheromone-calling Mediterranean fruit fly males (Diptera: Tephritidae). Oecologia 99: 88–94.

Hernández-Ortiz V & Morales-Valles P (2004) Distribución geográfica y plantas hospederas de *Anastrepha fraterculus* (Diptera: Tephritidae) en Venezuela. Folia Entomológica Mexicana 43: 181–189.

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.

Ludueña 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 Société Entomologique de France 46: 344–350.

Marcon HS, Coscrato VE, Selivon D, Perondini ALP & Marino CL (2011) Variations in the sensitivity of different

primers for detecting *Wolbachia* in *Anastrepha* (Diptera: Tephritidae). Brazilian Journal of Microbiology 42: 778–785.

Mayr E (1942) Systematics and the Origin of Species, from the Viewpoint of a Zoologist. Harvard University Press, Cambridge, MA, USA.

Morrone JJ (2006) Biogeographic areas and transition zones of Latin America and the Caribbean Islands based on pangeographic and cladistics analyses of the entomofauna. Annual Review of Entomology 51: 467–494.

Noor MAF & Ortíz-Barrientos D (2006) Simulating natural conditions in the laboratory: a re-examination of sexual isolation between sympatric and allopatric populations of *Drosophila pseudoobscura* and *D. persimilis*. Behavior Genetics 36: 322–327.

Nuñez L, Gómez Santos R, Guarín G & León G (2004) Fruits flies (Diptera: Tephritidae) and parasitoids associated with Psidium guajava L. and Coffea arabica L. in three municipalities of Vélez County (Santander, Colombia) -Part 2: Identification and evaluation of Hymenoptera parasitoids. Revista Corpoica 5: 13–21.

Oroño L, Paulin L, Alberti AC, Hilal M, Ovruski S 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.

Petit-Marty N, Vera MT, Calcagno G, Cladera JL, Segura DF et al. (2004a) Sexual behavior and mating compatibility among four populations of *Anastrepha fraterculus* (Diptera: Tephritidae) from Argentina. Annals of the Entomological Society of America 97: 1320–1327.

Ruiz-Arce R, Barr NB, Owen CL, Thomas DB & McPheron BA (2012) Phylogeography of *Anastrepha obliqua* inferred with mtDNA sequencing. Journal of Economic Entomology 105: 2147–2160.

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.

Rull J, Abraham S, Kovaleski A, Segura DF, Mendoza M et al. (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.

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.

Schliserman P, Ovruski S, Colin C, Norrbom AL & Aluja M (2004) First report of *Juglans australis* (Juglandaceae) as a natural host plant for *Anastrepha schultzi* (Diptera: Tephritidae) with notes on probable parasitism by *Doryctobracon areolatus*, *D. brasiliensis*, *Opiusbellus* (Braconidae) and *Aganaspis pelleranoi* (Figitidae). Florida Entomologist 87: 597–599.

Schutze MK, Krosch MN, Armstrong KF, Chapman TA, Englezou A et al. (2012) Population structure of *Bactrocera dorsalis*, *B. papayae* and *B. philippinensis* (Diptera: Tephritidae) in southeast Asia: evidence for a single species hypothesis using mitochondrial DNA and wing shape data. BMC Evolutionary Biology 12: 1–15.

Schutze MK, Jessup A, Ul-Haq I, Vreysen MJB, Wornoayporn V et al. (2013) Mating compatibility among four pest members of the *Bactrocera dorsalis* fruit fly species complex (Diptera: Tephritidae). Journal of Economic Entomology 106: 695–707.

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 & 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 AP, Ribeiro AF, Marino CL, Lima MM & Coscrato VE (2002) *Wolbachia* endosymbiont in a species of the *Anastrepha fraterculus* complex (Diptera: Tephritidae). Invertebrate Reproduction and Development 42: 121–127.

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.

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.