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Incipient speciation revealed in *Anastrepha fraterculus* (Diptera; Tephritidae) by studies on mating compatibility, sex pheromones, hybridization, and cytology

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It has long been proposed that the nominal species *Anastrepha fraterculus* is a species complex and earlier studies showed high levels of pre-zygotic isolation between two laboratory strains from Argentina and Peru. Further experiments were carried out on the same populations and on their reciprocal hybrids, including pre- and post-zygotic isolation studies, pheromone analysis, and mitotic and polytene chromosome analysis. A high level of pre-zygotic isolation had been maintained between the parental strains despite 3 years of laboratory rearing under identical conditions. The level of pre-zygotic isolation was reduced in matings with hybrids. There were also differences in other components of mating behaviour. There were quantitative and qualitative differences in the sex pheromone of the two strains with the hybrids producing a mixture. The pre-zygotic isolation barriers were complemented by high levels of post-zygotic inviability and sex ratio distortion, most likely not due to *Wolbachia*, although there was evidence of some cytoplasmic factor involved in sex ratio distortion. Analysis of polytene chromosomes revealed a high level of asynapsis in the hybrids, together with karyotypic differences between the parental strains. The combined results of the present study indicate that these two strains belong to different biological entities within the proposed *A. fraterculus* complex.

ADDITIONAL KEYWORDS: cryptic species – hybrid incompatibility – pre-zygotic/post-zygotic isolation – polytene chromosomes – speciation.

INTRODUCTION

Most biologists accept that speciation is a continuous process by which genetic variation becomes segregated between populations. Within Diptera, the Tephritidae family is an interesting field for evolutionary studies because species complexes have been identified and cases of sympatric speciation, host shifts, and host race formation have been documented

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(Feder *et al.*, 2003; Linn *et al.*, 2003). Many of these cases involve species of great economic significance, providing an important interface between basic and applied research.

The South American fruit fly *Anastrepha fraterculus* (Wiedemann) is a case in point. It is highly polyphagous (Norrbom, 2004) with a distribution from southern USA to Argentina (Steck, 1999). Early studies showed population differences in host preference (Malavasi & Morgante, 1983), karyotypes (Bush, 1962), isozymes (Morgante, Malavasi & Bush, 1980), and morphology (Stone, 1942) and subsequent studies on hybridization (Santos, de Uramoto & Matioli, 2001), egg morphology and embryonic development (Selivon, Morgante & Perondini, 1997; Selivon & Perondini, 1998; Selivon, Vretos & Perondini, 2003), mitochondrial DNA (Smith-Caldas *et al.*, 2001), highly repetitive DNA (Rocha & Selivon, 2004), mating compatibility (Vera *et al.*, 2006), and morphometrics (Hernandez-Ortiz *et al.*, 2004) have suggested that the nominal species *A. fraterculus* is a species complex (for a revision, see Steck, 1999; for additional discussion, see Hernandez-Ortiz *et al.*, 2004, Selivon, Perondini & Morgante, 2005; Goday *et al.*, 2006). Studies on reproductive isolation revealed both pre- and post-zygotic mechanisms (Selivon, Perondini & Morgante, 1999, 2005; Vera *et al.*, 2006).

Sex pheromones play a key role in mate recognition and mating in *Anastrepha* (Nation, 1989) and they may play a role in pre-zygotic isolation. In the *Bactrocera dorsalis* Hendel complex, there are distinct differences between *Bactrocera carambolae* Drew and Hancock and *Bactrocera papayae* Drew and Hancock in the volatile components of the male rectal gland (Perkins *et al.*, 1990); however, this does not prevent hybridization in the field (Wee & Tan, 2005).

Post-zygotic isolation can limit gene flow between hybridizing populations and this can lead to reduced fitness of the hybrids (Burke & Arnold, 2001) and sex ratio distortion (Haldane, 1922), which are both conditions already demonstrated in *A. fraterculus* (Selivon *et al.*, 1999). Hybridization can also reveal phenotypes resulting from interactions between differentiated regions of the nuclear genome and/or interactions between the nuclear genome and cytoplasmic components (Burke & Arnold, 2001). In some cases, symbiotic bacteria such as *Wolbachia* are the sole determinant of hybrid sterility (Bourtzis & Braig, 1999). *Wolbachia* has been found in Brazilian populations of *A. fraterculus* and was suggested as a cause of reproductive isolation (Selivon *et al.*, 2002, 2005). Polytene chromosome analysis can help to identify species complexes (Coluzzi *et al.*, 2002) and previous observations in *A. fraterculus* revealed significant chromosomal polymorphism at the karyotypic level (Bush, 1962; Solferini & Morgante, 1987; Goday *et al.*, 2006). However, the polymorphism reported in Argentina populations (Basso & Manso, 1998) was not associated with speciation because only one biological entity occurs (Alberti *et al.*, 2002).

The present study comprises a multi-disciplinary approach involving studies on, pre- and post-zygotic isolation, male sexual pheromones, cytology, and *Wolbachia* in two laboratory strains of *A. fraterculus*. The results obtained provide additional evidence that fully supports and strengthens earlier suggestions for this species being composed of an unknown number of cryptic species.

MATERIAL AND METHODS

Strains

The Argentina strain was derived from pupae sent from the Estación Experimental Agroindustrial Obispo Colombres, Tucumán, Argentina and the Peru strain from pupae sent from the La Molina facility, Lima, Peru (for details of the history of the strains, see Vera *et al.*, 2006). Both strains were identified as *A. fraterculus* by Dr R. Zucchi and Dr V. Hernandez-Ortiz. F_1 hybrids were obtained from Argentina males mated with Peru females (H_{AP}) and from the reciprocal cross (H_{PA}). In addition, a hybrid inbred strain was established in 2005 by mating H_{PA} females with H_{AP} males. Flies from Piracicaba, Brazil, as well as from a single population from South America of unknown origin, were also analysed for *Wolbachia*.

Pre-zygotic isolation studies

Field cage experiments (FAO/IAEA/USDA, 2003) were performed in Seibersdorf, Austria. In the bisexual test, 25 virgin males from each strain were released into the cage within 1 h after sunrise, and 15 min later, 25 virgin females from each of the two strains were released. In the unisexual test, 25 females from one strain were released in the cage together with 25 males of two strains. Matings were observed and the type of male and female was identified, mating duration was noted, as well as the time from the release of the females to the beginning of mating (i.e. latency).

Isolation was measured using the Index of Sexual Isolation (Cayol *et al.*, 1999) and departures from zero (indicating nonrandom mating) were evaluated using a chi-square test of independence (bisexual tests) and goodness of fit (unisexual tests). Eight replicates were run for the parental tests and five for the hybrid tests. Heterogeneity among replicates was assessed by a chi-square test (Zar, 1996). In bisexual tests, differences in latency were analysed using nonparametric analysis of variance (ANOVA) (Kruskal–Wallis test) and differences in mating duration using a one-way ANOVA. In unisexual tests, *t*-tests were used. For

both variables, data from all cages within each test were pooled. Statistical analyses were performed with STATISTICA (StatSoft, 2000).

Post-zygotic isolation studies

Reciprocal crosses were carried out in the laboratory between the two parental strains with approximately 50 virgin flies of each sex. Eggs were placed on a larval diet and egg hatch, percent pupation, percent adult emergence, and the sex ratio of the F_1 adults were noted. F_1 virgin hybrid males and females were backcrossed to the parental strains and also inbred. Eggs were placed on a larval diet and egg hatch, percent pupation, percent adult emergence, and the sex ratio of the F_2 adults were noted.

Cytology

Mitotic metaphase spreads were from third-instar larval neuroblasts (Zacharopoulou, 1987) followed by C-banding (Selivon & Perondini, 1997). Larvae from the two parental strains, F_1 hybrids and the hybrid strain at generation 20, were analysed. For each strain, more than 20 larvae were analysed. Polytene chromosome preparations were from third-instar larval salivary glands (Zacharopoulou, 1987). More than 50 larvae were used from each strain. Metaphase spreads and well spread polytene nuclei were photographed on negative film (100 ASA) at ×100 magnification using a phase contrast Leitz microscope. Photographs were edited using Microsoft Picture Manager.

Collection and analysis of volatile pheromones

Volatiles were collected between 05.30 h and 13.00 h under natural light conditions (Teal, Gomez-Simuta & Proveaux, 2000). The traps containing volatiles were eluted with methylene chloride containing 1 ng μL^{-1} of *n*-tetradecane (internal standard) and analysed chemically using a HP-5890 gas chromatograph (GC) equipped with EC-1 and EC-5 columns (both 30 m × 0.25 mm inner diameter × 0.25 μm film thickness; Alltech Associates), cool-on-column injectors and flame ionization detectors (Teal, Gomez-Simuta & Meredith, 1999). The oven temperature was programmed from 40 °C (hold for 4 min) to 210 °C (EC-1) or 200 °C (EC-5) at 10 °C min^{-1}. The identities of compounds were confirmed by both chemical (isobutane reagent gas) and electron impact mass spectroscopy using a HP 5890 GC interfaced to a 6890 mass spectrometer (MS). The GC had a cool-on-column injector and a 30 m × 0.25 mm inner diameter DB-1MS capillary column (J&W Scientific Inc.) as the analytical column and using the same conditions used for GC-flame ionization detector (FID) analyses. Authentic synthetic samples including isomers, obtained from the CMAVE chemical collection, were used to calculate retention indexes for FID and MS analyses and for determination of MS fragmentation patterns (Teal *et al.*, 1999).

Wolbachia analysis

DNA was extracted from whole insects (Nirgianaki *et al.*, 2003) and the *wsp* gene amplified using standard primers (Zhou, Rousset & O'Neill, 1998). Polymerase chain reaction (PCR) products were analysed on 1% agarose gels, stained with ethidium bromide, digested with *Alu*I (Minotech) and the restriction products separated on 2% agarose gels. Purified *wsp* gene PCR products were cloned into pGEM T-easy vector and then transformed into *Escherichia coli* DH5a competent cells. All sequencing reactions were carried out in Macrogen (http://www.macrogen.com) using T7 and SP6 universal primers. Direct sequencing was performed using the *wsp*-specific primers (Zhou *et al.*, 1998).

The phylogenetic relationship among *Wolbachia* strains was analysed using the *wsp* nucleotide sequences but excluding the hypervariable regions (Baldo, Lo & Werren, 2005). The sequences were aligned using the CLUSTALW multiple Sequence Alignment Program, version 1.81 (Higgins, Thompson, Gibson, Thompson & Higgins *et al.*, 1994). The phylogeny test was performed with bootstrap analysis, whereas tree inference was determined using the Neighbour-joining method. The substitution model was the Jukes–Cantor and all the sequences were nucleotide-coding. The tree was constructed with MEGA, version 3.1.

RESULTS

Pre-zygotic isolation

There were high levels of pre-zygotic isolation between the parental strains in the bisexual and unisexual tests as shown by the index of sexual isolation (ISI) values (Table 1). Although the mean ISI was lower in the unisexual tests with Argentina females than with Peru females, the difference was not significant ($t = 1.911$; d.f. = 14; $P = 0.078$). For F_1 hybrids, the level of pre-zygotic isolation was reduced with random mating in three cases (Table 1). However, Argentina females still significantly preferred Argentina males to H_{AP} males in two out of the five replicates ($\chi^2 = 3.83$; $P < 0.05$), although the five replicates were homogeneous ($\chi^2 = 4.93$; d.f. = 4; $P > 0.05$).

In bisexual tests, matings between Peru males and females had significantly longer latency periods

Table 1. Mating percentage (PM) and index of sexual isolation (ISI) in bisexual and unisexual field cage tests for two strains of *Anastrepha fraterculus* from Peru and Argentina (Arg) and F_1 hybrids

Test	Males		Females		PM	ISI	χ^2	*N*
Bisexual	Arg	Peru	Arg	Peru	63.0 ± 2.1	0.77 ± 0.05	142.74***	8
Unisexual Arg	Arg	Peru	Arg		73.5 ± 2.3	0.73 ± 0.05	76.96***	8
Unisexual Peru	Arg	Peru	Peru		61.0 ± 3.6	0.86 ± 0.04	88.66***	8
Unisexual H_{AP} – Arg	Arg	H_{AP}	Arg		63.2 ± 5.4	0.30 ± 0.12	6.70**	5
Unisexual H_{PA} – Arg	Arg	H_{PA}	Arg		55.2 ± 4.3	0.15 ± 0.11	1.17	5
Unisexual H_{AP} – Peru	Peru	H_{AP}	Peru		58.4 ± 6.9	0.10 ± 0.10	0.67	5
Unisexual H_{PA} – Peru	Peru	H_{PA}	Peru		64.0 ± 8.0	0.13 ± 0.09	1.80	5

H_{AP}, F_1 hybrid from matings between Argentina males and Peru females.
H_{PA}, F_1 hybrid from matings between Peru males and Argentina females.
Chi-square values are after pooling data from all replicates in each test. $^{**}P < 0.01$; $^{***}P < 0.001$.
Replicates within tests were homogeneous (χ^2 test of homogeneity; $P < 0.05$).

Table 2. Mean values of latency and mating duration for two strains of *Anastrepha fraterculus* from Peru and Argentina (Arg) and F_1 hybrids

Test	Mating combination (♀ × ♂)	Latency (min)	Duration (min)
Bisexual	Arg × Arg	32.7 ± 4.6 (147)*[a]	67.6 ± 4.4 (147)*[a]
	Peru × Arg	48.5 ± 36.2 (10)[a]	68.4 ± 20.3 (10)[a]
	Arg × Peru	31.5 ± 15.4 (19)[a]	65.1 ± 7.0 (19)[a]
	Peru × Peru	216.8 ± 15.9 (76)[b]	49.3 ± 4.9 (76)[a]
Unisexual Arg	Arg × Arg	17.8 ± 2.1 (127)[a]	67.0 ± 2.6 (127)[a]
	Arg × Peru	25.6 ± 5.9 (20)[a]	56.8 ± 4.6 (20)[a]
Unisexual Peru	Peru × Arg	132.0 ± 32.3 (9)[a]	28.0 ± 2.4 (9)[a]
	Peru × Peru	150.3 ± 10.5 (113)[a]	34.0 ± 1.4 (113)[a]
Unisexual H_{AP} – Arg	Arg × Arg	112.6 ± 6.5 (51)[a]	62.0 ± 7.0 (35)[a]
	Arg × H_{AP}	122.1 ± 3.2 (28)[a]	68.8 ± 4.4 (24)[a]
Unisexual H_{PA} – Arg	Arg × Arg	47.0 ± 6.5 (39)[a]	57.7 ± 7.0 (39)[a]
	Arg × H_{PA}	40.4 ± 5.1 (30)[a]	53.8 ± 5.8 (30)[a]
Unisexual H_{AP} – Peru	Peru × Peru	132.8 ± 4.5 (40)[a]	70.7 ± 5.0 (30)[a]
	Peru × H_{AP}	173.1 ± 3.9 (33)[b]	68.0 ± 3.3 (21)[a]
Unisexual H_{PA} – Peru	Peru × Peru	73.8 ± 6.8 (46)[a]	41.1 ± 9.5 (45)[a]
	Peru × H_{PA}	74.8 ± 5.3 (34)[a]	47.5 ± 6.0 (34)[a]

H_{AP}, F_1 hybrid from matings between Argentina males and Peru females.
H_{PA}, F_1 hybrid from matings between Peru males and Argentina females.
Means followed by the same superscript letter within each test are not statistically different ($P > 0.05$).
*Figures in brackets refer to the number of matings.

(Table 2) (Kruskal–Wallis test: $H = 87.461$; d.f. = 3; $N = 252$; $P < 0.001$; and Dunn's multiple comparisons; $P < 0.001$). In the unisexual tests, the type of male did not affect the latency, either for Argentina ($t = 1.486$; d.f. = 145; $P = 0.115$) or Peru ($t = 0.514$; d.f. = 120; $P = 0.608$). When data within tests were pooled without considering the origin of the male, there were differences in latency between Argentina and Peru females (Mann–Whitney *U*-test = 1453, $P < 0.001$) with Peru females mating much later in the day (Table 2).

For hybrids, there were no differences in latency between males within tests, except for Peru females with Peru and H_{PA} males (Table 2) where homotypic matings started earlier than heterotypic ($t = 2.23$; d.f. = 71; $P = 0.029$). Comparison of latency among tests showed significant differences (Kruskal–Wallis test: $H = 102.98$; d.f. = 3; $N = 301$; $P < 0.001$) even between tests that involved females from the same strain. Tests with H_{PA} males had shorter latency than those with H_{AP} males and significantly so for Peru females (Dunn's test, $P < 0.05$). Tests involving

Argentina females had lower values than those involving Peru females.

In bisexual tests, there were no differences in mating duration (Table 2) ($F = 2.417$; d.f. = 248; $P = 0.067$). In unisexual tests, there was no effect of the type of male on mating duration ($t = 1.516$; d.f. = 145; $P = 0.132$; and $t = 1.248$; d.f. = 120; $P = 0.214$, for Argentina and Peru, respectively) and Argentina females mated longer than Peru females ($t = 13.094$; d.f. = 267; $P < 0.001$). In the unisexual tests with hybrid males, mating duration involving parental or hybrid males did not differ (*t*-test: $P > 0.05$) (Table 2) and there were no differences between females. However, there was an effect of the hybrid involved and the interaction between these two factors was also significant [F (female) = 0.943; d.f. = 254; $P > 0.05$; F (type of test) = 20.983; d.f. = 254; $P < 0.001$; F (interaction) = 5.118; d.f. = 254; $P = 0.025$]. Matings with H_{PA} males were shorter than matings with H_{AP} males but only statistically so for Peru females (69.6 ± 5.2 min for H_{AP} males versus 43.8 ± 3.1 min for H_{PA} males; Tukey's test, $P < 0.001$).

POST-ZYGOTIC ISOLATION

All crosses produced some viable progeny with a high adult emergence (Table 3). In four crosses, Arg × Peru, $H_{PA} \times H_{AP}$, Arg × H_{AP}, and H_{PA} × Peru, there was a significant reduction in egg hatch combined with a reduced larval viability, although the latter was not statistically significant. The reduced egg hatch is restricted to specific crosses where the females were either from Argentina or H_{PA} hybrids and the males were either from Peru or H_{AP} hybrids (i.e. males from a cross between Peru females and Argentina males), suggesting a possible maternal effect of Peru females. In addition, one of these crosses, $H_{PA} \times H_{AP}$, showed a sex ratio distortion in favour of females, as was also observed in the Hybrid strain (Table 3). A similar sex ratio distortion was

Table 3. Mean ± SE (%) for egg hatch, larval survival, egg-pupal survival, adult emergence, and sex ratios for matings between two strains of *Anastrepha fraterculus* from Peru and Argentina (Arg) and their F_1 hybrids

	Egg hatch					
Number and cross (♀ × ♂)	Number of eggs	Hatch*	Larval survival†	Egg-pupal survival‡	Adult emergence§	Sex ratio (♀/♂)¶
1 Peru × Arg	4000	81 ± 6^{b}	87 ± 7^{a}	70 ± 5^{a}	98 ± 1^{b}	0.98 ± 0.04^{c}
2 Arg × Peru	4000	27 ± 3^{c}	84 ± 14^{a}	22 ± 3^{d}	97 ± 1^{c}	1.02 ± 0.32^{c}
3 Arg × Arg	6000	83 ± 6^{b}	87 ± 5^{a}	75 ± 3^{a}	96 ± 2^{b}	0.97 ± 0.17^{c}
4 Peru × Peru	6000	80 ± 3^{b}	85 ± 7^{a}	72 ± 4^{a}	98 ± 1^{b}	0.99 ± 0.12^{c}
5 $H_{PA} \times H_{PA}$	3169	78 ± 5^{b}	87 ± 4^{a}	68 ± 4^{a}	95 ± 7^{a}	1.01 ± 0.20^{c}
6 $H_{PA} \times H_{AP}$	4448	47 ± 6^{d}	75 ± 9^{a}	36 ± 6^{c}	92 ± 4^{a}	1.64 ± 0.20^{b}
7 $H_{AP} \times H_{PA}$	3361	80 ± 4^{b}	80 ± 15^{a}	64 ± 21^{a}	84 ± 12^{a}	0.98 ± 0.10^{c}
8 $H_{AP} \times H_{AP}$	2531	72 ± 9^{b}	91 ± 5^{a}	65 ± 9^{a}	92 ± 4^{a}	0.74 ± 0.10^{d}
9 Arg × H_{PA}	3160	79 ± 6^{b}	85 ± 15^{a}	67 ± 12^{a}	97 ± 1^{a}	0.97 ± 0.01^{c}
10 Arg × H_{AP}	3154	55 ± 6^{d}	90 ± 5^{a}	50 ± 7^{b,c}	97 ± 1^{a}	0.88 ± 0.01^{d}
11 Peru × H_{PA}	3011	92 ± 4^{a}	87 ± 12^{a}	81 ± 14^{a}	93 ± 8^{a}	1.00 ± 0.20^{c}
12 Peru × H_{AP}	3747	85 ± 3^{a,b}	79 ± 15^{a}	67 ± 21^{a}	80 ± 5^{a}	1.03 ± 0.10^{c}
13 H_{PA} × Arg	4080	74 ± 1^{b}	68 ± 24^{a}	50 ± 22^{b}	86 ± 12^{a}	2.33 ± 0.20^{a}
14 H_{AP} × Arg	3043	89 ± 2^{a,b}	88 ± 16^{a}	78 ± 15^{a}	96 ± 3^{a}	0.93 ± 0.01^{d}
15 H_{PA} × Peru	5470	27 ± 3^{e}	76 ± 15^{a}	20 ± 4^{d}	93 ± 2^{a}	1.00 ± 0.20^{c}
16 H_{AP} × Peru	4618	70 ± 5^{b}	88 ± 11^{a}	62 ± 9^{b}	95 ± 5^{a}	0.86 ± 0.10^{d}
Hybrid	3000	34 ± 9	57 ± 12	19 ± 2	91 ± 5	2.81 ± 0.38

F, female; M, male.
H_{AP}, F_1 hybrid from matings between Argentina males and Peru females.
H_{PA}, F_1 hybrid from matings between Peru males and Argentina females.
Means in the same column followed by the same superscript letter are not statistically different.
*($F = 77.41$, $P = 0.00$).
†($F = 0.82$, $P = 0.64$).
‡($F = 9.53$, $P = 0.00$).
§($F = 2.02$, $P = 0.027$.
¶($F = 31.48$, $P = 0.00$).

Table 4. Sex chromosome genotypes and cytotypes in offspring of matings between *Anastrepha fraterculus* strains from Argentina (Arg) and Peru and F_1 hybrids

No. and cross (♀ × ♂)	Parental genotypes	Expected genotypes	Cytoplasm
1. Peru × Arg	$X^PX^P \times X^AY^A$	X^AX^P, X^PY^A	Peru
***2.* Arg × Peru**	$\mathbf{X^AX^A \times X^PY^P}$	$\mathbf{X^AX^P}$, $\mathbf{X^AY^P}$	Arg
3. Arg × Arg	$X^AX^A \times X^AY^A$	X^AX^A, X^AY^A	Arg
4. Peru × Peru	$X^PX^P \times X^PY^P$	X^PX^P, X^PY^P	Peru
5. $H_{PA} \times H_{PA}$	$X^AX^P \times X^AY^P$	X^AX^A, X^AX^P, X^PY^P, X^AY^P	Arg
6. $\underline{H_{PA} \times H_{AP}}$	$\underline{\mathbf{X^AX^P \times X^PY^A}}$	$\underline{\mathbf{X^AX^P}, \mathbf{X^PX^P}, \mathbf{X^AY^A}, \boldsymbol{X^PY^A}}$	Arg
7. $H_{AP} \times H_{PA}$	$\underline{X^AX^P \times X^AY^P}$	$\underline{X^AX^A, X^AX^P, X^AY^P, X^PY^P}$	Peru
8. $H_{AP} \times H_{AP}$	$X^AX^P \times X^PY^A$	X^AX^P, X^PX^P, X^AY^A, X^PY^A	Peru
9. Arg × H_{PA}	$X^AX^A \times X^AY^P$	X^AX^A, X^AY^P	Arg
10. Arg × H_{AP}	$\mathbf{X^AX^A \times X^PY^A}$	$\mathbf{X^AX^P}$, $\mathbf{X^AY^A}$	Arg
11. Peru × H_{PA}	$X^PX^P \times X^AY^P$	X^AX^P, X^PY^P	Peru
12. Peru × H_{AP}	$X^PX^P \times X^PY^A$	X^PX^P, X^PX^P, X^PY^A, X^PY^A	Peru
13. $\underline{H_{PA} \times \text{Arg}}$	$\underline{X^AX^P \times X^AY^A}$	$\underline{X^AX^A, X^AX^P, \boldsymbol{X^PY^A}, X^AY^A}$	Arg
14. $H_{AP} \times$ Arg	$\underline{X^AX^P \times X^AY^A}$	$\underline{X^AX^A, X^AX^P, X^PY^A, X^AY^A}$	Peru
15. $\boldsymbol{H_{PA}}$ × Peru	$\mathbf{X^AX^P \times X^PY^P}$	$\mathbf{X^AX^P}$, $\mathbf{X^PX^P}$, $\mathbf{X^AY^P}$, $\mathbf{X^PY^P}$	Arg
16. H_{AP} × Peru	$X^AX^P \times X^PY^P$	X^AX^P, X^PX^P, X^AY^P, X^PY^P	Peru
17. <u>Hybrid</u>	$\underline{\mathbf{X^AX^P}, \mathbf{X^PX^P}, \mathbf{X^AY^A}, \boldsymbol{X^PY^A}, \mathbf{X^AX^A}}$		Arg

H_{AP}, F_1 hybrid from matings between Argentina males and Peru females.
H_{PA}, F_1 hybrid from matings between Peru males and Argentina females.
Bold indicates crosses with reduced egg hatch.
Underlined indicates crosses with distorted sex ratio.
Bold and underlined indicates crosses with reduced egg hatch and distorted sex ratio.
Italics indicates genotypes not identified cytologically.

observed in the cross, $\underline{H_{PA}} \times$ Arg, together with an egg-to-pupae survival of only 50%. The progeny in these last two crosses (as in the hybrid strain) have two common characteristics: Argentina cytoplasm and the male sex chromosome complement is expected to be X^AY^A, X^PY^A (Table 4).

CYTOLOGY

Mitotic chromosomes

The karyotype of the nominal *A. fraterculus* has six pairs of acrocentric chromosomes, including one pair of highly heterochromatic sex chromosomes with the male being XY, as shown by Giemsa and C-banding (Fig. 1). However, the two strains can be differentiated by size and morphology of sex chromosomes after Giemsa staining and C-banding. The Argentina strain has a large X-chromosome with two prominent C-bands located at the two tips, one being larger than the other. The Y-chromosome is smaller than the X-chromosome and also shows two C-bands, one on the proximal tip and the other in the sub-median region (Fig. 1A). In the Peru strain, both X- and Y-chromosomes are large and similar in size (Fig. 1B). The X-chromosome, at least in early metaphase or in less condensed chromosomes, exhibits a large gap (Fig 1D, F), approximately two-thirds from one end, surrounded by two C-bands. However, in more condensed chromosomes, only one large C-band was observed. In some metaphases, two types of X-chromosome were observed: one with one large C-band and the second with a gap and, subsequently, two C-bands, although smaller than the unique one. The Y-chromosome has a large C-band at the proximal end in both condensed and early metaphase spreads. These differences between the two parental strains can also be seen in the hybrids (Fig 1C, D, E).

In the hybrid strain, the expected sex chromosome cytotypes were X^AX^P, X^PX^P, X^AY^A, X^PY^A plus X^AX^A (Table 4) and some of these genotypes were observed but not X^PY^A and X^PX^P. However, X^AX^A female larvae as well as larvae with 13 chromosomes, either XXX or XXY, were observed both with X^A chromosomes (Fig. 1F).

Polytene chromosomes

The polytene chromosomes show poor banding, numerous weak points and inter- and intra-ectopic pairing. The polytene complement consists of five long chromosomes, probably corresponding to the five autosomes because sex chromosomes are highly heterochromatic, are not expected to form polytene elements as in other tephritids (Zhao *et al.*, 1998).

The banding pattern between the two parental strains is very similar, especially at the chromosome ends, making differentiation difficult. Figure 2A

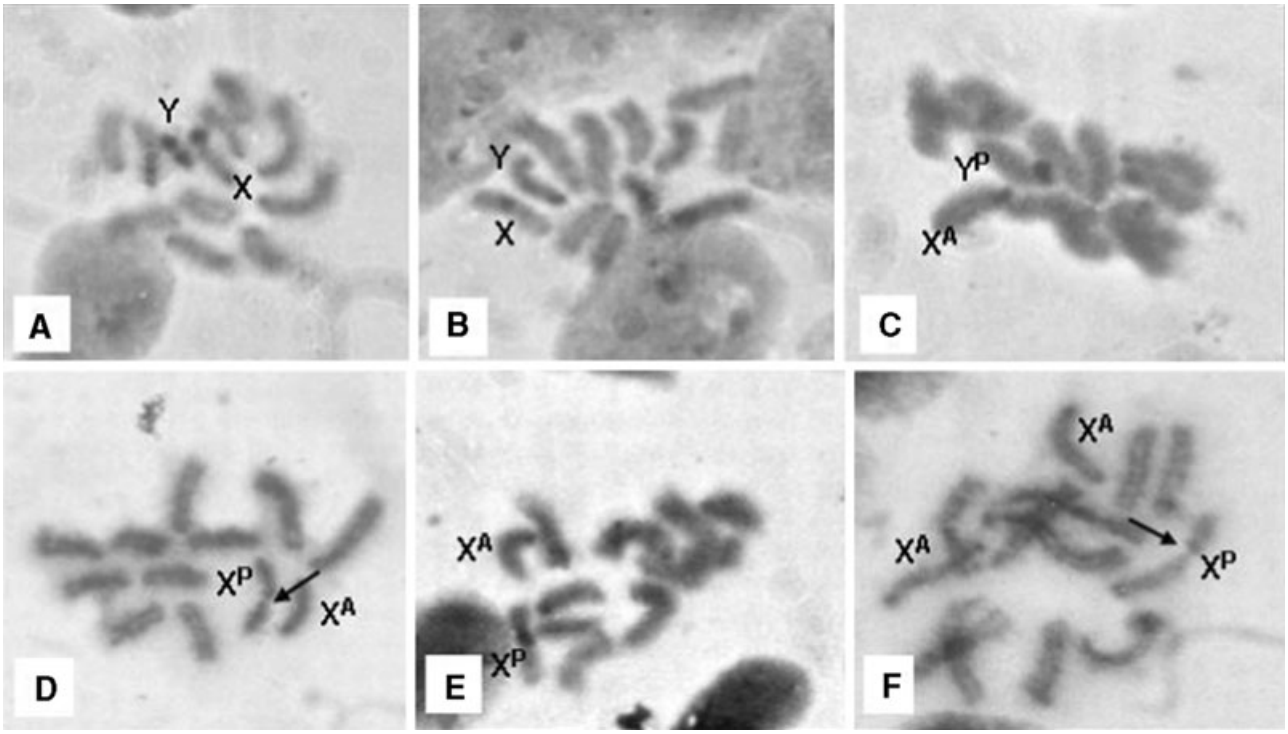

Figure 1. Mitotic chromosome spreads from: Argentina (A); Peru (B); H_{PA} (C, D); H_{AP} (E) and hybrid strain (F). Sex chromosomes, X and Y, are indicated. Arrows in (D) and (F) show a gap.

shows the same chromosome end for the two strains and indicates regions of homology and Fig. 2B shows another chromosome arm. The Argentina strain shows very little polymorphism; however, there is partial asynapsis (Fig. 2C) and many rearrangements, notably inversions (Fig. 2D), in the Peru strain. F_1 and F_2 hybrids showed extensive asynapsis (Fig. 3A, B, C), sometimes along the whole chromosome complement. Asynapsis is observed in almost all chromosome ends in spite of significant similarities in banding pattern. Homologous chromosomes also differ in size (Fig. 3D), as well as in the presence of inversion loops (Fig. 3E, G) and deletions (Fig. 3H), and differences in the puffing pattern in asynaptic areas (Fig. 3B) indicate differences in transcription. There was extensive asynapsis in the hybrid strain in the 14th and 20th generations (Fig. 4), similar to that observed in the F_1 and F_2 hybrids. The deletion observed in the F_1 (Fig. 3H) was also observed after 20 generations of inbreeding (Fig. 4D).

Pheromone analysis

There were significant qualitative and quantitative differences in the pheromone from Argentina and Peru males. Argentina males produced small amounts of (*E*)-β-ocimene, (*Z*)-nonanal and larger amounts of (*Z*)-3-nonen-1-ol, benzoic acid, suspensolide, (*Z*,*E*)-α-farnesene, (*E*,*E*)-α-farnesene, anastrephin and epianastrephin (Fig. 5A), but no detectable amounts of (*E*)-α-bergamontene or β-bisaboline. Peru males produced small amounts of limonene, (*Z*)-nonanal and (*Z*)-3-nonen-1-ol along with relatively large amounts of (*E*)-β-ocimene, (*E*)-α-bergamotene, (*E*,*E*)-α-farnesene and β-bisaboline along with suspensolide, anastrephin and epianastrephin but no (*Z*,*E*)-α-farnesene (Fig. 5B). Nonanol and benzoic acid are novel compounds for *Anastrepha* species. Volatiles from the two hybrid males were surprisingly similar in both the number and ratios of compounds released (Fig. 6) and contained all of the compounds identified in both parental strains. No statistical differences in average amounts of compounds released per hour by the parental strains were found ($t = 1.06$, $P > 0.05$) and the ratios of compounds released by males from different groups within each parent strain were not different. Thus, the ratio of pheromone components released by each strain was unique and did not vary significantly, despite collection of samples from males from different dates.

Wolbachia

Seven individuals from the strain of unknown origin, 12 from the Piracicaba strain, and ten from the Argentina and Peru strains carried *Wolbachia* based

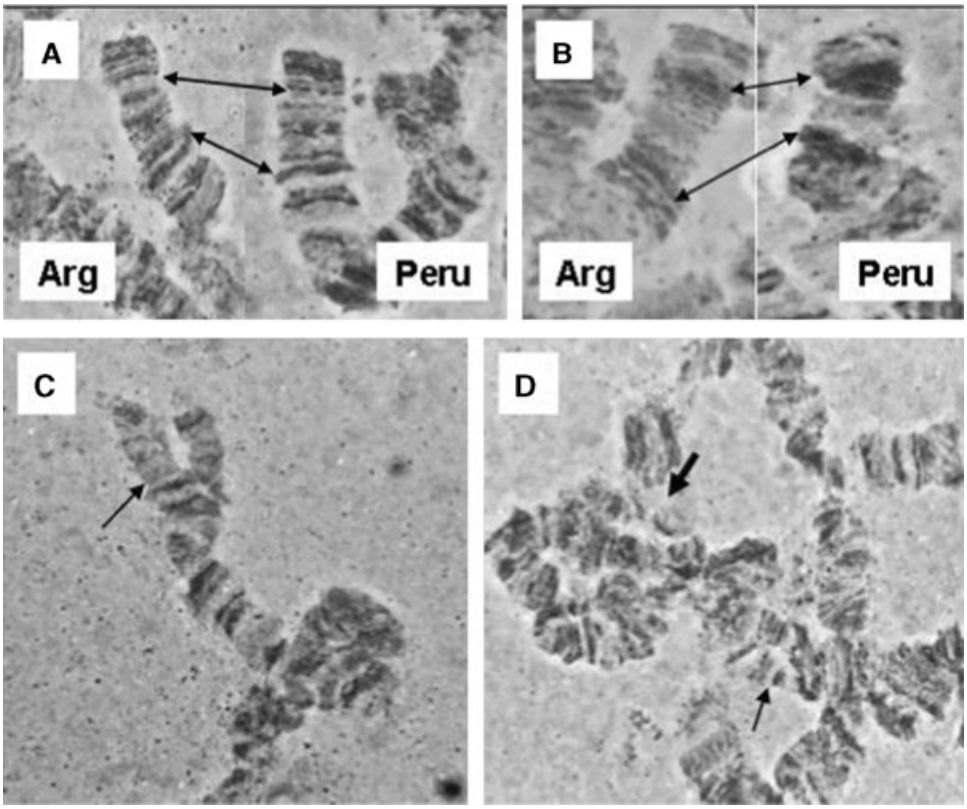

Figure 2. Polytene chromosome from: Argentina and Peru (A, B) showing homologous regions at the chromosome ends; Peru (C, D) showing asynapsis, (thin arrows) and chromosome inversion (thick arrow).

on the *wsp*-specific PCR assay (Zhou *et al.*, 1998). All flies tested were positive. *Alu*I-based restriction fragment length polymorphism (RFLP) analysis suggested that the strains from Piracicaba, Argentina and Peru were singly infected, whereas the strain of unknown origin was double infected (data not shown).

PCR products from the *wsp* gene from five males and females from the Piracicaba, Argentina, and Peru strains were directly sequenced, as were cloned *wsp* gene PCR products from two individuals from the population of unknown origin. The results confirmed the PCR-RFLP analysis with three strains being singly infected and one having a double infection. The singly-infected populations carry *Wolbachia* identical to *w*Spt (A *Wolbachia* supergroup) naturally infecting *Drosophila septentriosaltans* Magalhaes & Buck (Miller & Riegler, 2006) (Fig. 7), with the only difference being a conserved substitution in position 658 in the Argentina strain. The doubly-infected strain is infected with an A-supergroup *Wolbachia* strain identical to *w*AlbA naturally infecting the mosquito *Aedes albopictus* (Skűse) (Zhou *et al.*, 1998) and a B-supergroup strain closely related to the *w*Ma strain naturally infecting *Drosophila mauritiana* Tsacas & David (Zhou *et al.*, 1998).

DISCUSSION

Major differences were demonstrated in behaviour, chemistry, cytology, and genetics between two laboratory strains of *A. fraterculus* from Argentina and Peru. The differences are so significant that the two strains can be said to belong to different biological entities. The present study strongly supports and extends previous work which has provided an increasing body of evidence that this nominal species is a species complex (Hernandez-Ortiz *et al.*, 2004; Selivon *et al.*, 2004, 2005; Goday *et al.*, 2006). Although this work was conducteed using laboratory strains, it is unlikely that these differences were caused by laboratory adaptation because both laboratory populations have been shown to be fully compatible with their respective wild populations and the wild populations are incompatible with each other (Vera *et al.*, 2006).

The high level of pre-zygotic isolation found earlier (Vera *et al.*, 2006) was confirmed, and was unaffected by 3 years of identical laboratory rearing. A large component of the pre-zygotic isolation is due to differences in time of mating between the two parental strains (Vera *et al.*, 2006), which was again demon-

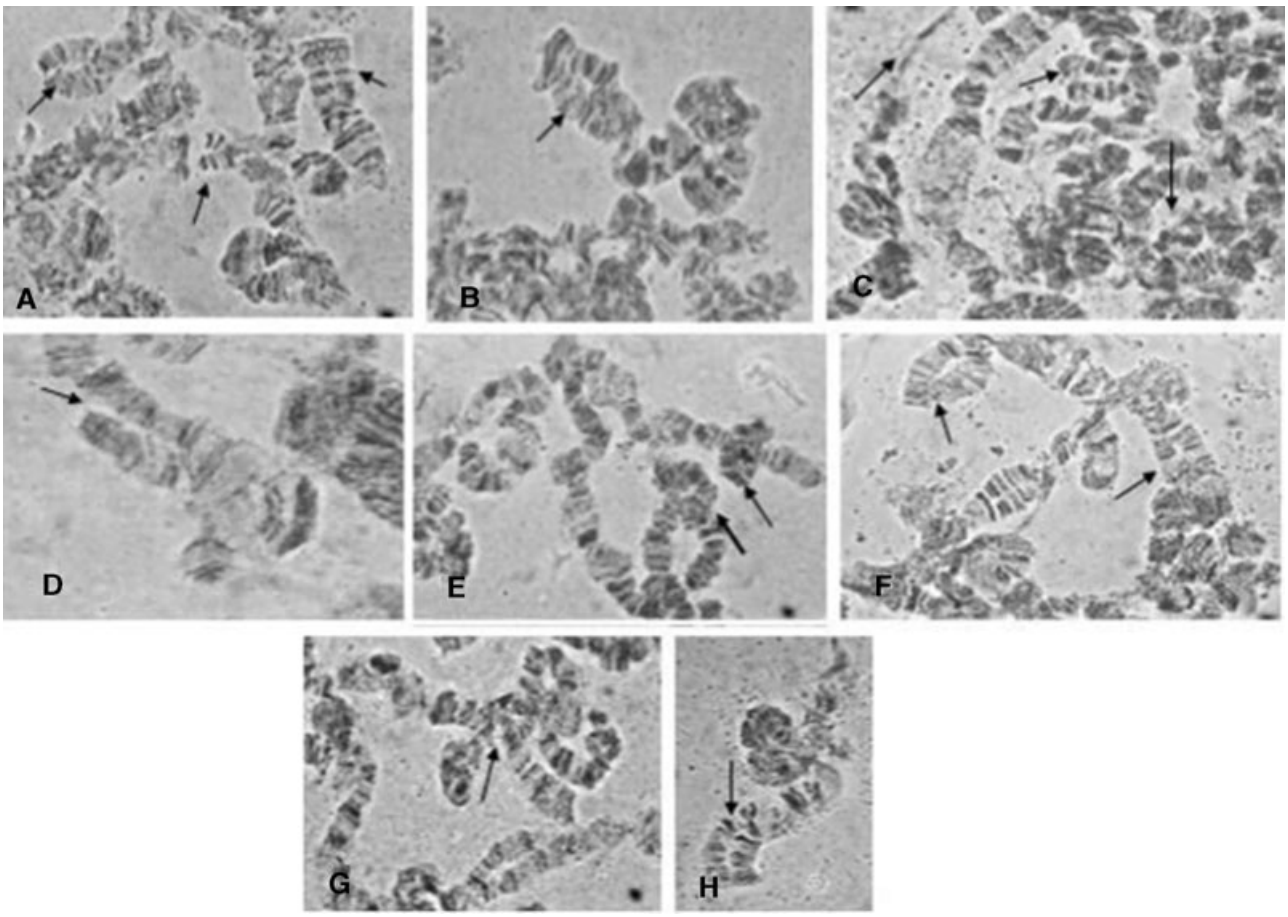

Figure 3. Polytene chromosomes from: H_{AP} (A) showing asynapsis (arrows) and (B) difference in puffing pattern (arrow); H_{PA} (C) showing asynapsis (arrows) and (D) difference in chromosome length (arrow); F_2 from $H_{PA} \times H_{PA}$ (E) showing asynapsis (thick arrow) and an inversion loop (thin arrow); F_2 from $H_{AP} \times H_{AP}$; (F) showing asynapsis (arrows); F_2 from $H_{AP} \times H_{PA}$ (G) showing an inversion loop (arrow) and hybrid strain (H) showing a deletion (arrow).

strated. This pre-zygotic isolation can also be attributed to the response of the females towards the sexual pheromone of the males because major differences in its composition were found between the two types male.

In the unisexual tests with H_{PA} males, neither Argentina nor Peru females showed a mating preference. However, for H_{AP} males, the Argentina females rejected them in the presence of Argentina males in two of the five replicates, whereas Peru females showed no preference. This asymmetric response is difficult to explain but may be related to the pheromone composition of the hybrid males because there are some quantitative differences between them (Fig. 6). However, both types of male produced all of the parental compounds and there were no statistical differences among them. Given that females respond to volatiles released by their own males, it is likely that parental females would not freely mate with a male from the other strain or with hybrid males because of the observed differences in pheromone composition (Figs 5, 6). However, the field cage data support the first assumption, but not the second because only in one case (and only in two out of five replicates) was nonrandom mating (favouring the parental males over the hybrid males) found. It should be noted, however, that these were unisexual tests and did not include hybrid females. It is likely that female hybrids would respond equally well to volatiles fron either type of hybrid male because the blends are virtually identical, but they would be unlikely to respond to volatiles released by parental males. Thus, if hybridization were to occur in nature, the hybrids would be pheromonally isolated from both parental strains and could constitute a new reproductively-isolated population (rapid one step speciation). Pre-zygotic isolation was also found in crosses involving a strain from Brazil (Vera *et al.*, 2006) and, again, male sexual pheromone may play a role because Lima, Howse & de Nascimento (2001)

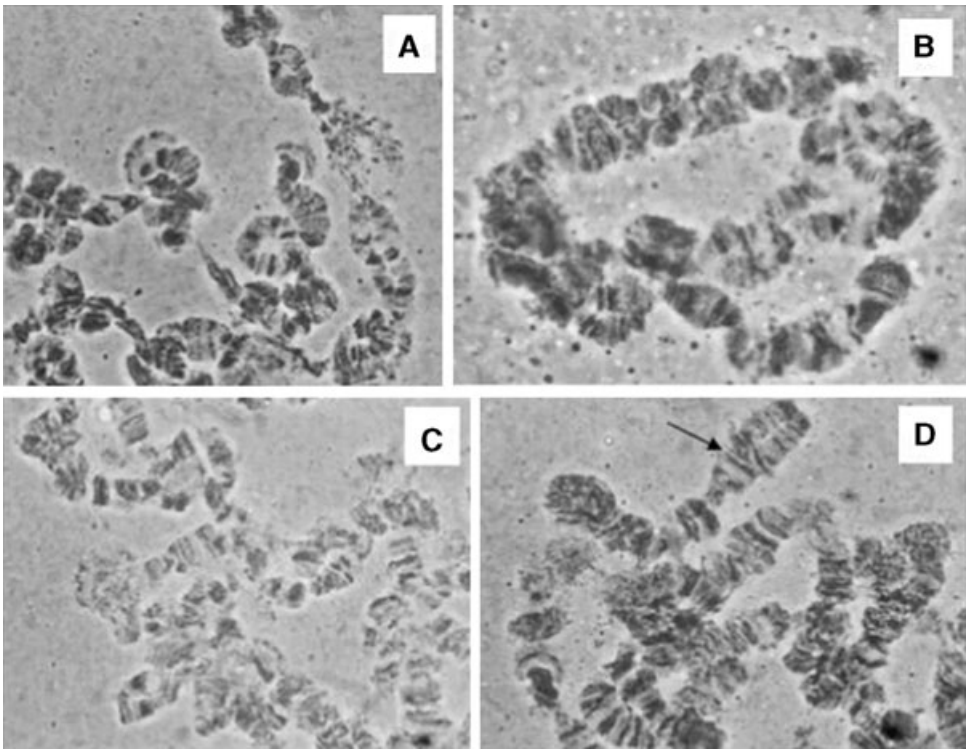

Figure 4. Polytene chromosomes from the hybrid stain analysed at the 20th generation after its establishment showing asynapsis (A, B, C) and a heterozygous deletion (D) (arrow).

identified several compounds in that strain that were not present in the strains from Argentina and Peru.

Argentina flies generally mated earlier than Peru flies, especially the Argentina females. The one exception to this is Peru females mating with Argentina males in the bisexual test. However, in this test, only ten matings were found in the eight replicates. In the bisexual test, the duration of mating was unaffected by the strain but matings involving Peru flies, in particular Peru females, were shorter and this was confirmed for the unisexual tests. The differences in mating duration may be attributed to the time at which mating started (Vera *et al.*, 2003). For hybrids, the influence of the females disappeared and the type of male became important. Surprisingly, when the females faced a hybrid of the same maternal origin, they behave as with the parental males but, when they faced a hybrid with a different maternal origin, the latency changed markedly. Females facing hybrid males with the maternal background of the other strain may detect critical differences in behaviour because the pheromones released by the two types of hybrid are almost identical.

Two phenotypes of relevance to post-zygotic isolation and hence potential speciation were found, namely reduced egg hatch and sex ratio distortion. However, the two phenotypes were not always observed together in the same cross, suggesting that they could have a different genetic basis. Reduced egg hatch was observed in four crosses in which females were either from Argentina or were hybrids from Argentina females (H_{PA}), whereas males were either from Peru or were hybrids from Peru females (H_{AP}). The reciprocal crosses did not show reduced egg hatch, indicating asymmetrical post-zygotic isolation. The observed decrease in egg hatch could be due to either unfertilized eggs or embryonic lethality but, because all these matings were carried out with large numbers of flies in laboratory cages, it is unlikely that many females remained unmated. The gross asynapsis in the hybrids, probably indicative of major genetic differences between the strains, is the most likely the cause of embryonic lethality. The asymmetry of the phenotype and together with the fact that identical genotypes did not show reduced hatch suggests some type of nuclear–cytoplasmic interaction is present, although probably not due to *Wolbachia*.

Sex ratio distortion was observed in cross 6 ($H_{PA} \times H_{AP}$), cross 13 ($H_{PA} \times$ Arg), and in the hybrid strain (Table 4) with the expected reduction in the proportion of males (Haldane, 1922). The expected progeny from these two crosses and in the hybrid strain have two common characteristics; they all have Argentina cytoplasm and the males have identical sex

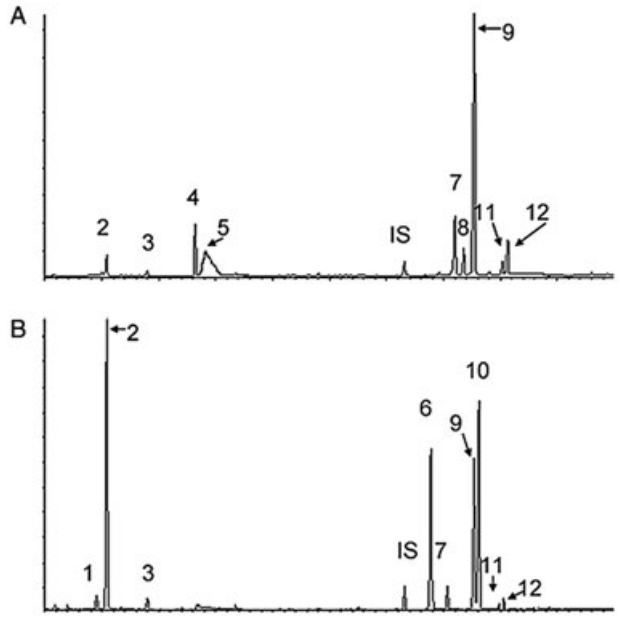

Figure 5. Comparisons of total ion chromatograms (electron ionization spectra) obtained from analysis of volatiles collected from groups of five males of the Argentina strain (A) or Peru strain (B). Compounds are: (1) limonene; (2) (*E*)-β-ocimene; (3) (*Z*)-nonanal; (4) (*Z*)-3-nonen-1-ol; (5) benzoic acid (IS, internal standard); (6) (*E*)-α-bergamontene; (7) suspensolide; (8) (*Z*,*E*)-α-farnesene; (9) (*E*,*E*)-α-farnesene; (10) β-bisaboline; (11) anastrephin; and (12) epianastrephin.

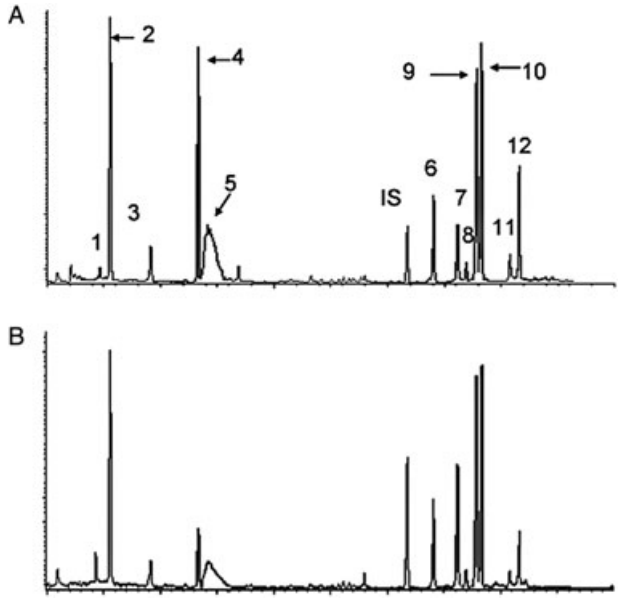

Figure 6. Comparisons of total ion chromatograms (electron ionization spectra) obtained from analysis of volatiles collected from groups of five hybrid H_{AP} males (A) or hybrid H_{PA} males (B). Numbers indicate the compounds described in the legend to Fig. 5.

chromosomes (i.e. X^AY^A and X^PY^A) (Table 4). There are other crosses producing males with the same sex chromosome genotype (Table 4) but these did not show sex ratio distortion. A low viability of X^PY^A males due to an interaction between nucleus and the cytoplasm could explain this sex ratio distortion. Indeed, individuals of this chromosomal type were never observed in larvae from the hybrid strain. This observation may be related to 'hybrid breakdown' observed in the F_2 and subsequent generations of inter-specific or inter-subspecific crosses (Burke & Arnold, 2001). Whatever the explanation for the sex ratio distortion, it contributes to post-zygotic isolation between the strains. Coyne & Orr (1989) demonstrated that Haldane's rule often results in a pattern of speciation where males from reciprocal crosses between two taxa show sterility or inviability before any effect is observed in females, indicating that complete sterility/inviability in hybrids is almost always preceded by the inviability/sterility in males only. Thus, according to Coyne & Orr (1989), Haldane's rule represents an obligatory initial step in the evolution of post-zygotic isolation. Sex ratio distortion was previously demonstrated by Selivon *et al.* (1999) in crosses of between *sp 2* females and *sp 1* males, with *sp 1* males probably being the same as the Argentina strain used in the present study based on karyotype analysis. Selivon *et al.* (1999, 2005) were the first to validate Haldane's rule in *A. fraterculus*.

The two strains clearly belong to different biological entities based on the mitotic karyotype and polytene chromosome analysis because they (and their hybrids) can easily be identified based both on size and C-banding of sex chromosomes. The Argentina karyotype has previously been reported in natural populations from Argentina (Basso & Manso, 1998; Basso *et al.*, 2003) and Brazil, referred to as *A.* sp.1 *aff. fraterculus* (Selivon *et al.*, 2005; Goday *et al.*, 2006). The Peru strain has not been analysed previously, but a similar karyotype (based on C-banding) was reported in a sample from Guayaquil, Ecuador and referred to as *A.* sp.4 *aff. fraterculus* (Selivon *et al.*, 2004; Goday *et al.*, 2006).

The extensive asynapsis in the polytene chromosomes in hybrids leaves no doubt that this results from inter-subspecific crosses as shown in *Drosophila* (Madi-Ravazzi, Bicudo & Manzato, 1997; Machado, Madi-Ravazzi & Tadei, 2006). It can be due to minute chromosomal rearrangements, specific interactions of genes that determine chromosome synapsis (Dobzhansky & Tan, 1936) or point mutations that disturb the identity of homologous loci (Kerkis, 1936). In the present study, considerable asynapsis was observed, even though the banding pattern of the two homologues was identical. The degree of asynapsis observed, especially in F_1 hybrids (Fig. 4), can be due

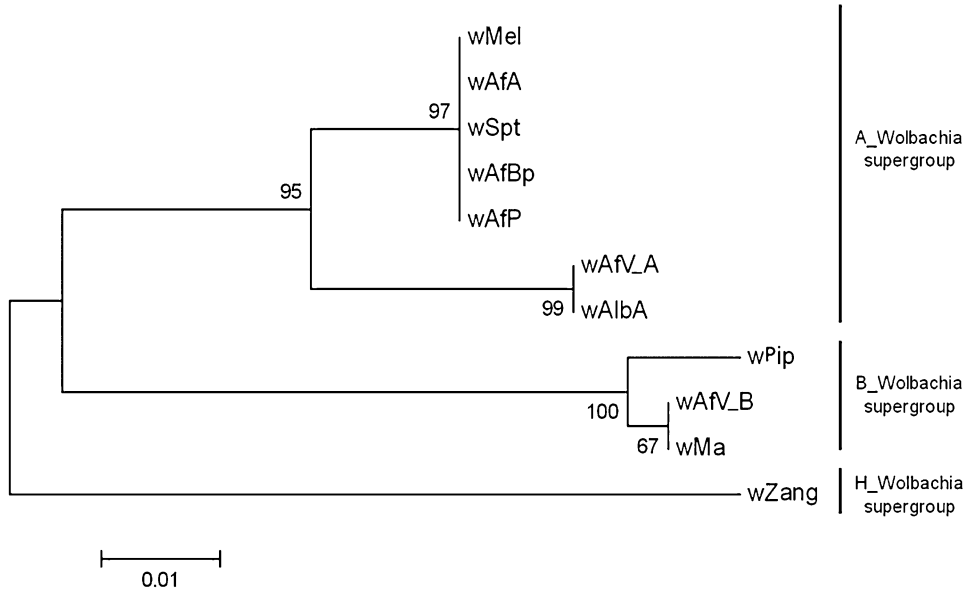

Figure 7. A Neighbour-joining phylogenetic tree based on *wsp* gene sequences (HVRs were excluded) from the *Wolbachia* strains: *w*Mel (host *Drosophila melanogaster*); *w*Spt (host *Drosophila septentriosaltans*); *w*AlbA (host *Aedes albopictus*); *w*AfA (host *A. fraterculus* Argentina); *w*AfBp (host *A. fraterculus* Piracicaba); *w*AfP (host *A. fraterculus* Peru); *w*AfV (host, an unknown *A. fraterculus* population); *w*Ma (host *D. mauritiana*); and *w*Pip (host *Culex pipiens*). The *w*Zang strain, which belongs to *Wolbachia* supergroup H (host *Zootermopsis angusticollis*), was used as outgroup. Numbers above nodes indicate bootstrap support (1000 replicates).

to significant genetic differentiation between the two strains, which is not restricted to chromosome structure, but also includes differences in gene activity, as indicated by differences in puffing pattern of homologous chromosomes in the asynaptic regions (Fig. 3B) (Zhimulev *et al.*, 2004).

Introgression of genes between the two strains is possible because the hybrids are partially fertile. In a hybrid strain created from a cross between homosequential *D. mauritiana* and *Drosophila simulans* Sturtevant, the proportion of fertile males derived from F_1 females backcrossed to either parent gradually increased; reaching 91% within eight generations, and this proportion was stable at least for a further ten generations (David *et al.*, 1976). The behaviour of the current hybrid strain is different as significant asynapsis and reduced egg hatch was maintained for at least 20 generations of inbreeding. The persistence of asynapsis is difficult to explain; however, the consistently reduced egg hatch could suggest some form of balancing selection is operating with heterozygous genotypes being favoured over homozygous genotypes. Genetic recombination may also be severely reduced because the level of somatic pairing in polytene chromosomes is correlated with meiotic pairing of chromosomes necessary for genetic recombination (Evgenev, 1971).

The data presented here for two *A. fraterculus* laboratory strains clearly show high levels of pre- and post-zygotic isolation, karyotypic and polytene chromosome differences, and qualitative and quantitative differences in male pheromones. Although each of these factors alone would be indicative of incipient speciation, taken together, they provide a very strong case for a taxonomic revision of this species complex, as suggested previously (Selivon *et al.*, 2005). The importance of this species as a major quarantine pest of fruit crops in many countries makes this revision essential and urgent.

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REFERENCES

Alberti AC, Rodriguero MS, Gomez-Cendra P, Saidman BO, Vilardi JC. 2002. Evidence indicating that Argentin-

ian populations of *anastrepha fraterculus* (Diptera: Tephritidae) belong to a single biological species. *Annals of the Entomological Society of America* **95:** 505–512.

Baldo L, Lo N, Werren JH. 2005. Mosaic nature of the *Wolbachia* surface protein. *Journal of Bacteriology* **187:** 5406–5418.

Basso A, Manso F. 1998. Are *Anastrepha fraterculus* chromosomal polymorphisms an isolation barrier? *Cytobios* **93:** 103–111.

Basso A, Sonvico A, Quesada-Allue LA, Manso F. 2003. Karyotypic and molecular identification of laboratory stocks of the South American fruit fly *Anastrepha fraterculus* (Wied) (Diptera: Tephritidae). *Journal of Economic Entomology* **96:** 1237–1244.

Bourtzis K, Braig H. 1999. The many faces of *Wolbachia*. In: Raoult D, Brouqui P, eds. *Rickettsiea and rickettsial diseases at the time of the third millennium*. Paris: Elsevier, 199–219.

Burke JM, Arnold ML. 2001. Genetics and the fitness of hybrids. *Annual Review of Genetics* **35:** 31–52.

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

Cayol JP, Vilardi J, Rial E, Vera MT. 1999. New indices and method to measure the sexual compatibility and mating performance of *Ceratitis capitata* (Diptera: Tephritidae) laboratory strains under field cage conditions. *Journal of Economic Entomology* **92:** 140–145.

Coluzzi M, Sabatini A, Della Torre A, Di Deco MA, Petraca V. 2002. A polytene chromosome analysis of the *Anopheles gambiae* species complex. *Science* **298:** 1415–1418.

Coyne JA, Orr HA. 1989. Patterns of evolution in *Drosophila*. *Evolution* **43:** 362–381.

David JR, Bocquet C, Leumunier F, Tsacas L. 1976. Persistence of male sterility in strains issued from hybrids between two sibling species: *Drosophila simulans* and *Drosophila mauritania*. *Journal of Genetics* **62:** 93–100.

Dobzhansky T, Tan CC. 1936. Comparison of the gene arrangements in two species of *D. pseudoobscura* and *D. miranda*. *Zeitschrift für Induktive Abstammungs und Vererbungslehre* **72:** 88–104.

Evgenev MB. 1971. The pattern of polytene chromosome conjugation and crossing-over in interspecific hybrids of *Drosophila*. *Theoretical and Applied Genetics* **41:** 249–254.

FAO/IAEA/USDA. 2003. *Manual for product quality control and shipping procedures for sterile mass reared tephritid fruit flies*, Version 5. Vienna: International Atomic Energy Agency.

Feder JL, Berlocher SH, Roethele JB, Dambroski H, Smith JJ, Perry WL, Gavrilovic V, Filchak KE, Rull J, Aluja M. 2003. Allopatric genetic origins for sympatric host-plant shifts and race formation in *Rhagoletis*. *Proceedings of the National Academy of Sciences of the United States of America* **100:** 10314–10319.

Goday C, Selivon D, Perondini ALP, Greciano PG, Ruiz MF. 2006. Cytological characterization and ribosomal DNA location in *Anastrepha* species. *Cytogenetics and Genome Research* **114:** 70–76.

Haldane JBS. 1922. Sex ratio and unisexual sterility in animal hybrids. *Journal of Genetics* **12:** 101–109.

Hernandez-Ortiz V, Gomez-Anaya A, Sanchez 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.

Higgins D, Thompson J, Gibson T, Thompson JD, Higgins DG. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting position specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22:** 4673–4680.

Kerkis J. 1936. Chromosome conjugation in hybrids between *D. melanogaster* and *D. simulans*. *American Naturalist* **70:** 81–86.

Lima IS, Howse PE, de Nascimento RR. 2001. Volatile substances from male *Anastrepha fraterculus* (Diptera: Tephritidae): identification and behavioural activity. *Journal of the Brazilian Chemical Society* **12:** 196–201.

Linn C, Feder JL, Nojima S, Dambroski HR, Berlocher SH, Roelofs W. 2003. Fruit odor discrimination and sympatric host race formation in *Rhagoletis*. *Proceedings of the National Academy of Sciences of the United States of America* **100:** 11490–11493.

Machado LPB, Madi-Ravazzi L, Tadei WJ. 2006. Reproductive relationships and degree of synapsis in the polytene chromosomes of *Drosophila buzzatii* species cluster. *Brazilian Journal of Biology* **66:** 279–293.

Madi-Ravazzi L, Bicudo HEMC, Manzato JA. 1997. Reproductive compatibility and chromosome pairing in the *Drosophila buzzati* complex. *Cytobios* **89:** 21–30.

Malavasi A, Morgante JS. 1983. Population genetics of *Anastrepha fraterculus* (Diptera: Tephritidae) in different hosts: genetic differentiation and heterozygosity. *Genetica* **60:** 207–211.

Miller WJ, Riegler M. 2006. Evolutionary dynamics of *w*Au-like *Wolbachia* variants in neotropical *Drosophila* spp. *Applied Environmental Microbiology* **72:** 826–835.

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.

Nation JL. 1989. The role of pheromones in the mating success of *Anastrepha* fruit flies. In: Robinson AS, Hooper G, eds. *Fruit flies: their biology, natural enemies and control*. Amsterdam: Elsevier, 189–205.

Nirgianaki A, Banks GK, Frohlich D, Veneti Z, Braig HR, Miller TA, Bedford JD, Markham PC, Savakis C, Bourtzis K. 2003. *Wolbachia* infections of the whitefly *Bemisia tabachi*. *Current Microbiology* **47:** 93–101.

Norrbom AL. 2004. *Fruit fly (Tephritidae) host plant database*. Version Nov., 2004. Available at: http://www.sel.barc.usda.gov:591/diptera/Tephritidae/TephHosts/search.html

Perkins MV, Fletcher MT, Kitching W, Drew RAI, Moore JC. 1990. Chemical studies of rectal gland secretions of some species of *Bactrocera dorsalis* complex of fruit flies

(Diptera: Tephritidae). *Journal of Chemical Ecology* **16:** 2475–2487.

Rocha LS, Selivon D. 2004. Studies on highly repetitive DNA in cryptic species of the *Anastrepha fraterculus* complex. In: Barnes BN, ed. *Proceedings of the sixth international symposium on fruit flies of economic importance*. Irene: Isteg Scientific Publications, 415–418.

Santos P, de 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.

Selivon D, Morgante JS, Perondini ALP. 1997. Egg size, yolk mass extrusion and hatching behaviour in two cryptic species of *Anastrepha fraterculus* (Wiedemann) (Diptera: Tephritidae). *Brazilian Journal of Genetics* **20:** 587–594.

Selivon D, Perondini ALP. 1997. Evaluation of techniques for C and ASG banding of the mitotic chromosomes of *Anastrepha* species (Diptera, Tephritidae). *Brazilian Journal of Genetics* **20:** 4–8.

Selivon D, Perondini ALP. 1998. Eggshell morphology in two cryptic species of the *Anastrepha fraterculus* complex (Diptera: Tephritidae). *Annals of the Entomological Society of America* **91:** 473–478.

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.

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

Selivon D, Vretos C, Fondes L, Perondini ALP. 2004. New variant forms in the *Anastrepha fraterculus* complex. In: Barnes BN, ed. *Proceedings of the sixth international fruit fly symposium*, Irene: Isteg Scientific Publications, 253–258.

Selivon D, Vretos C, Perondini ALP. 2003. Evaluation of egg morphology from ethanol preserved females of *Anastrepha* sp.2 *aff. fraterculus* (Diptera: Tephritidae). *Neotropical Entomology* **32:** 527–529.

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.

Solferini VN, Morgante JS. 1987. Karyotype study of eight species of *Anastrepha* (Diptera: Tephritidae). *Caryologia* **40:** 229–241.

StatSoft Inc. 2000. *STATISTICA for Windows*. User's manual. Tulsa, OK: StatSoft, Inc.

Steck GJ. 1999. Taxonomic status of *Anastrepha fraterculus*, pp. 13–20. In: *The South American fruit fly,* Anastrepha fraterculus *(Wied.): advances in artificial rearing, taxonomic status and biological studies*. IAEA-TECDOC-1064. Vienna: International Atomic Energy Agency, 202.

Stone A. 1942. The fruit flies of the genus *Anastrepha*. USDA Miscellaneous Publication No. 439. Washington, DC: US Department of Agriculture.

Teal PEA, Gomez-Simuta Y, Meredith JA. 1999. Isolation and identification of terpenoid sex pheromone components from extracts of hemolymph of males of the Caribbean fruit fly. *Archives of Insect Biochemistry and Physiology* **42:** 225–232.

Teal PEA, Gomez-Simuta Y, Proveaux AT. 2000. Mating experience and juvenile hormone enhance sexual signaling and mating in male Caribbean fruit flies. *Proceedings of the National Academy of Sciences of the United States of America* **97:** 3708–3712.

Vera MT, Caceres C, Wornoayporn V, Islam A, Robinson AS, de la Vega MH, Hendrichs J, Cayol JP. 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.

Vera MT, Cladera JL, Calcagno G, Vilardi JC, McInnis DO. 2003. Remating of wild *Ceratitis capitata* (Diptera: Tephritidae) females mated with wild and laboratory males in a one-day field cage trial. *Annals of the Entomological Society of America* **96:** 563–570.

Wee S-L, Tan K-H. 2005. Evidence of natural hybridisation between two sympatric sibling species of *Bactrocera dorsalis* complex based on pheromone analysis. *Journal of Chemical Ecology* **31:** 845–858.

Zacharopoulou A. 1987. Cytogenetic analysis of mitotic and salivary gland chromosomes in the medfly *Ceratitis capitata*. *Genome* **29:** 67–71.

Zar J. 1996. *Biostatistical analysis*. Upper Saddle River, NJ: Prentice-Hall.

Zhao JT, Frommer M, Sved JA, Zacharopoulou A. 1998. Mitotic and polytene chromosome analyses in the Queensland fruit fly, *Bactrocera tryoni* (Diptera: Tephritidae). *Genome* **41:** 510–526.

Zhimulev IF, Belyaeva ES, Semeshin VF, Koryakov DE, Demakov SA, Demakova OV, Pokholkova GV, Andreyeva EN. 2004. Polytene chromosomes: 70 years of genetic research. *International Review of Cytology* **241:** 203–275.

Zhou W, Rousset F, O'Neill S. 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. *Proceedings of the Royal Society of London Series B, Biological Sciences* **265:** 509–515.