Mating Incompatibility Among Populations of the South American Fruit Fly *Anastrepha fraterculus* (Diptera: Tephritidae)

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Ann. Entomol. Soc. Am. 99(2): 387–397 (2006)

ABSTRACT Mating compatibility among different populations of the South American fruit fly *Anastrepha fraterculus* (Wiedemann) was assessed through mating tests in pairwise combinations. Screened cages, inside a greenhouse, containing *Citrus limon* (L.) trees were used. Mating compatibility was determined using the index of sexual isolation. Most of the populations were noncompatible with each other and thus sexually isolated. Of these, Tucumán (Argentina) and Piracicaba (Brazil) populations showed a lower degree of isolation, whereas the other tested combinations were highly isolated. Full mating compatibility was detected only between two Argentinean (Concordia and Tucumán) and two Peruvian populations (La Molina and Piura + La Molina). Flies were sexually active at different times: Tucumán, Concordia, and Piracicaba populations presented an early morning peak, La Molina and Piura + La Molina were active around midday, and Ibague (Colombia) were active late in the afternoon. Manipulation of light phase conditions to match the times of maximum sexual activity did not increase the compatibility between La Molina and Tucumán. Based on these behavioral results, which confirm morphometric, genetic, and other evidence, the taxonomic revision of this cryptic species complex is warranted. One practical implication is that colonies of this pest to be used in any sterile insect technique approach should be derived from the target population or from a compatible population. Regional efforts should be initiated to determine the distribution of each subgroup and their relationship with each other in terms of compatibility.

RESUMEN Se evaluó la compatibilidad para el apareamiento entre distintas poblaciones de la mosca Sudamericana de la fruta, *Anastrepha fraterculus* (Wiedemann), mediante pruebas de apareamiento confrontando dos poblaciones por vez. Se utilizaron jaulas con árboles de *Citrus limon* (L.) en un invernadero. La compatibilidad para el apareamiento se determinó con el índice de aislamiento sexual. La mayoría de las poblaciones fueron no compatibles y consecuentemente estuvieron aisladas entre sí. Entre estas, las poblaciones de Tucumán (Argentina) y Piracicaba (Brasil) presentaron el menor grado de aislamiento, mientras que las otras presentaron alto aislamiento. Se detectó compatibilidad solamente entre dos poblaciones argentinas (Concordia y Tucumán) y dos poblaciones peruanas (La Molina y Piura + La Molina). Las poblaciones estuvieron sexualmente activas en distintos momentos del día: Tucumán, Concordia y Piracicaba presentaron un pico de actividad al amanecer, La Molina y Piura + La Molina al mediodía e Ibague (Colombia) al atardecer. La manipulación de los horarios del ciclo de luz-oscuridad, para coincidir la hora de máxima actividad sexual, no aumentó la compatibilidad entre La Molina y Tucumán. Basándonos en estos resultados comportamentales, que confirman las evidencias genéticas y morfológicas registradas, es necesaria una revisión taxonómica de este complejo de especies. Una consecuencia práctica sería que las colonias a utilizar para la cría masiva de insectos estériles para la implementación de la técnica del insecto estéril para el control de esta plaga deben originarse de la población a controlar o de una población compatible. Se deben iniciar esfuerzos regionales para determinar la distribución de cada subgrupo y su relación en términos de compatibilidad.

KEY WORDS sexual isolation, cryptic species complex, sterile insect technique, temporal isolation, reproductive compatibility

THE SOUTH AMERICAN FRUIT FLY *Anastrepha fraterculus* (Wiedemann) is present in most countries of America from the United States of America (Texas) to Argentina (Salles 1995, Steck 1999). On the South American continent, this species is found in two broad, apparently unconnected bands: one band along the western edge, including both highland and lowland areas of the Andean range; and the second band along the east coast. *A. fraterculus* originated in South America and has been reported to infest ≈80 host plants, including

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0013-8746/06/0387–0397$04.00/0

major fruit crops (Norrbom and Kim 1988). This highly destructive pest imposes quarantine restrictions for fruit export to many countries (Steck 1999).

The high levels of variability found in different populations of *A. fraterculus*, throughout its distribution range, has led, since the beginning of the past century, to the consideration that it may be a complex of cryptic species rather than a single biological entity (Stone 1942, Morgante et al. 1980, Solferini and Morgante 1987, Malavasi and Morgante 1982, Steck 1991, Steck and Sheppard 1993, Selivon 1996). Differences were reported mainly at the levels of morphology, pest status, and genetics (including karyotype, isozyme, and molecular analyses); these differences are reviewed in Steck (1999) and some aspects are discussed in subsequent studies (McPheron et al. 1999, Norrbom et al. 1999, Gomes Silva 2000, Smith-Caldas et al. 2001, Hernández-Ortiz et al. 2004). The presence of reproductive barriers leads to reproductive isolation and hence, under the biological concept of species, to speciation.

Reproductive isolation can take place either before or after insemination of the eggs, that is, pre- or postzygotic incompatibility, respectively. For prezygotic incompatibility, males and females of different populations do not mate with each other either because they occupy different habitats (ecological isolation), are active at different times of the day or even during different seasons (temporal isolation), or they encounter but reject each other (sexual or behavioral isolation) (Dobzhansky 1937). This latter case is frequent in animals with elaborate courtship, such as *A. fraterculus*, which has a lek mating system where males aggregate to attract females only for the purpose of mating (sensu Emlen and Oring 1977). Leks are formed in host and nonhost trees, and males locate on the underside of the leaves, near to the petiole or the stems and protrude their abdomen while they release a pheromone. Females arrive at the leks, and males display courtship behavior that includes wing movements and both visual and acoustical signaling (Malavasi et al. 1983, de Lima et al. 1994, Salles 1999). Females evaluate the possible partners and either remain there and mate with one of the males or relocate in search of other mates. Postzygotic incompatibility is expressed after egg fertilization and can cause inviability of the zygote or sterility of the progeny or in the progeny of the hybrids.

In spite of the morphological and genetic evidence indicating the potential existence of a complex of cryptic species in *A. fraterculus*, the level of reproductive isolation among populations from different origins at the continental level has not been investigated. Mating compatibility (Petit-Marty et al. 2004a) and postzygotic compatibility (Basso et al. 2003, Petit-Marty et al. 2004b) studies among Argentinean populations suggested that in this country, these populations belong to the same species, confirming results found at the genetic level (Vilardi et al. 1994, Sonvico et al. 1996, Basso and Manso 1999, Lifschitz et al. 1999, Alberti et al. 2002, Basso et al. 2003). However, among Brazilian populations, postzygotic studies (Selivon et al. 1999) suggested the occurrence of at least two different species, also supported by cytogenetic, isozyme, and molecular studies (Malavasi and Morgante 1982, Selivon 1996, Selivon et al. 2005).

To resolve the possible existence of multiple species within the complex, there is a need to assess populations from the entire distribution range to correlate the reported degree of genetic and morphological variability with levels of compatibility at different steps of the reproductive process. This issue is not only of interest from the evolutionary and taxonomic perspective but also has some practical relevance. Given the importance of *A. fraterculus* as a major fruit and quarantine pest, and the great success with which many tephritid populations are being suppressed, excluded, and in certain situations eradicated by using the sterile insect technique (SIT), the development of this technique has been proposed as a promising tool for integration into its management (Ortiz 1999). However, the existence of a sibling species complex would impose some difficulties to the implementation of any SIT program. Failure to identify sibling species could result in attempts to control one species with releases of a second species. Moreover, the benefits of the release of only sterile males, achieved in the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), as a consequence of the development of genetic sexing strains (Franz et al. 1996, Rendón et al. 2004), has resulted in the extensive use of only a small number of strains worldwide. This has been possible because of high levels of mating compatibility among worldwide populations of this species (Cayol et al. 2002). In the absence of such compatibility, even between populations of the nominal *A. fraterculus*, no extensive use of a single strain would be possible.

The aim of the present work was to evaluate mating compatibility of *A. fraterculus* populations, derived from six different locations from South America, tested in pairwise comparisons under seminatural conditions.

Materials and Methods

Source of Flies. Flies were obtained either from the wild or from laboratory-established strains. Mating compatibility tests were performed at the Entomology Unit, FAO/IAEA Agriculture and Biotechnology Laboratory, in Seibersdorf, Austria. This laboratory has the required quarantine security to permit working with a species with such taxonomical uncertainties. Import permits were obtained. Pupae from six locations (three wild populations and three laboratory strains) were sent by express airmail to Austria. The wild populations came from Peru (La Molina), Brazil (Piracicaba), and Argentina (Concordia). The population from La Molina was collected as pupae from infested cherimoyas, *Annona cherimola* Miller, in La Molina, southeastern Lima, and reared in the laboratory for one generation by using cherimoyas for larval rearing to enhance numbers. Pupae were shipped to Seibersdorf. The Piracicaba population was collected as pupae from infested guava, *Psidium guajava* L., in

Piracicaba, near Sao Paulo, and the pupae were sent directly to Seibersdorf. The population from Concordia was collected as pupae from guava in Concordia (northeastern Argentina) and sent directly to Seibersdorf. The laboratory strains came from Colombia (Ibague), Peru (Piura + La Molina), and Argentina (Tucumán). For Ibague, flies were originally sampled in 1992 from an unknown host, and in 1997 wild material from the same area and obtained from *Passiflora mollisima* (Bailey) was crossed to the laboratory strain to refresh the genetic background. Piura + La Molina is a strain originating in 2000 from infested mango, *Mangifera indica* L., and guava from Piura (northern Peru), which was refreshed with wild pupae obtained from cherimoyas collected in La Molina in 2002. The Tucumán strain was derived from infested guava collected in the vicinity of Tafi Viejo, Tucumán province (northwestern Argentina). The strain was established in 1997 and since then, no wild material was introduced to refresh the genetic background.

Once in Seibersdorf, pupae were placed under appropriate conditions to allow adult emergence. Colonies were established in the laboratory by using cages with low densities of flies (>7 cm^2 per fly). Mangoes were provided as oviposition substrate and larval rearing medium. This allowed synchronization of development for the sexual compatibility tests. For all the populations, specimens of both sexes were preserved in 70% ethanol and sent to Dr. Roberto Zucchi (Universidade de São Paulo, SP, Brazil) and Dr. Vicente Hernández-Ortiz (Instituto de Ecología, C. A., Mexico) for confirmation on the species identification. Voucher specimens are kept at the Entomology Unit, FAO/IAEA Agriculture and Biotechnology Laboratory, in Seibersdorf. Moreover, a sample of flies from each population was preserved in 100% ethanol for future DNA and morphological analysis.

Compatibility Tests. Pupae from the different populations were placed in emergence cages, and every 24 h adults were removed, sorted by sex, and placed in cages with adult food (3:1, sugar/hydrolyzed yeast) and water until sexual maturation (16 d old). Three days before the tests, flies were marked with a dot of water-based paint on the thorax (DEKA, Unterhaching, Germany). Flies were gently aspirated and placed in a bag made with a mesh cloth. They were immobilized by pressing the cloth bag against the table and with a fine brush the thorax was painted. Once the paint dried, flies were again aspirated and released back into the cages. Mating compatibility tests, involving pairwise comparisons among populations from two different origins, were carried out in a screened cylindrical cage (Calkins and Webb 1983) (3 m in diameter and 2 m in height) following standard procedures (FAO/IAEA/USDA 2003). In each cage, three potted *Citrus limon* (L.) trees, 1.7 m in height with ≈1.5-m-diameter canopy, provided the flies with surface for resting and mating activities. Because of unsuitability of weather during most of the experimental period (winter–spring 2003), tests were performed in a greenhouse with controlled temperature (24–29°C) and humidity (60–80%). On the day of the test, 30 sexually mature virgin males from each of the two test populations were released into the cage 10–15 min after sunrise. Approximately 15–20 min later, 30 virgin and sexually mature females from each of the same two populations were added. Dead or moribund flies were replaced. During the observation period, which lasted most of the day, mating pairs were collected as they formed by gently allowing the pair to walk into a small vial. The origin of both male and female, the location of the mating couple (either on the trees or cage), and the time at which copulation started were recorded. Vials were placed in the shade and the time of couple disengagement was noted. Although it would have been desirable to test all the possible pairwise combinations among populations (15 in total), because of availability of flies, it was possible to test eight. The total number of replicates for each pairwise comparison depended on the number of available flies, but at least four replicates were carried out for each combination.

Data Analysis. The degree of sexual activity in each cage was determined by the percentage of mating couples obtained in relation to the number of flies released. Mating compatibility between each pairwise comparison of populations was determined by means of the index of sexual isolation (ISI) (Cayol et al. 1999), which considers the number of couples obtained for each possible mating combination. ISI ranges from −1 (complete negative assortative mating, that is, all matings are with members of the opposite population) through 0 (random mating) to +1 (complete positive assortative mating, that is, total mating isolation of the two populations). The isolation index, proposed by Stalker (1942) and used in mating isolation studies in *Drosophila* species, equals the ISI when applied to situations where both males and females of the two strains or populations are released together and in equal proportions. This index also has been called the joint isolation index (Malagolowkin-Cohen et al. 1965). In spite of being a good and reliable index because of its simplicity in calculation and interpretation, Gilbert and Starmer (1985) have questioned this index because it does not take into account differences in vigor (mating propensity) between populations and the number of individuals available at the start of the test. Therefore, in our work, together with the ISI, the indices of relative performance for each sex (Cayol et al. 1999) were calculated. The male relative performance index (MRPI) is a relative measure of mating propensity of the males of one population versus the males of the other. It also ranges from −1 to +1. A value of +1 indicates that all matings in the cage were achieved by the males of one population (the first to be listed in the name of the test); a value of −1 indicates all matings were achieved by males of the other population. Zero indicates that males from both populations participated equally in matings. The female relative performance index (FRPI) is the counterpart of the MRPI and serves as a measure of mating propensity for female flies. The joint analysis of ISI, MRPI and FRPI, provides a complete and reliable picture of the sexual compatibility between the pop-

ulations (Cayol et al. 1999). Moreover, a chi-square test of independence was carried out for each cage test (i.e., replicate) to determine the significance of the ISI. Heterogeneity among replicates was determined by a chi-square heterogeneity test (Zar 1996).

General sexual behavior for each population was described by the location and time of the day of the male's calling activity, and by the location, start time, and duration of the homotypic copulations (both sexes coming from the same fly population). Because the experiments took place from January until June 2003 and sunrise occurred progressively earlier, mating start time was defined as the time from the release of the females to the beginning of a given copulation. This variable is referred to as latency. Copulation duration was estimated as the time the couple disengaged minus the time they started to copulate. Homogeneity among replicates within each combination was determined by means of a nonparametric analysis of variance (ANOVA) (Kruskal–Wallis test). In the cases where latency or copulation duration was homogeneous among replicates and the different mating combinations, data were pooled, and means obtained in the homotypic crosses were compared. In those cases where there were more than two groups, a Kruskal–Wallis test was performed, and if the test revealed significant differences, it was followed by a Dunn test for multiple comparisons, which considers Bonferroni's correction. A Mann–Whitney *U* test was performed in those cases where only two populations or sets of data were compared. All statistical analyses were performed with STATISTICA for Windows (StafSoft 2000).

Analysis of High ISI Values. Two of the populations tested exhibited a high value of ISI (and hence isolation) but also a different time of the day of maximum sexual activity. One population mated early in the morning and the other population at midday. These two populations were selected to determine whether the high ISI was because of the presence of purely temporal isolation (sensu Dobzhansky 1937, that is, flies are isolated only because they exhibit a different time of mating activity). Under this hypothesis, if temporal isolation was removed the ISI value should approach zero. However, if the value of ISI does not change, this indicates that another component of isolation is present (i.e., sexual incompatibility). To test this hypothesis, flies from the population that exhibited a peak of sexual activity during midday were sexed as they emerged, and one-half were placed in a room with a light cycle in a different phase. The lights turned on 4 h before the lights of the other room (light phase from 0200 to 1500 hours and referred to as "out of phase"). The other half of the flies, and the population that exhibited a peak of sexual activity in the early morning, were placed in the room in which the lights turned on at sunrise outside (light phase from 0600 to 1900 hours and referred to as "normal phase"). After sexual maturation, flies from the early morning population and flies from the midday population but out of phase were released together in the cage, and the mating compatibility was assessed. Two external controls were performed: one control involving the two populations held during the sexual maturation period at the same light regime (normal phase) and the other control involving flies from the midday population, one part matured with the normal phase (i.e., in agreement with external natural conditions) and the other part matured out of phase. The former was the "untouched" control, and the latter was used to assess whether creating temporal isolation between two identical populations could induce some level of prezygotic incompatibility. Compatibility and relative performance indices were computed and compared among the three tests. Moreover the observation period was broken down into four discrete intervals, and ISI was computed for each interval. The first three intervals lasted 0.5 h each (time in which >80% of the early morning matings took place) and the fourth interval lasted the remaining observation period (≈6 h).

Results

Percentage of flies involved in matings and indices of mating compatibility and performance are presented in Table 1. The general trend was of high levels of mating incompatibility, both in terms of the ISI and the chi-square values (Table 1). Full mating compatibility was detected only in the tests Tucumán–Concordia and La Molina-Piura + La Molina. La Molina and Concordia populations showed the highest degree of isolation. Within each test, replicates were homogeneous except for La Molina–Piura + La Molina, where in one of the six replicates there was a tendency toward positive assortative mating and in another there was a tendency toward negative assortative mating. Because these two populations were compatible, this heterogeneity is most likely because of random environmental effects. In Tucumán–Piracicaba, the degree of mating incompatibility was not as high as in other combinations, and one replicate showed a nonsignificant chi-square value (i.e., the type of male in each mating combination was independent of the type of female, suggesting mating compatibility). The other five replicates showed a statistically significant level of isolation. The FRPI value showed a greater mating propensity of Tucumán females compared with Piracicaba females, and the matings involving Tucumán males and Piracicaba females were less common than those involving Piracicaba males and Tucumán females (13 and 49 cases, respectively, of a total of 219 couples obtained in the six replicates performed). The Ibague population showed a lower mating propensity in both sexes compared with the La Molina population (high values of both MRPI and FRPI). For the other cases, MRPI and FRPI values revealed that, for both sexes, populations had equal mating performance.

The time at which populations started to mate (latency) was not equal for all the populations ($H = 622.4$, $df = 5$, $P < 0.0001$; Table 2). Concordia, Piracicaba, and Tucumán showed an early morning peak; the mean of La Molina and Piura + La Molina was around midday; and Ibague started mating late in the

Table 1. Sexual compatibility and performance of *A. fraterculus* populations tested in pairwise combinations

Pop tested	No. of couples				PM[a]	ISI[b]	MRPI[c]	FRPI[d]	χ^{2e}	N[f]
	AA	AB	BA	BB						
La Molina–Concordia	17 ± 1	1 ± 1	1 ± 0	20 ± 1	64.6 ± 0.4	0.92 ± 0.03	0.07 ± 0.07	0.10 ± 0.05	33.24 ± 1.55*	4
Tucumán–P+LM	16 ± 1	1 ± 0	3 ± 1	19 ± 2	63.3 ± 4.8	0.83 ± 0.06	−0.10 ± 0.06	0.00 ± 0.05	26.71 ± 3.49*	4
Tucumán–La Molina	16 ± 1	0 ± 0	3 ± 1	18 ± 2	62.2 ± 2.8	0.82 ± 0.03	−0.10 ± 0.08	0.04 ± 0.09	25.36 ± 1.96*	10
La Molina–Ibague	15 ± 0	2 ± 1	1 ± 1	6 ± 2	53.9 ± 1.7	0.78 ± 0.02	0.49 ± 0.06	0.37 ± 0.12	13.10 ± 2.02*	4
La Molina–Piracicaba	13 ± 2	4 ± 1	3 ± 1	15 ± 2	64.5 ± 3.8	0.55 ± 0.06	−0.04 ± 0.05	−0.04 ± 0.10	12.74 ± 2.61*	6
Tucumán–Piracicaba	16 ± 1	2 ± 1	8 ± 1	11 ± 1	60.8 ± 2.4	0.43 ± 0.08	−0.02 ± 0.03	0.30 ± 0.04	9.17 ± 2.45*	6
Tucumán–Concordia	12 ± 2	9 ± 1	12 ± 1	15 ± 2	80.4 ± 2.3	0.12 ± 0.10	−0.11 ± 0.06	−0.01 ± 0.02	2.30 ± 1.30 N.S.	4
La Molina- P + LM	8 ± 1	9 ± 1	8 ± 1	12 ± 2	60.6 ± 4.1	0.10 ± 0.12	−0.07 ± 0.05	−0.13 ± 0.05	2.61 ± 1.20 N.S.	6

Values are given as mean ± SE. P + LM, Piura+La Molina.

[a] Percentage of mating = number couples obtained/number potential couples × 100.

[b] ISI = [(AA +BB) − (AB + BA)]/N.

[c] Male relative performance index = [(AB + AA) − (BA + BB)]/N.

[d] Female relative performance index = [(BA + AA) − (AB + BB)]/N, where AA is the number of couples involving males and females from the first population mentioned (i.e., in the case of La Molina–Concordia, it refers to La Molina males × La Molina females) AB is the number of couples involving males of the first population mentioned and females from the second population (La Molina males and Concordia females) and so on.

[e] Mean independence χ^2 value obtained for all replicates in each test. This value is presented for illustration purposes only. The analysis was performed with the computation of the χ^2 value for each replicate and then the total χ^2 to test homogeneity among replicates within a test. *, $P < 0.001$; N.S., nonsignificant.

[f] N is total number of replicates performed for each test.

afternoon as light intensity decreased. In addition, for Piracicaba and La Molina the time at which these populations started to mate depended on the origin of the other population present in the cage (Table 3). Piracicaba flies started mating later when confronted with Tucumán flies than when confronted with La Molina flies. There was an interaction between this variable and the location of the couple. Piracicaba couples collected on the tree in the test in which they were confronted with Tucumán flies showed a longer latency period (2:05 ± 0:24, $n = 17$) than those couples collected on the screen in the same test (1:08 ± 0:10, $n = 41$) or either on the screen or the tree in the La Molina–Piracicaba test (0:46 ± 0:18, $n = 16$ and 0:49 ± 0:07, $n = 70$ for the screen and the tree, respectively) ($H = 18.92$, df = 3, $P < 0.001$, $n = 144$; the multiple comparison analysis revealed differences between the first group and the other three). La Molina flies started mating later in the test with Ibague flies and earlier in the presence of Piracicaba and Tucumán flies.

With respect to mating duration, mean times were different among homotypic crosses ($H = 186.2$, df = 5, $P < 0.0001$; Table 2). Ibague, Piura + La Molina, and La Molina flies showed significantly lower values, whereas for Tucumán, Piracicaba, and Concordia flies, mating duration was longer. Within each cross, La Molina and Tucumán flies showed a different mean time according to the origin of the other population present in the cage (Table 3). In the first case, mating duration was shorter in the presence of Tucumán flies in comparison with values obtained for La Molina in the presence of Ibague or Piura + La Molina flies. In the test Tucumán–Piura + La Molina mating duration of Tucumán flies was shorter than in the other tests.

Table 2. Latency and mating duration of the homotypic crosses (hours:minutes) in *A. fraterculus*

Pop	Latency[a]	Duration[a]
Ibague	10:18 ± 0:14a (22)	0:44 ± 0:06a (19)
La Molina	4:30 ± 0:07b (428)	1:04 ± 0:02a (389)
Piura + La Molina	3:15 ± 0:10b (145)	0:47 ± 0:03a (139)
Piracicaba	1:03 ± 0:05c (149)	1:28 ± 0:05bc (139)
Tucumán	0:46 ± 0:02cd (371)	1:13 ± 0:02b (367)
Concordia	0:33 ± 0:04d (141)	1:36 ± 0:03c (139)

For each population replicates from all the tests were pooled. Latency is time since the release of females and the beginning of the copulation. Means followed with a different letter differed according to a Dunn's (nonparametric) multiple comparison test ($P < 0.05$).

[a] Mean ± SE (number of matings).

For Piracicaba and Tucumán populations, mating location depended on the other population being tested (Table 3). In the presence of Tucumán flies, more couples of Piracicaba were collected from the screen than when La Molina flies were in the cage (Table 3). Tucumán flies also tended to mate on the screen and only when confronted with Piura–La Molina flies, the majority of the couples were collected in the tree (multiple comparisons with Bonferroni's correction, $P < 0.05$).

Levels of mating compatibility between Tucumán and La Molina with the manipulation of the light phase are presented in Table 4. In total, 93% of the matings involving La Molina males and females with the light phase modified took place during the range in which all the Tucumán homotypic matings took place. For the case of Tucumán versus La Molina flies with the normal light phase, the percentage of matings involving La Molina flies reached 68%. Restricting this to the time at which 75% of the Tucumán homotypic matings took place (third quartile), the percentages were 34% for the case of La Molina with the modified light cycle and 19% for La Molina flies with the normal phase. Mean latency values are presented in Table 5, and the number of couples collected for each mating type in each of the four discrete time intervals plus the ISI value obtained in each interval are presented in Fig. 1.

Table 3. Latency, mating duration and location of the homotypic crosses in *A. fraterculus*, according to the tested combination

Pop	Tested pop[a]	Latency[b] (h:min)	Duration[b] (h:min)	Location[c]			
				U	O	S	C
Ibague	La Molina	10:18 ± 0:14 (22)	0:44 ± 0:06 (19)	21	0	0	1
Concordia	La Molina	0:37 ± 0:07 (81)	1:32 ± 0:04 (80)	35	0	7	39
	Tucumán	0:27 ± 0:04 (60)	1:40 ± 0:05 (59)	31	0	7	22
	M-W test	$Z = -0.3$, $P = 0.754$	$Z = 0.8$, $P = 0.383$		$\chi^2 = 1.5$, $P = 0.21$		
Piracicaba	La Molina	0:49 ± 0:06 (88)	1:33 ± 0:06 (85)	70	0	2	16
	Tucumán	1:24 ± 0:10 (61)	1:20 ± 0:08 (54)	19	1	2	41
	M-W test	$Z = -3.5$, $P < 0.001$	$Z = 1.6$, $P = 0.099$		$\chi^2 = 36.7$, $P < 0.01$		
Piura + La Molina	La Molina	3:23 ± 0:13 (71)	0:51 ± 0:05 (68)	67	0	0	4
	Tucumán	3:06 ± 0:16 (74)	0:44 ± 0:04 (71)	73	0	0	1
	M-W test	$Z = 1.1$, $P = 0.270$	$Z = 2.0$, $P = 0.050$		$\chi^2 = 2.0$, $P = 0.16$		
La Molina	P+LM	4:51 ± 0:18ab (49)	1:10 ± 0:08a (41)	42	0	0	8
	Ibague	5:32 ± 0:17a (59)	1:13 ± 0:06a (59)	57	0	0	2
	Concordia	4:49 ± 0:16ab (68)	1:01 ± 0:06ab (63)	55	1	0	12
	Piracicaba	3:58 ± 0:19b (77)	1:06 ± 0:05ab (69)	71	2	0	4
	Tucumán	4:11 ± 0:11b (175)	0:59 ± 0:04b (157)	159	0	0	17
	K-W test	$H = 15.2$, $P = 0.004$	$H = 10.5$, $P = 0.033$		$\chi^2 = 11.3$, $P = 0.02$		
Tucumán	Concordia	0:36 ± 0:06a (49)	1:30 ± 0:04a (47)	18	0	1	30
	Piracicaba	0:40 ± 0:04a (94)	1:16 ± 0:03ab (92)	55	0	1	38
	P + LM	0:57 ± 0:08a (65)	0:51 ± 0:04c (65)	61	0	0	4
	La Molina	0:49 ± 0:04a (163)	1:16 ± 0:03b (163)	92	0	5	66
	K-W test	$H = 6.5$, $P = 0.088$	$H = 52.3$, $P < 0.001$		$\chi^2 = 41.5$, $P < 0.01$		

Latency is time since the release of females and the beginning of the copulation.

[a] Origin of the other population present in the test. P + LM, Piura + La Molina.

[b] Mean ± SE (N). M-W, Mann–Whitney test; K-W, Kruskal–Wallis test. Means followed with a different letter were statistically different (see text for a deeper explanation of the tests performed in each case).

[c] Number of couples collected at each location. U, underside of the leaf; O, over the leaf; S, stem; C, screen of the cage. For statistical analysis, only the couples collected in the underside of the leaf or the screen of the cage were considered.

Discussion

We analyzed the mating compatibility among several populations of *A. fraterculus* from South America and found that some of them are reproductively isolated. The populations analyzed involved lowland (Peru) and highland (Colombia) areas from the Andean region, and the southeastern part of the continent (Brazil and Argentina). Even though high levels of genetic and morphological diversity have been reported (Steck 1999, Hernández-Ortiz et al. 2004) and the status of a cryptic species complex was proposed many decades ago (Stone 1942), this is the first report of mating compatibility analysis among *A. fraterculus* populations from different countries.

Petit-Marty et al. (2004a) found complete compatibility among four Argentinean populations, concluding that only one species is present. This is sustained by isozyme (Vilardi et al. 1994, Alberti et al. 2002), molecular (Sonvico et al. 1996, Alberti et al. 2002), and cytogenetic (Basso and Manso 1999, Lifschitz et al. 1999, Basso et al. 2003) analysis. Our results confirm this for the two Argentinean populations (Tucumán and Concordia) but also indicate relatively higher levels of isolation among these populations and *A. fraterculus* populations from other countries in the region, providing strong additional evidence of the presence of a high degree of genetic and behavioral divergence at the continental level.

From the current study, populations can be classified into three groups: the early morning maters (Concordia, Piracicaba, and Tucumán), the noon or midday maters (La Molina and Piura + La Molina), and the

Table 4. Sexual compatibility and performance of La Molina flies with the time of maximum sexual activity changed through manipulation of the light phase conditions to match the times of maximum sexual activity of Tucumán flies

Pop tested	No. of couples				PM[a]	ISI[b]	MRPI[c]	FRPI[d]	χ^{2e}	N[f]
	AA	AB	BA	BB						
Tucumán–La Molina−4	20 ± 1	2 ± 0	3 ± 1	23 ± 1	79.7 ± 0.9	0.81 ± 0.04	−0.09 ± 0.03	−0.04 ± 0.03	31.9 ± 2.7*	6
La Molina–La Molina−4	10 ± 1	10 ± 1	9 ± 2	16 ± 1	73.8 ± 2.8	0.17 ± 0.09	−0.12 ± 0.04	−0.19 ± 0.06	2.1 ± 0.7 N.S.	4
Tucumán–La Molina	20 ± 2	1 ± 1	3 ± 1	12 ± 2	57.5 ± 4.1	0.82 ± 0.04	0.17 ± 0.07	0.28 ± 0.13	25.0 ± 3.6*	4

La Molina−4 refers to La Molina with a change in the light phase of 4 h. Values are given as mean ± SE

[a] Percentage of mating.

[b] ISI value.

[c] Male relative performance index.

[d] Female relative performance index; for detailed formulae of each index, see Table 1.

[e] Mean Independence χ^2 value obtained for all replicates in each test. This value is presented for illustration purposes only. The analysis was performed with the computation of the χ^2 value for each replicate and then the total χ^2 value to test homogeneity among replicates within a test. *, $P < 0.001$; N.S., nonsignificant.

[f] N is total number of replicates performed for each test.

Table 5. Latency period in homotypic matings in *A. fraterculus* in light phase manipulation experiment

Pop tested	Pop	Latency[a]	Mann–Whitney *U* test
Tucumán–La Molina−4	Tucumán	1:12 ± 0:08 (121)	$Z = 6.95$, $P < 0.001$
	La Molina−4	2:43 ± 0:09 (139)	
La Molina–La Molina−4	La Molina	3:33 ± 0:26 (38)	$Z = -1.58$, $P = 0.112$
	La Molina−4	2:32 ± 0:13 (65)	
Tucumán–La Molina	Tucumán	0:47 ± 0:07 (78)	$Z = 6.84$, $P < 0.001$
	La Molina	3:53 ± 0:19 (47)	

La Molina−4 refers to La Molina with a change in the light phase of 4 h. For each test, replicates were pooled. Latency: time (h:min) since the release of females and the beginning of the copulation.

late afternoon maters (Ibague). Daily rhythms in sexual activity have been found to be highly variable in *Anastrepha* spp. (see Aluja et al. 1999 for a revision), but apparently the majority of the species studied mate during twilight (dawn or dusk). Within *A. fraterculus*, the trend was to report it as an early morning mater. This evidence came from Brazilian (Malavasi et al. 1983, Morgante et al. 1983, de Lima et al. 1994, Salles 1999), Mexican (Aluja et al. 1999), and Argentinean (Petit-Marty et al. 2004a) populations. Only for a Peru population is there a brief report of a different behavior, where this species is referred to as an afternoon mater (Vargas 1971). Our results are in agreement with the previous reports for the early morning maters, although we obtained a different mating period for the Peruvian populations, and we include a new pattern represented by the Colombian population (Ibague), which showed no sexual activity in the morning and instead mated at dusk.

Avoidance of interspecific matings has been considered as the main driving force toward differential sexual activity peaks and is an example of temporal isolation (Morgante et al. 1993, Sivinski et al. 1999). However, in a scenario where either few species are present or a given species is colonizing a new area, environmental conditions (Aluja et al. 1997), or predation avoidance (Hendrichs et al. 1991, 1994; Hendrichs and Hendrichs 1998), could impose selection for a particular time of the day for sexual activity displays. It has been reported that *A. fraterculus* ceased or delayed its mating activity if the temperature in the morning is below 16 or 18°C (Malavasi et al. 1983 and de Lima et al. 1994, respectively). The population from Colombia (Ibague) that mated at dusk comes from a high-elevation region (1,600 m). It could be that early in the morning, temperatures are low enough to favor the selection for other times of mating activity. Populations coming from the Andean region at high elevations have revealed high levels of genetic differentiation with respect to other populations (Steck 1991, McPheron et al. 1999, Smith-Caldas et al. 2001), and evidence of morphological differences also has been reported (Hernández-Ortiz et al. 2004). Morphological characterization of the Ibague population has shown that this population is very similar to other Andean populations analyzed (V. Hernández-Ortiz personal communication). The finding that an Andean population exhibits a very distinct daily pattern provides more evidence to support the existence of a different taxonomic entity in this area.

In the test Tucumán–Piracicaba, the differences found with respect to mating location suggest that the tree and the screen were acting as two different mating arenas, thus reflecting some sort of ecological isolation (Dobzhansky 1937). Although Tucumán flies were active and mated on the tree, most Piracicaba couples formed on, and hence were collected from, the cage screen. When Tucumán flies decreased their activity, Piracicaba flies moved to the tree. This can be inferred by analyzing latency values. Piracicaba couples collected on the tree in this test showed statistically significant longer latency values than those couples collected on the screen in the same test or either on the screen or the tree in the La Molina–Piracicaba test. In this last test, because Peruvian flies were not so active early in the morning, Piracicaba flies occupied the tree as a mating arena. This tendency toward ecological isolation was a genuine observation as all the couples were included in the analysis. The standard protocol for this type of sexual behavior and compatibility test (FAO/IAEA/USDA 2003), suggests that any couple formed and collected outside the tree should not be included in the analysis because this may be because inappropriate testing conditions (i.e., the tree is too small or too hot or poor lighting conditions are provided). Nevertheless, considering the present data, it is recommended that mating compatibility tests, as part of research studies, should include those couples collected on the screen in the analysis because it may indicate some sort of spatial isolation or avoidance of mixed leks rather than inadequate testing conditions. Interestingly, males on the screen also were aggregated in leks and pheromone-called from places with direct sunlight and in the higher part of the cage, mainly the roof, resembling the behavior observed in nature (Malavasi et al. 1983).

Some differences in mating duration were detected among the populations. This finding has been reported for populations of the same species (Petit-Marty et al. 2004a) and for other species within this genus (for a revision, see Sivinski et al. 1999). In spite of these reports, there are no explanations for such variability and its implications (Sivinski et al. 1999). In addition, environmental temperature has often been reported as a modulator of copulation duration, making comparisons more difficult and speculative. Here, we assume that all the homotypic matings were successful in terms of ejaculate transfer. Possible reasons for the differences in mating duration in heterotypic crosses

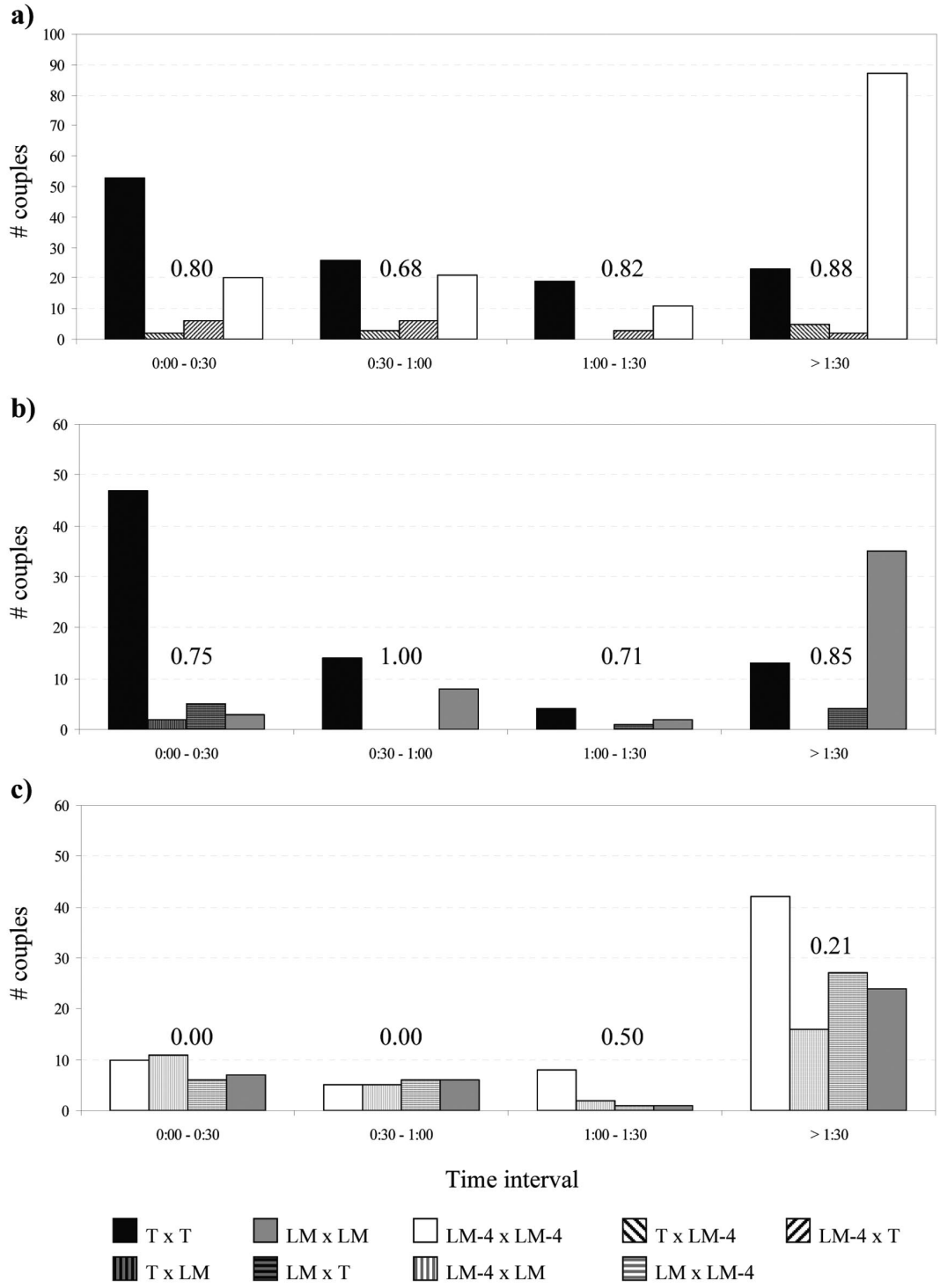

Fig. 1. Number of couples separated into four discrete time intervals obtained for each mating combination in the test where the light phase of La Molina flies was manipulated. (a) Tucumán (T) and La Molina out of phase (LM-4). (b) Tucumán and La Molina with the normal phase (LM). (c) La Molina out of phase and La Molina with the normal phase. ISI value provided for each mating combination and time interval computing all the replicates. Time intervals: first half an hour since beginning of the test (release of females); between the first half an hour and an hour; between the first hour and an hour and a half; and after the first hour and a half. Solid bars correspond to the homotypic crosses and dashed bars to the heterotypic crosses.

could not be evaluated because of the small sample size.

In the test in which Tucumán flies were released together with flies from La Molina that had a manipulated light phase, a shift in the latency was obtained for La Molina flies. Although it was not possible to move the peak fully to the early mating period of the Tucumán population to test the hypothesis that only temporal isolation is acting between the two populations, it would still have been expected to obtain at least a lower ISI value (i.e., less isolation). In fact the overall ISI values were equal and the ISI values obtained in each time interval were also high (Fig. 1). For flies that mated between half an hour and an hour after release, the number of couples obtained for each homotypic cross is similar, showing that even when sexual activity of the two populations was equal, the levels of isolation were high. Hence the isolation found between the two populations has an important component of sexual isolation and is not because of purely temporal isolation. The failure to move entirely the time of mating activity may be explained because 1) La Molina flies did not show a pronounced peak of sexual activity and they exhibited a greater plasticity or variability regarding the time at which they mate; and 2) the time at which lights turn on (sunrise) is not the only factor that modulates the time for mating; the quality of the light (e.g., in terms of intensity or polarization) or other environmental factors play an important role. Laboratory studies with controlled light quality may provide an answer.

Following earlier descriptions of high variability within *A. fraterculus* nominal species (Stone 1942), different approaches have provided evidence of a complex of cryptic species. The results reported here support some of these earlier suggestions. First, Andean populations are different from the other populations. Here, even though the single Colombian population was tested only against La Molina, it can be suggested that this population also is differentiated from the other populations tested because they either mate early in the morning (Argentinean and Brazilian populations) or at midday (Peruvian laboratory population). Second, Peruvian populations are different from southern Brazilian and Andean populations (Steck 1991) as La Molina flies tested against Piracicaba, Concordia, Tucumán and Ibague exhibited substantial levels of mating incompatibility. Contrary to expectations, we found some degree of mating isolation between Piracicaba (Brazil) and Tucumán (Argentina) populations. This seems to disagree with the general belief that populations from southern Brazil and Argentina belong to the same group or morphotype (Vilardi et al. 1994, Jaldo 2001, Smith-Caldas et al. 2001, Alberti et al. 2002, Basso et al. 2003, Hernández-Ortiz et al. 2004). In our case, matings involving Tucumán males and Piracicaba females were the most rare, whereas the other heterotypic matings, although still in a lower proportion compared with the two homotypic matings, were more frequent. This may indicate some reduction in competitiveness of Tucumán males, which could be explained by the fact that these originated from a laboratory population, whereas Piracicaba males were tested only after one generation of laboratory rearing, or the fact that some asymmetry in the isolation was detected. There is strong evidence that in southern Brazil more than one species of the complex occurs (Selivon et al. 1999, 2005). Thus, having tested only one population from this region, it seems premature to conclude that the different *A. fraterculus* populations from southern Brazil are all isolated from the Argentinean population. Moreover, a study evaluating postzygotic interactions and hybrid viability has been carried out in conjunction with this study to test evolutionary mechanisms that lead to mating incompatibility and will be the subject of another publication (N. Petit-Marty, unpublished data).

The increasing success of the SIT against several species within the Tephritidae indicates the potential of using this technique for *A. fraterculus* (Ortiz 1999). However, the levels of sexual isolation detected here highlight the need to consider each region individually and to carefully select which wild population is to be used as the source of flies to initiate a colony in each particular rearing facility. In the particular case of populations from southern Brazil and Argentina, further studies involving more populations from these countries, especially Brazil, and also from Uruguay and Paraguay, are needed to clearly identify the number of isolated populations or even different species. Detecting populations whose males are sexually compatible with females from as many populations as possible would be an advantage for any planned SIT program against this species.

Acknowledgments

We are extremely grateful to Nelson Canal (Universidad del Tolima, Colombia), Mónica Alburqueque Quiróz and Rafael Guillén (SENASA, Peru), and Julio Walder (CENA/USP, Brazil) for providing the material to perform the experiments. We also thank Pedro Rendón (USDA–APHIS–PPQ, Guatemala), Jorge Toledo and Pablo Liedo (Ecosur, Mexico), and Eutimio Gonzalez (LAMOFRU/UCV, Venezuela) for collaboration trying to provide samples that unfortunately were not available by the time of the study. We thank Roberto Antonio Zucchi and Vicente Hernandez-Ortiz for the taxonomic identification of the specimens. We also thank Natalia Petit-Marty for helpful comments on an early version of the manuscript.

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Received 25 January 2005; accepted 17 November 2005.