

Morphometric Differentiation Among *Anastrepha fraterculus* (Diptera: Tephritidae) Exploiting Sympatric Alternate Hosts

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Abstract

Anastrepha fraterculus (Wiedemann) is currently considered a complex of cryptic species infesting fruits from Mexico to Argentina and represents an interesting biological model for evolutionary studies. Moreover, detecting and quantifying behavioral, morphological, and genetic differentiation among populations is also relevant to the application of environment-friendly control programs. Here, phenotypic differentiation among individuals coexisting in the wild in a Northern region of Argentina was unveiled and associated with host choice. Six morphometric traits were measured in sympatric flies exploiting three different host species. Phenotypic variation was shown to be host-dependent regardless of geographical or temporal overlap. Flies collected from synchronous alternate hosts (peach and walnut) differed from each other despite the lack of geographical isolation. By contrast, flies emerging from guavas that ripen about two months later than peach and walnut showed no significant differentiation in comparison to flies collected from walnuts, but they differ significantly from flies originating from peaches. This result is consistent with the hypothesis that the same population of flies shifts from walnuts to guavas throughout the year, whereas the population of flies that uses peaches as a host is probably exploiting other alternate hosts when peach availability decreases. Further research is needed to study the underlying mechanism. Results are consistent with previous molecular markers (inter-simple sequence repeat-SSR) research on flies stemming from the same hosts and the same area, suggesting that differentiation among flies emerging from alternative hosts occurs at both genetic and phenotypic levels. The contribution of host preference in long-term genetic differentiation is discussed.

Resumen *Anastrepha fraterculus* (Wiedemann) es actualmente considerada un complejo de especies sinmórficas que infesta frutos desde México a Argentina y representa un modelo biológico interesante para estudios evolutivos. La detección y cuantificación de diferenciación morfológica, genética y de comportamiento entre sus poblaciones es también relevante para la aplicación de programas de control de bajo impacto ambiental. En este trabajo se reveló la ocurrencia de diferenciación fenotípica asociada con la elección del hospedero entre individuos que cohabitan en la naturaleza en una región del Norte de Argentina. Se midieron seis rasgos morfológicos en moscas simpátricas que utilizan tres especies de hospederos. La variación fenotípica observada es dependiente del hospedero e independiente del solapamiento espacial o temporal. Las moscas coleccionadas de hospederos alternativos sincrónicos (durazno y nogal) difirieron entre sí a pesar de la falta de aislamiento geográfico. En contraste, las moscas que emergen de guayabas, que maduran dos meses después que las anteriores, no se diferenciaron de las coleccionadas de nueces, pero sí de las emergidas de duraznos. Este resultado es consistente con la hipótesis de que a lo largo del año la misma población de moscas salta de nueces a guayabas, pero la población que usa duraznos explotaría otros hospederos alternativos cuando decrece la disponibilidad de durazno. Aunque los mecanismos subyacentes requieren estudios adicionales, estos resultados son consistentes con estudios previos con marcadores moleculares (fragmentos entre repeticiones de

secuencias simples ISSR) en moscas provenientes de los mismos hospederos y área, sugiriendo que la diferenciación entre las moscas que emergen de hospederos alternativos ocurre a nivel genético y fenotípico. Se discute la contribución de la preferencia de hospedero sobre la diferenciación a largo plazo.

Key words: South American fruit fly, morphometry, Bayesian analysis, speciation, host-race

Tephritid fruit flies represent interesting biological models for the study of speciation processes, with several examples of rapid radiation recorded within the family, mainly in genera involving species of economic importance such as *Rhagoletis* (Berlocher 2000), *Ceratitidis* (Virgilio et al. 2008), and *Bactrocera* (Clarke et al. 2005). *Anastrepha* (Schiner) is one of the main genera of the family Tephritidae, and includes >200 described species, some of which are fruit pests of economic importance (Norrbon 2004, Norrbom and Korytkowski 2011, Norrbom and Uchoa 2011).

Anastrepha fraterculus (Wiedemann), the South American fruit fly, is an insect pest of commercial fruits in most South American countries (Aluja et al. 2003). It was long considered to be a polyphagous fly attacking about 80 fruit species from >15 different families, many of them of economic importance (Malavasi et al. 2000, Norrbom 2004). Its taxonomic status represents a challenge, and available evidence indicates that *A. fraterculus* is actually a cryptic species complex (Steck 1991, Hernández-Ortíz et al. 2004, 2012; Vera et al. 2006; Cladera et al. 2014) with presence from Mexico to Argentina (Hernández-Ortíz and Aluja 1993). In Brazil, Selivon et al. (1996, 2002, 2005) identified three cryptic species based on diverse evidence including isozymes, karyotypic studies, morphometrics, and reproductive compatibility. Hernández-Ortíz et al. (2004, 2012), applying multivariate techniques to the measurements of 21 mesonotum, aculeus, and wing morphological traits, identified seven distinct morphotypes within the *A. fraterculus* complex, which appear to correspond to different cryptic species distributed allopatrically from Mexico to central Argentina. Vera et al. (2006) and Rull et al. (2013) established by mating compatibility assays under field cage conditions involving wild populations and laboratory strains from five different countries, the occurrence of reproductive isolation in most pairwise tests, supporting the existence of several cryptic species allopatrically distributed. In a more recent paper, Devescovi et al. (2014) demonstrated the occurrence of both pre- and postzygotic reproductive barriers between the so called Andean morphotype with populations from Mexico, Brazil, Peru, and Argentina, suggesting the existence of eight identifiable morphotypes within the *A. fraterculus* complex. In Argentina, *A. fraterculus* is present in the subtropical north-east and north-west regions where the weather is warm and humid (Vergani 1956). These two regions are separated by the biogeographic province of Chaco (Cabrerá and Willink 1980), a very arid region where *A. fraterculus* is normally absent except its occasional presence associated with human activity, i.e., commerce and migration, and is therefore restricted to urban or suburban areas (Alberti et al. 2002). Molecular evidence (Alberti et al. 2002, 2008; Cladera et al. 2014) and mating compatibility tests (Petit-Marty et al. 2004a,b; Cladera et al. 2014) indicate that in Argentina only one biological species is present, which would correspond to the *A. sp.1* aff. *fraterculus* described by Selivon et al. (1996) and renamed *Brazil-1* by Hernández-Ortiz et al. (2012) (Goday et al. 2006, Rull et al. 2012). In northwestern Argentina, the subtropical rainforest region (known as “Yungas”) has a high diversity of both native and exotic fruit fly hosts and *A. fraterculus* coexists with the introduced Mediterranean fruit fly, *Ceratitidis capitata* (Wiedemann) (Ovruski et al. 2005). Both tephritid species are

among the most serious pests affecting commercial fruit production in Argentina (Guillén and Sánchez 2007).

Due to the economic importance of these two fruit fly species in this country, a National Fruit Fly Control and Eradication Programme (PROCEM) was implemented in 1994. Currently, the program uses an integrated pest management (IPM) approach (Guillén and Sánchez 2007, Suárez et al. 2012) and its success in controlling *C. capitata* by applying the sterile insect technique (SIT; Knippling 1959, 1968) has encouraged the use of this method to control *A. fraterculus* as well (Ortiz 1999).

The SIT consists in mass production and release of gamma-irradiated sterile insects expected to mate with wild individuals, thereby preventing their reproduction. The evidence supporting the occurrence of only one *A. fraterculus* taxon in Argentina encourages SIT application to control this species since the presence of different morphotypes would reduce the SIT efficiency. Nevertheless, individual differences could be relevant because mating success partially depends on the multivariate phenotype (Segura et al. 2007, Sciuano et al. 2007). From the evolutionary biology perspective, the multivariate phenotype can be defined as a set of phenotypic traits that are potentially correlated in some way, and multivariate analysis are used to appropriately account for such correlations (Collyer et al. 2015). Phenotype usually reacts to differential environmental conditions (Nijhout 2002) with differential selection pressures that usually drive changes at the genetic level. Consequently, there is a chance that individuals reared on different hosts may have phenotypic differences which could influence mating compatibility among populations or mating male competitiveness. It has been previously established (Gómez-Cendra et al. 2014) that extremely different rearing conditions for *A. fraterculus* (laboratory vs. wild environments) produce some changes in morphology which constitutes one of the most readily interpretable assays of the potential for population differentiation (Simões et al. 2008). Different alternative hosts may provide diverse rearing environments in nature which may contribute to genetic and morphological variation in wild populations. With this background in mind, *A. fraterculus* constitutes an excellent biological model to evaluate the genetic and ecological basis of adaptation and possible consequences on genetic differentiation and isolation.

In a recent paper Oroño et al. (2013), analyzing ISSR (Inter-simple sequence repeat) loci, observed significant genetic differences among *A. fraterculus* adults that emerged from different host fruit species (peach, walnut, and guava) growing in a locality of Tucumán, Argentina. Genetic differentiation among individuals might also be expressed morphologically. It is expected that natural selection acts upon multiple, functionally related traits, and adaptation is an inherently multivariate process (Blows 2007). Thus, the phenotype as a whole include the information of multiple traits that are expected to be at least partially correlated. In the case of the population of *A. fraterculus* from Tucumán, the effect of possible host-mediated differentiation on the quantitative multivariate phenotype has not been explored so far. Therefore, in this paper, we compared several morphometric traits among adult flies collected from the same sampling site and involving the same host fruit

species studied by Oroño et al. (2013) to evaluate the relationship between host and multivariate phenotype of *A. fraterculus*.

Materials and Methods

Collection of Biological Material

The sampling site is located at Horco Molle, Tucumán Province, Argentina (Fig. 1). Geographically, it covers an area ranging from 26° 47'22.93" S to 26° 46'21.67" S and from 65° 20'27" W to 65° 19'32.19" W. In this site, host plants such as peach (*Prunus persica* L. (Batsch)) (Rosaceae), guava (*Psidium guajava* L.) (Myrtaceae), and walnut (*Juglans australis* Griseb.) (Juglandaceae) grow in sympatry without any horticultural care and are available as a tephritid feeding and breeding substrate.

Fruit showing evidence of natural infestation in the wild (oviposition holes) was recovered from 10 guava, 12 walnut, and 15 peach trees, whose geographic position was recorded with a GPS device (Garmin 12, Olathe, KS) for spatial analysis (as detailed below, Fig. 5A). Due to phenological differences in fruiting period, peaches and walnuts were collected in December 2004–January 2005, and guavas in March 2005. Collected infested fruits were transported to the laboratory at Planta Piloto de Procesos Industriales Microbiológicos y Biotecnología (PROIMI)—CCT Tucumán—CONICET facility and placed in a tray over a covered sandy litter where larvae could pupate. Trays were kept at $25 \pm 2^\circ\text{C}$ and 65% RH. The sand was sieved on a daily basis to obtain pupae, which were placed in plastic 0.5-liter flasks and kept under the same temperature and humidity conditions, and adults upon emergency were taxonomically identified with the key by Zucchi (2000), placed in a 2-ml eppendorf tube, and immediately frozen in an ultrafreezer at -80°C . Frozen individuals were then shipped in a 13-liter cryopreservation container (Air Liquide, Buenos Aires, Argentina) with liquid nitrogen to the Laboratorio de Genética de Poblaciones Aplicada (Buenos Aires University), Buenos Aires city, where all measurements were done.

Measures

Collected flies were dissected on a paraffin-filled Petri dish and the thorax, head, and wings were used to measure six traits related to body size, head shape, and flying ability: thorax length (THL), maximum head width (HW), minimum face width (FW), eye length (EL), wing length (WL), and width (WW) (Fig. 2).

Photographs of head and thorax were obtained when these structures were placed in a Petri dish filled with bacto agar 1% (DIFCO Laboratories, Detroit, MI) in distilled water. Head traits were recorded from the front and thorax length from the dorsal view. Special care was taken to place the pieces in horizontal position to reduce as much as possible parallax error.

Wings were mounted between slides and cover slips and then sealed with transparent nail polish (Mauricio J. Sztem & Cia. S.R.L, Buenos Aires, Argentina).

The chosen landmarks for wing measurements were located at the junctions of veins to the border of the wing or to another vein (Fig. 2). The left wing was used unless damaged, in which case the right wing was measured (no significant differences were observed between left and right wings measurements, data not shown). WW was defined as the distance between the R_1 vein intersecting the wing border and the point where CuA_1 vein joins the border (D13 segment in Selivon et al. 2005). WL measured the distance between the R_{4+5} vein intersecting the external border and the point where that radial vein joins R_1 .

All parts were photographed with a 3MP camera from a Leica EZ4HD stereoscopic microscope (Leica Microsystems (Switzerland) Ltd.). THL, WL, and WW were measured at $16\times$; HW, EL, and FW at $35\times$. Photographs were measured with a specifically created macro for ImageJ (Abràmoff et al. 2004, Rasband 1997–2012) image software.

The total number of individuals measured was 172, from which 65, 54, and 53 were retrieved from peach, guava, and walnut, respectively. All insects from guava and walnut were females, while those recovered from peach were 27 males and 38 females.

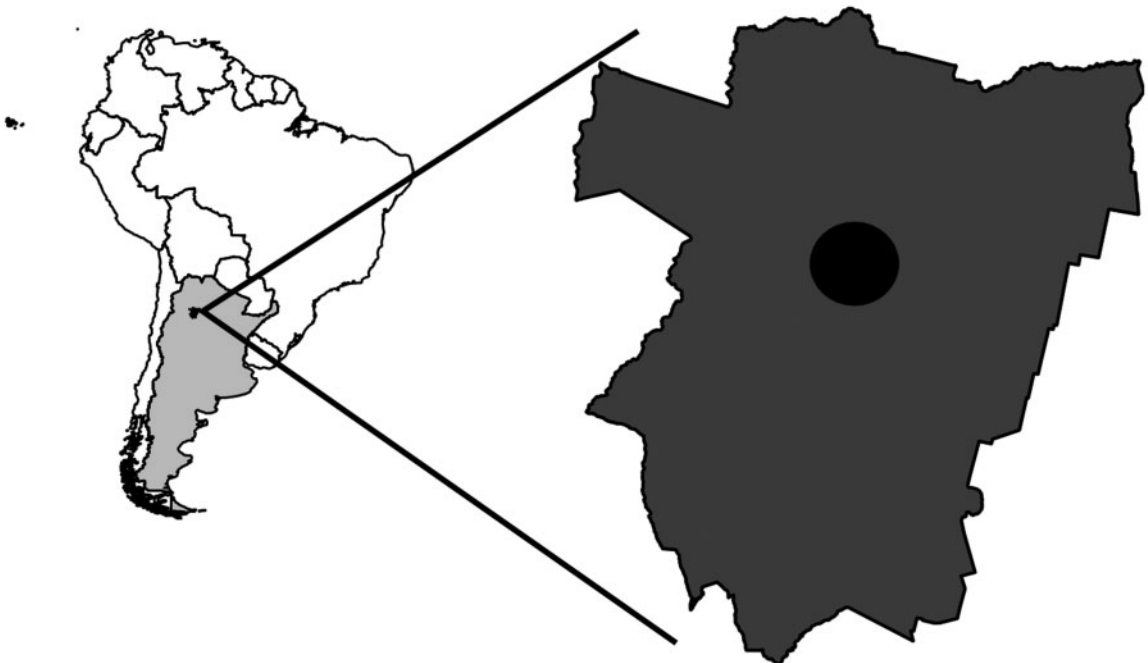


Fig. 1. Map of Argentina showing the localization of the sampling site at Horco Molle, Tucumán Province.

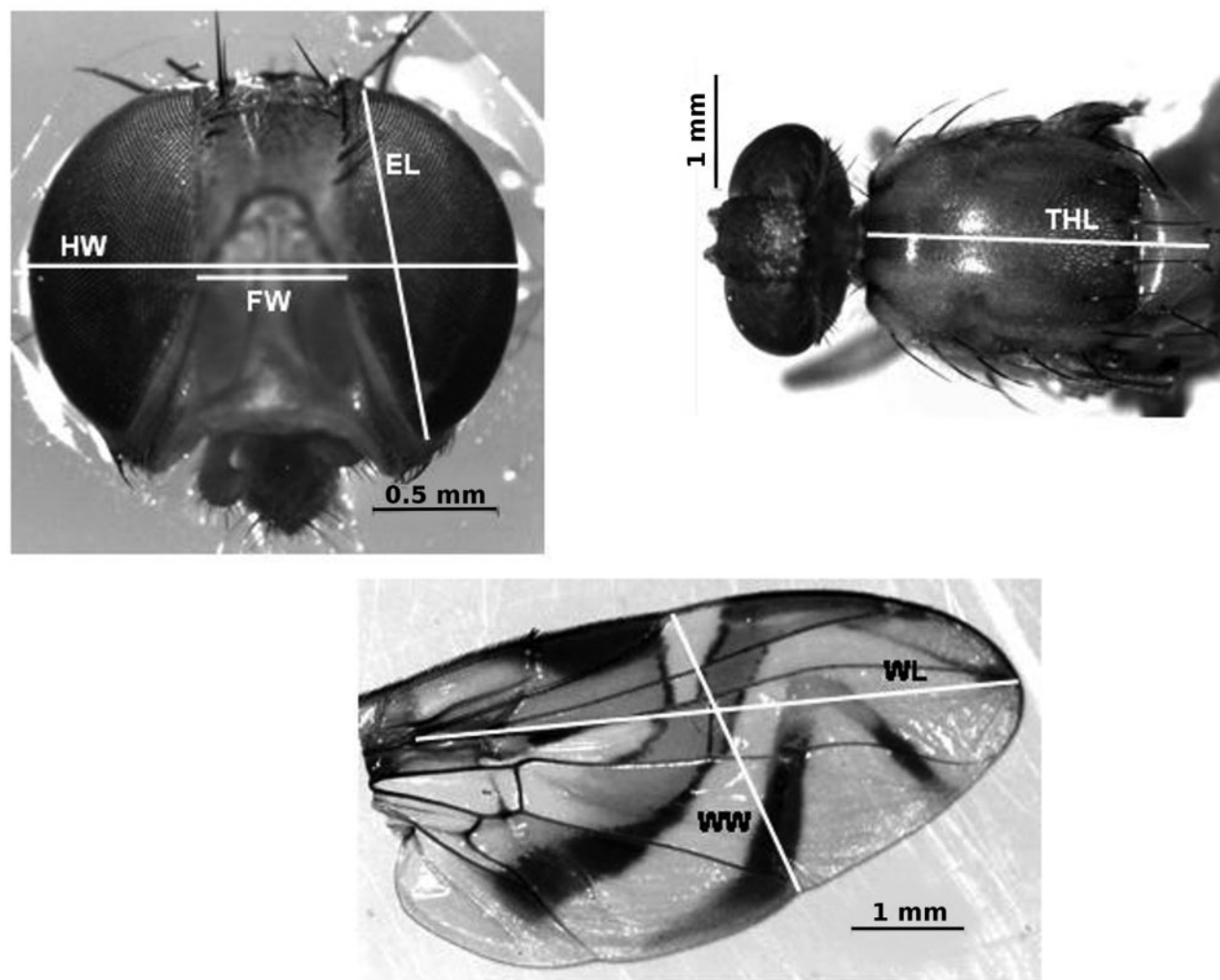


Fig. 2. Morphometric traits measured in *A. fraterculus*. EL: eye length. FW: face width. HW: head width. THL: thorax length. WL: wing length. WW: wing width.

Data Analysis

Phenotypic differences among flies emerged from different host plants were evaluated with generalized mixed-effect models taking into account host species and individual trees as explanatory factors for phenotypic variation. Although females are on average larger than males, the effect of sex could not be included in the models because males were present only in peach samples. To avoid bias attributable to differences in sex ratio among fruits two analyses were conducted: The first one (analysis 1) was based on female phenotype only. The drawback of this analysis is the reduction of sampling size. The second one (analysis 2) included all individuals. In this case male phenotype in the sample from peach was corrected by adding the mean difference between the sexes for each trait to each male measurement to eliminate the sex effect on body size-related traits. With this adjustment, differences between males and females within group became non significant (data not shown). A possible shortcoming of this adjustment is some unwanted effect on phenotypic variance.

For univariate analysis the model corresponds to the general expression

$$y_{ijk} = \mu + b_i + t_{ij} + e_{ijk} \quad (1)$$

where y_{ijk} represents the observation (measurement) of the trait for an individual fly from the tree j of host i and environment k , μ is the

overall mean, b_i is the (fixed) effect of the host species i , t_{ij} represents the (random) effect of the tree j nested in host i , and e_{ijk} is the random residual error. Components of variance and fixed effects were estimated with the package *lme4* (Bates et al. 2013) of the software R ver. 3.2.2 (R Core Team 2015), applying restricted maximum likelihood method. The significance of mean differences among host species was estimated by likelihood-ratio chi-square using the *Anova* function of the package *car* (Fox and Weisberg 2011).

The effect of host species on the multivariate phenotype was evaluated by two different approaches. The first one was a conventional multivariate analysis of variance (MANOVA), conducted with the package *stats* of the software R version 3.2.2 (R Core Team 2015). As this method does not take into account the (random) tree effect, we also applied the multivariate extension to equation (1), using the Bayesian approach implemented in the package *MCMCglmm* (Hadfield 2010) of R which approximates the estimates by Markov chain Monte Carlo simulations. To cope with the limitations due to multidimensionality of the dataset, we first conducted a principal component analysis (PCA) with the package *ade4* (Dray and Dufour 2007), and then we applied the *MCMCglmm* procedure on the first axes obtained. The analysis was based on 50,000 iterations, a thinning interval of 50, and a burnin of 1,000.

Table 1. Mean \pm SD (mm) for each trait measured on female *A. fraterculus* and results of individual analysis of variance for mean differences among insects collected from different host trees

Trait	Peach	Guava	Walnut	Chisq	P
EL	1.357 \pm 0.064	1.430 \pm 0.108	1.486 \pm 0.083	30.224	2.7 10^{-07}
FW	0.589 \pm 0.049	0.585 \pm 0.054	0.631 \pm 0.06	18.449	9.9 10^{-05}
THL	2.716 \pm 0.154	2.950 \pm 0.297	3.007 \pm 0.198	22.131	9.5 10^{-06}
HW	1.859 \pm 0.091	1.952 \pm 0.114	1.998 \pm 0.105	30.841	2.0 10^{-07}
WL	4.984 \pm 0.272	5.364 \pm 0.36	5.359 \pm 0.294	22.459	1.3 10^{-05}
WW	2.555 \pm 0.130	2.703 \pm 0.183	2.733 \pm 0.144	21.314	2.4 10^{-05}
N	38	54	53		

N, number of flies from each host. Traits: EL, eye length; FW, face width; HW, head width; THL, thorax length; WL, wing length; WW, wing width (See Fig. 2 for details).

Chisq: likelihood-ratio chisquare for the generalized linear model. P: test significance.

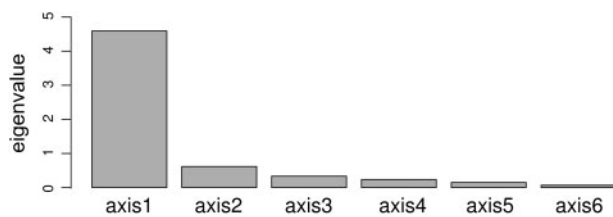


Fig. 3. Eigenvalues corresponding to the principal component analysis (PCA) of morphometric variation in *A. fraterculus*.

To study the spatial structure of the populations, the mixture model for phenotypic data was used by means of the package *Geneland* v 4.0.3 (Guillot et al. 2005, 2012) of R, where the coordinates for each sample (tree) were incorporated to the calculations. The model finds *a posteriori* clusters characterized by a mean μ_{kj} and a variance σ^2_{kj} , following a model which is a mixture of multivariate independent normal distributions (The *Geneland* Development Group 2012, Guillot et al. 2012). Therefore, the software utilizes the phenotypic differences among the individuals to place them in different putative clusters, beginning with a random assignation and modifying it with each iteration in order to find the result (number of clusters and individual assignation) that shows the highest posterior probability. No prior information about group membership (i.e. the host) is used in the analysis, so the clusters are generated *a posteriori* in each iteration.

Different parameter sets were tested for running *Geneland* and eight independent runs were made, with the number of populations (k) to test varying from 1 to 10, with 200,000 iterations and a thinning at 200. Based on these preliminary results, 40 new independent runs were made with k varying from 1 to 4, 1 million iterations, keeping the thinning in 200. The required burnin estimated so as to discard the runs prior to the convergence of the posterior density values was established at 1,000.

Results

The trends observed in the analysis including all individuals and using only female phenotype were consistent. For simplicity only results from analysis 1 (based on females only) are shown.

Basic statistics of the six measured traits on adults emerged from different host fruit species are given in Table 1. Univariate tests showed significant or highly significant differences among flies

Table 2. *MCMCglmm* results based on the first axis obtained in the PCA (Fig. 3)

	Peach	Guava	Walnut
Peach	–	<0.001***	<0.001***
Guava	–1.701	–	0.06
Walnut	–2.470	–0.752	–

Average differences among *A. fraterculus* populations are shown below and the corresponding P values above the diagonal.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 3. Results of morphological variation distribution analysis of *A. fraterculus* with *Geneland*

Run no.	k	%wb	%ab	Log-likelihood
2	3	88.62	98.53	–47745.69
30	3	88.68	98.53	–48546.68
29	3	88.76	98.58	–49477.55
31	3	69.48	74.55	–49692.31
14	3	88.46	98.35	–49725.34
24	3	57.66	70.18	–50096.18
8	2	67.96	79.83	–52494.37
9	3	88.78	98.53	–52631.90
39	3	88.78	98.65	–52784.55
10	3	88.48	98.35	–53698.77

Only the 10 runs with the highest mean of probability density are shown.

k : modal number of clusters, %wb: percentage of iterations where the number of clusters is equal to k without burnin, %ab: percentage of iterations where the number of clusters is equal to k after burnin.

Iterations: 1 million. Thinning: 200. Burnin: 1,000.

emerged from different host species for all traits (Table 1). In most cases individuals recovered from peach were the most differentiated, with mean values lower than those estimated for individuals emerged from guava and walnut. By contrast, mean values of individuals gathered from guava and walnut trees were rather similar to each other.

The conventional multivariate MANOVA test also showed highly significant differences among flies from different hosts (Pillai = 0.51, Approximated $F = 7.88$, $P = 1.22 \cdot 10^{-12}$).

The PCA produced six axes whose eigen values and percentage of explained variance are shown in Fig. 3. Most part of morphometric variance (76.5%) is explained by the axis 1, which is the only with an eigenvalue higher than one. As the contribution of the remaining axes is almost negligible, the *MCMCglmm* analysis was conducted on the first axis only. Pairwise contrasts between host species showed highly significant differences ($P < 0.01$) among individuals recovered from peach and those emerged respectively from walnut and guava (Table 2). By contrast, the differences between individuals recovered from walnut and guava were nonsignificant.

The preliminary analysis of spatial structure by means of the *Geneland* package testing the number of putative clusters from $k = 1$ to 10 indicated that the modal number of clusters retrieved was 3. Based on such observation, 40 new runs with k between 1 and 4 were performed with 1 million iterations each and a thinning of 200. In 36 runs the best result was $k = 3$. The modal number of clusters in the remaining runs were $k = 2$ (2 runs) and $k = 4$ (2 runs).

Results of the 10 runs with the highest mean of probability density are summarized in Table 3. Most runs yielded consistent results, where individuals retrieved from peach were mostly assigned to the same cluster (cluster 1), whereas most individuals recovered from

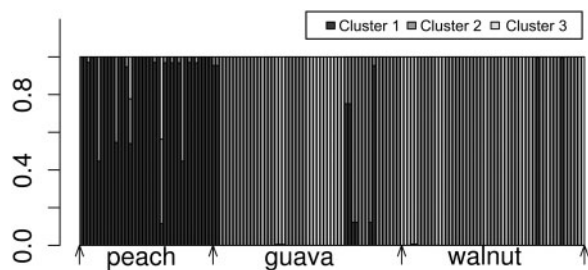


Fig. 4. Stacked bar plot representing the estimated individual population membership probability (considering the clusters found by *Geneland* in run no. 2 of Table 3) of females of *A. fraterculus*. Host species for each individual are indicated at the bottom.

Table 4. Numbers of females of *A. fraterculus* retrieved from each host species assigned to each cluster according to *Geneland* analysis

Cluster	Peach	Guava	Walnut	Cluster size
1	35	5	2	42
2	3	34	34	71
3	0	15	17	32
Sample size	38	54	53	145

Data for run no. 2 from Table 3 (best run according to probability density), with 1 million iterations.

guava and walnut fruits were assigned to either cluster 2 or 3. The run with the highest average posterior probability (no. 2) was selected to illustrate the results (Fig. 4). Table 4 shows that the number of individuals assigned to each cluster differs among flies emerged from different host species. An independence chisquare test indicated that the proportion of individuals included in each cluster differs from random expectations ($\chi^2 = 100.49$, $P < 2 \cdot 10^{-16}$). Finally, the maps of estimated population membership (Fig. 5) showed no clear spatial patterns. Rather they suggest that the differentiation among individuals is associated to host specificity.

Discussion

In most cases, where populations are geographically isolated speciation is usually a continuous process by which genetic variation becomes segregated between populations and reflected in phenotypic and behavioral differences (Cáceres et al. 2009). However, the abundance of polymorphisms, host races, and subspecies suggests that natural sympatric speciation is not uncommon (Drés and Mallet 2002, Mallet 2008). In such cases, sexual isolation can evolve by a mixture of pre and post zygotic barriers, involving behavioral (for instance, differences in courtship activity, temporal partition of mating activity, and mating duration), physiological (such as reduction in hybrid egg viability), and chemical aspects. The latter can be mediated by pheromone divergence, although adaptive divergence of chemosensory traits in response to factors such as hosts can also commonly drive the evolution of prezygotic barriers (Smadja and Butlin 2009). Phytophagous insects are excellent model systems to investigate the adaptation mechanisms and interspecific divergence, since their host plants constitute the most immediate environmental factor affecting early life cycle stages (Schoonhoven 2005). The remarkable diversity of phytophagous taxa might be partially explained by host shifts since modifications in diet or the rearing environment have been positively associated with reproductive isolation in insects (Soto et al. 2014). Tephritids in particular fit very

well in evolutionary studies because there is evidence of species complexes, sympatric speciation, host shifts, and host race formation (Feder et al. 2003, Linn et al. 2003, Cáceres et al. 2009). *Anastrepha fraterculus* constitutes one of such complexes and reproductive isolation among its morphotypes (Hernández-Ortiz et al. 2004, 2012; Vera et al. 2006, Rull et al. 2013) may probably follow an allopatric model driven by natural selection, as the differentiation occurs among populations spreading along thousands of kilometres allowing for geographic isolation. Moreover, differences in the diel pattern of mating activities have been recorded in allopatric populations which have been postulated to be, at least partially, the by-product of selection against maladaptive hybridization on adults of the Andean morphotype and local guilds of sympatric sister species with spatial overlap (Devescovi et al. 2014). Even when *A. fraterculus* can be seen as a complex of cryptic species, evidence shows that only one biological species exists in Argentina (Alberti et al. 2002, 2008; Petit-Marty et al. 2004a,b; Cladera et al. 2014). These studies, however, cannot rule out the occurrence of micro differentiation within particular areas associated with the heterogeneity in resource availability. Such heterogeneity can be transient or long term, and could be associated with a number of factors, including host exploitation, which could be the basis for a sympatric speciation process. Indeed, according to Drés and Mallet (2002) point of view, host races may be considered as just one (early) of a number of intermediates in the continuum between polymorphisms and full species. Previous studies have shown that the variation within the populations of *A. fraterculus* from Argentina is quite high (Alberti et al. 2002) and variation among individuals was found for some behavioral traits such as copula duration or preferred mating location in the trees (Petit-Marty et al. 2004a), indicating that populations are not totally homogeneous. Differences might be associated with host although different results were observed with different techniques and in different regions. Malavasi and Morgante (1983) did not find allozymic differences among flies that emerged from different host species in Brazil, whereas Oroño et al. (2013) analyzing molecular markers (ISSR) in the same population studied here observed significant differences in ISSR patterns between flies that emerged from peach as opposed to those retrieved from guava and walnut, while flies that emerged from these two latter hosts were indistinguishable. These authors attributed genetic differences among flies to host plant chemistry, which is consistent with the deme formation hypothesis for phytophagous insects (Mopper et al. 1995) that proposes that insects adapt to the defensive phenotypes of individual trees.

In the present work, *A. fraterculus* from the same three hosts species (peach, guava, and walnut) present in the same wild vegetation patch studied by Oroño et al. (2013) from Horco Molle (Tucumán, Argentina) were evaluated for six phenotypic quantitative traits. Significant differences were found from both univariate and multivariate analyses. When a Bayesian approach was used to find individual membership probabilities of posterior assignment to the identified clusters, it was clear that flies recovered from infested peaches were mostly assigned to a single cluster (cluster 1), whereas the flies emerging from guavas and walnuts were assigned to clusters 2 and 3 in similar proportions. Therefore, individuals using peach as a host are morphologically different from individuals from guava and walnut (which cannot be sorted out from each other). These results mirror those found by Oroño et al. (2013) by means of ISSR markers.

The differentiation between flies emerging from peaches in regards to the other two hosts cannot be explained on geographical grounds because peach and walnut trees are on average closer to

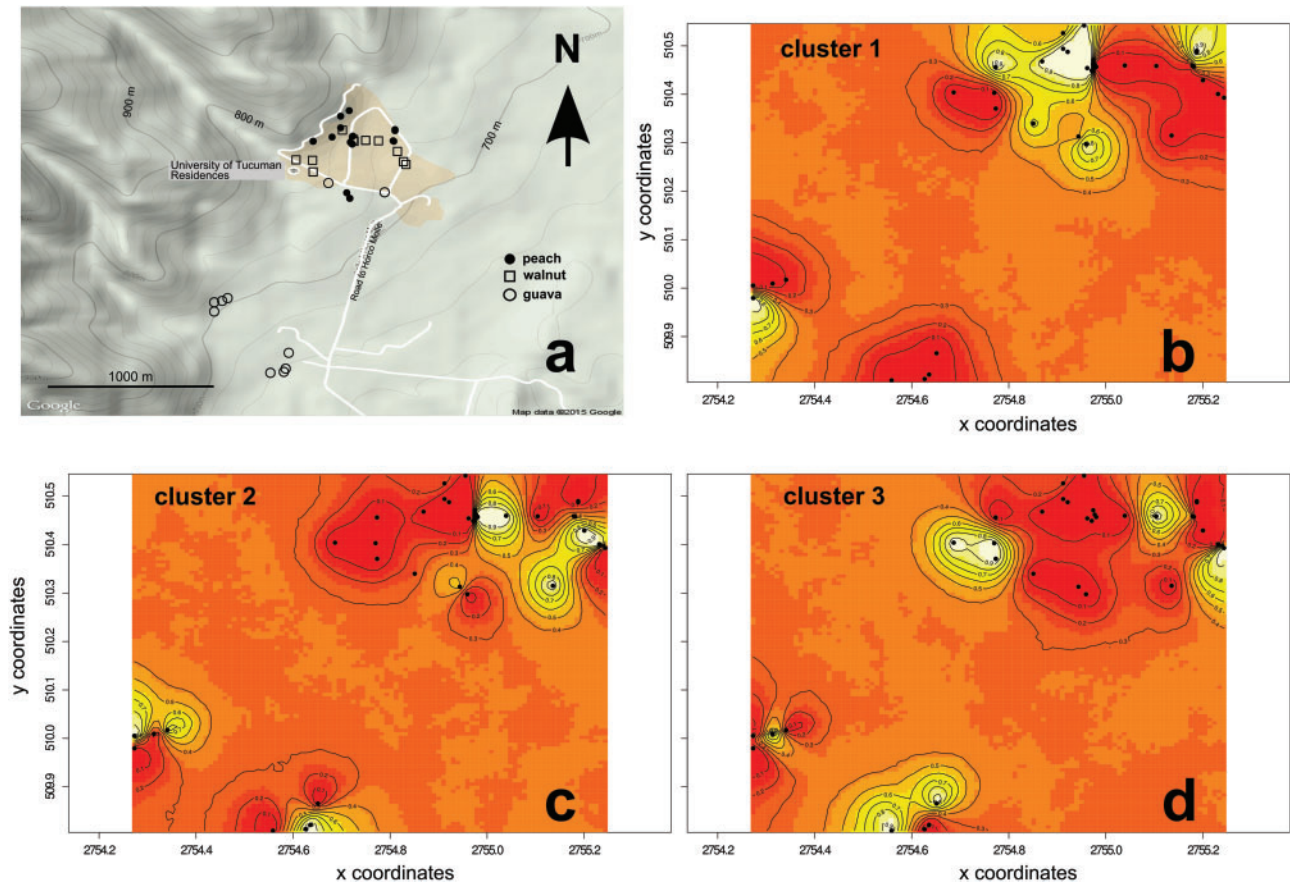


Fig. 5. Maps of the collection site at Horco Molle (Tucumán, Argentina) indicating the location of each sampled tree (a) and posterior probabilities of population membership for each cluster (b–d) (data for run no. 2 of Table 3).

each other than to guava trees. Differences in fruiting season are not a plausible explanation either, because peaches and walnuts in this locality ripen in December whereas guavas ripen in March, when neither peaches nor walnuts are available.

Larvae developing in different rearing environments (hosts) are facing diverse challenges and selection pressures. In fact, Gómez-Cendra et al. (2014) observed that wild *A. fraterculus* from the same geographical region studied in this paper showed significant morphometric changes after several generations of laboratory rearing. However, in that study laboratory-reared insects had not gene flow with the source wild populations for about 60 generations, and the observed morphological differences could be attributed to both environmental and genetic (drift, positive selection) causes. Differences in rearing could still explain phenotypic differences among flies emerged from different hosts in nature, since the adaptation to host chemical properties may have played a role in the differentiation and the developing of genetic differences and reproductive isolation among components of the *A. fraterculus* complex as evidenced by differences in host preferences in different countries (Hernández Ortíz et al. 2004). However, the trends observed in our work as well as in that by Oroño et al. (2013) are not easily explained on the grounds of differences in biochemical properties of the fruit species considered. In fact, guava and walnuts are highly differentiated in sucrose, protein, phenolic compound, and tannin contents, but no significant genetic (Oroño et al. 2013) nor phenotypic differences were detected between flies that emerged from these host species. By contrast, flies that emerged from peaches are genetically and morphologically differentiated from them. The patterns observed might be

at least partially explained on historical grounds, as walnuts and guavas are native, while peaches were introduced by Spanish colonists (Oroño et al. 2013). The differentiation of peach-hosted population in respect to the other populations might reflect the genetic consequences of a relatively recent host shift, similar to the host races of *Rhagoletis pomonella* associated to hawthorn (*Crataegus* spp.) and apple (*Malus pumila*) (Filchak et al. 2000) and rice and corn races of the armyworm *Spodoptera frugiperda* (Murúa et al. 2015). This view is consistent with our results from Geneland analysis in which most individuals that emerged from peach belong to only one cluster, whereas two clusters were identified for individuals emerging from both walnut and guava.

Throughout the year, *A. fraterculus* must shift hosts according to availability (Ovruski et al. 2003), and it was expected that all the flies present in the same area would show a similar behavioral pattern in regards to resource use, depending on host availability. Instead, the present results together with those by Oroño et al. (2013) show phenotypic and genetic differences among flies obtained from different host trees fruiting in sympatry (peach and walnut). Although flies are not geographically isolated the occurrence of sympatric and synchronous genetic and morphological differentiation between host-associated groups seems to indicate that there is little or no recent gene flow between them and that there is some factor, or factors, sustaining that differentiation through time. Furthermore, individuals obtained from peach are different from those obtained from a later fruiting host, guava. This suggests that the population of flies reared on walnut could shift to guava as walnut availability decreases, but flies using peach must shift to another

alternative host. According to fruiting seasons and fruit availability in the study area (Ovruški et al. 2003) those alternative hosts could be *Magnifera indica* L. (Anacardiaceae), *Annona cherimola* Mill (Annonaceae), *Ficus carica* L. (Moraceae), *Myrcianthes pungens* (Berg) Legrand, *Passiflora caerulea* L. (Passifloraceae), *Citrus paradisi* Macfadyn, and/or *C. aurantium* L. (Rutaceae). This hypothesis needs to be verified by further research.

The mechanism responsible of keeping sympatric populations partially isolated might be related to female choice of egg-laying sites or selection over larvae within host. The answer to this question requires a thorough research on behavior and genetic properties of samples collected from alternative hosts throughout the year. A mechanism that may be invoked to explain partial isolation between sympatric insects associated with different host species is assortative mating. This phenomenon is common in insects and can be generated by mechanisms such as mate choice, mate availability, and mating constraints (Cueva del Castillo et al. 1999). In parasitic insects using different host species population divergence is at least partially promoted if selection on traits between host-associated populations leads directly or indirectly to a reduction in gene flow between populations, thereby facilitating reproductive isolation (Mopper 1996). Development in contrasting environments can lead to differences in body size that contribute to pre-mating isolation through mechanisms such as size-assortative mating (Nagel and Schluter 1998), for instance studies in the fish *Pelvicachromis taeniatus* (Baldauf et al. 2009) suggest that the underlying mechanism of body size assortative mating may be mutual mate choice, which reduce sexual conflict. In the case of *A. fraterculus*, morphometric differences between flies emerged from different hosts might contribute to increase the likelihood of matings between individuals emerged from the same host, which might be the starting point for a sympatric differentiation process, whose final results are uncertain. Adaptation to a specific host can be reinforced by pheromones (Loxdale et al. 2011), so the study of the female response to pheromones, as suggested by Oroño et al. (2013) could help to explain the differentiation between populations associated with different hosts. An analysis of host switch throughout the year is necessary to improve the understanding of the unexpected differentiation among sympatric populations.

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