

Pheromone emission patterns and courtship sequences across distinct populations within *Anastrepha fraterculus* (Diptera-Tephritidae) cryptic species complex[†]

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Abstract

Sexual behavioural isolation can result from sexual selection and represents a relevant factor associated with the speciation process. We analysed the pheromone emission pattern and the courtship of males of five different populations of the *Anastrepha fraterculus* cryptic complex: Brazil (Vacaria, Tucumán and Piracicaba), Colombia and Peru. The time of pheromone emission was recorded in each population every 30 min during the day. The behavioural sequences of courting were video recorded and analysed using EthoSeq software. Males from different populations have showed different period of pheromone emission – Vacaria, Piracicaba and Tucumán executed calling only during the morning, Colombia only in the afternoon and Peru during both periods. The general frequencies of the courtship units of the males were distinct among the populations. Three groups were formed in the classification from the function of 14 behavioural routines: Vacaria, Piracicaba and Tucumán formed a single group (Brazil-1), while Colombia and Peru formed two distinct groups. In the probabilistic trees generated, the behavioural units that most contributed to the occurrence of copulation were distinct among the three groups formed: Brazil-1 (*Contact*, *Alignment* and *Arrowhead-1*); Colombia (*Flying*, *Mobile*, *Contact* and *Alignment*); Peru (*Flying*, *Arrowhead-1* and *Calling*). Our results indicated differences in sexual behaviour that may explain the behavioural isolation found between the distinct groups in addition with the temporal isolation found between the Brazil-1 and Colombia populations. The evolutionary implications for the *A. fraterculus* cryptic species complex are discussed.

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Introduction

Differences between populations in terms of sexual traits and/or mating preferences can result in speciation mediated by sexual selection (Butlin & Ritchie, 1994; Ritchie, 2007; Macedo & Machado, 2013). Sexual selection can favour characteristics that reinforce pre-zygotic reproductive isolation between subpopulations through the evolution of preferential mating associated with male secondary characters (Lande, 1981, 1982; Ritchie, 2007). Among cryptic species, pre-zygotic isolation tends to evolve either as a direct factor (Wu *et al.*, 1995) or by reinforcing already existing post-zygotic isolation (Noor, 1999). Sexual behaviour isolation is frequently a component of pre-zygotic isolation (Nanda & Singh, 2012), and male traits evaluated by the females during courtship are selected and enable reliable recognition of con-specifics, promoting isolation between related species and avoiding cross-mating (Boake *et al.*, 1997).

The nominal species *Anastrepha fraterculus* represents a cryptic species complex (Stone, 1942; Malavasi & Morgante, 1982; Steck, 1991; Selivon & Perondini, 1998; Hernández-Ortiz *et al.*, 2004; Selivon *et al.*, 2004; Selivon *et al.*, 2005; Vera *et al.*, 2006; Hernández-Ortiz *et al.*, 2012, 2015; Canal *et al.*, 2015; Manni *et al.*, 2015) that shows pre- and post-zygotic reproductive incompatibilities among lineages (Selivon *et al.*, 1999; Vera *et al.*, 2006; Cáceres *et al.*, 2009; Segura *et al.*, 2011; Rull *et al.*, 2013; Devescovi *et al.*, 2014; Vaničková *et al.*, 2015a). This species, known as the South American fruit fly, is a polyphagous species considered the most important native pest of native and commercial fruits in South America (Dutra *et al.*, 2007; Alberti *et al.*, 2008). It is widely distributed from northern Mexico to the south of South America (Stone, 1942; Aluja, 1994; Hernández-Ortiz *et al.*, 2015) and infests more than 116 hosts (Zucchi, 2016). Currently, eight distinct morphotypes has been postulated to constitute the *A. fraterculus* cryptic species complex known as the Peruvian, Andean, Mexican, Venezuelan, Brazil-1, Brazil-2, Brazil-3 and Ecuadorian morphotypes (Hernández-Ortiz *et al.*, 2012, 2015).

The pre-zygotic incompatibilities found between the morphotypes described for this cryptic species complex (Vera *et al.*, 2006; Cáceres *et al.*, 2009; Rull *et al.*, 2013; Devescovi *et al.*, 2014; Roriz *et al.*, 2017) suggest that reproductive barriers have evolved favouring isolation, comprising temporal and behavioural aspects associated with the mating system. The mating system in *A. fraterculus* is polygynous, in which the females discriminate among males congregated in displaying, lekking arenas (Aluja *et al.*, 1999). Male courtship behaviour was described by Gomez Cendra *et al.* (2011) as composed of 15 behavioural units arranged in three groups: pheromone emission, wing positioning and body movements. More recently, Passos-Roriz *et al.* (2018) reanalysed the courtship of *A. fraterculus* males and subdivided that behaviour into 26 behavioural units within eight groups. This repertoire of behaviours involves the emission of chemical, visual and acoustic signals.

Chemical communication, mediated by the emission of volatile sexual pheromones or the presence of certain hydrocarbons in the cuticle, can contribute to the recognition and

choice of reproductive partners in certain insect species (Antony & Jallon, 1982; Cobb & Jallon, 1990; Giglio & Dyer, 2013; Vaničková *et al.*, 2014). In the *A. fraterculus* complex, cuticular hydrocarbons differ between morphotypes as well as between males and females (Vaničková *et al.*, 2012, 2015b, c). Moreover, male-borne volatiles of distinct populations differ quantitatively even across populations belonging to the same morphotype (Břízová *et al.*, 2013). Juárez *et al.* (2015) reported that *A. fraterculus* females were attracted to male-borne volatiles of their respective populations; even though these authors argued that pair recognition probably occurred during courtship.

The evolution of differences in the time of sexual activity between populations may promote or accelerate reproductive isolation (Dobzhansky, 1937). The time of sexual activity within the *A. fraterculus* complex has been studied for Brazil-1, Brazil-3, Mexican, Peruvian and Andean morphotypes (Vera *et al.*, 2006; Cáceres *et al.*, 2009; Segura *et al.*, 2011; Rull *et al.*, 2013; Devescovi *et al.*, 2014; Dias *et al.*, 2015; Roriz *et al.*, 2017). Evidence shows that this trait varies between morphotypes and may contribute, in certain cases, to mating isolation.

The high complexity of *A. fraterculus* courtship (Aluja, 1994; Aluja *et al.*, 1999; Gomez Cendra *et al.*, 2011) and the fact that some components of male courtship can be important for mate recognition (Ryan & Rand, 1993) support our hypothesis that there are differences in the courtship behaviours of the different morphotypes within this cryptic species complex. As such, in this work, we pursued to determine the temporal pattern of pheromone emission as well as to analyse and characterize male courtship of some morphotypes within the *A. fraterculus* cryptic species complex. These analyses should help elucidate the pre-zygotic mechanisms underlying the reproductive isolation found between different lineages of this cryptic species complex, emerging as a useful tool for lineage discrimination.

Materials and methods

Flies

The distinct populations of *A. fraterculus* were maintained at the Insect Pest Control Laboratory of the IAEA/FAO, Seibersdorf, Austria. Flies were reared on artificial diets according to the methods described by Vera *et al.* (2006) and Rull *et al.* (2012). The colonies were maintained at $25 \pm 1^\circ\text{C}$, relative humidity $70 \pm 10\%$ and under a 12 h light:dark photoperiod (7:00 photophase; 19:00 scotophase). We used populations from San Miguel de Tucumán, Tucumán/Argentina ($26^\circ48'2''\text{S}$, $65^\circ13'3''\text{W}$; F1 generation), Vacaria, Rio Grande do Sul/south of Brazil ($28^\circ27'32''\text{S}$, $50^\circ59'44''\text{W}$; F32 generation), and Piracicaba, São Paulo/southeastern of Brazil ($22^\circ45'45''\text{S}$, $47^\circ50'33''\text{W}$; F35 generation) as representatives of morphotype Brazil-1, a population from La Molina/Peru ($12^\circ4'55''\text{S}$, $76^\circ55'41''\text{W}$; F30 generation) representing the Peruvian morphotype and a population from Ibagué, Tolima/Colombia ($4^\circ26'40''\text{S}$, $75^\circ14'32''\text{W}$; F29 generation) representing the Andean morphotype. The taxonomic

confirmations of the morphotypes were made by Dr Vicente Hernández-Ortiz of the Instituto de Ecología A.C. México.

After adult emergence, the flies were separated by sex and isolated in cylindrical acrylic cages, with free access to water and a diet composed of wheat germ, hydrolysed yeast and sugar (1:1:3) until sexual maturity (males: 10–25 days; females: 15–25 days). The experiments were undertaken in the Insect Pest Control Laboratory (IAEA, FAO, Seibersdorf, Austria) within arenas under these same colony maintenance conditions.

Temporal patterns of pheromone emission

To analyse the time of pheromone emission, at least 24 h before the test, ten marked (with Acrilex non-toxic paint) males of the same population were placed in cylindrical acrylic cages (29 × 20 cm). Ten females held in acrylic boxes (9 × 7 × 9 cm), with air flow in the top, were placed in the centre of each cylindrical male cage to stimulate male pheromone emission. Flies were kept for at least 12 h in darkness before the experiment to ensure the experiment began at the onset of the photophase. Pheromone emission frequency was recorded individually every 30 min, from 08:30 to 17:30 h. Males were considered to be emitting pheromone when they everted the anal epithelium at the distal portion of the abdomen and/or the prominent lateral glands (Lima *et al.*, 2001). Ten replicates were made for each population.

Male courtship sequences

To characterize male courtship sequence, two males and two females (simulating a small lek) of the same population were released into a small glass cage (9 × 7 × 9 cm) containing a lemon leaf (*Citrus* sp.) as a substrate for their sexual interactions (Briceño *et al.*, 2007 with modifications). Their behaviours were recorded for up to 30 min using a digital video camera (Geovision-GV-BX 220D-3, 2 M, variable focus lens, at 30 fps) positioned frontally to the glass cage under artificial light conditions. The videos were saved in an AVI format (GeoVision Multicam Surveillance version 8.5.4.0). Recordings were performed during the period of sexual pheromone emission. Only one male mated was analysed in each session and ten successful courtship replicates (i.e., that resulted in mating) were performed for each population.

Statistical analyses

To analyse temporal correlations in pheromone emission among populations, we generated an autologistic Generalized Linear Model (GLM) (Besag, 1972). This methodology compared the populations using time as a co-variable. We assumed a Poisson distribution for the number of males that emitted pheromones. The Log was utilized as a linking function of the GLM. An automodel was constructed using the co-variance of five consecutive data collection times, using the Vacaria population as the reference within the model.

Male courtship ethograms followed Passos Roriz *et al.* (2018) and Gomez Cendra *et al.* (2011) and are described in Appendix B. Only the behaviour of males that mated successfully was analysed. The frequency of the behavioural units was compared among the different populations of *A. fraterculus* through a GLM assuming that the data followed a Poisson

distribution, using Log as the linking function, and model hypothesis test for multiple comparisons.

The behavioural sequences were analysed using EthoSeq software (Japyassú *et al.*, 2006). The program produces a first-order transition matrix from the raw individual sequence data, and uses graph theory to produce hierarchical representations with directed trees (DiTree). The DiTree maximizes the sums of the transition probabilities between all of the behavioural units, generating the most parsimonious hierarchical graph, or trees (Japyassú *et al.*, 2006). To analyse the courtship in detail, we selected the tree whose root indicated copulation success (ending with mating) to produce courtship routines (most probable, linear sequences of behaviour). The most frequent routines were analysed through a canonical discriminant analysis to evaluate their contribution to the discrimination between populations. The behavioural routines were included in the analyses using the stepwise mode, with the Wilks' λ method to enter and remove the discriminant functions ($F = 0.05$ and $F = 0.10$, respectively).

The statistical analyses were performed using free R studio software, except the discriminant analyses which were executed on STATISTICA 7.1 software (Stat soft. 1984–2005).

Results

Temporal patterns of pheromone emission

The GLM analysed was significant, demonstrating differences in the pattern of pheromone emission across morphotypes (table 1). The initiation, ending and peak calling time varied among the morphotypes (fig. 1). Males from Vacaria, Tucumán and Piracicaba (morphotypes Brazil-1) start calling behaviour at the start of photophase and continued emitting pheromones for at least 5 h. There were also small within morphotype differences: Vacaria, Tucumán and Piracicaba males all displayed peak calling early in the day, but Vacaria males called at a relatively high level until noon, whereas calling by Tucumán, and Piracicaba decreased steadily through the morning. Males from Peru initiated pheromone emission behaviour 4 h after the onset of the lights and called at high levels over the rest of the day. This population showed the greatest mean abundance of calling in the morning. Colombia males initiated pheromone emission at the beginning of the afternoon (13:00 h) and showed a steady increase in calling behaviour until late afternoon (17:00) followed by a reduction of activity.

Male courtship sequences

Populations differed significantly in the frequency of male courtship behavioural units: some units were more frequent, while others were less frequent, or even non-existent in certain populations (fig. 2). *Mobile* (MO) was the most frequent behaviour in Colombia and Peru populations; within the Brazil-1 morphotype, it was more frequent for Vacaria than either Piracicaba or Tucumán ($\chi^2 = 103.39$; $P < 0.0001$). *Calling* (CALL) ($\chi^2 = 91.753$; $P < 0.0001$) and *Flying* (FL) ($\chi^2 = 217.24$; $P < 0.0001$) were also more frequent in the Colombia and Peru populations, while Tucumán showed the lowest levels. *Transversal* with pheromone emission (TR-call) was most frequent in Colombia, followed by Peru ($\chi^2 = 67.92$; $P < 0.0001$). *Spin* (SP-call) ($\chi^2 = 233.12$; $P < 0.0001$), *Alignment* (AL) ($\chi^2 = 85.43$; $P < 0.0001$), *Arrowhead-2* (AH2-call) ($\chi^2 = 65.03$; $P < 0.0001$) and *Contact* (CO) ($\chi^2 = 18.531$; $P = 0.0009$) were

Table 1. Generalized linear autologistic model of the data of the time of pheromone emission using Vacaria as reference population.

Population	Estimator	SE	Z	P
Colombia	0.993	0.101	9.859	<0.001*
Peru	0.774	0.107	7.242	<0.001*
Piracicaba	0.118	0.116	1.025	0.305
Tucumán	-0.225	0.129	-1.749	0.080
AutoCov (time)	0.252	0.010	25.168	<0.001*

*Significant differences.

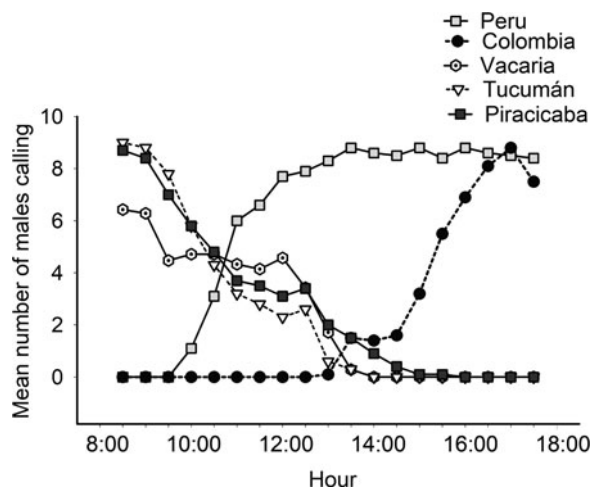


Fig. 1. Pattern of calling behaviour in *Anastrepha fraterculus* populations from Tucumán, Piracicaba and Vacaria (Brazil-1 morphotype), Peru and Colombia ($P < 0.05$, generalized linear autologistic model – GLM). The standard deviation can be seen in Appendix A (Supplementary data).

less frequent in Colombia and Peru. *Grooming* (GR) was less frequent in Piracicaba and Tucumán ($\chi^2 = 66.93$; $P < 0.0001$). *Hanation* with pheromone emission (HA-call) ($\chi^2 = 138.77$; $P < 0.0001$), *Arrowhead-1* (AH1-call) ($\chi^2 = 122.11$; $P < 0.0001$) and *Abdominal movement* with pheromone emission (AB-call) ($\chi^2 = 24.89$; $P < 0.0001$) were less frequent, while *Marking leaf* (ML) was more frequent ($\chi^2 = 9.73$; $P = 0.045$) in Colombia. *Enantion* with emission of pheromones (EN-call) was less frequent in Piracicaba ($\chi^2 = 56.86$; $P < 0.0001$). *Attempt* (AT) ($\chi^2 = 91.75$; $P < 0.0001$), *Enantion* (EN) ($\chi^2 = 144.7$; $P < 0.0001$), *Abdominal movement* (AB) ($\chi^2 = 16.55$; $P = 0.002359$), *Oscillation* (OC) ($\chi^2 = 18.03$; $P = 0.0012$) and *Stationary* (ST) ($\chi^2 = 105.4$; $P < 0.0001$) were more frequent, while *Fanning* (FA-call) was less frequent ($\chi^2 = 126.54$; $P < 0.0001$) in Peru. For all comparisons, the degrees of freedom were 4.

Of the 14 highly frequent behavioural routines included in the analysis, nine entered in the final discriminant analysis model (Wilks' $\lambda = 0.065$; $F_{36,159} = 4.74$; $P < 0.001$). AH1-call>AL, CALL>FA-call, VO>CALL, SP-call>FA-call and EN-call>MO were the routines that most often contributed to the discrimination between the populations (table 2). The scatter plot indicated three separate groups, with the first being formed by the Vacaria, Tucumán and Piracicaba populations, the second by Colombia and the third by Peru (fig. 3 and table 3). The centroids of Vacaria, Tucumán and Piracicaba, all of them populations of the morphotype

Brazil-1, were close to each other, while the morphotypes themselves were distant from each other in the discriminant space (table 4).

The probabilistic trees generated (TGS) by EthoSeq indicated distinct behavioural paths to mate among the populations (fig. 4). In the Colombia population (fig. 4a), nine behavioural units preceded *Attempt* (AT) and the units with the highest frequencies were *Flying* (FL) (22.7%), *Mobile* (MO) (18.8%), *Contact* (CO) and *Alignment* (AL) (13.64%). In the Peru population (fig. 4b), six behavioural units preceded *Attempt* (AT) and the units with the highest frequencies were *Flying* (FL) (48.9%), *Arrowhead-1* (AH1-call) (25.53%) and *Calling* (CALL) (10.92%). The three Brazil-1 populations (Piracicaba, Tucumán and Vacaria) showed the same units with the highest frequencies: *Contact*, *Alignment* and *Arrowhead-1*. The organization of these three units performed by Brazil-1 populations in the TGS was variable; also there were other units exclusive to one or another of these populations (figs 4c–e).

Discussion

In the present study, we found differences on the time of pheromone emission (fig. 1) and the sequence of courtship behaviours (table 3, fig. 4) between Brazil-1, Peruvian and Andean morphotypes but the trend of both parameters assessed have shown similarity within populations of the same morphotype (Brazil-1). This implies that differences in mating behaviours are relevant parameters for distinguishing lineages within the *A. fraterculus* cryptic species complex.

Thus, courtship difference could explain previously reported mating incompatibility within this cryptic species complex (Vera *et al.*, 2006; Cáceres *et al.*, 2009; Devescovi *et al.*, 2014). Our results make evident that male courtship behavioural units assessed serve to attract the female of the same population or morphotype, allowing the female to choose a potential partner (Aluja *et al.*, 1999; Gomez Cendra *et al.*, 2011). The reproductive incompatibilities found between the Brazil-1 morphotype and the populations from Colombia and Peru (Devescovi *et al.*, 2014) and between the Brazil-1 and Peruvian morphotypes (Cáceres *et al.*, 2009; Rull *et al.*, 2013; Devescovi *et al.*, 2014) may be consequence of this distinct courtship behaviour. Other pre-zygotic factors, such as variations in pheromone composition (Lima *et al.*, 2001; Cáceres *et al.*, 2009) and cuticle hydrocarbons (Vaničková *et al.*, 2014, 2015b, c), are probably also involved in conspecific recognition within this at cryptic species complex.

The differences in the time of pheromone emission between morphotypes is directly associated with differences in their mating times, as pheromone emission necessarily precedes mating (Vera *et al.*, 2006; Cáceres *et al.*, 2009; Segura *et al.*, 2011; Devescovi *et al.*, 2014; Dias *et al.*, 2015). Based on the time of pheromone emission, we confirmed the occurrence of time-based isolation in terms of mating activity between the Brazil-1 morphotype and the Andean morphotype (Vera *et al.*, 2006; Devescovi *et al.*, 2014). Also, we proved that there are overlapping periods of pheromone emission between Peru and the remaining populations. The lack of complete time-based isolation and the mating incompatibilities detected previously (Vera *et al.*, 2006; Cáceres *et al.*, 2009; Devescovi *et al.*, 2014), particularly between the Brazil-1 and Peruvian morphotypes, indicate that pheromone emission is not the only pre-zygotic factor to influence reproductive isolation. In all, the pattern of pheromone emission was observed to be an

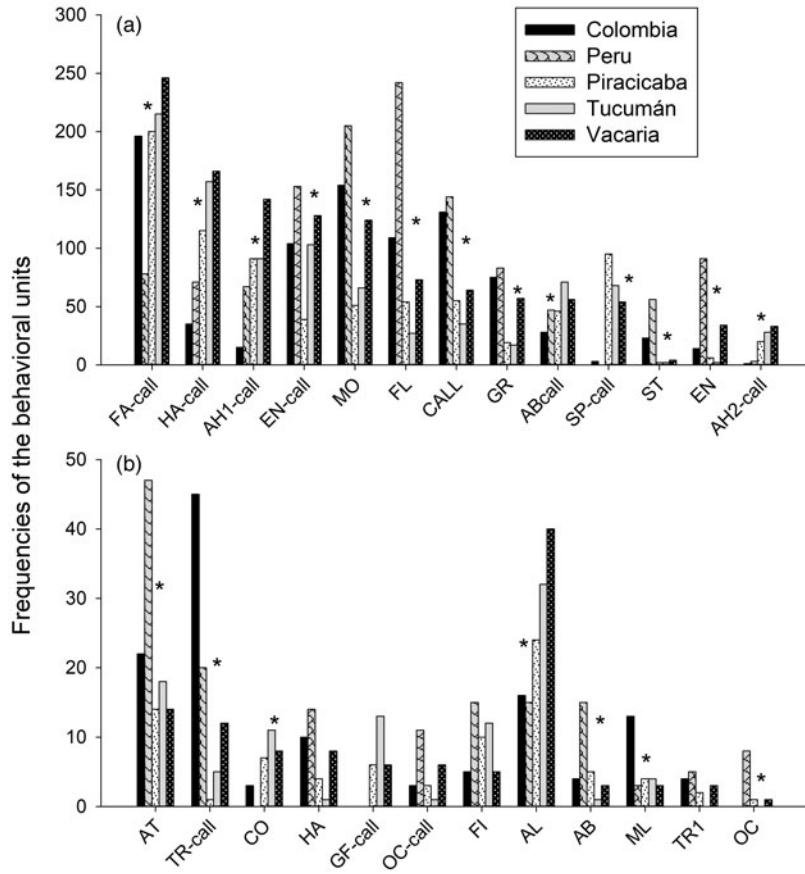


Fig. 2. Total frequencies of male courtship behavioural units in distinct *Anastrepha fraterculus* populations. Behavioural units with high (above 50) and low (below 50) frequencies are presented separately in plots a and b, respectively. Populations differ in the frequency of courtship units ($P < 0.05$, GLM).

Table 2. Frequencies of the 14 routines that participated in the discriminant analysis. The variables presented in light grey were included in the final model, while those in dark grey were not.

N = 55 Behavioural routines	Total frequency: TGS					Variables	
	Colombia	Peru	Piracicaba	Tucumán	Vacaria	Wilks' λ	P
FL > CALL	14	65	4	2	3	0.09	<0.001
CALL > FA-call	48	19	11	8	15	0.11	<0.001
AH1-call > AL	1	1	13	6	15	0.08	0.04
SP-call > FA-call	3	0	42	34	24	0.08	0.04
HA-call > AB-call	0	5	10	19	30	0.08	0.06
EN-call > HA-call	5	23	8	21	18	0.08	0.06
EN-call > MO	22	25	3	5	5	0.08	0.02
MO > EN	7	51	3	1	26	0.08	0.08
HA-call > AH1-call	0	5	10	19	30	0.08	0.1
FA-call > HA-call	11	8	41	45	59	0.06	0.5
FA-call > AH1-call	5	6	32	35	38	0.06	0.5
AH1-call > AH2-call	0	1	12	19	22	0.06	0.83
SP-call > HA-call	0	0	15	21	14	0.06	0.7
Ab-call > MO	11	8	2	6	1	0.06	0.68

important factor for classifying the biological lineages of *A. fraterculus*, but should be not considered as the unique factor involved in the reproductive isolation found between morphotypes.

The frequency of some behavioural units showed some peculiarities. The unit *Spin* (SP-call) was infrequent in the Peruvian and Colombian populations, and more frequent in the Vacaria, Tucumán and Piracicaba (Brazil-1) populations

Table 3. Discriminant functions, means of variables in the groups.

Group	Means of canonical variables			
	Root 1	Root 2	Root 3	Root 4
Colombia	1.72	1.92	-0.17	0.01
Peru	1.93	-1.61	0.22	0.06
Piracicaba	-1.61	0.26	0.56	0.67
Tucumán	-1.21	0.13	0.59	-0.72
Vacaria	-1.16	-0.47	-1.02	-0.03

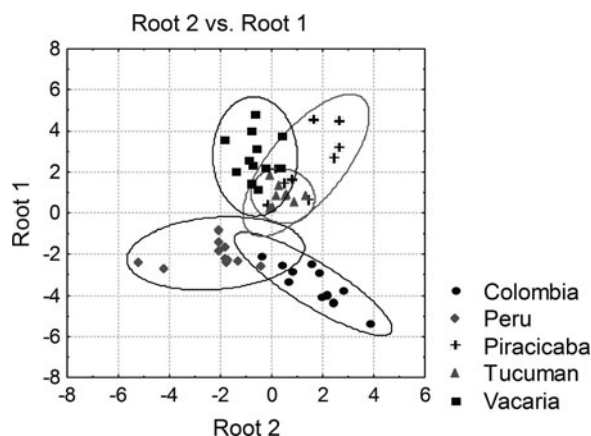


Fig. 3. Populations plotted within the discriminant function scatterplot obtained from behavioural routines. Ellipses confidence interval of 95%.

Table 4. Distances of Mahalanobis between the centroids of the populations obtained in the discriminant analysis.

Group	Colombia	Peru	Piracicaba	Tucumán	Vacaria
Colombia	0	12.62	14.83	12.94	14.72
Peru		0	16.47	13.63	12.37
Piracicaba			0	2.08	3.71
Tucumán				0	3.43
Vacaria					0

(fig. 2), thus seemingly representing a Brazil-1 courtship characteristic/peculiarity as reported previously (Gomez Cendra *et al.*, 2011; Passos Roriz *et al.*, 2018). Other behavioural units that allow morphotype distinction were the behavioural unit *Graceful* (GF-call), which was not observed in the Colombian and Peruvian populations and the behavioural unit *Flying* (FL), which presented high frequency in these two populations and low frequency in the remaining three populations. Behavioural units that were not observed in certain populations (such as *Oscillation* in Colombia and Tucumán and *Spin* in Peru) may reflect intrinsic characteristics of courting in those populations rather than lineage characteristics. Yet, this hypothesis needs confirmation.

Courtship routines allowed segregating the Peruvian and Colombian populations into two distinct groups, corroborating the observations of Devescovi *et al.* (2014) and Hernández-Ortiz *et al.* (2012). Furthermore, the courtship routines allowed/enable grouping the populations representing

the Brazil-1 morphotype (Vacaria, Piracicaba and Tucumán) (fig. 3). In contrast, other workers have reported reduced prezygotic compatibility between Piracicaba and Vacaria (Dias *et al.*, 2015) and between Piracicaba and Tucumán (Vera *et al.*, 2006) as well as morphometric differences between Piracicaba and Vacaria populations (Dias *et al.*, 2015). Although the present work did not identify strong differences among the three populations of the Brazil-1 morphotype, the behavioural routine MO>EN appears to differentiate Vacaria from the other two populations in the same way that the AH1-call>AL routine appears to separate Tucumán from the other two populations (table 2). Therefore, when one examines these populations in more detail, small differences can be seen between them. The probabilistic trees generated detected heterogeneous behavioural sequences among the populations. Nonetheless, the behavioural units that occurred most often in the behavioural probabilistic tree (TGS) of courtship that resulted in mating were the same in the biological lineages of this cryptic species complex. The behavioural units that occurred most in TGS of the Piracicaba, Vacaria and Tucumán (Brazil-1 morphotype) populations were the same (CO; AL; AH1-call), with some of the units being similar to those of the Colombia population (FL; MO; CO; AL) and with the Peru population (FL; AH1-call; CALL). In addition, the behavioural units *Contact* (CO) and *Alignment* (AL) were very important in both the Andean and the Brazil-1 morphotypes, where they were more frequent (fig. 2).

Contact (CO) and *Alignment* (AL) may present important functions in the courtship behaviour of *A. fraterculus*. In *Ceratitis capitata*, they were found to favour male mating success (Briceño & Eberhard, 2002; Briceño *et al.*, 2007) and are probably linked to female choice/evaluation during courtship. The behavioural unit *Arrowhead-1* (AH1-call), most often in populations belonging to the Brazil-1 and the Peruvian morphotype, may reflect the power of the male in a certain territory (Dodson, 1982). This behaviour unit has already been reported as important for *A. fraterculus* male courtship in a population from Argentina (Gomez Cendra *et al.*, 2011) and a population from Bento Gonçalves from Brazil (Passos-Roriz *et al.*, 2018), both belonging to the Brazil-1 morphotype. It is possible that the behavioural unit *Flying* (FL) most often in the Andean and Peruvian morphotypes has important function in fleeing from predators (Boller *et al.*, 1981; Cayol, 1999). During courtship, flying could be a sign of male aptitude, although it could also be an exploratory behaviour of the male to gain the attention of the female.

Despite the recent advances in describing the sexual behaviour and related traits of the different morphotypes, more studies are needed that link the analysed attributes of the morphotypes to different ecological factors. The eight morphotypes described were classified into three non-connected phenotypic lineages that evolved separately in three biogeographic regions: Meso-Caribbean, Andean and Brazilian (Hernández-Ortiz *et al.*, 2015). An important assumption is that the ecological niche of each of three lineages differed in terms of environment which could explain the different pheromone emission times.

In conclusion, in this work, it was possible to perceive that behavioural factors are distinct among the morphotypes, which likely reflects different selective pressures in the *A. fraterculus* cryptic species complex. Attributes of the males can be selected and remain in the population by female's preference (Andersson, 1994; Ritchie, 1996; Yamada *et al.*, 2008). Perhaps, female's preferences in this cryptic species complex

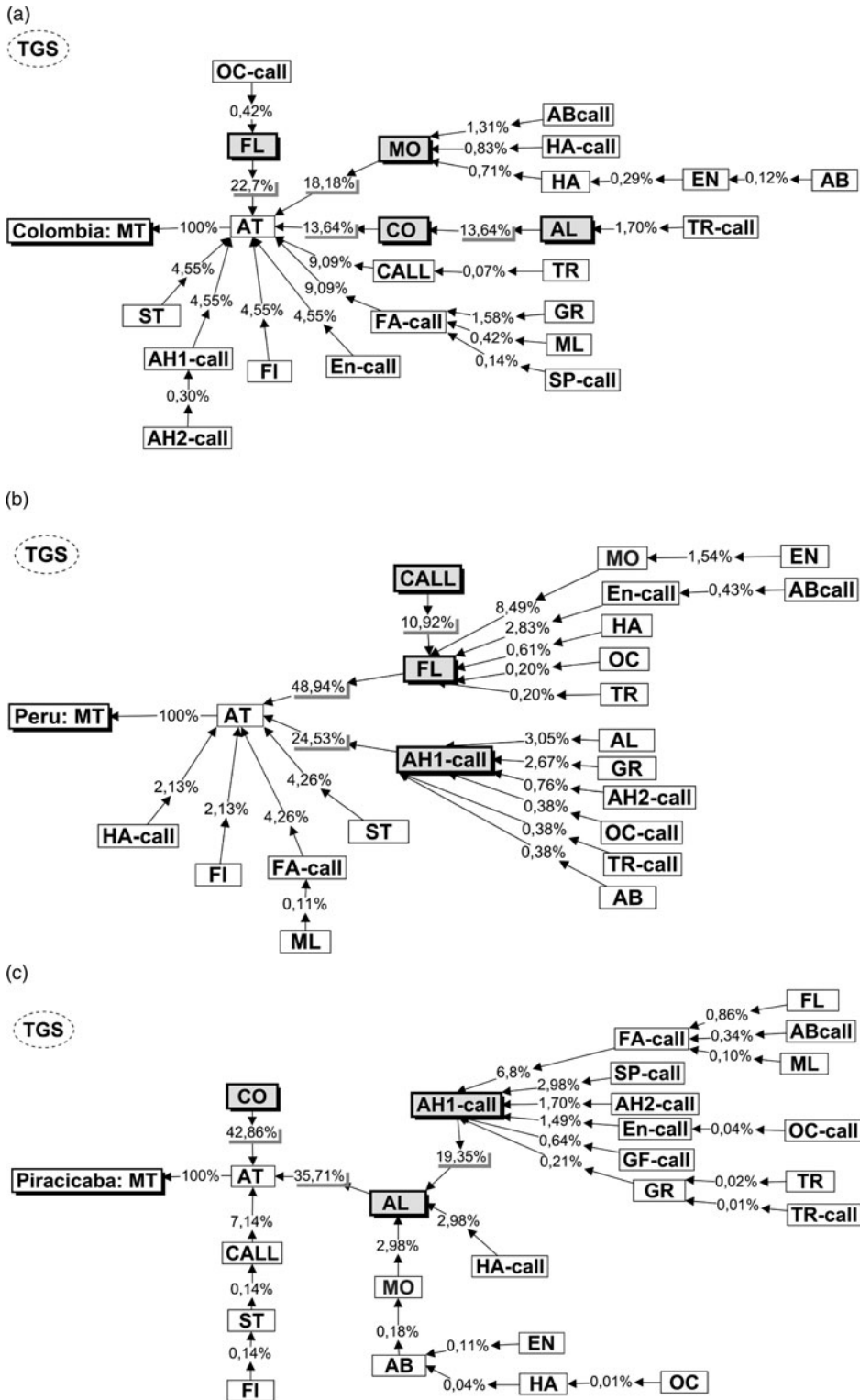


Fig. 4. Behavioural probabilistic tree of male courtship that resulted in mating (MT) in the populations (a) Colombia, (b) Peru, (c) Piracicaba, (d) Tucumán and (e) Vacaria. The arrows point to the next behavioural unit in sequence with the respective percentages of occurrence of each specific routine. The percentages are the probabilities of each path. The farther from the copulation (MT), the lower the percentages of occurrence of behavioural routines: the probability of FL/AT/MT is 22.7%, while that of OC-call/FL/AT/MT is 0.42% (a). The units highlighted were the most frequent ones in the tree generated sequences (TGS) of the courtship. The abbreviations of the behavioural units are described in the Appendix B.

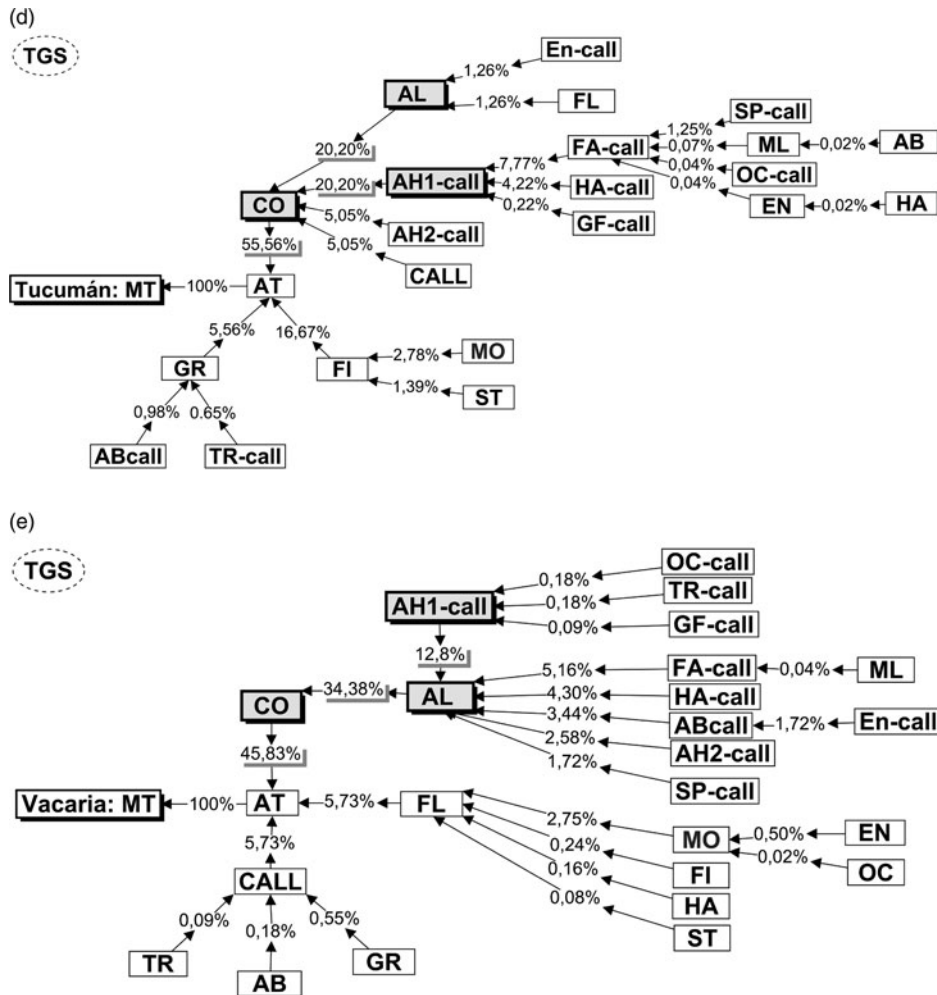


Fig. 4. Continued.

are directing the evolution of the courting behaviour specifically in each lineage. The results of this work indicate a tendency to behavioural isolation of courting among the analysed distinct lineages, and therefore abiotic factors that force to each lineage to have different courtship behaviour may be contributing to the evolution of the divergences between them. Studies that compare courtship behaviour in heterotypic mating in this cryptic species complex and analysis of ecological factors that drive behavioural trends will contribute to deepen our understanding of the ongoing speciation process.

Supplementary material

The supplementary material for this article can be found at <https://doi.org/10.1017/S0007485318000846>.

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