Geographic distribution of sex chromosome polymorphism in *Anastrepha fraterculus* sp. 1 from Argentina

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**Abstract**

**Background:** *Anastrepha fraterculus* is recognized as a quarantine pest in several American countries. This fruit fly species is native to the American continent and distributed throughout tropical and subtropical regions. It has been reported as a complex of cryptic species, and at least eight morphotypes have been described. Only one entity of this complex, formerly named *Anastrepha fraterculus* sp. 1, is present in Argentina. Previous cytogenetic studies on this morphotype described the presence of sex chromosome variation identified by chromosomal size and staining patterns. In this work, we expanded the cytological study of this morphotype by analyzing laboratory strains and wild populations to provide information about the frequency and geographic distribution of these sex chromosome variants. We analyzed the mitotic metaphases of individuals from four laboratory strains and five wild populations from the main fruit-producing areas of Argentina, including the northwest (Tucumán and La Rioja), northeast (Entre Ríos and Misiones), and center (Buenos Aires) of the country.

**Results:** In wild samples, we observed a high frequency of X1X1 (0.94) and X1Y5 (0.93) karyomorphs, whereas X1X2 and X1Y6 were exclusively found at a low frequency in Buenos Aires (0.07 and 0.13, respectively), Entre Ríos (0.16 and 0.14, respectively) and Tucumán (0.03 and 0.04, respectively). X2X2 and X2Y5 karyomorphs were not found in wild populations but were detected at a low frequency in laboratory strains. In fact, karyomorph frequencies differed between wild populations and laboratory strains. No significant differences among *A. fraterculus* wild populations were evidenced in either karyotypic or chromosomal frequencies. However, a significant correlation was observed between Y5 chromosomal frequency and latitude.

**Conclusions:** We discuss the importance of cytogenetics to understand the possible route of invasion and dispersion of this pest in Argentina and the evolutionary forces acting under laboratory conditions, possibly driving changes in the chromosomal frequencies. Our findings provide deep and integral genetic knowledge of this species, which has become of relevance to the characterization and selection of valuable *A. fraterculus* sp. 1 strains for mass rearing production and SIT implementation.

**Keywords:** Karyomorphs, Karyotypic polymorphism, Fruit fly pest, Dispersion patterns, Morphotypes, SIT
Background
The South American fruit fly, Anastrepha fraterculus Wiedemann (Diptera, Tephritidae), exhibits a broad geographic distribution in the American continent, ranging from 27° N to 35° S latitudes [1–5]. This pest has a wide range of host fruits, including wild and economically important plant species [5–7].

A. fraterculus constitutes a complex of cryptic species, with at least eight described morphotypes [8–11] and its putative center of origin is located in South America [12–14]. Integrative taxonomic studies have proposed a new perspective to study the members of A. fraterculus complex [15–19]. These studies have based their approaches on previous significant contributions, including the use of morphometry [9–11], cytogenetic analyses ([12, 20]; reviewed by Zacharopoulou et al. [21]), population genetics [12, 22–29], behavioral and physiological studies [30–35] and, pheromone and cuticle hydrocarbon composition analysis [36–38].

In Argentina, only one entity of this complex is present, formerly named Anastrepha fraterculus sp. 1 or Brazilian 1 morphotype [12, 20, 39]. This morphotype carries a karyotype composed of five pairs of acrocentric autosomes and a pair of sex chromosomes (2n = 12). Previous works performed in Argentinian wild populations described an occasional sex chromosome polymorphism ([40–42], reviewed by Cladera et al. [43]; Giardini et al. [44]). Particularly, these studies described the presence of five morphological variants of the X chromosome and four variants of the Y chromosome, with both types of polymorphism being detected at a low frequency [40–42]. Based on chromosomal size and staining patterns, later exhaustive studies have described cytotypes (or karyomorphs) composed of two variants of each sex chromosome (named X1, X2 and Y5, Y6) [45]. The X1 variant is a large submetacentric chromosome with two DAPI- positive bands located at each of its telomeres, the distal band being more prominent than the proximal one [20, 44–46]. The X2 variant is a large submetacentric chromosome with a DAPI- positive distal satellite. Its telomeric regions show the same DAPI staining patterns as the X1 chromosome [40, 41, 45, 47]. The Y5 is a small meta-submetacentric chromosome (40% shorter than X1) with an interstitial DAPI- positive region located in the long arm and a large DAPI- positive band in the short arm [44, 45]. The Y6 variant is a medium-size submetacentric chromosome 20% shorter than X1. This variant shows DAPI- positive bands in almost 50% of its length [45, 47]. It is worth noting that the karyomorphs identified in A. fraterculus sp. 1 from Argentina have shown cytological differences from those previously described for other members of the A. fraterculus complex [12, 20].

The existing partitioned information about the current distribution of A. fraterculus individuals carrying sex chromosomal variants of this morphotype, in conjunction with the uncertain taxonomic status of this species complex in America, carries important implications for the development of species- specific control strategies, such as the sterile insect technique (SIT) ([16, 17], reviewed in [13, 18]). In this context, cytogenetics plays a key role in the understanding of sex chromosome evolution and cryptic species resolution, and it is critical in the development and evaluation of SIT strategies (reviewed by Zacharopoulou et al. [21]).

In the present work, we studied the geographic distribution of sex chromosome variation in wild populations of A. fraterculus sp. 1 from Argentina and complemented this information by the analysis of laboratory strains in order to characterize chromosomal variants found at a low frequency. We discuss our results in the light of previous cytogenetic studies to understand the possible route of introduction and dispersion of this pest in Argentina. In addition, we propose some hypotheses about the possible origin of the sex chromosome variants detected so far in Argentinian populations of A. fraterculus. Our findings contribute to a better genetic knowledge of this species in the context of the identification of members in the A. fraterculus complex, thus providing tools to develop and apply environmentally safe control strategies against this fruit fly pest in Argentina and other South American countries.

Results
We analyzed 424 preparations of mitotic chromosomes of A. fraterculus (each made from the brain ganglia of an individual larva) and observed the presence of two size variants of X chromosome (X1 and X2; Fig. 1 a, b, and c and Fig. 2 a) and Y chromosome (Y5 and Y6; Fig. 1 d, e, and f and Fig. 2 a) in both, wild population and laboratory strain samples (Table 1; Additional File 1). In addition, no size polymorphism was detected in the autosomal complement.

Specifically, for wild population samples, La Rioja and Misiones ones showed only one of two mitotic karyomorphs (X1X1 and X1Y5 in females and males, respectively). Samples from Buenos Aires, Tucumán, and Entre Ríos showed the presence of four different karyomorphs (X1X1/ X1X2 and X1Y5/ X1Y6 in females and males, respectively) (Table 1; Fig. 3).

The presence of X2Y5 karyomorph was only observed in the laboratory strain Af-Cast-1, while X2X2 karyomorph was detected in two laboratory strains (Af-Cast-1 and Af-Cast-2 strains of A. fraterculus harboring different Wolbachia strains). X2Y6 was not found in any of the analyzed samples (Table 1).

No significant differences were found between observed and expected karyomorph frequencies in either wild populations or laboratory strains (Fisher’s Exact
Fig. 1 Sex chromosome karyomorphs detected in wild populations and laboratory strains of *A. fraterculus* sp. 1 from Argentina. a-e Cytological preparations of mitotic chromosomes stained with DAPI. a-c female metaphases, d-f male metaphases. Bar represents 10 μm.

Fig. 2 a. Schematic representation of sex chromosomes detected in wild and lab populations of *A. fraterculus*. Banding pattern corresponds to DAPI staining and C Bands. The line crossing all chromosome schemes shows the position of the centromere according to Giardini et al. [44]. b. Suggested chromosome rearrangements of X₁ and Y₅ to generate X₂ and Y₆, respectively.
Moreover, the analysis of chromosome incidence revealed homogeneity of X variant frequencies in both sexes in nature (Fisher’s Exact Test; \( p > 0.05 \) in all cases). Both results mentioned above agree with Hardy Weinberg Equilibrium within each population.

The presence of \( X_1X_1 \) and \( X_1Y_5 \) karyomorphs were observed at a high frequency in all wild populations (mean frequency values: 0.94 and 0.93, respectively) (Table 1). The analysis of geographic chromosome variation revealed that there were no significant differences in either \( X \) or \( Y \) variant frequencies among wild populations (Fisher’s Exact Test; \( p > 0.05; p = 0.34, p = 0.42 \), respectively). Additionally, non-significant differences were found in female karyomorph frequency among wild populations (Fisher’s Exact Test; \( p = 0.2847 \)).

The correlation analysis between chromosome frequencies from *A. fraterculus* wild populations and geographic variables (latitude and longitude) showed a significant and negative association between \( Y_5 \) frequency and latitude (Pearson’s Correlation; \( r = 0.88; p = 0.0498 \)). Conversely, \( Y_6 \) frequency increased with the latitude (Fig. 3).

The cytogenetic characterization of laboratory strains indicated some differences with respect to wild populations. After the analysis of 94 mitotic chromosome preparations (57 females and 37 males) from the Af-IGEAF strain, significantly lower frequencies of \( X_1X_1 \) (0.72) and \( X_1Y_5 \) (0.78) and higher frequencies of \( X_1X_2 \) (0.28) and \( X_1Y_6 \) (0.22) were observed with respect to wild samples (Table 1). In fact, Fisher’s Exact Test revealed that Af-IGEAF strain exhibited significant differences in \( X \) variants (\( p = 0.0034 \)) compared to its source wild population (Tucumán). The differences in \( Y \) variants between these samples were marginally significant (\( p = 0.06 \)) (Additional File 1).

In the Af-Y-short strain (purified *A. fraterculus* strain harboring \( Y_5 \) chromosome), 96% of females carried \( X_1X_1 \) and 4% carried \( X_1X_2 \) karyomorphs while 100% of males showed \( X_1Y_5 \), as expected for this line (Table 1). A significant increase in the frequency of the \( X_1 \) variant was verified in Af-Y-short strain in comparison with in relation to Af-IGEAF strain (Fisher’s Exact Test; \( p = 0.0004 \)) (Additional File 1).

Af-Cast-1 and Af-Cast-2 strains showed a differential distribution of karyomorphs (Table 1). For Af-Cast-1 strain, we detected the presence of \( X_1X_1 \) (76.5%), \( X_2X_2 \) (17.6%), and \( X_1X_2 \) (5.9%) in females and \( X_1Y_5 \) (73%) and \( X_2Y_5 \) (27%) in males (Table 1). For Af-Cast-2 strain, we detected the female karyomorphs \( X_1X_1 \) (93%) and \( X_2X_2 \) (7%), and no heterozygous females (\( X_1X_2 \)) were observed. Concerning male chromosome combinations, we observed 100% of \( X_1Y_5 \). In addition, the mentioned strains differed significantly in their \( X \) variant frequencies (Fisher’s Exact Test; \( p = 0.0328 \)) (Additional File 1).

### Discussion
In the present work, we studied the frequency and distribution of sex chromosome variants found in laboratory colonies and wild populations of *A. fraterculus* sp. 1 from different regions of Argentina by analyzing mitotic chromosome preparations.

The cytogenetic characterization of *A. fraterculus* sp. 1 wild populations located in different eco-climatic regions representing the main fruit-producing areas of Argentina allowed us to identify four sex chromosome cytotypes.
(or karyomorphs) \(X_1X_1/ Y_1X_1, X_2X_2/ Y_2\) and the absence of individuals harboring \(X_2X_2, Y_5, \) and \(X_2Y_6\) karyomorphs. These techniques were not useful in detecting chromosomal variation in the autosomes of the analyzed populations. Our results were slightly different from those previously reported by Lifschitz et al. [40], Manso and Basso [41], Basso et al. [42], and more recently by Basso et al. [48, 49]. These studies described the presence of several variants of \(X\) \((X_1, X_2, X_3, X_4)\) and \(Y\) \((Y_1, Y_2, Y_3, Y_4, Y_5, Y_6)\) chromosomes in \(A.\) *fraterculus*.
from Argentina. However, this variation was not observed in the extensive sampling of wild populations performed for the present work.

Concerning the karyomorph characterization of established laboratory colonies, we observed that Af-IGEAF laboratory strain showed significant differences in the distribution of chromosomal combinations compared to the current frequency of its founding wild population (Tucumán). This could be the consequence of stochastic and/or artificial selection effects driving changes in the chromosome and karyotypic frequencies. Similar processes were previously described for this species during the laboratory adaptation [50] and also observed in other Tephritidae species [51, 52]. Indeed, the other three laboratory strains analyzed here showed biased frequencies of chromosomal variants, as expected for these types of laboratory colonies, founded from Af-IGEAF strain with specific purposes and, using less than 50 parental crosses. In Af-Cast-1 and Af-Cast-2 strains (A. fraterculus colonies harboring different Wolbachia strains), we observed the presence of karyomorphs absent in wild populations (X2X2/ X2Y5). It is worth noting that X2Y6 was not observed in any of the colonies or wild populations analyzed, mainly explained by the low chromosomal frequency of Yp detected in them. However, these less frequent or absent karyomorphs in adult individuals and possible chromosome incompatibilities associated to the presence of Wolbachia need further analyses of paired-crosses, including parameters such as fecundity and larval survival as were previously evaluated in other insect species [53–56].

The analysis of both chromosome and karyomorph frequencies registered for wild A. fraterculus populations showed no differences among the studied localities but evidenced a significant trend of a differential distribution of the chromosome frequencies. In particular, a negative correlation was observed for the Y5 distribution according to latitude. The information available with respect to the distribution of A. fraterculus morphotypes in South America and the cytological studies previously performed, in conjunction with the results described here, can be of help to put forward some hypotheses about the introduction and dispersion of A. fraterculus sp. 1 in the Argentine territory. Recent studies proposed a possible non-monophyletic origin of A. fraterculus in South America. The expansion of this species to different regions of the South American subcontinent may have initiated by two unconnected routes of invasion: One arm extended along the western edge, including both highland and lowland areas of the Andean region, and the other along the eastern Brazilian coast [12–14]. In this sense, we consider that A. fraterculus Brazilian 1 morphotype could have entered Argentina through the northeast (Misiones) from Brazil. This movement is expected for this A. fraterculus morphotype, due to the geographic proximity, and it is evidenced by a conserved karyomorph (previously described by Selivon et al. [12] and Goday et al. [20] for A. fraterculus from Brazil and by Manso and Basso [41] for A. fraterculus from Argentina). Another probable route of invasion is through the northwest of the country (Jujuy-Tucumán) by the Peruvian A. fraterculus. The Peruvian karyotype was first described by Cáceres et al. [15] and is similar to that previously described for the Ecuatorian morphotype [20]. The cytological analysis of the Peruvian morphotype showed sex chromosomes of similar length, designated Xp and Yp. The Xp chromosome has a prominent interstitial heterochromatic block, whereas the Yp chromosome has a DAPI-positive block located at the centromeric region of the chromosome [15].

In our analysis of 173 A. fraterculus individuals belonging to Argentinian wild populations, we did not observe karyomorphs similar to those described for the Peruvian morphotype. Furthermore, the currently available information does not provide enough cytogenetic evidence to describe possible hybridization events between Brazilian 1 and Peruvian morphotypes, like those previously described by Selivon et al. [12, 57] and Cáceres et al. [15] through laboratory-controlled crosses. Although the results shown here support the assumption of a unique origin of this A. fraterculus sp. 1 in Argentina, further cytogenetic analysis (including populations from Brazil and western South American countries) in conjunction with genetic and morphological studies could contribute to our knowledge about possible routes of invasion of this pest in Argentina.

Another key point we address here is the potential source of the sex chromosome polymorphism detected in A. fraterculus from Argentina. We propose an explanation for the generation of less frequent X2 and Y6 variants as possibly caused by modifications in X1 and Y5 chromosomes, respectively. These sex chromosome variants were previously described as forming the unique karyomorph of A. fraterculus sp. 1 (X1Y5) [12, 20]. The X2 chromosome could be derived from X1 by a duplication of the proximal heterochromatic block followed by a chromosome breakage and a subsequent cohesion to the distal telomeric region, giving rise to the X2 heterochromatic satellite (Fig. 2b). This hypothesis is supported by previous studies on chromosome behavior during cell division [58, 59]. Throughout this cell process, centrocomers adopt a complex structure that makes them susceptible to be the site of chromosome rearrangements, as reviewed by Barra and Fachinetti [60]. These authors support the hypothesis that the most probable chromosome site to suffer duplication and/or breakage to form the X2 satellite is the proximal and pericentromeric zone of the X1 chromosome. On the other hand, the Y6 variant could be derived from Y5 by duplication and
expansion of the larger heterochromatic block (Fig. 2b). Previous studies described the behavior of constitutive heterochromatin as dynamically regulated [61]. In addition, transitions between both types of chromatin (euchromatin and heterochromatin) were previously described for telomeric heterochromatin and satellite DNA in *Drosophila* [62], supporting our hypothesis of interstitial heterochromatin expansion to form the Y₅ variant.

No further information regarding this type of intramorphotype variation has been reported in other members of this species complex so far. Future studies using integrated standard cytogenetic techniques, FISH (fluorescence in situ hybridization), CGH (comparative genomic hybridization), mapping of major ribosomal RNAs (rRNAs), and H3 histone genes will contribute to understand the nature of this variation and the chromosomal evolution of this morphotype. These techniques could also be useful to analyze the role of the detected polymorphism on the speciation process of *A. fraterculus* and the dispersion patterns of cryptic species in America.

Cytogenetics has played an essential role in integrative taxonomic studies that clarify relationships between closely related species and/or incipient speciation phenomena [21, 63, 64] and has been used in the development and application of SIT for major Tephritidae species [reviewed in 21]. In particular, the knowledge of mitotic and polytene chromosomes has been applied to the construction and characterization of classical genetic sexing strains [65–67]. In addition, the chromosome characterization has significantly contributed to recent genome projects of tephritid pest species and made it possible to identify the linkage groups facilitating genome assemblies [68, 69].

**Conclusions**

This study provides relevant information about the sex chromosome polymorphism in *A. fraterculus* sp. 1 from Argentina and describes possible routes of invasion and dispersion of this pest species in the territory. Although previous studies have not reported intramorphotype variation at the chromosomal level in other members of the *A. fraterculus* complex so far, we consider that a deeper cytogenetic analysis of these wild populations, including mitotic and polytene chromosomes analyses, will greatly contribute to shedding light on the origin and evolution of this complex. Moreover, the establishment of standardized protocols of integrative taxonomy for this cryptic species complex may allow the univocal identification of species and, therefore, the development of specific control strategies at the regional level. Detailed activities performed following the same guidelines in different laboratories of South America, organized in a common database and including multidisciplinary studies (e.g., morphometry, cytogenetics, phylogenetic, ecological and behavioral parameters, eco-chemistry, and genetics), in conjunction with the study of reproductive symbionts, seem to be the best strategy to address the complexity of the *A. fraterculus* complex.

**Methods**

**Insects**

Wild *A. fraterculus* individuals (larvae) were obtained from infested fruit species available in each sampling site, distributed in different eco-climatic regions and representing the fruit-producing area of Argentina (Table 1; Fig. 3). The fruit was collected during three consecutive fruiting seasons (2016–2018). The sampling sites, ordered by geographic coordinates were as follows: Montecarlo, Misiones ([26°33′58.32″ S 54°45′25.2″ W]; fruit species sampled: guava *Psidium guajava*); Horco Molle, Tucumán ([26°49′0″ S 65°19′0″ W]; fruit species sampled: peach *Prunus persica* and guava); San Blas de los Sauces, La Rioja ([28°24′37.84″ S 67°5′36.28″ W]; fruit species sampled: peach and plum *Prunus domestica*); Concordia, Entre Ríos ([31°23′34.66″ S 58°1′15.2″ W]; fruit species sampled: peach and guava); Hurplingham, Buenos Aires ([34°35′17.92″ S 58°38′20.58″ W]; fruit species sampled: peach and plum). The infested fruits were kept at a quarantine room with controlled conditions of temperature and relative humidity (25 ± 1 °C and 70 ± 10%) until *A. fraterculus* 3rd-instar larvae were recovered. The species identification was based on morphological characteristics (shape and number of tubules) of anterior spiracles, according to Frias et al. [70].

**Laboratory strains**

Immature stages of *A. fraterculus* from the following laboratory strains were included in the cytological analysis.

**Af-IGEAF strain**

This colony (named afterward Af IGEAF) was established in 2007 with approximately 10,000 pupae from the semi-mass rearing colony kept at Estación Experimental Agroindustrial Obispo Colombres, San Miguel de Tucumán, Tucumán, Argentina [71] and maintained to date (120 generations) under artificial rearing.

**Af-Y-short strain**

This strain was purified from the Af IGEAF strain and it harbors Y₅ chromosome (the shortest Y chromosome reported for this species). This colony was founded after the screening of 25 families, originally composed of one parental male and three females. After analyzing all the families, we pooled those with the Y₅ chromosome. This strain was maintained for 70 generations under laboratory conditions.
Af-Cast-1 and Af-Cast-2 strains
These two A. fraterculus lines were also purified from the A. fraterculus IGEAF strain, considering the Wolbachia strain they harbor (wAfraCast1_A and wAfraCast2_A, respectively) [72]. Each strain was maintained for 70 generations under laboratory conditions.

Preparations and staining of mitotic chromosomes
We followed the cytological technique described by Guest and Hsu [73] with minor modifications. Briefly, cerebral ganglia of A. fraterculus 3rd-instar larvae were dissected in Ringer solution and incubated in hypotonic solution (1% sodium citrate) for 10–15 min. The material was fixed for 1 min in freshly prepared fixative (methylene-acetic acid, 3:1) and then homogenized in 60% (v/v) acetic acid with a micropipette. For each preparation, the homogenized suspension was dropped onto a clean slide, which was placed on a hot plate to allow the tissue to spread, and then, air-dried. After drying, the preparations were immersed in DAPI solution (50 ng/ml in 2x SSC) for 5–7 min. Slides were mounted in antifade and observed under an Olympus BX40 (Olympus, Tokyo, Japan) microscope at 1000X magnification.

Data analysis
Analyses of chromosome and karyomorph frequencies among wild populations or laboratory strains were performed using Fisher’s Exact Test. Hardy Weinberg Equilibrium (HWE) for X chromosome variants, is characterized by both homogeneity of variant frequencies between sexes and Hardy Weinberg proportions in females [74]. We verified HWE deviations through Fisher’s Exact Tests by comparing both i) X chromosome variant frequencies between males and females and ii) observed and expected karyomorph frequencies in females. Fisher’s Exact Tests with p-value computed based on the network developed by Mehta and Patel [75] were implemented in the R package [76]. The relationship between chromosome variant frequencies and geographic variables (latitude and longitude) in wild populations was assessed through the analysis of Pearson’s correlation coefficient in Infostat Professional version 2014 [77].

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12863-020-00944-1.

Additional file 1. Relative frequency of sex chromosome variants detected in wild and laboratory strains of A. fraterculus sp. 1 from Argentina.

Abbreviations
CGH: Comparative genomic hybridization; DAPI: 4′,6-diamidino-2-phenylindole; FISH: Fluorescence in situ hybridization; HWE: Hardy-Weinberg equilibrium; min: Minutes; N: North; rRNA: Ribosomal RNA; S: South; SIT: Sterile Insect Technique; sp: Species; W: Western

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Authors’ contributions
MCG, JLC and SBL conceived the study. CAC and FHM helped with the maintenance of A. fraterculus laboratory strains and provided individuals for cytogenetic analysis. MES, MSF and MCGs were in charge of infested fruit sampling. MCG and MN conducted cytological assays. MIR conducted the statistical analysis. ACS helped in the acquisition, analysis and interpretation of data. MCG, ACS, MN, JLC and SBL drafted the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The wild material described in this work was obtained from infested fruit collections as it was mentioned in the Methods section. The laboratory lines studied were from the Laboratorio de Insectos de Importancia Agronómica, Instituto de Genética (INTA) Buenos Aires, Argentina.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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