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Evolutionary pathways in the Anastrepha fraterculus species complex revealed by karyotype variation and chromosomal differentiation: Implications for genetic pest management

María Cecilia Giardini

giardini.maria@inta.gob.ar

Instituto Nacional de Tecnología Agropecuaria

Mariela Nieves

Unidad Ejecutora CONICET

Elena Cancio-Martinez

Insect Pest Control Section, Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture,

Fabián H. Milla

Instituto Nacional de Tecnología Agropecuaria

M. Florencia Fourastié

Universidad de Buenos Aires

Graciela González

Universidad de Buenos Aires

M. Teresa Vera

Insect Pest Control Section, Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture,

Kostas Bourtzis

Insect Pest Control Section, Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture,

Silvia B. Lanzavecchia

Instituto Nacional de Tecnología Agropecuaria

Research Article

Keywords: Anastrepha fraterculus complex, comparative cytogenetics, sex chromosome polymorphism, heterochromatin variation, karyotype evolution

Posted Date: November 26th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11702017/v1

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Additional Declarations: No competing interests reported.

Abstract

Anastrepha fraterculus is a major fruit fly pest in the Neotropics and constitutes a complex of cryptic species. The eight recognized morphotypes differ in morphological, behavioural, genetic, and cytogenetic traits. Understanding evolutionary relationships and chromosomal diversity is important for pest management. Although all members share a conserved chromosome number (2n = 12), they exhibit considerable sex chromosome variability. Here, we performed a comparative cytogenetic analysis of laboratory colonies representing *A. fraterculus* morphotypes and the related species *A. ludens* and *A. obliqua*. Mitotic chromosomes were analyzed using sequential DAPI/CMA_3 staining. Autosomes were conserved, whereas the sex chromosomes differed in heterochromatin composition and distribution, with the Y chromosome showing marked variation in size and morphology. We provided here the first cytogenetic characterization of the Andean morphotype from Colombia and the Ecuadorian morphotype from the Peruvian highlands, identified an unrecognized karyotypic variant from Piracicaba, Brazil, and confirmed the karyotypes described for the Brazilian 1 morphotype and the Peruvian morphotype from the Peruvian and Ecuadorian lowlands. Comparative analyses performed in this study suggest that amplification, loss, and redistribution of heterochromatin may have contributed to sex chromosome diversification. We propose a hypothetical model in which a Piracicaba-like karyotype represents a putative ancestral condition, from which extant morphotypes may have diversified through two possible heterochromatin-remodelling trajectories. These findings provide a first evolutionary framework for karyotype diversification within the *A. fraterculus* complex, highlight the role of sex-chromosome evolution, and provide a cytogenetic foundation for species delimitation and genetic monitoring supporting control strategies for this important pest.

Key message

• Sex chromosome variation distinguishes morphotypes within the complex.

• New cytogenetic data reveal previously uncharacterized morphotypes and a karyotypic variant from Brazil.

• Heterochromatin remodeling may have contributed to sex chromosome diversification.

• Comparative cytogenetics provides a foundation for evolutionary studies and genetic monitoring to support sustainable pest management.

1. Introduction

The South American fruit fly, *Anastrepha fraterculus* (Diptera: Tephritidae), is one of the most economically important tephritid pests in the Neotropics, occurring from Mexico to northern and central Argentina (Norrbom et al. 1999). It is recognized as a cryptic species complex comprising at least eight morphotypes that differ in their geographic distribution, morphology, behavior and genetics (Hernandez-Ortiz and Aluja 1993; Hendrichs et al. 2015). These include the “Mexican” (Mexico, Guatemala, and Panama) (Hernández-Ortiz et al. 2004), “Venezuelan” (lowlands of Venezuela) (Steck 1991), “Andean” (highlands of Venezuela and Colombia) (Steck 1991), “Peruvian” (lowlands of Peru and Ecuador-*A*. sp.4) (Selivon et al. 2002), “Ecuadorian” (highlands of Peru and Ecuador) morphotypes (Hendrichs et al. 2015), and three Brazilian morphotypes: “Brazilian-1” (Brazil and Argentina), “Brazilian-2”, and “Brazilian-3”, corresponding to *A.f* sp.1, *A. f* sp.2 and *A. f* sp.3, respectively (Yamada and Selivon 2001; Selivon et al. 2002; 2005a, b). Initially distinguished through morphometric analyses (Hernández-Ortiz et al. 2004), these morphotypes have received additional support from behavioural (Segura et al. 2011; Roriz et al., 2018; Belliard et al. 2025), genetic (Cáceres et al. 2009; Prezotto et al. 2019; de Brito et al. 2025), and cytogenetic studies (Selivon et al. 2005b, a; Gariou-Papalexiou et al. 2016). Phylogeographic analyses have further revealed historical population expansions and geographic structuring during diversification in southern South America, including east-west differentiation among Brazilian populations (Vilardi et al. 2021; Congrains et al. 2023).

Cytogenetic analyses have provided valuable insights into genome organization and chromosomal evolution within the genus *Anastrepha* (Bush 1962; Solferini and Morgante 1987; Selivon et al. 2002, 2005a, b; Goday et al. 2006). Most species examined share a conserved diploid chromosome number (2n = 12), comprising five pairs of autosomes and one pair of sex chromosomes (Bush 1962; Solferini and Morgante 1987; Selivon et al. 2002, 2005 a, b; Goday et al. 2006). Within the *A. fraterculus* complex, the

autosomes are consistently acrocentric and highly conserved, whereas the sex chromosomes vary in morphology, relative size, and heterochromatin composition and distribution (Selivon et al. 2005, 2022; Goday et al. 2006; Giardini et al. 2015, 2025).

Heterochromatin variation, particularly on the sex chromosomes, have been proposed as a major driver of chromosomal differentiation within the complex (Goday et al. 2006; Giardini et al. 2015, 2025; Selivon et al. 2022). Differences in heterochromatin organization may reflect distinct evolutionary trajectories among morphotypes and populations and could be associated with genomic divergence and reproductive isolation (Hernández-Ortiz et al. 2012; Hendrichs et al. 2015). Chromosome banding techniques, including C-banding and DAPI/CMA_3 fluorochrome staining, allow the characterization of heterochromatin and the distinction of AT-rich and GC-rich regions, respectively (Selivon and Perondini 1997; Lifschitz et al. 1999; Goday et al. 2006). These methods have revealed variation in sex chromosome morphology and heterochromatin composition in the *A. fraterculus* complex and related species, providing cytogenetic characters for comparisons among taxa and for investigating their evolutionary relationships comparative information on patterns of sex differentiation within the genus (Bush 1962; Solferini and Morgante 1987; Selivon et al. 2002, 2005 a, b, 2022; Goday et al. 2006; Gariou-Papalexiou et al. 2016; Giardini et al. 2025).

Nevertheless, cytogenetic data remain limited or fragmentary for several morphotypes and geographic regions of the *A. fraterculus* complex. As a result, the extent of karyotype variation across the complex, the geographic distribution of chromosomal variants, and its evolutionary significance, therefore remain incompletely understood (Goday et al. 2006; Gariou-Papalexiou et al. 2016; Giardini 2022). In particular, a comparative assessment integrating sex chromosome morphology and heterochromatin organization across a broader range of morphotypes is still needed to evaluate patterns of chromosomal diversification within the complex (Bush 1962; Solferini and Morgante 1987; Selivon et al. 2002, 2005 a, b; Goday et al. 2006).

In this study, we investigated cytogenetic variation within the *A. fraterculus* complex, focusing on sex chromosome morphology and heterochromatin organization. Our objectives were to: i) characterize karyotype variation; ii) identify cytogenetic features associated with morphotype differentiation; and iii) develop an evolutionary framework for understanding karyotype diversification within the complex. We hypothesized that variation in these cytogenetic features reflects evolutionary divergence among morphotypes and provides phylogenetically informative characters for elucidating patterns of karyotype diversification. To test this hypothesis, we analyzed mitotic chromosomes from laboratory colonies using sequential DAPI/CMA_3 staining. Additionally, to place our findings in a broader comparative context we also characterized the karyotypes of two related species, *A. ludens* and *A. obliqua*.

2. Materials and methods

2.1 Biological material

Individuals from laboratory colonies of *A. fraterculus* from Ecuador, Colombia, Peru, Brazil, and Argentina, as well as the related species *A. ludens* and *A. obliqua* from Mexico were used in this study (Table 1). The colonies were maintained at the Insect Pest Control Laboratory (IPCL), Joint FAO/IAEA Centre, Seibersdorf (Austria) and reared under standardized biosafety conditions. Hereafter, the laboratory colonies are referred by two-letter abbreviations based on their locality and country of origin: GE (*A. fraterculus*, Guayaquil-Ecuador), IC (*A. fraterculus*, Ibagué-Colombia), PP (*A. fraterculus*, Piura-Peru), CP (*A. fraterculus*, Cusco-Peru), PB (*A. fraterculus*, Piracicaba-Brazil), VB (*A. fraterculus*, Vacaría-Brazil), YA (*Af* -Y-Short, Argentina), *A.o*M (*A. obliqua*, Mexico), and *A.l*M (*A. ludens*, Mexico).

2.2 Chromosome preparations

Mitotic chromosomes were obtained from cerebral ganglia of third-instar larvae from each laboratory colony following the standard cytogenetic protocol for tephritid flies, with minor modifications (Guest and Hsu 1973; Mavragani-Tsipidou et al. 2014). Briefly, larvae were dissected in insect Ringer's solution, and the brain tissues were incubated in 1% w/v sodium citrate for 10–15 min at room temperature. The tissues were then fixed in freshly prepared methanol: acetic acid (3:1) for 3 min, with three consecutive changes of fixative to ensure complete fixation. The fixed material was subsequently dissociated in 60% (v/v) acetic acid using a micropipette, and the resulting cell suspension was dropped onto clean microscope slides. Slides were placed on a hot plate (40–45°C) to promote chromosome spreading, air-dried, and aged at room temperature for at least 24 h before staining.

For each laboratory colony, ten independent chromosome preparations were examined. A minimum of 15 well-spread metaphases was analyzed per preparation, resulting in at least 150 metaphases evaluated for each population. Only metaphases with complete, well-resolved chromosome complements were included in subsequent analyses.

2.3 Sequential DAPI/CMA_3 staining

Sequential fluorochrome staining was performed to characterize chromosome morphology and heterochromatin composition and distribution following the protocol of Schweizer and Ambros (1994) with modifications adapted for *Anastrepha* species (Goday et al. 2006).

After aging, the chromosome preparations were immersed in phosphate-buffered saline (PBS) for 10 min and sequentially stained with fluorochromes. AT-rich heterochromatin was detected by staining with 4',6-diamidino-2-phenylindole (DAPI; 0.5 µg/ml in 2xSSC) for 15 min, followed by a 4 min rinse in PBS. GC-rich regions were then visualized with chromomycin A_3 (CMA_3; 0.6 mg/ml in McIlvaine´s buffer) by overnight incubation in a humid chamber containing 2xSSC. Finally, the preparations were rinsed in PBS for 4 min, mounted in antifade solution, sealed with rubber cement, and stored in the dark at room temperature until microscopy examination.

2.4 Image acquisition and karyotype analysis

Metaphase plates were examined under a fluorescence microscope (Leica DMLB) equipped with DAPI and CMA_3 filter sets. Images were captured using a high-resolution digital camera (Leica DFC350 FX) under standardized exposure conditions.

Chromosomes were identified based on relative length, centromere position, and banding patterns. Images were processed using Adobe Photoshop 2026©. Chromosome measurements, and idiograms were generated from mean values obtained from at least 15 metaphase plates per colony using MicroMeasure3.3 (Reeves 2001).

Table 1
Origin and characteristics of *Anastrepha* laboratory colonies maintained at the IPCL and analyzed in this study.

Colony ID	internal ID	Species	Origin (Locality-Country)*	Geographic coordinates*	Altitude (m a.s.l.)*	Host fruit/Type**	Arrival at IPCL	Generation***
GE	*Af* Ecuador	*A. fraterculus*	Chongón, Guayas, Ecuador	1°54'S 79°48'W	~ 50–80	*Mangifera indica* L (mango)/ Wild flies	March, 2024	G1
IC	*Af* Colombia	*A. fraterculus*	Ibagué, Tolima, Colombia	4°26'16"N 75°12'02"W	~ 1,285	*Coffea* sp. (coffee)/ Lab flies	May, 2019	G61
PP	*Af* Peru	*A. fraterculus*	Piura, Peru	5°12'3"S 80°37'31"W	~ 55	Unknown/ Lab flies	April, 2024	G1
CP	*Af* Cusco	*A. fraterculus*	Cusco, Valle Sagrado, Peru	13°31'20"S 71°59'W	~ 3,399	Unknown/Lab flies	April, 2024	G102
PB	*Af* Piracicaba	*A. fraterculus*	Piracicaba, São Paulo, Brazil	22°43'31"S 47°38'58"W	~ 580	Unknown/Lab flies	February, 2011	G96
VB	*Af* Vacaría	*A. fraterculus*	Vacaría, Rio Grande do Sul, Brazil	28°30'43"S 50°56'2"W	~ 971	Unknown /Wild flies	May, 2015	G89
YA	*Af* Y-Short	*A. fraterculus*	San Miguel de Tucumán, Argentina	26°49'00"S 65°13'00"W	~ 431	*Psidium guajava* (guava)/ Lab flies	—	G120
A. oM	*A. obliqua*	*A. obliqua*	Southern Mexico, Mexico	~ 16°20'10"N 95°14'30"W	~ 180–350	Unknown /Wild flies	July, 2016	G92
*A. l*M	*A. ludens*	*A. ludens*	Tapachula, Chiapas, Mexico	14°54'40"N 92°15'52"W	~ 177	Unknown /Lab flies	December, 2017	G64

GE= Guayaquil, Ecuador; IC= Ibagué, Colombia; PP= Piura, Peru; CP= Cusco, Peru; PB= Piracicaba, Brazil; VB= Vacaría, Brazil; YA = Af-Y-short, Argentina; *A. o*M= *Anastrepha obliqua*, Mexico; *A. l*M = *A. ludens*, Mexico. *Origin and geographic location of insect colonies, including coordinates and altitude. m.a.s.l: metres above sea level. ** Host fruit/Type. Host fruit refers to the fruit from which the individuals used to establish the colony were collected. Type indicates whether the colony was established from wild-caught individuals or from individuals originating from a laboratory colony previously established elsewhere and subsequently transferred to the IPCL. *** Generation of laboratory rearing at which the individuals were analyzed in this study. The *Anastrepha fraterculus* colonies were ordered according to their geographic coordinates, from north to south.

3. Results

3.1 General karyotypic features

All laboratory colonies exhibited the conserved karyotype typical of the genus *Anastrepha*, with a diploid chromosome complement of 2n = 12, comprising five pairs of autosomes and one pair of sex chromosomes. Following sequential DAPI/CMA staining, differential fluorescence patterns were observed exclusively in the sex chromosomes, whereas the autosomes showed no staining with these fluorochromes (Fig. 1A and 1B).

The X chromosome displayed a conserved submetacentric morphology and a uniform relative size across all colonies. The only variation detected among the analyzed colonies was restricted to the size and position of the DAPI-positive heterochromatic block(s), corresponding to AT-rich sequences (Fig. 1A and 1B; Fig. 2). In contrast, the Y chromosome exhibited marked variation in size, morphology, and the composition and distribution of heterochromatin. Based on Y- chromosome size and morphology, the analyzed *A. fraterculus* colonies were classified into two cytogenetic groups (Groups I and II) (Figs. 1 and 2).

3.2 Group I. Colonies with large submetacentric Y chromosomes

This group comprised the GE, PP, CP and PB colonies which shared a large submetacentric Y chromosome, with heterochromatin restricted to DAPI-positive blocks located at the terminal region of the short arm. These blocks were enriched in AT-rich sequences and their number, size, and position varied among the analysed colonies (Fig. 1A and 2). Notably, the Y chromosome of the CP colony exhibited a satellite containing one of the two DAPI-positive heterochromatic blocks observed in this karyotype (Fig. 1A and 2). The X chromosome was also highly conserved among the analyzed colonies, exhibited a large submetacentric morphology and a single DAPI-positive heterochromatic block. The size and position of this heterochromatic block varied among colonies (Fig. 1A and 2).

3.3 Group II. Colonies with small metacentric Y chromosomes

This group included the IC, VB, and YA colonies (Fig. 1B and 2). The Y chromosome was consistently small and metacentric and contained both DAPI and CMA_3-positive heterochromatic regions, indicating the presence of repetitive AT- and GC-rich sequences. The X chromosome was highly conserved across the group, exhibiting a submetacentric morphology and DAPI-positive heterochromatin. A single heterochromatic block of variable size was observed at the terminal region of the short arm in IC, whereas the VB and YA colonies exhibited two DAPI-positive blocks, located at the terminal regions of both arms (Fig. 1B and 2).

Figure 1. Mitotic metaphase chromosomes of male individuals from *Anastrepha fraterculus* colonies and related species. Columns show DAPI staining (blue), CMA_3 staining (yellow), and merged images (DAPI + CMA_3; purple). Rows correspond to the studied populations or colonies. **(A)** *A. fraterculus*: GE = Guayaquil, Ecuador; PP = Piura, Peru; CP = Cusco, Peru; PB = Piracicaba, Brazil. **(B)** *A. fraterculus*: IC = Ibagué, Colombia; VB = Vacaría, Brazil; YA = Af-Y-short, Argentina. **(C)** Related species: *A.l*M = *A. ludens*, Mexico; *A.o*M = *A. obliqua*, Mexico. The X and Y chromosomes are indicated in each panel. Scale bars = 10 µm.

3.4 Karyotypic analysis of related species

The *A.l*M colony showed a small Y chromosome with characteristic DAPI-positive heterochromatin regions (Fig. 1C and 2). The X chromosome was submetacentric and contained a single DAPI-positive heterochromatin block located at the terminal region of the short arm.

The *A.o*M karyotype exhibited a large submetacentric Y chromosome with a single DAPI-positive heterochromatin block located at the terminal region of the short arm (Figs. 1B and 2). The X chromosome was also large and submetacentric, with a single DAPI-positive heterochromatin block (Figs. 1C and 2).

4. Discussion

In the present study, we performed a comparative cytogenetic analysis of *A. fraterculus* laboratory colonies established from populations across different regions of the Americas, together with the closely related species *A. ludens* and *A. obliqua*. All analyzed karyotypes shared a conserved chromosomal complement of 2n = 12, comprising five pairs of autosomes and one pair of sex chromosomes, contrasting with substantial variation in the sex chromosomes. Our findings complement the cytogenetic information of de *A. fraterculus* complex and are consistent with previous studies (Bush 1962; Solferini and Morgante 1987; Selivon et al. 2005 a, b; Goday et al. 2006; Zepeda-Cisneros et al. 2014; Giardini et al. 2015).

4.1 Karyotypic dynamics across the *Anastrepha fraterculus* complex and related species

The results presented here provide the first cytogenetic characterization of the Ecuadorian and Andean morphotypes, represented by the Cusco (Peru) and the Ibagué (Colombia) populations, respectively. Both morphotypes had previously been identified based on morphological characters (Steck 1991; Hernández-Ortiz et al. 2004). We also identified a previously unrecognized karyotypic variant in the Piracicaba (Brazil) population. Although this population had been assigned to *A. fraterculus* sp. 1 based on mating compatibility (Vera et al. 2006) and cuticular hydrocarbon profiles (Vaníčková et al. 2015), it had not been cytogenetically characterized.

The Peruvian morphotype, represented here by the lowland populations of Piura (Peru) and Guayaquil (Ecuador), was previously described as *A. fraterculus* sp.4 based on Guayaquil populations (Selivon et al. 2002; Goday et al. (2006), and subsequently associated with the Peruvian morphotype based on specimens from Lima (Peru) (Cáceres et al. 2009). The Brazilian 1, 2 and 3 morphotypes, also referred as *A. fraterculus* sp. 1, sp. 2 and sp. 3, respectively, were previously cytogenetically characterized by Yamada and Selivon (2001) and Selivon et al. (2002). In the present study, the Brazilian 1 morphotype (*A. fraterculus* sp. 1) is represented by the Af-Y-Short colony from Argentina and the Vacaría population from Brazil. Overall, our results expand the comparative cytogenetic characterization of *A. fraterculus* complex, although the Mexican and Venezuelan morphotypes remain uncharacterized at the chromosomal level.

Comparison of the sex chromosomes size and morphology performed in the present study revealed two major cytogenetic groups within the analyzed *A. fraterculus* complex. The Ecuadorian morphotype exhibits the large-Y configuration characteristic of Group I, together with distinctive features, including a secondary constriction, chromosomal gaps, and a discontinuous heterochromatic block (Table 2). In contrast, the Andean morphotype shares with *A. fraterculus* sp. 1 and sp. 3 the small metacentric Y chromosome and a pronounced sex chromosome differentiation, placing it in Group II (Table 2). These patterns suggest that differences in sex chromosome morphology and organization are associated, at least in part, with variation in heterochromatin amount and distribution (Fig. 2). The Piracicaba population, in turn, displays a sex chromosome configuration distinct from those previously reported for the Brazilian morphotypes, supporting its identification as a novel cytogenetic variant within the complex.

The inclusion of *A. ludens* and *A. obliqua* in our analyses provides a broader comparative framework for interpreting sex chromosomes variation within the *A. fraterculus* complex. We confirmed the characterization of *A. obliqua* karyotype (Goday et al. 2006) and extended the one of Garcia-Martinez et al. 2009 for *A. ludens*. Both species autosomes showed no differential staining with DAPI/CMA, whereas their sex chromosomes displayed distinct morphological and heterochromatic features. In terms of Y-chromosome characteristics, *A. obliqua* showed greater similarity to Group I, while *A. ludens* shared features with Group II. These comparisons illustrate that the two configurations identified within the *A. fraterculus* complex also show parallels in related species (Fig. 2). These findings also contribute with the cytogenetic characterization and information related to chromosomal stability assessment of laboratory colonies which could potentially be used in the development of genetic sexing strains (GSS) for SIT programs.

Across the analyzed karyotypes, the patterns of sex chromosome differentiation are consistent with a role for heterochromatin remodeling in sex chromosome diversification, alongside the maintenance of the overall karyotypic organization (Selivon et al. 2002; Giardini et al. 2015; Moran et al. 2018). Changes in heterochromatin content and distribution may contribute to the differentiation of sex chromosome without requiring major changes in chromosome number or autosomal morphology. More broadly, heterochromatin differentiation and the dynamics of repetitive DNA have been proposed as important components of sex-chromosome evolution in insects (Charlesworth et al. 1994; Ugarkovic 2002; Bachtrog 2013; Garrido-Ramos 2017).

Table 2

Sex chromosome characteristics and karyotype description status of morphotypes within the *Anastrepha fraterculus* species complex. The table summarizes the colony ID, assigned morphotype, characteristics of the sex chromosome pair, and the current status of its karyotype description. DAPI and CMA_3 staining patterns are indicated where applicable.

Colony ID	Assigned morphotype	Sex chromosome pair characteristics	Reference
IC	Andean	Small metacentric Y-chromosome; DAPI/CMA+ over almost the entire chromosome. Large submetacentric X-chromosome; one centromeric DAPI+ block. Pronounced degree of sex chromosome differentiation.	This study
VB	*A. fraterculus* sp. 1	Small metacentric Y-chromosome; DAPI/CMA + at both ends. Large submetacentric X-chromosome; two terminal DAPI+ blocks. Pronounced degree of sex chromosome differentiation.	Yamada and Selivon, 2001
YA	*A. fraterculus* sp. 1	Small metacentric Y-chromosome; DAPI/CMA + at both ends. Large submetacentric X-chromosome; two terminal DAPI+ blocks. Pronounced degree of sex chromosome differentiation.	Giardini et al. 2020
*Af-sp.3-SS	*A. fraterculus* sp. 3	Small metacentric Y-chromosome; DAPI + at both ends and CMA_3+ in the centromeric region. Large submetacentric X-chromosome; two terminal DAPI+ blocks. Pronounced degree of sex chromosome differentiation.	Selivon et al. 2002
GE	*A. fraterculus* sp. 4 - Peruvian	Large submetacentric Y-chromosome; DAPI+ block at centromeric region. Large submetacentric X-chromosome; DAPI+ block in the interstitial region of the long arm.	Selivon et al. 2002
PP	*A. fraterculus* sp. 4 - Peruvian	Large submetacentric Y-chromosome; DAPI+ block at centromeric region. Large submetacentric X-chromosome; DAPI+ block in the interstitial region of the long arm.	Selivon et al. 2002
CP	Ecuadorian	Large submetacentric Y-chromosome; DAPI+ bifurcated-block at centromeric region. Large submetacentric X-chromosome; DAPI+ pericentromeric block extending into both arms.	This study
PB	Not assigned	Large submetacentric Y-chromosome; DAPI+ block at centromeric region. Large submetacentric X-chromosome; DAPI+ pericentromeric block extending into both arms.	This study
*Af-sp.2-SI	*A. fraterculus* sp. 2	Large submetacentric Y chromosome; DAPI+ block in the interstitial region of the long arm. Large submetacentric X chromosome; DAPI+ block in the interstitial region of the long arm and another DAPI+ block spanning the entire short arm.	Yamada and Selivon, 2001

Asterisks identify wild individuals collected at specific localities: Af-sp.2-SI, Santa Isabel, São Paulo, Brazil; Af-sp.3-SS, São Sebastião, São Paulo, Brazil. References are provided for previously described karyotypes.

4.2 Hypothetical evolutionary model of karyotype diversification within the *Anastrepha fraterculus* complex

Based on the cytogenetic patterns identified in the present study and previously reported data for *A. fraterculus* sp. 2 and sp. 3 (Goday et al. 2006), we propose a hypothetical model of karyotype diversification within the *A. fraterculus* complex focused on sex chromosome differentiation. The proposed model considers a putative ancestral condition characterized by relatively large and weakly differentiated sex chromosomes. As the Piracicaba population exhibits several of these features, its karyotype may serve as a starting point for exploring two alternative trajectories of sex chromosomes diversification (Fig. 3).

The first trajectory would involve the retention of relatively large and weakly differentiated sex chromosomes, followed by lineage-specific remodeling of their heterochromatic regions, as observed in *A. fraterculus* sp. 2 (Yamada and Selivon 2001; Goday et al. 2006) and in the Peruvian and Ecuadorian morphotype (Fig. 3, left). In the latter, secondary constrictions, chromosomal gaps, and heterochromatic regions appearing as two or more distinct blocks may reflect localized reorganization and/or amplification of repetitive DNA. The second trajectory would involve Y-chromosome size reduction accompanied by increasing X/Y differentiation, resulting in the small metacentric Y chromosomes observed in the Andean morphotype, *A. fraterculus* sp. 1, and sp. 3 (Fig. 3, right). This reduction may have resulted primarily from the loss and remodeling of heterochromatic and repetitive DNA, rather

than from major structural rearrangements, potentially accounting for the transition from larger submetacentric to smaller metacentric Y chromosomes (Bachtrog 2013).

Phylogeographic studies have provided evidence of historical range expansion and diversification of *A. fraterculus* in southern South America, as well as east–west patterns of population differentiation across Brazil (Vilardi et al. 2021; Congrains et al. 2023; Freilij et al. 2023). These patterns may reflect historical processes of population expansion and geographic connectivity rather than direct natural dispersal over long distances. Human-mediated transport of infested fruit through trade may also have contributed to the establishment and connectivity of geographically separated populations, as previously proposed for *A. fraterculus* populations in Argentina (Alberti et al. 2002; Petit-Marty et al. 2004). However, the contribution of these processes to the present-day distribution and differentiation of the morphotypes analyzed here remains to be determined. Considering this background, the geographic distribution of the karyotypes analyzed in the present work is compatible with a possible westward and southward expansion of ancestral *A. fraterculus* populations from southeastern Brazil (Fig. 4). Particularly, the Andean morphotype is geographically distant from sp. 1 and sp. 3, despite sharing with them the small metacentric Y-chromosome configuration. This pattern does not necessarily imply a shared evolutionary origin or a common monophyletic lineage. Instead, it may reflect independent evolution of similar chromosomal configurations through distinct evolutionary trajectories. Comparable diversity in the evolutionary histories of sex chromosomes and repetitive DNA has been documented across different taxa, including fish, plants, primates, and *Drosophila* (Mayr 1963; Schluter 2000; Charlesworth et al. 2005; Bachtrog 2013; Garrido-Ramos 2017).

Further studies combining DNA content measurements, total chromosome length, and quantitative estimates of heterochromatin amount and composition could provide additional insights into the chromosomal differentiation observed between the two groups. Therefore, the geographic pattern illustrated in Fig. 4 should be considered a working hypothesis that requires further testing through broader, geographically representative sampling and an integrative approach combining phylogeographic and genomic data.

Conclusion

- This study expands the cytogenetic knowledge of the *Anastrepha fraterculus* species complex by documenting karyotypic variation across several members and providing a comparative framework for generating hypotheses about sex chromosome diversification. - Heterochromatin remodeling and repetitive DNA turnover may have contributed to chromosome differentiation, although further studies are needed to test these mechanisms.

- The characterization of chromosome organization and variation in laboratory colonies provides a foundation for assessing their chromosomal stability and supports the development and evaluation of genetic sexing strains (GSS) for the Sterile Insect Technique (SIT).

- By contributing to the development of more reliable and effective strains, this knowledge can help strengthen SIT-based strategies for environmentally sustainable *A. fraterculus* control.

Declarations

Author contributions

MCG, KB and SBL conceived and designed the study. ECM, FHM, and MTV maintained the *Anastrepha fraterculus* laboratory colonies and provided biological material for cytogenetic analyses. MCG and MN performed the cytogenetic experiments. MCG, MFF, and SBL analyzed and interpreted the data. KB and MTV contributed to the interpretation of the cytogenetic findings through scientific discussions. KB and MTV provided laboratory facilities, reagents, and technical support at the IPCL IAEA. MCG prepared the original draft of the manuscript, with substantial contributions from MFF and SBL. MCG and MFF prepared the figures. GG provided laboratory facilities, reagents, and technical support at FCEyN, UBA. MCG and SBL secured funding and provided laboratory facilities and reagent at the Instituto de Genética, INTA. All authors critically reviewed and revised the manuscript and approved the final version.

Funding

This study was supported by the Agencia Nacional de Promoción de la Investigación, el Desarrollo Tecnológico y la Innovación through PICT 2021-00491 to SBL; the Instituto Nacional de Tecnología Agropecuaria (INTA) through the project and PD-E4-I079-008 to SBL; and by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) through project 11220150100835CO to MN. MCG received a BIRF fellowship through the INTA Postgraduate and Professional Development Program, which supported her research stay at the Insect Pest Control Laboratory (IPCL), Joint FAO/IAEA Centre, Seibersdorf, Austria. This work also received support from the Insect Pest Control Subprogramme of the Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture.

Competing interests

The authors declare no competing interests.

Data Availability Statement

The data that supports the findings of this study are available upon request from the authors.

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Figures

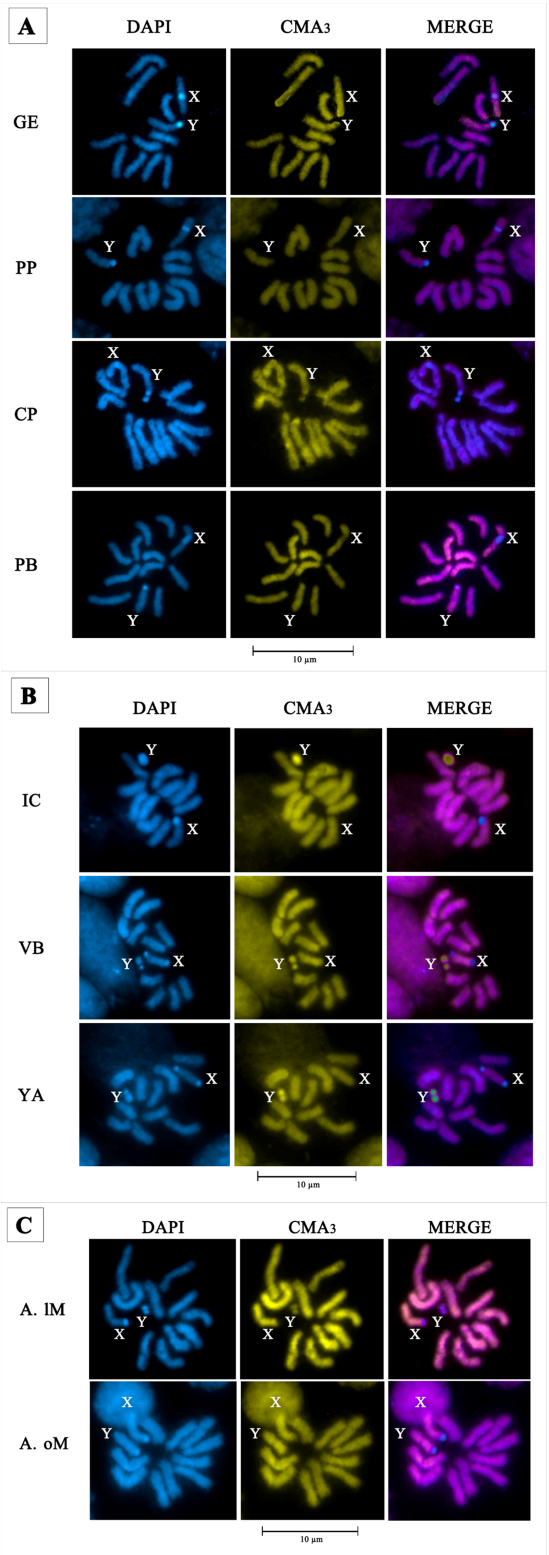


Figure 1

Mitotic metaphase chromosomes of male individuals from *Anastrepha fraterculus* colonies and related species. Columns show DAPI staining (blue), CMA_3 staining (yellow), and merged images (DAPI + CMA_3; purple). Rows correspond to the studied populations or colonies. **(A)** *A. fraterculus*: GE = Guayaquil, Ecuador; PP = Piura, Peru; CP = Cusco, Peru; PB = Piracicaba, Brazil. **(B)** *A. fraterculus*: IC = Ibagué, Colombia; VB = Vacaría, Brazil; YA = Af-Y-short, Argentina. **(C)** Related species: *A.l*M = *A. ludens*, Mexico; *A.*oM = *A. obliqua*, Mexico. The X and Y chromosomes are indicated in each panel. Scale bars = 10 µm.

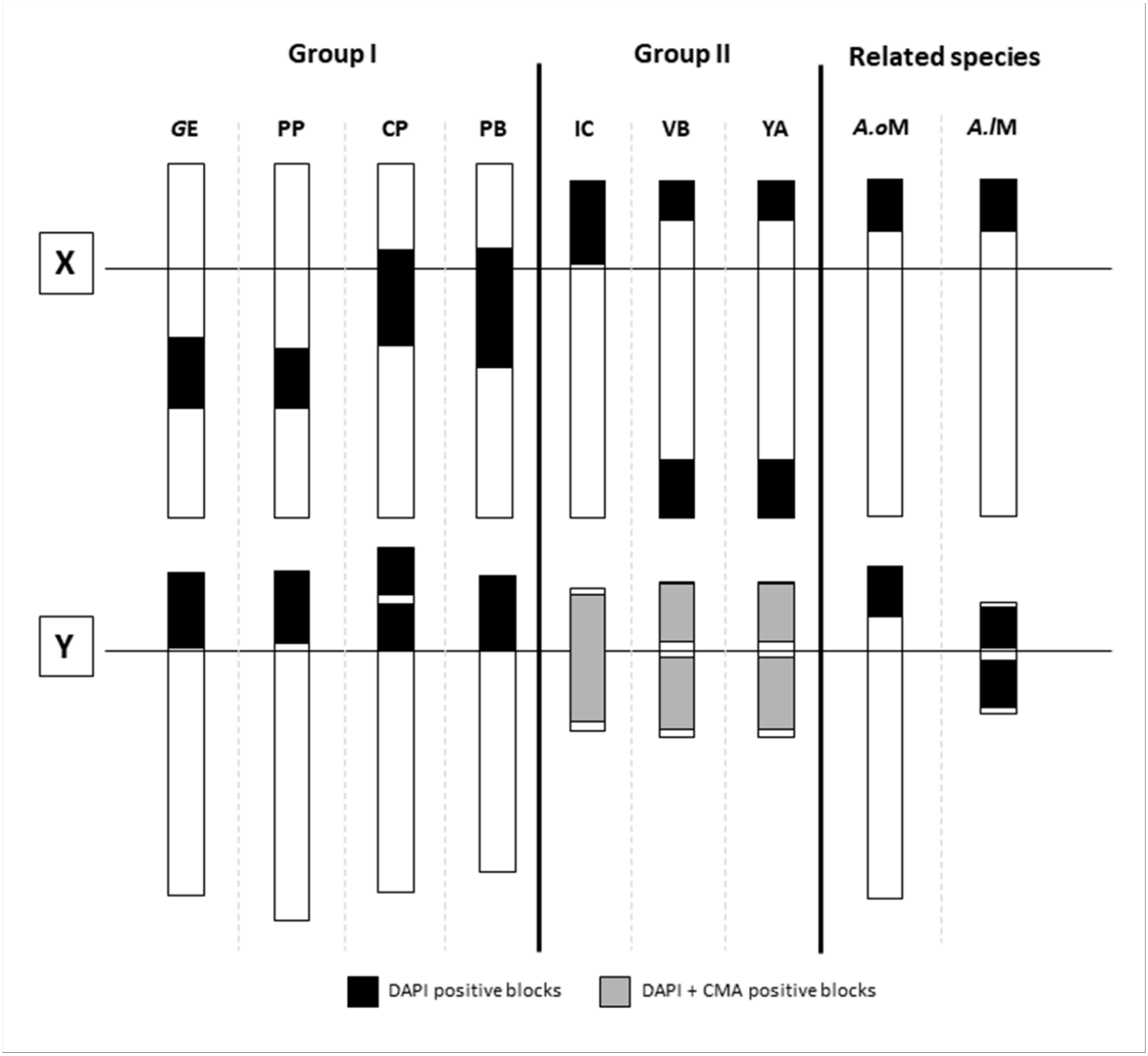


Figure 2

Idiograms of male sex chromosomes from the colonies analyzed in the present study grouped according to Y-chromosome size and morphology. Group I (left): GE, PP, CP and PB. Group II (middle): IC, VB and YA. Related species (right): *A./*M and *A.o*M. Chromosomes are drawn to represent relative length, with centromere position indicated by a horizontal line. Black blocks represent AT-rich heterochromatin (DAPI-positive), and grey blocks represent heterochromatin positive for both AT- and GC-rich sequences (DAPI+/CMA3+).

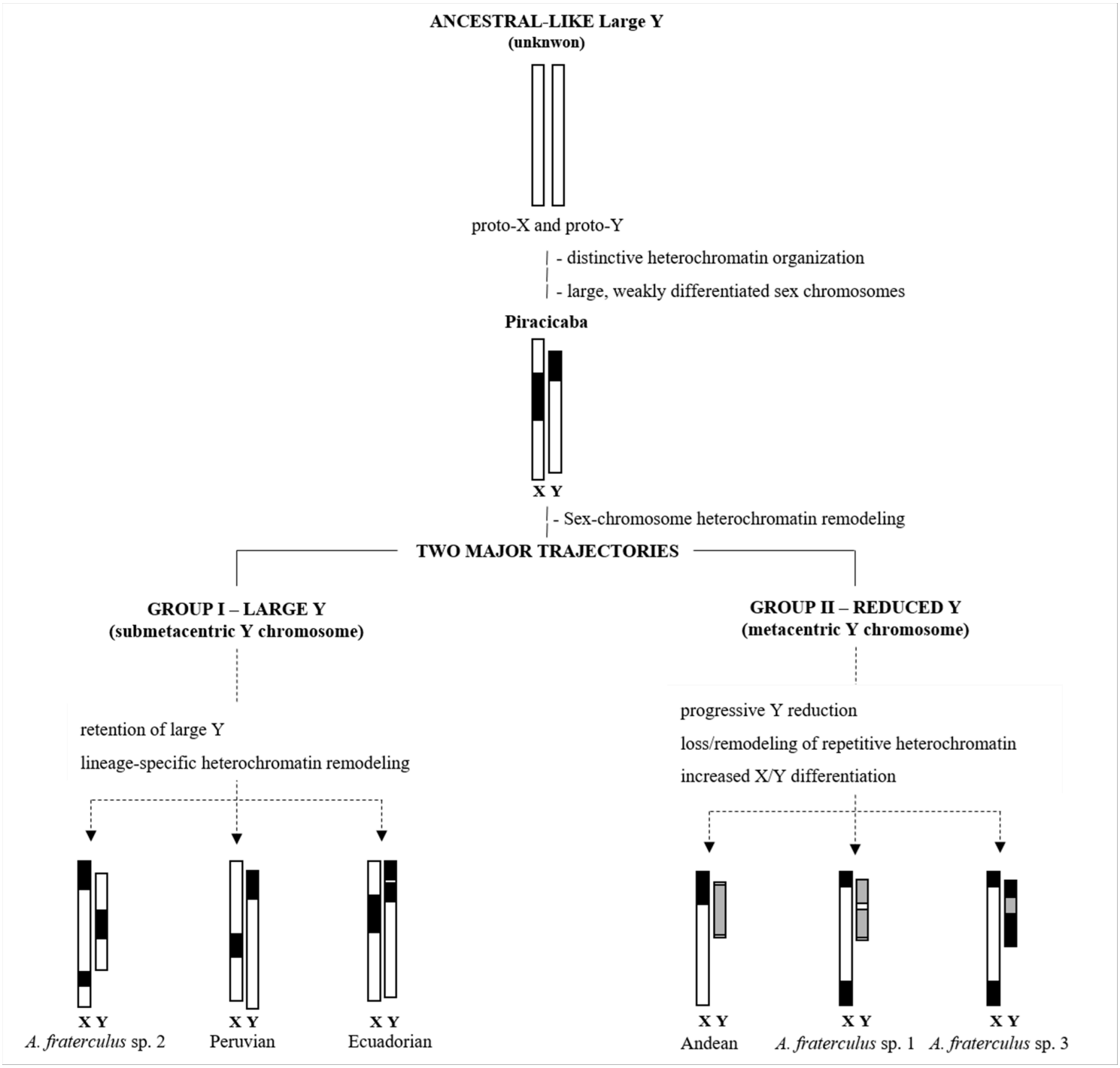


Figure 3

Hypothetical model of karyotype evolution within the *Anastrepha fraterculus* complex. A putative ancestral karyotype, characterized by large, weakly differentiated sex chromosomes and a distinctive heterochromatin organization, is proposed as the starting point for diversification, with the PB karyotype representing its closest extant approximation. From this ancestral condition, two major cytogenetic trajectories are inferred: a large-Y lineage (Group II), retaining relatively large, weakly differentiated sex chromosomes, and a reduced-Y lineage (Group I), characterized by progressive Y-chromosome reduction and increased X/Y differentiation. Diversification within each lineage is attributed mainly to changes in heterochromatin amount, distribution and repetitive DNA content.

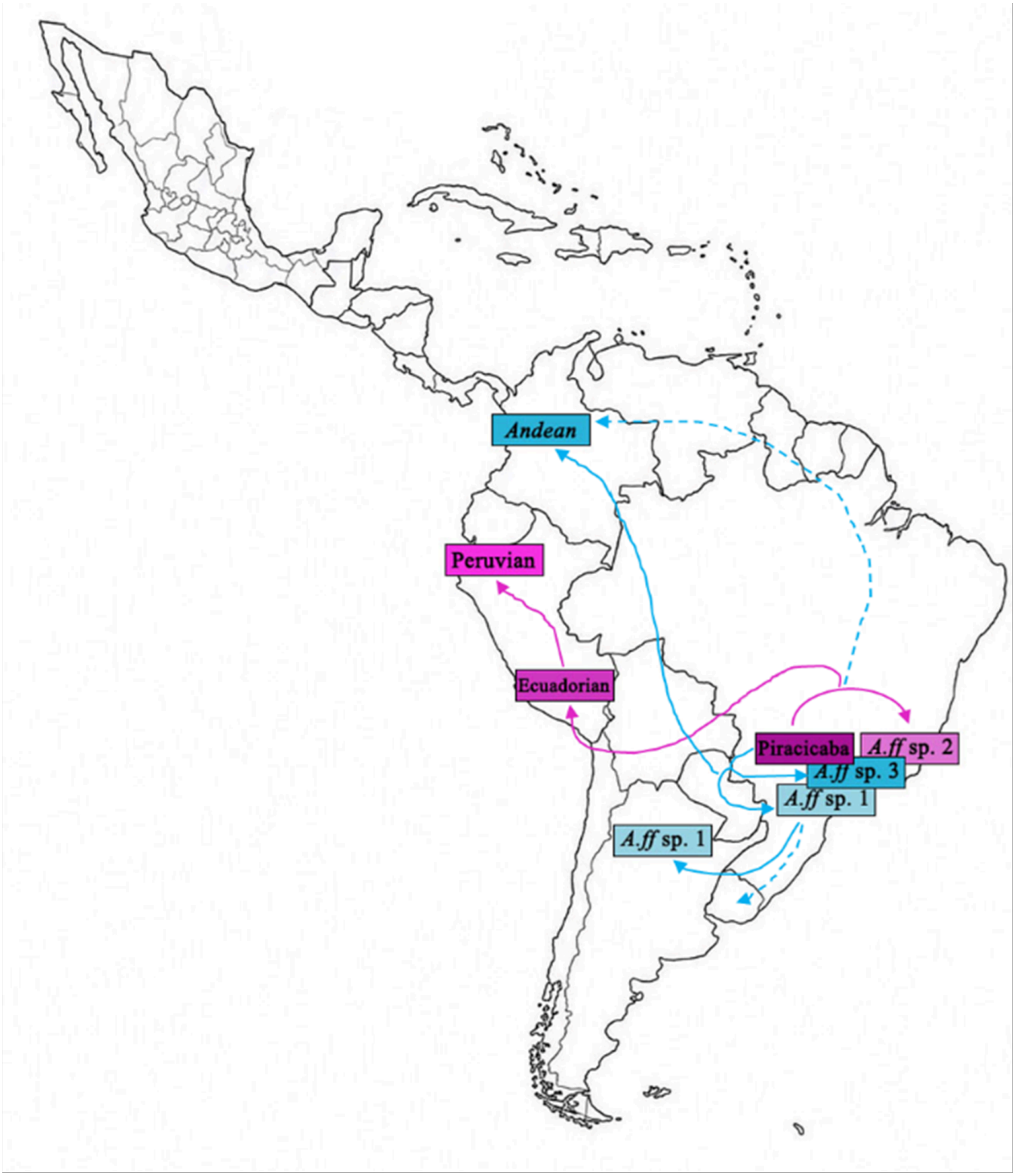


Figure 4

Hypothetical geographic pathways of karyotype diversification within the *Anastrepha fraterculus* complex. Map of Latin America showing hypothetical geographic pathways connecting the populations and cytogenetic groups analyzed in this study, including Piracicaba, *A. fraterculus* sp. 1, sp. 2, sp. 3, Ecuadorian, Peruvian and Andean populations. Blue arrows indicate hypothetical pathways associated with the reduced-Y lineage, whereas magenta arrows indicate those associated with the large-Y lineage. Solid and dashed arrows represent alternative pathways.