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SPECIAL ISSUE ARTICLE

Genetic consequences of domestication and refreshment on colonies of the South American fruit fly

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Abstract For almost a decade, natural populations of the South American fruit fly have been targeted for control through Sterile Insect Technique projects. To ensure a sustainable supply of competitive sterile flies for this approach, it is essential to understand the effects of domestication when strains of this pest are initially brought into the laboratory to establish colonies as well as the changes occurring after multiple generations of adaptation to conditions used for mass rearing. Using one colony established from a wild population of the Brazil-1 morphotype (WIL) and two from laboratory colonies in Brazil known as the *Piracicaba* (PL) and *Vacaria* (VL) strains, this study evaluated genetic diversity in samples from 10 generations after domestication and maintenance under semimass rearing conditions. Another aim of this study was to analyze changes in the genetic makeup of the colonies of the two laboratory strains after refreshment. Eight microsatellite markers were used for the genetic analyses. Results indicated a moderate but significant amount of genetic differentiation between the WIL population and the two laboratory strains. Results also showed that levels of genetic diversity in both the VL and PL strains were maintained at similar levels over a period of more than two years of rearing. Additionally, results suggest that successful creation of admixture via refreshment is more likely to be beneficial in relatively short-term domesticated colonies, and that performing refreshment approximately every six to eight generations could be beneficial to maintain the genetic diversity of *A. fraterculus* colonies under laboratory mass rearing conditions.

Key words tephritid fruit flies; mass rearing strains; genetic diversity; microsatellites; population genetics

Introduction

In neotropical regions of the world, there are many examples of successful suppression and eradication of tephritid pest fruit flies (Diptera: Tephritidae) through the use of the Sterile Insect Technique (SIT) (Pérez- Staples *et al.*, 2021). The South American fruit fly, *Anastrepha fraterculus* (Wiedemann, 1830), has for some time been

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a candidate species that could be controlled with implementation of the SIT method (Cladera *et al.*, 2014). In addition, recently both outright bans or drastic reductions in allowable levels of certain insecticides imposed by fruit importing countries has given new impetus to exploring the use of alternative and/or biologically based control measures such as SIT to deal with pest species (Urbaneja *et al.*, 2009; Kovaleski & Mastrangelo, 2021; Sarkar *et al.*, 2021). The use of SIT depends on the continuous rearing and release of large numbers of sterile males into a target region, aimed at impairing the fertility of the wild females over time. To obtain sufficient numbers of sterile insects, it is necessary to maintain large colonies in facilities designed for mass-rearing and also to carry out consistent and continuous assessments of quality control parameters for the batches of insects produced (Parker *et al.*, 2021).

In addition, a step that precedes the mass production of the target insect is the domestication or adaptation of the insects to artificial rearing conditions (Parker *et al.*, 2021). Laboratory strains are usually first established from a small collection of individuals from a wild population. Hoffmann & Ross (2018) found that laboratory domestication can result in very significant evolutionary changes in traits. When these genetic changes are detected across different generations, it suggests that the gene pool of the strain has been impacted by selection, genetic drift or inbreeding depression. Also, cases of selection and inbreeding in Diptera have already been correlated with loss of gene diversity and fitness in proportion to the colonization time in the laboratory (Jungen & Hartl, 1979; Briscoe *et al.*, 1992). A significant reduction of genetic diversity was also detected from a genetic "bottleneck" in a colony of the tsetse fly *Glossina pallidipes* (Diptera: Glossinidae) ca. 38 years old (Ciosi *et al.*, 2014). Loss of genetic variability during domestication has also been observed for Tephritidae species (Zygouridis *et al.*, 2009; Gillchrist *et al.*, 2012; Parreño *et al.*, 2014).

The laboratory populations will be further subjected to selection based on their ability to adapt to the new artificial rearing environment. In subsequent generations, larger and larger numbers of insects can be reared as the colony becomes better adapted to the rearing conditions (Hoffman & Ross, 2018). Concurrent with this laboratory adaptation, changes in biological and behavioral traits may arise due to factors such as the absence of predation, abundant food and water availability, and relaxed sexual selection. Risks exist that these laboratory pressures could lead to the selection individuals that are better adapted to laboratory conditions, ultimately resulting in a strain with life history traits that differ significantly from wild populations. Altered traits may also include early sexual maturation and mating, shorter life cycles, altered courtship behavior, and reduced tolerance to starvation or desiccation (Cayol, 2000; Hoffman *et al.*, 2001; Briceño & Eberhard, 2002). These are all major issues for area-wide integrated pest management (AW-IPM) programs that integrate the SIT, since their success depends on the ability of the laboratory reared sterile males to survive and outcompete wild males for wild females when they are released.

The presence or accumulation of undesirable traits in the mass-reared strain may be detected by the use of appropriate quality-control measurements. For fruit flies, there are standard procedures accepted internationally to evaluate such biological parameters for mass-reared flies, including their mating compatibility and competitiveness in field cages (FAO/IAEA/USDA, 2019). However, the detection and monitoring of levels of genetic variation, diversity and structuring among tephritid mass-reared strains compared to wild flies are not performed as often, despite the recognition of the importance of these parameters in supporting SIT strategies (Krafsur & Ouma, 2021).

For both laboratory reared and wild populations of insects, inbreeding and genetic distances between target founding populations, colonies and wild populations can be compared by quantifying variation in single Nucleotide Polymorphisms (SNPs) from mitochondrial DNA (mtDNA) or nuclear DNA, or through assessments of variation using anonymous markers such as microsatellites (Krafsur & Ouma, 2021). A set of species-specific microsatellites has been successfully developed for the assessment of genetic diversity in wild and laboratory populations of *A. fraterculus* populations in Argentina (Lanzavecchia *et al.*, 2014). The 14 loci used in this study revealed high levels of polymorphism and remarkably genetic variability in two wild populations and two laboratory strains of the Brazilian-1 morphotype of *A. fraterculus*, the prevalent morphotype of the *A. fraterculus* complex in southern Brazil and Argentina (Selivon *et al.*, 2022). In a related study, Parreño *et al.* (2014) used 10 of these microsatellite markers to assess the genetic variability and differentiation of a long-established laboratory strain and a wild population of the same morphotype recently domesticated for 6 generations under artificial rearing conditions. The authors observed that a high level of genetic variation appeared to be maintained in the old laboratory strain across generations, while the level of genetic variation declined in the strain more recently established from a wild population.

To gain a better understanding on the consequences of domestication on the genetic diversity and differentiation

of a wild strain (WIL) derived from flies of the Brazilian-1 morphotype of *A. fraterculus* and flies from two laboratory strains (VL and PL), we used a set of highly variable microsatellite markers (Lanzavecchia *et al.*, 2014) to record estimates of various population genetic parameters from colonies of these three strains at different time points in the domestication process (early, mid, and long). We also considered the impact that "refreshment" (i.e., the introduction of wild genetic material into laboratory colonies) might have on these parameters when genetic material from wild males was introduced into the colony.

Materials and methods

Insect strains and colonies

Individuals from three different strains of *Anastrepha fraterculus* (described below), all ultimately derived from the Brazilian-1 morphotype, were used in this study. With rare exceptions, the colonies used in this study were reared at 24–26 °C and 50%–80% RH under a 12 h/12 h light/dark cycle.

The strain designated Wild Vacaria (WIL) was started using wild pupae (∼1980 pupae) obtained in May 2020 from infested pineapple guava (*Feijoa sellowiana* Berg) in the municipality of Vacaria, Brazil (28°30′39″S, 50°55′47″W). The emerged adults were kept in screened cages (30 cm × 30 cm × 30 cm) with adult diet (a mix of sugar, wheat germ and yeast Bionis YE MF at 3 : 1 : 1) and water *ad libitum*. Females were allowed to oviposit in papaya fruits (*Carica* spp.) (Gayle *et al.*, 2013) over 10 generations. For the genetic analyses, fly samples were collected from 8 out of the 10 generations (P, F_1–F_4, F_6, F_8, and F_{10}), representing an ongoing domestication process.

Two other laboratory strains representing mid- and long-term mass rearing, *Vacaria* (VL) and *Piracicaba* (PL), respectively, of the Brazilian-1 morphotype were also used. The VL colony was initiated in late 2015 with wild pupae from infested native fruits from southern Brazil, domesticated following the procedures of Walder *et al.* (2014) and maintained under semimass rearing conditions in subsequent years (Mastrangelo *et al.*, 2021). The origin of the PL was described by Walder *et al.* (2014) and the strain has been maintained at the Food Irradiation & Radioentomology Laboratory of CENA/USP under continuous laboratory rearing with artificial conditions for more than 110 generations without any introduction of new wild flies. Adult flies were sampled from 9 generations of these two colonies (e.g., generations F_{23}, F_{27}, F_{31}, F_{35}, F_{39}, F_{41}–F_{43}, and F_{45} of VL, and F_{107}–F_{110}, F_{114}, F_{116}, F_{118}, F_{121} and F_{131} of PL). Four consecutive generations were analyzed in PL (F_{107}–F_{110}), because it passed through a severe bottleneck in generation F_{106}, with the mother colony being reduced to less than 800 individuals. After its recovery, the adults of this strain were kept in two medium ovipositing cages ($l \times w \times h = 50$ cm × 30 cm × 100 cm; with ca. 6300 flies/cage) over the generations.

For refreshment experiments, subsets of the PL and VL colonies were provided with wild males selected from the parental cage of the WIL line. For each strain, a cage (30 cm × 30 cm × 30 cm) was set with 560 females and 280 males of the laboratory strain, plus 280 males of the WIL strain (a total of 1120 flies/cage). Flies from generation F_{42} of the *Vacaria* strain and from the F_{121} of the *Piracicaba* strain were used for these cages. A single cage for each of the two refreshed strains was maintained over 10 generations under laboratory conditions (Mastrangelo *et al.*, 2021), with ca. 1980 flies/cage/generation. All strains were kept isolated from each other. Quality control parameters [i.e., egg hatch (%), sex ratio (♀/♂+♀), and fliers (%)] were monitored following the procedures of FAO/IAEA/USDA (2019). Adult samples from 7 of the 10 generations (F_1–F_4, F_6, F_8, and F_{10}) of the refreshed colonies were used for the genetic analyses.

DNA isolation

Individuals were randomly collected from the colonies, fixed in absolute ethanol and kept at −80 °C until further handling. Total DNA extractions were performed using a CTAB-based method (Doyle & Doyle, 1990), adapted for tephritids. We performed 12 to 20 DNA extractions (sex ratio 1 : 1, male : female) for most sampled populations (generation within a colony). Briefly, whole adult flies about 15 to 20 mg were rinsed in abundant distilled water, dried on towel paper, and homogenized in 650 μL of CTAB solution (2% CTAB; 1% PVP-40; 1.4 mol/L NaCl; 100 mmol/L Tris HCl pH 8; 20 mmol/L EDTA pH 8). Homogenizations were supplemented with 8 μL of Proteinase K (25 mg/mL, NEB), shortly vortexed and incubated at 65 °C for 2 h. Digestions were stopped by the addition of 650 μL of Chloroform: Isoamyl Alcohol (24 : 1, v/v) followed by vigorous vortex for 1 min. Mixtures were left at room temperature for 5 min, and centrifuged at 1300 r/min for 10 min at 4 °C. Resulting aqueous phases were transferred into clean tubes, supplemented with 8 μL of RNAse A (10 mg/mL, Sigma-Aldrich), and incubated at 37 °C for 15 min. RNAse treated DNA was extracted twice by the addition of 250 μL of CTAB solution and 650 μL of Chloroform: Isoamyl

Alcohol (24 : 1, v/v). For each extraction, mixtures were vortexed vigorously for 30 s, centrifuged at 1300 r/min for 10 min at 4 °C, and resulting aqueous phases transferred into clean tubes. Extracted DNA samples were precipitated in 650 μL of cold isopropanol and incubated at −20 °C overnight. On the next day, samples were centrifuged at 1300 r/min for 10 min at 4 °C, and pellets washed twice with cold 70% ethanol. Pellets were dried at room temperature for 30 min and resuspended with 30 to 40 μL of 1 × Tris-acetate EDTA buffer (TAE: 40 mmol/L Tris-acetate, 1 mmol/L EDTA). Extractions were visualized in 1 × TAE 1% agarose gels stained with GelRed (GLPBIO) and stored at −20 °C until further analysis.

Microsatellite amplification

DNA samples were 5- to 20-fold diluted in nuclease-free water and 2 to 3 μL used as template for microsatellite amplifications. Microsatellite markers included the highly polymorphic *loci* AfA10, AfA112, AfA120, AfA122, AfC103, AfD4, AfD12 and AfD105 (Lanzavecchia *et al.*, 2014). PCR amplifications were made for a final volume of 12 μL containing 0.2 μmol/L of each forward and reverse primer (Lanzavecchia *et al.*, 2014), 60 μmol/L of dNTPs, 10% Bovine Serum Albumin (BSA at 5 mg/mL), 1 × *Taq* buffer supplied with 1.5 mmol/L of $MgCl_2$ and 1 U of recombinant *Taq* DNA polymerase (Invitrogen). Amplifications were carried out as follows: 94 °C for 5 min, followed by 35 cycles of 94 °C for 20 s, 60 °C for 20 s, and 72 °C for 30 s, and a final extension at 72 °C for 30 min. All markers were amplified under same conditions with the exception of the AfA112 locus, which was amplified using 1 mmol/L of $MgCl_2$ and the touchdown PCR method (Korbie & Mattick, 2008) as following: 94 °C for 5 min, followed by 10 cycles of 94 °C for 20 s, 66–56 °C for 20 s (minus 1 °C every cycle), and 72 °C for 30 s, then 30 cycles of 94 °C for 20 s, 56 °C for 20 s, and 72 °C for 30 s, and a final extension at 72 °C for 30 min. All experiments included a non-template control (NTC), and amplifications were resolved in 1 × Tris-Borate-EDTA (TBE: 89 mmol/L Tris-borate, 2 mmol/L EDTA) 2% agarose gels stained with GelRed (GLPBIO).

Genotyping

Labeled microsatellite fragments were multiplexed according to the fluorescent dye incorporated in the forward primers (Lanzavecchia *et al.*, 2014). Each multiplexed microsatellite group contained up to 4 labeled fragments each. Genotyping mixtures were prepared for a final volume of 12 μL containing 9.5 μL of formamide, 0.5 μL of GeneScan 600 LIZ dye Size Standard v2.0 (Applied BioSystems) and 2 μL of the multiplexed microsatellite amplifications. Samples were denatured at 95 °C for 5 min in a preheated thermocycler and kept at −20 °C for 10 min prior to genotyping runs. Allele scoring was conducted in an ABI3130XL automatic sequencer (Applied BioSystems). Resulting fragment data files were analyzed and visually inspected in GeneMarker software v2.6.3 (Soft Genetics LLC) to assign sample genotypes per locus. Alleles were scored independently by two authors, and cross-validated before further analysis. Floating allele scores were transformed to integer numbers ("binning") using TANDEM V.1.09 (Matschiner and Salzburger, 2009) according to each microsatellite motif repeat size. Samples containing missing-data in ≥ 2 markers (25%) were excluded from further analysis. The final, binned genotyping data can be found in Data S1.

Genetic diversity and quality control parameters

Population genetic indices were obtained using the "basicStats" function implemented in the R/hierfstat package (Goudet, 2004). Allelic richness (AR) was calculated using the "allelic.richness" function from the same package, while unbiased expected heterozygosity (uH_e) was calculated as: $uH_e = H_e \times (2 \times n / (2 \times n - 1))$, where H_e is the expected heterozygosity and n the number of individuals in a given population. Inbreeding coefficient (F_{IS}) upper and lower confidence limits were assessed with 1000 bootstraps. Pairwise genetic distances (F_{ST}) between populations were estimated using the "genet.dist" function and the Weir & Cockerman (1984) model. Significance was assessed after 1000 bootstraps using the "boot.ppfst" function. Comparisons of F_{IS} and F_{ST} were considered statistically significant when the lower limit of the 95% confidence interval was different from zero. Deviations from the Hardy–Weinberg equilibrium (HWE) were tested using the "hw.test" function from the R/pegas package (Paradis *et al.*, 2017) under 10 000 Monte Carlo simulations.

Overall genetic diversity from WIL (parental), VL (F_{23}), and PL (F_{107}) were analyzed by one-way ANOVA, followed by the Tukey's test for multiple comparisons ($\alpha = 0.05$) using the R/stats functions "aov" and "TukeyHSD," respectively. To further test the hypothesis that the WIL population exhibits greater genetic diversity compared to VL and PL, one-sided Wilcoxon signed-rank test for paired samples, as implemented in the "wilcox.test" function (paired = TRUE, alternative = "greater"), was then used to verify significant differences ($\alpha = 0.05$) when considering pairs of microsatellite

markers (using the average of each $n = 7$ markers independently for each population). The same Wilcoxon test was used to verify the assumption for improvement in genetic diversity parameters after refreshment experiments. Comparisons were carried out between each parental laboratory strain (VL or PL) and refreshed lines (VLW and PLW, respectively) in the same moment in time (paired generations).

The one-way analysis of variance *F*-test was applied for the three quality control parameters [egg hatch (%), fliers (%), and sex ratio] obtained from the refreshed lines over 10 generations and their parental lines at the 5% of significance (ANOVA) and, when significant differences were detected, the Tukey's honestly significance difference (HSD) test ($\alpha = 0.05$) was applied to compare the means. Differences in egg hatch and fliers' parameters between the parental lines and each generation of the refreshed strains were also analyzed using the Kruskal–Wallis rank sum test, followed by Dunn's (1964) test for multiple comparisons, as implemented in the "kruskal.test" function from the R/stats package and the "dunnTest" function from the R/FSA package, respectively.

Linkage, selection, and population size

Non-random association of alleles at different *loci* (linkage disequilibrium) was tested with the functions "ia" (global) and "pair.ia" (pairwise) implemented in R/poppr package under 500 permutations. Effects of selection were tested for each microsatellite locus by applying the ln RH statistic (Kauer *et al.*, 2003) calculated as follows: $\ln RH = Ln\ [((1/(1 - H_{pop1}))^2 - 1)/((1/(1 - H_{pop2}))^2 - 1)]$. Ratios of expected heterozygosity were calculated for each locus using data from the WIL Parental population and subsequent generations: 3 (F_3 / P ratios), 6 (F_6 / P ratios), and 10 (F_{10} / P ratios) after laboratory foundation. Observed ln RH values for each locus were then standardized by the mean and standard deviation of ln RH values from *all loci* in the same population, following the formula: $Z = (x - \mu) / \sigma$, where x is the observed ln RH for the locus, μ is the mean, and σ is the standard deviation. Loci with standardized ln RH values falling outside the 95% confidence interval of the standard normal (Z) distribution (-1.96 to 1.96) were considered to be under selection. These were tested against the Z distribution using the "pnorm" function from the R/stats package. The contemporary effective population size (N_e) based on linkage disequilibrium was estimated using NeEstimator v2.1 (Do *et al.*, 2014), with a minimum allele frequency of 0.02 and a random mating model.

Genetic structure

Discriminant Analysis of Principal Components (DAPC) on allelic frequencies was performed using the "dapc" function implemented in R/adegenet package (Jombart *et al.*, 2010). The number of principal components (PCs) and genetic clusters to retain were estimated using the "xvalDapc" and "find.clusters" functions, respectively. Normal data ellipses were calculated using a multivariate *t*-distribution. Nei's genetic distances between populations were calculated using the "dist.genpop" function implemented in the R/adegenet package (Jombart *et al.*, 2010). Hierarchical cluster analyses (UPGMA) were then performed using "hclust" function from R/stats package, and node supports were calculated through 1000 bootstrap replicates using the "boot.phylo" function of the R/ape package (Paradis *et al.*, 2004). The presence of distinct genetic clusters (K) was investigated with STRUCTURE v.2.3.4 (Pritchard *et al.*, 2000). We used a no-admixture model, assuming correlated allele frequencies (Falush *et al.*, 2003) and using the sampling location information (LOCPRIOR) (Hubisz *et al.*, 2009) prior model. The most likely number of K was estimated through six independent replicate runs of 100 000 Markov chain Monte Carlo (MCMC) repetitions after an initial 100 000 burn-in period, assuming $K = 1$ to 6. Results were analyzed with Structure Harvester web version v.0.6.94 (Earl & vonHoldt, 2012), and the most likely K was determined according to Evanno's method (Evanno *et al.*, 2005).

Results

Domestication leads to genetic differentiation of A. fraterculus populations

It is generally accepted that the transitioning of new wild insect strains to rearing under artificial laboratory conditions (herein referred to as "domestication") promotes adaptation to the captive environment and a reduction in genetic diversity over time (Hoffmann & Ross, 2018). To test whether these assumptions apply to *A. fraterculus* populations from Brazil, we made estimates of various population genetics parameters in colonies at different stages of domestication process using flies of the Brazil-1 morphotype of *A. fraterculu*s. This included a wild-type colony (WIL) at the P or parental generation, the *Vacaria* laboratory strain (VL) at the F_{23} generation and the *Piracicaba* strain (PL) at the F_{107} generation. We reasoned that this approach would provide a "snapshot" of the genetic variability expected

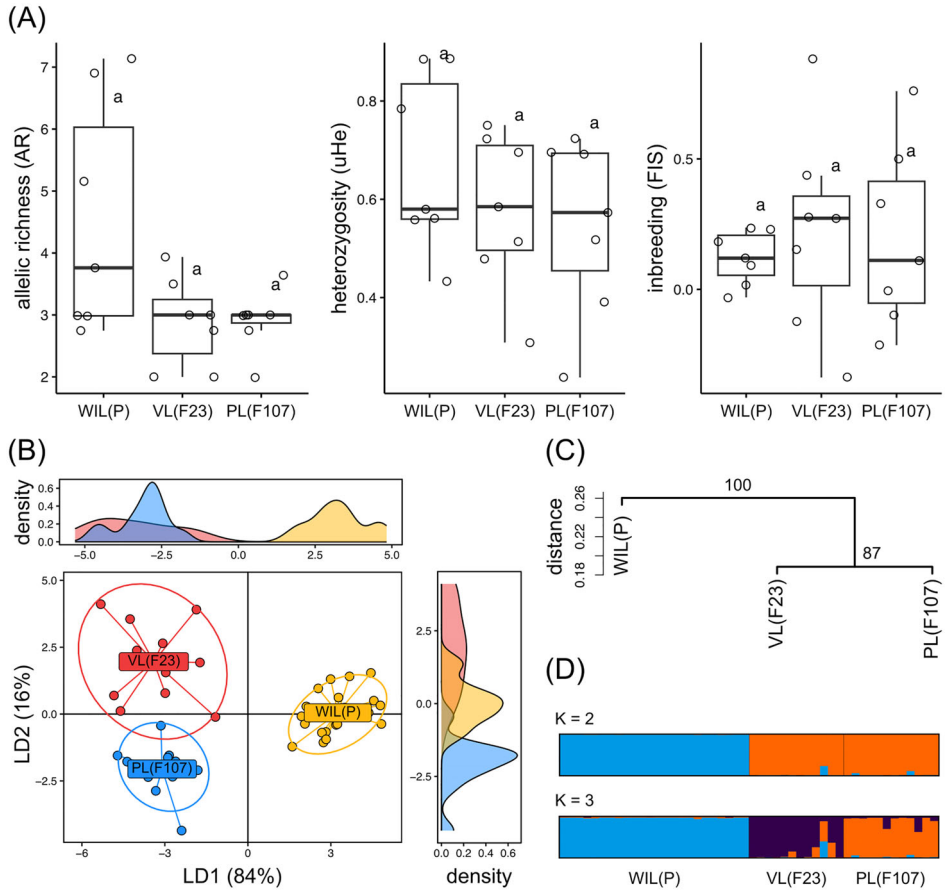

Fig. 1 Genetic variability and structure among *A. fraterculus* populations at different stages of domestication, including WIL (P) and the laboratory strains VL (F_{23}) and PL (F_{107}). (A) Genetic variability in terms of allelic richness (AR), unbiased expected heterozygosity (uH_e), and inbreeding coefficient (F_{IS}). Dots represent mean values for each microsatellite locus ($N = 7$) per population ($N = 12$–24 individuals). Significant differences between populations are indicated by different letters (one-way ANOVA with Tukey's HSD). (B) Discriminant Analysis of Principal Components (DAPC), where each dot represents an individual within population. (C) UPGMA clustering, with node support values calculated from 1000 bootstrap replicates. (D) Admixture analysis, where individuals are represented by vertical bars partitioned into $K = 2$ (optimal) or $K = 3$ (suboptimal) genetic clusters (components).

from the wild population as well as from strains after mid- and long-term domestication, respectively.

Estimates of genetic diversity (Table S1), including allelic richness (AR) and heterozygosity (uH_e), did not differ significantly between the samples of the strains analyzed here at the initial generations used for each experimental line (one-way ANOVA followed by Tukey's HSD, all P-adj ≥ 0.05), although a marginal significance difference in AR was observed between wild and laboratory flies [WIL (P) vs. VL (F_{23}), P-adj $= 0.049$; WIL (P) vs. PL (F_{107}), P-adj $= 0.053$]. The parental WIL strain had an overall higher level of diversity compared to its domesticated counterparts (Fig. 1A), and significant differences in AR were observed between wild and laboratory strains when each microsatellite pair was tested using the Wilcoxon matched-pair tests [WIL (P) vs. VL (F_{23}), $P = 0.039$; WIL (P) vs. PL (F_{107}), $P = 0.023$; and VL (F_{23}) vs. PL (F_{107}), $P = 0.531$]. Estimations of inbreeding (F_{IS}) also did not significantly differ between the samples analyzed. Nevertheless, as was seen in the genetic diversity results, the WIL strain showed smaller intervals for F_{IS} (mean, 95% CI = 0.121, 0.028–0.213), suggesting a moderate level of inbreeding. On the other hand, as expected, both laboratory colonies displayed a considerably higher level of inbreeding [F_{IS} mean, CI 95%: VL (F_{23}) = 0.223, (−0.128) – 0.575; PL (F_{107}) = 0.197, (−0.115) – 0.509]. This also resulted in a greater deficit of heterozygotes. Also, within populations, most of the loci (71.4% – 85.7%) examined here were found to be in HWE (Table S2 and Fig. S1)

The Discriminant Analysis of Principal Components (DAPC) of allelic frequencies revealed that the 3 strains appeared to be structured in three distinct genetic clusters (Fig. 1B). The WIL samples were all grouped in the positive range of values of linear discriminant 1 (LD1), while the VL and PL samples were grouped in the range of negative values for the same LD. The VL and PL samples were also grouped in distinct clusters by LD2. This pattern clearly indicates some degree of genetic differentiation among populations, despite their overall similar levels of genetic diversity. The UPGMA hierarchical clustering based on genetic distances according to Nei (1976), along with estimates of pairwise genetic variation (F_{ST}) based on the model of Weir & Cockerham (1984), also indicated a moderate but significant level of genetic differentiation among populations (Fig. 1C). This analysis also revealed that the VL and PL strains display closer genetic relationships (F_{ST}, CI 95% = 0.094, 0.037 – 0.149) compared to the WIL strain [F_{ST}, CI 95%: WIL (P) vs. VL (F_{23}) = 0.114, 0.056 – 0.178; WIL (P) vs. PL (F_{107}) = 0.110, 0.051 – 0.172] (Fig. 1C).

Analysis of molecular variance (AMOVA) further detected moderate genetic differences between the lines (phi-statistics = 0.173), with genotypic frequencies across all markers showing significant differences between each pair of populations (Exact G test, all *P-adj* $\leq$ 0.002). This level of population stratification was further supported by the admixture analysis (Fig. 1D). Here, when assigning $K = 2$ (best fit with $\Delta K = 51.25$), we found two distinct genetic clusters separating the wild-type from laboratory strains. Differences in the VL and PL genetic compositions were also observed when a suboptimal $K = 3$ was used.

Early stages of A. fraterculus domestication are marked by changes in genetic variability and gradual genetic differentiation

To gain a better understand of the dynamics leading to the differentiation observed in laboratory strains of *A. fraterculus*, we also looked at the level of genetic variability of the WIL population over 10 generations of domestication. No significant differences in terms of AR and uH_e (Fig. 2A) were found among generations of the WIL strain (one-way ANOVA followed by Tukey's HSD, all *P*-adj > 0.05), suggesting that only minimal genetic diversity is likely lost during the early stages of *A. fraterculus* domestication. However, when considering each microsatellite locus in paired tests, significant differences between the AR and the WIL parental generation sample were observed compared to samples from the F_6 generation and onward [Wilcoxon matched-pair tests, WIL (P) vs. WIL (F_6), $P = 0.023$] (Table S3). The mean inbreeding coefficient (F_{IS}) values indicated only moderate inbreeding level in the parental, F_1 and F_4 generations, but the F_3 population did display a significant level of inbreeding (mean, 95% CI = 0.179, 0.025 – 0.328) (Table S3) (Fig. 2A). Mean values of observed heterozygosity (H_o) were also relatively lower than mean uH_e (Table S3), a discrepancy generally attributed to some degree of inbreeding.

Overall, the DAPC analysis shows some overlap between the clusters representing the different strains sampled here (Fig. 2B), but it also reveals an apparent gradual genetic differentiation of the WIL strain over time as indicated by the clusters gradually shifting toward more positive values in both LD1 and LD2. This is consistent with the idea that once established in the laboratory, the WIL colony displayed a subtle yet consistent movement in the direction of genetic differentiation over each successive generation as indicated in Fig. 2B and Table S4. Similar trends were also observed in the UPGMA analysis (Fig. 2C) and pairwise F_{ST} comparisons (Table S4).

This gradual shift was also evident when visualizing population stratification in admixture analysis (Fig. 2D). We also identified two genetic clusters with the admixture analysis (when $K = 2$, best fit with $\Delta K = 25.33$), which also showed genetic composition shifting from one direction to another over time. Based on these results, it is possible to infer that the WIL population underwent two major episodes of genetic differentiation. The first occurred at generation F_3 [F_{ST}, lower limit CI 95%: WIL (P) vs. WIL (F_3) = 0.032, 0.012], where individuals start exhibiting a mixed genotype. The second event, involving more extensive differentiation, occurred between generations F_6 and F_{10} [F_{ST}, lower limit CI 95%: WIL (P) vs. WIL (F_6) = 0.057, 0.021; WIL (P) vs. WIL (F_{10}) = 0.069, 0.019] (Fig. 2D and Table S4). When considering a suboptimal $K = 4$, changes between the parental and initial generations in the laboratory became more apparent, with the most frequent wild-type genetic makeup (or alleles; blue bars in Fig. 2D) essentially disappearing after only four to six generations into laboratory conditions. Also consistent with the observations on AR (Table S3), the pairwise F_{ST} comparisons showed that the first detectable genetic differentiation in the laboratory generations occurred at F_6 [F_{ST}, lower limit CI 95%: WIL (F_1) vs. WIL (F_6) = 0.031, 0.001], showing moderate genetic differentiation between the WIL (P) and the F_6 generation (mean, CI 95% = 0.057, 0.093–0.021) (Table S4).

Interestingly, despite the clear indication of genetic differentiation, all WIL populations sampled conformed to

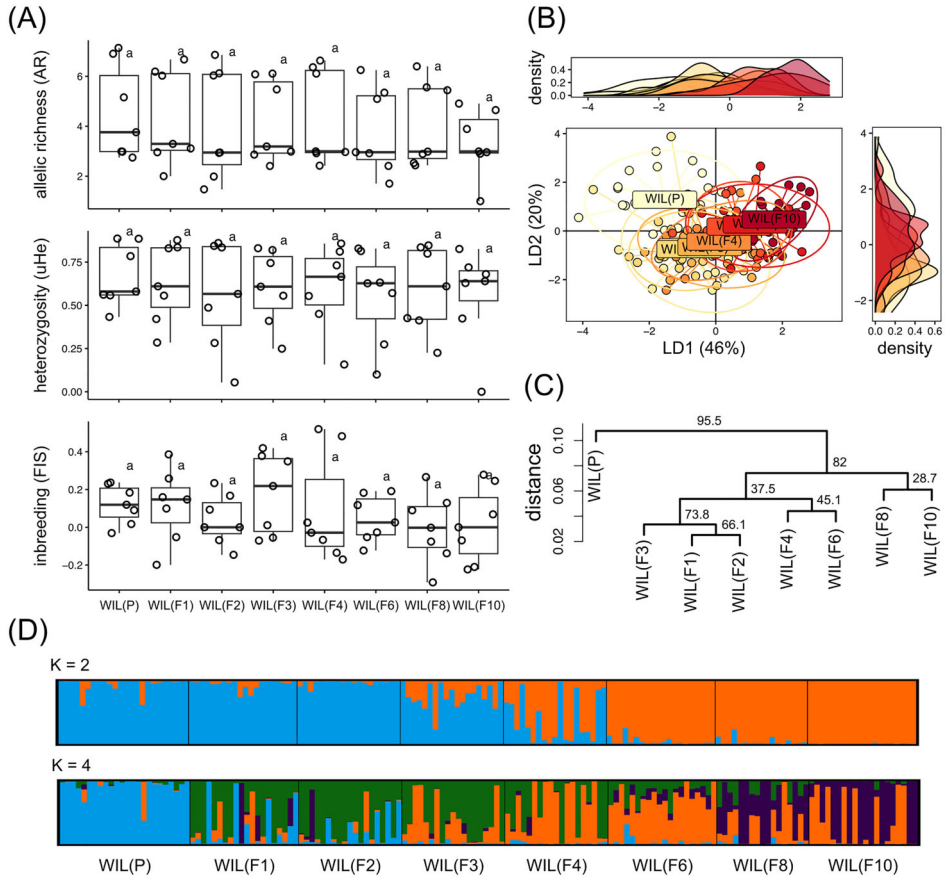

Fig. 2 Dynamics of genetic variability and structure in *A. fraterculus* during 10 generations of adaptation to laboratory rearing conditions. (A) Genetic variability in terms of allelic richness (AR), unbiased expected heterozygosity (uH_e), and inbreeding coefficient (F_{IS}). Dots represent mean values for each microsatellite locus ($N = 7$) per population ($N = 17-24$ individuals). Significant differences between populations are indicated by different letters (one-way ANOVA with Tukey's HSD). (B) DAPC clustering, where each dot represents an individual and populations are delimited by ellipsis. (C) UPGMA clustering. Node supports are given after 1000 bootstrap replicates. (D) Admixture analysis considering $K = 2$ (optimal) or $K = 4$ (suboptimal) genetic clusters (components).

HWE at most *loci* (Table S2). Since both genetic drift and directional selection are believed to occur during domestication, the genetic differentiation observed in laboratory populations may arise from the influence of these evolutionary forces over only a few *loci*. To identify possible deviations from neutral expectations, we calculated heterozygosity ratios (ln RH ratios) for each locus between the parental population and the F_3, F_6, and F_{10} generations of the WIL strain (Table S5). Typically, non-standardized ln RH ratios exhibit increasingly negative values over time [ln RH mean: WIL (F_3) / WIL (P) = −0.523; WIL (F_6) / WIL (P) = −0.881; and WIL (F_{10}) / WIL (P) = −1.789], indicating a loss of genetic variability relative to the parental colony in subsequent generations. Here, however, standardized ln RH ratios did not differ significantly from neutral expectations. The only exception was the microsatellite locus AfD105. This marker trended toward more negative values over generations (Table S5) and fell outside the 95% confidence interval of the standard normal distribution for the WIL (F_{10}) population ($P = 0.009$).

Significant linkage disequilibrium was observed in the WIL (F_6) (rbarD = 0.096, $P = 0.002$) and WIL (F_{10}) (rbarD = 0.100, $P = 0.002$) generations. Interestingly, the AfD105 locus appeared to be in linkage disequilibrium in both populations. This pattern seems to be related to the fixation of a single allele (151 bp), which increased from an initial frequency of 0.73 at the Parental generation, to 0.87 at F_3, 0.95 at F_6, and finally 1.0 at F_{10}. We also found evidence of a decrease in effective population size (N_e) in laboratory populations over time. Remarkably, the establishment of a laboratory colony

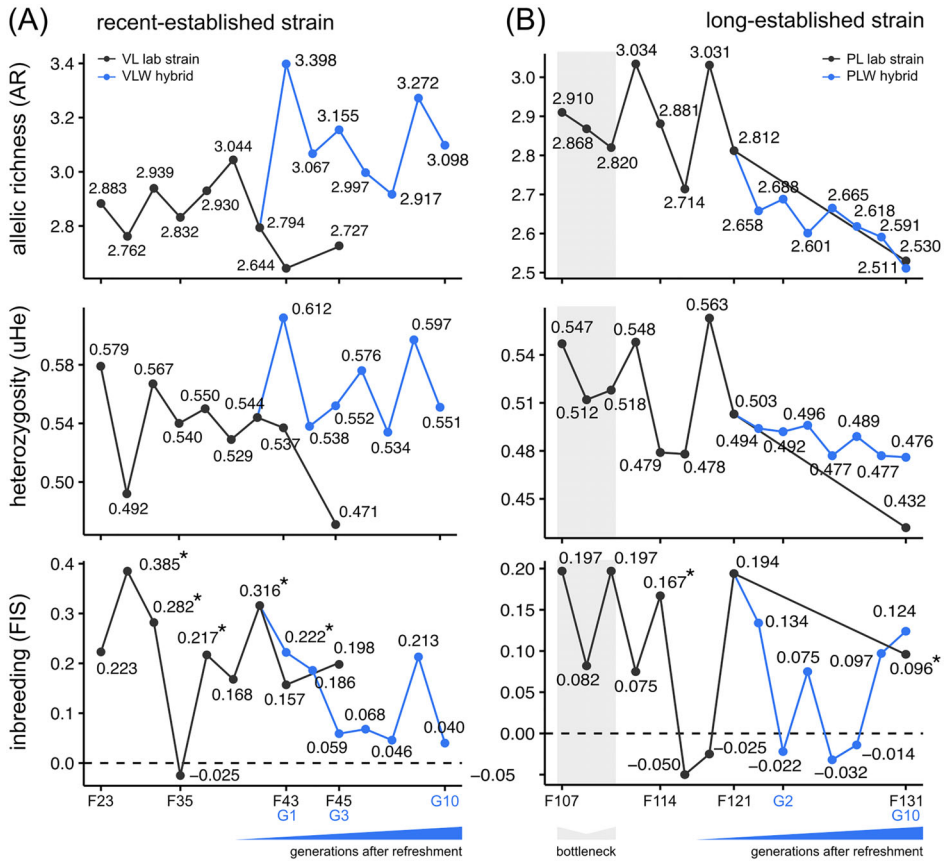

Fig. 3 Dynamics of genetic variability in the mid-term *A. fraterculus* strain of *Vacaria* (VL) (A) and the long-term inbred *Piracicaba* strain (PL) (B), with lines in terms of unbiased expected heterozygosity (uH_e), allelic richness (AR), and inbreeding coefficient (F_{IS}). The blue lines represent values for the refreshed colonies VLW [established with VL(F_{42}) vs. WIL males] and PLW [PL(F_{121}) vs. WIL males] at generations $G_{1\text{-}4}$, G_6, G_8, and G_{10}. Dots indicate the mean values per population ($N = 7$ microsatellite *loci*, and 9–32 individuals). F_{IS} values significantly different from zero are marked with "*," based on 95% CI derived from 1000 bootstrap replicates.

with wild individuals instantaneously reduced the N_e by more than a half in the 1st generation [N_e: WIL (P) = 571; WIL (F_1) = 234.9], with subsequent generations comprising only a fraction of the original wild population [N_e: WIL (F_3) = 78.3 (13.7% of the original population); WIL (F_6) = 20.3 (3.5%); and WIL (F_{10}) = 8.3 (1.4%)].

Temporal stability of genetic variation in laboratory strains of A. fraterculus

The genetic diversity parameters of the two *A. fraterculus* strains maintained under semimass rearing conditions at CENA for mid or long periods of time (VL and PL, respectively), both before and after refreshment, are presented in Fig. 3.

Despite some fluctuations, overall, the levels of genetic diversity in both laboratory strains appeared to be more stable across generations compared to that observed for the WIL colony. For instance, the largest drops in uH_e and AR in specific generations of VL were 16.4% ($F_{23} - F_{27}$) and 12.6% ($F_{41} - F_{42}$), respectively. In the PL strain, these parameters decreased by 13.5% ($F_{110} - F_{114}$) and 12.3% ($F_{110} - F_{116}$), respectively (Fig. 3). However, in both cases, the original levels were essentially restored within just a few generations following their decline, indicating that the changes were not permanent. In contrast, the WIL colony exhibited greater fluctuations in genetic diversity, with uH_e and, particularly, AR values decreasing substantially during the laboratory foundation phase by 18.2% ($P - F_6$) and 26.4% ($P - F_{10}$). Furthermore, for the WIL population, these values never returned to their original levels (Fig. 2).

The genetic parameters show stability in the VL strain, particularly for generations F_{31} through F_{42}, although as described this strain experienced a small reduction

of genetic variability between generations F_{23} and F_{27} (Fig. 3A). In contrast, genetic variability of PL does not seem to be affected in the same way, even after facing a bottleneck type reduction in population size. Here, the PL line experienced a strong decline in population size (from about 20 000 to less than 800 individuals) at generation F_{107}, which did not, however, result in an obvious loss of variability in all subsequent generations (Fig. 3B). A decrease in uH_e and AR in generation F_{109}, was observed, but both parameters also increased in the subsequent generations. Specifically, the allelic richness increased 13.5% (from 2.45 to 2.78) rapidly and returned to a normal level after the population size stabilized again at generation F_{110} (Fig. 3B).

Despite being 84 generations apart in laboratory culturing, both laboratory strains exhibited similar overall genetic diversity in terms of uH_e [mean ± SD: VL (F_{23} – F_{45}) = 0.512 ± 0.031; PL (F_{107} – F_{131}) = 0.486 ± 0.037; two-sided Student's *t*-test, df = 15.474, P = 0.138] and AR [mean ± SD: VL (F_{23} – F_{45}) = 2.839 ± 0.123; PL (F_{107} – F_{131}) = 2.844 ± 0.156; two-sided Student's *t*-test, df = 15.904, P = 0.942]. Additionally, these overall values of AR are lower and less variable than those observed for the WIL colony (P – F_{10}; 4.072 ± 0.373; two-sided Student's *t*-test, P < 0.00001 for both WIL vs. VL and WIL vs. PL). Therefore, considering the dynamics of AR observed for the WIL strain, our observations suggest that for the laboratory strains, stability of genetic variation—particularly in terms of allelic variability—arose early during the domestication process (∼20 generations, or approximately two years) and have been maintained over long periods of time.

Overall inbreeding coefficients (F_{IS}) indicated high levels of assortative mating (i.e., similar genotypes mate) in the VL strain [F_{IS} mean ± SD: VL (F_{23} – F_{45}) = 0.186 ± 0.107] but moderate levels in the PL strain [F_{IS} mean ± SD: PL (F_{107} – F_{131}) = 0.063 ± 0.093], with this difference being significant (two-sided Student's *t*-test, df = 15.715, P = 0.027). Interestingly, F_{IS} values were more uniform through the initial PL generations (excluding F_{110}, likely because of population recovery after bottleneck), while varying more in the VL line (Fig. 3). This was expected for PL as the line is more genetically homogeneous due to long-term inbreeding effects (uH_e = 0.49 ± 0.03).

The DAPC clustering analyses indicated low levels of genetic structure among generations in both PL and VL lines (Fig. 4A, B), although the VL line seems to be more resistant to the establishment of a homogeneous genetic identity (Fig. 4A, C). The STRUCTURE analysis revealed two distinct genetic groups within the VL strain (K = 2, best fit with a ΔK = 523.54; Fig. 4C), which seem to show little to no admixture. Curiously, the inferred ancestry proportions appear to remain relatively constant over generations of this strain (*f* mean ± SD: blue = 0.63 ± 0.10; orange = 0.33 ± 0.09; and hybrid = 0.05 ± 0.05), supporting the idea of favored assortative mating between individuals within the genetic clusters.

In contrast, the PL strain seems to be composed of three main genetic clusters (K = 3, best fit with ΔK = 2.81) (Fig. 4D). All individuals in the PL strain exhibit gene pools consistent with high levels of admixture. We detected a slight differentiation in PL individuals sampled from generations after a bottleneck. For example, the genetic composition from one generation to the next generation (F_{108}) seemed to change (see blue bars in Fig. 4D). A drastic change in allelic frequencies was observed between generations F_{116} and F_{118} of PL, during which the rarer genetic composition became dominant (Fig. 4D). This is also demonstrated by the low observed values of uH_e and AR (Fig. 3). This reduction in genetic diversity was followed by a rapid increase in subsequent generations (Fig. 3B), and the "typical" stable level of genetic variation was restored by generation F_{121}.

Effects of refreshment on mid- and long-established colonies of A. fraterculus

To investigate the effects of the refreshment strategy on the genetic variation in the *A. fraterculus* colonies, we performed crosses between males of the WIL (P) colony and females from the mid- and long-established laboratory strains VL (F_{23}) and PL (F_{107}). We then followed the changes of genetic variation in the refreshed colonies (referred here as VLW and PLW, respectively) for 10 generations. Simultaneously, we assessed some quality control parameters routinely verified in mass-rearing facilities. If the refreshment strategy is effective for *A. fraterculus*, we would expect to see an increase in genetic diversity (uH_e and AR), a decrease in inbreeding levels (F_{IS}), and an enhancement of quality control parameters in flies of the refreshed colonies.

We found that refreshment had different effects on each laboratory strain (blue lines in Fig. 3A, B). For the VL strain, we observed a significant increase in both uH_e [Wilcoxon matched-pair tests: VLW (G_1) vs. VL (F_{43}), and VLW (G_3) vs. VL (F_{45}), both P = 0.016] and AR [Wilcoxon matched-pair tests: VLW (G_1) vs. VL (F_{43}), and VLW (G_3) vs. VL (F_{45}), both P = 0.039] following the introduction of WIL males when compared to paired generations of VL without refreshment (Fig. 3A). In contrast, refreshment seemed to have little or no effect on

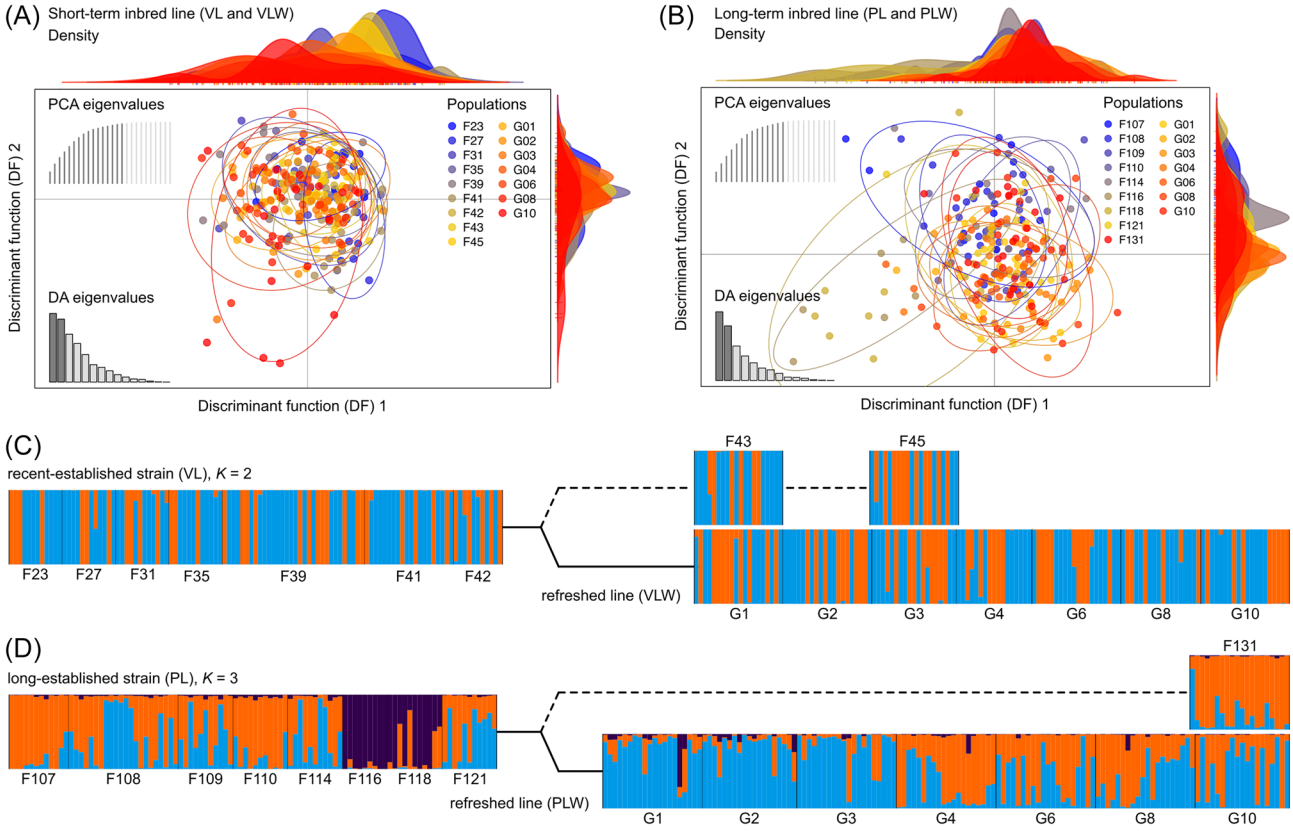

Fig. 4 Genetic structure of VL and PL lines of *A. fraterculus* during inbreeding generations and after refreshment with WIL genetic material. (A, B) Discriminant analysis of principal components (DAPC) clustering putative genetic groups within generations. Each dot represents a single genotyped individual, while the color indicates the generation from where the individual was sampled. Clusters are defined by ellipses and indicate the variance within the generation. (C, D) STRUCTURE admixture analysis showing the probability of assignment of each individual (represented by a vertical line) per generation to their optimal number of genetic cluster (*K*).

the genetic diversity of the PL strain [Wilcoxon matched-pair tests for both u_e and A_r: PLW (G_{10}) vs. PL (F_{131}), $P > 0.05$]. The measures of uH_e and AR seem to decline in both refreshed strains after about 6 to 8 generations of inbreeding (Fig. 3).

A strong effect of refreshment was also detected in terms of F_{IS} (Fig. 3). We observed a trend of F_{IS} values approaching to zero in the refreshed VLW line, indicating decreased inbreeding levels. This trend likely reflects the (re)introduction of wild new alleles and a corresponding increase in overall heterozygosity. Admixture analysis also showed that the VLW line is composed of two genetically groups, similar to its parents from the VL strain (Fig. 4C). On the other hand, changes in F_{IS} values for the PLW strain were less pronounced, although the genetic composition of the PL strain appeared to change following the introduction of WIL material, particularly during the first generations [PLW (G_1 – G_3); Fig. 4D]. This might suggest the (re)introduction of rare alleles or new genetic lineages into the long-established laboratory line.

To assess quality control parameters in the refreshed lines, we monitored egg hatch, fliers, and sex ratio between the domesticated parental colonies [VL (F_{42}) and PL (F_{107})] and samples from their refreshed lines (VLW and PLW) over 10 consecutive generations (Table 1). In both refreshed lines, no significant differences were found between the means of those three quality control parameters for the parental generations [VL (F_{42}) and PL (F_{107})], and the 1st and 10th generations of both refreshed strains. Significant differences, however, were noticed between the egg hatch means of the parental [VL (F_{42})] and G_6 generation of the VLW ($P < 10^{-3}$), and between mean percentages of fliers of the parental [VL (F_{42})] and G_7 generation of the same strain ($P = 0.01$) (Table S1).

Looking only at the comparisons between each generation and its parental group, the mid-established VL strain appeared to benefit the most from refreshment, with these

Table 1 Quality control parameters (means ± SE) observed for the refreshed colonies of the *Piracicaba* (PLW) and *Vacaria* (VLW) strains over 10 generations.

		Quality control parameter		
Strain	Generation	Egg hatch (%)	Fliers (%)	Sex ratio (♀/♂+♀)
PLW	Parental (PL–F_{121})	63.8 ± 3.0 b	78.0 ± 5.7	0.52 ± 0.04
	G_1	73.1 ± 2.7 ab	88.8 ± 2.4	0.53 ± 0.05
	G_2	71.8 ± 3.7 ab	98.3 ± 0.9	0.4 ± 0.03
	G_3	70.6 ± 0.4 ab	92.9 ± 3.6	0.5 ± 0.03
	G_4	60.2 ± 1.5 b	95.7 ± 1.7	0.49 ± 0.08
	G_5	81.1 ± 1.6 a	87.6 ± 8.1	0.6 ± 0.05
	G_6	69.5 ± 2.1 ab	89.7 ± 9.2	0.5 ± 0.05
	G_7	73.9 ± 4.6 ab	97.1 ± 2.9	0.5 ± 0.04
	G_8	66.8 ± 0.2 ab	99.3 ± 0.7	0.5 ± 0.08
	G_9	65.4 ± 4.1 b	94.6 ± 3.2	0.5 ± 0.07
	G_{10}	69.7 ± 3.6 ab	99.9 ± 0.1	0.47 ± 0.03
	ANOVA	$F = 3.7$	$F = 2.1$	$F = 0.72$
		$P = 0.006$	$P = 0.07$	$P = 0.69$
VLW	Parental (VL–F_{42})	77.9 ± 1.3 bc	95.0 ± 7.6 b	0.56 ± 0.03
	G_1	81.6 ± 2.6 abc	87.7 ± 7.4 ab	0.56 ± 0.02
	G_2	83.2 ± 2.7 abc	95.5 ± 1.6 ab	0.45 ± 0.01
	G_3	75.6 ± 2.1 c	79.5 ± 12.9 ab	0.52 ± 0.05
	G_4	76.6 ± 2.8 bc	93.3 ± 3.0 ab	0.46 ± 0.06
	G_5	78.1 ± 1.0 abc	95.5 ± 1.5 ab	0.59 ± 0.03
	G_6	88.5 ± 0.7 a	91.3 ± 4.8 ab	0.63 ± 0.02
	G_7	86.4 ± 1.9 ab	99.8 ± 0.1 a	0.45 ± 0.05
	G_8	86.6 ± 1.2 ab	97.7 ± 0.1 ab	0.61 ± 0.07
	G_9	76.8 ± 2.1 bc	98.6 ± 1.4 ab	0.53 ± 0.02
	G_{10}	85.1 ± 3.1 abc	95.5 ± 0.2 ab	0.49 ± 0.06
	ANOVA	$F = 4.9$	$F = 3.6$	$F = 2.1$
		$P < 10^{-3}$	$P = 0.01$	$P = 0.07$

Note: Means (± SE) followed by the same letters in the columns do not differ significantly by the Tukey's test ($P > 0.05$).

lines exhibiting better hatching (Kruskal–Wallis, $\chi^2 = 22.3$, df = 10, $P = 0.013$) and a higher number of fliers (Kruskal–Wallis, $\chi^2 = 20.3$, df = 10, $P = 0.026$) (Table S6). In contrast, the long-established PL strain displayed only marginal improvements, with hatching showing marginal significance (Kruskal–Wallis, $\chi^2 = 18.4$, df = 10, $P = 0.049$) and fliers only approaching significance (Kruskal–Wallis, $\chi^2 = 17.2$, df = 10, $P = 0.071$) (Table S6). Overall, refreshment apparently did not drastically alter the laboratory rearing fitness of the two strains, indicating that the improvements are minimal, although potentially beneficial.

Discussion

Despite the importance of mass reared colonies of insects for the successful application of SIT as part of effective biological control programs, to date many fundamental questions remain unanswered regarding the optimal methods to assess the genetic health of these colonies, especially as they are maintained over extended periods of time. Here, we used the South American fruit fly, *Anastrepha fraterculus* (Brazil-1 morphotype), to begin addressing some of those questions. Specifically, because it is generally believed that adaptation of wild strains to the captive environment promotes a reduction in genetic diversity over time (Hoffmann & Ross, 2018) via inbreeding, selection, and bottlenecking type events, we investigated if colonies of *A. fraterculus* would show such loss of genetic diversity during the process of domestication and the patterns of genetic variability resulting from long-term mass-rearing of different domesticated strains. We also investigated whether refreshment schemes designed to introduce material from wild flies into

mid- and long-term laboratory colonies would substantially alter their genetic makeup or quality parameters.

To address questions regarding the effects of initial domestication, we found multiple examples of evidence for moderate but significant genetic differentiation between a wild collected population (WIL) and two laboratory strains [VL (F_{23}) and PL (F_{107})]. The VL and PL strains were genetically more similar to each other (Fig. 1B), even though VL was originally founded by individuals from the same location as WIL, and thus expected to share a common origin. This suggests that much of the differentiation among these populations can be attributed to the domestication process. We did also observe significant differences in AR between wild and laboratory colonies when scores for each microsatellite locus were considered in pairwise comparisons, which would be consistent with the loss of rare (low frequency) wild alleles in laboratory colonies. However, despite some indications of differentiation, the average genetic diversity in terms of AR and uH_e did not significantly differ among the three populations, suggesting that domestication alone of *A. fraterculus* may have a modest impact on genetic diversity. This is somewhat surprising given that a variety of events associated with domestication such as bottlenecks, drift, founder effect, inbreeding or different selection pressures have been reported in drosophilids and other tephritids (Tsakas & Zouros, 1980; Loukas *et al.*, 1985; Briscoe *et al.*, 1992; Haymer, 1995; Simões *et al.*, 2008; Hernández *et al.*, 2009; Gilchrist *et al.*, 2012; Zygouridis *et al.*, 2014). However, as in our study, Ruiz-Montoya *et al.* (2024) also observed a low level of genetic differentiation among two mass-reared strains (both with more than 90 generations in laboratory) and a wild population of *Anastrepha ludens* (Loew) (Diptera: Tephritidae). According to these authors, mass-rearing conditions did not significantly reduce the genetic variability of laboratory colonies of *A. ludens* compared to a wild population. Finally, also in agreement with the genetic diversity findings, the estimations of F_{IS} suggested comparable inbreeding levels between laboratory colonies and WIL (Fig. 1A), but the WIL strain showed smaller intervals for F_{IS}. Furthermore, those populations largely conformed to the HWE, whereas loci not in HWE were not consistent among populations.

To further examine the WIL population for signs of genetic differentiation during the establishment of a new laboratory strain, we monitored levels of genetic diversity over 10 generations (approximately one year) of domestication under laboratory conditions. We divided the adaptation of WIL to laboratory conditions into three main categories, as illustrated by UPGMA dendrogram and admixture analysis (Fig. 2C). The initial period (parental to F_3) is characterized by a gradual decrease in genetic variability at specific microsatellite loci, increased levels of inbreeding, and noticeable changes in genotypic frequencies, all of which could result from a founder effect. Next, there is an intermediate period (F_4 to F_6) in which most of the wild genetic variability seem to be lost. This is reflected in significant differences in AR, uH_e and F_{IS} observed for samples from the F_6 generation onward (Table S3). In a final period (F_8 to F_{10}), the genetic variation in the laboratory colonies seems to be somewhat stabilized. Our results are similar to a study of the Tucumán wild population tested by Parreño *et al.* (2014) where both mean AR and H_e tended to decline during the first 6 generations under laboratory conditions of rearing. Similarly, in *Bactrocera* spp., considerable changes in genetic variability during domestication have been reported between generations F_4 and F_{10} for *Bactrocera tryoni* (Froggat) (Gilchrist *et al.*, 2012) and generations F_1 and F_5 for *Bactrocera oleae* (Rossi) (Zygouridis *et al.*, 2014).

As reviewed by Hopper *et al.* (1993), genetic drift, directional selection, and inbreeding are the main mechanisms driving genetic changes under laboratory conditions. Drift is often the dominant evolutionary force when populations are isolated and small, as is the case of small experimental colonies. However, according to population genetics theory, there is little concern for significant, long-term loss of genetic variability due to drift (Nei *et al.*, 1975), all of which is consistent with our data. We also found no strong evidence of directional selection, as ln RH ratios largely conformed to neutral expectations. In this scenario, genetic drift is likely to progressively purge genetic variation over time, as also suggested by ln RH ratio values.

Therefore, we conclude that our results largely align with Parreño *et al.* (2014), suggesting that genetic drift is the primary evolutionary force influencing genetic variability and differentiation in *A. fraterculus* during domestication, while intrinsic mechanisms help maintain the remnant genetic diversity over longer periods of time. Zygouridis *et al.* (2014) attributed that to the higher level of plasticity in generalist species (e.g., with a wide range of hosts for oviposition) that can allow for an easier adaptation to laboratory conditions compared to specialized species such as the olive fly. Specialized species may experience a more significant decrease in N_e, which accelerates the process of genetic drift, leading to faster genetic differentiation (Waples, 2013).

Considering the genetic variation of the VL and PL strains, which had been maintained under semimass rearing conditions at CENA/USP, we observed that the levels of genetic diversity of each strain were maintained at similar levels over a period of more than two years

of rearing (Fig. 3). Rare alleles tend to rapidly disappear in long-term breeding strains, where the dynamics of genetic diversity favors homogeneity (Fig. 4). The relatively lower and more uniform variability of the PL line through generations (Fig. 3B) are probably consequences of long-term inbreeding. The PL line was the first *A. fraterculus* strain to be domesticated in Brazil, and as such it has experienced more selective pressures compared to other more recent strains. Its founding population was very small (e.g., 262 parental females, and 42 F_1 females) and came from a single host (*Eugenia pyriformis* Cambess.) (Walder *et al.*, 2014). To obtain eggs more quickly, in some generations after the 10th, high densities of flies ($>$ 0.5 flies/cm^2) were used in the mother colony cages and the light was left on all night long. No refreshment was performed with wild flies at any time. The colony has gone through at least three major bottlenecks over the years (Costa, personal communication).

One bottleneck event occurred at the F_{107} generation of PL, which led to a decrease in uH_e and AR up to generation F_{109}, but both parameters increased in the next generation (Fig. 3B). Population bottlenecks can strengthen quantitative genetic differentiation resulting from lower genetic variation and increased inbreeding in Diptera (Bryant *et al.*, 1986). However, short duration bottlenecks (e.g., if population size increases rapidly right after the event) may have little effect on heterozygosity (Nei *et al.*, 1974; Nei *et al.*, 1975).

Besides the bottleneck event of PL at F_{107}, another change in the genetic structure of the population was noticed between generations F_{116} and F_{118} (Fig. 4D). For this colony, at various times such as during vacation and end-of-year recess periods, fluctuations in the maintenance of these mother colonies are common due to staff reductions and consequent less-careful handling that insects are subjected to. However, the only quality control parameter that appeared to be affected here was pupal weight, which was lower in F_{116} (10.9 mg) than in the F_{117} and F_{118} (12.4 and 13 mg, respectively), which could probably have been the result of improper management of the temperature (heating) of the larval rearing room in the F_{116} generation (M.L.Z. Costa, personal communication).

Another factor that may also be influencing the genetic diversity of the different strains of the same *A. fraterculus* morphotype is population size. The VL population has been maintained at high population levels and without bottlenecks since its establishment, which may have helped to promote a relatively greater genetic diversity than that of PL and even providing interesting mutant phenotypes for the development of genetic sexing strains (Meza *et al.*, 2020), including the black puparium, which recently was linked to mutations at the *ebony* gene in *A. ludens* (Paulo *et al.*, 2025). For other dipterans, such as mosquitoes, colonies maintained at small sizes ($\leq$ 100 individuals) can suffer more quickly from inbreeding depression and present fitness costs, compromising the success of the SIT (Ross *et al.*, 2019).

Despite those minor differences, our findings indicate that genetic variation in established laboratory strains of *A. fraterculus* show stability overall, as evidenced by consistent levels of AR over extended generations. While minor fluctuations were observed in specific generations (e.g., PL after the bottleneck at F_{107} and VL between F_{23} and F_{27}), the overall AR within each strain remained relatively low and constant, in contrast to that of higher and more variable levels of AR in the wild strain. These observations suggest that selective pressures associated with domestication, such as inbreeding and reduced population size, drive an initial reduction in allelic variability, which is later stabilized at relatively lower levels in domesticated strains. Notably, AR values are comparable between VL and PL strains, despite being 84 generations apart, indicating that this process occurs rapidly, within approximately 20 generations (or two years) under laboratory conditions.

Interestingly, PL and VL also showed lower genetic diversity values than those displayed by the TW and CL strains analyzed by Parreño *et al.* (2014). This may be explained by differences in founder populations or laboratory practices. Nevertheless, for the long-established CL line analyzed by Parreño *et al.* (2014), consistent with our results, no significant differences were found in genetic diversity indexes over several generations.

Regarding our study of refreshment, the introduction of wild males helped to reduce inbreeding in both laboratory strains, but most significant effects in genetic variation were noticed in VL (Fig. 3). Here, our results suggest that successful admixture from refreshment is more likely to occur in short-term domesticated colonies. It may also be true that the impact of admixture on refreshment is reduced in long-term domesticated colonies because these males may have adaptations to the laboratory environment that confer competitive advantages to them in terms of mating success inside the colony cages.

It has also been hypothesized that local adaptations may act against admixture, even in the presence of inbreeding depression (i.e., decrease in fitness and heterozygosity in the offspring of related parents), as the benefits of such possible adaptations would be greater than the cost of inbreeding (Verhoeven *et al.*, 2011). This concept might also be applied in the context of domestication, with adaptations arising in response to long-term rearing and which in turn may result in some degree of differentiation in genetic and behavioral traits

between domesticated and wild individuals (Rull *et al.*, 2005; Pereira *et al.*, 2007; Viscarret *et al.*, 2008; Hoffmann & Ross, 2018). Consistent with this, our results show that genetic diversity indexes (AR and uH_e) from both refreshed colonies showed a certain downward trend after 6−8 generations under laboratory rearing conditions (Fig. 3). According to the same hypothesis (Verhoeven *et al.*, 2011), newly established populations ("wildish" colonies) are less likely to have already established local adaptations, and therefore are expected to be more receptive to population admixture.

It is also important to notice that the founders of the recent established VL line came from the same municipium (Vacaria, Rio Grande do Sul state) as the wild-type flies used in the refreshment experiments. Therefore, we cannot discard the possibility of some level of genetic incompatibility between WIL and PL individuals. Using flies from the 50th generation of the PL strain, Dias *et al.* (2016) observed partial sexual incompatibility between PL and four populations from southern Brazil. It is worth mentioning that outcrosses between short-term established colonies instead of wild flies could potentially lead to similar genetic benefits, as demonstrated for *B. tryoni* (Gilchrist & Meats, 2014).

Overall, the genetic diversity parameters (Fig. 3) we estimated suggest that refreshments with wild material should be performed about every six to eight generations (i.e., about once a year), and that this would be beneficial to increase or at least maintain a more "wildish" genetic diversity of *A. fraterculus* colonies of the Brazil-1 morphotype in laboratory conditions. Similar conclusions were obtained for the olive fly, *B. oleae* (Zygouridis *et al.*, 2014). Also, because inbreeding depression is of major concern in the management of laboratory colonies used in SIT release programs, our data suggest that laboratory strains of *A. fraterculus* could benefit from refreshment via infusions using "new" genetic material. This would help to reduce the effects of genetic drift or inbreeding depression, especially in small or isolated populations such as laboratory colonies.

This study is the first to examine the dynamics of genetic variability during the domestication of a wild population of the *A. fraterculus* Brazil-1 morphotype over several generations, in addition to assessing the effects of long-term laboratory rearing and refreshment on genetic and biological parameters of different *A. fraterculus* colonies. Our results provide valuable insights into the genetic changes that can occur throughout domestication or that can appear in colonies of the same *A. fraterculus* morphotype if maintained under different laboratory pressures over time. Important recommendations for the management of *A. fraterculus* colonies were also given, such as the need to avoid population bottlenecks and to perform refreshments every six to eight generations. Future research should include different or a larger number of markers and replicate populations to further investigate the influence of population size on genetic diversity and to distinguish the effects of laboratory adaptation from genetic drift or founder effects.

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Disclosure

All the authors confirm there is no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Heatmap representation of *P*-values of Hardy–Weinberg Equilibrium (HWE) (0 to $\geq$ 0.05) for WIL (parental), VL (F_{23}) and PL (F_{107}) populations.

Table S1 Genetic variability estimates in the wild strain WIL(P) population and laboratory-established strains VL(F_{23}) and PL(F_{107}) of *Anastrepha fraterculus*.

Table S2 Estimations of Hardy–Weinberg Equilibrium (HWE) per microsatellite *loci* in the wild strain WIL and laboratory-established strains VL(F_{23}) and PL(F_{107}) of *Anastrepha fraterculus*.

Table S3 Genetic variability estimates in the *A. fraterculus* wild-type (WIL) population during 10 generations of domestication.

Table S4 Genetic differentiation (F_{ST}) among generations of the wild Vacaria strain (WIL).

Table S5 Expected heterozygosity (He) and ratios of expected heterozygosity for each microsatellite locus from samples of the parental (P), F_3, F_6 and F_{10} generations of the wild strain (WIL).

Table S6 Differences in egg hatch and fliers' parameters between the parental lines (PL and VL) and each generation of the refreshed strains (PLW and VLW).

