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ORIGINAL ARTICLE

Strong variation in frequencies of male and female determiners between neighboring housefly populations

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Abstract Sex-determination mechanisms evolve rapidly and vary between species. Occasionally, polymorphic systems are found, like in the housefly. Studying the dynamics and stability of such systems can provide a better understanding of the evolution of sex-determination systems. In the housefly, dominant male-determining loci (*M*) can lie not only on the Y chromosome (M^Y), but also on autosomes (M^A) or the X chromosome (M^X). *M* enforces male development by inhibiting the female-determining gene *transformer* (*tra*). A mutant *tra* allele, tra^D, is insensitive to *M* and is a dominant female determiner. M^Y prevails at high latitudes and polymorphic *M* loci together with tra^D at low latitudes. To get more insight into the stability and frequencies of these sex determiners with mutually exclusive dominance, we investigated 5 regional Spanish populations. We found strong variation among populations. Two populations with hemizygous M^{III} were found, 2 contained homozygous M^X with additional hemizygous M^I and M^{II} in 1 population. One population contained homozygous and hemizygous M^X with additionally hemizygous M^{II}. All females in populations with homozygous *M*, had tra^D, whereas no tra^D was found in populations without homozygous *M*. Our results indicate locally stable systems may either harbor a single hemizygous *M* and no tra^D, corresponding to a male heterogametic system, or homozygous and/or multiple *M* and heterozygous tra^D, reminiscent of a female heterogametic system. They support that *M* loci can accumulate in the presence of a dominant female determiner. Limited migration between populations may contribute to the stability of these systems.

Key words aneuploidy; housefly; polymorphic sex determination; recombination; regional variation; sex-determining loci

Introduction

Despite sexual reproduction being ubiquitous, the mechanisms that determine maleness and femaleness vary from one species to another (Beukeboom & Perrin, 2014). In most species, only a single sex-determination mechanism is present, but in some species multiple systems co-occur (Charlesworth & Mank, 2010; Bachtrog *et al*., 2014; Beukeboom & Perrin, 2014; Blackmon *et al*., 2017). Such polymorphic systems are of interest for identifying the factors that govern the evolution of sex determination and sex chromosomes. In fact, it is not well understood whether and how multifactorial (or polygenic) systems of sex determination can be stable, or whether they are a transient stage between an ancestral and derived state (Rice, 1986; Moore & Roberts, 2013; van Doorn, 2014). To gain a better understanding of the evolutionary dynamics of sex determination requires empirical data on the temporal and spatial distribution of male and female-determining factors in polygenic systems.

The housefly (*Musca domestica*, Diptera) is known to possess a polymorphic sex-determination system (Franco *et al*., 1982; Denholm *et al*., 1986; Tomita & Wada,

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1989; Feldmeyer *et al*., 2008; Kozielska *et al*., 2008). In houseflies, maleness is induced by an *M* locus that interferes with the function of the *transformer* (*tra*) gene that determines femaleness (Hediger *et al*., 1998a; Dübendorfer *et al*., 2002). *M* loci have been found not only on the Y chromosome but also on any of the 5 autosomes (M^I, M^{II}, M^{III}, M^{IV}, and M^V, collectively known as M^A) and even the X chromosome (M^X, together with M^Y known as M^{SEX}) (Franco *et al*., 1982; Feldmeyer *et al*., 2008; Kozielska *et al*., 2008; Hamm *et al*., 2015). *M* is dominant over the wild-type allele of *tra*, but there exists a mutant allele of *tra* (tra^D) that is insensitive to the action of *M* and acts as a dominant female determiner (Dübendorfer *et al*., 2002; Hediger *et al*., 2010). It is not understood which evolutionary forces drive this polymorphism of male and female determiners (Kozielska *et al*., 2010; Hamm *et al*., 2015; Meisel *et al*., 2016; Adhikari *et al*., 2021).

The genomic distribution of autosomal *M* loci and the frequency of the tra^D allele follow a latitudinal cline on a global scale (Franco *et al*., 1982; Feldmeyer *et al*., 2008; Kozielska *et al*., 2008). M^A and tra^D occur with higher frequency in low latitude regions, whereas at high latitudes, M^Y is mostly found and tra^D is absent (Tomita & Wada, 1989; Hamm *et al*., 2005; Feldmeyer *et al*., 2008). However, little is known about the spatial and temporal distribution of these sex-determining factors at a more local scale. Kozielska *et al*. (2008) and Meisel *et al*. (2016) investigated natural populations with a 25- and 22-year intermission and concluded that the polymorphic sex determiners, that is, similar frequencies of *M* loci and tra^D allele, had remained rather stable. This suggests that a stable equilibrium of multifactorial sex determiners had been established. Such an equilibrium seems in conflict with the theoretical prediction that the sex-determining system of populations that contain both dominant male and female determiners will eventually evolve toward one of the alternative single factor states (Bull, 1985; van Doorn, 2014). However, selective advantage of autosomal *M* loci (e.g., M^{II} and M^{III}) has also been reported from laboratory population experiments (Hamm *et al*., 2005; Kozielska, 2008), and may oppose this tendency. Moreover, Delclos *et al*. (2021) found thermal tolerance and preference to differ between males that carry M^Y and those that carry M^{III}. To assess whether polymorphism equilibria occur in field populations, further study of the dynamics of sex-determination gene variants in local housefly populations in various regions is therefore needed. This may be informative about the stability of this polymorphic sex-determination system and help identify evolutionary forces responsible for the observed differences in frequencies of male and female determiners.

Here, we assess the frequency and genomic distribution of sex-determining factors at a high spatial resolution, by investigating 5 neighboring housefly populations in the province of Catalonia, Spain. Specifically, we ask: (i) at which chromosomes *M* loci are present; (ii) whether tra^D is present; and (iii) how frequencies and homozygosity of *M* loci correlate with tra^D presence. To determine which chromosomes harbor *M* loci, we apply a previously used mapping method that relies on recessive autosomal marker segregation (Tomita & Wada, 1989; Kozielska *et al*., 2008; Feldmeyer *et al*., 2010; Meisel *et al*., 2016) and karyotyping. Presence of the tra^D allele is determined by diagnostic polymerase chain reaction (PCR). The experimental design also allowed us to check for non-Mendelian inheritance of markers and to determine male recombination frequencies and the occurrence of nondisjunction of sex chromosomes. We discuss how our results contribute to a better understanding of the population genetics and evolution of polymorphic sex-determination systems.

Materials and methods

Housefly populations and maintenance

Houseflies were collected from 5 cattle farms in an area spanning approximately 80 × 70 km, in the province of Catalonia in Spain (designated strains SPA1-5) in October 2015 (Fig. 1) and 1 cattle farm in the north of the Netherlands (strain NL1, collecting site 53°13′57.4"N/6°12′00.7"E) in September, 2016. Adults were caught by sweeping an insect net close to cows or calves. About 100–150 houseflies were captured per site, except for SPA5 of which approximately 30 houseflies were collected. Houseflies were taken back to the laboratory and reared at 25 °C with L : D 12 : 12 h photoperiod in a climate room according to Kozielska *et al*. (2008).

The long maintained laboratory strain *aabys* (Tomita & Wada, 1989) was used in linkage mapping crosses to locate *M* factors. The *aabys* strain is homozygous for 5 recessive mutations that yield visible phenotypes: *alicurve wings* (*ac*, autosome I), *aristapedia* (*ar*, autosome II), *brown body* (*bwb*, autosome III), *yellow eyes* (*ye*, autosome IV), and *snip wings* (*snp*, autosome V). These phenotypes can all be scored separately and do not affect each other. Heterozygous individuals with the wild-type allele have wild-type phenotype. The *aabys* mutant strain possesses an XX–XY sex chromosome pair with the *M* locus on the Y chromosome. There is no marker available for the X and Y chromosome. The Dutch strain with a presumed XX–XY system is used to determine

Fig. 1 Collecting sites of the 5 Spanish housefly populations (SPA1-5) in the province of Catalonia in Spain. All samples are from cattle farms in open landscapes.

autosomal marker recovery ratios and deviation from Mendelian segregation.

For the mapping crosses, single pair matings were set up in a plastic rearing cup (125 mL). Provided food consisted of 1 g milk powder in a small Petri dish and 5 mL 10% sugar water in a tube closed with a cotton plug. After 4 d, egg-laying medium was added in a small plastic cup (10 mL) with an opening. Females were allowed to lay eggs for 3 d after which the egg-laying medium was transferred to a new rearing cup with fresh medium up to approximately half the volume of the cup as food for the larvae. Egg-laying medium was prepared according to Hilfiker-Kleiner *et al*. (1994) with slight adjustment: food mix consisted of 150 g flour, 50 g yeast, 120 g milk powder, and 1 000 g bran. Each 200 g of food mix was dissolved in 196 mL water and 4 mL 10% (m/v) nipagin solution (dissolved in 96% ethanol).

Localization of M loci

Two rounds of crosses were performed for determining the chromosomal location of *M* loci, similar to the mapping scheme of Tomita and Wada (1989), Kozielska *et al*. (2008), Feldmeyer *et al*. (2010), and Meisel *et al*. (2016). In the first round, 20 randomly chosen males of each strain were individually mated with 3 virgin *aabys* females (Fig. S1). Offspring of all 3 female mates of each male were cultured in the same container. The sex ratios (proportion males) of the F1 generation were determined for each parental male to infer hemizygosity or homozygosity of the *M* locus. If progenies consist of 50% male and 50% female individuals, the father is hemizygous for a single *M* locus. Progenies of 100% males indicate homozygosity of at least 1 *M* locus in the male parent. If a male carries 2 hemizygous independently segregating *M* loci, 75% of the gametes will carry at least 1 *M*. Correspondingly, its progeny sex ratio will be 0.75. The F1 progeny of each parental male was tested for deviation of a 0.5, 0.75, or 1.0 sex ratio by a Chi-square test. Note that males that are homozygous for *M* on 1 chromosome pair can possess an additional *M* on another chromosome, either hemizygously or homozygously. As the F1 sex ratio will be already 100% for 1 homozygous *M* locus, such additional *M* loci cannot be inferred from the first generation cross. However, the existence of multiple *M* loci can be detected in the F2 backcross progeny through linked segregation of more than 1 phenotypic marker with sex (Fig. S1F).

The chromosomal locations of *M* loci were detected by the sex-specific segregation of autosomal markers in the F2 offspring. Two males were randomly picked from each F1 progeny and each was backcrossed with 3 *aabys* virgin females to produce F2 progenies. F1 males are

heterozygous for all 5 autosomal markers and have wild-type phenotypes. Marker alleles that are not linked with *M* have equal segregation in males and females of the F2 generation. In contrast, the dominant wild-type marker allele that is linked with an autosomal *M* segregates sex specifically. Thus, all males will have wild-type and all females will have mutant phenotypes. This assumes full linkage between the marker and the *M* locus. An example is shown in Fig. S1E, where *M* is on autosome III. All F2 males possess autosome III with *M* and are therefore heterozygous *bwb/+*, they have the black body (wild-type) phenotype. Conversely, F2 females are all homozygous *bwb/bwb* and have the brown body mutant phenotype. If multiple *M* loci are present on different autosomes, more than 1 *M*-linked marker will show sex-specific segregation; only males can have the wild-type linked phenotype but females will all have the mutant phenotypes. However, males with mutant phenotypes for any particular marker can also be observed because of independent assortment. Fig. S1F shows the case if *M* loci are located on autosome II and autosome III. For *ar* and *bwb*, all F2 females are homozygous and have the mutant phenotype for both markers. F2 males are heterozygous for at least 1 of the 2 markers. In males, the ratio of individuals that have the phenotypes "+/+," "ar/+," and "+/bwb" is 1 : 1 : 1. These ratios again assume no recombination between the *M* locus and marker, but see below.

If *M* is located on 1 of the sex chromosomes, none of markers in the F2 progeny will have sex-specific segregation, thus indicating an M^{SEX} locus. If M^{SEX} co-occurs with an autosomal *M* (M^{A}), the F2 sex ratio will be higher than 0.5, and only the autosomal marker linked with M^{A} will show sex-specific segregation in the F2 generation. Therefore, M^{SEX} can be detected by a combination of F2 sex ratio and phenotypical segregation.

As low sample size (i.e., low offspring number from unproductive crosses) may skew sex ratios or marker segregation ratios, we excluded all F1 (10 out of 95) and F2 (7 out of 150) crosses that produced less than 20 individuals from our analyses. The wild-caught Spanish flies had been maintained in the laboratory for ∼40 generations prior to conducting the crosses, and flies from the Dutch strain for ∼30 generations. Each new generation consisted of about 300 individuals (equal number of females and males), which minimizes the effects of drift during laboratory maintenance.

Categorization of progeny sex ratios

After preforming Chi-square tests on the progeny, F1 and F2 were classified into 3 sex-ratio categories: ∼0.5, ∼0.75, ∼1.0 as a proxy for the number of segregating *M* loci. The ∼0.5 category includes sex ratios that are not significantly higher than 0.5, indicating the presence of a single *M* locus. The ∼0.75 category includes sex ratios significantly higher than 0.5 but lower than 1.0, indicating the presence of more than 1 *M* locus segregating independently. The ∼1.0 sex-ratio category contains all-male progenies, which indicates the presence of at least 1 homozygous *M* locus.

Sex-specific segregation with 100% of the mutant phenotypes in females and 0% in males indicates complete linkage between the wild-type allele of a marker and the *M* locus. However, recombination may happen. This will be visible as a fraction (<<50%) of F2 females carrying the wild-type allele and having wild-type phenotype. The recombination rate can be estimated according to the formula: number of wild-type F2 females divided by the total number of females. The F2 males were not used to estimate the recombination rates because in the presence of homozygous M^{SEX}, presence of autosomal *M* loci cannot be discerned.

Determining tra^{D} presence

The tra^{D} allele is characterized by a 38 bp deletion in exon 3 (Hediger *et al*., 2010) as a molecular marker to identify via PCR whether an individual carries the tra^{D} allele. The SPA1 and SPA4 were tested with only 20 females as cross data revealed a universal presence of homozygous *M* loci in tested males, strongly indicating that the tra^{D} allele is present in all females. For SPA2, SPA3, SPA5, and NL1, 50 females were tested as cross results could not exclude the possibility that tra^{D} existed in the strain with low frequency. Theoretically, when sampling 50 females and tra^{D} is present in only 5% of females, the chance of not detecting tra^{D} is ∼0.04, which is acceptably low. At the time of the tra^{D} analysis, the Spanish strains had been maintained in the laboratory for approximately 100 generations and the Dutch strains for 90 generations with about 300 individuals per generation. Although tra^{D} allele frequencies may have changed during strain maintenance, we think that the rearing regime allows extrapolation of the observed frequencies to the field populations at the time of sampling.

Genomic DNA of each fly was extracted by the high-salt protocol (Aljanabi & Martinez, 1997). PCR was performed using the primer set traDF: 5′-GCAGTATACAACGCAAGACG-3′ and traDR: 5′-GCCTGTTAATGTGTTGAGTGG-3′ that amplifies a 319 bp fragment of the *tra* allele but a 281 bp fragment of the tra^{D} allele, as a result of a 38 bp deletion (Hediger

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et al., 2010). The PCR reaction was conducted according to DreamTaq DNA Polymerase (Thermo Fisher Scientific) manual. The PCR program started with 1 cycle of 3 min initial denaturation at 95 °C, followed by 35 cycles with 60 °C annealing temperature and 15 s extension at 72 °C, and ended with 1 cycle of 5 min final extension at 72 °C. PCR products were visualized by gel electrophoresis.

Karyotyping

Additional cytogenetic examination was conducted to identify sex chromosomes in each strain. Chromosome slides were prepared from brain tissue of 3rd instar larvae. Mitotic chromosomes were spread according to the method of Carabajal-Paladino *et al*. (2014) with slight modification. In short, larval brains were dissected in Ringer's solution (182 mmol/L KCl, 46 mmol/L NaCl, 3 mmol/L $CaCl_2$, 10 mmol/L Tris:HCl; pH 7.2), pretreated in hypotonic solution (75 mmol/L KCl) for 10 min and then fixed in Carnoy's fixative (ethanol : acetic acid, 3 : 1, v/v) for 10 min. Fixed tissues were then transferred to glass slides on a 45 °C heating plate, covered with a drop of 60% acetic acid (∼35 μL) and spread with a tungsten needle. Chromosomes were stained with ProLong™ Diamond Antifade Mountant with 4′,6-diamidino-2-phenylindole (Thermo Fisher Scientific) and visualized with a Leica epifluorescence microscope (DMI6000 B) equipped with a Leica charge-couple device camera (DFC365 FX). For each strain, 10 samples were examined. Chromosomes were numbered according to Hediger *et al*. (1998b).

Results

Marker segregation in the NL1 strain

As the cross-mapping scheme relies on Mendelian segregation of the used markers, we first tested autosomal marker segregation using the Dutch strain NL1. Our results confirmed that the NL1 strain indeed has a single *M* locus located on the Y chromosome. First, none of the crosses ($n = 12$ F1 and $n = 23$ F2) had F1 sex ratios significantly higher than 0.5 (Table 1). Second, in F2 crosses, none of the recessive phenotypic markers showed sex-specific segregation (Table 2). Karyotype analysis (see below) confirmed the presence of the Y chromosome, which is consistent with previously investigated Dutch populations (Franco *et al*., 1982).

As *M* is not located on an autosome, all F2 progenies of the NL1 strain were expected to have 50% segregation of autosomal markers, irrespective of sex. However, all 5 mutant alleles were recovered at a slightly lower than 50% rate, and this deviation was significant for all markers except *ye* (Table 3). The marker *snp* on chromosome 5 with 42.5% mutant allele discovery showed the highest deviation from Mendelian segregation.

Table 1 F1 and F2 progeny sex ratios of tested males of each housefly strain.

		Sex ratio†		
Strain	Generation	∼0.5	∼0.75	∼1.0
NL1	F1	12	0	0
	F2	23	0	0
SPA1	F1	0	0	19
	F2	38	0	0
SPA2	F1	2	9	6
	F2	13	6	0
SPA3	F1	7	0	0
	F2	10	0	0
SPA4	F1	0	0	14
	F2	7	21	0
SPA5	F1	18	0	0
	F2	26	0	0

Note: values indicate number of crosses.
† Proportion males.

Marker segregation values can also be estimated for the SPA strains, disregarding any possible linkage with *M* loci, thus summing the total values over females and males (Table S3). The results reveal the same pattern of overall segregation ratios for all 5 mutant marker alleles being lower than 50%. Deviations are typically only a few percent, except for marker *snp*. Marker *snp* showed the highest deviation but also the largest variation between strains, with SPA1 and SPA5 having values as low as 30.3% and 24.8% recovery of the mutant allele (Table S3), indicating variation at the population level. These results indicate incomplete penetrance of the *snp* phenotype. Note that these biased segregation values do not devaluate the methodology of linkage mapping (see Discussion).

M localization and genotypes in Spanish populations

Hemizygous M^{III} in SPA3 and SPA5 None of the F1 progeny sex ratios of SPA3 and SPA5 crosses deviated from 0.5, indicating that all tested males had 1 hemizygous *M* locus (Table 1). In the F2 offspring of both SPA3

Table 2 Number of F2 progenies with sex-specific segregation of autosomal markers in each of the tested housefly strains.

Strain	Total	*ac* autosome I	*ar* autosome II	*bwb* autosome III	*ye* autosome IV	*snp* autosome V
NL1	19	0	0	0	0	0
SPA1	37	0	0	0	0	0
SPA2	13	0	3	0	0	0
SPA3	10	0	0	10	0	0
SPA4	15	2	8	0	0	0
SPA5	23	0	0	23	0	0

Table 3 Segregation of marker alleles in F2 offspring of backcrosses between the NL1 and *aabys* strains.

Strain	Offspring number		*ac* (%)	*ar* (%)	*bwb* (%)	*ye* (%)	*snp* (%)
NL1	Male	1 564	45.2***	43.2***	45.5***	48.3	46.2**
	Female	1 558	44.4***	46.2**	47.2*	48.5	38.9***
	Total	3 126	44.8***	44.7***	46.4***	48.4	42.5***

Note: Percentages represent individuals that carry mutant phenotypes. Asterisks indicate deviation from Mendelian segregation. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

and SPA5 crosses, the *bwb* marker showed a clear biased segregation between males and females (Table 2). As the *bwb* marker is on autosome III, these results indicate the presence of an *M* locus on autosome III in these 2 strains. None of the other markers segregated in a sex-specific way, confirming the F1 results that no other *M* loci are present.

Homozygous M^X in SPA1 In SPA1 crosses, mostly all-male F1 progenies were produced (Table 1, Table S1B), indicating that SPA1 males were homozygous for at least 1 *M* locus. In the F2, all sex ratios were ∼50%, which corresponds with the presence of 1 homozygous *M* locus on a single chromosome. No markers showed sex-specific segregation (Table 2). These results indicate that all SPA1 males are homozygous for the *M* locus on the sex chromosome pair (XX, see karyotype analysis below).

Three SPA1 crosses yielded F1 progeny sex ratios close to, but not equal to 1.0 (nos. 3, 4, and 9, Table S1B). This cannot be explained by assuming the presence of multiple, independently segregating, *M* loci on different chromosome pairs as the F2 progeny sex ratio and marker ratios clearly indicated the presence of a single *M* locus. These results therefore indicate that, despite its homozygosity, the *M* locus occasionally did not segregate to all offspring. This could be the result of nondisjunction of sex chromosomes during spermatogenesis (see Discussion).

Homozygous and hemizygous M^X, M^I and M^{II} in SPA4 Most (12 out of 14) SPA4 crosses resulted in 100% male offspring in the F1 generation. This indicates that SPA4 males carried at least 1 homozygous *M* locus. In the F2 generation, 7 crosses had ∼0.5 sex ratios, indicating a single *M* on 1 chromosome. Additionally, 21 crosses produced sex ratios that were ∼0.75, indicating the presence of multiple *M* loci on different chromosomes. There are thus multiple autosomal *M* loci segregating in the SPA4 strain.

Among F2 progenies of 15 F1 males tested, the *ac* marker showed biased segregation for males and females in 2 (nos. 11-1 and 11-2 of parental male no. 11; Table S2E), indicating the presence of an *M* locus on autosome I. The *ar* marker showed biased segregation for males and females in progenies of 8 F1 males (nos. 3-1, 7-1, 10-2, 13-1, 16-1, 16-2, 18-1, and 19-1), indicating the presence of an *M* locus on autosome II. In 5 crosses (nos. 2-1, 2-2, 5-1, 8-1, and 8-2), no sex biased segregation for any of the phenotypic markers was observed, indicating the presence of an *M* locus on the sex chromosome. As the F1 progeny sex ratio of cross no. 2 was 1.0, it can be concluded that this male was homozygous for the *M* locus on the sex chromosome (XX, see karyotype analysis below). We further found evidence for hemizygosity of M^I and M^{II} loci. In the F2, sex biased segregation for the *ar* marker was observed in cross 12-1 indicating presence of M^{II}, yet it was not observed in 12-2. Thus, it can be concluded that male no. 12 was hemizygous for M^{II}. Overall,

9.1% of individuals carried M^{I}, 63.6% of individuals carried M^{II} and 100% of individuals carried M^{X} in the SPA4 strain. In terms of number of *M* loci per male, 27.3% of males carried *M* on 1 chromosome pair, 72.7% carried *M* on 2 chromosome pairs (Tables S4).

Two SPA4 crosses (nos. 7 and 8) yielded F1 sex ratios that were close to, but not equal to 1.0. Again, this cannot be explained by assuming the presence of more than 1 *M* locus on different chromosomes. F2 progenies of male no. 7 indicated the presence of *M* loci on autosome II and the sex chromosomes, which should yield a sex ratio of 0.75. F2 progenies of male no. 8 indicated the presence of *M* only on the sex chromosome which should yield a sex ratio of 0.5. However, the F1 sex ratios of crosses nos. 7 and 8 were significantly higher than 0.75 ($P < 0.05$). Furthermore, F2 progenies of F1 male no. 5-1 indicated the presence of a single *M* locus on the sex chromosome that theoretically should result in a sex ratio of 0.5. Yet, this cross yielded an F2 sex ratio of 0.84 (Table S2E). This male-biased sex ratio indicates more than 1 *M* locus segregating in the father. However, no marker in cross no. 5-1 showed sex-specific segregation, indicating the absence of an autosomal *M*. The skewed sex ratios of F1 cross nos. 7 and 8 and F2 cross no. 5-1 are most likely caused by nondisjunction of sex chromosomes during spermatogenesis, similar to what was observed in the SPA1 crosses (see Discussion).

Homozygous and hemizygous M^{SEX} and M^{II} in SPA2 males For the SPA2 strain, 6 crosses yielded all-male F1 progeny indicating the presence of at least 1 homozygous *M* locus (Table 1). Two crosses (nos. 2 and 6, Table S1C) produced F1 progenies with sex ratios that did not deviate from 0.5, indicating the presence of a single hemizygous *M* locus. Nine crosses yielded F1 sex ratios that were ∼0.75 or higher, indicating *M* loci on more than 1 chromosome. Males of SPA2 in these samples thus represented a mixture of homozygous *M* and hemizygous *M*, single *M* loci and multiple *M* loci. The percentage of individuals that carry homozygous *M* is 35.3%.

In F2 crosses, the *ar* marker (autosome II) had clear sex-specific segregation in 3 crosses (nos. 1-1, 14-2, and 19-1) (Table S2C). This means that the F1 males carried M^{II}. Two F2 crosses (nos. 11-2 and 19-2) indicated the presence of *M* on the sex chromosomes because no markers showed sex-specific segregation. Progeny no. 11-2 was generated from male no. 11 that produced all-male offspring in the F1. This indicated that SPA2 male no. 11 was homozygous for M^{SEX}. Progeny 19-2 was generated from male no. 19 that produced an F1 sex ratio of 76.4%, indicating that this male possessed hemizygous M^{SEX} in addition to M^{II}. Thus, the SPA2 samples contained males with M^{II} and both hemizygous and homozygous M^{SEX}. M^{SEX} was detected in all males and M^{II} was identified in 36.3% out of a total of 11 successfully tested males (Table S4).

Karyotypes

In NL1 samples, the XY chromosome pair was observed (Fig. 2A). Together with the cross-mapping results, this indicates the presence of M^{Y}. In all Spanish samples, the karyotypes of the sex chromosomes were XX (Fig. 2B–E). Cross data showed that SPA3 and SPA5 males possessed only a hemizygous M^{III} locus. Thus, the X chromosomes in SPA3 and SPA5 (Fig. 2D and F) did not carry an *M* locus. Cross data also revealed that SPA1 and SPA4 flies all carried homozygous M^{SEX}. Thus, both X chromosomes in these samples (Fig. 2B and E) carried an *M* locus. For SPA2, homozygous M^{SEX} loci were identified in some of the crosses. As only XX chromosomes were observed (Fig. 2C), it is likely that either both or one X carried an *M* locus. However, considering that hemizygous M^{SEX} and homozygous M^{SEX} co-occur in SPA2 males, the possible presence of Y-carrying individuals cannot be excluded as only a limited number of individuals were karyotyped.

Tra^{D} allele presence

PCR results showed that in none of the tested hemizygous *M* strains (NL1, SPA3 and SPA5) did females possess a tra^{D} allele (Table 4). For strains with homozygous and/or multiple *M* loci (SPA1, SPA2 and SPA4), all tested females possessed a tra^{D} allele (Table 4).

Recombination between marker alleles and M loci

The F2 mapping analysis allows for estimating recombination rates between marker alleles and *M* loci. In the SPA4 cross that revealed an M^{I} locus, not all F2 females had the mutant phenotype (no. 11-1, Table S2E). This indicates that recombination had occurred between M^{I} and the *ac* marker. The recombination rate between the *ac* marker and M^{I} was estimated at 4.44% ($N = 45$, Table 5). Recombination was also observed in 2 SPA2 (nos. 14-2, 19-1) and 6 SPA4 (nos. 3-1, 10-2, 13-1, 16-2, 18-1, 19-1) crosses that concerned an M^{II} locus. The recombination rate between M^{II} and the *ar* marker was estimated at 2.02% ($N = 99$) in SPA2 and 5.34% ($N = 281$) in SPA4 (binomial test, $P = 0.187$). In total, out of 380 females examined, 17 individuals had the wild-type *ar*

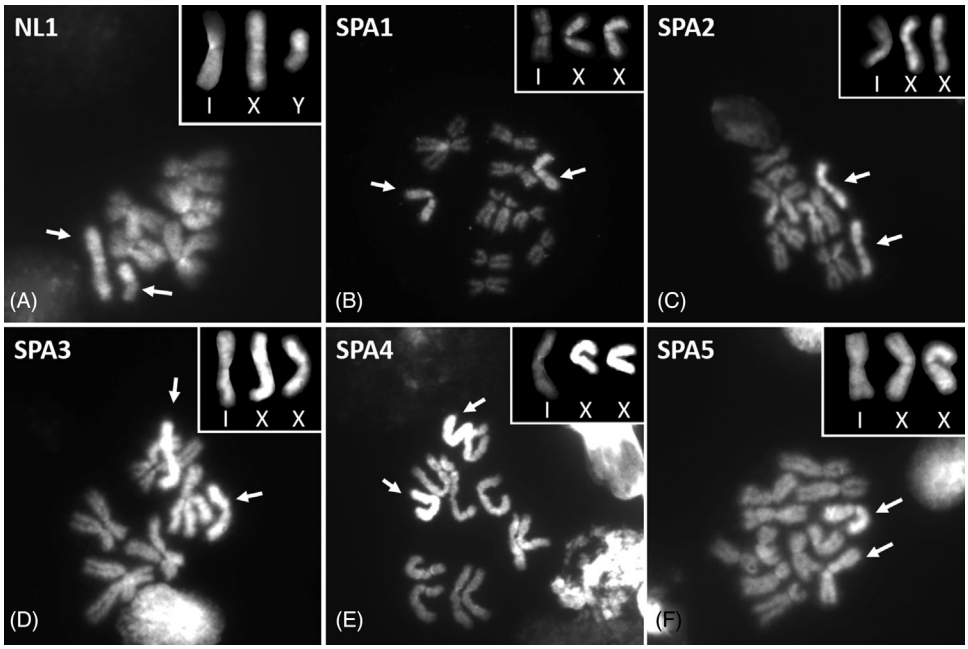

Fig. 2 Karyotypes of samples from each investigated housefly strain. Sex chromosomes are indicated by arrows and shown in the top right corner in comparison to autosome I.

Table 4 Percentage of females that possess the tra^D allele in each housefly strain.

Strain	Total females	$tra^D/tra^{\dagger}$	$tra/tra^{\dagger}$	Tra^D females (%)
NL1	50	0	50	0
SPA1	20	20	0	100
SPA2	50	50	0	100
SPA3	50	0	50	0
SPA4	20	20	0	100
SPA5	50	0	50	0

† tra^D/tra: number of females that possess the tra^D allele; tra/tra: number of females that do not possess the tra^D allele.

phenotype, indicating an overall recombination frequency of 4.47% between M^{II} and *ar*. No recombination was observed for *M* on autosome III as all females were of *bwb* type in SPA3 and SPA5 crosses ($N = 2234$). As *M* was not found on autosomes IV and V, recombination rates for these chromosomes cannot be determined. The same holds for the sex chromosomes because no markers are available on these chromosomes.

Discussion

Neighboring housefly populations from north Spain were investigated for number and chromosome locality of male-determining (*M*) loci using a mapping cross with visible, recessive markers, and karyotyping. Frequencies of the dominant female determiner tra^D were determined with PCR. Our aim was to investigate the diversity of sex-determination systems within a small geographical range. Additionally, we used a Dutch strain, which was confirmed to only carry the *M* locus on the Y chromosome, to test the fidelity of Mendelian inheritance of the 5 autosomal phenotypic markers in the linkage mapping cross, as well as for distinguishing the X and Y chromosomes in karyotype analyses. The autosomal marker segregation was expected to show a 1 : 1 ratio in F2 progenies regardless of sex. However, screening of the F2 revealed that the recessive mutant alleles of all 5 autosomal markers were recovered at less than 50%. Deviations from Mendelian segregation were only minor for the markers on autosomes I–IV, but higher for the *snp* marker on autosome V. Similar results were obtained for the Spanish strains

Table 5 Recombination rate between *M* loci and autosomal markers in Spanish housefly strains.

Autosome	Strain	Total females†	Wild-type females†	Recombination rate (%)
I	SPA4	45	2	4.44
II	SPA2	99	2	2.02
	SPA4	281	15	5.34
	Total	380	17	4.47
III	SPA3	745	0	0
	SPA5	1 489	0	0
	Total	2 234	0	0

† The numbers are summed from crosses that yielded over 20 females among F2 progenies and showed sex-specific segregation for markers on autosomes.

by pooling males and females. Notably, these lower ratios of *snp* phenotypes in F2 progenies varied between Spanish strains. Individuals with mutant phenotypes may have slightly reduced survival. Although this may apply to all markers, it is unlikely to fully explain the *snp* results. The snip wing phenotype appears to have reduced expressivity, which varies between strains (Table S5), reminiscent of the Curly mutation of *Drosophila melanogaster* (Ward, 1923). The slight modifications from Mendelian inheritance did not mitigate our ability to map the *M* loci as we still observed clear sex-specific segregation in F2. This holds even for the *snp* marker on autosome V, which despite its low expressivity would show sex-specific segregation when it is linked to an *M* locus; that is, more females will be homozygous for the *snp* mutation.

The composition of the sex determiners varied strongly between the Spanish strains. Frequency of tra^D in females showed 2 extremes: in 3 strains, all females carried tra^D and in 2 strains no females carried the dominant tra^D allele. The dominant nature of tra^D corrupts the male heterogametic sex determination, where males are hemizygotic for *M*. Although tra^D can never become homozygous and is present in females only, this system cannot be seen as female heterogamety in the strict sense, as long as the regular *tra* allele is still functional. Nevertheless, the presence of tra^D will lead to an increase of *M* frequencies, by allowing *M* to also be transmitted to females; it enables homozygosity of *M* in both males and females and sustains multiple *M* loci simultaneously. Indeed, homozygosity of *M* was found in 3 strains in which tra^D was fixed, a system that can be considered a quasi-female heterogametic system, and hemizygosity of *M* in 2 strains in which tra^D was absent, corresponding to a true male heterogametic system. In SPA2, although 100% females carried tra^D, both hemizygous (M^{II}, M^{SEX}) as well as homozygous (M^{SEX}) males were found. This can thus be considered a mixture of quasi-female and true male heterogamety. A similar stable mix of male determiners was found by Meisel *et al*. (2016) in a population from Chino (CA, USA). However, in contrast with this study, we did not observe females without tra^D in such mixed Spanish populations. One possible explanation is that we did not find non-tra^D females in the strains tested because of their very low frequency. Yet, the probability for such a scenario is low, as we estimate the chance of not detecting tra^D in this strain to be ∼0.04 when sampling 50 females and tra^D being present in only 5% of females. Alternatively, it is possible that upon sampling, only a low percentage of non-tra^D females existed in the field population and as a consequence was not represented in our sampling. A less likely possibility is that non-tra^D females were lost during laboratory maintenance due to drift. Given these possibilities, we conclude that in all strains with multiple and/or homozygous *M* loci, an equilibrium has established that involves all females to carry the tra^D allele.

Our results revealed that composition and frequency of the sex-determining factors varied strongly between neighboring populations. The Spanish populations were sampled within a distance of approximately 100 km in the province of Catalonia in northern Spain (SPA1, SPA2, and SPA3 near Girona within a range of approximately 25 km; and SPA4 and SPA5 6 km apart near Barcelona at a distance of approximately 80 km from SPA1-3). Yet there are striking differences in frequency and chromosomal locations of *M* in males as well as frequency of the tra^D carrying females. For example, despite their adjacent locations, in the SPA4 population, *M* loci are present on 3 chromosomes (M^I, M^{II}, and M^X) with M^X being homozygous in males and tra^D present in all females, whereas in the SPA5 population, all males are hemizygous M^{III} and tra^D is absent in females. It is worth noting that the tested strains in this study have been reared for many

generations in the laboratory (~40 generations upon testing for *M* and ~100 generations upon testing for tra^D) after collection from the field. Therefore, the original frequencies of various *M* loci and tra^D allele in the strains may have changed and could deviate from the actual situation in the field; for example, some variants may have been lost due to genetic drift. However, we are confident that our results do reflect the differences in sex-determining systems between the natural populations, such as male heterogamety versus quasi-female heterogamety. Mainly because all strains were maintained in the laboratory under similar conditions, it is unlikely that all of them were identical in harboring all 4 observed *M* loci and being mixed for *tra* and tra^D upon collection and subsequently developed completely different compositions of sex determiners.

Multiple factors have been proposed to maintain the polymorphic sex-determining factors in the housefly as evidence has shown a selective advantage to carrying autosomal *M*, such as M^{II} and M^{III} (Hamm *et al*., 2005; Kozielska, 2008). It has been proposed that autosomal *M* factors have spread due to their linkage with insecticide resistance genes (Kerr, 1970; Franco *et al*., 1982), but this explanation was rebutted, as Hamm *et al*. (2005) did not find association between high resistance levels and the presence of autosomal *M*. Climate conditions such as temperature or humidity were also proposed as potential factors for maintaining polymorphic sex-determining factors (Franco *et al*., 1982; Feldmeyer *et al*., 2008; Kozielska *et al*., 2008). Delclos *et al*. (2021) reported that thermal tolerance and preference differs between males that carry M^Y and M^{III}. Meisel *et al*. (2016) further proposed that sexually antagonistic selection, which is likely environmentally dependent contributed to maintaining the stability of polymorphic sex-determining systems. In the current study, all samples are from cattle farms in an open landscape without any obvious physical barriers between them. The environmental conditions are therefore also considered to be rather similar for these populations. Although environmental factors can explain the differences in sex-determining factors in populations that are geographically distinct (Feldmeyer *et al*., 2008), they do not seem to explain the variation we observed in neighboring Spanish populations. Our results indicate limited gene flow between housefly populations, as otherwise one would expect a similar distribution of sex determiners in all 5 sampled populations. Population isolation may enhance the effects of genetic drift, which will lead to loss of sex-linked alleles and fixation (Meisel *et al*., 2016). Limited dispersal is somewhat at odds with the widespread distribution of houseflies and the maximum estimated flight distance for the housefly being around 7 km (Quarterman *et al*., 1954; Wharton *et al*., 1962; Nazni *et al*., 2005). Our results suggest that houseflies are rather residential at farms and do not migrate as much as we thought. Resampling field populations during several periods is needed to provide more insights, and this is currently being performed by us.

In the Spanish populations, it seems that the invasion of tra^D leads to fixation of a quasi-female heterogametic system, whereas absence of tra^D or loss of tra^D results in the establishment (or maintenance) of a male heterogametic system. A complicating factor may be the fitness differences of the various *M*-carrying chromosomes (proto-Y chromosomes, because they are male-determining), as was reported by Adhikari *et al*. (2021). This may cause an ongoing competition of proto-Y among themselves and presence of tra^D as the condition for the invasion of multiple proto-Y in the population. The establishment and stability of this multifactorial sex-determination system appears therefore governed by multiple genetic and environmental factors. It is important to study temporal changes in population sizes as well as in population structure to gain a better understanding of the selection pressures acting on the polygenic sex-determination system in the housefly.

Occasionally, F1 female individuals were produced at very low frequency from SPA1 and SPA4 males that possessed homozygous M^X. The most likely explanation for this would be nondisjunction of X chromosomes during spermatogenesis, which results in some sperm cells with 2 X and others without an X chromosome and thus no *M* locus. Non-*M*-carrying sperm leads to female offspring. Nondisjunction of X chromosomes in houseflies has been reported before (Tsukamoto *et al*., 1980; Denholm *et al*., 1983) and was also observed in our karyotype analysis (Fig. S2). Apparently, individuals with odd sex chromosome numbers develop normally and are fertile (Tsukamoto *et al*., 1980; Denholm *et al*., 1983). F1 progeny resulting from 2 X-carrying sperm will have 3 X chromosomes (of which the 2 paternal ones carry an *M*, whereas the maternal one, from the *aabys* mother, does not carry an *M* locus) that develop into males. An F1 male with 3 X chromosomes (double *M* loci) will produce 83.3% (assuming no further disjunction in this male) of sperm with an *M* locus. When crossed with an *aabys* female, such males will produce F2 progenies with male-biased sex ratios. This can explain why the SPA4 cross no. 5-1 yielded a F2 sex ratio that was 0.84, yet only showed evidence for the presence of M^X. An alternative explanation for daughter production by homozygous *M* males can be loss of *M* function by unequal crossing over between the *M* loci. The *M* locus is large in sequence and contains one (or more) functional copies of the male-determining

gene *Mdmd* as well as multiple pseudogenes and repeats (Sharma *et al*., 2017). Unequal crossing over can happen between the functional *Mdmd* and a pseudogene resulting in a nonfunctional *M* locus and female development. However, this cannot explain cases with male-biased F2 sex ratios as an F1 male that produces sperms with nonfunctional hemizygous *M* would produce more daughters.

Recombination in males could be measured for chromosomes on which *M* loci were found and for which markers were available, such as autosomes I–III. The overall recombination rates between the marker allele and *M* locus were low for autosomes I and II (approximately 4.5%) and zero for autosome III despite the fact that over 2 000 individuals were examined. Whether recombination occurs in housefly males has been disputed. Recombination was rarely observed by some authors (Hiroyoshi, 1961; Tsukamoto *et al*., 1961; Tsukamoto, 1964; Hiroyoshi, 1977) but more frequently by others (Rubini *et al*., 1980; Feldmeyer *et al*., 2010) with frequencies as high as 30% for autosome II (Lester *et al*., 1979). Our study confirmed that recombination happens in male houseflies, even on the autosomes with sex-determining function. Observed absence of recombination between M^{III} and the *bwb* marker may be due to either close physical linkage or more ancient origin of the M^{III} locus. Interestingly, using similar *aabys* backcrosses, Feldmeyer *et al*. (2010) found recombination between the M^{III} locus and the *bwb* marker at 1% in a population collected from Warden, South Africa. Lack of recombination on autosome III in our crosses could indicate that the M^{III} locus has a different chromosomal location in Spanish flies compared to the African flies. Alternatively, reduced recombination may reflect the age of the proto-Y chromosome and may have evolved at different speeds in these populations.

In conclusion, our study demonstrates that the high diversity in housefly sex-determining systems is not only found on a global scale, but also exists at the regional level where neighboring populations can possess very different systems, such as male heterogamety and female heterogamety or a mixture of both. This indicates that housefly populations are genetically differentiated at a small spatial scale. Our results show coexistence of homozygous and multiple *M* loci in males and the tra^D allele in females. This confirms the prediction that *M* loci can accumulate in individuals of populations in which tra^D is present. As adjacent populations with or without the presence of tra^D allele are found, our results further suggest limited migration rates between populations. Unfortunately, very little is known about housefly population sex ratios. Such studies are needed, in addition to determining how population frequencies of sex-determination factors vary within and between seasons to understand the evolutionary dynamics of this polymorphic sex-determination system.

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Disclosure

The authors declare 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 Mapping cross schemes. In each case a wild-type male is crossed with an *aabys* female.

Fig. S2 Karyotypes of samples with 3 (A) or 1 (B) X chromosome. Sex chromosomes are indicated by arrows and shown in the top right corner in comparison to autosome I.

Table S1 Sex ratios (percent male) of F1 and F2 offspring in NL1 crosses.

Table S2 Percentage of individuals with mutant phenotypes in F2 offspring of NL1 crosses.

Table S3 Segregation of marker alleles in F2 offspring of back crosses between the SPA strains and *aabys* strain. Percentages represent individuals that carry mutant phenotypes. Numbers in italics indicate cases where the marker is linked with an *M* locus, meaning a higher recovery of the marker in females than males. Asterisks indicate deviation from Mendelian segregation.

Table S4 Overview of the chromosomal locations of *M* loci in males of the NL1 and SPA1-5 strains.

Table S5 Percentage of crosses with marker ratios that are significantly lower than 50% in the NL1 and SPA1-SPA5 strains.

