

# A microsatellite marker linkage map of the housefly, *Musca domestica*: evidence for male recombination

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## Abstract

We present the first molecular marker linkage map for *Musca domestica* containing 35 microsatellite plus six visible markers. We report the development of 33 new microsatellite markers of which 19 are included in the linkage map. Two hundred and thirty-six F2 individuals were genotyped from three crosses yielding a linkage map consisting of five linkage groups that represent the five autosomes of the housefly. The map covers a total of 229.6 cM with an average marker spacing of 4.4 cM spanning approximately 80.2% of the genome. We found up to 29% recombination in male houseflies in contrast to most previous studies. The linkage map will add to genetic studies of the housefly.

**Keywords:** *Musca domestica*, linkage map, microsatellites, male recombination, sex determination.

## Introduction

The housefly (*Musca domestica*) is a cosmopolitan species and an important disease vector for livestock and humans (Fotadar *et al.*, 1992). Besides its medical and economic importance, it is also of interest regarding the evolution of sex determination, as this species harbours several different sex determining systems (Dübendorfer *et al.*, 2002). Even though it has been studied for decades, remarkably little genomic mapping information is available of the housefly and there are strong calls for a genome sequenc-

ing project (Gao & Scott, 2006; Scott *et al.*, 2009). Thus far, linkage studies in the housefly are constrained to back crosses with mutants carrying visible mutations (Hiroyoshi, 1961, 1977; Tsukamoto *et al.*, 1961; Wagoner, 1967). These studies have mostly been aimed at localizing sex determining factors, but also at the mapping of other genes (Wagoner, 1969; Franco *et al.*, 1982; Denholm *et al.*, 1985; Tomita & Wada, 1989; Denholm *et al.*, 1990; Çakir & Kence, 1996; Kozaki *et al.*, 2002; Hamm *et al.*, 2005; Kandemir *et al.*, 2006; Feldmeyer *et al.*, 2008; Kozielska *et al.*, 2008; Hamm & Scott, 2009). There have also been several population genetic studies of houseflies based on mitochondrial sequences (Roehrdanz, 1993; Marquez & Krafur, 2002, 2003; Cummings & Krafur, 2005), but we know of only one study that used microsatellite markers (Krafur *et al.*, 2005). Here we develop 33 new microsatellite markers to augment the number of molecular markers that can be used in genetic studies.

In the housefly a diverse array of sex-determining factors occurs. In so-called 'standard' populations females are XX and males are XY (Dübendorfer *et al.*, 2002). All individuals are homozygous for the female-determining factor (*F*) on chromosome IV. Males additionally possess the dominant male-determining factor (*M*) on the Y chromosome which suppresses *F* and leads to male development (Hediger *et al.*, 1998a). In some populations individuals are homozygous for *M* on an autosome and sometimes males carry multiple *M* factors on different autosomes (Franco *et al.*, 1982; Tomita & Wada, 1989; Çakir & Kence, 1996; Hamm *et al.*, 2005; Feldmeyer *et al.*, 2008; Kozielska *et al.*, 2008; Hamm & Scott, 2009). In some populations with heterozygous autosomal *M* males, and in all populations with homozygous autosomal *M* males, females carry a dominant female-determining factor *F<sup>D</sup>*. *F<sup>D</sup>* is insensitive to suppression by *M*, leading to female development even in the presence of *M* (Dübendorfer *et al.*, 2002; Hediger *et al.*, 2010).

The overall consensus among housefly researchers has been that there is little or no recombination in male houseflies (Rubini *et al.*, 1980), similar to *Drosophila* where male recombination is completely absent (Morgan, 1914). Hiroyoshi (1961) found no recombination at all,

First published online 19 May 2010.

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**Table 1.** Overview of informative markers per cross. Prefix Md for *Musca domestica* has been omitted. Underlined markers were analysed in more than one cross. Markers in parentheses did not map to any of the linkage groups. Earlier published markers are described in Endsley *et al.* (2002) and Chakrabarti *et al.* (2004)

Cross	No. offspring	Polymorphic markers	
		Newly developed	Earlier published
M2	58	<u>CT238</u> , <u>CT291</u> , <u>CT289</u> , <u>CT297</u> , <u>CT322</u> , <u>CT339</u> , <u>CT364</u> , <u>CT373</u> , <u>AG224</u> , <u>AG227</u> , <u>AG290</u> , <u>AG324</u> , <u>AG422</u> ( <u>AG228</u> , <u>AG357</u> )	<u>CA104</u> , <u>CA117</u> , <u>CA119</u> , <u>CA121</u> , <u>CA148</u> , <u>CA154</u> , <u>CA155</u> , <u>CA224</u> , <u>CA226</u> , <u>HF25</u> , <u>HF31</u> , <u>HF33</u> , <u>HF44</u> ( <u>CAG34</u> )
M3	98	<u>CT238</u> , <u>CT289</u> , <u>CT297</u> , <u>CT302</u> , <u>CT339</u> , <u>CT364</u> , <u>CT373</u> , <u>AG224</u> , <u>AG324</u> , <u>AG328</u> , <u>AG357</u> , <u>AG372</u> , <u>AG422</u> ( <u>AG227</u> , <u>CT291</u> , <u>AG329</u> , <u>CT222</u> )	<u>CA104</u> , <u>CA154</u> , <u>CA170</u> , <u>HF33</u> , <u>HF44</u>
MY	80	<u>CT268</u> , <u>CT291</u> , <u>CT297</u> , <u>CT302</u> , <u>AG329</u> , <u>AG422</u> , <u>CAG78</u> ( <u>CT322</u> , <u>AG224</u> , <u>AG290</u> )	<u>CA104</u> , <u>CA117</u> , <u>CA170</u> , <u>CA202</u> , <u>CA224</u> , <u>CAG34</u> , <u>HF31</u> , <u>HF44</u> ( <u>CA06</u> )

whereas Sullivan (1961) and Milani (1967) observed some recombination in mutant strains with visible mutations, suggesting that recombination in males might be population dependent (Milani, 1967). In a later study, Lester *et al.* (1979) reported up to 31% male recombination in an Australian housefly strain. Rubini *et al.* (1980), however, attributed the rare occurrence of recombinants of heterozygous males and the appearance of mosaics to mitotic recombination. Hiroyoshi *et al.* (1982) also found male recombination in low frequency in several Japanese populations. One aspect that all these studies on male recombination have in common, as also noted by Hiroyoshi *et al.* (1982), is that they investigated populations with autosomal sex-determining factors. In this study, we use microsatellite markers to investigate male recombination rates in three populations, one with XY and two with autosomal *M* carrying males.

The aim of this paper is two-fold. We present the first genetic linkage map of the housefly using molecular markers. By combining microsatellite markers with traditional visible markers on each of the five autosomes we assign the molecular markers to each of five linkage groups. In addition, we provide further evidence for male recombination in houseflies. We expect that our linkage map will be instrumental for future genome studies, such as revealing the nature of autosomal sex-determining factors and for annotation of the housefly genome.

## Results

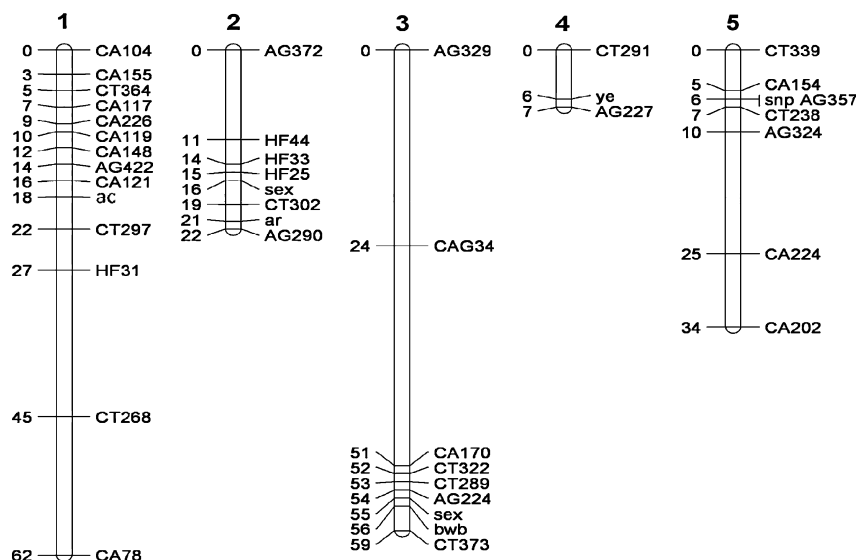
A total of 236 F2 progeny and backcross parents from three crosses (referred to as M2, M3, and MY) were genotyped with 58 microsatellite markers. Of the 33 newly developed microsatellite markers 20 turned out to be informative in at least one of the crosses analysed. Additionally, 17 of the previously published 25 microsatellite markers (Endsley *et al.*, 2002; Chakrabarti *et al.*, 2004), plus one marker developed from a GenBank sequence, were informative in at least one of the crosses (Table 1). A total of 35 microsatellite markers, six frequently used

visible mutations, plus the trait 'sex' were mapped onto five linkage groups, which correspond to the five autosomes of the housefly (Wagoner, 1967). None of the microsatellite markers mapped to the X or the Y chromosome. Three markers (MdCT222, MdAG228 and MdCA06) did not map to any of the linkage groups.

For the M2-cross (where males carry the *M* factor on autosome II) five linkage groups were found, representing all five autosomes ranging in size from 6–34 cM and consisting of 3–11 markers per group. The total linkage distance covered by these markers was 78 cM with an average spacing of 3.0 cM between markers for the whole framework map (Table 2). For the M3-cross (*M* factor on autosome III) linkage groups for autosomes I–III and V were found, ranging in size from 3–30 cM and consisting of 5–6 markers per group. The total map size was 64 cM with an average spacing of 3.2 cM between markers. The M2 and M3-crosses yielded recombination frequencies for males only, since the females are homozygous for almost all markers (see Experimental procedures for details). Although possible, we did not construct maps separately for females and males in the MY-cross, because the number of markers per linkage group in females was mostly too small. For the MY-cross we found linkage groups for autosomes I–III and V, ranging in size from 12–62 cM and consisting of 3–8 markers per group. The total distance covered was 165 cM, which is on average 2.3 times the size of the autosomal *M*-based maps, and with an average marker spacing of 9.2 cM. After joining the three maps, the combined map consisted of five

**Table 2.** Observed and estimated map lengths and coverage for each of the three crosses separately and the combined linkage map. Values are based on all five linkage groups for the M2-cross and the combined map, but for the M3- and MY-cross linkage group IV was not available

	M2	M3	MY	Combined
Observed map length (cM)	78	64	165	184
Estimated genome length (cM)	110.5	92.8	252.12	230.91
Coverage (%)	70.6	69.0	65.5	79.7
Number of markers	26	18	15	35



**Figure 1.** Linkage map of the housefly derived from combining three different mapping populations. Markers are indicated on the right; map distances (in Kosambi cM) on the left of a chromosome. Linkage groups are arranged by chromosome number according to Wagoner (1967). The number of linkage groups corresponds to the number of autosomes, no markers were found on the sex chromosomes. Each chromosome contains one visible marker (*ac* = *ali curve*, *ar* = *aristapedia*, *bwb* = *brown body*, *ye* = *yellow eyes*, *snp* = *snip wings*). The marker 'sex' occurs twice as it was once mapped with a strain that contained *M* on the second and once with a strain that contained *M* on the third autosome. All other markers are microsatellites.

linkage groups ranging in size from 7–62 cM, containing 3–14 markers per group and a total map size of 184 cM with an average spacing of 4.5 cM between markers (Fig. 1).

The estimated map length for the combined map was 230.9 cM, which is the average of two different methods (see Experimental procedures), 228.9 and 232.9 cM, respectively. The combined map covers about 79.7% of the genome, calculated as the observed length of 184 cM divided by the estimated length of 230.9 cM. The total size of the *M. domestica* genome is predicted to be 309–312 Mbp (Gao & Scott, 2006).

Based on 19 marker pairs which were distributed over four autosomes and mapped in both sexes, the average recombination rate was estimated to be 1.92 times higher in females than in males (23% compared with 12%). Single pairwise recombination rates in males between markers with LOD > 3 varied between 0 and 0.29. Average pairwise recombination rates for all mapped markers ranged from 0.04–0.28 per autosome (Table 3).

**Table 3.** Average male recombination frequencies for adjacent marker pairs per autosome and cross. Values in parentheses indicate the number of marker pairs

Cross	Autosome				
	I	II	III	IV	V
MY	0.17 (6)	0.28 (2)	0.19 (3)	–	0.18 (2)
M2	0.07 (10)	0.09 (5)	0.04 (4)	0.06 (2)	0.11 (5)
M3	0.14 (4)	0.15 (4)	0.06 (5)	–	0.13 (5)

Statistical analysis of recombination frequencies indicated that the full model was not significantly better than the additive model (Supporting Information Table S1). Further model reduction indicated that removing the variable 'chromosome' from the additive model had no significant effect, however, removing the variable 'cross' did. Specifically, it appeared that cross M2 showed significantly lower recombination rates than the other two crosses (Supporting Information Table S2; chromosome IV was not included in the analysis).

## Discussion

We present the first genetic linkage map of the housefly, *M. domestica*, based on molecular markers. With the help of visible markers that had previously been assigned to the five autosomes we were able to place 35 microsatellite markers on five linkage groups representing the five autosomes identified by Wagoner (1967). We did not find any markers linked to either the X or the Y chromosome. Similar to the medfly *Ceratitis capitata* (Stratikopoulos *et al.*, 2008), the X and Y chromosome of the housefly consist mainly of heterochromatin (Hediger *et al.*, 1998b). Heterochromatic regions are known to be refractory to cloning and sequencing strategies (International Human Genome Sequencing Consortium, 2004), which would explain their absence in our library.

The distribution of microsatellite loci along the linkage map appears to be non-random. In all five linkage groups we find clusters of markers towards one end of the linkage group. Non-random distribution of microsatellite markers

along linkage groups has also been observed in, for example, rice, zebrafish and the medfly (Shimoda *et al.*, 1999; La Rota *et al.*, 2005; Stratikopoulos *et al.*, 2008). In rice the accumulation of microsatellites in certain regions of the genome is correlated with gene-rich regions (La Rota *et al.*, 2005), but in zebrafish it was attributed to the accumulation of CA/GT sequences in these chromosomal regions (Shimoda *et al.*, 1999). At this point, we do not know the reason for aggregation of microsatellite markers in the housefly linkage map.

The recombination density found in this study is 0.74 cM/Mb (total map size of 229.6 cM estimated in this study divided by 310 Mb according to Gao & Scott, (2006)), thus comparable to other Dipteran insects where recombination densities range between 0.1 and 3.1 cM/Mb (reviewed and discussed in Wilfert *et al.*, 2007).

Studies on housefly male recombination have found varying results, ranging from no recombination (Hiroyoshi, 1961; Rubini *et al.*, 1980) up to 31% (Lester *et al.*, 1979). With our crosses we confirm the occurrence of recombination in males, thus supporting the claim of Lester *et al.* (1979) to revise the assumption of recombination absence in male houseflies. We did not only find recombination in crosses with autosomal *M* males but also in XY males on all autosomes (Table 3). However, it is not possible to discern whether this is attributable to the crossing of two different strains, i.e. two unrelated genomes disrupt recombination suppression in males, or whether recombination actually occurs widely in 'standard' XY populations.

Recombination frequencies in males may reflect the age of the sex-determining mechanism (Ohno, 1967; Rice, 1996; Charlesworth *et al.*, 2005). After a standard chromosome has acquired sex-determining function (so-called neo-sex chromosome), recombination will gradually reduce along the chromosome starting from the sex chromosome locus. Due to lack of recombination the sex-determining chromosome will gradually degrade. At some point another gene on a different chromosome might take over sex-determining function either by transposition of an existing, or the emergence of a novel sex determining gene. The 'old' sex chromosome may eventually vanish, if it does not contain essential genes anymore. Spread of recombination suppressors in the genome may eventually lead to genome-wide reduction in crossover frequencies. This process is believed to have general application to organisms with chromosomal sex determination, and may also act in the housefly where sex-determining factors can be found on different autosomes in different populations, turning these autosomes into neo-sex chromosomes. In this respect the housefly is an interesting study organism for sex chromosome evolution research, as it harbours different sex-determining mechanisms and male and female heterogametic systems can be compared within a single species.

We hope that this linkage map will serve as a starting point for further gene-mapping studies in the housefly, such as to identify economically important insecticide resistance genes, to localize and characterize sex-determining factors, and to further test hypotheses of sex chromosome evolution. We end with the wish that the linkage map will contribute to the realization of a housefly genome project (Gao & Scott, 2006; Scott *et al.*, 2009).

## Experimental procedures

### Crosses

We studied the segregation of 35 molecular markers in combination with six visible markers in three different housefly crosses. The molecular markers are a subset of 33 newly developed microsatellite markers that we report here, and microsatellite markers that have been published earlier (Endsley *et al.*, 2002; Chakrabarti *et al.*, 2004). For each cross we used a mutant marker strain (named 012345-1) recessive for visible traits on each of the five autosomes (*ali curve* (*ac*) on linkage group 1; *aristapedia* (*ar*) on 2; *brown body* (*bwb*) on 3; *yellow eyes* (*ye*) on 4; *snip wings* (*snp*) on 5) (see Tomita & Wada, 1989). This strain has been used by several authors to determine the position of the male-determining factor *M* in natural populations by back crossing wild-type males with mutant females (Tomita & Wada, 1989; Feldmeyer *et al.*, 2008; Kozielska *et al.*, 2008). Since the visible mutations have been cytologically assigned to chromosomes (Wagoner, 1967) we can directly associate the markers with linkage groups and chromosomes.

The wild-type males in our crosses came from populations which contained autosomal *M* factors and *M* located on the Y chromosome. We individually crossed wild-type males to mutant females. F1 male offspring were backcrossed to mutant females. Because of sex-linked inheritance of the phenotype, the F2 generation reveals the location of the *M* factor (for more details see Denholm *et al.*, 1983). For the linkage analysis we chose the strain FVG, collected in Faverges, France (2004), with autosomal *M* on chromosome II (this cross will be called M2-cross) and the strain WAD, collected in Warden, South Africa (2005), with autosomal *M* on chromosome III (this cross will be called M3-cross). Since females from the mutant strain are homozygous at almost all loci, these two crosses result in 'male only' linkage maps as recombination information will stem exclusively from males. The third cross involved mating a female from the strain UML, collected in Umhlali, South Africa (2005), to an XY male of the mutant strain (MY-cross). Males and females of the resulting F1 generation, thus brothers and sisters, were mated to create the F2 generation. This cross yielded recombination information for both females and males. We genotyped 58 offspring of the M2-cross, 98 offspring of the M3-cross and 80 offspring of the MY-cross, resulting in an overall number of 236 individuals for construction of the combined linkage map.

### Microsatellite development and genotyping

Genomic DNA of male houseflies was collected from four different strains; two laboratory strains (WHO, World Health Organization Standard Reference Strain and the 012345-1 mutant strain, both obtained from D. Bopp, University of Zürich, Switzerland) and two

wild caught strains (FVG, Faverges, France and MID, Midlaren, the Netherlands). Males of these strains carried the Y chromosome. DNA was extracted using a standard proteinase K/salt-chloroform protocol and pooled for all stains.

An enriched library was made by Ecogenics GmbH (Zürich, Switzerland) from size selected genomic DNA ligated into SAULA/SAULB-linker (Armour *et al.*, 1994) and enriched by magnetic bead selection with biotin-labelled (GA) 13 and (TAC) 8 oligonucleotide repeats (Gautschi *et al.*, 2000). Of 951 recombinant colonies screened, 271 gave a positive signal after hybridization. Plasmids from 192 positive clones were sequenced, of which 168 yielded microsatellite sequences. Forty-three out of the 168 sequences were duplicates leaving 125 sequences that were analysed with the software Tandem Repeat Finder (Benson, 1999) to identify the repeat motif, length and position of the repeat sequence. The microsatellite motifs were tandem repeats of either CT (52%) or AG (48%). Primers were designed using the software PRIMER3 (Rozen & Skaletsky, 2000). Forty-three sequences (34%) were either too small or the repeat flanking region was too small for primer design, leaving 82 sequences for which primers could be designed.

A total of six individuals (three females and three males from three different strains) were initially screened for marker amplification and polymorphism on a 5% agarose gel. Thirty-eight primer pairs failed to amplify or gave dubious amplification patterns and were discarded for further analysis. From the remaining forty-four markers the forward primers were labelled with a fluorescent dye (FAM, HEX or NED). PCR reactions were performed in 1X PCR buffer magnesium free (Promega, Madison, WI, USA) with 2.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs (Roche, Indianapolis, IN, USA), 0.2 µM of each primer, 0.4 units of Taq polymerase (Promega) and approximately 5 ng of template DNA. The PCR profile was 1 cycle of 15 min at 95°C followed by 25 cycles of 30 s at 94°C, 90 s at the primer specific annealing temperature (Supporting Information Table S3), 60 s at 72°C, and a final cycle of 10 min 72°C. Reactions were carried out in an Eppendorf mastercycler gradient machine. PCR products were analysed on an ABI 3730 automatic sequencer with ROX-500 as size standard. The size of the fragments was calculated using GeneMapper 4.0 software (Applied Biosystems, Foster City, CA, USA).

Of the 44 loci tested, 11 turned out to be monomorphic or gave unreliable results and 33 were polymorphic and suitable for use (Table 1). The nomenclature for the microsatellites is equivalent to Endsley *et al.* (2002), with Md referring to *M. domestica*, followed by the repeat type and the microsatellite sequence number. Additionally we developed one more microsatellite marker from available microsatellite sequences in GenBank (Supporting Information Table S3).

#### Linkage analysis

We constructed a linkage map for each of the three crosses separately using JoinMap 3.0 (Van Ooijen & Voorrips, 2001). We used the population type code 'CP' in JoinMap to allow for heterozygous and homozygous diploid parents and assigned genotype codes for each locus depending on the segregation type (for details see the JoinMap manual). All markers were tested for significant deviation from Mendelian segregation by  $\chi^2$  analysis ( $P < 0.01$ ). Markers that deviated significantly from Mendelian expectations were included in linkage groups if their presence did not alter the order established without them. Marker placement

was determined using a minimum LOD score (logarithm of odds) of 4.0. The Kosambi mapping function that incorporates the possibility of crossover interference was used to convert recombination frequencies into map distances (Kosambi, 1944). After establishing separate linkage maps per cross we joined the linkage maps by using the 'combine groups for map integration' command of JoinMap for groups that had enough overlapping markers and linkage was sufficient. This was not possible for linkage group (=chromosome) IV as only the M2-cross yielded more than two linked markers on this group.

We note that the conventional way of constructing a linkage map is to analyse both sexes separately when recombination frequencies differ. As the number of linked markers to construct a 'female only' map was too small and the number of linked markers increased by including female recombination information we included both sexes in one map (for the MY- and the combined map). Hence, our overall map reflects the ordering of markers, but the relative recombination frequencies differ per strain and sex. The recombination frequencies for all possible marker pairs in each cross were estimated using the LINKMFEX.exe module of the LINKMFEX v2.3 program (R. Danzmann, University of Guelph, <http://www.uoguelph.ca/~rdanzman/software/LINKMFEX>).

Recombination frequencies were analysed with logistic models, using the 'glm' procedure in R version 2.9.2 (R Development Core Team, 2009). To correct for overdispersion the 'family = quasibinomial' option was chosen and *F*-tests were used to assess statistical significance (Crawley, 2005, pp. 256).

#### Map length and coverage

Two approaches were used to estimate the map length of *M. domestica*: (1)  $G_{e1}$ : to compensate for the two chromosome ends beyond the outermost marker of the linkage group 2 *s* (*s* = average spacing of the linkage map) were added to the length of each group (Fishman *et al.*, 2001); (2)  $G_{e2}$ : each linkage group was multiplied by the factor  $(m + 1)/(m - 1)$ , where *m* is the number of markers in each linkage group, irrespective of markers mapping to the same location. The estimated map length is the sum of the revised length of all linkage groups (Chakravarti *et al.*, 1991). The final estimated map length ( $G_e$ ) is the average of the two estimated map lengths. The observed map length was calculated as the length of the framework map ( $G_{of}$ ). Map coverage then was calculated as  $G_{of}/G_e$ .

#### Acknowledgements

We thank Monika Hediger and Daniel Bopp (Developmental Biology, University Zürich) for providing the housefly WHO and 0-12345 mutant strains; Rense Veenstra for support in the lab, Irene Mateo-Leach for suggestions on data analysis, and Franjo Weissing for supervision. B.F. acknowledges a scholarship of the Robert Bosch Stiftung. We thank three anonymous referees for comments on the manuscript.

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### Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: DOI 10.1111/j.1365-2583.2010.01016.x

**Table S1.** Model reduction using *F*-tests for comparison between models of recombinant frequencies.

**Table S2.** GLM testing for significantly deviating recombination frequencies of chromosomes and crosses.

**Table S3.** Newly developed microsatellite markers with GenBank accession numbers, repeat length and annealing temperature.

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