



ORIGINAL ARTICLE

# Genetic vs environment influences on house mouse hybrid zone in Iran



Nima Hashemian, Hassan Rajabi-Maham\*, Maryam Edrisi

Department of Animal Sciences and Biotechnology, Faculty of Life Sciences, Shahid Beheshti University, G.C., Evin, Tehran 1983963113, IR, Iran

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**Abstract** Genetic divergence and environment influence on speciation process are the great deal studies over recent decades. One of the best ways for exploring the interaction of geography and genetics is the evaluation of hybrids in a contact zone. To understand if there is one or more hybrid zone between house mouse subspecies in Iran and what are the differences comparing these zones with European well-known hybrid zone, we performed this approach. Samples were live-trapped from Ilam city in west for *sensu lato M. m. domesticus* subspecies, and Neishabur city in north-east of Iran for *sensu lato M. m. musculus* subspecies. In five experimental groups, male and female mice of the two subspecies were crossed reciprocally to generate F1 hybrids, and then F1 offspring males and females were crossed also reciprocally between siblings to generate F2 hybrids. In the same manner as seen in European hybrid zone, hybridization between female *M. m. musculus* and male *M. m. domesticus* of all five groups showed male sterility in F1 generation, but intact female offspring. These sterile males comparing with a parent or healthy males showed low count and more abnormal sperm percentage in morphological and testis histological section studies. Comparing the results from this study with numerous studies carried out during several years on the European hybrid zone showed an equal condition of contact between two subspecies. Genetical elements have kept their same influence on postzygotic reproductive isolation more than environmental effects far from the Europe, here in Iran.

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## 1. Introduction

Speciation always is important in organism's evolution and genetic basis of reproductive isolation between closely related

taxa [1–3]. House mouse complex species (*Mus musculus*) is an excellent model to understand the genetic base of postzygotic reproductive isolation in the early stages of speciation. The house mouse is containing at least three main subspecies: *M. m. musculus* is distributed throughout eastern Europe and North Asia, *M. m. domesticus* in western Europe, the Middle East, Africa, Australia, and the America, *M. m. castaneus* in throughout southeastern Asia [4,5]. Because of the unique geographical location, Iran is a bridge between West and East and

\* Corresponding author.

E-mail addresses: [h\\_rajabi@sbu.ac.ir](mailto:h_rajabi@sbu.ac.ir), [smaham@gmail.com](mailto:smaham@gmail.com) (H. Rajabi-Maham).

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will be the main area of distribution of the *Mus musculus* subspecies. Three main subspecies, *M. m. musculus*, *M. m. domesticus* and *M. m. castaneus* of house mouse present in Iran and the Zagros and Alborz mountains have the key role as barriers to prevent freely gene flow between them. *M. m. musculus* and *M. m. domesticus* are the two most widespread subspecies of house mouse, which diverged from their common ancestor 0.3–0.5 million years ago [6–8]. Despite this short divergence time, reproductive isolation between two subspecies in their hybrid zone in the regions of secondary contacts across central Europe clearly observes [5,9]. Hybrid sterility has been known to modulate by multiple genes. Prdm9 (Hst1) is one of them that localizes to chromosome 17 and is the principal responsible for spermatogenic failure in house mice hybrids [10–12].

According to the Haldane's rule, hybrid sterility or inviability usually affects the heterogametic sex, which represents a central role of sex chromosomes in hybrid male sterility [13]. The introgression of sex chromosome genes was more limited than the autosomal genes between *M. m. musculus* and *M. m. domesticus* [14,15]. This suggests that sex chromosomes play an important role in the mechanism of reproductive isolation between the two subspecies [2,16]. In laboratory crosses between *M. m. musculus* and *M. m. domesticus*, asymmetric sterility in F1 hybrid males is routinely observed when *M. m. musculus* is the mother and this genetic incompatibility is observed in F1 hybrid males usually without affecting hybrid females [17–23]. The *M. m. musculus* X chromosome is an important contributor that plays a central role in hybrid male sterility [17,22,24–27]. According to the Dobzhansky–Muller incompatibility, genetic basis of hybrid male sterility in house mouse is caused by deleterious epistatic interactions between incompatible interacting genes [28]. This implies an important interaction of the *M. m. musculus* X chromosome with the other chromosomes, especially *M. m. domesticus* Y chromosome in genetic incompatibility [29–31]. Although the *M. m. domesticus* Y chromosome does not have any contribution in hybrid male sterility, but plays a role in sperm head abnormality in interaction with the *M. m. musculus* X chromosome (X–Y incompatibilities) [32,33].

In this study, we designed some examinations for evaluation the role of sex chromosomes in the hybrid genetic incompatibility between the two subspecies, *M. m. musculus* and *M. m. domesticus* and comparing the results with European house mice hybridization.

## 2. Materials and methods

### 2.1. Animal husbandry and crossing design

Samples were taken from Ilam province (33.6384°N 46.4226°E) for pure *domesticus* subspecies, and North Khorasan province (37.4761°N 57.3317°E) to have pure *musculus* subspecies. Parents of the two subspecies were crossed reciprocally: (*M. m. domesticus* × *M. m. musculus* and *M. m. musculus* × *M. m. domesticus*) to generate F1 hybrids, and F1 hybrids were crossed in sibling: (*M. m. domesticus* × *M. m. musculus*) F1 × (*M. m. domesticus* × *M. m. musculus*) F1 and (*M. m. musculus* × *M. m. domesticus*) F1 × (*M. m. musculus* × *M. m. domesticus*) F1 to generate F2 hybrids.

### 2.2. Genotyping of hybrids

After animal husbandry and crossing design, mice were dissected and liver tissues stored in 70% ethanol. Genomic DNA was extracted with the standard phenol–chloroform DNA extraction. In polymerase chain reaction, two markers for X chromosome (Intro2 and X15), and two markers for Y chromosome (Zfy2 and Ddx3y), were designed and used to determine the inheritance process of these chromosomes to genetic background of F1 and F2 hybrids generation.

### 2.3. Quantification of male fertility phenotypes

To check the parameters of male fertility, we quantified sperm concentration [23,34], sperm morphology and seminiferous tubules cross-sections [35]. In order to count sperm, the right and left cauda epididymis were dissected in 0.5 ml PBS (Phosphate Buffered Saline) medium solution and sperms diffused into media passively over 20 min, then 5 µl of sperm suspension was loaded in the Hemocytometer counting chamber. Measurement for each male was done with three times repetition and the mean concentration was reported million per cubic millimeter (10<sup>6</sup>/mm<sup>3</sup>).

The sperm suspension was also used to study sperm morphology. Sperm suspension air dried on a glass slide, painted with Diff-Quik solutions. For sperm abnormality report, sperm categorized in four abnormalities groups: proximal bent tail, distal bent tail, missing head or missing tail and amorphous head. The percentage of abnormal sperms for each male assessed by counting 200 sperm, randomly.

The data statistical analysis, Shapiro-Wilk method showed that the data do not follow a normal distribution for both sperm density and sperm morphology and the data was not normalized by none of the data conversion method. Kruskal-Wallis test was used for comparison between groups and Mann-Whitney to compare the difference between pair of groups in SPSS v19 software.

Right testis dissected immediately, weighed fresh upon dissection, fixed in Bouin for 24 h, and Washed in ethanol series for dehydration, cleared any ethanol with xylene, and embedded in 60 paraffin and allowed to harden overnight. The prepared tissue sectioned at 6 µm thickness, and stained with the Hematoxylin and Eosin standard staining protocol.

## 3. Results

### 3.1. X&Y-chromosome markers

Two markers for X chromosome (Intro2 and X15), and two markers for Y chromosome (Zfy2 and Ddx3y), were used to determine the inheritance of these chromosomes to genetic background of F1 and F2 hybrids generation. According to the Intro2 and X15 patterns, *M. m. musculus* female parent X chromosome inherited to genetic background of F1 male sterile hybrids and F2 male sterile hybrids. Results of the Zfy2 and Ddx3y patterns indicate *M. m. domesticus* male parent Y chromosome inherited to genetic background of F1 male sterile hybrids.

### 3.2. Male fertility parameters

#### 3.2.1. Sperm concentration

Mean sperm count of males in four experimental groups reported million per milliliter in Table 1. The results of Kruskal-Wallis test in sperm count average showed a significant difference between four male groups, and Mann-Whitney checked the significant differences between two comparing groups. As represented in Table 1, hybrid males with maternal origin of *M. m. musculus*, show significant decrease in mean sperm count comparing to other groups. The sperm count in hybrid males with maternal origin of *M. m. domesticus* is also significantly different from the mice with parental *M. m. musculus* (Mus) and the hybrids of *M. m. domesticus* maternal origin (Hdom-mus).

#### 3.2.2. Sperm morphology

Mean percentages of all considered abnormal sperms and the percentage of each abnormality in four males groups reported in Table 1 and Fig. 1. The results of Kruskal-Wallis analysis showed a significant difference for mean percentage of sperm abnormality in four male groups, and Mann-Whitney test represented significant increase in mean percentages of sperm abnormality in the hybrids with maternal origin of *M. m. musculus*, comparing to other groups ( $P < 0.05$ ); but no significant differences with parental subspecies (*M. m. musculus* and *M. m. domesticus*) ( $P > 0.05$ ) (Table 1).

#### 3.2.3. Testis histology

Seminiferous tubules in histological sections from testes of four group males has been studied, most of seminiferous tubules in hybrid males with maternal origin of *M. m. musculus* were characterized by a large reduction in complete spermatozoa numbers (Fig. 2c).

## 4. Discussion

In recent decades, using genetical and evolutionary studies created a way to understand the postzygotic reproductive isolation mechanisms that play an important role in the process of speciation in natural populations and laboratory samples. Postzygotic reproductive isolation in early stages, caused by heterogeneous interaction between nuclear genes, derived from the parental species or subspecies. This destructive interaction

is known as Dobzhansky-Muller (DM) incompatibility [16,28,36]. Based on the DM model, an evolutionary model of genetic incompatibility, at least two genes interaction is required to create hybrid genetic incompatibility. Many genetic studies in animal models detect genetic loci involved in the DM incompatibilities and attempt to reveal the genetic structure of the postzygotic reproductive isolation. The genetic laboratory studies in animal models such as house mice (*Mus musculus*) and fruit flies (*Drosophila*), sterility and inviability is more common in heterogametic sex (Haldane's rule), that suggested sex chromosomes play an important role in the mechanism of postzygotic reproductive isolation [13]. Results of this study and other studies related to genetic incompatibility between house mice subspecies, showed male sterility in hybrids as a key mechanism of postzygotic reproductive isolation assessment. The study of this genetic incompatibility between two subspecies, *M. m. musculus* and *M. m. domesticus*, referred to key role of the X chromosome and its interaction with autosomal chromosomes and also the role of the Y chromosome and its interaction with the X chromosome (XY incompatibility). Sex chromosome genes show limited introgression than autosomal chromosomes between *M. m. musculus* and *M. m. domesticus* subspecies, so it can be concluded that sex chromosomes play a key role in the reproductive isolation mechanism between the two subspecies [14,15]. Hybrid male sterility between *M. m. musculus* and *M. m. domesticus* subspecies indicates specific genetic patterns. In laboratory crosses between the two subspecies, asymmetric hybrids male sterility in F1 generation could be seen only in male hybrids with *M. m. musculus* female parent and genetic incompatibility is always observing just in hybrid males and the female are intact [17,23,27,37].

In this study, using Intro2 and X15 markers to investigate X chromosome introgression and inheritance to genetic background of hybrid males, it was shown that the *M. m. musculus* female parent X chromosome inherited to sterile males of F1 and F2 generations. This confirmed the key role of *M. m. musculus* X chromosome in male sterility and the influence of X-linked loci in increasing incidence of hybrid male sterility traits, without any incompatibility in females F1 generation. Also the *M. m. musculus* X chromosome introgression in the next generations such as F2 generation, leads to the same genetic incompatibility, which confirms earlier studies that highlight the influence of this incompatibility beyond the F1 generations [38]. In addition, using the Zfy and Ddx3y markers

**Table 1** Some important parameters mean in the quality of male fertility for parental subspecies males and their males offspring hybrids of F1.

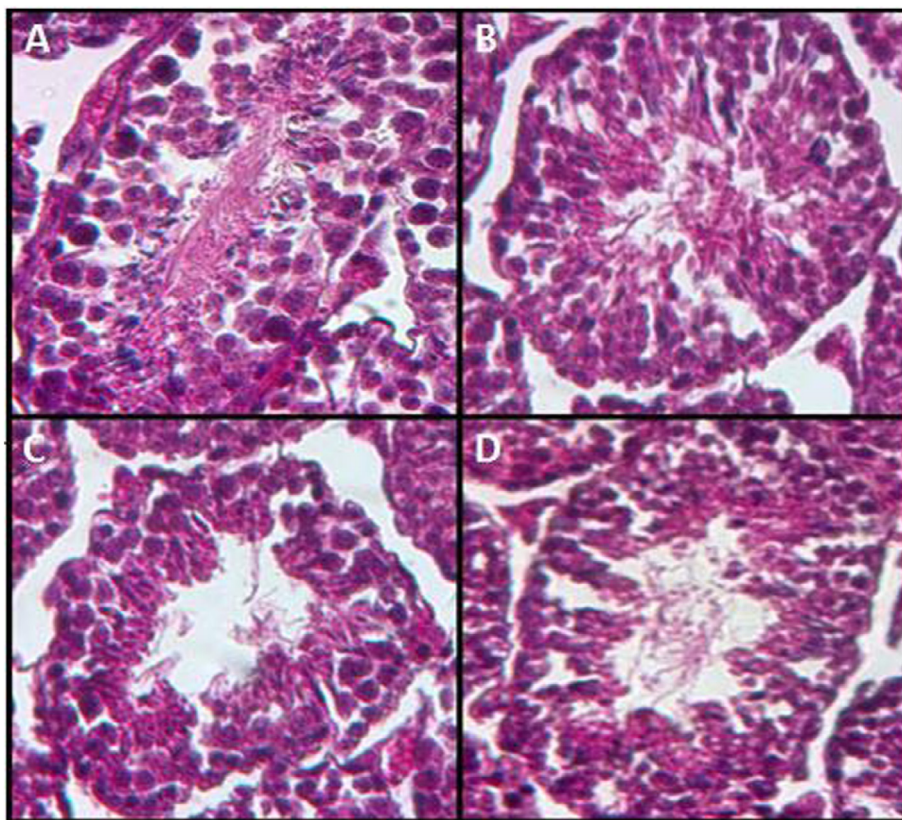
Parameters	♂ <i>M. m. domesticus</i>	♂ <i>M. m. musculus</i>	Males hybrids from ♀ <i>M. m. musculus</i> × ♂ <i>M. m. domesticus</i> cross	Males hybrids from ♀ <i>M. m. domesticus</i> × ♂ <i>M. m. musculus</i> cross
Sperm density (millions/ml)*	13.63 ± 0.4 <sup>a</sup>	10.31 ± 0.3 <sup>b</sup>	1.94 ± 0.06 <sup>c</sup>	13.7 ± 0.28 <sup>a</sup>
Percentages of all considered abnormal sperms*	8.3 ± 2.88 <sup>a</sup>	12.6 ± 5.24 <sup>a</sup>	65.8 ± 11.28 <sup>b</sup>	8.7 ± 4.08 <sup>a</sup>
Percentage of headless sperms*	4.3 ± 3.09 <sup>a</sup>	8.3 ± 3.75 <sup>a</sup>	30.8 ± 12.9 <sup>b</sup>	5.4 ± 4.31 <sup>a</sup>
Percentage of distal bent tail*	1.8 ± 7.2 <sup>a</sup>	1 ± 1.06 <sup>a</sup>	14.5 ± 9.25 <sup>b</sup>	1 ± 0.5 <sup>a</sup>
Percentage of proximal bent tail*	1.6 ± 0.65 <sup>a</sup>	1.8 ± 1.15 <sup>a</sup>	11.4 ± 5.56 <sup>b</sup>	1.5 ± 0.93 <sup>a</sup>
Percentage of amorphous sperm head*	0.6 ± 0.82 <sup>a</sup>	1.5 ± 1.06 <sup>a</sup>	9.1 ± 3.85 <sup>b</sup>	0.8 ± 0.76 <sup>a</sup>

\* For each factor on mean (people in each group) ± standard deviation expressed.

<sup>a,b,c</sup> Groups significantly different by Mann-Whitney ( $P < 0/05$ ).



**Fig. 1** (A) and (B) show respectively the morphology of normal sperm in *M. m. domesticus* and *M. m. musculus*. Most observed sperm abnormalities in hybrids; (C) Proximal bent tail; (D) Headless; (E) Amorphous head.



**Fig. 2** Testes histological cross sections and seminiferous tubules of the studied groups; (A) and (B) respectively *M. m. domesticus* and *M. m. musculus*; (C) and (D) hybrid males respectively with maternal origin of *M. m. musculus* and *M. m. domesticus*.

to investigate Y chromosome inheritance into genetic background of hybrid males, it was shown that the *M. m. domesticus* male parent Y chromosome inherited to genetic background of sterile hybrid males of F1 and F2 generations.

This is referring to the role of *M. m. domesticus* Y chromosome and its interaction with *M. m. musculus* X chromosome (XY incompatibility), to incidence of some sterility traits such as abnormal sperm head morphology and exacerbate this genetic

incompatibility, which confirms earlier studies on the role of *M. m. domesticus* Y chromosome and its interaction with the X chromosome and its effect on the sperm head morphology [32,33].

Studies on hybrids in *Drosophila* and mice showed genetic incompatibilities effect on spermatogenesis stages [39]. According to research in the European hybrid zone, hybrid male sterility due to the X-linked genes of *M. m. musculus* maternal origin and interacting with autosomal chromosomes impact in spermatogenesis and reducing sperm production [22]. In the present study, the lowest sperm counts belonged to hybrid males with *M. m. musculus* maternal origin. Genetic incompatibility caused by X-linked genes interaction with autosomal chromosomes, yielding abnormalities sperm head morphology; also different kind of sperm abnormality (headless, proximal bent tail, distal bent tail) because of destructive interaction with some autosomal chromosomes happen, it cause sperm abnormality and so reduce ability of sperm fertility [26,38,40].

In this study, abnormal sperm percentage in hybrids with *M. m. musculus* maternal origin had significantly increased comparing with parental subspecies while *M. m. domesticus* maternal origin hybrids hadn't. This bias in sperm abnormality percentage represents a destructive interaction of the X chromosome with the autosomal chromosomes. The overall results obtained in this study in the Iran mice hybrid zones are in coherent with the studies in hybrid zones of Europe. In other word, the speciation mechanism in geographic level between two subspecies have had the same act in both regions. Despite the thousands kilometers distance between these zones, genetic incompatibility and environmental influences on mice phenotypes, sexual behavior and so on are too identical. Depending on distribution of *Mus musculus* subspecies in Iran, the key role of the Alborz and the Zagros mountains in distribution of subspecies in different parts of the country is indisputable. Since thousands of years, these mountains have acted as barriers to contact these subspecies to each other and have prevented hybridization and freely gene flow. According to the present study and previous studies on *Mus musculus* complex in Iran [41–43], it can be assumed that genetic incompatibility process in *Mus musculus* complex is the most important part of reproductive isolation. As the case of the European hybrid zone, Iranian geographic barriers of gene flow accelerate the process of speciation between subspecies of house mouse complex species.

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