Research Article

Morphological, cranial and coat colour variations in *Mus musculus castaneus* populations of Pothwar, Pakistan

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Abstract

The species diversities of *Mus musculus* populations in Pakistan were not examined well till today, irrespective of its geographic importance being situating in a midpoint of the homeland of house mouse diversity. Cytochrome b gene analysis of *M. musculus castaneus* populations of northern Pakistan revealed that this South Asian subspecies is highly diverse genetically, harboring four known sub-lineages. Here we performed the morphological analysis of mice samples from the Pothwar Plateau, northern Pakistan which have already been genetically characterized by Cytochrome b gene analysis. Discriminant Function Analysis (DFA) of 18 morphometric, cranial and coat colour variables was applied to 140 of these genetically characterized adult specimens. Discriminant Function Analysis (DFA) showed 50-70% morphological isolation between four sub-lineages of *M. m. castaneus* identified through molecular analyses. These results thus suggest that the house mice experienced the genetic admixing of the four *castaneus* sub-lineages in this plateau, perhaps reflecting the rapid growth of local human populations at some prehistorical times.

Keywords: Cytochrome b; Discriminant Function Analysis; Morphological variations; *Mus musculus castaneus*

Introduction

Of all the mice species within sub-genus *Mus*, the commensal house mouse *Mus musculus* is well known for its well debated evolution and radiation [1]. It has been the subject of many researchers for its wide range of genetic differentiation and history of worldwide colonization and ecological adaptation. Attempts to classify house mouse subspecies on morphological basis

resulted in many ambiguities and it was only after the advent of molecular techniques and their use in taxonomy that the differentiation was made between the different species and morphological variant populations of the same species. *Mus musculus* is now regarded as a polytypic species represented by three closely related and morphologically similar subspecies (*Mus musculus musculus*, *Mus musculus domesticus* and *Mus*

musculus castaneus) having varying degree of gene flow and sexual incompatibility which have been reported to be radiated about 0.5 million years ago [2]. Of these *M. musculus castaneus* is widespread mainly in Southeast Asia [1, 3] and is more polytypic as well as diverse genetically e.g. [4] than *M. m. domesticus* or *M. m. musculus*. The origin, genetic constitution and distribution of *M. m. musculus* and *M. m. domesticus* is becoming reasonably well documented and these two subspecies represent genetically well-defined homogeneous entities [5, 6]. This is not the case for *M. m. castaneus* which is far from completely elucidated and still remains poorly documented [6]. However, these studies which investigated the genetic diversity and distribution of *M. m. castaneus*, didn't study the Castaneus populations of Pakistan to the extent it was studied in other areas of its distribution as very few samples from Pakistan were included, so its biogeography is still not known properly. We presented the first major effort to resolve this issue and added to the knowledge of genetic constitution, phylogeography and colonization history of *M. m. castaneus* in one of the most important part of its range [7]. The aim of

the present study was to make an attempt to identify the genetically characterized taxa of *Mus* on the basis of their body measurements, skull and pelage characters and to reveal whether morphometric differentiation corresponds to molecular data.

Materials and Methods

Study area and sample collection

House mouse sampling carried out in the Pothwar Plateau of Punjab Province, Pakistan, a region bounded by the Murree hills and foothills of the Himalayas in the north, the Jhelum River in the east, the Salt range in the south, and the Indus River in the west (roughly latitudes 32°22' to 34° N and longitudes 71°30' to 73°30' E) (Fig. 1). Seventeen localities were surveyed between May 2011 and April 2013 using a mixture of live and snap traps (Table 1). Sampling localities encompassed a range of habitats, including houses (inside or near human habitations), agricultural fields (wheat, groundnut and sweet pea), and uncultivated areas characterized by dry pebble and stone substrates, scrublands with sparse shrub cover, or open forests dominated by *Acacia* and *Olea* trees and *Ziziphus* shrubs.

Figure 1. Map of the study area showing capturing sites(numbers) in each district of the Pothwar region

Table 1. Collection localities, sample size and geographical coordinates of mice specimens analyzed in this study

District	Locality/Village	<i>Mus cookii</i>	<i>Mus terricolor</i>	<i>Mus musculus castaneus</i>	Latitude (N)	Longitude (E)
Attock	Rangli	----	----	12	33° 29'37.9"	72°06'9.5"
	Kisran	----	----	13	33°28' 14.8"	72°15'07.1"
Chakwal	Bari	----	----	12	32°55' 46.1"	72°43'45.8"
	Khokharzare Dam	----	----	9	32°48' 48.6"	72°51'36.7"
	Mumdot	----	----	11	32°52' 26.6"	72°27'24.2"
	Makhial	----	----	12	32°39' 43.9"	72°39'20.77"
Jhelum	Ahmadabad	----	----	12	32°31' 11.9"	72°51'42.0"
Rawalpindi	Usmanzada Adra	----	01	9	33°11' 27.3"	73°10' 52.1"
	Koont Farm	01	----	10	33°06' 58.0"	73°00' 52.2"
	Gujar Khan	----	----	10	33°15'31.15"	73°18'19.1"
	Kalar Syedan	----	----	8	33°23' 6.37"	73°20' 13.1"
	Chakri	----	----	7	33°35'22.53"	72°58'42.7"
	Rata amral	----	----	7	33°36'20.88"	73° 2'37.49"
Islamabad Capital Territory	Sihala	----	----	3	33°32'38.66"	73°11'48.8"
	Pind Bhegwal	----	----	02	33° 43' 7.2"	73° 15' 44.4"
	Taramri	----	----	02	33°38'39.13"	73° 9'51.69"
	Mohra nur	----	----	01	33° 42' 0"	73° 10' 0"

Morphological analysis

All the genetically characterized specimens were subjected to morphometric, cranial and coat colour analysis. In addition to body weight (BW), four body measurements included in the morphometric analysis were head and body length (HBL), tail length (TL) from rump to the last tail vertebra, ear length (EL) from basal notch to distal tip, and hind foot length without claw (HFL) were recorded in mm. To minimize the effect of on to genetic growth, only adult specimens were used. Apart from the external body measurements, skull measurements of the specimens were also taken using a digital caliper accurate to the

nearest 0.05 mm [8, 9] The cranial measurements that were included are greatest skull length (GSL), nasal length (NL) zygomatic breadth (ZB), incisive foramen length (IFL), cranial depth (CD), and cranial breadth (CB). To determine the pelage colour and texture, round skins of the specimens were prepared. Pelage colour of dorsal and ventral side of the body was determined by using Munsell colour chart [10]. Munsell colour chart uses tripartite colour scores i.e. hue, value and chroma to describe a colour. Hue depicts whether a colour looks yellow, green, red, blue or purple. Value indicates lightness of the

colour while chroma indicates its departure from a neutral colour of the same lightness.

Statistical analysis

Discriminant function analysis (DFA) of morphometric, cranial and coat colour variables was performed in SPSS16 to predict membership of each specimen to the respective species/sub-lineage identified on the basis of molecular analysis. Discriminant score of two linear functions for each specimen was used to classify each individual.

Results

According to Cytb analysis [7] *Mus* fauna of Pothwar region comprises of three *Mus* species, namely *Mus musculus castaneus* (Accession numbers KY661759 to KY661856), *Mus terricolor* (Accession no KX790792) and *Mus cookii* (Accession no KX790791). Out of these species *M. m. castaneus* was reported to be the most abundant one and further genetic analysis concentrating on *M. m. castaneus* (CAS) proved this sub-species highly diverse and diverged into four sub-lineages named (Fig. 2) hereafter CAS-1, CAS-2, CAS-3 and CAS-4 [3].

Morphological variations

As according to genetic characterization *M. m. castaneus* turned out to be the most abundant species recorded from Pothwar region which was further differentiated into four sub-lineages, while all other species were represented by just one or two specimens so those species were not included in further morphological analyses. So variations in external body characters of 140 collected *M. m. castaneus* specimens were compared among four sub-lineages. Highest BW was recorded for CAS 2 (14.9 ± 2.65 g), followed by CAS 4 (14.0 ± 3.28), and CAS 3 (13.4 ± 3.6) while lowest BW was observed in CAS-1 (11.8 ± 4.57). In the case of HBL, trend of highest and lowest was similar as for BW with CAS 1 being smallest with 73.5 ± 8.90 and CAS 2 largest

with 77.8 ± 8.17 . But for CAS 3 and CAS 4 trend was somewhat different with CAS 3 (76.3 ± 11.35) larger than CAS 4 (75.5 ± 9.02). TL was measured 65.4 ± 7.71 in CAS1, 70.4 ± 8.43 in CAS2, 68.7 ± 6.01 in CAS3 and 66.9 ± 6.32 in CAS4.

Variation in EL was not much prominent among four sub-lineages of *M. m. castaneus*. It was measured as 13.2 ± 1.76 , 12.9 ± 1.43 , 13.3 ± 1.42 and 13.7 ± 2.04 from CAS 1 to CAS 4, respectively. Unlike other characters mentioned above, HFL was smallest in CAS 2 (15.8 ± 2.32) and largest in CAS3 (17.0 ± 1.03). In CAS 1 HFL was measured 16.7 ± 0.16 and in CAS 4 it was 16.5 ± 1.74 . Discriminant function analysis (DFA) based on five morphological measurements was 46.7% accurate in assigning the individual specimens to the correct sub-lineage identified on the basis of molecular analysis (Table 2). From the values of standardized canonical discriminant coefficients in (Table 3) it can be concluded that HFL, BW and HBL are the variables which are playing important role in discrimination between CAS sub-lineages as larger the standardized coefficient, greater is the contribution of the respective variable to the discrimination between groups. Position of group centroids in canonical discriminant functions graph (Fig. 3) also shows that morphological differentiation between the sub-lineages is not complete as clusters for all sub-lineages overlap to varying degree.

Results of DFA show that classification of specimens into respective sub-lineage on the basis of cranial characters was 69.6% correct (Table 4) and according to standardized canonical discriminant function coefficients IFL and GSL were the most important skull characters for classification into respective group (Table 5). Position of group centroids in canonical discriminant functions graph (Fig. 4) for skull variables shows that there was considerable differentiation between the sub-lineages as

there was less overlap between clusters for each sub-lineage and group centroids were also separated considerably.

Tremendous variation was observed in the dorsal and ventral coat colour of *castaneus* which varied from light orange brown and dark golden brown to dark grey dorsally and light orange/peach and fawn colour to pure white ventrally. There was also variation in foot colour from light pinkish brown to pure white and to black. A discriminant function analysis based on dorsal and ventral coat colour variables (taken together) was 47.1%

accurate in assigning the individual specimens to the correct sub-lineage identified on the basis of molecular analysis (Table 6). Values of standardized canonical discriminant coefficients for these variables are given in (Table 7) which shows that ventral coat colour plays important role in discrimination between CAS sub-lineages. Position of group centroids in canonical discriminant functions graph (Fig. 5) also shows that differentiation between the sub-lineages is not complete as clusters for all sub-lineages overlap to varying degree.

Table 2. Percentage count of the number of specimens correctly classified to the respective sub-lineage on the basis of Discriminant Function Analysis (DFA) of External Morphological Variables

% Count	Sub-lineage	Predicted Group Membership				Total
		C1	C2	C3	C4	
	C1	41.7	20.8	25.0	12.5	100.0
	C2	0.0	74.1	12.1	13.8	100.0
	C3	15.6	12.5	56.3	15.6	100.0
	C4	8.3	33.3	8.3	50.0	100.0

46.7% of original grouped cases correctly classified

Table 3. Standardized Canonical Discriminant Function Coefficients of morphological variables for *M. m. castaneus* sub-lineages. CAS-1 (N= 22), CAS-2 (N=56), CAS3- (N=31) and CAS-4 (N=31)

Variable	Function 1
BW	.485
HBL	.301
TL	.296
EL	-.165
HFL	-.664

Table 4. Percentage count of the number of specimens correctly classified to the respective sub-lineage on the basis of Discriminant Function Analysis (DFA) of cranial variables

% Count	Sub-lineage	Predicted Group Membership				Total
		C1	C2	C3	C4	
	C1	75.0	.0	25.0	0.0	100.0
	C2	7.7	61.5	7.7	23.1	100.0
	C3	0.0	0.0	75.0	25.0	100.0
	C4	7.7	15.4	7.7	69.2	100.0

69.6% of original grouped cases correctly classified

Table 5. Standardized Canonical Discriminant Function Coefficients of cranial variables for *M. m. castaneus* sub-lineages. CAS-1 (N= 22), CAS-2 (N=56), CAS3- (N=31) and CAS-4 (N=31)

Variable	Function 1
GSL	-.652
NL	-.249
CB	.181
CD	.420
ZB	-.279
BFM	.349
IFL	.774

Table 6. Percentage count of the number of specimens correctly classified to the respective sub-lineage on the basis of Discriminant Function Analysis (DFA) of coat colour variables

% Count	Sub-lineage	Predicted Group Membership				Total
		C1	C2	C3	C4	
	C1	44.7	29.4	25.9	0.0	100.0
	C2	4.2	85.4	.0	10.4	100.0
	C3	7.1	3.6	71.4	17.9	100.0
	C4	0.0	64.3	0.0	35.7	100.0

47.1% of original grouped cases correctly classified

Table 7. Standardized Canonical Discriminant Function Coefficients of coat colour variables for *M. m. castaneus* sub-lineages. CAS-1 (N= 22), CAS-2 (N=56), CAS3- (N=31) and CAS-4 (N=31)

Variable	Function 1
(D)V	-.152
(D)C	-.327
D(Hue)	.110
(V)V	.429
(V)C	.537
(V)HUE	.304

Figure 2. ML tree for Cytb sequences from *M. m. castaneus* sub-lineages [31] along with *M. m. domesticus* used as out group. (Green CAS1, yellow CAS2, blue CAS3, pink CAS4)

Figure 3. Discriminant Function Analysis (1st two functions) of 5 morphological variables. Overlapping of the clusters for different sub-lineages indicate that sub-lineages are not completely distinguishable on the basis of morphological characters. CAS-1 (N= 22), CAS-2 (N=56), CAS3- (N=31) and CAS-4 (N=31)

Figure 4. Discriminant Function Analysis (1st two functions) of cranial variables. Less overlapping of the clusters for different sub-lineages indicates that sub-lineages are considerably distinguishable on the basis of cranial characters. CAS-1 (N= 22), CAS-2 (N=56), CAS3- (N=31) and CAS-4 (N=31)

Figure 5. Discriminant Function Analysis (1st two functions) of coat colour variables. Overlapping of the clusters for different sub-lineages indicate that sub-lineages are not completely distinguishable on the basis of coat colour characters. CAS-1 (N= 22), CAS-2 (N=56), CAS3- (N=31) and CAS-4 (N=31)

Discussion

Analysis of genetic and morphological diversity by present study provided valuable knowledge about the mice populations of northern Pakistan. Principal findings of this study is that *M. m. castaneus* populations of this region have substantial morphological variation depicted by more than one Cytb sub-lineages which may coexist and are

distributed throughout Pothwar region and may not show geographical segregation. In this way this study explores the diversity and “polytypic character” of *M. m. castaneus* i.e. it is represented by more than one genetic entity and morphological forms. This view has been strengthened by tremendous coat colour variation found in this sub-species.

As the basic structure and transformation of the morphological character has the genetic basis, there has been a growing trend of analyzing the morphological species by genetics and molecular markers [11-17]. Biosystematics studies the taxonomical status and evolution of taxa based upon statistical data, determines how a species differentiates into sub-species through time and space under the effect of genetic information [18]. Although in recent years there has been tremendous increase in the use of molecular techniques in the field of phylogenetics, however study of morphological characters still remains an important tool in inferring phylogenies [19-24] as morphological data can be collected more easily from large number of preserved museum specimens and also from fossil record [25]. Moreover, morphometric data can also be used to study the selection pressures and trends for adaptations within a taxa or lineage [26-29].

In present study, morphological analysis of all body measurements for three sub-species including external body characters and skull variables were in good agreement with previous reports [8] except very clear disagreement in the case of tail length for *M. m. castaneus*. According to previous reports *M. m. castaneus* has been characterized by tail length longer than body length [8] but according to present study tail length was never longer than body length rather specimens from Pothwar populations of *M. m. castaneus* always have tail length equal or shorter than body length. This disagreement about the *M. m. castaneus* populations of Pothwar can be attributed to the absence of data specific to *M. m. castaneus* populations of Pakistan as *M. m. castaneus* populations in Pakistan have never been the subject of any comprehensive study characterizing this population genetically and morphologically.

Visibly shorter tail and comparatively larger

skull in *M. m. castaneus* from Pothwar can also be attributed to the life history and adaptations according to the habitat as study of morphometric variations among commensal and non-commensal mice populations belonging to same species (*M. musculus*) by [30] explained the effect of life history and ecology on the morphological characters and according to them populations exhibiting commensal mode of life had smaller skulls and much longer tails than non-commensal populations of the same sub-species. Shorter tail length is an ancestral character in *M. musculus* as it is proved by the fact that other close relatives of *M. musculus* (*M. spicilegius*, *M. macedonicus* and *M. spretus*) have shorter tail length. So, the longer tail length is an adaptation to commensal mode of life as tail movements contributes to the better survival in three dimensional commensal habitats [30].

Craniometric data revealed that *M. m. castaneus* populations of study area are characterized by broader zygomatic plate and a clearly notched tip of the upper incisor as observed in 100% captured samples. Considerable variations observed in *M. m. castaneus* coat colour by present study are suggestive of either strong selection in the different habitats or partially segregated gene pools. Traditionally, *M. m. castaneus* has been reported as dark coloured mice while *M. m. bactrianus* is identified as having golden brown or bright yellow dorsal fur but in present study tremendous coat colour variation in *castaneus* was observed which approaches the description of *bactrianus*. So, this study supports the view discussed by some previous authors e.g. [6] that what had been called *bactrianus* now seems to be part of high mtDNA diversity that exists among *castaneus* populations from Pakistan and northwest India. So, coat colour variation within *castaneus* can be an interesting topic for future research in

Pakistan.

Conclusion

This study provided validated information about the *Mus* fauna of Pothwar region, and led to the conclusion that more than one clades of South East Asian subspecies *M. m. castaneus* are intermingled in this area which were previously known to have a non-overlapping geographical distribution. This view has been strengthened by the tremendous coat colour variation found in this sub-species. This study supports the view held by some other authors that long debated *M. m. bactrianus* seems to be the part of high mtDNA diversity present among *M. m. castaneus* populations of Pakistan and northwest India. Capture of *M. cookii* specimen during this study may represent a case of previously unknown mice or it may provide a clue about major range expansion of this species which was previously never reported from Pakistan or from western parts of Asia and thus provides insight for future endeavors focusing on this subject.

Authors' contributions

Conceived and designed the experiments: MS Nadeem, Performed the experiments: S Bibi, Analyzed the data: T Mahmood & Franc Janzekovic, Contributed materials/analysis/ tools: AR Kayani, Wrote the paper: S Bibi & M Mushtaq.

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