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THE VARIATIONS OF CARBONIC ANHYDRASE IN SPECIES OF THE GENERA OF *MUS* LINNAEUS, 1758 AND *RATTUS* FISCHER, 1803 (MAMMALIA: RODENTIA) IN THE LINE OF ANKARA - ZONGULDAK PROVINCES

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ABSTRACT. Carbonic anhydrase (CA_1) enzyme variations of one hundred and forty six specimens of *Mus domesticus, Mus macedonicus* (House mice) and one hundred and twenty specimens of *Rattus rattus* (Roof rat) and *Rattus norvegicus* (Brown rat) were examined by the field work conducted in 23 localities in the line of Ankara-Bolu-Zonguldak. It was determined that CA_1 was fixed to two different homozygous alleles in both house mouse species. In the roof rat samples, CA_1 was fixed to two homozygous alleles at different frequencies in two groups formed according to the colour of the back fur. A single homozygous allele was detected at the CA_1 locus of brown rat samples. It was observed that these four species were separated in the UPGMA tree made according to allele frequencies. In this respect, it was evaluated that CA_1 could be a molecular marker that can be used to distinguish these species.

1. INTRODUCTION

Rattus and *Mus* species are not native to Türkiye, they come to the country in different ways such as land and sea transportations. Such species are called synanthropic or agrophilic species [1], and they were sometimes considered as invasive species [2]. In the new locations where they have just settled, over time, they have undergone differentiation as a result of the evolutionary process. Taxonomically, although there are scientific papers showing that *Mus domesticus* Rutty, 1772 is a different species from *Mus musculus* Linnaeus,1758, *M. domesticus* is now accepted as a subspecies of *M. musculus* in recent studies, thus *M. musculus domesticus* and *Mus macedonicus* Petrov and Rozic, 1983 are distributed in Türkiye [3, 4, 5, 6, 7, 8, 9]. Allozyme variations are used as molecular markers to demonstrate genetic differentiation and distinguish taxa from each other. In this connection, various studies have been conducted on the allozyme of the genus *Mus* [8, 10, 11, 12, 13, 14].

Also, it was reported that *M. domesticus* (Syn.; *M. musculus*) and *M. macedonicus* range in the line of Ankara - Zonguldak based on morphological, karyological, and morphometric characteristics, as well as 2 alleles of isocitrate dehydrogenase [8].

Keywords. Mus, Rattus, carbonic anhydrase, variation

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It is known that two *Rattus* species are widely distributed in Türkiye. The first distribution records on *Rattus rattus* (Linnaeus, 1758) and *Rattus norvegicus* (Berkenhout, 1769) recorded from areas neighbouring Türkiye have been provided by [15, 16, 17, 18, 19]. The most recent detailed, morphological, karyological and allozyme studies on rat species were given by [20, 21, 22, 23, 24, 25, 26]. The taxonomic importance of non-specific esterase variations in *R. norvegicus*, and the diagnostic power of the patterns of blood serum proteins in *R. rattus* and *R. norvegicus* were enlightened in the detected 4 different colour variations in *R. rattus* specimens of Türkiye [21, 22, 26]. On the other hand, *R. norvegicus* samples were found to be quite homogeneous in terms of colour variation.

Also, Yiğit et al. [22, 23] reported seven of 22 allozyme loci studied were found to be polymorphic in Turkish *R. rattus*, and eight of 22 loci were polymorphic in the 4 sub-populations of Turkish *R. norvegicus*. Various studies have been performed on allozyme of the genus *Rattus* [27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39]. Baverstock et al. [34, 35] conducted a study on *R. rattus* and *R. norvegicus*, found differences in many enzyme loci in the species of the genus *Rattus*, including the Carbonic Anhydrase enzyme. In this study, carbonic enzyme variation in samples of two genera collected from Ankara-Zonguldak line and whether this enzyme can be used as a genetic marker was investigated.

2. MATERIALS AND METHODS

In this study, the blood samples of *Mus* and *Rattus* collected from Ankara-Zonguldak line before 2002 and stored in the Mammals Research Collection of Ankara University (AUMAC, www.mammalia.ankara.edu.tr) were used (Table1).

Protocol of starch gel electrophoresis

1. Procedure of erythrocytes haemolysis were performed in accordance with [40].

2. Electrophoretic procedures were carried out as given by [41, 42] with small modifications; the starch gel percentage was 11 % and the samples loaded on gel were run at 12 v / cm with 9-15 mA for 5 hour.

3. Histochemical staining: After the electrophoresis was completed, the reaction mixture for the carbonic anhydrase enzyme was prepared by method below;

ß-Naphthyl acetate: The reaction mixture was prepared by adding 40 mg of Fast Blue RR (Sigma F-0500) to 96 ml of 0.05 M phosphate buffer and 4 ml of a 1 % ß-Naphthyl acetate stock solution prepared previously in 50 % acetone [42]. Staining was done by incubating at 37 °C in the dark until bands were seen on the gel.

4. Gel fixation: After the enzyme bands were seen, the reaction was stopped by washing with gel fixation solution [45 parts of 13 methanol, 55 parts of acetic acid solution (1acetic acid: 5 H2O)].

5. Documentation of results: When staining was complete, the gel was photographed by placing it on the light box, and alleles were located by plotting the observed band patterns. Then, the calculation of allele frequencies was done using these zymograms. Allozyme was numbered according to the most common allele. The most common allele was given numbers 100, slow movers from this allele were given numbers less than 100, and fast movers were given numbers greater than 100. Allele frequency was calculated according to Baverstock et al. [35] (p = f (A) = (2 x number of AA homozygotes) + (number of Aa heterozygotes) / (2 x total number of individuals)).

6. Data analyses: the computer program NTSYS-pc (version 1.80) was used in all data analyses (allele frequencies, similarity coefficient and phylogenetic tree). UPGMA Dendrograms were formed based on the similarity coefficients of Manhattan Distance.

3. RESULTS

Distribution of studied samples of four species according to localities is given in Figure 1 and Table 1. Specimens of *Mus* spp. were generally caught from areas close human settlements in rural areas, while *Rattus* spp. were caught in urban areas. Although these four species are caught in the same area, there is niche specialization in biotope use such as *M. m. domesticus* in human settlements, *M. macodenicus* in the grain field, *R. rattus* in the roof of wooden buildings and barns, *R. norvegicus* in infrastructure.

Carbonic anhydrase (CA_1) enzyme variations in *M. m. domesticus* (n: 49); according to laboratory studies with blood hemolysates, two different alleles, CA_1^{100} and CA_1^{90}, were observed at the CA_1 locus in this species, and their frequencies were found to be 0.84 and 0.16, respectively. Among the samples examined, no individual heterozygous for the CA_1 locus was found. It was determined that the samples examined in terms of CA_1 locus were homozygous (Table 2, Figure 2).

Carbonic anhydrase (CA_1) enzyme variations in *M. macedonicus* (n: 7); CA_1 was studied in the blood hemolsylates of 7 of the 24 captured samples. Two different alleles including CA_1^{100} and CA_1^{90}, were observed in this locus of *M. macedonicus* and their frequencies were found to be 0.57 and 0.43, respectively. As in the previous species, heterozygous allele for the CA_1 locus was not observed and the all samples examined in terms of CA_1 locus were homozygous (Table 2, Figure 2).

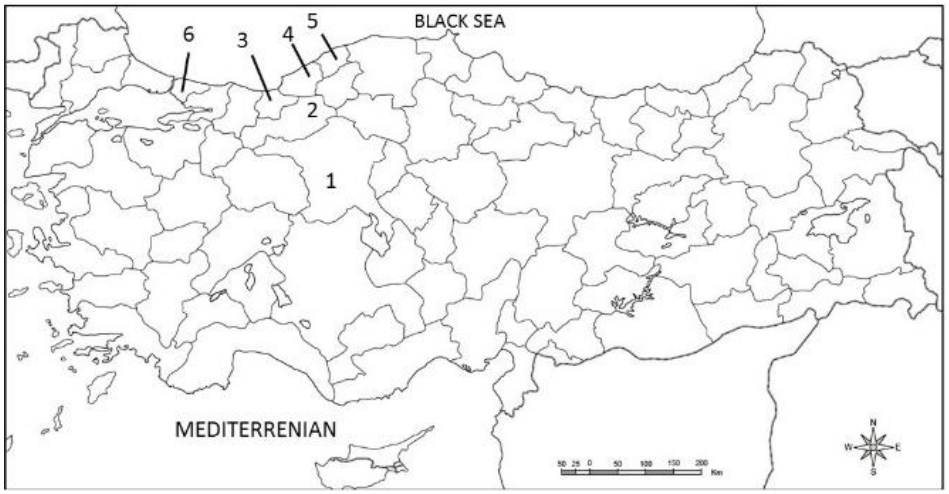

FIGURE 1. Sampling provinces of *Mus* spp. and *Rattus* spp. (1: Ankara, 2: Bolu, 3: Düzce, 4: Zonguldak, 5: Bartın, 6: Asiatic part of İstanbul)

Carbonic anhydrase (CA_1) enzyme variations in *R. rattus* (group1 n: 7, group2 n: 33); In the examined samples, two groups were determined for the dorsal fur colour and the samples were evaluated under these two groups; Group1 with blackish gray back fur (*R. rattus* (1)) and Group2 with brownish-grey dorsal fur (*R. rattus* (2)). *R. rattus* (1) with blackish gray back fur, the abdomen fur is a mixture of gray-white, in other words, smoke-coloured, and no variation was found within the group. Three types of variations were detected in the belly fur in R2: taupe white, light gray and yellowish white.

Two different alleles including CA_1^{80} and CA_1^{70} were observed in the CA_1 locus according to the blood haemolysis studies of the samples belonging to both groups, and among the samples examined, the allele frequencies were determined as 0.57 and 0.43 in the *R. rattus* (1) group, and 0.33 and 0.67 in the *R. rattus* (2) group, respectively. Among the samples examined, no individual heterozygous for the CA_1 locus was found. It was determined that the samples examined in terms of CA_1 locus were homozygous (Table 2, Figure 2).

Carbonic anhydrase (CA_1) enzyme variations in *Rattus norvegicus* (n; 13); According to laboratory studies with blood hemolsylates of 13 *R. norvegicus* specimens, only the CA_1^{80} allele was observed in the CA_1 locus in this species and its frequency was found to be 1.00 in the samples examined. Among the samples examined, no individual heterozygous for the CA_1 locus was found. It was determined that the samples examined in terms of CA_1 locus were homozygous (Table 2, Figure 2).

Comparison of allele variations in four species studied;

The positions and zymograms of CA1 enzyme locus alleles observed in the studied groups on the gel were given in figure 2. According to comparative statistical analyses based on the allelic frequencies and distance matrix of the Ca1 locus in two *Mus* species (*M. m. domesticus* and *M. macedonicus*) and two *Rattus* species (*R. rattus* (1), *R. rattus* (2) and *R. norvegicus*) (Table 2,3) UPGMA Dendrograms were established using the NTSYS-pc program (Figure 3).

According to the UPGMA Dendrograms established based on the similarity matrix (Table 3), *Mus* spp. and *Rattus* spp. Species were completely separated from each other by forming two different clusters. The cluster formed by *Mus* spp. is connected to the *Rattus* spp. cluster with the value D: 0.180. The genetic distance (D) between *M. m. domesticus* and *M. macedonicus* was determined as D: 0.045, and it appeared D: 0.040 between the *R. rattus* (1) group and the *R. rattus* (2). High D value was found to be 0,111 between *R. rattus* (2) and *R. norvegicus*, and these two species are completely separated from each other by the CA_1^{80} allele.

According to the UPGMA Dendrograms based on CA frequencies, the ancestral species among *Mus* spp. was *M. macedonicus*, and *Rattus* spp. as *R. norvegicus.* According to these results, in other words, *M. m. domesticus* and *R. rattus* (2) can be said to be more differentiated species. In addition, these results supported the idea that the CA_1 locus is a taxonomic marker that can be used to distinguish these species.

TABLE 1. Distribution of examined samples according to localities (numbers indicate the sample Numbers)

Locations	***M. m. domesticus***	***M. macedonicus***	***R. rattus***	***R. norvegicus***
1. Beykoz / İstanbul (Asiatic part)	-	-	26	-
2. Kargalar village / Zonguldak	-	-	3	-
3. Saka village / Zonguldak	2	-	4	-
4. The campus of Karaelmas Univesity of Zonguldak	5	-	11	19
5. City center of Zonguldak	1	-	2	-
6. Kilimli / Zonguldak	-	-	1	1
7. Çerde village / Ulus / Bartın	10	-	7	-

8. Melenağzı village / Düzce	1	1	1	-
9. Köprübaşı village / Düzce	12	-	3	-
10. Samandere village / Düzce	7	-	1	-
11. Hacıyakup village / Düzce	1	2	8	-
12. Mudurnu-Bolu	3	2	-	-
13. Kürkçüler village / Gerede / Bolu	1	1	-	-
14. Sapanlı village / Gerede / Bolu	-	3	1	-
15. Ömerler village / Abant / Bolu	4	-	-	-
16. Bürnük village / Bolu	-	-	3	-
17. Tandoğan Campus of Ankara University / Ankara	16	-	7	-
18. Maltepe-Ankara	-	-	1	7
19. İskitler-Ankara	-	-	-	7
20. City center / Ankara	28	7	2	5
21. Şereflikoçhisar / Ankara	-	1	-	-
22. Gölbaşı-Ankara	1	7	-	-
23. Bala-Ankara	17	-	-	-

TABLE 2. Frequencies of alleles observed for CA_1 loci in starch gel electrophoresis

Alleles / Species	***M. macadenicus***	***M. m. domesticus***	***R. rattus* (1)**	***R. rattus* (2)**	***R. norvegicus***
CA_1^{100}	0,57	0,84			
CA_1^{90}	0,43	0,16			
CA_1^{80}			0,57	0,33	1,00
CA_1^{70}			0,43	0,67	

TABLE 3. Distance matrix obtained from CA1 allele frequency data of *M. m. domesticus*, *M. macedonicus* and *R. rattus* (1), *R. rattus* (2) and *R. norvegicus*.

	R. norvegicus	*R. rattus* (1)	*R. rattus* (2)	*M. macedonicus*	*M. m. domesticus*
R. norvegicus	0				
R. rattus (1)	0,0717	0			
R. rattus (2)	0,1117	0,040	0		
M. macedonicus	0,0950	0,167	0,206	0	
M. m. domesticus	0,1400	0, 212	0,251	0,045	0

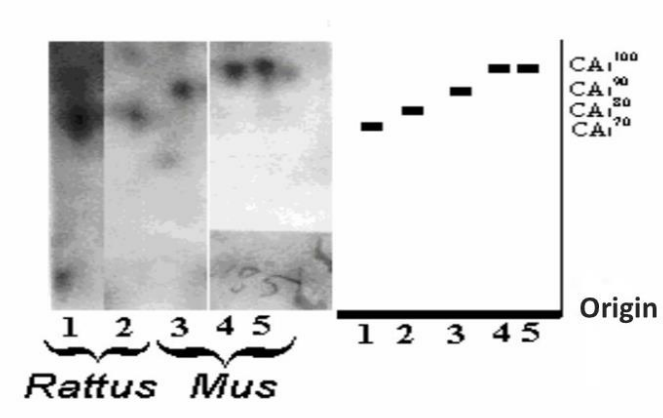

FIGURE 2. Alleles observed for CA_1 loci on the starch gel electrophoresis.

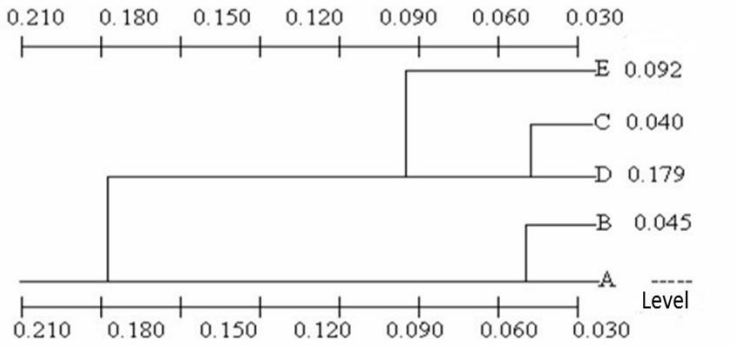

FIGURE 3. UPGMA Dendrograms generated from CA1 allele frequencies of *M. m. domesticus* (A), *M. macedonicus* (B), *R. rattus* (1) (C), *R. rattus* (2) (D) and *R. norvegicus* (E).

4. DISCUSSION

***Mus* spp.;** although fur colour, external and cranial characteristics were used in the identification of *Mus* spp., strong taxonomic characteristics could not be revealed in the distinguishing of species, and the zygomatic index (ZI), a cranial metric characteristic, is used to differentiate between *M. m. domesticus* and *M. macedonicus*.

Regarding ***Mus spp.,*** while fur colour, external features, and cranial characteristics have been used for their identification, robust taxonomic characteristics for species differentiation have not been discerned. However, the zygomatic index (ZI), a cranial metric characteristic, is utilized to distinguish between *M. m. domesticus* and *M. macedonicus*. [3, 4, 5, 6, 7, 8, 9, 14, 18, 44]. In this study, the specimens with a ZI parameter of less than 0.5 are identified as *M. m. domesticus*, and those with a ZI parameter of greater than 0.5 are identified as *M. macedonicus*. Depending on the difficulty of distinguishing species by looking at morphological and statistical characteristics, the genetic markers are also used in species identification and in establishing evolutionary relationships.

For this purpose, enzyme electrophoresis for taxonomy of *Mus* spp. were performed by [10, 11, 12, 13, 14]. In the research of Thaler et al. [10] using enzyme electrophoresis, Romanian *Mus* samples were divided into two different biochemical groups and identified 6 loci with allelic differences without assigned the samples to the certain taxa. Mezhzherin et al. [14] in their study on Transcaucasian *Mus* samples, stated that esterase 1-2 and isocitrate dehydrogenase enzymes were diagnostic in the differentiation of *M. musculus* and *M. domesticus*, and described of the hydride zone of three parapatric *Mus* species. Awasthi et al. [13] reported the maximum heterogeneity of *M. musculus* in the study performed by enzyme electrophoresis on 4 *Mus* species in India. Gözcelioğlu et al. [8] revealed that there were two alleles of isocitrate dehydrogenase enzyme in *M. m. domesticus* and *M. macedonicus*, and not distinctive among these taxa. Our results showed that CA1 alleles can be used to reveal the relationship between species and effectively distinguish between *Mus* and *Rattus* species.

Taxonomic studies with CA allozyme electrophoresis on *Mus* spp. are notably scarce. Thaler et al. [10] identified allelic differences in CA_2 enzyme in *Mus* samples from different locations from Europe. Bonhomme et al. [11, 12] observed a single allele for the CA_2 locus in *M. m. domesticus* in European. In this study, two different alleles were observed in *M. m. domesticus* and *M. macedonicus* for the CA_1 locus examined. In this respect, it can be said that the CA_2 locus may be more distinctive for *M. domesticus* and there is more variation within the species in the CA_1 locus, and CA_1 did not distinguish Türkiye samples of *M. m. domesticus* and *M. macedonicus*.

***Rattus* spp.;** *R. rattus* species has a very heterogeneous structure in terms of colour. The colour variations and identification characteristics of Turkish *Rattus* species were described in detail by [20]. Unlike *R. rattus*, another species in the

genus *Rattus, R. norvegicus* has a very homogeneous morphology structure, and morphological, biometric and karyological features of this species were given by [20]. The morphological features of both *Mus* and *Rattus* species examined in this study are consistent with [8, 9, 20]. In the study on non-specific esterase variations of *R. norvegicus*, the enzyme patterns were found to be tissue-specific polymorphic [24] also the blood serum proteins of *R. rattus* and *R. norvegicus* were revealed by using SDS-page electrophoresis [21]. It was stated in both papers that non-specific esterase and the patterns of blood serum proteins are not genetic markers in distinguishing species.

Baverstock et al. [34] in their study among the karyotypic forms (2n: 38, 40, 42) of *R. rattus*, it was stated that single allele migrated to most anodal were observed in these karyological forms for the CA_1 enzyme. However, it was also stated that in the one of samples with 2n: 42 (only in Southeast Asian type (Japan)) from two different locations, CA_1 was found to be fixed two alleles. While Baverstock et al. [34] observed a single allele in 2n: 38 karyological forms of *R. rattus*, according to Yiğit et al. [20] in Türkiye samples with 2n: 38, two different alleles were observed in the CA_1 locus. Also, Samollow et al. [33] in their study with Australian rats reported that the carbonic CA_1 enzyme distinguished *R. rattus* and *R. norvegicus* species. The single allele detected in *R. norvegicus* in Türkiye is consistent with the findings of [34, 35, 45]. Accordingly, it can be said that the CA_1 enzyme does not show intraspecific variation in *R. norvegicus* and is fixed by a single allele. As a result, it has been revealed that CA_1 enzyme can be used as a genetic marker, and it can distinguish especially *R. rattus* and *R. norvegicus* species in terms of fixed allelic differences.

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Declaration of Competing Interests The authors declare no conflict of interest.

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