



# Non-Metric Epigenetics Reveals no Geographic Differences in Eurasian Red Squirrel (*Sciurus vulgaris* Linnaeus, 1758) (Rodentia: Sciuridae) in Bulgaria

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**Abstract:** The research aimed to study the degree of population epigenetic variability and divergence of the Eurasian red squirrel (*Sciurus vulgaris balcanicus* Heinrich, 1936) populations by evaluating its cranial polymorphism in its main habitats in Bulgaria: Western, Central and Eastern Stara Planina, Kraishte region, Rila, Vitoshka and the Rhodopes. Eighteen non-metric characteristics of the skull were studied, whose condition does not depend on the sex and age of the individuals. The epigenetic variability of red squirrels in different geographical groups in Bulgaria was similar. The statistical analysis revealed no significant population differentiation of the species in Bulgaria, indicating homogeneity in terms of epigenetic characteristics. The obtained results were consistent with the taxonomic concept of the subspecies status and homogeneity of the squirrel population in Bulgaria. This was based on craniometric characteristics and an evaluation of the coat's colour phases' frequency distribution. The revealed pattern of population epigenetic diversity of the subspecies *Sciurus vulgaris balcanicus* Heinrich, 1936 expanded the knowledge of the species diversity within its range. A future large-scale study should address the intraspecific taxonomy of *Sciurus vulgaris* and the differentiation and uniqueness of its subspecies structure in Europe and Asia.

**Key words:** epigenetic variation, red squirrel, Bulgaria

## Introduction

The red squirrel (*Sciurus vulgaris* L.) is an arboreal herbivorous rodent found in Europe and Asia (PETER et al. 2005). Over 40 subspecies have been described at different times due to variations in coat colour and minor morphological differences among representatives of various populations (ELLERMAN & MORRISON-SCOTT 1965, CORBET 1978). However, assigning a subspecies status to individual geographic groups of squirrels solely by these characteristics makes their subspecies taxonomic status uncertain (SIDOROWICZ 1971, CORBET 1978, LOWE & GARDINER 1983).

Subsequent reviews of the red squirrel's subspecies diversity in its Palaearctic range (SIDOROWICZ 1971) showed that although there were some differences in skull morphology, many overlaps between morphological characteristics in several previously described subspecies were seen, as well as a low correlation between coat polymorphism and skull morphology. This was why SIDOROWICZ (1971) distinguished only 18 subspecies within the species range.

Over the years, various studies have been conducted on the distribution and subspecies status of the red squirrel in Bulgaria. These studies have accumulated data on the squirrel's presence in the

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country, as well as opinions on its subspecies status. (KOVACHEV 1925, BOETTICHER 1925, PETKOV 1929, HEINRICH 1936, WOLF 1940, MARKOV 1949, NIETHAMMER & BOHMANN 1950, RUSKOV, 1951, MARKOV 1955, MARKOV 1957, MARKOV 1960, MARKOV 1961). For details, see MARKOV 1960.

The detailed study of the distribution of the squirrel fur colouration and skull morphology in Bulgaria (MARKOV, 1960) showed that it is almost ubiquitous in the high-stem coniferous and deciduous forests of Rila, Pirin, Rhodopes, Stara Planina, Vitosha, Osogovo, Milevska, Rui Mountain, Strandzha, Dobrudzha. As in the other parts of its Palearctic range, the colour of the thick and soft coat is highly variable, but it can still be reduced to two colour phases – dark and light (MARKOV 1960, 1961)

Different authors work on the subspecies belonging to the squirrel in Bulgaria, and the number of subspecies described in different periods reaches 8 (MARKOV 1960). MARKOV (1960), who carried out a major revision of the subspecies taxonomy and geographical differentiation of the squirrel population in Bulgaria based on skull morphology, concluded that the red squirrel in the country belongs to a single subspecies – *S. v. balcanicus* Heinrich 1936.

Differentiating the red squirrel populations from various mountain massifs in the country through classical metric morphological and colour studies of the coat is difficult. Therefore, other methods must be applied to describe and potentially differentiate the populations. The study of minor non-metric characteristics of the skeleton, often referred to as ‘epigenetic’ traits (CORRUCCINI 1976), is particularly effective in such cases. These traits are variable and can be used to assess the diversity of populations and species.

Non-metric characters are discontinuous variants in different parts of the skeleton. Their appearance results mainly from basic genetic control, and they are independent of growth and direct external environmental influences, qualifying them as morphological genetic markers (BAUCHAU 1988). Therefore, they are suitable for assessing genetic variability and population divergence (SJØVOLD 1977).

Although the heritability of non-metric traits, mainly the presence of foramina and similar structures for blood vessels and nerves or dental occlusal variants, has been evaluated as rather low, the estimation of epigenetic variation in time and space as a result of genetic relationships can be made by considering several traits simultaneously. Thus, the main use of non-metric characters has been to assess epigenetic variability and divergence among populations. The problems lie in the genetic isolation of

populations, the lack of reproductive contact, the detection of genetic drift, and systematic studies to clarify species taxonomy for phylogenetic interpretation (ANSORGE 2001).

The absence of research on the epigenetic characteristics of the red squirrel, not only in Bulgaria but also in its European range, makes it challenging to take unambiguous opinions on the degree of geographical differentiation of its population and the presence of groups with specific epigenetic characteristics. There is a lack of insight into the epigenetic characteristics of the species in the country and their importance for the proper differentiation of existing population groups, especially in a species subject to hunting and economic exploitation.

This study aims to provide the first report on the population variation of non-metric skull characteristics in the red squirrel. Comparing the epigenetic divergence among them will allow the determination of their epigenetic geographic variability and the analysis of the mutual geographical epigenetic relations. These findings will lead to the discovery of the population epigenetic distance and differentiation of the species *Sciurus vulgaris* L. in its main localities in Bulgaria.

## Materials and Methods

To clarify the systematic position of the red squirrel (*Sciurus vulgaris* L.) in Bulgaria, Dr G. N. Markov conducted its craniological and dental measurements using extensive skull and fur materials from all over the country from 1948 to 1958. In the same years, he gathered information on the biology, ecology, and abundance of this species on the territory of Bulgaria. His results established that the red squirrel in Bulgaria belongs to a single subspecies with two coloured morphs.

The study of the epigenetic polymorphism of the red squirrel (*Sciurus vulgaris* L.) in Bulgaria was carried out based on the investigation of squirrel skulls from seven localities across the country, collected by Dr G. N. Markov and used in the monograph “The Squirrel in Bulgaria” (MARKOV 1960).

Subsequently, this craniological material and individual specimens provided by hunters were deposited in the scientific collection of the Institute of Zoology at the Bulgarian Academy of Sciences. After the Institute of Zoology was integrated into the Institute of Biodiversity and Ecosystem Research at the Bulgarian Academy of Sciences, the material was stored there.

A total of 251 squirrel skulls were examined to assess the epigenetic polymorphism of the species in Bulgaria. The craniological study of each specimen involves assessing the state of 18 cranial epigenetic fea-

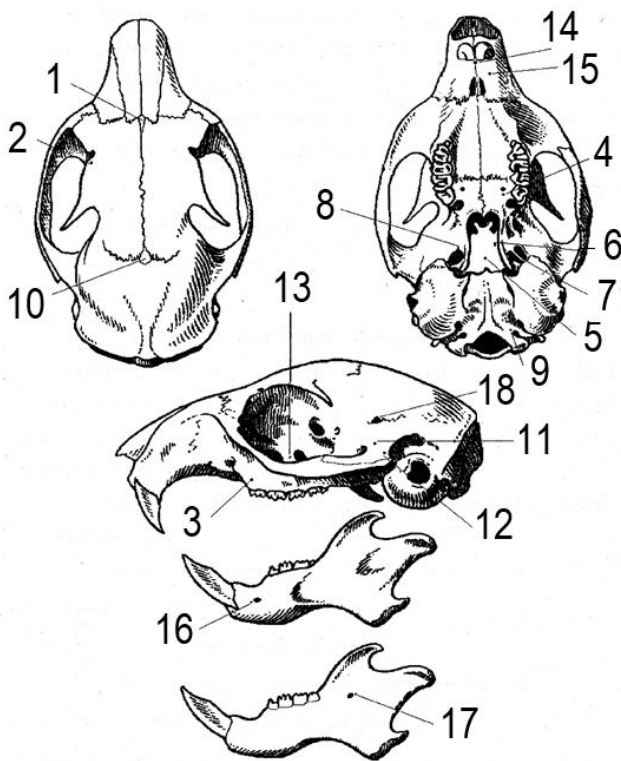


Fig. 1. The topographical location of the investigated epigenetic traits on the skull of the red squirrel.



Fig. 2. An overview map of the localities of the examined red squirrel skulls in Bulgaria. The composition of the studied groups is described in the Materials and Methods section.

tures. These traits represent openings for blood vessels and nerves and additional bony elements. Including a sign in the created scheme (Figure 1) requires that its state be clearly and unambiguously distinguishable on the red squirrel's skull and be independent of the sex and age of the individual. The selection of these traits was based on a deep review of the characteristics used and recommended in the study of epigenetic polymorphism in rodents by BERRY (1963), BERRY & SEARLE (1963), HEDGES (1969), and MARKOWSKI (1992). The topographical location of the epigenetic characters examined is shown in Figure 1.

Table 1. The observed skull traits and their examined status in the red squirrel *S. v. balcanicus* (Heinrich, 1936) from Bulgaria.

№	Trait	Examined status
1	Os interfrontale	presented
2	Foremen frontale	double
3	Foremen maxillare I	presented
4	Foremen palatinum major	double
5	Foremen sphenoidale medium	presented
6	Foremen sphenoidale laterale	presented
7	Foremen ovale	double
8	Foremen ovale	open
9	Foremen hypoglossum	double
10	Os interparietale	accessorial
11	Foremen temporale	presented
12	Foremen postzygomaticum	presented
13	Foremen opticum	accessorial
14	Foremen intermaxillare major	presented
15	Foremen intermaxillare laterale	presented
16	Foremen mentale	double
17	Foremen mandibulare	double
18	Fenestra parieto-temporalis	complete

The condition of the studied traits was observed on the skull's left side, and their status is shown in Table 1.

The present study examined the frequency distribution of red squirrel epigenetic craniological characters independent of individuals' sex and age. This allows all red squirrels, regardless of sex and age, to be combined into a joint group reflecting their numbers in each studied locality.

Based on the place of origin information of each individual held in the collection, the skulls were distributed among the following geographical groups (Figure 2): Western Stara Planina (G1 – 31 skulls), Central Stara Planina (G2 – 14 skulls), Eastern Stara Planina (G3 – 16 skulls), Kraishte region (G4 – 16 skulls), Rila Mountain (G5 – 79 skulls), Vitosha Mountain (G6 – 29 skulls), Rhodope Mountains (G7 – 66 skulls).

Based on the established frequency distribution of the 18 epigenetic characteristics, the Epigenetic variability ( $V_i$ ) was calculated according to SMITH (1981). The Mean Measure of Divergence (MMD)

after SJØVOLD (1973) was determined between each group and the others. The Measure of Uniqueness (MU), according to BERRY (1963), was calculated for each sample as the sum of its statistically significant ( $P < 0.05$ ) epigenetic distances from the other samples.

## Results

Table 2 presents the frequency distribution of epigenetic craniological traits for each studied geographical group (G) in Bulgaria.

No significant differences exist in the frequency distribution of non-metric craniological traits within geographical groups of red squirrels from Bulgaria. There are also no characteristics that assign individuals to specific geographical groups. The manifestation of the traits Foremen sphenoidale medium (5), Foremen sphenoidale laterale (6), Foremen ovale (8), Foremen temporale (11) и Foremen postzygomaticum (12) in all groups was zero. The signs Os interfrontale (1), Foremen maxillare I (3) and Foremen mandibulare (17) in the different groups had a weak manifestation, with zero or close to zero value. Foremen intermaxillare major (14) had a maximum appearance in all groups, and in 4 of the groups – Central Stara Planina (G2), Eastern Stara Planina (G3), Kraishte (G4), Rila (G5) it occurred in all studied specimens. The occurrence of the traits Foremen palatinum major (4), Foremen hypoglossum (9), Foremen intermaxillare laterale (15), and Fenestra parieto-temporalis (18) varied greatly in individual samples. On the contrary, the frequency distribution of the signs Foremen frontale (2), Foremen ovale (7), Os interparietale (10), Foremen opticum (13), and 16 – Foremen mentale, varied in double.

The average epigenetic variability of the geographical groups of the red squirrel in Bulgaria is presented in Table 3.

The estimation of the epigenetic variation of the studied geographical groups showed a similar pattern of variation: it was most pronounced in the group from Western Stara Planina and lowest in the group from Eastern Stara Planina, while in Central Stara Planina, it was 0.073; or within one mountain range, the entire range of 20.5% of the epigenetic variability of the species in Bulgaria was realised. In the other localities studied, it occupied intermediate values. Variability between the populations showed only minor differences and did not indicate any geographical or altitude trend.

The analysis of the statistical significance of the epigenetic distances (MMD) in red squirrels in Bulgaria (Table 4) showed that none of the paired groups achieved a statistically significant epigenetic distance.

The Measure of uniqueness (MU) for each sample as the sum of its statistically significant ( $P < 0.05$ ) epigenetic distances from the other samples showed the lack of epigenetic uniqueness of all the populations studied. This result indicated that none of the studied geographical groups of the red squirrel in Bulgaria have epigenetic uniqueness.

## Discussion

Studies of the non-metric skull characteristics of mammals provide an additional tool for quantitative interpretations to assess the epigenetic variability of a species across their recent and historical areas. The statistical evaluation of the reliability of epigenetic distances found between species populations also helps to assess the presence or absence of epigenetic homogeneity of the studied species in the investigated region. This approach is particularly applicable to the study of species for which extensive craniological collections have been established in several museums (ANSORGE et al. 2012).

It is widely accepted that several species inhabiting isolated forest fragments suffer from genetic isolation. In these cases, analysing variability using non-metric features is attractive for studying conservation problems (BARANOV et al. 1997, PERTOLDI et al. 2000).

So far, the red squirrel's main non-metric epigenetic cranial characteristics were unknown. Described for the first time through the 18 non-metric craniological traits, an opportunity was revealed to determine the epigenetic variability and the mean measure of divergence between the main geographical groups on the territory of Bulgaria.

The red squirrel is protected in most of Europe and is listed in Appendix III of the Bern Convention. It is classified as Least Concern in the IUCN Red List (IUCN 2023). However, in some areas, such as Bulgaria, hunting of the species for its valuable fur still occurs, according to "Newspaper of State", issue 102, dated December 8 (2023). It is possible that, based on this law, red squirrels in various parts of the country will be subject to mass hunting sometime in the future. The dual evaluation of its protected status requires constant observation of its epigenetic variability.

The epigenetic distances of the studied geographical groups of red squirrels in Bulgaria show no significant population differences. Additionally, the statistical measure of Uniqueness did not identify any group as distinct from the others, indicating an equal epigenetic profile for all squirrel groups in the country.

The summarised review of the results obtained in the study of the epigenetic polymorphism of non-metric signs in the red squirrel (*Sciurus vulgaris* L.)

**Table 2.** Frequency distribution of the non-metric craniological traits within geographic groups (G) of the red squirrel from Bulgaria. The geographical distribution and composition of the studied groups are described in the Materials and Methods section.

Trait	Geographic group						
	G1	G2	G3	G4	G5	G6	G7
1	0.000	0.000	0.000	0.000	0.012	0.000	0.000
2	0.193	0.000	0.000	0.133	0.113	0.107	0.106
3	0.068	0.000	0.000	0.000	0.013	0.000	0.000
4	0.666	0.833	0.937	0.800	0.756	0.586	0.666
5	0.000	0.000	0.000	0.000	0.000	0.000	0.000
6	0.000	0.000	0.000	0.000	0.000	0.000	0.000
7	0.000	0.071	0.133	0.133	0.080	0.000	0.098
8	0.000	0.000	0.000	0.000	0.000	0.000	0.000
9	0.230	0.428	0.214	0.200	0.313	0.320	0.150
10	0.133	0.214	0.071	0.066	0.121	0.071	0.125
11	0.000	0.000	0.000	0.000	0.000	0.000	0.000
12	0.000	0.000	0.000	0.000	0.000	0.000	0.000
13	0.142	0.153	0.142	0.285	0.200	0.259	0.158
14	0.967	1.000	1.000	1.000	1.000	0.965	0.967
15	0.133	0.230	0.375	0.071	0.083	0.172	0.096
16	0.333	0.230	0.333	0.200	0.240	0.080	0.172
17	0.037	0.100	0.000	0.000	0.014	0.000	0.000
18	0.344	0.142	0.307	0.214	0.508	0.440	0.315

**Table 3.** Epigenetic variability ( $V_i$ ) within geographical squirrel groups (G) in Bulgaria. The geographical distribution and composition of the studied groups are described in the Materials and Methods section.

$V_i$	Geographic group						
	G1	G2	G3	G4	G5	G6	G7
	0.083	0.073	0.066	0.067	0.077	0.072	0.070

**Table 4.** Mean measures of divergence (MMD) between the geographical groups (G) in the upper matrix and their standard deviations in the lower matrix. The geographical distribution and composition of the studied groups are described in the Materials and Methods section.

Groups	G1	G2	G3	G4	G5	G6	G7
G1		0.020	0.058	0.010	0.005	0.006	0.011
G2	0.022		0.071	0.041	0.008	0.030	0.016
G3	0.036	-		0.045	0.019	0.052	0.016
G4	-	-	-		0.039	0.022	0.058
G5	0.007	0.012	0.018	-		0.011	0.008
G6	-	0.027	0.035	-	0.011		0.004
G7	0.011	0.018	0.017	-	0.007	-	

in Bulgaria confirms MARKOV 1960's claim that the red squirrel in Bulgaria belongs to only one subspecies, *S. v. balcanicus* Heinrich, 1936, and is homogeneous in its craniological characteristics.

This study established the Bulgarian squirrel's average epigenetic variability ( $V_i = 0.0725$ ), which provides a starting point for future research and assessment of the species' epigenetic variability not only in Bulgaria but also in its populations across Europe.

The results of this study expand and enrich the

knowledge about the population biology of the red squirrel in Bulgaria and could serve as a basis for future studies on this species. The epigenetic characteristics can be used to observe the variability and diversity of the red squirrels, especially in areas in Europe with low population densities. This is particularly relevant in isolated woodland areas where the red squirrels are known to suffer from genetic isolation or in zones with intensive hunting pressure. Further studies of the non-metric skull epigenetic characters of red squir-

rel populations from the Balkan Peninsula could be combined with molecular genetic studies on suitable markers to reveal their genetic structure and phylogenetic relationships with other European subspecies.

Although the heritability of non-metric traits, mainly the presence of foramina and similar structures for blood vessels and nerves or dental occlusal variants, has been evaluated as rather low, the simultaneous consideration of several traits allows for an estimation of epigenetic variation in time and space as a result of genetic relationships (ANSORGE 2001). Thus, the main use of non-metric characters has been aimed at assessing epigenetic variability and population divergence among the red squirrel subspecies *Sciurus vulgaris balcanicus* (Heinrich, 1936).

The expected rapid progress in genetics and genomics will shed more light on nonmetric traits' genetic and epigenetic basis. So, in the future, it may be better understood what epigenetic distances indicate and how they can potentially be used to detect population differences within a species.

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