Department for Animal Breeding, Nutrition and Laboratory Animal Science
Division Animal Breeding and Genetics
University of Veterinary Medicine Budapest

# Comparison of mtDNA control region among three Zaupel sheep descendant breeds

By:
**Shane McCarvill**

Supervisor:
Dr Andras Gaspardy

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## 1. List of abbreviations used in this thesis

| | |
|---|---|
| MtDNA | Mitochondrial Deoxyribonucleic Acid |
| DNA | Deoxyribonucleic Acid |
| CR | Control Region |
| D-Loop | Displacement Loop |
| L | Light Strand |
| HG | Haplogroup |
| HT | Haplotype |
| dNTP | Deoxyribonucleotide Triphosphate |
| MgCl | Magnesium Chloride |
| BSA | Bovine Serum Albumin |
| ATP | Adenosine triphosphate |
| Cyt B | Cytochrome B gene |

## 2. Abstract

The consideration of the descendants is indispensable in the preservation of endangered animal breeds.

The author compared mitochondrial DNA control region sequence in three descendant breeds of the extinct Zaupel sheep. The investigation was carried out in order to prove the common origin of the Waldschaf breed from Austria, Bovec sheep from Slovenia and Cikta sheep from Hungary.

A total of 118 biological samples were taken from non-related representatives of the three breeds between 2015 and 2017. A newly designed primer pair and a former one (Hiendleder et al., 1998) were used to amplify the segment (1179 bp) to be tested.

The total number of haplotypes in the whole study population was 49. The majority of which fell into haplogroup B. As a novel observation haplogroups, C and D appeared in Cikta and Bovec sheep, respectively. The Tajima D-test value in the entire study population was -0.914 ($P>0.10$), meaning that the separation of the three descendant breeds did not cause genetic drift, these are collectively in genetic equilibrium.

The genetic information confirmed the origin of the breeds known from the breed history.

## 3. INTRODUCTION

### 3.1 Extinct Zaupel sheep

The origin of the Zaupel sheep (Zaupelschaf) can be traced back to the Neolithic peat sheep (*Ovis aries palustris*; Mason, 1967), which, as a former companion of the Indo-Germanic tribes, populated areas stretching from the Balkans, through the Alps, to the Pyrenees (Bohm, 1878). Excavations show that in the European Early Middle Ages, the fallow sheep existed as its only successor throughout Europe (Bökönyi, 1958). This was then split into several types per region as a result of selection and improvement. The ancient German fallow sheep existed in five variants (Golf, 1939), one of which was the prolific, mixed-wool Zaupel sheep (*Ovis aries Germanicus rusticus*; Heyne, 1916). With the arrival of larger, better muscled, yet finer-wooled foreign sheep, the fate of the Zaupel sheep was sealed from the middle of the 15th century (Adlung, 1912). The breed was permanently lost by the middle of the 20th century, owing to this we do not possess a complete genetic profile on the Zaupel breed and therefore we have to rely on historic sources in order to describe the Zaupel breed.


Figure 1: The extinct Zaupel sheep (Bohm, 1978)

Johannes Heyne in 1909 in “Die Schafzucht” described the Zaupels dispersion as being found from the middle of Germany to the north till Belgium, to the south till Italy, the East till Hungary and to the west over the Rhein. Mostly it is breed in Oberschwaben, Ober- and Unterbayern, in Steiermark, Kärnten, Krain and Tirol. Bohm (1878, Figure 1) described the Zaupel as being a

small breed with a mature ewe weighing between 30 to 35 kg and measuring 55 to 56 cm at the withers, males were slightly larger measuring 60 cm height at the wither. Bohm also describes the males as being horned with the horn curving backwards and downwards and ending in line with the eye. The Zaupel was described as a lily-white colour with a black muzzle and more or less regular rings around the eyes. Brown and black colour variants could also be found but were not as common. The wool was described as being long and rough with many animals exhibiting downy hairs underneath the fleece. The weight of the fleece after shearing was approximately 1.5 kilograms.

### 3.2 Living descendant breeds of Zaupel

Of the extinct but historically important Zaupel sheep, four independent, still existing gene reserve breeds originated in Central Europe (Seibold, 1990). Today, as transboundary breeds, there are the Bavarian Waldschaf in Germany (versions of this is Waldschaf in Austria and the Sumavska in the Czech Republic), the Alpine Steinschaf in Austria (versions of this are Steinschaf in Bavaria, and Bovec sheep (or Bovska in Slovenia) and Mountain Sheep (Bergschaf; also, with brown colour variations living as Engadiner Fuchsschaf in Switzerland, and as Braunes Bergschaf in Germany and Austria). The fourth Zaupel sheep descendant who came to Hungary with Swabian immigrants of the 18th century is the Cikta (Koppány, 2000).
It is highly probable that each descendant breed of Zaupel sheep would also have also became extinct decades ago if the interest in conserving native farm animals had not been strengthened.

Figure 2: Bavarian Waldschaf sheep
(http://bib.ge/sheep/open.php?id=426 (Accessed 29/03/21))

The Bavarian Waldschaf (Figure 2), also known as the Bavarian Forest sheep. They are a small to medium size breed, males weigh between 60 and 70 kg, while females weigh 40 to 55kg. The Bavarian forest sheep is usually white in colour, black and brown colour variants are also found. The fleece weight is approximately 3.5kg. The Bavarian forest sheep can be kept in unfavourable climatic conditions. They are a fertile prolific breed with an average lambing percentage of between 180 and 200%. Lambs can achieve a weight gain of between 180 and 200 grams a day without supplementary feeding. The Bavarian Waldschaf is currently classified as an endangered breed.

The Alpine Steinschaf (Figure 3) is a small to medium-sized breed. Males weigh between 55 to 80 kg and females weigh between 40 and 60 kg. The Alpine Steinschaf is predominantly found in the white colour variant, while other colours such as brown and black are also commonly found. Males and females are in the most part horned, the males have strongly curved spiral horns, while the female has smaller lightly curved horns. The fleece weight is approximately 3.5kg in females and 4.5kg in males.

The Alpine Steinschaf are a prolific breed with lambing percentages of 180 to 200% being common, they are capable of lambing twice in a twelve-month period and often exhibit a heat within four weeks of lambing.

The Alpine Steinschaf is currently classified as an extremely endangered livestock breed.

Figure 3: Alpine Steinschaf

(https://learnnaturalfarming.com/alpines-steinschaf-sheep-the-rare-breed/ (Accessed 29/03/21))

Bovec sheep (Figure 4) are a small breed with males weighing between 45 and 50 kg, while females weigh between 35 and 40kg. They reach a shoulder height of between 55 and 60 cm. The Bovec sheep can be found in a variety of colours including white, brown, black or a combination of these colours. They are predominantly used for milk production and produce an average of 221kg of milk per 210-day lactation, composed of 6.3 % fat and 5.5 % protein. A well-managed flock can produce over 300kg of milk.

The Bovec sheep breed is currently classified as an endangered livestock breed.

Figure 4: Bovec Sheep

(https://www.heritagesheep.net/Bovec%20sheep.htm (Accessed 29/03/21))

The Cikta (Figure 5) is a small sheep breed, Males weigh between 35 to 40 kg and females 40 to 45 kg. They are white in colour; no other colours are found in the breed. Rams usually have small horns while females are polled. The Cikta are used to produce meat with ewes having an average of 1.1 lambs per litter. The Cikta breed is currently classified as endangered.

Figure 5: Cikta sheep

(http://www.livestockoftheworld.com/Sheep/Breeds.asp?BreedLookupID=1340&SpeciesID=10 (Accessed 29/03/21))

# 4. LITERATURE REVIEW

## 4.1 Mitogenome of sheep

Mitochondrial DNA (mtDNA) is an extranuclear, non-recombining material of inheritance in the mitochondria. This circular genome is mainly used in evolutionary research, in the phylogenetic study of mammalian species and populations by maternal origin (e.g. Tapio et al., 2006), but also in forensic genetics (e.g. Zenke et al., 2017).

Table 1. shows features of the Ovis aries mitochondrial genome (Hiendleder et al., 1998).

Table 1: Features of the *Ovis aries* mitochondrial genome[a] (Hiendleder et al., 1998)

| Feature | From | To | Size | Start codon | Stop codon[b] | 3' spacer |
|---|---|---|---|---|---|---|
| tRNA-Phe | 1 | 68 | 68 | | | |
| 12S rRNA | 69 | 1,026 | 958 | | | |
| tRNA-Val | 1,027 | 1,093 | 67 | | | |
| 16S rRNA | 1,094 | 2,667 | 1,574 | | | |
| tRNA-Leu (UUR) | 2,668 | 2,742 | 75 | | | AA |
| NADH1 | 2,745 | 3,700 | 956 | ATG | TAa | |
| tRNA-Ile | 3,701 | 3,769 | 69 | | | |
| tRNA-Gln (L) | 3,767 | 3,838 | 72 | | | AT |
| tRNA-Met | 3,841 | 3,909 | 69 | | | |
| NADH2 | 3,910 | 4,951 | 1,042 | ATA | Taa | |
| tRNA-Trp | 4,952 | 5,018 | 67 | | | A |
| tRNA-Ala (L) | 5,020 | 5,088 | 69 | | | A |
| tRNA-Asn (L) | 5,090 | 5,162 | 73 | | | |
| Origin of L-strand repl. | 5,163 | 5,194 | 32 | | | |
| tRNA-Cys (L) | 5,195 | 5,262 | 68 | | | |
| tRNA-Tyr (L) | 5,263 | 5,330 | 68 | | | C |
| COI | 5,332 | 6,876 | 1,545 | ATG | TAA | |
| tRNA-Ser (UCN) (L) | 6,874 | 6,944 | 71 | | | TAAAC |
| tRNA-Asp | 6,950 | 7,017 | 68 | | | T |
| COII | 7,019 | 7,702 | 684 | ATG | TAA | AAT |
| tRNA-Lys | 7,706 | 7,773 | 68 | | | T |
| ATPase8 | 7,775 | 7,975 | 201 | ATG | TAA | |
| ATPase6 | 7,936 | 8,615 | 680 | ATG | TAa | |
| COIII | 8,616 | 9,399 | 784 | ATG | Taa | |
| tRNA-Gly | 9,400 | 9,468 | 69 | | | |
| NADH3 | 9,469 | 9,815 | 347 | ATA | TAa | |
| tRNA-Arg | 9,816 | 9,884 | 69 | | | |
| NADH4L | 9,885 | 10,181 | 297 | ATG | TAA | |
| NADH4 | 10,175 | 11,552 | 1,378 | ATG | Taa | |
| tRNA-His | 11,553 | 11,621 | 69 | | | |
| tRNA-Ser (AGY) | 11,622 | 11,681 | 60 | | | A |
| tRNA-Leu (CUN) | 11,683 | 11,753 | 71 | | | |
| NADH5 | 11,754 | 13,574 | 1,821 | ATA | TAA | |
| NADH6 (L) | 13,558 | 14,085 | 528 | ATG | TAA | |
| tRNA-Glu (L) | 14,086 | 14,154 | 69 | | | ACTA |
| Cyt b | 14,159 | 15,298 | 1,140 | ATG | AGA | CAA |
| tRNA-Thr | 15,302 | 15,371 | 70 | | | |
| tRNA-Pro (L) | 15,371 | 15,436 | 66 | | | |
| Control region | 15,437 | 16,616 | 1,180 | | | |

[a] Nucleotide number 1 is the 5' end of the tRNA-Phe-specifying gene. Anticodons for the two tRNA-Leu and the two tRNA-Ser are given in parentheses. (L) denotes light-strand sense. Positions include the 58 and 38 nt of each feature. ATPase6 and ATPase8, genes encoding subunits 6 and 8 of ATPase; COI-III, genes encoding subunits I–III of cytochrome c oxidase; Cyt b, gene encoding cytochrome b; NADH1–6, genes encoding subunits 1–6 of nicotinamide adenine dinucleotide dehydrogenase.

[b] Incomplete stop signals are denoted by lowercase letters.

MtDNA, which, like in humans, is about 16,500 base pairs in sheep, is also used to study the history of domestication, and in this respect, we can get an idea of the relative history of the breeds. The mitochondrial DNA (mtDNA) which is not enveloped like nuclear DNA in chromosomes, is located in the mitochondrial matrix which can be found inside the inner mitochondrial membrane. The outer compartment of a mitochondrion is surrounded by the outer and inner membrane. The outer membrane contains porins through which smaller or larger proteins can enter the mitochondria. While, the inner mitochondrial membrane has all the elements of the electron transport system and the ATP synthase complex (Chial et al., 2008) The mitochondrial genome mutates more frequently than the nuclear genome (approximately 100 times more often) this causes divergence in mtDNA at within-mitochondrion and between-mitochondrion level (Gáspárdy, 2021). Owing to the frequent mutation rate of mitogenome, it has been widely used as a phylogenetic marker for both cladogram building and molecular dating (Brown et al., 1979).

As stated by Gáspárdy (2021) the complete mitochondrial genome (such as the one above) provide complex information for the phylogeographic and population genetics in sheep. The complete mitochondrial genomes of several species of domestic sheep (Ovis aries) have now been mapped by Davenport et al. (2018).

### 4.2 Haplogroup and haplotype investigations in sheep

According to the sheep mtDNA the control region (CR) also known as the mitochondrial displacement loop (D-loop), there are several major maternal variants (haplogroups A, B, C, D, E, F and G; the latter two are currently extinct). The CR is composed of a triplex DNA structure at the site of origin of the heavy strand (Gáspárdy, 2021).

### 4.3 Other notable regions of mtDNA

Along with the Cr another quite mutable segment of the mtDNA is the cytochrome b gene (Cyt B). By comparison of the CR or Cyt B either individually or together it is possible to distinguish the 5 haplogroups from each other (Meadows et al., 2011).

### 4.4.1 Haplogroups in Europe

According to (Dymova et al., 2017) group B is found mainly in European breeds, Haplogroup B appeared in Europe approximately 6400 years ago and reached western Europe before haplogroup A (Sanna et al., 2015). Haplogroup C was observed only on the Iberian Peninsula (Portugal and Spain) (Pedrosa et al., 2007) and on the southernmost countries of the Balkan peninsula Albania and Greece (Pariset et al., 2011). In Italy (Mariotti et al., 2013) observed haplogroup D in the breeds Laticauda and Bergamasca.

### 4.4.2 Haplogroups in Asia

The Mongolian plateau was of the utmost importance of the spread of sheep to eastern Asia, owing to this very small genetic change was observed in sheep from Mongolia and from China. Ganboldi et al. (2019) observed the occurrence of haplogroups A, B and C in native Mongolian sheep. Moghani sheep from the Iranian plateau were identified as belonging to haplogroup a by Mohammadhashemi et al. (2012). Haplogroups D and E were least frequently observed by (Tapio et al., 2006) and (Meadows et al., 2007) in a number of samples from Turkey and the Caucasus (the region between the black sea and the Caspian Sea, encompassing Armenia Azerbaijan and Georgia).

### 4.4.3 Haplogroups in Africa

It has been documented in numerous publications that the dominant haplogroup in Africa is B. Othman et al. (2015) identified haplogroup B in Egypt in the Barki and Ossimi breeds, Alvarez et al. (2013) observed haplogroup b in Mauritania in the Touareg and Peul breeds. Haplogroup C was observed in a small number of samples in a study of Siroua sheep by Kandoussi et al. (2020) in Morocco. Haplogroup B however was the dominant haplogroup in the breed.

### 4.4.4 Haplogroups in America

Campos et al. (2020) conducted a study analysing the Mitochondrial control region in 40 unrelated domestic Mexican sheep.31 haplotypes were observed along with 74 polymorphic sites. The pertaining phylogenetic analysis revealed that all Mexican sheep belonged in Haplogroup B. Revelo et al. (2020) conducted a study on the Creole sheep and identified that

both the noticeably different hairy and woolly variety both belonged to haplogroup B and were derived from an Iberian and African ancestor.

### 4.4.5 Haplogroups in Australia

Hiendleder et al. (2002) makes the observation that the large proportion of haplogroup A in New Zealand in addition to haplogroup b is due to the early importation of fat tailed Indian sheep alongside the importation of mouflon into Australia in accordance with the sheep stream hypothesis.

## 4.5 Haplogroup Dispersion

Table 2 lists genetic diversity observed between domestic and wild sheep mitogenomes (Meadows et al., 2011).

Table 2: Genetic diversity observed between domestic and wild sheep mitogenomes (Meadows et al., 2011)

| | HA | HB | HC | HD | HE | Mouflon | Urial | Argali |
|---|---|---|---|---|---|---|---|---|
| HA | — | 0.57 | 0.93 | 0.75 | 0.90 | 0.58 | 2.19 | 2.53 |
| HB | 93 | — | 1.01 | 0.81 | 1.00 | 0.07 | 2.31 | 2.59 |
| HC | 150.5 | 163.5 | — | 1.00 | 0.36 | 1.00 | 2.33 | 2.65 |
| HD | 122.5 | 131.5 | 162 | — | 0.98 | 0.81 | 2.27 | 2.61 |
| HE | 147 | 162 | 58.5 | 159.5 | — | 0.98 | 2.30 | 2.63 |
| Mouflon | 94 | 11 | 162.5 | 131.5 | 160 | — | 2.31 | 2.60 |
| Urial | 357.7 | 377 | 380.3 | 370.5 | 375.7 | 377.3 | — | 2.32 |
| Argali | 413 | 423 | 433 | 425.5 | 429 | 424 | 379 | — |

The average number of nucleotide differences (D) is given below the diagonal and nucleotide substitutions per site (K, given as a percentage) are given above the diagonal for the full mitochondrial sequence after removal of both indels and the repetitive component of the control region.

## 5. AIM

The hypothesis was that, with modern genetic knowledge, I would be able to show common roots and connections in breeds considered to be of common origin. If this is true, then statistical processing of the data should not justify a significant genetic difference between descendant breeds of Zaupel. In this sense, we surveyed the diversity of maternal genetic background in the following three breeds: Waldschaf, Bovec sheep, and Cikta based on the sequence order of the mutable control region (CR) of the mitochondrion. With the results I wanted to genetically prove the common origin of the related varieties known from their history, and on the other hand to create a basis for the further maintenance of the genetic characteristics of the representatives in the Zaupel breed group.

## 6. MATERIALS AND METHODS

Biological samples were taken from non-closely related individuals in Waldschaf (Austria, n = 27) and Bovec sheep (Slovenia, n = 21) breeds. In Bovec sheep, blood samples were collected from potential breeding rams included in the regular prion genotyping program, determined by order of the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia. All rams selected were offspring of different ewes. In Cikta sheep (Hungary, n = 70), the so-called "founder sampling" was used (Maróti-Agóts et al., 2008). In the Cikta breed, we collected samples from the living female representatives of the oldest families (with 4-5-6 ancestral generations) according to the pedigree found in the flock-book which was re-established in 2000 (Figure 6). All biological samples were taken between 2015 and 2017.

Figure 6: Sample collection location Nagydorog 2015

DNA was isolated from the obtained samples using the SIGMA GenElute Blood Genomic DNA Kit according to the manufacturer's instructions. The 25 µl PCR reaction mixture prepared for each sample contained 2.5 µl dNTP, 2.5 µl buffer, 1.5 µl MgCl, 2 µl primer, 1 µl BSA, 0.4 µl Taq polymerase (ThermoFisher Scientific) and PCR grade water. Two primer pairs were used to amplify the segment to be tested: a newly designed primer for the beginning of the region, and with a second pair as described by Hiendleder et al. (1998). First, the following amplification protocol was used for the newly designed primers (MtOA_F15400 5'-ACACCCAAAGCTGAAGTTCTAC-3' and MtOA_R16087 5'-

GTTGGTTTCACGCGGCATGGT-3'): an initial cycle of 94°C for 20 sec, 94°C for 30s and 62°C for 30 s; then 72°C 45 s 34 cycles and a final extension step 72°C for 7 min product: 688 bp. The second amplification protocol was used as follows (MtOA_F15983 5'-AACTGCTTGACCGTACATAGTA-3' and MtOA_R592 5'-AGAAGGGTATAAAGCACCGCC-3'): an initial cycle of 94°C for 30 sec, 94°C for 30s and 6 cycles 54°C, 6 cycles 53°C, 21 cycles 52°C each for 30 s; and a final extension step 72°C for 7 min product: 1246 bp. A programmable Thermal Cycler 2720 PCR equipment (Applied Biosystem) was used to amplify the DNA sequence. PCR products were purified with the SIGMA GenElute PCR Clean-up Kit according to the protocol.

The length of aligned and trimmed CR sequences was 1179 bp long and corresponded to positions 15437–16616 on the reference sequence (AF010406; Hiendleder et al., 1998).

Mutations were evaluated using the test proposed by Fu and Li (1993) first, and then, we used the Tajima D-test, developed by Tajima (1989) as a method for population genetic evaluation, to analyse the detected sequence mutations.

Using DNAsp v6.0 software (Rozas et al., 2017), the number of polymorphic sites in the entire assay sample was determined and the mean nucleotide difference within and between groups was calculated. The resulting sequences were arranged with MEGAX (Kumar and Stecher, 2018), aligned, and the related dendrogram was drawn.

The corrected number of base substitutions within the sequences was determined by the method of Jukes and Cantor (Jukes and Cantor, 1969; Jukes, 1990).

The distribution of haplotypes by descendent breeds was plotted using Network 10.2.00 software (fluxus-engineering.com; Bandelt et al., 1999). The sorting of the samples into haplogroups was performed by comparing the obtained sequences with gene bank reference ones (A-HM236174, B-HM236176, C-HM236178, D-HM236180, E-HM236182 Meadows et al., 2005; *O. musimon Mouflon* HM236184, *O. ammon Argali* HM236188, *O. vignei Urial* HM236186 Hiendleder et al., 2002).

## 7. RESULTS

In the CR region of the whole study sample, the number of monomorphic base sites was 1048, while that of the polymorphic base sites (mutations) was 131. Regarding the polymorphic base sites, 13 single (singleton site positions: 140 286 312 462 499 632 696 913 962 1093 1145 1148 1183) and 118 parsimony mutations occurring in more individuals were found.
In another Hungarian native sheep breed, the Tsigai (81 individuals), 98 variable base sites were found in the CR region by Annus et al. (2015), of which 47 singleton and 51 parsimony mutations were detected. From these, it can be comprehended that several related breeds show an overall more diverse genetic composition than a single breed, e.g., the Tsigai which is also a gene reserve breed.

Table 3 shows the number of individuals sampled and polymorphic positions per breeds. It can be observed that the number of mutations observed shows a positive relationship with the number of individuals included in the study. The average number of mutations per individual reveals that the Waldschaf and Bovec sheep samples show significantly greater relative diversity than the Cikta sample.

Table 3: Sample size and number of polymorphic positions

| Parameter | Waldschaf | Bovec sheep | Cikta | total |
|---|---|---|---|---|
| No. of individuals/sequences | 27 | 21 | 70 | 118 |
| No. of parsimony sites/mutations | 68 | 52 | 108 | 131 |
| Avg. no. of mutations per individual | 2.52 | 2.48 | 1.54 | 1.11 |

The total number of haplotypes in the whole study population was 49.
The comparison of related breeds is reported in Table 4 for an average number of nucleotide differences (k) and average nucleotide diversity ($\pi$). The average nucleotide diversity ($\pi$) is the number of different nucleotides at a given base site of two randomly selected mtDNA, which in turn determines the genetic diversity of the stock. It can be seen from the data obtained that the Cikta is characterized by the greatest diversity, with the highest number of average nucleotide

differences (21.251) and average nucleotide diversity (18.02 * $10^{-3}$). This breed is followed by Waldschaf and then Bovec sheep which exhibit the least diversity.

Table 4: Values of k and π according to related breeds

| Parameter | Waldschaf | Bovec sheep | Cikta |
|---|---|---|---|
| Average number of nucleotide differences, k | 18.661 | 17.152 | 21.251 |
| Average nucleotide diversity, π | $15.83*10^{-3}$ | $14.54*10^{-3}$ | $18.02*10^{-3}$ |

Table 5 reports the values of the average number of nucleotide variations (k) and the average nucleotide diversity (π) in comparison between breeds. In the comparison of Waldschaf and Bovec sheep, the average number of nucleotide differences was 18.773. Here, the corrected number of base substitutions calculated according to Jukes and Cantor (Dxy (JC)) was 16.77 * $10^{-3}$ with a standard deviation (sd) of 2.86 * $10^{-3}$. The average number of nucleotide differences between Waldschaf and Cikta was 20.806, while the corrected number of base substitutions (Dxy (JC)) according to Jukes and Cantor was 17.84 * $10^{-3}$ with a standard deviation of 2.37 * $10^{-3}$. In the third comparison (Bovec sheep and Cikta), the average number of nucleotide differences was 21,383, the Jukes and Cantor (Dxy (JC)) value with a standard deviation of 19.18 * 10-3 and 2.36 * $10^{-3}$, respectively.

Table 5: Pairwise averages of k and π for the three related breeds

| Parameter | Waldschaf and Bovec sheep | Waldschaf and Cikta | Bovec sheep and Cikta |
|---|---|---|---|
| Average number of nucleotide differences, k | 18.773 | 20.806 | 21.383 |
| Average nucleotide diversity, π | $15.91*10^{-3}$ | $17.65*10^{-3}$ | $18.14*10^{-3}$ |
| Overlapping mutations, n and % | 43, 36% | 51, 29% | 42, 26% |

According to the Tajima test performed in the entire study population, the average number of pairwise nucleotide differences (k) was 20.880 and the average nucleotide diversity ($\pi$) was 17.71 * $10^{-3}$. The Tajima D-test value was -0.914, not statistically significant (P>0.10). A significant positive value would be an indicator of genetic narrowing (bottle neck effect) or disintegration into subpopulations of a given population. Meanwhile, a significant negative value would be an indicator of selection that intends to eliminate an undesirable gene or, suitably, demographic expansion.

In the evaluation of Kusza et al. (2015), the average nucleotide diversity of both breeds Gyimesi Racka and Turcana was lower (both $5*10^{-3}$) than that of all breeds currently studied, and the Tajima D-values of both breeds were statistically proven to be in the minus range (Gyimesi Racka -1.734 and Turcana -1.814).

The Fu and Li's D* and F* tests performed on the entire pool yielded the non-significant values of 1.217 (P>0.10) and 0.562 (P>0.10), respectively. Furthermore, the Fu and Li's test for haplotype diversity (Hd) gave 0.973 with a standard deviation of $5*10^{-3}$. In contrast, the Fu's Fs statistic resulted in a significant value of -3.296 (P=0.013).

In the variant of the Hungarian Racka bred in Romania Dudu et al. (2016) found similar values for Hd = 0.958 and sd = $9*10^{-3}$ taking into account the control region and cytochrome b gene together. The more frequently mutated control region, not just the longer cytochrome b sequence, may also have contributed to the relatively high values of that single breed variant.

The number of haplogroups identified was four. The most populous of the haplogroups was B, followed by A, with 35, and 10 haplotypes, respectively. Figure 7 illustrates the separation of haplogroups D and C from these, which include one and three haplotypes represented by one Bovec sheep and six Cikta individuals, respectively. The network of the connections between the found CR haplotypes and the reference CR haplogroups calculated by the median-joining method clearly shows that the haplotypes are mainly located around the reference haplogroup B.

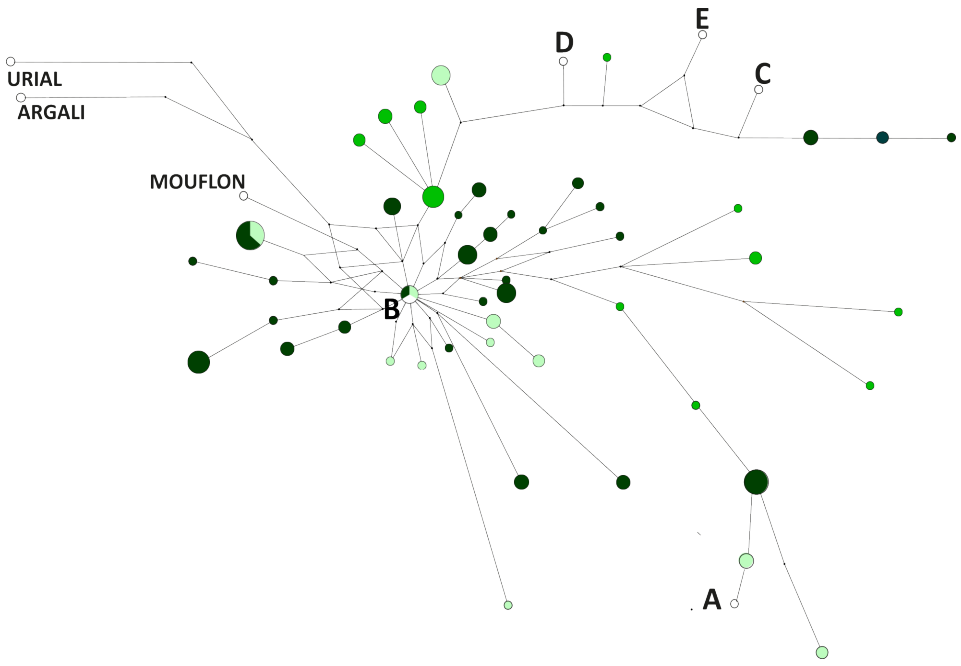


Figure 7: Connections between sample CR haplotypes and reference CR haplogroups by median-joining network

Legend: Waldschaf samples – light green, Bovec sheep samples – green, Cikta samples – dark green. Different white coloured spots with denomination are reference samples (A-HM236174, B-HM236176, C-HM236178, D-HM236180, E-HM236182 Meadows et al., 2005; *O. musimon Mouflon* HM236184, *O. ammon Argali* HM236188, *O. vignei Urial* HM236186 Hiendleder et al., 2002).

## 8. DISCUSSION

Wassmuth et al. (2002) found that all individuals of Steinschaf (including Bovec sheep) belong to haplogroup B. However, in the Waldschaf, haplogroup A was found in more than half of the animals. It shows that not all the Zaupel sheep descendants can be traced back to the same domesticated ancestor (peat sheep or *Ovis aries palustris*). Rather, the present study confirms that Waldschaf is also likely to be members of haplogroup B.

Among 11 Austrian sheep breeds, the shortest genetic distance was proven by Baumung et al. (2006) between Alpines Steinschaf and Waldschaf based on microsatellites, nuclear information.

The Copper Age sheep, which were found by Oliveri et al. (2012) to also belong to haplogroup B is the possible link between the peat sheep and its distant ancestor, a certain Asiatic subspecies of *Ovis ammon*.

The common genetic origin confirming the breed history may be the reason for the similarity of the three studied breeds.

The complete identity between the breeds is supported by the value of the Tajima D-test (-0.914, $P>0.10$) and the values of the Fu and Li's D* and F* tests (1.217, $P>0.10$ and 0.562, $P>0.10$, respectively). According to these values there is a limited difference in number of polymorphic sites and the mean number of nucleotides pairwise differences among the breeds. These results indicate that the community of Zaupel descendants studied are in a position of genetic equilibrium, deviation from this does not endanger it, and there is no force supporting population demographic expansion.

Our joint population of Zaupel sheep descendants shows significant negative value of Fu's Fs statistic (-3.296, $P=0.013$), which demonstrates foreign gene flow after a spatial expansion. The value of Fs statistic can be considered as low in this case, since in the clusters of other similar sheep research, it was decreased to a greater extent within the negative range with a much lower probability of error: e.g., -7.48 ($P=0.001$, Liu et al. 2016), -10.88 ($P<0.001$, Guo et al. 2005), and -76.28 ($P<0.001$, Sulaiman et al. 2011). A conclusion can be drawn that the demographic history of the breeds differs slightly from each other, which is a sign of a diverse maternal

background, i.e., involvement of ewes with other genetic backgrounds in the breed. From the development of our domestic animal breeds, we have experienced that the use of the improver sires on the local dam population for several generations (grading up) creates a new foreign breed in a given area.

In processing this data, four haplogroups were distinguished. The haplogroup B (typical for European sheep domesticated in Near East) prevailed in all breeds (Waldschaf, Bovec sheep, and Cikta) with 81%, 62%, and 80%, respectively. This was followed by the frequency of haplogroup A (characteristic of the Indian subcontinent, 77%; Lv et al., 2015). In contrast to the former observation of (Wassmuth et al., 2002), this haplogroup was found in much less than half of the animals in the Waldschaf (19%). Bovec sheep is distinguished by a remarkable proportion of haplogroup A (33%), while this haplotype is also present in the Cikta, but less typically (11%).

The today presence of haplogroup A in Central Europe should not be surprising. The region of Hungary, which is most likely amongst the earliest continental centres of wool production, served by far with the oldest samples of sheep remains in Europe assigned to haplogroup A. Sabatini et al. (2019) speculated that the haplogroup A Bronze Age sheep (1500 B.C.) in Hungary (Százhalombatta-Földvár) may represent evidence of new sheep tentatively introduced into Europe during the Bronze Age in order to improve productions traits.

The haplogroup C was first separated during phylogenetic development. The peculiarity of the processing result is that we detected haplogroup C in the Cikta breed (with six individuals). A 9% incidence of haplogroup C indicates an even more complex maternal background of that breed. This means that ewes have entered Hungary in the past from the closer and farther areas, either from Asia Minor (Meadows et al., 2007), Caucasus (Tapio et al., 2006), Georgia (Kunelauri et al., 2019) and Caspian Sea region (Lv et al., 2015) or from Mongolia (Ganbold et al., 2019) and China (Guo et al., 2005; Chen et al., 2006) where, to the best of our knowledge, haplotype C can be found outside Europe. Additionally, Lv et al. (2015) found a significantly higher frequency of haplotype C in fat-tailed breeds than in short-tailed breeds, and the highest level of haplotype C variability in the breeds of North China.

Research conducted in the neighbouring countries of the Balkan Peninsula has dealt with the

classification of domestic sheep breeds into haplogroups. For example, Cinkulov et al. (2008) found that in the West Balkan Pramenka sheep, the B haplogroup is predominant, but in addition, haplogroup A occurred in traces. The investigation of Ferencakovic et al (2013) pointed out that the East Adriatic sheep breeds have a homogenous maternal origin of haplogroup B, while Dubrovnik Ruda sheep and Istrian sheep differentiate from them with few individuals displaying haplogroup A. Three Romanian breeds (Turcana, Tsigai and Black Head Ruda) were clustered by Dudu et al. (2016) exclusively in haplogroup B. In the fourth breed of their work, the Romanian Racka which is a variant of Turcana the haplogroup A was also detectable, but none of the Racka haplotypes was associated with C, D or E lineages.

Within Europe haplogroup C has been found, so far, only on the Iberian Peninsula (in Portugal: Pereira et al., 2006 and in Spain: Pedrosa et al., 2007) and in in the southern countries of the Balkan Peninsula (in Albania and Greece: Pariset et al., 2011). The haplotype C, also identified in Egyptian breeds (Othman et al., 2015) is also in agreement with the assumption of early spread in sheep. The Iberian Peninsula was a springboard for the appearance of Asia Minor sheep on the European stage since it was connected directly to North Africa and Asia Minor through the Mediterranean Sea.

The European spread of sheep could have occurred on several highly probable routes, partly along the Eastern European plain and along the Danube Valley, through the Carpathian Basin. (Schmölke et al., 2018).

The other novelty is the discovery of the very rare haplogroup D at 5% level in the Slovenian Bovec sheep breed, which thus also consists of three haplogroups. Although Mariotti et al. (2013) have recently detected its presence in breeds Bergamasca (17%) and Laticauda (7%) in the neighbouring Italy.

Haplogroups D and E are the least frequent and have only been identified in samples from Turkey and the Caucasus (Tapio et al., 2006; Meadows et al. 2007). In the study of Liu et al. (2016) on 15 indigenous Tibetan sheep populations the frequency of the rarest haplogroup D was less than 0.2%. Fossils in Anatolia showed a 3% presence of haplogroup E in the Bronze Age (Demirci et al. 2013).

Dymova et al. (2017) carried out archaeological mitochondrial DNA CR analysis based on about 4,000-1,000 years old sheep bone remains in Altai and found all the five recent haplogroups,

including lineages D and E. That richness of diversity led them to conclude that the Altai region had been a migratory area for many sheep and peoples in the past.

Haplogroup E was detected in Iran in 10 Iranian native breeds (Rafia and Tarang, 2016).

Kirikci et al. (2019) stated the Karayaka breed from Northern Anatolia cannot be categorized as a genetically homogeneous population, but even has four different haplogroups (A, B, C, and E).

In our study, the Slovenian Bovec sheep, although considered to belong to haplogroup D, had fallen between D and E, as it is 33 mutations away from Genbank D and 37 mutations separate it from Genbank E.

## 9. CONCLUSION

The working hypothesis, which was based on the breed histories, was verified by genetic analyses and their statistical testing. The high proportion of haplotype B and the overlapping mutations found within the three breeds, supplemented by the Tajima D-, Fu and Li's D* and F* tests giving non-significant values (in each case $P>0.10$), confirmed that these breeds in question do indeed originate from a common ancestor, the Zaupel.

The Fu's Fs statistic based on haplotype frequencies is a control for rare allele's excess. Its significant negative value (Fs statistic=-3.296, $P=0.013$) is an indicator of population expansion (genetic hitchhiking), which demonstrates foreign gene flow after a spatial expansion.

In addition to the haplogroups B and A typical of Europe, I was also able to detect the presence of haplogroups C and D in Central Europe. I have to say that domestic forms carrying haplogroups C and D as well from the first domestication centre of sheep located in the Middle East subsequently spread into Europe throughout the Mediterranean and Ponto-Caspian regions, or simply following the coastline extending all around the pre-Flood Black Sea's freshwater lake (before about 5600 BC). Sheep belonging to haplogroup C and D may have either appeared in Europe with the prehistoric man or may have later came from Asia during migration periods. Concerning the native sheep of Hungary, the last such "incomplete recorded" period can be considered to be the Ottoman Empire in the 16-17th century. This is when, fat-tailed sheep, such as the Karagül and Kivircik (with haplogroup C frequency of 6% and 4%, respectively; Demirci et al., 2013) may have appeared which then merged into local populations. The D haplotype found in Bovec sheep is most likely explained by the genetic acquisition of Bergamasca Alpine sheep due to its geographical proximity. Therefore, it is conceivable that the haplogroup C and D is also detectable in other native breeds in Hungary and Slovenia or other surrounding countries. It is clear that haplogroup E has not been detected by this study or even by others in Europe.

Knowledge of pedigree data is essential for the identification of maternal lineages in mitochondrial genome examinations. For representative sampling, it important to use the

founder sampling method because the values of true mitochondrial diversity can be calculated in this way. Furthermore, finding a unique haplotype for rare families with a high probability is likely to be the only approach to do so. It is an interesting question whether individuals with specific haplotypes should be excluded as outliers from the maintenance of autochthonous breeds.

In addition, this work draws attention to the importance of maintaining families and within-family selection. The increased emphasis on the maternal side is also justified by the fact that females are present in a higher proportion than males and remain in breeding for a longer period of time, thus they can be a greater custodian of the implementation and maintenance of genetic diversity.

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## 12. Appendices

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- increase citation numbers of publications authored by Hungarian veterinarians, thus improve the impact factor of Hungarian veterinary journals.
- present the knowledge base of the University of Veterinary Medicine Budapest and its partners in a focussed way in order to improve the prestige of the Hungarian veterinary profession, and the competitiveness of the organizations in question.
- facilitate professional relations and collaboration.
- support open access.

## Appendix 4. Supervisor counter-signature form

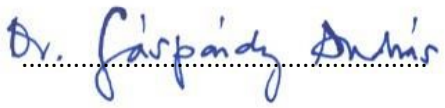
I hereby confirm that I am familiar with the content of the thesis entitled

**Comparison of mtDNA control region among three Zaupel sheep descendant breeds**

.....

written by ..... **Shane McCarvill**..... (student name) which I deem suitable for submission and defence.

Date: Budapest, ....09....day...04.....month ....2021.....year



.....

Dr. Gáspárdy András

Supervisor name and signature