

Thesis

Leonie Fogarassy

2022



Figure 1: emblem of the University of Veterinary Medicine Budapest

<https://univet.hu/hu>

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

## **Comparison of Forest sheep and Hungarian Cikta based on their mtDNA Control Region**

by

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2023

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# Thesis Topic Declaration Form

University of Veterinary Medicine

Student name: Fogarassy, Leonie

## THESIS TOPIC DECLARATION FORM

I hereby request approval from the Head of Department of the Department of/and.. animal...  
breeding, Nutrition and laboratory animal science  
to prepare a thesis based on a topic announced and supervised by said Department as follows.

Date: Budapest, 17 November 2021

  
Student signature

Thesis topic:

Comparison and experimental series of  
the different breeds of sheep in view  
of their ancestors, the Zaupeel sheep.

Title of Thesis (English title as well):

Comparison of Forest sheep and Hungarian  
Cikta based on their mtDNA

Supervisor signature:



Approved by:



Head of Department signature

Dept. for Animal Breeding and Genetics

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## 1. Introduction and aim

Today we live in a time of constant progress and development, but also, it is one of the essential aims these days to go back and reconnect with nature. Our planet is located in a total climate change and crisis, and humans brought us up to this point. On the way to assure success, society made a lot of sacrifices. Not only concerning the natural environment, but massive changes can also be observed regarding the natural living of animals. Humans overpopulate the earth; more animals are needed to produce foodstuffs, and more space is required to keep those animals. So one of the sad secondary effects is that many wild animals are threatened with extinction, or animals with less use for human living are also endangered species. The breeding focus gets shifted concerning the different breeding goals of these animals like milk, wool, or meat. Important aspects like the health or the body conditions of these animals are pushed in the background, and this results in, for example, higher infection rates. After looking at all these aspects it gets clear to go back to the roots, focus again on older or maybe extinct breeds to enlarge the genetic pool and get the possibility of producing more healthy and resistant animals.

For this intention, we took and investigated blood samples from different individuals out of a herd of sheep which may be related to the extinct Zaupel sheep. During this investigation, the essential point is comparing mutated mitochondrial DNA (mtDNA) and genomic DNA. In connection with this investigation, it was necessary to analyze the control region of the maternal mtDNA. The results allow us to suppose the different investigated sheep races descent and find similarities in the DNA.

In our case and investigation, the Forrest sheep was used as the descendant of the Zaupel sheep.

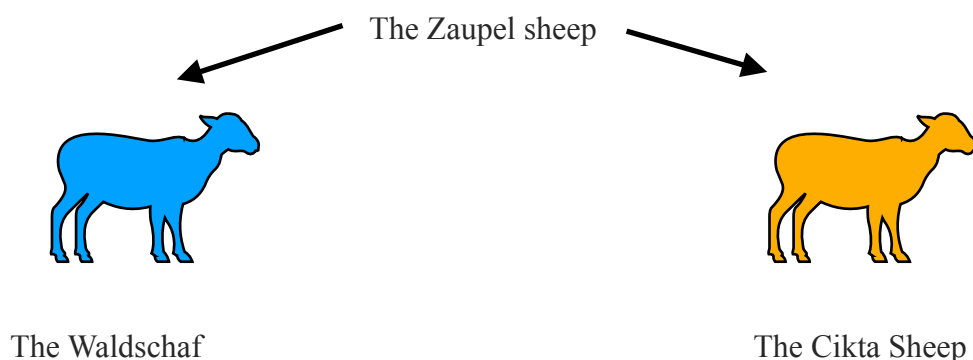


Figure 2: The Zaupel sheep and their descendants; personal chart



## **2. Literature review**

### **2.1 The mitochondrial DNA**

The Deoxyribonucleic acid, short DNA, is the total basic module for every existent living like animals or plants. It is a simple polymer built of four bases (Adenine, Cytosine, Thymine and Guanine).

The mitochondrial DNA, shortened with mtDNA, is the DNA inside the mitochondrial Matrix, and you can not find it in Procaryots. Mitochondria are cell organelles surrounded by a double-layered membrane existing in eukaryotic cells, and they are the most critical organelle for energy production. In animals, the mtDNA is very small and formed like a ring. They produce energy via cellular respiration in two ways. The first one is via the citrate cycle, which is located in the matrix, and the second is via the respiratory chain, which is located in the membrane [1]. The produced energy shows up in the form of Adenosine-triphosphate (ATP). The mtDNA replicates through DNA-Polymerase  $\gamma$  [2].

In humans, an own genome of 16.500 base pairs can be found, which is used for evolutionary research and strictly maternally inherited. The paternal parentage does not assist mtDNA to the offspring. After penetration and in the early embryogenesis, the spermatocytes get destroyed. So the paternal part is lost during the first embryonic cell divisions. [3,4].

About sheep, the mtDNA is used to explore the history of domestication and the development and further development of the breed. In general, there are four described mitochondrial lineages for sheep: haplotype group A, B, C and D. Group B can be mainly found in the European region, especially in European mouflon sheep [5,6].

## 2.2 The control region

The control region (CR), also called the "Displacement-loop containing region" or just „D-loop" [7], is a non-coded section of the mitochondrial DNA (mtDNA). This area is the largest non-coded area that can be found in the mtDNA. The control region is responsible for the control of the replication and the transcription of the mitochondrial DNA. A mitochondrial genome of a vertebrate shows a structure with 16 000 base pairs. (bp), which are encrypted to 37 compact genes. These 37 genes are composed of two rRNAs, 13 protein open reading frames, and 22 tRNAs [8]. Two of the essential facts about the mtDNA of animals are the absence of introns and the capability for replication [9].

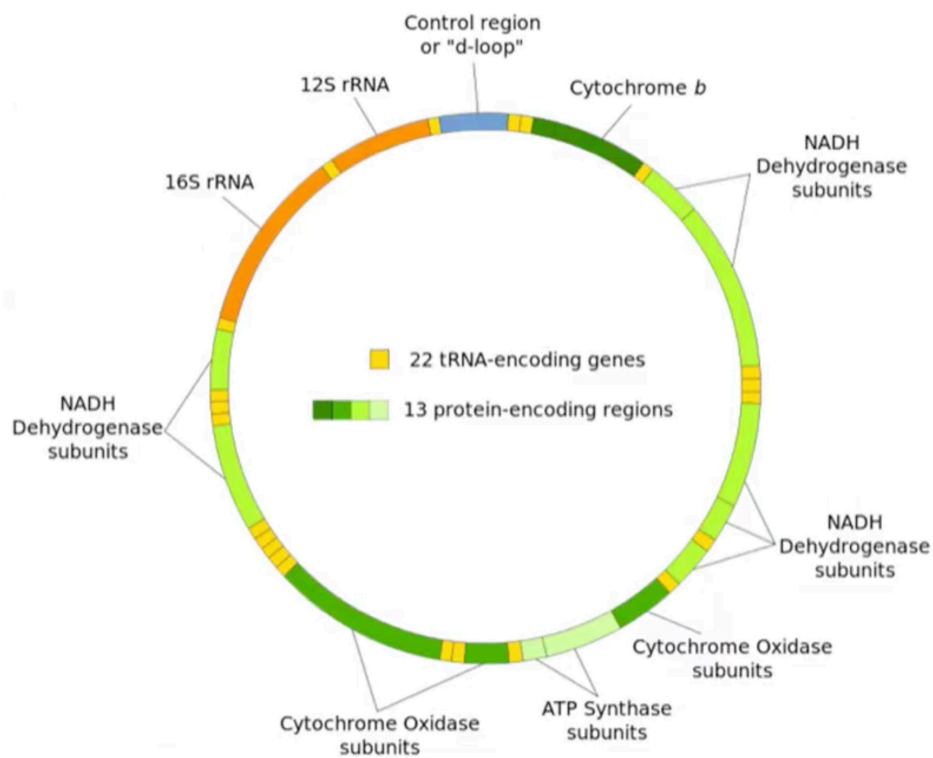


Figure 3: chart of the mtDNA with control region; <https://hu.weblogographic.com/difference-between-y-dna>

### 2.3. Breed characteristics

One of the earliest domesticated animals is the sheep. First indications of domestication are referable to the year 9000-8000 BC and the south-west Asian region. The mutton originates all sheep that are domestic today. One of the main reasons for the domestication of the sheep was wool. Meanwhile, the sheep are also used to produce milk and meat. We can differentiate between milk and meat breeds, and there are many differences between the species. Through domestication, a vast diversity of different breeds exists. These breeds exist because there are a lot of other criteria for breeding sheep, like the quality of the wool (rate of the hair, growth, length, regularity), phenotype (body height and form), the colour of the thread (black, brown, white, tricolours), tail (length), ears (long, hanging, underdeveloped), horn growth (typically horned, gender-dependent horn growth, diverse horn growth, non-horn growth), reproduction characteristics (mono-oestrus, poly oestrus, fertility), milk accomplishment and herd character. These days there are 500-600 breeds of sheep [10,11,12].

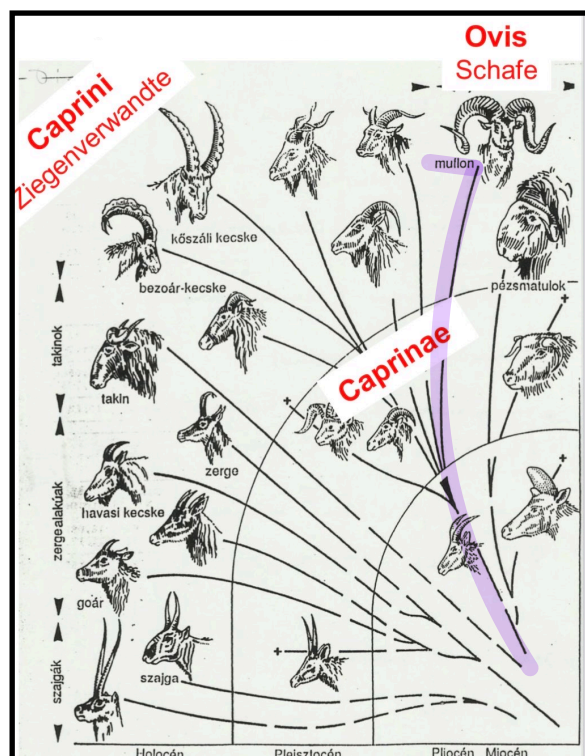


Figure 4: taxonomic classification of the sheep; [https://univet.hu/files/courses/dt/7664/files/07\\_Schaf-%20Ziegenzucht%20Einführung.pdf](https://univet.hu/files/courses/dt/7664/files/07_Schaf-%20Ziegenzucht%20Einführung.pdf)

## 2.4 The Zaupel Sheep

The Zaupel Sheep (*Ovis aries germanicus rusticus*) is a mixed wool sheep most common in medieval times. The colour is primarily dirty white, but sometimes also brown and black. It was very unpretentious, robust and fertile. Because of the character of the sheep, farmers had the opportunity to put cows, pigs and the Zaupel sheep together on the meadow. The main disadvantage was the rough wool and the small amount of meat, so the Zaupel was replaced by the merino sheep, a famous sheep because of its high-quality wool. They have been suppressed in out-lands and critical climatic regions. The Zaupel became extinct; descendants survived in the Bavarian woods today; we call this breed "The Waldschaf". During medieval times' emigrant's brought the Zaupel sheep from the Swabian alp to southwest Europe. Since today we can find descendants of the Zaupel on this route. The Waldschaf in German regions and the Cikta sheep in Hungary [13].

Today the definition of Zaupel sheep isn't the correct term for a particular breed; it is a general term for all sheep that developed from this breed. In the book "Die Schafzucht" written in 1906 by Johannes Heyne, the Zaupel has been described as the following: "The Zaupel sheep (*Ovis aries germanicus rusticus*) is 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 Kärnten, Krain and Tirol. The Zaupel seep is not largely framed; he mentions 58-60 cm withers height in rams and 55-56 in ewes; an adult ewe only weighs 30-35 kg. The bones are medium-hard, and the head is long but slender. The ears are moderately long, the withers sharp and arcuate. He also mentions the horns, which you can find mostly in male animals. These are curved flat to the back and down; the end is on one line with the eye and the ear. The slender small of the back is sanded off. Neck and trunk are covered with solid and shiny wool; in many animals, you can find downy hair under the long and rough thread. The weight of the yarn after searing is about 1.5 kg. You can separate in Hanoverian and Pomeranian sheep, the Wallis sheep in Switzerland and the refined black Swiss sheep" [14,15].

In the case of the Zaupel, we can talk about a spectacular comeback. Due to the Šumavská ovce and the Cikta sheep, it was possible to receive a small stock. The three breeds are summarised under the generic term "Zaupel sheep" with the Bavarian Forrest sheep.



Figure 5: Zaupel sheep ram; Tanja Kutter/ Schwäbisches Bauernhofmuseum Illerbeuren [16]



Figure 6: Zaupel sheep ewe with lamb; Tanja Kutter/ Schwäbisches Bauernhofmuseum Illerbeuren [16]



Figure 7: Herd of Zaupel sheep; Tanja Kutter/ Schwäbisches Bauernhofmuseum Illerbeuren [16]

## 2.5 Descendants of the Zaupel sheep

### 2.5.1 The Cikta

The Cikta sheep, also called Swabian sheep, is an old traditional German breed descended from the Zaupel sheep. After the Ottoman occupation in the 18 century, this breed settled in Hungary. The stock decreased because of the small commercial benefit and the advance of the merino sheep. These days there is just a low amount of these animals, but mainly in Hungary. Because of their smaller size and their frugal lifestyle, they are the perfect budget-side breed. The actual aim is the preservation of the species and the genes [17,18].



Figure 8: Cikta sheep; <https://www.ferto-hansag.hu/de/cikta-schaf.html>

### 2.5.2 The Waldschaf (Forest sheep/ Sumavska)

The Waldschaf is a small to medium-sized mixed wool sheep, the head of the females is straight males one is lightly oval. Ears are sharply formed medium-large and are attached laterally to the head. They can occur with or without horns, primarily common in a whitish colour, but they also exist in black, grey, brown or brindled. The male weighs 55-88 kilograms and has a withers height of 67-70 centimetres. Females are smaller and lighter; they only weigh 35-60 kilograms and have a width of 60-65 centimetres. They are very robust, frugal, weatherproof and very resistant to parasites and diseases. This calm sheep breed prefers to live in a closed group on a meadow. Usually, it is enough to feed them with meadow grass; you can add silage during pregnancy. They have good fertility and an aseasonal heat cycle. Commonly most of the births take place in winter. Females are sexually mature with 9-15 months, males with 4-5 months [21,22].

The meat of this breed is very delicate, finely fibrous, low in fat content and without the unwanted sheep taste. It is not possible to use the Waldschaf for milk production because the udder is too small. The wool has a high quality, is close and light, and tweed fabric is made out of this wool [19,21].



Figure 9: Waldschaf ram; <https://www.oebisz.at/rassen/schafzassen/waldschaf/>



Figure 10: Waldschaf ewe; <https://www.oebz.at/rassen/schafassen/waldschaf/>



### 3. Material and Methods

#### 3.1 Sample Collection

The sample collection, which Prof. Dr Gaspardy organised, started in autumn 2015. He and his scientific team choose which herd and particular animals are the best choices for taking a sample. The sample recovery has taken place on three different farms. Duna-Dráva National Park, which is state-owned, is located in Nagydorog. There are living 20 families of sheep. The other two places are private farms. One of them, situated in Pénzesgyőr, has eleven families and is owned by Mr T. Nagy. The other private farm is located in Szécsénke and is owned by Mr J. Janosi. Their stock consists of five families. The team chose the animals out of their family to represent the optimal breed characteristics, leaving a total specimen count of 72. The sheep had 6-5-4 ancestral generations distributed over the 36 oldest sheep families (maternal lineages). Out of each family, the scientific team chose two representatives for sample taking. The taken samples are blood samples out of the jugular vein and preserved in an EDTA (Ethylenediaminetetraacetic) tube, so the blood is not coagulable [22].



Figure 11: Taking samples from a Cikta sheep with a white fleece; personal photo taken by Dr. András Gáspárdy on 22nd October 2015 in Szécsénke



Figure 12: sampling point; personal photo taken by Dr. András Gáspárdy on Nagydorog from Cikta ewes on 10th November 2015

Related to the Zaupel sheep, we can find relative descendants, the Austrian Waldschaf. Dr Beate Berges from Öngene added her scientific results in terms of isolated DNS for this study. In this case different biological samples were taken from non-closely related individuals in Waldschaf (Austria, n= 27) and were made available for research functions in 2017. Dr Endre Kovács travelled to Wels to meet Dr Beate Berger in person, transfer the samples and bring them to Hungary.

Öngene is the austrian national coalition for gen reserves in case of farm animals. The aim is to protect and preserve different hereditary disposition [23,24].

### **3.2 DNA preparation and sequencing**

The blood samples were collected in autumn 2015. After venepuncture of the vena jugularis the preserved blood was collected in tubes containing EDTA (anticoagulation) and was stored at -20 °C at the Genetic Laboratory of the Department. Then the DNA was isolated by the use of SIGMA GenElute Blood Genomic DNA Kit according to the manufacturer's instructions. PCR reaction mixtures, 25 µl of each contained 2.5 µl dNTP, 2.5 µl buffer, 1.5 µl MgCl, 2 µl primer, 1 µl BSA, 0.4 µl Taq-polymerase (Thermo Scientific) and distilled water. For intensification of Cytochrome B mitochondrial genome a primer (1140 bp) designed after Meadows (2005) was the following: CYTB-F 5'-GTCATCATCATTCTCACATGGAATC-3' and CYTB-R 5'-CTCCTTCTCTGGTTTA

CAAGACCAG-3'. Amplification of DNA was done by means of programmable Thermal Cycler 2720 PCR machine (Applied Biosystem).

The resulting PCR product purified by use of SIGMA GenElute PCR Clean-up Kit according to the protocol. The product was checked by gel electrophoresis [25].

### **3.3 Statistical Evaluation**

For the evaluation of the different sequences, we performed various tests. The review aims were to compare genetic changes or genetic differences between the individuals/population [26].

#### **3.3.1 Tajima D Test**

The Tajima test or Tajima D test was developed by Mr Fumio Tajima and is dedicated to the statistic of population genetics. The test is used for detecting and plotting sequence mutations and their correlations. The main focus is separating random mutations from non-random mutations. In contrast to random mutations, non-random ones are not generated during selection. These kinds of transformations have an impact on the constitution of an individual. The most revealing point is the point of Equilibrium of the genetic frequency through the genetic drift. In the case of the constant size of the population and the continuous rate of mutations, results of the Tajima's test will reach this point anytime.

During the test, three individuals are chosen, and the trial will compare their homologous DNA. Selected individuals are tested on their identical mutation sequences and sorted by non-equal mutation within their DNA. The test should identify these arrangements that are not relatable to the Equilibrium or the genetic drift, and these sequences are mutations. So this will show particularly where the genetic drift was provoked and caused a difference in each breed [27].

### **3.3.2 Fu and Li's Test**

The Fu and Li's test is a scientific test for different gene selection or demographic changes in any kind of population. The test is based on the certainty estimates of the number of a nucleotide of segregating/ polymorphic sites and an average number of nucleotide differences. Genealogy mutations can be external or internal; which means: External mutations are mutations that occur in the outer branches, and internal mutations are mutations that occur in the internal components of the genealogy [27].

### 3.3.3. Jukes & Cantor Method

The Jukes and Cantor method is an eased equation, named after the academics Jukes and Cantor, responsible for correcting distance data of the DNA or creating a genealogical tree. It supposes that all sites are independent and have identical mutation rates. Also, it assumes that all possible nucleotides (A, T, C and G) substitutions occur at the same rate  $\alpha$  per time. It is based on the rate of mutation and backwards mutation and the idea that all nucleotides have the same value and are mutated equally between the ancestors [28].

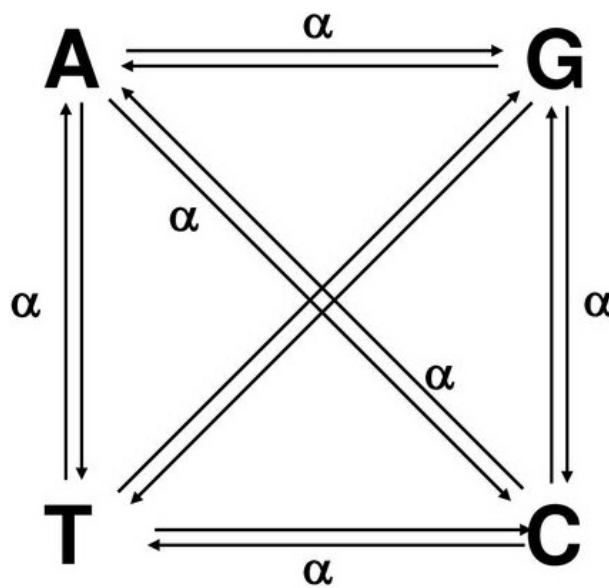


Figure 13: Chart Jukes and Cantor method; <https://slidetodoc.com/phylogeny-ii-parsimony-ml-semphy-phylogenetic-tree-branch/>

### 3.3.4 Median-joining network

The median-joining network is based on informative mutations of the haplotype, and it is possible to represent the genetic connection and correlation graphically. Working with the median-joining network is one of the most popular courses of action to detect reticulation [29].

#### 4. Results and discussion

In the complete study, the number of used sequences/individuals was 97. The total number of investigated sites was 1179, 1054 were monomorphic, and 125 were polymorphic. The Polymorphic sites comprise ten singleton variable sites (140 286 312 462 499 696 813 962 979 1183) and 115 parsimony informative sites. The number of haplotype was 38. In Table 1, the number of sequences, number of polymorphic sites and the total number of mutations per breed are visible.

Parameter	Waldschaf	Cikta
Number of sequences	27	70
Number of polymorphic sites	68	108
Total number of mutations	68	108

Table 1: DNA divergence between populations

Table 2 shows the total data of table 1 plus the shared mutations between the Waldschaf and the Cikta sheep.

Parameter	Total data
Number of sequences	97
Number of polymorphic sites	125
Total number of mutations	125
Shared mutations	51

Table 2: total data of table 1

The comparison between the investigated breeds is reported in Table 3. The number of nucleotide differences ( $k$ ) and the nucleotide diversity ( $\pi$ ). Both of these values can show us impressions about the polymorphism inside of the population.

It can be determined that the most remarkable diversity characterises the Cikta sheep, with also the highest number of average nucleotide differences (21.25135) and average nucleotide diversity of 0.01802.

In case of the Waldschaf, the data shows a value of 18.66097 for the average nucleotide differences and 0.01583 for the average nucleotide diversity.

Parameter	Waldschaf	Cikta
Average number of nucleotide differences (k)	18.661	21.251
Average number of nucleotide diversity ( $\pi$ )	0.01583	0.01802

Table 3: Values of k and  $\pi$  to related breeds

Table 4 shows a report above the value of the average number of nucleotide variations (k) and the nucleotide diversity ( $\pi$ ) concerning the examined breeds. The first value, the average number of nucleotide differences between the Cikta and the Waldschaf, is 20.635. In this measurement, the remediated number of base substitutions calculated by the Jukes and Cantor method was 0.01799 with a standard deviation (SD) of 0.00157. The number of overlapping mutations between the Waldschaf and the Cikta sheep occurred inverted proportion to k and  $\pi$ .

Parameter	Waldschaf and Cikta
Average number of nucleotide differences, (k)	20.635
Average number of nucleotide diversity, ( $\pi$ )	0.0175
Overlapping mutations	51

Table 4: Average of k and  $\pi$ , overlapping mutations

Under the Tajima test's provisions, the study's pairwise nucleotide difference (k) was 20.86584, and the average nucleotide diversity ( $\pi$ ) was 0.01765. The performed test was statistically not significant ( $p > 0.10$ ) because the value was -0.47672. Genetic constriction (bottleneck effect) or a break up into subpopulations is indicated if the merit is positive and significant. The bottleneck effect is a genetic drift that reduces the population by a random event, resulting in a minor genetic variability. On the other hand, a significant negative value would be a sign of selection.

The next performed test, the Fu and Li's test, is performed on the whole pool, which resulted in non-significant values of 1.45676 ( $p > 0.10$ ) and 0.76212 ( $p > 0.10$ ).

Through the presentation, via the median-joining (chart 1) method, the composition between found CR haplotypes and the reference CR haplotypes can be shown. The chart indicates explicitly that the haplotypes are mainly located around reference haplotypes B. The size of the circles clarifies the dimension of the population, which represents a haplotype. The yellow colour represents the Cikta sheep, the red colour the Waldschaf, and the black colour shows references to haplogroup [30, 31].

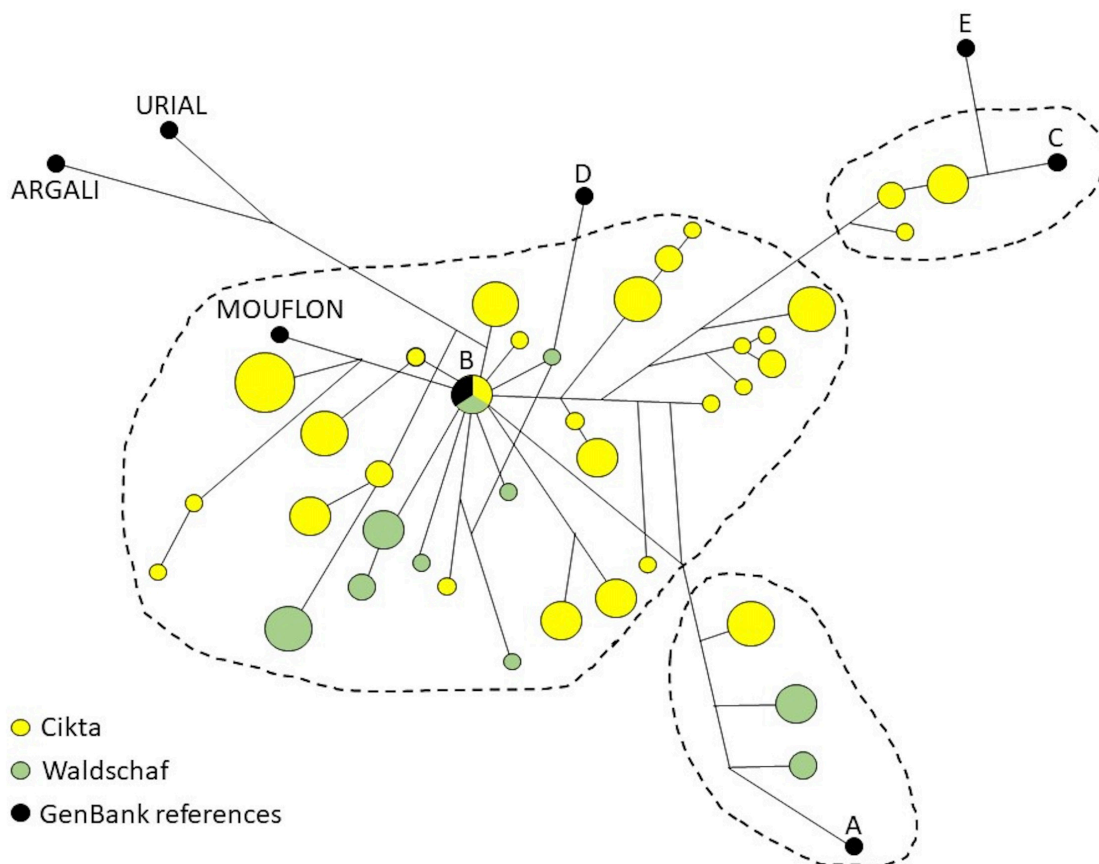


Figure 14: composition between found CR haplotypes and CR haplotypes calculated by median- joining network



Parameter	Waldschaf	Cikta
Number of sequences	27	70
Number of segregating sites (S)	68	108
Number of haplotypes (h)	11	29
Haplotype diversity (Hd)	0.88889	0.96025
Average number of difference (k)	18.66097	21.25135
Nucleotide diversity ( $\pi$ )	0.01583	0.01802
Nucleotide diversity with JC ( $\pi$ JC)	0.01616	0.01837

Table 5: Overview of measured values

Parameter	Total Data
Number of sequences	97
Number of segregating sites (S)	125
Number of haplotypes (h)	38
Haplotype diversity (Hd)	0.96478
Nucleotide diversity with JC ( $\pi$ JC)	0.01765

Table 6: Total data of table 5 (Overview of measured values)

## **5. Conclusion and recommendation**

This study discusses partial mitochondrial sequences, mtDNA and the control region between two different breeds of sheep. Both performed tests, the Tajima's D and the Fu and Li's tests, resulted in a not significant result. Due to the median-joining network, the CR haplotypes and the reference CR haplotypes can be shown.

The aim of this scientific work was the genetic investigation between the two sheep breeds, Forrest sheep and Cikta sheep, in correlation to their common ancestor, the Zaupel sheep.

The Zaupel is an extinct breed with three descendants, the Waldschaf, the Cikta and the Suma. Zaupel sheep is an umbrella term that includes all of these three breeds, which are genetically the closest to the Zaupel.

Each breed that is related to the Zaupel sheep shows excellent robustness and perfect fertility. All of them are not so sensitive against some diseases like other breeds of sheep. The wool quality is not the best compared to the merino sheep, for example, but the benefits predominate. So the stock of all three breeds is permanent increasing.

During my work, I focused on the complexity of DNA analysis and the statistical, mathematical evaluations of these methods. Through the accomplished results of the tests, it is possible to give a prognosis for these breeds. In my introduction, I talked about constant progress and development but sometimes going back to the origin is also a solution.

## **6. Acknowledgement**

First, I would like to thank my supervisor Dr. András Gáspárdy for his understanding and help during the research. He always encouraged me and supported me and this work.

Also, I would like to thank Dr. Beate Berger and the "Österreichische Nationalvereinigung für Genreserven" for adding their scientific results.

Mainly I want to thank my family, especially my father. Papa, you were always my biggest support and best friend. Without you, this work and much more would not have been possible.

Besides, I want to thank my friends which I met during my studies. We started as strangers and ended up as lifetime friends.

In loving memory of Dr. med. Géza Fogarassy.

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## Thesis progress report for veterinary students

Name of student: Leonie Fogarassy

Neptun code of the student: DDZBIE

Name and title of the supervisor: Dr. András Gáspárdy, Associate Professor, Head of Department

Department: Department of Animal Breeding and Genetics, Institute for Animal Breeding, Nutrition and Laboratory animal Science

Thesis title: Comparison of Forest sheep and Hungarian Cikta based on their mtDNA Control Region

### Consultation – 1st semester

Timing				Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2021	11	17	First time to speak about the possible topic. We are decision on final title and filled the application form.	
2.	2022	02	08	The aim of the study is to comparison the Forest sheep and the Cikta sheep. Both are descendants of the extinct Zaupeel sheep. Despite the fact that they are bred <i>inter se</i> in two countries the research aimed to reveal the close genetic relationship based on mtDNA CR sequences. Student began research of literature and compiling data of breed history.	
3.	2022	04	12	For the purpose of this investigation we collected biological samples at Hungarian farms and received isolated DNA form Austria. GenBank resources, in addition to sequences obtained from previous investigations into other indigenous Hungarian sheep breeds, will be used as reference sequences. The results from this study may prove	



				the similarities between the Forest sheep and Cikta. Student became familiar with the DNA-laboratory technics.	
4.	2022	06	28	<p>Discussion of thesis progress report.</p> <p>The sequencing of the CR is successfully ended, we shall start with the statistical analysis of the sequences. She submitted first draft of introduction chapter for my review. The statistical processing and analysis a CR-sequences successfully finished. I provided her with these to write chapters Material and Method and Results.</p> <p>The processing resulted in a scientific presentation (in Croatia) and communication:</p> <p>Fogarassy, Leonie – Berger, Beate – Zenke, Petra – Kovács, Endre – Sáfár, László – Becskei, Zsolt – Maróti-Agóts, Ákos – Gáspárdy, András (2022): Vergleich zweier Nachfolgerassen des Zaupel-Schafs, Cikta und Waldschaf, basierend auf ihrer mtDNS Kontrollregion. Danubian Animal Genetic Resources, 7(2): 43-50.</p>	

**Grade achieved at the end of the first semester: .....excellent (5)..**

#### Consultation – 2nd semester

Timing				Topic / Remarks of the supervisor	Signature of the supervisor
	year	month	day		
1.	2022	09	15	The last two chapters of the thesis (Discussion and Conclusion) were prepared.	
2.	2022	11	02	We have started the final review of the thesis.	



**Grade achieved at the end of the second semester: ...excellent (5).....**

The thesis meets the requirements of the Study and Examination Rules of the University and the Guide to Thesis Writing.

I accept the thesis and found suitable to defence,

.....  
signature of the supervisor

Signature of the student: *L. Fogarassy*

Signature of the secretary of the department: .....

Date of handing the thesis in.....