# VYTAUTAS MAGNUS UNIVERSITY

**FACULTY OF NATURAL SCIENCES**

**DEPARTMENT OF BIOLOGY**

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# MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF *LAELAPS JETTMARI* (MESOSTIGMATA: LAELAPIDAE) MITES FROM SMALL RODENTS

Bachelor final Thesis

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(signature) (date)

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# ABBREVIATIONS

- BLAST – Basic Local Alignment Search Tool
- COI (COX1) – cytochrome c oxidase subunit I
- dd$H_2O$ – double-distilled water
- DNA – deoxyribonucleic acid
- dNTP – deoxyribonucleotide triphosphate
- EDTA – ethylenediaminetetraacetic acid
- $MgCl_2$ – magnesium chloride
- ML – Maximum-Likelihood
- NCBI – National Center for Biotechnology Information
- $NH_4OH$ – ammonium hydroxide
- NJ – Neighbor-Joining
- Nt - nucleotide
- PCR – polymerase chain reaction
- RNA – ribonucleic acid
- rDNA – ribosomal deoxyribonucleic acid
- TAE – tris-acetate-EDTA
- 28S rRNA – 28 subunit of ribosomal ribonucleic acid

# SUMMARY


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Information about Laelapidae mites infesting small rodents is scarce. In this study, we investigated morphological and molecular methods for identifying *Laelaps jettmari* mites, as well as carrying out phylogenetic analysis of these mites using nuclear rRNA 28S region (domains 1-3). Mites were first identified morphologically under the microscope. Afterwards, DNA was extracted using 2.5 % $NH_4OH$ solution and the rRNA 28S region (domains 1-3) was amplified by conventional PCR using the primers 43F and 929R. Then the PCR products were analysed by horizontal electrophoresis on 1.5% agarose gel and the positive samples were sent for sequencing to the sequencing centre in Macrogen, Amsterdam (Netherlands). After getting the sequence of *L. jettmari*, it was uploaded to MEGA X software for analysing it using evolutionary principles. The results of the study suggest that there is no difference between *L. jettmari* sequences collected from various rodent hosts in different geographical locations. The *L. jettmari* sequence obtained in this study and the sequences of *Laelaps agilis* collected from GenBank presented the shortest interspecific genetic distances of 28S rRNA region (domains 1-3), showing the slight evolutionary distance between these species. This paper gives novel molecular information for the identification and phylogenetic analysis of *L.jettmari* mites based on 28S rRNA region.

# INTRODUCTION

Laelapidae is a diverse and numerous family, including over 90 genera and 1000 species (Berlese, 1892; Beaulieu et al., 2011). Mites from the family Laelapidae are spread all around the world and are normally found on the nests or bodies of small rodents, being the most frequent ectoparasites infesting rodents (Mašán and Fenďa, 2010; Hawlena et al., 2013; Guo et al., 2013).

Many taxonomic classifications for the superfamily Dermanyssoidea have been proposed, and they are sometimes conflicting since they are based primarily on host relationships, ecology, and morphological characteristics (Vitzthum, 1943; Zumpt and Patterson, 1951; Radovsky, 1985; Lindquist et al., 2009; Dowling and OConnor, 2010a). These inconsistencies are caused by the morphological variety of the members of the superfamily and their extensive ecological amplitude. Due to these and other constraints, a precise molecular tool for phylogenetic analysis would be immensely beneficial. The discovery of phylogenetic relations of a number of mite species using DNA sequence analysis has been made possible by scientific advances in molecular biology techniques.

Small rodents serve as carriers and/or reservoirs for bacteria, viruses, protozoa, and even helminths that may infect humans and domestic animals. Mites are vectors for these germs, and as a result, they play an essential part in the transmission of the diseases these pathogens cause, as well as causing dermatitis and other skin issues. That is the reason why mites' study is gaining great medical importance, being their morphological and molecular identification the basis for further analyses (Moro et al., 2005).

**Aim of the work** was to morphologically and molecularly identify *Laelaps jettmari* mites infesting small rodents in Lithuania.

**Task**:

1. To morphologically identify *Laelaps jettmari* mites collected from small rodents in Lithuania.
2. To identify and confirm *Laelaps jettmari* mite species using molecular methods based on rRNA 28S region (domains 1-3) amplification.
3. To characterize obtained sequences and make a phylogenetic analysis of *Laelaps jettmari* mites.

# 1. LITERATURE REVIEW

## 1.1. *Laelaps jettmari* (Mesostigmata: Laelapidae) mites taxonomy and biology

Mesostigmata is the most geographically spread, as well as the most diversified order of Parasitiformes, with 109 families and over 11,000 species (Zhang, 2011). Mesostigmata order is also referred as Gamasida.

One of the tasks of this research is the description and characterization of the mite species *Lalelaps jettmari*. In terms of its lineage, the different taxonomic categories in which it is included are as follows:

- Superorder Parasitiformes
- Order Mesostigmata
- Suborder Monogynaspida
- Cohort Gamasina
- Subcohort Dermanyssiae
- Superfamily Dermanyssoidea
- Family Laelapidae

The subfamilies comprised in the family Laelapidae are the followings (showing in brackets the genera comprised in each): Haemogamasinae (*Eulaelaps* and *Haemogamasus*), Hirstionyssinae (*Hirstionyssus*), Laelapinae (*Androlaelaps*, *Hyperlaelaps*, *Laelaps* and *Ondatralaelaps*) and Myonyssinae (*Myonyssus*) (Mašán and Fend′a, 2010).

Laelapidae (laelapid mites) is a diverse and numerous family. 90 genera and 1316 species have been identified to belong to Laelapidae family (Berlese, 1892; Beaulieu et al., 2011). The species that are part of it demonstrate a wide range of strategies and associations.

As described above, *L. jettmari* is included in the superfamily Dermanyssoidea (Dermanyssoid mites), which comprises free-living predators as well as facultative and obligatory ectoparasites of vertebrates and insects and their nests (mammals, birds, and several arthropod species most commonly) or as endoparasites in marsupials (although this associations have not been studied deeply) (Radovsky, 1985; Gettinger et al., 2005; Dowling and Oconnor, 2010a, b; Lindquist et al., 2009b).

Ectoparasite species may be linked to host habitat. Based on that, after habitat disruption the composition of rodents will most likely vary, which might lead to ectoparasites choosing alternative hosts and hence transferring to them (Poláčiková, 2013).

## 1.2. Morphology of gamasid mites

Gamasid mites range in size from 0.2 to 0.5 millimeters to 1.5 to 2.5 millimeters, but seldom reach 5 millimeters. The color of the chitin coating is normally yellow or brown, however it might be orange or pink on rare instances. Parasites that have sucked blood will appear redish at first, then brownish as time passes (once the blood has been digested by the mite) (Bregetova, 1956).

Gamasids' bodies are characterized by layers of chitin integument, with the outermost layer serving as a protective barrier and a means of securing the muscles. The initial segments of these mites' legs are normally cylindrical and movable. A pair of stigmas or spiracles are seen commonly one on each side of the ventral face. Also, it must been remarked that gamasid mites do not have eyes or abdominal suckers (Bregetova, 1956).

The family Laelapidae contains 92 recognised genera and over 1300 documented species, making it the most diversified family of Mesostigmata in terms of morphology and ecology (Beaulieu et al. 2011; Kazemi et al. 2014; Nemati and Gwiazdowicz 2016; Kazemi and Beaulieu, 2016).

Members of the family Laelapidae have slender and more elongate stylet form chelicerae that penetrate the skin of the vertebrates and insects they parasitize, allowing them to feed on their blood quickly and efficiently. This also gives them the ability to replenish their blood reserves in order to have food to withstand periods of shortage (Miťková et al., 2015). In Figure 1.1 we appreciate a drawing of chelicerae of *Laelaps jettmari*.

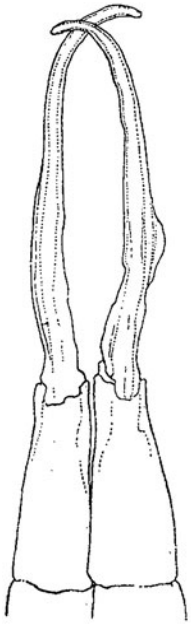

**Figure 1.1.** Chelicerae *from male Laelaps jettmari (Zakhvatkin, 1948)*

## 1.2. Development cycle of gamasid mites

The development cycle of gamasid mites includes the following phases: egg, larva, nymph I (protonymph), nymph II (deutonymph) and adult.

**Eggs** of parasitic mites are relatively big (0.1 to 0.35 millimeters), occupying the majority of the female's body when completely developed. They have a spherical or oval form, a very thin shell, with a color that ranges from white to yellow (Bregetova, 1956).

The **larvae** of gamasid mites have three pairs of legs, unlike adults and nymphs, and a small number of setae. In addition, larvae have neither spiracles nor peritrema (structures used for respiration), so they breathe through the skin (Bregetova, 1956).

The fundamental differences between parasitic and free-living larvae are related to body shields and mouthparts. In parasitic forms, body shields are usually absent, and mouthparts are underdeveloped and soft. Whereas in free living forms, body shields are present (cephalothoracic and

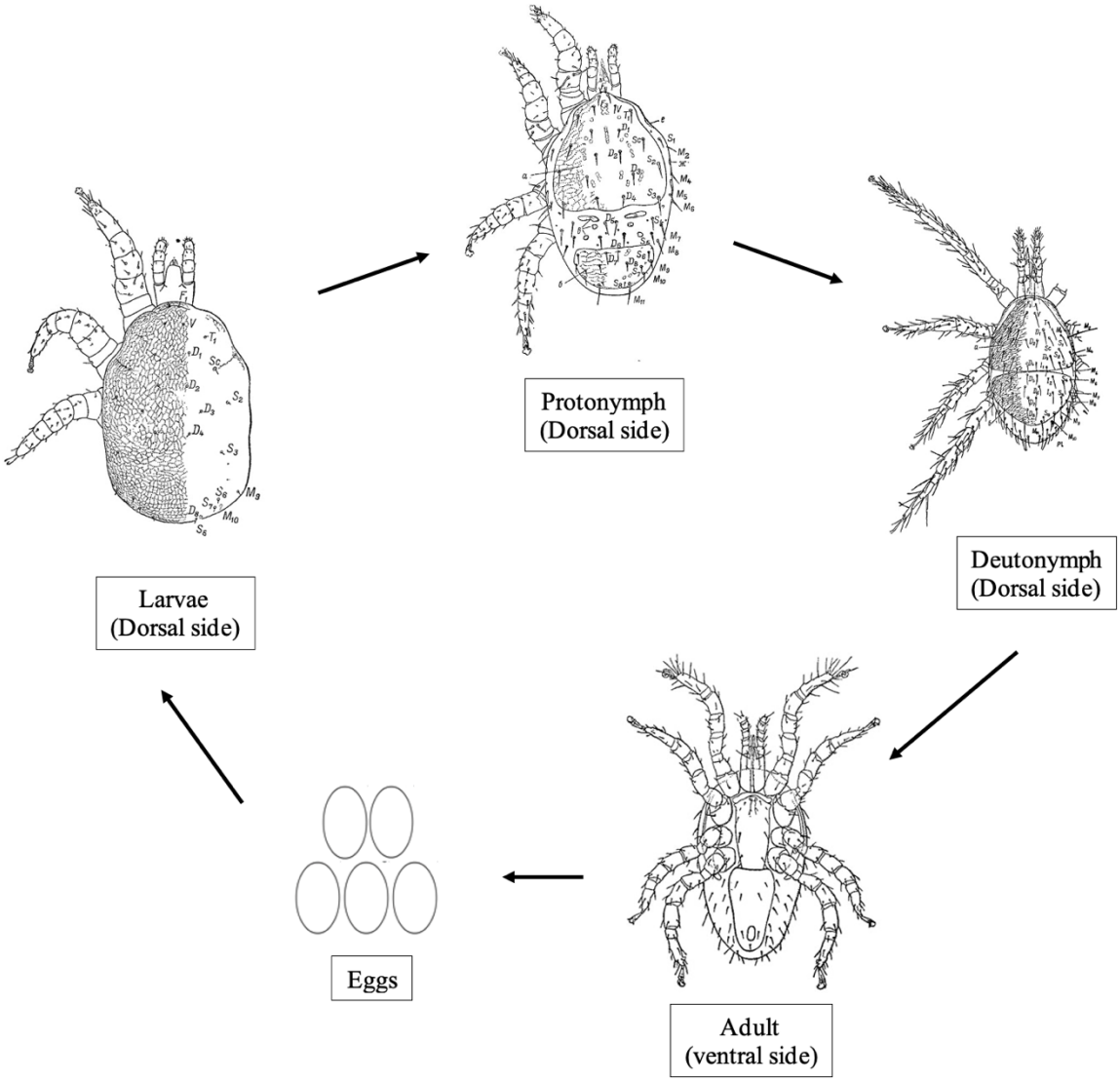


**Figure 1.2.** Development cycle of Gamasid mites (Bregetova, 1956)

pygidial on dorsal side, and thoracic and anal on abdominal side) and mouthparts are highly developed (Bregetova, 1956).

**Protonymphs** have four pairs of legs, one more than larvae, and more setae (body hairs) than larvae. On the ventral side of the body peritrema is visible (Bregetova, 1956).

The integument of the body may vary, in some species it is soft and slightly colored, while in others it may be sclerotized with developed shields. Generally, in protonymphs sclerotization of the body and oral parts is seen (Bregetova, 1956).

**Deutonymph** stage is characterized by the appearance of thicker and denser body shields, as well as a yellowish or brownish coloration. The number of setae in deutonymphs is greater than in protonymphs (Bregetova, 1956).

Furthermore, deutonymphs have a large peritrema, similar in size to adults, but differing in that deutonymphs have no peritremal shield. Another distinction between the deutonymph and the adult stage is that the deutonymph lacks an external reproductive apparatus (Bregetova, 1956).

However, in those mites in which the deutonymph phase cannot suck blood, the peritrema remains short and underdeveloped, and they lack body shields. (Bregetova, 1956).

Only females in the deutonymph and adult phases of the mite being studied in this thesis, *Laelaps jettmari*, can suck blood. For this reason, deutonymphs of *L.jettmari* would have a large peritrema and body shields (Bregetova, 1956).

The exterior anatomy of the mite helps in the sex determination at this stage, as females are often bigger than males (Bregetova, 1956).

**Adult**. The shields mites have can be used to determine their sex in adult stages. Female mites have one or more ventral shields between the sternal and anal shield, while males have one common shield that contains all ventral shields or is separated into two ventral shields. Furthermore, males have a genital entrance on the anterior part of the ventral shield (Bregetova, 1956).

Adult forms of Gamasid mites can be oviparous (if they are free-living mites or transitory parasites) or viviparous (if permanent parasites) (Bregetova, 1956).

In Gamasid mites matures a single egg at a time. The period between the laying of each egg is 3 - 8 hours, and they are placed individually or in small groups and attached to the substrate using cement grease, which hardens in air (Bregetova, 1956).

Eggs can be laid in the host and its nest, as well as in other hidden places (like behind rocks or under fallen leaves) (Bregetova, 1956).

## 1.3. Reproduction of Gamasid mites

During reproduction, the Gamasid male attaches the sperm sac, also known as spermatophore, to the genital opening of the female (Bregetova, 1956).

Fertilization is normally only possible once the female emerges from the deutonymph stage. At this point, the male clings to her and stays with her until she transforms into adult, at which point copulation occurs. Repeated copulations of one male with one female were reported (Bregetova, 1956).

There is a relationship between blood digestion and egg maturation, egg maturation and laying occur when a female drinks blood. Oviposition begins when there is still undigested blood in the female stomach and finishes before it is entirely digested, when only trace amounts remain. If the female does not receive enough blood, the eggs develop but are deposited in less amounts. Females frequently go through this cycle numerous times (Bregetova, 1956).

Gamasid mites have limited fecundity, which is offset by their rapid development at all stages. They spread quickly if there is plenty of food and right combination of other conditions (optimal temperature and humidity) (Bregetova, 1956).

Blood sucking is not only a feeding mechanism for Gamasid mites, but it is also a carrier of various illnesses (Bregetova, 1956). Gamasid mites' role in the spread of a variety of diseases prompted researchers to explore their medical significance.

It is hard to determine the total number of gamasid mite species due to the limited research in this field. They have been found in numerous broad locations, such as almost all southern Asia, different parts of South America, and even a few European nations. Despite this, little is known about the gamasid mites' fauna (Bregetova, 1956).

This variety is reflected by a wide range of life strategies correlated to morphological adaptations. Regarding to haplodiploidy, both arrhenotoky and pseudoarrhenotoky appear in species in the Laelapidae family (Di Palma, 2022).

Haplodiploidy is a phenomenon in which males are haploid, whereas females are diploid. The causes for this are varied, principally arrhenotoky and pseudoarrhenotoky. (Di Palma, 2022).

Arrhenotoky, a process in which men develop from unfertilized eggs and females develop from fertilized eggs, is the most common cause. Another reason is pseudoarrhenotoky, which happens when males emerge from fertilized eggs, but the paternal genome is lost prior to spermatogenesis, resulting in their progeny not inheriting this genome (Cruickshank and Thomas, 1999).

Phylogenetic analyses indicate that arrhenotoky derives from pseudoarrhenotoky (De Jong et al., 1981; Cruickshank and Thomas, 1999).

The change from pseudoarrhenotoky to arrhenotoky allow females to reproduce without mating. This process is known as facultative asexuality. Females produce unfertilized eggs from which males arise that can mate with the female mother. This will lead to the mother producing fertilized eggs from which more females will arise, that can mate with the males. In this case, a new colony can arise only starting with one female (Di Palma, 2022).

## 1.4. Habitats of gamasid mites

Gamasid mites are most commonly found on land environments. A few species, however, dwell in watery locations. As these mites cannot survive in dry environments, humidity is an important component in their survival. They usually hide themselves to avoid being exposed to the sun's rays. These mites are often quite mobile and move fast from one place to another (Bregetova, 1956).

Types of gamasid mites:

- Free living. They live in a wide range of environments: on fungus, rotting wood, fallen leaves, behind stones or wet meadows. Normally this type of gamasid mites are predators.
- Parasitic. Gamasid mites of this kind live on the host's body (ectoparasites) or in the host's internal organs (endoparasites), feeding themselves at the expense of the host. They have been found in rodents, bats, seals, sea lions, monkeys, insects and spiders (Bregetova, 1956).

As for ectoparasites, they can be permanent or temporary, although the last ones tend to predominate.

Temporary ectoparasites suck blood from the host and can stay on the host for several hours to days. After feeding on the blood of the host, they develop outside of the host's body, generally in a hole or nest. That means that oviposition and hatching of larvae and nymphs occur outside the host's body (Orlova et al., 2012).

The mites of the family Laelapidae are permanent ectoparasites. Hosts of this family are principally rodents, but they can also parasite beetles, ants, spiders, snakes or insectivores. Many Laelapid mites have a broad host range (they can feed on a variety of animals), whereas others, like those in the genus *Laelaps*, can only feed on one or a few closely related species. Some mites in this family can bite humans transferring various diseases (acting as vectors) or, can cause dermatitis and other skin problems (Bregetova, 1956).

## 1.5. Hosts of *Laelaps jettmari* mites

A parasite's host specificity is an essential characteristic that reflects its ability to exploit multiple host species.

Conventionally, a parasite's host specificity has been defined as the number of host species in or on which the parasite has been found (Poulin et al., 2011). This presupposes that parasite utilize all hosts similarly (both locally and beyond its geographic range), and that the hosts are equivalent from the parasite's point of view. However, parasites preferentially target some hosts over others and the pattern of attacking various hosts may vary throughout space (Van der Mescht et al., 2017).

To round out this concept, host specificity may be defined ecologically as the diversity of host species used by a parasite and its variation over its geographic range (Futuyma and Moreno, 1988).

Besides, host specificity is characterized from an evolutionary standpoint by not only the number of host species utilized but also their relatedness (Poulin and Mouillot, 2003).

However, the relationships between these different approaches to explaining host specificity remain largely unstudied, since they have seldom been quantified concurrently in the same host-parasite interaction and in the same location. Understanding these connections, on the other hand, may can be of great importance in achieving a better understanding of host-parasite ecology and evolution (Van der Mescht et al., 2017).

Laelapidae mites are distributed all over the world and are frequently found on the nests or bodies of small rodents, making them the most prevalent ectoparasites infesting rodents. (Mašán and Fenďa, 2014; Hawlena et al., 2013; Guo et al., 2013).

Small rodents belong to the class Mammalia and to the order Rodentia. Rodentia is the most numerous and diverse order of Mammalia, accounting for over 40% of all mammal species, being an order organized into 28 families with over 2,050 species (Huchon et al. 2002; Poláčiková, 2013).

Small rodents are widely spread in different types of habitats (Fleming, 1975) and live closely to humans and animals (both domestic and wild) (Miťková et al., 2015). Wild rodents as well as other small animals and ectoparasites are a major source of transmission of various zoonotic diseases (Huang et al. 2010). These make ectoparasites located on the surface of small mammals (principally rodents) have medical interest as they can act as zoonotic vectors transmitting certain diseases (Huang et al., 2010).

Among the infections transmitted by the superfamily Dermanyssoidea are bacterial infections (such as *Rickettsia*, *Bartonella* or *Salmonella*) and viral infections (such as equine encephalitis virus or West Nile virus). It has also been reported that mites are a source of transmission of some protozoa and filaria (Moro et al. 2005, Pratt 1976).

Nevertheless, the rate of mite's infestation in rodents depends on the species of rodent we are studying and the habitat it is located. In addition, not all rodent species are equally infested by certain types of mites (Paulauskas et al., 2009; Van der Mescht et al., 2017).

Some members of the Laelapidae family, such as the mites *Haemogamasus*, show low host specificity, allowing them to choose from a diverse variety of hosts. Others, such as *Laelaps* and *Hirstionyssus*, exhibit higher host specificity, choosing among a smaller range of hosts (Mašán and Fend′a, 2010).

## 1.6. Geographical distribution of Dermanyssoid mites from small mammals

There have not been many previous studies on Dermanyssoid mites infesting small mammals. Some of those that have been conducted are as follows: China (Guo et al., 2013), (Guo, 1998), (Liu et al., 2020); Czech Republic (Netušil, 2005); Russia (Korneev, 2003); Siberia (Mal'kova, 2010); Asiatic Russia (Vinarski, 2017); Slovakia (Mašán and Halliday, 2010), (Mašán and Fend′a, 2010, 2014); Bulgaria (Davidova et Vasilev, 2011); Norway (Edler et Mehl, 1972); Korea (Khaing et al., 2014); Austria (Mahnert, 1971); Latvia (Grinbergs, 1959), Turkey (Çicek et al., 2008); Hungary (Haitlinger, 1979), Spain (Haitlinger, 2004), Malaysia (Mohd-Taib et al., 2021) and Brazil (Gettinger et al., 2005).

Regarding to the situation in Lithuania, Podenaite (1979) reported studies in conference in which species of Dermanyssoid mites were described. Furthermore, Paulauskas et al., (2009) found *Laelaps* mites on rodents (but they were not identified until the species level).

Except articles Kaminskienė et al. (2017, 2020) and Radzijevskaja et al. (2018), no additional information about mites in small rodents has been released in Lithuania.

## 1.7. Gamasid mite's infestation of pathogens

Waage (1979) described two routes of parasitism evolution: A and F. In route A, parasite-host associations occurred prior to morphological adaptation of these parasites to feed at the expense of hosts. In contrast, F pathways are based on morphological adaptations in parasites to feed at the expense of hosts prior to actual parasite-host associations. In the case of the superfamily under study, Dermanyssoidea, the evolution of parasitism may have occurred by a combination of both routes, A and F. Furthermore, members of the order Mesostigmata are characterized by their high parasitic pre-adaptation. Even in more primitive forms, both in free-living members and in facultative or obligate parasites, cheliceraes are very well adapted to feed at the expense of the host they parasitize, mainly on secretions, scales, scabs or blood (Dowling and Oconnor, 2010a).

One of the diseases transmitted by Gamasid mites is Lymphocytic choriomeningitis. The reservoir of the virus causing this infection are mostly rodents, and the mites of the Laelapidae family have been linked to its transmission (Bregetova, 1956).

*Laelaps jettmari* is an ectoparasite that most likely acts as a vector for the Epidemic Haemorrhagic Fever virus, capable of infecting people, horses, and even monkeys. Its reservoir are mostly rodents and it has been observed in north-east Korea and China (Bregetova, 1956).

Western equine encephalomyelitis is another illness spread by Gamasid mites that can affect both people and horses. This disease has been mostly found in North America. The virus spreads naturally among birds and blood-sucking arthropods, such as Gamasid mites, resulting in the infection of people and horses accidently (Bregetova, 1956; Moro et al., 2005; Durden et al., 1993).

## 1.8. Laelapidae mite's genetic characterization

Many taxonomic classifications have been suggested under the superfamily Dermanyssoidea, and they are usually contradictory since they are based mostly on host associations, ecology and morphological characteristics (Vitzthum, 1943; Zumpt and Patterson, 1951; Radovsky, 1985; Lindquist et al., 2009; Dowling and OConnor, 2010a).

These inconsistencies are caused by the considerable morphological variety and extensive ecological amplitude of the members of the superfamily. Furthermore, the small size of mites, the fact that immature stages present indistinguishable traits for species identification (thus they must be allowed to grow until adulthood to be reliably recognized), the large number of species and the similar morphologic characteristics among different species make morphological identification difficult. As a result, a clear phylogenetic analysis based only on morphological characteristics has proven to not be effective (Kaminskienė, 2021)

Because of these limitations, a precise molecular tool for phylogenetic analysis would be extremely useful. As DNA sequences from numerous mites are now available in worldwide databases, molecular-based species identification has become easier.

Scientific developments in molecular biology techniques have permitted the identification of phylogenetic relationships of a variety of mite species using DNA sequence analysis. The mitochondrial cytochrome c oxidase subunit I (COI) region is extremely conserved and has been exploited for this purpose. DNA barcoding with the COI gene can be used to classify mite species at molecular level, since the COI sequence has been shown to be a valid molecular marker for the identification and phylogenetic analysis of mites (Ernieenor et al., 2018).

The nuclear rRNA 28S region (domains 1-3) is used to investigate phylogenetic relationships among Dermanyssoidea as well as the evolution of parasitism, as in Dowling and OConnor (2010a). In that study a novel set of overlapping primers was synthesized to boost primer specificity, based on

primer sequences used to target 28S gene from Park and Ó Foighil (2000). In Figure 1.3, one of the hypothesis for Dermanyssoidea mites' phylogenetic relationships is shown (Dowling and OConnor, 2010a). In the figure, thick black lines represent probability and bootstrap values greater than 85%, while the rest lines represent probability ranging from 70 to 84 percent. On the tree, all branches below 70% were collapsed.

Though one single PCR technique takes longer than morphological identification of an individual mite by microscopy, it has several advantages. The main one is the capacity to execute many tests at the same time, allowing for the identification of a large number of mites in a single procedure (Ernieenor et al., 2017; Zhao et al., 2020)

There are still significant challenges, despite the adoption of cutting-edge molecular biology methods, for mite classification and identification, (Zhao et al., 2020).

The most significant one is the lack of mite sequences in genomics databases. More than 50,000 mite species have been described, but only around 100 of these have their sequence uploaded on GenBank. As a result, most species cannot be identified using molecular approaches since there is no access to the gene sequence template for synthesizing the primers (Zhao et al., 2020).

Also, there is limited number of research in this topic, as well as a lack of clarity on the efficacy of primers for species identification, because target sequences in different species are located at different locations in the same gene (Zhao et al., 2020).

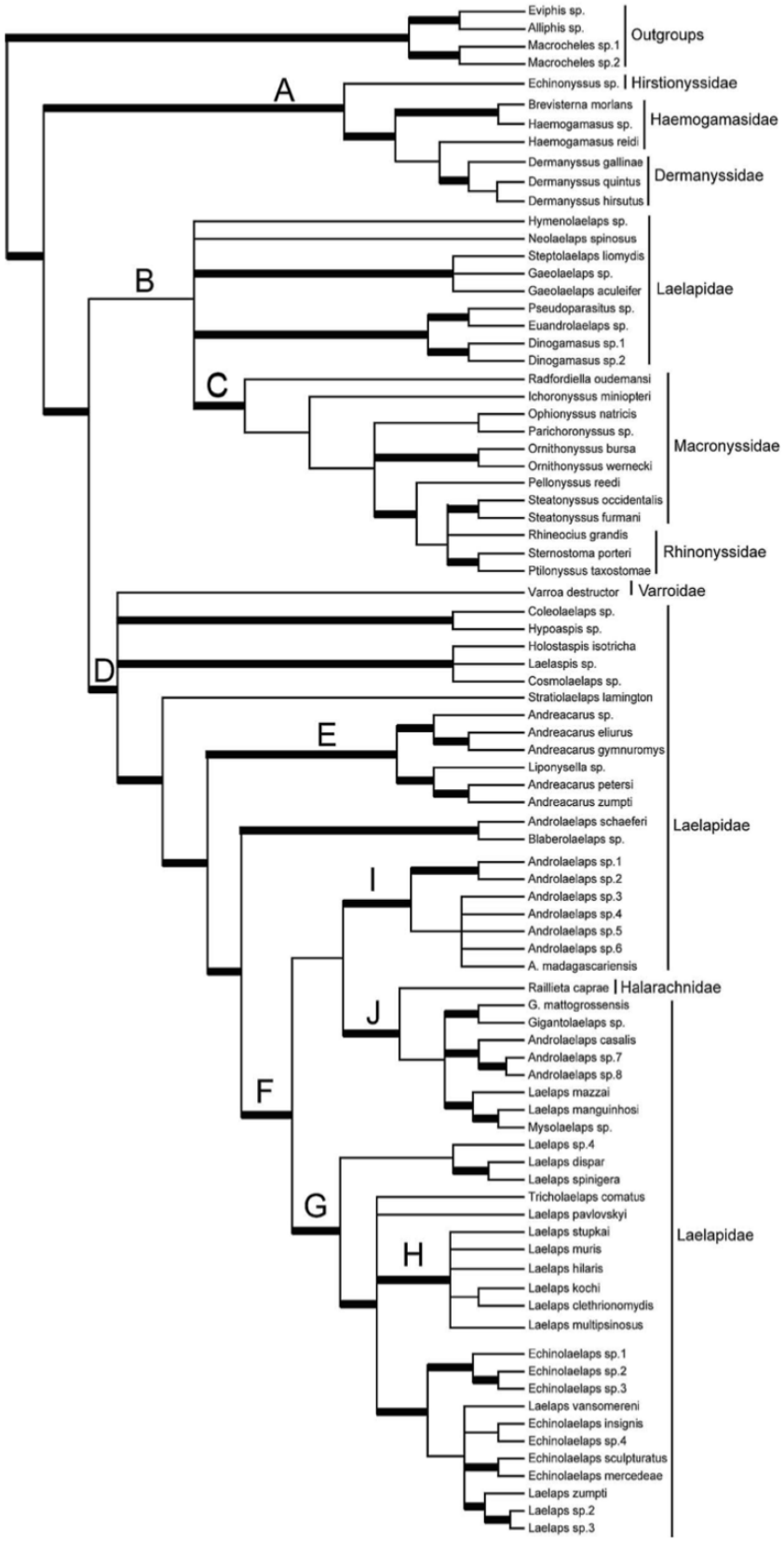


**Figure 1.3.** One hypothesis of phylogenetic relationships of Dermanyssoid mites (Dowling and OConnor, 2010a).

# 2. MATERIAL AND METHODS

## 2.2. Rodents trapping and sampling

Small rodents were captured from Rusnė (Lithuania) on 10th October 2018.

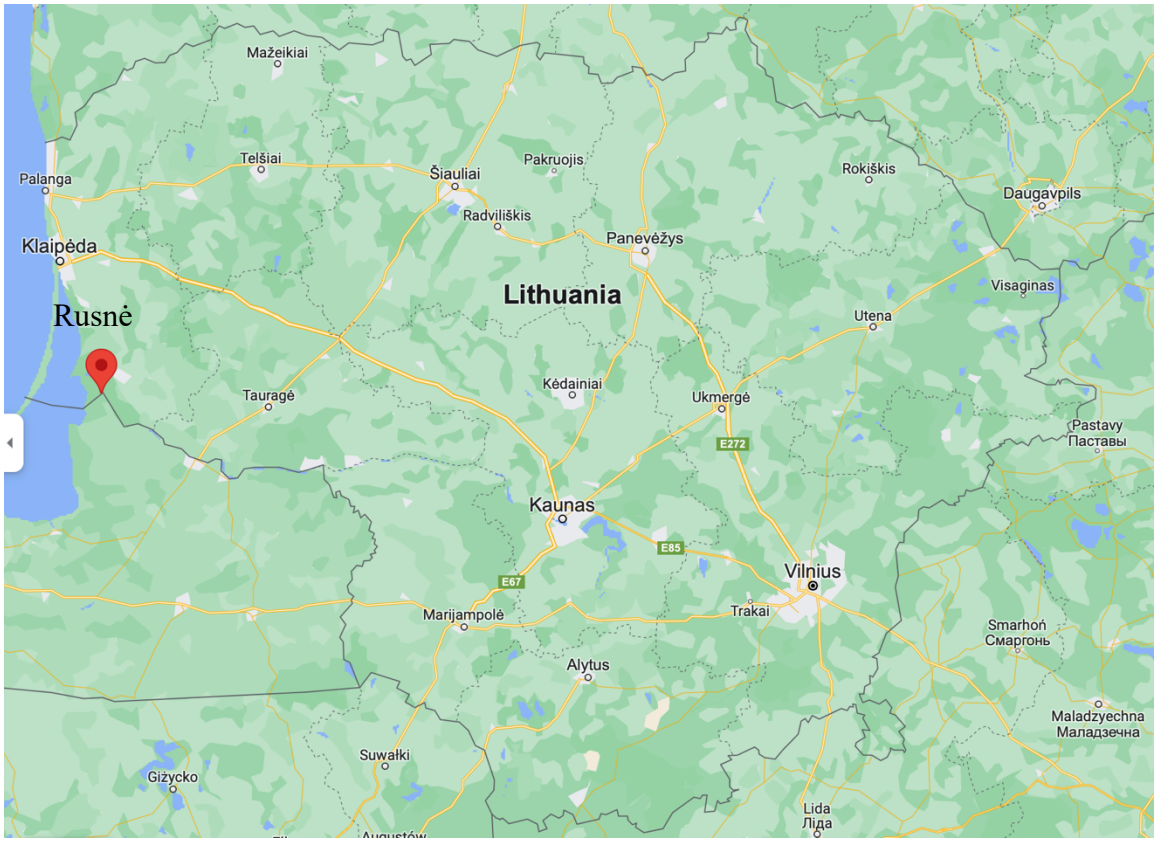


**Figure 2.1.** Lithuanian map showing the location of Rusnė (Picture taken from Google maps, https://www.google.com/maps/place/Lituania/@55.4487394,22.9602294,8.16z/data=!4m5!3m4!1s0x46dd94140f33be13:0xf30a54d3a55dbab9!8m2!3d55.169438!4d23.881275).

The red figure points at where Rusnė is located in the map of Lithuania.

## 2.3. Collection of Gamasid mites from rodents

Small rodents were captured with live traps using black bread dipped in sunflower oil to attract the rodents. Traps were placed for 3 days and they were usually put in the night-time and examined every morning. However, it is advised that traps are checked every 2-3 hours after they are placed, so mites have not had enough time to leave the host.

If rodents were alive after being removed from traps, they were killed by decapitation (neck vertebrae fractured) before being placed in plastic bags. Captured rodents were identified to the genus and species level in the laboratory.

When manipulating the rodents, care must be taken to ensure that mites do not transmit from one host to another. Each rodent should be placed in a separate plastic bag that is clearly labelled.

It is preferable to wait a few hours before collecting mites from rodents, since when the corpse cools after death, mites leave the host's body and become visible in the bag. Although information about where the mite was in the host is lost, because Gamasid mites frequently move location in the host, this information is not really important for this study. The majority of Gamasid mites were located in the rodent's head, with only a few detected on the paws and tails.

Then mites were collected, and the rodent was further examined for mites that stayed in the host skin. The mites were then removed with soft tweezers and placed in tubes containing 70% ethanol, which were properly labelled with the site where they proceed and the date of collection and stored at 4°C until further studies.

Laboratory personnel must be careful as these mites act as vectors for the transmission of certain diseases. Therefore, during the extraction of mites from their hosts, they might bite laboratory employees and spread infections to them.

As a result, the following precautions should be taken:

• All animals and nests transported to the laboratory should be in adequately closed bags, boxes, or jars.

• Handling of these animals and nests should be done with gloves, forceps and tweezers, being cautious that mites are not spread in the laboratory.

• Once the mites have been removed, the animal's lifeless corpse should be placed in a deep pit.

## 2.4. Morphological identification of mites

Identification of mites consists of determining to which genus and species they belong. For this purpose, identification keys are used (Bregetova, 1956; Baker, 1999; Mašán and Fend'a, 2010; Kaminskienė et al., 2017).

Gamasid mites can be morphologically identified using monocular or binocular microscope, microscope with a high magnification or even with magnifying glasses.

### 2.3.1. Morphological characteristics of *Laelaps jettmari* (VITZTHUM 1930)

To identify mites, both the dorsal and ventral surfaces must be examined. Because of this, it is more convenient to use preparations in which some mites are put on the ventral side and others on the dorsal side.

The body of Gamasid mites is divided into the body itself (called the idiosome), which contains 4 pairs of legs in the adult stage, and the oral organ complex (called the gnathosome). The gnathosome is a structure with the ability to move, attached to the anterior end of the body on the ventral side (Baker, 1999).

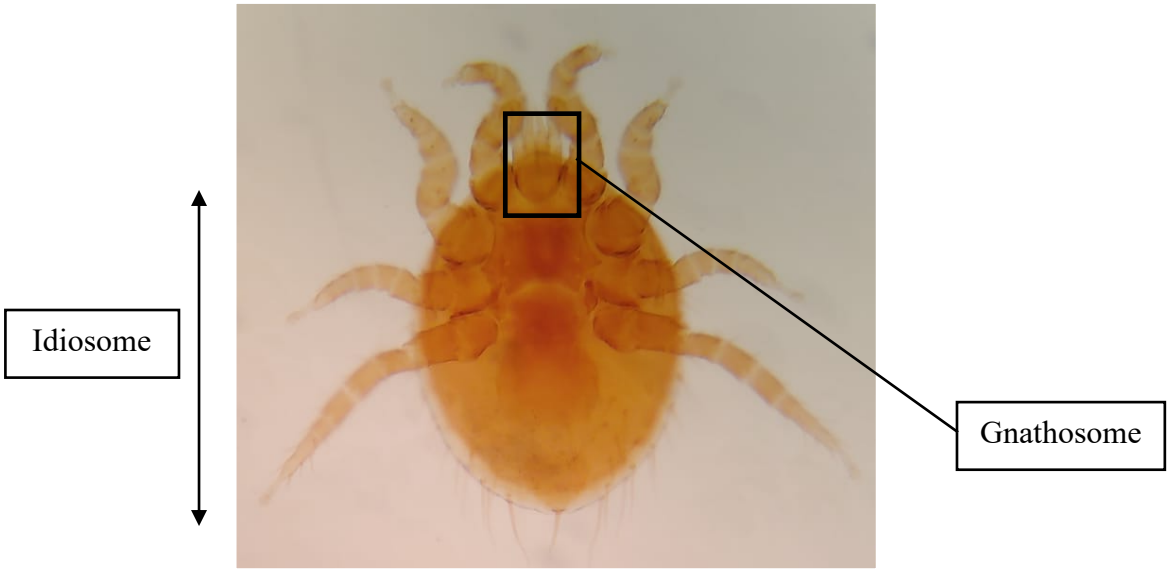


**Figure 2.2.** Ventral view of *Laelaps jettmari* female (photo by author).

The main difference among female and male of *Laelaps jettmari* is that the female has separated shields (sternal, genitoventral and anal shields) on ventral view, while the male has all shields combined together in one single shield, called genitoventral shield.

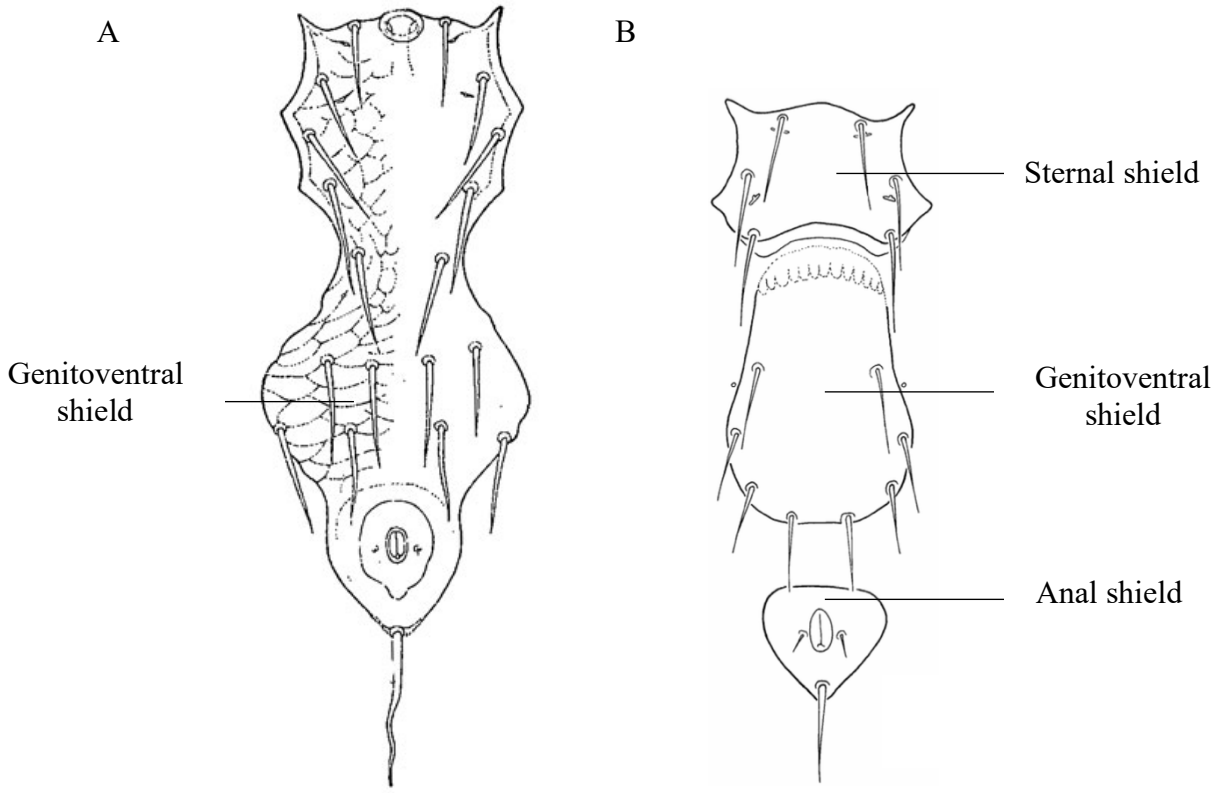


**Figure 2.3.** Shields of *Laelaps jettmari* seen from ventral view: A – male (from Lange, 1955); B - female (Mašán and Fend'a, 2010).

**Female *Laelaps jettmari***

The sternal shield is longer in the center, nearly square-like. The anterior setae (st1) of the sternal shield do not reach the shield's posterior edge. All sternal setae are long and thin, with tips that overlap the insertion of setae st2. The shield's posterior edge is concave.

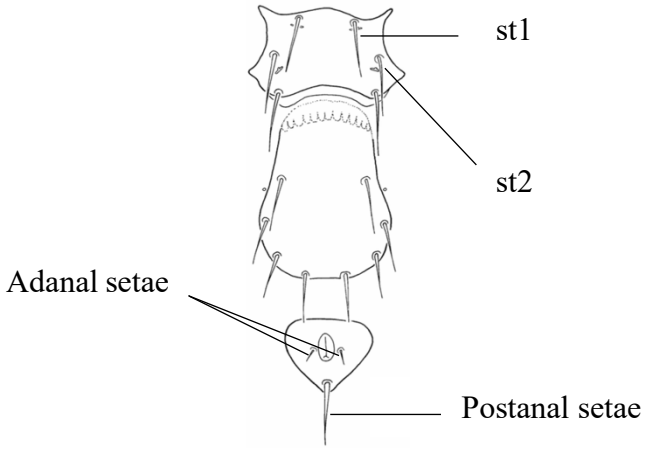


**Figure 2.4**. Setae 1 and 2 of sternal shield, adanal setae and postanal setae of *Laelaps jettmari* female (Mašán and Fend'a, 2010).

The genitoventral shield is usually large and has a straight or heavily rounded posterior border. This shield has no constriction in the center. The genitoventral shield is relatively wide and has a pair of pores close to the genital setae (Mašán and Fend′a, 2010).

The anal shield is subtriangular in shape. The adanal setae are closer to the anus and are shorter, or at least as long as the anal opening. The postanal seta is much longer than the adanal setae (Mašán and Fend′a, 2010).

**Male *Laelaps jettmari***

On tarsus IV, the *L. jettmari* male has three thickened spur-like setae (Bregetova, 1956).

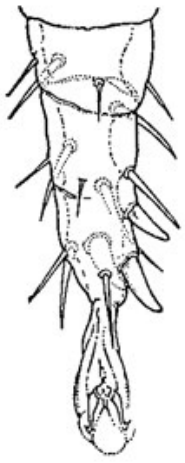

**Figure 2.5.** Tarsus IV of *L. jettmari* male (*Bregetova, 1956*).

## 2.5. Molecular methods

Molecular methods have been used for identification and phylogenetic analysis of *L.jettmari* mites.

### 2.5.1. DNA extraction from mites

Mite's DNA is extracted using a 2.5 % $NH_4OH$ solution, being the mites processed individually.

Laelapid mites are removed from the ethanol solution with soft tweezers and dried for 3 to 5 minutes on a paper cloth at room temperature. After, they are separately transferred to 0.5mL Eppendorf tubes. The tubes are then filled with 40 µL of 2.5 % $NH_4OH$ solution (or 70-100 µL on pooled mites). The mites are smashed in the tube with a sterile plastic pestle and kept at room temperature for 30 minutes. Next, they are incubated at 100ºC for 30 minutes, which is a critical step to ensure maximum DNA recovery.

Following that, the tubes are centrifuged at 13.000 rpm for 1 minute to collect the condensate from the cap and the walls of the tube. The tubes are then opened and returned to the heating block for a 20-minute incubation at 100ºC. This procedure is performed to volatilize the leftover ammonia solution. After, the tubes are sealed and put on an ice cube for 2 or 3 minutes. Following that, the tubes are centrifuged at 13.000rp for 30 seconds, and the extracted mite's DNA is kept at -20ºC for future use.

### 2.5.2. PCR amplification based on *28S rRNA*

One of the tasks of this research is to carry out the molecular identification of mites based on domains 1-3 of the 28S nuclear ribosomal RNA (rRNA) gene. For this purpose, conventional PCR is used to amplify a region of 856 bp using the primers 43F and 929R described in the table below (Dowling and OConnor, 2010a).

**Table 2.1.** Primers used for molecular identification of Laelapid mites.

| Gene | Primers | Primer's sequence (5'→3') | Length of the fragment amplified | Reference |
|---|---|---|---|---|
| *28S rRNA* | 43F | GCTGCGAGTGAACTGGAATCAAGCCT | 856 bp | Dowling and OConnor, 2010a |
| | 929R | AGGTCACCATCTTTCGGGTC | | |

The reagents as well as the amounts of reagents used and the temperature cycles to which the samples are subjected in PCR are listed in the following tables. Amplification was done using primers of 28S rRNA and protocols by Dowling and OConnor (2010a), though annealing temperature was modified for this research.

**Table 2.2**. Reagents and quantities used per sample for PCR analysis

| Reagent | Quantity per sample (µL) |
|---|---|
| dd $H_2O$ | 15.5 |
| 5 x MyTaq Reaction Buffer | 5 |
| 43 F primer (10pm / µL) | 1 |
| 929 R primer (10pm / µL) | 1 |
| MyTaq DNA polymerase (54/ µL) | 0.5 |
| extracted DNA | 2 |
| **Total volume per reaction** | **25** |

MyTaq Reaction Buffer and MyTaq DNA polymerase were both acquired from Thermo Fisher Scientific, Lithuania.

**Table 2.3**. PCR protocol used for amplification of domains 1-3 of *28S rRNA* from mites (protocol from Dowling and OConnor, 2010a).

| Cycle | Temperature | Time | Number of repetitions | Description |
|---|---|---|---|---|
| C1 | 94 ºC | 2 min | 1 X | Initial Denaturation |
| C2 | 94 ºC | 25 s | 40 X | Denaturation |
| | 57 ºC | 1 min | | Annealing |
| | 72 ºC | 1 min | | Extension |
| C3 | 72 ºC | 7 min | 1 X | Final extension |
| C4 | 4 ºC | hold | 1 X | |

### 2.5.3. Electrophoresis and PCR products visualization

To evaluate PCR products, they are subjected to horizontal electrophoresis on 1.5% agarose gel (Thermo Fisher scientific, Vilnius, Lithuania) and analysed in ultra-violet UVP GelDoc-IT 310 Imaging System (Ultra-Violet Products Ltd., Cambridge, The United Kingdom). To prepare the agarose gel, agarose and 0.5 X Tris-acetate-EDTA (TAE) buffer are used. For PCR product visualization Ethidium Bromide solution is added, which is at a stock concentration of 10 mg/ mL. GeneRulerTM 50bp DNA Ladder (Thermo Fisher Scientific, Vilnius, Lithuania) is used as a

molecular marker to determine the molecular weight and size of the double stranded DNA during gel electrophoresis.

To begin, the protocol is organized and we arrange which PCR product is added to which well of the gel. Afterwards, PCR products are taken out of the freezer to defrost. The electrophoresis instruments are then prepared. The electrophoresis apparatus is cleaned, and plastic scallops are inserted in the gel solution to generate the tracks. Next, the buffer for the electrophoresis is prepared. To prepare 250 ml of 1.5% of agarose gel, 3.75 g of agarose are weighted and then mixed in an Erlenmeyer with 250 mL of 0.5 X TRIS-acetate-EDTA (TAE) buffer. This buffer is stable at room temperature (+18ºC-+25ºC).

Then, the limit of liquid inside the Erlenmeyer is marked. After, the solution is heated in the microwave until the agarose is completely dissolved and boiled. Due to evaporation of the liquid during heating, distilled water is added to the specified limit. The agarose solution is then cooled to +55ºC, and 10 µL of Ethidium Bromide (at a stock concentration of 10 mg/mL) are added and mixed in.

The agarose solution is then poured into the electrophoresis prepared bath. After this, the bubbles formed are moved to the sides (avoiding the side in which the samples are going to be loaded). The scallops are then added, and the agarose is let to polymerizes for roughly 30 minutes. After the agarose solution has set, the scallops are removed from the gel, and 0.5X TAE buffer is poured into the electrophoresis equipment to equally cover the gel.

After that, 3 µL of 6X Loading dye are added to the PCR products, followed by centrifugation for about 20 seconds to let the dye settle in the tubes. The samples are loaded into the wells, loading 5 µL of 50bp GeneRuler Marker in the first and last well of each row. Then, 10 µL of Ethidium Bromide (from stock concentration of 10 mg/ mL) are added in both sides of the bath in the buffer.

Finally, the electrophoresis apparatus is covered, and the wires are connected to the current source. To know the voltage required to the start of the apparatus, the distance between the electrodes of the electrophoresis apparatus is multiplied by 5. Once voltage required is known, the electrophoresis is started and let run for 40 to 60 minutes.

### 2.5.4. DNA purification for sequencing

Thermo Fisher Scientific GeneJET Gel Extraction Kit is used for the DNA purification for sequencing. To begin, a clean scalpel or razor blade is used to cut the gel slice containing the DNA fragment. We try to cut as close to the DNA as possible to reduce the amount of gel taken. After the gel slide is placed in a pre-weighted 1.5 mL Eppendorf and weight recording the weight of the gel slide.

Following that, Binding buffer is added to the gel slide at a volume-weight ratio of 1:1 (for example, for every 100 mg of agarose gel slide, 100 µL of Binding buffer are added).

After that, incubation at 50-60 ºC for 10 minutes follows, until the gel slide is completely dissolved, with constant inversion mixes of the tube to facilitate the melting process. When it is completely dissolved, the tube is vortexed briefly before loading to the column. The mix's yellow colour indicates that the pH is optimal for DNA binding. If the colour is orange or violet, 10 µL of 3M sodium acetate are added, which turn the solution yellow.

The solubilized gel solution is then transferred to a GeneJET purification column and centrifuged for 1 minute. The flow-through is removed, and the collection tube is reinserted into the column.

The purification column is subsequently filled with 100 µL of Binding Buffer and centrifuged for 1 minute. The flow-through is then discarded, and the collection tube is reinserted into the column.

After that, 700 µL of Wash Buffer are added to the purification column, and it is centrifuged for 1 minute. Then the flow-through is discarded and the collection tube is placed back in the column. The purification column is centrifuged again for 1 minute to completely remove the residual wash buffer.

Afterwards, the column is transferred into a 1.5 mL Eppendorf and 28 µL of Elution Buffer are added, after which it is centrifugated for 1 minute. Next, the purification column is discarded and the Eppendorf containing the DNA is stored at -20ºC.

The purified PCR products are sent for DNA sequencing to the sequencing centre in Macrogen, Amsterdam (Netherlands).

## 2.6. Sequence analysis

A phylogenetic tree is a model that calculates the relationships between sequences and their most likely common ancestors (Kumar et al., 2012). The majority of phylogenetic trees are created using DNA or protein sequences.

The mite’s DNA sequences were uploaded to MEGA X software package (Molecular Evolutionary Genetics Analysis). This program comprises a variety of tools for statistic comparative analysis of molecular sequences using evolutionary principles (Kumar et al., 2012).

Then, homologous sequences were identified in GenBank using the Basic Local Alignment Search Tool (BLAST), both being part of the National Centre for Biotechnology Information (NCBI)

After this, the sequences were aligned using the multiple sequence alignment program ClustalW. Next, the phylogenetic trees were constructed using the Neighbor-Joining (NJ) and Maximum Likelihood (ML) methods using General Time Reversible model (Gamma Distributed

with Invariant Sites (G+I)), Kimura 2-parameter (Gamma Distributed) and Tamura-Nei models with bootstrap analysis of 1000 replicates.

Afterwards, genetic distances were estimated to determine the average evolutionary divergence across all sequencing pairings.

# 3. RESULTS

## 3.2. Morphological Identification

After morphological identification, a total of 22 *L. jettmari* were found, of which 22 were females and 0 males. All *L. jettmari* mites were taken from *Apodemus agrarius*. A total of 9.9% of *A. agrarius* analyzed carried *L. jettmari* mites.

**Figure 3.1**. Ventral shields of *Laelaps jettmari* female (photo by author).

The morphological identification of Gamasid mites is mainly done with females and only in some cases with males. That is because female parasites are the ones that suck blood for maturation of eggs and oviposition, and thus are found more frequently parasitizing rodents. Males do not normally suck blood, as their main function is copulate with females to have offspring.

## 3.3. Molecular identification and phylogenetic analysis

A total of 13 DNA samples of *L. jettmari* collected from small rodents in Rusnė (Lithuania) were analysed using domains 1-3 nuclear 28S rRNA gene amplification by conventional PCR. From these, three samples showed amplification in the electrophoresis gel and were sent to sequencing to Macrogen in Amsterdam (Netherlands). Among these, only one good quality sequence was obtained.

In Picture 3.2 it is shown the electrophoresis gel of the 28S rRNA domains 1-3 gene sequence amplification by conventional PCR from *L. jettmari* mites. This gene is found in all *L. jettmari* mites, so in theory in all samples there must be amplification, so all wells should be positive. However, not always the DNA is amplified, sometimes the concentration of DNA is very little or in other cases as the mite is very small the quantity of DNA extracted is not enough to show up in the gel.

In the conventional PCR we amplify an approximately 856 pb fragment of 28S rRNA gene.

In the positive control there should be amplification as the sample used is known to contain the gene amplified by PCR. This control tests all parameters of PCR (the master mix, $MgCl_2$ concentration, primer annealing temperature, extension times…). If there is amplification it means that the parameters and solutions used are working correctly. In our case, the amplified fragment is seen around 800bp in all positive controls, so we concluded that our PCR reaction had worked properly. As positive control pool of *L. agilis* was used.

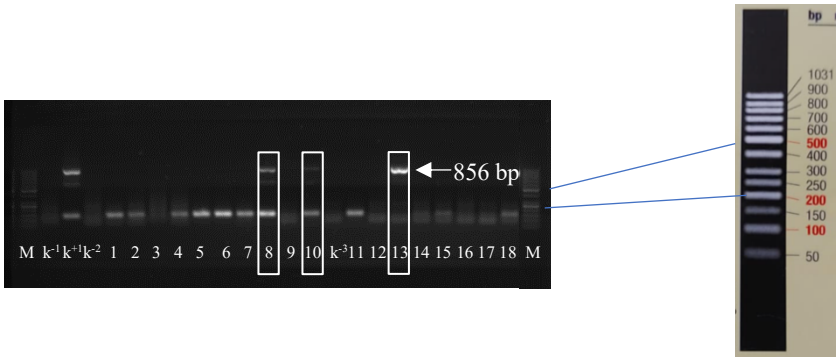


**Figure 3.2**. Electrophoresis gel of the amplification of 28S rRNA gene in *Laelaps jettmari* mites by conventional PCR (17/2/22). M: GeneRulerTM 50bp DNA Ladder, $K^-$: negative control, $K^+$: positive control, 1-18: DNA of *Laelaps jettmari* mites (photo by author).

As negative control, double distilled water was used. In the negative control there shouldn't be amplification, as the sample used does not contain the gene amplified as it is plain distilled water. Consequently, no bands will be observed in the electrophoresis gel. If some bands are observed, it means that some reagent is contaminated with foreign DNA, so the PCR needs to be repeated changing the reagents used. In our case no band showed up in negative controls, so we concluded that none of our reagents was contaminated with unspecific DNA.

As the marker and all controls showed up as predicted in the previous paragraphs, we could relate that the PCR and electrophoresis went well, and the results obtained were conclusive.

In picture 3.2, amplification of *L. jettmari* sequences is seen in the wells 8, 10 and 13, which correspond to the sequences 18 Rus 5-3, 18 Rus 104-1 and 18 Rus 144-2 respectively.

The band observed around 100 bp is due to primer duplicates formed during PCR. These are usually less than 100 bp in length, and do not influence gel interpretation as their length is much shorter than that of the 28s rRNA amplification product (which is between 800 and 900 bp).

The samples 18 Rus 5-3, Rus 104-1 and 18 Rus 144-2 were sent to sequence. From these, 18 Rus 104-1 was the one we used for phylogenetic analysis for its high quality, as the others were not suitable for phylogenetic analysis because of high background noise.

### 3.3.1. Phylogenetic analysis of *Laelaps jettmari* mite

The DNA sequence from *L. jettmari* mite (18 Rus 104-1) was collected from the host *A.agrarius* and was analysed based on the 28S rRNA domains 1-3 gene region.

The following Laelapid mites 28S rRNA gene sequences were obtained from GenBank and aligned with our *L. jettmari* sequence (104-1): *Laelaps hilaris* (MZ043846, MZ043845, GU440637), *Laelaps stupkai* (GU440596), *Laelaps muris* (GU440638), *Laelaps clethrionomydis* (GU440636), *Laelaps kochi* (GU440626), *Laelaps* sp. (GU440628), *Laelaps spinigera* (GU440613), *Laelaps agilis* (MZ043840, MZ043844, MZ043842), *Laelaps jettmari* (GU440635, MZ043834, MZ043833), *Laelaps zumpti* (GU440623), *Laelaps vansomereni* (GU440619), *Echinolaelaps echidninus* (MT813463), *Androlaelaps* sp. (GU440592) and *Ondatralaelaps multispinosus* (FJ911778). *Dermanyssus gallinae* (FJ911771) was used as an outgroup.

The neighbour-joining (NJ) and maximum likelihood (ML) approaches were used to reconstruct evolutionary relationships among Laelapid mites and the trees obtained are shown in Figure 3.3 (ML) and 3.4 (NJ).

The NJ and ML obtained trees have comparable topologies and branching patterns. The 28S rRNA gene sequences of different Laelapid mite species were clearly split into distinct clusters in all phylogenetic trees, as shown in Figures 3.3 and 3.4.

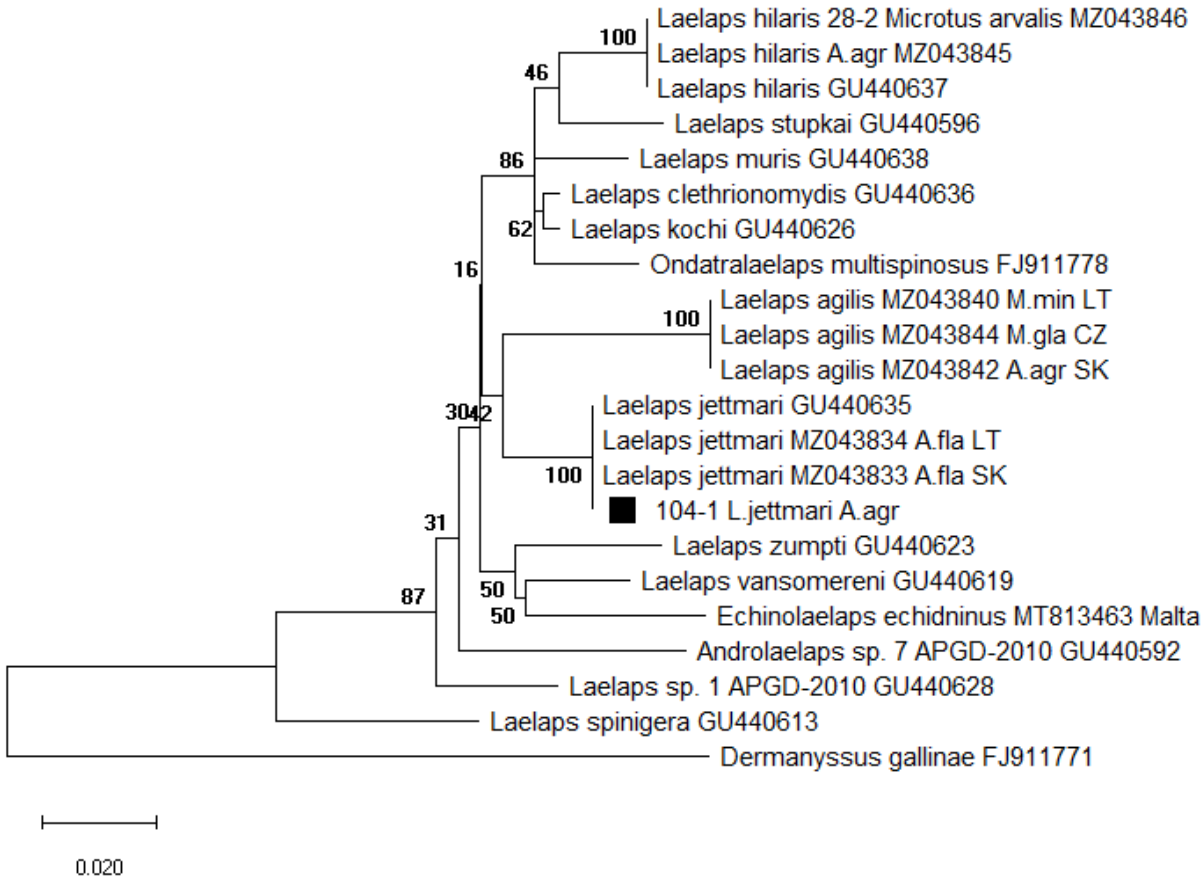


**Figure 3.3**. Phylogenetic tree of 28S rRNA gene sequences of Laelapid mites generated using the Maximum Likelihood method and Tamura-Nei model and bootstrap analysis of 1000 replicates. The sample sequenced in the present study is marked (■). Abbreviations: A. fla – *Apodemus flavicollis*, A.agr – *Apodemus agrarius*, M. gla – *Myodes glareolus*, M. min – *Micromys minutus*, LT – Lithuania, CZ – Czech Republic and SK – Slovakia.

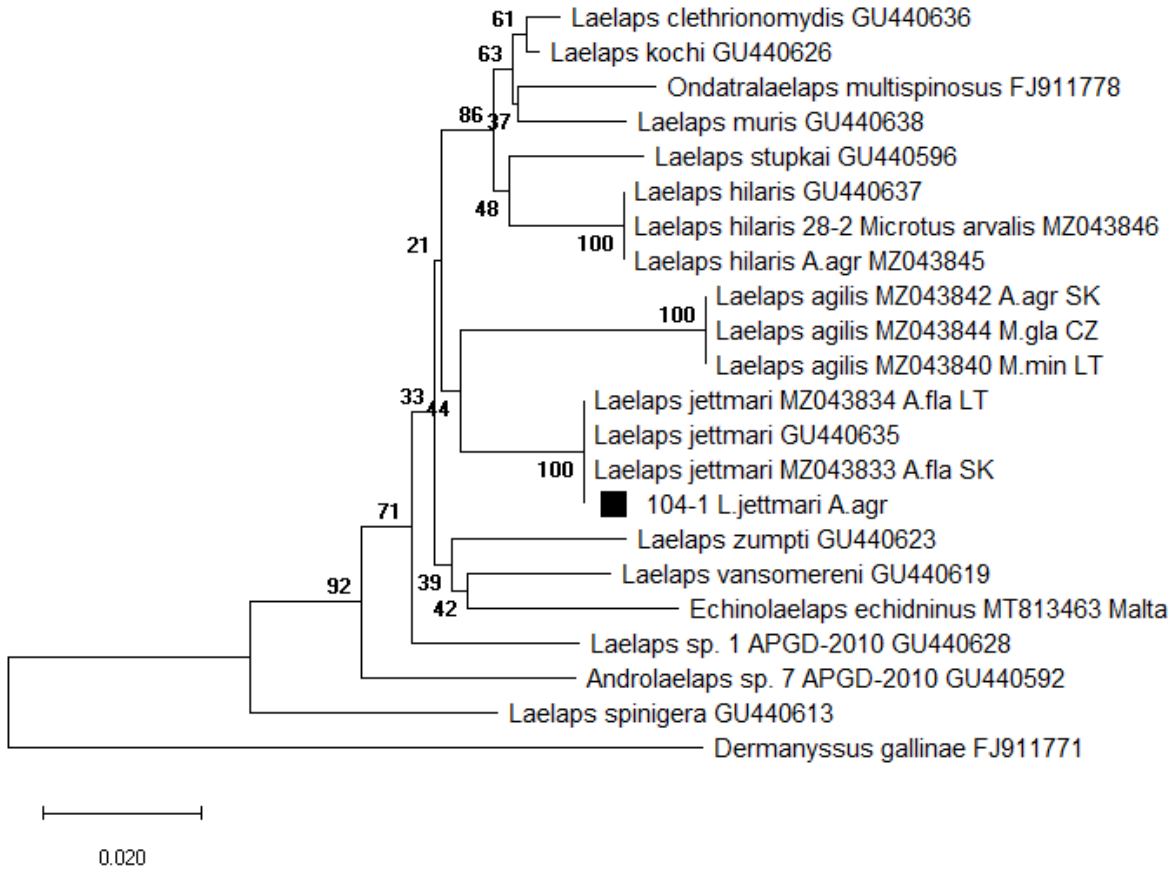


**Figure 3.4**. Phylogenetic tree of 28S rRNA gene sequences of Laelapid mites generated using the Neighbor Joining method and Tamura-Nei model and bootstrap analysis of 1000 replicates. The sample sequenced in the present study is marked (■). Abbreviations: A. fla – *Apodemus flavicollis*, A.agr – *Apodemus agrarius*, M. gla – *Myodes glareolus*, M. min – *Micromys minutus*, LT – Lithuania, CZ – Czech Republic and SK – Slovakia.

The table 3.1 shows information about the sequences obtained from GenBank that were aligned to the sequence obtained in this study, *Laelaps jettmari* (Rus 104-1).

**Table 3.1.** Hosts species, localities, length and GenBank nucleotide accession numbers of the 28S rRNA gene sequences of Laelapid mites used to construct the phylogenetic trees shown in Figures 3.3 and 3.4.

| Genus | Taxonomic status of species | Host species | Locality | Length (bp) | GenBank Accession No. |
|---|---|---|---|---|---|
| *Laelaps* | *Laelaps hilaris* | *Microtus arvalis* | Lithuania | 805 | MZ043846 |
| | | *Apodemus agrarius* | Lithuania | 814 | MZ043845 |
| | | - | - | 833 | GU440637 |
| | *Laelaps stupkai* | - | - | 810 | GU440596 |
| | *Laelaps muris* | - | - | 833 | GU440638 |
| | *Laelaps clethrionomydis* | - | - | 833 | GU440636 |
| | *Laelaps kochi* | - | - | 812 | GU440626 |
| | *Laelaps sp.* | - | - | 814 | GU440628 |
| | *Laelaps spinigera* | - | - | 807 | GU440613 |
| | *Laelaps agilis* | *Micromys minutus* | Lithuania | 818 | MZ043840 |
| | | *Myodes glareolus* | Czech Republic | 805 | MZ043844 |
| | | *Apodemus agrarius* | Slovakia | 818 | MZ043842 |
| | *Laelaps jettmari* | - | - | 832 | GU440635 |
| | | *Apodemus flavicollis* | Lithuania | 802 | MZ043834 |
| | | *Apodemus flavicollis* | Slovakia | 803 | MZ043833 |
| | *Laelaps zumpti* | - | - | 818 | GU440623 |
| | *Laelaps vansomereni* | - | - | 820 | GU440619 |
| *Echinolaelaps* | *Echinolaelaps echidninus* | Mouse | Malta | 827 | MT813463 |
| *Androlaelaps* | *Androlaelaps* sp. | - | - | 832 | GU440592 |
| *Ondatralaelaps* | *Ondatralaelaps multispinosus* | - | - | 1208 | FJ911778 |
| *Dermanyssus* | *Dermanyssus gallinae* | - | - | 1255 | FJ911771 |

The percentage of identity of the sequence *L. jettmari* (18 Rus 104-1) with the other sequences collected from GenBank used in the phylogenetic study is shown in the table 3.2.

**Table 3.2.** Percentage of identity of the sequence under study, *L. jettmari* (18 Rus 104-1), with each sequence used in the alignment in MEGA X software to make the phylogenetic trees shown in Figures 3.3 and 3.4.

| Sequence | Percentage of identity |
|---|---|
| *Laelaps jettmari* GU440635 | 100% |
| *Laelaps jettmari* MZ043834 | 100% |
| *Laelaps jettmari* MZ043833 | 100% |
| *Laelaps clethrionomydis* (GU440636) | 97.21% |
| *Laelaps kochi* (GU440626) | 96.93% |
| *Laelaps* sp. (GU440628) | 96.08% |
| *Laelaps vansomereni* (GU440619) | 95.94% |
| *Laelaps muris* (GU440638) | 96.09% |
| *Laelaps hilaris* (MZ043846) | 95.95% |
| *Laelaps hilaris* (MZ043845) | 95.95% |
| *Laelaps hilaris* (GU440637) | 95.95% |
| *Laelaps zumpti* (GU440623) | 95.80% |
| *Laelaps agilis* (MZ043844) | 95.52% |
| *Laelaps agilis* (MZ043842) | 95.52% |
| *Laelaps agilis* (MZ043840) | 95.52% |
| *Laelaps spinigera* (GU440613) | 92.73% |
| *Echinolaelaps echidninus* (MT813463) | 95.10% |
| *Androlaelaps* sp. (GU440592) | 94.97% |
| *Ondatralaelaps multispinosus* (FJ911778) | 95.23% |
| *Laelaps stupkai* (GU440596) | 95.26% |

In the analysis, *Dermanyssus gallinae* was used as an outgroup.

The sequence alignment showed no variable sites between the 28S rRNA sequence from *L.jettmari* (104-1) and the *L. jettmari* sequences collected from GenBank (GU440635, MZ043834 LT, MZ043833 SK). Hence, the genotype of *Laelaps jettmari* is identical in both the mites found in Slovakia and Lithuania. Therefore, it can be concluded that there is no difference in sequence among different host or location. This is because universal primers were used to amplify 28S rRNA region. If other primers had been chosen, like the ones to amplify COI from mitochondrial DNA, more variability would have been seen inside species depending on the host and location.

Different parameters related to the genetic distance between the sequences under study were calculated. As for this, the overall mean genetic distance between the *Laelaps* mites 28S rRNA gene

sequences was 0.06. The genetic distance between the 28S rRNA gene sequence of *L. agilis* and *L.jettmari* was 0.03.

According to the nucleotide composition, the nucleotide frequencies among all sequences used are 25.00% of Adenine, 25.00% of Thymine/Uracil, 25.00% of Cytosine, and 25.00% of Guanine. Furthermore, the composition of nucleotides of the sequence *Laelaps jettmari* (18 Rus 104-1) is 29.8% of Thymine/Uracil, 16.6 % of Cytosine, 27.1 % of Adenine and 26.4 % of Guanine, being its length 715 nucleotides.

The phylogenetic analysis carried in this paper involved 22 nucleotide sequences. The codon positions included in it were 1st+2nd+3rd+Noncoding, being a total of 720 positions in the final dataset.

As we can see in the phylogenetic trees (Figures 3.3 and 3.4), *L. jettmari* and *L.agilis* have a common ancestor meaning that they originated from the same branch.

In the alignment of the sequences *Laelaps agilis* MZ043840 *M.min* LT, *Laelaps jettmari* MZ043834 *A.fla* LT, *Laelaps jettmari* GU440635, *Laelaps jettmari* MZ043833 *A.fla* SK and 104-1 *L. jettmari A.agr* LT, the total variable of nucleotide sites among the 720 sites aligned are 32, as shown in Table 3.3.

**Table 3.3**. Variable nucleotide sites in the alignment among the sequences MZ043840, MZ043834, GU440635, MZ043833 and 104-1. Abbreviations: A.fla – *Apodemus flavicollis*, M.min - *Micromys minutus,* A.agr - *Apodemus agrarius* and LT - Lithuania.

| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Laelaps agilis* MZ043840 *M.min* LT | A | G | T | C | A | T | G | A | A | G | C | T | T | C | G | G | A | G | A | A | C | C | T | G | T | A | T | T | A | C | C | C |
| *Laelaps jettmari* MZ043834 *A.fla* LT | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Laelaps jettmari* GU440635 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Laelaps jettmari* MZ043833 *A.fla* SK | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 104-1 *L. jettmari A.agr* LT | T | A | G | T | T | C | A | G | G | A | T | G | A | A | A | A | G | A | T | G | T | G | C | A | C | C | C | C | T | T | T | T |

# DISCUSSION

Mites of the family Laelapidae are frequent ectoparasites of small animals, particularly rodents. Mites infesting small rodents have not been deeply studied and little is known about them in Lithuania.

Concerning the situation in Lithuania, in Podenaite (1979) article and diverse conferences sixteen species of Dermanyssoidea mites were reported. However, the frequency of infestation or the abundance of mites in different host species was not described. *Laelaps* mites were also reported on rodents by Paulauskas et al., 2009 but were not identified until species level. Aside from Kaminskienė et al. (2017, 2020) and Radzijevskaja et al. (2018) papers, no new information concerning mites in small rodents has been published in Lithuania.

Among the Laelapidae family, ectoparasites from the *Laelaps* genus are frequently restricted to a certain host species or genus, infesting a narrower variety of hosts, and their presence in the nest is rather low (Mašán and Stanko 2005, Mašán and Fend′a, 2010).

In other studies on host specificity of *Laelaps* genus, grey hamster and common vole were found as the hosts of *L. jettmari* (Bregetova, 1956). Furthermore, in the countries that conformed the USSR, *Cricetulus migratorius* was the major host for *L. jettmari* mites (Bregetova, 1956).

In a study conducted in Turkey, the most common mite infesting the rodents analysed was *L.jettmari* and it was mainly found on *Mus musculus* and *C.s migratorius,* being found on one occasion in *Citellus citellus* (Cicek et al., 2008). Also, it has been reported as ectoparasite in *Pitymys subterraneus* (Sélys-Longchamps, 1835).

*L. jettmari* has been documented in Korea, Japan, and Manchuria as a parasite on rodents of many genera, particularly *Apodemus* (Cicek et al., 2008). This is in line with our results, as in Rusnė (Lithuania) we have only found *L. jettmari* mites in *Apodemus agrarius*.

The associations between Laelapidae mite species and small rodents have not been studied in detail. Kaminskienė et al., 2017 reported associations between different Laelapidae mites and small rodents from Lithuania, in which *L. jettmari* was identified as one of the ectoparasites infesting Lithuanian rodents.

Molecular identification of Laelapidae mites from small rodents was carried out in Lithuania and presented in dissertation (Kaminskienė, 2021). In this study both 28S rRNA and COI gene sequences were used for molecular identification, being edited, aligned, and subjected for phylogenetic analysis in Mega X software.

The domains 1-3 of the 28S region of the nuclear rRNA analysed in the present study have been used to investigate phylogenetic relationships within Dermanyssoid mites and the evolution of parasitism in Dowling and OConnor (2010a). In that research, based on primers described by Park

and Ó Foighil (2000), a novel set of overlapping primers were developed to boost primer specificity for the domain 1-3 28S rRNA region. These analyses of the 28S rRNA gene sequences demonstrated that this method may be used to identify Dermanyssoid mites with similar morphological characteristics. Dowling and OConnor (2010a) previously reported phylogenetic relationships among Dermanyssoid mites based on 28S rRNA gene sequences. According to the findings, parasitism of vertebrates and arthropods evolved at least eight times independently, with the majority of these events occurring outside of the Laelapidae family.

Furthermore, the phylogenetic analyses provide no reason for *Laelaps*, *Echinolaelaps*, and *Tricholaelaps* to remain as distinct taxonomic entities and they should all be grouped into one genus, as several authors have done (Dowling and OConnor, 2010a). This is also in agreement with the results of this study, since as shown in both trees, NJ and ML (Figures 3.3 and 3.4), *Echinolaelaps* is included in the same branch as *Laelaps vansomereni* and *Laelaps zumpti.* However, in this study *Tricholaelaps* sequences have not been included in the phylogenetic trees, so we have not been able to verify that it is grouped in the same genus with *Laelaps*.

Genetic distances between species were considerably higher than genetic distances within species. This is consistent with the logic that a species is characterised by a number of genetic characteristics that differentiate it from other species.

The phylogenetic study based on the 28S rRNA gene shows genetic similarities among the mite species analysed. *L. jettmari* and *L. agilis* presented the shortest interspecific genetic distances of 28S rRNA, indicating that they have a common ancestor and so they originated from the same branch. There is no difference in *L. jettmari* sequences collected from different rodent hosts in different geographical regions, because universal primers to amplify 28S rRNA were used. Thus, the genotype of *L. jettmari* found in Slovakia (MZ043833) and Lithuania (MZ043834) are identical. 100% identity is found within the species *L. hilaris*, *L. agilis* and *L. jettmari*. The two *L.hilaris* sequences which provide information about host and country were taken from Lithuania but from different hosts, *Microtus arvalis* (MZ043846) and *Apodemus agrarius* (MZ043845). Additionally, the sequences from *L. agilis* used were taken from different locations and hosts: Lithuania and *Micromys minutus* (MZ043840), Czech Republic and *Myodes glareolus* (MZ043844), and Slovakia and *Apodemus agrarius* (MZ043842). Therefore, we could conclude that the genotype of the *Laelaps hilaris* and *Laelaps agilis* sequences under study does not vary depending on host and location. This is in concordance with Kaminskienė (2021) results.

The 28S rRNA gene sequences of Laelapidae mites analyzed showed an average of 25.00% composition of each nucleotide. However, the sequence of *Laelaps Jettmari* studied (18 Rus 104-1) was rich in thymine (29.8%) and adenine (26.4%). Other research yielded comparable findings (Ros and Breeuwer, 2007; Bhowmick et al., 2019).

# CONCLUSIONS

1. Using morphological characteristics, twenty-two *Laelaps jettmari* females collected from *Apodemus agrarius* mice were identified. A total of 9.9% of *A. agrarius* carried *L. jettmari* mites.

2. The DNA of a total of 3 *Laelaps jettmari* was successfully visualized using molecular methods based on rRNA 28S region (domains 1-3) amplification.

3. Phylogenetic analysis based on 28S rRNA region showed that there is no difference among different host species or locations when comparing the *Laelaps jettmari* sequence obtained in this study and *L. jettmari* sequences collected from GenBank (GU440635, MZ043834 LT, MZ043833 SK).

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# APPENDIX 1

## Polymerase chain reaction (PCR)

The Polymerase chain reaction (PCR) technique has been a breakthrough both for mite identification and for establishing evolutionary relationships between different mite species, that traditionally were made based on morphological characteristics as we mentioned before.

PCR was invented by Kary Mullis in 1983, patented in 1985, and awarded with the Nobel Prize in Chemistry in 1993. This method allows the synthesis of billions of copies of a specific target DNA or RNA fragment.

PCR is a method widely used in molecular biology, and is based in 3 steps:

1) Denaturation: separation of the two DNA strands into single-stranded DNA when the temperature rises above 90°C, because the hydrogen bonds cannot be maintained at such high temperature. Normally, the denaturation temperature ranges between 94-96 ºC .

1) Hybridization: Primers are added to the mix, which are single stranded DNA of about 21-25 nt. Two primers flank the target DNA sequence and hybridize with opposing strands of DNA, one to the left (5′) and one to the right (3′) of the target region to be amplified.

This stage is typically performed at a temperature of 50-70ºC (the higher the temperature, the more selective is the hybridization). At this temperature, hydrogen bonds can rebuild, and complementary sequences hybridize, resulting in primers hybridizing to target DNA.

3) Elongation: In this step, a thermostable DNA polymerase, most commonly Taq polymerase, catalyzes the addition of dNTPs present in the reaction mixture to the growing strand. This step is performed at 72 ºC since this is the optimal temperature for the Taq polymerase.

This cycle is repeated between 20 and 50 times in a thermo cycler, that contains a heating block in which temperature varies very quickly due to Pelier effect.

The components needed to carry out PCR are the followings:

- DNA template, containing the target sequence.
- Desoxyribonucleotide tryphosphates (dNTPs) in equal proportion: A, T, C and G.
- Taq DNA polymerase: thermostable DNA polymerase, first isolated from *Thermus aquaticus*, able to whistand over 90º. It carries out the synthesis of the new strand of DNA using as template the target DNA sequence.
- Primers: single stranded short DNA sequences (20-25 nt) that flank the target sequence.
- Reaction buffer that provides a stable pH. It contains $MgCl_2$, as $Mg^{2+}$ is the cofactor of Taq DNA polymerase, boosting its activity.

PCR is extremely sensitive to non-target DNA contamination. As a result, a negative control is included, to ensure that reagents used are not contaminated (if a band appears in agarose gel electrophoresis in the negative control, there is contamination during the process).

A positive control is normally added, which is a sample where DNA sequence of interest is known to be present, so it will work under the given conditions and a band will be seen in the electrophoresis gel. The positive control put the master mix, $MgCl_2$ levels, primer annealing temperature, and extension periods to the test. If the positive control doesn't show in the gel, it means that something is incorrect with the previously mentioned parameters.

The detection and analysis of PCR products is normally made by agarose or acrylamide gel electrophoresis. DNA is revealed by ethidium bromide staining, visible by ultraviolet transillumination (280– 320 nm).

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