MOLECULAR DESCRIPTION OF MEDICINAL LEECH (*HIRUDO VERBANA* CARENA, 1820) BASED ON DNA SEQUENCES

MASTER THESIS

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Supervisor
Prof. Dr. Mehmet Oğuz ÖZTÜRK

DEPARTMENT OF
MOLECULAR BIOLOGY AND GENETICS

February 2023

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AFYON KOCATEPE UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

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ABSTRACT

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In this study, 641-736 bp partially distinctive nucleotide sequences belonging to the mtCOI and 18S rRNA gene locus from *Hirudo verbana* samples were identified as identical and registered in GenBank (OM010329, OM010332-37, OM033510, OK091623-32).Among the mtCOI nucleotide sequences of 6 of these isolates (OM010329, 32, 33, 35, 37, OM033510), 1 variation (A > G) was observed at nucleotide 431. The nucleotide percentages in the mtCOI gene sequences of *Hirudo verbana* samples are (A 24.5-26.0%; T 24.3-25.2%; G 28.1-29.6%; C 20.9-21.6%). The nucleotide percentages in the 18S rRNA gene sequences are (26.9-27.2% A; 26.7-26.8% T; 28.3-28.6% G; 17.4-17.9% C). According to the mtCOI sequence dataset distance matrix obtained by the Pairwise comparison method, there was a complete agreement between the *H.verbana* isolates identified in this study and the *H.verbana* isolate registered in the GenBank. mtCOI gene sequence results of the isolates confirmed the taxonomic position of *H.verbana*, which was defined according to its anatomical and morphological features. As a result, mtCOI and 18S rRNA gene sequences were defined for the first time in this study on the samples belonging to *H.verbana* species in Karamık Lake, and it contributed to the determining the genetic characteristics of this species on a local and global scale.

2023, xii + 72 pages

Keywords: COI, *Hirudo verbana*, DNA, Karamık Lake.

ÖZET

Yüksek Lisans Tezi

DNA DİZİLERİNE DAYALI OLARAK TIBBİ SÜLÜK (*HIRUDO VERBANA* CARENA, 1820)'ÜN MOLEKÜLER TANIMLAMASI

Al-Hussein Hameed Hussein OKBI

Afyon Kocatepe Üniversitesi

Fen Bilimleri Enstitüsü

Moleküler Biyoloji ve Genetik Anabilim Dalı

Danışman: Prof. Dr. Mehmet Oğuz ÖZTÜRK

Amaç: Bu çalışmada amaç; Karamık Gölü'nden *Hirudo verbana* örneklerinin anatomik, morfolojik özellikleri ile 18S rRNA ve mitokondriyal sitokrom c oksidaz alt ünite I lokuslarının (mtCOI) sekans verilerinitanımlamaktır. *Hirudo verbana* türünün moleküler taksonomik konumu, 18S rRNA ve mt COI genetik dizi verileri ile test edilecektir. Sonuçlar NCBI veri tabanındaki *Hirudo verbana* izolatları ile uyumluluk analizine tabi tutulacaktır.Araştırma sonuçları ile*Hirudo verbana* türünün genetik özelliklerine, DNA barkod kütüphanesine, taksonomik kökenine ve evrimsel konumunun belirlenmesine katkıda bulunulacaktır.

Yöntemler: *Hirudo verbana* örnekleri Karamık Gölü'nden toplandıktan sonra, fiksasyon ve muhafaza için %95 etanol içine konulmuştur. *Hirudo verbana* örneklerinin anatomik ve morfolojik incelemeleri için Olympus SZ30 stereo mikroskop, Olympus CH20 ışık mikroskobu ve ZEISS Axio Imager mikroskobu kullanılmıştır.

Moleküler çalışmalar için *Hirudo verbana*'ya ait 16 örneğin kaudal vantuzları çıkarılmış ve Gene Matrix Tissue kiti kullanılarak DNA ekstraksiyonu yapılmıştır. DNA izolasyonundan sonra, 18S rRNA ve mt COI'nin yaklaşık 700 bazlık bölgesini çoğaltmak için tek adımlı PCR yapılmıştır. 18S bölgesi için C (5′-CGG TAA TTC CAG CTCCAATAG-3′) ve Y (5′-CAGACAAATCGCTCCACCAAC-3′) primerleri ve mt COI bölgesi için LCO 1490 ve HCO 2198 primerleri kullanılmıştır.

ABI 3730XL Sanger dizileme cihazı (AppliedBiosystems, Foster City, CA) ve BigDyeTerminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) (Macrogen-Holland) kullanılarak mt COI dizilemesi için doğrudan LCO 1490 ve HCO 2198 primerleri ile *Hirudo verbana*'nın saflaştırılmış PCR ürünlerinden dizileme yapılmıştır. Nükleotid dizileri, Mega 7.0 çoklu dizi hizalama ClustalW2 yazılımı yardımıyla hizalanmıştır.

Gen dizilerinin homoloji araştırması için FASTA programı ve BLAST algoritması kullanılmıştır. Genetik uzaklıkları hesaplamak için Mega 7.0'da Tamura Nei'nin çift yönlü silme yöntemli iki parametreli modeli kullanılmıştır. Filogram analizinde Mega 7.0'da Maksimum Olabilirlik (ML) yöntemi kullanılmıştır.

Bulgular: Bu çalışmada incelenen*Hirudo verbana* örnekleri Karamık Gölü'nden toplanmıştır. Örnekler anatomik ve morfolojik özelliklerine göre tanımlanmıştır. Daha sonra, aynı örneklerin mtCOI (OM010329, OM010332-37, OM033510) ve 18SrRNA (OK091623-32) gen lokuslarına ait 641-736 bp kısmen anlamlı nükleotid dizileri özdeş olarak tanımlanmış ve GenBank'a kaydedilmiştir.

İkili karşılaştırma yöntemiyle elde edilen mtCOI dizilerine göre, bu çalışmada tanımlanan *Hirudo verbana* izolatları ile GenBank'ta kayıtlı *Hirudo verbana* izolatları arasında tam bir uyum gözlenmiştir. Böylece, bu çalışmada tanımlanan *Hirudo verbana* izolatlarının mt COI sekans sonuçları, Karamık Gölü'nden toplanıp anatomik ve morfolojik özelliklerine göre tanımlanan *Hirudo verbana* türünün taksonomik konumunu doğrulamıştır.

Bu araştırmada üretilen 6 izolatın (OM010329, 32, 33, 35, 37, OM033510) mtCOI lokusunun 431 nolu nükleotidinde, 1 varyasyon (A > G) gözlenmiştir. Bununla birlikte, OM010334 ve OM010336 izolatların mtCOI dizilerinde ve diğer tüm 18SrRNA gen dizilerinde herhangi bir nükleotid varyasyonu gözlenmemiştir. *Hirudo verbana* örneklerinin mtCOI gen dizilerindeki nükleotid yüzdeleri (A %24,5-26,0; T %24,3-25,2; G %28,1-29,6; C %20,9-21,6) dir.18S rRNA gen dizilerindeki nükleotid yüzdeleri (%26,9-27,2 A; %26,7-26,8 T; %28,3-28,6 G; %17,4-17,9 C) dir.

Maksimum olabilirlik analizi değerleri Hirudo izolatları arasında filogram oluşumunu güçlü bir şekilde desteklemiştir. ML analizi sonucunda, bu çalışmada elde edilen Hirudo verbana izolatına ait OM010337.1 ve OM033510.1 kendi aralarında yakın konumlanma göstermiştir. Ardından, çalışma izolatımız (OM010336.1) ile bir diğer çalışma izolatımız (KU216244.1) arasındaki ilişkinin de aynı yakın konumlara sahip olduğu gösterilmiştir. OM010334.1 izolatından sonra tek bir dal (JN083798.1) ve ardından bir grup diğer çalışmalardan AY763150.1 ve EF446694.1 izolatları yakın konumlanma göstermiştir. Bu konumlandırmayı tek bir dal (AY63151.1) takip etmiştir. Ardından diğer *Hirudo* türleri (KY989460.1 *Hirudo orientalis*, AY763148.1 *Hirudo medicinalis*, AY763155.1 *Hirudo troctina* ve KU216240.1 *Hirudo sulukii*) her biri tek dal olarak konumlanmıştır. Son dal olan Erpobdella montezuma (GQ368760.1) dış grup olması nedeniyle ağaçta en sonda yer almıştır.

Sonuç: Çalışma sonucunda Karamık Gölü'nden elde edilen *Hirudo verbana* türünün anatomik ve morfolojik özelliklerinin yanı sıra, mtCOI ve 18SrRNA gen dizileri ilk defa bu çalışmada tanımlanmıştır.

Mevcut çalışma sürecinde elde edilen *Hirudo verbana* örneklerinin mtCOI dizileri kendi aralarında (OM721927-30 ve OM033510) ve NCBI veritabanındaki *Hirudo verbana* izolatlarının mtCOI lokus dizileri (AY763151, AY763150, JN083801, KT692947, EF446694, KU216244)ile tam bir uyum göstermiştir. Böylece, anatomik ve morfolojik özelliklerine göre türü tanımlanan Karamık Gölü *Hirudo verbana* örneklerinin moleküler taksonomik konumu, mtCOI sekans verileri ile doğrulanmıştır. Ayrıca, bu çalışmasonuçları ile*Hirudo verbana* türünün genetik özelliklerinin, DNA barkod kütüphanesinin, taksonomik kökeninin ve evrimsel homojenliğinin belirlenmesine katkıda bulunulmuştur.

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Anahtar Kelimeler: COI, *Hirudo verbana*,DNA, Karamık Gölü.

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Al-Hussein Hameed Hussein OKBI

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LIST OF SYMBOLS and ABBREVIATIONS

Symbols

Bp	Base pair
dH_2O	Distilled water
HCl	Hydrochloric acid
K	Potassium
M	Molar
mL	Milliliter
µM	Micromolar
mM	Milimolar
$MgCl_2$	Magnesium chloride
Na^+ iyonları	Sodium ions
NaCl	Sodium chloride
µg	Microgram
µm	Micromolar
µL	Microliter
Nm	Nanometer
V	Volt

Abbreviations

A	Adenine
BLAST	Basic Local Alignment Tool
C	Cytosine
DNA	Deoxyribonucleic acid
Cox	Cytochrome c oxidase subunit
DNA	Deoxyribonucleic acid
dNTP	Di-nucleotide triphosphate
EtBr	Ethidium Bromide
ETDA	Ethylenediamine tetraacetic acid
F	Forward
FASTA	Fast-all, FastA
G	Guanine
ITS	Integrative Tension Sensor
MT	Mouth Tube
mtCOI	Mitochondrial Cytochrome Oxidase
mtDNA	Mitochondrial DNA
ML	Maximum Likelihood
MP	Maximum Parsimony
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide Hybrid
ND	NanoDrop
PFI	Parasitic Frequency Index
PH	Potential Hydrogen
Ppm	Units per Million
PS	Anterior Mouth Pre-Oral Barb
PZR	polymer chain reaction

R	Reverse
RAPD	Randomly amplified polymorphic DNA
rDNA	Nuclear Ribosomal DNA
rRNA	Ribosomal RNA
RFLP	Restriction fragment length polymorphism
RPM	Revolutions per minute
SDS	Sodium dodecyl sulfate
SEM	Scanning Electron Microscope
SNPs	Single nucleotide polymorphisms
spp	Types
SS	Spinal Sheath
SVCV	Vector of Carp Virus
T	Thymine
TAE	Tris Acetate Acid
TBE	Tris Boric Acid
Tris	Trisaminomethane
UPGMA	Unweighted Double Group Method with Arithmetic Mean
UV	Ultraviolet

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1. INTRODUCTION

In the monophyletic Hirudinea, particular emphasis is defined on ectoparasitic taxa whose nutrition depends on vertebrate blood. The best known among these are the ectoparasitic medicinal leech species, which have been used for medicinal purposes for a long time in human history (Živić et al. 2015).

Three species of medicinal leeches (*Hirudo medicinalis*, *Hirudo verbana*, and *Hirudo troctina*) are widely in the Eurasian region (Utevsky et al. 2010). Among these species, *Hirudo medicinalis* and *Hirudo verbana* were named "*Hirudo medicinalis*" because of insufficient knowledge about their biology until proven definitively in the context of reproductive and molecular analysis data (Kutschera et al. 2007, Siddall et al. 2007). In this context, since all medicinal leech forms in Europe were thought to represent varieties of a species, the protection status (IUCN, CITES, Bern Convention) was established for *H. medicinalis* (Hechtel et al. 2002). As a result of detailed taxonomic studies carried out in the following years, it was revealed that the distribution area of *H. medicinalis* did not include the regions specified in this protection status (Utevsky et al. 2008, Westendorff et al. 2008, Utevsky et al. 2010, Elliot and Kutschera 2011, Živić et al. 2015). In the past decades, medicinal leeches in the wetlands of Turkey were identified as *Hirudo medicinalis*, consistent with research results in Europe (Balık 2006, Kasparek et al. 2000, Akbulut et al. 2012). However, in recent studies, it has been proven that the majority of leeches in Turkey belong to *Hirudo verbana* (Apakupakul et al. 1999, Saglam 2011, Siddall et al. 1998).

In this study; anatomic-morphologic features and 18S rRNA and mitochondrial cytochrome c oxidase subunit I (mt COI) loci sequences of *Hirudo verbana* samples from Karamık Lake will be described for the first time. The results will be subjected to compatibility analysis with *Hirudo verbana* isolates from the NCBI database. The molecular taxonomic position of *Hirudo verbana* from Karamık Lake samples will be tested with genetic sequence data of 18S rRNA and mt COI. The research results will contribute to the DNA barcode library, taxonomic origin, and evolutionary position of the species *Hirudo verbana*.

2. GENERAL INFORMATION

2.1 Taxonomic Position of *Hirudo verbana* Species

The leech class (Hirudinea) has 680 defined species under the phylum Annelida which are small monophyletic species. About 71% of the identified species are associated with freshwater habitats. other parts of the latter are accustomed to living in sea and wetland biomes. Numerous leeches are predators, but particular emphasis is laid upon ectoparasitic lifestyles in which their nutrition depends on vertebrate blood. The most familiar are the ectoparasitic medicinal leech species (*Hirudo* spp.), which has long been used for medicinal purposes and was considered the main form of therapy in the past. From a historic point of view, *Hirudo verbana* has been known as *Hirudo medicinalis* (Živić et al. 2015). But *Hirudo verbana* has lately been identified as a different organism from the European medicinal leech *Hirudo medicinalis* (Table 2.1).

Table 2.1 Taxonomic classification of *Hirudo verbana* species (Int Ref. 1).

Taxonomic Category	**Taxon**
Kingdom	Animalia
Phylum	Annelida
Class	Clitellata
Subclass	Hirudinea
Infraclass	Euhirudinea
Order	Arhynchobdellida
Suborder	Hirudiniformes
Family	Hirudinidae
Genus	*Hirudo*
Species	*Hirudo verbana* Carena, 1820
	Parent hirudo Linnaeus, 1758
Synonymized names	*Hirudo officinalis* (Savigny, 1822)

2.2 Anatomical, Morphological and Pysiological Features of *Hirudo verbana*

2.2.1 External Structure of *Hirudo verbana*

Body structure of *Hirudo verbana* is slightly flattened dorsoventrally. The body consists of segments and the middle segments have five rings. The pigmentation of *Hirudo verbana* consists of yellow, red, green, and black colors. It has reddish-orange rosary-like or narrow black mottled stripes on its back. There is a dark green or sometimes brownish band in the middle of the back. There are two more orangish stripes on each side interrupted by a pair of oval dots. On ventral part, there are two dark bands near the lateral on a yellowish-green back ground. Between these two dark bands, there is a light-colored region without spots. There are five pairs of eyes in the anterior part of these leeches (Saglam 2019).

The species contains oval suckers at the anterior and posterior ends. The front sucker is formed by the fusion of 6 parts. The front sucker is smaller than the caudal sucker. The five pairs of eyes are arranged in an arc. Male and female genital pores are ventral to the body and in the sub-anterior terminal position. There are five rings between these genital pores (Saglam 2019).

2.2.2 Excretory system of *Hirudo verbana*

The excretory organs of Hirudinea consist of 10-17 pairs of nephridium, one in each segment, located in the middle part of the body. The structure of the nephridium consists of a ciliated funnel, the nephridium capsule, the nephridium duct, the bladder, and the excretory hole. The organelle, which looks like a ciliated funnel, opens into the body cavity on one side and into the nephridium capsule on the other (Gönenç 2000).

The nephridium capsule is shaped like a bowl filled with cells called amocyte. Amebocytes phagocytize the residues they take from the capsule cavity. After the posterior end of the nephridium duct expands and shapes the bladder, it opens out with the drainage hole located in the anterior part of the segments (Gönenç 2000).

2.2.3 Nervous System of *Hirudo verbana*

The central nervous system in medicinal leeches consists of 34 ganglia connected by a pair of ventral nerve cords. Six of these ganglia are located in the head region, 21 are spaced along the ventral 10 cords of the body, and seven are fused into a central mass in the posterior drawer. The nervous system of leeches isa stimulant specialized to their invertebrate structure. The cells forming the nerve nodes are grouped in a follicular fashion. Each ganglion consists of six follicles. The ganglionic nerve, which is located up to the first sixth segment of the leech, forms the brain. Although leeches have two ventral nerve strips, the segmental ganglia fused. Apart from this structure that forms the central nervous system, leeches also have a peripheral sympathetic nervous system. There are seven peripheral nerves associated with the sense organs in the head region. The sympathetic nervous system contains a nerve ring on the pharyngeal wall in front of the main border ring (Shain et al. 1998).

2.2.4 Circulation and Respiration in Medicinal Leeches

In leeches, circulation is provided by coelomic spaces, and in some species, this structure assumes the role of auxiliary circulation. In medical leeches with a well-developed circulatory system, the coelomic space has turned into a real blood vessel system. In medical leeches, blood is transported from one place to another by the contraction of the lateral longitudinal canals within the regular canal system of the coelomic cavity. In medical leeches, the body surface is in a structure to provide gas exchange. When leeches tremble and shake, they can breathe, as well as by attaching their posterior recess to a place and making their body fluctuate. Medical leeches have hemoglobin in their blood (Govedich et al. 2010).

2.2.5 Digestive System of Medicinal Leeches

In *Hirudo verbana*, the mouth is located at the anterior end, in the center of the anterior sucker. The leeches have three keratinized jaws with smooth or toothed edges. The jaws converge around a center and are equidistant towards the periphery. It is possible to see a three-pointed star-shaped jaws mark on the skin when they make an incision while sucking blood. The jaws is wide and takes up the entire front sucker (Saglam 2019).

The mouth is followed by a muscular pharynx. The salivary glands are located around the pharynx. The channels belonging to these are opened between the teeth. The salivary glands of medicinal leeches fed while sucking blood contain hirudin secretion, which prevents blood from clotting. In jawed leeches, the stomach (mid-intestine, which is the largest part of the digestive tract, comes after the pharynx (Govedich et al. 2010).

The stomach is in the form of a wide tube with a thin wall. There are many sacs, usually in pairs, on the sides of the stomach. The number of these sacs is 11 pairs in the medicinal leech *Hirudo verbana*. This part of the digestive tube acts as a crop and helps to store the food taken from the outside. Digestion is done in the stomach and caecum. There may be protrusions in the form of pouches on the sides of the caecum intestine. The hindgut is short. The anus is located in front of the posterior retraction from the dorsal leech (Govedich et al. 2010).

2.2.6 Reproductive System of *Hirudo verbana*

The male reproductive system of *Hirudo verbana* consists of 10 pairs of testicles, epididymis, sperm sac, atrium, penis, and penile sheath. The female reproductive system consists of the vagina, fallopian tube and ovary (Kerimova 2020). The epididymis of *Hirudo verbana* is somewhat small. The excretory bulb is long. The penis sheath is long and prominent. Testicular sacs are elongated and oval in shape. The vagina is tubular, but typically free of swelling and sharp folds. The atrium is oval-shaped and bulging. Ovarian canal is long (Sağlam 2019).

2.2.7 Chromosome characteristics of *Hirudo verbana*

The cytogenetic research findings demonstrated that the leeches' chromosomal preparations were rich in cells at the late prophase phases of spermatogenesis, but the metaphase plates were quite backward and poorly distributed. The identical haploid chromosome number, n=13, was discovered in all of the tested specimens (Figure 2.1), confirming the identification of the species as *Hirudo verbana*. The three types of chromosomes are acrocentric, submetacentric, and metacentric (Utevsky et al. 2009).

2.2.8 Some Bioactive Substances of *Hirudo verbana*

By the salivary glands of *Hirudo* leeches; Bioactive substances with a bacteriostatic, analgesic, solvent, anti-edematous, immunostimulant, relieving microcirculation disorders, vasodilator, anticoagulant, anti-inflammatory and antibiotic effects are secreted (Baskova et al. 2008).

Bioactive molecules found in the salivary glands of medicinal leeches are used in the treatment of many diseases. Reducing the foci of inflammation in the blood requires the use of high concentrations of drugs, which causes allergies, and adverse effects on the liver and kidneys. In these cases, hirudotherapy is beneficial by regulating the blood flow in the inflammation focus, providing immune protection and regeneration of tissues (Mory et al. 2000).

Hirudin is the main salivary gland secretion of medicinal leeches. It prevents coagulation by binding to thrombin. The substance known as Bufrudin, which is a thrombin inhibitor like Hirudin and differs structurally and immunologically, has been isolated from *Hirudinaria manillensis* (Papavramidou and Christopoulou-Aletra 2009).

Hyaluronidase is the most specific enzyme identified for hyaluronic acid. It reduces the viscosity and allows the fluids injected into the tissues to pass more easily and increases the rate of absorption. In addition, Hyaluronidase helps to increase the resorption of extravasated blood and excess fluid from the tissues, secretion in the salivary glands, and the effectiveness of local anesthetics (Hovingh and Linker 1999).

Apyrase is a non-specific platelet aggregation inhibitor that acts on Adenosine 5'diphosphate, arachidonic acid, platelet-activated factor (PAF), and epinephrine, and two different apyrase enzymes have been extracted from the salivary secretions of *Hirudo* species (Rigbi et al. 1996).

Bioactive substances in leech secretions and saliva are used in the treatment of many diseases. In some studies, it has been determined that hirudin has bacteriostatic and bactericidal effects in addition to its other effects (Graham 1995).

The bioactive substances carried by medical leeches are important in the treatment of dental diseases, gynecological diseases, eye diseases, respiratory system disorders, musculoskeletal system diseases, cardiovascular system diseases, otolaryngological diseases, and dermatological diseases (Wollina et al. 2016).

Hirudotherapy is effective in the treatment of venous congestion after free tissue transfer, replantation, or local flap applications, it has been recommended as an easy and safe treatment method for the patient (Porshinsky et al. 2011).

Modern hirudotherapy has been widely used today, it is possible to develop some complications after treatment. Bacterial infections and allergic reactions are the most common complications (Whitaker et al. 2009).

Aeromonos hydrophila is also seen as a factor in infections that develop after leech therapy. *Aeromonas hydrophila* is a Gram-negative bacterium found in the intestinal flora of the leech and aids in the digestion of the absorbed blood. Infectious agents other than *Aeromonas hydrophlia* have also been reported in hirudotherapy. While *Aeromonos veronii* was reported in 6% of these reported cases, *Serratia marcescens* and *Vibrion fluvialis* were also encountered (Wilmer et al. 2013).

2.3 Life cycle of *Hirudo verbana*

The medicinal leech, *Hirudo verbana* are hermaphrodites. But the leeches require another member of their species for reproduction. The positioning of their reproductive organs in *Hirudo verbana* makes it impossible to reproduce by itself. March, April and May spring months are the feeding period. In reproducing period, which normally starts in early June to August (Ceylan et al. 2015).

Adult *Hirudo verbana* lays eggs in the dark wet soil area of the warm-water lakes. *Hirudo verbana* species pick dark, moist areas to lay their eggs in wet soil (Kutschera and Wirtz 2001).

Leeches have been shown to exude a white, frothy girdle that covers the clitellum area (Figure 2.2 A), then leeches put a cocoon (Figure 2.2 B and 2.2 C) in the midpoint of the foamy. When that happens, it becomes harder and changes the membrane around the cocoon, and becomes spongy. (Figure 2.2 D). Also, On the touching spots of the cocoon, the spongy membrane does not emerge. Around the soil where cocoons had been laid, frothy residuals of varied sizes were seen (Figure 2.2 E). It was found that recently laid cocoons are brick-colored when they first appear and turn whiter within 1-2 days (Figure 2.2 F). The exterior spongy layer of the cocoon was found to have a complicated network structure, and because of how intertwined this structure is, it is hard to see the cocoon's inner membrane (Ceylan et al. 2015).

In *Hirudo verbana*, based on embryo growth and albumin consumption, air holes progressively emerge inside the cocoon, and after about 10 days, the opercula of the cocoon begin to be open. It was discovered that even though opercula are broken down and albumin source as nutrition, is present, in the last of the 30-day hatching season, only 11.69% of cocoons hatchlings out of the cocoons, just 36.36% of the cocoons hatchlings were alive all; the remaining cocoons still contain at least one individual (Ceylan et al. 2015).

In reaction to a drop in temperature, *Hirudo verbana* typically forms dense clusters. In the surroundings of the young leeches, no additional stimuli were found. When the temperature dropped below 10 °C, adult leeches would cluster (Kutschera and Roth 2006).

2.4 *Hirudo verbana,* Searching for Host

Medical leeches swim normally but occasionally pause to wave their bodies or heads. This behavior was formerly broken down into two categories head movement and body waving. Which refer to as scanning. This scanning may be fully explained by these acts, which are all a part of a continuum. Although the behavior associated with scanning is thought to have a part in orientation, this has not yet been confirmed. Contrary to earlier research that stated scanning was engaged in social behavior, behavioral experiments demonstrate the importance of scanning in helping the leech navigate toward prey cues.

When these cues are present, the leech uses scanning activity to reposition itself toward stimuli that resemble prey. Whether prey is present or not, scanning still normally occurs, but when prey-like stimuli are present, scanning is targeted to the source of the stimulus. The leech is most likely able to gain a deeper grasp of its prey target through this action. The presentation of scanning, whether prey stimuli are present or not, is suggestive of a muscular response that is internally regulated and is not released in response to sensory inputs (Harley and Wagenaar 2014)

2.5 Nutrition of Medicinal Leeches

Ceylan (2016) determined that Hirudo verbana populations feed on cattle, sheep, goats, dogs, fish, frogs, turtles, crayfish, water beetle larvae and water snakes in natural environments. The researcher supported his study with pictures of the host diversity of Hirudo verbana as a parasitic species. These visual data are important in terms of understanding the host organism diversity that Hirudo verbana prefers in natural environments for nutrition and to see the traumatic parasitic effect on host organisms (Figure 2.3-2.9).

Hirudinea taxon was investigated by Kaçmaz (2020) in the inland waters of Edirne province. In this context, leech samples from the Hirudinea taxon were found in 144 of 254 localities examined. Within the scope of the research, 6 species were identified, including *Hirudo verbana*. In the study, the ecological characteristics of the habitats in which the leeches live were also defined.

Ceylan and Erbatur (2012) investigated the eating habits of the therapeutic leech *Hirudo verbana* under controlled conditions. Leeches were fed with recent cow blood. Bulk weighing the following feeding allowed for the measurement of a 97.74% weight gain. Some pleased leeches who had lost some of their ability to move were attacked by malnourished leeches, showing a cannibalistic predisposition. However, cannibalism was not properly demonstrated. Furthermore, some leeches were found to vomit a part of the food even after 2 months of the feeding day. Some leeches were stimulated by the vomited blood, however, after a brief period of investigation, the excited leeches acted disinterestedly and left the area.

Kerimova (2020) investigated the production of the medicinal leech *Hirudo verbana*, fed with cattle and chicken blood, under laboratory conditions. The researcher used heparinized and homogenized chicken and bovine blood for feeding the leeches. The nutrients were given to the leeches in a heparinized and homogenized format. The researcher determined the production and aquaculture performance by giving the blood of two different animals to the medical leeches with two different methods. In this context, 14 cocoons and 211 young Hirudo verbana were reported from medicinal leeches fed with homogenized bovine blood. He collected 14 cocoons and 177 young Hirudo varbana individuals from leeches fed on homogenized chicken blood. It produced 46 cocoons and 370 leeches from medicinal leeches fed with the heparinized bovine blood method. 20 cocoon numbers and 270 young *Hirudo verbana* individuals were collected from leeches fed heparinized chicken blood.

According to the findings of Manav et al. (2019), One of the neglected species in studies on animal reproduction is medicinal leeches. Throughout their investigation, they looked at the effects of giving the *Hirudo verbana* a variety of different kinds of blood on reproduction, growth, and survival. The findings revealed that the kind of blood provided did not influence the growth performance or survival of leeches. Leeches given chicken blood performed 2.5 times better than those fed cattle blood. Gravidity began in both groups in the seventh month; however, while all gravidities in the leeches fed chicken blood occurred within one month, gravidity in the leeches given bovine blood happened within three months.

2.6 Molecular Studies on *Hirudo verbana*

The study conducted by Siddall et al. (2007) exhibited at least three European medicinal leech species. And the leeches marketed as *Hirudo medicinalis* were in fact, *Hirudo verbana*.

The study by Trontelj et al. (2004), showed that a second European taxon based on RAPD molecular markers, *Hirudo verbana*, constitutes a distinct organism. The eight populations' main coordinate analysis and phenetic clustering both showed the same fundamental structure, although taxonomically rather than geographically divided.

Genome sequences were investigated in the study of *Hirudo medicinalis* and *Hirudo verbena*'s two unfinished mitochondrial genome sequences in particular (Annelida, Hirudinea). The DNA sequences were 14,729 and 14,604 base pairs long, but they were missing the non-coding section. They had mitochondrial DNA (13 protein-coding genes, 22 tRNAs, and two rRNAs), but they did not have the non-coding region (Nikitina et al. 2016).

Trontelj and Utevsky (2005) claim that despite the confusion in the taxonomy of the differentiations in morphologic shapes of *Hirudo* spp. many of the latter have been described in the past.

Hirudo verbana was found in Serbia, according to morphological research on the *Hirudo* spp. shown *Hirudo medicinalis* is entirely going extinct in Serbia by Živić et al. (2015). Instead, only specimens of congener *Hirudo verbana* species can be found. Additionally, the analyzed samples were found to be associated with five haplotypes (Živić et al. 2015).

Phylogeography and phylogeny of *Hirudo verbana*, *Hirudo medicinalis* and *Hirudo orientalis* were comparatively studied by Trontelj and Utevsky (2012). Analysis of populations from various regions in Europe, the Caucasus, Asia Minor, and Central Asia was performed using a variety of phylogenetic and population genetic approaches through both mitochondrial (COI and 12S) and nuclear DNA sequences (ITS1, 5.8S, and ITS2). *Hirudo verbana* was the only specie that portrayed clear structure. The Balkans and Italy make up the Western phylogeny group for this species, whereas Southern Ukraine, the North Caucasus, Turkey, and Uzbekistan make up the Eastern phylogeny group. Because the two phylogenetic groups did not overlap, it was clear that distinct lineages had colonized in quite different ways (Trontelj and Utevsky 2012).

The correct interpretation of the conducted study by Živić et al. (2015) brings a significant improvement to the phylogeographic distribution of *Hirudo verbana*. A subdivision of the Eastern and Western phylogeny groups belonging to the *Hirudo* taxon has been definitively supported. The boundary between them passed through the

Balkan Peninsula, where the distribution of the Western phylogeny group roughly coincides with the Adriatic and Aegean drainage basins, and the Eastern phylogeny group roughly coincides with the Danubian drainage basin (Utevsky and Trontelj 2016).

In the study by Saglam et al. (2016), 18 samples were collected of *Hirudo* spp, from six populations from Turkey. Using Maximum Likelihood (ML) and Bayesian Inference (BI) analyses of mtCOI, 12S rRNA, and 18S rRNA gene sequences, their resolved phylogenetic relationships were examined. Then, morphological characteristics were scored. The result showed *Hirudo sulukii* which is a discovery in Turkey. It was identified from Adıyaman Kara Lake, Gaziantep Sülüklü Lake, and Batman Segirkan wetland. Phylogenetic divergence eg, in COI, is 10-14%.

The study conducted by Babenko et al. (2018) investigated salivary cell secretion (SCS). It was said that it is essential for the blood supply of therapeutic leeches. That allows the latter to be used for some medicinal purposes. To salivary cells obtained from three closely linked leech species, *Hirudo medicinalis*, *Hirudo orientalis* and *Hirudo verbana*, the genome and RNA sequencing of the leech species *Hirudo medicinalis* was used.

Differential expression analysis was used to identify genes unique to salivary cells, and the results were confirmed by proteomics. Numerous cell-specific genes were found to encode previously unidentified components of saliva. Furthermore, salivary cells did not exhibit any differences in the expression of genes encoding well-known anticoagulants. The idea of interaction between salivary proteins and hemostasis-related components has been updated as a result of a functional investigation of distinct salivary cell genes (Babenko et al. 2018).

Trontelj et al. (2004) said that the general opinion is that the medicinal leeches in Europe are varieties of *Hirudo medicinalis*, but in their study with RAPD (Randomly Augmented Polymorphic DNA) molecular markers, it was determined that the taxon *Hirudo verbana* is a separate species from the poppular species *Hirudo medicinalis* (Trontelj et al. 2004).

In their article, Elliott and Kutschera (2011) used molecular methodology and stated that the phylogenetic relationships indicated that there are at least two independent medicinal leech lineages closely related to *Hirudo medicalis*. These lineages were *Hirudo verbana* and *Hirudinaria manillensis*. *Hirudo medicinalis* were once assumed to be abundant in Europe. However, now they are considered rare and endangered in many countries. Genetic studies have confirmed that *Hirudo verbana* has been incorrectly marketed as *Hirudo medicalis*. *Hirudo verbana* has most likely already disappeared into the wild. *Hirudo verbana* lacks legal rights, in contrast to *Hirudo medicinalis*. It may be inferred that the loss of wetlands in general, particularly eutrophic ponds and marshes, across Europe is the primary cause of the fall of medicinal leech populations. A drop in amphibians, a vital resource of food for *Hirudo* spp. and essential for the younger organisms has also been brought on by the loss of these aquatic bodies (Elliott and Kutschera 2011)

Siddall et al. (2007) emphasized the mitochondrial sequence and nuclear microsatellite findings that European medicinal leeches consist of at least three species and that the leeches marketed as *Hirudo medicinalis* are *Hirudo verbana*.

Trontelj and Utevsky (2012) and Utevsky and Trontelj (2016) stated that there is not enough information about the biogeography and ecology of medicinal leeches (*Hirudo* sp.). It has been reported that *Hirudo verbana*, *Hirudo medicinalis*, and *Hirudo orientalis* species were detected in the genus of *Hirudo* by mitochondrial and nuclear DNA sequence analysis. It was emphasized that the *Hirudo verbana* species is divided into two sub-phylogroups eastern and western countries (Trontelj and Utevsky 2012, Utevsky and Trontelj 2016).

Siddall and Burreson (1998) used molecular data to investigate the evolutionary relationships of leeches for the first time. A total of twenty-one species from seven of the ten traditionally recognized hirudinean families were studied. Acanthobdella peledina, a branchiobdellid, four oligochaetes, and two polychaetes made up the species. One tree was created by a cladistic analysis of the mitochondrial cytochrome c oxidase subunit I gene. The analysis's monophyletic groupings were found to be largely

congruent with current taxonomic classifications of leeches into higher categories. It has been established that the piscicolidae and Arhynchobdellida have an odd relationship, and the haemopids are classified as Hirudinidae.

Kutschera and Elliott (2014) reported that *Hirudo verbana* Carena 1820, a Mediterranean taxon, was often mistaken for *Hirudo medicinalis* which is known as species of Hirudinea because of its usages. The genetic difference between *Hirudo verbana* and *Hirudo medicinalis*, as determined by CO-I analysis results, was 9.4% using recently discovered mitochondrial DNA sequence data from samples obtained in Germany. As a result, the first *Hirudo* population existed around 10 million years ago. Currently, two geographically distinct biospecies coexist in a small number of natural environments. When the behavior of adult *Hirudo medicinalis* was examined in comparison to that of its sister taxon *Hirudo verbana*, no differences were discovered.

2.7 Studies on the Medicinal Importance of *Hirudo verbana*

Sobczak and Kantyka (2014) claimed that the saliva of medicinal leeches (*Hirudo medicinalis* and *Hirudo verbana*) is widely used in therapy. With over 100 bioactive substances, *Hirudo* saliva has various therapeutic effects. These effects include but are not limited to properties related toanticoagulation, vasodilation, thrombosis, anti-inflammation, and anesthetic. In recent years, leeches have been widely and effectively utilized in veterinary medicine to treat a wide range of animal ailments, particularly those affecting cats, dogs, and horses. The most typical indications for the use of leeches include spinal disorders, scars, related diseases, hip and elbow dysplasia, tendons, ligaments, fascia inflammation, acute and chronic arthritis, and hip and elbow dysplasia. Depending on the size of the animal, leech treatment is thought to be a painless operation that takes between 30 and 120 minutes. Every leech utilized in a medical operation must come from a licensed bio farm. Animals must be protected from microbial illnesses by having sterile maintenance medical leeches cultured, transported, and stored under the proper conditions. In veterinary medicine, hirudiotherapy has also been utilized effectively, particularly when other treatments are ineffective, take too long to take action, or pose a risk to the tissues due to postoperative venous congestion.

In the study by Mumcuoglu (2014), claimed that during each treatment, an average of 1-10 leeches are used. According to the research, an average patient may need two or more treatments per day with a duration that is carried on to up to 6 days. Leech treatment is used up until angiogenesis produces venous capillary return along the wound's edge. When the patient's hemoglobin level is below 8 g/dL, hematological tests should be carried out every 4 hours, and a blood transfusion should be administered.

Siddall et al. (2007) claimed that *Hirudo medicinalis* has a lot of benefits that can contribute to new treatment methods. The United States Food and Drug Administration has approved *Hirudo medicinalis* as a prescription medical device despite the historical relevance of many protease inhibitors purified from leech saliva. It should be highlighted that correct species identification is necessary for the description of bioactive chemicals. Uniformity within a model type employed in laboratory settings is also necessary for the interpretation of developmental and neurophysiological aspects.

Abdullah et al. (2012), Hirudotherapy (HT) looked into the benefits of *Hirudo* species for treatment. That is one of the oldest tools to be used by various medical practitioners. Methodologically, HT involves attaching culture leeches to the injured places. Gastrointestinal disorders, dermatology, and gynecological abnormalities have been added to the scope where HT is being used newly. More recently, new applications have been found in hypersensitivity conditions such as cancer therapy, asthma, male/female infertility, and diabetes. Based on the presented facts, it can be said that HT efforts are made to optimize the benefits of medical leech treatments in clinics.

The etymology of the word "leech" and the practice it tries to establish were investigated by Robert et al. (2000). Their study revealed interlinked social histories. In this study, the linguistic and medical history of the word "leech" were examined.

Jahangir et al. (2016) claimed that leech therapy has been accepted worldwide because of its effect on transplantation. However, its effectiveness goes beyond dermatological implications and extends to a holistic approach. Its reputation comes from saliva filled with bioactive enzymes. With over 3000 skin diseases present. These conditions are

considered to impose significant damage on body health. Not only that, but they also affect physical, emotional, and financial consequences. Hence, leeches can be considered a support for various dermatological diseases.

Various treatments are applied to control the symptoms of osteoarthritis and prevent the progression of the disease. One of these treatments is hirudotherapy. In this context, a 68-year-old female patient, who has been receiving hirudotherapy treatment for 6 years due to unbearable pain in both knees. Following hirudotherapy, a serious skin infection due to *Acinetobacter* sp. developed. They reported that this *Acinetobacter* infection that occurred following hirudotherapy was the first record for Turkey (Gönen et al. 2013).

Alam et al. (2016) reported that leeches were utilized by ancient Egyptian, Indian, Greek, and Arab doctors to treat a range of illnesses. These included both conventional practices and systemic conditions like skin disorders, nervous system abnormalities and many diseases.

Elliott and Kutschera (2011) emphasized that over-collection of wild *Hirudo medicinalis* leeches in Europe has led to population decline and the need for other species, particularly those associated with *Hirudo verbana*.

2.8 Some Taxonomic Studies on the Medicinal Leeches Species

A study was conducted by Elliott and Tullett (1984) that analyzed the status of *Hirudo medicinalis* in European countries, particularly in the British Isles. In the study, it was stated that the existence of medicinal leeches is known in many European countries, but the status of medicinal leeches in Belgium, Portugal, and Turkey is uncertain. It has been reported that medical leeches are likely to be found in Turkey's wetlands.

Neubert and Nesemann (1999) reported that due to the taxonomic confusion of *Hirudo verbana* with *Hirudo medicinalis*, the ecology of the species is not known enough and it is distributed in the Balkans, Greece, Turkey, and Eastern Mediterranean countries. It has been reported that *Hirudo verbana* is found in floodplain forests in the northwest of Turkey, which is caused by floods caused by small rivers.

Demirsoy et al. (2001) wrote about the ecological and geographical distribution (Kasparek et al., 2000) of studies conducted in Turkey on behalf of *Hirudo medicinalis*, it was emphasized that the leeches were highly likely to be *Hirudo verbana*.

Kutschera (2006) reported that although the European conservation legislation (Bern Convention, EU Habitats Directive, CITES, and IUCN) protected the *Hirudo medicinalis* species, the *Hirudo verbana* species was not included in any protected status. It has been emphasized that *Hirudo verbana* may be among the rare species shortly due to over-collecting, intensive exportation, and habitat destruction, therefore, the species should be taken under protection status urgently. It has been stated that the studies needed on the ecology of *Hirudo verbana* populations in nature will contribute to the management and conservation processes.

A total of eight leech species were identified by Kazanci et al. (2015) from various rivers in Turkey (Yeşilırmak River, Yedigöller National Park, Büyük Menderes River, Acıgöl Lake) and lakes (Işıklı Lake, Beyşehir Lake, and Karamuk Lake). Among these species is *Hirudo verbana*. In this process, parameters of these fields such as temperature, pH, dissolved oxygen, conductivity, nitrite, ammonium, orthophosphate, and sulfate were defined. According to the results of this study, *Hirudo verbana* lives in alpha-mesosaprobic streams and oligotrophic, mesotrophic, eutrophic and polytrophic lakes.

Utevsky et al. (2010) investigated the distribution of *Hirudo* leeches. In the study, Switzerland, Austria, Germany, Italy, Slovenia, Croatia, Bosnia and Herzegovina, Serbia, Montenegro, Macedonia, Greece, Hungary, Moldova, Ukraine, Russian Federation, Uzbekistan, and Turkey, located in the south of the geography where *Hirudo verbana* and *Hirudo medicinalis* species are distributed. It has been stated that the *Hirudo verbana* species survives in some parts of Central Asia by adapting to arid conditions. Considering their geographical location, it is stated that ecological factors rather than historical and anthropogenic effects play a role in the distribution of *Hirudo* leeches.

Elliott and Kutschera (2011) reported that quantitative information about medicinal leeches in nature is quite limited, whereas laboratory studies are relatively more abundant. Habitats of medicinal leeches are typically defined as eutrophic lakes with mud substrate and littoral vegetation.

Buczyński et al. (2014) investigated the presence of the medicinal leech, *Hirudo medicinalis* in bird nests. The presence of medicinal leeches was detected in the nests of 4 of 11 bird species. In addition, cocoons, the reproductive material of medicinal leeches, were also found in bird nests. It has been predicted that leeches' preference for bird nests may be a result of their search for safe areas against predators and human pressure, especially fish.

Trontelj and Utevsky (2005) find *Hirudo nipponia* in East Asia. Also, they said that *Hirudo orientalis* is easily identified by its coloring patterns from Transcaucasia and Iran. Also, *Hirudo verbana* is a species, which is present in Turkey and Southeast Europe that is currently mostly farmed in leech farms.

Kutschera (2007), collected specimens from leeches in Turkey and they were kept in artificial ponds at ZAUG Blutegelzucht in Biebertal, Germany. About 1000 samples of black-pigmented leeches were regularly examined. Based on a few criteria, these black medicinal leeches are here, *Hirudo verbana* defined as nigra. Low-density hybrids were seen among these variations.

Kutschera and Roth (2006) said that, although *Hirudo verbana* was utilized medicinally in Europe, little is known about its biology and ecology related to reproduction. In this study, the cocoon-forming mode was defined and the behavior of young specimens was examined in how adult samples responded to low temperatures (5–10 °C). Individuals of *Hirudo verbana* momentarily group as thick clumps in cold soil or water. In this species of leech, aggregation is adaptively significant for individual survival.

Kutschera and Shain (2019) write that in one of his publications, Lamarck documented every species of leech known at the time and coined the word "Hirudinea." In this

paper, the life and accomplishments of Lamarck are discussed about the genus *Hirudo* of leeches. The article provided information on the development of these parasitic annelids, including their origin (somewhere in Asia, 15–20 million years ago), formation, patterns of speciation, systematics, and specific use.

Todorov et al. (2016) found that there remained uncertainty over the taxonomic validity of the many morphological variants of these leeches up to the end of the 20th century. In Europe, it was widely believed that all types of therapeutic leeches belonged to the same species, called *Hirudo medicinalis*. Lately, the using of molecular mechanisms in medical leech research has guided it to new positions in their taxonomy of *Hirudo* spp., and four species with clearly defined and non-overlapping ranges have proven their existence in the Western Palearctic. For the explanation of the taxonomic information of *Hirudo* spp. in Bulgaria, 56 areas were observed, and recorded samples were analyzed using morphological and karyological methods during the period 2005-2014.

In this study by Ceylan et al. (2015), the reproduction of *Hirudo verbana* was investigated. A positive correlation was defined between the weight of the rootstock individuals and the number of cocoons released and the cocoon size. It was determined that the reproductive efficiency of the *Hirudo verbana* species was related to rootstock weight. In this context, the researchers said that rootstock leeches must be protected to use leeches in farm production and to protect natural leech environments.

Saglam et al. (2018) said the density changes and some water quality parameters of *Hirudo verbana* were investigated monthly in Akpınar Marshes in Eastern Anatolia for one year. *Hirudo verbana* specimens were collected using a modified unit square design and unit time collection method as an indicator of population density. Some water parameters such as water temperature, dissolved oxygen, pH, some cationic elements (lithium, sodium, ammonium, potassium, magnesium, calcium), and anionic elements (fluoride, chloride, sulfate, nitrate, phosphate, bromide) were determined. These parameters were analyzed to determine their effects on the population density of this medicinal leech. *Hirudo verbana* populations are most intense in May-June; The species was found in minimum numbers in November and hibernate in December-March. A

significant correlation ($p < 0.05$) between the number of medicinal leeches per unit area and monthly water temperature, ammonium, and fluoride content was determined using the Pearson correlation coefficient.

The study of Sağlam (2011), medical leech samples in the Samsun region were examined. Also, leech trade quotas were examined. Medicinal leeches in the Kızılırmak delta and its basin were identified as *Hirudo verbana* species.

According to Saglam et al. (2016), there are now five *Hirudo* species recognized in Eurasia, with Turkey being one of the largest exporters of the medical leech, especially *Hirudo verbana*. In this study by Saglam et al. (2016), also aimed to define the range of *Hirudo* species in Turkey. For this reason, 18 samples from six different groups were gathered from around the country. Following dorsal and ventral dissections, morphological traits were evaluated, and phylogenetic relationships were determined by analyzing mitochondrial cytochrome c oxidase subunit I (COI), 12S ribosomal RNA (rRNA), and nuclear 18S rRNA gene segments. A new species of therapeutic leeches called *Hirudo sulukii* has been discovered in Turkey's Segirkan Wetland in Batman, Kara Lake in Adiyaman, and Sülüklü Lake in Gaziantep.

Kasparek et al. (2000) surveyed all of the major possible habitats in western Turkey. It demonstrated that medical leeches, *Hirudo medicinalis* are not uncommon but rather widespread throughout the nation. It also demonstrated that they may be found in nearly all appropriate settings. However, the species does not appear to be present in the country's big river deltas in the south (Çukurova deltas, Göksu delta). This might be due to zoogeographic factors (Taurus mountains barrier). This quick evaluation in a wide number of wetlands was made possible by the use of a semi-quantitative survey approach based on collection efficiency (the number of leeches gathered per hour by a single person). The latter revealed that leech density varied significantly from wetland to wetland, allowing a ranking of Turkish wetlands based on their value for therapeutic leeches to be produced. The largest populations were found in inner and south-west Anatolia (Lakes Eber and Karamik, and Sultan Marshes) and also along the Black Sea coast (Kizilirmak delta, Yeşilirmak delta, and Karagöl Marshes near Sinop). This study

also revealed that commercial exploitation for the pharmaceutical business and other purposes occurs in only a few locations and does not appear to have a significant impact on the population. Nonetheless, many populations are threatened by habitat reduction.

Another study, conducted by Akbulut et al. (2012), sought to establish the yearly export limit for *Hirudo medicinalis* (the medicinal leech), which is traded worldwide under the framework of the CITES Convention, to which the nation is also a signatory, more about and routinely monitor the existing population. 18 distinct wetlands with significant *Hirudo medicinalis* potential were investigated, and it was discovered that the Kızılırmak Delta and Yeşilırmak Delta are the regions where nearly all medicinal leeches shipped from Turkey are gathered. Wetlands, particularly shallow wetlands with dense surfaces and submerged macrophyte flora were surveyed for habitats that may harbor therapeutic leeches. The levels of *Hirudo medicinalis* discovered in these wetlands at various times were studied in terms of density.

Ceylan and Çetinkaya (2021) researched the populations of Mediterranean medicinal leeches (*Hirudo verbana*) living in marshes around Lake Eirdir (Turkey). Removal methods were employed to determine population size, and age classes in leeches were estimated for the first time. Bhattacharya's approach was used to determine the body length frequencies. Three age groups were recognized, with the 0+ age group dominating with 78.6%. 89.9% of the population weighed less than 1g. The broodstock was estimated to account for just 7% of the total body weight. This research found that the *Hirudo verbana* communities in the marshes around Lake Egirdir are reasonably well conserved. It also suggests that there is no danger to the long-term viability of the therapeutic leech communities.

According to Ceylan (2020), report, medical leeches can be utilized to satisfy urgent needs in the medical, veterinary, pharmaceutical, and cosmetic industries. But these leeches are a threatened species. Uncertainty surrounds the reproduction processes of therapeutic leeches. The purpose of the current study was to ascertain how reproductive age affected the ability of therapeutic leeches to reproduce. The findings revealed that the youngest maternal leeches have better reproductive success. Furthermore, it was shown that younger leeches produced more progeny. As a result, as the age of the

leeches forming the cocoon grew, so did the number of cocoons from which no leeches were created, the rate of abnormalities in cocoons, and the morphological abnormalities of offspring.

Ceylan et al. (2019) investigated the effects of rootstock leech density on the reproductive efficiency and survival of *Hirudo verbana* in this study. 93 pregnant leeches with a weight of 5.94±1.06 g were stored in 2L pet jars containing moist peat in 5 different densities (1, 2, 4, 8 and 16 leech L-1). The highest breeding performance was obtained at 2 and 1 leech L-1 stocking densities. There were deaths as a result of cannibalism in the L-1 leech groups of 8 and 16, which are the densest groups. As the rootstock density increases, deformations occur in the cocoons, which causes drying during the incubation period, embryo deaths and a decrease in the number of offspring. With the results of this study, it was determined that high rootstock leech density caused a significant decrease in reproductive efficiency and cannibalistic mortality in *Hirudo verbana* production.

Ceylan et al. (2015) in the research, the authors assessed the reproductive effects of the therapeutic leech *Hirudo verbana*. The reproductive effort of *Hirudo verbana* is considerable, and it can replicate even after losing 70% of its initial weight. The mature individual weight of the medicinal leech is directly inversely correlated with reproductive success. In this context, heavier rootstock leeches should be selected and used in artificial production areas. However, to preserve natural populations, the collection of heavier leeches from native environments must be forbidden.

The study performed by Saglam (2019), investigated to the morphological components on the inside and outside of the two therapeutic leeches, *Hirudo sulukii* and *Hirudo verbana*. Between 1995 and 2016, *Hirudo* spp was gathered from different locations in Turkey for the latter purpose. The leeches were initially studied while still alive, then shocked with 10% ethyl alcohol, with 70% ethyl alcohol for fixing or fixed in 4% formaldehyde. The leeches were then dissected, and the internal morphology was examined using a stereo microscope. Both types of leeches appeared to have flattened dorsoventral surfaces. On the dorsolateral part of the body of *Hirudo sulukii*, there are black, segmentally structured unitary ellipsoid and elongated dots, as well as two zigzag

black longitudinal lines. The ventral skin of *Hirudo sulukii* is kinda between green to brown in hue with a few random black spots. *Hirudo verbana*'s dorsal skin is coated in broad, diffuse orange stripes. *Hirudo verbana* may be differentiated from other species from the rubble of dark ventrolateral lines ranging in hue from green to yellow. As a result of the study, it was defined that these two leech species have completely different morphologies from each other.

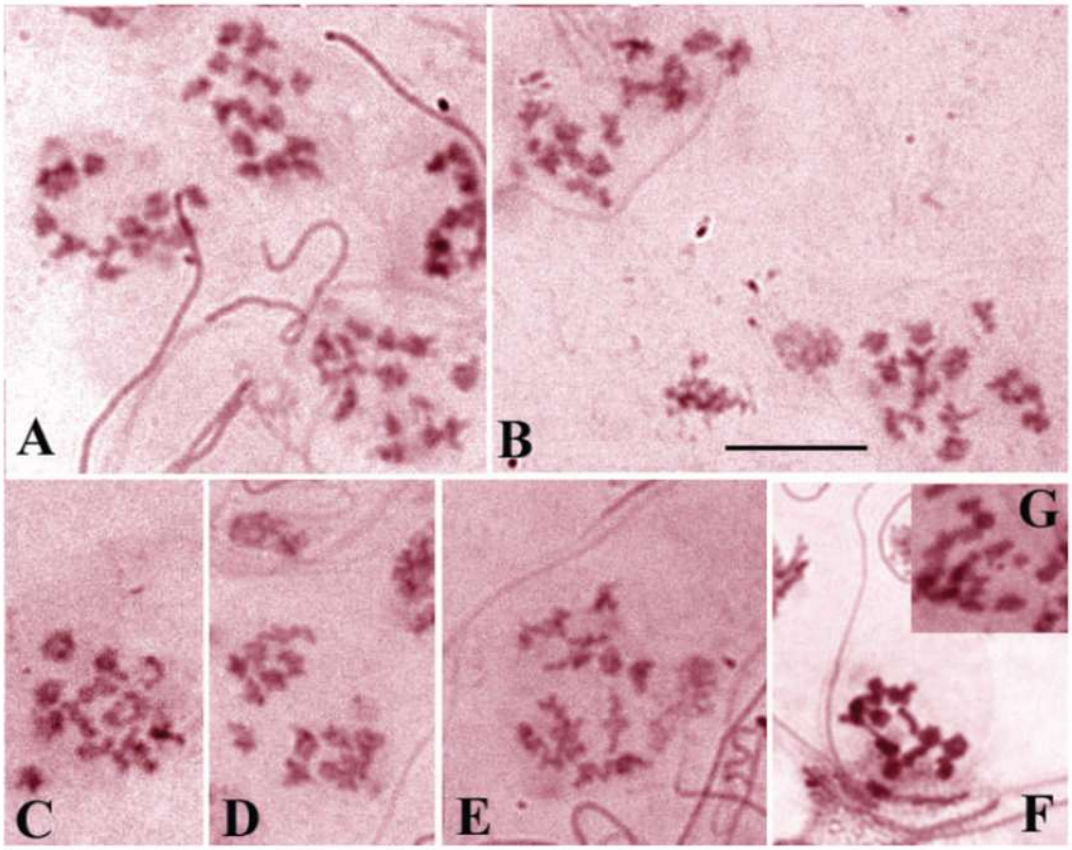

Figure 2.1 *Hirudo verbana*: A-E – Diakinesis phase, F-G – Metaphase I plates. Chromosomes can be easily counted as n=13. Scale-bar (A-G): 10μm (Utevsky et al. 2009).

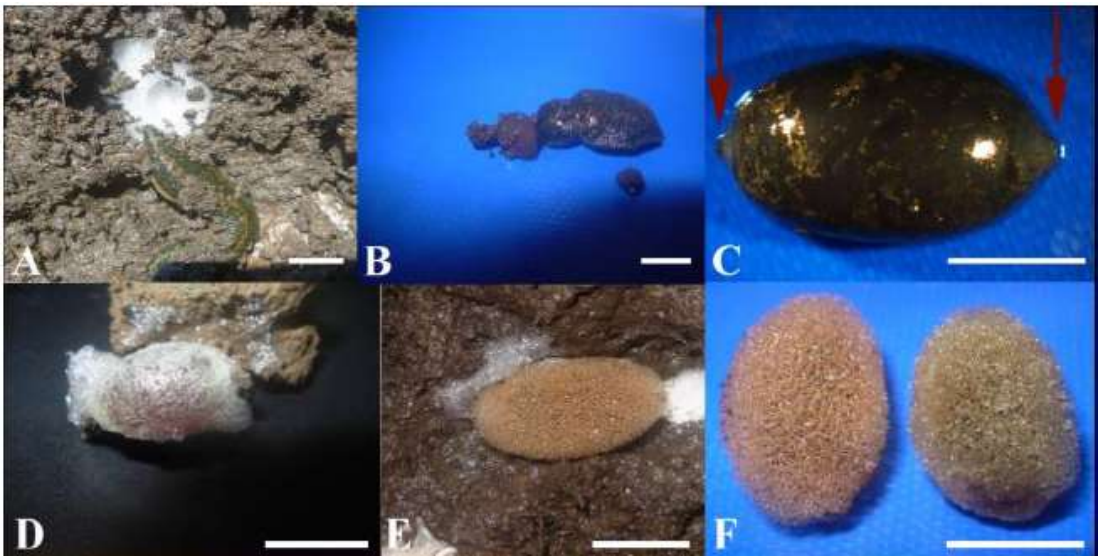

Figure 2.2 Cocooning stages: A: *Hirudo verbana* sample that secretes foamy. B, C: *Hirudo verbana* sample that cocoon occurrence. D, E: Strong cocoon. F: deposited cocoons. (Ceylan et al. 2015).

Figure 2.3 Feeding *Hirudo verbana* from a fish (Ceylan 2016).

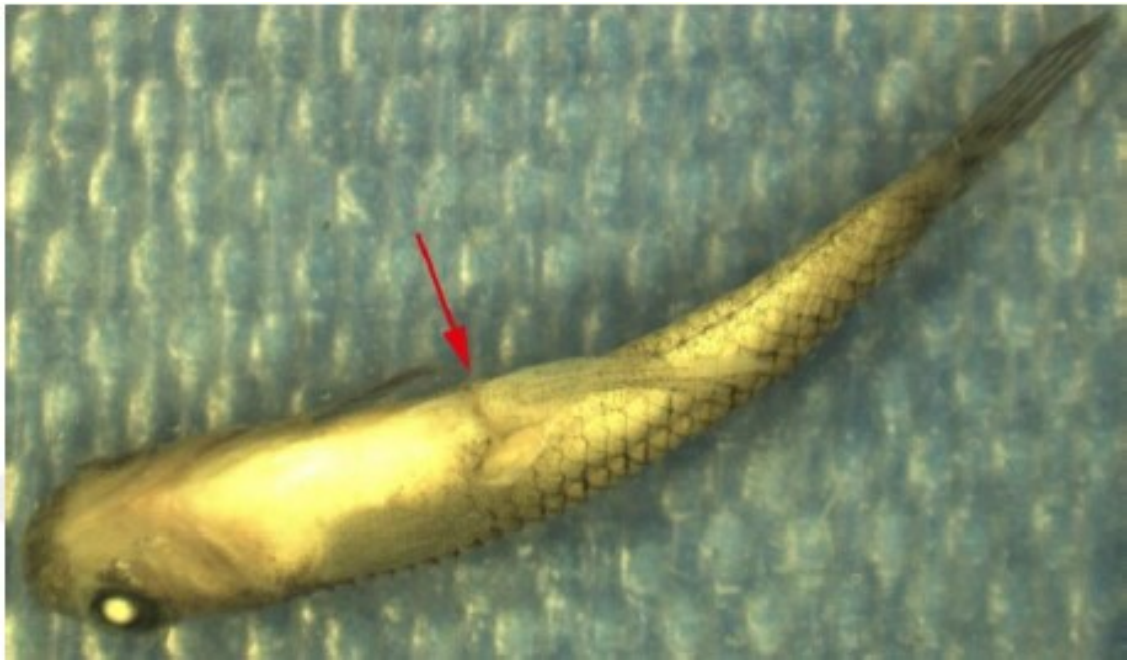

Figure 2.4 "Y" shaped leech jaw section, ventral region of a host fish (Ceylan 2016).

Figure 2.5 *Hirudo verbana* feeding on a frog tadpole larva (Ceylan 2016).

Figure 2.6 A dead frog with leech jaw Marks, ventral side (Ceylan 2016).

Figure 2.7 Blood leakage on the leg of a lamb after the feeding process of the leech (Ceylan 2016).

Figure 2.8 A case of *Hirudo verbana* parasitism on the left leg of a spotted turtle (Ceylan 2016).

Figure 2.9 *Hirudo verbana* feeding from a broken chelicerae base of a crayfish (Ceylan 2016).

3. MATERIALS and METHODS

3.1 General Characteristics of Lake Karamık

Lake Karamık is located in the inner part of the Aegean Region, opening the province of Afyonkarahisar (Figure 3.1). Lake Karamık is situated in the southern region of the tectonic origin basin, which extends north-south to the east of the Sultandağları and west of the Kükürt Mountain (Ozdamar and Kiyak 2017).

The lake measures 16 km in length from northeast to southwest, 7.8 km in width from east to west, and 1002 m in height. Lake Karamık has a surface size of around 56 km^2. The drainage area of Lake Karamık is 342 km^2 (Figure 3.2). The lake is quite shallow between two and three meters and eutrophic (Hasbek and Arı 2018).

The movements of the Alpine tectonic caused formed the Lake Karamık. The faulting motions brought on by Alpine tectonic movements in the Upper Oligocene led to the formation of Karamık Lake. A pit was created along the faults around the western, northern, and northeastern portions of the Sultandalar and the southern and southwestern portions of the Emirdalar during the area's paroxysm phase of tectonic activities in the Upper Oligocene. Southwest of this pit, Karamık Lake was created. An area of gravel, sand, and partly clay were developed on the northern borders of the Sultan Mountains in the basin's south (Atalay 1977).

The area in question participated in the Alpine Orogeny as a solid mass, partial faulting occurred in Emirdağ and Sultandağları in the later stages of the mentioned orogeny, and as a result of all these, while Sultandağları and Emirdağlar were rising, Karamık Basin collapsed and formed; it has become very close to what it is today (Atalay 1977).

Lake Karamık feeds Eğirdir Lake from under the Karakuş Mountains with two sinkholes. These sinkholes, which provide water circulation in the form of a natural formation, were closed using regulators because of the decrease in the water amount of Lake Karamık (Taş and Yakar 2010).

3.2 Collecting of *Hirudo verbana* Samples

The *Hirudo verbana* samples used in the study were collected from Lake Karamık. For the aim, it was made some noise in the water to collect the leeches which were coming to the place towards the activity area, clinging to their boots and the board of the boat. After the *H. verbana* samples were caught with a plastic scoop, they were transferred to plastic bottles in containing medium water (Figure 3.3). *H.verbana* samples brought to the research laboratory were fixed at 37C in 70% ethanol and then stored in a 95% ethanol medium for anatomical, morphological, and molecular analyzes (Figure 3.4).

Species identification of the leech samples were defined according to their anatomic and morphological marks using an Olympus SZ30 stereomicroscope, an Olympus CH20 light microscope, and a ZEISS Axio Imager microscope (Figure 3.5 and Figure 3.6) (Neubert and Nesemann 1999).

3.3 DNA Isolation

The all leeches were stored in 95% ethanol at room temperature until used for total DNA extraction. 16 samples of *Hirudo verbana* were selected for the DNA extraction (Figure 3.7). Their weight and height were measured before starting the processes (Figure 3.8 and Figure 3.9). Each of the samples' caudal suckers was removed and used for all analyses (Figure 3.10 and Figure 3.11). The posterior sucker was used for the DNA of the leech samples using a Gene Matrix Tissue kit (Int Ref. 2).

Other parts of the samples of *Hirudo verbana* were preserved in an ethanol medium (Figure 3.12). DNA extraction was done by partially modifying the procedure of Trontelj and Utevsky (2005) which include the following steps:

1. In 1.5mL Eppendorf tubes, the samples stored in 95% ethyl alcohol buffer were transferred.
2. The tubes were filled with 500 ml of solution I, which is composed of (pH 8, 50 mM Tris-HCl, pH 8, 20 mM EDTA, and 2% SDS).

3. Proteinase K (20 mg/ml) was added after the tissue had been thoroughly homogenized using a sterile micro pestle, and the mixture was quickly vortexed. The sample was incubated for two hours at 55 °C with periodic mixing in a water bath.
4. After incubation, the sample was chilled for 10 minutes on ice before 250 ml of solution II (6 M NaCl) was added and properly mixed.
5. The tube was centrifuged for 15 minutes at 8000 rpm after cooling on ice for 5 minutes.
6. To precipitate the DNA, double the amount of 100% AR grade ethanol was used.
7. The supernatant was collected without contacting the pellet after the precipitate was centrifuged at 8000 rpm for 5 minutes.
8. The DNA pellet was centrifuged at 11,000 rpm for 5 minutes after being washed with 500 ccs of cold ethanol. A pipette was used to gently extract the supernatant (top) and drain any extra liquid.
9. On a heating block at 55 °C, the pellets (bottom) were partly left to dry (ethanol-free) while the lid was locked.
10. Depending on the size of the pellet (100 mL on average), the pellet (bottom) was gently pipetted with a large diameter filter tip until dissolved in 50–200 mL of fresh, sterile water. The polymerase chain reaction used the template from this dispersed DNA.

3.4 Polymerase Chain Reaction (PCR)

After obtaining targeted DNA isolation, to amplify the approximately 700 base region of 18S rRNA and mtCOI one-step PCR was done. Solis Biodyne (Estonia) FIREPol® DNA Polymerase Taq polymerase enzyme was added to carry out the PCR reaction. Primers C and Y were used for the 18S region targeted in the PCR study (Apakupakul et al. 1999). Also, Primers LCO 1490 and HCO 2198 (Table 3.1) were used for the mtCOI region targeted in the PCR study (Folmer et al. 1994).

The propagation media properties of Solis Biodyne (Estonia) DNA Polymerase applied in the PCR process are listed below. PCR content was applied in 25 ml volumes at the following rates:

PCR Buffer (10 X-1 X)
MgCl2 (25 mM-1.5 mM)
dNTP mix (20 mM-0.2 mM)
F and R Primers (10 µM- 0.3 µM)
Taq DNA Polymerase (5U/ul- 2 U)
DNA template (3 µl) It was completed to 35 µl with PCR grade water.

For PCR amplification performing, buffer, Mg2+, dNTP, primers, Taq polymerase, DNA template, and HO, were mixed to make a 25 µL mixture in the tube. According to the results of the preliminary experiments, the annealing temperature of the primers was optimized. The PCR cycler conditions used were initial denaturation at 95 °C for 5 minutes, 40 cycles at 95 °C for 45 seconds for the duration, 57°C for 45 seconds for annealing, 72 °C for 1 minute for extension, lastly, for final extension tempter were set at 72°C and for 5 minutes. The results acquired from amplification by PCR were carried out in 1.5% agarose gel which was mixed with 1x TAE buffer at 100 volts current for 90 minutes and their images were taken in UV light using ethidium bromide dye.

3.5 Agarose Gel Preparation and Electrophoresis

A 100 mL loading buffer solution was prepared to be used in electrophoresis-gel medium reactions. Solution components: 10 X PCR buffer, 25 mM MgCl2, 20 mM dNTP mix, 10 µM F. Primer, 10 µM R. Primer, 5U/ µl Taq DNA Polymerase, 3 µl DNA template (Int Ref. 4).

1.5 g agarose, 100 mL1 X TAE and EtBr were added to prepare a 1.5% agarose gel in 100 mL volume. For the mixture to dissolve well, it was gently shaken and placed in the microwave oven, and the flask was removed from the microwave and shaken at intervals until its contents melted. After the molten agarose solution was cooled to 55 °C at room temperature, EtBr was added and shaken, and dispersed into the solution. Agarose was poured slowly into a horizontal electrophoresis cassette with a 10-well comb and allowed to polymerize for 30 minutes. The comb was removed from the polymerized gel and made ready for use (Int Ref. 3).

The agarose gel was placed in a horizontal electrophoresis tank containing 1XTBE gel buffer and filled with TBE to cover the gel. For sample loading, a small parafilm was taken and loading buffer (approximately 1 µl) for each sample was placed on the parafilm, and each of them was mixed with 5 µl of sample loading buffer and loaded onto the gel.

The PCR product size was determined by adding a DNA standard marker (GeneRuler DNA ladder mix, Catalog No: SM0338Fermentas) to the first well and turned on at 100 V for 90 minutes until it was 6 cm away from the well. The gel was measured and photographed under the UV trans-illuminating gel doc system v3.1 (ABI, Abilene, TX) after stopping the reaction.

It was checked that the band size of the PCR products belonged to the desired gene regions by comparison with the DNA standard criterion. The purification kit MAGBIO "High Prep™ PCR Clean-up System" (AC-60005) procedures were followed for purifying the single band samples of the PCR product (Int Ref. 2).

3.6 Sequencing, Data Analysis and Phylogenetic tree

For COI locus, purified PCR isolates of Hirudo verbana directly with primers LCO 1490 and HCO 2198 using the ABI 3730XL Sanger sequencing device (Applied Biosystems, Foster City, CA) and the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) (Macrogen-Holland) sequencing has been done. Nucleotide sequences were aligned with the help of Mega 7.0 multi-sequence alignment ClustalW2 software and manually adjusted (Int Ref. 5).

The FASTA program (EMBL; http://www.ebi.ac.uk/Tools/fasta33/nucleotide.html) and the BLAST algorithm were used for the homology search of 18S rRNA gene sequences. Tamura Nei's two-parameter model with a bidirectional deletion method in Mega 7.0 was used to calculate genetic distances (Tamura et al. 2011). In the phylogram analysis (Table 3.2), the Maximum-Likelihood (ML) method was used in Mega 7.0 (Newman et al. 2016).

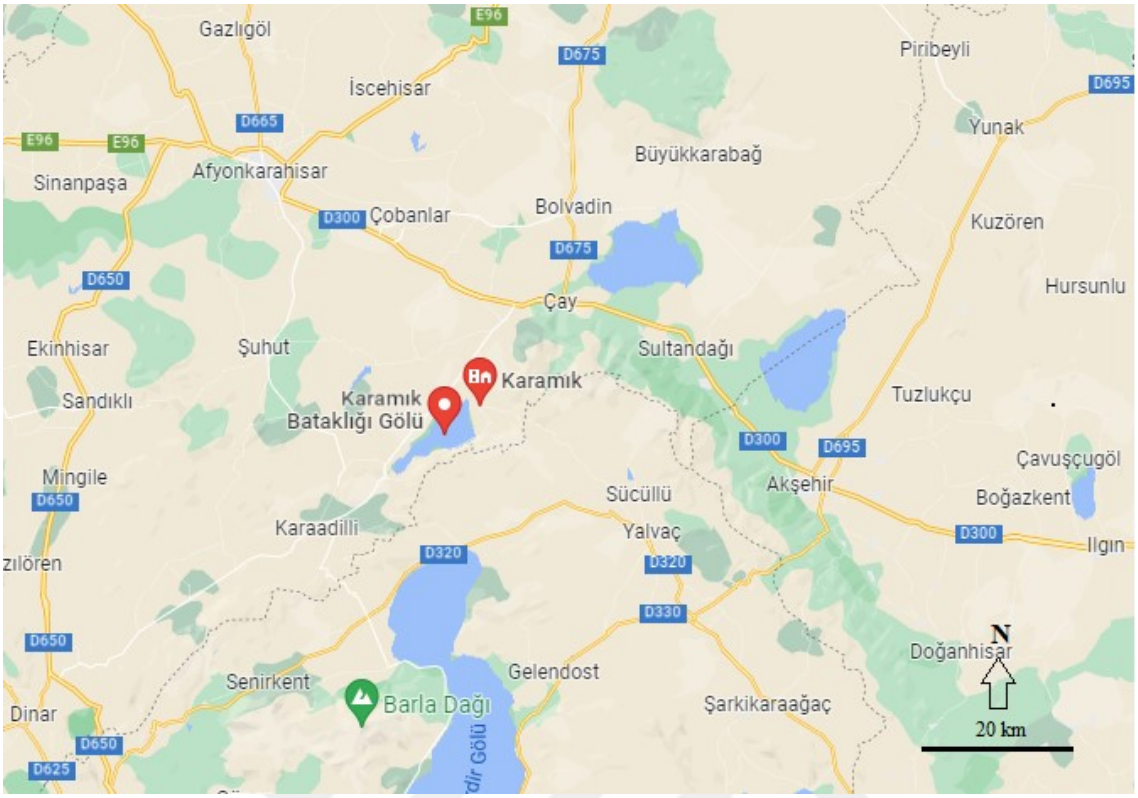

Figure 3.1 Map of Lake Karamık (Int Ref. 3).

Figure 3.2 Koçbeyli Location in Lake Karamık (original).

Figure 3.3 Collecting of *Hirudo verbana* samples from Lake Karamık (original).

Figure 3.4 Relaxing and fixation of the specimens in warmish ethanol medium (original).

Figure 3.5 Examination of *Hirudo verbana* specimens with a stereo microscope (original).

Figure 3.6 *Hirudo verbana* specimens flattened from dorso-ventral side (original).

Figure 3.7 General view of *Hirudo verbana* samples used in the study (original).

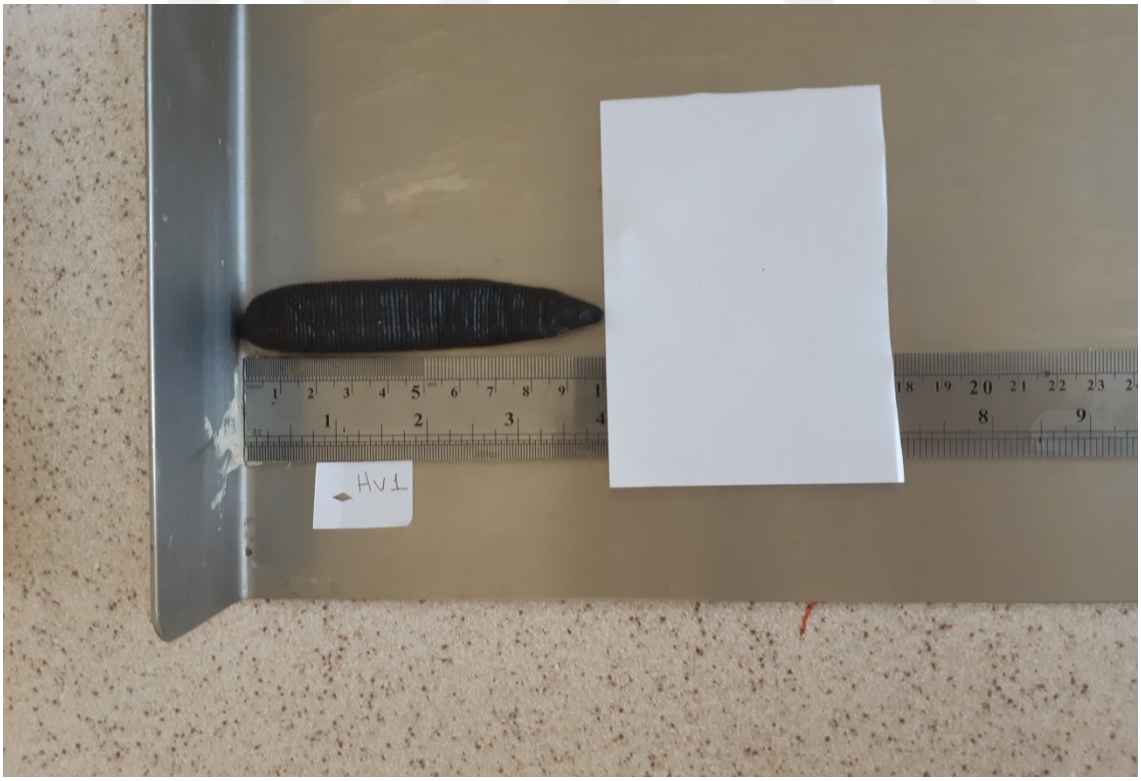

Figure 3.8 Height measurement of *Hirudo verbana* specimens (original).

Figure 3.9 Weight measurement of *Hirudo verbana* specimens (original).

Figure 3.10 Tissue dissection for isolation of DNA from a *Hirudo verbana* sample (original).

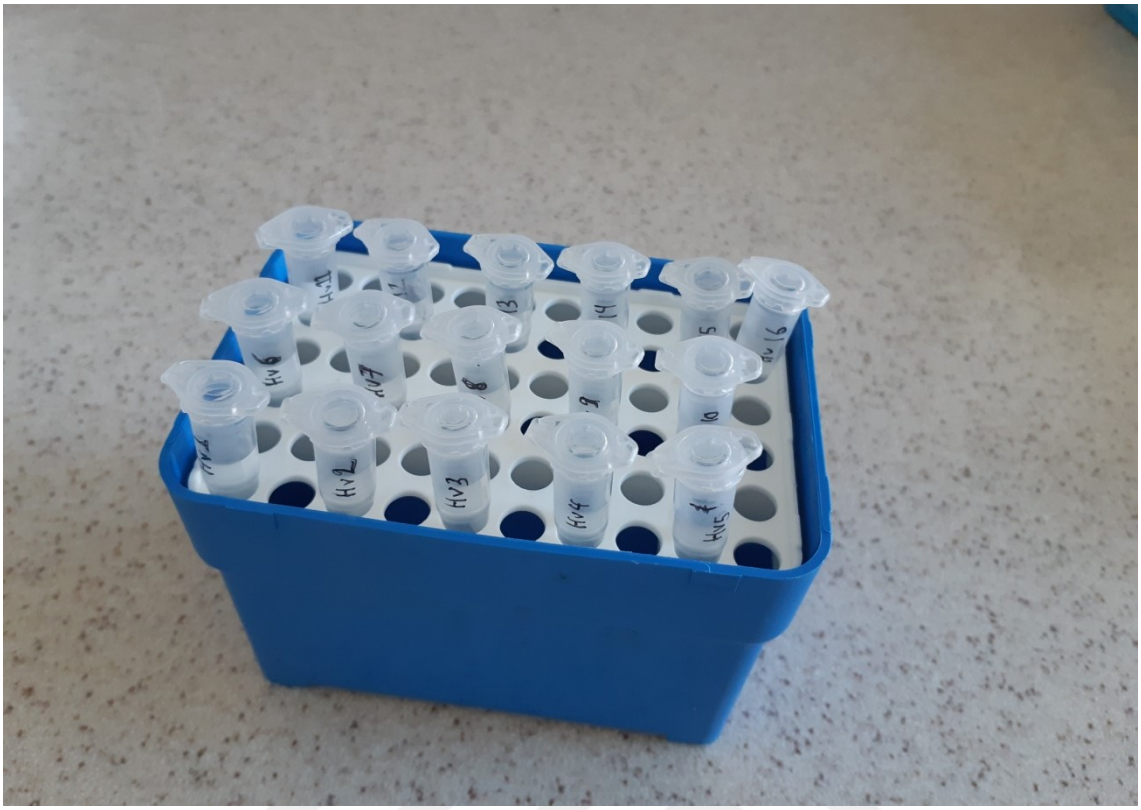

Figure 3.11 The tissues taken from *Hirudo verbana* samples for DNA isolation (original).

Figure 3.12 Samples of *Hirudo verbana* preserved in ethanol medium (original).

Table 3.1 Primers used for amplification and sequencing of mt COI and 18S rRNA gene loci at *Hirudo verbana* specimens.

Target gen	Primers	Sequence (5′–3′)	References
mtCOI	LCO 1490 HCO 2198	GGTCAACAAATCATAAAGATATTGG TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)
18S rRNA	C Y	CGGTAATTCCAGCTCCAATAG CAGACAAATCGCTCCACCAAC	Apakupakul et al. (1999)

Table 3.2 Data of *Hirudo* spp isolates used in phylogram analysis.

Species	Gene locus	NCBI Numbers	Locations	References
Hirudo verbana	mtCOI	OM010329 OM010332 OM010333 OM010334 OM010335 OM010336 OM010337 OM033510	Karamık Lake, Turkey	This study
Hirudo verbana	mtCOI	AY763151	**Sežana, Slovenia**	Trontelj and Utevsky (2005)
Hirudo verbana	mtCOI	JN083798	Odesa Region, Ukraine	Trontelj and Utevsky (2012)
Hirudo verbana	mtCOI	AY763150	Lecce, Italy	Trontelj and Utevsky (2005)
Hirudo verbana	mtCOI	EF446694	Lake Ohrid, Macedonia	Trontelj and Utevsky (2012)
Hirudo verbana	mtCOI	KU216244	Lake Uluabat, Bursa,Turkey	Sağlam et al. (2016)
Hirudo verbana	mtCOI	KT692947	Lake Balik, Samsun, Turkey	Sağlam et al. (2016)
Hirudo verbana	mtCOI	JN083801	Izmir, Turkey	Trontelj and Utevsky (2012)
Hirudo medicinalis	mtCOI	AY763148	Saale River, Halle, Germany	Trontelj and Utevsky (2005)
Hirudo orientalis	mtCOI	KY989460	Urmia, Iran	Darabi-Darestania et al. (2018)
Hirudo sulukii	mtCOI	KU216240	Lake Suluklu, Gaziantep, Turkey	Sağlam et al. (2016)
Hirudo nipponia	mtCOI	GQ368749	Korea	Phillips and Siddall (2009)
Hirudo troctina	mtCOI	AY763155	Marrakech, Morocco	Trontelj and Utevsky (2005)
Erpobdella montezuma **-Out group**	mtCOI	GQ368760	Arizona, USA	Phillips and Siddall (2009)

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4. RESULTS

4.1 Anatomical and Morphological Characteristics of *Hirudo verbana* Specimens

Within the scope of the research 16 *Hirudo verbana* specimens were examined. The largest specimen was 10.3 cm in length and 6.80 g in weight, while the smallest specimen was 3.8 cm in length and 0.26 g in weight were measured (Table 4.1). The anatomical and morphological features of the samples were defined as follows: The body shape of *H. verbana* is slightly flattened dorso-ventrally side. The body consists of segments. The medicinal leech has reddish-orange rosary-like or narrow black mottled stripes on its back. Paramedian lines are broad and orange. The ventral portion has yellowish-green pigmentation with black or dark brown stripes on the sides. Between these two dark bands is a light-colored region without spots (Figures 4.1, 4.2, 4.9).

Hirudo verbana samples contains oval suckers at the anterior and posterior ends. The caudal sucker is larger than the anterior sucker but does not exceed the maximum with the body (Figure 4.3, Figure 4.4). Five pairs of eyes form black dots shapes on the anterior-dorsal terminal part. Each pair of eyes is in the same ring. The eyes are arranged in an arc symmetry (Figure 4.5). The mouth is located in the center of the anterior sucker. The mouth is followed by a muscular pharynx. The medicinal leech has three keratinized jaws located within 2-3 mm of the anterior sucker. The jaws have fine teeth on the edges (Figure 4.6 and Figure 4.7). The stomach (mid-intestine), which is the largest part of the digestive tract. The stomach is in the form of a wide tube with a thin wall. There are 11 sac pairs on the sides of the stomach. The hindgut is short. The anus is located in front of the posterior sucker at the dorsal side (Figure 4.8).

Genital holes are located in the clitellar region on the XI and XII segments. The male genital opening is located in the groove between the fourth and fifth rings of the XI segment. The female genital hole is located between the fourth and fifth rings of the XII segment (Figure 4.9). The epididymis of *Hirudo verbana* is small and the discharge bulb is long. The penis sheath is long and prominent. The vagina of *Hirudo verbana* is tubular. The species has 10 pairs of testicular sacs that are oval structure (Figure 4.10).

Figure 4.1 General view of *Hirudo verbana* specimens from dorsal and ventral (original).

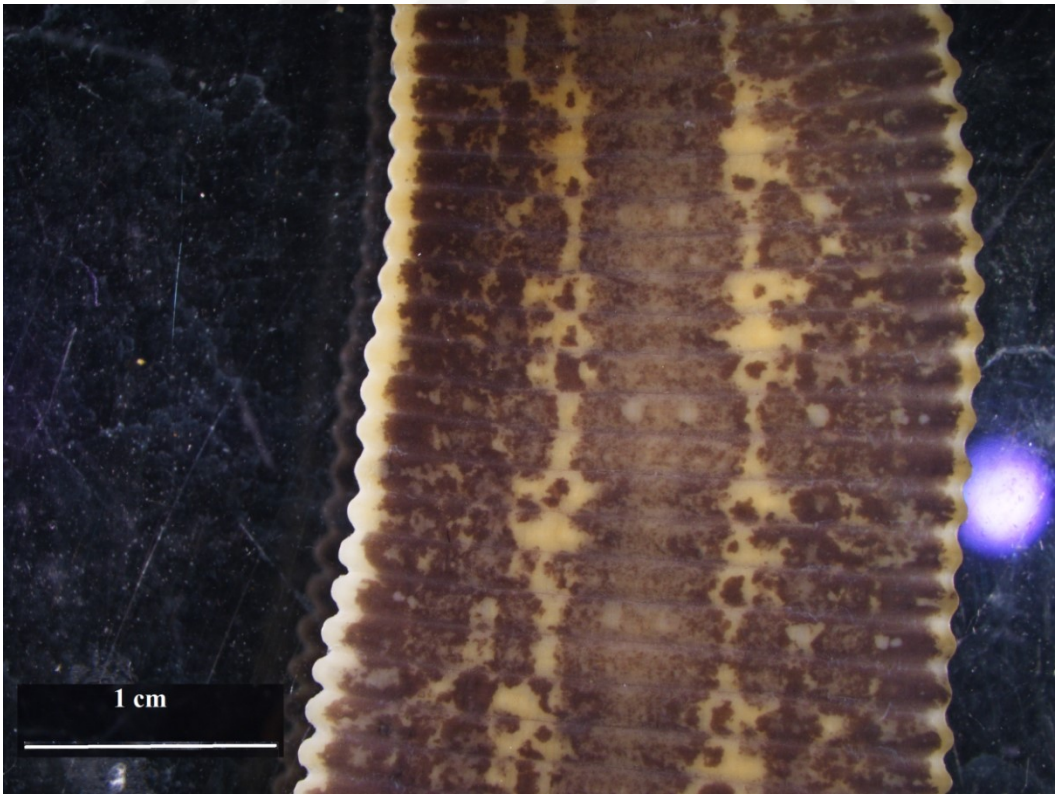


Figure 4.2 Dorsal view of *Hirudo verbana* (original).

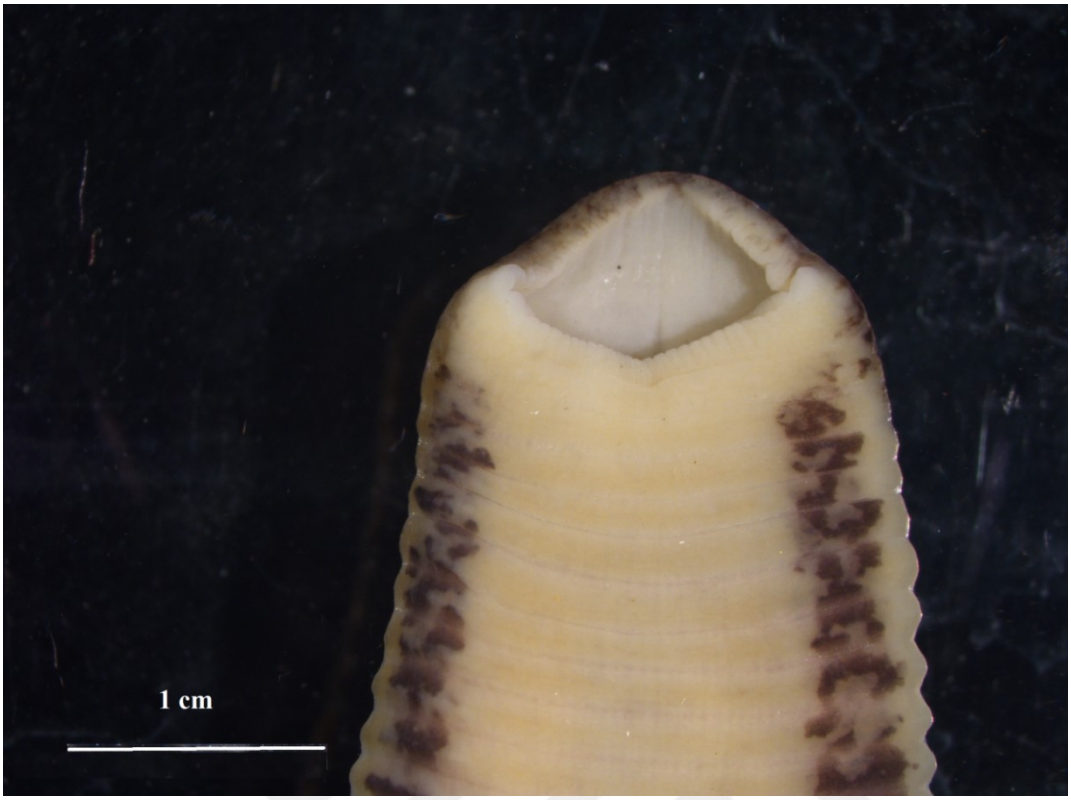


Figure 4.3 Ventral view of mouth sucker of *Hirudo verbana* (original).

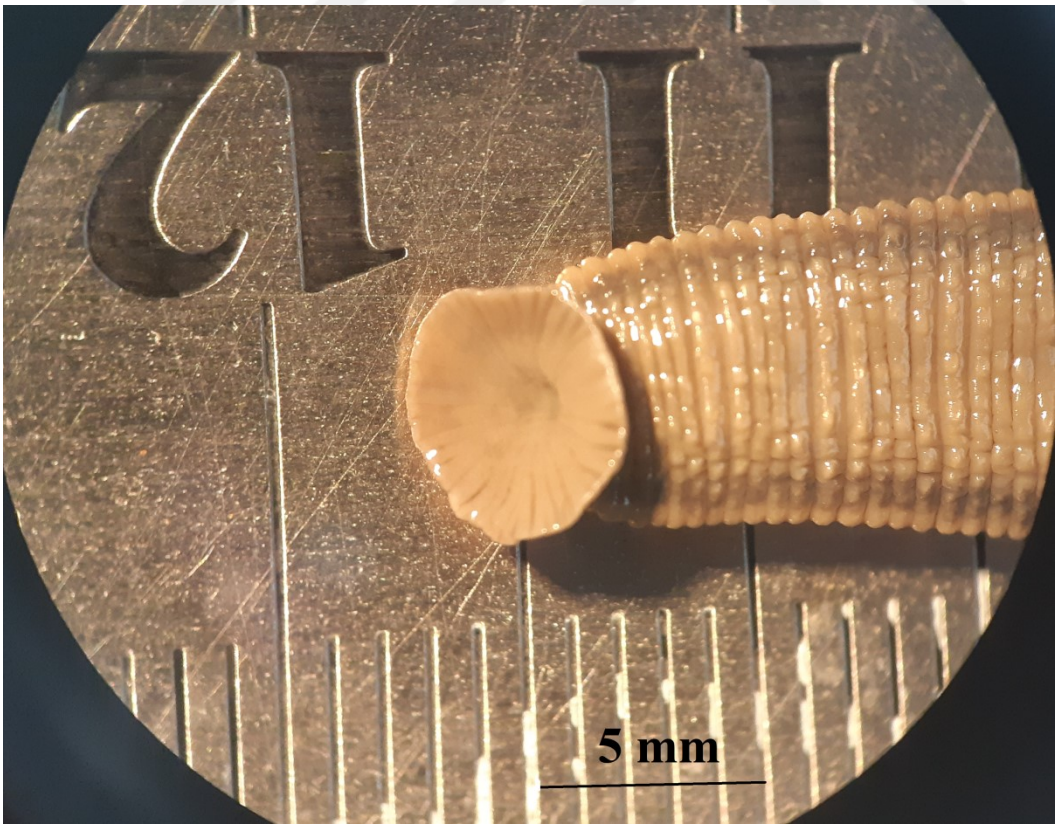


Figure 4.4 Ventral view of posterior sucker of *Hirudo verbana* (original).

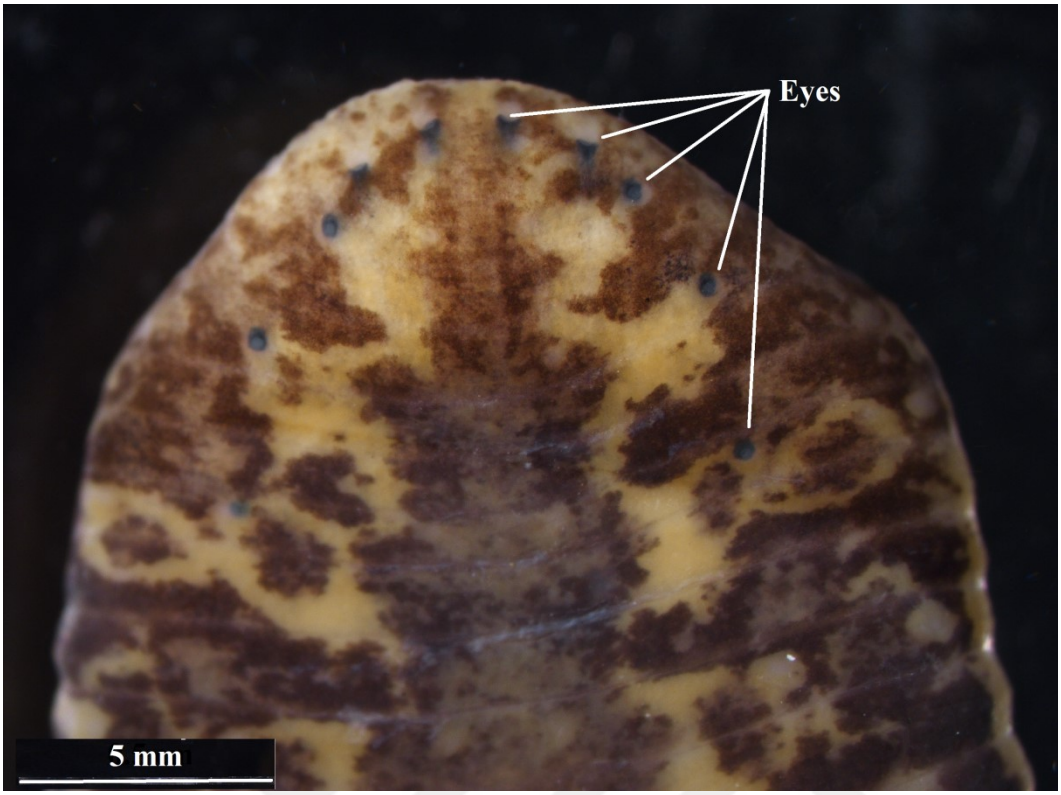


Figure 4.5 Eye spots from the anterior dorsal side of *Hirudo verbana* (original).

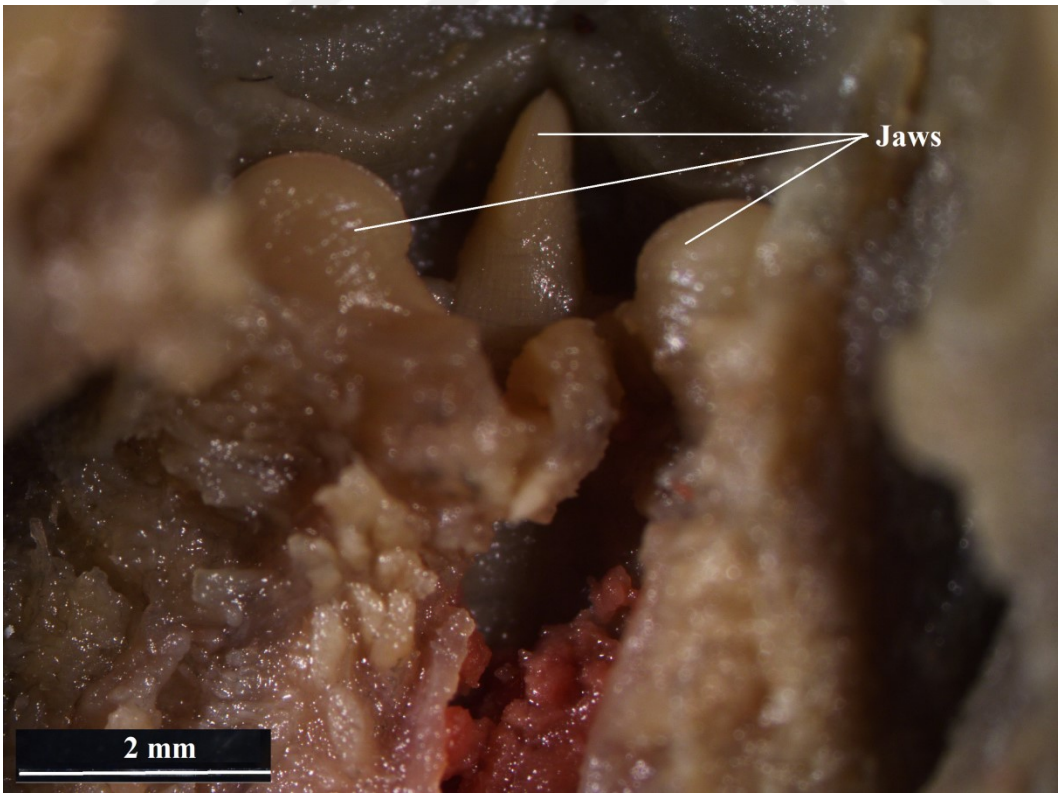


Figure 4.6 Position of jaws of *Hirudo verbana* (original).

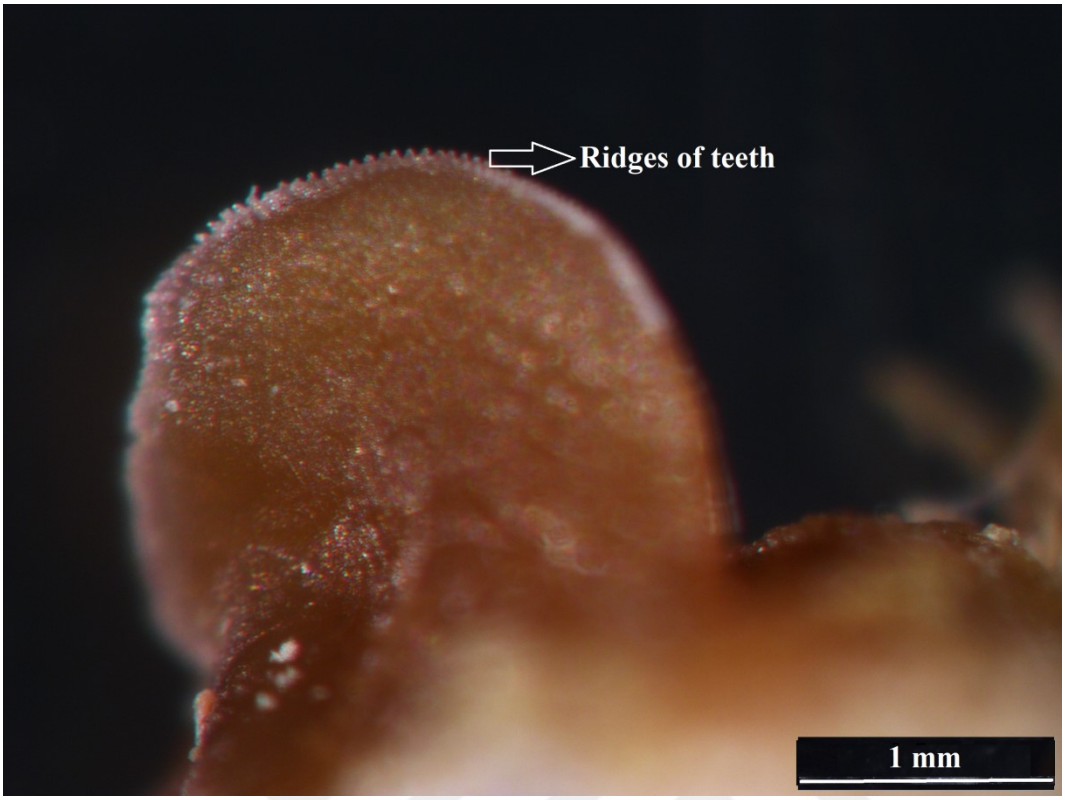


Figure 4.7 Ridges of teeth on jaws of *Hirudo verbana* (original).

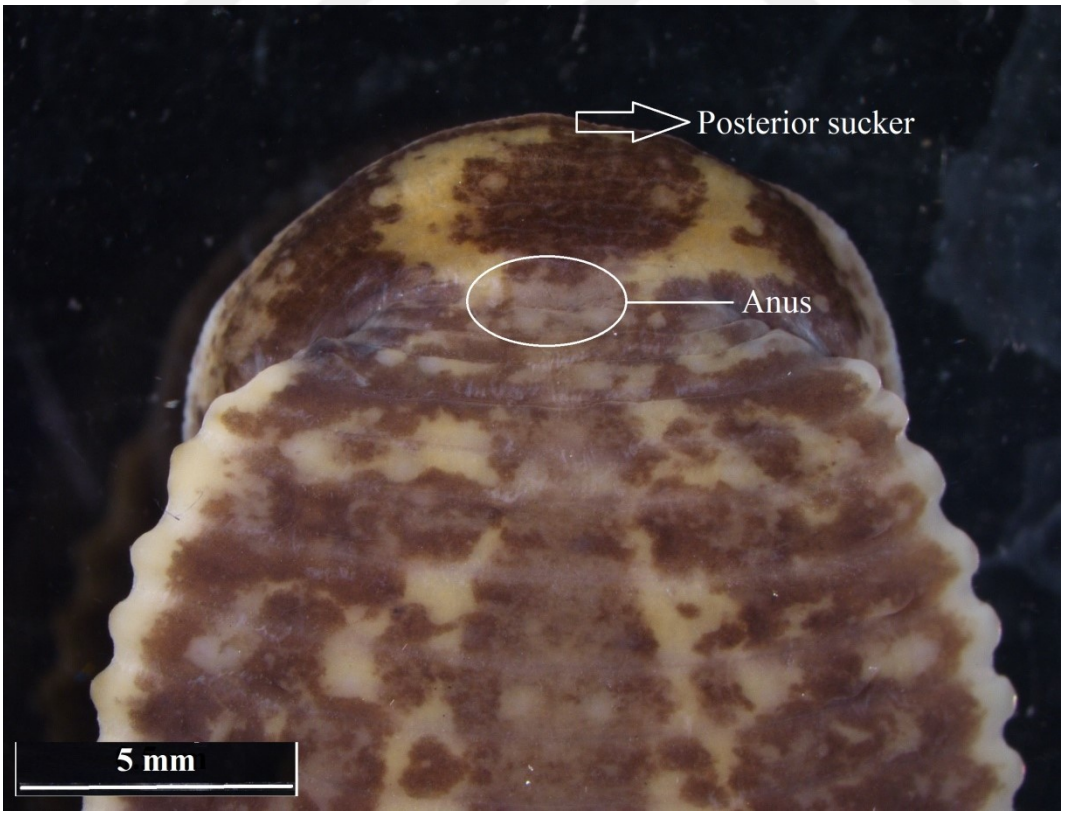


Figure 4.8 Anus opening on posterior region of *Hirudo verbana* (original).

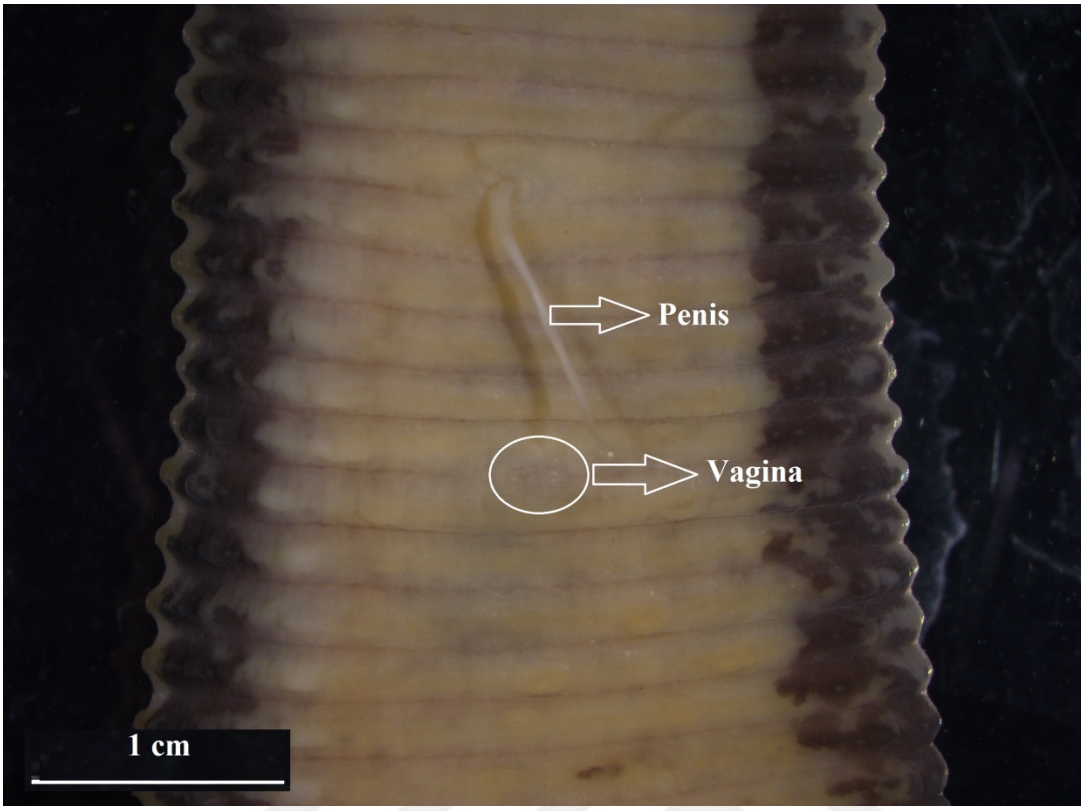


Figure 4.9 Position of genital organs of *Hirudo verbana* (original).

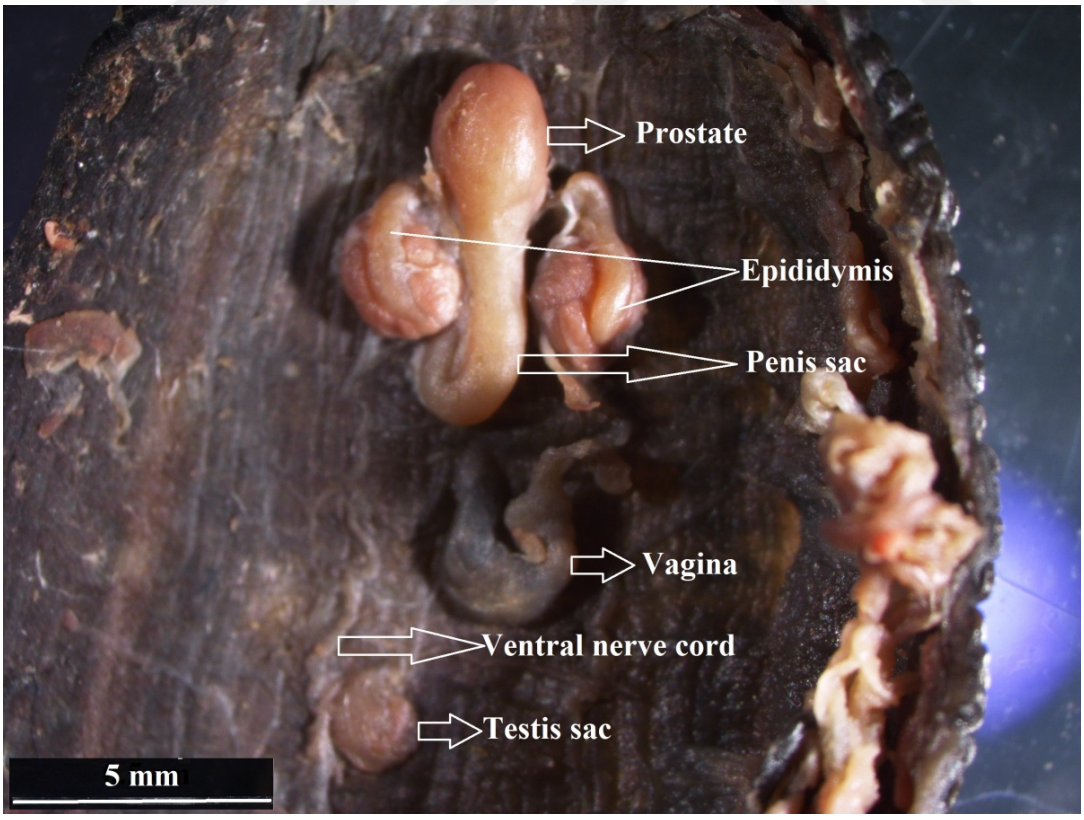


Figure 4.10 Position of genital organs in body cavity of *Hirudo verbana* (original).

4.2 DNA Isolation

In this study, the molecular identification of *Hirudo verbana* species was made on the mtCOI and 18S rRNA gene loci of 16 samples which were collected from Lake Karamık. The band size of the isolates mtCOI was determined as 641-696 bp and 18S rRNA as 679-736 bp (Figures 4.11 and 4.12). The nucleotide sequences of mtCOI of 8 Hirudo verbana samples and the 18S rRNA locus of 10 H.verbana samples were identified and their sequences were registered in GenBank (Tables 4.2 and 4.3).

4.3 Sequencing Data

The mtCOI sequences of the *Hirudo verbana* isolates examined in the study showed exact similarity to the closest isolate on NCBI. Thus, it has been determined that the existing isolates are individuals belonging to the same taxon. Also, the mtCOI and 18S rRNA locus sequences of the 10 *Hirudo verbana* samples examined in the study showed a complete homology agreement among themselves (Table 4.4).

The base values in the mtCOI sequences of the examined *Hirudo verbana* samples were defined: T 40.19-40.58; A 27.91-28.45%; G 15.18-15.44%; C 15.91-16.28%. Accordingly, the lowest A value was observed in isolate Hv9 (OM010335), while the highest A ratio was found in Hv5 (OM010329). While the lowest T case was Hv11 (OM010336), the highest T was recorded in Hv7 (OM010333) and Hv13 (OM010332) samples. The lowest G value was observed in isolate Hv7 (OM010333), while the highest G ratio was found in Hv8 (OM010337). the lowest C case was Hv13 (OM010332) and Hv17 (OM010333), the highest C was recorded in Hv12 (OM010334) and Hv9 (OM010335) samples (Table 4.4).

The base values in the 18S rRNA of the examined *Hirudo verbana* samples were defined: T 24.23-25.17%; A 24.54-26.02%; G 28.13-29.65%; C 20.92-21.61%. Accordingly, the lowest A value was observed in isolate Hv7 (OK091630), while the highest A ratio was found in Hv1 (OK091623). While the lowest T case was Hv9 (OK091631), the highest T was recorded in Hv10 (OK091627). The lowest G value was

observed in isolate Hv5 (OK091624), while the highest G ratio was found in Hv7 (OK091630). the lowest C case was Hv8 (OK091626), the highest C was recorded in Hv5 (OK091624) samples (Table 4.4).

The nucleotide diversity in the mtCOI sequences of *Hirudo verbana* samples and the distribution of nucleotides according to their positions were defined. Accordingly, no nucleotide variation was observed in OM010334 and OM010336 isolates. However, at the 431st isolated nucleotide of the (OM721929, OM010332, OM010333, OM010335, OM010337, OM033510) replaced the Adenine nucleotide with the Guanine nucleotide at 0,00151% variation was observed (Table 4.5).

The mtCOI locus of *Hirudo verbana* isolates were obtained in this study and other *Hirudo* spp Pairwise comparison (%) of mtCOI sequences of the isolates was made (Table 4.6). In terms of mtCOI sequences, no differences were observed between *Hirudo verbana* isolates of the present study and *Hirudo verbana* isolates from other studies. The present isolates obtained in the current study process showed full agreement among themselves and with the other *Hirudo verbana* samples from other studies (AY763151.1, AY763150.1, JN083801.1, KT692947.1, EF446694.1, KU216244.1).

Also among the other *Hirudo* species, In the distance matrix analysis, *Hirudo medicinalis* (AY763148.1) was identified as the closest species to *Hirudo verbana* isolates in terms of mtCOI gene sequence characteristics. This was followed by *Hirudo troctina* (AY763155.1) and *Hirudo orientalis* (KY989460.1) which come before *Hirudo nipponia* (GQ368749.1), in the last *Hirudo sulikii* (KU216240.1) takes place in the relationships with this study, respectively.

In this context, a difference of (0.204 - 0.112)% was found between the mtCOI gene sequences of different *Hirudo* species which include *Hirudo medicinalis* (AY763148.1), *Hirudo troctina* (AY763155.1), *Hirudo orientalis* (KY989460.1), *Hirudo nipponia* (GQ368749.1) and *Hirudo sulikii* (KU216240.1) with *Hirudo verbana* isolates. *Erpobdella montezuma* (GQ368760.1) was used as the outgroup (Table 4.6).

4.4 Filogram Analysis Data

Findings of the present *Hirudo verbana* isolates obtained in this study and *Hirudo* spp phylogram analysis was performed using the maximum likelihood (ML) method using the mtCOI gene sequences of the isolates (Table 4.7).

Maximum likelihood analysis values strongly supported phylogram formation among Hirudo isolates. As a result of ML analysis, OM010337.1 and OM033510.1 of Hirudo verbana isolate obtained in the present study showed close positioning among themselves, this positioning was followed by the remaining isolates (OM010335.1), the same positions shown for the (OM010333.1) from our study and (JN083801.1) from other study and was followed by (OM010332.1), this group of samples was followed by singles branches which are (OM010329.1) and (KT692947.1).

This branch was followed by the relationship between (OM010336.1) from our study and (KU216244.1) from another study which have the same close positions. Then it was followed by single branches (JN083798.1) after it (OM010334.1) followed by a group have a close positioning (AY763150.1 and EF446694.1). This positioning was followed by a single branch (AY63151.1) from another isolate. All these branches were followed by the remaining isolates which are single branches from other Hirudo species and other isolates (KY989460.1 Hirudo orientalis, AY763148.1 Hirudo medicinalis, AY763155.1 Hirudo troctina, and KU216240.1 Hirudo sulukii). It was followed by the last branch Erpobdella montezuma (GQ368760.1) formed a different branch in the tree due to being an outgroup (Table 4.7).

4.5 Haplotype variations of *Hirudo verbana*

Only one haplotype variation was recorded in the COI sequences of Hirudo verbana isolates identified in this study. In this context, haplotype (gene) diversity, Hd: 0,4286, the variance of haplotype diversity: 0,02846, and the standard deviation of haplotype diversity: 0,169. Also, haplotype variations were not shown at 18S rRNA sequences of Hirudo verbana isolates identified in this study.

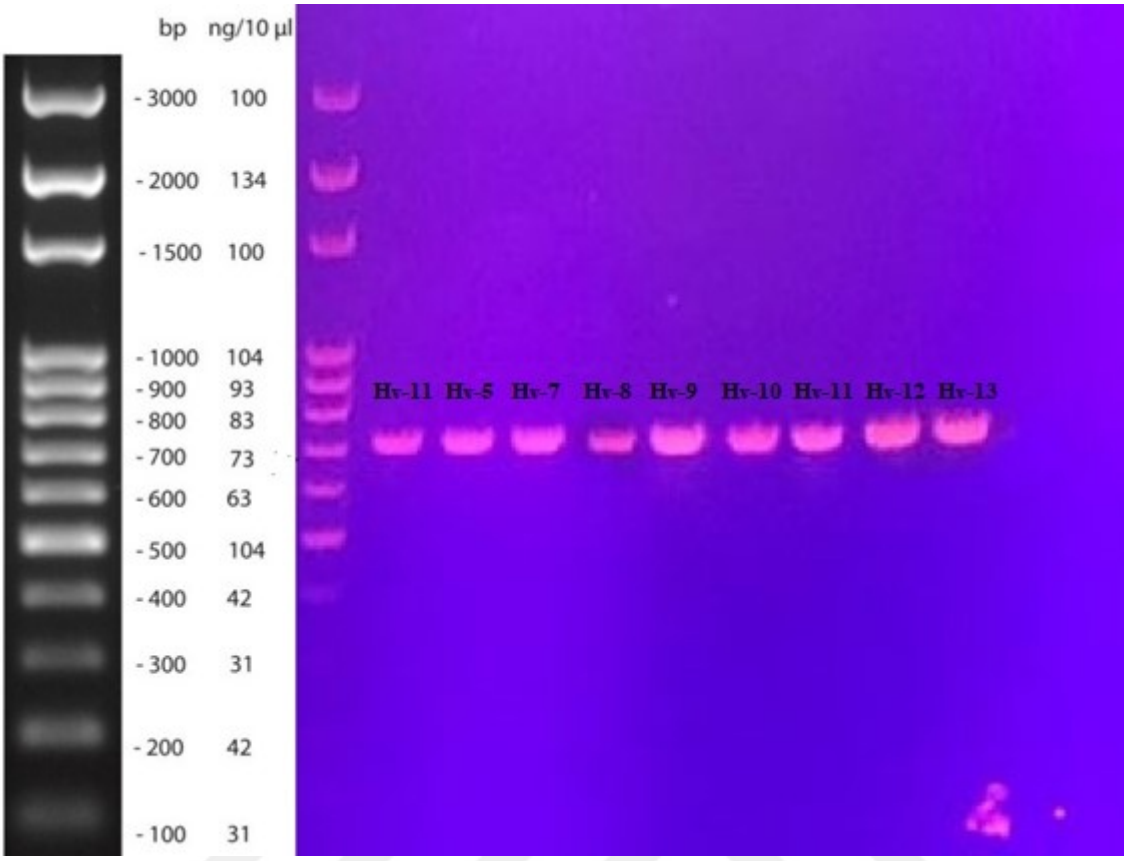


Figure 4.11 Electrophoresis images of mtCOI locus of *Hirudo verbana* specimens and Marker DNA (original).

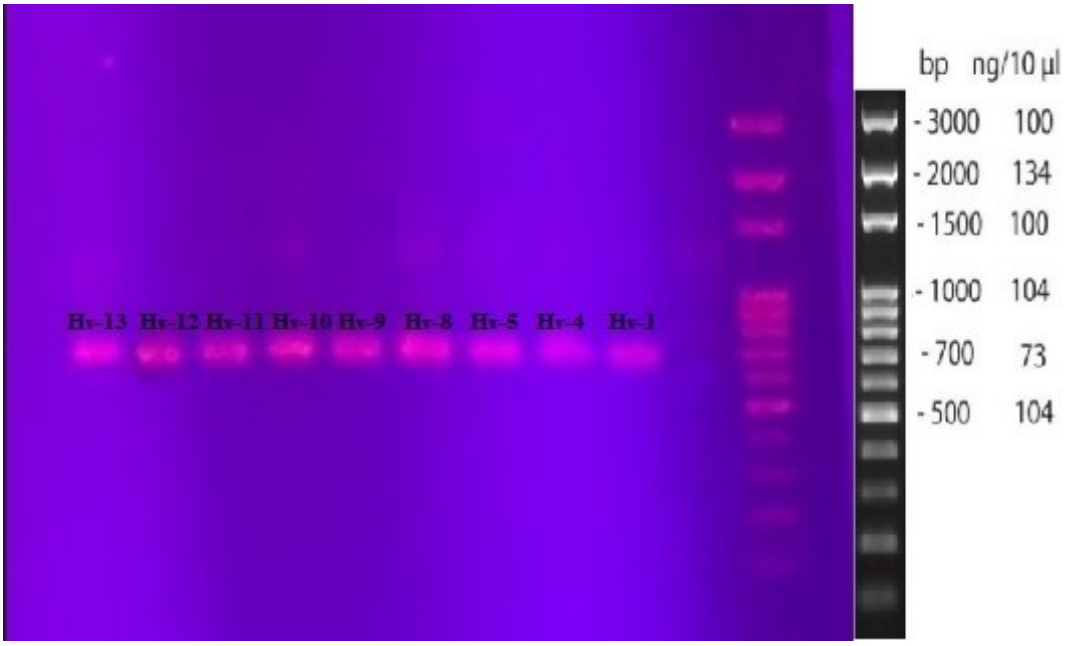


Figure 4.12 Electrophoresis images of 18S rRNA locus of *Hirudo verbana* specimens and Marker DNA (original).

Table 4.1 Body measurement values of *Hirudo verbana* samples.

Collected Date	Location	Samples No	Length (cm)	Width (cm)	Weight (gr)
13. 09. 2021	Karamık Lake, Koçbeyli location	Hv-1	10.3	1.90	6.80
		Hv-2	9.0	1.55	4.20
		Hv-3	8.4	1.80	2.82
		Hv-4	8.4	1.50	3.00
		Hv-5	8.3	1.65	2.90
		Hv-6	7.5	1.60	2.17
		Hv-7	7.4	1.20	1.86
		Hv-8	6.9	1.20	1.86
		Hv-9	6.4	1.55	1.58
		Hv-10	6.3	0.90	1.30
		Hv-11	6.1	0.95	1.10
		Hv-12	5.4	0.85	0.56
		Hv-13	4.9	0.70	0.78
		Hv-14	4.0	0.60	0.42
		Hv-15	4.0	0.55	0.38
		Hv-16	3.8	0.45	0.26

Table 4.2 The sequence data of mtCOI of *Hirudo verbana* samples.

Sample No	GenBank No	Base Pair No	The sequence data
Hv-5	OM010329	696	1 gtcaacaaat cataaagata ttggtacatt atatcttatt cttggttctt gatcagctat 61 attaggttct tcaataagat ctattattcg aattgaatta gcacaacctg gaaaattttt 121 aggtgatgat caattatata attctttagt gactgctcat ggattagtaa taatcttctt 181 tatggtaata ccaattttaa ttggtggttt tggaaattga cttttacctc taatagttgg 241 tgctattgat atatcatttc ctcggttaaa taactttaga ttttgattat tgccaccatc 301 aataattata ttattaagtt catctataat tgaaaatgga gtaggaacag gatgaaccat 361 ttatcctcca ttggctgata gtgtctctca ttcaggccca tctgtagata tggctatctt 421 ctccctacat atagctggag cttcatcaat tcttggatct ttaaacttta tctcaactat 481 tattaatata cgtatttccg gtataagatc tgaacgagta cctttattcg tgtggtcggt 541 agtaattact accattttat tacttctctc tttaccagtt ttagctgcag ccattactat 601 attgttaact gatcgtaatt taaatactac ttttttgat ccaattggag ggggggatcc 661 aatcttattt caacatctat tttgattttt tggtca
Hv-7	OM010333	688	1 ataaagatat tggtacatta tatcttattc ttggttcttg atcagctata ttaggttctt 61 caataagatc tattattcga attgaattag cacaacctgg aaaattttta ggtgatgatc 121 aattatataa ttctttagtg actgctcatg gattagtaat aatcttcttt atggtaatac 181 caattttaat tggtggtttt ggaaattgac ttttacctct aatagttggt gctattgata 241 tatcatttcc tcggttaaat aactttagat tttgattatt gccaccatca ataattatat 301 tattaagttc atctataatt gaaaatggag taggaacagg atgaaccatt tatcctccat 361 tggctgatag tgtctctcat tcaggcccat ctgtagatat ggctatcttc tccctacata 421 tagctggagc ttcatcaatt cttggatctt taaactttat ctcaactatt attaatatac 481 gtatttccgg tataagatct gaacgagtac ctttattcgt gtggtcggta gtaattacta 541 ccattttatt acttctctct ttaccagttt tagctgcagc cattactata ttgttaactg 601 atcgtaattt aaatactact ttttttgatc caattggagg gggggatcca atcttatttc 661 aacatctatt ttgatttttt ggtcaccc
Hv-8	OM010337	641	1 gctatattag gttcttcaat aagatctatt attcgaattg aattagcaca acctggaaaa 61 tttttaggtg atgatcaatt atataattct ttagtgactg ctcatggatt agtaataatc 121 ttctttatgg taataccaat tttaattggt ggttttggaa attgactttt acctctaata 181 gttggtgcta ttgatatatc atttcctcgg ttaaataact ttagattttg attattgcca 241 ccatcaataa ttatattatt aagttcatct ataattgaaa atggagtagg aacaggatga 301 accatttatc ctccattggc tgatagtgtc tctcattcag gcccatctgt agatatggct 361 atcttctccc tacatatagc tggagcttca tcaattcttg gatctttaaa ctttatctca 421 actattatta atatacgtat ttccggtata agatctgaac gagtaccttt attcgtgtgg 481 tcggtagtaa ttactaccat tttattactt ctctctttac cagttttagc tgcagccatt 541 actatattgt taactgatcg taatttaaat actacttttt ttgatccaat tggagggggg 601 gatccaatct tatttcaaca tctattttga ttttttggtc a
Hv-9	OM010335	688	1 aaagatattg gtacattata tcttattctt ggttcttgat cagctatatt aggttcttca 61 ataagatcta ttattcgaat tgaattagca caacctggaa aatttttagg tgatgatcaa 121 ttatataatt ctttagtgac tgctcatgga ttagtaataa tcttctttat ggtaatacca 181 attttaattg gtggttttgg aaattgactt ttacctctaa tagttggtgc tattgatata 241 tcatttcctc ggttaaataa ctttagattt tgattattgc caccatcaat aattatatta 301 ttaagttcat ctataattga aaatggagta ggaacaggat gaaccattta tcctccattg 361 gctgatagtg tctctcattc aggcccatct gtagatatgg ctatcttctc cctacatata 421 gctggagctt catcaattct tggatcttta aactttatct caactattat taatatacgt 481 atttccggta taagatctga acgagtacct ttattcgtgt ggtcggtagt aattactacc 541 attttattac ttctctcttt accagtttta gctgcagcca ttactatatt gttaactgat 601 cgtaatttaa atactacttt ttttgatcca attggagggg gggatccaat cttatttcaa 661 catctatttt gatttttttgg tcaccctg

Table 4.2 The sequence data of mtCOI of *Hirudo verbana* samples (continuation).

Hv-10	OM033510	642	1 agctatatta ggttcttcaa taagatctat tattcgaatt gaattagcac aacctggaaa 61 atttttaggt gatgatcaat tatataattc tttagtgact gctcatggat tagtaataat 121 cttctttatg gtaataccaa ttttaattgg tggttttgga aattgacttt tacctctaat 181 agttggtgct attgatatat catttcctcg gttaaataac tttagatttt gattattgcc 241 accatcaata attatattat taagttcatc tataattgaa aatggagtag gaacaggatg 301 aaccatttat cctccattgg ctgatagtgt ctctcattca ggcccatctg tagatatggc 361 tatcttctcc ctacatatag ctggagcttc atcaattctt ggatctttaa actttatctc 421 aactattatt aatatacgta tttccggtat aagatctgaa cgagtacctt tattcgtgtg 481 gtcggtagta attactacca ttttattact tctctcttta ccagttttag ctgcagccat 541 tactatattg ttaactgatc gtaatttaaa tactactttt tttgatccaa ttggaggggg 601 ggatccaatc ttatttcaac atctattttg atttttggt ca
Hv-11	OM010336	641	1 gctatattag gttcttcaat aagatctatt attcgaattg aattagcaca acctggaaaa 61 tttttaggtg atgatcaatt atataattct ttagtgactg ctcatggatt agtaataatc 121 ttctttatgg taataccaat tttaattggt ggttttggaa attgactttt acctctaata 181 gttggtgcta ttgatatatc atttcctcgg ttaaataact ttagattttg attattgcca 241 ccatcaataa ttatattatt aagttcatct ataattgaaa atggagtagg aacaggatga 301 accatttatc ctccattggc tgatagtgtc tctcattcag gcccatctgt agatatagct 361 atcttctccc tacatatagc tggagcttca tcaattcttg gatctttaaa ctttatctca 421 actattatta atatacgtat ttccggtata agatctgaac gagtaccttt attcgtgtgg 481 tcggtagtaa ttactaccat tttattactt ctctctttac cagttttagc tgcagccatt 541 actatattgt taactgatcg taatttaaat actacttttt ttgatccaat tggaggggggg 601 gatccaatct tatttcaaca tctattttga ttttttggtc a
Hv-12	OM010334	685	1 ataaagatat tggtacatta tatcttattc ttggttcttg atcagctata ttaggttctt 61 caataagatc tattattcga attgaattag cacaacctgg aaaatttta ggtgatgatc 121 aattatataa ttctttagtg actgctcatg gattagtaat aatcttcttt atggtaatac 181 caattttaat tggtggtttt ggaaattgac ttttacctct aatagttggt gctattgata 241 tatcatttcc tcggttaaat aactttagat tttgattatt gccaccatca ataattatat 301 tattaagttc atctataatt gaaaatggag taggaacagg atgaaccatt tatcctccat 361 tggctgatag tgtctctcat tcaggcccat ctgtagatat agctatcttc tccctacata 421 tagctggagc ttcatcaatt cttggatctt taaactttat ctcaactatt attaatatac 481 gtatttccgg tataagatct gaacgagtac ctttattcgt gtggtcggta gtaattacta 541 ccattttatt acttctctct ttaccagttt tagctgcagc cattactata ttgttaactg 601 atcgtaattt aaatactact ttttttgatc caattggagg gggggatcca atcttatttc 661 aacatctatt ttgatttttt ggtca
Hv-13	OM010332	685	1 ataaagatat tggtacatta tatcttattc ttggttcttg atcagctata ttaggttctt 61 caataagatc tattattcga attgaattag cacaacctgg aaaatttta ggtgatgatc 121 aattatataa ttctttagtg actgctcatg gattagtaat aatcttcttt atggtaatac 181 caattttaat tggtggtttt ggaaattgac ttttacctct aatagttggt gctattgata 241 tatcatttcc tcggttaaat aactttagat tttgattatt gccaccatca ataattatat 301 tattaagttc atctataatt gaaaatggag taggaacagg atgaaccatt tatcctccat 361 tggctgatag tgtctctcat tcaggcccat ctgtagatat ggctatcttc tccctacata 421 tagctggagc ttcatcaatt cttggatctt taaactttat ctcaactatt attaatatac 481 gtatttccgg tataagatct gaacgagtac ctttattcgt gtggtcggta gtaattacta 541 ccattttatt acttctctct ttaccagttt tagctgcagc cattactata ttgttaactg 601 atcgtaattt aaatactact ttttttgatc caattggagg gggggatcca atcttatttc 661 aacatctatt ttgatttttt ggtca

Table 4.3 The sequence data of 18S rRNA of *Hirudo verbana* samples.

Sample No	GenBank No	Base Pair No	The sequence data
Hv-1	OK091623	684	1 ggtaattcca gctccaatag cgtatattaa agttgttgca gttaaaaagc tcgtagttgg 61 atctcgggtt cggactggtt gcacgcctcg cggcgtagcg acccgctccg acctgttttt 121 cggttctgcg cccggttctc ttaaccgagt gccgggtgcg gccgagacgt ttactttgaa 181 aaaattagag tgcttaaagc aggctcgaca gcctgaatag tagcgcatgg aataatagaa 241 taggacctcg gttctatttt gttggttttc ggagctcgag gtaatgatta agagggacag 301 acgggggcat tcgtattacg gtgttagagg tgaaattctt ggatcgccgt aagacgaact 361 actgcgaaag catttgccaa gaatgttttc attaatcaag aacgaaagtc agaggttcga 421 agacgatcag ataccgtcgt agttctgacc ataaacgatg tcgactggcg atccgcggga 481 gttgatcgta cgaccccgcg ggcagcctcc gggaaaccaa agtctttgga ttccgggggga 541 agtatggttg caaagccgaa acttaaagga attgacggaa gggcaccacc aggagtggag 601 cctgcggctt aatttgactc aacacgggaa aactcacccg gtccggacac cggaaggatt 661 gacagattga tagctctttc ttaa
Hv-4	OK091625	715	1 ccagctccaa tagcgtatat taaagttgtt gcagttaaaa agctcgtagt tggatctcgg 61 gttcggactg gttgcacgcc tcgcggcgta gcgacccgct ccgacctgtt tttcggttct 121 gcgcccggtt ctcttaaccg agtgccgggt gcggccgaga cgtttacttt gaaaaaatta 181 gagtgcttaa agcaggctcg acagcctgaa tagtagcgca tggaataata gaataggacc 241 tcggttctat tttgttggtt ttcggagctc gaggtaatga ttaagaggga cagacggggg 301 cattcgtatt acggtgttag aggtgaaatt cttggatcgc cgtaagacga actactgcga 361 aagcatttgc caagaatgtt ttcattaatc aagaacgaaa gtcagaggtt cgaagacgat 421 cagataccgt cgtagttctg accataaacg atgtcgactg gcgatccgcg ggagttgatc 481 gtacgacccc gcgggcagcc tccgggaaac caaagtcttt ggattccggg ggaagtatgg 541 ttgcaaagcc gaaacttaaa ggaattgacg gaagggcacc accaggagtg gagcctgcgg 601 cttaatttga ctcaacacgg gaaaactcac ccggtccgga caccggaagg attgacagat 661 tgatagctct ttcttaattc ggtgggtggt ggtgcatggc cgttcttagt tggtg
Hv-5	OK091624	679	1 ccagctccaa tagcgtatat taaagttgtt gcagttaaaa agctcgtagt tggatctcgg 61 gttcggactg gttgcacgcc tcgcggcgta gcgacccgct ccgacctgtt tttcggttct 121 gcgcccggtt ctcttaaccg agtgccgggt gcggccgaga cgtttacttt gaaaaaatta 181 gagtgcttaa agcaggctcg acagcctgaa tagtagcgca tggaataata gaataggacc 241 tcggttctat tttgttggtt ttcggagctc gaggtaatga ttaagaggga cagacggggg 301 cattcgtatt acggtgttag aggtgaaatt cttggatcgc cgtaagacga actactgcga 361 aagcatttgc caagaatgtt ttcattaatc aagaacgaaa gtcagaggtt cgaagacgat 421 cagataccgt cgtagttctg accataaacg atgtcgactg gcgatccgcg ggagttgatc 481 gtacgacccc gcgggcagcc tccgggaaac caaagtcttt ggattccggg ggaagtatgg 541 ttgcaaagcc gaaacttaaa ggaattgacg gaagggcacc accaggagtg gagcctgcgg 601 cttaatttga ctcaacacgg gaaaactcac ccggtccgga caccggaagg attgacagat 661 tgatagctct ttcttaatt
Hv-8	OK091626	736	1 ggtaattcca gctccaatag cgtatattaa agttgttgca gttaaaaagc tcgtagttgg 61 atctcgggtt cggactggtt gcacgcctcg cggcgtagcg acccgctccg acctgttttt 121 cggttctgcg cccggttctc ttaaccgagt gccgggtgcg gccgagacgt ttactttgaa 181 aaaattagag tgcttaaagc aggctcgaca gcctgaatag tagcgcatgg aataatagaa 241 taggacctcg gttctatttt gttggttttc ggagctcgag gtaatgatta agagggacag 301 acgggggcat tcgtattacg gtgttagagg tgaaattctt ggatcgccgt aagacgaact 361 actgcgaaag catttgccaa gaatgttttc attaatcaag aacgaaagtc agaggttcga 421 agacgatcag ataccgtcgt agttctgacc ataaacgatg tcgactggcg atccgcggga 481 gttgatcgta cgaccccgcg ggcagcctcc gggaaaccaa agtctttgga ttccgggggga 541 agtatggttg caaagccgaa acttaaagga attgacggaa gggcaccacc aggagtggag 601 cctgcggctt aatttgactc aacacgggaa aactcacccg gtccggacac cggaaggatt 661 gacagattga tagctctttc ttaattcggt gggtggtggt gcatggccgt tcttagttgg 721 tggagcgatt tgtctg

Table 4.3 The sequence data of 18S rRNA of *Hirudo verbana* samples (continuation).

Hv-9	OK091631	685	1 ccagctccaa tagcgtatat taaagttgtt gcagttaaaa agctcgtagt tggatctcgg 61 gttcggactg gttgcacgcc tcgcggcgta gcgacccgct ccgacctgtt tttcggttct 121 gcgcccggtt ctcttaaccg agtgccgggt gcggccgaga cgtttacttt gaaaaaatta 181 gagtgcttaa agcaggctcg acagcctgaa tagtagcgca tggaataata gaataggacc 241 tcggttctat tttgttggtt ttcggagctc gaggtaatga ttaagaggga cagacggggg 301 cattcgtatt acggtgttag aggtgaaatt cttggatcgc cgtaagacga actactgcga 361 aagcatttgc caagaatgtt ttcattaatc aagaacgaaa gtcagaggtt cgaagacgat 421 cagataccgt cgtagttctg accataaacg atgtcgactg gcgatccgcg ggagttgatc 481 gtacgacccc gcgggcagcc tccgggaaac caaagtcttt ggattccggg ggaagtatgg 541 ttgcaaagcc gaaacttaaa ggaattgacg gaagggcacc accaggagtg gagcctgcgg 601 cttaatttga ctcaacacgg gaaaactcac ccggtccgga caccggaagg attgacagat 661 tgatagctct ttcttaattc ggtgg
Hv-10	OK091627	735	1 ggtaattcca gctccaatag cgtatattaa agttgttgca gttaaaaagc tcgtagttgg 61 atctcgggtt cggactggtt gcacgcctcg cggcgtagcg acccgctccg acctgttttt 121 cggttctgcg cccggttctc ttaaccgagt gccgggtgcg gccgagacgt ttactttgaa 181 aaaattagag tgcttaaagc aggctcgaca gcctgaatag tagcgcatgg aataatagaa 241 taggacctcg gttctatttt gttggttttc ggagctcgag gtaatgatta agagggacag 301 acgggggcat tcgtattacg gtgttagagg tgaaattctt ggatcgccgt aagacgaact 361 actgcgaaag catttgccaa gaatgttttc attaatcaag aacgaaagtc agaggttcga 421 agacgatcag ataccgtcgt agttctgacc ataaacgatg tcgactggcg atccgcggga 481 gttgatcgta cgaccccgcg ggcagcctcc gggaaaccaa agtctttgga ttccgggggga 541 agtatggttg caaagccgaa acttaaagga attgacggaa gggcaccacc aggagtggag 601 cctgcggctt aatttgactc aacacgggaa aactcacccg gtccggacac cggaaggatt 661 gacagattga tagctctttc ttaattcggt gggtggtggt gcatggccgt tcttagttgg 721 tggagcgatt tgtct
Hv-11	OK091632	679	1 ccaatagcgt atattaaagt tgttgcagtt aaaaagctcg tagttggatc tcgggttcgg 61 actggttgca cgcctcgcgg cgtagcgacc cgctccgacc tgtttttcgg ttctgcgccc 121 ggttctctta accgagtgcc gggtgcggcc gagacgttta ctttgaaaaa attagagtgc 181 ttaaagcagg ctcgacagcc tgaatagtag cgcatggaat aatagaatag gacctcggtt 241 ctattttgtt ggttttcgga gctcgaggta atgattaaga gggacagacg ggggcattcg 301 tattacggtg ttagaggtga aattcttgga tcgccgtaag acgaactact gcgaaagcat 361 ttgccaagaa tgttttcatt aatcaagaac gaaagtcaga ggttcgaaga cgatcagata 421 ccgtcgtagt tctgaccata aacgatgtcg actggcgatc cgcgggagtt gatcgtacga 481 ccccgcgggc agcctccggg aaaccaaagt ctttggattc cgggggaagt atggttgcaa 541 agccgaaact taaaggaatt gacggaaggg caccaccagg agtggagcct gcggcttaat 601 ttgactcaac acgggaaaac tcacccggtc cggacaccgg aaggattgac agattgatag 661 ctctttctta attcggtgg
Hv-12	OK091628	728	1 cagctccaat agcgtatatt aaagttgttg cagttaaaaa gctcgtagtt ggatctcggg 61 ttcggactgg ttgcacgcct cgcggcgtag cgacccgctc cgacctgttt ttcggttctg 121 cgcccggttc tcttaaccga gtgccgggtg cggccgagac gtttactttg aaaaaattag 181 agtgcttaaa gcaggctcga cagcctgaat agtagcgcat ggaataatag aataggacct 241 cggttctatt ttgttggttt tcggagctcg aggtaatgat taagagggac agacgggggc 301 attcgtatta cggtgttaga ggtgaaattc ttggatcgcc gtaagacgaa ctactgcgaa 361 agcatttgcc aagaatgttt tcattaatca agaacgaaag tcagaggttc gaagacgatc 421 agataccgtc gtagttctga ccataaacga tgtcgactgg cgatccgcgg gagttgatcg 481 tacgaccccg cgggcagcct ccgggaaacc aaagtctttg gattccgggg gaagtatggt 541 tgcaaagccg aaacttaaag gaattgacgg aagggcacca ccaggagtgg agcctgcggc 601 ttaatttgac tcaacacggg aaaactcacc cggtccggac accggaagga ttgacagatt 661 gatagctctt tcttaattcg gtgggtggtg gtgcatggcc gttcttagtt ggtggagcga 721 tttgtctg

Table 4.3 The sequence data of 18S rRNA of *Hirudo verbana* samples (continuation).

Hv-13	OK091629	727	1 cagctccaat agcgtatatt aaagttgttg cagttaaaaa gctcgtagtt ggatctcggg 61 ttcggactgg ttgcacgcct cgcggcgtag cgacccgctc cgacctgttt ttcggttctg 121 cgcccggttc tcttaaccga gtgccgggtg cggccgagac gtttactttg aaaaaattag 181 agtgcttaaa gcaggctcga cagcctgaat agtagcgcat ggaataatag aataggacct 241 cggttctatt ttgttggttt tcggagctcg aggtaatgat taagagggac agacgggggc 301 attcgtatta cggtgttaga ggtgaaattc ttggatcgcc gtaagacgaa ctactgcgaa 361 agcatttgcc aagaatgttt tcattaatca agaacgaaag tcagaggttc gaagacgatc 421 agataccgtc gtagttctga ccataaacga tgtcgactgg cgatccgcgg gagttgatcg 481 tacgaccccg cgggcagcct ccgggaaacc aaagtctttg gattccgggg gaagtatggt 541 tgcaaagccg aaacttaaag gaattgacgg aagggcacca ccaggagtgg agcctgcggc 601 ttaatttgac tcaacacggg aaaactcacc cggtccggac accggaagga ttgacagatt 661 gatagctctt tcttaattcg gtgggtggtg gtgcatggcc gttcttagtt ggtggagcga 721 tttgtct

Table 4.4 Percentage values (%) of A, T, G, and C nucleotides at COI and 18S rRNA locus of *Hirudo verbana* isolates identified in this study.

Samples no	Isolat no	Locus	A (%)	T (%)	G (%)	C (%)
5	OM010329	COI	28,45	40,23	15,23	16,09
13	OM010332	COI	28,18	40,58	15,33	15,91
7	OM010333	COI	28,32	40,58	15,18	15,91
12	OM010334	COI	28,05	40,41	15,26	16,28
9	OM010335	COI	27,91	40,41	15,41	16,28
11	OM010336	COI	28,19	40,19	15,42	16,20
8	OM010337	COI	28,08	40,25	15,44	16,22
10	OM033510	COI	28,24	40,25	15,29	16,22
1	OK091623	18S rRNA	26,02	24,27	28,22	21,49
5	OK091624	18S rRNA	25,92	24,30	28,13	21,65
4	OK091625	18S rRNA	24,90	24,76	29,09	21,26
8	OK091626	18S rRNA	24,73	25,14	29,21	20,92
10	OK091627	18S rRNA	24,76	25,17	29,12	20,95
12	OK091628	18S rRNA	24,73	25,00	29,26	21,02
13	OK091629	18S rRNA	24,76	25,03	29,16	21,05
7	OK091630	18S rRNA	24,54	24,82	29,65	20,99
9	OK091631	18S rRNA	25,69	24,23	28,47	21,61
11	OK091632	18S rRNA	25,77	24,30	28,57	21,35

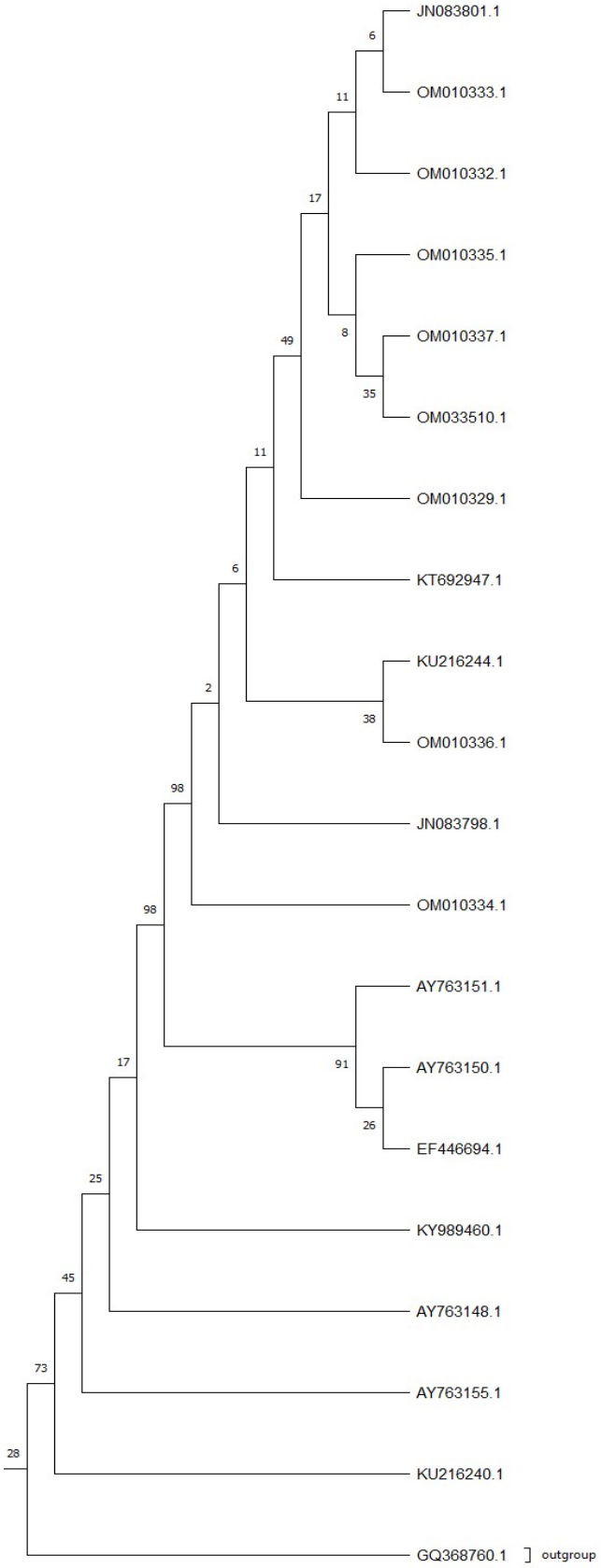
Table 4.5 Nucleotide variations number and percentage values (%) at COI sequences of *Hirudo verbana* isolates identified in this study according to the reference isolate, JN083793.

Samples no	İsolat no	Nucleotide variations number and percentage values (%)	Locations of nucleotide variations	Name of nucleotide variations
5	OM010329	1 (0,00151)	431	A > G
13	OM010332	1 (0,00151)	431	A > G
7	OM010333	1 (0,00151)	431	A > G
12	OM010334	0	-	-
9	OM010335	1 (0,00151)	431	A > G
11	OM010336	0	-	-
8	OM010337	1 (0,00159)	431	A > G
10	OM033510	1 (0,00159)	431	A > G

Table 4.6 Pairwise comparison of COI nucleotide sequences in some *Hirudo* spp isolates from the GenBank database and the present study.

	AY763151.1	AY763150.1	JN083798.1	JN083801.1	KT692947.1	OM010329.1	OM010332.1	OM010334.1	OM010333.1	OM010335.1	OM010336.1	KU216244.1	EF446694.1	OM010335.1	OM010333.1	OM010334.1	OM010332.1	OM010329.1	KT692947.1	JN083801.1	JN083798.1	AY763150.1	AY763151.1	KU216240.1
Hirudo verbana AY763151.1		0,002	0,023	0,022	0,022	0,022	0,022	0,020	0,022	0,022	0,020	0,022	0,002	0,022	0,022	0,020	0,022	0,022	0,022	0,022	0,022	0,020	0,127	0,188
Hirudo verbana AY763150.1			0,022	0,020	0,020	0,020	0,020	0,018	0,020	0,020	0,018	0,020	0,000	0,020	0,020	0,018	0,020	0,020	0,020	0,020	0,020	0,020	0,124	0,184
Hirudo verbana JN083798.1				0,009	0,006	0,006	0,006	0,005	0,006	0,006	0,005	0,006	0,021	0,006	0,006	0,005	0,006	0,006	0,006	0,006	0,006	0,006	0,146	0,196
Hirudo verbana JN083801.1					0,006	0,003	0,003	0,005	0,003	0,003	0,005	0,011	0,019	0,003	0,003	0,005	0,003	0,003	0,003	0,003	0,003	0,003	0,141	0,200
Hirudo verbana KT692947.1						0,003	0,003	0,001	0,003	0,003	0,002	0,006	0,019	0,003	0,003	0,001	0,003	0,003	0,003	0,003	0,003	0,003	0,142	0,198
Hirudo verbana OM010329.1							0,000	0,001	0,000	0,000	0,002	0,008	0,019	0,000	0,000	0,001	0,000	0,000	0,000	0,000	0,000	0,000	0,142	0,202
Hirudo verbana OM010332.1								0,001	0,000	0,000	0,002	0,008	0,019	0,000	0,000	0,001	0,000	0,000	0,000	0,000	0,000	0,000	0,142	0,202
Hirudo verbana OM010334.1									0,001	0,001	0,000	0,006	0,017	0,001	0,001	0,002	0,000	0,000	0,000	0,000	0,000	0,000	0,139	0,198
Hirudo verbana OM010333.1										0,000	0,002	0,008	0,019	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,142	0,202
Hirudo verbana OM010335.1											0,002	0,008	0,019	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,142	0,202
Hirudo verbana EF446694.1												0,025	0,019	0,008	0,008	0,006	0,008	0,008	0,008	0,008	0,008	0,008	0,121	0,184
Hirudo verbana KU216244.1														0,003	0,003	0,003	0,005	0,005	0,005	0,005	0,005	0,005	0,146	0,198
Hirudo verbana OM010336.1																	0,002	0,002	0,002	0,002	0,002	0,002	0,140	0,198
Hirudo verbana OM010337.1																		0,000	0,000	0,000	0,000	0,000	0,142	0,202
Hirudo verbana OM033510.1																			0,000	0,000	0,000	0,000	0,142	0,202
Hirudo orientalis KY989460.1																				0,000	0,000	0,000	0,138	0,182
Hirudo troctina AY763155.1																					0,000	0,000	0,111	0,180
Hirudo medicinalis AY763148.1																						0,000	0,794	0,150
Hirudo nipponia GQ368749.1																							1,711	0,923
Erpobdella montezuma **Out group** GQ368760.1																								1,391
Hirudo sulukii KU216240.1																								

Table 4.7 Maximum likelihood (ML) tree based on COI nucleotide sequences in some *Hirudo* spp isolates from the GenBank database and the present study.



5. DISCUSSION and CONCLUSION

Medicinal leeches are used effectively in the treatment of many diseases in many countries of the world. The leeches are the main hematophagous organisms used in the treatment of post-operative sites of humans and animals such as cats, dogs and horses, as well as in the treatment of inflammatory diseases, edema, and painful areas. Among the bioactive substances in the saliva of the leech, hirudin is the strongest anticoagulant with a similar effect to heparin. More, besides facilitating wound healing, has positive effects in the elimination of diabetic effects, and in the treatment of blood-related diseases such as intravascular coagulation syndrome and polycythemia (Abdisa 2018).

In the process of using leeches directly in postoperative procedures, bacteria in the digestive systems of these creatures may cause secondary infections. For this reason, antibiotic treatment after medical leech application is considered an important step. The bacterial flora of medicinal leeches varies from species to species within the scope of symbiotic life ecology. Therefore, the type of antibiotic used in the treatment differs according to the type of leech (Laufer et al. 2008). In line with this information, the exact and correct identification of the taxonomic positions of medical leeches is one of the prerequisites for a reliable and healthy treatment.

In the Hirudinea taxon, three species of medicinal leeches (*Hirudo medicinalis*, *Hirudo verbana*, and *Hirudo troctina*) are widely in the Eurasian region (Utevsky et al. 2010). Until recent years, the species, *Hirudo medicinalis* and *Hirudo verbana* were named "*Hirudo medicinalis*" because of insufficient knowledge about their biology until proven definitively in the context of molecular analysis data (Siddall et al. 2007).

In recent years, the distribution area of *Hirudo medicinalis* has been defined more accurately (Utevsky et al. 2008, Westendorff et al. 2008, Utevsky et al. 2010, Elliot et al. 2011, Živić et al. 2015). Similarly, in recent studies, it has been proven that the majority of the leech populations in Turkey belong to the *Hirudo verbana* species rather than *H. medicinalis* (Apakupakul et al. 1999, Sağlam 2011, Siddall and Burreson 1998, Sağlam et al. 2018, Ceylan et al. 2019, Sağlam 2019, Ceylan and Çetinkaya 2021).

The genetic data and phylogeographic structure of a species provide the basic information needed to understand its evolution and biogeographic history (Marchandin et al. 2003). Mitochondrial DNA and Different regions of rDNA have been recognized as stable and specific markers for revealing relationships between closely related species or populations due to different evolutionary rates (Hwang and Kim 1999).

The recent molecular systematic investigations of the medicinal leeches have discriminated four European species of the genus *Hirudo* and showed that each of those species has a specific coloration pattern (Trontelj et al. 2004, Trontelj and Utevsky 2005, Siddall et al. 2007, Utevsky et al. 2009, Elliott and Kutschera 2011, Trontelj and Utevsky 2012).

The identified morphological characters are typical of the medicinal leech and distinguish clearly this species from other European leeches of the genus *Hirudo*, in particular from *Hirudo medicinalis* (Trontelj and Utevsky 2005, Siddall et al. 2007, Utevsky et al. 2009, 2010, Elliott and Kutschera 2011, Trontelj and Utevsky 2012, Kutschera and Elliott 2014). Based on the similar coloration and patterns of the dorsal and ventral sides, the present study showed that the medicinal leeches from the population studied belong to only one species. Also, the medical leech population in Karamık Lake was identified as belonging to the *Hirudo verbana* species within the scope of mt COI and 18SrRNA locus sequence data. These data support the knowledge that there is *Hirudo verbana* widely distributed in Anatolia.

Although there have been studies on *Hirudo verbana* in the last 10 years (Trontelj and Utevsky 2005, 2012, Siddall et al. 2007, Borda et al. 2008, Kvist et al. 2010, Utevsky et al. 2010) are still unresolved regarding the biology and distribution of this species. As a contribution to this problem, the findings of Živić et al. (2015) support the suggestion by Trontelj and Utevsky (2012) that *Hirudo verbana* is genetically divided into eastern and western clades. The fact that the haplotype results in this study were more similar to *Hirudo verbana* isolates in the eastern region (Azerbaijan, Iran, Turkey) supports the view of Trontelj and Utevsky (2012) to "divide *Hirudo verbana* into eastern and western clades".

The small subunit (18S) ribosomal gene is one of the most frequently used genes in phylogenetic studies and an important marker in environmental biodiversity screening. The 18S rRNA gene is part of the ribosomal functional core and is exposed to similar selective forces in all living beings. For that reason, biodiversity studies are commonly conducted using 18S rRNA genes (Meyer et al. 2010, Wu et al. 2015). *Hirudo verbena* samples from Karamık Lake showed full compatibility among themselves in terms of 18SsRNA sequences. According to these results, it can be said that the *Hirudo verbana* population in the study area is stable in terms of the 18S rRNA locus.

The mitochondrial gene, cytochrome c oxidase subunit 1 (COI), is also an important gene used in animal classification. Former works have validated the ability of COI sequences to diagnose species in certain taxonomic groups (Hebert et al. 2003). The isolates obtained in the present study process showed full agreement among themselves and with the other *Hirudo verbana* samples from other localities (AY763151.1, AY763150.1, JN083801.1, KT692947.1, EF446694.1, KU216244.1) (Trontelj and Utevsky 2005, 2012, Sağlam et al. 2016). Thus, it has been shown that the COI locus can be used as a reliable indicator for the identification and confirmation of the taxonomic position of *Hirudo verbana*.

Živić et al. (2015) stated that all medical leech samples analyzed from Serbia could be collected in five haplotypes. Two of these haplotypes were previously known from Ukraine (Trontelj and Utevsky 2012), while three haplotypes were newly identified. These three haplotypes were found to be very similar to the eastern clade comprising specimens from Eastern Europe, Turkey, and Uzbekistan (Trontelj and Utevsky 2005). Also, Sağlam et al. (2016), stated that isolated sequence data from their *Hirudo verbana* showed high identity with the mtCOI sequence. The isolates in this study and the isolates of the researchers given above showed complete agreement among themselves.

As an ecological finding, Ceylan et al. (2015) determined that the smallest individual from *Hirudo verbena* samples collected from Lake Eğirdir was 1.85 g. Wilkin and Scofield (1991) recorded that the smallest mature medicinal leeches, which were 3.07 g, decreased to 1.30 g after laying the cocoon, and thus continued to reproduce despite

70% weight loss. Petrauskienė et al. (2011) and Kovalenko and Utevsky (2012) suggested that *Hirudo verbana* be included in the r-strategist species group due to its ability to survive under variable environmental conditions and its high growth rate (Ceylan et al. 2015, Karataş et al. 2022). According to the weight values of the smallest mature *Hirudo verbana* samples examined in the present study, consistent with the data of the researchers above, the *Hirudo verbana* populations in Karamık Lake also have similar bio-ecological characteristics. It is important to know the population dynamics of medicinal leeches collected from their natural habitats for medical treatment to protect their sustainable bio-ecological characteristics. For example, Turkey holds the majority of world medical leech exports. Studies on the protection of medicinal leeches are scarce. However, it has been traded under a limited quota in recent years (Karataş and Dernekbasi 2018).

From a different perspective, cannibalism, which is defined as intraspecies predation, is defined as an individual killing and eating his fellows. *Hirudo medicinalis* was classified as a cannibalistic annelid (Kutschera and Roth 2005). Ceylan and Erbatur (2012), who investigated the reproduction and bio-ecology of *Hirudo verbana* can be categorized as a cannibalistic trait, although they did not describe the blood-sucking and killing action from its saturated specimens (Ceylan and Erbatur 2012). In accordance with the findings of Ceylan and Erbatur (2012), no cannibalistic behavior was found in the *Hirudo verbana* population during the field studies in Karamık Lake.

In conclusion, the end of the study was identified out on the anatomic and morphologic properties and mt COI and 18S rRNA sequence of *Hirudo verbana* samples from Lake Karamık. The mtCOI data of identified *Hirudo verbana* isolates were in full agreement with the mtCOI locus sequence of the other *Hirudo verbana* isolates in the NCBI database. Thus, the molecular taxonomic position of the *Hirudo verbana* samples, whose species were identified according to their anatomical and morphological features, was confirmed by the sequence data. The present study results have contributed to the establishment of the DNA barcode library, taxonomic origin, and evolutionary homogeneity of *Hirudo verbana*. However, studies on more locations and more genes are needed to better understand the genomic features of *Hirudo verbana*.

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