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THE APPLICATION AND BENEFITS OF FORENSIC PALYNOLOGY IN ROBBERY EVENTS IN TURKEY

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ABSTRACT. Forensic science is required to elucidate crime, and it has been carried out through various induction methods. Forensic palynology, a branch of forensic science, is used to find offenders by investigating the connection between the crime scene, evidence, and offender, solving events, proving the reliability of the victim's narratives, building up the profile of the offender, reducing the number of suspects, assisting security forces in conducting accurate investigations and detecting smuggling of drugs and other materials. Palynological evidence is known to be extremely resistant to extremely high temperatures (over 400°C), strong acids (such as HCl-HF), and fungal and bacterial activity. One of the most important reasons for using palynological evidence as evidence in court is its high resistance. Property crimes account for most judicial cases in Turkey. Burglary accounts for a significant portion of property crime. We aimed to solve various burglary events in Turkey using palynological evidence in this study. We used various methods to analyze palynological particles found in forensic samples sent to our laboratories between March 2016 and May 2017. The findings were distributed to law enforcement officers and legal experts working in courtrooms (lawyers/attorneys/judges).

1. INTRODUCTION

"Forensic Palynology" deals with the spores, pollen, and palynomorphs found within biological evidence collected at the crime scene (such as soil, weapons, clothing, shoes, scarves, socks, etc.). Since the 1950s, palynological evidence has been utilized in numerous forensic cases and continues to be used up to the present day [1]. Studies in the field of palynology began with Grew's discovery of pollen in 1682 when he referred to these structures as "spermatic globules." [2]. Palynomorphs comprise microfossils such as acritarchs, dinoflagellate cysts, colonial algae, and chitinozoans with organic wall structures resistant to acids [3].

Concerning forensic palynology, Klaus Wilhelm conducted palynological examinations on mud samples from boots belonging to a suspect in a murder case in Austria in 1959. As a result of their research, they managed to determine the location where the murder had been committed [4].

Keywords. Forensic science, forensic palynology, robbery, palynomorphs, pollen, spore

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The same year, palynological tests were performed on mud samples taken from the victim's clothing in another murder case that happened in Sweden. As a result of these examinations, the location and time of the murder were determined. These two cases became the first documented examples in official court records [5].

During the 1960s and 1970s, Max Frei [6] utilized palynological data to solve numerous criminal cases in Switzerland, enabling the identification of crime scenes and suspects [1].

Forensic palynological studies in Turkey started in the 2000s. In 2004, the first article was published under the title "Biocriminal Palynology". In the article, a palynological study on sheep wool in Elmadağ district of Ankara province was carried out and the comparison of pollen and their percentages in the wool was given [7].

Dogan at al. (2006) contributed to the resolution of a case by using forensic palynology for the first time in Turkey with a study on a burglary case in Sakarya province [8]

Forensic palynological evidence can be collected from a wide range of sources. Some of these sources include various tools (residues on tools like guns, rifles, forks, knives, shovels, rakes, hoes, etc., suspected to have been used in the crime), pollen traces on packaging materials, mud and soil particles on the soles of shoes, sticky substances like honey, beeswax, pine resin, textiles such as blankets, carpets, rugs, paints, both painted and unpainted wood or similar materials, different parts of a corpse in homicide cases (respiratory passages, intestinal contents, skin, gaps between fingers and toenails), stains on various objects, dust, papers, and more.

In Türkiye, most criminal incidents involve crimes against property. Among these, theft cases constitute a significant proportion. This study aims to solve various theft cases in Türkiye using palynological evidence. Palynological particles discovered in forensic samples between March 2016 and May 2017 that were sent to the laboratory were analyzed using various techniques and the incidents tried to be solved.

2. MATERIALS AND METHODS

Obtaining Samples

Samples for which investigations were requested from various public prosecutors' offices or courts from various judicial events were sent and examined. The origins of the examined requests in this study are shown in Table 1.

TABLE 1. The origins of the examined requests.

Sending Unit	Sending Year	Incident and Date
Turgutlu Chief Public Prosecutor's Office	Manisa	Bovine theft in Manisa province Ahmetli district, Dereköy Neighborhood Matdere Locality (Incident 1)-26.03.2017
Vezirköprü Chief Public Prosecutor's Office	Samsun	Attempt to steal animals from a house in Samsun province Vezirköprü district Aydınlı District (Incident 2)- 09.03.2017
Gordes Chief Public Prosecutor's Office	Manisa	A total of six cattle theft incidents from victim's barn in Salur Mahallesi Yaka Mevkii of Gördes district of Manisa province (Incident 3)- 04.01.2017
Posof Chief Public Prosecutor's Office	Ardahan	Attempted animal theft incident in Savaşır Village of Posof district of Ardahan province (Incident 4)-15.11.2016
Havza Public Prosecutor's Office	Samsun	Theft incident in the Celikhan District of Havza district of Samsun province (Incident 5)-16.04.2016
Çaycuma Chief Public Prosecutor's Office	Zonguldak	Four different cattle theft incidents occurred in Filyos Çaycuma district in Zonguldak province (Incident 6)-09.03.2016

Obtaining Palynomorphs, Spores, and Pollens from Samples

HF Acid Method

This method is used to obtain palynomorphs, spores, and pollen from soil samples. The process begins by weighing the sample to 10 g and initiating the drying step if the quantity is less than 10 g or drying the entire sample. The soil sample is placed in a plastic container and treated with HCl (hydrochloric acid), which is performed under a fume hood to protect from HCl vapor. This step aims to remove calcareous materials. After allowing the sample to settle for 24 hours, HCl is poured slowly onto the settled portion of the sample to continue the HCl reaction. It's added while monitoring the progress of the reaction. Completed samples are centrifuged, and washed, thus contamination is prevented [9].

Samples where the HCl reaction has finished are treated with HF (hydrofluoric acid) to remove mineral materials. Subsequently, the samples are allowed to settle with water, centrifuged, and subjected to acid treatment. Neutralized samples are treated with KOH (potassium hydroxide), washed, and filtered using fine mesh sieves.

After another centrifugation of these samples, distilled water and HNO_3 are added. The samples are mixed with sodium hexametaphosphate solution, and the

tubes are shaken after another centrifugation. The samples are centrifuged for ten minutes following this procedure. After that, KOH is applied to the samples once more. Following another washing of this mixture, the samples are liberated from surplus liquid. Following staining the samples, permanent preparations are made. By using this technique, soil samples are used to extract palynomorphs, spores, and pollen.

Wodehouse Method

This method is also applied in the preparation of pollen and spore slides for the analysis of samples such as clothing, textiles, soft furnishings, ropes, carpets, baskets, straws, etc.

Samples collected from the scene and sent to our laboratory have been washed with distilled water without contamination [10]. The distilled water containing pollen and spore samples is filtered through rice mesh screens with a diameter of 150-200 μm and transferred to centrifuge tubes. These tubes are centrifuged at 3500 rpm for 15 minutes. If the sediment level in the centrifuge tubes is high, the process is repeated 2-3 times; if the sediment level is low, it's repeated once more. After these steps, the distilled water in the samples is poured off. The tubes are inverted, placed on drying paper, and left for 1-2 minutes for the distilled water inside the tubes to drain. Following this stage, the samples undergo the staining process, and permanent slides are prepared.

Preparation of Permanent Slides

Preparation of Glycerin-Gelatine with Basic-Fuchsin

First, the gelatine is softened in distilled water. The softened gelatine is mixed with glycerin. To stain the pollen, basic fuchsin is added to prepare glycerin gelatine. Phenol is added to prevent mold. The mixture is heated, poured into petri dishes, and left to cool [11].

Pollen in tubes is transferred to slides using glycerin gelatine with basic fuchsin. The heated slides are covered with coverslips, labeled, and placed upside down on two parallel glass rods. Slides are awaited until freezing to diagnose spores and pollen [10].

Microscopic Examination of Slides

A binocular OLYMPUS CX41 light microscope was used to diagnose and count the pollen and spores. For counting, a 10x eyepiece and an x40 plan objective were utilized. The OLYMPUS E-330 camera (x100 plan oil-immersion lens) was used to take microphotographs of the palynological particles on the slides.

In the cases examined, two preparations were prepared for each sample sent by the prosecutor's office. Examples examined in the cases are given below in tables (Table 2-7). Diagnosis of pollen was made according to reference slides available

or prepared in the Hacettepe University Palynology Laboratory and publications on palynology [5, 11-15].

TABLE 2. Incident 1.

Sample No.	E.G. Content	Quantity
1	A sawdust sample from the incident.	1 Piece
2	A sawdust sample from the suspicious vehicle's body.	1 Piece

TABLE 3. Incident 2.

Sample No.	E.G. Content	Quantity
1	Wheat samples from the wheat Warehouse.	1 Piece
2	The black boots of the suspect.	1 Pair
3	Yellow-colored work gloves belonging to the suspect.	1 Pair
4	Brown-colored striped fabric pants belonging to the suspect.	1 Piece
5	The black coat belonging to the suspect.	1 Piece

TABLE 4. Incident 3.

Sample No.	E.G. Content	Quantity
1	Plant/soil samples from the area where the animals were loaded.	4 Package
2	Soil/straw/litter samples from the place where the animals were stolen.	3 Package
3	Samples from various parts of the suspect's vehicle.	5 Package
4	A soil sample from the left tool chest of the suspect's vehicle.	1 Package

TABLE 5. Incident 4.

Sample No.	E.G. Content	Quantity
1	Shoe samples of the suspects.	6 Pair
2	Sample from the floor mats of the suspects' Ford truck.	1 Package
3	Sample from the floor mats of the suspects' pickup truck.	1 Package
4	Sample from the victim's barn.	1 Package

TABLE 6. Incident 5.

Sample No.	E.G. Content	Quantity
1	Blue-colored jeans and a burgundy-colored hoodie worn by suspects.	1 Piece
2	Blue-colored jeans and cream-colored cardigan from the suspects.	1 Piece
3	Black jeans and a yellow coat from the suspects.	1 Piece
4	Wheat sample from wheat storage.	200 g

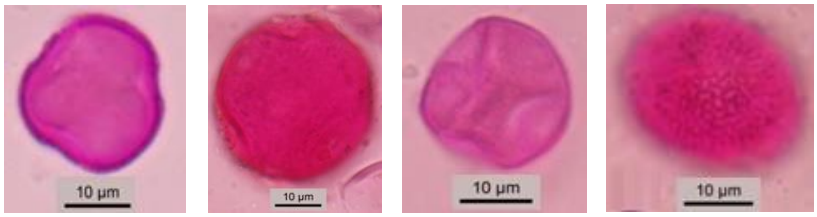
TABLE 7. Incident 6.

Sample No.	E.G. Content	Quantity
1	Sawdust samples found on the ground where the animals were loaded.	1 Package
2	Sawdust samples from the KIA truck body.	1 Package

3. RESULTS

In our study, forensic samples sent to our laboratory regarding the theft and attempted theft that occurred in various regions in Turkey between March 2016 and May 2017 were examined and evaluated from a palynological point of view.

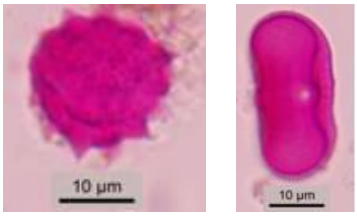
Various numbers of pollen belonging to Cupressaceae/Taxaceae, Pinaceae, *Plantago*, and Poaceae taxa were found in the examination made from the sawdust sample taken from the crime scene in incident 1. Brassicaceae, *Campanula*, *Cannabis*, and Oleaceae pollen found in the sawdust taken from the vehicle's body were not found in the sawdust sample taken from the crime scene (Figure 1). When these findings are evaluated together, the probability of the Fort Transit vehicle, which is suspected to have been used in the incident, to be found at the crime scene is very low.



a. b. c. d.

FIGURE 1. Microphotographs of pollen obtained from sawdust sample taken from the body of the suspect vehicle (a: Brassicaceae, b: *Campanula*, c: *Cannabis*, d: Oleaceae).

In the second incident, various numbers of pollen belonging to *Corylus*, Cupressaceae/Taxaceae, Pinaceae, and Poaceae taxa were found in the examinations made in the sample taken from the wheat warehouse. Pollens belonging to the insect-pollinated (zoogamous) *Bellis* and *Daucus* found in the samples taken from the wheat barn were not found in any of the suspect's belongings (Figure 2). When the results of our investigations are evaluated together, the probability of the suspect being present at the crime scene is seen as very low.



a. b.

FIGURE 2. Microphotographs of pollen obtained from a wheat sample taken from a wheat barn (a: *Bellis*, b: *Daucus*).

Pollens of taxa such as Cupressaceae/Taxaceae, Pinaceae, and Poaceae were found in the samples taken from the place where the animals were loaded and from the inside of the suspect vehicle, the front and rear plate, and various parts of the safe in the incident 3. In one of the samples taken from the place where the animals were stolen, starch grains that were found in uncountable concentrations were never found in the samples taken from various parts of the suspect vehicle. In addition, to consider the possibility of the suspect vehicle at the crime scene, it was expected that pollen belonging to taxa such as *Artemisia*, Boraginaceae, Caryophyllaceae, *Daucus*, and *Quercus* found in the samples taken from the crime scene would also be found in the samples taken from the vehicle. On the other hand, it is expected to encounter the shield hairs of the *Olea* (Olive) plant, which is abundant in the vegetation of the region (Figure 3). When these findings are evaluated together, the probability of the suspect being present at the crime scene is very low.

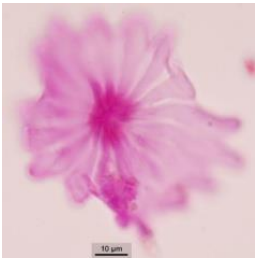


FIGURE 3. Microphotographs of the shield hairs of the Olea (Olive) plant.

Samples from the shoes and the mats of the Ford brand truck and pickup truck that belonged to the suspects in the fourth incident contained varying amounts of pollen from different taxa. Numerous *Chaetomium* spores and varying amounts of pollen from different taxa were found in the samples taken from the victim's barn (Figure 4). Pollen grains of Zoogamous taxa, Apiaceae, *Artemisia*, *Bellis*, and *Taraxacum*, were found in all samples examined. However, in this case, the absence of *Chaetomium* spores found in the sample taken from the victim's barn in any sample belonging to the suspects reduces the probability of the suspects being present at the crime scene. When these findings are evaluated together, the probability of the suspects being present at the crime scene is very low.

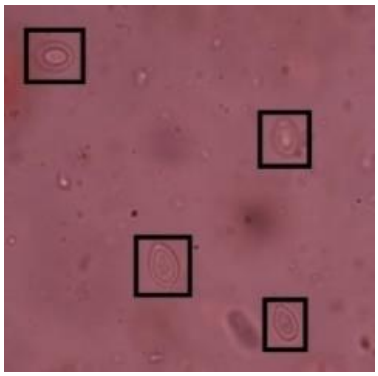
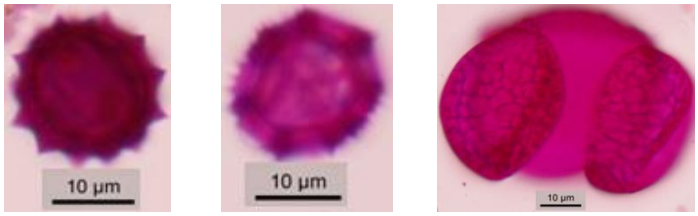


FIGURE 4. Microphotographs of the *Chaetomium* spores.

In the fifth incident, various pollen from multiple taxa was identified in the suspects' clothing. Pollen samples found in the examples were compared with the taxa they belong to, to determine whether the pollen from these taxa is similar or not. In this comparison, it was determined that the common taxa with pollen found on the suspects' clothing are *Bellis*, *Carex*, *Cistus*, Cupressaceae/Taxaceae, *Juglans*, Oleaceae, Pinaceae, Poaceae, *Quercus*, and Rosaceae. Another notable feature in this comparison is the high presence of pollen belonging to Pinaceae. Taxa from Pinaceae disperses their pollen by wind pollination and their pollen exhibits a wide distribution over large areas. The significant presence of Pinaceae pollen in the examined samples regarding this incident indicates that the suspects were in proximity to an environment where these taxa were present. The fact that Bellis, *Taraxacum*, and Pinaceae pollen were found in samples taken from the suspect individuals suggests that both these taxa and the individuals were present in the same environment (Figure 5). Due to the lack of detailed and sufficient photographs sent from the crime scene and its immediate surroundings, it has not been possible to establish a connection between the crime scene and the suspected individuals.



a. b. c.

FIGURE 5. Microphotographs of the suspects' clothes and pollen common to the area where the incident took place (a: *Bellis*, b: *Taraxacum* c: Pinaceae).

Furthermore, in all samples sent to our laboratory, wheat starches were found (Figure 6). These starch grains are most likely to have been transferred to the belongings of the suspect individuals from wheat storage. When these findings are considered together, the likelihood of the suspect individuals being present at the crime scene is quite high. To verify the accuracy of this possibility, we believe that our conclusion needs to be supported by additional evidence as well.

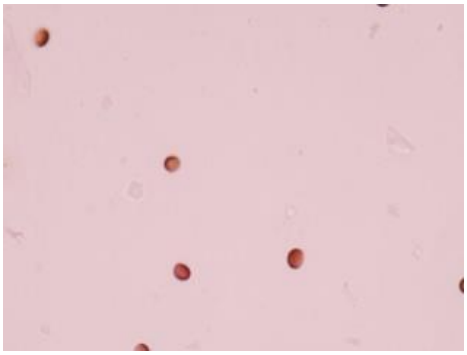


FIGURE 6. Microphotographs of wheat starches.

In the sixth incident, various pollen from different taxa were identified in the samples taken from the sawdust found at the location where the animals were loaded. Similarly, in the sawdust sample taken from the back of the suspect's truck, various quantities of pollen from different taxa were detected. In both examined samples, the common taxa with pollen were determined to be Cupressaceae/Taxaceae, *Lamium*, Pinaceae, and *Salix*. Cupressaceae/Taxaceae and Pinaceae are anemophilous plants, and their pollen can be found almost everywhere. The other common taxa were identified as *Salix* and *Lamium*. It can be stated that the pollen quantity of *Salix* is insufficient for comparison. While the pollen count for *Lamium* in the sample taken from the crime scene was 1229 grains, it was only 6 grains in the sample taken from the truck (Figure 7). These pollen quantities indicate a lack of consistency between the examined samples.

If the amount of *Lamium* pollen in the truck sample had been similar to the quantity in the sample from the crime scene, a clear connection could have been established between the truck and the crime scene. Additionally, taxa like *Alnus*, Asteraceae, *Fraxinus*, and *Populus*, whose pollen was found at the crime scene, were not observed in the truck sample. Although Oleaceae and Rosaceae pollen were found in the truck sample, they were not present in the sample taken from the crime scene.

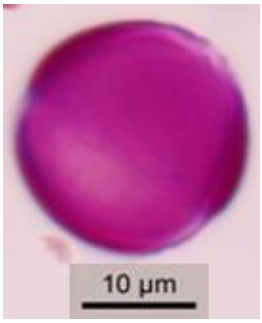


FIGURE 7. Microphotographs of *Lamium* pollen.

When these findings are considered together, the likelihood of the suspected vehicle being present at the crime scene appears to be a very weak possibility. To verify the accuracy of this possibility, we believe that our conclusion needs to be supported by additional evidence as well.

4. DISCUSSION

A burglary case from February 2006 was the first in Turkey to be solved using forensic palynology [8]. Since then, many cases have arrived at our laboratory, and research has been conducted on them. We have informed the legal system of our thoughts on these cases using the knowledge we have gained from these experiences. These cases' outcomes not only led to the capture of the offender but also saved the lives of innocent bystanders. In the studied cases, palynomorphs and spores were also used in addition to pollen. In case number five, starch grains were used. This demonstrates the need for those in this field to possess a strong grasp of both botanical science and palynology.

In addition, when we look at the examples of forensic palynology in the world [1, 16-20] we do not know how much our contribution is to the results of the cases, although it is known that it affects the results of the cases one-to-one.

Today, the United Kingdom is at the forefront of the use of forensic palynology, followed by New Zealand, Australia, Canada, a few Asian countries and the USA continue to use forensic palynology, albeit rarely [20]. In our country, the use of palynology in forensic cases is rarely utilized compared to these countries.

5. CONCLUSIONS

According to the study's data, pollen can be utilized as evidence in forensic investigations. It has been found that palynology can be utilized in forensic cases to establish a connection between the suspect's person or vehicle and the crime scene. Furthermore, our research will serve as a resource to enlighten scientists working on related problems.

Author Contribution Statements YH- specimen identification, data analysis, manuscript writing and editing, CD- supervising, specimen identification, data analysis

Declaration of Competing Interests The authors declare no conflict of interest.

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DRABA VERNA L. (BRASSICACEAE/CRUCIFERAE): A SALT AVERSE TAXON

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ABSTRACT. In this study, it was aimed to determine whether *Draba verna* L. (=*Erophila verna* (L.) Chevall. subsp. *verna* (L.) DC.), which is a relative of model organism *Arabidopsis thaliana* (L.) Heynh. and distributing around saline areas, is a halophyte or not and to research the salinity tolerance during germination period. *D. verna* seeds were germinated at distilled water and different NaCl concentrations (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 mM) at 8ºC/16ºC 12/12 h photoperiodism (light intensity 12000 lux ±%10) for 20 days. The NaCl concentrations and the germination percentages were as follows distilled water 100%, 100 mM NaCl 83%, 200 mM NaCl 2%, 300 mM NaCl 1% and no germination over 300 mM NaCl. Un-germinated seeds were taken into recovery and show 87.3% mean germination, and the ones still ungerminated were tested for viability. Increase in salinity, cause decrease in germination rate which means that *D. verna* is not resistant to salinity but salinity has important pressure on germination. The tolerance of *D. verna* seeds to salinity, although it has a wide distribution area at saline areas, is result of decrease in soil salinity during germination period. It can be concluded that *D. verna* is not a halophyte it is a salt avoider.

1. INTRODUCTION

Plants have adaptations that enable them to survive in very variable and extreme ecosystems ranging from habitats with wide distribution and favourable living conditions such as temperate forests, steppes, meadows to tundras, and deserts and saline areas. Plants are categorized in different ways, and when it is based on soil salinity they are seperated in two main groups; halophytes that tolerate salinity and glycophytes that avoid salinity [1]. Seeds of halophytic and glycophytic plants exhibit a response to salt stress similar to each other, and their germination process is delayed under salt stress conditions [2,3].

Halophytes possess distinctive adaptation mechanisms that enable them to germinate, thrive, and accomplish their life cycles in high-salinity environments, where majority of plants fail to survive [4, 5]. Despite such adaptations, they display superior germination abilities under unsaline or less saline conditions [6-9]. However, an increase in salinity levels leads to a decline in the germination rate and ratio and/or retards germination [10-17, 18-21].

Keywords. Brassicaceae/Cruciferae, *Draba verna*, germination, salinity

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The majority of halophyte seeds that fail to germinate due to salinity are able to germinate after salt is removed from the environment and when stress conditions are reduced [22], reflecting the physiological response of plants under high stress [23]. Under field conditions, seed germination of halophytes occurs after rain or floods, which provide moisture to the soil and cause salt to leach into the lower layers of the soil. The germination stage of most species occurs during this period [24].

Seed germination in saline conditions typically occurs in the spring or during seasons with excessive rainfall when the soil salt levels decrease [23]. Salinity has a drought-like effect on plants, known as 'physiological drought'. It obstructs the plants' osmotic activities and hinders root water and mineral uptake from the soil. While annual plants are more vulnerable to salt, perennial plants are more adaptable to saline conditions [25].

Brassicaceae/Cruciferae, commonly known as the mustard family, is a widely distributed monophyletic group consisting of 338 genera and 3709 species. Among them, *Arabidopsis thaliana* (L.) Heynh. is the most well-known species and serves as a model organism for flowering plants [26].

Draba verna L. is an annual, small, and delicate plant belonging to the Brassicaeae/Cruciferae family. It grows along roadsides, in swamps and fields, as well as in forest clearings where the vegetation cover is not dense. Typically, this taxon colonizes these areas following light or moderate disturbances. Thus, throughout the growing season from autumn to early spring, the plant faces minimal competition from larger plants and benefits from ample access to sunlight throughout its life cycle [27]. This widely distributed species was first described from Europe. In Türkiye, it can be found in various regions including Edirne, Istanbul, Çankırı, Sinop, Samsun, Trabzon, Artvin, Izmir, Ankara, Niğde, Muğla, Muş, Mardin, Konya, and Gaziantep [28, 29]. *D. verna* is a plant with multiple seeds, each ranging from 0.3-0.5 mm in size.

D. verna was chosen for this study due to its relation to *A. thaliana*, a model organism for seed plants that grows in saline environments. The aim was to ascertain whether the *D. verna* is a halophyte or salt-averse taxon.

2. MATERIALS AND METHODS

2.1. Study Area

Draba verna seeds were collected from Bolluk Lake in Konya-Cihanbeyli (N 38º 32' 59.8"-E 32º 55' 33.6") in May of 2014 (Figure 1). Bolluk Lake, located west of Tuz Lake, contains high levels of sodium sulphate, making it a saline water body. The lake is encompassed by infertile hills and steppe lands. Surface currents and a sulfurous water spring in the north provide the lake's main sources of water.

FIGURE 1. General view of Bolluk Lake

2.2. Collection of Seeds and Germination Trials

Draba verna seeds (Figure 2 and Figure 3) were kept at +4ºC until they were examined. The dimensions of the seeds were evaluated by BAB stereo binocular microscope and BAB image processing and analysis system (Bs200Pro). Mean weight of seeds were measured. Randomly selected one hundred seeds were randomly grouped in 500 to weighed using a precision balance for 5 times. Then the average weight of a *D. verna* seed was measured as $3.0\times10^{-5}\pm0.0008$ g.

To prevent fungal infection, the seeds underwent treatment with a 0.1% sodium hypochlorite solution for 3 minutes. Subsequently, they underwent three rounds of washing with sterile distilled water to ensure no contamination was present. Then four replicates of 25 seeds were placed in petri dishes on two layers of Whatman No.1 filter paper dampened with 4 ml of distilled water. The petri dishes were sealed with parafilm and then incubated at daily temperatures of 6/18°C, 8/20°C and 10/22°C in light (12-hour daily photoperiod) and continuous darkness (petri dishes were kept in black bags) for 20 days. Germination was monitored every two days for the light trial and at the end of the trial for the dark treatment. The temperatures recorded represent the mean maximum and minimum daily temperatures of the distribution area during the germination period in March and April. Germination is regarded as the emergence of radicle. The rate of germination in light was calculated using the modified Timson index of germination rate [12].

FIGURE 2. *Draba verna* (Photograph by Dr. İsa Başköse)

FIGURE 3. *Draba verna* seeds

Twenty-five seeds with 4 replicates were incubated at optimum light and temperatures for 20 days at the following NaCl concentrations: distilled water, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 mM NaCl solutions. Seeds

that failed to germinate during the salinity trials were washed using distilled water and then were incubated at optimum temperature for another 20 days with 4 ml of distilled water. The viability of the non-germinated seeds was determined with the TTC (Triphenyl Tetrazolium Chloride) test [13].

The germination index was calculated using the formula ΣG/t, where G is the percentage of seed germination at 2-day intervals and t is the total germination time. The maximum value obtained with this equation is 50 and indicates a high germination velocity [30].

Recovery percentage was calculated with the formula: [(a−b) / (c−b)] x 100 here a: total number of germinated seeds tested (all the seeds germinated in NaCl treatment and germinated after recovery); b: number of seeds germinated under saline conditions; c: total number of seeds tested [31]. The last germination was calculated with the formula: (a / c) x 100 [13]. Seed viability was calculated with the formula: [(a + d) / c] x 100. Here d is the number of embryos that were stained pink in the TTC solution [13].

Ungerminated seeds were treated with a 1% tetrazolium solution for 24 hours at 30°C. The viability of the seeds was then observed under a binocular microscope [32, 33]. Red staining of the seed was considered a positive indicator of viability as it has activity at the cellular level. In contrast, TTC does not react with non-viable seeds and therefore they do not stain [34].

Decreasing germination percentage (DGP) gives information about salinity tolerance and calculated by using the following formula:

DGP = [(Germination percentage at distilled water – Germination percentage at salinity) / (Germination percentage at distilled water)] x 100 [35].

2.3. Statistical Analysis

All the data were arcsin transformed and then evaluated by SPSS (IBM SPSS Statistics Version 25) with One Way ANOVA for the comparison of the influence of trials. T test was used for importance control ($p < 0.05$).

2.4 Bioclimatic Analysis of the Distribution Area of the Taxon

In order to determine the climatic characteristics of Bolluk Lake, which is the research region, the meteorological data of Cihanbeyli district, which is the closest station to the region, were used.

Precipitation and temperature data for Cihanbeyli were obtained from the General Directorate of Meteorology, Ministry of Forestry and Water Affairs. Bioclimatic interpretations were made according to Emberger (1955) [36, 37]. Ombrothermic diagrams of the study area were constructed according to Gaussen (1955) [38].

2.5 Soil Analysis of the Distribution Area of the Taxon

Soil samples of 1-2 kg were taken from appropriate root depths (rhizosphere) in the Konya-Cihanbeyli Bolluk Lake area where *D. verna* is distributed. All the soil analyses were accomplished at the 'General Directorate of Agricultural Research Central Soil, Fertilizer, and Water Resources Research Institute (Ankara)'.

3. RESULTS AND DISCUSSION

3.1. Germination Features

Temperature, salinity and alkalinity are some of the factors that affect the initial and maybe the most important life cycle stage of plants; germination [18,20]. The response of seeds to salinity, whether salinity cause death of seeds or prevent germination, depends on species. Halophyte plants, like glycophyte plants, show the highest germination rate in distilled water, but the percentage of germination decreases for both halophytes and glycophytes as the salt concentration increases, but halophyte seeds have higher salt resistance compared to glycophytes [2, 3, 6-17, 39].

D. verna is a widespread taxon and its range includes the vicinity of saline lakes. Being related to *Arabidopsis thaliana*, which is the model organism of seed plants, and its presence in saline areas makes it a suitable candidate for the study of salt resistance mechanisms at molecular level. The restricted distribution of this plant to saline areas requires further investigation into whether it is a halophyte or an ecotype suited to halophytic areas. Halophytes refer to plants that can germinate in NaCl solutions of 200 mM concentration and beyond [13, 20].

Based on the temperature experiments conducted, the germination percentages were observed to be 100% at 6 ºC/18ºC, 92% at 8ºC/20ºC and 95% at 10ºC/22ºC. Considering these germination percentages, it was determined that the most suitable temperature conditions for the germination of *D. verna* seeds are 6ºC/18ºC (12/12 h photoperiodism) (Figure 4). The impact of varying salt concentrations (distilled water, 100, 200, 300, 400, 500, 500, 600, 700, 800, 900, 1000 mM NaCl) on the sprouting of *D. verna* seeds was evaluated by subjecting them to 12/12 h photoperiod and 6ºC/18ºC temperature conditions. All the germination results, germination percentages, germination rates, last germination percentages, viability percentages after TTC test DGP ratios at changing salinities were given at Table 1.

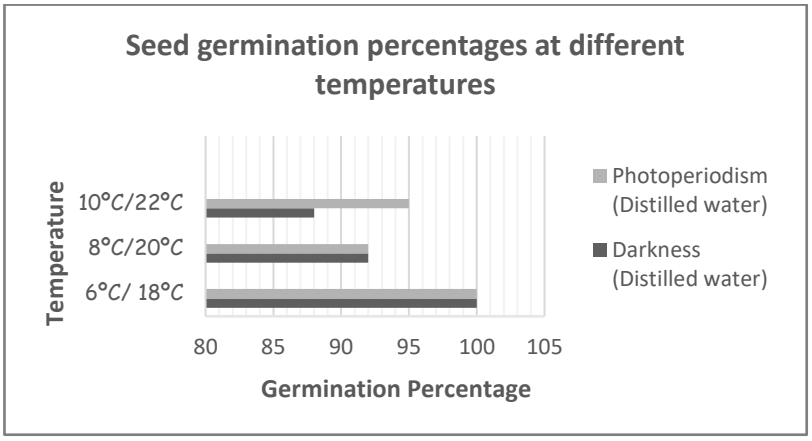

FIGURE 4. Seed germination percentages of *Draba verna* taxon at different temperatures

TABLE 1. Germination results of *D. verna*

	Germination (%)	Germination rate	Recovery (%)	Last germination (%)	Viability (%)	DGP
Darkness (Distilled water)	**100**	-	-	-	-	-
Photoperiodism (Distilled water)	**100[a]**	**34.85**	-	**100**	**100**	-
100 mM NaCl	**83[b]**	**23.95**	**47.06**	**91**	**94**	**16.16**
200 mM NaCl	**2[c]**	**0.45**	**93.88**	**94**	**95**	**97.98**
300 mM NaCl	**1[c]**	**0.1**	**97.97**	**98**	**98**	**98.99**
400 mM NaCl	**0[c]**	**0**	**97**	**97**	**97**	**100**
500 mM NaCl	**0[c]**	**0**	**89**	**89**	**89**	**100**
600 mM NaCl	**0[c]**	**0**	**95**	**95**	**95**	**100**
700 mM NaCl	**0[c]**	**0**	**89**	**89**	**89**	**100**
800 mM NaCl	**0[c]**	**0**	**93**	**93**	**93**	**100**
900 mM NaCl	**0[c]**	**0**	**84**	**84**	**84**	**100**
1000 mM NaCl	**0[c]**	**0**	**87**	**87**	**87**	**100**

D. verna showed the highest germination in distilled water like other taxa (Table 1). However, even in *D. verna*, which boasted a 100% germination rate in distilled water, the percentage of germinating seeds rapidly declined with increasing salt concentrations. At concentrations of 400 mM NaCl and beyond, no seeds germinated.

After conducting the improvement experiments, it was found that *D. verna* seeds exhibited an average germination rate of 87.3%. The results demonstrate that these seeds are capable of successful germination even after exposure to salt stress. The seeds germinate when environmental conditions improve, particularly when the salt level reduces.

The average percentage of viability of mature seeds of *D. verna* individuals was 92.36%. Such a high percentage of viability of the seeds and high germination percentages in the result of the recovery experiment after exposure to salt indicate that the low germination in salinity trials is due to the increased salt concentration.

Zhang et al. (2015) established the concept of 'Decreasing Germination Percentage (DGP)' and examined the germination patterns of 12 halophytes. Their study concluded that the highest reduced germination percentage meant the lowest salt tolerance. The results of this study have supported the idea that salt tolerance decreases with increasing salinity levels (Table 1).

Seeds stayed non-germinated at the end of the salinity trials first taken into recovery and if they did not germinate, then the viability test was conducted [13]. The color change observed in Figure 5 was an indicator of the viability.

According to Moore's (1972), Delouche's (1976), and Grabe's (1976) classification, seeds are considered viable if their embryos are entirely light pink or bright red without any milky white or yellowish staining at the end of the radicle [40-42].

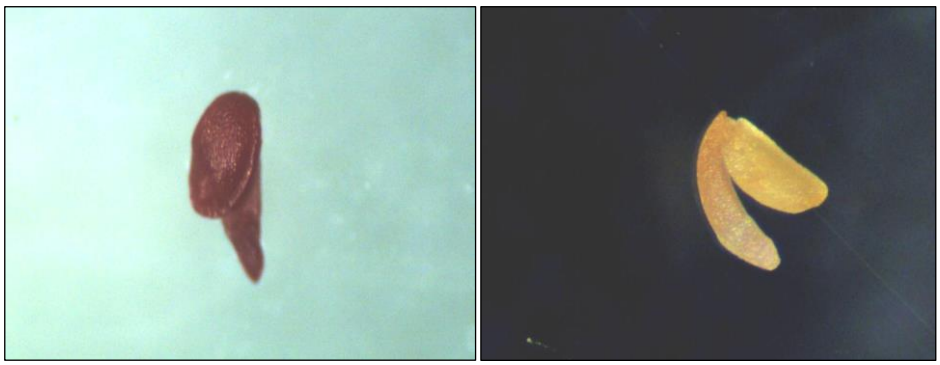

FIGURE 5. Viable seed (left-completely satined red) and un-viable seed (right-not stained red) after TTC test

As outlined in Parretio-de Guzman et al. (2011) study, the distinctions between viable and non-viable seeds post-TTC treatment were categorised in the following manner: The seeds were deemed viable if the entire embryo was stained or if there were minimal unstained regions in the plumule. Conversely, seeds were considered non-viable if more than the tip of the radicle was unstained, more than half of the radicle was unstained, the entire radicle and the junction of the plumule and radicle axis were unstained, the entire radicle and more than half of the plumule were unstained, the radicle and more than half of the plumule were unstained and greenish in colour, or the entire embryo was unstained [43, 44].

3.2. Bioclimatic Features

Bioclimatic analysis of the study area was performed according to Emberger method (Table 2). Ombrothermic diagrams of the study areas were created according to Gaussen method (Figure 6) [38]. The dry periods determined in the ombrothermic diagram start with May in Cihanbeyli and continue until the beginning of October.

TABLE 2. Bioclimatic analysis of the study area

Stations / Parameters	Cihanbeyli (Konya)
P (mm)	326.5
M (°C)	30.7
m (°C)	-4.9
Q	32.04
PE (mm)	40.1
S	1.31
Rainfall regime	Sp.W.F.Su. (Eastern Mediterranean Type 2)
Bioclimatic layers	Semi-arid, very cold in winter, Mediterranean climate

P: Mean total annual rainfall (mm), **M:** Mean maximum. temperature of the warmest month (°C), **m:** Mean mininum temperature of the coldest month (°C), **Q:** Rainfall-temperature coefficient, **PE:** Summer rainfal total (mm), **S:** Drought index, **Sp:** Spring, **W:** Winter, **F:** Fall, **Su:** Summer [36].

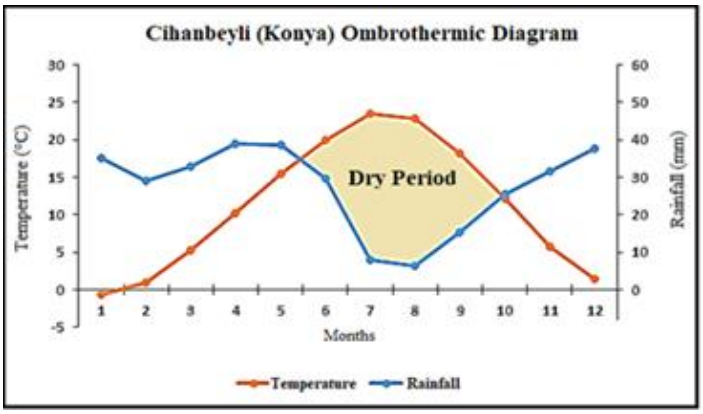

FIGURE 6. Cihanbeyli Ombrothermic Diagram

3.3. Soil Features

The salinity tolerance of certain species determines their habitat types and range [45]. Germination is typically the most vulnerable stage of a plant's life cycle as it defines the conditions that the plant must face in the soil [46].

The results of the analyses of the soil samples taken from the area of distribution of the target taxon in Bolluk Lake are presented in Table 3. The salt concentration in the soil is determined by the electrical conductivity of the saturated solution. The electrical conductivity of a solution is proportional to the salinity of the soil. Accordingly, the EC (electrical conductivity) is below the lower limit of 4 dS/m during the germination period in the distribution area. This value can be interpreted as non-saline or slightly saline soil [47]. In addition, when the total salt content of the soil is examined, it can be said that the soil is very low in salt.

TABLE 3. Analysis of soil samples from the research area

Soil samples	**Texture**	**Electrical conductivity (dS/m)**	**Salt (%)**
I	CL (Clay loam)	1.439	0.062
II	CL (Clay loam)	1.482	0.064

4. CONCLUSIONS

As a result, a statistically significant difference was determined between distilled water and 100 mM NaCl ($p<0.05$). The same situation was observed between 200 mM NaCl and higher salt concentrations ($p<0.05$). A value between 100-200 mM NaCl can be considered as the threshold value for *D. verna* seeds. *D. verna*, an ephemeral taxon like *Holosteum umbellatum* L., avoids salt by germinating during the period of decreasing salt concentration, when the salt in the soil is washed away by snowmelt and spring rains, and by completing its development before the dry summer months when salt concentration increases. Seed germination at low salinity also coincides with climatic conditions. The germination period coincides with the period when the salt is washed out of the soil as a result of snowfall and rain, and the plant completes its development bear and shed seeds before the start of the summer season when the salt rises again. In this case, the taxon *D. verna* is not halophyte, but it can complete its life cycle in saline areas with an ecological adaptation that allows it to avoid salt, so it can be classified as salt averse taxon.

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ANATOMICAL AND MORPHOLOGICAL CHARACTERISTICS OF ENDEMIC *BARBAREA AURICULATA* TAXA (VAR. *AURICULATA* AND VAR. *PALUDOSA*)

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ABSTRACT. The aim of this study was to determine the anatomical and morphological characteristics of two endemic taxa of *Barbarea auriculata* naturally growing in Türkiye. In morphological studies, characteristics of the vegetative (root, stem, and leaf) and generative (flower, fruit, and seed) structures of both taxa were determined and detailed descriptions were given. Furthermore, according to the morphological data obtained, the identification key was revised again. In anatomical analyses, cross-sections were taken manually from the roots, stems, and leaves of the two taxa. The sections were made into permanent preparations and examined under a binocular light microscope. Sections were then photographed with an integrated camera system. The morphological and anatomical characteristics of both species are discussed in detail according to their similarities and differences.

1. INTRODUCTION

The family Brassicaceae is represented by 321 genera in the world [1] and 96 genera in Türkiye [2]. The genus *Barbarea* is represented by 29 species in the world [3] and 19 taxa belonging to 14 species in Türkiye, 11 of which are endemic [4,5]. Members of this genus are distributed in the warm regions of Eurasia, Australia, and North America, and in some countries of South America and the eastern parts of Africa.

Members of the genus *Barbarea* are biennial and perennial, rhizomatous or herbaceous plants with normal root structures, mostly distributed in humid environments or along riversides. Stems are erect, spreading, creeping, and sometimes recumbent. The basal leaves are simple, entire, lyrate, pinnatifid, or pinnatisect and the stem leaves are short-petiolate or sessile, auriculate, and enveloping (amplexicaul). *Barbarea* taxa are usually glabrous, but some have dense or sparse simple hairs on the stem and leaves. All taxa of the genus in Türkiye have yellow flowers and the inflorescence is racemose or paniculate.

Keywords. Anatomy, Barbarea, Brassicaceae, endemic, morphology.

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The fruit is silique, characteristic for the family. Seeds are in single rows, elliptic, ovate, brown, and swollen or slightly flattened.

Upon conducting a review of the relevant literature, it was concluded that there are no comprehensive studies of the anatomy of *Barbarea* taxa in either the world or in Türkiye to date. There are only a few limited studies of the taxa of this genus in Türkiye [6,7,8]. Therefore, in the present study, morphological and anatomical examinations of two *Barbarea auriculata* taxa (var. *auriculata* and var. *paludosa*) distributed in the Eastern Anatolia and Black Sea regions of Türkiye were carried out on specimens collected from different localities. All the data obtained are given in detail and discussed comparatively.

2. MATERIALS AND METHODS

2.1. Fieldwork and Collection of Samples

Field studies were carried out between 2006 and 2009 during the vegetation period from April to August. Samples for morphological studies were collected at both flowering and fruiting times of the taxa. While collecting samples, it was taken into account that they should be as numerous as possible to properly represent the respective populations. The collected specimens were pressed and dried according to general herbarium techniques [9] and prepared for identification. They were then identified using the relevant literature [10,11,12]. The specimens used in anatomical analyses were collected at maturity during field studies from the natural habitats of the taxa and preserved in 70% ethanol.

2.2. Morphological Studies

Morphological examinations were carried out primarily on specimens collected from field studies together with other specimens deposited in national herbariums (ANK, GAZI, EGE, HUB, and KNYA). All morphological and morphometric measurements were made on dry specimens and an average of at least 50 measurements were made for each taxonomic character to determine the limit of variation. Detailed descriptions were prepared according to the data obtained. Photographs of the morphological characteristics of the species were taken during field studies or in the laboratory.

2.3. Anatomical Studies

Anatomical examinations were carried out on specimens that were collected during field studies and placed in 70% ethyl alcohol. Anatomical sections of the root, stem, and leaf parts of the *Barbarea auriculata* taxa were prepared by hand. The sections were examined under a BX51 Olympus microscope with an integrated camera system and photographed in detail. The sections were organized using a computer, and tissue and cell parts were identified and shown in photographs. The specimens used in the anatomical studies and their localities are given in Table 1.

TABLE 1. Specimens used to anatomical studies and localities

Taxa	Collector No	Locality
Barbarea auriculata var. *auriculata*	Bağcı-3728	B7 Erzincan, Kemaliye, above Ergü village, stream and field banks, 29 June 2007, 1550-1600 m a.s.l
Barbarea auriculata var. *paludosa*	Bağcı-3698	B7 Erzincan, above Koçyatağı village, moist meadow areas, 26 June 2007, 2150 m a.s.l

3. RESULTS

In this section, the morphological and anatomical characteristics of endemic *Barbarea auriculata* taxa (var. *auriculata* and var. *paludosa*) distributed in Türkiye are given in detail. The root and stem anatomies of the taxa were evaluated separately, but since the leaf anatomies of the taxa are similar according to the data obtained, their leaf anatomical features are given together in the discussion section in detail.

3.1. Morphological Characteristics

3.1.1. *Barbarea auriculata* Hausskn. ex Bornm., in Mag. Bot. Lap. 30:55 (1931).

Perennial herb, usually with fibrous petiolar remains. Flowering stem erect, simple or branched, 15-55 cm, stems sometimes slightly hispid-setose or glabrous. Rosette leaves long petiolate, slightly retrorse-pilose, up to 14 cm, with orbicular to ovate terminal leaflets, 0.2-5.0 x 0.2-3.5 cm, slightly lobed or entire. Lower leaves with a large terminal leaflet and usually 0-1 pairs of lateral leaflets, sometimes 1-2 pairs of lateral leaflets, and distant, semi-amplexicaul auricles, larger than the lateral leaflets. Stem leaves petiolate or sessile, entire or repand-dentate to lobed, with large auricles. Upper stem leaves amplexicaul, crenate to dentate, sparsely pubescent, and sometimes glabrous. Inflorescence racemose or panicle, up to 20 cm, ebracteates or bracteates, if present similar to uppermost stem leaves. Nectaries 2, conspicuous. Flower buds glabrous or at least with one white simple hair at tip on some flowers. Sepals 3.5-4.5 x 2-2.5 mm, petals yellow, 6-8 x 2.0-2.5 mm, filaments 4-5.5 mm long, anthers 1.5-2.0 x 0.7-1.0 mm, pedicels 3-7 mm, slightly hispid or glabrous. Silique strict, torulose, glabrous, rarely slightly pubescent, linear at maturity, greenish or yellowish, valves conspicuously veined, 9-32 x 1.0-2.1 mm. Seeds in one row in each cell. Style (1.5-) 2-4 mm, stigma capitate, 2-lobed.

In the "Flora of Turkey", this species is given as two varieties according to whether the lower part of the stems are glabrous or retrorsely hispid-setose hairy. However, as a result of the examination of the specimens of both taxa, it was determined that both hairy and glabrous lower parts of the stems of both taxa

were present and it was understood that these characters could not be used in variety distinction. In this study, according to the new findings, pubescence of the fruit and stylus characteristics were used to distinguish the two taxa.

Diagnostic key to *Brabarea auriculata* taxa;

1. Stems glabrous below; fruit glabrous, stylus (2-) 2.5-3.5 (-4) mm …….. var. *auriculata*
1. Stems retrorsly hispid-setose below; fruit hairy, stylus 1.5-2.5 (-3) mm …… var. *paludosa*

3.1.1.1. *Barbarea auriculata* Hausskn. ex Bornm. var. ***auriculata***, in Mag. Bot. Lap. 30:55 (1931) (Figure 1).

The taxa is endemic to Türkiye and distributed only in the Eastern Anatolia region within the borders of the Kemaliye district of Erzincan province at 1500-600 m in fields and along stream banks (Figure 2). It usually blooms in April and May. The species is categorized as EN (Endangered) according to IUCN criteria. The endemic var. *auriculata* is known only from the type locality (Erzincan, Kemaliye) (Figure 2). It was not collected again after the year it was first described and was categorized as "Extinct in the wild-EX" in the Red Data Book of Turkish Plants published in 2000 [13]. However, it was collected again from the type locality both within the scope of this study (in 2007) and earlier by other botanists working in the region (in 2005) and was accordingly removed from the EX category.

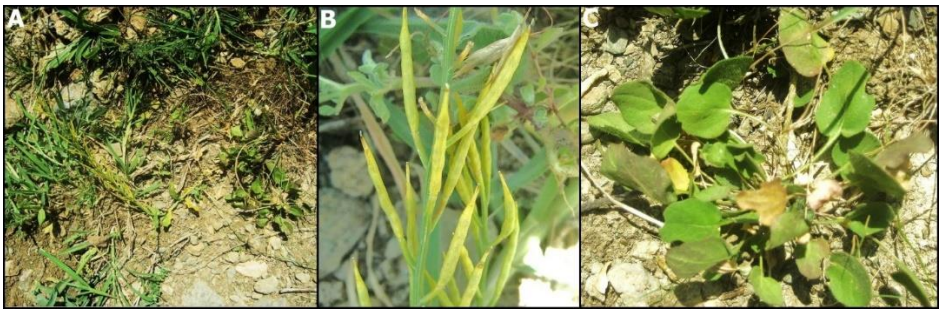

FIGURE 1. Morphological characteristics of *Barbarea auriculata* var. *auriculata*, A) habitus, B) fruit, C) rosette leaves

Distribution in Türkiye: B7 Erzincan: Kemaliye, above of Ergü village, stream and field edges, 29 June 2007, 1550-1600 m a.s.l. 39˚ 12' 828" N-38˚ 31' 428" E, *Bağcı-3728, Savran ve Başköse*; Kemaliye, above of Ergü village, 1580 m a.s.l., 10 April 2005, 39°12.59′N, 38°31.20′E, *Kandemir-6960*; Armenia Turcica: Egin (ad fluvium Euphratem) ad Argubaschi, Habenus Erek, 3 June 1890, *Sintenis-2460* (K!).

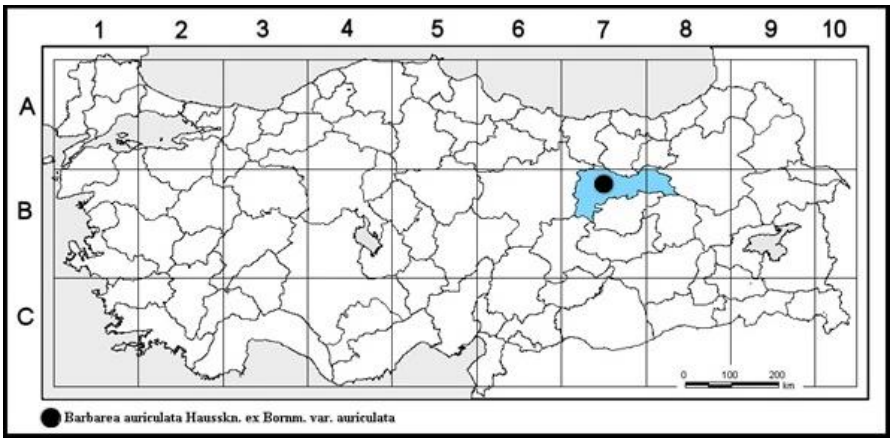

FIGURE 2. Distribution map of *Barbarea auriculata* var. *auriculata* in Türkiye

3.1.1.2. ***Barbarea auriculata*** Hausskn. ex Bornm. var. ***paludosa*** Coode & Cullen in Notes R.B.G. Edinburg. 26:000 (1964) (Figure 3 and 4).

FIGURE 3. Morphological characteristics of *B. auriculata var. paludosa,* A) inflorescence, B) flower, C-D) fruit, E) stem leaves, F) rosette leaves

The taxa is endemic to Türkiye and distributed in the provinces of Trabzon, Ardahan, Kars, Elazığ, and Erzincan in the Eastern Anatolia and Black Sea regions (Figure 5) between 1500 and 2650 m in moist meadows and wetlands

and along slopes and roadsides. The taxon usually blooms in May and June. It is categorized as VU (Vulnerable) according to IUCN criteria [13].

FIGURE 4. Habitus of *B. auriculata* var. *paludosa*

Distribution in Türkiye: A8 Trabzon: Between Trabzon and Gümüşhane, 2-3 km after the Zigana tunnel, to the right of the road, damp places, 1750 m a.s.l., 12 July 2009, 40˚ 38' 727" K – 39 22' 734" D, *Savran, Bağcı-4092*. **A9 Ardahan:**

Ardahan-Artvin road, 1-2 km before Çamlıbel pass (from Ardahan), roadside, 2600-2650 m a.s.l., 28 June 2007, 41˚ 12' 335" N - 42˚ 30' 469" E, *Bağcı-3714a, Savran*. **A9 Kars:** Arpaçay, Karakale Village, meadow, moist places, 1800-1900 m a.s.l., 27 June 2007, 40˚ 52' 000" N- 43˚ 27' 225" E, *Bağcı-3707, Savran*; Kars-Şavşat road, 15. km moist places, 2220 m a.s.l., 11 July 2009, 41˚ 11' 820" K – 42˚ 33' 171" D, *Bağcı-3979, Savran*, **B7 Elazığ:** Kuşakçı Mountain, around Yedipınar village, slopes and roadside, 1520-1780 m a.s.l., 21 May 2002, *Türkoğlu-3106*, **B7 Erzincan**: Above Koçyatağı village, moist meadow areas, 2150 m a.s.l., 26 June 2007, 39˚ 54' 483" N - 39˚ 13' 658" E, *Bağcı-3698, Savran*; Between Erzincan and Gümüşhane, Ahmediye Pass, east of BTV station, wetlands, 2100 m a.s.l., 20 June 2009, *Savran-3606*; Erzincan-Sivas Kızıldağ pass, eastern roadsides, wetlands, 2190 m a.s.l., 24 June 2009, *Savran-3680*.

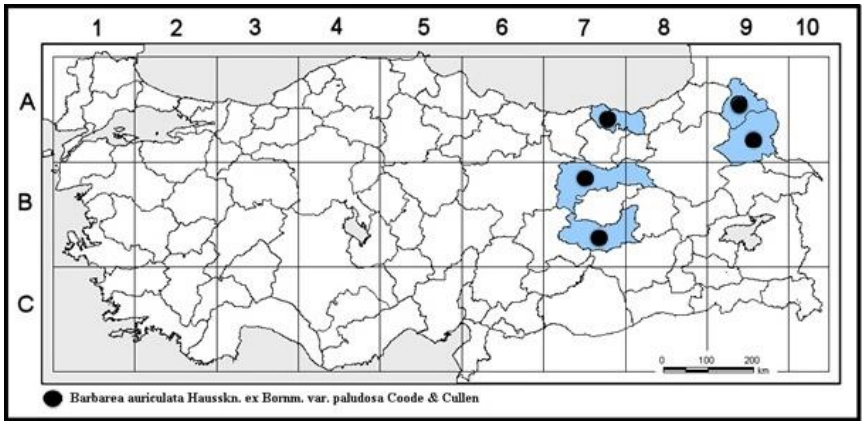

FIGURE 5. Distribution map of *B. auriculata* var. *paludosa* in Türkiye

3.2. Anatomical characteristics

3.2.1. *Barbarea auriculata* var. *auriculata*

Root Anatomy: Root is cylindrical. Periderm (exodermises) is multilayered (3-4) on the outer surface of the root (Figure 6B). Cortex tissue is very wide, 20- to 25-layered with parenchyma tissue under the periderm (Figure 6A). In the cortex tissue, cells store dense starch grains and are orbicular or oval-shaped (Figure 6B). There are also clustered sclerenchymatous cells in the parenchyma tissue in the cortex. There are no significant lines between the pith region and the cortex. The cambium is formed between the phloem and xylem in the order of 2-3 cells. In addition, the presence of three separate rings in this taxon indicates that the plant is perennial. The xylem was found as concentric rings below the cambium (Figure 6C). The cambium periodically produces lignified and unlignified tissues. Sclerenchyma cells were seen in the lignified xylem area. The

sclerenchyma tissue fills intensely between xylem tissues. Parenchyma tissue is also observed in the pith region (Figure 6D).

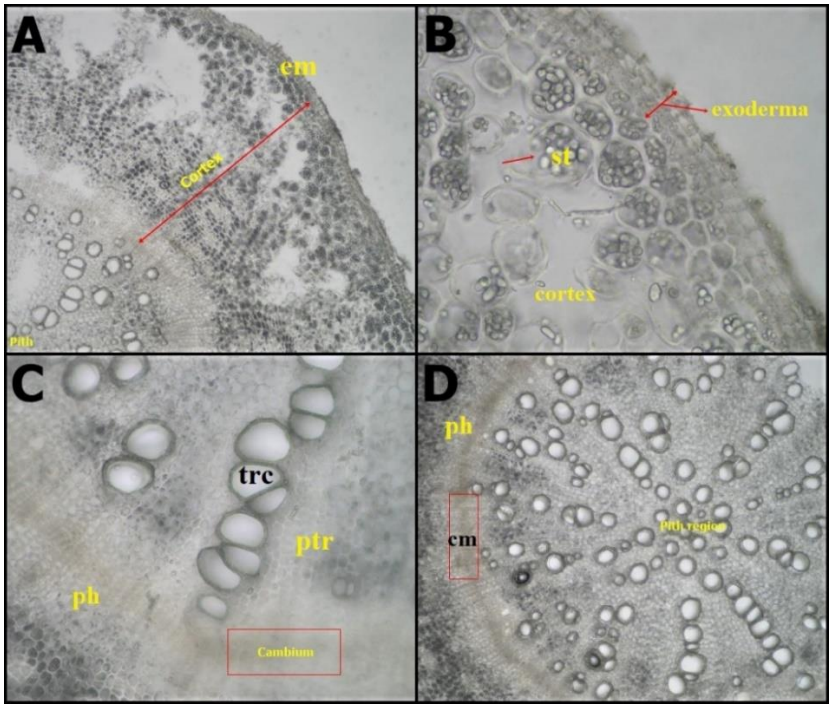

FIGURE 6. Root Cross-section of *Barbarea auriculata* var. *auriculata*, A) cortex and pith region (10X), B) cortex and exoderma (40X), C) trachea, pith rays and cambium (40X), D) pith region (10X), (cm: cambium, em: exoderma, ph: phloem, ptr: pith ray, st: starch, trc: trachea)

Stem Anatomy: It can be understood that the stem structure is not cylindrical but canaliculated (grooved) due to the outward projections (Figures 7A, 7D). In transverse section, the outermost part of the stem has a single layer of epidermis with a thick cuticle (Figure 7B). In some sections, the epidermis appears to be 2-rowed, in which case the second lower layer is called the collenchyma (Figure 7C). Stomata of the amphistomatic type are present on the epidermis. The cortex layer consists of parenchyma in 5-6 layers, thin-walled, with regularly oval or rounded cells. The outer parenchyma cells of the cortex contain chloroplast, while inner cortex cells contain dense starch granules. Below the cortical parenchyma is the endodermis, which consists of a single layer of orbicular or oval-shaped cells. There are no starch granules in the endodermic cells. In vascular bundles, the phloem consists of 4-5 cell layers (Figures 7E, 7F) and there is also lunar-shaped sclerenchyma tissue with 1-2 cell rows on the outer part of the phloem (Figures 7D, 7E). Tracheid cells are especially evident in the area of the xylem. The cambium is distinguished between the xylem and phloem

tissues. The sclerenchymatous tissue is located between vascular bundles, in 5-6 layers and consisting of irregular and squashed cells (Figure 7G). Cambium cells are not clear (Figure 7E). The pith region occurs in the center of the stem, composed of large orbicular or polygonal parenchymatic cells (Figures 7H, 7I). In the anatomical structure of mature stems, these cells fragment in the center to form rexigenous spaces (Figure 7H), but this is not seen in young stems (Figure 7I).

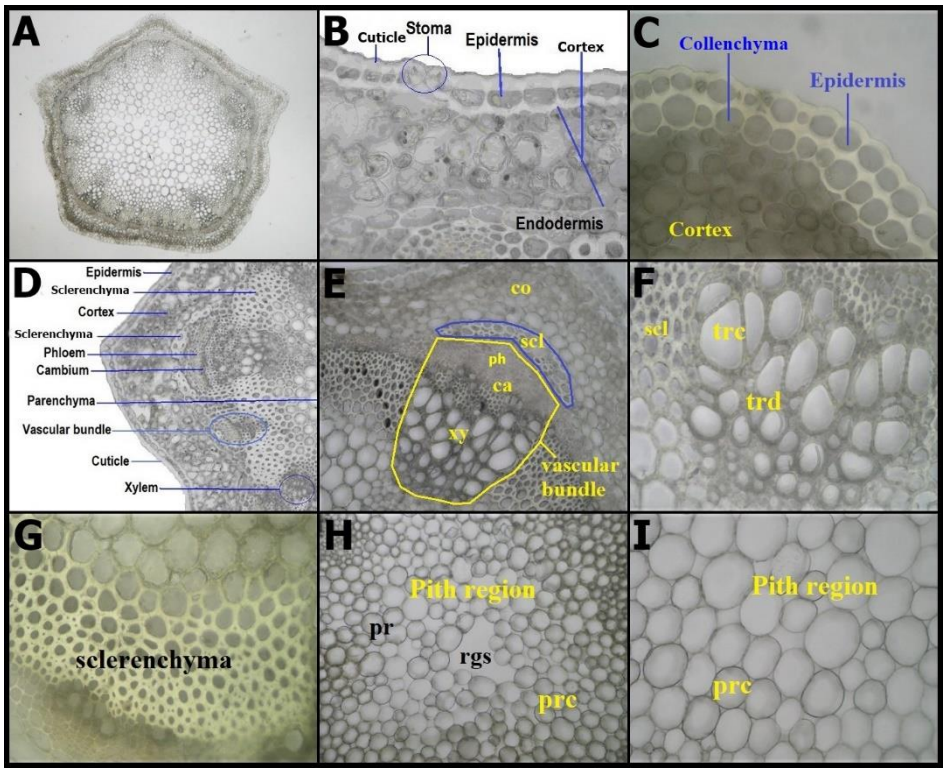

FIGURE 7. Stem Cross-section of *Barbarea auriculata* var. *auriculata*, A) general stem section (4X), B) epidermis and cortex (20X), C) epidermis and collenchyma (20X), D) general anatomic parts (10X), E) vascular bundle (20X), F) trachea and tracheid (40X), G) sclerenchyma tissue (40X), H) pith region (adult plant, 20X), I) pith region (young plant, 40X). (ca: cambium, co: cortex, fl: phloem, pr: parenchyma, prc: parenchymatic cells rgs: rexigenous spaces, scl: sclerenchyma, trc: trachea, trd: tracheid xy: xylem)

3.2.2. *Barbarea auriculata* var. *paludosa*

Root Anatomy: Root is cylindrical. Exodermis is multilayered (3-4) on the outer surface of the root. A secondary structure was seen, far from the periderm, with the cortex layer below. Cortex tissue is very wide, 15- to 20-layered, with parenchyma tissue under the periderm. In the cortex, tissue cells store dense

starch grains and are orbicular or oval-shaped. There are also clustered sclerenchymatous cells in the parenchyma tissue in the cortex. There are no significant lines between the pith region and the cortex. The cambium is formed between the phloem and xylem in the order of 3-4 cells. The presence of three separate rings in this taxon indicates that the plant is perennial. The xylem was found as concentric rings below the cambium, which periodically produces lignified and unlignified tissues. Sclerenchyma cells were seen in the lignified xylem area. The sclerenchyma tissue fills intensely between xylem tissues. Parenchyma tissue is also observed in the pith region (Figures 8A-8D).

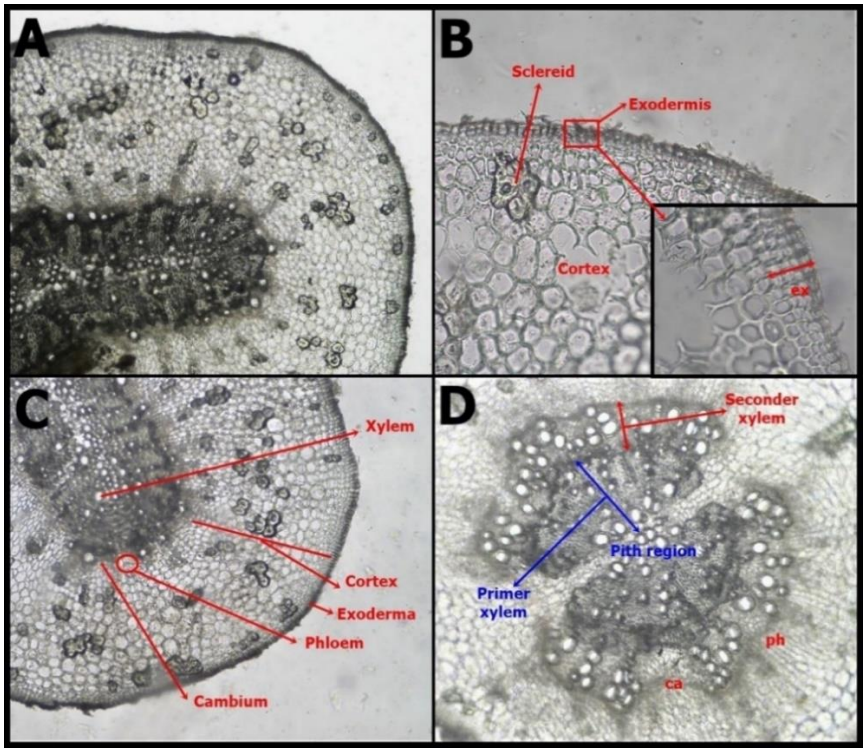

FIGURE 8. Root Cross-section of *Barbarea auriculata* var. *paludosa* A) general stem section, B) exodermis and cortex, C) general anatomic parts of root, D) pith region and vascular bundle, (ca. cambium, ex: exodermis, ph: phloem)

Stem Anatomy: It can be seen from the stem cross-section that the body structure is not cylindrical (Figure 9A). In transverse section, the single-layered epidermis was found with thick cuticle on the outermost part of the stem. The cells of the epidermis are cubic (Figure 9B). Stomata of the amphistomatic type are present on the epidermis. Cortex layer consists of parenchyma in 3-5 layers, thin-walled, with regularly oval or round cells. The outer parenchyma cells of the cortex contain chloroplast, while inner cortex cells contain dense starch granules. Underneath the cortex parenchyma, there is a single-layered

endodermis with cells orbicular or oval-shaped. The endodermal cells store starch granules. In vascular bundles, the phloem consists of 4-5 cell layers and xylem tissue externally surrounds it. Tracheid cells are especially evident in the area of the xylem. The cambium is distinguished between the xylem and phloem tissues (Figures 9E, 9F). Cambium cells were not clear. Sclerenchymatous tissues is located between vascular bundles (Figures 9C, 9D) in 5-6 layers and consists of irregular and squashed cells (Figure 9G). There is also lunar-shaped sclerenchyma tissue with 1-2 cell rows on the outer part of the phloem (Figure 9E). In the center of the stem is the pith region, composed of large orbicular or polygonal parenchymatic cells (Figures 9H, 9I). These cells may disintegrate in the center to form rexigenous spaces (Figure 9H). Sometimes, in mature stems, the central part may be completely hollow (Figure 9I).

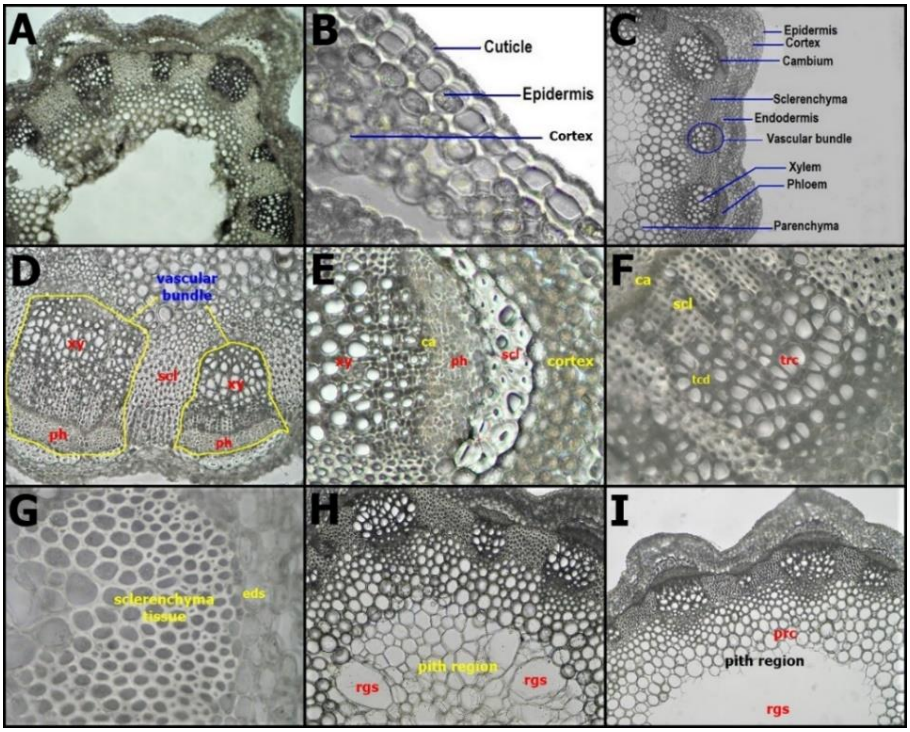

FIGURE 9. Stem Cross-section of *Barbarea auriculata* var. *paludosa* A) general stem section (4X), B) epidermis and cortex (40X), C) general anatomic parts (10X), D) vascular bundle (20X), E) vascular bundle parts (40X), F) trachea and tracheid (40X), G) sclerenchyma tissue (40X), H) pith region (young plant, 20X) I) pith region (adult plant, 20X). (ca: cambium, eds: endodermis, ph: phloem, pr: parenchyma, prc: parenchymatic cells, rgs: rexigenous spaces, scl: sclerenchyma, trc: trachea, tcd: tracheid xy: xylem)

4. DISCUSSION

4.1. Morphology

The geographical distribution areas and altitudes of these taxa are different. The only common distribution area occurs in Erzincan province (Figure 2 and Figure 5). *Barbarea auriculata* var. *paludosa* has a wider distribution area and is distributed at higher altitudes than var. *auriculata* (Figure 2 and Figure 5). Var. *auriculata* is known only from its type locality, the Kemaliye district of Erzincan province, and is categorized as CR (Critically Endangered) according to IUCN criteria [13].

Although these taxa of *Barbarea auriculata* (var. *auriculata* and var. *paludosa*) are morphologically similar, they differ in stem length, terminal leaflet structure of the rosette leaves (Figures 10A, 10C), and fruit and style size (Figures 10B, 10D). Comparative differences between the taxa are given in Table 2.

TABLE 2. Morphological differences between the two varieties

Taxa► Characters▼	***Barbarea auriculata* var. *auriculata***	***Barbarea auriculata* var. *paludosa***
Stem lenght	18-43 cm	15-55 cm
Rosette leaves length	up to 12 cm	up to 14 cm
Terminal leaflets size	0.2-3.5 x 0.2-2.3 mm	0.7-5.0 x 0.5-3.5 cm
Silique hair type	usually glabrous, rarely slightly pubescent	usually pubescent, sometimes glabrous
Silique size	9-26 x 1.0-2.06 mm	15-32 x 0.5-1.7 mm
Style size	(2-) 2.5-3.5 (-4) mm	1.5-2.5 (-3) mm

4.2 Anatomy

4.2.1 Root Anatomy

In anatomical studies, it was determined that although the two taxa are similar in their root, stem, and leaf anatomies, there are some differences between them.

Based on root anatomy data, it was determined that the root anatomies of the taxa were similar. On the other hand, the existence of some anatomical differences was observed in detail. The most important difference is the presence of a secondary structure in the root of *Barbarea auriculata* var. *paludosa* (Figure 8D). There are therefore both primary and secondary xylem elements in the central cylinder of the root of this taxon. In var. *auriculata*, there are only primary xylem elements in the pith region due to the presence of the primary structure (Figure 6D). The second important difference is the number of layers of parenchymatic cells forming the cortex. In var. *auriculata*, the cortex consists of 20-25 layers of parenchyma cells (Figure 6A), while in var. *paludosa* it consists of 15-20 cell layers (Figures 8A, 8C). Third, there is a difference in the number

of cell layers of the cambium between the xylem and phloem. In var. *auriculata* the cambium consists of 2-3 cell layers (Figures 6C, 6D), whereas in var. *paludosa* it consists of 3-4 cell layers (Figure 8D).

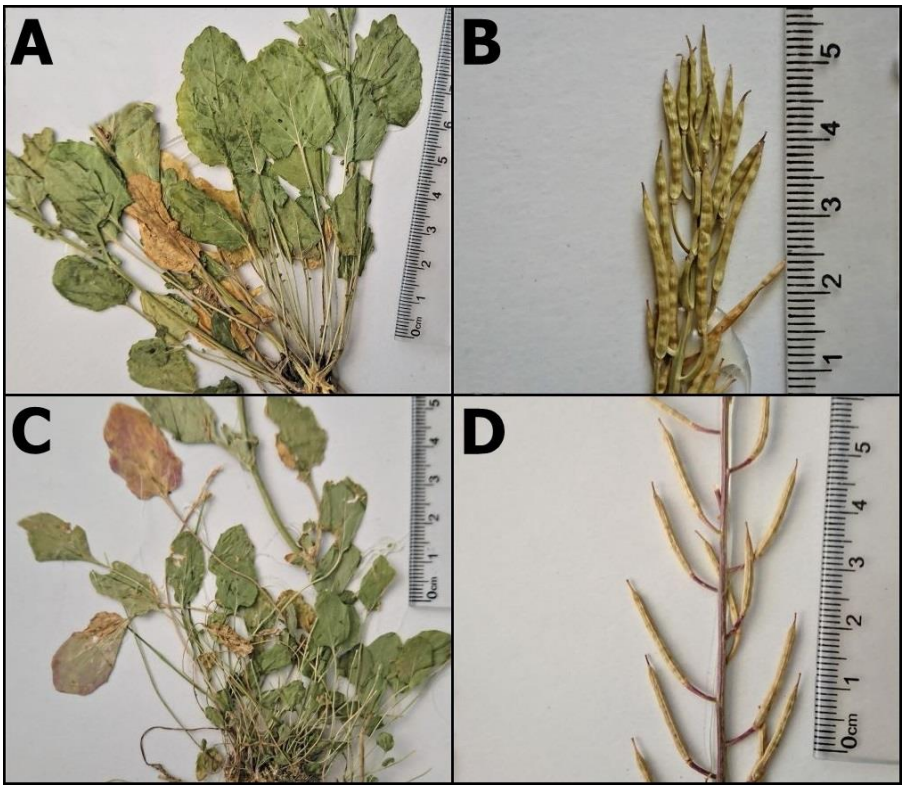

FIGURE 10. Morphological differences between the taxa, A-B) *Barbarea auriculata* var. *auriculata*, C-D) *Barbarea auriculata* var. *paludosa*, (A and C rosette leaves, B and D fruit structures)

Sclereids are found in dense, scattered, or small clusters among the parenchyma cells in the cortex layer of var. *paludosa*. This is rarely seen in var. *auriculata*. The presence of supportive tissue elements such as sclerenchyma in the root cortex structure of many plants is consistent with the literature data [14].

4.2.2 Stem Anatomy

Although the stem anatomical structures of the taxa are similar, some structural differences were also observed. The most important difference is in the epidermis layer. Both uni-layered and bi-layered epidermis can be observed in var. *auriculata*. In the latter case, this second layer is called a collenchyma (Figures

7B, 7C). In contrast, in var. *paludosa*, the epidermis consists of only a single layer of densely arranged cubic or round cells (Figure 9B).

Another difference is the number of parenchymatic cell layers of the cortex. In var. *auriculata*, the parenchyma cells of the cortex consist of 5-6 cell layers (Figures 7B-7D), while in var. *paludosa* this number is 3-5 (Figures 9B, 9C). In plants, the width of the cortex layer and the number of cell layers vary from species to species and according to the developmental conditions of the plant [14].

According to the literature, starch can be found intensively in the endodermis cells of many species [15]. However, this was not observed in the endodermis cells of these two *Barbarea auriculata* varieties.

In addition, sclerenchyma tissue is also densely located between vascular bundles in both taxa. This has also been observed in other taxa of the family [16].

4.2.3. Leaf Anatomy

Since the studied varieties belong to the same species, the anatomical study revealed that their leaf anatomical structures and characteristics were the same. This is true for the majority of the other taxa of the genus *Barbarea*. For this reason, the leaf anatomy of both varieties is described here for a single specimen.

When the leaf anatomy was examined, it was determined that the leaves are of the type referred to as bifacial or dorsiventral. In this type, the leaf mesophyll layer consists of two separate parenchyma tissues, the palisade layer and sponge layer, and it is the most common type among species in nature. Although anatomical studies have shown that leaf anatomy differs between taxa of both Brassicaceae and other families [14,16–19], the mesophyll structures of the two considered varieties were found to be similar in this study.

Although the cells forming the upper and lower epidermis layer are densely arranged, they differ in cell size (large or small) and shape (round, cubic, or oval). There is a thin layer of cuticle over the epidermis layer. There are stomata between the epidermis cells (upper or lower). The palisade parenchyma is located under the upper epidermis layer and consists of 2-3 layers with densely arranged long cylindrical cells with abundant chloroplasts. The sponge parenchyma is located above the lower epidermis layer and consists of 4- to 5-layered, scattered, differently shaped cells with few chloroplasts and intercellular spaces. There are conduction bundles at the junction of both tissues. The general leaf anatomy and parts are detailed in the figure below (Figure 11).

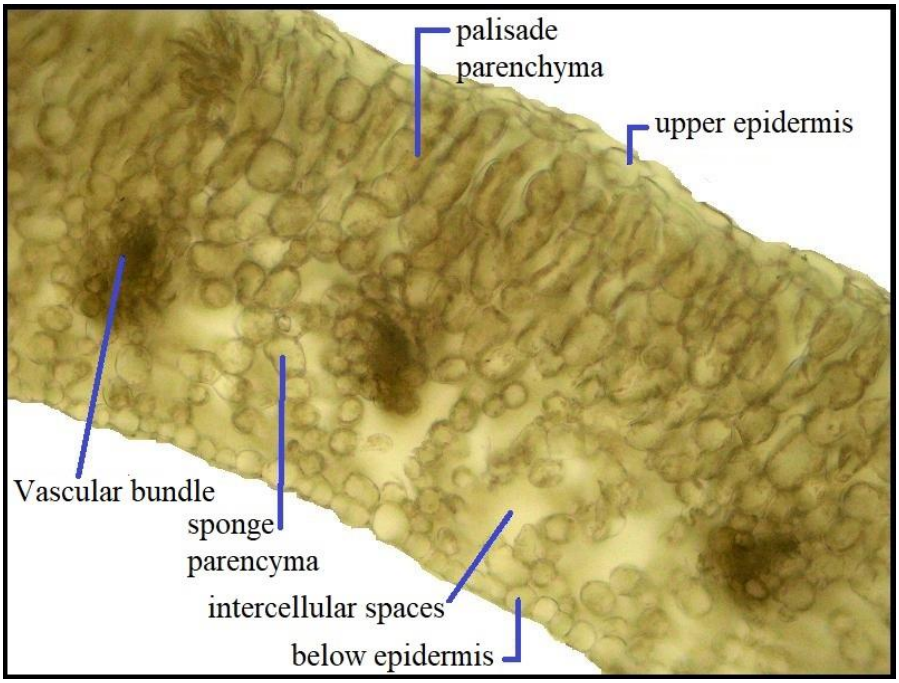

FIGURE 11. *Barbarea* leaf cross-section, anatomical parts, (magnification 20X)

5. CONCLUSIONS

In this study, the morphological characteristics of the taxa *Barbarea auriculata* var. a*uriculata* and *Barbarea auriculata* var. *paludosa* were determined in detail. Base on the data obtained, the differences between the two taxa were discussed and the diagnostic key was revised to identify the taxa. In addition, the comprehensive anatomy of endemic varieties of *Barbarea auriculata* was determined for the first time in this study. Anatomical differences between the two taxa are discussed.

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Author Contribution Statements IB-fieldwork, laboratory work, literature review, data analysis, conceptualization, writing, editing, YB-project development, fieldwork, conceptualization, review, editing, AS-project development, fieldwork, data analysis, literature review, article editing. All authors have read and approved the article.

Declaration of Competing Interests The authors declare no conflict of interest.

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A COMPREHENSIVE STUDY ON THE INFECTIVITY *OF GALBA TRUNCATULA* (O. F. MÜLLER, 1774), AN INTERMEDIATE HOST OF *FASCIOLA* SP. REPORTED IN DİYARBAKIR HOSPITALS

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ABSTRACT. This study aims to comprehensively investigate the trematode infection in *Galba truncatula* (O. F. Müller, 1774) a widely distributed intermediate host in the Dicle River, following the previously reporting of Fascioliasis cases in Diyarbakır hospitals. While previous literature has documented the presence of *F. hepatica* and *F. gigantica* in individuals treated in Diyarbakır hospitals, the origin of the trematodes remains unspecified. In this study, *G. truncatula* samples were collected from three densely populated areas along the Dicle River, specifically Hevsel Gardens, and subjected to histopathological examination to determine the parasite's infectivity. The analysis revealed that the snails were not infected with trematodes; however, the presence of the host and its wide dispersion in the Dicle River pose a significant risk of disease in the future. Hevsel Gardens, a location where sewage and wastewater from the northern suburbs of Diyarbakır converge with the river, is extensively used for activities that involve direct contact with *G. truncatula*. These activities include vegetable cultivation, fishing, farming, husbandry, and swimming, and pose an escalating risk of potential contagion. The study acknowledges the undetermined source of the parasite but highlights its preliminary nature, emphasizing the urgency of proactive measures. The findings will enhance our understanding of the health risks linked to *G. truncatula* and underscore the significance of implementing effective control measures in a timely manner.

1. INTRODUCTION

Galba truncatula (O. F. Müller, 1774), an aquatic pulmonate gastropod, is believed to have originated in Europe but has since expanded its distribution to various regions worldwide and not typically considered an exotic or invasive species. It can now be found in all European countries, including numerous Mediterranean islands such as Corsica, Malta, the Azores, Madeira, the Faroe Islands, the Balearic Islands, and the Canary Islands [1]. Furthermore, recent discoveries have identified its presence in North and South America, Africa, and Asia, including countries such as Russia, Iran, Pakistan, and India [1]. While *G. truncatula* is predominantly found in northern Africa, encompassing Morocco,

Keywords. Galba truncatula, trematode infection, histopathological examination, the Dicle River

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Algeria, Tunisia, and Egypt, it has also been documented in South Africa, Ethiopia, Kenya, and Tanzania [1–3]. *Notably, comprehensive distribution maps detailing the exact range of G. truncatula across the globe are currently unavailable* [1].

G. truncatula is characterized by its conical, slightly turreted shell with swollen whorls and deep sutures. The shell, thin and mid-brown in colour, consists of four distinct whorls with prominent sutures (Fig. 2). Typically, the height of the shell ranges between 6 and 11 mm, while the width falls within 3 to 6 mm. This amphibious snail species inhabits areas near small streams, rivers, and various wetlands, exhibiting a preference for still or slow-moving water. Interestingly, it demonstrates adaptability by venturing onto land, frequently occupying damp field margins, wet hollows, and even dried-up puddles. In terms of diet, *G. truncatula* feeds on algae as well as fresh or decomposed plant matter. Its ability to crawl considerable distances from open water contributes to its widespread distribution and presence in diverse environments.

Trematodes, commonly referred to as flukes, are parasitic organisms that have a distinct life cycle requiring multiple hosts. They are obligate internal parasites, meaning they rely on other organisms for their survival and reproduction. The life cycle of trematodes involves an intermediate host, often a snail, where asexual reproduction takes place, and a definitive host, typically a vertebrate, where sexual reproduction occurs. These parasites have the ability to cause diseases in a wide range of vertebrates, including mammals, birds, amphibians, reptiles, and fish. The impact of trematode infections on various vertebrate classes highlights the significant health risks associated with these parasites [4]. Within the family Lymnaeidae, freshwater snails have a critical role as obligatory hosts for trematodes. In Central and South America, species such as *Lymnaea viator, L. neotropica, L. cubensis,* and *Pseudosuccinea columella* fulfill this role. In Australia, *L. tomentosa* is involved in the transmission of trematodes, while in the United States, *Fossaria modicella* and *Stagnicola bulimoides* are relevant. However, it is *G. truncatula* that serves as the primary vector responsible for transmitting trematodes to humans across Europe, Asia, Africa, and South America [4–6].

G. truncatula plays a crucial role as the intermediate host for several trematode species, including *Fasciola hepatica, F. gigantica, Fascioloides magna, Haplometra cylindracea, Plagiorchis spp.,* and *Paramphistomum daubneyi.* These parasites have been extensively studied and documented in researches [1,7–9]. These studies have provided valuable insights into the transmission dynamics and biology of these trematodes, furthering our understanding of the complex interactions between *G. truncatula* and these parasite species.

Fascioliasis, a parasitic trematode infection caused *by F. hepatica* and *F. gigantica*, typically occurs when individuals unintentionally ingest the parasite. The most prevalent mode of transmission involves the consumption of contaminated water or the ingestion of tainted vegetables, particularly watercress or meats. Moreover, individuals can acquire the infection by consuming foods

that have been washed or irrigated with contaminated water [10–12]. In rare circumstances, people may become infected by consuming undercooked sheep or goat liver containing immature forms of the parasite. It is important to note that direct person-to-person transmission of *Fasciola* does not occur. For the parasite to infect another individual, the eggs excreted in the feces of infected individuals (and animals) must develop within specific types of freshwater snails under favorable conditions [10–12].

In an aquatic or highly moist environment, the eggs of *Fasciola* undergo a three-week incubation period at 15°C and hatch when temperature conditions become favorable, releasing miracidia [13]. These miracidia actively swim for several hours, seeking out a suitable freshwater snail host for penetration. While many snails may be receptive, only a select few can support the complete larval development of the parasite. *G. truncatula* fulfills this crucial role, as its presence is vital to the parasite's life cycle [7,9,10,13]. Upon penetration of the snail, the majority of miracidia enter the snail's tissue near the pulmonary cavity, shedding their epithelial plates and cilia in the process. Within 12 hours, a miracidium undergoes metamorphosis into a mother sporocyst. The mother sporocyst develops and produces the first generation of eight daughter rediae, subsequently rupturing the sporocyst's wall. These initial rediae can give rise to a second generation of rediae, both of which generate a significant number of cercariae. The cercariae exit the rediae through the birth pore and must mature within the snail's tissues before emerging. Typically, the cercariae emerge from their intermediate snail host, *G. truncatula*, during autumn, often triggered by bright light [14]. After emerging, the cercariae swim to the water's surface, shed their tails, and encyst themselves, transforming into metacercariae. These metacercariae attach to vegetation near the water's edge [14].

The findings of this study will contribute to a better understanding of the epidemiology and transmission patterns of *F. hepatica* and *F. gigantica* in the region. By pinpointing the prevalence of these trematodes in *G. truncatula*, it will be possible to assess the potential risks of human infections and implement appropriate control measures, if necessary. Additionally, the research outcomes will help in devising strategies for the effective management and prevention of trematode infections associated with *G. truncatula* in the Dicle River area of Diyarbakır.

2. MATERIALS AND METHODS

Sample collection

The study involved the collection of *G. truncatula* samples from three distinct locations along the Dicle River, each with specific coordinates for precise identification. Location 1 was situated under the Dicle University Bridge (37°55'15.5"N, 40°14'56.3"E), while location 2 was in the center of Hevsel Garden (37°53'34.4"N, 40°15'06.5"E). Lastly, location 3 was near the Ongözlü Bridge (37°53'22.9"N, 40°13'47.8"E). The snail sampling was conducted during autumn, a critical period characterized by the increased reproduction and

multiplication of trematodes. To ensure personal safety, protective gloves were used during the collection process, and the snails were carefully placed in plastic containers filled with river water. Approximately 300 snail samples were collected throughout the designated search period. To preserve the snail samples, they were immediately transferred to universal tubes containing moist tissue paper and kept cool. Within a maximum of 5 hours, the samples were transported back to the laboratory. Once in the laboratory, the snails were stored in a deep freeze at a temperature of -20°C, ensuring their preservation for subsequent analysis. Within 12 hours after collection, the snails underwent morphological recognition, employing specific criteria described in earlier references [15–18]. These criteria included shell shape and measurements, the number of whorls, aperture shape and position, and width. The assistance of malacologist Rıdvan Şeşen from the Department of Biology at Dicle University facilitated accurate identification of the samples. To document the shells of the collected samples, high-resolution digital microscope cameras (Jiusion wifi USB Digital Microscope 50 to 1000×) were employed, enabling detailed and clear photographs (Fig. 2). For parasitic analysis, 40 mature snails were selected from each of the three locations, resulting in a total of 120 snails being utilized for this specific aspect of the study.

Histopathological preparation

After a period of three days, the collected snails were transported to the laboratory at Gazi Yaşargil Training and Research Hospital under controlled temperature conditions of 21°C. During the necropsy procedure, the shells of the snails were delicately removed. Subsequently, the snail tissues underwent a dehydration process and were embedded in paraffin at a temperature of 56°C. To ensure proper histopathological examination, the whole tissues were immersed in a neutral buffered 10% formalin solution and sent to the pathology laboratory. The tissues were fixed in the formalin solution for a duration of 24 hours. Following fixation, the tissues were embedded in paraffin blocks using a Leica ASP® 300 automatic vacuum tissue tracking device manufactured by Leica Microsystems in Germany. Sections of the prepared paraffin blocks, with a thickness of 4 μm, were obtained using a Leica RM® 2135 rotary microtome device, also from Leica Microsystems. These sections were then mounted on slides and subjected to staining using Hematoxylin-Eosin (H&E) stains, enabling normal histological evaluation. The prepared slides were carefully examined under an Olympus BX53F light microscope (Olympus, Tokyo, Japan). To document the findings, photographs were taken using an Olympus E-330 camera, ensuring detailed visual representation of the histopathological specimens.

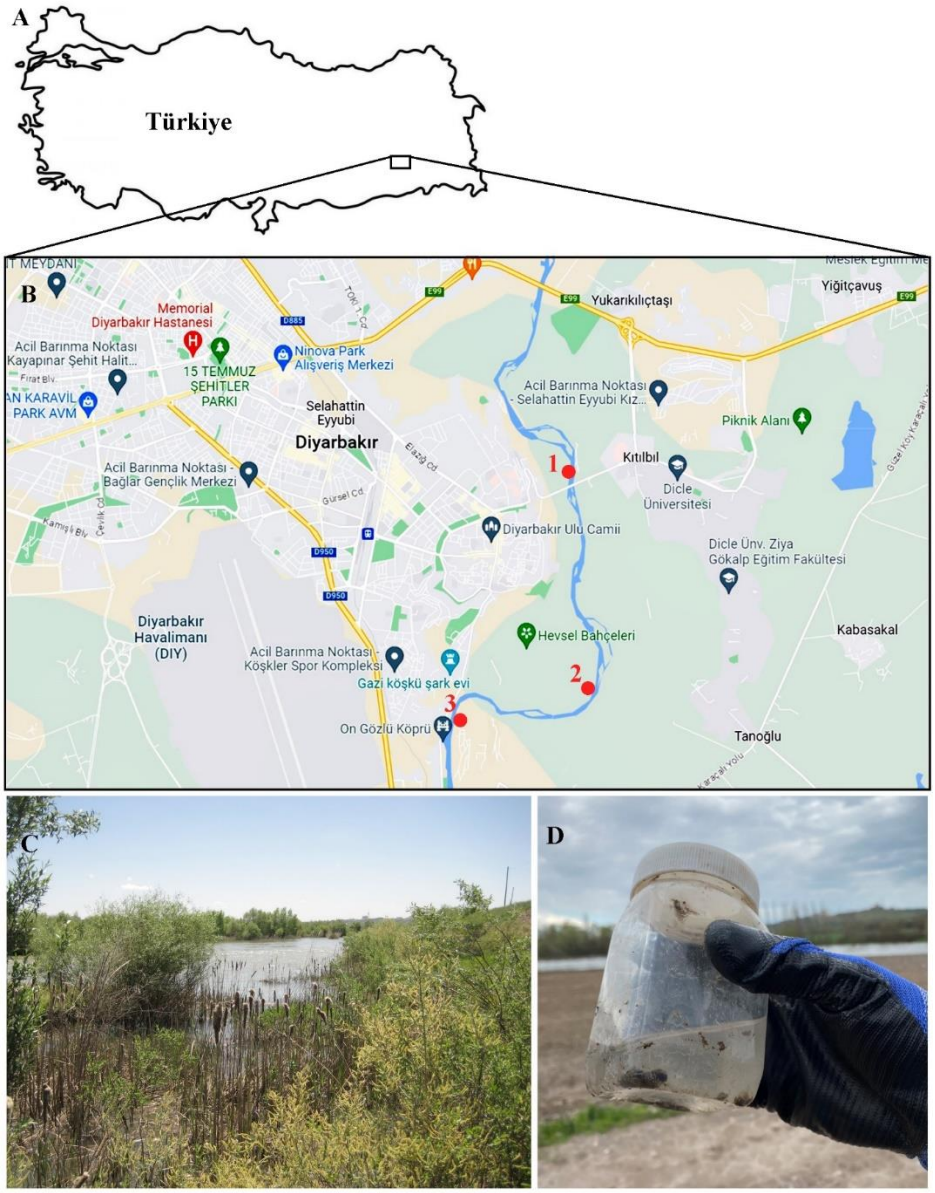

FIGURE 1. A. The map of Türkiye, B. The map of Diyarbakır, and three collection areas of *Galba truncatula*, C. The bank of the Dicle River, D. The samples in a plastic container.

FIGURE 2. The morphology of *Galba truncatula*, focusing on key features such as the shape of the shell, the number of whorls, the aperture, apex, body whorl, umbilicus, and sutures. The image includes a black bar measuring 1 mm, serving as a reference for scale.

3. RESULTS AND DISCUSSION

In terms of infection dynamics, once the miracidium enters a snail host, it undergoes a transformation into a sporocyst. Depending on the outcome, the sporocyst either survives the initial week of infection and gives rise to first-generation rediae (snails with active infections), or it perishes within the first week (snails with abortive infections) [19]. While the presence of living parthenitae in dissected snails easily confirms active infections, identifying abortive infections requires histological investigation, which detects residual parasites and identifies any tissue lesions within the snail's organs. Histological investigation also enables differentiation between snails with abortive infections and uninfected snails that have not experienced miracidia penetration [19]. In general, the presence of rediae serves as an indicator of snail infection, and their observation in histological sections helps assess the extent of the infection. *However, in the current study's histopathological sections of G. truncatula, no cercariae, sporocyst, or notably rediae of F. hepatica and F. gigantica were observed* (Fig. 3). Previous investigations of the *G. truncatula-Fasciola hepatica* model have documented histological alterations in snails with active infections. Notably, the albumen gland, digestive gland, gonads, and kidney of these snails displayed epithelial necrosis followed by regeneration [19,20]. However, in the present study, no signs of epithelial necrosis or regeneration were observed in the albumen gland, digestive gland, gonads, or kidney of *G. truncatula*, indicating the absence of infection with *F. hepatica or F. gigantica* (Fig. 3).

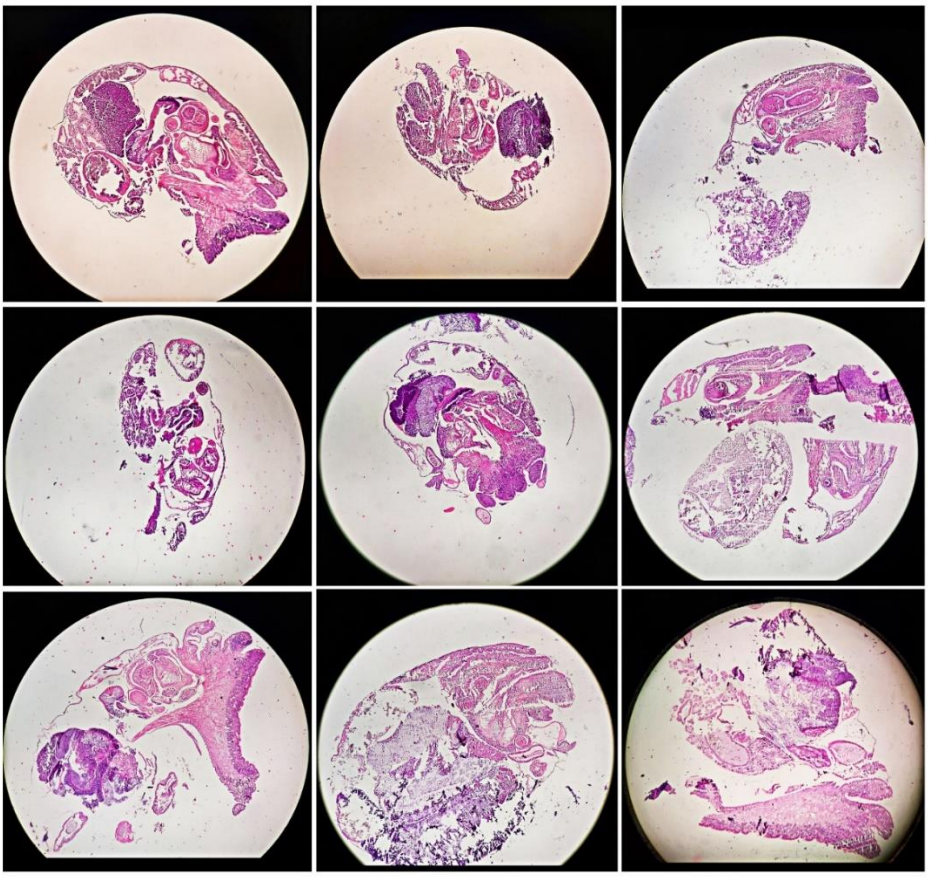

FIGURE 3. Microscopic images of different histological sections of *G. truncatula*. Notably, no evidence of trematodes was detected in any of the examined sections.

No recorded literary works have specifically explored *F. hepatica* and *F. gigantica* infestations in *G. truncatula* within the context of the Dicle River. Nevertheless, some documented cases of Fascioliasis treated in hospitals in Diyarbakır have been reported in the literature. In a published paper, a 44-year-old female patient presented to the Department of Gastroenterology at Dicle University Medical Faculty with symptoms of abdominal pain, nausea, vomiting, anorexia, and weight loss. Following comprehensive diagnostic assessments involving laboratory tests and imaging techniques, the patient was initially admitted to the hospital with a provisional diagnosis of cholangitis-cellular carcinoma. However, after a meticulous examination, the patient was ultimately diagnosed with *F. hepatica* through the utilization of Endoscopic Retrograde Cholangiopancreatography (ERCP) [21]. In another study conducted at the Department of Gastroenterology, Dicle University Faculty of Medicine, a 46-

year-old woman sought medical attention for right upper quadrant abdominal pain and jaundice. Upon evaluation, the patient was diagnosed with *F. gigantica* [22]. Additionally, a separate article reported the examination and treatment of several patients with Fascioliasis who presented at the Surgery Clinic of Dicle Medical Faculty [23]. A study focusing on *F. hepatica* in small ruminants in the vicinity of Diyarbakır, particularly Siirt, revealed the presence of *F. hepatica* in six out of 3,193 cattle and one out of 3,659 small ruminants [24]. Moreover, reports of Fascioliasis in small ruminants have emerged from neighbouring regions close to Diyarbakır, including Van, Tatvan, Elazig, Malatya, and Hakkari [24–26]. However, there is a lack of available information in the literature regarding the precise origins of these parasites. It is plausible that patients acquired the trematodes through the consumption of undercooked meat, such as from goats, sheep, or cattle sourced from various locations. Fascioliasis can be transmitted from large livestock, such as cattle or sheep, to humans through a specific transmission pathway. The life cycle begins with these animals grazing on pastures contaminated with Fasciola metacercariae, the infectious stage of the parasite. Upon ingestion, the metacercariae develop into adult parasites within the host's liver. Humans can become infected when they consume raw or undercooked animal products derived from these infected animals or when they ingest water or plants contaminated with metacercariae. This transmission pathway highlights the zoonotic potential of Fascioliasis and the importance of proper food safety practices and the control of contaminated water sources in preventing human infections. Another potential source could be the ingestion of contaminated vegetables, particularly watercress, or the consumption of water containing parasites in their rural areas. It is worth noting that the hospitals in Diyarbakır attract patients from neighbouring provinces and districts due to their comprehensive and advanced medical facilities. This raises the possibility that the parasites may have originated from outside of Diyarbakır. The patients could have potentially come into contact with *G. truncatula*, which typically inhabits lakes, rivers, canals, and stagnant water ponds in areas surrounding Diyarbakır. According to the literature, it is known that the parasite can persist within the human body for up to 13.5 years [27]. Hence, it is also possible that some patients may have been harboring the parasite for an extended period. Another consideration is that the cited literature was published in 2014, suggesting that patients might have been infected prior to 2014, and the infection may have resolved in the intervening time. Considering these various possibilities, it becomes exceedingly challenging to identify the precise source of the parasite. However, by obtaining information on the residences of patients with Fascioliasis who seek treatment in hospitals in Diyarbakır, it may be possible to gain further insights into the potential sources of the infection.

4. CONCLUSIONS

In summary, the significance of *G. truncatula* becomes evident as it serves as an essential intermediate host for Fasciola species in parasitology research. Moreover, its presence and wide distribution in the Hevsel Gardens along the Dicle River, a habitat it has inhabited since 1992 [28], underscore the potential risks associated with its presence in this ecosystem. This highlights the importance of closely monitoring the implications of its presence for both parasitological studies and the conservation of freshwater ecosystems. The discharge of sewage waters from suburban areas into the river, coupled with the lack of infrastructure, creates an environment conducive to the occurrence of Fascioliasis. Limited data on the prevalence of the disease further accentuate the need for immediate action. To safeguard the local population from these diseases, it is crucial to detect the presence of *F. hepatica* and *F. gigantica* in the river and *G. truncatula* in future studies before it becomes too late. Additionally, addressing the issue of wastewater discharge from neighborhoods without a wastewater network is essential. Moreover, further investigation of the aquatic environments in the districts where treated patients reside would provide valuable insights into the potential as an intermediate host for *G. truncatula*. Despite the challenges posed by patient rights and privacy, it is crucial to gather more information to better understand and control the spread of this species. Overall, this study serves as a preliminary call to action, emphasizing the importance of proactive measures to mitigate the risks associated with the presence of *G. truncatula* and protect the well-being of the local community.

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Author Contribution Statements İE contributed extensively to the manuscript, including the approval of the final version, critical literature review and interpretation, material collection and preparation, manuscript critical review, preparation and writing. AY contributed specifically to the histopathological analysis and section investigations.

Declaration of Competing Interests The authors declare no conflict of interest.

Ethics Committee Approval As this article does not involve any studies with human or vertebrate animal subjects, it was not necessary to obtain approval from an ethics committee.

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THE VARIATIONS OF CARBONIC ANHYDRASE IN SPECIES OF THE GENERA OF *MUS* LINNAEUS, 1758 AND *RATTUS* FISCHER, 1803 (MAMMALIA: RODENTIA) IN THE LINE OF ANKARA - ZONGULDAK PROVINCES

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ABSTRACT. Carbonic anhydrase (CA_1) enzyme variations of one hundred and forty six specimens of *Mus domesticus, Mus macedonicus* (House mice) and one hundred and twenty specimens of *Rattus rattus* (Roof rat) and *Rattus norvegicus* (Brown rat) were examined by the field work conducted in 23 localities in the line of Ankara-Bolu-Zonguldak. It was determined that CA_1 was fixed to two different homozygous alleles in both house mouse species. In the roof rat samples, CA_1 was fixed to two homozygous alleles at different frequencies in two groups formed according to the colour of the back fur. A single homozygous allele was detected at the CA_1 locus of brown rat samples. It was observed that these four species were separated in the UPGMA tree made according to allele frequencies. In this respect, it was evaluated that CA_1 could be a molecular marker that can be used to distinguish these species.

1. INTRODUCTION

Rattus and *Mus* species are not native to Türkiye, they come to the country in different ways such as land and sea transportations. Such species are called synanthropic or agrophilic species [1], and they were sometimes considered as invasive species [2]. In the new locations where they have just settled, over time, they have undergone differentiation as a result of the evolutionary process. Taxonomically, although there are scientific papers showing that *Mus domesticus* Rutty, 1772 is a different species from *Mus musculus* Linnaeus,1758, *M. domesticus* is now accepted as a subspecies of *M. musculus* in recent studies, thus *M. musculus domesticus* and *Mus macedonicus* Petrov and Rozic, 1983 are distributed in Türkiye [3, 4, 5, 6, 7, 8, 9]. Allozyme variations are used as molecular markers to demonstrate genetic differentiation and distinguish taxa from each other. In this connection, various studies have been conducted on the allozyme of the genus *Mus* [8, 10, 11, 12, 13, 14].

Also, it was reported that *M. domesticus* (Syn.; *M. musculus*) and *M. macedonicus* range in the line of Ankara - Zonguldak based on morphological, karyological, and morphometric characteristics, as well as 2 alleles of isocitrate dehydrogenase [8].

Keywords. Mus, Rattus, carbonic anhydrase, variation

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It is known that two *Rattus* species are widely distributed in Türkiye. The first distribution records on *Rattus rattus* (Linnaeus, 1758) and *Rattus norvegicus* (Berkenhout, 1769) recorded from areas neighbouring Türkiye have been provided by [15, 16, 17, 18, 19]. The most recent detailed, morphological, karyological and allozyme studies on rat species were given by [20, 21, 22, 23, 24, 25, 26]. The taxonomic importance of non-specific esterase variations in *R. norvegicus*, and the diagnostic power of the patterns of blood serum proteins in *R. rattus* and *R. norvegicus* were enlightened in the detected 4 different colour variations in *R. rattus* specimens of Türkiye [21, 22, 26]. On the other hand, *R. norvegicus* samples were found to be quite homogeneous in terms of colour variation.

Also, Yiğit et al. [22, 23] reported seven of 22 allozyme loci studied were found to be polymorphic in Turkish *R. rattus*, and eight of 22 loci were polymorphic in the 4 sub-populations of Turkish *R. norvegicus*. Various studies have been performed on allozyme of the genus *Rattus* [27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39]. Baverstock et al. [34, 35] conducted a study on *R. rattus* and *R. norvegicus*, found differences in many enzyme loci in the species of the genus *Rattus*, including the Carbonic Anhydrase enzyme. In this study, carbonic enzyme variation in samples of two genera collected from Ankara-Zonguldak line and whether this enzyme can be used as a genetic marker was investigated.

2. MATERIALS AND METHODS

In this study, the blood samples of *Mus* and *Rattus* collected from Ankara-Zonguldak line before 2002 and stored in the Mammals Research Collection of Ankara University (AUMAC, www.mammalia.ankara.edu.tr) were used (Table1).

Protocol of starch gel electrophoresis

1. Procedure of erythrocytes haemolysis were performed in accordance with [40].

2. Electrophoretic procedures were carried out as given by [41, 42] with small modifications; the starch gel percentage was 11 % and the samples loaded on gel were run at 12 v / cm with 9-15 mA for 5 hour.

3. Histochemical staining: After the electrophoresis was completed, the reaction mixture for the carbonic anhydrase enzyme was prepared by method below;

ß-Naphthyl acetate: The reaction mixture was prepared by adding 40 mg of Fast Blue RR (Sigma F-0500) to 96 ml of 0.05 M phosphate buffer and 4 ml of a 1 % ß-Naphthyl acetate stock solution prepared previously in 50 % acetone [42]. Staining was done by incubating at 37 °C in the dark until bands were seen on the gel.

4. Gel fixation: After the enzyme bands were seen, the reaction was stopped by washing with gel fixation solution [45 parts of 13 methanol, 55 parts of acetic acid solution (1acetic acid: 5 H2O)].

5. Documentation of results: When staining was complete, the gel was photographed by placing it on the light box, and alleles were located by plotting the observed band patterns. Then, the calculation of allele frequencies was done using these zymograms. Allozyme was numbered according to the most common allele. The most common allele was given numbers 100, slow movers from this allele were given numbers less than 100, and fast movers were given numbers greater than 100. Allele frequency was calculated according to Baverstock et al. [35] (p = f (A) = (2 x number of AA homozygotes) + (number of Aa heterozygotes) / (2 x total number of individuals)).

6. Data analyses: the computer program NTSYS-pc (version 1.80) was used in all data analyses (allele frequencies, similarity coefficient and phylogenetic tree). UPGMA Dendrograms were formed based on the similarity coefficients of Manhattan Distance.

3. RESULTS

Distribution of studied samples of four species according to localities is given in Figure 1 and Table 1. Specimens of *Mus* spp. were generally caught from areas close human settlements in rural areas, while *Rattus* spp. were caught in urban areas. Although these four species are caught in the same area, there is niche specialization in biotope use such as *M. m. domesticus* in human settlements, *M. macodenicus* in the grain field, *R. rattus* in the roof of wooden buildings and barns, *R. norvegicus* in infrastructure.

Carbonic anhydrase (CA_1) enzyme variations in *M. m. domesticus* (n: 49); according to laboratory studies with blood hemolysates, two different alleles, CA_1^{100} and CA_1^{90}, were observed at the CA_1 locus in this species, and their frequencies were found to be 0.84 and 0.16, respectively. Among the samples examined, no individual heterozygous for the CA_1 locus was found. It was determined that the samples examined in terms of CA_1 locus were homozygous (Table 2, Figure 2).

Carbonic anhydrase (CA_1) enzyme variations in *M. macedonicus* (n: 7); CA_1 was studied in the blood hemolsylates of 7 of the 24 captured samples. Two different alleles including CA_1^{100} and CA_1^{90}, were observed in this locus of *M. macedonicus* and their frequencies were found to be 0.57 and 0.43, respectively. As in the previous species, heterozygous allele for the CA_1 locus was not observed and the all samples examined in terms of CA_1 locus were homozygous (Table 2, Figure 2).

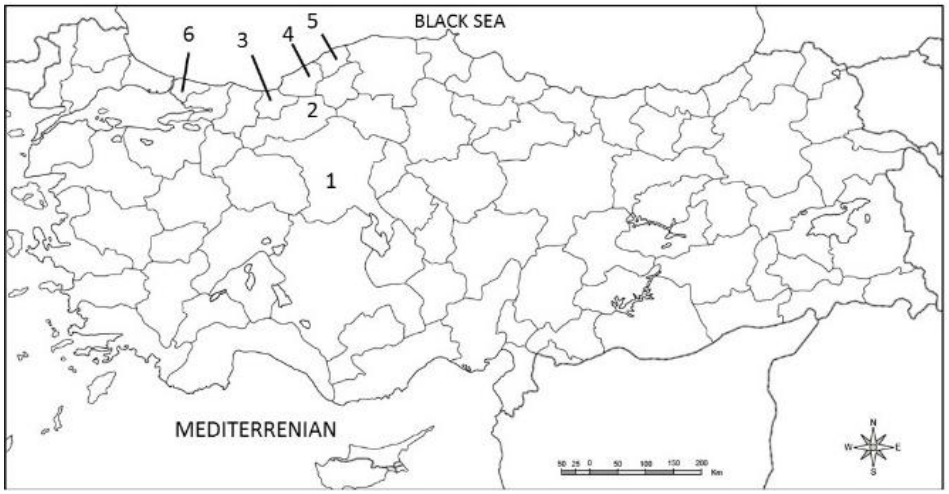

FIGURE 1. Sampling provinces of *Mus* spp. and *Rattus* spp. (1: Ankara, 2: Bolu, 3: Düzce, 4: Zonguldak, 5: Bartın, 6: Asiatic part of İstanbul)

Carbonic anhydrase (CA_1) enzyme variations in *R. rattus* (group1 n: 7, group2 n: 33); In the examined samples, two groups were determined for the dorsal fur colour and the samples were evaluated under these two groups; Group1 with blackish gray back fur (*R. rattus* (1)) and Group2 with brownish-grey dorsal fur (*R. rattus* (2)). *R. rattus* (1) with blackish gray back fur, the abdomen fur is a mixture of gray-white, in other words, smoke-coloured, and no variation was found within the group. Three types of variations were detected in the belly fur in R2: taupe white, light gray and yellowish white.

Two different alleles including CA_1^{80} and CA_1^{70} were observed in the CA_1 locus according to the blood haemolysis studies of the samples belonging to both groups, and among the samples examined, the allele frequencies were determined as 0.57 and 0.43 in the *R. rattus* (1) group, and 0.33 and 0.67 in the *R. rattus* (2) group, respectively. Among the samples examined, no individual heterozygous for the CA_1 locus was found. It was determined that the samples examined in terms of CA_1 locus were homozygous (Table 2, Figure 2).

Carbonic anhydrase (CA_1) enzyme variations in *Rattus norvegicus* (n; 13); According to laboratory studies with blood hemolsylates of 13 *R. norvegicus* specimens, only the CA_1^{80} allele was observed in the CA_1 locus in this species and its frequency was found to be 1.00 in the samples examined. Among the samples examined, no individual heterozygous for the CA_1 locus was found. It was determined that the samples examined in terms of CA_1 locus were homozygous (Table 2, Figure 2).

Comparison of allele variations in four species studied;

The positions and zymograms of CA1 enzyme locus alleles observed in the studied groups on the gel were given in figure 2. According to comparative statistical analyses based on the allelic frequencies and distance matrix of the Ca1 locus in two *Mus* species (*M. m. domesticus* and *M. macedonicus*) and two *Rattus* species (*R. rattus* (1), *R. rattus* (2) and *R. norvegicus*) (Table 2,3) UPGMA Dendrograms were established using the NTSYS-pc program (Figure 3).

According to the UPGMA Dendrograms established based on the similarity matrix (Table 3), *Mus* spp. and *Rattus* spp. Species were completely separated from each other by forming two different clusters. The cluster formed by *Mus* spp. is connected to the *Rattus* spp. cluster with the value D: 0.180. The genetic distance (D) between *M. m. domesticus* and *M. macedonicus* was determined as D: 0.045, and it appeared D: 0.040 between the *R. rattus* (1) group and the *R. rattus* (2). High D value was found to be 0,111 between *R. rattus* (2) and *R. norvegicus*, and these two species are completely separated from each other by the CA_1^{80} allele.

According to the UPGMA Dendrograms based on CA frequencies, the ancestral species among *Mus* spp. was *M. macedonicus*, and *Rattus* spp. as *R. norvegicus.* According to these results, in other words, *M. m. domesticus* and *R. rattus* (2) can be said to be more differentiated species. In addition, these results supported the idea that the CA_1 locus is a taxonomic marker that can be used to distinguish these species.

TABLE 1. Distribution of examined samples according to localities (numbers indicate the sample Numbers)

Locations	***M. m. domesticus***	***M. macedonicus***	***R. rattus***	***R. norvegicus***
1. Beykoz / İstanbul (Asiatic part)	-	-	26	-
2. Kargalar village / Zonguldak	-	-	3	-
3. Saka village / Zonguldak	2	-	4	-
4. The campus of Karaelmas Univesity of Zonguldak	5	-	11	19
5. City center of Zonguldak	1	-	2	-
6. Kilimli / Zonguldak	-	-	1	1
7. Çerde village / Ulus / Bartın	10	-	7	-

8. Melenağzı village / Düzce	1	1	1	-
9. Köprübaşı village / Düzce	12	-	3	-
10. Samandere village / Düzce	7	-	1	-
11. Hacıyakup village / Düzce	1	2	8	-
12. Mudurnu-Bolu	3	2	-	-
13. Kürkçüler village / Gerede / Bolu	1	1	-	-
14. Sapanlı village / Gerede / Bolu	-	3	1	-
15. Ömerler village / Abant / Bolu	4	-	-	-
16. Bürnük village / Bolu	-	-	3	-
17. Tandoğan Campus of Ankara University / Ankara	16	-	7	-
18. Maltepe-Ankara	-	-	1	7
19. İskitler-Ankara	-	-	-	7
20. City center / Ankara	28	7	2	5
21. Şereflikoçhisar / Ankara	-	1	-	-
22. Gölbaşı-Ankara	1	7	-	-
23. Bala-Ankara	17	-	-	-

TABLE 2. Frequencies of alleles observed for CA_1 loci in starch gel electrophoresis

Alleles / Species	***M. macadenicus***	***M. m. domesticus***	***R. rattus* (1)**	***R. rattus* (2)**	***R. norvegicus***
CA_1^{100}	0,57	0,84			
CA_1^{90}	0,43	0,16			
CA_1^{80}			0,57	0,33	1,00
CA_1^{70}			0,43	0,67	

TABLE 3. Distance matrix obtained from CA1 allele frequency data of *M. m. domesticus*, *M. macedonicus* and *R. rattus* (1), *R. rattus* (2) and *R. norvegicus*.

	R. norvegicus	*R. rattus* (1)	*R. rattus* (2)	*M. macedonicus*	*M. m. domesticus*
R. norvegicus	0				
R. rattus (1)	0,0717	0			
R. rattus (2)	0,1117	0,040	0		
M. macedonicus	0,0950	0,167	0,206	0	
M. m. domesticus	0,1400	0, 212	0,251	0,045	0

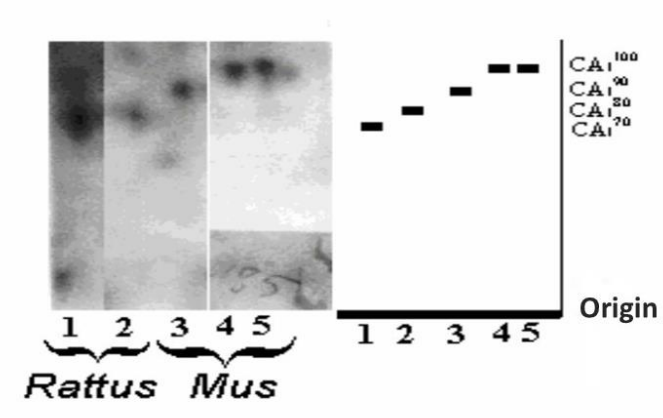

FIGURE 2. Alleles observed for CA_1 loci on the starch gel electrophoresis.

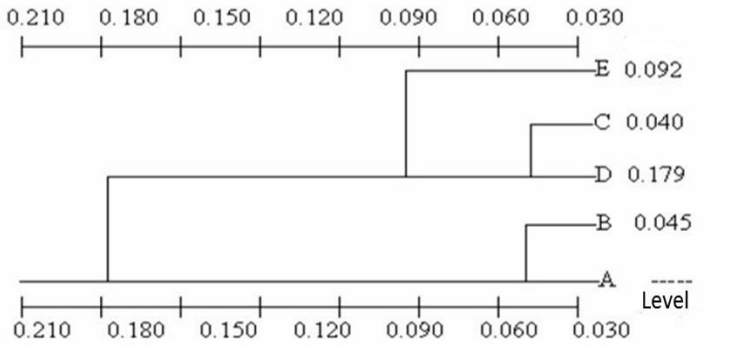

FIGURE 3. UPGMA Dendrograms generated from CA1 allele frequencies of *M. m. domesticus* (A), *M. macedonicus* (B), *R. rattus* (1) (C), *R. rattus* (2) (D) and *R. norvegicus* (E).

4. DISCUSSION

***Mus* spp.;** although fur colour, external and cranial characteristics were used in the identification of *Mus* spp., strong taxonomic characteristics could not be revealed in the distinguishing of species, and the zygomatic index (ZI), a cranial metric characteristic, is used to differentiate between *M. m. domesticus* and *M. macedonicus*.

Regarding ***Mus spp.,*** while fur colour, external features, and cranial characteristics have been used for their identification, robust taxonomic characteristics for species differentiation have not been discerned. However, the zygomatic index (ZI), a cranial metric characteristic, is utilized to distinguish between *M. m. domesticus* and *M. macedonicus*. [3, 4, 5, 6, 7, 8, 9, 14, 18, 44]. In this study, the specimens with a ZI parameter of less than 0.5 are identified as *M. m. domesticus*, and those with a ZI parameter of greater than 0.5 are identified as *M. macedonicus*. Depending on the difficulty of distinguishing species by looking at morphological and statistical characteristics, the genetic markers are also used in species identification and in establishing evolutionary relationships.

For this purpose, enzyme electrophoresis for taxonomy of *Mus* spp. were performed by [10, 11, 12, 13, 14]. In the research of Thaler et al. [10] using enzyme electrophoresis, Romanian *Mus* samples were divided into two different biochemical groups and identified 6 loci with allelic differences without assigned the samples to the certain taxa. Mezhzherin et al. [14] in their study on Transcaucasian *Mus* samples, stated that esterase 1-2 and isocitrate dehydrogenase enzymes were diagnostic in the differentiation of *M. musculus* and *M. domesticus*, and described of the hydride zone of three parapatric *Mus* species. Awasthi et al. [13] reported the maximum heterogeneity of *M. musculus* in the study performed by enzyme electrophoresis on 4 *Mus* species in India. Gözcelioğlu et al. [8] revealed that there were two alleles of isocitrate dehydrogenase enzyme in *M. m. domesticus* and *M. macedonicus*, and not distinctive among these taxa. Our results showed that CA1 alleles can be used to reveal the relationship between species and effectively distinguish between *Mus* and *Rattus* species.

Taxonomic studies with CA allozyme electrophoresis on *Mus* spp. are notably scarce. Thaler et al. [10] identified allelic differences in CA_2 enzyme in *Mus* samples from different locations from Europe. Bonhomme et al. [11, 12] observed a single allele for the CA_2 locus in *M. m. domesticus* in European. In this study, two different alleles were observed in *M. m. domesticus* and *M. macedonicus* for the CA_1 locus examined. In this respect, it can be said that the CA_2 locus may be more distinctive for *M. domesticus* and there is more variation within the species in the CA_1 locus, and CA_1 did not distinguish Türkiye samples of *M. m. domesticus* and *M. macedonicus*.

***Rattus* spp.;** *R. rattus* species has a very heterogeneous structure in terms of colour. The colour variations and identification characteristics of Turkish *Rattus* species were described in detail by [20]. Unlike *R. rattus*, another species in the

genus *Rattus, R. norvegicus* has a very homogeneous morphology structure, and morphological, biometric and karyological features of this species were given by [20]. The morphological features of both *Mus* and *Rattus* species examined in this study are consistent with [8, 9, 20]. In the study on non-specific esterase variations of *R. norvegicus*, the enzyme patterns were found to be tissue-specific polymorphic [24] also the blood serum proteins of *R. rattus* and *R. norvegicus* were revealed by using SDS-page electrophoresis [21]. It was stated in both papers that non-specific esterase and the patterns of blood serum proteins are not genetic markers in distinguishing species.

Baverstock et al. [34] in their study among the karyotypic forms (2n: 38, 40, 42) of *R. rattus*, it was stated that single allele migrated to most anodal were observed in these karyological forms for the CA_1 enzyme. However, it was also stated that in the one of samples with 2n: 42 (only in Southeast Asian type (Japan)) from two different locations, CA_1 was found to be fixed two alleles. While Baverstock et al. [34] observed a single allele in 2n: 38 karyological forms of *R. rattus*, according to Yiğit et al. [20] in Türkiye samples with 2n: 38, two different alleles were observed in the CA_1 locus. Also, Samollow et al. [33] in their study with Australian rats reported that the carbonic CA_1 enzyme distinguished *R. rattus* and *R. norvegicus* species. The single allele detected in *R. norvegicus* in Türkiye is consistent with the findings of [34, 35, 45]. Accordingly, it can be said that the CA_1 enzyme does not show intraspecific variation in *R. norvegicus* and is fixed by a single allele. As a result, it has been revealed that CA_1 enzyme can be used as a genetic marker, and it can distinguish especially *R. rattus* and *R. norvegicus* species in terms of fixed allelic differences.

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