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Esterase polymorphisms in relict endemic *Liquidambar orientalis* Mill. var. *orientalis* and *L. orientalis* Mill. var. *integriloba* Fiori populations in Turkey

Belgin Göçmen Taşkın[1*], Vatan Taşkın[1], Köksal Küçükakyüz[1], Ömer Varol[1], Bekir Çöl[1] and Tülin Arslan[2]
[1]*Muğla University, Department of Biology, 48000 Kötekli, Muğla, TURKEY.*
[2]*Muğla University, Department of Aquaculture, 48000 Kötekli, Muğla, TURKEY.*
*(*author for correspondence: belgingocmen@hotmail.com)*

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Abstract

In this study, esterase isozyme polymorphisms in Boreal-Tertiary origin relict endemic *L. orientalis* populations were investigated. Four populations located in the Southern and the Southwestern regions of Turkey were screened. Two of these populations (Fethiye-Günlüklü and Fethiye-İnlice) belong to subspecies *L. orientalis* Mill.var. *orientalis*. The other two (Muğla-Köyceğiz and Antalya-Gebiz) belong to subspecies *L. orientalis* Mill.var. *integriloba* Fiori. A total of 12 esterase bands were detected and named according to their increasing relative mobilities on the polyacrylamide gels. Six of them were α-esterases, four of them were β-esterases and the remaining two were α / β-esterases. These 12 esterase bands resulted in 37 different banding patterns in four populations. Only four of these 37 patterns were common among the populations. The levels of variations detected within and among the populations were surprisingly high. According to the results obtained from this study, subspecies *L. orientalis* Mill. var. *orientalis* and subspecies *L. orientalis* Mill. var. *integriloba* Fiori were not distinguished from each other in any respect. Being the first in the specialized literature of the studied plant species, this preliminary study carried out using esterase isozymes as a biochemical marker is important.

Key Words: *Liquidambar orientalis,* relict endemic, esterase polymorphism, genetic diversity, esterase isozymes

Türkiye'deki relikt endemik *Liquidambar orientalis* Mill. var. *orientalis* ve *L. orientalis* Mill. var. *integriloba* Fiori populasyonlarındaki esteraz polimorfizmi

Özet

Bu çalışmada, Boreal-Tertiary orijinli relikt endemik *L. orientalis* populasyonlarında esteraz izoenzim polimorfizmleri incelenmiştir. Türkiye'nin güney ve güney batı bölgelerinden dört populasyon taranmıştır. Bu populasyonlardan ikisi (Fethiye-Günlüklü ve Fethiye-İnlice) *L. orientalis* Mill.var. *orientalis* alt türüne aittir. Diğer ikisi ise (Muğla-Köyceğiz ve Antalya-Gebiz) *L. orientalis* Mill.var. *integriloba* Fiori alt türüne aittir. Poliakrilamid jelde artan göreceli yürüme değerlerine göre adlandırılan toplam 12 esteraz bandı tespit edilmiştir. Bunların altısı α-esteraz, dördü β-esteraz ve ikisi de α / β-esterazdır. Bu 12 esteraz bandı, dört populasyonda 37 farklı bant deseni oluşturmuştur. Bu 37 farklı bant deseninden sadece dördü bütün populasyonlarda ortaktır. Populasyon içi ve populasyonlar arası çeşitlilik şaşırtıcı derecede yüksek bulunmuştur. Çalışmadan elde edilen sonuçlar, *L. orientalis* Mill. var. *orientalis* ve *L. orientalis* Mill. var. *integriloba* Fiori alt türlerinin birbirlerinden ayırt edilemediklerini göstermektedir. Esteraz izoenzimlerini biyokimyasal bir belirteç olarak kullanan bu ön çalışma, bu bitki türüne ait literatürde ilk olma özelliği nedeniyle önemlidir.

Anahtar Sözcükler: *Liquidambar orientalis,* relikt endemik, esteraz polimorfizmi, genetik çeşitlilik, esteraz izozimleri

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Introduction

Liquidambar L. is the only genus in the family of Hamamelidaceae (Li and Bogle, 1997). It has a fossil record throughout the Tertiary Period, but is considered to have reached its widest distribution during the Miocene (Lancucka-Srodoniowa; 1966, Uemura, 1983). Four species are currently recognized in this woody genus (Li and Bogle, 1997). All of these species are wind-pollinated deciduous trees found in similar habitats in Eastern Asia, Eastern and Central America, and Southwest Asia (Hoey and Parks, 1991). Two species, *L. formosana* and *L. acalycina*, are present in Eastern Asia. *L. formosana* has a distribution over much of eastern, central, and southern mainland China as well as Taiwan. *L. acalycina* is often found at much higher elevations than *L. formosana* and has a more restricted range in China that overlaps the range of *L. formosana*. *L. styraciflua* is common to the Coastal Plain and Piedmont of eastern North America as well as to the cloud forests of Mexico and Central America (Hoey and Parks, 1991). The fourth species, *L. orientalis* is native only to southwest of Turkey (Davis, 1972; Hoey and Parks, 1991). *L. orientalis* Mill. var. *orientalis* and *L. orientalis* Mill. var. *integriloba* Fiori are the two subspecies of *L. orientalis* found in the same locations together.

In addition to its ecological and biogeographical importance, production of the balsam, "liquid storax" (in Turkish "Günlük" or "Sığla"), by wounding the bark, makes this species economically important, as well. Besides its traditional use for more than seven hundred years as an all purpose drug, particularly as the most effective cure for stomach ulcers by the local population, the liquid storax has also been widely used in perfume and cosmetic industries and in pharmacy. At the beginning of 20th century, the species was covering a 6321 ha area in Turkey, however, today its distribution is restricted to only a 1337 ha area resulting in about 79 percent decrease (Akman, 1995).

Because of its relict endemism and economic importance, several morphological, anatomical and palinological studies have been carried out in *L. orientalis* (Akman, 1995; Efe, 1987). However, no information has been noticed about genetic markers and the genetic variability of this species in the specialized literature. Studies of genetic diversity are important in terms of the conservation of this species. Estimates of plant genetic diversity are necessary if we are to understand the forces that affect the genetic organization of natural plant populations (Gitzendanner and Soltis, 2000). Maintaining the existing genetic diversity in populations is one of the most important measures for species conservation.

The use of isozyme variation is a long tradition in population genetics, and a more recent application in conservation biology (Gitzendanner and Soltis, 2000). Most of our knowledge on genetic variation in forest species has been developed over the last two decades using molecular markers, especially isozymes (Ledig, 1998). Nonspecific esterases are usual markers in genetic studies of animals, plants and microorganisms because they are easy to detect and appear to be highly polymorphic (Davis, 1964; Machado, 2001; Mangolin, 1997; Resende, 2000). For most esterases, rather general substrate specificity is observed, indicating that they may have a broad biological function.

In the present study, we report esterase polymorphism, as a biochemical marker, in order to obtain preliminary data on the present genetic polymorphism within and among two populations of *L. orientalis* Mill. var. *orientalis* and two *L. orientalis* Mill. var. *integriloba* Fiori. Since these are the first results of an inclusive project carried out on the determination of genetic diversity of *L. orientalis* populations based on isozyme markers, this study is significant.

Materials and methods

Plant material

Young leaf samples of *L. orientalis* were collected from four different populations located in the Southern and the Southwestern regions of Turkey (Figure 1). Two of these populations (Fethiye-Günlüklü and Fethiye-İnlice) belong to subspecies *L. orientalis* Mill.var. *orientalis*. The other two (Muğla-Köyceğiz and Antalya-Gebiz) belong to subspecies *L. orientalis* Mill.var. *integriloba* Fiori (Figure 1). All of these populations were selected among the populations

Table 1. The geographical conditions of the locations from which the plant samples were collected.

Location	Longitude	Latitude	Altitude
Muğla-Fethiye-Günlüklü	36° 44´ 4,49´´ N	28° 55´ 7,14´´ E	0-2 m
Muğla-Fethiye-İnlice	36° 44´ 13,43´´ N	28° 58´ 27,56´´ E	5-10 m
Muğla-Köyceğiz	36° 59´ 37,00´´ N	28° 38´ 50,00´´ E	10-15 m
Antalya-Gebiz	37° 14´ 21,00´´ N	30° 58´ 34,00´´ E	500-1200 m

reported by Davis (1972). The geographical conditions of the locations from which the plant samples were collected were given in Table 1. Also, the average and extreme climatic values obtained from the meteorology stations nearby the study areas were summarized in Table 2.

Individual trees in each population were selected by using the transect method. Transect can be defined as a sampling unit laid out to different parts of study area to represent the population properly. In this study, a transect was a long straight line. In each population, the number and directions of transects were determined depending on the size of the population and the other characteristics of the location such as slope, topography and uniformity. Depending on the size of the population, one tree was sampled at every 50 to 80 m of a transect. Samples from 70, 30, 17 and 16 individual trees were collected from Muğla-Köyceğiz, Fethiye-Günlüklü, Fethiye-İnlice and Antalya-Gebiz locations, respectively. All of these 133 samples were used in the study.

Figure 1. A map showing the locations of the *Liquidambar orientalis* Mill. var. *orientalis* and *L. orientalis* Mill. var. *integriloba* Fiori populations screened in the study. Numbers indicate the following: 1. Fethiye-Günlüklü: *L. orientalis* Mill. var. *orientalis*, 2. Fethiye-İnlice: *L. orientalis* Mill. var. *orientalis*, 3. Muğla-Köyceğiz: *L. orientalis* Mill.var. *integriloba* Fiori, 4.Antalya-Gebiz: *L. orientalis* Mill.var. *integriloba* Fiori .

Table 2. The average and extreme climatic values obtained from the meteorology stations nearby the study areas.

	Meteorological Elements	Observation Periods (years)	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.	MEAN
FETHİYE	Mean Temp. (°C)	32	10.1	10.6	12.6	16.0	20.3	24.8	27.5	27.3	23.7	19.0	14.2	11.2	18.1
	Max. Mean Temp. (°C)	32	15.8	16.2	18.6	21.9	26.3	31.2	34.2	34.3	31.2	26.5	21.0	17.1	24.5
	Min. Mean Temp. (°C)	32	5.3	5.6	6.9	10.0	13.7	17.4	20.1	20.0	16.7	12.9	9.0	6.6	12.0
	Mean Rainfall (mm)	32	160.6	117.4	85.4	50.7	22.7	3.3	1.0	0.5	8.9	60.2	125.6	177.4	813.7
	Mean Relative. Humidity	32	67	65	66	66	64	59	58	60	61	66	68	69	64
KÖYCEĞİZ	Mean Temp.(°C)	32	9.2	9.8	12.2	15.9	20.9	26.2	28.8	28.1	24.6	19.4	13.8	10.4	18.3
	Max. Mean Temp. (°C)	32	15.5	16.1	18.9	22.5	27.8	33.4	36.3	35.9	32.8	27.6	21.1	16.5	25.4
	Min. Mean Temp. (°C)	32	3.9	4.0	5.8	9.1	13.2	17.5	20.2	19.8	16.2	12.1	7.8	5.2	11.2
	Mean Rainfall (mm)	32	211.2	151.8	111.9	61.1	30.7	14.8	2.6	2.5	14.7	75.5	172.5	230.5	1079.8
	Mean Relative Humidity	32	70	66	66	64	60	50	50	53	56	63	68	71	61
BUCAK	Mean Temp. (°C)	24	3.8	4.6	7.3	11.9	16.5	21.6	25.3	25.1	20.9	15.0	8.8	5.1	13.8
	Max. Mean Temp. (°C)	24	9.6	10.6	13.8	18.5	23.6	28.6	32.4	32.8	29.3	23.2	16.0	10.7	20.8
	Min. Mean Temp. (°C)	24	-0.5	0.0	1.9	5.9	10.1	14.5	18.2	17.9	13.3	8.3	3.5	1.0	7.8
	Mean Rainfall (mm)	22	92.1	81.9	85.1	59.0	63.4	39.5	16.0	9.0	19.7	51.9	67.1	118.8	703.5
	Mean Relative Humidity	24	67	64	60	57	54	44	36	38	40	50	62	69	53

From each individual tree, approximately 100 g of young leaves were collected in a 22 cm x 16 cm, properly labeled nylon bag by using a special forester tree knife. Leaf samples were stored in flaked ice kept in ice boxes till they were brought to the laboratory. Then, the samples were stored at -20 ˚C freezer until the enzyme extraction procedure.

Extraction of general esterases

One hundred milligrams of leaf tissue were ground in a chilled mortar and pestle with one milliliter of extraction buffer. The extraction buffer was the Tris-malate buffer of Soltis et al. (1983) which was modified by Hoey and Parks (1990) containing 0.05 M sodium tetraborate, 0.02 M sodium metabisulfite, 0.25 M ascorbic acid, 0.005 M diethyldithiocarbamic acid, 0.1 M maleic acid, 0.1 M Tris, 4 % w/v PVP 40, 0.5 % β-mercaptoethanol, 1 % Tween 80 and 2 % polyethylene glycol 400. Extracts were used immediately or stored in a -80 ˚C freezer for later use.

Analysis of general esterases

Esterase patterns were determined upon analyzing in polyacrylamide gels using a 12 % separating gel and 4 % stacking gel (Nascimento and Bicuda, 2002). For identification of esterases, the gels were soaked for 30 min in 150 ml 0.1 M sodium phosphate buffer at pH 6.2, on a shaker at 25 ˚C and placed for 1 h at dark in a staining solution (150 ml of 0.1 M sodium phosphate at pH 6.2, 90 mg of α-naphthyl acetate, 45 mg of β-naphthyl acetate, 180 mg of Fast Blue RR salt and 10 ml of N-propanol) (Nascimento (for those that hydrolyze β-naphthyl acetate) and magenta (for those that hydrolyze both, α and β-naphthyl acetates). The gels were destained for 24-48 h in a solution containing ethyl alcohol, acetic acid, and water in a proportion of 2:1:8 (Lima-Catalani et al., 2004). Then, the band patterns produced by the enzymes were photographed on a white illumination source using a digital camera. An example of such photograph is shown in Figure 2.

Evaluation

Esterase band patterns of each individual samples in the populations were determined by calculating the relative mobilities (Rm) of the bands detected on the gels. Then, the zymograms of each population were constructed and the frequencies of each band as well as band patterns in the populations were calculated to evaluate the degree of variations within and among the populations in terms of general esterases.

Results

In all four *L. orientalis* populations screened in this study, a total of 12 esterase bands were detected and

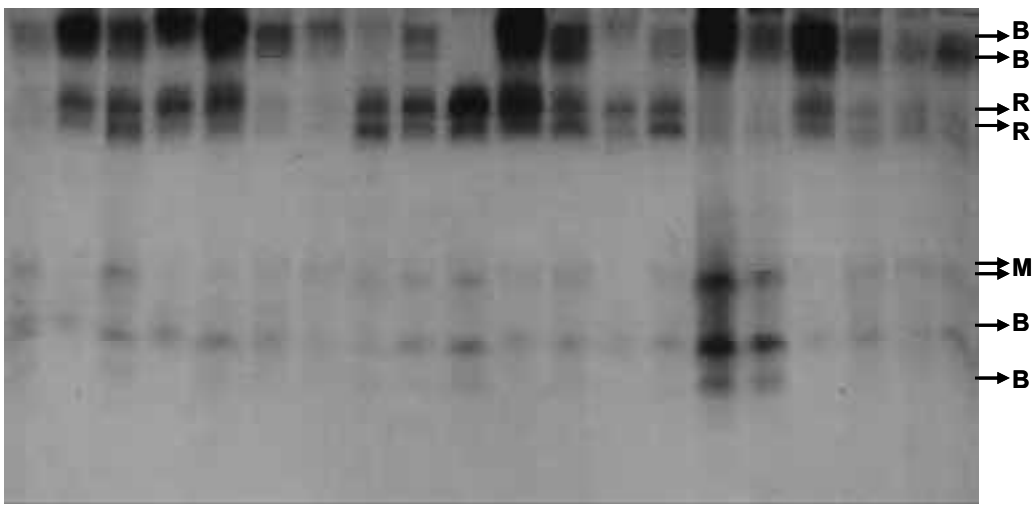

Figure 2. A photographic illustration of esterase band patterns for the Günlüklü Population. The bands indicate the hydrolysis products created by the α-, β- and α / β-esterases, which are shown on the figure by Black (B), Red (R) and Magenta (M), respectively.

named according to their increasing relative mobilities on the gel (Table 3). Six of them (Est-1, Est-2, Est-7, Est-10, Est-11 and Est-12) were black indicating the hydrolysis of the substrate α-naphthyl acetate and thus these proteins were classified as α-esterases. Four of them (Est-3, Est-4, Est-5 and Est-6) were red, produced upon the hydrolysis of the β-naphthyl acetate and were classified as β-esterases; and the remaining two (Est-8 and Est-9) were magenta resulted from the hydrolysis of both α- and β- naphthyl acetates, hence classified as α / β-esterases (Figure 2).

Among these bands, two β-esterase bands (Est-3 and Est-4) were specific to the Köyceğiz Population with very low frequencies (0.04 for both of the bands). One α-esterase band (Est-7) was specific to the Gebiz Population with a frequency of 0.31 in the population (Table 3). On the other hand, one α-esterase band (Est-10) and one α / β-esterase band (Est-9) were found to be common in all the populations screened, except in the Gebiz Population (Table 3). The highest frequency of Est-10 band was 0.88 and identified in the İnlice Population while the lowest frequency of the same band was 0.57 and identified in the Günlüklü Population (Table 3). The frequencies of Est-9 the Günlüklü Population (Table 3).

The remaining four α-esterase bands (Est-1, Est-2, Est-11 and Est-12), two β-esterase bands (Est-5 and Est-6) and one α / β-esterase band (Est-8) were detected in all of the four populations studied. The highest frequency of Est-1 band was 1.00 and found both in İnlice and Gebiz Populations, and the lowest frequency of the same band was 0.39 and found in the Köyceğiz Population. The lowest frequency of Est-2 band (0.04) was detected in the Köyceğiz Population, and the highest frequency of the same band (1.00) was detected in the Gebiz Population. The frequencies of Est-11 and Est-12 bands differed between 0.69 (in the Gebiz Population) and 1.00 (in the Günlüklü Population) and between 0.10 (in the Köyceğiz Population) and 0.88 (in the İnlice Population), respectively (Table 3). For all populations, both Est-5 and Est-6 bands had the same frequencies. The highest frequency value of these bands was 1.00 and detected in the Gebiz Population; the lowest frequency value of them was 0.06 and detected in the Köyceğiz Popu-

Table 3. Esterase bands and detected frequencies in the four *L. orientalis* populations.

Band[1]/Lokasyon	POPULATIONS			
	Günlüklü[2]	İnlice[2]	Köyceğiz[3]	Gebiz[3]
Rm=0.08, B, EST-1	0.73	1.00	0.39	1.00
Rm=0.1, B, EST-2	0.50	0.76	0.04	1.00
Rm=0.12, R, EST-3	-	-	0.04	-
Rm=0.15, R, EST-4	-	-	0.04	-
Rm=0.17, R, EST-5	0.67	0.59	0.06	1.00
Rm=0.2, R, EST-6	0.67	0.59	0.06	1.00
Rm=0.23, B, EST-7	-	-	-	0.31
Rm=0.32, M, EST-8	1.00	0.59	0.40	0.69
Rm=0.35, M, EST-9	1.00	0.94	0.40	-
Rm=0.39, B EST-10	0.57	0.88	0.71	-
Rm=0.45,B, EST-11	1.00	0.88	0.86	0.69
Rm=0.52, B, EST-12	0.57	0.88	0.10	0.12

[1]B: Black (α-Est), R: Red (β-Est), M: Magenta (α / β-Est), Rm: Relative mobility.
[2]Populations of *L. orientalis* Mill.var. *orientalis* (Fethiye-Günlüklü, Fethiye-İnlice)
[3]Populations of *L. orientalis* Mill.var. *integriloba* Fiori (Muğla-Köyceğiz, Antalya-Gebiz).

lation (Table 3). Est-8 band had the frequencies between 0.40 (in the Köyceğiz Population) and 1.00 (in the Günlüklü Population) (Table 3).

With these 12 esterase bands, 37 band patterns were detected in four populations: 14 in Köyceğiz, 9 in Günlüklü, 7 in İnlice and 7 in Gebiz Populations (Figure 3). Four of these 37 patterns were common among the populations (Figure 3, Table 4): Pattern 2 was observed in both Köyceğiz and Gebiz Populations, but frequency of the pattern was considerably higher in the Gebiz Population (0.19) than in the Köyceğiz Population (0.04). Pattern 14 was observed in both Köyceğiz and Günlüklü Populations. With the frequency of 0.23, it was the second common pattern in the Günlüklü Population while it was quite rare in the Köyceğiz Population (0.09). Patterns 16 and 19 were observed in both Günlüklü and İnlice Populations. They were the two most common patterns in the İnlice Population with the same frequencies of 0.29, but they were rare in the Günlüklü Population. The frequency of pattern 16 was 0.03 and the frequency of pattern 19 was 0.10 in this population (Table 4). Among others, pattern 11 in the Köyceğiz Population, pattern 18 in the Günlüklü Population and pattern 28 in the Gebiz Population were the most common patterns with the frequencies of 0.19, 0.33 and 0.25, respectively (Table 4). The number of unique band patterns encountered in Köyceğiz, Günlüklü, İnlice and Gebiz Populations were 12, 6, 5 and 6, respectively (Figure 3).

Discussion

One of the most important environmental pressure factors resulting in a decrease in population sizes of a species is human activities. As known, since the beginning of 20th century, relic endemic *L. orientalis* populations have been under the influence of intense human activities. If we consider the genetic drift as the main evolutionary power working in small populations to reduce the total genetic variation in time, our expectation has been to observe lower variations in these populations. However, a number of variations in general esterase patterns within and among the populations were detected in the four *L. orientalis* populations screened in this study.

The main explanation for these high levels of polymorphisms detected in these populations could be the nature of esterases. They are highly variable and multifunctional hydrolytic enzymes (Miller and Novak 1983, Wagner et al., 2002). The evolutionary process might have been acting in a way of preserving the variation in esterases for the continuity of their broad biological functions in this endemic species. In order to make more realistic and reliable judgments about the current situation of esterase polymorphisms of the studied populations, we would have to take into account the

Table 4. Frequencies of esterase band patterns in the four *L. orientalis* populations.

Band Pattern No	Köyceğiz	Band Pattern No	Günlüklü	Band Pattern No	İnlice	Band Pattern No	Gebiz
1	0.01	**14**	**0.23**	**16**	**0.29**	**2**	**0.19**
2	**0.04**	15	0.07	**19**	**0.29**	28	0.25
3	0.17	**16**	**0.03**	23	0.06	29	0.06
4	0.04	17	0.03	24	0.12	30	0.13
5	0.10	18	0.33	25	0.12	31	0.19
6	0.10	**19**	**0.10**	26	0.06	32	0.06
7	0.04	20	0.10	27	0.06	33	0.13
8	0.04	21	0.07				
9	0.03	22	0.03				
10	0.10						
11	0.19						
12	0.01						
13	0.01						
14	**0.09**						

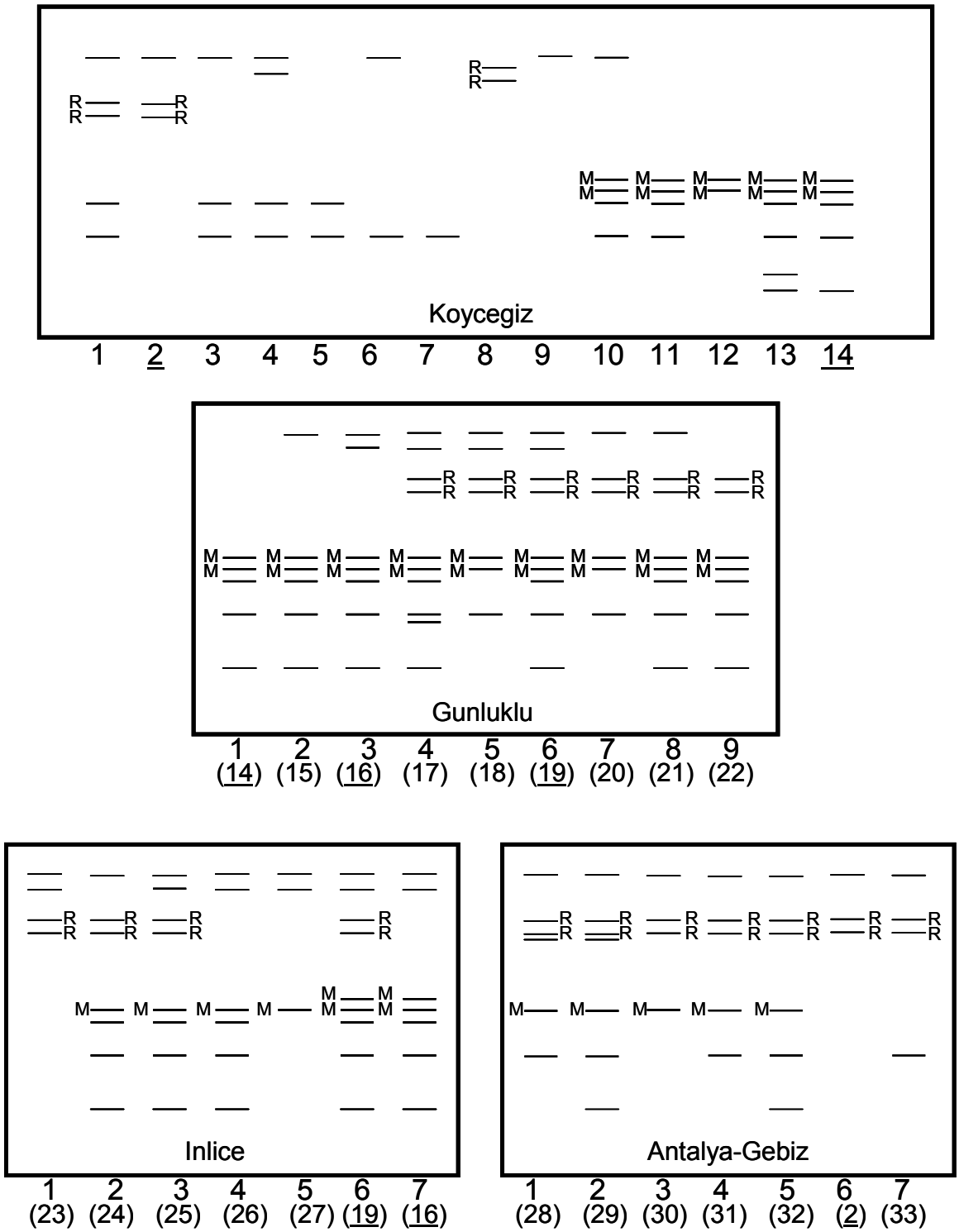

Figure 3. Zymograms of esterase band patterns in the four *L. orientalis* populations. Numbers in bold indicate different kinds of the enzyme patterns seen within the particular population. Numbers in parentheses indicate the pattern number in all of the four populations. Underlined numbers indicate the patterns detected in more than one population. The letter "R" stands for red which shows the presence of a β-esterase band, whereas the letter "M" stands for magenta which shows the presence of a α / β-esterase band. Bands that are not accompanied with a letter are all α-esterases.

previous levels of polymorphisms which might have been higher or lower than the present level. Yet we can definitely conclude that among the 12 esterases detected in populations, four α-esterases (Est-1, Est-2, Est-11 and Est-12), two β-esterases (Est-5 and Est-6) and one α / β-esterase (Est-8) were essential for the species, since they are common in all populations.

Based on esterase polymorphism results obtained from this study, the two subspecies of *L. orientalis* could not be distinguished from each other, because the esterase patterns observed were highly different from each other. Also, a relationship between esterase band patterns and geographical and climatic conditions of the regions was sought. However, we could not conclude the presence of any relationships that stand out based on the results of this study.

Studies of genetic diversity in *L. orientalis* are important to gain knowledge for the conservation of this Boreal-Tertiary origin relict endemic species. Being the first in the specialized literature, this preliminary study carried out using esterase isozymes as a biochemical marker is important. However, for better evaluation and understanding of the ongoing situation in *L. orientalis* populations in the Southwestern part of Turkey, there is an urgent need to screen not only these four populations but also the other populations located in the region in many other isozyme loci as well as DNA loci.

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