Turk J Pharm Sci 2020;17(5):480-485
DOI: 10.4274/tjps.galenos.2018.24572

ORIGINAL ARTICLE

Determination of the Genetic Relationships Among *Salvia* Species by RAPD and ISSR Analyses

Salvia Türleri Arasındaki Genetik İlişkilerin RAPD ve ISSR Analizleriyle Belirlenmesi

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ABSTRACT

Objectives: *Salvia* L. is the largest genus of the family Lamiaceae, which includes approximately 1000 species. According to recent studies, 100 *Salvia* species in total grow in Turkey. At the same time, 53% of them are endemic. The purpose of this study was to investigate the genetic relationships among 15 *Salvia* species that grow in wild conditions in Turkey's Eastern Anatolia region.

Materials and Methods: The genetic relationships among 15 *Salvia* species were investigated using inter-simple sequence repeat (ISSR) and random amplified polymorphic-DNA (RAPD) profiles in the present study. Thirteen ISSR primers and 11 RAPD primers were utilized. The ISSR and RAPD data were combined to construct the unweighted pair group method using arithmetic average cluster.

Results: Based on the RAPD and ISSR data, the *Salvia* species were classified into six groups. As a result of the combined analysis, it was shown that similarities between the species varied between 0.54 (*S. rosifolia-S. sclarea, S. rosifolia-S. limbata, and S. staminea-S. verticillata*) and 0.93 (*S. sclera-S. divaricata*).

Conclusion: The findings show that the two markers represent powerful instruments for assessing the genetic diversity and relationships among *Salvia* species.

Key words: *Salvia* species, genetic diversity, ISSR, RAPD

ÖZ

Amaç: *Salvia* L., yaklaşık 1000 tür içeren Lamiaceae familyasının en büyük cinsidir. Son çalışmalara göre, Türkiye'de toplam 100 *Salvia* türü yetişmektedir. Aynı zamanda, türlerin %53'ü endemiktir. Bu çalışmada, Türkiye'nin Doğu Anadolu Bölgesi'nde doğal olarak yetişen 15 *Salvia* türü arasındaki genetik çeşitliliğin değerlendirilmesi amaçlanmıştır.

Gereç ve Yöntemler: On beş *Salvia* türü arasındaki genetik çeşitlilik, random amplifiye polimorfik DNA (RAPD) ve ISSR DNA profilleri kullanılarak araştırıldı. On üç ISSR primeri ve on bir RAPD primeri kullanıldı. Aritmetik ortalamayı kullanan ağırlıksız çift grup metodu kümelenmesi ISSR ve RAPD verilerinin kombinasyonu ile gerçekleştirildi.

Bulgular: RAPD ve ISSR verilerinin sonuçlarına göre, *Salvia* türleri altı gruba ayrılmıştır. Kombine analiz sonucunda türlerin benzerliklerinin 0,54 (*S. rosifolia-S. sclarea, S. rosifolia-S.limbata ve S. staminea-S. verticillata*) ve 0,93 (*S. sclera-S. divaricata*) arasında değiştiği görülmüştür.

Sonuç: Bu çalışmada, hem RAPD hem de ISSR belirteçlerinin *Salvia* türleri arasındaki genetik çeşitliliği incelemek için kullanılabileceği gösterilmiştir.

Anahtar kelimeler: *Salvia* türleri, genetik çeşitlilik, ISSR, RAPD

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Received: 31.08.2018, **Accepted:** 15.11.2018

INTRODUCTION

Salvia L. is the largest genus of the family Lamiaceae. Central and South America, Western Asia (Iran, Afghanistan, Turkey, and Russia), Eastern Asia, Africa, and Europe are the main distribution regions of *Salvia* species. Western Asia is the second richest region with ca. 200 species of the genus after America with ca. 500 species.[1,2] Turkey is among the important diversity centers of *Salvia*.[1] According to the Flora of Turkey there are 86 species.[3] According to recent studies, 100 *Salvia* species in total grow in Turkey. At the same time, 53% of them are endemic.[2]

Species have been utilized as traditional medication for the mild sickness of the stomach and the common cold since ancient times.[4] Moreover, their volatile oils are utilized as an anti-inflammatory agent and antiseptic and, at the same time, a pleasurable sensory feeling is produced by them in the mouth and throat.[5,6] *Salvia* species have antibacterial, antifungal, antimycobacterial, cytotoxic, antitumor, cardiovascular, antifeedant, and insecticidal effects.[7] *Salvia* species have the following essential oil composition: α-pinene, β-pinene, 1.8-cineole, camphor, borneol, b-thujone, thymol, caryophyllene, caryophyllene oxide, and 1-octadecanol.[5,8] Several *Salvia* species were also studied to investigate their antioxidant and antibacterial properties.[5,7,9,10] Different properties of *Salvia* species growing around Erzincan Province were investigated in previous studies. Polat et al.[11] investigated the micromorphological and anatomical characteristics of three endemic *Salvia* species. The ethnobotanical uses of some *Salvia* species were investigated in and around Erzincan by Korkmaz and Karakuş.[12]

It has been confirmed that molecular markers are highly estimable for judicial, biodiversity, and mapping practices. Sufficiently high polymorphism enables the component bands of the fingerprints to function as genetic markers, with the distinction and recombination of the markers, which are utilized for the building of genetic maps.[13]

Various kinds of molecular markers, for example, random amplified polymorphic DNA (RAPD),[14] inter-simple sequence repeats (ISSRs),[15] and amplified fragment length polymorphism (AFLP),[16] have been improved and utilized widely in the investigation of genetic relationships, germplasm management, and genetic diversity, together with the developments achieved in plant molecular biology. Scientists extensively utilize ISSRs, which take place among the above-mentioned molecular markers, in different spheres of plant improvement due to their being simple and cost effective.[17] Variation in species is also studied by RAPD.[14]

The aim of the present study was to evaluate the genetic diversity present in 15 *Salvia* species, employing two marker systems.

MATERIALS AND METHODS

The material used

Leaf specimens of 15 *Salvia* taxa from Turkey were examined. The following species were analyzed: *S. cryptantha* Montbret and Aucher, *S. caespitosa*, *S. candidissima*, *S. nemorosa* L., *S. sclarea* L., *S. verticillata* L., *S. verticillata* L. subsp. *amasiaca* (Freyn & Bornm.) Bornm., *S. staminea* Montbret and Aucher ex Benth., *S. multicaulis* Vahl, *S. limbata* C.A.Mey., *S. aethiopis* L., *S. rosifolia* Sm., *S. virgata* Jacq., *S. pachhystachya* Trauv., and *S. divaricata* Montbret and Aucher ex Benth. Eleven examples of Irano-Turanian elements, 1 Euro-Siberian, and 3 of unknown region were among the samples examined. Table 1 includes information on the phytogeographical regions, endemism, and record numbers of *Salvia* taxa.

In field studies, plant specimens gathered from various regions of Turkey represent the species. Scientific names of the plant specimens were determined with the help of Davis[18] and Guner[19] after herbarium studies had been carried out. All of the taxon names were checked in the literature.[20,21] Furthermore, identification of the phytogeographical regions and endemic taxa was performed. Irano-Turanian elements constitute the majority of the taxa (11 taxa). There were 4 endemic taxa (27%). Herbarium samples of all taxa were deposited at the Herbarium of Erzincan University.

Chemicals

The DNA isolation of *Salvia* species was performed by the combination of cetyltrimethyl ammonium bromide isolation methods with minor changes.[22] Nanodrop (QIAGEN) was used for the determination of the quantity and purity of genomic DNA and 0.8% agarose gel electrophoresis was utilized against the known concentrations to prove this.

Random amplified polymorphic DNA

Eleven primers out of 23 were amplified polymorphic amplicons and used for the analyses of genetic diversity in RAPD-polymerase chain reactions (PCR) (Table 2). PCR amplifications were performed in SENSEQUEST Thermal Cycle in a total volume of 20 µL, 1X PCR buffer (without $MgCl_2$) 0.25-µM deoxyribonucleoside triphosphate (dNTP), 0.5 mM primer, 2.5 mM $MgCl_2$, and 1.5 U Taq DNA polymerase (BioLab M0267S). Initial denaturation at 95 ˚C for 5 min with the following 46 cycles at 94 ˚C for 1 min at various annealing temperatures for all primers for 1 min, 72 ˚C for 2 min, a penultimate step of 15 min at 72 ˚C, and a final extension of 10 min at 4 ˚C constituted the amplification profile. The PCR products (20 mL) were blended with 6X gel loading buffer (3 mL) and exposed to agarose. Then electrophoresis was applied to separate them by means of 1% agarose gel in 0.5X tris, borate, and EDTA (TBE) buffer with 80 V constant voltages for 150 min. The gels were dyed with Etd-Br visualized under ultraviolet light and the gel visualization system was used to take photographs of them.

Inter-simple sequence repeats

Fifteen primers were used for ISSR amplifications. Two primers of these (ISSR UBC844B and ISSR UBC822) did not give amplification in PCR reactions. The other 13 primers were amplified polymorphic amplicons (Table 3). The PCR mixture (20 µL) was prepared as follows: 2.0 µL of 10X PCR buffer, 0.5 µL of dNTPs (10 mM), 2 µL of magnesium chloride (25 mM), 1.0 µL of primer (5 mM), 0.5 µL of polymerase enzyme (Taq)

Table 1. Taxonomic information about the *Salvia* species studied

No	Taxon name	Record no	Locality	Phytogeographical region	Endemic
1	***S. cryptantha*** Montbret & Aucher ex Benth.	Korkmaz 3649	Erzincan, Çayırlı	Ir.-Tur.	+
2	***S. caespitosa*** Montbret & Aucher ex Benth.	Korkmaz 3891	Erzincan, Çayırlı	Ir.-Tur.	+
3	***S. candidissima*** Vahl	Korkmaz 2887; 3896	Erzincan, Çayırlı	Ir.-Tur.	-
4	***S. nemorosa*** L.	Korkmaz 2805	Erzincan, Çayırlı	-	-
5	***S. sclarea*** L.	Korkmaz 2795	Erzincan, Çayırlı	-	-
6	***S. verticillata*** L.	Korkmaz 2994; 2792	Erzincan, Çayırlı	Eur.-Sib.	-
7	***S. verticillata*** L. subsp. ***amasiaca*** (Freyn & Bornm.)	Korkmaz 107	Erzincan, Çayırlı	Ir.-Tur.	-
8	***S. staminea*** Montbret & Aucher ex Benth.	Korkmaz 3858	Erzincan, Çayırlı	Ir.-Tur.	-
9	***S. multicaulis*** Vahl	Korkmaz 3516	Erzincan, Çayırlı	Ir.-Tur.	-
10	***S. limbata*** C.A.Mey.	Korkmaz 2992	Erzincan, Çayırlı	Ir.-Tur.	-
11	***S. aethiopis*** L.	Korkmaz 3221	Erzincan, Çayırlı	-	-
12	***S. rosifolia*** Sm.	Korkmaz 3268	Erzincan, Çayırlı	Ir.-Tur.	+
13	***S. virgata*** Jacq.	Korkmaz 3217	Erzincan, Çayırlı	Ir.-Tur.	-
14	***S. pachhystachya*** Trautv.	Korkmaz 2228	Erzincan, Ergan Mountain	Ir.-Tur.	-
15	***S. divaricata*** Montbret & Aucher ex Benth	Korkmaz 2641	Erzincan, Ergan Mountain	Ir.-Tur.	+

Ir.: Iran, Tur.: Turkey, Sib.: Siberian, Eur.: Europe

Table 2. Primers and sequences used in RAPD amplification

RAPD primers	Sequence (5'-3')	Length of amplified bands	No of bands	No of monomorphic bands	No. of polymorphic bands	Polymorphism ratio (%)
OPA-1	CAGGCCCTTC	2238-444	15	0	15	100
OPA-2	TGCCGAGCTG	2941-433	16	0	16	100
OPA-4	TGCCGAGCTG	1649-400	12	0	12	100
OPA-6	AATCGGGCTG	2088-600	11	0	11	100
OPB-8	CTGCTGGGAC	2439-419	18	0	18	100
OPB-10	GGTCCCTGAC	1500-232	11	0	11	100
OPH-18	GTCCACACGG	958-183	8	0	8	100
OPW-8	GAATCGGCCA	1945-566	13	0	13	100
OPY-6	GACTGCCTCT	1873-353	15	0	15	100
OPY-16	AAGGCTCACC	1174-200	10	0	10	100
OPH-17	CACTCTCTCTC	1790-351	14	0	14	100

RAPD: Random amplified polymorphic DNA

(250 units), 13 µL of distilled water, and 1.0 µL of genomic DNA sample (100 ng/µL). Initial denaturation at 94 °C for 4 min with the following 35 cycles at 94 °C for 0.30 min, at various annealing temperatures for all primers for 0.35 min, 72 °C for 1 min, a penultimate step of 5 min at 72 °C, and a final extension of 10 min at 4 °C constituted the amplification profile. The PCR products (20 mL) were blended with 6X gel loading buffer (3 mL) and exposed to agarose. Then electrophoresis was applied to separate them by means of 2% agarose gel in 0.5X TBE buffer with 80 V constant voltage for 150 min. The gels were dyed with Etd-Br visualized under UV light and the gel visualization system was used to take photographs of them.

Table 3. Primers and sequences used in ISSR amplification

ISSR primers	Sequence (5'-3')	Length of amplified bands	No. of bands	No. of. monomorphic bands	No. of polymorphic bands	Polymorphism ratio (%)
ISSR UBC811	GAGAGAGAGAGAGAGAC	1935-276	15	0	15	100
ISSR UBC815	CTCTCTCTCTCTCTCTG	1246-408	11	0	11	100
ISSR UBC826	ACACACACACACACACC	936-362	7	0	7	100
ISSR UBC840	GAGAGAGAGAGAGAGATT	1303-241	12	0	12	100
ISSR UBC844A	CTCTCTCTCTCTCTCTGC	1592-378	13	0	13	100
ISSR UBC845	CTCTCTCTCTCTCTCTTT	2878-391	18	0	18	100
ISSR UBC852	TCTTCTTCTCTCTCTCAA	1432-592	7	0	7	100
ISSR 8081	GAGAGAGAGAGAGAGAGAC	2040-303	14	0	14	100
ISSR 8082	CTCTCTCTCTCTCTCTCTG	3483-441	14	0	14	100
ISSR 17889A	GTGTGTGTGTGTCC	1268-337	17	0	17	100
ISSR HB12	CAGCAGCAGGC	1392-408	10	0	10	100
ISSR HBS10	GAGAGAGAGAGACC	1362-344	11	0	11	100
ISSR UBC834	AGAGAGAGAGAGAGAGTT	1442-458	11	0	11	100

ISSR: Inter-simple sequence repeat

Statistical analysis

The Total Lab TL120 program was utilized for the assessment of the ISSR and RAPD patterns. The scoring of PCR products was performed as presence (1) and absence (0) of bands. The Jaccard (1908) similarity index was calculated by using the data, and a dendrogram was created based on the unweighted pair group method using arithmetic average.

RESULTS AND DISCUSSION

First, screening of 23 RAPD primers was performed against *Salvia* species, and 143 distinct reproducible bands in total with 10.2 bands on average per primer were produced by 11 primers. The products amplified varied between 183 and 2941 bp in size. All (100%) of the 143 bands acquired were polymorphic. Their division into four clusters was enabled by the construction of a dendrogram in accordance with the RAPD data of *Salvia* species (Figure 1).

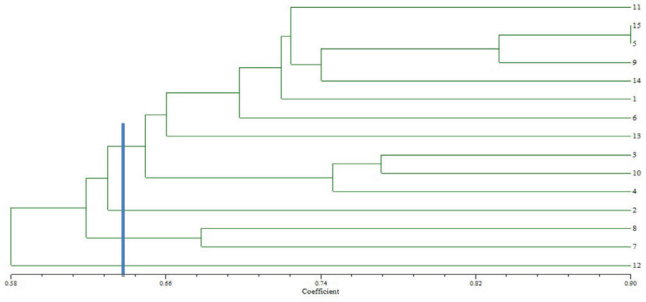

Figure 1. RAPD marker-based UPGMA clustering for *Salvia* species

1. *Salvia cryptantha*, 2. *S. caespitosa*, 3. *S. candidissima*, 4. *S. nemorosa*, 5. *S. sclarea*, 6. *S. verticillata*, 7. *S. verticillata subsp. amasiaca*, 8. *S. staminea*, 9. *S. multicaulis*, 10. *S. limbata*, 11. *S. aethiopis*, 12. *S. rosifolia*, 13. *S. virgata*, 14. *S. pachhystachya*, 15. *S. divaricata*

RAPD: Random amplified polymorphic DNA, UPGMA: Unweighted pair group method using arithmetic averag

The first cluster included *S. cryptantha*, *S. candidissima*, *S. nemorosa*, *S. sclarea*, *S. verticillata*, *S. multicaulis*, *S. limbata*, *S. aethiopis*, *S. virgata*, *S. pachhystachya*, and *S. divaricata*.

The second one included *S. caespitosa*.

The third one included *S. verticillata* subsp. *amasiaca* and *S. staminea*.

The fourth cluster included *S. rosifolia*.

The most significant likeness was identified between *S. rosifolia* and *S. caespitosa* (0.50), while the most significant difference was determined between *S. divaricata* and *S. sclarea* (0.90).

According to the RAPD data, most of the species (seven from 11 species) in the first cluster were Irano-Turanian phytogeographical region elements of Turkey (Table 1; Figure 1). Both of the most similar species (*S. rosifolia* and *S. caespitosa*) are Irano-Turanian phytogeographical region elements and endemic species.

To perform cultivar identification, 13 ISSR primers that demonstrated reproducible and polymorphic patterns were selected and produced 160 bands (polymorphic) in total, with 12.3 bands on average per primer. There was a variation from 241 to 3483 base pairs in size. The ISSR data of *Salvia* species were used to construct a dendrogram, which enabled their division into six clusters (Figure 2).

The first cluster included *S. cryptantha*, *S. candidissima*, *S. sclarea*, *S. multicaulis*, *S. limbata*, *S. aethiopis*, *S. virgata*, *S. pachhystachya*, and *S. divaricata*.

The second one included *S. verticillata*.

The third cluster included *S. nemorosa*.

The fourth cluster included *S. verticillata* subsp. *amasiaca* and *S. staminea*.

The fifth cluster included *S. rosifolia*.

The sixth cluster included *S. caespitosa*.

The most significant likeness was identified between *S. caespitosa* and *S. divaricata* (0.47), while the most significant difference was determined between *S. divaricata* and *S. sclera* (0.95).

According to the ISSR data, most of the species (seven from nine species) in the first cluster were Irano-Turanian phytogeographical region elements of Turkey (Table 1; Figure 2). Both of the most similar species (*S. divaricata* and *S. caespitosa*) are Irano- Turanian phytogeographical region elements and endemic species.

The construction of a dendrogram was performed according to the combined data acquired from the RAPD and ISSR marker analyses (Figure 3).

The first cluster included *S. cryptantha*, *S. candidissima*, *S. sclarea*, *S. multicaulis*, *S. limbata*, *S. aethiopis*, *S. virgate*, *S. pachhystachya*, and *S. divaricata*.

The second cluster included *S. verticillata*.

The third cluster included *S. nemorosa*.

The fourth cluster included *S. staminea* and *S. verticillata* subsp. *amasiaca*.

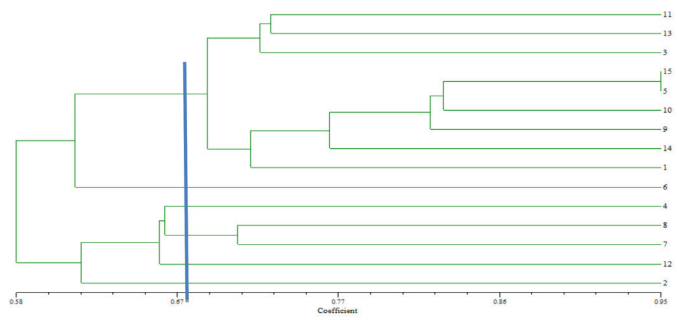

Figure 2. ISSR marker-based UPGMA clustering for *Salvia* species

1. *Salvia cryptantha*, 2. *S. caespitosa*, 3. *S. candidissima*, 4. *S. nemorosa*, 5. *S. sclarea*, 6. *S. verticillata*, 7. *S. verticillata subsp. amasiaca*, 8. *S. staminea*, 9. *S. multicaulis*, 10. *S. limbata*, 11. *S. aethiopis*, 12. *S. rosifolia*, 13. *S. virgata*, 14. *S. pachhystachya*, 15. *S. divaricata*

ISSR: Inter-simple sequence repeat, UPGMA: Unweighted pair group method using arithmetic averag

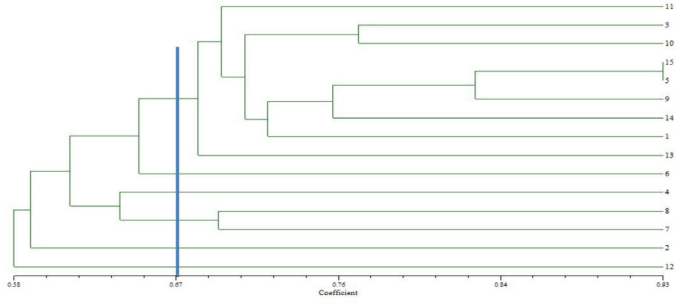

Figure 3. RAPD-ISSR marker-based UPGMA clustering for Salvia species

1. *Salvia cryptantha*, 2. *S. caespitosa*, 3. *S. candidissima*, 4. *S. nemorosa*, 5. *S. sclarea*, 6. *S. verticillata*, 7. *S. verticillata subsp. amasiaca*, 8. *S. staminea*, 9. *S. multicaulis*, 10. *S. limbata*, 11. *S. aethiopis*, 12. *S. rosifolia*, 13. *S. virgata*, 14. *S. pachhystachya*, 15. *S. divaricata*

RAPD: Random amplified polymorphic DNA, ISSR: Inter-simple sequence repeat, UPGMA: Unweighted pair group method using arithmetic averag

The fifth cluster included *S. caespitosa*.

The sixth cluster included *S. rosifolia*.

As a result of the combined analysis, it was determined that similarities of the species varied between 0.54 (*S. rosifolia-S. sclarea*, *S. rosifolia-S. limbata*, and *S. staminea-S. verticillata*) and 0.93 (*S. sclera-S. divaricata*).

The most significant difference was determined between *S. sclera* and *S. divaricata* in RAPD, ISSR, and the combined data produced from RAPD and ISSR. Differently from *S. sclera*, *S. divaricata* is an Irano-Turanian phytogeographical region element and endemic species of Turkey.

The descriptions of morphological and agronomic properties and isozyme examination were constituted for evaluating the relationship among *Salvia* species. Morphological examinations have a number of limitations, which can be eliminated by the use of molecular markers with good reproducibility and high sensitivity.[23] Molecular markers showing polymorphism at the level of DNA have been regarded as an important instrument used to assess plant genetic diversity characterization.[24,25]

To characterize *Salvia* species, different types of molecular markers, for example, RAPD, ISSR,[26,27] AFLP,[28,29] SSR,[28] and sequence-related amplified polymorphism (SRAP),[27] have been employed with great success. Agar et al.[26] used RAPD profiles to study genetic relationships in eight *Salvia* taxa. They showed that RAPD profiles were useful for the determination of genetic profiles that can be used to identify *Salvia* species. In another study, Song et al.[27] used ISSR and SRAP markers to assess the level of genetic diversity in *S. miltiorrhiza*. The results showed that these markers were effective and reliable in evaluating the degree of genetic variation in *S. miltiorrhiza*.

RAPD and ISSR represent an easy and effective marker system used to assess and determine genetic diversity among plant species. The ISSR technique has a number of benefits, such as combining the majority of the advantages of AFLP and SSR markers, providing higher reproducibility when compared to RAPD, identifying a higher rate of genomic polymorphisms when compared to RFLP, and being more cost-efficient when compared to AFLP.[29-31]

RAPD and ISSR markers were used to measure the genetic diversity among eight species of *Salvia* collected from different locations in Iran.[32] In our study, RAPD and ISSR combined data obtained from marker assays showed that *S. limbata* and *S. aethiopis* were in the same group just like in that study. *S. verticillata* and *S. nemorosa* species were also observed in separate groups.

ISSR and RAPD markers revealed findings that were almost independent of each other among *Salvia* species. Therefore, an especially positive correlation was determined for the ISSR and RAPD analysis of genetic relations among *Salvia* species.

CONCLUSION

The findings show that these two markers represent powerful instruments used to assess the genetic diversity and relations among *Salvia* species.

ACKNOWLEDGEMENTS

The authors would like to thank the Office of Scientific Research Projects of Erzincan University (EUBAP-SAGE-080715-0166) for its financial support.

Conflicts of interest: No conflict of interest was declared by the authors. The authors alone are responsible for the content and writing of the paper.

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