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Research Article
(Araştırma Makalesi)

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Anahtar Kelimeler: Çiftlik hayvanları, UPGMA dendogramı, filogeni, RFLP, SSR.

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Comparing Autosomal SSR and PCR-RFLP Markers to Determine Phylogenetic Relationship Based on Genetic Distances in Livestock

Çiftlik Hayvanlarında Genetik Mesafe Temelli Filogenetik İlişkinin Belirlenmesinde Otozomal SSR ve PCR-RFLP Markerlerinin Karşılaştırılması

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ABSTRACT

Objective: Many molecular tools are available to analyse phylogenetic relationships in livestock. Nowadays, Simple Sequence Repeats and Single Nucleotide Polymorphisms are commonly used molecular techniques to determine phylogenetic relationships in livestock breeds or types. However, alternative molecular techniques may be preferred to conduct phylogenetic analysis in case of limiting conditions such as budget and time. In this context, in the present study, Simple Sequence Repeats and Polymerase Chain Reaction- Restriction Fragment Length Polymorphism techniques were compared to reveal phylogenetic relationship based on genetic distances.

Material and Methods: In this study, 11 different layer pure chicken lines represented by 30 individuals for each line were genotyped based on 11 Polymerase Chain Reaction- Restriction Fragment Length Polymorphism and 17 Simple Sequence Repeats loci to analyse phylogenetic relationship.

Results: Both techniques showed almost similar results in terms of Unweighted Pair Group Method with Arithmetic Mean dendrogram created based on genetic distances. White and brown chicken lines were separated by both Polymerase Chain Reaction- Restriction Fragment Length Polymorphism and Simple Sequence Repeats techniques in harmony with their genetic origins and breeding history.

Conclusion: It is suggested that Polymerase Chain Reaction- Restriction Fragment Length Polymorphism technique may be preferred to analyse phylogenetic relationship based on genetic distance, when the budget, time and laboratory infrastructure are limited.

ÖZ

Amaç: Çiftlik hayvanlarında filogenetik ilişkinin analizi için çok sayıda moleküler araç bulunmaktadır. Günümüzde çiftlik hayvanı ırk ve tiplerinde filogenetik ilişkinin belirlenmesinde Basit Dizi Tekrarları ve Tek Nükleotid Polimorfizmleri en yaygın kullanılan moleküler tekniklerdir. Bununla birlikte, bütçe ve zaman gibi sınırlayıcı koşullarda filogenetik analiz yapabilmek için alternatif moleküler teknikler tercih edilebilir. Bu bağlamda, mevcut çalışmada genetik mesafe temelli filogenetik ilişkinin ortaya çıkarılmasında Basit Dizi Tekrarları ve Polimeraz Zincir Reaksiyonu-Restriksiyon Parça Uzunluk Polimorfizmi teknikleri karşılaştırılmıştır.

Materyal ve Metot: Bu çalışmada filogenetik ilişki analizi için 11 farklı yumurtacı saf hattın her birinden 30'ar birey 11 PCR-RFLP ve 17 SSR lokus temelinde genotiplendirilmiştir.

Bulgular: Genetik mesafe temelinde oluşturulan UPGMA dendogramı bakımından her iki teknik benzer sonuçlar göstermiştir. Hem Polimeraz Zincir Reaksiyonu-Restriksiyon Parça Uzunluk Polimorfizmi hem de Basit Dizi Tekrarları tekniği ile beyaz ve kahverengi yumurtacı hatlar genetik köken ve yetiştirilme geçmişlerine uygun olarak ayrılmıştır.

Sonuç: Bütçe, zaman ve laboratuvar alt yapısı kısıtlı olduğunda, genetik mesafe temelli filogenetik ilişkinin incelenmesinde Polimeraz Zincir Reaksiyonu- Restriksiyon Parça Uzunluk Polimorfizmi tekniğinin kullanılabileceği önerilmektedir.

INTRODUCTION

Goat (*Capra hircus*), sheep (*Ovis aries*), cattle (*Bos taurus*) and pig (*Sus scrofa*) were first domesticated nearly 11.000 YBP in Fertile Crescent (Zeder 2008), while multiple independent domestication centres including Southern China, South Asia and Southeast Asia were proposed for chicken domestication (Liu et al. 2006; Kanginakudru et al. 2008). After livestock species were domesticated, a long history of many factors such as mutation, selective breeding, genetic drift, isolation and adaptation led to the emergence of huge diverse animal breeds (Groeneveld et al. 2010; Hailu and Getu 2015). In worldwide, a large number of local livestock breeds including 1.019 cattle, 576 goat, 1.155 sheep, 543 pig and 1.514 chicken breeds were reported till today (FAO 2015). Discovering the origin of livestock breeds has gained interest in the last decade. Indeed many studies have been conducted to reveal the origin of different local animal breeds including chicken (Meydan et al. 2016), turkey (Vergara et al. 2019), goose (Ren et al. 2016), cattle (Xia et al. 2020), sheep (Ganbold et al. 2019), goat (Al-Araimi et al. 2017) and pig (Touma et al. 2019). In livestock, studies not only focus on breeds but also on eco-types (Zhu et al. 2019) and lines (Seo et al. 2013). Genetic diversity studies integrated with the origin of animal breeds may facilitate sustainable use of local livestock populations, maintenance of genetic diversity and managing conservation programs.

In this context, phylogenetic analysis has been a part of genetic diversity studies in order to discover the origin of livestock species. In livestock breeds or types, phylogenetic relationships may be revealed based on genetic distance or differentiation by which Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram, Neighbor Joining (NJ) tree and Factorial Correspondence Analysis (FCA) could be conducted. Until today many autosomal markers such as Random Amplified Polymorphic DNA **(**RAPD) (Balcıoğlu et al. 2010), Restriction Fragment Length Polymorphism (RFLP) (Elmacı et al. 2008; Öner et al. 2012), Amplified Fragment Length Polymorphism (AFLP) (Anila et al. 2010), Simple Sequence Repeats (SSR) (Demir and Balcioglu 2019) have been applied in order to determine genetic distance, diversity or differentiation at a molecular level.

Nowadays, however, SSR and SNPs are commonly used molecular techniques to determine phylogenetic relationships in livestock breeds or types. Although data obtaining from SNP chips or Next Generation Sequencing (NGS) analysis are more informative compared to other methods, their use is limited due to requiring more budget, laboratory infrastructure and knowledge. On the other hand, RFLP data can be obtained at low cost compare to SSR markers in which fluorescent labelled primers and capillary electrophoresis systems are needed.

The present study aimed to determine the phylogenetic relationship among 11 layer pure chicken lines coming from three different genetic origins based on data obtained from SSR and PCR-RFLP techniques. In this context, in case of a limited budget, the application of the PCR-RFLP technique was assessed by comparing to the SSR technique.

MATERIAL and METHODS

Studied chicken lines and their breeding history

Brown layer pure chicken lines were originated from Rhode Island Red (RIRI and RIRII) and Plymouth Rock (BARI, BARII, COL and L-54), whereas white layer pure chicken lines (Black, Brown, Blue, Maroon and D-229) were derived from White Leghorn. It is known that L-54 is a synthetic line containing approximately 15% White Leghorn blood to increase egg yield and to decrease body weight. D-229 line was obtained from Czechia in 2010 while the rests of all chicken lines were imported from Canada in 1995. Since then selection studies have been conducted by Ankara Poultry Research Institute for many yield traits (Göğer et al. 2017; Karslı et al. 2017).

Data collection

In the present study, PCR-RFLP and SSR data were provided from three different previously completed projects in order to construct UPGMA dendrogram in eleven different layer pure lines raised by Ankara Poultry Research Institute. PCR-RFLP data were provided from the project namely "Detection of Polymorphisms in Some Candidate Genes Associated with Egg Yield and Quality in Layer Pure Lines Raised in Ankara Poultry Research Institute by Using PCR-RFLP Method". In the project, a total of 17 loci were investigated in six brown and five white chicken lines. We used only 11 of 17 loci [Growth Hormone Receptor (GHR-intron-2/*Hind*III and GHR-intron-5/*Nsp*I); Dopamine Receptor (DRD1/ *Bse*NI and DRD2/ *Bse*GI), Vasoactive Intestinal Peptide (VIP-501/*Vsp*I and VIP-I2/*Hinf* I); Vasoactive Intestinal Peptide Receptor (VIPR-1/ *Hha*I and VIPR-2/*Taq*I); Ovocalyxin-32 (OCX32-exon4/*Nco*I and OCX32-exon2/*HpyCH4*IV) and Melatonin Receptor (MR1C/*Mbo*I)] in data for phylogenetic relationship analysis. SSR data were provided from the project namely "Determination of Genetic Diversity of Brown Layer Pure Lines in the

Ankara Poultry Research Station by Using Microsatellite Markers" (Karslı and Balcıoğlu 2019) for six brown chicken lines and from the other project namely "Assessment of Genetic Diversity, Population Structure and Conservation Priorities of Five Different White Pure Layer Line by Microsatellite Markers" (Karslı and Fidan 2019) for five white chicken lines. We used a total of the same 17 SSR loci (ADL0112, ADL0268, LEI0094, LEI0116, LEI0192, MCW0020, MCW0037, MCW0067, MCW0069, MCW0078, MCW0081, MCW0111, MCW0123, MCW0183, MCW0248, MCW0301 and MCW0330) for both two projects. A total of 330 data belonging to six Brown pure layer chicken lines including RIRI (n=30), RIRII (n=30), BARI (n=30), BARII (n=30), COL (n=30) and L-54 (n=30), and five White pure chicken lines including Black (n=30), Brown (n=30), Blue (n=30), Maroon (n=30) and D-229 (n=30) raised in Ankara Poultry Research Institute were used.

DNA Isolation, SSR and PCR-RFLP Analysis

DNA extraction and SSR analysis (including used markers, PCR reaction and protocol together with fragment analysis) were previously described by Karslı and Balcıoğlu (2019) and Karslı and Fidan (2019).

Similarly, a salting out method described by Miller et al. (1988) was used to extract DNA from blood samples for PCR-RFLP analysis. A common PCR reaction (50 ng template DNA, 1.2 µL HQ buffer-GeneAll, 2 µL 10X buffer-GeneAll, 2.5 mM dNTPS, 10 pM of each primer, 2.5 U *Taq* DNA Polymerase (GeneAll) and 11.4 µL nuclease free water) and PCR protocol (initial denaturation at 9℃ for 5 min followed by 30 cycles of denaturation at 94℃ for 45 s, annealing at 50-62 ℃ for 45 s, extension at 72℃ for 50 s with a final extension at 72 ℃ for 5 min) were used to amplify PCR-RFLP loci with specific primers (Table 1). Amplified PCR products were digested with specific endonucleases (Table 1) by using a common RFLP reaction (8 µL of amplified PCR products, 2.5 U restriction enzyme and 8 µL 10X buffer). To detect the genotypes, digested RFLP products were visualized on 3% agarose gel electrophoresis.

Data analysis

In this study, detection of genetic distance values and construction of UPGMA dendrograms were performed by using Popgene version 1.31 software (Yeh et al. 1997). Output file obtained from Popgene software was further processed by Mega version 6 software (Tamura et al. 2013) to construct sharper phylogenetic tree.

Table 1. Some descriptive information about PCR-RFLP process
Çizelge 1. *PCR-RFLP işlemine ait bazı tanımlayıcı bilgiler*

Gene	Primers (5'-3')	Ann.Temp. (°C)	PCR Size	Restriction Enzyme	References
GHR-intron 2	F:GGCTCTCCATGGGTATTAGGA R: GCTGGTGAACCAATCTCGGTT	59	718	*Hind*III	Li et al. (2008)
GHR-intron 5	F: ACGAAAAGTGTTTCAGTGTTGA R: TTTATCCCGTGTTCTCTTGACA	56	740	*Nsp*I	Li et al. (2008)
DRD1	F:CACTATGGATGGGGAAGGGTTG R: GCCACCCAGATGTTGCAAAATG	62	283	Bse*N*I	Xu et al. (2010)
DRD2	F:TGCACATAAAAGCCCACTCACTG R:GCCTGAGCTGGTGGGGGG	60	248	*BseG*I	Xu et al. (2011)
VIP/501	F:GAAACCCATCTCAGTCATCCTA R:ACCACCTATTTTTCCTTTTCTACA	55	306	*Vsp*I	Zhou et al. (2010)
VIP/I2	F: GCTTGGACTGATGCGTACTT R: GTATCACTGCAAATGCTCTG	58	520	*Hinf*I	Zhou et al. (2010)
VIPR-1	F:CCCCGTTAAACTCAGCAGAC R:CCCAAAGTCCCACAAGGTAA	58	434	*Hha*I	Xu et al. (2011)
VIPR-2	F:CTCCTCAGGCAGACCATCATG R:CTTGCACGTATCCTTGGGTAGC	58	486	*Taq*I	Xu et al. (2011)
OCX32-exon4	F: TGTTTCTGATGAAGAGCCAGA R: CTTTGCCACTCTGTAGGCTGT	58	250	*Nco*I	Uemoto et al. (2009)
OCX32-exon2	F: GCCCACTGGTCAGAAAAGAA R: CCTGCAGAGGAAAAGAGCTG	58	405	*HpyCH4*IV	Uemoto et al. (2009)
MR1C	F: GGTGTATCCGTATCCTCTAA R: GACAGTGGGACAATGAAGT	50	372	*Mbo*I	Li et al. (2013)

RESULTS

In this study, genetic distance and genetic identity values in chicken lines were created based on both PCR-RFLP (Table 2) and SSR (Table 3) data. A total of 11 PCR-RFLP loci revealed that genetic distance value ranged from 0.023 to 0.277, whereas genetic identity value varied from 0.758 to 0.977 in studied populations. The lowest and highest genetic distance were detected between BARI-BARII (0.023) and BARI-Brown (0.277), respectively. Accordingly, the lowest and the highest genetic identity were observed between BARI-Brown (0.758) and BARI-BARII (0.977) based on PCR-RFLP data.

Based on a total of 17 SSR markers, genetic distance and genetic identity values were between 0.152-4.547 and 0.011-0.859, respectively. The lowest and highest genetic distance were detected between D-229-Brown (0.152) and BARI-Blue (4.547), respectively. Accordingly, the lowest and the highest genetic identity were observed between BARI-Blue (0.011) and D-229-Brown (0.859), respectively.

Table 2. Genetic distance (below the diagonal) and genetic identity values (above the diagonal) obtained in studied chicken lines based on PCR-RFLP data
Çizelge 2. *Çalışılan tavuk hatlarında PCR-RFLP verileri temelinde elde edilen genetik mesafe (köşegenin altı) ve genetik benzerlik (köşegenin üstü) değerleri*

	Blue	Brown	D-229	Black	Maroon	RIRI	RIRII	COL	BARI	BARII	L-54
Blue	****	0.928	0.935	0.941	0.834	0.870	0.777	0.851	0.817	0.812	0.954
Brown	0.074	****	0.893	0.945	0.945	0.806	0.776	0.837	**0.758**	0.770	0.898
D-229	0.067	0.114	****	0.967	0.834	0.889	0.822	0.807	0.855	0.890	0.964
Black	0.060	0.056	0.034	****	0.914	0.859	0.805	0.822	0.834	0.857	0.966
Maroon	0.181	0.056	0.182	0.090	****	0.826	0.823	0.883	0.774	0.784	0.867
RIRI	0.139	0.215	0.118	0.152	0.192	****	0.961	0.883	0.917	0.913	0.904
RIRII	0.253	0.254	0.196	0.218	0.195	0.040	****	0.863	0.857	0.881	0.840
COL	0.162	0.178	0.215	0.196	0.124	0.124	0.148	****	0.872	0.868	0.899
BARI	0.202	**0.277**	0.156	0.182	0.256	0.086	0.154	0.137	****	**0.977**	0.904
BARII	0.208	0.262	0.117	0.155	0.244	0.092	0.127	0.142	**0.023**	****	0.929
L-54	0.048	0.108	0.037	0.035	0.143	0.102	0.174	0.107	0.101	0.074	****

RIRI: Rhode Island Red I. RIRII: Rhode Island Red II. BARI: Barred Rock I. BARII: Barred Rock II. COL: Colombian Rock. L-54: Line-54

Table 3. Genetic distance (below the diagonal) and genetic identity values (above the diagonal) in studied chicken lines based on SSR data
Çizelge 3. *Çalışılan tavuk hatlarında SSR verileri temelinde elde edilen genetik mesafe (köşegenin altı) ve genetik benzerlik (köşegenin üstü) değerleri*

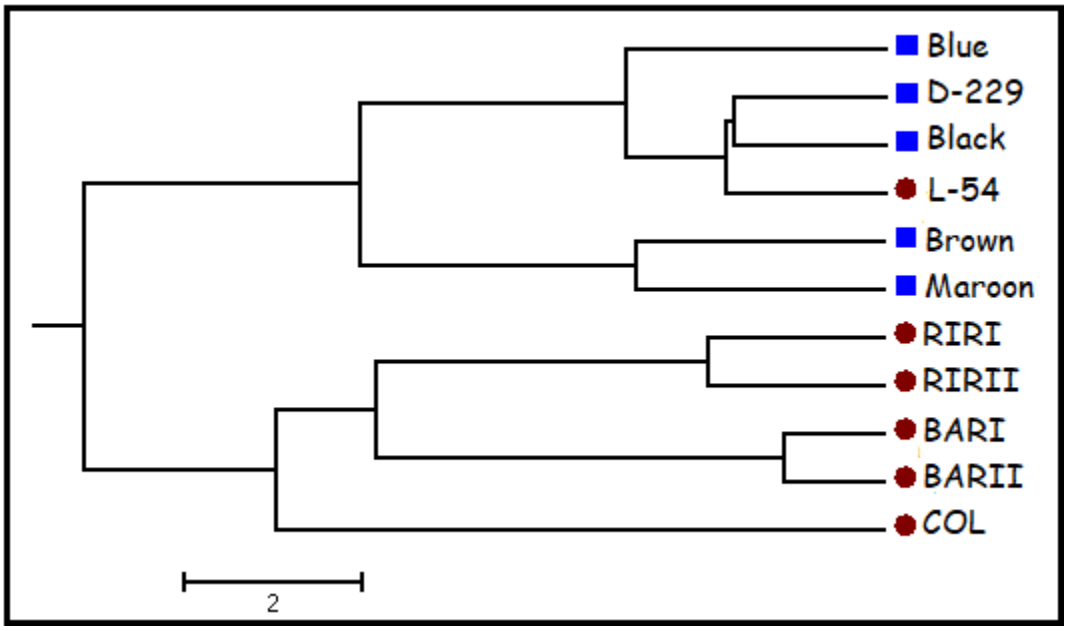
	Blue	Brown	D-229	Black	Maroon	RIRI	RIRII	COL	BARI	BARII	L-54
Blue	****	0.816	0.739	0.676	0.675	0.022	0.017	0.031	**0.011**	0.068	0.021
Brown	0.204	****	**0.859**	0.725	0.665	0.080	0.067	0.035	0.012	0.116	0.022
D-229	0.302	**0.152**	****	0.735	0.728	0.021	0.015	0.044	0.015	0.081	0.032
Black	0.392	0.321	0.308	****	0.792	0.037	0.044	0.049	0.043	0.064	0.076
Maroon	0.393	0.408	0.318	0.234	****	0.025	0.026	0.029	0.023	0.023	0.044
RIRI	3.826	2.528	3.868	3.285	3.682	****	0.639	0.377	0.362	0.325	0.577
RIRII	4.091	2.699	4.222	3.124	3.640	0.448	****	0.343	0.391	0.404	0.433
COL	3.466	3.356	3.114	3.011	3.545	0.977	1.071	****	0.833	0.341	0.402
BARI	**4.547**	4.394	4.180	3.153	3.781	1.017	0.938	0.183	****	0.412	0.432
BARII	2.682	2.152	2.510	2.755	3.772	1.125	0.907	1.075	0.886	****	0.278
L-54	3.859	3.798	3.434	2.583	3.115	0.550	0.838	0.910	0.841	1.282	****

RIRI: Rhode Island Red I. RIRII: Rhode Island Red II. BARI: Barred Rock I. BARII: Barred Rock II. COL: Colombian Rock. L-54: Line-54

In the present study, the UPGMA dendrogram was constructed for both PCR-RFLP (Figure 1) and SSR (Figure 2) data based on genetic distance values. According to PCR-RFLP data, except L-54, white and brown chicken lines were clustered separately. It is observed that Brown and Maroon as well as D229 and Black lines clustered closely in White chicken lines. In addition, RIRI and RIRII besides BARI-BARII clustered

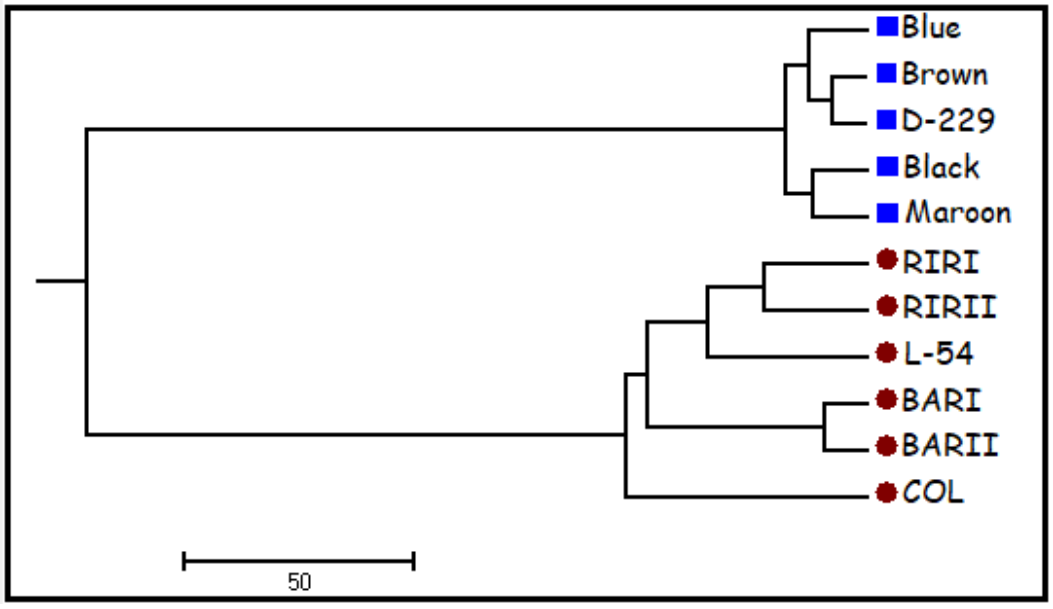
together as expected. On the contrary, L-54, which is one of the brown chicken lines, clustered with white chicken lines rather than Brown ones.

Based on SSR data, White and Brown chicken lines clustered separately in two distinct branches. Additionally, RIRI and RIRII besides BARI and BARII clustered together.



RIRI: Rhode Island Red I. RIRII: Rhode Island Red II. BARI: Barred Rock I. BARII: Barred Rock II. COL: Colombian Rock. L-54: Line-54

Figure 1. UPGMA dendrograme constructed among studied chicken lines based on PCR-RFLP data
Şekil 1. *Çalışılan tavuk hatları arasında PCR-RFLP verileri temelinde oluşturulan UPGMA dendogramı*



RIRI: Rhode Island Red I. RIRII: Rhode Island Red II. BARI: Barred Rock I. BARII: Barred Rock II. COL: Colombian Rock. L-54: Line-54

Figure 2. UPGMA dendrograme constructed among studied chicken lines based on SSR data
Şekil 2. *Çalışılan tavuk hatları arasında SSR verileri temelinde oluşturulan UPGMA dendogramı*

DISCUSSION

According to both PCR-RFLP and SSR data, the genetic distances observed among Brown and White chicken lines were higher than the values observed within the lines. Brown and White chicken lines were clearly separated by the UPGMA dendrogram based on both data. These results are in accordance with genetic origins and breeding history of studied chicken lines. Surprisingly, L-54 was assigned into White chicken lines according to PCR-RFLP data. It is thought that there are two main reasons for this situation. Firstly, as mentioned above L-54 contains 15% White Leghorn blood. On the other hand, L-54 which is still thought to possess 85% Plymouth Rock (Colombian) blood, was expected to cluster together with brown layer lines. Secondly, it may occur due to the nature of PCR-RFLP and SSR markers representing a different part of the genome. The PCR-RFLP data belonged to 11 different gene regions related to egg yield and quality, while SSR markers randomly distributed across the genome.

In studied 11 chicken lines obtained genetic distance values based on PCR-RFLP (ranging from 0.023 to 0.277) were lower than the values based on SSR markers (ranging from 0.152 to 4.547). On the contrary, genetic identity values based on PCR-RFLP (ranging from 0.011 to 0.859) were higher than the values based on SSR markers (ranging from 0.758 to 0.977). As expected, SSR markers were found more informative than PCR-RFLP in terms of investigation of phylogenetic relationships in livestock such as chicken.

The results of the present study were accordant to findings reported in previous studies conducted for Brown and White chicken lines raised in Ankara Poultry Research Institute (Karslı and Balcıoglu 2019; Karslı and Fidan 2019). RIRI and RIRII besides BARI and BARII were reported clustering together in Neighbour Joining (NJ) tree (Karslı and Balcıoglu 2019), while Brown and D-229 besides Black and Maroon reported clustering together in UPGMA dendrogram based on SSR data (Karslı and Fidan 2019). In the present study, 17 SSR loci showed similar results to finding reported by Karslı and Balcıoğlu (2019) and Karslı and Fidan (2019), who used 22 and 19 SSR loci, respectively.

It is reported that as the present study, PCR-RFLP and SSR markers are good tools in order to reveal the phylogenetic relationships. For instance, Nagaraju et al. (2001) compared RFLP and three PCR based techniques (RAPD, SSR and ISSR) to distinguish diapausing and non-diapausing silkworm varieties. It was reported that all genetic marker techniques separated diapausing and non-diapausing silkworm varieties based on the UPGMA dendrogram (Nagaraju et al. 2001).

CONCLUSION

In the present study, phylogenetic trees constructed based on 11 PCR-RFLP and 17 SSR loci were compared in eleven pure layer chicken lines in which similar results were observed. Based on 11 PCR-RFLP loci, constructed UPGMA dendrogram separated white and brown chicken lines in harmony with genetic origins and breeding history with very low differences. The results obtained from this study revealed that although SSR markers are more informative, PCR-RFLP markers may be used to construct phylogenetic tree in case of limited budget, time and laboratory infrastructure.

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