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GENETİC DIVERSITY ANALYSIS OF *Hericium* ISOLATES BY ISSR and SRAP MARKERS

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ABSTRACT

Inter Simple Sequence Repeats (ISSR) and Sequence-Related Amplified Polymorphism (SRAP) markers were applied for genomic fingerprinting of 8 *Hericium* isolates of Turkey and American origin. The ISSR, SRAP and combined ISSR/SRAP analyses based dendrograms were generated by the UPGMA method. Fifteen ISSR primers amplified a total of 154 bands of which 151 (97.4%) were polymorphic, whereas 16 SRAP primer combinations amplified 164 bands of which 156 (95.1%) were polymorphic. All the *Hericium* isolates analyzed was divided into three major clusters and *H. americanum* independently located on the one cluster in all dendograms. The dendrograms indicated a clear pattern of clustering and the 8 *Hericium* isolates showed significant differences suggesting that they were far to each other in genetic relation. These results demonstrated that both methods were highly effective for discriminating among species and strains of *Hericium*. Finally, to our knowledge, this is the first application of ISSR and SRAP markers on the systematics of *Hericium* isolates and these findings would benefit future research in *Hericium* spp.

Keywords: *Hericium* spp; genetic diversity; moleculer markers; Inter Simple Sequence Repeats (ISSR); Sequence-Related Amplified Polymorphism (SRAP)

INTRODUCTION

Being in a temperate climatic zone, Turkey is rich in mushroom diversity because of its unique geomorphology, topography and climate. *Hericium erinaceus* and *Hericium corallodes* are resident of decaying trees and logs throughout the Turkey **(Aşkun, 2002; Afyon *et al.*, 2004; Afyon *et al.*, 2005; Demirel *et al.*, 2010)**belonging to the family *Hericiaceae* and the phyla *Basidiomycota***(Kuo, 2016)** *H. erinaceus* is certainly rare in nature**(Moril *et al.*, 2008)** It is red-listed in 13 of the 23 European countries because its natural habitats are beginning to disappear (**Govaerts *et al.*, 2011**). All members of the genus produce white fruiting bodies covered in downward cascading spines. Fruitbodies of all *Hericium* spp. look like a mass of icicle and can be identified by its long spines that rise from a white central. Fleshy is changed from white to off-white (**Kuo, 2016)**

Hericium spp. are delicious with a subtle citrus-floral flavor and a musky delicate smell (**Imtiaj *et al.*, 2008**). It is important not only for its nutrition and taste, but also for its medical value **(Moril *et al.*, 2008; Keyzers and Davies-Coleman, 2005; Shang *et al.*, 2012).***H. erinaceus* has been used as a medicine for hundreds of years in China **(Han *et al.*, 2013)**.Then, *H. erinaceus* is one of the most popular species among the cultivated mushrooms for commercial production.

The traditional methods for the identification of mushrooms are problematical. Commercial species and strains of *Hericium* are classified based on the morphological characters. But morphological characters tend to be influenced by environmental factors in mushrooms. When environmental factorsare combinated with close genetic relation between the isolates make their identification difficult and sometimes impossible. Molecular markers are plentiful, independent of tissue or environmental effects, and allow cultivar identification in the early stages of development **(Esposito *et al.*, 2007).** These features make them useful complements to morphological and phenological characters. PCR-based markers that require for relatively a small amount of template genomic DNA to reveal polymorphism at DNA level are very useful tools for characterization and genetic diversity estimation.

A PCR-based method for detecting *Hericium* species using ribosomal internal transcribed spacer (ITS) sequences has been reported by **Lu *et al.*,(2002) andPark *et al.* (2004).**However, the applicability of this technique is limited principally to the classification of fungi at the species level **(Garnica *et al.*, 2003; Peintner *et al.*, 2004).** On the other hand, DNA fingerprinting techniques such as RAPD, ISSR, SRAP, AFLP have proven their capacity to verify closely related species or strains belonging to the same species **(Zervakis *et al.*, 2001; Lopandic *et al.*, 2005).**

Although different molecular markers based on nucleic acid polymorphisms such as Random Amplified Polymorphic DNA (RAPD) **(Yan *et al.*, 2004; Chandra *et al.*, 2010)** Inter Simple Sequence Repeats (ISSR) **(Yu *et al.*, 2008; Du *et al*, 2011)** and Sequence-Related Amplified Polymorphism (SRAP) **(Sun *et al.*, 2006; Ma *et al.*, 2010)**have been exploited in assessing the genetic diversity among cultivars and strains in several edible and medicinal fungi. However, to the best of our knowledge, there is no study about using these molecular markers on determination of the molecular systematics of *Hericium* spp. For the reason, in the study, concerning the genotyping of 8 isolates of *Hericium*, the genetic variability was investigated using ISSR, SRAP and combined ISSR and SRAP tecniques.

MATERIAL AND METHODS

Mushroom strains

A total of 8 isolates of *Hericium* representing three different species (*H. erinaceus*, H. *americanum* and *H. coralloides)* were provided by Agroma Limited Company, Denizli, Turkey. All 8 isolates are presented at Table 1. This study was conducted at the Mushroom Production Unit and Biotechnology Laboratories of Faculty of Agriculture, Ege University.

Table 1 Species, geographical origin and cod of *Hericium* isolate

Species	Geographical origin	Isolate Code
Hericium erinaceus	Denizli-Turkey	HE- Denizli
Hericium erinaceus	İzmit, Turkey	HE-İzmit
Hericium erinaceus	Trabzon, Turkey	HE-Trabzon
Hericium erinaceus	Ankara, Turkey	HE-Ankara
Hericium erinaceus	USA	HE-America
Hericium erinaceus	Commercial var.,Turkey	HE
Hericium corolloides	USA	HC
Hericium americanum	USA	HA

DNA extraction

Genomic DNA was extracted from dry fruitbodies according to the modified SDS (sodium dodecyl sulfate) method **(O'Donnel *et al.,* 1998)**and the quality confirmed by 1.0% (w/v) agarose gel elektrophoresis at 100V. The concentration of DNA was determined with the Quantity One software (Bio-Rad). Samples were diluted to 50 ng μl^{-1} and stored at -20 ^oC for for PCR amplification.

ISSR analysis

Fifteen ISSR primers were selected based on our own preliminary works to generate data for analysis in this study (Table 2). ISSR amplification was carried out using a Eppendorf Thermal cycle in 25 µl reaction mixture contained 2.5 µl 10 X PCR buffer, 1 µM primers, 400 µM dNTPs, 2.5 mo1 MgCl2, 1U *Taq* DNA polymerase and 50 ng μl^{-1} template DNA. Using ISSR primers and their annealing temperatures (^oC) were summerized in Table 2.
The amplifications were performed according to the protocol of **Malekzadeh *et al.* (2011)**with some modifications. Amplification conditions were; an initial step of 3 min at 94 ^oC, followed by 30 cycles of 45 s at 94 ^oC, 60 s at 52-56 ^oC, 60 s at 72 ^oC, and 5 min final extension step at 72 ^oC. The PCR products were separated on a 2.5% agarose gels in 1x TAE buffer (40 mM Tris-Acetate, 1 mM EDTA, pH:8.0) running at 70 V constant voltage for 2.5 h, then stained with 0.5 µg/ml ethidium bromide (EtBr) and photographed under UV. To confirm the reproducibility of the banding patterns, the PCR experiments were repeated twice.

SRAP analysis

18 SRAP primer pairs were employed using 10 forward primers and 10 reverse primers (Table 3). Sixteen SRAP primer combinations were selected for SRAP analysis. The SRAP-PCR reaction mixture with a total volume of 25 µl consisted 2.5 µl 10 X PCR buffer, 250 µM dNTPs, 2.5 mo1 $MgCl_2$, 1 U of *Taq* polymerase, 50 ng each primer and 50 ng μl^{-1} template DNA at 0.5 mmol. DNA amplifications were performed with an initial step at 94 ^0C for 4 min and five cycles of 45 s at 94 ^oC, 45 s at 35 ^oC and 60 s at 72 ^oC. The following 35 cycles consisted of 94 ^oC for 45 s, 50^oC for 45 s and 72 ^oC for 60 s, with a final extension at 72^0C for 8 min **(Li and Qurios, 2001)** The amplification products were separated by electroforesis in 2.5% agarose gels in 1x TAE buffer (40 mM Tris-Acetate, 1 mM EDTA, pH:8.0) at 70 V for 2.5 hours and stained with 0.5 µg/ml ethidium bromide (EtBr) and photographed under UV. 100-2000 bp DNA Ladder (Invitrogen 100 bp, Termo Fisher Scientific) was used as standard for molecular size determinations

Data analysis

Reproducible bands from individual marker systems were scored as present "1" and absent "0" which were compiled as a binary matrix. Only those bands amplified consistently were considered. The results were then converted to a similarity matrix, and a dendrogram was constructed using the Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) **(Sneath and Sokal, 1973)** with the Numerical Taxonomy and Multiware Analysis System software, version 2.0 **(Rohlf, 1992)**.

Table 2 ISSR primers and corresponding annealing temperatures

Primers	Sequence (5´_3)	Annealing Temperature (ºC)	Primers	Sequence (5´_3)	Annealing Temperature (ºC)
P3	(GA)8 T	54	**P22**	(AG)8YC	56
P4	(GA)8C	54	**P25**	(GA)8YC	56
P5	(AG)8G	56	**P26**	(GA)8YT	54
P6	(AG)8C	56	**P27**	(GA)8YG	56
P9	GS(GT)8	54	**P32**	(GA)8A	52-54
P10	(AG)8T	54	**ISSR1**	BDB(ACA)5	52-54
P16	(TC)8C	54	**ISSR18**	VHV(GT)8	54
P21	(AG)8YA	52			

Table 3SRAP forward (me) and reverse (em) primers

Primers	Sequence (5´_3)	Primers	Sequence (5´_3)
me1	TGAGTCCAAACCGGATA	**em 1**	GACTGCGTACGAATTAAT
me2	TGAGTCCAAACCGGAGC	**em 2**	GACTGCGTACGAATTTGC
me3	TGAGTCCAAACCGGAAT	**em 3**	GACTGCGTACGAATTGAC
me4	TGAGTCCAAACCGGACC	**em 4**	GACTGCGTACGAATTTGA
me5	TGAGTCCAAACCGGAAG	**em 5**	GACTGCGTACGAATTAAC
me6	TGAGTCCAAACCGGACA	**em 6**	GACTGCGTACGAATTGCA
me7	TGAGTCCAAACCGGACG	**em 7**	GACTGCGTACGAATTCAA
me8	TGAGTCCAAACCGGTGC	**em 8**	GACTGCGTACGAATTCAC
me9	TGAGTCCAAACCGGAGG	**em 9**	GACTGCGTACGAATTCAT
me10	TGAGTCCAAACCGGTGT	**em 10**	GACTGCGTACGAATTCTA

RESULTS

In total, 15 ISSR and 16 SRAP primer pairs gave reproducible results that were further considered for data analysis. Table 4 and 5shows the total number of bands and the percentage of polymorphisms for each primer or primer pair.

ISSR analysis

150 polymorphic bands make it possible for a researcher to reliably estimate genetic similarities among genotypes within the same species (Pejic et al., 1998). In the study, fifteen ISSR primers amplified a total of 154 scorable bands (Table 4), of which 151 were polymorphic and accounted for 97.4%.

The number of polymorphic bands detected with each primer ranged from 5 $(GA_8)A$ to 18 $(AG)_8G$, with an average of 10.3. Figure 1 represents the extent of polymorphism observed among the eight isolates as revealed by ISSR1

Table 4 Polymorphism obtained by ISSR analysis in *Hericium* isolates

	Primer/Primer combination	Total bands	Polymorphic bands	Percentage of polymorphism
ISSR	P3	12	12	100
	P4	5	4	80
	P5	18	18	100
	P6	7	7	100
	P9	8	8	100
	P10	17	17	100
	P16	7	7	100
	P21	9	9	100
	P22	12	11	91.7
	P25	11	11	100
	P26	10	9	90
	P27	9	9	100
	P32	5	5	100
	ISSR1	17	17	100
	ISSR18	7	7	100
Total		**154**	**151**	**-**
Avarage		**10.3**	**10.1**	**97.4**

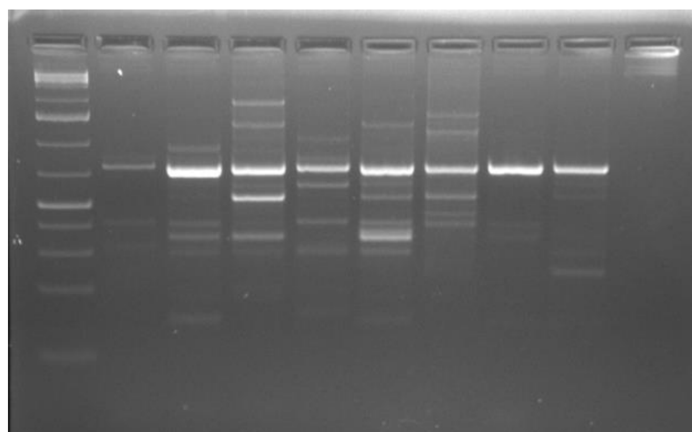

Figure 1 Representative ISSR amplification profile using ISSR1

The genetic similarity derived from the data of ISSR marker analysis varied from 0.039 between to 0.262. The co-phenetic correlation for the ISSR dendogram was estimated at 0.85. Figure 2 shows the dendrogram based on the ISSR data. UPGMA grouped the 8 isolates into three main clusters at a similarity index value of 0.07.

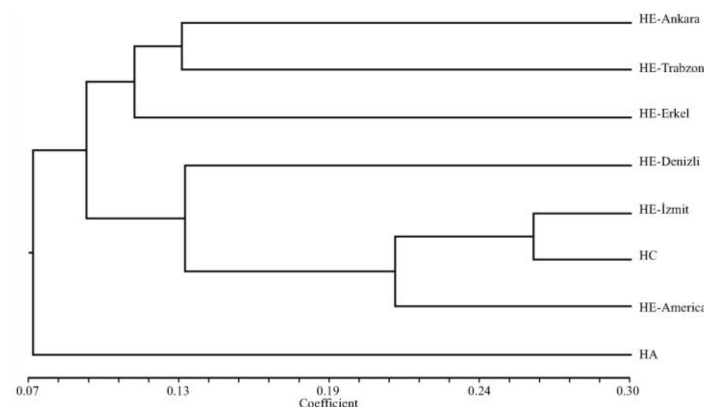

Figure 2 UPGMA dendogram based on data from ISSR analysis of 8 *Hericium* isolates

Cluster I comprised 3 isolates that were subdivided into two sub-clusters. Sub-cluster 1 comprised 2 genotypes, with HE-Ankara and HE-Trabzon (with a 0.13 similarity coefficient) while sub-cluster 2 comprised only one isolate, HE. Cluster II consisted of 4 isolates that were subdivided into two sub-clusters. Sub-cluster 1 comprised 3 genotypes, HE-İzmit, HCand HE-America, while sub-cluster 2 comprised only one isolate, HE-Denizli. Cluster III consisted of single isolate *H. americanum*.

SRAP analysis

A total of 100 different combinations of primers were employed using 10 forward and 10 reverse primers and 16 combinations were chosen in the following analysis. A total of 164 bands were scored and of which 156 were polymorphic (Table 5). The extent of polymorphism revealed by the me1-em3 primer is as shown in Figure 3.

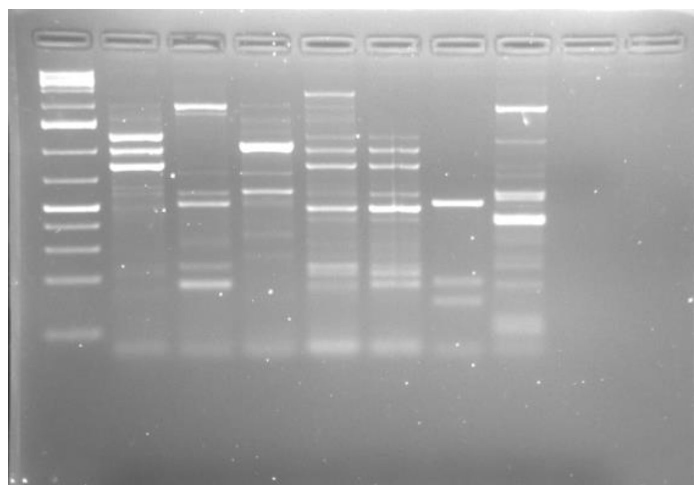

Figure 3 Representative SRAP amplification profile using me1-em3

Percentage polymorphism ranged from 72.7% to a maximum of 100% . The number of bands varied from 7 (me5-em7) to 22 (me1-em3), with an average of 9.75 polymorphic fragments per primer. Genetic similarities among the 8 isolates ranged from 0.056 to 0.762 and the co-phenetic corelation fort he SRAP dendogram was estimated at 0.96. Figure 4 shows the dendrogram based on the SRAP data. The genetic similarity derived from the data of SRAP marker analysis varied from 0.056 to 0.762. The UPGMA clustering algorithm from SRAP analysis grouped the isolates into three major clusters (Figure 4).

Table 5 Polymorphism obtained by SRAP analysis in *Hericium* isolates

	Primer/Primer combination	Total bands	Polymeric bands	Percentage of polymorphism
SRAP	me1-em2	11	11	100
	me1-em3	22	22	100
	me1-em4	8	7	87.5
	me1-em9	8	7	87.5
	me3-em2	10	10	100
	me3-em3	10	9	90
	me3-em4	7	7	100
	me3-em6	12	12	100
	me5-em3	10	10	100
	me5-em4	8	7	87.5
	me5-em7	7	6	85.7
	me5-em9	8	7	87.5
	me5-em10	12	12	100
	me6-em5	15	14	93.3
	me7-em6	8	8	100
	me10-em3	8	7	87.5
Total		**164**	**156**	**-**
Avarage		**10.25**	**9.75**	**95.1**

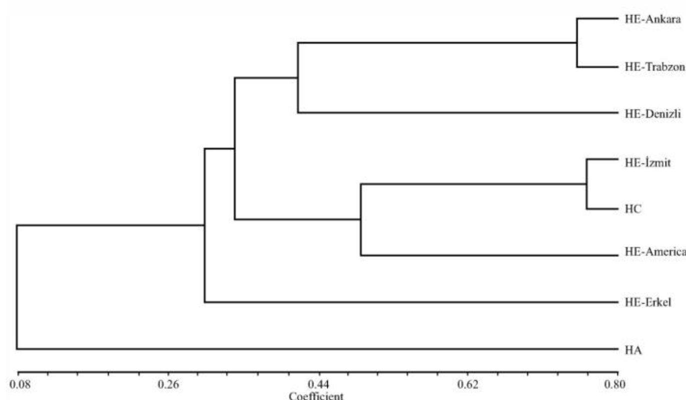

Figure 4UPGMA dendogram based on data from SRAP analysis of 8 *Hericium* isolates

Cluster I comprised 6 isolates that were subdivided into two sub-clusters. The first sub-group of Cluster I consisted of isolates collected from Turkey, HE-Ankara, HE-Trabzon and HE-Denizli. The second sub-group of Cluster I consisted of *H. erinaceus* isolates HE-İzmit, HE-America and *H. coralloides* isolate. With in this group, isolate HE-İzmit and HC with a 0.76 similarity coefficient appeared to be closly realed. Cluster II and Cluster III each comprised a single isolate, HE and *H. americanum*, respectively. Groupings identified by UPGMA analysis also revealed that *H. americanum* isolate was genetically very distinct from other isolates.

Combined ISSR and SRAP analysis

Analysis of combined ISSR and SRAP data shows genetic similarities among all the 8 isolates ranged from 0.110 to 0.679 (Figure 5).

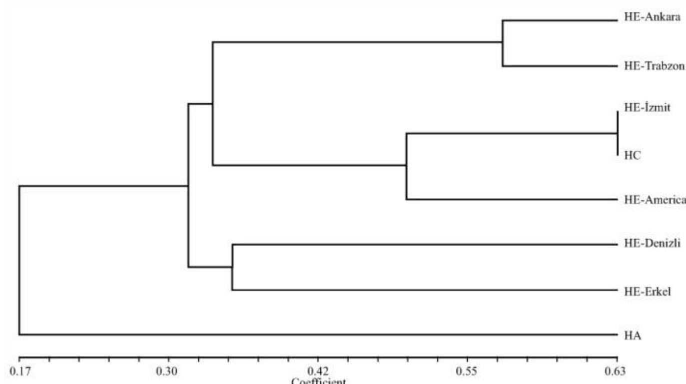

Figure 5 UPGMA dendogram based on data from combinated ISSR and SRAP analysis of 8 *Hericium* isolates

The cophenetic correlation for combinated SRAP-ISSR dendogram was estimated 0.94. The dendrogram of SRAP showed higher similarity to that of the combined data set than to those of ISSR. UPGMA grouped the 8 genotypes into three main clusters. Cluster I comprised 5 isolates that were delineated into two sub-clusters. Sub-cluster I comprised 2 genotypes, HE-Ankara and HE-Trabzon(with a 0.58 similarity coefficient), while sub-cluster II comprised 3 genotypes, HE-İzmit, HC and HE-America. It is appeared that HE-İzmit and HC has closest relation genetically between all of *Hericium* isolates. Cluster II consisted of two isolate HE and HE-Denizli. However, Cluster III consisted of single isolate *H. americanum*that appeared to be distinct from all the other species.

Morphologically, *H. americanum* alsodiffered from *other Hericium isolates* (Figure 6). However, morfological evidences indicated that *H. coralloides* and *H. erinaceus isolates* had similar morphologies and size (data not shown).

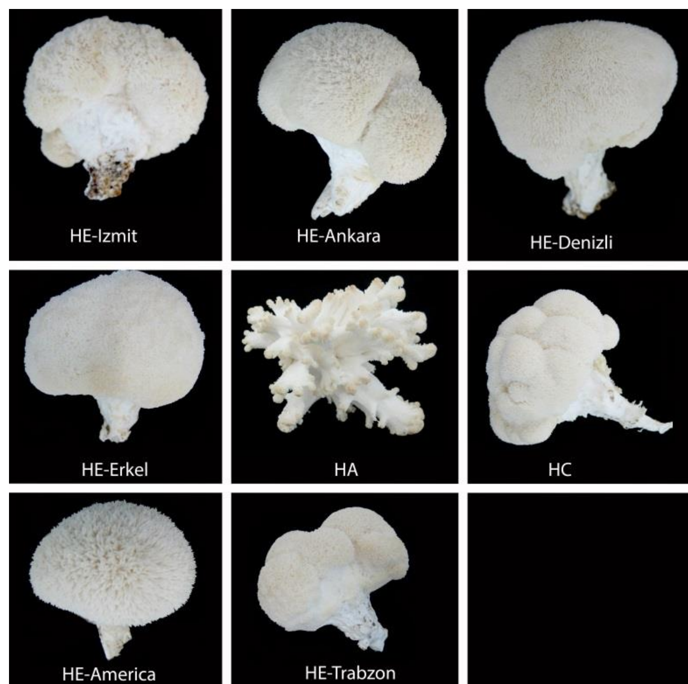

Figure 6 Fruiting bodies of *Hericium* spp

DISCUSSION

Information about the genetic diversity of mushrooms is critical for designing optimal breeding strategies and obtaining a continuous breeding progress. In this study, two markers ISSR, SRAP and combinationed ISSR and SRAP were simultaneously used to investigate genetic diversity of 8 isolates of *Hericium* representing: *Hericium erinaceus* (6 isolates), *H. americanum* (1 isolate), *H. coralloides* (1 isolate).

The high ratio of polymorphism obtained from ISSR (97.4%) and SRAP (95.1%) resulted from the fact that the *Hericium* isolates used in the study. Although **Wen *et al.*(2005)** and **Sun *et al*. (2006)** reported that the genetic similarity seems to have no relation to the geographical factor, the high ratio of polimorphism in the study may be relate different geographical factors and genetic backround of *Hericium* isolates.In the present study, compared with ISSR and SRAP data, it can be found that the avarage polymorphic band produced by ISSR primers (10.1) are more than those produced by SRAP primer pairs (9.75) , although the number of total band produced by SRAP primer pairs were more than that of ISSR. **Tang *et al* (2010)** and **Yu *et al.* (2008)** reported that SRAP technique better reflects the diversity of the genotypes than ISSR.

From the UPGMA clustering results, three groups were obtained using SRAP primers and combined data set based on clustering analysis. Similarly three groups were obtained using ISSR primers. Although similar genetic relationships among the tested *Hericium* isolates were revealed by ISSR and SRAP markers, there were some differences in the positioning of some isolates among the two dendrograms. By cluster analysis, *Hericium americanum* isolate was strongly separated from other *Hericium* isolates in each of dendograms. The low genetic similarity values may reflect different populations in far regions. But, there was genetic similarity between HE-İzmit and HE-America more than local *Hericium* isolates collected from different cities of Turkey. Morever, the high similarity between morphological characteristics of *H. coralloides* and *H. erinaceus* isolates also indicates that this isolate can be closely related to the other *H. erinaceus* isolates analyzed (**Figure 6**).

Nevertheless, there were some differences in branching of the dendrogram between the marker types. HE isolate formed a separate cluster, while HE-Denizli was in the same cluster with HE-Ankara and HE-Trabzon isolates in SRAP analysis. Contrastly, HE-Erkel was together with HE-Ankara and HE-Trabzon in Cluster I in ISSR analysis. ISSR and SRAP techniques target different parts of the genome. ISSR marker amplification targets are located in the region between simple sequence repeats (SSRs) whereas microsatellite DNA is not a general transcription region **(Song *et al.* 2010).** On the other hand, amplification target of SRAP markers is the Open Reading Frame (ORF), including the intron(s) and promoter region **(Li and Quiros, 2001).**It is reasonable that the principle and virtues of ISSR and SRAP are different, and they can be used to amplify a different region of genome. **Tang *et al*. (2010)** reported that integration of ISSR and SRAP markers derived from different amplification regions is more effective in detecting genomic variation and in realizing a more complete analysis of resource diversity.Therefore, we can say that combining ISSR and SRAP analysis were effective and reliable for accurately assessing the genetic relationship of *Hericium* isolates.

On the other hand, in this study, similar average Jaccard's similarity coefficients **(Jaccard, 1908)** were detected as 0.313, 0.107 and 0.318 by SRAP, ISSR and combining ISSR and SRAP analysis, respectively. Morever, the widest range of similarity was found in SRAP analysis (0.056 to 0.762) and combined data set (0.110 to 0.679) suggesting that higher genetic variations existed in the target genome regions than those targeted by ISSR (0.039 to 0.262) markers.

Dendrograms indicated a clear pattern of clustering and the 8 *Hericium* isolates shared great difference suggested that they were far to each other in genetic relation. **Nei and Li (1979)** suggested that genetic variation in population could be well reflected by the average similarity coefficients, there were great variations within group HA and other *Hericium* isolates.

The cophenetic correlation coefficient values show the correlation between similarity indices and dendrogram. In the study, the cophenetic correlation coefficient values were found to be 0.96, 0.85 and 0.94 for SRAP, ISSR and combinated ISSR and SRAP methods, respectively. The value is deemed reasonably very good good if the cophenetic correlation coefficient is larger than 0.9 **(Mohammadi and Prasna, 2003).** Accordingly, it is seemed that the dendrograms represent similarity indices a very high level in the present study.

This results showed that SRAP and ISSR markers were suitable for genetic diversity research on *Hericium* spp. SRAP technique was similarly reported to be highly effective in genetic diversity analysis of *Ganoderma* populations **(Sun *et al.*, 2006),** *Auricularia polyricha* **(Yu *et al.*, 2008; Du *et al.*, 2011)**,*Tricholoma matsutake* **(Ma *et al.*, 2010).** Morever, high polymorphism of ISSR was also reported in many previous studies, for examples, in *Agaricus bisporus* **(Malekzadeh *et al.*, 2011; Nazrul and Yin-Bing, 2010)** *Auricularia auricula* **(Tang *et al.*, 2010).** In our study, individually, the ISSR and SRAP marker systems showed a high correlation with the combined data sets. The similar structure of dendrogram also suggests that both techniques are suitable for genetic polymorphism research on fungi. Significant genetic variations within *Hericium* isolates tested in the present study were observed, and this would be useful for the selection of core collections for the purpose of crossbreeding or germplasm conservation.

CONCLUSION

To conclude, the results of the present study show that the SRAP and ISSR technique is an effective and reliable additional method for analyzing the genetic relationship and similarities among *Hericium* isolates. Morever, the study provided valuable information for potential applications of these two marker systems in molecular breeding of *Hericium* species. Morever, the study provided valuable information for potential applications of these two marker systems in molecular breeding of *Hericium* species.

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