Journal of Medicinal Plants Research Vol. 6(9), pp. 1590-1595, 9 March, 2012
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR11.1186
ISSN 1996-0875 ©2012 Academic Journals

Full Length Research Paper

Species-specific touch-down DAMD-PCR markers for *Salvia* species

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Accepted 7 October, 2011

Identification of genotypes and in some cases species in *Salvia* L. is complicated due to the morphologic similarities. This study utilized a touch-down directed amplification of minisatellite DNA polymerase chain reaction (Td-DAMD-PCR) technique to develop species-specific Td-DAMD-PCR markers for *Salvia* L. A total of 22 minisatellite core sequences as primers, and individuals and bulked samples from five species of *Salvia* L. along with one *Origanum* L. and one *Sideritis* L. species were used. A total of 70 species-specific markers differentiating each species utilized were determined. Td-DAMD-PCR markers were obtained at higher annealing temperature and were reproducible. These markers could be used as diagnostic markers for identification of *Salvia* L. species. Utilization of Td-DAMD-PCR markers will be useful in germplasm characterization, plant genetic resources conservation and utilization in *Salvia* L. Furthermore integration of these DNA markers and analytical methods in *Salvia* L. improvement programs will lead to the development of a comprehensive system which can be conveniently applied at the industry level.

Key words: Directed amplification of minisatellite (DNA) markers, minisatellites, species identification, touch-down polymerase chain reaction (PCR).

INTRODUCTION

Plant species in the genus *Salvia* L. are widely used as spices, drug and fragrance industries. The genus *Salvia* L. contains about 900 species distributed throughout many areas in the world and more than 40 species of the genus are endemic to the Mediterranean region (Hedge, 1992; Karaca et al., 2008). Species morphologies in the genus *Salvia* L. is remarkably variable due to the effect of environmental factors including temperature, precipitation and altitude along with the stage of development (Bertea et al., 2006). Furthermore, existence of inter-specific hybridization among the species makes species identification difficult in *Salvia* L. using the conventional morphological markers (Reales et al., 2004; Bertea et al., 2006).

Deoxyribonucleic acid (DNA) based methods have potentials to become widely employed techniques for a rapid and precise identification of species, genotypes and lines (Karaca et al., 2004). The use of DNA markers for genotype and species identification is important for plant breeders and taxonomists because DNA markers do not have the disadvantages of morphological and chemical characteristics. Furthermore, DNA based methods offer cost-effective, safe and efficient way to determine the genetic purity of genotypes and species, and map the economically important quantitative traits (Karousou et al., 1998). Among the DNA marker techniques, restriction fragment length polymorphism (RFLP) is one of the oldest DNA techniques used in plant identification and genetic studies (Williams and Clair, 1993). However, this technique posses several limitations in comparison to various PCR based DNA marker techniques. The RFLP

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Abbreviations: AFLP, Amplified fragment length polymorphism; **DAMD-PCR**, directed amplification of minisatellite DNA region polymerase chain reaction; **PCR,** polymerase chain reactions; **RAPD,** random amplified polymorphic DNA; **RFLP,** restriction fragment length polymorphism; **SSR,** simple sequence repeat; **TBE,** TRIS-borate EDTA; **Td-PCRs,** touch-down polymerase chain reactions; **BSA,** bulked segregant analysis; **TLC**, thin layer chromatography; **HPLC**, high performance liquid chromatography.

Table 1. Localities of 5 *Salvia* species.

Sample name	Location I	Location II	Location III
Salvia tomentosa Mill.	Goynuk	Kemer	Kanyon
Salvia sclarea L.	Alanya	Antalya	Mahmutlar
Salvia virgata Jacq.	Elmali	Bayindir	Antalya
Salvia dichroantha Stapf.	Gebiz	Bozburun	Bozdogan
Salvia fruticosa Mill.	Kemer	Cibilli	Kesme

technique requires higher quality and quantity of genomic DNA but produces less polymorphic markers in comparison to simple sequence repeat (SSR), amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) (Vos et al., 1995; Karaca et al., 2002; Ince et al., 2010a).

Simple sequence repeats also known as microsatellites is one of the most promising molecular marker techniques to identify genotypes and molecular mapping studies since microsatellites produce high level of polymorphisms, usually generate co-dominant markers and easy to handle (Bilgen et al., 2004). However, the application of the microsatellites in *Salvia* species is not common due to paucity of the primer pairs.

Two other most commonly used DNA marker techniques in plant species are AFLP and RAPD (Vos et al., 1995; Ince et al., 2010b). AFLP is much reproducible than RAPD since the AFLP uses restriction site-specific adapter pairs, adapter-specific primers, and stringent polymerase chain reaction (PCR) amplification conditions (Vos et al., 1995). In comparison to AFLP and SSR, PCR artifacts and reproducibility problems have been reported in RAPD (Ellsworth et al., 1993; Karaca and Ince, 2008).

Minisatellites are tandemly or almost tandemly repeated regions of eukaryotic genomes, many of which show high levels of allelic length variations due to the differences in the number of repeat units (Jeffreys et al., 1985). Heath et al. (1993) reported a technique called direct or directed amplification of minisatellite region DNA (DAMD) to direct the PCR based amplification of minisatellite regions.

It has been speculated that when a portion of a minisatellite array is involved in an inversion, a single primer makes PCR possible for the amplification of minisatellite region (Heath et al., 1993). However, in one of our studies we observed that not all the sequences of DAMD-PCR amplicons contained minisatellites but they were polymorphic and useable markers for genotype and species identification (Karaca and Ince, 2008). Furthermore, our previous studies indicated that DAMD-PCR could be improved to better resolution and reproducibility when a touch-down PCR procedure (Td-DAMD-PCR) used in the amplification of minisatellite regions (Ince and Karaca, 2011).

In the present study, we used individual and bulked samples of five *Salvia* L. species to identify species-specific Td-DAMD-PCR markers which could be utilized in species identification, germplasm characterization and plant genetic resources conservation.

MATERIALS AND METHODS

Plant materials

Leaf samples of the plant materials used in the present study were collected from 5 different *Salvia* species grown at 3 different locations in the district of Antalya in the Mediterranean Basin of Turkey (Table 1). A total of five individual plants from each location (a total of 15 individual samples for a species) and four bulked samples consisting of 10 individual plant leaves (a total of 40 samples for a species) were used.

The species studied consisted of *Salvia tomentosa* Mill., *Salvia sclarea* L., *Salvia virgata* Jacq., *Salvia fruticosa* Mill., and *Salvia dichroantha* Stapf. Leaf samples of *Origanum majorona* L. (collected from Alanya) and *Sideritis pisidica* Boiss. and Heldr. Apud Bentham (collected from Antalya) were also collected and used for DNA extraction studies.

DNA extraction

Plant genomic DNAs from bulked and individual leaf samples were extracted according the protocol described in Karaca et al. (2005). All reagents used in DNA extraction and subsequent analyses were molecular biology grade purchased from Amresco and Bioron. Concentration and purity of the extracted DNAs were determined using a spectrophotometer and quality of the extracted DNAs was determined using standard agarose gel electrophoresis technique.

Td-DAMD-PCR

Amplification of minisatellite regions were performed using touch-down polymerase chain reactions (Td-PCRs). Amplification reactions were carried out in 25 µl reaction volume containing 120 nano gram genomic DNAs from individual or bulked samples as templates, 2 µM each of the minisatellite primer (listed in Table 2), 80 mM TRIS-HCl (pH 8.8), 19 mM $(NH_4)_2SO_4$, 0.009% Tween-20 (w/v), 0.28 mM each dNTP, 3 mM $MgCl_2$, and 2 units *Taq* DNA polymerase. Td-DAMD-PCR was carried out in a 96-well Px2 Thermal Cycler (Thermo Hybaid) with the following cycling profile: 3 min hold at 94°C, followed by a ten cycle of pre-PCR consisting of 30 s at 94°C for denaturing, 45 s at 60°C for annealing, and 3 min at 72°C for primer extension reaction. Annealing temperature was reduced 0.5°C per cycle for the first 10 cycles. The PCR amplification was then continued for 30 more cycles at a constant 55°C in annealing temperature, and the rest of the pre-PCR cycling parameters were kept unchanged. At the end of the PCRs, samples

Table 2. List of minisatellite primers used in the study.

*Primes**	*5→3' Sequence*
URP1F	ATCCAAGGTCCGAGACAACC
URP2F	GTGTGCGATCAGTTGCTGGG
URP2R	CCCAGCAACTGATCGCACAC
URP4R	AGGACTCGATAACAGGCTCC
URP6R	GGCAAGCTGGTGGGAGGTAC
URP9F	ATGTGTGCGATCAGTTGCTG
URP13R	TACATCGCAAGTGACACAGG
URP17R	AATGTGGGCAAGCTGGTGGT
URP25F	GATGTGTTCTTGGAGCCTGT
URP30F	GGACAAGAAGAGGATGTGGA
URP32F	TACACGTCTCGATCTACAGG
URP38F	AAGAGGCATTCTACCACCAC
FVIIEX8	ATGCACACACACAGG
FVIIEX8C	CCTGTGTGTGTGCAT
33.6	GGAGGTGGGCA
14C2	GGCAGGATTGAAGC
HBV3	GGTGAAGCACAGGTG
HBV5	GGTGTAGAGAGGGGT
M13	GAGGGTGGCGGCTCT
6.2H1	CCCTCCTCCTCCTTC
6.2H2	AGGAGGAGGGGAAGG
YNZ22	CTCTGGGTGTGGTGC

*Detailed information about the sources of primers was published in Ince et al. (2009).

were kept for 10 min at 72°C for final extension reaction.

Agarose gel electrophoresis

After Td-PCRs completed, 5 µl DNA-loading buffer consisting of 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, and 40% (w/v) sucrose in sterile water were added to each amplified reaction, and 10–12 µl of these mixtures were loaded on 2-3% (w/v) high resolution agarose gels (Serva) containing 0.6 µg/ml ethidium bromide and electrophoresed at 5 V/cm at constant voltage for 8 to 12 h in the presence of 1X TRIS Borate EDTA buffer consisting of 89 mM TRIS–Borate, 2 mM EDTA (pH 8.3). After electrophoresis, PCR products were visualized and photographed on a UV transilluminator for further analysis.

Data analysis

Determination of species-specific Td-DAMD-PCR markers in this study was based on the following very basic criteria: a species-specific marker was defined as a distinct and reproducible DNA fragments presented in all the individuals and in all the bulked samples of one species but it was absent in the other species as well as in *Origanum* and *Sideritis* samples (Karaca et al., 2008).

RESULTS

Amplification of DAMD-PCR products from plant samples listed in Table 1 were performed under relatively high stringency condition using a touch-down PCR (Td-DAMD-PCR). The annealing temperature in the first 10 cycle of Td-PCRs decreased from 60 to 55°C to eliminate PCR artifacts. After the touch-down steps, template DNAs were amplified at 55°C. All the 22 primers shown in Table 2 were initially used to investigate their amplification patterns using two individual samples of five species along with the two species (*Origanum* and *Sideritis*). Analyses indicated that all the primers tested amplified *Salvia*, *Origanum* and *Sideritis* genomic DNAs. With the exception of primers 33.6, URP32F and FVIIEX8C, rest of the primers produced repeatable major and minor amplicons within and between different PCR runs.

Total number of Td-DAMD-PCR amplicons varied from 4 to 25 depending on the primers and types of templates (individuals vs. bulked). In general the number of Td-DAMD-PCR amplicons in bulked samples was higher than that of individual samples. This was as expected since bulked samples had more number of primer annealing sites than the individual samples. Higher number of amplicons obtained from bulked sample was not only an indication of genetic diversity within a species but also was an indication of quality and quantity of bulked DNA samples.

Td-DAMD-PCR amplification patterns of 15 individual from a species and 4 bulked samples of the same species were compared. Results indicated that there was a high level of polymorphisms among the individual samples collected from different locations. Reproducible and monomorphic Td-DAMD-PCR amplicons for individual samples of each species were identified. Monomorphic amplicons of the individual samples were investigated to see whether they were polymorphic between the five species of four bulked samples. Comparison of amplicons of individual and bulked samples is critical since amplicons of bulked samples may have been derived from only one of the individuals in a bulk. In that case, the marker is not at all diagnostic for the species or population, but may merely be a rare (private) allele. Results indicated that monomorphic amplicons of a species were polymorphic between the species indicating that the occurrence of above mentioned criticisms were not the case in the present study and bulked samples can be efficiently used in identification of species-specific DNA markers.

Based on the criteria used in the present study a total of 70 species-specific markers ranging in size from 186 base pairs to 2,000 base pairs were obtained among the primers listed in Table 2. Figure 1 shows some of the species-specific Td-DAMD-PCR markers identified in this study. The highest number of species-specific markers, 20, was obtained for *S. virgata* while nine were determined for *S. tomentosa* (Table 3). Higher number of species-specific markers of *S. virgata* indicated that *S. virgata* distinctly related to other *Salvia* species studied. On the other hand, relatively lower numbers of species-specific markers were obtained for *S. fruticosa* (13) and

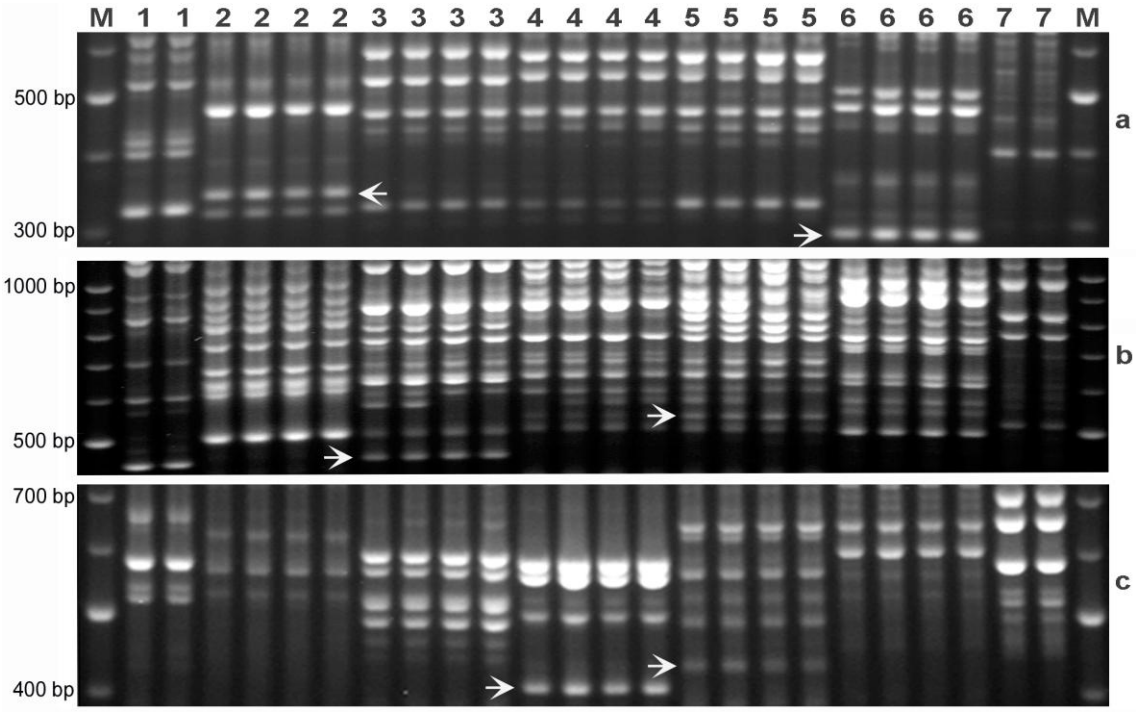

Figure 1. Species specific Td-DAMD-PCR markers. Panels a through c are the amplified products of primers 6.2H1, 33.6, and HBV5, respectively. Lane numbered; M: DNA size markers, (1) *Origanum majorona* L., (2) *Salvia sclarea* L., (3) *Salvia fruticosa* Mill., (4) *Salvia tomentosa* Mill., (5) *Salvia dichroantha* Stapf., (6) *Salvia virgata* Jacq., and (7) *Sideritis pisidica* Boiss. and Heldr. Apud Bentham. Arrows show species-specific markers being detectable at this region of the gel. Please note that not all regions of the gel are shown.

S. dichroantha (11). These two species were the most related species studied in the present study (Karaca et al., 2008).

DISCUSSION

Isolation of good-quality DNA suitable for subsequent analyses is usually difficult from medicinal and aromatic plant species which usually contain higher amount of alkaloids, flavanoids, phenols, polysaccharides, terpenes and quinines (Jobes et al., 1995; Suman et al., 1999; Karaca et al., 2005). Contaminations of polysaccharides, proteins and secondary metabolites usually interfere with Subsequent DNA analyses dealing with enzymatic reactions. The amount and quality of the DNAs extracted especially from bulked samples are critical in PCR analyses. In the present study extracted DNAs were analyzed using spectrophotometric method and restriction enzyme digestion studies. Analyses clearly indicated that all the DNA samples were free from proteins and polysaccharides and completely digestible with three endonucleases utilized.

Conventional bulked segregant analysis (BSA) developed by Michelmore et al. (1991) and modified versions of BSA (Yang et al., 1997) have been used for identification of simple and quantitative traits. In BSA usually two bulks of 10 to 40 individuals are mixed from individuals representing extreme phenotypes. The DNA obtained from these bulks and parental genotypes are screened with the molecular markers for the detection of polymorphism. The DNA markers showing the polymorphism between the bulks are then selected and screened on the DNA of individual lines of the bulks, and subsequently on the complete set of lines of the mapping population (Zhang et al., 2009). In the present study the use of bulked samples in one hand increased the number of plants analyzed and in the other hand reduced the cost of analyses while saving the time to complete the study.

The use of species-specific DNA markers will definitely provide several advantages over the other techniques used for characterization and standardization of medicinal and aromatic plant species. The use of chromatographic and spectroscopic techniques such as TLC, HPLC, gas chromatography, column chromatography along with volumetric and gravimetric methods have been routinely used as valuable tools for quality control and standardization of medicinal and aromatic plant samples (Joshi et al., 2004).

Macroscopic techniques use shape, size, color, texture, surface and fracture characteristics while microscopic techniques involve comparative inspections of broken and powdered materials for identification studies. Most of the chromatographic techniques and marker compounds

Table 3. Species-specific Td-DAMD-PCR markers.

***Salvia* species**					
Primers	***S. sclarea***	***S. fruticosa***	***S. tomentosa***	***S. dichroantha***	***S. virgata***
URP1F	315; 186	-	-	-	210
URP2F	-	280	-	-	370
URP2R	2000	200	-	226	-
URP4R	-	270	380	368	600
6.2H1	340	-	2000	320	290
URP6R	410	380	-	450	950; 368
URP9F	226	-	400	280	250
URP13R	1700	-	360	-	2000; 350
14C2	450	-	700	790	-
URP30F	-	980	-	300	320
URP17R	-	-	-	-	950; 368
URP25F	274	-	-	2700; 250	580; 290
URP32F	1600	-	-	370	350
33.6	-	480	-	-	-
URP38F	335	480; 325	-	-	-
6.2H2	325	-	-	-	-
FVIIEX8	680	-	-	480	450
FVIIEX8C	470	500	520	-	550
HBV3	-	590	560	-	1500
HBV5	310	500	300	-	1400
M13	1600; 390	270	-	-	-
TNZ22	-	300	480	-	400
Total	17	13	9	11	20

have limitations, since many factors may affect the ultimate chemical profiles of any medicinal and aromatic herbs. Chemical profiles and the observations based on macroscopic and microscopic methods can be easily affected by genetic and extrinsic factors such as harvesting, drying and storage conditions. On the other hand, genetic factors determined using DNA markers are not affected by physiological conditions as well as environmental factors since the genetic composition is unique for each species (Bhutani, 2000; Chan, 2003; Joshi et al., 2004).

Species-specific DNA markers are not only useful in quality-related issues but also could be useful in the identification and selection of the desired plants which may be known by different binomial names in different regions and countries. Furthermore, certain rare and expensive medicinal and aromatic plant species are often substituted with morphologically similar, easily available or less expensive species. Species-specific DNA markers could be used in identification of morphologically and/or phytochemically indistinguishable species. DNA based estimation of genetic diversity are also important in designing crop improvement programs for management of germplasm and evolving conservation strategies (Teletchea et al., 2005; Ince et al., 2009).

In conclusion, this study reported a total of 70 Td-DAMD-PCR markers which were very useful in differentiation of 5 *Salvia* species collected from there different locations. Although, species-specific Td-DAMD-PCR markers determined in this study were based on a total of 60 samples consisting of 15 individuals and 4 bulked samples, further research using more species of *Salvia* is required to test whether these Td-DAMD-PCR markers could still differentiate these *Salvia* species collected from other locations and could be used as diagnostic marker for species identification in *Salvia* L. Since Td-DAMD-PCR markers were produced at higher annealing temperature, they are highly reproducible than most widely used RAPD markers and, therefore, Td-DAMD-PCR markers will be useful in germplasm characterization, plant genetic resources conservation and utilization.

ACKNOWLEDGEMENTS

This work was supported in part by and The Scientific and Technological Research Council of Turkey, and the Scientific Research Projects Coordination Unit of Akdeniz University.

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