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Td-DAMD-PCR assays for fingerprinting of commercial carnations

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Abstract: *Dianthus* L. is one of the highly valued plant species in the family Caryophyllaceae. More than 30,000 cultivars of commercial carnations have been recorded and there is a need for an effective and cheap method to reveal their genetic diversity and identify the cultivars. To the best of our knowledge this is the first report of implementation of the touchdown direct amplification of minisatellite-region DNA polymerase chain reaction (Td-DAMD-PCR) technique in the genus *Dianthus*. A total of 12 core minisatellite primers empirically selected from 22 primers were used in fingerprinting and phylogenetic studies of some commercial carnation cultivars. Analysis revealed that commercial carnations have a wide genetic base and they were probably obtained using inter-crosses between and among different species of the genus *Dianthus*. A total of 17 DNA markers were variety specific and most of the remaining markers obtained in the present study were useful in fingerprinting of the commercial varieties studied. Commercial varieties were differentiated in spray and standard carnation groups in a principal coordinate analysis and a Bayesian 50% majority-rule consensus tree. Td-DAMD-PCR markers reported in this study could be very useful in species identification, determination of genetic relationships, and phylogenetic studies of species of *Dianthus*.

Key words: Bayesian analysis, commercial varieties, *Dianthus caryophyllus*, DNA markers, genetic relationship, spray and standard type carnations

1. Introduction

Dianthus L. is one of the highly valued plant genrea in the family Caryophyllaceae. The genus contains both annual and perennial plant species. It is considered native to the Mediterranean region and is grown in several parts of the world. Some species of this genus are valued for their ornamental, volatile, aromatic, and medicinal properties. There are approximately 300 species in this genus, but *D. caryophyllus*, *D. barbatus*, and *D. chinensis* are the most widely cultivated ones. Carnation (*Dianthus caryophyllus* L.), an extremely popular genus in floriculture, is of great commercial value in the world flower industry as a cut flower in ornamental plants due to its excellent keeping quality and its wide range of colors and forms (Kimura et al., 2009; Farsi et al., 2013; Maitra and Roychowdhury, 2013; Yagi, 2013; Jarda et al., 2014).

Species of *Dianthus* typically outcross and the absence of self-pollination causes highly heterozygote varieties. Crosses within and between different species, mutation breeding, the development of doubled haploid techniques, and the applications of recombinant DNA technology in carnations resulted in the development of more than 30,000 commercial varieties worldwide (International *Dianthus* Register). However, the pedigrees of many varieties are not known. Carnation varieties are mainly identified using morphological characteristics. It has been pointed out, however, that phenotypic traits are often influenced by environmental conditions and human judgments. For instance, some standard- and spray-type varieties do not have easily distinguishable flower characteristics due to phenotypic overlaps caused by environmental conditions (Fu et al., 2008; Kimura et al. 2009; Farsi et al., 2013; Wojcik et al. 2013).

In the last 30 years DNA based molecular marker systems have been found very useful in the studies of plants in terms of their systematic and phylogenetic structure and their breeding, conservation, maintenance, and production (Ince et al. 2009, 2014; Izzatullayeva et al., 2014). Application of DNA based molecular markers in plant breeding has been the choice of method worldwide. DNA based molecular marker systems are simple, quick, and relatively cheap in comparison to many other methods (Karaca et al., 2004; Balao et al., 2010; Yagi et al., 2012; Wojcik et al., 2013; İnce and Karaca, 2014).

Random amplified polymorphic DNA (RAPD) (Wojcik et al., 2013), amplified fragment length polymorphism (AFLP) (Balao et al., 2010), sequence characterized amplified region (SCAR), inter-simple

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sequence repeat (ISSR) (Wojcik et al., 2013), and simple sequence repeat (SSR) (Yagi et al., 2012) systems have been used in *Dianthus* for different aims such as identification, characterization, diversification, and phylogenetic studies. However, the touchdown direct amplification of minisatellite-region DNA polymerase chain reaction (Td-DAMD-PCR) technique has not yet been applied in the genus *Dianthus*. Some could argue that there is no need for it in carnations because the other systems have been used successfully. However, previous comparison studies among DNA marker systems have demonstrated that the best results are produced when data from different marker systems are combined (Karaca et al., 2002; Wojcik et al., 2013). Therefore, new DNA systems are always useful in research.

Minisatellites are tandemly or almost tandemly repeated regions of eukaryotic genomes (Jeffreys et al., 1985). They show high levels of allelic length variations due to the differences in the number of repeat units. Heath et al. (1993) reported the DAMD technique for direct amplification of minisatellite regions of DNA using a single core primer. It has been speculated that when a portion of a minisatellite is involved in an inversion, a single primer makes PCR possible for the amplification of the minisatellite region (Heath et al., 1993; Karaca and Ince, 2008; Ince and Karaca, 2011a). Conventional DAMD-PCR markers, compared with RAPD markers, are more reproducible and dependable due to their powerful amplification and relatively high PCR firmness (Karaca and Ince, 2008). Further studies revealed that using touchdown polymerase reaction conditions can enhance the reproducibility of DAMD-PCR and its utilization rate (Karaca and Ince, 2008; Ince et al., 2009).

This study was undertaken to identify and assess suitable minisatellite primers to be used in the Td-DAMD-PCR method, to characterize 16 commercial carnation varieties in terms of phylogenetic and spatial relationships, and to investigate whether the Td-DAMD-PCR method can be useful to fingerprint spray and standard type carnation varieties.

2. Materials and methods

2.1. Plant materials

"Aktiffide Üretim Pazarlama ve Tic. Ltd. Şti. Antalya/Türkiye", a seedling company in Antalya, gifted 16 commercial carnation varieties for our study: Zivago (spray type), Hornet (spray type), Consuela (standard type), Lior (spray type), Messalina (spray type), Brunello (spray type), Betsy (standard type), Rocio (spray type), Carim (spray type), Vital (spray type), Delicia (standard type), Omaggio (standard type), Roble (standard type), Turbo (standard type), Karaoke (spray type), and Berry (spray type).

2.2. DNA extraction

Prescreening studies using several RAPD markers indicated that all the seedlings belonging to a particular commercial carnation variety were pure. Thus, bulked samples consisting of 10 individual plant leaves from each variety were used in the DNA extraction studies. Plant genomic DNA samples from bulked leaf samples were extracted according to a protocol described by Karaca et al. (2005). Some of the DNA samples of plants were further purified according to protocols described by Ince and Karaca (2009) and by Ince et al. (2011) because there was some pigment and protein contamination of the extracted DNA.

Tissues were completely frozen in liquid nitrogen and ground to a powder with a mortar and pestle. Tissues (1 g) were homogenized in a solution consisting of 2.48 mL of extraction solution [0.4 mL of 2 M Tris-HCl, pH 8.0, 0.4 mL of 0.5 M ethylene diamine tetra acetic acid (EDTA), pH 8.0, 1.6 mL of 5 M NaCl, 0.08 mL of Triton-X 100] and 1.52 mL of lysis buffer [5.6% cetyltrimethylammonium bromide, 0.1 mL of β-mercapto ethanol] and incubated at 65 °C for 2 h before being divided in 2 phases by centrifugation. There were 2 chloroform steps (chloroform:isoamyl alcohol 25:24:1 v/v) in the subsequent centrifugations (Ince and Karaca, 2009). Following the last chloroform centrifugation, the supernatant was precipitated with NaCl and isopropanol. Following RNase and proteinase K treatments, DNA was again precipitated with potassium acetate (pH 5.5) and ethanol.

All reagents used in DNA extraction and subsequent analyses, including polymerase chain reactions, were molecular biology grade purchased from Amresco (Solon, OH, USA) and Bioron. Concentration, amount, quality, and purity of the extracted DNA samples were determined using a spectrophotometer and agarose gel electrophoresis technique (Ince and Karaca, 2011b).

2.3. Td-DAMD-PCR

Amplification of minisatellite regions was performed using touchdown polymerase chain reactions (Td-PCRs) based DAMD profile. Amplification reactions were carried out in a 25 μL reaction volume containing 0.06–0.1 μg of genomic DNA as a template, 2 μM of each minisatellite primer listed in Table 1, 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 *Taq* units of DNA polymerase as described in Ince and Karaca (2012).

Td-DAMD-PCR was carried out in a Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA) with the following cycling profile: 3 min hold at 94 °C, followed by 10 cycles of pre-PCR, consisting of 30 s at 94 °C for denaturing, 30 s at 55 °C for annealing, and 2 min at 72 °C for primer extension reaction. Annealing temperature was reduced by 0.5 °C per cycle during the first

10 cycles. The PCR amplification was then continued for 30 more cycles at a constant 50 °C annealing temperature, and the rest of the pre-PCR cycling parameters were kept unchanged. At the end of the PCRs, the samples were kept for 10 min at 72 °C for the final extension reaction (Ince and Karaca, 2012).

2.4. Agarose gel electrophoresis

Following the completion of Td-PCRs, 5 µL of 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 was added to each amplified reaction, and 10 µL–12 µL of these mixtures were loaded in 2% (w/v) high resolution agarose gels (Serva) containing 0.6 µg/mL of ethidium bromide and electrophoresed at 5 V/cm of constant voltage for 8–12 h in the presence of 1X Tris Borate EDTA buffer consisting of 89 mM Tris, 89 mM borate, and 2 mM EDTA (pH 8.3). Following electrophoresis, amplicons were visualized and photographed on a UV transilluminator for further analysis (Ince and Karaca, 2012).

2.5. Data analysis

Each DAMD-PCR marker was scored as present (1) or absent (0). Markers showing the same mobility were assumed to be the same and no attempts were made to code for band intensity in this study. Obtained scores were used in the calculation of Nei and Li's genetic similarity indices and the determination of polymorphic information content (PIC) values of each DAMD-PCR primer pair. The Nei and Li's genetic similarity index values were calculated using the following formula: GSI_{XY}: 2a/(a + b) + (a + c), where X and Y are the numbers of markers in commercial carnations X and Y, respectively, a is the number of markers shared between individuals X and Y, b is the number of markers present in individual X but absent in individual Y, and c is the number of markers absent in individual X but present in individual Y, with Multi Variety Statistical Package software (MVSP 3.13O, Kovach Computing Services, Pentraeth, UK). The PIC value of each primer was calculated according to Rana and Bhat (2004) using the following formula: PIC = 2PiQi, where Pi is the frequency of presence and Qi is the frequency of absence of a particular marker.

Principal coordinate analysis (PCoA) was used to visualize the spatial relationships among commercial varieties using MVSP software. In the PCO axes each carnation variety was represented as a point in space.

Td-DAMD-PCR data were also analyzed in the Bayesian method using the MrBayes v3.2.1 x64 software program to construct a consensus tree. One cold and 3 heated chains were run, starting from a random tree for 10 million Markov chain Monte Carlo (MCMC) generations, with chains sampled every 100th cycle (Ronquist and Huelsenbeck, 2003). The nucleotide model used was 4 by 4 and the number of substitution type was selected as "1" (F81 model). Binary data were executed using MCMC methods that included random walk Monte Carlo methods algorithms and a 50% majority-rule consensus phylogenetic tree was constructed with the posterior probabilities indicated at the nodes (Ronquist and Huelsenbeck, 2003; Karaca et al., 2013; Ince et al., 2014).

3. Results

3.1. Assessment of Td-DAMD-PCR primers

A total of 22 DAMD-PCR primers reported in Ince et al. (2009) were used, and from these 12 were selected as listed in Table 1. Although all 22 DAMD-PCR primers produced amplified products within the same PCR set, there were differences in intensity of some markers between DNA templates. In other words, 10 DAMD-PCR primers from 22 were found to be template dependent, producing very sharp or less intense bands in some other carnation varieties. On the other hand, 12 DAMD-PCR primers (Table 1) were selected since they produced similar marker densities across the 16 carnations used in the present study. Selected primer pairs were originally developed from different organisms ranging from human to phage. For instance, primers URP1F, URP2F, URP2R, URP9F, URP13R, URP17R, and URP38F were developed from rice (*Oryza sativa* L.); 14C2, 6.2H1, 33.6, and YNZ22 were developed from human (*Homo sapiens*); and primer M13 was developed from the phage M13. The polymorphic information content value of each core minisatellite primer varied from 0.120 to 0.341, with an average of 0.249/primer, indicating that these primers have moderate levels of polymorphism.

Amplification of carnation genomic DNA samples was conducted under relatively high annealing temperature and produced distinct markers amongst the 16 commercial carnations. Representative banding patterns of selected Td-DAMD-PCR primers are shown in Figure 1. A total of 858 bands were observed across all commercial varieties studied; 570 (66.43%) of these were polymorphic (Table 1). The percentage of polymorphic markers produced by each primer ranged from 37.3% to 100%. The number of alleles by each Td-DAMD-PCR primer ranged from 4 to 20, with an average of 8.33. The size of the amplified fragments across the 16 carnations varied from 0.15 kb to 3 kb.

In the present study we determined a total of 17 variety specific Td-DAMD-PCR markers that can easily identify 9 varieties within the 16 carnation cultivars. Primers producing variety specific markers were as follow: Zivago (14C2), Hornet (6.2H1), Consuela (YNZ22, URP13R, and 14C2), Lior (URP9F), Brunello (URP17R, URP17R, and 6.2H1), Delicia (YNZ22), Roble (URP17R, Turbo (URP2F), and Berry (M13, YNZ22, URP2F, 33.6, and

Table 1. Primers used in the Td-DAMD-PCR technique. Total number of bands (TNB), number of polymorphic bands (NPB), polymorphism (P), amplification size range (ASR), variety-specific markers (VSM), alleles per primer (APP), and polymorphism information content (PIC).

Primer	Sequence (5′⇒3′)	TNB	NPB	P	ASR	VSM	APP	PIC
URP1F	ATCCAAGGTCCGAGACAACC	36	36	100	0.15–1	0	4	0.332
URP2F	GTGTGCGATCAGTTGCTGGG	96	80	83.3	0.15–1	2	16	0.318
URP2R	CCCAGCAACTGATCGCACAC	51	19	37.3	0.3–0.9	0	4	0.232
URP9F	ATGTGTGCGATCAGTTGCTG	60	28	46.7	0.15–0.9	1	7	0.212
URP13R	TACATCGCAAGTGACACAGG	66	50	75.6	0.22–1	1	6	0.219
URP17R	AATGTGGGCAAGCTGGTGGT	112	80	71.4	0.22–1.2	3	12	0.215
URP38F	AAGAGGCATTCTACCACCAC	35	19	54.3	0.32–0.7	0	4	0.318
14C2	GGCAGGATTGAAGC	40	24	60	0.25–3	2	5	0.191
6.2H1	CCCTCCTCCTCCTTC	69	37	53.6	0.48–1	3	8	0.120
33.6	GGAGGTGGGCA	54	22	40.7	0.35–0.9	1	5	0.197
YNZ22	CTCTGGGTGTGGTGC	168	120	71.4	0.4–3	3	20	0.293
M13	GAGGGTGGCGGCTCT	71	55	77.5	0.32–1	1	9	0.341

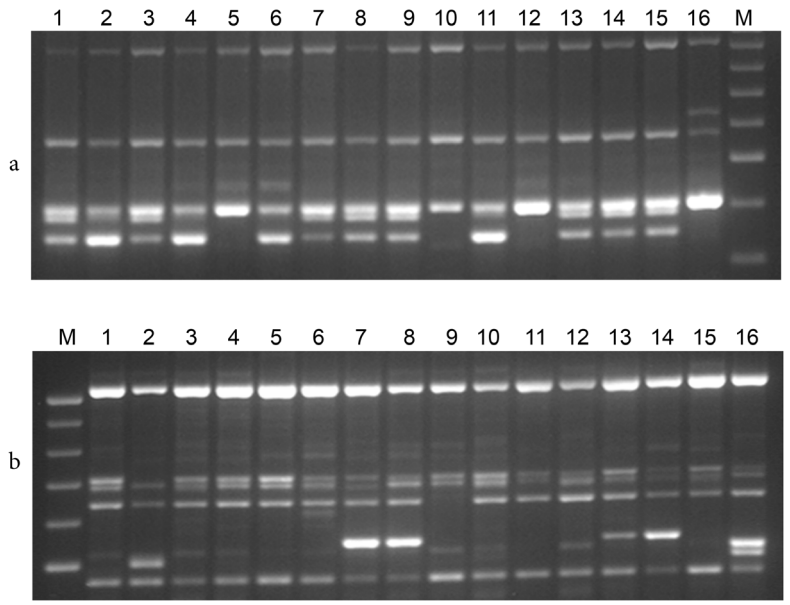

Figure 1. Representative Td-DAMD-PCR markers. M: DNA size markers ranging from 100 bp to 1000 bp. Panels a and b show Td-DAMD-PCR markers obtained using 33.6 and 6.2H1 primer, respectively. Td-DAMD-PCR markers are separated in 2% high resolution agarose gels.

6.2H1). There were no variety specific Td-DAMD-PCR markers for the varieties Messalina, Betsy, Rocio, Carim, Vital, Omaggio, or Karaoke. Although URP38F, URP1F, and URP2R primers produced polymorphic markers among the commercial carnation varieties, none of these products was variety specific. The remaining primers listed in Table 1 produced at least one variety specific marker.

3.2. Genetic similarity

The presence or absence of a band was considered a marker and the relationship between 2 varieties was calculated

using the Nei and Li's formula. The mean genetic similarity index (MGS) was 0.743 (±0.055) among the 16 commercial carnation varieties used in this study. The MGS of the standard type carnation varieties was 0.780 (±0.048) and the MGS of the spray type commercial carnation varieties was 0.761 (±0.056). These findings indicated that both types of commercial varieties used in this study have high levels of genetic diversity. Lower MGS values indicated that the studied commercial varieties were probably obtained via hybridization between or among different species of *Dianthus*. Moreover, the standard deviation values (shown in parentheses after the MGS values shown above) indicated the degree of genetic differences within standard or spray type commercial carnation varieties.

As shown in Table 2, the highest genetic similarity (GS) value of 0.873 was observed between Betsy and Consuela. Both are known as standard type commercial cultivars according to the commercial company that provided the carnations. On the other hand, the lowest genetic similarity value of 0.593 was observed between Berry and Betsy. Berry is a spray type while Betsy is a standard type.

Further studies were undertaken to identify genetic relationships within and between standard and spray type commercial varieties. Within the spray type carnations, Carim and Zivago were the most closely related, while Berry and Hornet were the least related varieties. Within the standard type carnations, Betsy and Consuela were the most closely related, while Roble and Consuela were the least related carnations (Table 2).

3.3. Spatial relationships

PCoA, which is an ordination method similar to principal component analysis, uses the distance matrix (rather than the values) to plot the axes (Manly, 1994). PCoA was used to determine and reveal spatial relationships among the commercial varieties as well as within and between the spray and the standard types. In the PCoA analysis axes 1 and 2 represented 17.98% and 12.9% of the variation among the commercial carnations, respectively. From the 10 spray type carnations used, 9 were represented with well separated points in space on the left axis, while all 6 standard type carnations were represented with well separated points in the right axis (Figure 2). The variety Berry was the most distantly related commercial carnation used in this study.

Within the standard type commercial varieties, Betsy and Consuela showed a close spatial relationship, while Omaggio, Turbo, Delicia, and Roble showed distant spatial relationships. Within the spray type carnations, Brunello, Karaoke, Messalina, Carim, Hornet, and Zivago showed close spatial relationships, while Lior, Vital, and Rocio varieties showed distant spatial relationships (Figure 2).

Table 2. Similarity matrix for commercial varieties of carnation determined on the basis of Td-DAMD-PCR markers and Nei & Li's coefficients.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	Zivago	1.000															
2	Hornet	0.766	1.000														
3	Consuela	0.708	0.722	1.000													
4	Lior	0.796	0.741	0.789	1.000												
5	Messalina	0.710	0.765	0.704	0.778	1.000											
6	Brunello	0.807	0.771	0.748	0.835	0.771	1.000										
7	Betsy	0.734	0.769	0.873	0.764	0.731	0.739	1.000									
8	Rocio	0.796	0.777	0.789	0.844	0.699	0.782	0.819	1.000								
9	Carim	0.818	0.857	0.739	0.811	0.724	0.804	0.748	0.849	1.000							
10	Vital	0.722	0.699	0.771	0.844	0.757	0.745	0.781	0.808	0.736	1.000						
11	Delicia	0.673	0.747	0.724	0.686	0.707	0.660	0.752	0.740	0.765	0.700	1.000					
12	Omaggio	0.752	0.673	0.745	0.764	0.692	0.703	0.755	0.743	0.710	0.800	0.772	1.000				
13	Roble	0.734	0.712	0.691	0.636	0.673	0.649	0.774	0.686	0.710	0.629	0.752	0.792	1.000			
14	Turbo	0.714	0.692	0.779	0.708	0.692	0.684	0.826	0.796	0.745	0.704	0.827	0.789	0.844	1.000		
15	Karaoke	0.815	0.777	0.789	0.789	0.660	0.836	0.743	0.788	0.811	0.731	0.700	0.743	0.724	0.722	1.000	
16	Berry	0.703	0.660	0.661	0.714	0.679	0.690	0.593	0.673	0.697	0.692	0.718	0.704	0.648	0.739	0.710	1.000

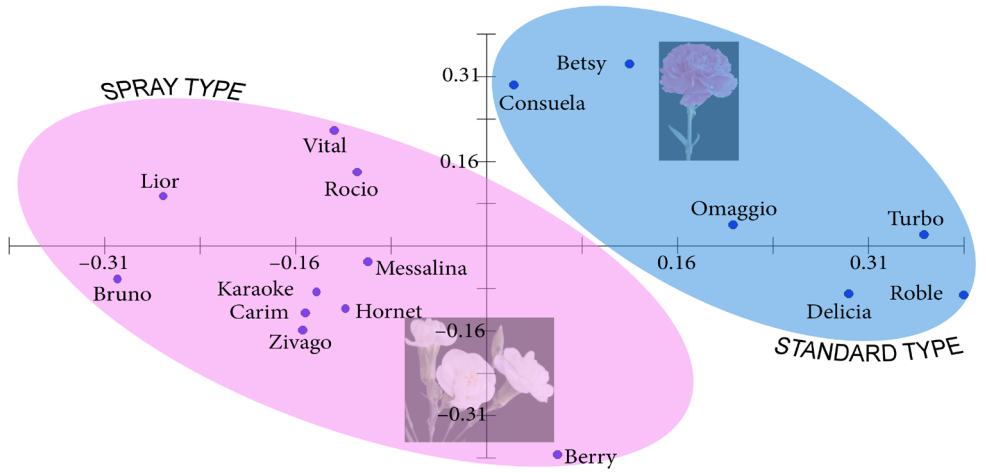

Figure 2. Principal coordinate analysis (PCoA) of the 16 commercial carnations. The 1st and 2nd axis explained 17.98% and 12.9% of the variance, respectively.

3.4. Molecular phylogeny

The 2 most commonly used distance-based methods in cluster analysis are the unweighted group method with arithmetic mean (UPGMA) and the neighbor joining (NJ) method. These methods produced similar trees in our studies (data not shown). However, additional analyses are required in UPGMA and NJ methods to obtain bootstrap values. Since the Bayesian method shows posterior probabilities at the nodes, indicating the reliability of the branches in a phylogenetic tree, it was performed on the 16 commercial carnation varieties in the present study. A phylogenetic consensus tree using the Td-DAMD-PCR data set based on the Bayesian analysis was constructed and the Bayesian posterior probability values, which could be treated as bootstrap values, were shown on the branches of the consensus tree (Figure 3).

As shown in Figure 3, all 16 commercial carnation varieties studied were separated into 2 major groups of spray and standard types. This Bayesian based consensus tree also confirmed that standard type carnation cultivars could be phylogenetically separated from the spray type carnation varieties using the Td-DAMD-PCR markers reported in this study. The number of characters used in the construction of the consensus tree were 1584 (present + absent) on the 16 commercial varieties amplified with the 12 primers. This number is greater than any morphological data used to differentiate varieties.

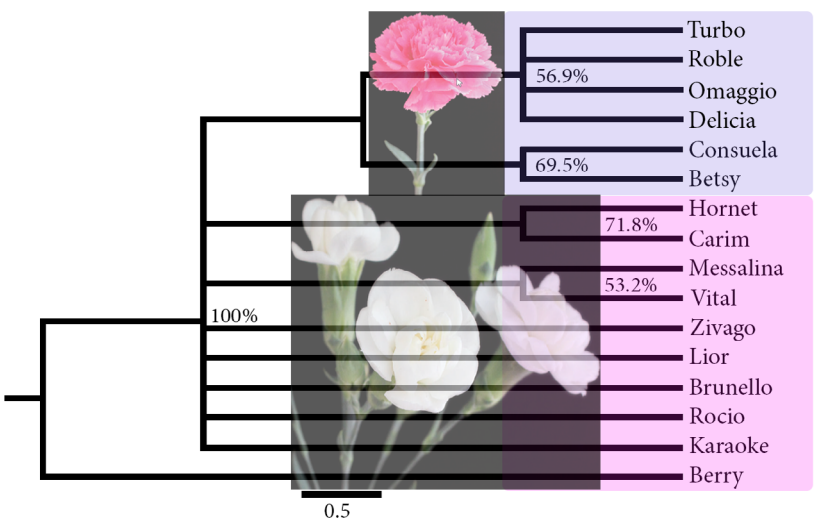

Figure 3. A 50% majority-rule consensus tree of the 16 commercial carnations. The number on the branch represents Bayesian posterior probabilities. Scale shows the branch lengths measured in the expected substitutions per site.

The DAMD-PCR markers based analyses indicated that the standard type varieties Consuela, Betsy, Delicia, Omaggio, Roble, and Turbo were genetically more related and their relatives might have arisen from a common ancestor. This group, called standard type, was supported with a Bayesian posterior probability value of 68.73%. The remaining varieties could not be formed into a single group (Figure 3). Among the 16 varieties, Berry was separated from the rest of the sample with a Bayesian posterior probability value of 100%.

4. Discussion

The effectiveness of a molecular marker system is mainly determined by its ability to differentiate variations among lines, varieties, or species. Among DNA marker systems, RAPD and ISSR have found the widest application for DNA fingerprinting and population genetic studies (Fu et al., 2008; Izzatullayeva et al., 2014) since these molecular marker systems are simple, quick, and relatively inexpensive. Their main advantage comes from the fact that their application does not need any prior genomic information about the target sequences of organisms under study. However, these systems have some disadvantages, including PCR artifacts, low level of polymorphism, and repeatability problems. The Td-DAMD-PCR system also does not require information about the target sequences in genomes, but produces more polymorphism with higher reproducibility. In other words, Td-DAMD-PCR techniques possess all the advantages of RAPD and ISSR techniques and are not limited by their disadvantages. This system has been successfully applied to many plant species including *Salvia*, *Origanum*, *Thymus*, *Capsicum*, and *Cynodon*, to name a few (Karaca et al., 2002; Karaca and Ince, 2008; Ince et al., 2009; Ince and Karaca, 2012). In this study we showed that the Td-DAMD-PCR system was not only suitable for the genetic study of *Dianthus* but was also useful in the differentiation of commercial carnations with high levels of polymorphism, little or no PCR artifact formation, and a high level of reproducibility.

The amplification reactions of the commercial carnation varieties used touchdown (Td) cycling conditions, which offered a simple and rapid means of PCR optimization, and increased specificity, sensitivity, and efficiency without the need for lengthy optimizations of each core minisatellite primer. The Td-DAMD-PCR markers were obtained at 55 °C annealing temperature, which is a temperature higher than those used in RAPD and ISSR systems. Although RAPD and ISSR systems have been effectively used in *Dianthus* (Fu et al., 2008; Behroozian et al., 2013; Wojcik et al., 2013; Jarda et al., 2014), the development of a new DNA marker system is always useful for a crop. For instance, new DNA markers (like Td-DAMD-PCR in carnations) can be useful to support the variety registration system under the seeds and seedlings law based on UPOV (The International Union for the Protection of New Plant Varieties, Switzerland) and for fingerprinting studies.

Genetic relationships within and between species of the genus *Dianthus* have been analyzed by several DNA marker systems (Vainstein et al., 1991; De Benedetti et al., 2003), but the information regarding genetic diversity within commercial carnation varieties is very limited. Analyses based on the SSR marker system by Kimura et al. (2009) indicated that carnation varieties could be identified using this marker system. However, Kimura et al. (2009) reported that the SSR markers generated by 13 primer pairs could not separate 41 standard carnation varieties from spray type carnation varieties. The Td-DAMD-PCR marker system reported in the present study successfully differentiated standard and spray type commercial varieties. Such differentiation is important since these 2 groups are the major classes of carnations with economic importance (Tah and Mamgain, 2013). It is well documented that standard type carnations usually produce larger blooms on longer flower stems, whereas the spray types produce many smaller flowers with weaker stems. Spray carnations grow better at higher temperatures, while standard types perform well in cool climates (Crespi et al., 2007). Dividing carnations into these groups was one of the most important findings of this study at the molecular level.

Both spatial relationships and phylogenetic groupings of the 16 commercial varieties could not arrange the varieties according to their flower color, which is one of the most important commercial factors in carnations. For this reason, flower color is considered important for assessing the genetic variability of the germplasm using molecular markers (Fu et al., 2008). Therefore, new marker systems are needed to link the genes responsible for the synthesis of pigments such as carotenoids and flavonoids. Although many carnations do not have carotenoid pigments, carotenoids are known to be responsible for colors ranging from yellow to orange in different plant species. In carnations, cyanidins and pelargonidins, which are water soluble pigments of flavonoids, are responsible for red or magenta color, and orange, pink, or brick red color, respectively. Comparative analyses within different color groups of carnations using Td-DAMD-PCR markers developed in the present study could be very useful to find color linked markers, which in turn could be used in marker assisted selection in carnation breeding and production programs.

Although the number of markers and varieties used were limited, molecular phylogeny based on Td-DAMD-PCR markers indicated that spray type carnations might have evolved from standard type carnations. As shown in the Bayesian consensus tree in Figure 3, all the standard

type of carnations clustered together while the spray types could not be grouped in one clade. Based on the molecular phylogeny analysis of this study, it is possible to claim that the variety Berry is the oldest carnation variety within the 16 commercial carnations. According to Kimura et al. (2009), Hosoya (1999) reported that the first spray type variety was a mutant obtained from William Sim in 1952. The findings of our study using Td-DAMD-PCR markers are not in contrast to previous findings indicating that spray type carnations originated from the standard type (Hosoya, 1999; Kimura et al., 2009; Wojcik et al., 2013).

Development of varieties with higher productivity and more desirable floral characteristics is very important in carnation production. Various agronomical characters such as growth parameters, flowering parameters, flower quality parameters, and yield parameters usually vary from variety to variety. These parameters serve as key traits for carnation variety improvement studies in which plant breeding plays a vital role (Scovel et al., 1998). Td-DAMD-PCR markers developed in the present study could be used as marker assisted selection (MAS) for these traits in carnation. The MAS then could be used to monitor breeding programs at early stages of plant development without depending on growth conditions.

In conclusion, to the best of our knowledge this is the first report of application of the Td-DAMD-PCR technique in the genus *Dianthus*. The Td-DAMD-PCR technique could differentiate the spray and standard carnations used in the present study. Further studies are required to confirm the discrimination power of the Td-DAMD-PCR markers between the standard and spray types using a large number of carnation varieties and more markers. Nonetheless, DAMD-PCR markers reported in this study could be very useful in species identification, determination of genetic relationships, and phylogenetic studies of species of *Dianthus* L.

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References

Balao F, Valente LM, Vargas P, Herrera J, Talavera S (2010). Radiative evolution of polyploid races of the Iberian carnation *Dianthus broteri* (Caryophyllaceae). New Phytol 187: 542–551.

Behroozian M, Jafari A, Farsi M (2013). RAPD analysis of genetic variation within and among natural populations of two species of *Dianthus* L. (Caryophyllaceae) in NE Iran. Iran J Bot 19: 194–201.

Crespi A, Fernandis CF, Castro A, Berrnardos S, Amich F (2007). Morpho-environmental characterization of the genus *Dianthus* (Caryophylaceae) in the Iberian Peninsula: *D. pungens* group. Ann Bot Fenn 44: 241–255.

De Benedetti L, Burchi G, Bruna S, Mercuri A, Schiva T (2003). Molecular markers and cut flower longevity in carnation. Acta Hort 683: 437–443.

Farsi M, Behroozian M, Vaezi J, Joharchi MR, Memariani F (2013). The evolution of *Dianthus polylepis* complex (Caryophyllaceae) inferred from morphological and nuclear DNA sequence data: one or two species? Plant Syst Evol 299: 1419–1431.

Fu XP, Ning GG, Gao LP, Bao MZ (2008). Genetic diversity of *Dianthus* accessions as assessed using two molecular marker systems (SRAPs and ISSRs) and morphological traits. Sci Hort 117: 263–270.

Heath DD, Iwama GK, Devlin RH (1993). PCR primed with VNTR core sequence yields species specific patterns and hypervariable probes. Nucleic Acid Res 21: 5782–5785.

Hosoya M (1999). Transition and trend in carnation cultivars. Agric Hort 74: 23–30 (in Japanese).

Ince AG, Karaca M (2009). The MAGi RNA extraction method: highly efficient and simple procedure for fresh and dry plant tissues. J Sci Food Agric 89: 168–176.

Ince AG, Karaca M, Onus AN (2009). Development and utilization of diagnostic DAMD-PCR markers for *Capsicum* accessions. Genet Resour Crop Ev 56: 211–221.

Ince AG, Karaca M (2011a). Genetic variation in common bean landraces efficiently revealed by Td-DAMD-PCR markers. Plant Omics 4: 220–227.

Ince AG, Karaca M (2011b). Early determination of sex in jojoba plant by CAPS assay. J Agri Sci 149: 327–336.

Ince AG, Yildiz F, Karaca M (2011). The MAGi DNA extraction method for fresh tissues and dry seeds. J Med Plants Res 5: 5458–5464.

Ince AG, Karaca M (2012). Species-specific touchdown DAMD-PCR markers for Salvia species. J Med Plants Res 6: 1590–1595.

İnce AG, Karaca M (2014) E-microsatellite markers for some *Salvia* species naturally occurring in the Mediterranean region. Turk J Biol doi: 10.3906/biy-1404-29.

Ince AG, Karaca M, Elmasulu SY (2014). New microsatellite and CAPS-microsatellite markers for clarifying taxonomic and phylogenetic relationships within *Origanum* L. Mol Breeding 34: 643–654.

Izzatullayeva V, Akparov Z, Babayeva S, Ojaghi J, Abbasov M (2014). Efficiency of using RAPD and ISSR markers in evaluation of genetic diversity in sugar beet. Turk J Biol 38: 429–438.

Jarda L, Butiuc-Keul A, Höhn M, Pedryc A, Cristea V (2014). Ex situ conservation of *Dianthus giganteus* d'Urv. subsp. *banaticus* (Heuff.) Tutin by in vitro culture and assessment of somaclonal variability by molecular markers. Turk J Biol 38: 21–30.

Jeffreys AJ, Wilson V, Thein SL (1985). Individual specific 'fingerprints' of human DNA. Nature 332: 278–281.

Karaca M, Saha S, Zipf A, Jenkins JN, Lang DJ (2002) Genetic diversity among forage bermudagrass (*Cynodon* spp.): evidence from chloroplast and nuclear DNA fingerprinting. Crop Sci 42: 2118–2127.

Karaca M, Saha S, Callahan FE, Jenkins JN, Read JJ, Percy RG (2004). Molecular and cytological characterization of a cytoplasmic-specific mutant in pima cotton (*Gossypium barbadense* L.). Euphytica 139: 187–197.

Karaca M, Ince AG, Elmasulu SY, Onus AN, Turgut K (2005). Coisolation of genomic and organelle DNAs from 15 genera and 31 species of plants. Anal Biochem 343: 353–355.

Karaca M, Ince AG (2008). Minisatellites as DNA markers to classify bermudagrasses (*Cynodon* spp.): confirmation of minisatellite in amplified products. J Genet 87: 83–86.

Karaca M, Ince AG, Ay ST, Turgut K, Onus AN (2008). PCR-RFLP and DAMD-PCR genotyping for Salvia species. J Sci Food Agric 88: 2508–2516.

Karaca M, Ince AG, Aydin A, Ay ST (2013). Cross-genera transferable e-microsatellite markers for 12 genera of Lamiaceae family. J Sci Food Agric 93: 1869–1879.

Kimura T, Yagi M, Nishitani C, Onozaki T, Ban Y, Yamamoto T (2009). Development of SSR markers in carnation (*Dianthus caryophyllus*). J Japan Soc Hort Sci 78: 115–123.

Maitra S, Roychowdhury N (2013). Performance of different standard carnation (*Dianthus caryophyllus* L.) cultivars in the plains of West Bengal, India. Int J Bio Resour Stress Manage 4: 395–399.

Manly BFJ (1994) Multivariate statistical methods: a primer. London, UK: Chapman & Hall.

Rana MK, Bhat KV (2004). A comparison of AFLP and RAPD markers for genetic diversity and cultivar identification in cotton. J Plant Biochem Biot 13: 19–24.

Ronquist F, Huelsenbeck JP (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.

Scovel G, Ben-Meir H, Ovadis M, Itzhaki H, Vainstein A (1998). RAPD and RFLP markers tightly linked to the locus controlling carnation (*Dianthus caryophyllus*) flower type. Theor Appl Genet 96: 117–122.

Tah J, Mamgain A (2013).Variation in different agronomical characters of some carnation (*Dianthus caryophyllus*) cultivars. Res J Biol 1: 10–23.

Vainstein A, Hillel J, Lavi U, Tzuri G (1991). Assessment of genetic relatedness in carnation by DNA fingerprints analysis. Euphytica 56: 225–229.

Wojcik M, Dresler S, Jawor E, Kowalczyk K, Tukiendorf A (2013). Morphological, physiological, and genetic variation between metallicolous and nonmetallicolous populations of *Dianthus carthusianorum*. Chemosphere 90: 1249–1257.

Yagi M, Kimura T, Yamamoto T, Isobe S, Tabata S, Onozaki T (2012). QTL analysis for resistance to bacterial wilt (*Burkholderia caryophylli*) in carnation (*Dianthus caryophyllus*) using an SSR-based genetic linkage map. Mol Breeding 30: 495–509.

Yagi M (2013). Application of DNA markers for breeding carnations resistant to bacterial wilt. Jpn Agr Res Q 47: 29–35.