



Molecular Characterization and TRAP Analysis of Gene in *Dendranthema morifolium*

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Abstract

The molecular marker system - TRAP was applied to develop a novel and more accurate method to identify the variety and establish the evolutionary relationship of different categories of *Dendranthema morifolium*. A software - GENESIS 2.4 was used to conduct the cluster analysis and genetic dendrogram establishment. The results showed that 202 different fragments were amplified with 6 pair primers using the TRAP marker system. The polymorphic fragments number is 45, which takes up to 22.3%. The cluster analysis showed that 4 materials used in this study can be classified into 2 main groups and 3 subgroups. The genetic identity is 0.0767 and the average genetic distance is 0.9236 among the four materials. A new tool using the TRAP marker system is more accurate and can be used to identify different categories of *Dendranthema morifolium* at molecular level.

Key words: *Dendranthema morifolium*, TRAP; sequence analysis, molecular marker.

Introduction

Dendranthema morifolium is the most unique natural medicine, food and natural resources in China. The medicinal parts of *Dendranthema morifolium* are the dry flower heads, the active extract of which has the functions as heat clearing and detoxifying, protecting the liver, improving eyesight, anti-aging, enhancing human immunity, etc. It has remarkable effects on hypertension, coronary heart disease, and arteriosclerosis. Modern pharmacological studies have shown that *Dendranthema morifolium* has very positive effects on the cardiovascular system, and has become one of the most commonly used Chinese medicine for treating cardiovascular and cerebrovascular diseases. Due to gradual reduction of wild resources, *Dendranthema morifolium* in many areas is planted by artificial cultivation, thus the difference in quality of *Dendranthema morifolium* resulting from different origins and cultivation techniques are very significant. Therefore, it is very important to identify and study the genetic variation of *Dendranthema morifolium*.

In 2003, Hu and Vick in US Northern Crop Science Laboratory proposed a new type of PCR based on molecular biomarker system, namely targeted region amplification polymorphism (TRAP) (1). TRAP technology is based on known cDNA or EST sequence information, it is different from SRAP, RAPD and AFLP and other labeling techniques which can directly conduct PCR amplification without any sequence information. It has been successfully applied in the studies of various plants (1-6). Currently, many species of genome sequences have been obtained, including human, Arabidopsis, and the genome sequence of important crop rice and various microorganisms. A large number of EST sequences are available to be used, which makes it possible to apply bioinformatics and EST database to generate a series of TRAP markers, furthermore, the properties and markers can be easily correlated with TRAP.

In this paper, TRAP was used to analyze and investigate the molecular and biological properties of *Dendranthema morifolium*. A novel method was introduced for identification of *Dendranthema morifolium* from different origins. It can provide a scientific fundamental for the research of *Dendranthema morifolium* resources and genetic diversity.

Materials and methods

Materials

Four various *Dendranthema morifolium* were collected from Changling in Hebei Province, Tonghui in Anhui Province, Luocheng in Guangxi Province, and Fangchi in Guizhou Province, respectively. The origins of *Dendranthema morifolium* were identified by Prof Wenhai Xu in Pharmaceutical college, Guangxi Medical University.

EST Screening

In the dbEST database (database for expressed sequence tags) of NCBI (The National Center for Biotechnology Information), 10,259 tags of registered *Dendranthema morifolium* can be found. These tags were copied and then pasted tag by tag to KEGG pathway system of kegg (Kyoto Encyclopedia of Genes and Genomes) for metabolic pathway analysis of EST in *Dendranthema morifolium*. 117 tags of EST which are related to metabolism of nucleotide and hydroxylase were selected. Among these tags, 20 tags of EST with length between 400-900 bp were further selected for primer design.

Primer Selection

The primers were fixed, a software oligo 6.44 was used for the 20 EST tags to design 20 primers with one primer for each tag. Random primers applied four original primers designed by Zhao et al (7). Four random primers collocated mutually with 20 fixed primers to form

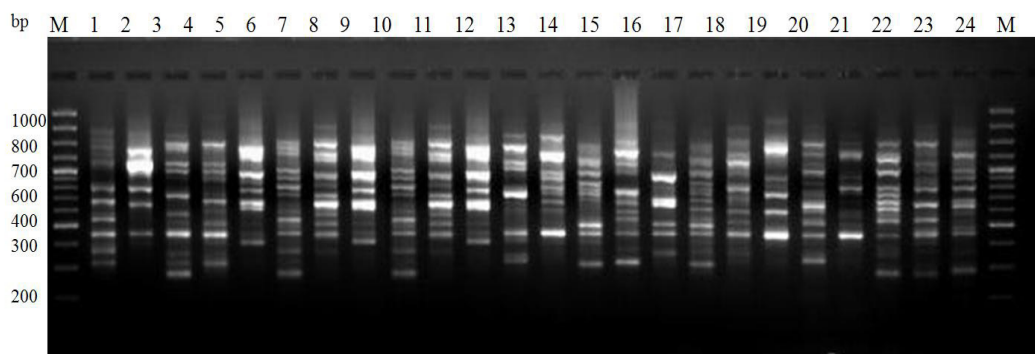


Figure 1. PCR amplified results of 6 pair primers for *Dendranthema morifolium* samples. 1, 5, 9, 13, 17, 21: Hebei samples; 2, 6, 10, 14, 18, 22: Anhui samples; 3, 7, 11, 15, 19, 23: Guangxi samples; 4, 8, 12, 16, 20, 24: Guizhou samples.

80 pairs of primer. After screening, 6 pairs of primer with abundant amplified bands, clear signals and good repeatability were selected for TRAP analysis.

All random primers used were described as follows: A1: 5' GGAATCACACACATGAAGA 3'; A2: 5' TTCTCCTTCACTGGACACTT 3'; A3: 5' CTA-TATCTCGGTACTAAAC 3'. The sequence and NCBI login account of used fixed primers are: E1: 5' AACCTAGCACCTGACCGTACT 3'/CV172318; E2: 5' ATTCCGCTGATTCCACGAGA 3'/CV171606; E3: 5' TACCTGGACCAGACGACACCT 3'/CV171904. The collocation of random and fixed primers are A1/E1, A1/E2; A2/E1, A2/E3; A3/E1, A3/E2.

The above 6 pairs of primers were amplified 1-4, 5-8, 9-12, 13-16, 17-20, 21-24 samples with the sequence of Hebei, Anhui, Guangxi and Guizhou *Dendranthema morifolium*. That is, number 1, 5, 9, 13, 17, 21 samples are Hebei *Dendranthema morifolium*, number 2, 6, 10, 14, 18, 22 samples are Anhui *Dendranthema morifolium*, number 3, 7, 11, 15, 19, 23 samples are Guangxi *Dendranthema morifolium* and number 4, 8, 12, 16, 20, 24 samples are Guizhou *Dendranthema morifolium*.

DNA Extraction

DNA of *Dendranthema morifolium* was extracted using CTAB method (8).

PCR Reaction

The volume of reaction system was 25 μ L, the procedures of amplification were: denaturation for 5 min at 92°C followed by 5 cycles (denaturation for 50 s at 92°C, annealing for 2 min at 37°C, extension for 1.7 min at 75°C), then proceeded with another 35 cycles (denaturation for 1.2 min at 92°C, annealing for 2 min at 37°C, extension for 1.7 min at 75°C), lastly extended 10 min at 75°C.

12 μ L denaturant (98% deionized formamide, 0.6 mol/L EDTA, 0.025% xylene cyanol, 0.025% bromophenol blue) was added into 15 μ L amplified PCR product at 92°C. The mixture was loaded into ice bath after 15 min. 5 μ L supernatant of the denatured sample was taken for 35 min pre-SDS-PAGE at 85 W and 1.6 h SDS-PAGE at 100 W using 6% polyacrylamide gel (1 L of which contains 100 mL TBE (tris-borate-EDTA), 48.3 g polyacrylamide, 2.7 g methylene bis-acrylamide and 472.36 g urea) and 1 X TBE electrophoretic buffer. After SDS-PAGE, 3 L stationary liquid was used for 25 min oscillating dipping, washed three times with water, silver-stained 35 min, washed 15 s with water, developed 6-12 min until spectral bands showed up, again

fixed for 6 min, washed 6 min with water, the sample was then taken, dried naturally, photographed and recorded.

Data Collection and Analysis

The bands displayed on TRAP polyacrylamide gel were analyzed statistically and transferred to data matrix based on requirements of computational analysis. With-band was assigned 1 (for both strong band and weak band), without-band was assigned 0, to yield an 1-0 data matrix and import into computer. For polymorphic loci, the bands having difference and stably appeared by repeating 3 times or more should be used for data analysis. GENESIS 2.4 analysis software was used to calculate the genetic identity and genetic distance of *Dendranthema morifolium*, the cluster branching dendrogram was also established.

Results

PCR Amplification Results

Dendranthema morifolium from 4 origins were amplified by 6 pairs of primers (total 24 samples with number 1-24) to yield 202 amplified bands. Of these bands, 45 were polymorphic, and the percentage of polymorphic bands was 22.3 %. Number 1 sample did not show electrophoretic bands at 330, 450, 460, 710 and 740 bp, number 2 sample showed characteristic bands at 440, 720 and 730 bp (in Group 1, *Dendranthema morifolium* from one origin uniquely processed the bands which could not be observed by *Dendranthema morifolium* from other three origins), number 3 and 4 samples did not show electrophoretic bands at 450, 720 and 730 bp, number 8 sample did not show electrophoretic bands at 250 and 520 bp, number 10 sample showed one characteristic band at 630 bp, number 13 sample did not show electrophoretic bands at 430 bp. There was no significant difference for Group 5. Number 22 sample showed one characteristic band at 610 bp. The total number of bands for each pair of primer was between 30-39, the length of fragment was between 45-980 bp, on average each primer could produce 32.6 bands and 6.8 polymorphic bands (see Figure 1).

Analysis of Genetic Identity and Genetic Distance

Genetic identity and genetic distance are important parameters to estimate the genetic diversity of populations. A genetic analysis software - GENESIS 2.4 was used to calculate genetic identity and genetic distance of *Dendranthema morifolium* from 4 populations. The

Table 1. Genetic identity (above diagonal) and genetic distance (below diagonal) from four populations of *Dendranthema morifolium*.

Origin	Hebei	Anhui	Guangxi	Guizhou
Hebei	-	0.9483	0.9115	0.9153
Anhui	0.0427	-	0.9021	0.9586
Guangxi	0.0938	0.1042	-	0.9054
Guizhou	0.0837	0.0361	0.0995	-

results were listed in Table 1.

Table 1 showed that genetic identity of Anhui *Dendranthema morifolium* and Guangxi *Dendranthema morifolium* is the smallest, which equals 0.9021, genetic identity of Anhui *Dendranthema morifolium* and Guizhou *Dendranthema morifolium* is the largest, which equals 0.9586; genetic distance of Anhui *Dendranthema morifolium* and Guangxi *Dendranthema morifolium* is the largest, which equals 0.1042, genetic distance of Anhui *Dendranthema morifolium* and Guizhou *Dendranthema morifolium* is the smallest, which equals 0.0361. Average genetic identity of 4 populations is 0.9236, and average genetic distance of 4 populations is 0.0767, suggesting that the relationships between populations are very close, there are not only genetic variation but also high genetic stability present in different populations of *Dendranthema morifolium*.

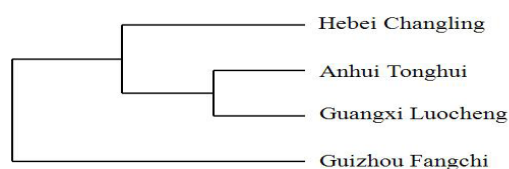
Cluster Analysis

The clustering branch tree of system was established through population genetic analysis software GENESIS 2.4 and UPGMA clustering method (see Figure 2).

As shown in the figure 2, 4 origins of *Dendranthema morifolium* were divided into two groups and three sub-groups by cluster analysis, Anhui Tonghui *Dendranthema morifolium* and Guangxi Luocheng *Dendranthema morifolium* cluster at first, then cluster with Hebei Changling *Dendranthema morifolium*, and finally cluster with Guizhou Fangchi *Dendranthema morifolium*. There is a long distance between Anhui and Guizhou provinces, however *Dendranthema morifolium* from the two provinces cluster together, suggesting that it is not obvious for the geographical boundaries of *Dendranthema morifolium*.

Discussion

In recent years, the rapid development of molecular biology has penetrated into all fields of life sciences, including traditional Chinese medicine, which provides a new method for the identification and genetic studies of Chinese herbal medicine (9, 10). DNA molecular markers directly analyze the genotype of the organism, instead of the phenotype. This technique is not affected by environmental factors, sample shape and material sources, which can provide a more accurate and reliable

**Figure 2.** Dendrogram of 4 cultivars of *Dendranthema morifolium* on TRAP data.

method for the identification of Chinese traditional medicine (11, 12). At this moment, the most commonly used molecular marker techniques are RAPD (random amplified polymorphic DNA) and AFLP (amplified fragment length polymorphism). RAPD method is simple with lower cost, but the repeatability is poor and there are only few detection sites. AFLP have many spectra bands, but the experimental steps are complicated with higher cost. In 2007, Xu et al (13) proposed a new molecular marker technology – SRAP (sequence related amplified polymorphism). TRAP technique was developed from SRAP technique, which uses the random primers of SRAP, but the fixed primers of TRAP were designed according to EST sequence in gene bank. Comparing with SRAP, RAPD and AFLP marker techniques, TRAP technique is based on known EST sequence or cDNA information, thus a variety of databases can be applied to locate the target genes, and the obtained polymorphism markers are easily associated with the properties. Therefore, the technology has been rapidly applied since the introduction in 2003 (14-16). Scientists have successfully applied this technique for the research on *Brassica napus* (13) and *Ctenopharyngodon idellus* (17). To the best of our knowledge, there was little to no studies on use of TRAP technology for Chinese medicine. In this paper, this technology was used to conduct the research for molecular markers using *Dendranthema morifolium* from different origins, the results show that the TRAP technology has the advantages of high sensitivity, specificity, clear bands and stable data. It is a novel molecular marker technology with the advantages of RAPD and AFLP markers, and simultaneously avoiding the shortcomings of the two. DNA dosage of TRAP template is very few, one extraction can meet the requirement of more than 10000 PCR amplification, the requirements of DNA purity of template and fragment size are not high, a certain amount of impurities will not affect the results, this technology is very suitable for Chinese herbal medicine especially dry and processed medicine which are very difficult to extract DNA. TRAP is highly efficient, rapid, simple, stable, and easier to program comparing conventional PCR, therefore it can be used to make reagent box and commercialize to reduce the research cost.

6 pairs of primers were selected from 4 different cultivars of *Dendranthema morifolium* using TRAP technology for identification. From the PCR amplification product map and genetic clustering analysis map, it can be seen that the 6 pairs of primers can effectively distinguish the 4 different cultivars of *Dendranthema morifolium*. For A1/E1 primer pairs, lack of electrophoretic bands at 450, 470, 730 bp and appearance of one characteristic band at 420 bp can separate Hebei Changling *Dendranthema morifolium* and Anhui Tonghui *Dendranthema morifolium* from other genotype regions. For A1/E2 primer pairs, Guangxi Luocheng *Dendranthema morifolium* and Guizhou Fangchi *Dendranthema morifolium* can be separated by lack of electrophoretic band at 530 bp for the former and observation of electrophoretic band at 530 bp for the latter. Therefore, applying the PCR amplification of only A1/E1 and A1/E2 primer pairs can efficiently identify the 4 different cultivars of *Dendranthema morifolium*. In combination with the other primer pairs, the genetic dif-

ferentiation of the 4 species of *Dendranthema morifolium* was further determined, and the TRAP technique provided a new method for the identification of *Dendranthema morifolium*.

The detection rate of TRAP in 4 species of *Dendranthema morifolium* was only 22.3%, and the genetic identity and the genetic distance were more than 0.05 and 0.8, respectively. It can be seen that the genetic diversity of *Dendranthema morifolium* is very low, and the genetic relationship among different species is very close. It is usually considered that the difference among different groups of Chinese traditional medicine is internal variation, which is the primary cause of the quality of Chinese traditional medicine. *Dendranthema morifolium* has a greatly far and wide distribution in China – from Hainan Province in the south to Jilin Province in the north, from Sichuan Province in the west to the coast area in the east, it should possess great genetic variations between different populations. In this study, it has shown that the genetic variation among different cultivars was not very significant, and the reasons need to be further investigated. In addition, the results of clustering in this study showed that there is a large genetic difference between Guizhou Fangchi *Dendranthema morifolium* and *Dendranthema morifolium* from other cultivars.

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