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*Full Length Research Paper*

# Genetic diversity of the genus *Gnaphalium* in China assayed using random amplified polymorphic DNA analysis

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**We have analyzed the genetic diversity and affinity relationships among five species and eight populations of the Genus *Gnaphalium* from the Jiangsu and Zhejiang Provinces of China. The random amplified polymorphic DNA (RAPD) technique was used to analyze genetic diversity, and a dendrogram figure was constructed using the Unweighted Pair Group Method (UPGMA). Thirty RAPD primers were tested, the amplified DNA fragments range from 0.25 to 2 kb. Seven from 30 primers were selected for polymorphic analysis, a total of 328 DNA bands were detected. Cluster analysis using the UPGMA method showed that eight samples could be classified into three types, which is in agreement with traditional morphological classification. In summary, RAPD markers provided a basis for the molecular identification of Herb *Gnaphalii*, and can be further used to study detailed genetic relationships within the Genus *Gnaphalium*.**

**Key words:** Gnaphalium, random amplified polymorphic DNA (RAPD), genetic diversity, cluster analyses.

## INTRODUCTION

The Genus *Gnaphalium* contains medicinal and edible plants and consists of up to 200 species around the world, of which 19 are distributed in China, mainly in the Yangtze River and Pearl River basins. In Chinese traditional medicine, whole *Gnaphalium* plants can be used to treat cough, sputum and dyspnea. However, the species traits of the genus *Gnaphalium* are difficult to distinguish morphologically and in traditional Chinese medicine markets herb *Gnaphalii* often consists of Radix *Pulsatillae Chinensis* (Lu et al., 2005), and is often mixed with *Gnaphalium hypoleucum* DC. Therefore, it is important that a simple and efficient method for the identification of Genus *Gnaphalium* species is developed.

The origins of and relationships between some wild *Gnaphalium* species that are native to China have been inferred from morphological and microscopic features (Chen et al., 2009). DNA marker data offer an alternative approach to explore the relationships among plant species. Genetic distance measures obtained from DNA marker data have been shown to reflect known genetic relationships, for example in *Camellia (Theaceae)* cultivars (Wang et al., 2011), the genus *Cattleya* (Michael et al., 1995) and *Pongamia pinnata* (Vigyak and Latha, 2011). DNA marker studies of wild *Gnaphalium* species native to China have not yet been used to assess genetic diversity. Pedro et al. (2010) analyzed *Gnaphalium* DNA and showed a high degree of polymorphism using ISSR markers in UV-B radiated plants. Our previous study described a DNA extraction method and preliminary RAPD analysis (Lu et al., 2009). This study investigates the feasibility of using RAPD technology to test taxonomic relationships among wild Chinese *Gnaphalium* species, and estimates the taxonomic values of RAPD markers.

RAPD was developed by Welsh and McClelland (1990) and Williams et al. (1990). Currently, researchers frequently use molecular genetic markers for plant identification and genetic relationship analyses. RAPD has been used in many studies, including analysis of the genetic diversity of *Geranium* (Yin et al., 2008), identification of *Ophiopogon japonicus* ker-gawl (Lei et al., 2006), investigating the genomic instability of the medicinal plant *Codonopsis lancelolata* Benth (Guo et al.,

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**Table 1.** Origins and places of purchase.

| No. | Species | Collecting time | Places |
|---|---|---|---|
| 1 | *Gnaphalium affine* D.Don | January 2008 | Collected from Suzhou University New Campus in Jiangsu province |
| 2 | *Gnaphalium hypoleucum* DC. | June 2008 | Collected from Hangzhou Botanical Garden in Zhejiang province |
| 3 | Herb Gnaphalii | March 2009 | Purchased from Bozhou medicine market in Anhui province |
| 4 | *Gnaphalium hypoleucum* DC. | May 2009 | Collected from mountain Fenghua Xuedou in Zhejiang province |
| 5 | *Gnaphalium pensylvanicum* Willd. | May 2009 | Collected in Suzhou Dongshan in Jiangsu province |
| 6 | *Gnaphalium luteo-album* L. | March 2009 | Purchased from Bozhou medicine market in Anhui province |
| 7 | *Gnaphalium japonicum* Thunb. | May 2009 | Collected from Suzhou Golf Garden in Jiangsu province |
| 8 | *Gnaphalium pensylvanicum* Willd. | June 2009 | Collected from Hangzhou Botanical Garden in Zhejiang province |

**Table 2.** Quality and concentration of genomic DNA ( $\bar{X}$ ±SD，n=3).

| Samples | A260/A280 | Concentration (μg/ml) |
|---|---|---|
| 1 | 1.89±0.0012 | 1643.733±1.65 |
| 2 | 1.52±0.0025 | 303.3367±2.38 |
| 3 | 1.46±0.0022 | 1179.663±0.66 |
| 4 | 1.81±0.0054 | 556.39±0.44 |
| 5 | 2.17±0.00047 | 942.37±0.97 |
| 6 | 1.25±0.0014 | 431.4867±2.13 |
| 7 | 1.17±0.00047 | 227.9467±0.61 |
| 8 | 2.07±0.0148 | 207.0767±0.57 |

2006), and for the identification of *Phyllanthus emblica* (Warude et al., 2006) and *Tinospora cordifolia* (Rout, 2006), which are traditional Chinese medicines similar to ginseng.

## MATERIALS AND METHODS

### Plant material

Leaves from plants of the genus *Gnaphalium* were obtained in the field from Jiangsu and Zhejiang provinces where *Gnaphalium* species are very common. Fresh and dry leaves were collected and stored at -70°C until DNA extraction. Table 1 shows the *Gnaphalium* species used in this study, their origins, and time and place of collection. Where samples were purchased, suppliers are listed.

### DNA isolation and spectrophotometric analysis

Genomic DNA was extracted from young leaves using a modified CTAB (cetyl trimethyl ammonium bromide) method (Doyle and Doyle, 1987). Leaves (stored at -70°C) were rapidly crushed in 20% polyvinylpyrrolidone (PVP) with a pestle and mortar, and then 0.1-0.2 g leaf powder was immediately transferred to 1.5 ml Eppendorf tubes. 1000 µL preheated CTAB extraction buffer (1% CTAB, 10 mM EDTA, 0.7 M NaCl, 50 mM Tris-HCl pH 8.0, 1% PVP) was then added and mixed several times by gentle inversion. Samples were then incubated for 30 min at 65°C. Tubes were gently inverted every 5 min, then cooled to room temperature. Subsequently, 450 µL of cold chloroform: isoamyl alcohol (24:1) were added, and tubes were spun for 2 min at 12 000 rpm/min in a refrigerated centrifuge. Supernatants were transferred into new tubes and 450 µL of cold chloroform: isoamyl alcohol (24:1) was added for a second extraction. Supernatants were poured into new tubes and then 800 µL of cold alcohol was added and left at room temperature for 5 min. Samples were spun for 10 min at 12 000 rpm/min in a refrigerated centrifuge, supernatants were discarded using a pipette and the DNA pellet was washed twice with 1ml of 70% ethanol. DNA's were dried and re-suspended in 100 µL TE solution and stored at -20°C until use.

The concentration was determined using spectrophotometric analysis (DU730 nucleic acid protein analyzer, Scientific Instrument Co., Hangzhou, China), and the quality of DNA samples was analyzed by 1% agarose gel electrophoresis. For spectrophotometric analysis, optical density values at 230, 260 and 280 nm (Table 2) of each DNA sample were determined.

### RAPD amplification and electrophoresis analysis

30 random primers (obtained from Shanghai Sangon Biological Engineering Technology and Service Co., China) were used for RAPD amplification. Each 25 µL reaction mixture contained 0.5 µL (10 to 20 ng) template DNA with 0.5 µL primer (10 µmol/L) and 12.5 µL 2×Taq Master Mix (Biological Technology Co., Nanjing Boer Di, China) and 11.5 µL distilled water. Amplification was performed on a TC-48/T/H(a) Peltier thermal cycler (Hangzhou, China) programmed for 28 cycles (initial denaturation step at 94°C for 3 min; 94°C for 45 s ; 38°C for 45 s ; 72°C for 2 min) followed by an extension at 72°C for 10 min.

PCR products were separated on 1.0% agarose gels in 0.5 X TBE buffer with 0.5 µg/ml ethidium bromide at 100 V constant voltages for 0.5 h. To estimate the size of separated fragments, DNA ladder was loaded in the first lane of each gel. Eight samples were processed simultaneously for each primer. Gels were observed using an ultraviolet imaging system. In all cases, a PCR marker and λDNA/*Hin*d III were used as reference.

### RAPD primer screening and genetic polymorphism analyses

Primers that produced less than three bands were discarded. Further screening identified seven primers that amplified clear, reproducible, and polymorphism rich bands. The number and sequence of these seven primers are listed in Table 3. RAPD amplification of all samples is shown in Figure 3.

Gels were visualized using a Gel imaging system (Shanghai

**Table 3.** Number and base sequence of the seven selected primers.

| Number | Base sequence (5'-3') | G+C (%) |
|---|---|---|
| 10 | GAC GAG CAG G | 70 |
| 13 | AAA GTG CGG C | 60 |
| 15 | AGG CCG GTC A | 70 |
| 23 | ACT TCG CCA C | 60 |
| 25 | CAA TCG CCG T | 60 |
| 28 | CTG CTG GCA C | 70 |
| 29 | TCG GCC ACA T | 60 |

Tanon, China); analysis of RAPD bands was performed using NT.SYSpc2.1 software (Numerical Taxonomy and Multivariate Analysis System. Version 2.1). The RAPD bands (markers) were scored as 1 if present and 0 if absent. Only clear and reproducible bands were used for the binary data matrix and the dendrogram was constructed using POPGEN32 according to coefficients and the UPGMA algorithm (Using Arithmetic Averages) was used for hierarchical clustering analysis.

## RESULTS

### Quality of isolated DNA

The DNA quality of eight samples is different. The A260/A280 of DNA of *G. affine* D.Don and G*. hypoleucum* DC. was in between 1.7 and 1.9. The concentration of DNA of *G. affine* D.Don is 1643.73 µg/ml. The A260/A280 of DNA of *G. pensylvanicum* Willd. was higher than 1.9, while that of the other samples was lower than 1.7 (Table 2).

### Polymorphism amplification results

We amplified the DNA of *G. affine* D.Don with 30 random l0 bp oligonucleotide primers, and selected polymorphic primers 9, 10, 11, etc. (Figure 1). Followed by screening the other samples one by one, we finally obtained seven polymorphic primers 10, 13, 15, 23, 25, 28 and 29 (Figure 2). The number and sequence of these polymorphic primers are listed in Table 3. Using these seven selected primers to amplify DNA from test samples, 328 bands were obtained, 87.2% of which were polymorphic; each primer produced an average, of 41 DNA fragments. Figure 3 shows that bands amplified using primer 10 were polymorphic among samples 1-8.

### Genetic similarity coefficient

The similarity coefficients of eight samples ranged from 0.509 to 0.909 (Table 4), the average similarity coefficient was 0.613. This result indicated that small genetic differences exist between *Gnaphalium* species. The similarity coefficients of *G. pensylvanicum* Willd. from Suzhou Dongshan and Hangzhou Botanical Garden is 0.909, indicating a very close genetic relationship; a low similarity coefficient of *G. pensylvanicum* Willd. from Hangzhou and *G. japonicum* D.Don are 0.509, indicating a relatively distant genetic relationship. Overall, all similarity coefficients are higher than 0.509, suggesting close genetic relationships among the five species and eight populations of the genus *Gnaphalium*.

### Cluster analysis

We used the UPGMA clustering method on five species and eight populations of genus *Gnaphalium* for genetic cluster analysis. The genetic relationships among the various species are illustrated in the derived dendrogram (Figure 4). The eight samples can be clustered into class I, II or III. Firstly, class I included *G. affine* D.Don from Suzhou, *G. hypoleucum* DC. from Ningbo Fenghua and *G. luteo-album* L. from Anhui Bozhou. The similarity coefficient is 0.7454, *G. affine* D.Don from Suzhou and *G. hypoleucum* DC. from Ningbo were further clustered into a group, followed by polymerization with *G. luteo-album* L. from Anhui Bozhou while the similarity coefficient is 0.6363; secondly, class II included *G. hypoleucum* DC. from Hangzhou, herb Gnaphalii from Bozhou, *G. pensylvanicum* Willd. from Suzhou and *G. pensylvanicum* Willd. from Hangzhou. The similarity coefficient is 0.90, *G. pensylvanicum* Willd. from Suzhou and Hangzhou cluster into a group, followed by aggregation with herb *Gnaphalii* from Bozhou while the similarity coefficient is 0.7636, and finally forming a class with *G. hypoleucum* DC. from Hangzhou while the similarity coefficient is 0.6363; thirdly, the similarity coefficient is 0.56, class I, class II and *G. japonicum* Thunb. clustered into class III.

## DISCUSSION

The quality of DNA is important for PCR amplification. Typically, the A260/A280 of high-quality DNA is between 1.7 and 1.9, but the A260/A280 of DNA from the herb *Gnaphalii* and *G. luteo-album L.* purchased from ‘Bozhou’ was lower than 1.7, indicating that degradation of DNA in dried herbs may occur. However these samples were of sufficient quality for following RAPD amplification and genetic distance analysis.

The similarity coefficient among five species and eight populations of genus *Gnaphalium* were higher than 0.509 (Figure 4), indicated that the genetic background of different *Gnaphalium* species from different regions is similar. But their similarity coefficient ranged from 0.509 to 0.909, indicated that these species have certain differences. On the one hand, *G. pensylvanicum* Willd. from Suzhou and *G.pensylvanicum* Willd. from Hangzhou

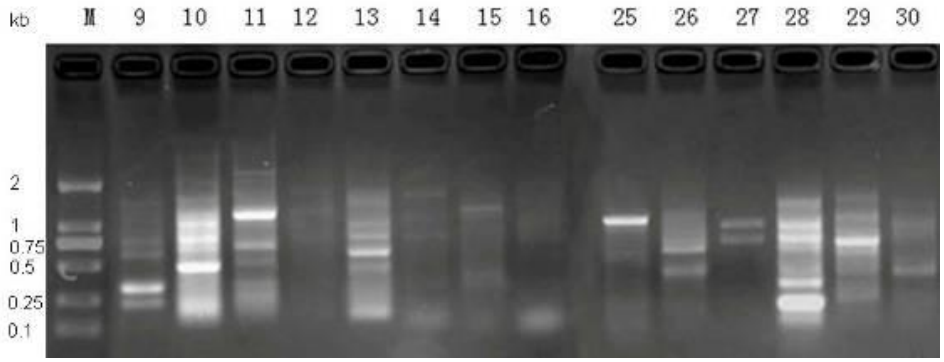


**Figure 1.** Amplification results of *Gnaphalium affine* D.Don using primers 9-16, 25-30.

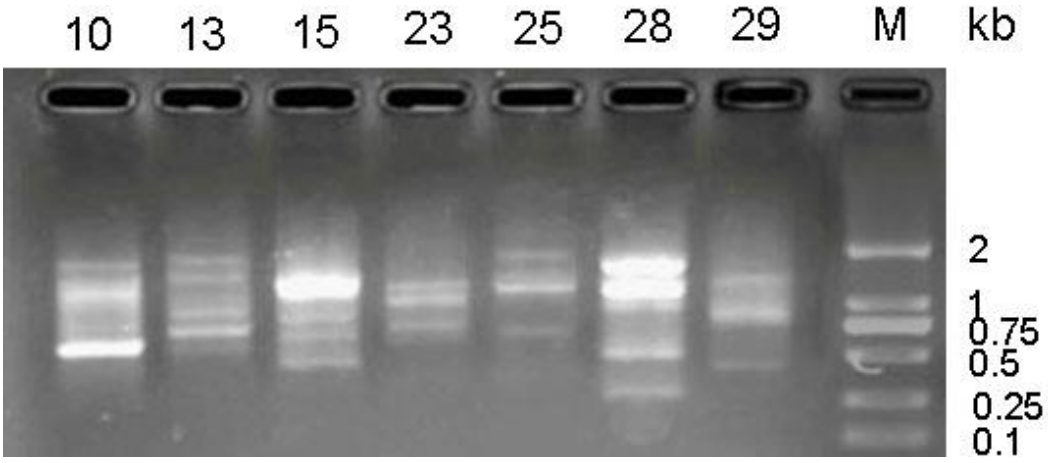


**Figure 2.** Amplification of *Gnaphalium pensylvanicum* Willd. using primers 10, 13, 15, 23, 25, 28 and 29.

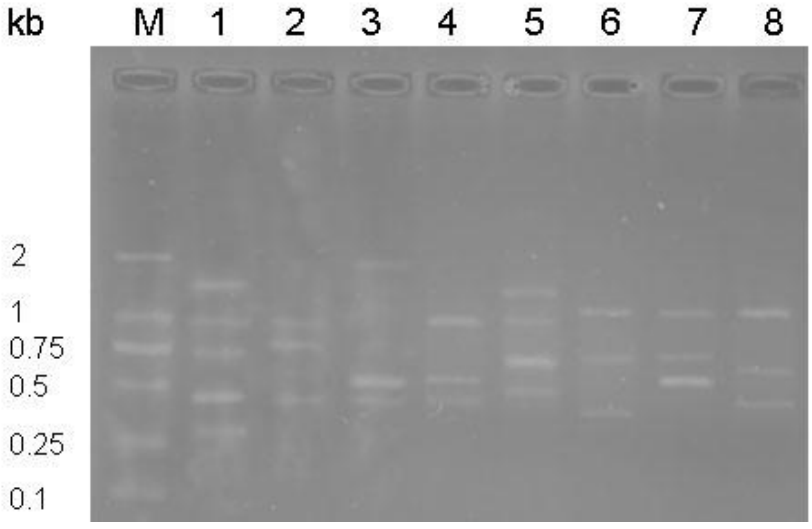


**Figure 3.** PCR amplification of samples 1-8 using primer 10. 1. *Gnaphalium affine* D.Don, 2. *Gnaphalium hypoleucum* DC. from Hangzhou, 3. Herb Gnaphalii, 4. *Gnaphalium hypoleucum* DC. from Fenghua, 5. *Gnaphalium pensylvanicum* Willd. from Suzhou, 6. *Gnaphalium luteo-album* L., 7. *Gnaphalium japonicum* Thunb., 8. *Gnaphalium pensylvanicum* Willd. from Hangzhou.

**Table 4.** Genetic similarity coefficients of 8 samples based-on RAPD markers.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| 1 | 1.0000000 | | | | | | | |
| 2 | 0.5636364 | 1.0000000 | | | | | | |
| 3 | 0.5636364 | 0.6363636 | 1.0000000 | | | | | |
| 4 | 0.7454545 | 0.7090909 | 0.6363636 | 1.0000000 | | | | |
| 5 | 0.5818182 | 0.6181818 | 0.7636364 | 0.6181818 | 1.0000000 | | | |
| 6 | 0.6363636 | 0.4909091 | 0.4909091 | 0.6363636 | 0.5454545 | 1.0000000 | | |
| 7 | 0.5818182 | 0.5454545 | 0.6181818 | 0.5454545 | 0.5636364 | 0.5818182 | 1.0000000 | |
| 8 | 0.5636364 | 0.6727273 | 0.6727273 | 0.6000000 | 0.9090909 | 0.5636364 | 0.5090909 | 1.0000000 |

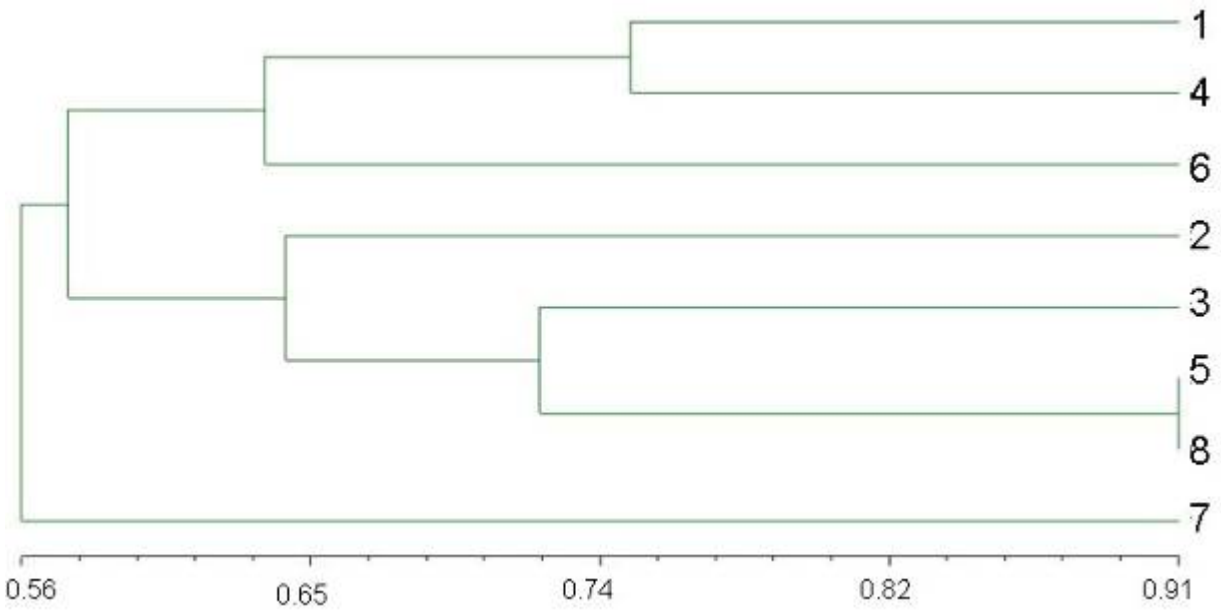


**Figure 4.** Dendrogram of genus *Gnaphalium* constructed using RAPD markers.

cluster as a class, they are the same species from different locations, and have the closest relationship. This result showed geographical isolation not caused changes of genetic characteristics of *G. pensylvanicum* Willd. Many scholars have supported this result. Sajeev et al. (2011) supported that forty-nine ginger clones cultivated in North-East India group into six hypothetical populations based on their source or location of collection using RAPD markers. On the other hand, our results showed that *G. hypoleucum* DC. from Fenghua and from Hangzhou are traditionally in a different cluster, suggesting different habitats can affect the genetic characteristics. Because different geographical environment may cause the genetic variation of *G. hypoleucum* DC., this phenomenon also demonstrated that *G. hypoleucum* DC. exist in rich genetic diversity among populations. Li et al. (2010) also demonstrated this phenomenon; his research results showed that geographical location appears to have affected genetic diversity due to adaptation of the plants to the different environments. Meanwhile, *G. affine* D.Don and *G. hypoleucum* DC. also showed a close genetic relationship and cluster together as a class, while *G. luteo-album* L. is in a different cluster, which is consistent with traditional classification based on morphology. In morphology, the involucres of *G. luteo-album* L. are bright brown, but of *G. affine* D.Don and *G. hypoleucum* DC. are yellow and white .Others, class I, class II and *G. japonicum* Thunb. clustered into class III. This is consistent with the traditional morphological classification. Because involucres of *G. japonicum* Thunb. are red-brown, but the involucres of the other four species are yellow.

In addition, the clustering results indicated the herb *Gnaphanii* purchased from Anhui 'Bozhou' market can be classified as *G.pensylvanicum* Willd. However, the herb *Gnaphanii* comparing with the morphological and

microscopic characteristics of *G. hypoleucum* DC., our results support that herb *Gnaphanii* was from *G. hypoleucum* DC. This indicated that cluster analysis based on RAPD may not be used as a source of reference for the identification of medicinal herbs; sometimes need to combine with morphological and microscopic characteristics.

Many species of Genus *Gnaphalium* are distributed in China. Our experiment involved five species, and provides an important foundation for further study of the genetic relationships among *Gnaphalium* species. Results from this study indicate that the RAPD technique is useful for the taxonomic study of wild *Gnaphalium* species. The level of polymorphism revealed from this study also suggested that RAPD could be a useful tool for determining the serious mixed status of traditional Chinese medicines at the molecular level (Tian et al., 2010). RAPD can also be used in the study of Geo-herbalism (Zhu et al., 2010). Relationships represented in the dendrogram are basically consistent with the available pedigree information and this was proved by DNA identification. This technology has also been successfully used with other medicines, especially precious traditional Chinese medicines (Wang et al., 2007; Zhou et al., 2007).

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