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Full Length Research Paper

Study of specific random amplification of polymorphic DNA- sequence characterized amplified region (RAPD-SCAR) marker for the endangered Chinese endemic herb *Atractylodes lancea*

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The phylogenetic relationships of five *Atractylodes* species were examined by random amplification of polymorphic DNA (RAPD) assay. A total of 298 bands were amplified by 24 RAPD primers, of which 244 bands (81.88%) were polymorphic. The results indicated that: 1) *Atractylodes chinensis* had a close genetic relationship with *Atractylodes lancea*, and *A. chinensis* should be treated as *A. lancea* var. *chinensis*; 2) *Atractylodes coreana* was closely related to *A. lancea* and *A. chinensis*; 3) *Atractylodes macrocephala* was distantly related to other species of *Atractylodes*. One primer which produced an *A. lancea*-specific band with a length of 1800 bp was obtained from 80 RAPD primers. Subsequent analysis showed that all of the 38 individuals of *A. lancea* could produce the sequence characterized amplified region (SCAR) bands, while the other four species of *Atractylodes* could not, indicating that *A. lancea* can be exactly distinguished from the other four species by the RAPD-SCAR marker.

Key words: *Atractylodes*, *A. lancea*, endangered herb, random amplification of polymorphic DNA (RAPD), sequence characterized amplified region (SCAR).

INTRODUCTION

According to the Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2010), the dried rhizome of 2 species in the *Atractylodes* genus *A. tractylodes lancea* (Thunb) DC. (with the Chinese name Nancangzhu) and *A. tractylodes chinensis* (DC.) Koidz (Beicangzhu), can be used as *A. tractylodis Rhizoma*, a traditional Chinese crude drug. Flora Repubulicae Popularis Sinicae (Lin and Shi, 1987) records 5 species in *A.tractylodes* DC., including *A. tractylodes lancea* (Thunb.) DC., *A.tractylodes coreana* (Nakai) Kitam., *A. carlinoides* (Hand.-Mazz.) Kitam., *A. tractylodes japonica* Koidz. Cx Kitam. and *A. macrocephala* Koidz., while *A. chinensis* (Bunge) Koidz. is considered as a synonym of *A. lancea* (Thunb.) DC.. Nancangzhu and Beicangzhu are thought to be just the trade names of *A. Rhizoma*. *A. lancea* produced in north China is called Beicangzhu, while *A. lancea* distributing in south China is called Nancangzhu in which the drug from Jurong of Jiangsu has the best quality. There are 4 species in the *Atractylodes* genus in the modified English version Flora of China (Shi and Greuter, 2011), including *A. lancea*, *A. coreana*, *A. carlinoides* and *A. macrocephala*, while *A. japonica*, which is included in Flora Repubulicae Popularis Sinicae as one species, is herein treated as a synonym of *A. lancea*.

As described in the Chinese Pharmacopoeia 2010, Nancangzhu is also called Maocangzhu, mainly distributing in the narrow region of Jiangsu, Zhejiang, Anhui and Hubei. Traditionally, Mt. Maoshan in Jurong Region in Jiangsu province is known as the genuine producing area of *A. lancea* (Lu, 2000). Because of environmental degradation and excessive harvest, *Atractylodes* plants especially *A. lancea* in Jiangsu and Zhejiang are endangered.

The key to the species of *Atractylodes* in Flora of China (Lin and Shi, 1987) is mainly based on variable characters,

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Table 1. The plant materials used for the analysis.

Symbol	Species	Population	Individual numbers	Locality	Collection number
N	*A. lancea* (Thunb) DC.	NX	6	Xiaojiuhuashan, Jiangsu	Geyanfeng200606001-200611006
		NH	6	Hushan, Jiangsu	Xuxiaolan200311002-200311007
		NT	10	Xiaotangshan, Jiangsu	Zhouyifeng200604004- Zhouyifeng200604013
		NB	5	Baokang, Hubei	Xuxiaolan200311004-200311018
		NY	8	Yingshan, Hubei	Xuxiaolan200311020-200311027
		NJ	2	Jinzhai , Anhui	Geyanfeng200606021-200606022
		NW	1	Wuhu , Anhui	Geyanfeng200606031
B	*A. chinensis* (DC.) Koidz		5	Yangtaishan, Beijing	Liuyuehui200607009-200607013
G	*A. japonica* Koidz. cx Kitam		3	Fushun, Liaoning	Xu'enguo200605010-200605012
CX	*A. coreana* (Nakai) Kitam		5	Kunyushan, Shandong	Geyanfeng200607011-200607015
BZ	*A. macrocephala* Koidz		3	Sheyang, Jiangsu (cultivated)	Zhouyifeng200511012-200511014

such as leaf shape, texture, divided degree, lobe number. The Picture Index of Senior China Plant (1975), Flora of Taiwan (1998) and the Chinese Pharmacopoeia 2010 acknowledge that *A. lancea* and *A. chinensis* should be treated as two species; while Liu (1959) classified *A. coreana* as a variety of *A. chinensis*. A classification proposa1 based on geographic environment was put forward by Hu et al. (2000), he classified herb into three major categories: Maocangzhu distributing in Jiangsu as *A. lancea* var. *maoshanensis* Hu et Feng, Luotiancangzhu distributing in south of Qinling Mountains as *A. lancea* subsp. *luotianensis* Hu et Feng; and Beicangzhu distributing in north of Qinling Mountains as *A. lancea* var. *chinensis* (Bunge) Kitam. Delimitation of species in the genus *Atractylodes* remains unsolved.

The phylogenetic relationships of *Atractylodes* were analyzed by molecular biological methods in many researches. Ren et al. (2000) classified 10 populations into 3 groups according to distinct genetic differences, Dabieshan group, Nancangzhu group and Beicangzhu group. Accoriding to Kohjyouma et al. (1997), random amplification of polymorphic DNA (RAPD) fingerprinting can be used for discriminating *A. lancea*, *A. chinensis*, *A. coreana*, *A. japonica* and *A. macrocephala*. Mizukami et al. (1998) found that *A. lancea, A. macrocephala* and *A. Japonica* varied distinctly in their RFLP maps. In addition, *A. chinensis* and *A. coreana* were found to have exactly the same ITS sequence which was different from those of *A. lancea*, *A. japonica* and *A. macrocephala* (Cheng et al., 1997; Shiba et al., 2006). Sequences of region 1 of *trn*K gene, *mat*K (Mizukami et al., 1998), *rbc*L (Yi, 1998), *trn*L-F (Ge, 2007; Ge et al., 2007) of *A. lancea* and *A. japonica* were totally the same, and different from those of *A. chinensis*, *A. coreana* and *A. macrocephala*. Being contradictory to each other, these results not only didn't solve the problem, but also made the relations of *Atractylodes* species more complicated. But all the above molecular biological studies supported to treat *A. lancea* and *A. chinensis* as two species.

SCAR has the advantage of a good reproducibility and stability, and some researchers have already applied it in the authentication of genuine medical plants, such as *Panax* species (Wang et al., 2001), *A. japonica* and *A. macrocephala* (Hu et al., 2006), *Prunella vulgaris* L. (Sun et al., 2011). In this study, we used RAPD-SCAR markers for identification of *Atractylodes* species, regarding populations collected from the main distributing areas of *A. lancea* from Jiangsu, Hubei and Anhui.

MATERIALS AND METHODS

Plant materials

54 individuals from 11 populations of 5 *Atractylodes* species were collected (Table 1) and identified based on the morphological features according to 'Flora of China'. (*A. lancea* and *A. chinensis* were defined according to Chinese pharmacopoeia combined with their distribution,

and *A. japonica* was listed as a species).

DNA extraction

Total DNA was extracted by the cetyltrimethyl ammonium bromide (CTAB) method (Paterson et al., 1993) with some modification. 1 to 3 g leaves of each sample were frozen in liquid nitrogen, ground into powders and then filtered. Samples were treated with 650 μL 2% CTAB extraction buffer preheated to 65°C (5% β-mercaptoethanol) for 1 h at 65°C and shook every 10 min, then centrifuged at 12000r/min for 12 min after mixing with chloroform-isoamylalcohol (24: 1). 2-fold vol. of anhydrous ethanol was added into the upper solution and allowed to stand at -20°C for 2 h. The resulting pellet was washed with 70% ethanol, dried and then resuspended in sterilized distilled water. The extracted DNA was purified using E.Z.N.A.TM Cycle Pure Kit (Beijing Bio-lab Materials Institute) and dissolved in sterile double distilled water and stored at -20°C for further use.

RAPD procedures

The preliminary RAPD analysis was carried out using 80 primers，then 24 primers with multiple bands, strong signals and stable amplified products were chosen (Table 2). PCR amplification reactions were carried out in a volume of 20 μL solution containing 1 × *Taq* buffer (10 mmol/L Tris-HCl, 50 mmol/L KCl, 0.1% Trion X-100, pH 8.4), 2.5 mmol/L $MgCl_2$, 1 U *Taq* DNA-polymerase, 40 ng template DNA, 0.4 μmol/L each primer and 0.15 mmol/L dNTPs. The polymerase chain reaction was conducted with the following program: a premelt for 3 min. at 94°C, followed by 38 cycles of 45 s denaturation at 94°C, 30 s annealing at 38°C, 1 min extension at 72°C, plus a final extension of 5 min at 72°C. All PCR products were separated on a 1.4% agarose gel, visualized with ethidium bromide staining under ultraviolet light.

Data analysis

For each individual sample, bands on agarose gels were scored as present (1) or absent (0) and genetic similarity of *Atractylodes* was studied by POPGENE.

Cloning and sequencing of RAPD products

A specific band to *A. lancea* was obtained in RAPD amplification. The PCR products were recovered, purified and cloned into plasmid vectors. 2 clones were randomly chosen to be sequenced (Invitrogen Biotechnology, Shanghai). A pair of specific primers was designed for the SCAR reaction according to the DNA sequence.

RAPD-SCAR marker

DNA from 54 individuals was amplified using the synthesized primers. The conditions were the same with the RAPD analysis except using 0.3 μmol/L primers, 60 ng DNA and annealing temperature of 64°C.

RESULTS

Polymorphism of RAPD

Twenty-four primers producing polymorphic bands strong and clear enough were selected from eighty 10-mer random primers (Table 2). There were 298 clear bands in total, in which 244 bands were polymorphic. Each primer produced 12.42 bands and 10.17 bands on average. The length of DNA fragments varied from 250 to 1800 bp. The results indicated the high polymorphism and big interspecific divergence of the RAPD marker of *Atractylodes*.

Genetic identity and distance

Genetic identity and distance reflect the genetic similarity and divergence between species respectively. Genetic identities and distances of 5 *Atractylodes* species were obtained using POPGENE (Table 3). Genetic identities between the 5 *Atractylodes* species are ranging from 0.5201 to 0.7651, and the genetic distances ranged from 0.2677 to 0.6537, in which *A. lancea* and *A. chinensis* had the maximum genetic identity (0.7651) and the minimum genetic distance (0.2677), indicating the closest relationship. And *A. macrocephala* and *A. coreana* have the minimum genetic identity (0.5201) and the maximum genetic distance (0.6537), indicating the remotest relationship. It can be concluded that *A. lancea* is most related to *A. chinensis*, then *A. coreana* and *A. japonica*, and *A. macrocephala* is the last.

RAPD-SCAR screening, analysis of sequence of *A. Lancea*-specific fragment and primer design

A specific primer AB5 (5′-CCCGAAGCGA-3′) was screened from the 80 RAPD primers for authentication of *A. lancea*. The distinctive band of *A. lancea* was about 1800 bp (Figure 1).

The *A. lancea*-specific fragment amplified with AB5 was cloned and sequenced (Figure 2). The total length was 1756 bp, and we found no homologous sequence in the GenBank.

A pair of SCAR primers F_1 (5'-AACAGAGAGAGCAGCGAAGG-3') and R_1 (5'-TCCCCAGTGTCATCCTCTTC-3') were designed corresponding to site 1112-1131 bp and site 1523-1542 bp. The amplification product was 431 bp.

RAPD-SCAR marker of *A. lancea*-specific DNA fragment

PCR amplification results of 54 individuals of 5 *Atractylodes* species using the SCAR primers were shown in Figure 3. *A. chinensis*, *A. coreana*, *A. japonica* and *A. macrocephala* had no SCAR band, while 38 individuals of *A. lancea* showed the SCAR band, in spite of the band width and brightness varied among different origins of *A. lancea*. For example, amplification bands of samples from Baokang in Hubei and Tangshan in Jiangsu populations were narrower than that of populations

Table 2. List of primers of RAPD and the amplification results.

Primers	Sequence 5'-3'	Total bands	Polymorphic bands	Percentage of polymorphic bands
AB2	GGAAACCCCT	9	4	44.44
AB4	GGCACGCGTT	12	11	91.67
AB5	CCCGAAGCGA	17	15	88.24
AH5	TTGCAGGCAG	10	7	70.00
AH6	GTAAGCCCCT	10	5	50.00
AH8	TTCCCGTGCC	9	9	100
AL17	CCGCAAGTGT	14	12	85.71
AL18	GGAGTGGACT	12	9	75.00
AL20	AGGAGTCGGA	11	6	54.55
AQ2	ACCCTCGGAC	8	5	62.50
AQ3	GAGGTGTCTG	17	15	88.24
AQ4	GACGGCTATC	17	16	94.12
AQ5	ACGGAGCTGA	14	10	71.43
AR11	GGGAAGACGG	10	10	100
AR13	GGGTCGGCTT	11	8	72.73
B04	GGACTGGAGT	13	11	84.62
B06	TGCTCTGCCC	8	6	75.00
B07	GGTGACGCAG	11	9	81.82
B08	GTCCACACGG	17	16	94.12
B11	GTAGACCCGT	9	7	77.78
B19	ACCCCCGAAG	12	11	92.67
F13	GGCTGCAGAA	18	15	83.33
G15	ACTGGGACTC	15	14	93.33
N13	AGCGTCACTC	14	13	92.86
Total	--	298	244	--
Average	--	12.42	10.17	81.88

Table 3. Genetic identities and distances of five species of *Atractylodes.*

Species	*A. japonica*	*A. coreana*	*A. chinensis*	*A. lancea*	*A. macrocephala*
A. japonica	****[a]	0.5906	0.6040	0.5705	0.5336
A. coreana	0.5266	****	0.6980	0.6376	0.5201
A. chinensis	0.5041	0.3596	****	0.7651	0.5268
A. lancea	0.5613	0.4501	0.2677	****	0.5268
A. macrocephala	0.6282	0.6537	0.6408	0.6408	****

[a] Nei's genetic identities (above diagonal) and genetic distances (below diagonal).

from Hushan in Jiangsu; Bands of Yingshan in Hubei and Xiaojiuhuashan populations were brighter than that of populations from Anhui and Hushan in Jiangsu, indicating that sequence diversity might exist among different populations of *A. lancea*.

DISCUSSION

Genetic relationship of *Atractylodes* species

The viewpoints concerning the systems, classification, and kinship of herbs are different. Lin and Shi (1987) recorded that *A. coreana* leaves were entire and sessile; lower and middle leaves of *A. japonica* and *A. macrocephala* were pinnatisect and long petiolate while the upper leaves were undivided. Xiao (2002) found that the entire leaves are lobate, sessile and petiolate and they coexisted in individuals of *A. lancea* and *A. chinensis*. Peng and Wang (2006) observed that the shapes of epidermal cells of *A. chinensi* can not be distinguished because of a continuous transition. Shi (1981) stated that *A. lancea* (Thunb) DC. and *A. chinensis* (DC.) Koidz should be merged into same

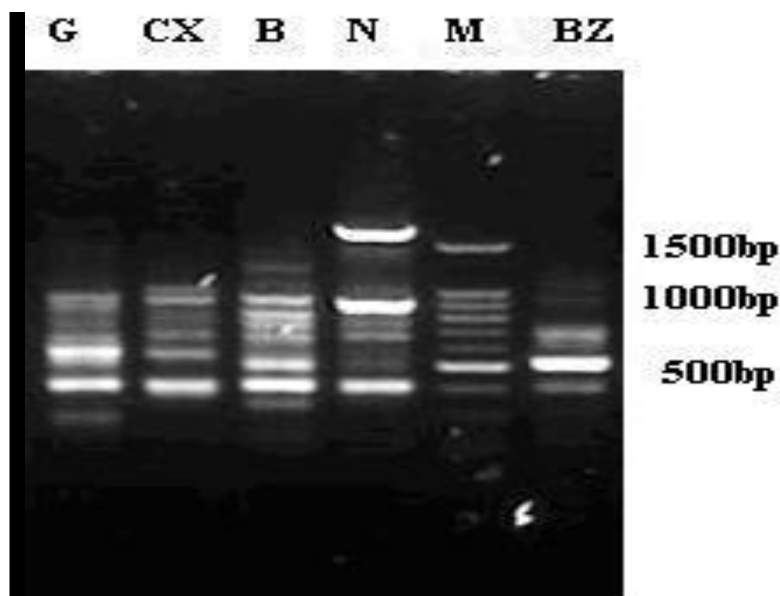

Figure 1. RAPD amplication of five *Atractylodes* species with primer AB5. G: *A. japonica*; CX: *A. coreana*; B: *A. chinensis*; N: *A. lancea*; M: DNA ladder; BZ: *A. macrocephala.*

5’-**CCCGAAGCGA**GGATCTCATGCTATCATTGAACGGCTACCGAACCGCCATACTCAGG
TAACCGGTCTAGGTTCCAAGTACATCAACACCCCATAGCTACTTAGGGCCGCAACGCAA
TAAGGCTTTTCGCTCTAGTTGGGCAATGCAAGGAGGCCTCTTTCGCCATTCTTTCTAAA
TGATAAACTATTATTAGTTCAGTTCGGTAGAACATCGATGAGAGGATAATTTTTTCACAG
AAGCATATGATTGCCCCTCAATCGCCAGGTGGCTCGCTCCAGGTGCATGAGTAGCTTTG
CTGGCTCTTATACGAAAGGTACCAGCCTCTTTCAGTGGTTCTTGCTTGCTCTTTAAAGC
TGATCTGGTGCTGTCTCGTTCCTTCAGTTTGTTCTGATTCCGATGAAGGATACTATATTC
CGCCTACACAACTACCTTGCTTGGTGGTTCAAAATGGAAGTCTAGGTATCTGCTTAAGT
GGTCTGCTCGTACCGTTCCAAAAAGAGCTGCGCTGACAGGCGTATTTGAAAATGTGTT
TCCGGGCTCACATATCTAAATTTCAGCGAGATAGGGTGCGGATAGGTGGAATGGAAATT
CCACTCCAATCCCTTTAATGGATCCCCGAAGGAGACGGGTGCAAATGGAATTTCAATCA
AAGGCGCTATGACCACTGAGCATGCCAACAAGGCAAACTCAGAAAATCGTCTCCTGTC
CCAGGTCACTGAAAGGGAGATACTCGCAGCCGAGCTCTCGTCCCACTCCCGCTGCCAA
CTTCGTGATAGAACGACCATGCGAAGGGATACGGATAGGCTGCCCTCCACACTTAATG
GCATTGTGTTACACCTTAATGTCTGGATTTAGCGTTATTGTCAAGCATCTCATTCACTACT
AAAGAAAGGTATGGATATTCTGCTCGAAAGCTTGACGAAGAAGCGTAAGCAAAAAGC
ATATTTTAGGAAAGAGGTCCAGTCAGCTGGTTACAGCTAATGAATGAAAAAACGTGTG
TGCCGATTTAGATTTCCCGTCTTAAGCCCCACCTCGGTAGCTCAGCGGTAGAGCGGCCT
CTTGTATAGTTCAACAGGTAGTCGGACGTGGGTTCAAATCCCGCTCGAGGGAAAA**AAC
AGAGAGAGCAGCGAAGG**GGCCTTTCCTCGCTTCTGTCTTTTTTTTTTTATATGATGCAT
CGAATGCTTCCTCTGCTTTCAGTAAAAATCCCAAAAGCCTATTCTCGAGGCACTCTTTC
TCTTATAGCGCTCTTTCCTTCCCTTCGAGCTAGCCGGAATGAATCCATTTCTTTTGTCTG
TAAGAGAAAAGGCGCTTATTCCATGAAATAGGAGCGCAGCGGAGTCATGAACAAAACA
AGAATAGCCACTTCCTTATCTACGCTATTTACTTTCCTCCCCTCGTGGGAAGTAGCAAG
AGCCTTTATCTAATCTACTATTGAACCATCTCACCTGAAAGTCAGTCGCTACCTTAAGGC
ATGCCTTGACTCCAAGAGCTAACTCGATGGGACTTGCTTTCCTAAAGAGAAGACGAAG
(3’**CTTC**
AGGATGACACTGGGGATCGTTCTTACTTAAAGAAATTCCCATGAGCTGCCTTGAGCT
TCCTACTGTGACCCCT5’)
GGTAACTCCAAAATCGATGATTCTTGGCCACTGACTTACTGCTTTCACTCAAATGCCAC
TGATGCGTAAGACATTGAGTTGGACTACTTTCCTTCTGGTAATGCCTTTCCCTGTGGTC
ACCTTGCTCTTCTACTTACTGGCTTGGCTATTTCGAAGGATTCGCTTCGGGAATC-3’
(3’**AGCGAAGCCC**5’)

Figure 2. Complete sequence of the *A. lancea*-specific DNA fragment. Sequences included in brackets are the complementary sequences; sequences underlined are the primers of SCAR marker; sequences bolded are the primer AB5.

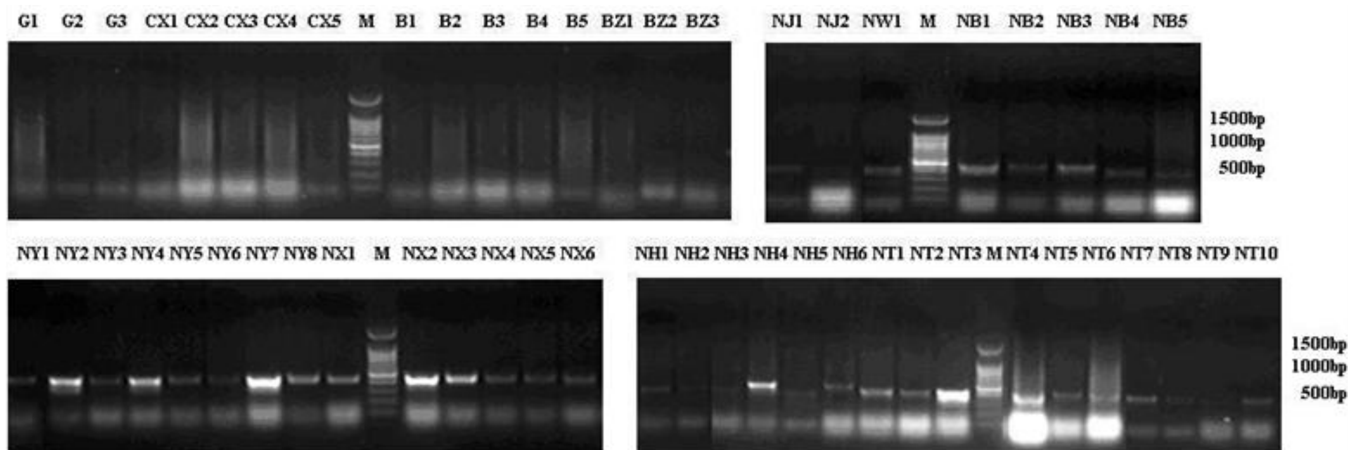

Figure 3. RAPD-SCAR analysis of five *Atractylodes* species *A. lancea* individuals. M: DNA ladder; G1-G3: *A. japonica*; CX1-CX5: *A. coreana*; B1-B5: *A. chinensis*; BZ1-BZ3: *A. macrocephala*; the left individuals are all *A. lancea*, of which NJ1-NJ2 are from Jinzhai in Anhui; NW1 is from Wuhu in Anhui; NB1-NB5 are from Baokang in Hubei; NY1-NY8 are from Yingshan in Hubei; NX1-NX6 are from Xiaojiuhuashan in Jiangsu; NH1-NH6 are from Hushan in Jiangsu; NT1-NT10 are from Xiaotangshan in Jiangsu.

species, while distribution regions for the *A. lancea* (Thunb) DC. and *A. chinensis* (DC.) Koidz didn't overlap. Ge (2007) came to the conclusion that the leaf shape and petiole showed a transition state and thus can't be used as the evidence for identification of *Atractylodes* species.

The distributing areas of *A. lancea* and *A. chinensis* are not overlapped, so the chemical components are not completely same (Kohjyouma et al., 1997; Hu et al., 2000; Ji et al., 2001; Nakai et al., 2006). For example, *A. lancea*, *A. chinensis* and *A. coreana* have a higher level of atractylodin, making them different from *A. japonica* which contains a large amount of atractylon (Fu, 1981; Ma, 1982). The volatile oil extracted from *A. chinensis* mainly contains β-eudesmol or β-eudesmol and atractylon, while the main components of *A. lancea* oil are hinesol, β-eudesmol and atractylon.

Sequences of region 1 of *trn*K gene, *matK* (Mizukami et al., 1998), *rbcL* (Li, 1998), *trnL-F* (Ge, 2007; Ge et al., 2007) of *A. lancea* and *A. japonica* were identical as ITS sequences of *A. chinensis* and *A. coreana* (Cheng et al., 1997; Shiba et al., 2006) did, though Ge et al. (2007) found stable variable sites in *trnL-F* of *A. lancea* and *A. chinensis*. Though sequences of DNA fragments can only reflect a reasonable level of genetic relationships, and stable intraspecific variations are likely to appear, it should be noted that identify of the corresponding fragments from *A. lancea* and *A. japonica* and the morphological characteristics seemed to contradict each other. Therefore, further study on transition and cross of leaf shape or petiole need to be considered.

The RAPD fingerprinting of this research demonstrated that *A. lancea* was close to *A. chinensis* while remoting with *A. macrocephala*. The genetic identities between *A. lancea*, *A. chinensis*, *A. corean* and *A. japonica* are nearly equal to their genetic distances, indicating the relationship of the 4 species and the similarity and transition of morphological characters further prove that. As stated above, Hu et al. (2000) suggested classifying Nancangzhu from different habitats as geo-varieties.

Identification of *A. lancea* and *A. chinensis*

It has been proposed that *A. lancea* and *A. chinensis* were distinguished according to the geographical distribution that is, populations from Jiangsu and Hubei being identified as *A. lancea*, while populations from Hebei, Shaanxi and shanxi being identified as *A. chinensis*, which has been supported by the studies of chemical composition (Kohjyouma et al., 1997; Hu et al., 2000; Ji et al., 2001; Nakai et al., 2006). Though our research has proved that the closet genetic distance and relationship existed between *A. lancea* and *A. chinensis*, the SCAR marker could separate them from each other. So it is reasonable to treat *A. chinensis* as the variety of *A. lancea* (Thunb.) DC. var. *chinensis* (Bunge) Kitam (Hu et al., 2000).

The morphological classification standard of *Atractylodes* has been changing but is still far from perfect. For example, Flora of China classifies the species according to the presence or absence of petioles of cauline leaves. But Ge (2007) found that many individuals of the species included into the petiolate group had no petioles. The facts also indicate the difficulty to differentiate species according to morphological characteristics. So it's more appropriate to identify the species of *Atractylodes* based on their distribution.

In this research an *A. lancea*-specific fragment was obtained and SCAR primers F_1 and R_1 were designed. It has been proved that *A. lancea* could be well distinguished from other *Atractylodes* species based on

this method. The *A. lancea*-specific RAPD-SCAR marker provides a simple, cheap and reliable procedure to authenticate the herb *A. lancea*.

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