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Full Length Research Paper

Inter-simple sequence repeats (ISSR) molecular fingerprinting markers for authenticating the genuine species of rhubarb

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Rhubarb is prescribed as the roots and rhizomes of *Rheum officinale* Baill., *Rheum palmatum* L., and *Rheum tanguticum* Maxim. ex Balf. in Chinese Pharmacopoeia. These three species are difficult to discriminate due to the morphological and anatomical similarity of the aerial parts and herbal medicines. In the present paper, inter-simple sequence repeats (ISSR) molecular fingerprinting markers have been employed to authenticate three genuine species of rhubarb using 15 primers to discriminate *R. officinale*, *R. palmatum*, and *R. tanguticum*. A total of 155 DNA fragments were amplified, of which 132 were polymorphic (85.2% of all bands). Four specific authentication markers have been found to authenticate three species of rhubarb. The UBC807 and UBC811 primers each generated one species-specific fragment that was clearly amplified in *R. officinale* (400bp from UBC807 and 248bp from UBC811). The application of the UBC807 primer also produced a 520bp DNA fragment from *R. palmatum* and *R. tanguticum*, and UBC816 primer generated a 620bp fragment that was specific for *R. palmatum* and *R. officinale*. Therefore, three loci combinations from primer UBC816 with any one of the primer UBC807and UBC811, that is UBC816-620bp with UBC807-520bp, UBC816-620bp with UBC807-400bp, and UBC816-620bp with UBC811-248bp, served the purpose of distinguishing three genuine species of rhubarb. To enhance the efficiency of authentication, ISSR fingerprinting codes have been constructed using four polymorphic bands for authenticating three genuine species of rhubarb. The present study could be applied to distinguish the genuine species of rhubarb at the molecular level.

Key words: Rhubarb, inter-simple sequence repeats-polymerase chain reaction, molecular marker, authentication.

INTRODUCTION

Definitive identification of original plant species is important for standardizing herbal medicine and quality assurance. Rhubarb is one of the most well-known and frequently used herbal medicines for thousands of years in China. As described in Chinese Pharmacopoeia, rhubarb consists of the roots and rhizomes of *Rheum officinale* Baill., *Rheum palmatum* L., and *Rheum tanguticum* Maxim. ex Balf. (Polygonaceae), (Chinese Pharmacopoeia Committee, 2010) all of which are endemic to China (Bao and Grabovskaya-Borodina, 2003). Considering that *R. officinale* is mainly grown in the Southern part of China, while the other two species are indigenous to the North, they were differentiated into two herbs under the name of “South Rhubarb” and “North Rhubarb”, respectively, in local herbal markets for hundreds of years. The rhubarb can benefit human health in many aspects, such as purgation, anti-inflammatory, antibacterial, purging heat, curing renal disorders, antitumor and antimutagenicity, etc. (Chinese Pharmacopoeia Committee, 2010; Huang et al., 2007). Ye et al. (2007) compared the compounds of *R. tanguticum*, *R. palmatum*, and *R. officinale* and suggested that due to the significant differences in chemical composition of three rhubarbs, the different

Table 1. Sources of *R. officinale*, *R. palmatum*, and *R. tanguticum.*

Species	Species code	Location	Voucher	Altitude (m)	Longitude	Latitude	No. of individuals
R. palmatum	RP	Wuwei, Gansu, China	Xu-mei Wang 08071807 (SANU)	2575	101°26.000′	38°10.000′	5
R. officinale	RO	Nanchuan, Chongqing, China	Xu-mei Wang and Xiao-qi Hou 09072609 (SANU)	1832	107°11.769′	29°00.008′	5
R. tanguticum	RT	Maqin, Qinghai, China	Jian-quan Liu Q99155 (LZU)	3580	100°14.189′	34°38.821′	5

rhubarb from different species should be separately used in clinical practices. On the other hand, comparing with the other two species, the rhubarb from *R. tanguticum* has the best quality, and widely cultivated, so it is not only commonly used in Chinese and Tibetan medicine products, but also in Chinese community all over the world and some other Asia countries. However, the three genuine species of rhubarb are difficult to be identified in morphology. Traditionally, species delimitation of three genuine species of rhubarb is mainly based on parameters of morphological features, such as depth of leaf division and color of perianth according to the description of Flora of China (Bao and Grabovskaya-Borodina, 2003). For example, the leaves of *R. officinale* and *R. palmatum* are lobed, and there is tiny difference between the lobed parts. For *R. officinale*, the lobed parts of blade are broadly triangular and the perianth is white, and the lobed parts of blade of *R. palmatum* are narrowly triangular and the perianth is purple-red. Whereas, the leaves of *R. tanguticum* are parted, lobed parts of blade are narrowly lanceolate and the perianth is usually purple-red. However, the parameters of three genuine species of rhubarb are judged subjectively and different individuals of the same species may present a variation in their morphology such as color of perianth according to our field survey from July, 2008 to August, 2009. The anatomical research also indicated that three genuine species were similar to and can not be distinguished from one another (Li and Zhang, 1983). Therefore, a technique to differentiate between the three species of rhubarb is needed. Recently, various types of DNA-based molecular techniques such as hybridization, polymerase chain reaction (PCR), and sequencing techniques provide more objective and reliable methods for authenticating herbal medicines (Joshi et al., 2004; Shcher and Carles, 2008). Among PCR-based methods, inter-simple sequence repeats (ISSRs) has been found to be an efficient and reliable technique established by Zietkiewicz et al. (1994) for the identification of species or varieties, population authentication and population genetic structure, etc. (Shen et al., 2006; Liu et al., 2009). Compared with random amplified polymorphic DNA (RAPD) method, amplified fragment length polymorphism (AFLP), and other molecular markers, the main advantages of ISSR are: no need for DNA sequence information prior to amplification, low cost, simple operation, high stability, and abundance of genomic information. ISSR analysis has been applied in herbal medicine to discriminate between species in the same genera (Qiu et al., 2003; Liu et al., 2009). The previous studies mainly focused on the discrimination of the medicinal rhubarb from the adulterants using chloroplast *trnL*/*trnF* sequences and RAPD markers (Yang et al., 2001, 2003) and the genetic relationship of the three genuine species of rhubarb (Chen et al., 2008; Suo et al., 2010). In the present study, ISSR markers were undertaken with the objective of establishing specific molecular markers for authenticating three genuine species of rhubarb and therefore guaranteeing the standardization of clinical prescriptions, chemical and pharmacological research of rhubarbs, and the local herbal markets.

MATERIALS AND METHODS

Plant materials

Five wild individuals from each of *R. officinale*, *R. palmatum*, and *R. tanguticum*, collected from main production areas of genuine medicinal materials of rhubarb in China, were used in this study (Table 1). The samples of three genuine species of rhubarb were collected from the main distribution regions in China. The identification of species was performed based on the Flora of China. Fresh leaf samples for each species were gathered and preserved in zip-lock bags with silica gel until required for DNA isolation.

DNA extraction

Complete genomic DNA was extracted from dried leaves using the modified cetyl trimethyl ammonium bromide (CTAB) method followed by Wang (2010). DNA concentration and purity were determined by spectrophotometry (ND-2000, NanoDrop, USA) and electrophoresis in the 1% agarose gels with known standards. For PCR amplification, the final concentration of each DNA sample was diluted to approximately 50 ng/µl with sterilized double distilled water.

Selection of primers

To select suitable primers for the study of the three genuine species

Table 2. ISSR primers used for ISSR-PCR of three genuine species of rhubarb.

Primer code	Sequence (5'→3')	Annealing temperature (°)	No. of amplified bands	No. of polymorphic bands
UBC807	$(AG)_8T$	51	11	8
UBC808	$(AG)_8C$	53	11	9
UBC810	$(GA)_8T$	51	13	11
UBC811	$(GA)_8C$	53	14	11
UBC816	$(CA)_8T$	52	12	10
UBC835	$(AG)_8YC$	52	10	9
UBC841	$(GA)_8YC$	52	7	7
UBC842	$(GA)_8YG$	56	11	9
UBC847	$(CA)_8RC$	51	9	6
UBC848	$(CA)_8RG$	51	10	9
UBC857	$(ACA)_5CYG$	52	9	9
UBC873	$(GACA)_4$	52	7	6
UBC880	$(GGAGA)_3$	52	8	7
UBC881	$(GGGGT)_3$	52	12	10
UBC890	$VHV(GT)_7$	56	11	11

R = (A, G); Y = (C, T); B = (C, G, T); D = (A, G, T); H = (A, G, T); V = (A, C, G).

of rhubarb, 100 ISSR primers synthesizes by Shanghai Sangon Biological Engineering Technology and Service (China), according to the primer set published by University of British Columbia, Canada (UBC set No.9), were screened using three DNA samples. And each DNA sample is from one of the three species. From the preliminary screening, 22 primers that could amplify visible bands were selected for further examination. Eventually, 15 ISSR primers that produced clear and reproducible bands were selected for the amplification of all DNA samples (Table 2).

ISSR profiling

ISSR amplifications were carried out in a 25 µl volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.4 µM primer, 1 unit *Taq* Plus DNA polymerase (Tiangen, Beijing, China), and approximately 50 ng template DNA.

Amplification was performed in a in a DNA Thermocycler PTC-200 (MJ Research, USA) with modifications of the annealing temperature to optimize the reaction conditions for individual primers. The reaction mixtures were denatured at 94℃ for 5 min, followed by 40 cycles of 1 min at 94℃, 1 min at 51 - 56℃ (Table 2), extension at 72℃ for 1 min, and a final extension step of 10 min at 72℃ and eventually stored at 4℃.

Amplified products were electrophoresed in 2% agarose gels with 1X TBE Buffer at 110 V for 1.5 h, and stained with ethidium bromide (0.5 µg/ml). Gels with amplification fragments were visualized and photographed in ultraviolet (UV) light by using Bio-Rad Gel Documentation System (Bio-Rad Laboratories, UK). A DL2000 ladder (TaKaRa Biotechnology, China) was used as DNA molecular weight standards.

Data analysis

The amplification products were scored in terms of a binary code as present (1) or absent (0), each of which was treated as a unit character regardless of its intensity. Genetic parameters including the polymorphism (the proportion of polymorphic loci to the total number of loci scored in all samples of the three species) and Nei's genetic distance were calculated using the computer program POPGENE (Yeh et al., 1997).

The obtained genetic distance matrix was then used to perform the cluster analysis and construct the UPGMA (unweighted pair group method with arithmetic mean) dendrogram using MEGA 4.0 (Tamura et al., 2007).

RESULTS

Concentration and purity of genomic DNA

DNA quantifications were performed by UV-spectrophotometer (ND-2000, NanoDrop, USA) and the purity was then determined by calculating the ratio of absorbance at 260 nm to that of 280 nm, with all ratios of OD_{260}/OD_{280} ranged from 1.6 to 1.9.

DNA concentration and purity was also determined by electrophoresis on 1.0% agarose based on the intensities of band when compared with l kb plus DNA ladder as marker. Through electrophoresis neither lagging nor degradation was found in each lane. The fragment size was about 30kb.

Products of ISSR amplification

Optimization of the annealing temperature for each primer was essential to obtain the best PCR performance. To control for individual variations, the author examined five samples per species. Consequently, a total of 155 clear and reproducible bands were amplified from fifteen individuals of three genuine species of rhubarb using the 15 selected ISSR primers, of which 132 were polymorphic. The loci numbers varied from 7 to 14 per primer (Table 2), with fragment size ranging from

200 - 1700 bp. ISSR fingerprinting of fifteen individuals of three species of rhubarb using primers UBC807, UBC811, and UBC816 is shown in Figures 1A - C. The lowest pair wise genetic distance was between RO-3 and RO-4, and the highest was between RO-5 and RT-4 (Table 4).

The mean Nei's genetic distance between *R. palmatum* and *R. tanguticum*, *R. officinale* and *R. tanguticum*, *R. officinale* and *R. palmatum* was 0.4139, 0.5717, and 0.5341, respectively. The fifteen individuals were clustered into two groups by UPGMA analysis based on Nei's genetic distance (Figure 2). Group I included five samples of *R. officinale*, while Group II comprised five samples of *R. palmatum* and five individuals of *R. tanguticum*, indicating that *R. palmatum* and *R. tanguticum* was similar.

Selection of specific authentication markers for the three genuine species of rhubarb

Valuable fingerprints were obtained using the primers, in which the reliable and highly polymorphic bands were useful as markers for species authentication. To authenticate the genuine species of rhubarb efficiently, three primers of the selected 15 primers can be used as identification markers based on four polymorphic bands. The identification markers were converted into 1 and 0, based on the presence or absence of the four polymorphic bands. Then the specific ISSR authentication codes of the three genuine species of rhubarb were constructed (Table 3; Figures 1A - C).

DISCUSSION

The discrimination of genuine species has become a key issue in the standardization of herbal drugs in traditional medicine because most of these medications are produced from herbs collected in wild habitats or cultivated on farms. However, the traditional identification depends on morphological and histological examinations. It is often difficult to accurately identify medicinal plants, or to differentiate and authenticate species within the same genus based on this subjective evaluation. This study was designed to produce a rapid genetic test for identification of three genuine species of rhubarb. In present study, the ability of 15 primers to generate polymorphic DNA fragments using ISSR markers was investigated. In total, 155 bands in fingerprinting were obtained, 132 of which were polymorphic (85.2%). This indicates that inter-simple repeat sequences are abundant and highly dispersed through the genome of the three genuine species of rhubarb and highly polymorphic. However, the polymorphic loci were low at the species level ranging from 10.32 to 56.87%. As the wild species, *R. officinale*, *R. palmatum*, and *R. tanguticum* are endemic to China, and the natural resource is limited. In recent years, the usage of rhubarb has been extended into the functional food. Therefore, the wild resource is decreasing significantly. The results shown above are consistent with the viewpoint that the level of genetic diversity is low in many endangered species (Li and Jin, 2007). To authenticate the genuine species of rhubarb efficiently and to control for individual variations, the author only selected the bands that were present in all the five individuals per species. The UBC807 and UBC811 primers each generated one species-specific fragment that was clearly amplified in *R. officinale* (400 bp from UBC807 and 248 bp from UBC811). The application of the UBC807 primer also produced a 520 bp DNA fragment from *R. palmatum* and *R. tanguticum*, and UBC816 primer generated a 620bp fragment that was specific for *R. palmatum* and *R. officinale*.

Therefore, three loci combinations from primer UBC816 with any one of primer UBC807 and UBC811, that is, UBC816-620 bp with UBC807-520 bp, UBC816-620bp with UBC807-400 bp, and UBC816-620 bp with UBC811-248 bp, served the purpose of distinguishing three genuine species of rhubarb. The present study is the first application of ISSR method to the medicinal rhubarb (that is, the genuine species of rhubarb) and the results make it possible to compare differences between rhubarb species at the molecular level.

DNA molecular markers used in the identification of traditional Chinese medicine and its cultivars, etc. has showed a very good development prospects. In previous studies, Yang et al. (2001) found a specific DNA fragment in the medicinal rhubarb which could be used to discriminate medicinal rhubarb from the adulterants through sequencing the *trn*L/*trn*F regions of chloroplast DNA. This method is accurate and reliable, but all sample need to be sequenced, thus, the cost is higher. RAPD analysis was also performed to authenticate the official from the unofficial genuine species of rhubarb, and the three officinal genuine species could also be distinguished using four DNA fragments (Yang et al., 2003). However, RAPD marker has poor reproducibility. In this study, the present author tried to find a quick and easy method of identifying the three genuine species of rhubarb. ISSR-PCR method is the same quick, simple operation as RAPD, while more accurate, and does not require sequencing, especially for the identification of multiple samples, therefore, the cost is reduced greatly.

Based on Nei's genetic distance between individuals, a cluster analysis was carried out and a dendrogram was generated that represented the genetic relationship among fifteen individuals. Interestingly, in the dendrogram, all the individuals of each species formed a monophyletic clade. Through comparing the Nei's genetic distance of all samples of three species, the genetic relationship between *R. palmatum* and *R. tanguticum* was

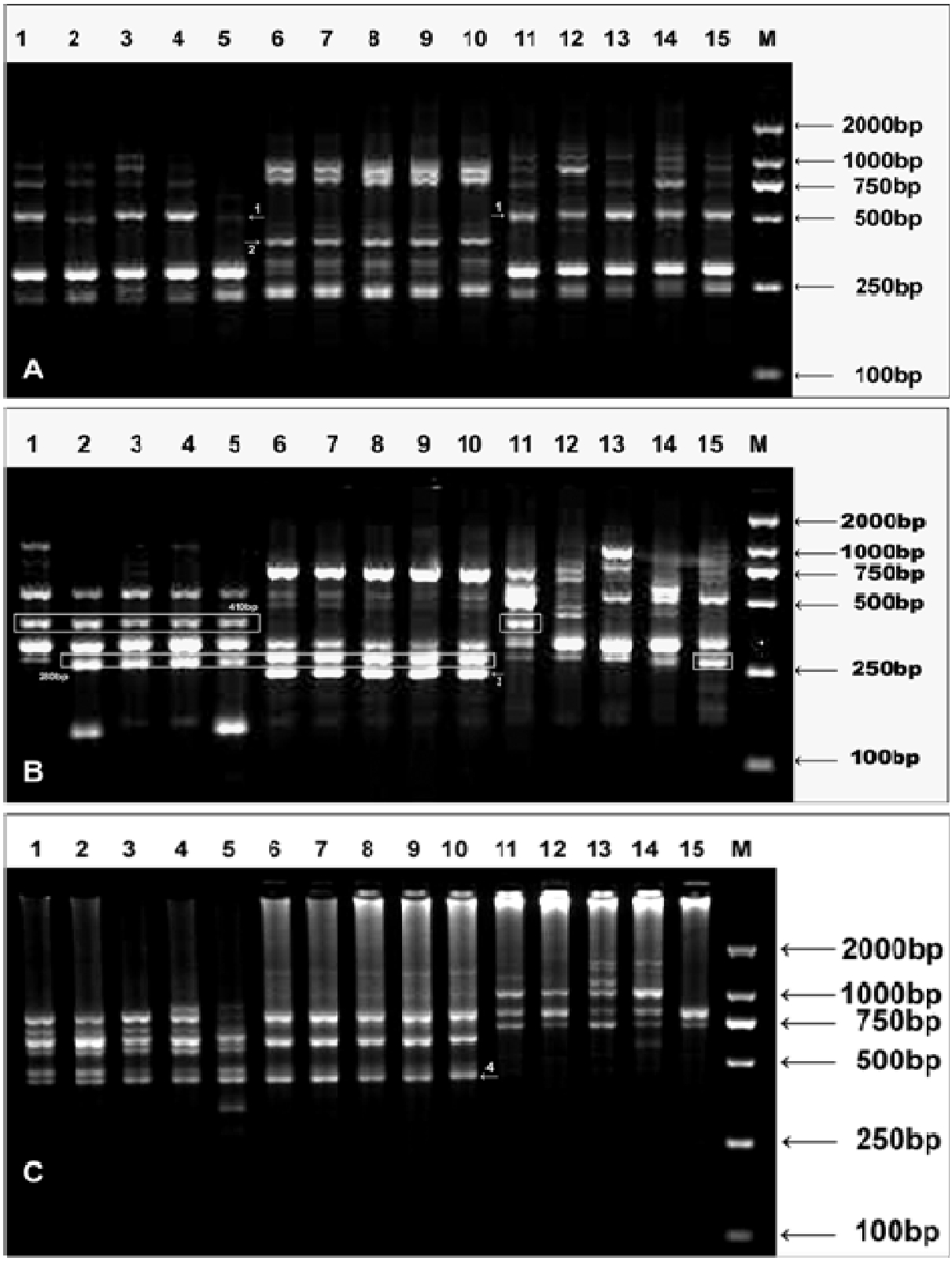

Figure 1. The ISSR fingerprinting of *R. palmatum*, *R. officinale*, and *R. tanguticum* using primer UBC-807(A), Primer UBC-811(B), and Primer UBC-816 (C). The lane numbers 1−5 are the individuals of *R. palmatum*, 6−10 are the individuals of *R. officinale*, and 11−15 are the individuals of *R. tanguticum*; M represents a DL2000 DNA marker; the bands marked with white arrows are the polymorphic bands used in Table 3, boxes indicate ISSR amplicons with the size of 410 and 280 bp.

Table 4. Pair-wise genetic distance of fifteen samples of three genuine species of rhubarb.

	RP-1	RP-2	RP-3	RP-4	RP-5	RO-1	RO-2	RO-3	RO-4	RO-5	RT-1	RT-2	RT-3	RT-4	RT-5
RP-1	—														
RP-2	0.1162	—													
RP-3	0.1531	0.1606	—												
RP-4	0.1682	0.1914	0.2312	—											
RP-5	0.2394	0.1682	0.3249	0.1914	—										
RO-1	0.4895	0.5001	0.5108	0.5108	0.5436	—									
RO-2	0.5001	0.5108	0.5001	0.5216	0.5548	0.0462	—								
RO-3	0.5326	0.5436	0.5108	0.5326	0.5661	0.0806	0.0462	—							
RO-4	0.5108	0.5216	0.4895	0.5326	0.5891	0.0667	0.0328	0.0130	—						
RO-5	0.5661	0.5775	0.5216	0.5661	0.6490	0.0736	0.0530	0.0462	0.0328	—					
RT-1	0.2727	0.3160	0.2898	0.3429	0.4283	0.5001	0.5108	0.5001	0.4791	0.5108	—				
RT-2	0.3990	0.3895	0.4185	0.4185	0.5108	0.5891	0.5775	0.5436	0.5436	0.5326	0.2813	—			
RT-3	0.5001	0.4687	0.4383	0.4383	0.5548	0.6126	0.6490	0.6367	0.6367	0.6246	0.3895	0.3895	—		
RT-4	0.3990	0.4087	0.4383	0.4383	0.4483	0.6126	0.6490	0.6367	0.6367	0.6490	0.2985	0.3339	0.3339	—	
RT-5	0.3895	0.3800	0.3521	0.4687	0.4383	0.5326	0.5436	0.5108	0.5326	0.5436	0.3249	0.3249	0.3072	0.3429	—

closer, while that of *R. officinale* and *R. tanguticum* wasar. The results were consistent with sequence-related amplified polymorphism (SRAP) marker by Chen et al. (2008). However, AFLP analysis among these three species showed that *R. officinale* and *R. palmatum* had closer genetic relationship (Suo et al., 2010).

The results indicated that the genetic relationship among three species is interlaced. In the present study, for example, the primer UBC811 reproducibly produced the distinct polymorphism. All samples of *R. palmatum* and one sample of *R. tanguticum* showed amplified product of 410bp (enclosed by upper boxes). And almost all samples of *R. officinale* and *R. palmatum* showed amplified band of 280bp (enclosed by lower boxes) except for one sample of *R. palmatum*. Meanwhile, one sample of *R. tanguticum* also had a 280bp fragment. This indicated that these three species are closely related and probably comprise a natural species complex within the total genus (Yang et al., 2001). In fact, their distribution ranges do overlap in a few sites according to our field survey. Therefore, for better understanding of the genetic relationship among three species, the analysis of much more samples of *R. officinale*, *R. tanguticum* and *R. palmatum* from the whole distribution is necessary. The establishment of a stable ISSR-PCR reaction system and amplification procedure is crucial for ISSR application.

Optimal conditions were selected based on the reproducibility and relatively high polymorphism bands of the PCR products. The annealing temperature plays a significant role in amplification conditions. The lower annealing temperature will result in the primers mismatch, while the higher temperature may enhance the specificity of binding, but it will reduce the combination degree between the primers and template. Although, the annealing temperature was based on the melting temperature (T_m) of the primers, four annealing temperatures 51, 52, 53, and 56°C were examined for all the primers in this study. Reliable ISSR-PCR systems for rhubarb have been established by analyzing the amplified bands emergence and successively ideal reaction conditions.

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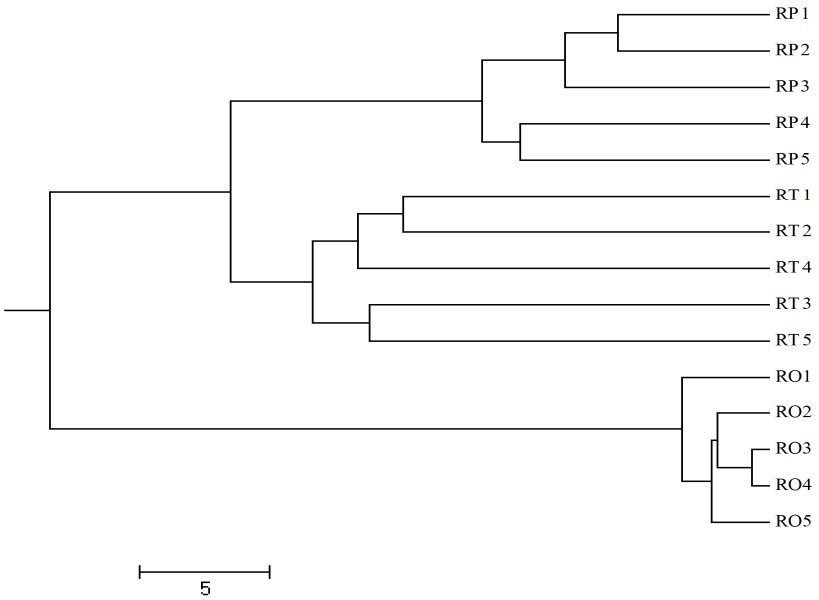

Figure 2. UPGMA dendrogram analysis based on Nei's genetic distance.

Table 3. ISSR Authentication codes of three genuine species of rhubarb.

Species code	ISSR authentication marker				Authentication code
	1(UBC807-520 bp)	2(UBC807-400 bp)	3(UBC811-248 bp)	4(UBC816-620 bp)	
RP	1	0	0	1	1001
RO	0	1	1	1	0111
RT	1	0	0	0	1000

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