Application of ISSR Markers to Fingerprinting of Elite Cultivars (Varieties/Clones) From Different Sections of the Genus *Populus* L.

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Abstract

The Inter-Simple Sequence Repeat (ISSR) was used in this study for genetic fingerprinting and identification of 28 important *Populus L.* (poplar) cultivars (varieties/clones), and determination of the genetic relationships among these cultivars. These 28 cultivars belonged to sections *Aigeiros, Tacahamaca, Leuce, Turanga,* and hybrids between sections *Aigeiros* and *Tacahamaca.* Out of 27 ISSR primers tested, eight primers generated clear multiplex profiles. The best three primers produced 154 easily detectable fragments, 129 (84%) of which were polymorphic among the cultivars. Each of these 3 primers produced fingerprint profiles unique to each of the accessions studied, and thus could be solely used for their identification. Twenty-five markers, unique to 10 of the cultivars studied, were detected. These markers may be converted into cultivar-specific probes for identification purposes. Genetic relationships among the cultivars were evaluated by generating a similarity matrix based on the simple matching coefficient and the unweighted pair group method with arithmetic average (UPGMA) dendrogram. The results showed a clear-cut separation of cultivars among different sections of poplar, and were in agreement with the genealogy of the sampled cultivars. The present study shows that ISSR markers could generate abundant polymorphism, are reproducible, and are quick for characterization of poplar cultivars. In the future, the markers used in this study, in combination with other molecular techniques, could provide a useful panel of ISSR markers for large-scale DNA fingerprinting of poplar cultivars and determination of the genetic relationships among these cultivars.

Key words: Poplar, ISSR, Cultivars identification, Genetic relationships.

Introduction

The genus *Populus L.* (Salicaceae), a genus of deciduous trees (rarely semi-evergreen), has a wide natural distribution in the Northern hemisphere. Various classifications have been suggested, the most recent recognizing 29 species that are grouped under six separate sections (ECKENWALDER, 1996). The economically most important species are in the sections *Aigeiros* Duby, *Tacamahaca* Spach., and *Leuce* Duby. These species and their interspecific hybrids constitute more than 90% of the cultivated poplars of the world (DICKMANN and STUART, 1983). In China, poplars are not only economically important for pulp, paper, architecture, and lumber industries, but also have been widely used for windbreaks and erosion control. Many poplar clones, cultivars, and varieties are widely cultivated, and many of them are endemic to China (ZHAO and CHEN, 1994; WANG *et al.,* 1984). Moreover, several new cultivars are being registered annually.

Most of the poplar species and hybrids are cultivated clonally through vegetative propagation. The unit of cultivation and breeding in poplars is clone, and the individual cultivars are represented by a single clone. Accurate identification of poplar cultivars and knowledge of their genetic interrelationships are essential for breeding, variety control and registration, stock handling, and protection of plant breeders' rights. The poplar seedling industry and breeding programs require especially reliable identification means that can be applied routinely to large numbers of samples. Traditionally, poplar clone and cultivar identification has been attempted with the combination of morphological and phenological characteristics. The method adopted by the International Poplar Commission for identification, registration, and certification of poplar clones is based on a total of 64 morphological, phenological and floral traits (IUPOV, 1981). However, this method of clone identification is time consuming and subjective.

Since the late 1980s, several molecular marker approaches have been successfully used for fingerprinting and identifying poplar clones, cultivars and species, and for determining the relationships among them. These molecular marker approaches include allozyme (RAJORA, 1989; JANSSEN, 1997; HEINZE, 1998), Randomly Amplified Polymorphic DNA (RAPD) (CASTIGLIONE *et al.,* 1993; LIN *et al.,* 1994; SIGURDSSON *et al.,* 1995), Amplified Fragment Length Polymorphism (AFLP) (CHAUHAN *et al.,* 2004; STORME *et al.,* 2004), and Simple Sequence Repeat (SSR) (RAHMAN *et al.,* 2000; RAHMAN and RAJORA, 2002; RAJORA and RAHMAN, 2003). ISSR has not yet been used for this purpose.

ISSR overcomes many of the technical limitations of RFLP and RAPD approaches (TSUMURA *et al.,* 1996) and has higher reproducibility than RAPDs (MEYER *et al.,* 1993; FANG and ROOSE, 1997). ISSR markers involve the PCR amplification of DNA, using single primers based on microsatellite sequences. These primers target microsatellites that are abundant throughout the eukaryotic genome (TAUTZ and RENZ, 1984; KIJAS *et al.,* 1995) and evolve rapidly (LEVINSON and GUTMAN, 1987). ISSR analysis has been used to assess genetic diversity in maize (KANTETY *et al.,* 1995) and bean (MÉTAIS *et al.,* 2000), as well as to identify cultivars of potato (PREVOST

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and WILKINSON, 1999), barley (FERNÁNDEZ *et al.,* 2002) and citrus (FANG and ROOSE, 1997).

In the present study, we report the use of ISSR markers for fingerprinting and identification of a selection of important poplar cultivars and for determination of the relationships among these cultivars.

Materials and Methods

Plant material

One clone (abscised branches) of each of the 28 cultivars was collected from the Chinese of cities of Beijing Drs., Hebei province, Liaoning province, and Shanxi province (*Table 1*). All of these poplar clones were planted in the collection of the Research Institute of Forestry, the Chinese Academy of Forestry (Beijing). Ten of these clones belonged to section *Aigeiros*, seven to section *Tacahamaca*, five to section *Leuce*, and one to section *Turanga*. Five were interspecific hybrids between sections *Aigeiros* and *Tacahamaca*.

DNA extraction

DNA was isolated using the CTAB method according to REICHARDT and ROGERS (REICHARDT and ROGERS, 1993), with minor modifications. Briefly, 0.2–0.5 g of young leaf tissue was ground in liquid nitrogen. Then, 2 ml of Solution I (2% w/v CTAB, 100 mM Tris-HCl, 20 mM EDTA, 1.4M NaCl, 2% v/v β-mercaptoethanol, 2% w/v polyvinylpyrrolidone, pH 8.0) was added and incubated at 65 °C for 60 min. The homogenate was then extracted with an equal volume mixture of chloroform:isoamylalcohol (24:1) and centrifuged at 8000 rpm for 10 min. The upper aqueous phase was recovered and incubated with 1/10 volume of Solution II (10% w/v CTAB, 0.7 M NaCl), prewarmed to 65 °C. The aqueous phase was then extracted with 1 volume mixture of chloroform:isoamylalcohol (24:1) and recovered as before. To the recovered aqueous phase, 1 volume of Solution III (1% w/v CTAB, 50 mM Tris-HCl, 10 mM EDTA, pH 8.0) was added and incubated 2 hours at 37 °C. The mixture was then centrifuged for 5 min at 3500 rpm, and the supernatant removed. The DNA pellet was then re-dissolved in Solution IV (10 mM Tris-HCl, 0.1 mM EDTA, 1 M NaCl, pH 8.0). Later it was treated with RNase A (200 ng/µl) for 60 min at 37 °C, and afterwards was extracted with 1 volume mixture of chloroform:isoamylalcohol (24:1) and recovered as before. After ethanol precipitation, the DNA pellet was washed with 70% ethanol, dried, and re-suspended in 0.1 ml of TE buffer (pH 8.0). The high molecular weight DNA was checked for quality and quantity using 0.8% agarose gel electrophoresis and fluorimetry (ND-1000 Spectrophotometer, NanoDrop).

PCR amplifications and electrophoresis of PCR products

PCR reaction mixture (20 µl) contained the following concentration/components: 1 U of Taq DNA polymerase

Table 1. – Poplar materials used in this study.

Code[a]	Cultivar(varity/clone)	Section	Species	Country of origin
L1	Maobaiyang(Datong)	*Leuce*	*P. tomentosa*	Datong, Shanxi
L2	Hebeiyang-1		*P. hopeiensis*	Datong, Shanxi
L3	Yinbaiyang(Datong)		*P. alba*	Datong, Shanxi
L4	Yinxingyang-2		*P. alba* × *P. bolleana*	Datong, Shanxi
L5	Xingjiangyang(Aertai)		*P. bolleana*	Datong, Shanxi
T1	Xiaoyeyang(328)	*Tacamahaca*	*P. simonii*	Datong, Shanxi
T2	Qinghaiqingyang(107)		*P. cathayana*	Datong, Shanxi
T3	Wutaiqingyang(77)		*P. cathayanai*	Datong, Shanxi
T4	BeiJingqingyang(Huairou)		*P. cathayana*	Huairou, Beijing
T5			*P. trichocarpa*	Datong, Shanxi
T6	Maoguoyang(309)		*P. cathayana*	Datong, Shanxi
T7	Zhongqing-10		*P. cathayana*	Datong, Shanxi
A1	Zhongqing-48	*Aigeiros*	*P.* × *canadensis*	Gu'an, Hebei
A2	Jiayang(Gu'an)		*P.* × *euramericana*	Shunyi, Beijing
A3	Oumei-107		*P. deltoides*	Datong, Shanxi
A4	Liaoheyang		*P. deltoides*	Langfang, Hebei
A5	Langfangyang-2		*P.* × *euramericana*	Dalian, Liaoning
A6	Gaiyang		*P. deltoides*	Fengning, Hebei
A7	Liaoningyang(Fengning)		*P. deltoides*	Dalian, Liaoning
A8	Liaoningyang(Dalian)		*P. deltoides*	Gu'an, Hebei
A9	Liaoningyang(Gu'an)		*P. nigra*	Datong, Shanxi
A10	Jianganyang(shanyin)		*P.* × *euramericana*	Datong, Shanxi
TA1	Oumei-13	*Tacamahaca*	*P. nigra* × *P. simonii*	Datong, Shanxi
TA2	Hezuoyang	×	*P. nigra* × *P. cathayana*	Changping, Beijing
TA3	Beijingyang-2	*Aigeiros*	*P. nigra* × *P. cathayana*	Datong, Shanxi
TA4	Zhongshang-8		*P. maximowiczii* × *P. deltoides*	Jianshi, Hubei
TA5	Gemeiyang(Hubei)		*P. nigra* × *P. simonii*	Datong, Shanxi
TU	Qunzhongyang		*P. euphratica*	Datong, Shanxi
	Huyang(Xingjiang)	*Turanga*		

[a]: Codes were used along of the article.

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Table 2. – Fingerprint patterns generated using 27 ISSR primers.

No.	Sequence	Amplification pattern	No.	Sequence	Amplification pattern
1	$(CA)_8$WG[a]	Smeared with bands	15	$(AC)_8$SC	Smeared with bands
2	$(CA)_8$GW	Smeared with bands	16	$(AC)_8$SG	No products
3	$(GA)_8$WC	Good	17	$(AG)_8$WA	No products
4	$(GA)_8$WT	Smeared with bands	18	$(AG)_8$WT	Good but Less Bands
5	$(TG)_8$SG	Smeared with bands	19	$(AG)_8$WC	Good but Less Bands
6	$(TG)_8$SC	No products	20	$(AG)_8$SA	Good
7	$(GT)_8$WG	Smeared	21	$(AG)_8$SC	Good but Less Bands
8	$(GT)_8$WA	No products	22	$(AG)_8$SG	No products
9	$(AC)_8$ST	Smeared with bands	23	$(TG)_8$SA	No products
10	$(TC)_8$SG	No products	24	$(TG)_8$WA	No products
11	$(AC)_8$WA	Good but Less Bands	25	$(TG)_8$WT	Smeared
12	$(AC)_8$WT	Good but Less Bands	26	$(GT)_8$SC	Smeared
13	$(AC)_8$WG	Smeared	27	$(GT)_8$SG	No products
14	$(AC)_8$SA	Good			

[a]: W = A or T, S = C or G.

(Toyobo, Japan), 10 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.4 µM of primer, 0.2 mM of each dNTP (Shanghai Sangon, China), 2.5% formamide and 30 ng of template genomic DNA. DNA amplifications were performed in a Mastercycler Gradient 5331 (Eppendorf, Germany) using the following touchdown programme: 3 min at 94°C for 1 cycle; 30 s at 94°C, 60 s at 62°C and 80 s at 72°C for 1 cycle; annealing temperature at 62°C being subsequently reduced by 1°C for the next 10 cycles and remained at 52°C for the remaining 24 cycles; 7 min at 72°C for 1 cycle. For primer screening, PCR products were analyzed on 1.5% agarose gels and stained with ethidium bromide. The gels were visualized with ultraviolet light, and the information of amplification patterns was recorded manually. Amplification products of the selected primers were separated on 4% denaturing polyacrylamide gels (230 mm x 300 mm x 0.4 mm). After silver staining (BASSAM *et al.,* 1991), the gel was dried at room temperature and photographed. A 100-bp ladder was used to estimate the molecular size of fragments.

Primers and band profile reproducibility

Out of 27 primers tested (*Table 2*), 3 were selected for fingerprinting poplar cultivars, according to their amplification patterns. All the generated patterns were repeated twice in order to verify reproducibility. Two replicate DNA extractions of a subset of 5 samples, including L5, T2, T6, A2, and A8, were used to assess the consistency of the band profiles. Negative controls, consisting of a complete reaction mixture without the template DNA, were used to test for the presence of non-specific amplification.

Analysis of amplification profiles

ISSR bands that behave as dominant markers were scored for the presence (1) or absence (0) of homologous fragments for all cultivars. Only reproducible bands that ranged from 200 to 1500 bp were scored. The genetic relationships among the cultivars were determined by calculating the simple matching coefficient, estimated as $SM_{ij} = (a+d)/(a+b+c+d)$, where SM_{ij} is the measure of SM between sample i and j, a is the number of shared present fragments by i and j, d is the number of shared absent fragments by i and j, b is the number of fragments present in i and absent in j, and c is the number of fragments present in j and absent in i. The resultant pair-wise similarity matrix was employed to construct cluster plots by UPGMA. Data were analyzed with SPSS program (version 11.5). The 0/1 matrix is available to readers upon request.

Results

Primer selection

To select primers useful for poplar genotyping, 27 ISSR primers (*Table 2*) were tested using a subset of the DNA templates or all DNA templates. These primers had either different dinucleotides core repeats or anchored nucleotides. Eight of the 27 primers generated clear multiplex profiles (Good/Good but Less Bands), among which $(GA)_8$WC, $(AC)_8$SA and $(AG)_8$SA produced the best ISSR profiles. In addition, the results also showed that the most of primers based on GA/AG and AC/CA dinucleotides core repeats generated good profiles, which seem to indicate that the more frequent microsatellites in poplar contain the repeated dinucleotides $(AG/GA)_n$ and $(AC/CA)_n$. However, the other 19 primers produced no products at all, or only smears or smears with bands that could not be scored. Modification on the annealing temperature, primers concentration, and template concentration did not improve their patterns.

Fingerprint patterns and pattern reproducibility

ISSR amplification for all samples resulted in multiple band fingerprint profiles for the 3 selected ISSR primers (for example, *Figure 1*). The average number of scorable fragments per primer was 51, with a range from 42 to 59, while the average number of polymorphic fragments per primer was 43, with a range from 36 to 49. Out of the total 154 scorable fragments, 129 (84%) were polymorphic among the cultivars (*Table 3*). In addition, 25 fragments unique to 10 of the sampled cultivars were identified. These may be developed into cultivar-specific probes useful for identification purposes. Each of the selected primers produced fingerprint profiles unique to each of the 28 cultivars, and it was possible to distinguish all cultivars.

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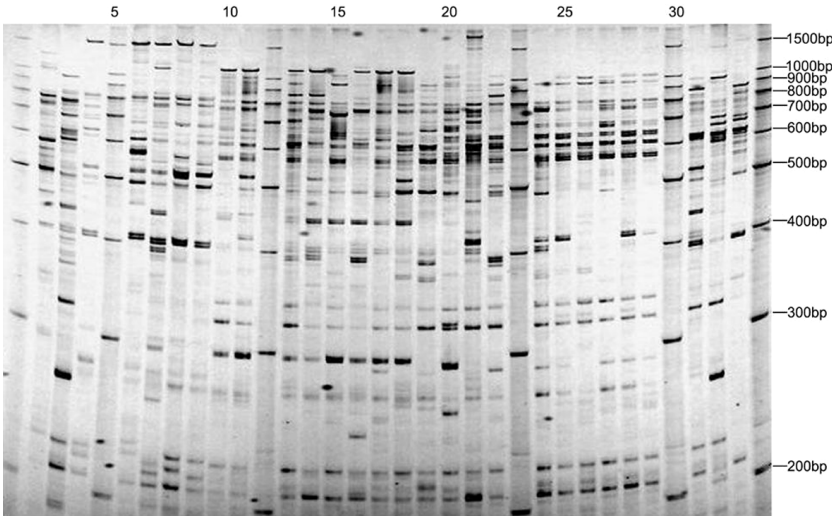

Figure 1. – Fingerprint patterns generated using primer $(GA)_8RC$. Lanes 1, 5, 12, 23, 30, 34 are DNA Marker, the remaining lanes from left to right correspond to poplar samples TU, TA5, L1, L2, L3, L4, L5, T5, TA1, T2, T3, T7, T4, T6, TA2, A2, TA4, A1, A10, A3, A8, A5, A4, A7, A6, TA3, A9, T1.

Table 3. – The polymorphism detected by using the 3 selected ISSR primers.

Primer	Total fragments	Polymorphic fragments	Percent polymorphic fragments	Unique fragments
$(GA)_8RC$	53	44	83%	9
$(AC)_8YA$	42	36	86%	6
$(AG)_8YA$	59	49	83%	10
Total	154	129	84%	25
Average	51	43	84%	8

In the current experiment, the amplification of ISSR markers was consistent across 2 replicate DNA extractions from 5 samples, with over 98% of the scoreable fragments reproducible (data not shown). Very faint fragments and a few well-amplified fragments were not reproducible, and such fragments were not scored in this study. The negative control without the template DNA always failed to show the presence of any amplified product, and was consequently not shown here in the results.

Genetic relationships among cultivars

The simple matching coefficients (data not shown) for the 378 possible pairs of 28 poplar cultivars ranged from 0.513 to 0.961. Cultivars in *Turanga* and *Leuce* and cultivars in T*acamahaca*, *Aigeiros* and *Tacamahaca* x *Aigeiros* shared very low genetic similarity with the SM value ranging from 0.513 to 0.695.

The dendrogram (*Figure 2*) obtained using SM coefficients had two main clusters (0.603 of similarity): cultivars from *Turanga* and *Leuce* (a) and cultivars from *Tacamahaca*, *Aigeiros* and *Tacamahaca* x *Aigeiros* (b). The first main cluster (a) was divided clearly into two sub-clusters (0.704 of similarity), i.e, a1 that contained only cultivar *Tu* and a2 that contained all *Leuce* cultivars. Similarly, two sub-clusters, (b1) and (b2), (0.654 of similarity) were assigned within the main second cluster (b). Sub-cluster (b1) including 5 cultivars in *Tacamahaca* was further discriminated into two groups (0.702 of similarity): group (c) and group (d). Group (c) had cultivar TA2 TA3, TA5, A9, and (d) had cultivar T1, T2, T3, T4. Similarly, sub-cluster b2 including 9 cultivars in *Aigeiros* was divided into two groups (0.749 of similarity): group (e) and group (f). Group (e) had cultivar T5, T6, T7, TA1, TA4 and group (f) had cultivar A1, A2, A3, A4, A5, A6, A7, A8, A10.

Discussion

In this study, all 3 ISSR primers produced highly polymorphic band profiles. Furthermore, each of these primers produced fingerprint profiles unique to each of the cultivars. Therefore, each primer can be used separately to identify these cultivars in the future.

Our results indicate that the genetic relationships among closely related poplar cultivars, inferred by using ISSR markers, were in accordance with their genetic origin and genealogy. A6, A7 and A8 belong to "Liaoningyang", which comes from a cross "I-69 (*P. deltoides* Bartr. cv. 'Lux' ex I-69/55) x *P. deltoides* cv. Shanhaiguanensis" and consists of 6 clones difficult to discriminate morphologically (ZHENG *et al.,* 2003b). In terms of the cluster plot, the 3 cultivars were very closely related (0.961 of similarity), and thus they might be different "Liaoningyang" clones. A4 also showed a close relationship (0.944 of similarity) with "Liaoningyang" because they originate from the same cross (ZHENG *et al.,* 2003b).

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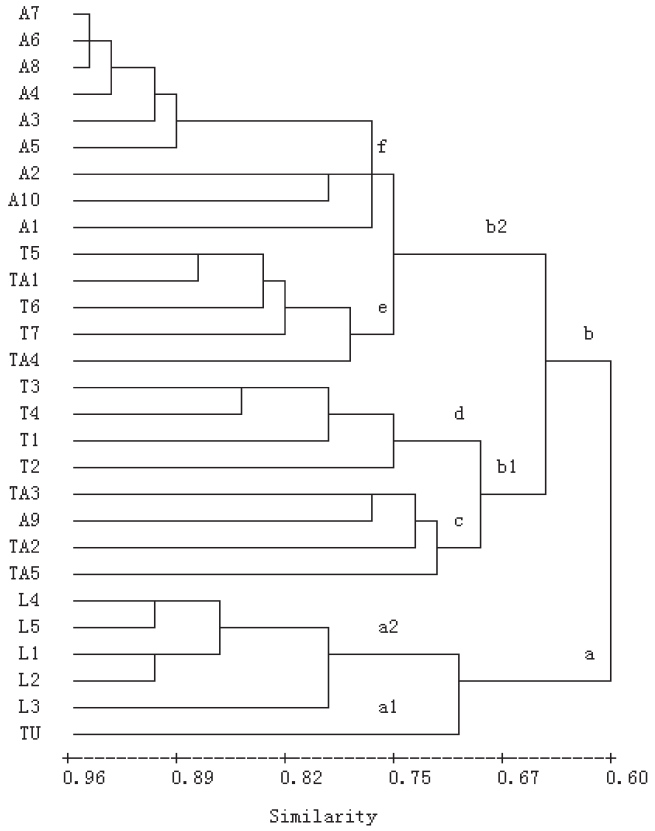

Figure 2. – UPGMA dendrogram based on the simple matching similarity coefficient.

"Liaoningyang", A4, A3, and A5 all originat from a half-sib family, of which *P. deltoides* cv. Shanhaiguanensis is their common parent. Their other parents are I-69, I-69, I-63 (*P. deltoides* Bartr. cv. 'Harvard' ex I-63/51) and I-72 (*P.* x *euramericana* (Dode) cv. 'San Martino' I-72/58), respectively (ZHENG *et al.,* 2003b). I-69 and I-63 belong to southern *P. deltoides* genotypes while I-72 is an interspecific hybrid genotype between *P. deltoides* and *P. nigra.* These three varieties, however, possess very similar agronomic traits and are cultivated in the same area (ZHENG *et al.,* 2003a). Therefore, "Liaoningyang", A3, A4 and A5 were grouped into a cluster with a higher similarity level (0.892 of similarity). The similar breeding program applied to these six cultivars also likely led to their high level of similarity. In practice, "Liaoningyang", A3 and A5 were bred by the same breeders (ZHENG *et al.,* 2003b).

TA1 originated from a cross *P. simonii* x *P. nigra* var. *Pyramidalis* (DUAN and ZHANG, 1997) was more closely related with T5 (0.877 of similarity) native to North America than with TA5 (0.662 of similarity) coming from the similar cross *P. simonii* x *P. nigra* var. *Pyramidalis* + *Salix matsudana* (ZHENG *et al.,* 2002). Although some researchers thought that TA5 got genetic influence from *Salix matsudana* Koidz. (DUAN and ZHANG, 1997), further experiments are needed to clarify this issue by using more ISSR primers or different molecular marker systems.

In UPGMA dendrogram (*Figure 2*), all *Leuce* cultivars (L1, L2, L3, L4, L5, 0.795 of similarity) were clustered into one cluster (a2) while TU separated as a unique cluster representing *Turanga* section (a1). Group f (0.767 of similarity) was mainly composed of *P. deltoids* cultivars (A3, A4, A6, A7, A8) and hybrids cultivars (A1, A2, A5, A10) between *P. deltoids* and *P. nigra*, which all belonged to *Aigeiros* section. Group e (T5, T6, T7, TA1, TA4, 0.779 of similarity) and group d (T1, T2, T3, T4, 0.702 of similarity) contained different *Tacamahaca* cultivars. These two groups, however, were not further grouped into a larger group, but separately clustered with group f and group c (0.719 of similarity), which included 3 interspecific hybrids (TA2, TA3, TA5) between *Aigeiros* and *Tacahamaca*, and 1 *Aigeiros* cultivars (A9) native to northwest of China. Our results indicated that genetic relationships of poplar cultivars inferred from the ISSR matrix were in agreement with the genealogy of the poplar cultivars studied. Thus, ISSR can be used to study genetic relationships of poplar cultivars and distinguish closely related poplar cultivars.

In the current experiment, the amplification of ISSR markers was consistent across DNA samples extracted from different leaves of the same tree, with over 98% of the scoreable fragments reproducible. This was in concordance with previous studies where the reliability of the protocol was demonstrated between PCRs, DNA extractions and even laboratories when applied to oilseed rape (CHARTERS *et al.,* 1996).

In the standard PCR analysis of microsatellites, sequencing and primer development are time consuming and expensive for a single marker. In contrast, it is very quick to screen and optimize for many ISSR primers. In our experiment, primarily screening and optimization of 27 ISSR primers only took about a week. Furthermore, by using touchdown PCR strategy, which could also improve specificity of PCR, screening and optimization of primers became quicker and simpler.

It is essential for future breeding programs to determine genetic diversity and genetic relationships of native and exotic germplasm resources in poplar by using various molecular markers. On the other hand, the poplar seedling industry requires an especially reliable mean of cultivar identification that can be applied routinely to large numbers of samples. The present work has shown that ISSR analysis is quick and reproducible, can generate sufficient polymorphisms, and has potential for large-scale DNA fingerprinting purposes of poplar cultivars, although most ISSR alleles are dominant rather than co-dominant.

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