Full Length Research Paper

Sequence characterized amplified region (SCAR) markers-based rapid molecular typing and identification of *Cunninghamia lanceolata*

Aihua Shen[1,2#], Haibo Li[2#], Ke Wang[1], Hongmei Ding[3], Xin Zhang[4], Lin Fan[4] and Bo Jiang[2*]

[1]College of Environmental and Resource Sciences, Zhejiang University, Hangzhou, 310027, China.
[2]Zhejiang Forestry Academy, Zhejiang Provincial Key Laboratory of Biological and Chemical Utilization of Forest Resource, Hangzhou, 310023, China.
[3]Zhejiang Chinese Medical University, Hangzhou, 310053, China.
[4]School of Forestry and Biotechnology, Zhejiang Agriculture and Forestry University, Hangzhou, 311300, China.

Chinese fir (*Cunninghamia lanceolata* (Lamb.) Hook.) is the main timber and afforestation tree species in South China. *C. lanceolata* clones, with similar morphological characteristics, make their exact identification very difficult. A simple and effective method is required to distinguish these clones in the clonal selection and breeding of *C. lanceolata*. However, *C. lanceolata* clones have so far been identified only through traditional phenotypic analysis. In the present study, 20 *C. lanceolata* clones from different geographic provenances were collected and analyzed using randomly amplified polymorphic DNA (RAPD) technique. Nine polymorphic RAPD bands were cloned and sequenced. Six stable informative dominant sequence characterized amplified region (SCAR) markers were developed by designing six pairs of specific SCAR primers from six sequenced polymorphic RAPD bands, respectively. On the basis of the differences in SCAR phenotypes detected among the 20 *C. lanceolata* clones, 10 individual clones were identified from 20 studied clones at first, and the other 10 clones were divided into 4 distinguishable groups. Some *C. lanceolata* clones from the same geographic provenances were also distinguished from one another. The experiment was the first report that SCAR markers-based molecular method was used in the research on molecular identification of *C. lanceolata*. By developing and jointly using specific useful SCAR makers, rapid molecular typing of *C. lanceolata* clones was accomplished, which could be used as a tool for the identification of *C. lanceolata* clones.

Key words: Chinese fir, *Cunninghamia lanceolata*, tree breeding, SCAR markers, molecular typing, identification.

INTRODUCTION

Chinese fir (*Cunninghamia lanceolata* (Lamb.) Hook) is an important timber and afforestation species in South China. *C. lanceolata* widely grows in various types of ecological environment and has long been cultivated in different natural areas of China (Figure 1). *C. lanceolata* is one of several species with strong sprout capacity in needle-leaved trees and appropriate for vegetative propagation. *C. lanceolata* is distributed across nine geographic provenance regions in China. Remarkable genetic variation exists among *C. lanceolata* from these different regions. However, in the past decade, the frequent exchange of *C. lanceolata* seeds among different provenance regions and the introduction of *C. lanceolata* seeds from one provenance region to another made the seed resource confusing. The fast developing clonal selection and breeding of *C. lanceolata* in different areas of China have not only made the source of these clones uncertain but also led to confusion in the intellectual property rights of *C. lanceolata* breeding materials. In the

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*Corresponding author. E-mail: 1178646251@qq.com. Tel: +86 571 87798226; Fax: +86 571 87798206.
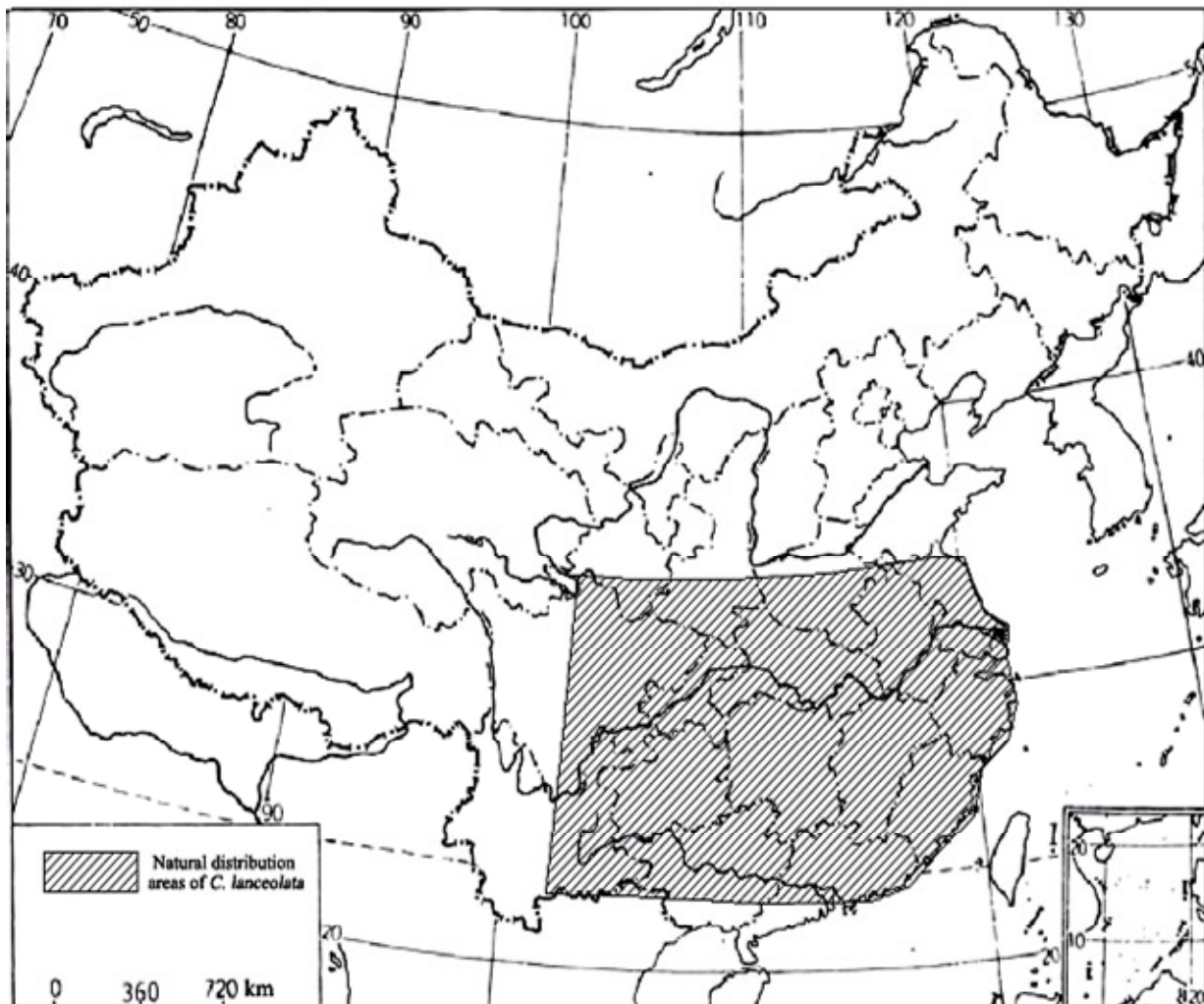
#These authors contributed equally to this study.

Figure 1. The natural distribution of *Cunninghamia lanceolata* in China.

cultivation areas, identical *C. lanceolata* seeds or clones were commonly named after the different provenance regions. The exact identification of *C. lanceolata* clones with similar morphological characteristics, using only the traditional phenotypic analysis was very difficult. Thus, the search for a simple and effective method to distinguish *C. lanceolata* clones from one another, especially in identifying superior *C. lanceolata* clones from other clones, is of great importance.

Since the end of the last century, PCR-based molecular markers, mainly the randomly amplified polymorphic DNA (RAPD), have been widely used in the identification of germplasm resources, genetic mapping, quantitative characteristic site location, and marker-assisted selection of *C. lanceolata* (You, 1998; You and Hong, 1998; Hang et al., 2000; Chen, 2001; Wang and Ma, 2005). In recent years, inter-simple sequence repeats (ISSR) markers have also been used in the hybrid identification of *C. lanceolata* × *Platycladus orientalis* and the molecular polymorphic analysis of the geographic provenances of *C.*

lanceolata (Qi, 2008; Yang et al., 2009). As a precious industrial timber species, *C. lanceolata* is characterized by fast growth, high-quality timber, high production, and wide use. Recent research work have also been done on gene cloning relative to wood-forming of *C. lanceolata*, using reverse transcription polymerase chain reaction (RT-PCR), differential-display reverse Transcription-PCR (DDRT-PCR), and complementary DNA amplified fragment length polymorphism (cDNA-AFLP) techniques, and constructing cDNA library derived from the bark of *C. lanceolata*. Some genes, such as CCoAOMT and cinnamoyl-CoA reductase (CCR), were cloned, and some expressed sequence tag (ESTs) were annotated (Yang and Shi, 2005; Fu et al., 2008, 2009; Jiang et al., 2009; Li et al., 2010; Cheng et al., 2010).

Although the use of RAPD and ISSR techniques promoted the research of *C. lanceolata* germplasm resources to the molecular level, these markers were not the most ideal techniques in practical application. The RAPD method often detects between five and ten bands

Table 1. *Cunninghamia lanceolata* clones surveyed in this study.

Sample number	Clone Name	Geographic provenance (China)	Collect area (China)
1	Long15	Zhejiang	Zhejiang
2	Xiang5	Hunan	Hunan
3	Qian2	Guizhou	Guizhou
4	Fujian1	Fujian	Guizhou
5	Zhejiang15	Jiangxi	Zhejiang
6	Xiang23	Hunan	Zhejiang
7	Xiang4	Hunan	Hunan
8	Zhejiang16	Jiangxi	Zhejiang
9	Xiang3	Hunan	Zhejiang
10	Zhejiang5	Fujian	Zhejiang
11	Zhejiang13	Guangdong	Zhejiang
12	Kai3	Zhejiang	Zhejiang
13	Qian4	Guizhou	Guizhou
14	Zhejiang4	Hunan	Zhejiang
15	Xiang1	Guizhou	Guizhou
16	Xiang12	Guizhou	Guizhou
17	Zhejiang8	Guangxi	Zhejiang
18	Zhejiang14	Sichuan	Zhejiang
19	Zhejiang7	Fujian	Zhejiang
20	Zhejiang2	Hunan	Zhejiang

but can produce some false positives and negatives bands because of its competitive reaction (Heun and Helentjaris, 1993). The complex patterns generated by RAPD made the analysis of gels tedious and time-consuming. Alternatively, the ISSR technique needs more time in establishing optimal reaction conditions, which often varies with different ISSR primers. Thus, a simple and effective method is still required, which can serve as a tool for distinguishing the *C. lanceolata* clones in routine procedure.

To overcome the reproducibility problem associated with the RAPD technique, a sequence characterized amplified region (SCAR); (Paran and Michelmore, 1993) was developed. A SCAR marker was detected through PCR using specific oligonucleotide primers designed on the basis of the sequence data of one RAPD band and following PCR amplification under more stringent conditions. In recent years, the informative dominant SCAR markers have been widely and successfully used in the identification of grapevine cultivar (Vidal et al., 2000), bamboo species (Das et al., 2005), medicinal herbs species (Dnyaneshwar et al., 2006; Choi et al., 2008), and *Lentinula edodes* strains (Li et al., 2008; Wu et al., 2010). In *C. lanceolata*, Li et al. (2007) developed two SCAR markers for the identification of satellite chromosomes in *C. lanceolata* seeds.

The present study aims to develop a simple PCR-based method for rapid molecular typing and identification of *C. lanceolata* clones in China. This goal was achieved by screening differential RAPD bands among studied *C. lanceolata* clones from different provenances in China,

developing stable SCAR markers, and jointly using the SCAR markers to distinguish the studied clones. The present study is the first to describe the joint use of SCAR markers for the molecular typing of *C. lanceolata*.

MATERIALS AND METHODS

Sample collection

Twenty samples of high-quality *C. lanceolata* representing different provenances in China were surveyed and sampled (Table 1). Young leaf material from these randomly selected trees was collected and stored with silica gel in zip-locked plastic bags until use.

Isolation of genomic DNA

Genomic DNA was isolated from the young leaf following the protocol of Möller et al. (1992). The concentration and purity of the DNA sample were determined through agarose gel electrophoresis and absorbance in a GeneQuant Pro DNA/RNA spectrophotometer (GE Healthcare, Cambridge, UK). Only DNA samples with $OD_{260}/OD_{280} > 1.8$ were diluted in ultra-pure sterile water and stored in $-20°C$ for PCR analysis.

RAPD analysis

RAPD amplification was performed in a 20 μl reaction mixture containing 1 × PCR buffer, 75 ng genomic DNA, 2.5 mM $MgCl_2$, 250 μM of each dNTP, 0.3 μM of RAPD primer, and 1.5 U of *Taq* DNA polymerase (Hangzhou Bioer, China). The 10-mer RAPD primers were synthesized by Sangon Biological Engineering Technology and Services (Shanghai, China) Co., Ltd. Amplification was performed in a DNA thermal cycler (TC-XP, Hangzhou Bioer, China) using the

following parameters: 5 min at 94°C, followed by 30 cycles of 40 s at 94°C, 40 s at 36°C, 2 min at 72°C, and a final extension cycle of 7 min at 72°C. The amplified PCR products were resolved electrophoretically in a 1.5% agarose gel in a Tris-Acetate-EDTA buffer. Gels were visualized through ethidium bromide staining and recorded using an automatic gel imaging analyzer (JS-380A, Shanghai PeiQing, China).

Cloning and sequencing of RAPD bands

The polymorphic RAPD bands were excised in agarose gels and purified using the BioSpin Gel Extraction Kit (Bioer, Hangzhou, China). The bands were then ligated into the pMD-18T vector (Takara, Dalian, China). The inserted DNA fragments were sequenced using ABI 377 automated DNA sequencer with a pair of sequencing primers, BcaBEST RV-M and BcaBEST M13-47. The nucleotide sequences of the cloned PCR products were determined using at least two samples isolated from different colonies by the GeneCore BioTechnologies (Shanghai, China) Co., Ltd.

SCAR primers design and SCAR markers

Based on the sequenced RAPD bands, the oligonucleotide primer pairs were designed as SCAR primers using the Oligo 6.54 software (MBI, USA). The sequences of SCAR primers were synthesized by the Sangon Biological Engineering Technology and Services (Shanghai, China) Co., Ltd. The PCR reaction conditions for genomic DNA amplification with SCAR primers were the same as those of RAPD primers, except for the annealing temperatures. The optimal annealing temperatures to achieve specific amplifications were determined by gradually increasing this parameter in successive trial runs. The amplified PCR products were analyzed.

RESULTS

Screening and sequencing of polymorphic RAPD bands

Eight of the seventy-five RAPD primers screened produced distinct, reproducible, polymorphic profiles within the 20 studied *C. lanceolata* clones listed in Table 1. The approximate size range of these polymorphic bands was from 500 to 1500 bp. Of the eight RAPD primers, three primers (S63, S90, and S500) amplified a single bright polymorphic band, one primer (S511) amplified two polymorphic bands, three primers (S88, S1002 and S1015) amplified three polymorphic bands, and one primer (S2059) amplified four polymorphic bands (Figure 2). The nine polymorphic RAPD bands were named RACu90, RACu511, RACu2059_S, RACu2059_X, RACu63, RACu1002, RACu1015, RACu88, and RACu500; and were selected for transformation into specific SCAR markers. These differential bands were subsequently cloned and sequenced. Sequencing indicated that their actual lengths were 717, 796, 919, 758, 1168, 1562, 1203, 924, and 1211 bp, respectively.

Design of SCAR primers and development of SCAR markers

Nine (9) pairs of specific SCAR primers were designed

and synthesized based on the sequences of the 9 differential RAPD bands. For each pair of SCAR primers, the optimal annealing temperature for specific amplification was determined (Table 2). All sequences of SCAR primers contained the original whole bases of the corresponding 10-mer RAPD primers. The 9 expected SCAR markers were named SCAR1 to SCAR9, and the expected size of each marker was determined (Table 2).

The genomic DNA of the 20 studied *C. lanceolata* clones were amplified by 9 corresponding pairs of SCAR primers. SCAR9 of 1211 bp in size was amplified only from one clone Kai3 (No.12) and SCAR5 of 1168 bp in size was amplified from Xiang3 (No. 9) and Zhejiang2 (No. 20) (Figure 3), which indicates that the two differential RAPD markers (RACu500 and RACu63) were successfully converted into specific SCAR markers (SCAR9 and SCAR5). Four SCAR markers SCAR1 (717 bp in size), SCAR6 (1562 bp in size), SCAR7 (1203 bp in size), and SCAR8 (924 bp in size) were also amplified from different clones, but the polymorphism of the SCAR amplification were not similar to those of the original RAPD amplification (Figure 2). However, the amplification of SCAR2 (796 bp in size), SCAR3 (919 bp in size), and SCAR4 (758 bp in size) failed to display the original polymorphism detected by RAPD among the 20 studied *C. lanceolata* clones, which indicates that the three RAPD markers (RACu511, RACu2059_S, and RACu2059_X) were not successfully converted into specific SCAR markers (Figure 4).

For each pair of SCAR primers, the result was stable and reproducible at their respective optimal annealing temperatures using two different *Taq* DNA polymerase (Hangzhou Bioer and Dalia TaKaRa) and thermal cyclers (TCXP, Hangzhou Bioer, China and T-Gradient, Biometra, Germany).

Molecular typing of *C. lanceolata* clones based on SCAR markers

Based on the 6 SCAR markers developed in this study, a molecular classification diagram of 20 *C. lanceolata* clones was established (Figure 5). This result showed that the joint use of the 6 SCAR markers created 10 clones, including Zhejiang15 (No. 5), Xiang23 (No. 6), Zhejiang16 (No. 8), Xiang3 (No. 9), Zhejiang13 (No. 11), Kai3 (No. 12), Zhejiang4 (No. 14), Xiang1 (No. 15), Xiang12 (No. 16), and Zhejiang2 (No. 20), were identified from the 20 studied clones. The other 10 clones were divided into 4 groups, which could also be distinguished from each other. The four groups included: A) Long15 (No.1) and Zhejiang8 (No.17); B) Xiang5 (No.2) and Qian2 (No.3); C) Fujian1 (No.4) and Zhejiang14 (No.18), and D) Xiang4 (No.7), Zhejiang5 (No.10), Qian4 (No.13), and Zhejiang7 (No.19).

In the collected *C. lanceolata* clones listed in Table 1, six clones, including Xaing5, Xaing23, Xaing4, Xaing3, Zhejiang4 and Zhejiang2, were from Hunan provenance,
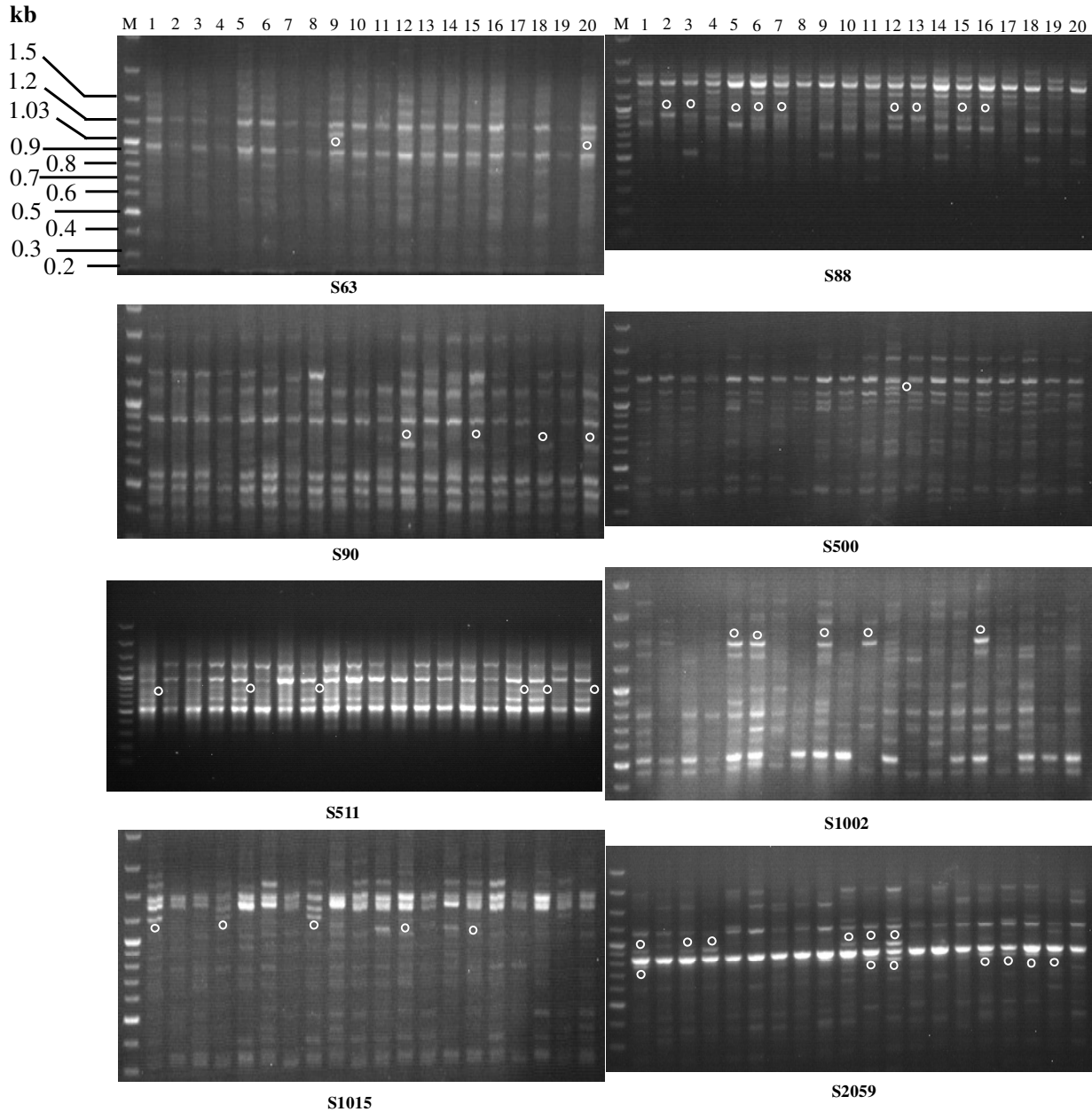
Figure. 2 RAPD electrophoretic profiles of 20 *Cunninghamia lanceolata* clones amplified by 8 RAPD primers. Lanes from l to 20 correspond to the 20 *C. lanceolata* clones listed in Table 1. Lane M is the molecular marker. The *numbers* on the left of the figure indicate the DNA size markers in kilobases (kb). An RAPD band specific to some clones is indicated by a white dot.

and three clones, including Fujian, Zhejiang5, and Zhejiang7 were from the Fujian provenance. The current study was the first to show that the *C. lanceolata* clones from the same geographic provenance were distinguished from one another based on molecular markers. Four *C. lanceolata* clones from Guizhou provenance (Qian2, Qian4, Xaing1 and Xaing12), two clones from Jiangxi provenance (Zhejiang15 and Zhejiang16), and two clones from Zhejiang provenance (Long15 and Kai3) could also

be distinguished from each other. These results show that the 6 SCAR markers developed in this study could be used not only in identifying or distinguishing the 20 *C. lanceolata* clones from one another but also in describing in detail the molecular type of *C. lanceolata* clones of same provenance by developing and using specific molecular markers. The study suggested the development and joint use of specific SCAR markers as a helpful tool for the identification of *C. lanceolata* clones, traditio-

Table 2. Specific SCAR primer sequences derived from cloned RAPD bands and their optimal annealing temperature.

RAPD primer	RAPD band[a] polymorphic	SCAR primer[b]	Sequence (5′→3′)[c]	Annealing temperature (°C)	SCAR marker (length in bp)
S90	RACu90	SC1_U	AGGGCCGTCTAGTGTCCAAACATT	67	SCAR1 (717)
		SC1_L	AGGGCCGTCTCCTTCGATGGG		
S511	RACu511	SC2_U	GTAGCCGTCTAAGCATACAAAAAA	60	SCAR2 (796)
		SC 2_L	GTAGCCGTCTATTGGTGGGT		
S2059	RACu2059_S	SC 3_U	ACAAGCGCGAAACACCAAT	66	SCAR3 (919)
		SC 3_L	ACAAGCGCGACTCGGATCTGGACA		
	RACu2059_X	SC 4_U	ACAAGCGCGAATGTACATTTGGC	65	SCAR4 (758)
		SC 4_L	ACAAGCGCGAACCCCAAATCTTT		
S63	RACu63	SC 5_U	GGGGGTCTTTGTAGAAATCA	62	SCAR5 (1168)
		SC 5_L	GGGGGTCTTTCGGTGCAAT		
S1002	RACu1002	SC 6_U	CACTTCCGCTCTGATACCACTTCG	65	SCAR6 (1562)
		SC 6_L	CACTTCCGCTAATCAATCTTCAAC		
S1015	RACu1015	SC 7_U	CTACAGCGAGTGGAGACAAGCAA	64	SCAR7 (1203)
		SC 7_L	CTACAGCGAGTAAATCTTTTAGC		
S88	RACu88	SC 8_U	TCACGTCCACCCATGTATATCAGA	61	SCAR8 (924)
		SC 8_L	TCACGTCCACACTACCATACATTT		
S500	RACu500	SC 9_U	TCGCCCAGTCGATCAGATAA	63	SCAR9 (1211)
		SC 9_L	TCGCCCAGTCCACCCTCA		

[a]The letters RA and Cu refer to RAPD and *Cunninghamia lanceolata*, respectively.
[b]The letter SC preceding the U (upper primer) and L (low primer) refer to SCAR.
[c]The nucleotide underlined represents the original sequence of the RAPD primer used.

nally by phenotypic characteristics.

DISCUSSION

The SCAR amplification profiles of *C. lanceolata* clones in the current study were stable and reproducible at their respective optimal annealing temperature (higher than 60°C) using two different *Taq* DNA polymerase. However, in the polymorphism of SCAR1, the SCAR6-8 amplification was not similar to those of the corresponding original RAPD markers (RACu90, RACu1002, RACu1015, and RACu88). The RAPD markers RACu511, RACu2059_S, and RACu2059_X were not converted into specific SCAR markers (SCAR2–4). The difference in polymorphism between RAPD and the SCAR markers or the lost polymorphism of converted SCAR markers were also shown in the studies of cucumber (Horejsi et al., 1999), grapevine (Vidal et al., 2000), wheat (Yan et al.,

2003), *Populus deltoides* (Zhang et al., 2007), and common carp (Gao et al., 2007). RAPD polymorphism was mostly caused by mismatches on the primer sites or molecular rearrangement. SCAR polymorphism originated from the variations within the amplified fragment (Bodenes et al., 1997). SCAR amplification resulted in the loss of the original RAPD polymorphism, which indicated that RAPD polymorphism resulted from the mismatches at the original priming sites. It was also possible that the non-specific bands detected by RAPD were of the same length with the specific bands, which could not be resolved by electrophoresis due to its low concentration. Thus, the non-specific bands were regarded as polymorphic bands (Hernandez et al., 1999). In the present study on *C. lanceolata* clones, the RAPD polymorphic loci that failed to convert the RAPD into SCAR markers might be located at the 3' end of the 10-mer primers. One or two bases at the 3′ end of the 10-mer primers were very important for the conversion from RAPD into SCAR
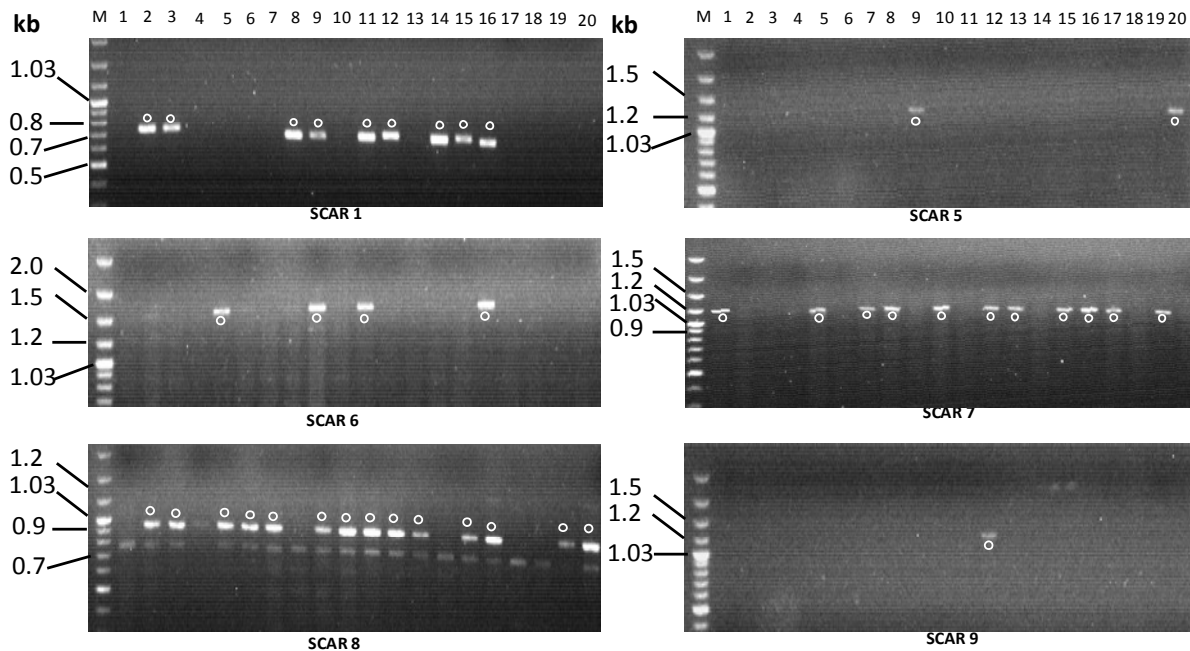
Figure. 3 SCAR electrophoretic profiles of 20 *Cunninghamia lanceolata* clones with amplified six pairs of SCAR primers. Lanes from I to 20 correspond to the 20 *C. lanceolata* clones listed in Table 1. Lane M is the molecular marker. The numbers on the left of the figure indicate the DNA size markers in kilobases (kb). A SCAR marker specific to some clones is indicated by a white dot.
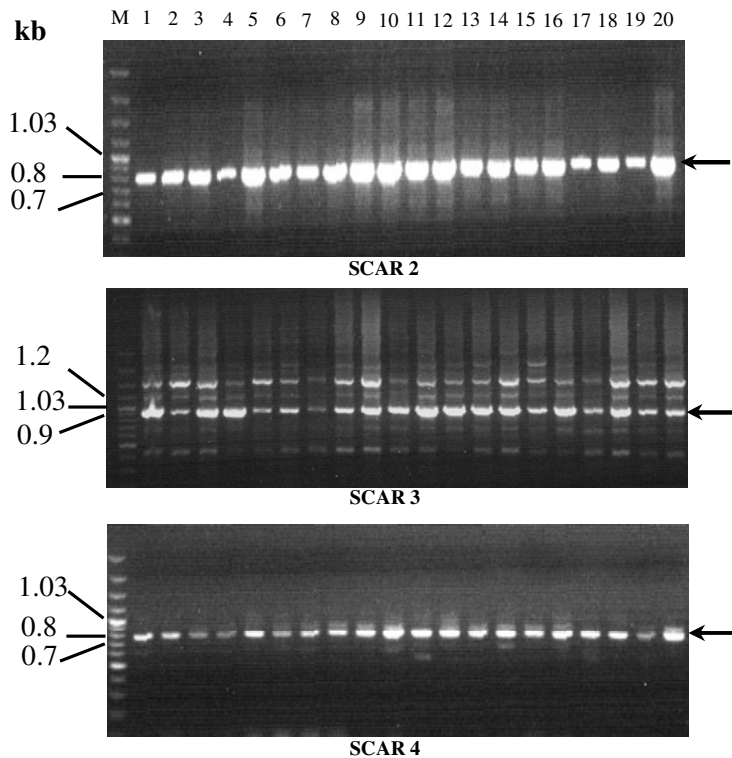

Figure 4. SCAR electrophoretic profiles of 20 *Cunninghamia lanceolata* clones with three pairs of SCAR primers. Lanes from I to 20 correspond to the 20 *C. lanceolata* clones listed in Table 1. Lane M is the molecular marker. The numbers on the left of the figure indicate the DNA size markers in kilobases (kb). The SCAR marker is indicated by a white arrow.
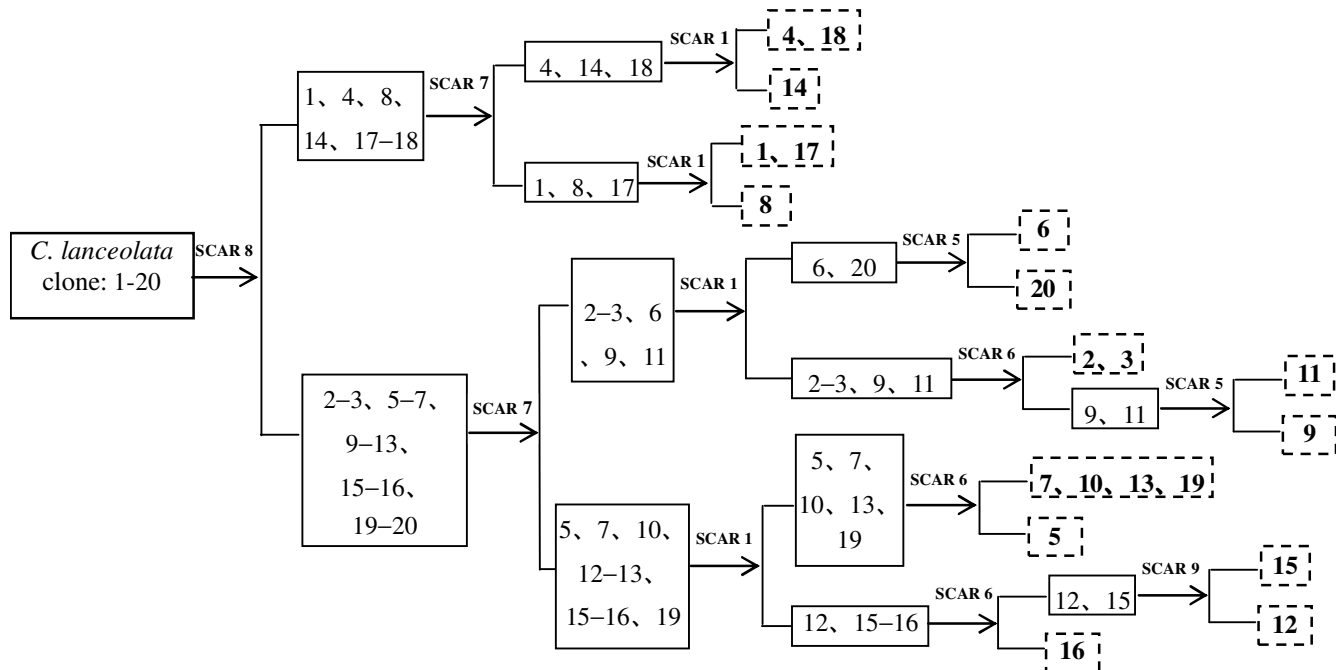
Figure 5. Molecular classification diagram of 20 *Cunninghamia lanceolata* clone with SCAR markers. The *C. lanceolata* clones listed in Table 1 are included in the line boxes. The solid line box represents a distinguishable *C. lanceolata* group using SCAR markers, while the dashed line box represents *C. lanceolata* clone (s) identified by SCAR markers.

markers (Vanichanon et al., 2000). The study of Vidal et al. (2000) on grapevine indicated that the original RAPD polymorphism could be successfully detected in the SCAR amplification, and the stability of SCAR amplification could be improved by adding between four and eight nucleotides at the 3′ end of 10-mer primers.

C. lanceolata possesses a complex genome structure with many repetitive sequences (Gan and Sun, 2006). In the present study, it is also possible that the difference in the polymorphism between RAPD and SCAR markers or the lost polymorphism of converted SCAR markers were mainly because of the complex structure of the *C. lanceolata* genome and the highly repetitive sequences within the genome. Hence, it is relatively difficult to detect reliable polymorphic bands in the *C. lanceolata* genome using only the RAPD technique, and then converting them into stable SCAR markers. Some high polymorphic markers, such as the amplified fragment length polymorphism (AFLP), ISSR, simple sequence repeats (SSR), and the sequence-related amplified polymorphism (SRAP) could be considered to be used or jointly used in the identification of *C. lanceolata* clones in future studies.

Multiplex PCR assay can detect multi loci phenotype at a single PCR, and has been widely applied to the detection of multiplex viruses and bacteria in clinical specimens (Hendolin et al., 1997; Corless et al., 2001). The multiplex PCR assays were established successfully for the joint use of some SCAR markers for distinguishing *L. edodes* strains (Li et al., 2008; Wu et al., 2010).

Therefore, further work should be done in establishing a multiplex PCR assay to be jointly used with these SCAR markers for the rapid molecular typing of *C. lanceolata* clones.

One main advantage of the SCAR markers is that they can be co-dominant generally when polymorphism is revealed after digestion of the PCR product by restriction enzymes, which enables the co-dominant SCAR markers to be more useful in high resolution mapping and genetic studies because the heterozygous genotype can be distinguished from the homozygous dominant individuals (Paran and Michelmore, 1993). Therefore, it is necessary to develop the co-dominant SCAR markers to be used in genotypic study in the future, which is important for the late *C. lanceolata* breeding program in China. The six dominant SCAR markers that were developed in the current study can be useful as the DNA fingerprints to help in the identification of *C. lanceolata* clones distributed over larger areas in China. Further work should also be done in the verification of more collected *C. lanceolata* clones based on these SCAR markers in the future.

ACKNOWLEDGEMENTS

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