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DEVELOPMENT AND CHARACTERISATION OF EST-SSR MARKERS FOR GENETIC ANALYSIS OF *CASUARINA* SPECIES

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Simple sequence repeats (SSR) markers have greater advantages relative to other molecular markers in genetic analysis due to their Mendelian inheritance and co-dominance expression features. Numerous expressed sequence tags (EST) of genus *Casuarina*, now available from public databases, make it possible to develop EST-SSR markers with high efficiency at low cost. From the analysis of 12,063 EST derived from casuarinas, 367 SSR loci were identified from 352 unigenes, and the distribution frequency of SSR loci was 3.0%. The di- and trinucleotide accounted for 57.77 and 34.06% respectively. They comprised overwhelming majority in five types of repeat motifs of SSR, and AG/CT was the most predominant motif type of SSR. A total of 79 primer pairs were designed based on the non-redundant EST-SSR sequences, but only 21 were successfully amplified and generated polymorphic SSR products in four different *Casuarina* species and eight clones of *C. equisetifolia*. Eight primer pairs out of the 21 were applied for paternity analyses of open-pollinated progenies of one female individual of *C. equisetifolia*. At 95% confidence interval, 76 male individuals were identified as pollen parents of 262 out of 461 progenies, but at 80% confidence interval, 84 male individuals were identified as father trees of 328 out of the 461 progenies.

Keywords: Mendelian inheritance, cross-species transferability, parentage analysis

INTRODUCTION

The family Casuarinaceae comprises four genera and 96 species occurring naturally from Australia to the Pacific islands and in South-east Asia (Turnbull 1990). *Casuarina* are nitrogen-fixing trees of considerable importance in many tropical and subtropical regions of the world. Over 2 million ha have been planted for wood production, shelterbelts, land rehabilitation and for ornamental purposes around the world (Zhong et al. 2011). The most important *Casuarina* in international plantings are limited to a few species, viz. *C. equisetifolia*, *C. cunninghamiana*, *C. glauca* and *C. junghuhniana*. Due to their important roles in coastal protection, sand dune stabilisation and agroforestry systems, researches on genetic diversity, reproductive biology and breeding system, genetic improvement, and ecological function have been carried out in many countries (Nagarajan et al. 1998, Warren & Zou 2002, Huang et al. 2009, Broadhurst 2011, Samarakoon et al. 2013, Zhang et al. 2014, Zhang et al. 2016).

Development of molecular marker techniques has provided efficient tools for genetic analyses of plants. Molecular markers such as RAPD (random amplified polymorphic DNA) (Ho et al. 2002), AFLP (amplified fragment length polymorphism) (Gaskin et al. 2009, Huang et al. 2009) and ISSR (inter simple sequence repeat) (Yasodha et al. 2004, Ho & Lee 2011) have been used for genetic relationship analysis, genetic diversity assessment and hybrid identification in *Casuarina*. However, all the aforementioned molecular markers are dominant markers with limitations such as difficulty in distinguishing heterozygotes as well as poor transferability and repeatability, making them inadequate for parentage analyses in *Casuarina*.

Microsatellites, known as simple sequence repeats (SSR), are short (1–6 bp long), tandemly repeated DNA sequences widely dispersed throughout eukaryotic genomes (Powell et al. 1996). The co-dominant microsatellites are robust markers as they are hypervariable and multiallelic, highly reliable, uniformly distributed

in plant genome, and transportable among closely related species. The positive features of microsatellites make it an efficient tool for genetic diversity assessment, parentage analysis, variety protection, gene mapping and marker-assisted selection in plants (Ho et al. 2006, Huang et al. 2016, Lee et al. 2018).

Unlike traditional methods of SSR discovery, new advances in genomic technologies have generated a large number of ESTs (express sequence tags) in many plant species, which contain numerous microsatellite sequences. Exploring and identifying microsatellites from EST is now available in plants, as EST databases become increasingly common in public databases such as EMBL and Genbank. Development of EST-SSR markers through mining EST databases has become a fast, efficient, and low-cost option for many plant species. A large number of ESTs derived from *C. equisetifolia* and *C. glauca* are available in the Genbank (Hocher et al. 2006). These sequence data offer a prerequisite for developing EST-SSR markers of plant in the *Casuarina* genus. Though some EST-SSR markers of *Casuarina* genus have been developed (Xu et al. 2018), only limited markers are actively employed in casuarina improvement programmes. Developing more highly polymorphic EST-SSR markers is necessary, especially for purposes like parental analysis in *Casuarina*.

The objectives of this study were: (1) to analyse the frequency and distribution of SSR in EST of *Casuarina*, (2) to develop and validate EST-SSR markers for polymorphism in *Casuarina* and assess their cross-species transferability, and (3) to explore their application in parentage analysis of *Casuarina* progeny seedlings.

MATERIALS AND METHODS

SSR retrieval and pre-treatment

EST sequences were downloaded from EST database at the NCBI (National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) using *Casuarina* as search keyword. A total of 34,897 EST sequences of *C. equisetifolia* and *C. glauca* were obtained for this study. Redundancy EST sequences were aligned by EST-trimmer software (http://pgrc.ipk-gatersleben.de/misa download/esttrimmer.pl) to remove the 5′ or 3′ end of poly A or poly T stretches within 50 bp, and EST sequences less than 100 bp were discarded. After pretreatment, the software SSR Identification Tool (http://archive.gramene.org/db/markers/ssrtool) was used to search for SSR from these EST sequences. The identification criteria were dinucleotide repeats ≥ 9, trinucleotide ≥ 6, and tetra- to hexanucleotide ≥ 5.

Non-redundant EST sequences containing SSR were used to design primers with the software PRIMER 5.0. The parameters were set as follows: GC content 40–60%, annealing temperature 53–57 °C, expected amplified product size 100–500 bp, and primer length 18–24 nucleotides. All primers were synthesised by the Beijing Genomics Institute.

DNA extraction and SSR primer screening

Genomic DNA of *Casuarina* for primer screening was extracted from fresh young branchlets of *C. equisetifolia* clone "A8" using a modified CTAB method (Doyle & Doyle 1990). PCR amplification system for screening of primers was 10 µL in volume, which consisted of 1.0× buffer (100 mM Tris-HCl pH 9.0, 80 mM $(NH4)_2SO_4$, 100 mM KCl–0.5% NP-40), 2.0 mM $MgCl_2$-200 µM dNTP, 0.5 µM forward primer, 0.5 µM reverse primer, 1 U Taq DNA polymerase, and 10–40 ng genomic DNA. The PCR reaction program was: pre-denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and finally 72 °C for an extension of 10 min. PCR products were examined by electrophoresis on a 1.0% agarose gel stained with 1:20 GoldView in 1× TAE and photographed with Photoprint 215 SD.

EST-SSR polymorphism and cross-species transferability detection

Primer pairs that produced clear SSR product bands were selected to detect their polymorphism and cross-species transferability using 12 individuals belonging to four species of the genus *Casuarina* (*C. equisetifolia, C. glauca, C. cunninghamiana, C. junghuhniana*) and eight clones of *C. equisetifolia*. The aforementioned PCR reaction system with 10 µL in volume was used, and extra 10 pmol (0.01 µL) Fluorescent-dUTP was added into the system for SSR genotyping in ABI 3130xl genetic analyser. Touchdown PCR program was employed to the ABI 3130xl genetic analyser. The Touchdown program consisted of

(1) 94 °C for 5 min, 20 cycles at 94 °C for 1 min, 60–50 °C for 30 s with a decrease of 0.5 °C per cycle and 72 °C for 1 min; (2) 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min; and (3) a final extension at 72 °C for 10 min. Detection of each SSR was performed on the ABI 3130xl genetic analyser when PCR products were confirmed through agarose gel electrophoresis.

The detection procedure followed the standard module using software GeneMapper 4.0, and each allele and its fluorescence intensity (units) were verified by visual inspection. In order to verify the existence of sequence repeat variation in SSR locus among different clones of *Casuarina*, some PCR products were recovered for sequencing using the ABI PRISM 3730 sequencing equipment at the Beijing Genomics Institute.

Paternity analyses of open-pollinated progenies of *C. equisetifolia*

To carry out paternity analysis of open-pollinated progenies of a female individual using the new-developed SSR markers, a dominant female individual in a 5-year-old provenance test plantation of *C. equisetifolia* was selected, and its open-pollinated seeds were collected. This provenance test plantation is surrounded by even-aged mixed plantations of *Acacia auriculiformis* and *C. equisetifolia*. Young branchlets of the selected female and 169 male trees which could be the potential pollen donors (father trees) were sampled and dried on silica gel in the field and then transferred to an ultra-low temperature freezer for storage prior to DNA extraction. The open-pollinated seeds were sown in a tray and kept in a greenhouse for germination and seedling growth.

Genomic DNA of 461 progeny seedlings, one mother tree and the 169 potential father trees was extracted from young branchlets using modified CTAB method mentioned above. Eight SSR primer pairs (EST-C01 to EST-C08) with relatively high polymorphism were used to genotype the 631 individuals. A likelihood-based paternity analysis approach was applied to the 461 progenies based on multilocus genotypes for progenies, potential fathers and mother using CERVUS Version 3.0 (Kalinowski et al. 2007). Two confidence intervals of 95 and 80% were set for determination of the most likely father by parentage analyses using CERVUS 3.0 software. In this study, the simulation parameters required by the program were set as follows: 10,000 cycles, 169 candidate father individuals, 0.90 and 0.01 as the proportions of loci typed and mistyped respectively.

RESULTS

Characterisation of frequency and distribution of SSR in EST sequences

A total of 12,063 unigenes (non-redundant sequences) were obtained after alignment of redundant EST sequences using EST-trimmer software. Within these unigenes, 367 SSR loci were identified from 352 unigenes containing SSRs. There were five types of repeat motifs in the 367 SSR loci, namely, di-, tri-, tetra-, penta- and hexanucleotide repeat motifs (Figure 1). This result suggested that only 2.9% unigenes contained SSRs, which represented an average density of one SSR in every 19.83 kb. Of the five types of motifs, di- and trinucleotide presented an overwhelming majority, accounting for 57.77 and 34.06% respectively. By contrast, the remaining three kinds of repeat motifs (tetra-, penta- and hexanucleotide) only accounted for 3.27, 0.82 and 3.54% respectively.

In view of their overwhelming majority, only di- and trinucleotide motifs were used for analysis of motif type. As shown in Table 1, within 212 dinucleotide motifs, the motif AG/CT had the highest frequency of 93.87%, followed by motifs AT/TA (3.30%) and AC/TG (2.83%). Of the 127 trinucleotide motifs, AAG/TTC was the most abundant, with a frequency of 44.09%, followed by the AGT/TCA (11.02%), AGT/TCA (10.24%), ACC/TGG (7.87%), ACG/TGC (7.09%), AAC/TTG and AAC/TTG (both 5.51%), and AAT/TTA and ACT/TGA (both 3.94%). The motif CCG/GGC (0.79%) had the lowest frequency in all trinucleotide motifs.

Development of EST-SSR markers

Thirty-two of 79 primer pairs designed for *Casuarina* were amplified and generated clear SSR products in 1.0% agarose gel under the given PCR system and reaction conditions. The remaining 47 primer pairs either had no amplified product or produced a number of faint bands indicative of non-specific SSR amplified products and were discarded.

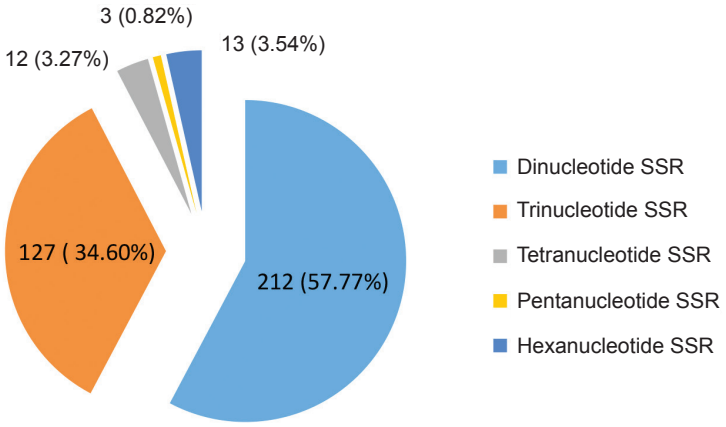

Figure 1 Frequency distribution of five types of SSRs in 367 SSR loci within 352 EST sequences; numbers outside brackets are the SSR numbers identified from 367 SSR loci and numbers between brackets are their percentages

Table 1 Types and proportions of repeat motifs in two kinds of SSRs obtained from *Casuarina* ESTs

SSR type	Type of repeat motif	No. of SSR	Proportion (%)
Dinucleotide	AG/TC	199	93.87
	AT/TA	7	3.30
	AC/TG	6	2.83
	Overall	212	100.00
Trinucleotide	AAG/TTC	56	44.09
	AAT/TTA	5	3.94
	AAC/TTG	7	5.51
	ACC/TGG	10	7.87
	AAC/TTG	7	5.51
	ACT/TGA	5	3.94
	CCG/GGC	1	0.79
	AGT/TCA	14	11.02
	AGT/TCA	13	10.24
	ACG/TGC	9	7.09
	Overall	127	100.00

The 32 primer pairs were further examined by amplifying the SSR loci in genomic DNA of eight clones of *C. equisetifolia* and four *Casuarina* species, aimed at detecting polymorphism among different cultivars (clones) and cross-species transferability. In total, 21 primer pairs were found to present polymorphism among eight clones and fours species, and all 21 performed excellent cross-species transferability within *Casuarina* genus based on successful amplification with polymorphic loci. The characterisations of 21 primer pairs based on 12 individuals are shown in Table 2.

According to genotyping results for eight clones of *C. equisetifolia* and four *Casuarina* species, the number of alleles per locus ranged from 3 to 5, and in total 38 alleles were found for the 12 individuals. Allelic profiles of the 12 individuals genotyped by the first 10 SSR loci are presented in Table 3.

PCR products amplified by primer pair EST-C10 from different clones were recovered and sequenced, and the four DNA fragment sequences containing variable simple sequence repeat GTT are presented in Figure 2. The results revealed that the numbers of repeat units GTT in

Table 2 Characterisations of EST-SSR marker primer pairs for PCR amplification based on DNA samples of one individual in *Casuarina glauca, C. cunninghamiana, C. junghuniana* and *C. equisetifolia* and eight clones of *C. equisetifolia*

Locus	Repeat motif	Primer sequences (5′–3′)	Expected product size (bp)	Annealing temperature (°)
EST-C01	$(AGA)_6$	F: TGCAGCATCATCACTACT R: ACTCCAACCAACTCTATTC	297	54
EST-C02	$(CTTCT)_5$	F: TTTGTCTTCCCTACTCCG R: AACCCTTTTCCACTTTCTTA	162	52
EST-C03	$(CTT)_6$	F: TTCAAAACCCTAGCATCT R: CATACCATTAACCAAAGC	200	50
EST-C04	$(CT)_{14}$	F: GCTGGAGGTGGTGGTGTT R:TATGGAATAGACGAGAAGTGAG	256	56
EST-C05	$(TCGCAC)_3$	F: CATCTGAACTTTTGAAACCCTA R: GGCATGGCTTCGTCTTGG	197	56
EST-C06	$(TAG)_6$	F: GCCGAGTTATGGGGACGA R: GGTGTTTGTGACGACGCT	240	52
EST-C07	$(CGT)_6$	F: GCACGGTCGTCTTATTCT R: TCGCTTCCCATACAAATC	265	54
EST-C08	$(GAC)_{12}$	F: GCTTTGTCCTACCGTTTC R: ATCACCACCATCCTCGTC	148	52
EST-C09	$(TCT)_{10}$	F: CTATTGTTGTGCTTCATCCT R: CAATAGTCCTAGCACCATT	110	57
EST-C10	$(GTT)_9$	F: AAAGAGAGGCTCAGAAAGA R: GCACGAAGCAAGAGATAGA	165	55
EST-C11	$(CAACGACAA)_3$	F: CCTCAAACCAAGACCACC R: CCGACTTCCATGCTCAAT	320	52
EST-C12	$(TG)_9$	F: TGCCGCTGAACAAAATGA R: ATGGTCTCGCCTGGAATG	246	54
EST-C13	$(CATCTT)_3$	F: ATGGGACATTTTGGTGAT R: CTTTGCTTTAGGCGTTTT	282	50
EST-C14	$(TC)_9$	F: CCCTGCTTCTGGTCATTC R: GATCTGTGGCTTTGCTTG	226	56
EST-C15	$(AG_{)13}$	F: CTTCGCCGTTTCCTCAGA R: ATATTTGCTTCGCAGGTCA	195	55
EST-C16	$(GAA)_5$	F: ATGATGAAGACGAGGATC R: CTTCTTCTTCTTCCACCAC	165	54
EST-C17	$(AG)_{17}$	F: GAATCAAGAACCGCGAAC R: TCCGAATACCAGACTCCAG	311	56
EST-C18	$(CT)_{12}$	F: AAAGGCACAAGTTAGGAGAG R: GCTGGTGCTGTTGAAATG	214	56
EST-C19	$(CT)_7$	F: CGACCCAACCAAAATCTC R: AAGCGACAATCTGAAAGAAG	260	55
EST-C20	$(AAGAAC)_4$	F: GAAATGCTTATACAGAGAGG R: AATCTTCACGATAACTGAGG	239	56
EST-C21	$(TCTT)_6$	F: AATCTAACAACTGCTTTGGC R: GGGATGCTGATCGTAACAT	286	57

four clones were 9, 10, 8 and 2 respectively. Both flanking regions of repeat motifs GTT in each clone presented a high degree of uniformity, confirming the high conservation of non-microsatellite nucleotide sequences between closely related species (Figure 2).

Paternity analyses of open-pollinated progenies of *C. equisetifolia*

The first eight SSR primer pairs (EST-C01 to EST-C08) in Table 2 were used to genotype the 461 progeny seedlings originating from one mother tree of *C. equisetifolia*. In the paternity analyses using CERVUS Version 3.0 (Kalinowski et al. 2007), at 95% confidence interval, 262 out of 461 progenies were assigned to 76 male individuals as pollen donors. However, at 80% confidence interval, 328 progenies were assigned to 84 male individuals as father trees. In the remaining 199 and 133 progenies, at 95 and 80% confidence intervals respectively, the paternity analysis failed to identify their father trees, and this may be due to external pollen flow or missing data at some loci.

Table 3 Allelic profiles of eight clones of *C. equisetifolia* and four species within *Casuarina* genus

SSR locus	EST-C01	EST-C02	EST-C03	EST-C04	EST-C05	EST-C06	EST-C07	EST-C08	EST-C09	EST-C10
individual	A1/A2	A1/A2	A1/A2	A1/A2	A1/A2	A1/A2	A1/A2	A1/A2	A1/A2	A1/A2
C1	297/306	148/158	217/199	240/240	197/197	244/244	264/264	144/140	110/119	165/165
C2	294/294	158/158	217/199	240/240	197/197	235/235	264/264	144/144	125/110	165/174
C3	297/306	148/158	217/199	240/240	185/197	244/244	252/264	null	110/110	162/165
C4	297/306	158/158	220/220	240/240	197/197	244/244	260/264	144/144	110/110	162/165
C5	294/294	158/158	217/217	240/240	197/197	246/246	264/264	144/144	110/110	165/165
C6	297/306	160/160	199/217	240/240	197/197	246/246	264/264	144/144	125/125	165/165
C7	297/306	160/160	217/217	null	197/197	240/240	264/264	144/144	125/125	165/165
C8	294/294	160/160	217/217	240/240	197/197	240/240	264/264	144/144	125/125	165/165
Sp1	297/306	158/158	217/217	242/242	185/197	242/242	264/264	136/136	119/125	159/165
Sp2	303/303	158/158	217/199	240/256	179/179	244/244	264/264	140/140	110/119	165/165
Sp3	297/306	158/158	217/199	240/240	197/197	244/244	258/264	144/144	110/119	158/165
Sp4	297/306	158/158	217/217	242/242	197/197	244/244	240/264	128/140	110/119	165/165
No. of allele	4	3	3	3	3	5	5	4	3	5

C1 to C8 represent clones "Zhanjiang3", "Pingtan 2", "A13", "A8-2", "A13", "701", "Hui 76" and "Hui 98" respectively; Sp1 to Sp4 represent individuals of *C. equisetifolia*, *C. glauca*, *C. cunninghamiana* and *C. junghuhniana* respectively; A1 = allele 1, A2 = allele 2

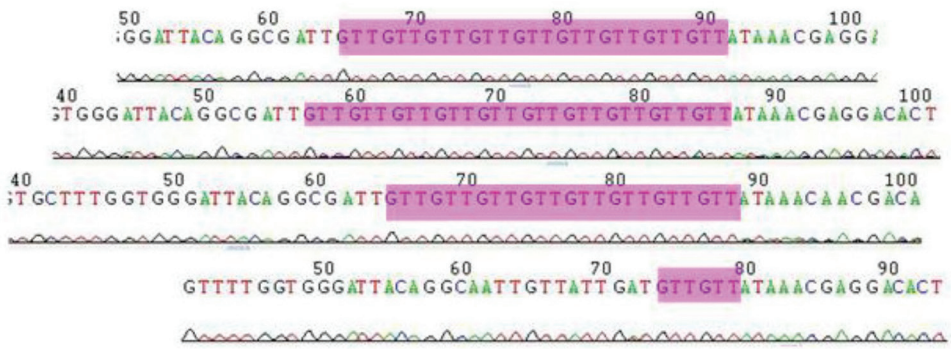

Figure 2 DNA fragment sequences containing variable repeat motifs GTT obtained by amplifying four different cultivars (clones) using primer pair EST-C10

DISCUSSION

Frequencies and distribution of SSRs in *Casuarina* EST sequences

Owing to the rapid progress in functional genomics research in plants, the number of ESTs in public database is increasing exponentially. Developing SSR markers by means of searching for SSR loci in EST sequences has become a focal point for development of new SSR markers. In this study, a total of 367 SSR loci were identified from 12,063 unigenes of *Casuarina* indicating that the distribution frequency of SSR loci was only 3.0%. Based on published reports, the abundance of SSRs in ESTs vary widely in different plants. Areshchenkova and Ganal (2002) detected 250 SSR loci from 27,000 EST sequences of tomato, with only 0.93% distribution frequency. In *Liriodendron* sp. (Xu et al. 2010) and rubber tree (*Hevea brasiliensis*) (Feng et al. 2009), relatively higher SSR distribution frequencies with 6.0% (394 SSRs out of 6520 unigenes) and 12.3% (1233 SSRs out of 10,018 unigenes) respectively were detected. Furthermore, the average density of one SSR per 19.83 kb obtained in *Casuarina* EST sequences was far lower than that of other plants, such as rice (3.40 kb), maize (8.10 kb), soybean (7.40 kb) (Cardle et al. 2000) and barley (6.3 kb) (Thiel et al. 2003), but similar to cotton (20.00 kb) (Cardle et al. 2000). However, the abundance level of SSRs in EST sequences can be influenced by differences in search tools and identification criteria. For example, Rota et al. (2005) found that the frequency of SSRs in rice was reduced from 50 to 1% when SSR searching parameter of minimum length was changed from 12 to 30 bp.

Di- and trinucleotide repeat motifs have been widely reported in plants. The two most common motif types found in *Casuarina* EST database were di- and trinucleotide repeats, which accounted for 92.4% of all motif types. Within dinucleotide repeat, AG/CT was the most predominant motif type in *Casuarina* EST sequences, which was consistent with *Arabidopsis* (Morgante et al. 2002), rice (Temnykh et al. 2001) and rubber tree (Roy et al. 2004).

Polymorphism and cross-species transferability of SSR markers

Polymorphism of SSRs originates from the length variations caused by the different number of repeat units. Generally, it was thought that the occurrence of slipped mispairing during DNA replication and repair and unequal crossing-over between repeat units during meiosis accounted for the length variation in SSRs (Strand et al. 1993, Park et al. 2009). In this study, 47 EST-SSR primer pairs either failed to amplify or produced non-specific amplification of SSR products, resulting in exclusion from further polymorphism examination. Causes for amplification failure can be the presence of an intron within the primer sequences that prevents primer annealing, or a large intron in the flanking region that disrupts PCR extension (Cardle et al. 2000, Saha et al. 2004, Park et al. 2009).

Of the remaining 32 primer pairs with successful amplification, 21 produced polymorphic products in the 12 genotypes of *Casuarina*. All the12 individuals analysed by 10 SSR markers had unique SSR fingerprints (Table 3), validating the high efficiency of these markers in variety discrimination. Furthermore, all the 21 primer pairs presented high cross-species transferability among four *Casuarina* species in this study. Transferability of microsatellite loci between closely related species is a consequence of homology of flanking regions of simple sequence repeats (Collevatti et al. 1999). EST-SSRs usually demonstrate higher transferability than genomic SSRs due to their relatively more conservative genic regions (Yu et al. 2004). It was reported that 43 of 78 EST-SSR markers exhibited transferability from *Triticum* to *Hordeum* (Gupta et al. 2003), but only 2 of 11 SSR markers from an enrichment library of *Swietenia humilis* showed amplification across the Meliaceae family (White & Powell 1997). The high cross-species transferability of *Casuarina* EST-SSR markers make them an excellent tool for parentage analysis of inter-specific hybrids and other genetic studies involving different species of *Casuarina*.

Application for parentage analysis and retrospective selection of *Casuarina*

SSR markers can be used to determine pollen dispersal distance (Broadhurst 2015), mating system (Zhang et al. 2016), and pollen donors (Wei et al. 2015, Ramos et al. 2018) by paternity analysis. In this study, 8 of the 21 newly developed SSR markers were used to determine the father trees of 461 open-pollinated progenies of one female tree. The paternal analysis showed that the

pollen donors (father trees) of 56.8% (262 out of 461) and 71.1% (328 out of 461) progenies could be confirmed at 95 and 80% confidence intervals respectively. Such percentages are similar with results obtained from *Pinus massoniana* (48.9%) (Ai et al. 2006), *P. tabulaeformis* (44.8%) (Zhang et al. 2009) and *Eucalyptus grandis* (54%) (Jones et al. 2008). Failure in identification of paternal parent for the remaining progeny at 95 and 80% confidence intervals might be attributed to two reasons: (1) there were external pollen sources besides the male trees sampled within the provenance trial to pollinate the female tree selected; and (2) some missing alleles in some loci due to amplification failure or genotyping errors caused paternity analysis failure of some progenies.

Highly informative SSR markers also can be used to develop new breeding strategies for *Casuarina* by means of retrospective selection. The conventional breeding strategies are to establish progeny trials involving each parent tree, and then evaluate parental contribution to progeny performances by estimating general combining ability (GCA) and specific combining ability (SCA) of the parent trees. However, full-sib progenies of *Casuarina* obtained from controlled pollination are costly and laborious. An alternative method is to carry out paternity analyses of superior progenies using SSR marker technique in progeny trials established with open-pollinated seeds. Parent trees that produce a high frequency of superior offspring mean they possess high GCA and SCA. These hybridogenic parent trees obtained from retrospective selection can be used to establish a new seed orchard for production of superior hybrid seeds.

CONCLUSIONS

In this study, 21 SSR markers were developed from EST sequences of *Casuarina*. High polymorphism and cross-species transferability of these SSR markers were confirmed in four species within *Casuarina* genus and eight clones of *C. equisetifolia*. Meanwhile, the Mendilian inheritance and co-dominance expression features of SSR marker were verified by means of paternity analysis of open-pollinated progenies of one *C. equisetifolia* individual. These SSR markers would be efficient tools for further use in parentage analysis, genetic diversity assessment, variety protection, gene mapping and marker-assisted selection of *Casuarina* tree species in the future.

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