Indian J. Genet., 69(3): 256-259 (2009)

Short Communication

Development and characterization of polymorphic chloroplast microsatellite markers in sweet flag (*Acorus calamus* L.)

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(Received: April 2009; Revised: July 2009; Accepted: August 2009)

The history of medicinal plants and their uses in India is as old as human civilization. These are viewed as a possible bridge between sustainable economic development, affordable health care and conservation of vital biodiversity. WHO (2002) estimates that about 80% of the world's population depends on herbal medicines for their primary healthcare [1, 2]. Out of these, one of the highly valued herbal and medicinal plant species, which commonly exists in India, is *Acorus calamus* L. This species is known as Vasa Bach (Hindi) and Sweet flag (English) and considered endangered in Kerala and vulnerable in Tamil Nadu [3]. It is a highly valued herbal and economically important plant species in India and other European countries from very early times as a stimulant, brain tonic, aphrodisiac, laxative, emetic, expectorant, emmenagogue and diuretic. It is therefore used as an ingredient in the several drugs of the Unani, Ayurveda and Local Health Care Systems [4].

Previously marker based studies on *A. calamus* have been confined to RAPDs [5] and ISSR markers [6]. However, both of these techniques generate dominant markers and there are serious questions concerning about their reproducibility. Whereas, microsatellites or simple sequence repeats (SSRs) represent an ideal class of molecular genetic marker in plant genome analysis, which would be able to disclose multiple alleles and with even distribution throughout the genome and relatively easy to score [7]. These markers prove highly polymorphic in nature between the individuals of a population and among the populations [8]. Also these can be easily shared between laboratories, thus providing a common tool for collaborative research by acting as universal genetic markers [8]. The chloroplast genome is found a useful source of markers for genetic studies of plants because of conserved gene order and general lack of heteroplasmy and recombination. Its generally uniparental mode of inheritance makes it a powerful tool to elucidate relative contributions of seed and pollen flow to identify the genetic structure of natural populations in comparison to the nuclear markers. Chloroplast microsatellites (cpSSRs) revealed to be of special value in studies of plant population geographic structure and differentiation as well as for the paternity analysis. However, in case of nuclear markers, a high level of homoplasmy is expected [9]. There are no SSR markers available for *A.calamus* till date. Perhaps this will be the first report wherein cpSSR marker sets are made available for the *A.calamus* for population genetic studies and molecular characterization of germplasm with precision.

Different samples of *A.calamus* collected from various parts of the state of Uttarakhand, Himachal Pradesh, Jammu and Kashmir, (India) during 2007-08 and assembled at the experimental area of Division of Genetics and Tree Propagation, Forest Research Institute, Dehradun, Uttarakhand. DNA was extracted from young leaves of *A. calamus* plants using modified CTAB method [10]. The method was modified to obtain suitable quantity and quality of DNA for SSR analysis. The concentration and yield of the extracted DNA was measured by using a Biophotometer (Eppendorf, 6131) at A_{260}/A_{280} nm wavelength. The quality of DNA was

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determined by running the samples on 0.8% (w/v) agarose gel.

The search for chloroplast-microsatellites (cpSSR) was greatly facilitated by the availability of the completely sequenced chloroplast genome of *A.calamus* available at NCBI gene data bank (www.ncbi.nlm.nih.gov) sequenced by Goremykin et al., in 2005 [11] having accession no. AJ879453. The sequenced chloroplast genome of *A. calamus* was used for primer designing using web based computer software programme SPUTNIK available at (http://hornbill.cspp.latrobe.edu.au/cgi-binpub/ssrprimer/indexssr.pl). The parameters kept in view for the primer development were: primer length 20 bp; Tm 50-60^{o}C; GC content 40-60%; maximum Tm difference between forward and reverse primer of 4^{o}C. As a result, a total of 18 cpSSR markers were developed. The selected primers were synthesized commercially through the Operon Biotechnologies (Germany) and the annealing temperatures for the primers were standardized.

To evaluate amplification and polymorphism of the microsatellites, 10ng of DNA samples were amplified in a 25 µl reactions containing 2.5 µl of 1X reaction buffer (100mM Tris pH 9.0; 500 µM KCl; 0.1% gelatin), 2.5 mM $MgCl_2$, 200mM (0.2 mM) of each dNTPs (Bangalore Genei, Bangalore, India), 0.2mM of each forward and reverse primer (Operon Biotechnologies, Germany) and 1 unit of *Taq* DNA polymerase (Bangalore Genei, Bangalore, India). The polymerase chain reaction protocol used the 5min denaturation at 94^{o}C; then samples were incubated for 35 cycles of denaturation at 94^{o}C for 30s, optimized annealing temperatures for different primer pairs ranging from 53^{o}C - 58^{o}C for 1 min (Table 1) and extension at 72^{o}C for 2 min. The reactions were completed by incubating the samples at final extension for 10 min at 72^{o}C. PCR reactions were conducted in a Mastercycler Gradient PCR system (Eppendorf, Germany).

The amplified fragments were resolved by electrophoresis on 3% metaphor agarose gel stained with ethidium bromide (0.5 µg / ml) and run in 1X TBE buffer (89mM Tris, 89mM Boric acid and 2.24 mM Na_2 EDTA, pH 8.0) for 2.5 – 3 h at 50V. Marker ϕ x174 *HaeIII* digest (Fermentas) was included (Figs. 1 and 2) in the gel, and it was photographed with the gel documentation system (UVP Model LMS-20E, Upland, USA).

Table 1. Repeat motif, forward and reverse primer sequences, annealing temperature and expected product size of the 18 developed chloroplast microsatellite markers for sweet flag (*A. calamus*)

Marker name	Repeat type	Repeat Motif	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Temp. (^{0}C)	Product size (bp)
AC-01	Pentanucleotide	$(GAAGG)_3$	tacgttctcctttatggacc	attattgatcgatttggacg	56	271
AC-02	Dinucleotide	$(AT)_6$ $(TA)_5$	ttcggattagaattcacaaa	caaagaaagtttgtcccttg	54	182
AC-03	Tetranucleotide	$(AAGC)_3$	aaggtttacattggacgaaa	acaaccagaagcagaaggta	56	337
AC-04	Trinucleotide	$(ATT)_4$	agaaatcagtggattcatgg	attcgaaacaaagaaacgaa	54	274
AC-05	Dinucleotide	$(AT)_7$	actattccctcccgtatgtt	gaaccaatccaattaatcca	53	188
AC-06	Pentanucleotide	$(AATAA)_3$	ttacaaatgcgatgctctaa	ggaatcctgctctgctataa	56	373
AC-07	Pentanucleotide	$(TTTATT)_4$	cgatggataagaatcctgag	ttcatatgtatgacgcaacc	53	382
AC-08	Pentanucleotide	$(AAGGG)_3$	aaggattgagccgaataaa	aagtttctcttgcaatacgg	53	325
AC-09	Tetranucleotide	$(AAAT)_3$	aaccaatctactgccaaatc	catcagctgtgacatcaatc	56	276
AC-10	Dinucleotide	$(AT)_7$	ctcctccacatccacatagt	cattccaaacctaattcgtc	58	385
AC-11	Trinucleotide	$(GAT)_4$	tcaaatagtttgggctccta	cagaagaagctttgaaagga	54	315
AC-12	Trinucleotide	$(GAA)_4$	tcaaatagtttgggctccta	cagaagaagctttgaaagga	54	320
AC-13	Trinucleotide	$(GAA)_7$	tgggtatcaagataattggg	ttctaactgtcctggacctg	58	180
AC-14	Pentanucleotide	$(ATTAA)_3$	atctttcacattcggctaga	ccgctgcatctttatttatt	54	315
AC-15	Trinucleotide	$(TAA)_4$	cggaataagcgagataaatg	gccatattcggtatctgaag	56	385
AC-16	Trinucleotide	$(TTC)_7$	tctcttcattgttggaaacc	tgggtatcaagataattggg	54	344
AC-17	Trinucleotide	$(TTC)_4$	ctctttaaagtgaaagcgga	agaaactgaacgaaacctca	54	317
AC-18	Trinucleotide	$(ATC)_4$	cagaagaagctttgaaagga	tcaaatagtttgggctccta	54	320

The chloroplast genome of *A. calamus* produced a total of 18 pair of SSR markers. All the developed cpSSR primers were found perfect type except the AC-02 microsatellite marker, which is compound type and consisted of two repeat motifs i.e. AT and TA. Out of the eighteen SSR primers, three comprised dinucleotide repeat (DNRs) types (16.66%), eight trinucleotide repeat (TNRs) types (44.44%), two tetranucleotide repeat (TtNRs) types (11.11%) and five pentanucleotide repeat (PtNRs) types (27.77%) [9]. In terms of the repeat motif, only the AT/TA repeat motif was found in the DNRs, whereas GAA/CTT and TTC/AAG repeat motifs were the most common, accounting for 50% of all the repeat motifs in TNRs (Table 1). The maximum repeat unit number of dinucleotide repeat motifs (AT/TA) was 7, whereas; repeat unit number of trinucleotide repeat motifs GAA/CTT and TTC/AAG was also found 7. The overall repeat motif number ranged from 3 to 7. Similarly the average repeats unit number for the tetranucleotide and pentanucleotide repeat motifs were found between 3 and 3.2, respectively.

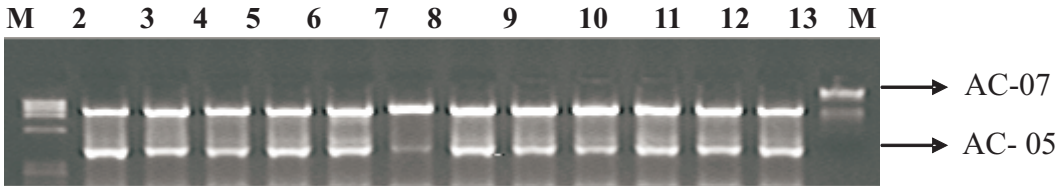

Fig. 1(a). SSR pattern obtained from twelve different DNA samples (Lane 2-13) using primer AC-07 and AC-05. Lane 1 and Lane 14 is ϕX-174 DNA/BsuRI (HaeIII) molecular weight marker

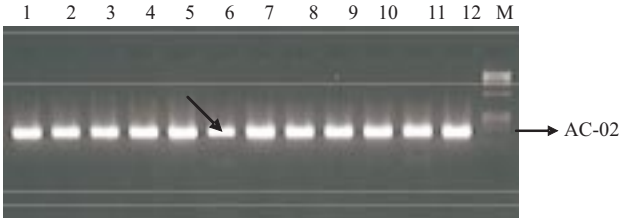

Fig. 1(b). SSR pattern obtained from twelve different DNA samples (Lane 1-12) using primer AC-02. Lane 13 is ϕX-174 DNA/BsuRI (HaeIII) molecular weight marker. Lane 6 is showing the allelic variation (indicated with arrow)

For ascertaining the useful attributes of genetic markers, all the developed cpSSR markers were tested on 12 genomic DNA samples of *A.calamus* originating from 6 different populations. Out of the 18 primer pairs, 17 produced good amplification. The optimum annealing temperature for 13 primer pairs (AC-01, AC-02, AC-03, AC-04, AC-06, AC-09, AC-11, AC-12, AC-14, AC-15, AC-16, AC-17, AC-18) was found between 54^0C & 56^0C, for 3 primer pairs (AC-05, AC-07 & AC-08) 53^0C and for remaining 2 primer pairs (AC-10, AC-13) it was 58^0C (Table 1). Good allelic amplification was obtained for all the developed cpSSR markers across the tested samples, except AC-10, that did not given any amplifications (Fig.1a & b). In general, the developed cpSSR markers revealed low to medium allelic diversity when tested across different genotypes of *A.calamus*. Population variation in *A.calamus* has also been analyzed using RAPD [5] and ISSR markers [6], but these markers have shown low level of polymorphism in comparison to the presently developed cpSSR markers. The cpSSR markers can detect intra- and interspecific genetic diversity with high precision, because it does not genetically recombine and exist in a heterozygous state [12].

Individualization of the plant germplasm resources has become important in the present day scenario for their proper management and utilization, as well as for the IPR protection [13]. This can be achieved easily by using the DNA typing techniques involving use of highly polymorphic markers like SSRs. The germplasm characterization is essentially required for the species like *A.calamus*, which has revealed very limited diversity in its available gene pool [13]. The study provides a set of SSR markers that have been proven beneficial for both intra and inter population variation in *A.calamus*. Till date, no SSR markers have been described and developed for the *A.calamus* in literature. Hence, these developed markers can suitably be used for studies related to population genetics and characterization of the elite lines of genetic material.

References

1. **Dhar U., Manjkhola S., Joshi M., Bhatt A., Bisht A. K. and Joshi M.** 2002. Current status and future strategy for development of medicinal plants sector in Uttaranchal, India. Curr. Sci., **83**: 956-964.

2. **Kokate C. K., Purohit A. P., Gokhale S. B.** 2005. Pharmacognosy, vol. XXXI. Nirali Prakashan Pune: 1-57.

3. **Maiti S.** 2005. Harnessing herbal heritage of India. News Letters- National Research Center for Medicinal and Aromatic plants. **6**: 1-6.

4. **Ravindran P. N. and Balachandran I** 2004. Under utilized medicinal spices. Spice India, **17**: 2-14.

5. **Chauhan H. S.** 2006. A high rhizome and oil yielding variety "CIM-Balya" of Vach (*Acorus calamus*). J Med. Arom. Plant. Sci., **28**: 632-634.

6. **Pai A. and McCarthy B. C.** 2005. Variation in shoot density and rhizome biomass of *Acorus calamus* L. with respect to environment. Castanea., **70**: 263-275.

7. **Buteler M. I., Jarret R. L. and La Bonte D. R.** 1999. Sequence characterization of microsatellites in diploid and polyploid *Ipomoea*. Theor. Appl. Genet., **99**: 123-132.

8. **Ogliari J. B., Boscariol R. L. and Camargo L. E. A.** 2000. Optimization of PCR amplification of maize microsatellite loci. Genet. Mol. Biol., **23**: 395-398.

9. **Deguilloux M. F., Pemonge M. H. and Petit R.** 2004. Use of chloroplast microsatellites to differentiate oak populations. Ann. For. Sci., **61**: 825-830.

10. **Stange C., Prehn D., and Johnson P. A.** 1998**.** Isolation of *Pinus radiata* Genomic DNA suitable for RAPD analysis. Plant. Mol. Biol. Rep., **16**: 1-8.

11. **Goremykin V. V., Holland B., Hirsch-Ernst K. I. And Hellwig F. H.** 2005. Analysis of *Acorus calamus* chloroplast genome and its phylogenetic implications. Mol. Biol. Evol., **22**: 1813-1822.

12. **Echt C. S., Deverno L. L., Anzidei M. and Vendramin G. G.** 1998. Chloroplast microsatellites reveal population genetic diversity in red pine, *Pinus resinosa* Ait. Mol. Eco., **7**: 307-316.

13. **Hendre P. S., Phanindranath R., Annapurna V., Lalremruata A. and Aggarwal R. K.** 2008. Development of new genomic microsatellite markers from robusta coffee (*Coffea canephora* Pierre ex A. Froehner) showing broad cross-species transferability and utility in genetic studies. BMC. Plant Biology., **8**: 1-19.