**Isolation and characterization of microsatellite markers from Hibiscus rosa-sinensis (Malvaceae) and cross-species amplifications**

**Simona Bruna ⊙Ezio Portis ⊙Luca Braglia ⊙Laura De Benedetti ⊙Cinzia Comino ⊙ Alberto Acquadro ⊙Antonio Mercuri**

# Isolation and characterization of microsatellite markers from *Hibiscus rosa sinensis* (Malvaceae) and cross-species amplifications

---

**Simona Bruna · Luca Braglia ·Laura DeBenedetti ·Antonio Mercuri**

CRA-FSO Research Unit for Floriculture and Ornamental Species, Corso Inglesi 508, I-18038, Sanremo, Italy

**Ezio Portis* ⊙Cinzia Comino ⊙Alberto Acquadro**

Di.Va.P.R.A. Plant Genetics and Breeding, University of Torino, via L. da Vinci 44, I-10095 Grugliasco (Torino), Italy

* corresponding author (e-mail: ezio.portis@unito.it)

**Abstract** We report on the development of 10 microsatellite markers in *Hibiscus rosa-sinensis* (Hrs). Three markers were obtained from sequences available in GenBank and seven were isolated using a two-step 'primer extension' procedure, based on the microsatellite-AFLP (M-AFLP) technique. Polymorphism was explored in 21 Hrs genotypes representing the genetic variation within commercial varieties. Inter-specific amplification was assessed on 12 *Hibiscus* wild species. A total of 45 and 56 alleles (ranging from 1 to 10 for each locus) was amplified respectively from the 21 Hrs varieties and among the full *Hibiscus spp.* genotype set. Primers and conditions for polymerase chain reaction (PCR) amplification of the detected loci are reported.

**Keywords** *Hibiscus* spp. ⊙ SSRs ⊙Microsatellite-AFLP ⊙Database search ⊙Cross-species amplification

*Hibiscus* is the largest genus of the Malvaceae family and includes at least 250 species growing in tropical and sub-tropical regions throughout the world. Within this genus the chromosome number varies from n = 12 to ~144, but most commonly is 14 or 18 (Fryxell 1968, 1988). The species belonging to this genus are characterised by a wide range of uses (Anderson and Pharis 2003; Cobley 1976; Wilson and Menzel 1964; Ugborogho and Shofoyeke 1983). Most of *Hibiscus* species are grown as ornamentals, among which *H. syriacus*, *H. storckii*, *H. arnottianus* and *Hibiscus rosa-sinensis* (Hrs), which is the most common and widespread group. Hrs varieties are largely cultivated in tropical areas but they are also widely grown as garden or potted plant in Europe, in the USA and in other countries with a temperate climate.

The high variability observed in Hrs is certainly due to its genetic origin. Singh and Khoshoo (1989) suggested that hybridization occurred between two main groups of species with different centre of origin (South-Indian Ocean Islands and southern coasts of Africa the first one, Pacific Islands the second one). To date, genetic variation within *Hibiscus* spp. and their genetic relatedness have been investigated by means of RAPD markers (Barik et al. 2006) and AFLPs (Tang et al. 2003, Van Huylenbroeck et al. 2000, Cheng et al. 2004, Bruna et al. submitted); moreover, a set of microsatellites markers have been developed for *H. tiliaceus* (Takayama et al. 2006). Microsatellites are highly represented in any plant genomes and their polymorphism can be detected with a simple polymerase chain reaction (PCR) (Morgante and Oliveri 1993).

In this work we report on the development and characterization of the first set of SSRs markers in *Hibiscus rosa-sinensis*. Two approaches have been followed: *in silico* analysis of publicly available *Hibiscus* DNA sequence and a two step "primer extension" procedure based on the microsatellite-AFLP (M-AFLP) technique (Van Eijk et al. 2001).

Using the *Taxonomy Browser* resource present in NCBI (www.ncbi.nlm.nih.gov/sites/entrez?db=taxonomy) we isolated 13 DNA sequences belonging to *Hrs*, and 23 sequences of other *Hibiscus* species. The sequences were analysed using Sputnik, a microsatellite motif identification program (http://cbi.labri.fr/outils/Pise/sputnik.html), by applying minimum length criteria of 10 repeat units for mononucleotides and 5 repeat units for all higher order repeats. Three microsatellite motifs were found, showing non-compound repeats, and primers were designed on the flanking regions (from H-DAT1 to H-DAT3, Table 1) using *Primer 3* software (Rozen & Skaletsky, 2000).

M-AFLP technique was carried out according to Acquadro et al. (2005a) as reported in Figure 1. Briefly, total DNA (200 ng) was co-digested with *Eco*RI (or *Pst*I) and *Mse*I, and ligated to standard adapters (Vos et al. 1995). The ligation reaction was used as a template for pre-amplification by means of primers complementary to the adapter sequences plus one selective nucleotide: *Eco*RI+A (or *Pst*I+A) and *Mse*I+C. M-AFLP selective amplifications were carried out using 10pmol of an *Eco*RI (or *Pst*I) adapter directed primer with three selective bases in combination with 10pmol of an 5'-anchored microsatellite primer [5'-AM primer: GTCG$(AG)_7$, GACG$(TG)_7$, and CAGC$(CA)_7$,]. The 20 µl PCR reaction contained also 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1U of GoTaq polymerase (Promega) in the manufacturer-supplied buffer. Amplified fragments were resolved on 5% denaturing poly-acrylamide gels and silver stained. Selected M-AFLP fragments were excised from gel, re-amplified, cloned by TA cloning kit (Invitrogen) and recombinant colonies were sequenced (BMR Genomics, Padova; www.bmr-genomics.it). From the derived sequences of each positive amplicon, two primers (forward and forward-nested primers, Table 1) directed towards the microsatellite motif were designed using *Primer 3* software (Rozen & Skaletsky 2000). The opposite microsatellite flanking sequence was amplified using the restriction-ligation reaction as template for the amplification, combining the previously developed forward primer with the *Mse*I primer with no selective nucleotide. Reactions were performed in a 20µl mixture containing 1µl of restriction ligation, as template, 10pmol *Mse*I primer, 10pmol forward primer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 1U of FastStart *Taq* polymerse® (Roche Applied Science), in the manufacturer-supplied buffer. Hot Start PCR was performed with the following profile: 94°C for 5min, followed by 35 cycles of 30s at 94°C, 30s at 55°C and 60s at 72°C, and ending with a 10min extension at 72°C. For nested PCR 1µl of 100-fold diluted first PCR reaction was used as template using the forward-nested primer and the *Mse*I primer with the same PCR conditions described above. Only single band products were sequenced. A second primer (reverse) was designed for each positive sequence. Seven M-AFLP-derived SSR primers were

designed (from H-MAFLP1 to H-MAFLP7), three of them showed compound while 4 showed non-compound repeats (Table 1).

The ten developed SSRs were tested for their informativeness on 21 *H. rosa sinensis* varieties which are representative of the genetic variation present in commercial varieties (Bruna et al. submitted). DNAs from 12 *Hibiscus* wild species (*H. schizopetalus, H. liliiflorus, H. fragilis, H. boryanus, H. arnottianus, H. storckii, H. kokio, H. denisonii, H. syriacus, H. tiliaceus, H. panduriformis, H. cannabinus)* were used to test transferability of the developed Hrs primers (Table 1). Genotyping was performed as described in Acquadro et al. (2005b). Briefly, PCRs were carried out in a volume of 20µl using 10pmol of both the forward and the reverse primer in the presence of 25 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1U of GoTaq polymerase (Promega) in the manufacturer-supplied buffer. A touchdown PCR protocol was used: 94°C for 5 min, then 11 cycles at 94°C for 30s, 60°C for 30s decreasing of 0.5°C every cycle, 72°C for 60s, followed by 24 cycles at 94°C for 30s, 55°C for 30s and 72°C for 60s.

A total of 45 alleles (ranging from 1 to 10 for each locus) was amplified from the 21 Hrs varieties assayed and six loci were polymorphic. All primer sets also amplified from 1 to 10 alleles without additional optimization in the other *Hibiscus* species, and 56 alleles were amplified from the full genotype panel (Table 1). Five out of the six polymorphic loci showed clear multi-banding patterns, as individuals presenting from one to four alleles per locus (Table 1) revealing tetrasomic inheritance. These banding patterns were shown to be highly reproducible in repeated amplifications.

The polymorphic information content (PIC) was estimated as by Anderson et al. (1992) and both the PIC among the 21 Hrs varieties ($PIC_a$) and among the full 33 genotypes set ($PIC_b$) were calculated for the polymorphic SSRs. $PIC_a$ ranged from 0.362 to 0.608 (mean 0.521±0.04), while $PIC_b$ varied from 0.469 to 0.801 (mean 0.628±0.05). The highest PIC values were produced by H-DAT2 while H-DAT1 and 4.6 H-MAFLP3 had respectively the lowest PICa and PICb values.

The high level of cross species amplification and the relatively high number of allele detected suggest that these markers can be used for both inter and intra-specific studies as well as to determine the presence of polyploidy within the *Hibiscus* genus.

## References


Acquadro A, Portis E, Albertini E, Lanteri S (2005a) M-AFLP based protocol for microsatellite loci isolation in *Cynara cardunculus* L. (Asteraceae). Mol Ecol Notes 5:272–274

Acquadro A, Portis E, Lee D, Donini P, Lanteri S (2005b) Development and characterisation of microsatellite markers in *Cynara cardunculus* L. Genome 48:217–225

Anderson N and Pharis J (2003) Kenaf fibre-A new basket liner. Minnesota Commercial Flower Growers Bulletin 52(3):7-9.

Barik S, Senapaki SK, Aparajita S, Mohapatra A, Rout GR (2006) Identification and genetic variation among Hibiscus species (Malvaceae) using RAPD markers. Z Naturfosch 1(2):123-128

Cheng Z, Rong Lu B, Sameshima K, Fu D, Chen J (2004) Identification and genetic relationships of kenaf (*Hibiscus cannabinus* L.) germplasm revealed by AFLP analysis. Genetic Resources and Crop Evolution 51:393–401.

Cobley LS (1976) An introduction of the Botany of Tropical Crops 1976, 2nd Edition, revised by Steel WM, Longmans Ltd., London, pp. 272

Fryxell PA (1968) A redefinition of the tribe Gossypieae. Bot Gaz 129:296-308.

Fryxell PA (1988) *Malvaceae* of Mexico. Syst Bot Monogr. 25:1-522.

Morgante, M. and Olivieri, A. M. 1993. PCR-amplified microsatellites as markers in plant genetics. Plant J. 3: 175-182.

Rozen S, Skaletsky HJ (2000) Primer 3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics. Methods and protocols: methods in molecular biology. Humana Press, Totowa, New Jersey, USA, pp 365–386

Singh F, Khoshoo TN (1989) Cytogenetic basis of evolution in garden Hibiscus. The Nucleus 32:62–67.

Takayama K, Kajita T, Murata J, Tateishi Y (2006) Isolation and characterization of microsatellites in the sea hibiscus (*Hibiscus tiliaceus* L., Malvaceae) and related hibiscus species. Molecular Ecology Notes 6:721–723

Tang T, Zhong Y, Jian S, Shi S (2003) Genetic diversity of *Hibiscus tiliaceus* (Malvaceae) in China assessed using AFLP markers. Annual of Botany 92:409-414.

Ugborogho RE, Shofoyeke BA (1983) Observations on the morphology, reproductive biology and economic importance of *Hibiscus esculentus* L. and *H. sabdariffa* L. (Malvaceae) in Nigeria. Nigrian Field 48:79–80.

Van Eijk M, De Ruiter M, Broekhof J, Peleman J (2001) Discovery and detection of polymorphic microsatellites by microsatellite-AFLP. In: Heller SR (ed) Plant and Animal Genome IX Abstracts, p. 143. Scherago International, New York.

Van Huylenbroeck JM, De Riek J, De Loose M (2000) Genetic relationships among *Hibiscus syriacus*, *Hibiscus sinosyriacus* and *Hibiscus paramutabilis* revealed by AFLP, morphology and ploidy analysis. Genetic Resources and Crop Evolution 47:335–343.

Vos P, Hogers R, Sleeker M, Reijans M, Lee T, Homes M, Freiters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new concept for DNA fingerprinting. Nucleic Acids Res 23:4407-4414.

Wilson FD and Menzel MY (1964) Kenaf (*Hibiscus cannabinus*) Roselle (*Hibiscus sabdariffa*). Econ Bot 18:80–91.

*Figure 1.* Diagram of the M-AFLP technique: A) First step: forward primer design; B) second step: reverse primer design

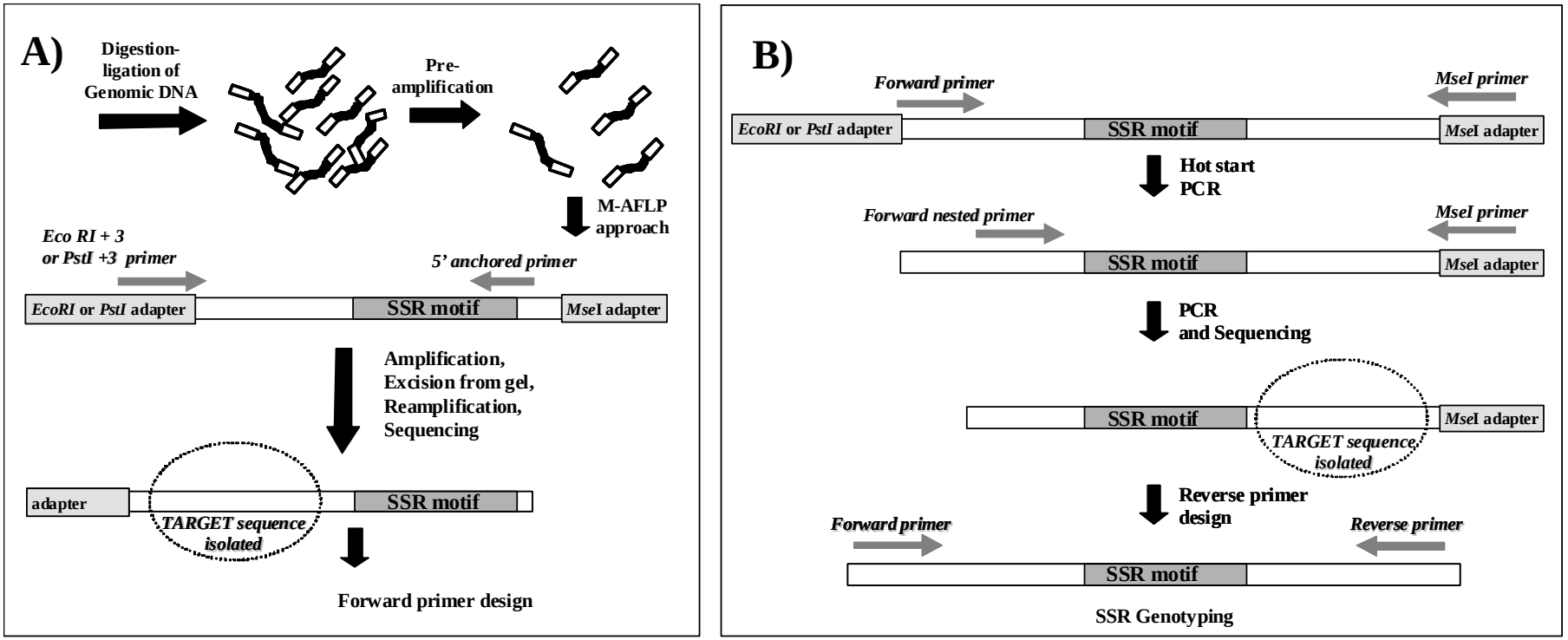

*Table1*. Characteristics of 10 microsatellite markers isolated from *Hibiscus rosa sinensis*.

| Locus name | Repeat motif | Primer sequence (5'-3') | Size range (bp) | Ta | NA1 | NA2 | NA/Ind | PICa | PICb | Gene bank Accession |
|---|---|---|---|---|---|---|---|---|---|---|
| H-DAT1 | $(TGC)_6$ | F: CCCTTCAAGTGCTCCTCT<br>R: TCAATTCACCTTCCGTACCC | 115-160 | 50° C | 10 | 10 | 1-4 | 0.362 | 0.479 | AY286052 |
| H-DAT2 | $(TTA)_6$ | F: TGTCAAGCTGTCAAGGGTGA<br>R: CCGATCCGTGTTTTTCAAGT | 400-480 | 55° C | 9 | 9 | 1-2 | 0.608 | 0.801 | AB233232 |
| H-DAT3 | $(TA)_{20}$ | F: AAGCGAAATCGACTGAAGGA<br>R: TGTCGTAGAAACTTCCAATCCA | 450 | 48° C | 1 | 1 | 1 | 0 | 0 | AY727162 |
| H-MAFLP1 | $(CT)_8$ | F: AGCCTGTCACCAACAAA<br>Fn: CCCCACTCACCTATTCC<br>R: GAGAGCTTACGAAGCGGAGA | 159-169 | 55° C | 5 | 9 | 1-4 | 0.570 | 0.703 | EU862808 |
| H-MAFLP2 | $(AC)_3(AT)_4$ | F: ACCGTTCTTTGTTTTAGATT<br>Fn: CAAGGGAACATTTATGATTAT<br>R: CAGGGAAAACCACAAAGGAA | 174-252 | 55° C | 6 | 10 | 1-4 | 0.559 | 0.613 | EU862809 |
| H-MAFLP3 | $(TA)_6$ | F: CTCCCAGTAGCTTTCTGTG<br>Fn: TCAGGCGTCTAATGTATATTTA<br>R: TGGGGTTTTGTTTCTTGCTT | 150-164 | 55° C | 5 | 5 | 1-4 | 0.450 | 0.469 | EU862810 |
| H-MAFLP4 | $(AC)_2(AT)_4$ | F: CACCNCAAACATACTCACAC<br>Fn: CATGCAAAAAGGTTACTAGA<br>R: ACTGTGCAGCCACTTCAACA | 154-192 | 55° C | 5 | 8 | 1-3 | 0.580 | 0.701 | EU862811 |
| H-MAFLP5 | $(TA)_5(GT)_{12}$ | F: GCCCTTGGGATAATGTT<br>Fn: CCCAACTAATGTAAATTGTGT<br>R: AGTGACCAGCGTAACTCT | 124 | 55° C | 1 | 1 | 1 | 0 | 0 | EU862812 |
| H-MAFLP6 | $(CA)_3$ | F: AACAAACTAGTAAAGAACGTACC<br>Fn: ACCGGTGATCAAAGTGTA<br>R: CGTAACGTATATCGGTTGTGC | 124 | 55° C | 1 | 1 | 1 | 0 | 0 | EU862813 |
| H-MAFLP7 | $(CA)_2$ | F: TTGGGAGGTTCGTTGT<br>Fn: AGGCAATCAACCTTTGA<br>R: TGCTTCCGCTTTCCATTATT | 138-140 | 55° C | 2 | 2 | 2 | 0.500 | 0.500 | EU862814 |

Ta: annealing temperature; NA: number of alleles, detected among 21 *Hibiscus rosa-sinensis* varieties (NA1) and in the whole set of genotypes; (NA2); Na/ind: number of alleles per individual; PIC: polymorphic information content calculated among 21 *Hibiscus rosa-sinensis* varieties (PICa) and among the whole set of genotypes (PICb).