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PRIMER NOTE

M-AFLP-based protocol for microsatellite loci isolation in *Cynara cardunculus* L. (Asteraceae)

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Abstract

Nine microsatellite markers for *Cynara cardunculus* L. were developed using a two-step 'primer extension' procedure, based on microsatellite-amplified fragment length polymorphism (M-AFLP) technique. In the first step, highly enriched SSR gel profiles were produced and, from the derived sequences of selected bands, forward primers directed towards the microsatellite motif were designed. In the second step, the opposite microsatellite flanking sequence was isolated using a nested approach on a restricted-ligated genomic fraction. Polymorphism was explored in 24 plants of wild cardoon (*Cynara cardunculus* L. var. *sylvestris*) as well as two accessions of both globe artichoke (*Cynara cardunculus* L. var. *scolymus*), and cultivated cardoon (*Cynara cardunculus* L. var. *altilis*).

Keywords: AFLP, artichoke, cardoon, microsatellite-AFLP, SSRs

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Cynara cardunculus L. is a diploid ($2n = 2x = 34$) species native to the Mediterranean basin, which includes varieties such as globe artichoke (H. var. *scolymus* L.), cultivated cardoon (var. *altilis* DC) and wild cardoon [var. *sylvestris* (Lamk) Fiori]. The latter is considered the ancestor of both cultivated forms on the basis of molecular (Lanteri *et al*. 2004), cytogenetic and isozyme (Rottenberg *et al*. 1996) studies.

We have previously generated a set of 23 microsatellite primer pairs in globe artichoke through either an enriched library approach or database sequence searches (Acquadro *et al*. 2003) and a novel approach, named MAL (microsatellite amplified library, Acquadro *et al*. 2005) which is a combination of the amplified fragment length polymorphism (AFLP) methods (Vos *et al*. 1995) with a primer–extension-based enriched library. From this set, five selected SSRs were recently used, together with AFLP markers, for the estimation of genetic diversity and relatedness between Italian and Spanish accessions of cultivated cardoon (Portis *et al*. 2005) and for assessing the genetic variation in seven Italian populations of wild cardoon from the islands of Sicily and Sardinia (Portis *et al*. 2005).

Here we report the development of a new set of nine polymorphic SSR markers in *Cynara cardunculus*, using a two-step 'primer extension' procedure, based on the microsatellite-AFLP (M-AFLP) technique (Van Eijk *et al*. 2001).

In the first step, total DNA (200 ng) was restricted-ligated (with *Eco*RI and *Mse*I restriction enzymes) and pre-amplified according to Vos *et al*. (1995); M-AFLP amplifications were performed as reported by Albertini *et al*. (2003). Briefly, selective amplifications were carried out using 10 pmol of an *Eco*RI adapter directed primer with three selective bases in combination with 10 pmol of an 5′-anchored microsatellite primer [5′-AM primer: $GTCG(AG)_7$, $GACG(TG)_7$, and $CAGC(TC)_7$] in a 20-μL polymerase chain reaction (PCR) consisting of 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 U of *Taq* polymerase (Promega) in the manufacturer-supplied buffer.

Amplified fragments were resolved on 5% denaturing polyacrylamide gels and silver stained. Selected M-AFLP fragments were excised from gel with a razor blade, boiled for 10 min in 20 μL of sterile double-distilled water, re-amplified and directly sequenced on an ABI3100 (Applied Biosystem) platform. From the derived sequence of each positive amplicon, a primer (the forward primer, Table 1) directed towards the microsatellite motif and a nested primer (forward nested, data not shown) were designed using the OLIGO software (Biogene). For two loci (CMAFLP-07, CMAFLP-11) additional internal SSRs were detected and internal specific microsatellite-flanking primers were designed.

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Table 1 Characterization of microsatellites developed from *Cynara cardunculus* ($n = 24$ wild cardoon accessions). N_A is number of alleles detected; H_E and H_O are expected and observed heterozygosities, respectively; F_{IS} is Wright's fixation index and *PI* is probability of identity

Locus	Repeat motif	Primer pair sequence (5′–3′)	Allele size range (bp)	N_A	H_E	H_O	F_{IS}	*PI*	GenBank Accession no.
CMAFLP-01	$(AT)_6(AC)_3(GA)_2$	F: TCATGGCATTTATGAATGTT	210–230	10	0.84	0.77	0.09	0.08	AY795822
		R: ATAAATATTTTGATTGTTTTCT							AY795823
CMAFLP-04	$(TC)_{11}(TA)_9(GT)_{13}$	F: CGATAGCTCTTTCCCTTT	310–360	10	0.87	0.77	0.11	0.06	AY795824
		R: ATGGGTGAGATTGGTTTAC							AY795825
CMAFLP-05	$(TC)_4(TA)_3(CA)_{12}$	F: GGATTTCTGATTCAGATGTTAT	150–152	2	0.48	0.50	−0.05	0.61	AY795826
		R: CACCCCAACTCTGAATGT							AY795827
CMAFLP-07	$(GA)_{12}(GA)_9$	F: GGCTCCACCGTACTCCTA	210–235	8	0.85	0.75	0.12	0.07	AY795828
		R: TCTCTCGTCAGTAAACCC							
CMAFLP-08	$(AG)_6(ATC)_4(CAT)_6$	F: AGGTGTGAAGGCTTCATC	380–420	6	0.71	0.67	0.06	0.21	AY795829
		R: TCCCGAGATCCTTGACTCAG							AY795830
CMAFLP-11	$(GCA)_6$	F: CGGCATTCAGGGAAGGAA	165–175	2	0.18	0.19	−0.06	0.88	AY795831
		R: AGGGGGAACAAGCCAT							
CMAFLP-13	$(GA)_4(TA)_6$	F: TTTGATCTTGTCCCTATATATATA	260–320	6	0.74	0.71	0.04	0.23	AY795832
		R: TCGGCTTTTCTGAATATC							AY795833
CMAFLP-15	$(GA)_{11}$	F: TTGAGAGGGTTTTCCGAGAG	80–95	6	0.80	0.71	0.11	0.13	AY795834
		R: TAGGATGAGTCCTGAGTAAT							AY795835
CMAFLP-18	$(CA)_8(TA)_6(CA)_5(TA)_4(GT)_5$	F: AAGTGTTGCATAATAACTTACC	200–250	13	0.89	0.69	0.23*	0.04	AY795836
		R: CCGAACAAATTGCTTACAA							AY795837

*Significant departure ($P < 0.05$) from Hardy–Weinberg equilibrium.

In the second step, the opposite microsatellite flanking sequence was amplified using the approach developed by Acquadro *et al*. (2005). The restriction–ligation reaction was used as a template for the amplification with the previously developed forward primer, in combination with the *Mse*I primer with no selective nucleotide. Reactions were performed in a 20-μL mixture containing 1 μL of restriction ligation as a template, 10 pmol *Mse*I primer, 10 pmol forward primer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 1 U of FastStart *Taq* polymerase (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 30 s at 94 °C, 30 s at 55 °C and 60 s at 72 °C, and ending with a 10-min extension at 72 °C. For nested PCR, 1 μL of 100-fold diluted first PCR was used as a template using the nested primer and the *Mse*I primer with the same PCR conditions described previously. Only single-band products where directly sequenced. A second primer (reverse) was designed for each positive sequence. The sets of primers designed are listed in Table 1.

The nine SSRs were then tested for their informativeness on 24 *C. cardunculus* L. var. *sylvestris* accessions collected in Palazzolo (Sicily, Italy) together with two accessions of globe artichoke (varietal types 'Spinoso sardo' and 'Romanesco'), and two of cultivated cardoon (varieties 'Gobbo di Nizza' and 'Lleno de España'). Genotyping was performed as described in Acquadro *et al*. (2005). Briefly, PCRs were carried out in a volume of 20 μL using 10 pmol of both the forward and the reverse primer (Table 1) in the presence of 25 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 U of *Taq* 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 30 s, 60 °C for 30 s decreasing of 0.5 °C every cycle, 72 °C for 60 s, followed by 24 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s. Seven microsatellites showed compound repeats, three of which were 'perfect' and four were 'imperfect'; only two SSR loci showed noncompound repeats (Table 1). All loci were polymorphic in the 24 wild cardoon individuals assayed and the number of alleles per locus varied from two to 13. The calculations of observed (H_O) and expected (H_E) heterozygosities, Wright's fixation index (F_{IS}) and the probability of identity (*PI*) were estimated with the program IDENTITY 1.0 (Wagner & Sefc 1999). Exact tests of Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were made by means of the software GENEPOP 3.4 (Raymond & Rousset 1995).

Observed and expected heterozygosities (H_O and H_E) ranged from 0.19 to 0.77 and from 0.18 to 0.89, respectively. Fixation index (F_{IS}) values ranged from 0.06 to 0.23. Only one of the nine loci (CMAFLP-18) showed significant deviation from HWE. This can be attributed to genotyping artefacts, such as the presence of null alleles. No significant pairwise LD was found among loci.

Allele frequencies ranged from 0.02 to 0.87. Nineteen (30%) were rare alleles ($P < 0.05$), of which eight were

exclusive of one genotype. CMAFLP-18 was the most informative locus (*PI* = 0.04) and CMAFLP-11 was the least informative (*PI* = 0.88). The total *PI* was 3.2×10^{-8}. Analysis of the 63 alleles detected by the nine SSRs allowed the unambiguous discrimination of the 24 wild cardoon genotypes included in this study. The same level of discrimination was also obtained by using only three of the nine SSRs: CMAFLP-01, CMAFLP-04 and CMAFLP-18.

All primer sets also amplified more than one allele per locus without additional optimization in globe artichoke and cultivated cardoon accessions, suggesting that these markers are potentially useful for characterizing genetic variation in the *Cynara cardunculus* species. Ongoing research is involved in applying these markers for the construction of a genetic-molecular map of the species.

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