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PRIMER NOTE

Isolation and characterization of polymorphic microsatellite markers in Galapagos prickly pear (*Opuntia*) cactus species

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Abstract

The *Opuntia* (prickly pear) genus contains over 200 species. Six of them are endemic to the Galapagos archipelago. Although these cacti are 'keystone' species of the Galapagos' semi-arid ecosystem, they have never been studied in detail. Because of their current threatened status and their important role in the ecosystem, we developed 16 microsatellite markers to study the population genetic structure of some of these species. These markers display a high level of polymorphism with numbers of alleles per locus ranging from six to 53. Results also revealed possible polyploidy in these cacti.

Keywords: Cactaceae, Galápagos, microsatellite markers, *Opuntia*, polyploidy

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Island archipelagos are crucial in the study of evolutionary processes. Galapagos, a classic example of such an island group, houses many endemic organisms that have been studied extensively in order to resolve evolutionary questions. In spite of their keystone role in the ecology of the Galapagos semi-arid ecosystem, the endemic *Opuntia* cactus species have, however, received little attention. The current classification of these *Opuntia* species is based on few overlapping morphological measurements and recognizes six species, further divided in 14 varieties (Anderson & Walkington 1971). Recent introductions of herbivorous mammals caused enormous damage to *Opuntia* populations (Grant & Grant 1989; Campbell & Donlan 2005). By consequence, many of these cacti are nowadays threatened with extinction and appear on the IUCN Red List. The population genetic structure of *Opuntia* species of Galapagos as studied by molecular markers, will therefore not only be informative in the interest of evolutionary processes but can also play an important role in the development of conservation plans.

Browne *et al*. (2003) were the first to study the genetic differentiation among Galapagos' *Opuntia* species using genetic (allozyme) data. Because of the low number of scorable loci and the complete absence of genetic variability they found, we decided to search for more variable markers. Here we describe the development of 16 microsatellite markers that will be useful to study population genetic aspects of at least the *Opuntia echios* varieties. Owing to their distribution patterns, these varieties will be informative about gene flow on different scales within an archipelago, and possible hybridization (Verdyck & Tye, personal observations). These markers will also give us some information about polyploidization, an important factor in cactus evolution (e.g. Pinkava *et al*. 1985).

We isolated genomic DNA from three *Opuntia echios* varieties (*O. gigantea* from Santa Cruz, *O. zacana* from North Seymour, and *O. echios* from Baltra and Santa Cruz) using the DNeasy Plant Mini Kit protocol (QIAGEN). In addition to the standard procedures, we conducted an extra centrifugation step of 5 min to the lysate at full speed to remove most of the precipitates. To maximize overall DNA yield, extraction products were dissolved in 100 µL of water. The three extraction products were pooled to develop microsatellite markers. A TOPO shotgun subcloning kit (Invitrogen) was used to construct a genomic library according to the manufacturer's instructions. Literature study showed that CA, TC and GA microsatellite repeats are most commonly used in recent plant studies. To screen recombinant colonies $(CA)_{10}$, $(TC)_{10}$ and $(GA)_{10}$ probes were ^{32}P radioactively end-labelled using a T4 polynucleotide kit (Invitrogen). Hybridization was performed overnight in a church buffer at 50 °C. Subsequent washing took place in 5× SSC, 15 min at room temperature, and 15 min at 50 °C. Twenty thousand colonies were screened by colony hybridization, 45 colonies were sequenced, of which 95% contained microsatellites.

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Positive clones were sequenced with M13 forward and M13 reverse vector primers. Sequences were analysed until 20 microsatellite loci were found which contained sufficiently long flanking regions and number of repeats.

PRIMER 3 software (Rozen & Skaletsky 2000) was used to develop primers. Manual adjustments were made to minimize primer–dimer formation in multiplex combinations. To optimize annealing temperatures, gradient polymerase chain reactions (10 °C) of five samples were run for all loci in monoplex and in the proposed multiplex combinations (Table 1). Microsatellite DNA amplifications were performed in 5 μL total reaction volume. Each reaction mixture contained: 0.5 μL extract, 1× REDTaq Thermophylic Reaction Buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.01% gelatine), 1.875 mM $MgCl_2$, 0.175 U REDTaq DNA polymerase (Sigma-Aldrich), 320 μM of each dNTP, and 0.25 μL of each primer. Samples were amplified on a TGradient instrument (Biometra) using the following parameters: after an initial denaturation of 2 min (94 °C), 34 cycles were performed each consisting of 1 min of denaturation (95 °C), 90 s of annealing (temperatures in Table 1), and 2 min of extension (72 °C). A final extension of 9 min (72 °C) was the last step in this protocol. The resulting products were run on a 1.2% agarose TBE gel to check the quality and quantity of each reaction. Two of the proposed multiplex combinations (indicated with * in Table 1) did not work well and we recommend monoplexing these loci to obtain better results. Forward primers were then Cy5-labelled, and reverse primers were 5′-GTTT pigtailed to reduce nonspecific amplification (Brownstein *et al*. 1996).

To estimate degree of polymorphism, total DNA from 231 individuals representing two *O. echios* varieties (*O. echios* and *O. gigantea*) was isolated using DNeasy Plant Mini Kits (QIAGEN). Microsatellite DNA amplifications were performed as described before. ALF express sequencer and ALLELELOCATOR 1.03 (both Amersham Pharmacia Biotech) were, respectively, used to visualize and analyse PCR products. Product sizes were determined by comparison with M13mp8DNA standards, as described by Van Oppen *et al*. (1997). Sixteen microsatellite loci showed clear multibanding patterns presenting one to eight bands per locus. These banding patterns were shown to be reproducible in repeated amplifications. The number of alleles ranged from six to 53 per locus. Possibly due to the low variability between Galapagos' *Opuntias*, primers also successfully amplified microsatellites for other endemic species on the archipelago (*O. galapageia* var. *macrocarpa*, *O. saxicola*, and *O. megasperma*).

Polyploidy has been important in the evolution of Cactaceae (Gibson & Nobel 1986), particularly in the genus *Opuntia* (Pinkava *et al*. 1985). Earlier studies revealed chromosome numbers in *Opuntia* cacti ranged from $2n = 11$ to $2n = 88$ (e.g. Pinkava *et al*. 1998; Palomino & Heras 2001; Powell & Weedin 2001; Cota-Sánchez 2002). The multiple banding patterns observed at each locus in this study can indeed be an indication of these earlier findings. However, because the exact ploidy level of Galapagos' *Opuntia* species is not yet known, we are not able to calculate H_O at this point.

The high level of polymorphism, with number of alleles ranging from six to 53, makes these markers very suitable to investigate the population genetic structure of these varieties and possibly many other *Opuntia* species.

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Table 1 Characterization of 16 microsatellite markers isolated from *Opuntia echios* based on 231 collected individuals

Marker name	Repeat motif in library	Primer sequences 5′–3′	Multiplex (T_a in °C)	Size range (bp)	No. successfully genotyped	Total no. of bands	No. of bands per ind.	Mean no. of alleles per ind. (± SD)	Pr un ge
Opuntia6	$(TC)_{14}$ $(CA)_{10}(CT)_5$	F: Cy5-ATCTCATTGTATCATCTATTTCCTG R: GTTT-AGCACAAAGACACTTCATCG	1 (55.2)	127–152	205	15	1–7	3.2 (± 1.3)	0.
Opuntia2	$(AG)_{14}(CG)_4$	F: Cy5-CACATACGCAAATACATGG R: GTTT-GCTTCATTTTCCAGGTTACT	1 (55.2)	211–258	192	35	1–8	4.3 (± 1.3)	0.
Opuntia4	$(GA)_{12}$	F: Cy5-GATGATTCCGCCATTCACC R: GTTT-CGTCGATCTGACTCACACC	2 (53.5)	105–152	210	32	1–8	4.8 (± 1.3)	0.
Opuntia14	$(TA)_{17}$ $(GA)_{13}$	F: Cy5-TCAGGATTCAAGAAGATTTGC R: GTTT-CGATTCAATTGATGATGGGC	2 (53.5)	206–258	184	44	1–8	4.3 (± 1.9)	0.
Opuntia11	$(CT)_{13}TT$ $(CT)_2$	F: Cy5-CCTACACCTGCTGCCAATC R: GTTT-CGAGACAAACATCAGAGGAG	3 (53)*	110–138	212	17	1–5	2.6 (± 1.1)	0.
Opuntia13	$(AG)_{12}$	F: Cy5-CCAAATACCCAGCCCATAC R: GTTT-CGAGAACCTAACTTCCGATG	3 (53)*	247–301	210	37	1–8	4.6 (± 1.5)	0.
Opuntia15	$(TC)_{10}$	F: Cy5-GCGGTGGAAGCAGTTAGG R: GTTT-TCAGTCGATCATACCCAAGG	3 (53)*	343–357	191	10	1–4	1.64 (± 0.58)	0.
Opuntia8	$(CT)_5(TC)_{12}$ $GC(TC)_5$	F: Cy5-ACCGCCATCACCAGCTATC R: GTTT-CTCACCCACAATTCCAAACC	4 (57)	136–178	218	34	1–7	3.4 (± 1.4)	0.
Opuntia12	$(TC)_4C$ $(TC)_{12}$	F: Cy5-TAATCTTATTCTCAGGTCAGTTAC R: GTTT-GGTATCTTGTTATTCGTTCG	4 (57)	226–294	202	53	1–8	6.6 (± 1.5)	1.
Opuntia9	$(AG)_{15}$	F: Cy5-CTAGGCTTCATCCCACATTAGG R: GTTT-TCCAAATTCACCTCCTCTGC	5 (59.3)*	147–185	180	28	2–8	4.7 (± 1.2)	0.
Opuntia16	$(GA)_8$	F: Cy5-GTCAATCCCGAGCAATTTAGG R: GTTT-CTCATTAGTGAGGCCCAACG	5 (59.3)*	322–350	200	17	2–8	4.1 (± 1.3)	0.
Opuntia21	$(TC)_{14}$	F: Cy5-AAAGGGAAGACCTTGCTCTC R: GTTT-TCTATTCTCAGCCCTCCTCTC	6 (59)	75–144	191	42	1–8	6.1 (± 2.1)	0.
Opuntia10	$(CT)_9$	F: Cy5-ACCAACATCAAACCTTCAATACC R: GTTT-CATGCTTCATCTTGTTCATTGG	6 (59)	191–247	204	24	2–8	3.6 (± 1.0)	0.
Opuntia3	$(AG)_{19}$	F: Cy5-GTGAGTGCCCAGATGAAACT R: GTTT-TCCTCAACTTTATTGTAGCAAGAG	6 (59)	317–344	200	15	1–7	3.1 (± 1.2)	0.
Opuntia1	$(CT)_{13}(AC)_3$	F: Cy5-CCATCTACTTCCCACTTTGC R: GTTT-CTCCTGTGTTTCTCTGTGCTC	7 (61.5)	100–126	214	17	1–7	3.1 (± 1.1)	0.
Opuntia5	$(TAC)_5$	F: Cy5-TATGCACAAAGCACCATGC R: GTTT-CCAACCATACCAACTGTACTGAC	7 (61.5)	352–367	199	6	1–4	1.97 (± 0.70)	0.

*Primers that were originally developed for multiplex purpose, but we recommend to PCR them separately to obtain best results; T_a, annealing temp