Development and Characterization of Microsatellite Markers (SSR) in *Cucurbita* Species

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Introduction: At present, microsatellite (SSR) markers are the most useful tools for relationship studies as well as mapping, due to their easy handling, co-dominant inheritance, and multiallelic and highly polymorphic nature. They provide stable (anchor) points of physical orientation in the plant genome. Their major drawbacks are the high amount of labour and high costs required for their development. So far, no published SSR marker exists for *Cucurbita* species. Conventional methods of SSR isolation consist of screening partial genomic libraries with appropriate probes (4) followed by large amounts of sequencing work. Meanwhile, to reduce time requirement and costs, several alternative isolation strategies have been introduced for SSR marker development (8). We adapted a method, which relies on the digestion-ligation reaction of the amplified fragment length polymorphism procedure (AFLP, 7). The DNA-fragments are then fished by Streptavidin coated Dynabeads (5, and personal communication of T.C. Glenn). In this communication we describe the first results of applying this technique for SSR isolation in *C. pepo*.

Material and Methods: *Plant material:* For SSR development genomic DNA was extracted from the Austrian oil-pumpkin variety "Gleisdorfer Ölkürbis". For the relationship study using the newly developed SSR markers, 48 genotypes were selected. This included representatives of the species *C. pepo*, *C. maxima*, *C. moschata* and *C. ecuadorensis*. Within the *C. pepo* group, beyond representatives of all cultivar types a described by Paris (3), emphasis was put on hull-less pumpkin genotypes (numbers 18 to 36 in Fig. 1).

DNA isolation: Genomic DNA was extracted from young leaves of oil-pumpkin plants according to the procedure of the Wizard® Genomic DNA Purification Kit, supplied by Promega Corp., WI, USA (www.promega.com).

Microsatellite enrichment, screening and sequencing: SSR isolation was done following a slightly modified procedure of Schable et al. (5). After digestion with *RsaI*, fragments were ligated to SuperSNX24 linkers and hybridized to biotinylated microsatellite oligonucleotides. These were captured with streptavidin coated paramagnetic beads (Dynal). Uncaptured DNA was washed away and the remaining DNA was amplified using the SuperSNX24 primer. The product was ligated into AccepTor Vector (Novagen), inserted into NovaBlue Singles Competent Cells and screened for inserts using the β-galactosidase gene. Positive clones were amplified using T7 and SP6 primers and screened for their size. Sequencing of fragments with a size above 500 bp was done by an outside company (IBL, Vienna). For automated microsatellite sequences the search sequences were exported to SSRIT (http://www.gramene.org/gramene/searches/ssrtool). All sequences were checked against each other using the FASTA program (http://www.ebi.ac.uk/fasta33/nucleotide.html). PCR primers were designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), in the 18-25 bp range. They were synthesised by MWG Biotech AG (D-85560 Ebersberg). PCR

amplifications for primer testing were performed in 15µl volume using an Eppendorf Mastercycler Gradient thermal cycler. The final PCR concentration was 40 ng of genomic DNA, 2.25 µM of each primer, 1x reaction buffer containing 1.5 mM $MgCl_2$, 3.75 mM dNTP and 0.5 U *Taq* polymerase. Cycling parameters were 94°C for 60 s, followed by 32 cycles of 94°C for 25 s, an annealing step between 48°C and 58°C for 25 s and elongation at 72°C for 25 s, finally 72°C for 5 min. PCR products were scored on an 12% acrylamide gel stained with silvernitrate (6) (Fig.1). The annealing temperature, which gave the best result, was chosen for follow up experiments.

Primer evaluation and relationship study: Amplification using 22 primers was performed in 10µl volume (26 ng of genomic DNA, 1.49 µM of each primer, 1x reaction buffer containing 1.5 mM $MgCl_2$, 2.5 mM dNTP and 0.35 U *Taq* polymerase) with a MWG Primus 96 plus Thermocycler. Fragment separation and staining was done according to Stift et al. (6), allele scoring and sizing by manual screening.

Statistical analysis: The SPSS software package was used for data processing. Distance between clusters was calculated as the average distance between all pairs of genotypes (UPGMA). The relationship between genotypes was measured by squared Euclidean distance.

Results and Discussion: The library enrichment in microsatellite repeats resulted in 1704 positive clones. 621 had a size above 500 bp and had been sequenced. 334 (54%) contained a repeat. Duplicates or repeats too close to the cloning site were eliminated. Primer pairs were designed for 81 (13%) sequences. 56 (9%) primer pairs amplified a product of the expected size, 25 did not give any amplification product. The 56 primer pairs were tested for polymorphism and quality on a set of 12 selected *Cucurbita* genotypes. 29 of the primers gave only monomorphic bands. From the remaining 27, the best 22 primer pairs were used for the relationship study (Fig. 2). The number of alleles per locus ranged from 2 to 6 with an average of 4.4. The relationship study of the selected genotypes using 22 *Cucurbita* specific SSR markers is in full agreement with previous results of Decker (1) and Katzir et al. (2), by grouping the *C. pepo* genotypes into the two subspecies *ovifera* and *pepo* (1). The Cocozelle genotype "Striato d'Italia" (Z6) is far away from the Zucchini genotypes, as was also found by Katzir et al. (2). Within the *ssp. ovifera* in this sample of genotypes, no correspondence to the cultivar groups as established by Paris (3), can be seen. The analysis clearly differentiated the species *C. maxima* and *C. moschata,* from each other and from the *C. pepo* group. As expected, most of the oil-pumpkins bred by the company "Gleisdorf" are clustered together. Other oil-pumpkins in the vicinity might be derivatives of Styrian material.

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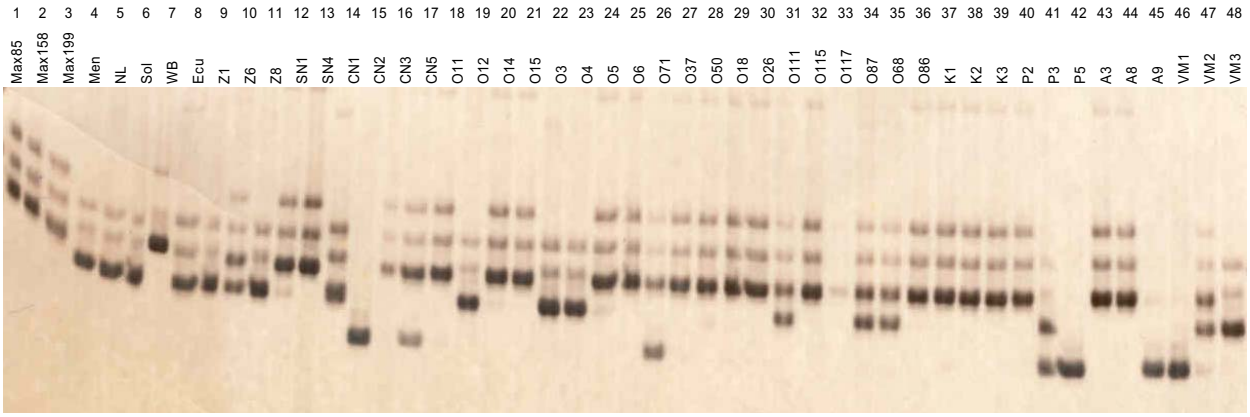

Fig. 1: Characterization of 48 *Cucurbita* genotypes for SSR marker SGA3 separated in 12% polyacrylamide, stained with silvernitrate. The marker detected one amplification site per genotype (smallest fragment) with 4 alleles. Eight of the genotypes are heterozygous, e.g., 10, 17, 26 etc.

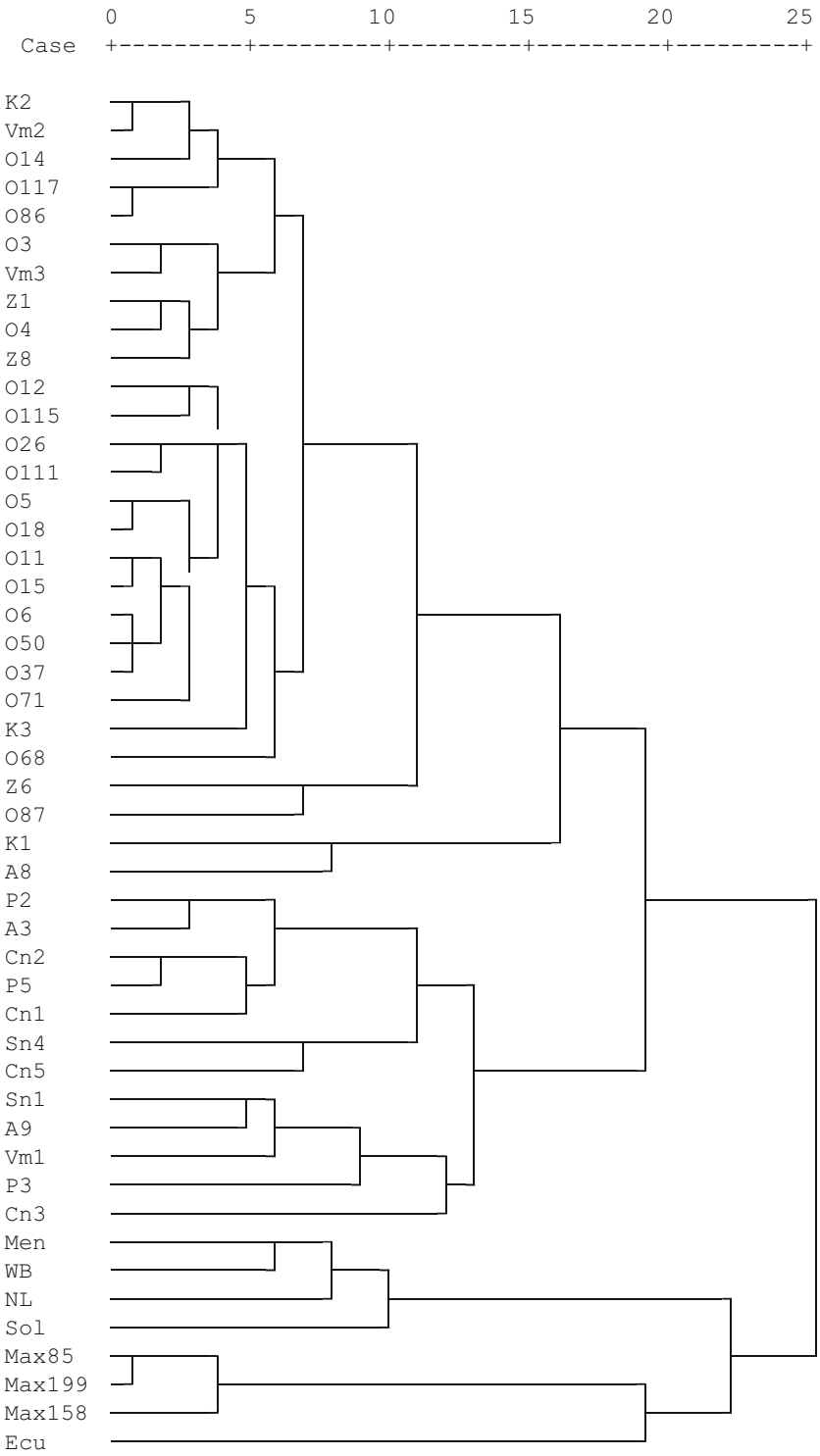

Fig. 2: Dendrogramm based on *C. pepo* SSR markers scoring 48 genotypes of *C. pepo*, *C. moschata*, *C. maxima* and *C. ecuadorensis*. The abbreviations are as in Table 1.

Max85	*C. maxima*	Max85
Max158		Max158
Max199		Max199
Men	*C. moschata*	Menina
NL		Nig. Local
Sol		Soler
WB		Waltham Buttern.
Ecu	*C. ecuadorensis*	Grif 9446 01 SD
	C. pepo	
Z1	**Zucchini**	True French
Z6		Striato d' Italia (Cocozelle)
Z8		Erken
SN1	**Straightneck**	General Patton
SN4		Sunray
CN1	**Crookneck**	Bianco Friulano
CN2		Courge Cou Tours
CN3		ohne Namen
CN5		Sundance
K1	**Pumpkin**	Pomme d'Or
K2		Tondo di Padana
K3		Chinese Miniatur
O11		Retzer Gold
O12		Gleisdorfer Ölk.
O14		Sepp
O15		Markant
O3		Chinesischer
O4		S-Afrika
O5		Lady Godiva
O6		Estancia Bugar
O71		Georgica
O37		Miranda
O50		Slovenska Golica
O18		Kakai
O26		Lu's Ölkürbis
O111		Szentesi Oliva
O115		09H4 CZ
O117		PM 18
O87		Pulawska
O68		Anton Berger
O86		PI 285611
P2	**Scallop**	unknown
P3		Early White Bush
P5		Galeux
A3	**Acorn**	Tay Belle
A8		Thelma Sanders Sweet Potato
A9		Yugoslavian finger
VM1	**Veg. Marrow**	unknown
VM2		Bulgarian Summer
VM3		Alba

Table 1: List of *Cucurbita* genotypes used for the realtionship study with *Cucurbita* specific SSRs.
(O = Oil-pumpkin)