Europ.J.Hort.Sci., **74** (2). S. 86–94, 2009, ISSN 1611-4426. © Verlag Eugen Ulmer KG, Stuttgart

Genetic Diversity of *Osteospermum* Genotypes Analysed by AFLP and Chloroplast SSR Markers

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Summary

Osteospermum cv. belongs to the so called 'New ornamental plants', which gained much popularity over the last few years. A collection of 111 *Osteospermum* and nine *Dimorphotheca* genotypes were evaluated by AFLP (Amplified Fragment Length Polymorphism) analysis. In the present study we analysed the genetic diversity within breeding pools from different breeders together with a selection of wild species.

The genotypes could be grouped into four main clusters. While Faccioli et al. (2000) were able to classify the genotypes by origin of the cultivar, no such relation was found in the present work. Yellow flower colour, assumed to originate from only a few genetic sources was found in all four major clusters. However, within the clusters these genotypes were grouped together. Overall genetic distances among *Osteospermum* genotypes turned out to be small emphasizing the need to optimise the choice of parental genotypes in breeding programs aiming at low inbreeding depression.

Three different cluster methods (UPGMA, Fitch and Margoliash and Neighbour-joining) were used to calculate the phenograms. To estimate the reliability of dendrograms a bootstrap-analysis has been carried out with 100 replicates. The phenograms revealed only marginal differences between the methods. These differences are mainly found for groups with small bootstrap-values, including interspecific hybrids.

In addition to cluster analyses, the data set was analysed by principal component analysis (PCA) to display similarities among genotypes. The same four major groups could be distinguished supporting the cluster analyses.

The AFLP data are complemented with data from two chloroplast SSR markers which allows the differentiation of eight cytoplasmic lineages among the genotypes investigated.

Key words. *Asteraceae* – genetic resources – dendrograms

Introduction

Osteospermum cv. is a so called "new ornamental" plant and became a very popular ground cover and bedding plant over recent years. It belongs to the family *Asteraceae* and originates from tropical regions of Africa, St. Helena, and the southwest of Saudi-Arabia (Bremer 1994). The growth habit varies from erect to prostrate and decumbent and the plants develop numerous flowers grouped in a capitulum with tubular central disk florets and a ring of ligulated ray florets (Faccioli et al. 2000). *Osteospermum* and *Dimorphotheca* are taxonomically very closely related genera of the *Asteracea* family joined into the tribe *Calendulae* (Norlindh 1943). Due to the close relationship several species were reclassified into one or the other genus in the past so that for example *Osteospermum ecklonis* is also cited as *Dimorphotheca ecklonis* in the literature (Norlindh 1943; Vaira et al. 2000)

The *Osteospermum* varieties known today are mainly interspecific hybrids (Faccioli et al. 2000). They were developed from English, Danish, German and South African varieties and are continuously improved by crosses between varieties

In the work presented here we evaluated the molecular diversity of genetic resources for *Osteospermum* and *Dimorphotheca* breeding comprising a selection of wild species. Due to synonyms and similarities among the genotypes a classification on the basis of morphological characters can be very difficult. Faccioli et al. 2000 and Berio et al. (2001) applied RAPD markers to distinguish *Osteospermum* varieties from different breeding gene pools and found a high level of genetic diversity among the varieties. In addition they could group most of the genotypes according to their origin. AFLP's display a high potential to distinguish very similar genotypes, have a higher information content per analysis and are technically more reliable then RAPDs (Debener et al. 2000; Vosman et al. 2004; Linde et al. 2007). Therefore, we applied AFLPs to a larger set of *Osteospermum* and *Dimorphotheca* genotypes to improve the reliability and the resolution of estimates about polymorphisms in these taxa. Chloroplast microsatellites (cpSSR) consist of short poly(A) and poly(T) repeats with sizes around 20bp from the chloroplast genome (Weising and Gardner 1999). As plastid sequences are generally highly conserved the published primer sequences can be used across a large number of Angiosperm taxa (Weising and Gardner 1999). Advantages of cpSSRs are their maternal inheritance in most Angiosperms so that inheritance of cytoplasmic components or maternal lineages can be followed in taxonomic

Table 1. List of *Osteospermum* and *Dimorphotheca* genotypes analysed in the present study.

Code	Series name	Cultivar name	Origin[1]	Flower colour	Maternal line
1	Cape Daisy	not defined	G	light violet	I
2	Cape Daisy	not defined	G	light violet / white	II
3	Cape Daisy	not defined	G	dark red	I
4	Cape Daisy	not defined	G	white	I
5	Cape Daisy	not defined	G	unknown	I
6	Cape Daisy	not defined	G	unknown	II
7	Cape Daisy	not defined	G	yellow	II
8	Cape Daisy	not defined	G	yellow	III
9	Sunnyside	not defined	F	white	I
10	Sunnyside	not defined	F	light violet	II
11	Sunnyside	not defined	F	unknown	II
12	Sunnyside	not defined	F	unknown	I
13	Sunny	not defined	J	unknown	I
14	Tradewinds	not defined	K		I
15	Tradewinds	not defined	K	light violet / white	I
16	Tradewinds	not defined	K		I
17	Dalina	not defined	L		I
18	Dalina	not defined	L		I
19	Sunsation	not defined	H	blue	I
20	Sunsation	not defined	H	white	II
21	Sunsation	not defined	H	white	I
22	Sunadora	not defined	H	orange	II
23	Springstar	not defined	D	unknown	I
24	Jamboana	not defined	E	white	II
25	Jamboana	not defined	E	purple / white	II
26	Astra	not defined	B	white	II
27	Astra	not defined	B	rose	II
28	Astra	not defined	B	violet	II
29	Impressario	not defined	C	rose- purple	II
30	Impressario	not defined	C	white	II
31		'Whirling'	P		I
32		'La Mortola'	P		I
33		'Buttermilk'	P		I
34		*O. ecklonis*	P		I
35	Flower Power	not defined	I		II
36	Flower Power	not defined	I		II
37	Flower Power	not defined	I		I
38	Flower Power	not defined	I		II
39	Flower Power	not defined	I		II
40	Flower Power	not defined	I		I
41	Cape Daisy	not defined	G	yellow	III
42	Sunnyside	not defined	F	unknown	II
43	Sunnyside	not defined	F	unknown	II
44	Symphony	not defined	D	yellow	I
45	Symphony	not defined	D	yellow	I
46	Symphony	not defined	D	yellow	I
47	Symphony	not defined	D	yellow	I
48	Summer Daisy's	not defined	C	salmon	I
49	Summer Daisy's	not defined	C	yellow/orange	I
50		'Sparkler'	P		II
51		'Silver Sparkler'	P		II
52	Flower Power	not defined	I		I
53	Flower Power	not defined	I		I

Table 1. Continued

Code	Series name	Cultivar name	Origin[1]	Flower colour	Maternal line
54	Flower Power	not defined	I		I
55	Flower Power	not defined	I		I
56	Flower Power	not defined	I		II
57	Flower Power	not defined	I		II
58	Flower Power	not defined	I		I
59	Flower Power	not defined	I		I
60	Flower Power	not defined	I		II
61	Flower Power	not defined	I		III
62	Flower Power	not defined	I		I
63	Flower Power	not defined	I		II
64	Flower Power	not defined	I		I
65	Flower Power	not defined	I		I
66	Flower Power	not defined	I		I
67		*O. ecklonis* 'Prostratum'	P		I
68		*O. ecklonis* 'Giant'	P		I
69		*O. jucundum* 'Compactum'	P		IV
70	Symphony	not defined	D	cream / white	I
71	Symphony	not defined	D	cream	I
72	Springstar	not defined	D	unknown	I
73	Springstar	not defined	D	unknown	I
74	Sunadora	not defined	H	yellow	II
75	Sunadora	not defined	I	violet / salmon	II
76		'Chris Brickell'	P		not defined
77		'Giles Gilbey'	P		I
78		'Cannington Joyce'	P		I
79		'Cannington John'	P		I
80		'Hampton Court'	P		VII
81		'Blue Streak'	P		I
82		'Coconut Ice'	P		III
83		'Cannington Kira'	P		I
84	Dalina	not defined	L		II
85	Sunny	not defined	J	light violet	II
86	Impressario	not defined	C	purple	II
87		'Weetwood'	P		IV
88		'Gweek'	P		III
89	Cape Daisy	not defined	G	unknown	I
90	Sunny	not defined	J	white / rose	I
91	Cape Daisy	not defined	G	yellow	III
92	Crescendo	not defined	F	orange	I
93	Crescendo	not defined	F	white	I
94	Sunny	not defined	J	vanilla / orange	II
95	Sunny	not defined	J	pink / orange	II
96	Symphony	not defined	D	orange	I
97	Flower Power	not defined	I		II
98	Flower Power	not defined	I		I
99		*O. ecklonis* 1	A	unknown	I
100		*O. ecklonis* 2	A	unknown	I
101		*O. ecklonis* 3	A	unknown	I
102		*D. cuneata* 1	A	unknown	VI
103		*D. cuneata* 2	A	unknown	VI
104		*D. cuneata* 3	A	unknown	VI
105		'Sky & Ice' 1	A	white / blue centre	II
106		'Sky & Ice' 2	A	white / blue centre	II

Table 1. Continued

Code	Series name	Cultivar name	Origin[1]	Flower colour	Maternal line
107		'Sky & Ice' 3	A	white / blue centre	II
108		*O.* 'Passion Mix' 1	A	white to pink/violet	II
109		*O.* 'Passion Mix' 2	A	white to pink/violet	II
110		*O.* 'Passion Mix' 3	A	white to pink/violet	II
111		*O. vaillantii* 1	A	unknown	II
112		*O. vaillantii* 2	A	unknown	II
113		*O. vaillantii* 3	A	unknown	II
114		*D. sinuata* 1	A	yellow	V
115		*D. sinuata* 2	A	yellow	V
116		*D. sinuata* 3	A	yellow	V
117		*D. pluvialis* 1	A	white or yellow	II
118		*D. pluvialis* 2	A	white or yellow	II
119		*D. pluvialis* 3	A	white or yellow	II
120	Sunny	not defined	J	yellow	III

[1]A: B & T; B: Florensis; C: Grünewald; D: Kientzler; E: S & G; F: Sakata; G: Sörensen; H: Schmülling; I: Selecta; J: Sunny; K: Goldsmith; L: Young Plants; P: private collection

studies (Provan et al. 2001) and their high reproducibility in comparison to RAPD markers. We included cp SSRs into our analysis to get information about the genetic diversity of the maternal ancestors of the varieties under investigation. For mainly outcrossing crops as *Osteospermum* it is important to evaluate genetic resources to improve breeding of new varieties by avoiding inbreeding depression. Our results demonstrate a strategy to circumvent this problem by using genetic distance data for selection of crossing partners.

Materials and Methods

Plant material: We used 120 *Osteospermum* and *Dimorphotheca* genotypes from various breeding companies. Some cultivars belong to so called "cultivar series" differing for example in flower colour while other traits are mostly similar. The genotypes are listed in Table 1 with the name of the series and the origin (company that released the variety). Most cultivars are freely available, but some of them are newly bred and not available yet. Therefore, these genotypes are only listed with their code number and, in some cases, the name of the variety series.

DNA extraction

Genomic DNA was isolated from 9.5–10.5 mg of dried leaf material. The isolation was done with the Qiagen DNeasy® plant Mini Kit according to the suppliers' instructions. Quantification of the genomic DNA was achieved by electrophoresis through 1 % Agarose gels and independently by spectrophotometry following standard procedures (Sambrook et al. 2000)

AFLP-analysis

AFLP analyses were performed as described by von Malek et al. (2000) with minor modifications. After restriction and ligation, the preamplification reaction was conducted with primers lacking any selective nucleotides (Table 2). The final reaction was carried out with primers containing three selective nucleotides at the 3'-end. *Hin*dIII primers where end-labelled either with the IRD 800 or IRD 700 dye (MWG Biotech, Ebersberg, Germany). In total, ten selective primer combinations were used for the analyses (Table 2). The PCR-products were separated on 6 % polyacrylamide gels and analysed on a Licor-DNA-Analyser Gene ReadIR 4200 (MWG Biotech). A subset of the reactions was performed two to three times on independent DNA extractions to evaluate pattern reproducibility.

Data analysis

For each genotype the banding pattern was evaluated by visual inspection and fragment patterns were digitally encoded with 0 for absent and 1 for present marker bands. Bands which could not be identified clearly received an ambiguity score of 2. The pairwise distances between the OTU's (Operational Taxonomic Units = analysed Genotypes) were calculated using the modified similarity method of Nei and Li (Nei and Li 1979) from the PHYLIP software package (http://evolution.genetics.washington.edu/phylip.html).

We used three different clustering methods to construct the phenograms. All three methods (UPGMA, Fitch and Margoliash and Neighbour joining) are available in the PHYLIP software package. A bootstrap analysis (Felsenstein 1985) with 100 replicates was conducted to estimate the reliability of dendrograms. The dendrograms were displayed with the TreeView software program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

In addition, a Principal Component Analysis (PCA) was carried out using NTSYSpc version 2.10 s (Rohlf 2000).

Chloroplast microsatellites (cpSSR)

The PCR reaction for chloroplast microsatellite analysis was carried out according to a protocol of Vogel et al.

Table 2. Selected primer combinations and polymorphism rates for AFLP-analysis of 120 Genotypes belonging to the genera *Osteospermum* and *Dimorphotheca*.

Primer pairs	Total number of bands (mean)	Scored bands	Polymorphic bands	Polymorphism (%)
H800ACA_MACC	104	61	60	98.36
H700CAT_MGCA	84	34	32	94.12
H700CAT_MCCG	60	47	47	100.00
H700CAT_MACC	97	46	45	97.83
H800ACA_MGCA	60	23	23	100.00
H700CGA_MCTC	89	32	32	100.00
H800ACA_MCTC	63	34	34	100.00
H800ACA_MACG	38	21	20	95.24
H700CAT_MACG	52	21	21	100.00
H800ACA_MCCG	40	21	21	100.00
Total	687	340	334	
Mean	69	34	33	98.00

(2003) with 4ng of template DNA using primer pairs ccmp1, ccmp2, ccmp6, ccmp7 and ccmp10 as describe in WEISING and GARDNER (1999). The PCR-products were separated on 6 % polyacrylamide gels and analysed on a Licor-DNA-Analyser Gene ReadIR 4200 (MWG Biotech).

Results

AFLP reactions conducted with 11 different primer combinations produced 334 polymorphic and six monomorphic distinguishable marker-fragments across all genotypes (degree of polymorphism 98 %). On average, 47.8 % of the amplified fragments were polymorphic between any pair of genotypes. No pattern variation occurred between three repeat experiments. The genetic distance between genotypes ranged from a minimum value of 0.000515 (6 different fragments) between genotype 45 and 46 and the maximum value of 0.101957 (192 different fragments) between genotype 6 and 119. The genotypes 45 and 46 originated from the same series and have a similar growth habit and flower colour. In comparison genotype 119 belongs to the wild species *Dimorphotheca pluvialis* while genotype 6 is a newly bred variety of *Osteospermum*.

Three phenograms were computed by: UPGMA, Fitch and Margoliash and Neighbour joining (FELSENSTEIN 1988). Differences between topologies of the different phenograms were only marginal. The phenogram constructed by the UPGMA method is shown in Fig. 1. In this phenogram four major and two minor clusters can be distinguished (highlighted as A to F).

Almost all varieties are grouped into the main clusters A to D. Cluster E comprises Genotypes of *Dimorphotheca cuneata*, *Osteospermum ecklonis* and a variety. *Osteospermum vaillanthii* forms a very distinct sub cluster within the cluster F that also contains genotypes *Dimorhphotheca sinuata* and *Dimorphotheca pluvialis* each forming sub clusters. Genotypes with yellow flower colour are represented in every cluster but form groups within these clusters (see dark bars at Fig. 1).

Cluster A is the largest cluster with small distances from 0.000807 to 0.036028 between genotypes, comprising most of the varieties. The smallest distances within this cluster are between genotype 50 ('Sparkler') and 51 ('Silver Sparkler') both belonging to the same series. Cluster B is the cluster with the lowest genetic diversity represented by extremely small distances of maximally 0.004344 and clearly separated from all other clusters. It mainly consists of 'Symphony' and 'SummerDaisy' varieties. With distances ranging between 0.09353 and 0.18516, clusters C, D, and E are more diverse then the other clusters.

Analysis of the data with principal component analysis

The results of the PCA are shown in Fig. 2. The letters A–E indicate genotypes comprised in the clusters of the phenogram in Fig. 1. The PCA grossly reflects the groups that can be distinguished within the phenogram with two exceptions: genotypes of cluster D are located together with genotypes of cluster A and genotypes of cluster C overlap with those of cluster A. However, most of the genotypes of clusters A and C from the phenogram form very distinct groups.

Chloroplast Microsatellites (cpSSR)

Four of the five Primers used (ccmp1, ccmp2, ccmp6, ccmp10) generated detectable bands but only two of them (ccmp1 and ccmp2) revealed polymorphisms between the OTU's. Primer ccmp1 produced a distinct polymorphism separating the three genotypes of the *O. vaillanthii* (111, 112, and 113) from all other genotypes. With primer ccmp2 seven variable band sizes (204, 206, 203, 189, 200, 190, 205bp) could be detected. Together these two SSRs distinguished eight different cytoplasmic groups (Table 3). They comprise all six *O. ecklonis* genotypes and 46 varieties (group1), all three *D. pluvialis* genotypes and 44 varieties (group 2), three *O. vaillanthii* genotypes (group 3), 8 varieties (group 4), one variety and a genotype of *O. jucundum* (group5), all three *D. sinuata* genotypes (group 6), all three *D. cuneata* genotypes (group 7) and a single variety (group 8). In contrast to cluster B which only comprises genotypes with chloroplast patterns from group 1 and the species clusters E and F the clusters A, C and D comprised genotypes with different chloroplast patterns.

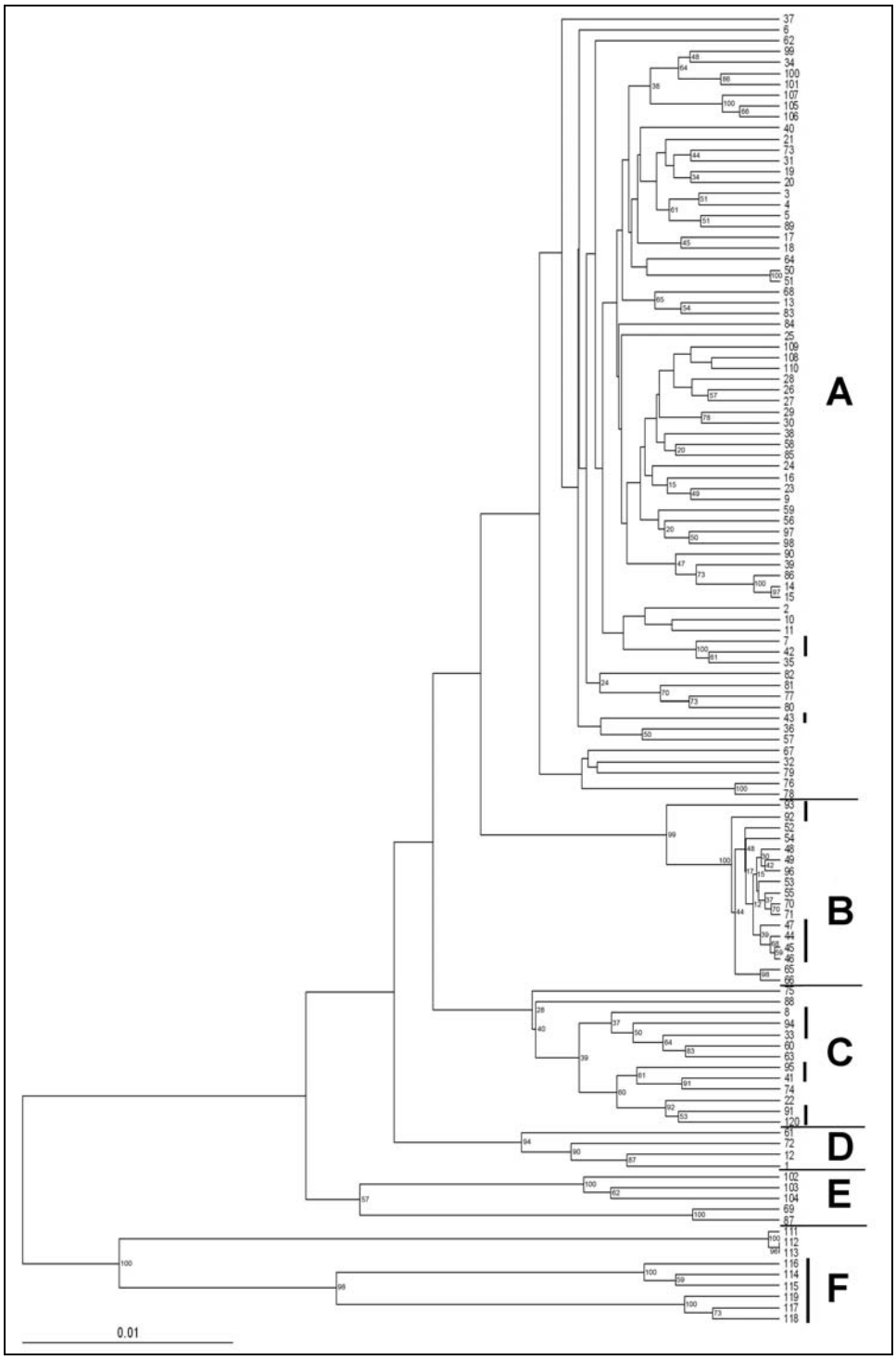


Fig. 1. Phenogram for all 120 genotypes of *Osteospermum* and *Dimorphotheca* based on AFLP marker data for 340 marker fragments. The Phenogram was computed via the UPGMA method and relative genetic distances according to the method of Nei and Li (Nei and Li 1979). Numbers at the nodes indicate bootstrap values in % from 100 repeats. Letters at the right margin and vertical lines mark the main clusters and bars label those genotypes with yellow flower colour. The scale bar indicates relative genetic distances.

Discussion

The molecular analysis with AFLPs revealed a very close genetic relationship among all *Osteospermum* varieties and the *Osteospermum* and *Dimorphotheca* species that we analysed. For example, the maximal distances among genotypes in the largest cluster A comprising the majority of genotypes has a maximum of 0.04 which is much lower then in several other outcrossed crops as e.g. *Dahlia* with distances around 0.3 (Wegner and Debener 2008) and also roses with distances between 0.2 and 0.6 (Debener et al. 1996). For cluster B the distances are much lower. The *Osteospermum* and *Dimorphotheca* species display distances of maximally 0.12 even between genera. Even under the assumption that the taxonomic differentiation between *Osteospermum* and *Dimorphotheca* might be incorrect two conclusions can be drawn from our results. Firstly, the species analysed here are very closely related to each other even if only a limited number of genotypes are considered. Secondly, a very small gene pool is available for breeding new varieties. This means that parents have to be selected with special care if inbreeding has to be avoided.

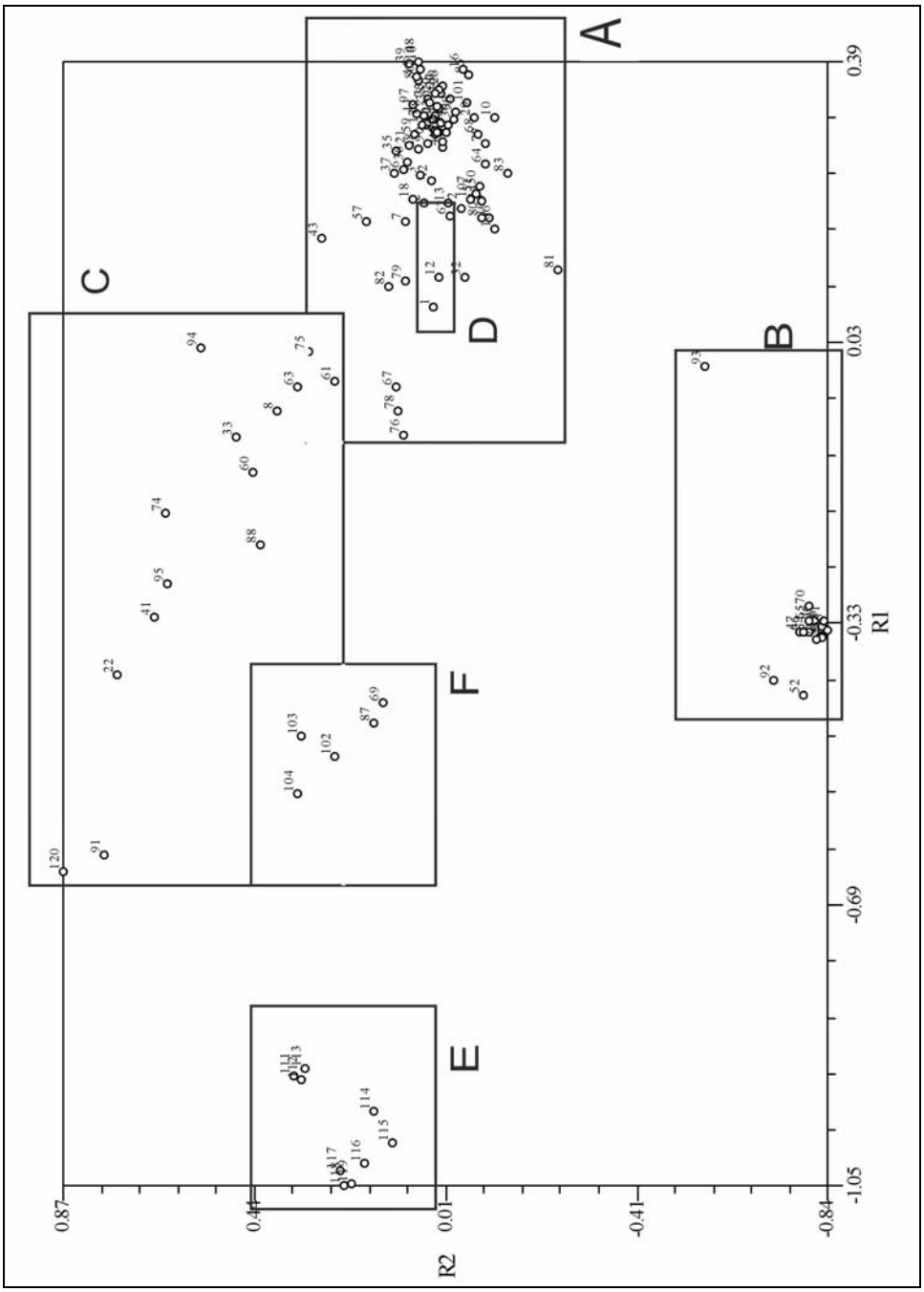


Fig. 2. Principal-component-analysis (PCA) of *Osteospermum* and *Dimorphotheca* genotypes based on 340 AFLP markers. Principal components were calculated and printed in NTSYS with default options (module Eigen, three dimensions and module Matrix plot for display). Boxes are drawn around the major clusters as characterised in Fig. 1.

The small distances found in our study contrast the results of Faccioli et al. (2000) who found extensive genetic variation among 39 *Osteospermum* varieties. However their analyses was only based on a small number of genotypes and only 71 RAPD markers for which reproducibility has not been demonstrated in this publication. Furthermore, they used simple matching coefficient for computation of relative genetic similarities which may lead to differences as compared to other indices more suitable to diversity analyses as that of Nei and Li (1979). Faccioli et al. (2000) also reported some correlation between breeding groups of varieties and clusters that we could not detect in our study. One reason for a lack of correlation in our analyses might be due to intensive breeding efforts during which breeders utilised available genotypes from other breeders as parents for their own breeding programs and therefore homogenised the breeding pool for *Osteospermum*. As *Osteospermum* gained economic importance since the publication of Faccioli et al. (2000) breeding efforts intensified and the tendency to homogenise the gene pool might have increased as well.

Genotypes with yellow flower colour are located in every cluster. Within these clusters they are grouped together indicating a common origin of the yellow colour in these sub clusters. An important breeding aim is to develop genotypes with yellow flower colour combined with a compact growth habit. Since genotypes with yellow flowers are distributed over all four clusters, it should be possible to identify potential parents who inherit both, the yellow flower colour and a compact growth habit, without the undesired inbreeding depression.

Table 3. Groups of *Osteospermum* and *Dimorphotheca* genotypes as defined by DNA fragments from two cpSSR markers.

Group	Fragment size (in bp)		Genotypes comprised in each group
	ccmp1	ccmp2	
A	138	204	1, 3, 4, 5, 9, 12, 13, 18, 19, 21, 23, 31, 32, 33, 34, 37, 40, 44, 45, 46, 47, 48, 49, 52, 53, 54, 55, 58, 59, 62, 64, 65, 66, 67, 68, 70, 71, 72, 73, 77, 78, 79, 81, 83, 89, 92, 93, 96, 98, 99, 100, 101
B	138	206	2, 6, 7, 10, 11, 14, 15, 16, 17, 20, 22, 24, 25, 26, 27, 28, 29, 30, 35, 39, 43, 50, 51, 56, 56, 57, 60, 63, 74, 75, 84, 85, 86, 94, 95, 97, 105, 106, 107, 108, 108, 110, 111, 112, 113, 117, 118, 119
B'	130	206	111, 112, 113
C	138	203	8, 41, 61, 82, 88, 91, 120
D	138	189	69, 87
E	138	200	114, 115, 116
F	138	190	102, 103, 104
G	138	205	80

The comparison of the different methods to calculate the phenograms revealed only marginal differences (data not shown). The bootstrap values strongly support clusters B, D E and F while some parts of clusters A and C display low values. However, the PCA analyses constitutes independent evidence for most of the clusters especially clusters A and B which form distinct groups. Here only cluster D falls within the genotypes of cluster A. However, the main findings about low genetic diversity and the distribution of genotypes of the same breeder or yellow flower colour being distributed over several strongly supported clusters remain unchallenged.

Molecular fingerprint analyses of some vegetatively propagated varieties and their sports do not reveal any differences in marker patterns even when large numbers of markers are analysed (Debener et al. 2000; De Riek et al. 2001).

However, in the present study we found differences between the AFLP patterns of 'Sparkler' (50) and 'Silver Sparkler' (51) a derived variety of 'Sparkler'. The distance value of 0.000937 between these genotypes is based on 340 AFLP-markers from which only seven are polymorphic. As the AFLP-technique is a highly reliable marker technique and independent repeat experiments with these varieties did not show any pattern differences between repeats it can be concluded that *Osteospermum* displays a higher genetic variability among vegetatively propagated clones then for example roses or Azaleas where polymorphisms between sports and varieties are rarely detected (Debener et al. 2000; De Riek et al. 2001; Vosman et al. 2004). Our results however correspond to those of Trigiano et al. (1998) who detected differences between Sports of *Dendranthema grandiflora* Tzelev with molecular markers. One consequence will be that essentially derived varieties based on sport mutations must be defined by a threshold distance value rather then by genetic identity between sports and original varieties.

The narrow genetic base of *Osteospermum* varieties is further supported by the observation that only two of the four chloroplast SSR markers revealed polymorphisms among the genotypes and only one of these could distinguish more then two groups. The polymorphisms revealed by two ccmp-primer pairs allowed us to distinguish eight groups within our data set which represent maternal lineages. It is hypothesised that ornamental *Osteospermum* originated from multiple interspecific hybridisation between various *Osteospermum* species namely *O. ecklonis*, *O. barberiae*, *O. caulescens*, *O. fruticosum*, *O. jucundum* and *O. chrysanthemifolia* (Faccioli et al. 2000). In our analyses all species (*O. ecklonis*, *O. vaillantii*, *O. jucundum*, D. *pluvialis*, *D. sinuata*, *and D. cuneata*) displayed distinct chloroplast SSR patterns as expected. Only two of the putative parental species were included in our analyses for which *O. ecklonis* shared its cpSSR pattern with the largest group of 46 varieties. This is in agreement with the hypothesis that *O. ecklonis* is an ancestor of present day varieties. The observation that 90 out of 120 varieties are derived from only two maternal lineages is in agreement to the other data on the limited genetic variability of *Osteospermum*. However, further statements about the origin of varieties can't be made as we did not include most of the suspected ancestral species in our analyses.

As a conclusion from all our experiments the following strategies should be employed in future *Osteospermum* breeding: New crosses should include those with individuals from different similarity groups of our analyses e.g. between clusters A and B. In addition, it would be advisable to include more diverse germplasm in the pool of present day varieties which might be found among wild relatives. As a consequence, for the identification of distinct varieties and especially for differentiation of essentially derived varieties from original varieties genetic distances much less then 0.1 have to be considered as significant.

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Received April 24, 2008 / Accepted December 09, 2008

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