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Sequence-characterized Markers from *Begonia* x *tuberhybrida* Voss.

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Summary

We developed 12 new sequence characterized markers from *Begonia* x *tuberhybrida* Voss. Total of 37 alleles ranged from two to six with expected heterozygosity H_E=0.52, observed heterozygosity H_O=0.58, and probability of identity P_I=0.31, in average per a locus. Probability of identity combined over all 12 loci was P_I=0.03. Four out of 12 loci expressed significant departures from HWE. Cross-species transferability was evaluated on the panel of 15 botanical species of *Begonia*. Total of 25.3 % cross-species amplification indicated rather low transferability of markers from *B.* x *tuberhybrida* to other *Begonia* species, which confirms the requirement of species-specific approach to development of *Begonia* markers.

Key words. *Begonia* x *tuberhybrida* – *Begoniaceae* – cross-species amplification – cultivar identification – DNA marker polymorphism –SCAR markers – sequence characterized markers

Introduction

Begonia has a long and distinguished history in cultivation since its introduction to Kew Gardens in 1777. This genus belongs to world top-ten plant genera in species richness at present estimated to be around 1550 (Tebbitt 2005). Excitingly, additional new species are frequently described even these days (Hughes 2007). Among vast amount of species especially Andean begonias got particular horticultural merit to produce diverse tuberous group of *Begonia* x *tuberhybrida* (Haegeman 1979).

Development of molecular markers to elucidate complex *Begonia* systematics, to reconstruct its phylogeny or to understand genetic composition of hybrids remains rather neglected area. Hitherto, DNA markers from *Begonia* were reported only for wild species.

Species-specific microsatellite markers were developed for endemic *Begonia socotrana* (Hughes et al. 2002a), and *Begonia sutherlandii* Hook (Hughes et al. 2002b). Later, universal DNA markers from chloroplast *trnL* intron and nuclear ribosomal internal transcribed spacer (ITS) were used for reconstruction of molecular phylogeny of African begonias (Plana et al. 2004). However, till now no DNA markers were developed for ornamental begonias like quite popular complex hybrid group of *Begonia* x *tuberhybrida* Voss.

Therefore, we wanted in this study to develop new sequence characterized markers from cloned products of RAPD reactions made on *Begonia* x *tuberhybrida* DNA template.

Materials and Methods

Plant material

The panel of eight contrast accessions of *Begonia* x *tuberhybrida* Voss. used for rapid pre-screening (see bellow) was composed of 'TBHpo0704', 'TBHpo0904', 'TBHpo1004', 'TBHma0504', 'TBHma0704', 'TBHma0904', 'TBHma1004', and 'TBH Melissa Blackmore & Langdon'.

The panel of 32 ornamental cultivar and breeding germplasm accessions of *Begonia* x *tuberhybrida* Voss. used for genetic analysis of marker loci was composed of 'TBHma0104', 'TBHma0204', 'TBHma0304', 'TBHma0504', 'TBHma0604', 'TBHma0704', 'TBHma0804', 'TBHma0904', 'TBHma1004', 'TBH crispa-marginata yellow-red Nohel', 'TBH picotee white AB Nohel', 'TBH picotee yellow Nohel', 'TBH pendula pink AB Nohel', 'TBH pendula red Nohel', 'TBH fimbriata orange Nohel', 'TBH pendula orange Nohel', 'TBH pendula red Nohel', 'TBH erecta red Holice', 'TBH gigantea yellow Holice', 'TBH gigantea orange Holice', 'TBH erecta orange Holice', 'TBH gigantea white Holice', 'TBH po0204', 'TBH po0404', 'TBH po0604', 'TBH po0704', 'TBH po0804', 'TBH po0904', 'TBH po1004', 'TBH Primrose Blackmore & Langdon', 'TBH Melissa Blackmore & Langdon', 'TBH Matador Blackmore & Langdon'.

The panel of wild *Begonia* species used for cross-species reference was composed of the species listed in Table 3, obtained by courtesy of the American Begonia Society, Botanical Garden WPIV Leyman Belgium, and Jardin Botanique de Montreal Canada.

All plant material used in our analyses is maintained in the BEGOBIO Begonia living and DNA collection located at the Biology Centre AS CR Ceske Budejovice, and in the Begonia Collection of Sempra Flora Ltd. Holice, Czech Republic.

Cloning and sequencing

The RAPD fragments from eight contrast accessions of *Begonia* x *tuberhybrida* Voss. were produced to pre-screen for band polymorphism using 29 RAPD primers (Operon Technologies), (Table 1). RAPD reaction conducted in

Table 1. Initial pre-screening for polymorphic RAPD fragments to be cloned and sequenced for conversion to SCAR markers was performed using 29 RAPD primers.

RAPD primer	Primer sequence (5'–3')	Annealing temp. (°C)
OPA01	C A G G C C C T T C	37
OPA02	T G C C G A G C T G	37
OPA03	A G T C A G C C A C	37
OPA04	A A T C G G G C T G	37
OPA05	A G G G G T C T T G	37
OPA06	G G T C C C T G A C	37
OPA07	G A A A C G G G T G	37
OPA08	G T G A C G T A G G	37
OPA09	G G G T A A C G C C	37
OPA10	G T G A T C G C A G	37
OPA11	C A A T C G C C G T	37
OPA12	T C G G C G A T A G	37
OPA13	C A G C A C C C A C	37
OPA14	T C T G T G C T G G	37
OPA15	T T C C G A A C C C	37
OPA16	A G C C A G C G A A	37
OPA17	G A C C G C T T G T	37
OPA18	A G G T G A C C G T	37
OPA19	C A A A C G T C G G	37
OPA20	G T T G C G A T C C	37
OPX02	T T C C G C C A C C	35
OPX10	C C C T A G A C T G	35
OPY12	A A G C C T G C G A	35
OPZ09	C A C C C C A G T C	35
OPZ20	A A T T T G G C G G	35
OPAB05	C C C G A A G C G A	35
OPAB09	G G G C G A C T A C	35

40-μL volumes contained 0.53 mM each dNTPs, 1x Optimized DyNAzyme EXT buffer, 3 mM $MgCl_2$, 1 U DyNAzyme EXT DNA polymerase (Finnzymes), 1 μM RAPD primer, and 40 ng genomic DNA. The reaction conditions were: 1 min at 94 °C, followed by 35 cycles of 0.5 min at 94 °C, 0.5 min annealing at 37 °C, and 1 min at 72 °C with final extension of 72 °C for 5 min on PTC-150 MiniCycler (MJ Research).

The RAPD reaction products obtained with individual primers were separated on 1.5 % agarose gel. Individual clearly separated highly intensive polymorphic bands from arbitrarily selected gel line of *Begonia* x *tuberhybrida* Voss., the accession 'TBHma1004' were cut out of the gel, extracted and purified by Wizard SV Gel and PCR Clean-Up System (Promega), and AT-cloned using the pGEM-T Easy Vector System (Promega). The RAPD fragments cloned into the recombinant *Escherichia coli* clones were then sequenced on ABI 3130 sequencer.

Locus-specific primer pairs from these novel sequences were designed using PRIMER3 (Rozen and Skaletsky 2000). One primer of each pair was always labeled with the fluorescent dye 6-FAM, VIC, NET or PET.

PCRs with new locus-specific primers conducted in 40-μL volumes contained 0.53 mM each dNTPs, 1x Optimized DyNAzyme EXT buffer, 3 mM $MgCl_2$, 1 U DyNAzyme EXT DNA polymerase (Finnzymes), 1 μM of each primer, and 40–120 ng genomic DNA. The reaction conditions were: 1 min at 94 °C, followed by 35 cycles of 0.5 min at 94 °C, 0.5 min annealing at 37 °C, and 1 min at 72 °C with final extension of 72 °C for 5 min on PTC-150 MiniCycler (MJ Research). We found that amplification by enormously processive DyNAzyme EXT DNA polymerase was quite critical point to achieve high quality PCR products.

The fragment analysis of amplification products from PCR reactions were analyzed on ABI 3130 sequencer running GENESCAN and GENOTYPER software using GS 500 LIZ size ladder (Applied Biosystems).

Data analysis

Genetic parameters, the expected heterozygosity (He), observed heterozygosity (H_O), and probability of identity (P_I) were calculated for each of 12 loci using IDENTITY ver. 3.01 (Wagner and Sefc 1999), and GENCAP ver. 1.2.2 (Wilberg and Dreher 2004).

Results and Discussion

The RAPD reaction products obtained with 29 primers were separated on 1.5 % agarose gel to pre-screen for band polymorphism. We identified total of 243 RAPD bands when faint or diffused bands were neglected. From this, 109 bands (44.8 %) were classified as polymorphic when complied with the rule of at least one absence of a band (dominant inheritance assumed) on the basic eight-accession panel.

From total of 29 RAPD primers, 12 primers (41.4 %) giving unambiguous clearly interpretable band polymorphism on a gel profile, were selected for the next step of band cloning and sequencing.

Arbitrarily, for all 12 best RAPD primers the gel line of the accession 'TBHma1004' was selected to cut out the bands for cloning and sequencing. Isolated and purified bands were then cloned into the recombinant *Escherichia coli* clones and sequenced on ABI 3130 sequencer by double reading. Total of 94 raw sequences were obtained to yield after editing 28 (29.8 %) unique high quality sequences (identical, fragmental or artificially recombined sequences were removed from the analysis).

Due to economical reasons, we reduced these 28 edited sequences finally to only 12 sequences and designed locus-specific primer pairs so that one primer of each pair was always labeled with the fluorescent dye. These primer pairs were then used in fragment analysis to survey the panel of 32 cultivars and breeding genotypes of *Begonia* x *tuberhybrida* Voss. germplasm for genetic parameters (Table 2). Moreover, the panel of 15 wild-species relatives of *Begonia* x *tuberhybrida* Voss. was surveyed by these primer pairs for cross-species analysis (Table 3). Altogether the products of 976 PCR reactions were analyzed by fragment analysis on ABI 3130 giving the data for description of 37 alleles with 3.08 alleles per a locus, in average, and with the size range of alleles from 114 bp to 464 bp with 185.6 bp, in average.

For quantitative evaluation of genetic data we accepted metapopulation concept (Dobson 2003) in that we assumed significant genetic connectivity and gene flow

Table 2. Set of 12 sequence-characterized primer pairs developed from *Begonia* x *tuberhybrida* Voss. accession 'TBHma1004' and their genetic characterization on the panel of 32 *Begonia* x *tuberhybrida* cultivar and breeding accessions including observed heterozygosity (H_O), expected heterozygosity (H_E), and probability of identity (P_I).

Locus	Primer sequence (5'–3')	Accession no.	Annealing temp.(°C)	H_E	H_O	P_I	Fragment size (bp)
D89opa02	F: G A C C C T T T T C T A C G G C G A T C T	EF606693	60	0.34	0.44	0.49	364, 381
	R: T C T T C G C T T T C T T T C T T T C T T C A A						
D67opa03	F: C A C A A G T T T C A C A A T T C T G C A T A G	EF606694	58	0.56	0.34	0.30	341, 398, 464
	R: G T C G A C A T G T G C A T C T T T A T G T C						
D40opa05	F: A T G T A T T G G A A G A A A T C G G T C C G	EF606696	60	0.65	0.59	0.20	y267, 275, 341
	R: T G G T C C A G G A A T T C C T A A A A A T C						
D46opa11a	F: A T A G A G C T G T T G G A A C G G C T C C A A	EU035986	55	0.58	0.72	0.25	169, 212, 293
	R: G A T A A T G A C G G C A T T G C C G A T A A A G						
D46opa11b	F: C T C A C A G C C G C C A T C T G G G C T G C G A	EU035986	55	0.53	0.72	0.26	114, 138, 150, 159
	R: C C T G T T C C G C C A G A C C T C T C T T C C G						
D46opa11c	F: C A G G C T G A C C G T C T C G G A G A G A T A	EU035986	55	0.50	0.38	0.37	y158, 223
	R: G C C C T T G C T C C G G T A A T G C G G G A C T						
D57opa13	F: T C C A T A G C T T C T G A C C T G C A A G T	EF606698	65	0.48	0.47*	0.39	230, 297
	R: C C A C C A T A G T C G A A G C T G C C A						
D43opa14	F: G T G A T C C G G C C G G T T A C C G T	EF606699	57	0.66	0.81*	0.16	323, 392, 403, 424
	R: A C C T G A T T T T A C A C A C C T G C G T T						
D22opa18	F: A C T G A G C T G A T G G A T T C C T C G T C	EF606701	55	0.34	0.44	0.49	160, 191
	R: G G G C T T A T T G A C C A G A T C C A G T T						
D49opa19b	F: A G C G G C G C A T G A A C C G T A C C T T C G C	EU035987	57	0.69	0.63*	0.13	y155, 164, 175, 186, 217, 228
	R: A G T G A A C A T G G G C G G T A T C C T G T C						
D91opab05b	F: A T G C C A C T A T T A A C T A C A G A G C A G T	EU035990	50	0.43	0.63	0.42	120, 127
	R: T G A T A C G T C T A G C C A T A A C A A C T G T						
D80opz20a	F: C G G A T G G G A G A C T T T C A G A A T C G G	EU035989	55	0.53	0.81*	0.31	138, 189, 199, 207
	R: T G G A C T G C A T G G C C G A G A T G G G A C						
mean				0.52	0.58	0.31	

y amplified PCR fragments differ from expected size in *Begonia* x *tuberhybrida* Voss., accession 'TBHma1004';
* significant departures from HWE (P<0.01)

among cultivated *Begonia* x *tuberhybrida* genotypes and cultivars due to long-lasting interrelated breeding programs involving closely related subpopulations of *Begonia* x *tuberhybrida* germplasm. Then the characteristics of marker loci were calculated and are given in Table 2. The number of alleles ranged from two to six. The expected heterozygosity (H_E = 0.34–0.69, mean 0.52±0.06 at 95 % confidence limit), observed heterozygosity (H_O = 0.33–0.81, mean 0.58±0.09 at 95 % confidence limit) and probability of identity (P_I = 0.13–0.49, mean 0.31±0.07 at 95 % confidence limit) were calculated for each of 12 loci (Wagner and Sefc 1999; Wilberg and Dreher 2004). According to these parameters, D49opa19b was revealed as the most informative locus (P_I=0.13). Probability of identity combined over all 12 loci was P_I=0.03. It may be interpreted in that P_I=0.03 is the probability that the two *Begonia* x *tuberhybrida* Voss. accessions drawn at random from the panel of 32 accessions will have the same genotype over the set of 12 marker loci. In other words, we can say that three out of 100 accessions will have identical genotype using 12 markers generated in this study. This means that our marker set unambiguously (uniquely) identifies unknown *Begonia* x *tuberhybrida* Voss. accession within 33 unknown samples, based on probabilistic measure.

Nine loci out of 12 designed primer pairs amplified products of the expected size of *Begonia* x *tuberhybrida* Voss., accession 'TBHma1004'.

GENEPOP ver. 4.0 was used to test for deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) within populations using the Markov chain parameters (Raymond and Rousset 1995). Four out of 12 loci (33.3 %) expressed significant departures from HWE (P<0.01). Linkage disequilibrium (LD) was identified in three out of 12 described loci (25 %) (P>0.001), D46opa11a, D46opa11b, and D46opa11c, which is in full

Table 3. Cross-species transferability of 12 sequence characterized primer pairs with indication where primer pair amplified products (+) or failed to amplify products (–).

Locus	D89 opa 02	D67 opa 03	D40 opa 05	D46 opa 11a	D46 opa 11b	D46 opa 11c	D57 opa 13	D43 opa 14	D22 opa 18	D49 opa 19b	D91 opab 05b	D80 opz 20a
B. semperflorens	–	–	+	–	–	–	+	–	+	–	+	+
B. elatior 48	–	–	–	+	+	–	+	+	+	+	+	–
B. elatior 49	–	–	–	+	+	–	+	+	+	+	+	–
B. convolvulacea	–	–	+	–	–	–	–	–	–	–	+	+
B. dregei	–	–	+	–	–	–	–	–	–	–	+	–
B. holtonis	–	–	+	–	–	–	–	–	–	–	+	–
B. incarnata	–	–	+	–	–	–	–	–	–	–	+	–
B. malabarica	–	–	+	–	–	–	–	–	–	–	+	+
B. meyeri	–	–	+	–	–	–	–	–	–	–	+	+
B. odorata	–	–	+	–	–	–	–	–	–	–	+	–
B. oxyanthera	–	–	+	–	–	–	–	–	–	–	+	+
B. peltata	–	–	+	–	–	–	–	–	–	–	+	–
B. radicans	–	–	+	–	–	–	–	–	–	–	+	+
B. roxburghii	–	–	+	–	–	–	–	–	–	–	+	–
B. cinnabarina	+	–	+	+	+	+	+	+	+	+	+	+

compliance with the fact that these were derived from three adjacent regions of a single original locus (sequence EU035986).

The cross-species amplification was tested on the panel of 15 botanical species of *Begonia* obtained from Clayton M. Kelly Seed Fund of American Begonia Society, Seed Fund of the Association of Australian Begonia Societies Inc., and from the Service des Graines AFABEGO France (Table 3). Only primer pair from D91opab05b locus amplified PCR product in all 15 *Begonia* species. Locus D40opa05 was amplified in 13 from 15 species (86.7 %), and locus D80opz20a in seven from 15 (46.7 %) species. Template from *Begonia cinnabarina* was amplified in 11 from 12 loci, which corresponds to the genetic proximity of *Begonia cinnabarina* to *Begonia* x *tuberhybrida* (Haegeman 1979).

Obtained 25.3 % cross-species amplification indicated rather low transferability of markers between *Begonia* species. This is in compliance with reported fact that *Begonia* species are usually restricted to very small and isolated geographical areas, which efficiently prevents allele exchange between evolving populations (Tebbitt 2005).

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