Identification and Genetic Variation among *Hibiscus* Species (Malvaceae) Using RAPD Markers

Suvakanta Barik, Sunil Kumar Senapati, Subhashree Aparajita, Anuradha Mohapatra, and Gyana Ranjan Rout*

Plant Biotechnology Division, Regional Plant Resource Centre, Bhubaneswar-751015, Orissa, India. Fax: 0091-674-2550274. E-mail: grrout@rediffmail.com

* Author for correspondence and reprint requests

Z. Naturforsch. **61c**, 123–128 (2006); received September 5, 2005

Germplasm identification and characterization is an important link between the conservation and utilization of plant genetic resources. Traditionally, species or cultivars identification has relied on morphological characters like growth habit or floral morphology like flower colour and other characteristics of the plant. Studies were undertaken for identification and determination of genetic variation within the two species of *Hibiscus* and 16 varieties of *Hibiscus rosa-sinensis* L. through random amplified polymorphic (RAPD) markers. Primer screening was made by using the DNA of variety "Prolific". Genetic analysis was made by using ten selected decamer primers. A total of 79 distinct DNA fragments ranging from 0.3 to 2.5 kb were amplified by using ten selected random decamer primers. The genetic similarity was evaluated on the basis of presence or absence of bands. The cluster analysis indicated that the 16 varieties and two species formed one cluster. The first major cluster consisted of three varieties and a second major cluster consisted of two species and 13 varieties. The genetic distance was very close within the varieties and also among the species. Thus, these RAPD markers have the potential for identification of species/varieties and characterization of genetic variation within the varieties. This is also helpful in *Hibiscus* breeding programs and provides a major input into conservation biology.

Key words: Genetic Relationship, *Hibiscus* sp., RAPD Marker

Introduction

The molecular approach for identification of plant species or varieties seems to be more effective than traditional morphological markers because it allows direct access to the hereditary material and makes it possible to understand the relationships between plants (Williams *et al.*, 1990; Paterson *et al.*, 1991). Random amplified polymorphic DNA (RAPD) technique has been widely used in many plant species for varieties analysis, population studies and genetic linkage mapping (Williams *et al.*, 1990; Yu *et al.*, 1993; Rout *et al.*, 2003). Optimization of the RAPD analysis depends on selection of primers. Although, the RAPD method uses arbitrary primer sequences, many of these primers must be screened in order to select primers that provide useful amplification products.

The genus *Hibiscus* belonging to the family Malvaceae comprises of about 50 species of shrubs and trees and numerous hybrids (Bose and Choudhury, 1991). *H. rosa-sinensis* L., the tropical hibiscus, has glossy heavy foliage with large, brilliant and spectacular flowers. *H. schizopetalus* Hook., f. also called "Fringed *Hibiscus*" with orange-red flowers has been popularly cultivated in the Asian countries. *H. sabdariffa* L. with yellow flowers is cultivated for garden hedges and for medicinal purpose. A large number of hybrids and mutants is grown in many parts of the world as well as in Indian subcontinent. A large number of varieties with spectacular flowers has been evolved but they are not properly documented. Particularly for the floricultural crops, morphological characteristics such as flower shape, size and colour were used to discriminate the species or varieties. Often, long periods of vegetative growth elapse before such evaluation can take place. In contrast, the molecular markers facilitate research on genetic variation at the DNA level. In this communication, we report the optimization of primer screening and evaluation of genetic variability among two *Hibiscus* species and 16 varieties of *Hibiscus rosa-sinensis* L. by using RAPD markers.

0939–5075/2006/0100–0123 $ 06.00

Materials and Methods

Plant material

Two species (*Hibiscus schizopetalus* Hook., f. "Japanese Lantern", *Hibiscus sabdariffa* L.) and 16 varieties of *Hibiscus rosa-sinensis* L. ("Australian Rose", "Acc. no. 1", "Prolific", "H. D. Maity", "Albus", "Scarlet Brilliant", "Scarlet", "Snow Flake", "Tiki", Acc. no. 2", "Juno", "Moorea", "Red Satin", "Acc. no. 3", "Toreador", "Acc. no. 4") were selected from the garden of Regional Plant Resource Centre, Bhubaneswar, Orissa, India. The characteristics of the varieties/species used for RAPD analysis are enlisted in Table I.

DNA extraction

DNA was extracted from fresh leaves collected from garden raised plants of different species/varieties by the CTAB method (Doyle and Doyle, 1990). Approx. 200 mg of fresh leaves were ground to powder in liquid nitrogen using a mortar and pestle. The ground powder was transferred to a 50 ml falcon tube with 10 ml of cetyltrimethyl ammonium bromide, EMERK (CTAB) buffer [2% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris(tris(hydroxymethyl)aminomethane)-HCl, pH 8.0, and 0.2% (v/v) β-mercaptoethanol]. The homogenate was incubated at 60 °C for 2 h, extracted with an equal volume of chloroform/iso-amyl alcohol (24:1 v/v) and centrifuged at 10,000 × *g* for 20 min (Kubata KR-2000 C, Rotor-RA-3R, Japan). DNA was precipitated from the aqueous phase by mixing with an equal volume of isopropanol. After centrifugation at 10,000 × *g* for 10 min, the DNA pellet was washed with 70% (v/v) ethanol, air-dried and resuspended in TE (10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA) buffer. DNA quantifications were performed by visualizing under UV light, after electrophoresis on 0.8% agarose gel. The resuspended DNA was then diluted in TE buffer to 5 μg/μl concentration for use in polymerase chain reaction (PCR) reactions.

PCR amplification

A set of 30 random decamer oligonucleotides (Series A, C, D and N) purchased from Operon Technologies Inc. (Alameda, USA) was used as primers for the amplification of RAPD fragments. Amplification reactions were performed in 25 μl [2.0 μl of 1.25 mM each of dNTP's, 15 ng of the primer, 1 × Taq polymerase buffer, 0.5 U of Taq DNA polymerase (Genei, Bangalore, India), and 20 ng of genomic DNA]. DNA amplification was performed in a PTC-100 DNA Thermal Cycler (M. J. Research Inc., Watertown, MA, USA) programmed for 45 cycles: 1st cycle of 3.5 min at 94 °C, 1 min at 38 °C and 2 min at 72 °C; then 44 cycles each of 1 min at 94 °C, 1 min at 38 °C, 2 min at 72 °C followed by one final extension cycle of 5 min at 72 °C. Amplified products were electrophoresed in a 1.2% (w/v) agarose (Sigma, USA) gel with 1 × TAE buffer stained with ethidium bromide and photographed under UV light. Gel photographs were scanned through a Gel Doc System (Gel Doc. 2000, Biorad, California, USA) and the amplification product sizes were evaluated using the software Quantity One (Biorad, USA). The sizes of the amplification products were estimated from a 3.0 kb (100 bp to 3.0 kb) ladder (MBI Fermentas Inc., Amherst, USA). All the reactions were repeated at least three times.

Data analysis

Data were recorded as presence (1) or absence (0) of band products from the examination of photographic negatives. Each amplification fragment was named by the source of the primer (Operon Technologies Inc.), the kit letter or number, the primer number and its approx. size in base pairs. Bands with similar mobility to those detected in the negative control, if any, were not scored. Similarity indexes were estimated using the dice coefficient of similarity (Nei and Li, 1979). Cluster analyses were carried out on similarity estimates using the unweighted pair-group method arithmetic average (UPGMA) by means of NTSYS-PC version 1.8 (Rohlf, 1995).

Results and Discussion

The present investigation offers an optimization of primer screening for evaluation of genetic relationship of two species of *Hibiscus* and sixteen varieties of *Hibiscus rosa-sinensis* L. through RAPD analysis. The variety "Prolific" was used for screening primers (derived from series A, C, D and N) obtained from different series for amplification by using polymerase chain reactions. The results showed that D- and N-series primers produced relatively more amplification fragments compared to A- and C-series primers. The amplification generated by primers OPD-11, OPD-18 and

Table I. *Hibiscus* species/varieties used for the RAPD analysis.

No.	Species/varieties	Morphological characteristics
H1	*Hibiscus rosa-sinensis* "Acc. no. 1"	The plant is short, leaves are small oval and the margin is entire; flower is short, single and size is up to 5–5.5 cm.
H2	*Hibiscus rosa-sinensis* "Australian Rose"	Medium size plant; flower is large, single, pinkish in colour having white patches on the side of each petal; size of the flower ranges from 10–11 cm; each petal have ridge on the upper part.
H3	*Hibiscus rosa-sinensis* "Prolific"	The plant is a very hardy, well shaped bush; leaves are roundish, cordate at base, undulate margin thick up to 15 cm long; flower is yellow with a deep center and band of white; flower size is 12–16 cm.
H4	*Hibiscus schizopetalus* "Japanese Lantern"	Glabrous evergreen shrub, up to 4 m high with splender dropping branches; leaves are smooth, toothed; flower is orange-red hanging from slender stalks; petals are deeply silt and recurved and have a long projecting pendulous staminal column.
H5	*Hibiscus rosa-sinensis* "H. D. Maity"	Medium size plant grown in pot; leaves are shiny green; flower is single, large, glistering reddish purple; blooming year around.
H6	*Hibiscus rosa-sinensis* "Albus"	Medium size plant; free flowering; white colour.
H7	*Hibiscus rosa-sinensis* "Scarlet Brilliant"	Evergreen tropical shurb; leaves are green ovate, strongly toothed at margins; free blooming with showy vivid crimson red flower; petals have frilled margins and bloome year around.
H8	*Hibiscus rosa-sinensis* "Scarlet"	The plant is tree-like semi-standard; glossy foliage; flower is scarlet crimson and bold staminal; blooming in spring and winter.
H9	*Hibiscus rosa-sinensis* "Snow Flake"	The plant is a vigorous branching shurb with willowy reddish stems dense with rough ovate; toothed leaves, grayish-green, variegated mainly towards margin with cream white, crimson veins and centre.
H10	*Hibiscus rosa-sinensis* "Tiki"	Attractive exotic Hawaii cultivar with large single flowers, vivid yellow, shading to apricot-salmon inside and a constructing maroon red centre at base of long staminal column.
H11	*Hibiscus rosa-sinensis* "Acc. no. 2"	Woody branched; leaves are medium sized with serrated margin; flower is small (6–6.5 cm), whitish in colour.
H12	*Hibiscus rosa-sinensis* "Juno"	Dwarf busy plant; flower is double compact, cherry red in colour.
H13	*Hibiscus rosa-sinensis* "Moorea"	The plant is woody, branched, having medium size leaves with margin serrated; large magnificent single pink colour flower with 10–12 cm in diameter.
H14	*Hibiscus sabdariffa*	Medium size shrub has simple ovate leaves, mostly deeply 3-lobed with mid lobe broadest, serrate margins; stems and petioles are often red with yellow flowers 7 cm in diameter.
H15	*Hibiscus rosa-sinensis* "Red Satin"	The plant is medium size; leaves are large and succulent; spectacular variety with large double blooms, pendulous attractive red in colour; petal thick in center forms white patch; the anther tubes are arranged in three groups.
H16	*Hibiscus rosa-sinensis* "Acc. no. 3"	Magnificent shrub with woody branches, stem is brownish green in colour; leaves are bistipulate, petiolate, margin serrated; flowers are double with pinkish colour.
H17	*Hibiscus rosa-sinensis* "Toreador"	The plant is tall with pubescent, serrate leaves; free blooming with 12–15 cm flower size; maize yellow in colour, in center a contrasting definite eye of ruby-red with straight effect.
H18	*Hibiscus rosa-sinensis* "Acc. no. 4"	The plant is a small shrub ideal for potting; leaves are small with margin serrated; flowers are small, magnificent single pure pink in colour; size ranges from 4–4.5 cm in diameter.

Table II. Total number of amplified fragments and number of polymorphic fragments generated by PCR using selected random decamers.

Name of primer	Sequence of the primer 5′ – 3′	Total number of amplified products	Number of polymorphic products	Size range [kb]
OPA-16	5′-AGCCAGCGAA-3′	6	5	0.5 –2.1
OPC-06	5′-GAACGGACTC-3′	5	3	0.9 –2.5
OPC-16	5′-CACACTCCAG-3′	5	4	0.5 –2.0
OPC-18	5′-TGAGTGGGTG-3′	3	3	0.5 –1.0
OPD-11	5′-AGCGCCATTG-3′	7	5	0.35–1.0
OPD-18	5′-GAGAGCCAAC-3′	9	8	0.55–2.1
OPN-09	5′-TGCCGGCTTG-3′	11	9	0.4 –2.4
OPN-12	5′-CACAGACACC-3′	13	9	0.5 –2.0
OPN-16	5′-AAGCGACCTG-3′	12	10	0.3 –2.0
OPN-19	5′-GTCCGTACTG-3′	8	5	0.45–1.0

OPN-09 produced small number of fragments by using the variety "Prolific". The primers OPD-18 and OPN-12 produced maximum number of DNA fragments; the size of the DNA fragments ranged from 400 to 2,500 base pairs. Primer OPN-12 amplified 13 fragments whereas OPN-09 produced 11 bands in *Hibiscus rosa-sinensis* var. Prolific. It was also noted that some decamer primers did not show any amplification by using the variety Prolific (data not shown). The number of fragments varied from one series of primers to other series. The twenty decamer primers produced good amplification of RAPD fragments ranging from 350 to 2,500 base pairs. Subsequently, ten decamer primers were selected and used to analyze the genetic relationship among two species of *Hibiscus* and sixteen varieties of *Hibiscus rosa-sinensis* through polymerase chain reaction (Table II).

The ten decamer primers (OPA-16, OPC-06, OPC-16, OPC-18, OPD-11, OPD-18, OPN-09, OPN-12, OPN-16, OPN-19) showed good polymorphism within the sixteen varieties of *Hibiscus rosa–sinensis* and two species of *Hibiscus*. The reproducibility of the amplification product was tested on rose DNA from three independent extractions of the varieties. Most of the amplification reactions were duplicated. Only bands that were consistently reproduced across amplifications were considered for the analysis. Bands with the same mobility were considered as identical fragments, receiving equal values, regardless of their staining intensity. When multiple bands in a region were difficult to resolve, data for that region of the gel was not included in the analysis. As a result, ten informative primers were selected and used to evaluate the degree of polymorphism within two

Table III. Similarity matrix of two species and sixteen varieties of *Hibiscus*.

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18
H1	1.00																	
H2	0.65	1.00																
H3	0.63	0.49	1.00															
H4	0.59	0.69	0.71	1.00														
H5	0.58	0.72	0.49	0.71	1.00													
H6	0.50	0.45	0.76	0.63	0.48	1.00												
H7	0.62	0.73	0.48	0.56	0.59	0.40	1.00											
H8	0.73	0.73	0.55	0.65	0.71	0.49	0.74	1.00										
H9	0.67	0.63	0.53	0.69	0.60	0.41	0.71	0.71	1.00									
H10	0.72	0.56	0.47	0.50	0.51	0.43	0.50	0.69	0.53	1.00								
H11	0.63	0.44	0.44	0.38	0.49	0.38	0.41	0.57	0.44	0.77	1.00							
H12	0.62	0.57	0.55	0.65	0.59	0.42	0.58	0.65	0.62	0.57	0.52	1.00						
H13	0.50	0.43	0.71	0.58	0.48	0.72	0.40	0.47	0.48	0.48	0.45	0.49	1.00					
H14	0.65	0.56	0.56	0.50	0.47	0.50	0.55	0.69	0.56	0.65	0.65	0.59	0.52	1.00				
H15	0.56	0.65	0.49	0.69	0.67	0.50	0.62	0.69	0.58	0.49	0.44	0.64	0.38	0.47	1.00			
H16	0.62	0.62	0.62	0.70	0.66	0.60	0.51	0.63	0.55	0.59	0.55	0.58	0.56	0.55	0.71	1.00		
H17	0.77	0.63	0.53	0.57	0.58	0.48	0.55	0.71	0.60	0.77	0.65	0.64	0.57	0.70	0.58	0.64	1.00	
H18	0.59	0.62	0.57	0.72	0.69	0.51	0.56	0.67	0.66	0.55	0.52	0.56	0.51	0.59	0.66	0.60	0.62	1.0

species of *Hibiscus* and sixteen varieties of *Hibiscus rosa-sinensis*. The selected primers generated distinctive products in the range of 0.3–2.5 kb. Maximum and minimum number of bands was produced by the primers OPN-12 (13), OPN-09 (11) and OPD-18 (9), respectively (Table II). A total of 79 amplified fragments was scored across sixteen varieties of *H. rosa-sinensis* and two species of *Hibiscus* for the selected primers, and was used to estimate genetic relationships among themselves. The patterns of RAPD produced by the decamer primers OPD-11, OPD-18 and OPN-09 are shown in Fig. 1. The genetic variation through RAPD markers has been highlighted in a number of ornamental plants including rose (Vainstein *et al.*, 1993; Mohapatra and Rout, 2005), *Vanda* sp. (Hoon-Lim *et al.*, 1999), *Pelargonium* (Renou *et al.*, 1997), and *Ixora* cultivars (Rajaseger *et al.*, 1997). The present findings show the narrow variation within the varieties and close relationship between species. The similarity matrix was obtained after multivariant analysis using Nei and Li's coefficient and is presented in Table III. The similarity matrix was then used to construct a dendrogram with the unweighted UPGMA method (Fig. 2). The dendrogram shows two major clusters. The first major cluster had three varieties (*Hibiscus rosa-sinensis* var. Prolific, Albus and Moorea) and the second major cluster included 13 varieties of *Hibiscus rosa-sinensis* and two species of *Hibiscus* (*Hibiscus schizopetalus* "Japanese Lantern" and *Hibiscus sabdariffa*). The second major cluster again consisted of two minor clusters, one having four varieties of *Hibiscus rosa-sinensis* ("Acc. no. 1", "Tiki", "Acc. no. 2" and "Toreador") and one having the species *Hibiscus sabdariffa.* Another minor cluster had 10 varieties

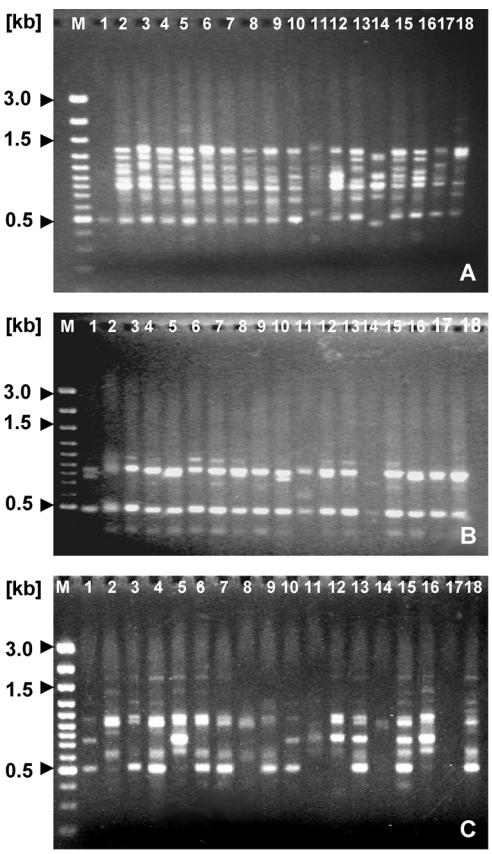

Fig. 1. RAPD patterns of two species and sixteen varieties of *Hibiscus* generated by the primer OPD-18 (5′-GAGAGCCAAC-3′) (A), OPD-11 (5′-AGCGCCATTG-3′) (B) and OPN-09 (5′-TGCCGGCTTG-3′) (C). M, molecular weight ladder (kb), H1–H18 reflect the different varieties/species of *Hibiscus*.

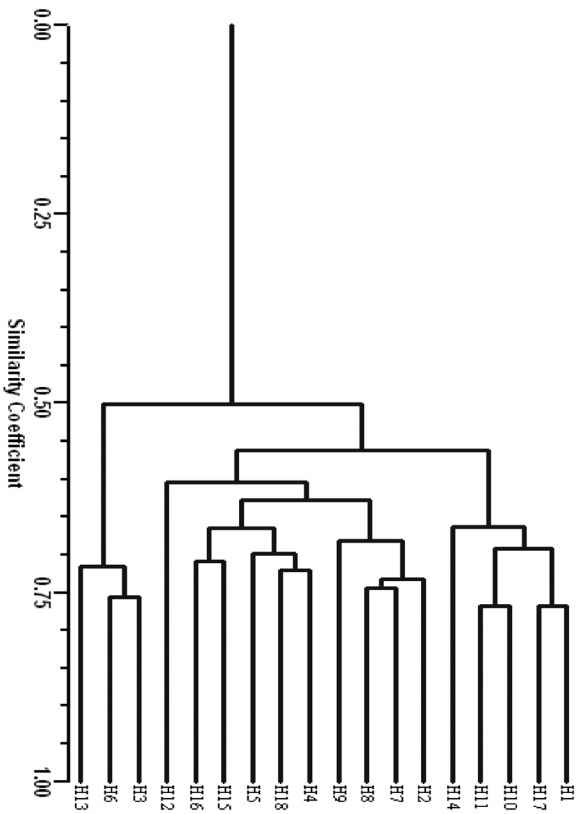

Fig. 2. Dendrogram of cluster analysis of RAPD markers. The scale indicates the fractional similarities among the varieties/species.

of *H. rosa-sinensis*. *Hibiscus sabdariffa* was not forming any cluster among themselves but *H. schizopetalus* "Japanese Lantern" having 72% similarity with *Hibiscus rosa-sinensis* var. Acc. no. 4 because of type of plant and growth habit. There was a narrow variation within the varieties. Our results were similar to the findings of Ben-Meir and Vainstein (1994). Vainstein *et al.* (1993) reported that the genetic similarities are small within the cultivated rose groups by using 28 DNA fragments from microsatellite fingerprints. They also indicated a higher genetic similarity between the hybrid tea and the floribunda group than within each group.

The present findings include the identification and genetic variation within sixteen varieties of *Hibiscus rosa-sinensis* and two species of *Hibiscus*. The dendrogram shows that the distant within the varieties was not significantly different. The genetic distances indicate that the variety "Prolific" has 72% similarity with "Albus". The varieties "Albus" and "Prolific" have 72% similarity with "Moorea". The variety "H. D. Maity" has 70% similarity with "Acc. no.4" and *H. schizopetalus* "Japanese Lantern". "Red Satin" has 72% similarity with "Acc. no. 4". The "Australian Rose", "Scarlet" and "Scarlet Brilliant" have 72% similarity among themselves and 67% similarity with "Snow Flake". The close relationships within the cultivars of celery were also reported by using RAPD markers (Yang and Quiros, 1993). This indicates that the RAPD markers provide a more reliable method for identification of varieties/species than morphological characters. It would allow a more quantitative assessment of genetic distances between varieties and species. This investigation as an understanding of the level and partitioning of genetic variation within the varieties/species would provide an important input into determining appropriate management strategies. Furthermore, this approach might be useful in future breeding programs in *Hibiscus*.

Acknowledgement

The authors wish to acknowledge the Department of Forest and Environment, Govt. of Orissa for providing the facility.

Ben-Meir H. and Vainstein A. (1994), Assessment of genetic relatedness in roses by DNA fingerprint analysis. Sci. Hortic. **58**, 158–164.

Bose T. K. and Choudhury B. (1991), Tropical Garden Plants. Horticulture and Allied Publishers, Kolkata, India, pp. 129–135.

Doyle J. J. and Doyle J. L. (1990), Isolation of plant DNA from fresh tissue. Focus **12**, 13–15.

Hoon-Lim S. A. W., Teng P. C. P., Lee Y. H. and Goh C. J. (1999), RAPD analysis of some species in the genus *Vanda* (Orchidaceae). Ann. Bot. **83**, 193–196.

Mohapatra A. and Rout G. R. (2005), Identification and analysis of genetic variation among rose cultivars using random amplified polymorphic DNA. Z. Naturforsch. **60c**, 611–617.

Nei M. and Li W. H. (1979), Mathematical modes for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA **76**, 5269–5273.

Paterson A. H., Tanksley S. D., and Sorreis M. E. (1991), DNA markers in plant improvement. Adv. Agron. **46**, 39–90.

Rajaseger G., Tan H. T. W., Turner I. M., and Kumar P. P. (1997), Analysis of genetic diversity among *Ixora* cultivars (Rubiaceae) using random amplified polymorphic DNA. Ann. Bot. **80**, 355–361.

Renou J. P., Aubry C., Serveau M., and Jalouzot P. (1997), Evaluation of the genetic variability in the genus *Pelargonium* using RAPD markers. J. Hortic. Sci. **72**, 229–237.

Rohlf F. J. (1995), NTSYS-PC – Numerical Taxonomy and Multivariate Analysis System, Version 1.80. Exter Software, Setauket, New York.

Rout G. R., Bhatacharya D., Nanda R. M., Nayak S., and Das P. (2003), Evaluation of genetic relationship in *Dalbergia* species using RAPD markers. Biodiversity Conserv. **12**, 197–206.

Vainstein A., Ben-Meir H., and Zucker A. (1993), DNA fingerprinting as a reliable tool for the identification and genetic analysis of ornamentals. Proceedings of the XVIIth Eucarpia Symposium "Creating Genetic Variation in Ornamentals", San Remo, pp. 63–68.

Williams J. G. K., Kubelik A. R., Livak K. J., Rafalski J. A., and Tingey S. V. (1990), DNA polymorphisms amplified by arbitary primers are useful as genetic markers. Nucl. Acids Res. **18**, 6531–6535.

Yang X. and Quiros C. (1993), Identification and classification of celery cultivars with RAPD markers. Theor. Appl. Gen. **86**, 205–212.

Yu K., Van Deynze A., and Pauls K. P. (1993), Random amplified polymorphic DNA (RAPD) analysis. In: Methods in Plant Molecular Biology and Biotechnology (Glick B. R. and Thompson J. E., eds). CRC Press, Boca Raton, USA.