HAYATI Journal of Biosciences December 2013
Vol. 20 No. 4, p 196-200
ISSN: 1978-3019

Available online at:
http://journal.ipb.ac.id/index.php/hayati
DOI: 10.4308/hjb.20.4.196

SHORT COMMUNICATION

The Genetic Relationships of Grevillea Hybrids Determined by RAPD Marker

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Received January 28, 2013/Accepted October 17, 2013

Grevillea **(Proteaceae) is native to Australia and is known as garden plant. The flowers have high diversity colors, which range over orange, yellow, pink, red and green. It is a large genus, consisted of 357 species. This genus is economically important and there are hundreds of Grevillea hybrids with beautiful color and shape of flower. Information of the genetic relationships of Grevillea hybrids is not yet available. Ten Grevillea hybrids were used in this study to evaluate the genetic relationships between hybrids and with their parents. PCR-RAPD technique was employed in this study with seven RAPD primers. The dendrogram of Grevillea hybrids was performed using Neighbor-Joining analysis based on genetic distance. The analysis revealed that 'Moonlight', 'Caloundra Gem', and Little Pink Willie' were clustered with** *Grevillea banksii***. The three hybrids were hybrid of** *G. banksii* **and other Grevillea species. 'Superb' was closely related to 'Robyn Gordon' as they are hybrid of** *G. banksii* **and** *G. bipinnatifida***.**

Keywords: genetic relationships, *Grevillea*, hybrids, RAPD

INTRODUCTION

Grevillea is member of Proteaceae that comprised of 357 species (Makinson 2000). Grevillea species are distributed mostly in the Southern hemisphere, including Papua New Guinea, Indonesia and New Caledonia (Wrigley & Fagg 1991). However, most species are endemic to Australia (Wrigley & Fagg 1991). *Grevillea* is well known as garden plants, with various color of flowers and high variation of leaf shape (Olde & Marriott 1994).

The flower arrangements of *Grevillea* are classified into the umbel-like or "spider" flower arrangement where the flower styles arise from a rounded inflorescence and the elongated raceme or "brush" arrangement. The attractive flowers of *Grevillea* lead to its use as cut flowers (Joyce *et al*. 1996). The breeding of *Grevillea* is directed at modifying existing species and varieties to develop plants with larger flower and a greater color range (Wrigley & Fagg 1991).

Latest taxonomic grouping divided *Grevillea* into 33 groups and 16 subgroups based on comparative morphology (Makinson 2000). Molecular study using RAPD and ISSR markers, involving 16 genotypes representing 12 species and 3 subspecies of *Grevillea*, generally supported the grouping suggested by Makinson (Pharmawati *et al*. 2004).

Grevillea species hybridized readily and hundreds of *Grevillea* hybrids are available (ANPSA: http://anpsa.org.au/greville.html; Costin & Costin 1988). Breeding through hybridization, improve flower color and flower production (Martin & Brown 2007). Hybrid of *Grevillea* often arose by chance near its two parent species. Therefore, confirmation of hybrid parentage is needed. For example, *Grevillea* 'Robyn Gordon' was predicted to arise from a cross between *G. banksii* that was planted close to *G. bipinnatifida* (ANPSA: http://anpsa.org.au/g-super1.html). *Grevillea* 'Superb' and *Grevillea* 'Ned Kelly' were believed to have same parentage to *Grevillea* 'Sandra Gordon'. *Grevillea* 'Moonlight', 'Key Williams', Caloundra Gem', and 'Litte Pink Willie' derived from crosses between *G. banksii* and other hybrid species (Martin & Brown 2007).

Molecular marker can be used to determine hybrid species and their genetic relationships. Among several molecular markers available, random amplified polymorphic DNA (RAPD) is the simplest method (William *et al*. 1990). RAPD has

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been widely used in determination of intraspecific as well as interspecific variations (Pharmawati *et al*. 2004; Noormohammadi *et al*. 2012), plant cultivar and varietal identification (Chaudhary *et al*. 2010; Sun *et al*. 2012). RAPD marker was proved to be able to identify hybridity of putative hybrids and their parents both in natural or spontaneous hybridizations (Tovae-Sanchez & Oyama 2004; El-Kamali *et al*. 2010; Clarkson *et al*. 2011; Yulita & Mansur 2012) as well as in artificial crosses (Chen & Mii 2012; Narendrula & Nkongolo 2012). In this study, PCR-RAPD was employed to examine genetic variations and molecular relationship of several Grevillea hybrids.

MATERIALS AND METHODS

Plant Materials and DNA Extraction. Leaf samples of ten *Grevillea* hybrids were obtained from commercial plant nursery in Sydney, NSW, Australia, while leaf of *G. banksii*, *G. bipinnatifida*, and *G. olivacea* were collected from Mt. Annan Botanic Garden, NSW, Australia (Table 1). Due to limited availability of *Grevillea* species, not all parents of *Grevillea* hybrids were included in this study.

DNA was extracted based on the method of Doyle and Doyle (1990) with modifications (Pharmawati *et al*. 2004). Leaf tissue (0.5 g) was grounded in liquid nitrogen to a fine powder. Leaf powder was added to 5 ml preheated modified extraction buffer and incubated at 55 °C overnight with gentle shaking. The modified CTAB buffer contained 2% w/v CTAB, 1.4 M NaCl, 50 mM EDTA, 100 mM Tris-HCl (pH 8), and 2% (v/v) 2-mercaptoethanol.

DNA Electrophoresis and Quantification. Gel electrophoresis in 1% agarose in TAE buffer (Tris acetate-EDTA) with ethidium bromide (0.5 µg/mL) was used to examine DNA. Electrophoresis was performed at 100 V for 45 min and DNA was visualized with a UV transilluminator. The concentration of DNA was determined by comparison to known concentration of lambda DNA (MBI, Fermentas, Richlands B.C., Qld).

PCR-RAPD Assay. PCR-RAPD was done in 25 µL reaction mixtures containing 10 ng template DNA, 12.5 µL of HotStar*Taq*™ Master Mix (Qiagen, Clifton Hill, VIC, Australia) which contained 1 x PCR buffer, 1.25 unit HotStar*Taq*™ polymerase, 200 mM of each dNTP, 2.5 mM MgCl2 and 0.2 µM primer in a FTS-960 microplate fast thermal sequencer (Cobert Research, Sydney, Australia). The PCR program was as follows: initial activation step for 15 min at 95 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C, with 10 min final extension step at 72 °C. Seven primers (OPC 10, OPC 11, OPC 15, OPD 5, OPD 8, OPD 18, and OPD 20 from Operon Technologies, Alameda, CA) were tested. The PCR-RAPD products were visualized in 1.8% agarose gel electrophoresis in TAE buffer stained with 0.5 µg/mL ethidium bromide. A 1.0 kb plus ladder (Invitrogen Australia, Mt Waverly, VIC, Australia) was run in each gel as a size marker.

Data Analysis. The average percentage of fragment polymorphism was calculated from all fragments of PCR-RAPD products. Fragments were scored as presence (1) or absence (0) of a band. The size of each fragment was determined using Quantity One Software (BioRad). A distance matrix

Table 1. *Grevillea* hybrids and species used in this study

Species and hybrid	Parental origin	References
G. banksii R.Br.	-	Makinson (2000)
G. bipinnatifida R. Br.	-	Makinson (2000)
G. olivacea A.S.George	-	Makinson (2000)
Caloundra Gem	*G. banksii* R.Br. x G. 'Coochin Hill' (*G. hodgei* Olde & Marriott)	APNI*; Brwon (2004)
Key Williams	*G. banksii* R.Br. x G. 'Sandra Gordon'	Farihill Native Plant & Botanic Garden
Little Pink Willie	*G. banksii* R.Br. x G. 'Caloundra Gem'	Plant Nursery, Sydney
Moonlight	*G. banksii* R.Br. x *G. whitena*	Downing and Dowing, 2010
Ned Kelly	*G. banksii* R.Br. x *G. bipinnatifida* R.Br.	ANPSA#
Robyn Gordon	*G. banksii* R.Br. x *G. bipinnatifida* R. Br	ANPSA
Sandra Gordon	*G. sessilis* C.T.White & W.D.Francis x *G. pteridifolia* Knight	ACRA∞
Superb	*G. banksii* R.Br. x *G. bipinnatifida* R. Br.	ACRA, ANPSA
Winpara Gem	*G. olivacea* A.S.George x *G. thelemaniana* Hügel & Endl	ACRA
Winpara Gold	*G. olivacea* A.S.George x *G. thelemaniana* Hügel & Endl	

*APNI (Australian Plant Name Index, http:// http://www.anbg.gov.au/apni/); #ANPSA (Australian Native Plant Society Australia, http://anpsa.org.au/greville.html); ∞ACRA (Australian Cultivar Registration Authority Inc, http://www.anbg.gov.au/acra/acra-list-2009.html).

based on mean character differences from PCR-RAPD data was developed using Multi Variate Statistical Package (MVSP) version 3 (Kovack 2005; Sun & Lo 2011). Cluster analysis was done using the unweighted pair group method (UPGMA)

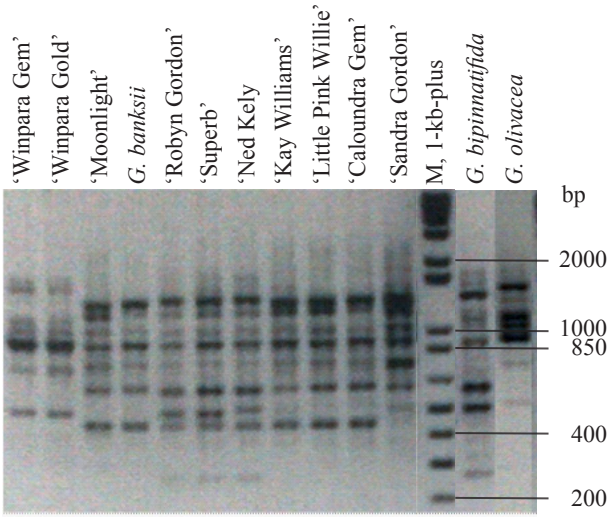

Figure 1. Amplification profile of ten *Grevillea* hybrids and three *Grevillea* species using PCR-RAPD using primer OPD 18.

RESULTS

PCR-RAPD. PCR-RAPD using seven RAPD primers was successfully conducted and showed clear and scorable products. Figure 1 shows results obtained for amplification of *Grevillea* DNA using OPD 18.

A total of 105 bands were produced with an average 15 bands per primer. The range of band sizes was 264 to 2580 bp. Primer amplified the least number of bands was OPD 5, while primer amplified largest number of bands was OPC15. Table 2 shows the number and the size of DNA fragments produced by each RAPD primers and the percentage of polymorphism.

Grevillea **Hybrids Relationships.** The pairwise distance matrix based on mean character differences between genotypes was generated (Table 3). The UPGMA dendrogram (Figure 2) based on distance matrix revealed that the 13 genotypes were grouped into two main groups. The first group (Group 1) was further divided into two sub group (1A and 1B). Subgroup 1A included, 'Robyn Gordon', 'Superb', 'Ned Kelly' and *G. bipinnatifida*. Sub Group

Table 2. RAPD primer sequences, number and sizes of amplified fragments and the percentage of polymorphism

Primer	Sequence (5' - 3')	Fragment (bp)	Number of fragments	Number of polymorphic fragments	Polymorphism (%)
OPC 10	TGTCTGGGTG	355-2170	14	14	100.0
OPC 11	AAAGCTGCGG	382-2520	16	16	100.0
OPC 15	GACGGATCAG	410-2580	19	19	100.0
OPD 5	TGAGCGGACA	468-2300	11	10	90.9
OPD 8	GTGTGCCCCA	285-2000	15	15	100.0
OPD 18	GAGAGCCAAC	264-1730	13	12	92.3
OPD 20	ACCCGGTCAC	520-1830	17	17	100.0
Total			105	103	
Average			15		97.6

Table 3. Pairwise distance matrix between ten *Grevillea* hybrids and three *Grevillea* species based on mean character differences (Kovach 2005)

Hybrid/Species	1	2	3	4	5	6	7	8	9	10	11	12	13
'Winpara_Gem'	0.00												
'Winpara Gold'	0.04	0.00											
'Moonlight'	0.51	0.47	0.00										
G. banksii	0.51	0.47	0.16	0.00									
G. olivacea	0.44	0.44	0.49	0.49	0.00								
'Robyn Gordon'	0.41	0.39	0.28	0.23	0.54	0.00							
'Superb'	0.41	0.37	0.26	0.21	0.52	0.02	0.00						
'Ned Kelly'	0.46	0.41	0.30	0.26	0.59	0.13	0.11	0.00					
'Kay Williams'	0.52	0.49	0.19	0.19	0.52	0.29	0.27	0.33	0.00				
'Little Pink Willie'	0.53	0.49	0.13	0.13	0.53	0.30	0.28	0.30	0.19	0.00			
'Caloundra Gem'	0.50	0.46	0.12	0.17	0.46	0.29	0.27	0.31	0.16	0.12	0.00		
'Sandra Gordon'.	0.48	0.43	0.21	0.26	0.49	0.31	0.29	0.36	0.18	0.26	0.18	0.00	
G. bipinnatifida	0.42	0.40	0.44	0.42	0.53	0.32	0.30	0.30	0.48	0.44	0.43	0.39	0.00

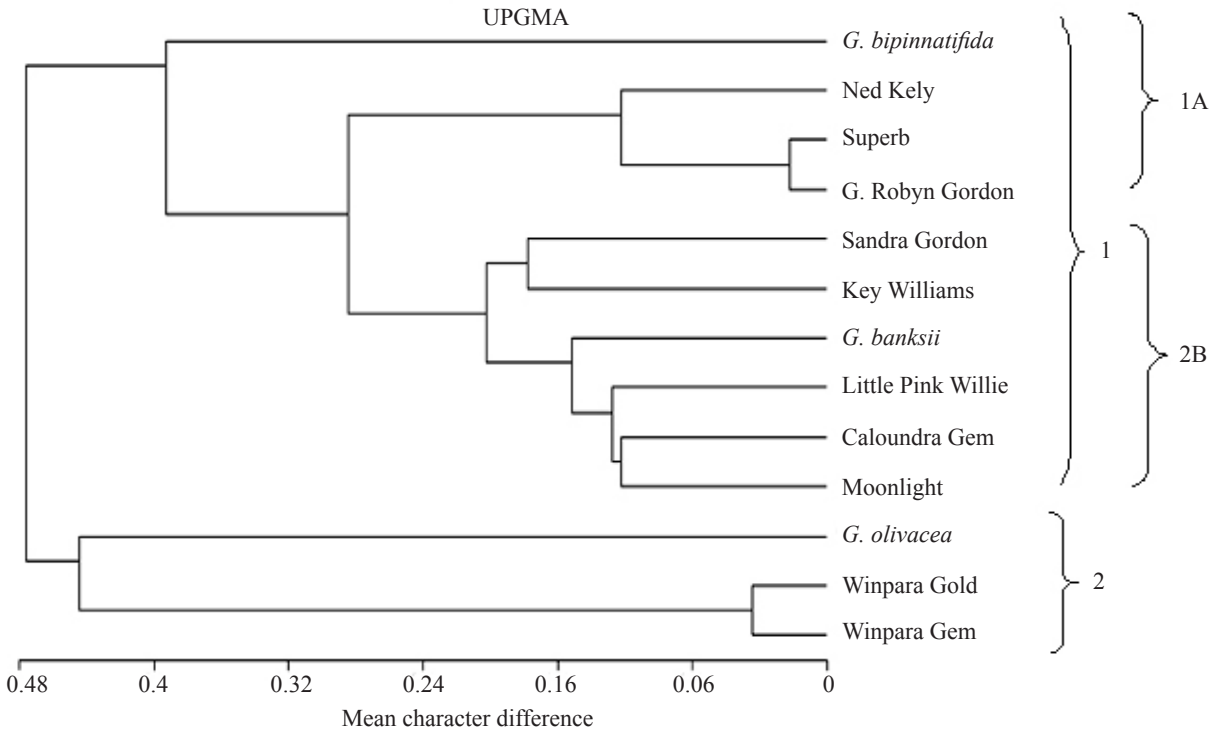

Figure 2. Dendrogram using UPGMA of distance matrix based on mean character of differences of 13 *Grevillea* genotypes reprensenting ten hybrids and three species.

1B consisted of *Grevillea* 'Little Pink Willie', 'Caloundra Gem', 'Moonlight', 'Sandra Gordon', 'Key Williams', and *G. banksii*. The second group (Group 2) included *Grevillea* 'Winpara Gem', 'Winpara Gold' and *G. olivacea*.

DISCUSSION

The cluster analysis of RAPD showed relatedness of *Grevillea* hybrids and *Grevillea* species. This analysis can be used to infer hybrids parentages (Srivastava *et al.* 2009). The divergent of the hybrids from each other suggested that they have less relationship because of different parentage (Ali *et al*. 2008).

The PCR-RAPD assay showed high average polymorphism within ten *Grevellia* hybrids and three *Grevillea* species, i.e. 97.6%. Based on polymorphism detected, the *Grevillea* genotypes studied were clustered into two groups. Group 1 was characterized by *G. banksii* as one parent of the hybrids. *Grevillea* 'Ned Kelly', 'Robyn Gordon' and 'Superb' developed a subcluster 1A. This result confirmed their well known close relationships. Those three hybrids are hybrids of *G. banksii* and *G. bipinnatifida* (ANPSA: http://anpsa.org.au/g-super1.html). *Grevillea bipinnatifida* is grouped in Group 1 together with *G. banksii*. This grouping is supported by Makinson (2000) which grouped *G. bipinnatifida* and *G. banksii* in Pteridifolia group based on their morphology and habitus.

In Subgroup 1B, *Grevillea* 'Caloundra Gem' was closely related to *Grevillea* 'Little Pink Willie'. This is in agreement with information of their parentage where *Grevillea* 'Caloundra Gem' is a hybrid between *G. banksii* and *Grevillea* 'Coochin Hill' (*G. hodgei*) [APNI: http://www.anbg.gov.au/apni/; Brown (2004)] and 'Little Pink Willie' is a hybrid of *G. banksii* and *Grevillea* 'Caloundra Gem'. *Grevillea* 'Moonlight' is clustered in SubGroup 1B because 'Moonlight' is believed to be crossed of *G. banksii* and *G. whiteana* (Downing & Dowing 2010). Based on morphological characters, *G. banksii* and *G. whiteana* were member of same subgroup i.e. Banksii Subgroup (Makinson 2000). *Grevillea* 'Kay Williams' and 'Sandra Gordon' were closely related. 'Kay Williams' is hybrid of *G. banksii* and *Grevillea* 'Sandra Gordon'. *Grevillea* 'Sandra Gordon' is results of a cross of *G. sessilis* and *G. pteridifolia* (ACRA: http://www.anbg.gov.au/acra/acra-list-2009.html). The position of 'Sandra Gordon' in same subgroup with *G. banksii* based on RAPD markers was supported by grouping by Makinson (2000), where *G. sessilis* was place at Banksii Subgroup. Moreover, *G. pteridifolia* was at same group with *G. banksii* in Pteridifolia Group (Makinson 2000).

In group 2, *Grevillea* 'Winpara Gem' is sister to 'Winpara Gold' (ACRA accession no 340 and 427 respectively), and closely related to *G. olivacea*. Those two hybrids are resulted from crosses between *G. olivacea* and *G. thelemaniana* (ACRA: http://www.anbg.gov.au/acra/acra-list-2009.html).

This study proved that PCR-RAPD has a potential as a method to determine the relationships of *Grevillea* hybrids and their parents. PCR-RAPD as dominan marker was able to detect a few co-dominan markers (Shasany *et al*. 2005; Sun & Lo 2011). Therefore, PCR-RAPD will be helpful to identify relationships between hybrids and their parents, especially when co-dominan markers are not available. However, in *Grevillea*, further study involving more parent species is needed to further resolve the position of hybrids.

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