Genetic diversity in *Banksia* and *Dryandra* (Proteaceae) with emphasis on *Banksia cuneata*, a rare and endangered species

TINA L. MAGUIRE & MARGARET SEDGLEY*

Department of Horticulture, Viticulture and Oenology, The University of Adelaide, Waite Campus, Plant Research Centre, PMB 1, Glen Osmond, SA 5064, Australia

Random amplified polymorphic DNA (RAPD) markers were investigated as a tool for estimating genetic diversity within 33 species of *Banksia* and three species of *Dryandra*. Three primers were used on DNA from 10 seeds per species, and band data were pooled to give between 52 and 89 bands per species, most of which were polymorphic. Genetic diversity was calculated using six published metrics on three species, for which allozyme data were also available. Based on between-method consistency, three metrics were chosen for analysis of the full data set. Levels of genetic diversity in *Banksia* and *Dryandra* ranged from 0.59 to 0.90. Based on this information, a detailed study was conducted on all 10 known populations of *B. cuneata*, a rare and endangered species, with a restricted geographical distribution in south-western Australia. Estimates of genetic diversity ranged from 0.65 to 0.74, which is high for a rare and endangered species. Analysis of molecular variance (AMOVA) was used to partition RAPD variation within and between populations. Nearly all of the variation was attributable to individuals within populations, indicating a lack of population divergence. It is suggested that the combination of bird pollination and high outcrossing rates in *B. cuneata* maintain genetic diversity and cohesion between the populations.

Keywords: AMOVA, *Banksia*, *Dryandra*, population genetics, Proteaceae, RAPDs.

Introduction

Scientific approaches to the conservation and exploitation of plant genetic resources require a detailed knowledge of the amount and distribution of genetic diversity within a species. Allozymes have been used in *Banksia* to estimate genetic diversity and high levels have been reported, amongst the highest recorded for plants (Scott, 1980; Schemske & Lande, 1985; Carthew *et al.*, 1988; Coates & Sokolowski, 1992). More recently many workers have moved to DNA markers such as RAPDs, which have been useful for population genetic studies in a number of genera (Chalmers *et al.*, 1992; Huff *et al.*, 1993). It has also been suggested that RAPDs may be an appropriate technique to monitor diversity in plant populations (Anderson & Fairbanks, 1990; Virk *et al.*, 1995).

Banksia cuneata A. S. George is a rare and endangered species known only from 10 populations (≈ 550 plants) in the central area of south-western Australia in an area of ≈ 90 km². It is found in deep yellow sands which occupy 10–15 per cent of the area, giving it a fragmented distribution. Associated with these soils is a rich and diverse flora dominated by species of the families Proteaceae, Myrtaceae and Leguminosae. In the last 50–60 years land clearing for agriculture and other disturbances have reduced the *B. cuneata* population size to about 7 per cent of its original distribution, and it now occurs in remnants of native vegetation. The mating system and patterns of genetic variation for six populations of *B. cuneata* have been determined using allozyme electrophoresis (Coates & Sokolowski, 1992), and estimates of outcrossing based on six loci ranged from 0.67–0.95, with low levels of selfing. The populations were divided into two groups, with gene flow within groups but not between them. It was

*Correspondence. E-mail: msedgley@waite.adelaide.edu.au

suggested that an ecological barrier to pollinator movement may be responsible for the restriction of between-group gene flow.

This study investigated levels of genetic diversity within species of *Banksia* and *Dryandra* using RAPDs, and evaluated six methods of data analysis. Based on this information, a detailed study determining patterns of genetic variation within and between all known populations of *B. cuneata* was conducted. Ten populations were included in this study, four of which had not been previously investigated.

Materials and methods

Plant material

Seeds of 33 species of *Banksia* and three species of *Dryandra* collected from natural populations were obtained from a commercial seed source (Nindethana Seed Service, WA). Seeds represent bulk collections from single or multiple sites of wild populations. The species chosen represented the two sister genera *Banksia* and *Dryandra*, and two subgenera, two sections and 13 series within the genus *Banksia* (George, 1981, 1988; Table 1). Ten seeds of each species were randomly selected for RAPD analysis.

Banksia cuneata seed material was collected from all 10 known populations (Fig. 1). Twenty seeds were randomly selected, one per plant, from each of four populations with in excess of 50 plants, and 10 seeds were collected, one per plant, from each of three populations with fewer than 50 plants. In three cases, the populations comprised fewer than 10 plants, so a one-seed sample was taken from each. In total 125 plants, ≈ 25 per cent, of the remaining 550 plants were sampled.

DNA isolation

DNA was extracted from each seed using a modification of the method of Weining & Langridge (1991) comprising: phenol/chloroform/isopropanol extraction for 5 min on ice, DNA precipitation for 1 min with ice-cold isopropanol and sodium acetate, with DNA recovered by centrifugation at 11 600 *g* for 10 min. The pellet was washed twice with 70 per cent ethanol, dried and dissolved in 50 μ L of TE buffer, with 1.0 μ L of RNAase (R40: 40 mg/mL RNAase A in TE), and stored at 4°C for up to 1 month.

DNA was subjected to gel electrophoresis on 1.6 per cent agarose gels in TBE buffer (Sambrook *et al.*, 1989), and stained with ethidium bromide. DNA

concentration was estimated by visual assessment of band intensities, compared to salmon sperm genomic DNA standards.

DNA amplification and documentation

DNA amplification was performed in a MJ Research Thermal Cycler. The programme commenced with a denaturation step at 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, 36°C for 1 min, 72°C for 2 min, and terminated with an extension step at 72°C for 5 min. Optimized reaction conditions were carried out in a 25 μ L total volume containing 1 $\times$ Taq buffer (Gibco-BRL), 3 mM MgCl₂, 200 μ M each of dNTP (dGTP, dATP, dCTP, dTAP), 1 unit of Taq polymerase (Gibco-BRL), 0.5 μ L T4 gene 32 protein (Boehringer Mannheim), 1 μ M 10 mer primer (Operon Technologies) and 10 ng template DNA. Each reaction mix was overlaid with PCR grade paraffin oil. DNA amplification fragments were separated by 2 per cent agarose gel (Seakem, Promega) electrophoresis using TBE buffer (Sambrook *et al.*, 1989). A negative control was added in each run to test for contamination. In order to test reproducibility, the selected primers were tested three times on the same sample, for a random subset of three DNA samples. To aid interpretation of band identity between gels, each contained a standard DNA sample and pGEM DNA marker. Gels were stained with ethidium bromide and fragment patterns were photographed under UV light with Polaroid 667 film. Polaroid photographs were scanned using a transmission scanner (Hewlett Packard Scanjet IIcx/T). The intensity and molecular weight of each visible band was determined using the software CREAM (Kem-En-Tec Software Systems, Blue Sky Scientific). Based on the faintest visible band of the molecular weight marker (pGEM) a minimum intensity threshold was established. Bands with an intensity greater than the minimum threshold were scored as present.

Sixty primers were evaluated for their suitability in a pilot survey (series OPA, OPB and OPC, Operon Technologies). Three primers were selected for the initial study of genetic diversity in 33 species of *Banksia* and three species of *Dryandra*. The primers OPA-20 (GTTGCGATCC), OPB-03 (CATCCCTG) and OPB-04 (GGACTGGAGT) gave reproducible and informative markers. Five primers were selected for the detailed study on *B. cuneata*, OPA-1, OPA-4, OPA-9, OPA-11 and OPA-16 (Table 3). Band fragments included in the final analysis ranged between 2.5 kb and 100 bp in length (Fig. 2), were scored as present (1) or absent (0) for

Table 1 Band data and estimates of diversity for 33 species of *Banksia* and three of *Dryandra* using RAPDs. Taxonomy follows George (1981, 1988)

Species	Number of bands			Diversity (standard deviation)		
	Polymorphic	Monomorphic	Total	Nei & Li (1979)	Jaccard (1901)	Russell & Rao (1940)
Genus <i>Banksia</i>						
<i>Subgenus Banksia</i>						
<i>Section Banksia</i>						
<i>Series Salicinae</i>						
<i>B. integrifolia</i> *	82	0	82	0.77 (0.07)	0.74 (0.09)	0.86 (0.04)
<i>B. robur</i>	61	0	61	0.79 (0.06)	0.77 (0.07)	0.88 (0.04)
<i>Series Grandes</i>						
<i>B. grandis</i>	63	0	63	0.72 (0.06)	0.68 (0.07)	0.82 (0.03)
<i>B. solandri</i> **	52	2	54	0.67 (0.07)	0.62 (0.09)	0.75 (0.05)
<i>Series Quercinae</i>						
<i>B. quercifolia</i> **	51	1	52	0.73 (0.06)	0.69 (0.07)	0.83 (0.04)
<i>Series Bauerinae</i>						
<i>B. baueri</i>	86	2	88	0.72 (0.06)	0.68 (0.08)	0.81 (0.05)
<i>Series Banksia</i>						
<i>B. baxteri</i>	84	0	84	0.71 (0.06)	0.67 (0.07)	0.82 (0.03)
<i>B. candolleana</i>	60	0	60	0.74 (0.06)	0.71 (0.08)	0.83 (0.04)
<i>B. menziesii</i>	79	5	84	0.65 (0.06)	0.59 (0.07)	0.77 (0.04)
<i>B. serrata</i>	53	1	54	0.73 (0.07)	0.70 (0.08)	0.84 (0.04)
<i>Series Crocinae</i>						
<i>B. burdettii</i> **	89	0	89	0.71 (0.08)	0.67 (0.07)	0.83 (0.04)
<i>B. prionotes</i>	57	1	58	0.73 (0.08)	0.69 (0.09)	0.82 (0.06)
<i>Series Cyrtostylis</i>						
<i>B. ashbyi</i>	69	1	70	0.67 (0.07)	0.61 (0.09)	0.77 (0.04)
<i>B. attenuata</i>	60	1	61	0.77 (0.07)	0.74 (0.08)	0.86 (0.05)
<i>B. audax</i>	69	0	69	0.79 (0.06)	0.77 (0.07)	0.88 (0.04)
<i>B. elderiana</i>	52	1	53	0.73 (0.07)	0.69 (0.09)	0.83 (0.06)
<i>B. laevigata</i> *	58	0	58	0.75 (0.07)	0.72 (0.09)	0.85 (0.05)
<i>B. lindelyana</i> *	69	2	71	0.72 (0.05)	0.69 (0.06)	0.82 (0.03)
<i>B. praemorsa</i> **	57	4	61	0.65 (0.10)	0.59 (0.13)	0.79 (0.05)
<i>Series Prostratae</i>						
<i>B. blechnifolia</i> **	55	4	59	0.69 (0.07)	0.65 (0.08)	0.80 (0.04)
<i>B. repens</i>	77	1	78	0.73 (0.07)	0.69 (0.09)	0.83 (0.04)
<i>Series Tetragonae</i>						
<i>B. caleyi</i>	57	1	58	0.74 (0.06)	0.70 (0.07)	0.82 (0.04)
<i>B. lemanniana</i>	82	0	82	0.81 (0.07)	0.79 (0.08)	0.90 (0.04)
<i>Series Coccineae</i>						
<i>B. coccinea</i>	65	0	65	0.78 (0.06)	0.76 (0.07)	0.85 (0.04)
<i>Section Oncostylis</i>						
<i>Series Spicigeræ</i>						
<i>B. ericifolia</i>	51	1	52	0.76 (0.06)	0.73 (0.07)	0.85 (0.05)
<i>B. occidentalis</i> **	74	3	77	0.69 (0.08)	0.65 (0.09)	0.81 (0.04)
<i>B. tricuspis</i> **†	54	0	54	0.80 (0.07)	0.78 (0.09)	0.89 (0.05)
<i>Series Dryandroideae</i>						
<i>B. dryandroides</i> **	66	0	66	0.74 (0.07)	0.70 (0.09)	0.83 (0.04)
<i>Series Abietinae</i>						
<i>B. meisneri</i> **†	59	0	59	0.73 (0.06)	0.69 (0.08)	0.83 (0.05)
<i>B. pulchella</i>	66	1	67	0.71 (0.07)	0.67 (0.09)	0.80 (0.07)

Table 1 Continued

Species	Number of bands			Diversity (standard deviation)		
	Polymorphic	Monomorphic	Total	Nei & Li (1979)	Jaccard (1901)	Russell & Rao (1940)
<i>Subgenus Isostylis</i>						
<i>B. illicifolia</i>	60	1	61	0.71 (0.08)	0.67 (0.09)	0.80 (0.05)
<i>B. oligantha</i> *	48	0	48	0.73 (0.07)	0.69 (0.09)	0.85 (0.04)
<i>B. cuneata</i> ***†	78	0	78	0.80 (0.09)	0.78 (0.10)	0.90 (0.04)
<i>Genus Dryandra</i>						
<i>D. formosa</i>	55	4	59	0.64 (0.09)	0.59 (0.11)	0.75 (0.05)
<i>D. polycephala</i>	76	1	77	0.77 (0.09)	0.74 (0.11)	0.87 (0.04)
<i>D. carlinoides</i>	59	3	62	0.66 (0.08)	0.61 (0.10)	0.76 (0.05)

Conservation status of *Banksia* and *Dryandra* species (George, 1987):

*species which are rare but not currently considered endangered or vulnerable;

**vulnerable species not presently endangered but at risk in the longer term;

***endangered species which may disappear from the wild within one or two decades if present land use and other causal factors continue;

†Declared rare flora under the Western Australian Wildlife Conservation Act 1950–79.

all individuals, and a matrix of RAPD phenotypes was assembled.

Analysis of genetic diversity of *Banksia* and *Dryandra*

Utilizing the matrix of RAPD phenotypes for each DNA sample, an index of genetic distance (D) was calculated, ($D = 1 - F$) where F is similarity, using the following six methods: (1) $2n_{11}/(2n_{11} + n_{01} + n_{10})$ from Nei & Li (1979), where n = number of band positions, n_{11} = the number of positions where $x = 1$ and $y = 1$, n_{00} = the number of positions where $x = 0$ and $y = 0$, n_{01} = the number of positions where $x = 0$

and $y = 1$, n_{10} = the number of positions where $x = 1$ and $y = 0$ (x and y = individuals compared); (2) $n_{11}/(n - n_{00})$ from Jaccard (1901); (3) n_{11}/n from Russell & Rao (1940); (4) $(n_{11} + n_{00})/n$ from Apostol *et al.* (1993); (5) $n(1 - (n_{11}/n))$ from Excoffier *et al.* (1992); (6) $(n_{11} + n_{00})/(n_{11} + 2(n_{10} + n_{01}) + n_{00})$ from Rodgers & Tanimoto (1960).

Fig. 1 Geographical range of *Banksia cuneata* in south-west Australia, showing locations of the 10 populations from which seed was collected.

Fig. 2 Agarose gel showing RAPD markers produced using primer OPA-9 for individuals of *Banksia cuneata*. Lane 1 shows the DNA size standard pGEM (Promega), lane 2 shows the standard individual to aid in band identity for between-gel comparisons, lanes 3–6 show individuals in population 7, lanes 7–16 show individuals in population 3 and lane 17 is the negative control. Bands within the molecular size range 2.5 kb to 100 bp are scored as present (1) or absent (0), and a matrix with elements of 1 or 0 is established for each individual, for all primers used in the study. The matrix is used in genetic distance and AMOVA analyses.

Distance matrices were calculated using the statistical package RAPDISTANCE (Amstrong *et al.*, 1994). The mean distance value for each species was taken as an estimate of genetic diversity, and the standard deviation of the mean was calculated for each data set.

Analysis of genetic diversity of *B. cuneata*

Based on the initial analysis of genetic diversity using six published metrics, the method of Nei & Li (1979) and the statistical package RAPDISTANCE (Amstrong *et al.*, 1994) were used for analysis of *B. cuneata* data. Analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) was used to estimate variance components attributable to differences within and between populations. Significance levels for variance component estimates were calculated by permutational procedures. The number of permutations for significance testing was set at 100 for all analyses. An unweighted pair group mean average (UPGMA) clustering analysis was carried out using a distance matrix based on the Phi statistic (PhiST) produced by the AMOVA analysis for between-population distances.

Results

Genetic diversity of *Banksia* and *Dryandra*

Band data for the three selected primers were pooled such that each species had total band numbers ranging from 48 to 89. The number of monomorphic bands was low, with most bands polymorphic (Table 1). Analysis of RAPD data for three *Banksia* species with each of the six published statistical methods gave a wide range of estimates of genetic diversity (Table 2). The species were chosen on the basis that there were reported estimates of outcrossing rates and genetic diversity based on allozyme electrophoresis data (Scott, 1980; Coates & Sokolowski, 1992). With high outcrossing rates and

animal pollination in these *Banksia* species high levels of genetic variation are expected within a species, with most variation occurring within rather than between populations. This suggests that the methods of Nei & Li (1979), Jaccard (1901) and Russell & Rao (1940) are most appropriate and these were subsequently used for analysis of the total data set.

The estimates of genetic diversity for all species with the three chosen metrics were high, ranging from 0.59 for *Banksia praemorsa*, *B. menziesii* and *Dryandra formosa*, up to 0.90 for *B. lemanniana* and *B. cuneata* (Table 1).

Genetic diversity of *B. cuneata*

The five primers yielded a total of 169 polymorphic bands (Table 3). No monomorphic bands were scored and the number of markers per primer ranged from 30 (OPA-4) to 37 (OPA-9, OPA-16). Of the 169 RAPD markers scored, 33 (19.5 per cent) were present in all populations and there were no fixed differences between populations, with the remaining 136 (80.5 per cent) bands variable between populations. Most bands occurred at a frequency of 0–20 per cent, with relatively few bands in the 80–100 per cent category (Table 4). The total number of bands present in each population was similar, with the exception of population 10 with only three individuals, which had only half the number of bands (Table 4).

The total genetic diversity for *B. cuneata* of $D = 0.70$, calculated by this study, was high for a rare species (Table 5). The diversity within each population ranged from 0.65 (population 10) to 0.74 (population 2), with greatest genetic diversity estimates in the larger populations (2, 3, 4) compared to the smaller populations (7, 8, 10). AMOVA analysis (Table 6) attributed nearly all of the variation to individuals within populations. Analysis between populations showed a small negative variance component, indicating lack of population structure,

Table 2 Genetic diversity of three species of *Banksia* calculated from RAPD data using six different methods

Species	RAPD analysis					
	Nei & Li (1979)	Jaccard (1901)	Russell & Rao (1940)	Apostol <i>et al.</i> (1993)	Excoffier <i>et al.</i> (1992)	Rodgers & Tanimoto (1960)
<i>B. attenuata</i>	0.77	0.74	0.86	0.41	0.25	0.57
<i>B. menziesii</i>	0.65	0.59	0.77	0.35	0.29	0.51
<i>B. cuneata</i>	0.80	0.78	0.90	0.36	0.28	0.52

with some plants being more related between populations than within. That there was no distinction between populations of *B. cuneata* was reflected by the absence of fixed differences between populations in RAPD markers. UPGMA clustering with low levels of genetic diversity between populations, reflected the lack of genetic differentiation between the 10 populations.

Discussion

This study shows that by using RAPDs the 33 species of *Banksia* and the three species of *Dryandra* tested have high levels of estimated genetic diversity. The levels of expected genetic diversity in *B. cuneata* populations are in agreement with results from enzyme electrophoresis (Coates & Sokolowski, 1992) which estimated outcrossing rates ranging from 0.67–0.95, with low levels of selfing. Genetic diversity using RAPDs is high for a geographically restricted rare and endangered species.

Table 3 Summary of data obtained by RAPD analysis for five primers with 125 individuals of *Banksia cuneata*

Primer	Primer sequence 5' to 3'	Number of polymorphic bands
OPA-1	CAGGCCCTTC	33
OPA-4	AATCGGGCTG	30
OPA-9	GGGTAACGCC	37
OPA-11	CAATCGCCGT	32
OPA-16	AGCCAGCGAA	37
	Total	169
	Mean per primer	33.8

The 10 populations of *Banksia cuneata* show only slight differences in the levels of genetic diversity. Populations 1, 6, 7 and 10 occur in highly disturbed roadside vegetation, populations 3, 4, 8 and 9 show low levels of disturbance, whereas populations 2 and 5 have virtually untouched vegetation. Population 10 was a highly disturbed small population of only three individuals, and showed the lowest diversity of 0.65. The larger populations with low levels of disruption had the highest diversity estimates ranging from 0.71 to 0.74.

The lack of population differentiation in *Banksia cuneata* using RAPDs contrasts with evidence of population structure using allozymes (Coates & Sokolowski, 1992). Their study using six populations and six polymorphic loci found significant differentiation of populations into east and west groups, with a salt river system acting as an ecological barrier to pollinator movement. The westerly populations were reported to be heterogeneous, and some populations which were geographically close were more related to distant populations. In the eastern populations, gene flow was reported to be relatively high, suggesting that birds as pollinators (Ayre & Whelan, 1989) were effective in maintaining genetic cohesion and diversity. Birds are able to travel considerable distances, and associated with areas of *B. cuneata* is a rich and diverse vegetation system for bird foraging and nesting. Since clearing about 50–60 years ago, there has been no significant genetic differentiation between the populations, although population sizes have been reduced. It is possible that after clearing the populations are still visited by pollinators because of surrounding vegetation, which supports the bird population. It is also possible that

Table 4 Summary of band frequencies for 10 populations of *Banksia cuneata*

Population	Number of samples	Number of bands	Band frequency				
			0–20%	20–40%	40–60%	60–80%	80–100%
1	10	131	91	28	23	15	12
2	20	157	93	37	19	13	7
3	20	161	78	43	24	18	6
4	20	145	83	47	20	11	8
5	10	128	90	28	24	16	11
6	6	115	94	29	11	18	17
7	10	135	96	24	23	12	14
8	6	118	90	20	23	14	22
9	20	145	91	32	23	18	5
10	3	66	103	34	0	15	17
Mean		130.1	90.9	32.2	19.0	15.0	11.9

the populations are ageing and there have been too few generations since clearing to see any significant genetic differentiation.

The relationships between the six distance metrics tested have been discussed by Gower (1985). Some of the metrics are related by simple monotonic functions and the distances they produce are linearly or curvilinearly related. Thus the metrics of Nei & Li (1979) and Jaccard (1901) form one group, and Rodgers & Tanimoto (1960), Excoffier *et al.* (1992) and Apostol *et al.* (1993) form another, with the metric of Russell & Rao (1940) producing distances that are poorly related to those produced by any of the others. Our results are consistent with Gower's (1985) expectations, and suggest that the Nei & Li (1979), Jaccard (1901) or Russell & Rao (1940) methods are the most appropriate for RAPD data sets. AMOVA is not strictly rigorous with non-Euclidean metrics, but as Excoffier *et al.* (1992) point out, the metric of Nei & Li (1979) differs only in the choice of denominator and the metric is nearly interchangeable with the Euclidean metric.

Table 5 Genetic diversity of 10 populations of *Banksia cuneata* calculated using the similarity (F) metric of Nei & Li (1979), where distance $D = 1 - F$

Population	Number of plants sampled	Mean distance (diversity)	Standard deviation
1	10	0.68	0.085
2	20	0.74	0.096
3	20	0.73	0.067
4	20	0.73	0.053
5	10	0.68	0.073
6	6	0.70	0.065
7	10	0.67	0.070
8	6	0.67	0.032
9	20	0.71	0.080
10	3	0.65	0.017
Total	125	0.70	0.031

Table 6 Analysis of molecular variance (AMOVA) for 125 individuals in 10 populations of *Banksia cuneata* using 169 RAPD bands. The data show the degrees of freedom (d.f.), sum of squared deviation (SSD), mean squared deviation (MSD), variance component estimate, percentage of total variance contributed by each component and the probability (P) of obtaining a more extreme component estimate by chance alone

Source of variation	d.f.	SSD	MSD	Variance component	% total	P -value
<i>Analysis among populations</i>						
Between populations	9	1.446	0.161	-0.0160	-4.73	0.57
Within populations	115	40.821	0.355	0.3549	104.73	<0.01

The AMOVA method, designed for other molecular data, has recently been used to analyse RAPD data for buffalo grass (Huff *et al.*, 1993), *Eucalyptus* (Nesbitt *et al.*, 1995) and *Grevillea* (Rossetto *et al.*, 1995).

It is important for future conservation and persistence of *B. cuneata* and other rare species that the remaining populations are protected. Even though they have reasonably high levels of diversity, this study shows that genetic diversity may decline in very small populations, such as population 10 with only three individuals. Pollinator maintenance is essential for plant genetic diversity, and reserves which have other coexisting vegetation to support pollinators must also be protected. The distribution of these reserves, to allow gene flow between populations, is important for the survival of *B. cuneata*, as well as maintaining genetic diversity in other plant species.

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