GENE DIVERSITY IN GREVILLEA POPULATIONS INTRODUCED IN BRAZIL AND ITS IMPLICATION ON MANAGEMENT OF GENETIC RESOURCES[1]

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ABSTRACT – We describe isoenzymes variability in six populations of *Grevillea robusta* from a provenances and progenies test established in a randomized block design with five plants per replication in Southern Brazil. The population genetic structure was examined by using biochemical markers in 5-year old trees, specifically at *MDH-3, PGM-2, DIA-2, PO-1, PO-2, SOD-1*, and *SKDH-1* loci. The northern provenances (Rathdowney and Woodenbong) showed a strong divergence in relation to the average of provenances when alleles per locus (*Ap*), allele richness (*Rs*), Nei's gene diversity (*H*), and inbreeding coefficient (*f*) were considered. Inbreeding in varying degrees was detected. The commercial control showed the highest inbreeding coefficient, (*f* = 0.4448), whereas the provenance average was *f* = 0.2306, possibly due to insufficient sampling of populations in their origin (Australia). Despite its restricted natural range, a positive correlation between genetic divergence and geographic distance among original populations was detected. Genetic distance and cluster analyses based on the Bayesian model revealed three distinct provenance groups: 1) Rathdowney-QLD and Woodenbong-QLD; 2) Paddy's Flat-NSW; and 3) Mann River-NSW, Boyd River-NSW and the commercial control (material used in Brazil). The grouping of the control to both Mann River-NSW and Boyd River-NSW provenances suggests that the northern provenances have the highest potential for genetic improvement of wood productivity in Brazil, due to their high genetic diversity and low inbreeding coefficient.

Keywords: *Isoenzymes, Provenances/progenies trial, Tree breeding*

DIVERSIDADE GENÉTICA EM POPULAÇÕES DE GREVÍLEA INTRODUZIDAS NO BRASIL E SUA IMPLICAÇÃO NO MANEJO DE RECURSOS GENÉTICOS.

RESUMO – A variabilidade isoenzimática para seis populações de Grevillea robusta*, oriundas de um teste de procedências/progenies, implantado no delineamento em blocos casualizados com 5 plantas por parcela, no Sul do Brasil, é descrita. A estrutura genética da população foi analisada utilizando-se marcadores bioquímicos, aos 5 anos de idade, especificamente para os locos* MDH-3, PGM-2, DIA-2, PO-1, PO-2, SOD-1, *e* SKDH-1. *As procedências do norte de ocorrência natural (Rathdowney e Woodenbong) apresentaram divergência genética superior, em relação à média das progênies, considerando o número de alelos por locus, (*Ap*), a riqueza alélica (*Rs*), a diversidade genética de Nei (*H*), e o coeficiente de endogamia (*f*). A endogamia foi detectada em diversos graus. A testemunha comercial apresentou o maior coeficiente de endogamia, (*f = 0,4448), comparativamente à média das procedências (*f = 0,2306), possivelmente devido à insuficiente amostragem populacional na região de origem (Austrália). Apesar de sua ocorrência natural restrita, observou-se correlação positiva entre divergência genética e distância geográfica entre as populações originais. A distância genética e análise de cluster, baseada no modelo bayesiano, mostrou três grupos de procedências distintos: 1) Rathdowney-QLD e Woodenbong-QLD; 2) Paddy's Flat-NSW; e 3) Mann River-NSW, Boyd River-NSW e a testemunha comercial (material utilizado no Brasil). O agrupamento da testemunha com as procedências Mann River-NSW e Boyd River-NSW sugere um maior potencial das procedências do norte para o melhoramento genético visando à produção de madeira no Brasil, devido a sua elevada diversidade genética e baixo coeficiente de endogamia.*

Palavras-Chave: *Isoenzimas; Testes de procedências/progênies; Melhoramento florestal.*

1. INTRODUCTION

Grevillea (*Grevillea robusta* A. Cunn.) is a tree species of Proteaceae family occurring naturally in Queensland, Australia, in areas extending from northern Gympie (latitude 25°50' S) to Guy Fawkes and Orara river areas (30°10' S). Orara River is a tributary of Clarence River that runs mostly through New South Wales at altitudes varying from sea level to 1,100 m at Bunya Mountains in Queensland (Harwood et al., 1997). This species occurs in two main habitats: in narrow bands associated with *Eucalyptus* along Richmond and Clarence rivers, and mixed with *Araucaria cunninghamii* Aiton ex. Don forests (Webb, 1959).

Grevillea populations are geographically discontinuous and separated by Sclerophyllous forests that are prone to fire, or by climax forests where they struggle to survive forest fires and the disadvantage of their typically small population size (Harwood et al., 1997). In general, there is a small number of effectively reproducing individuals. In Emu Creek, Queensland (28°58' S; 152°10' E) for example, only 90 trees were breeding in an elongated population stretching 2.4 km next to a stream. Population density of less than one individual per hectare is observed in association with *Araucaria cunninghamii* forests (Webb et al., 1967). Also, according to a survey by Harwood (1992), grevillea is naturally distributed in a number of small populations distant two to 10 km apart. This situation leads to genetic structures with strong differentiation among and weak within populations (Moran and Hoper, 1983, 1992).

Natural grevillea stands have been continuously depleted through exploitation for wood (Maiden, 1911) and deforestation for agricultural and pasture lands since the early days of the European colonization. Remnant populations are represented by young dispersed trees in degraded sites as well as in areas where *Araucaria* forests were removed for agriculture practice.

Grevillea has been introduced into many countries in South America, Africa and South Asia, especially for shade trees in tea and coffee plantations. It is also an important source of firewood and timber in small upland farms in tropical regions of eastern and central Africa (Harwood, 1989). It was first brought to Brazil in the late 19th Century (Instituto Brasileiro do Café,

1981) and disseminated throughout the southern and southeastern regions where climatic conditions are favorable for the species. Other uses of grevillea in Brazil include wood production for timber (Iwakiri et al., 2004), and as windbreak around croplands and pasture (Baggio, 1983; Durigan and Simões, 1987; Campanha et al., 2007) as well as in urban forestry (Beloto and De Angelis, 2003; Kramer and Krupek, 2012). According to Ribaski (2006), grevillea plays an important role in agroforestry systems. It contributes to improve environmental conditions in the field and provide comfort to animals and better pasture development. Moreover, it produces valuable logs for processing by small landowners (Radomski and Ribaski, 2011). Grevillea is known not to compete for resources with agricultural crops, while maintaining fast growth even in low fertility soils (Pinto Júnior et al., 2013) and is one of most popular tree species planted in pasture lands in Brazil (Almeida, 2011).

Efforts in genetic improvement of grevillea in Brazil started with provenance/progeny tests in the early 1980's. Important variations among provenances were reported by Shimizu et al. (1998) and Martins et al. (2000). Thereafter new provenances were reintroduced in Brazil and institutions as Instituto Florestal de São Paulo (IF-SP), Embrapa Florestas (CNPF) and Instituto Agronômico do Paraná (IAPAR) which demonstrated it potential Paraná, São Paulo and Mato Grosso do Sul States. Differences between them have been reported (Sebbenn et al., 1999; Martins and Neves, 2003; Martins and Neves, 2004; Martins et al., 2004; Martins et al., 2005; Martins et al., 2006; Leal and Ramos, 2011).

Population genetics studies are important elements in supporting genetic improvement and conservation programs, especially in the case of grevillea. These are vital in understanding the genetic structure in the remnant populations in Australia as well as in populations introduced into different countries. Studies have revealed high outcrossing rates (0.92; Hadwood et al., 1990) and evidence of self-incompatible breeding system in natural populations (Hadwood, 1992) and also in Kenya (Kalinganire et al., 1996, 2000).

So far, no study on population genetics had been carried out in grevillea populations introduced into Brazil. The present work addresses this issue in order to generate elements in support to the species breeding and conservation programs.

2. MATERIAL AND METHODS

2.2 Populations and sampling

The study was based on a five-year old combined provenance and progeny trial involving Australian sources (Table 1) and a control from commercial populations disseminated in Brazil, presumably of NWS origin. The trial was established in Ponta Grossa, State of Paraná, Brazil (latitude 25° 05' 42" S, longitude 50° 09' 43" W, and elevation 900 m above sea level). The local climate is classified according Koeppen's system as Cfb (mild summers). Average summer temperatures remain below 22 °C and winters are mild, although severe frosts can sometimes occur. Annual rainfall exceeds 1,500 mm and no soil water deficit is observed. The local soil is classified as Cambissol (equivalent to Inceptisol) and the relief varies from flat to a slightly rolling topography.

The trial was established in a randomized blocks design with seven replications of 5-plant plots. The treatments were represented by five Australian provenances and one local commercial control of unknown original provenance (Table 1).

Natural populations were sampled in areas extending from latitudes 28°13' 00"S to 29° 53 00"S, longitudes 152°26'00"E to 152°52'01"E, and altitudes 15 m to 100 m above sea level. The maximum distance (based on Ersts, 2017) among sampled populations was 171.21 km between Rathdowney and Boyd River, and the minimum distance of 25.08 km between Rathdowney and Woodenbong.

2.3. Isoenzyme electrophoresis

Isoenzyme technique played an important hole in characterizing plants, in general, and also forest species studies since the years 1970 have been largely replaced by molecular techniques, they are still informative and maintain their applicability, especially in studies of population genetics. Nowadays articles using isoenzymes as main technique (Litkowiec et al., 2013; Mathiasen and Premoli, 2013; Harter et al.; 2015; Medoukali et al., 2015; Chlumský et al., 2016; Kutlunina et al., 2016; Luo et al., 2016; El-Esawi et al., 2017; Konzen and Martins, 2017) as well in association with molecular markers (Streiff et al., 1998; De Woody et al., 2009; Ekart et al., 2016; Reichmann et al., 2017; Quipildor et al., 2018;) are still published worldwide on population genetic studies. Although molecular markers are more informative and currently used, in some cases isozymes is still helpful to screen population and individuals

Table 1 – Geographic coordinates of natural *Grevillea robusta* populations included in the study.
Tabela 1 – Coordenadas geográficas das populações naturais de Grevillea robusta incluídas no estudo.

Population	Longitude(E)	Latitude(S)	Altitude(m)	Tree number(n)
1. Rathdowney-QLD	28° 13' 00"	152° 52' 01"	100	83
2. Woodenbong-NSW	28° 26' 00"	152° 45' 00"	200	72
3 Paddy's Flat-NSW	28° 44' 00"	152° 26' 00"	180	80
4 Mann River-NSW	29° 24' 00"	152° 29' 00"	60	79
5 Boyd River-NSW	29° 53' 00"	152° 27' 00"	15	74
6. Commercial control-Brazil (ex-NSW)			85	

Table 2 – Genetic parameters based on isozyme loci in *Grevillea robusta* provenances.
Tabela 2 – Parâmetros genéticos de procedências de Grevillea robusta baseados em locus isoenzimáticos.

Population	n	Ap	Rs	He	Ho	H	f	F_{ST}
1. Rathdowney	83	3.14	3.02	0.5338	0.3497	0.5350	0.3465	0.0434
2. Woodenbong	72	3.00	2.88	0.5441	0.4374	0.5449	0.1973	0.0695
3. Paddy's Flat	80	2.86	2.84	0.5317	0.4573	0.5319	0.1414	0.0781
4. Mann River	79	2.86	2.67	0.5022	0.4576	0.5023	0.0896	0.0708
5. Boyd River	74	2.86	2.77	0.4870	0.4052	0.4876	0.1688	0.0644
6. Commercial control *	85	2.86	2.85	0.4849	0.2699	0.4861	0.4448	0.0583
Overall mean		2.93		0.5140	0.3962		0.2306	0.0641

Genetic material previously introduced into Brazil; n= sample size, Ap = average number of alleles per locus; Rs = allele richness per locus and per sample; H_e = expected heterozygosity; H_e, = observed heterozygosity; H = genetic diversity (Nei, 1987); f = inbreeding coefficient; F_{ST} = fixation index (Wright, 1965).

generating results to support decisions on genetic improvement and conservation.

Fresh leaves were labeled and stored at -30 °C until enzyme extraction. They were homogenized in an extraction buffer with germanium dioxide (Mitton et al., 1979). Electrophoresis was conducted using lithium-borate buffer 8.3 (Scandalios, 1969) for peroxidase (PO) and diaphorase (DIA) in a constant 60 mA system for four hour; histidine 8.0 (Brown et al., 1975), malate dehydrogenase MDH), and phosphoglucomutase (PGM) in a constant 50 mA for five hours; and tris-citrate 8.6 (Fowler and Morris, 1977), superoxide dismutase (SOD), and shikimate dehydrogenase (SKDH) in 60 mA for five hours.

2.4. Descriptive statistics and clustering

Allele frequencies, number of alleles per locus (A), expected heterozigosity (H_e), and observed heterozigosity (H_o) were estimated through GDA (Lewis and Zaykin, 2001) software. Allele richness (Rs), both per locus and per sample (Petit et al., 1998), unbiased gene diversity (H) (Nei, 1987), fixation index (F_{ST}), inbreeding coefficient (f or F_{IS}), and geographic distance (Ersts, 2017) were also estimated in this study. Hamilton's (1971) relatedness was calculated by using FSTAT program, version 2.9.3.2. (Goudet, 2002). Divergence among populations was inferred by plotting estimates of pairwise differences (Rousset's distance (1997) and geographic distance (Ersts, 2017).

Genetic structure of populations was performed by using Admixture analysis using Bayesian assignment procedures (Structure 2.2.3 (Pritchard et al., 2000) using 500.000 burn-in periods and Evanno method (Evanno, 2005) implemented by Earl (2012), a website program, for estimating K partitions (population groups).

Tree plot of Nei´s genetic distance was perfomed using NTSYSpc Version 2.10 z (Rohlf, 2002).

3. RESULTS

3.1. Genetic diversity

Genetic diversity parameters obtained from isoenzymes analysis are summarized in Table 2. Measures of genetic variation within populations (Table 2) indicated an average of 2.93 alleles per locus (Ap) across loci. Rathdowney and Woodenbong populations showed higher numbers of alleles per locus (3.14 and 3.00, respectively) than the average of other populations ($Ap = 2.86$). These provenances are geographically closer than the distances between other provenances.
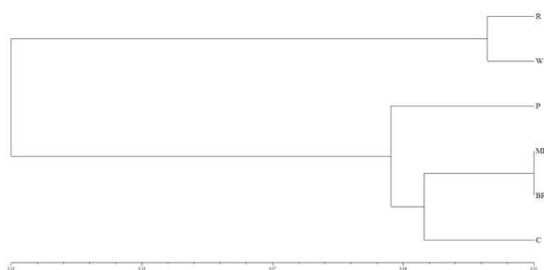
The expected heterozygosity (H_e) varied from 0.4849 to 0.5441 with an average of 0.5140. They were higher from those reported by Harwood et al. (1997) varying from 0.080 to 0.131) using isoenzymes. Heterozygous deficiency was observed in all populations. Woodenbong ($H_o = 0.3497$ against $H_e = 0.5338$) and the commercial control ($H_o = 0.2699$ against $H_e = 0.4849$) were the most outstanding in that respect. The highest inbreeding coefficients were observed in Rathdowney and the commercial control (0.3465 and 0.4448, respectively) when compared to other provenances (average of 0.2314).

Private alleles (number four) were found both in the commercial control at locus *MHD3* and in Rathdowney at locus *PO1*, suggesting that the local population contains other than the Australian northernmost provenances.

By comparing Rathdowney, the northernmost provenance, with Woodenbong the southernmost provenance, it becomes evident that they represent two distinct groups, as reported by Harwood et al. (1997). The local commercial control showed a high inbreeding level. Therefore, in spite of their common presence in small farms in Brazil, they must be used with caution, due to inbreeding depression.

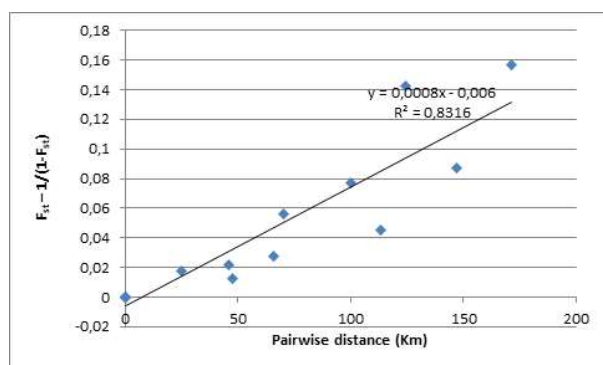### 3.2. Genetic structure and variation among populations

Two most distinct groups are shown in Figure 1 by using Nei's (1978) genetic distance. The first includes populations from the northern region of the species range (Rathdowney and Woodenbong); the second is formed by all other Australian provenances. Within the second group, Paddy's Flat differs from Mann River and Boyd River. Also, a clear difference was observed between the second group and the commercial control. In general the grouping of provenances followed a pattern close to their geographical distribution. This evidence is confirmed in Figure 2. There was an indication of a close association (Rousset's distance) between the population variation and their geographic distances ($r^2 = 0.8316$).

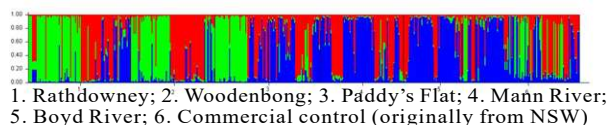R = Rathdowney; W = Woodenbong; P = Paddy´s Flat; MR = Mann River; BR = Boyd River; C = Control

Figure 1 – Genetic distance (Nei, 1978).
Figura 1 – Distância genética (Nei, 1978).

Figure 2 – Differentiation among populations considering the pattern of variation in Rousset's distance (F_{ST}/1-F_{ST})) in relation to geographic distances of grevillea populations. R² = 0.83, P< 0.0001 *.
*Figura 2 – Diferenciação entre as populações considerando o padrão de variação da distância de Rousset ((F_{ST}/ 1- F_{ST})) em relação às distâncias geográficas das populações de grevílea. R² = 0,83, P <0.0001 *.*

1. Rathdowney; 2. Woodenbong; 3. Paddy's Flat; 4. Mann River; 5. Boyd River; 6. Commercial control (originally from NSW)

Figure 3 – Baysian model- based clustering analysis (2000).
Figura 3 – Análise de cluster baseado no modelo de análise Bayesiana (Pritchard et al., 2000).

3.3. Admixture analysis

Three groups were shown by K value. These groups are represented in the admixture analysis (Figure 3). Populations from the northern distribution range (Rathdowney, Woodenbong and Paddy´s Flat) showed similar mixture patterns. Within the group including Mann River and Boyd River also similar admixture

patterns were observed. The commercial control showed a high degree of admixture, in similar pattern to that observed for the southern provenances.

4. DISCUSSION

The present study demonstrated clear variations among provenances. Also, correlation of provenance variation with their populations' geographic distribution became clear. The evidence of a high inbreeding rate and other aspects in variation patterns in the local commercial control raises concerns about continued use of this material. The main issue seems to be the restricted genetic variation in this material because of the small number of trees initially sampled for introduction into Brazil (Shimizu et al., 1998). This fact, over the years, has promoted damaging effects caused by inbreeding, such as the reduction of germination rate and loss of vigor and malformation of planted trees (Ferreira and Martins, 1998). This explains the higher inbreeding coefficient observed for ancient Grevillea germplasm introduction (in the mid-19th century) in relation to new populations introduced after 1990 (late 20th century).

For this reason, Embrapa Forest, in partnership with CSIRO, from Australia, introduced a new Australian germplasm base considering a better sampling including more provenances and individuals within provenances. This material were established as several trials and has been showing a higher potential for wood production, with genetic gains from 23 % to 33 % compared to original (commercial) introduced material (Martins et al., 2003, 2004).

Although apparently seed production in Brazil is normal, from 70,000 to 110,000 seeds per kg (Lamprecht, 1990) an accurate study about seed production is lacking. A high seed production in regions close to Curitiba city has been observed (Kalil Filho, personal communication) however information on literature were not found. Seed production among others depends on mating system and pollinators. *Grevillea robusta* is known as an outcrossing species not only in the natural occurrence region (Harwood et al., 1992) but also in introduced areas, as Kenya (Kalinganire et al., 2000) because of its protandrous (Brough, 1933) and self-incompatible (Hadwood, 1992; Kalinganire et al., 1996; Kalinganire et al., 2000) characteristics. Moreover it is pollinated by birds and bats (Jøker, 2000; Kalinganire et al., 2001)) not by insects (Brough, 1933; Kalinganire

et al., 2001). It was still observed by Kalinganire et al., (2001) a non-efficient seed production derivate from absence of a higher pollinator activity in Kenya. Thus a desirable seed production depends not only from environmental suitability to produce flowers but also from adequate and abundant pollinators to reach a higher seed production. In absence of specific pollinators in adequate amounts seed production can be increased through controlled pollination. Kalinganire et al. (2001) has observed experimentally, in Kenya, that controlled pollination produced more seeds than open pollination. Therefore in Brazil we have to be aware about pollinators issue and in some cases to consider controlled pollination as a tool to increase seed production aiming the advance in grevillea breeding programs.

Data from a provenance tests carried out in northern State of Paraná and elsewhere in Brazil (Sebbenn et al., 1999; Martins et al., 2006; Leal and Ramos, 2011) have demonstrated higher performances of recently introduced provenances in comparison to previously introduced material.

The pioneer work on population genetics in grevillea as described by Harwood et al. (1997) offered the basic elements to establish genetically sound breeding programs for a more efficient use and conservation of this valuable species. The multitude of knowledge that is being gradually amassed on the species breeding system, geographic variation, genetic parameters, silvicultural requirements, wood quality, and its role in agroforestry systems is a key to a better use and efficient conservation measures for this species.

5. CONCLUSIONS

The information gathered on different aspects of population genetics in grevillea introduced into Brazil represents an asset of vital importance for the best use of this forest resource. Pioneer introductions made in the early 20th century raised interest in this species for its value as shade tree and wind break around agricultural crops. However, low wood production and bad stem form discouraged any other form of utilization. Genetic studies have demonstrated that undesirable performances were basically due to inbreeding depression because of a very narrow genetic base.

Further introductions of well sampled populations from the northern areas of the species range are seen as means to place this species in focus as a multiple use alternative, especially in small farms. If ample genetic variation is preserved, there will always be opportunities for genetic improvement toward different end uses and suited for production in different ecologic sites.

6. ACKNOWLEDGMENT

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