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Genetic similarities among four species of the *Plectranthus* (L'Hér.) genus

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ABSTRACT. The *Plectranthus* genus comprises several species generally referred to as boldo, which are highly used in popular medicine due to their anti-dyspeptic, analgesic and digestion-stimulating properties. The amount of natural active principles can vary greatly in the related genotypes, which may lead to the inappropriate use of these plants. This work aimed to analyze the interspecific diversity among four species of the *Plectranthus* genus (*P. grandis, P. barbatus*, *P. neochilus* and *P. amboinicus*) and the intraspecific diversity of *P. barbatus* collected from different places in southern Brazil, by means of the RAPD technique. A higher genetic similarity was observed between the *P. neochilus* and *P. amboinicus* species (80%), followed by *P. grandis* and *P. barbatus* (77%). *P. barbatus* genotypes from Passo Fundo and Porto Alegre showed a genetic similarity which was close to 100%, while the genetic similarity for *P. barbatus* genotypes from other locations was higher than 96%. Although a low variability among genotypes of this species was found in this study, RAPD markers allowed a clear differentiation among the analyzed genotypes, showing a 53% mean genetic similarity, with a high correlation value (r = 0.99), which proves a high agreement between the genetic similarity and clustering data.

Key words: RAPD, boldo, genetic diversity, medicinal plants.

RESUMO. Similaridade genética de quatro espécies do gênero *Plectranthus*. O gênero *Plectranthus* abrange plantas de diversas espécies, referidas como boldo, que são utilizadas na medicina popular pelas suas propriedades antidispépticas, analgésicas e estimulantes da digestão. Genótipos relacionados podem diferir amplamente na quantidade de princípios ativos naturais, resultando no uso de plantas não-apropriadas à saúde da população. O objetivo do presente estudo foi avaliar a diversidade genética interespecífica de quatro espécies do gênero *Plectranthus* (*P. grandis*, *P. barbatus*, *P. neochilus* e *P. amboinicus*) e intraespecífica de *P. barbatus*, coletadas em diferentes localidades da região Sul do Brasil, por meio da técnica de RAPD (*Random Amplified Polymorphic DNA*). Foi observada maior similaridade genética entre as espécies *P. neochilus* e *P. amboinicus* (80%), seguido de *P. grandis* e *P. barbatus* (77%). Genótipos de *P. barbatus* provenientes de Passo Fundo e Porto Alegre apresentaram similaridade genética próximo a 100%, enquanto nas demais regiões foi superior a 96%. Embora no presente trabalho tenha sido detectada baixa variabilidade entre genótipos desta espécie, a técnica de RAPD permitiu clara separação entre as quatro espécies analisadas, apresentando uma similaridade genética média de 53%, com um valor de correlação alto (r = 0,99), demonstrando elevada representatividade dos dados de similaridade genética com os de agrupamento.

Palavras-chave: RAPD, boldo, diversidade genética, plantas medicinais.

Introduction

The Lamiacea family is extended, widely spread and adapted to nearly every habitat. It is characterized by the occurrence of various aromatic and medicinal species and the presence of essential oils produced in the glandular trichomes or scales which cover stems and leaves (WEBERLING; SCHWANTES, 1986). The *Plectranthus* genus belongs to the Mepetoideae subfamily, Ocimaeae tribe, Lamiaceae family and is thought to be one of the richest in essential oils, which include mono and sesquiterpenes as their main components. This genus includes many plants of medicinal and economic interest; their chemical composition, however, is little known (ABDEL-MOGIB et al., 2002).

Several species belonging to the *Plectranthus* genus are used in popular medicine for their antidispeptic, analgesic and digestion-stimulating properties (VIGANÓ et al., 2007). Due to the presence of bitter substances, the leaf macerate in aqueous solution has a hiposecretory gastric activity,

which acts by reducing the production of gastric juice, in addition to decreasing its acidity, and can be used in the treatment of gastritis, dyspepsia and heartburn (SIMÕES et al., 1998).

According to Lorenzi and Matos (2002), there are four species of the *Plectrantrus* genus, popularly known as boldo, which have medicinal properties. *Plectranthus barbatus* Andrews and *Plectranthus grandis* (Cramer) R.H. Willemse, labeled respectively false boldo and 'boldo-grande', are very similar and, for this reason, usually mixed up; both are used for the same purpose in popular medicine (LORENZI; MATTOS, 2002; MILANEZE-GUTIERRE et al., 2007). *Plectranthus Neochilus* (Schlechtre) ('boldo gambá') is a highly aromatic herbal plant according to Lorenzi and Matos (2002), used similarly to *Plectranthus barbatus*. According to Lukhoba et al. (2006), *Plectranthus amboinicus* (Lour) Spreng. also has medicinal properties similar to those of the previously mentioned species.

Because of their taxonomic similarities, several terminologies have been used to refer to the same species of the *Plectranthus* genus, which interferes with the collection of information about the ethnobotanic use of this genus. Besides, species of the *Plectranthus* genus usually used for medicinal purposes show a number of synonyms (LUKHOBA et al., 2006).

One alternative that may contribute to solving the terminology problems with species of the *Plectranthus* genus is the molecular analysis of the bold plant genome. Several molecular marker techniques have made it possible to accurately point out DNA genetic variations of organisms, thus elucidating synonymy and homonymy cases when an estimate of the morpho-phenologic characteristics does not show polymorphism. When molecular markers are used, the likelihood of genotype identification greatly increases for all species (MULCAHY et al., 1993; OLIVEIRA et al., 2008). To illustrate this, studies with *Vitis rupestris* Scheele (PAVEK et al., 2003), *Camellia sinensis* (L) O. Kuntze (KAUNDUN; PARK, 2002), *Malpighia emargunata* D.C. (SALLA et al., 2002), *Campanula microdonta Koidz* (OIKI et al., 2001), Orchidaceae (SUN; WONG, 2001), Ocimeae and Libiatae (PATON et al., 2004) and *Commelina benghalensis* L. (VIEIRA et al., 2007) can be mentioned.

The Random Amplified Polymorphic DNA (RAPD) technique, based on the polymerase chain reaction (PCR), which promotes DNA sequence amplification, offers advantages for being relatively simple and fast; furthermore, it does not demand previous information on the target sequence because it uses random sequence short primers (WELSH; MCCLELLAND, 1990; WILLIAMS et al., 1990; NAKAJIMA et al., 1998).

Due to the difficulty in finding morpho-phenologic markers to discriminate boldo species, the aim of the present study was to evaluate the interspecific genetic diversity of four species of the *Plectranthus* genus (*P. grandis*, *P. barbatus*, *P. neochilus* and *P. amboinicus*) and the intraspecific diversity of *P. barbatus*, collected from different locations in southern Brazil by means of the RAPD technique. Thus we intended to investigate the occurrence of DNA markers for one or more boldo species so that they could be properly identified.

Material and methods

Four *Plectranthus* species from different locations (Table 1) were used, with their botany identification confirmed by means of a specific identification key for Lamiaceae. A specimen of each species, collected by biologist Juliana de Magalhães Bandeira, was stored at the Botany Department PEL herbarium of the Federal University of Pelotas, registered under numbers 24586 (*P. grandis*), 24587 (*P. barbatus*), 24585 (*P. neochilus*) and 24584 (*P. amboinicus*).

Approximately 150 mg young leaves (from the second or third node, counting from the apex) of the *Plectranthus* species under study were collected and stored at -80°C until sample processing for DNA extraction.

Table 1. *Plectranthus* genotypes used in the genetic similarity analysis and their respective collection sites.

Code	Genotypes	Source of material
PgPel	*P. grandis*	Pelotas/Rio Grande do Sul State
PgFlo	*P. grandis*	Florianópolis/Santa Catarina State
PnPel	*P. neochilus*	Pelotas/ Rio Grande do Sul State
PnPOA	*P. neochilus*	Porto Alegre/Rio Grande do Sul State
PaPel	*P.amboinicus*	Pelotas/Rio Grande do Sul State
PbPel	*P. barbatus*	Pelotas/Rio Grande do Sul State
PbPOA	*P. barbatus*	Porto Alegre/Rio Grande do Sul State
PbPF	*P. barbatus*	Passo Fundo/Rio Grande do Sul State
PbRIPF	*P. barbatus*	Passo Fundo/ Rio Grande do Sul State Indian Reservation
PbFlo	*P. barbatus*	Florianópolis/Santa Catarina State

Genomic DNA was extracted from previously isolated *Plectranthus* species leaves by the CTAB 2% method, according to Doyle and Doyle (1990). DNA quantification was performed following electrophoresis in 0.8% agarose gel, and comparing band intensity to λ DNA/*Hin*d III fragments at a 0.5 μg μL^{-1} concentration, later diluted in MlilliQ water to an approximate 10 ng μL^{-1} concentration, so that it could be used for the PCR (Polymerase Chain Reaction).

Thirty-six primers were used for the PCRs, namely: Operon Kit decamers – OPX (01 to 20), OPA (01 and 07), OPAC (07, 16 and 19), OPB (01, 05, 18, 19 and 20), OPF (07 and 19), OPI 07 and UBC (50, 53, 410) from British Columbia University.

The amplification reactions were performed in a model PTC-100 MJ Research Inc. thermocycler following the thermal profile: first cycle at 94°C for 2 min. 30 seconds, 36°C for 30 seconds and 72°C for 2 min.; second cycle repeated 19 times at 94°C for 20 seconds, 36°C for 15 seconds, 45° C for 15 seconds and 72°C for 2 min.; third cycle repeated 18 times at 94°C for 30 seconds, 36°C for 15 seconds, 45°C for 45 seconds and 72°C for 2 min., and a final cycle at 72°C for 10 min.

PCR reactions were performed in 0.2 mL polypropylene tubes containing 2.5 µL 10 X buffer (10 mM Tris-HCl pH 9.5, 50 mM KCl), 2.0 mM $MgCl_2$, 180 µM of each dNTP, 1.2 µM primer, 1U Taq DNA polymerase - Invitrogen, 20 ng genomic DNA and enough sterilized Milli Q water to obtain 25 µL.

Amplification reproducibility material was tested twice using DNA originated from two distinct extractions of all genotypes under study (Table 1).

PCR material was separated by horizontal electrophoresis in 1.5% 65 V agarose gel for 90 min. After electrophoresis, the gel was immersed in ethidium bromide (5 µg mL^{-1}) and analyzed under UV light in an E-BOX-100 model Vilber Lourmat photodocumentation system.

PCR reaction fragments were recorded as present (1) or absent (0), making up a binary data matrix. For the genetic similarity calculation, the Dice coefficient (NEI; LI, 1979) was used. Based on the similarity matrix, the cluster analysis was done by the Unweighted Pair-group Method with Arithmetic Means (UPGMA) for further dendrogram elaboration with the support of version 2.1 NTSYSpc software (ROHLF, 2000). Cluster data were used for the calculation of the cophenetic matrix in order to check dendrogram representation in relation to similarity data, measured by the correlation coefficient (r). Besides, with the use of the Winboot computer software, the binary data matrix was used for *bootstrapping* analyses (with 1000 replications), searching to infer the confidence of each cluster graphically represented in the dendrogram.

Results and discussion

Of the 36 tested primers, 27 produced polymorphic bands out of 284 amplified fragments; of these, only 29 (10.21%) were monomorphic and 255 (89.79%) were polymorphic (Table 2). The mean polymorphism generated by each primer was 9.44. Some polymorphic products are shown in Figure 1, where an approximately 1350 pb band generated by the OPX20 primer can be observed (Figure 1A). This band permits the differentiation of *P. amboinicus* from the other genotypes; in addition, two bands of approximately 1350 and 700 pb generated by the OPX19 primer (Figure 1B) differentiate *P. grandis* from *P. barbatus*.

Table 2. Polymorphism detected by selected primers for RAPD reactions of the four species of the *Plectranthus* genus.

Primer	Number of amplified fragments		
	Monomorphic bands	Polymorphic bands	Total
OPX01	2	15	17
OPX05	0	6	6
OPX06	0	13	13
OPX07	0	17	17
OPX08	0	10	10
OPX09	1	12	13
OPX12	3	18	21
OPX13	1	2	3
OPX14	1	7	8
OPX16	0	6	6
OPX17	0	8	8
OPX18	2	10	12
OPX20	0	11	11
OPA01	3	8	11
OPA07	2	7	9
OPAC07	2	7	9
OPAC19	2	6	8
OPB01	0	13	13
OPB05	1	8	9
OPB18	2	11	13
OPB19	1	10	11
OPB20	1	7	8
OPF07	0	11	11
OPF19	1	5	6
OPI07	3	12	15
UBC53	1	8	9
UBC410	0	7	7
Total	29	255	284

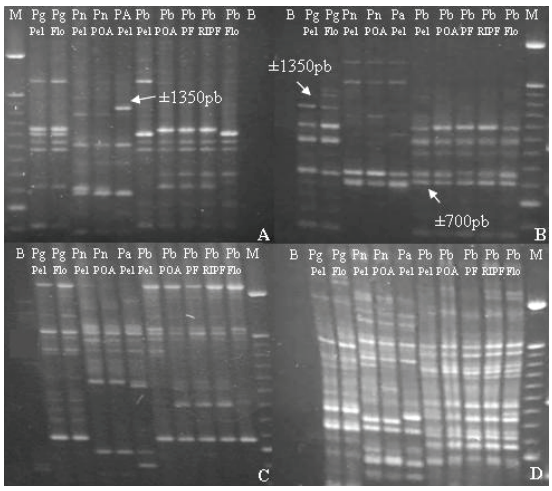

Figure 1. RAPD molecular markers in four *Plectranthus* species generated by OPX20 (A), OPX19 (B), OPX09 (C) and OPX01 (D) primers, respectively. B – blank sample, without DNA addition; M – λ 100 pb molecular marker; Pg – *P. grandis*; Pn – *P. neochilus*; Pa – *P. amboinicus*; Pb – *P. barbatus*. Other abbreviations correspond to the location of each genotype (Table 1).

In the electrophoretic profiles of the instraspecific analysis of *P. barbatus* plants from different locations, polymorphism was observed in 8 out of 27 primers used, resulting in a 92 band total, 11 of which (11.96%) were polymorphic (Table 3).

Table 3. Polymorphism dectected by RAPD primers among *P. barbatus* genotypes from five different locations.

Primer	Number of amplified fragments		
	Monomorphic bands	Polymorphic bands	Total
OPX08	9	1	10
OPX09	12	1	13
OPX12	20	1	21
OPX20	7	4	11
OPAC07	8	1	9
OPB05	8	1	9
OPB20	7	1	8
OPF07	10	1	11
Total	81	11	92

From the polymorphisms obtained by 27 RAPD markers, a 53% mean genetic similarity was identified and, in the data analysis between the similarity and cophenetic matrixes, a 0.99 correlation value (r) was found, which demonstrates a high data representation in the dendrogram.

Therefore, it became evident by the present study that the use of RAPD - type markers allowed a clear interspecific separation of the four analyzed *Plectranthus* species (Figure 2).

The *bootstrapping* values in most nodes in the dendrogram were also high, which indicates the consistency and the correct separation among the different species of the *Plectranthus* genus and *P. barbatus* genotypes analyzed. The lowest *bootstrapping* values were those between *P. barbatus* from Pelotas and *P. barbatus* from Florianópolis (49.1) and between genotypes of *P. barbatus* from Passo Fundo and *P. barbatus* from the Passo Fundo Indian Reservation (51.7) (Figure 2). On the other hand, these two genotypes presented a higher genetic similarity (99.61%), which can probably be accounted for by the proximity of these areas (Table 4).

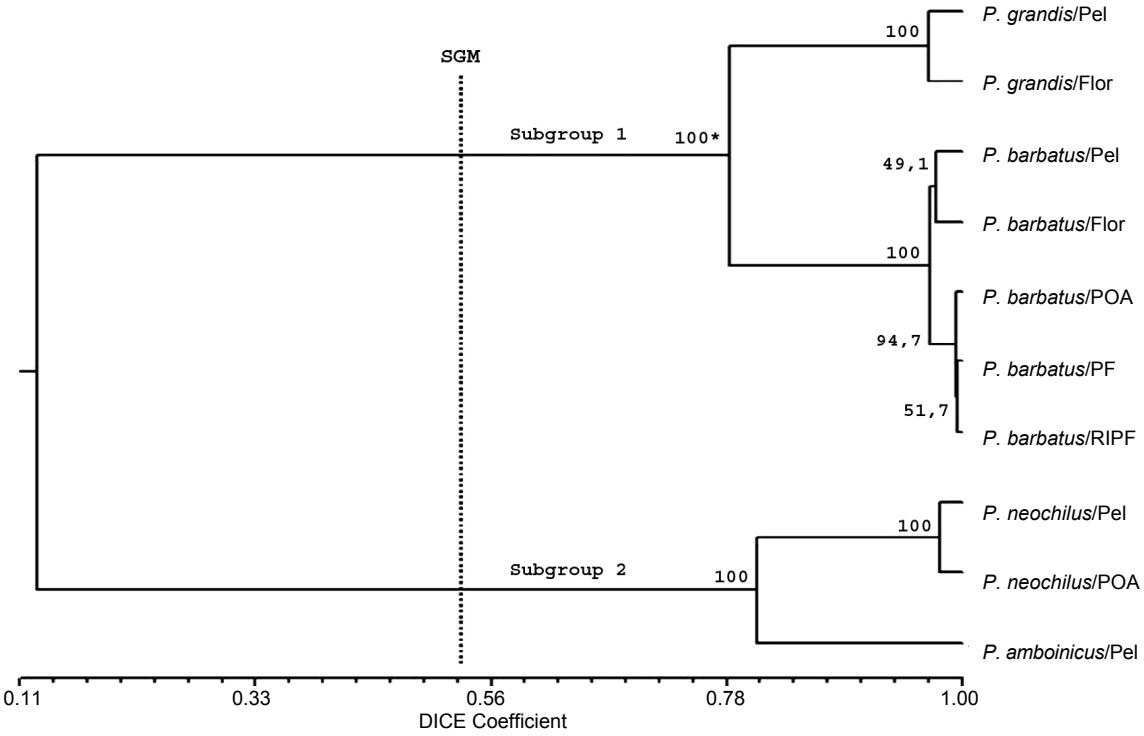

Figure 2. A UPGMA dendrogram of genetic similarity of the ten *Plectranthus* spp. (SMG - average genetic similarity of 53%). **Bootstrapping* values.

Table 4. Genetic similarity values calculated by the Dice coefficient using polymorphism data generated by 27 RAPD primers.

P.grandis/Pel	1.000									
P.grandis/Flor	0.968	1.000								
P.neochilus/Pel	0.129	0.130	1.000							
P.neochilus/POA	0.113	0.113	0.979	1.000						
P.amboinicus/Pel	0.110	0.111	0.800	0.812	1.000					
P.barbatus/Pel	0.786	0.789	0.138	0.114	0.086	1.000				
P.barbatus/POA	0.773	0.785	0.152	0.128	0.102	0.968	1.000			
P.barbatus/PF	0.770	0.781	0.159	0.136	0.101	0.964	0.996	1.000		
P.barbatus/RIPF	0.775	0.778	0.167	0.143	0.109	0.961	0.992	0.996	1.000	
P.barbatus/Flor	0.781	0.785	0.160	0.136	0.110	0.976	0.976	0.973	0.977	1.000
	Pg/Pel	Pg/Flor	Pn/Pel	Pn/POA	Pa/Pel	Pb/Pel	Pb/POA	Pb/PF	Pb/RIPF	Pb/Flor

Considering that *Plectranthus* spp. are cross-pollinated plants, a high interspecific genetic variability was expected. By dendrogram inference (Figure 2), even on observing a clear separation among the studied genotypes, *P. grandis* and *P. barbatus* were found to be genetically closest, which points to an eventual common origin. A similar pattern was found between *P. amboinicus* and *P. neochilus*, which showed about 80% genetic similarity; however, they showed a low similarity in relation to other species analyzed (Table 4).

According to Lorenzi and Matos (2002), *P. barbatus* and *P. grandis* are morphologically very similar, and because of this they are easily mixed up. Luckhoba et al. (2006) did an ethnobotany review with 62 *Plectranthus* species and found that about 30% of the literary citations consider *P. grandis* synonymous with *P. barbatus*, mistaking the two species for one. In the present study, a clear separation between the two species is evidence which is in agreement with a classification by Passinho et al. (2000) who, by means of the AFLP (Amplified Fragment Length Polymorphism) technique, demonstrated the occurrence of genetic variability between the two species, attesting to its authenticity.

Twenty-four primers have permitted the differentiation between the *P. grandis* and *P. barbatus* species, presenting a total of 164 bands where 37.20% were polymorphic and 62.80%, monomorphic; among these, OPX01 primer, with 8 polymorphic bands, showed the highest polymorphism (Figure 1D), while 32.84% of the 134 bands generated from 21 RAPD primers allowed the differentiation between *P. neochilus* and *P. amboinicus.*

According to Casas et al. (1999), molecular markers are highly used both to study the genetic variability among species and to identify the similarity among different intraspecific accesses. However, other molecular approaches can be used such as that by Paton et al. (2004) who, through phylogenetic analysis of the TrnL-TrnF and rps16 regions, devised a dendrogram that connects the *P. barbatus* and *P. amboinicus* species at a 67% *bootstrapping* value. In the present paper, a 53% mean genetic similarity was found by which the four species were clearly separated in a first subgroup represented by *P. grandis* and *P. barbatus*, while *P. neochilus* and *P. amboinicus* are included in a second subgroup (Figure 2). This clustering is directly related to the type of genomic approach, once the RADP analysis detects random DNA fragments in the genome. In a study by Paton et al. (2004), specific sequences were analyzed which, when referring to related species, can perform this type of classification.

Although it is estimated that a minimum of 12 polymorphic primers are needed for the genetic polymorphic analysis using the Dice coefficient (LANDRY; LAPOINT, 1996) probably if more primers had been used in addition to the 27 used in this research, a higher intraspecific genetic variability would have been detected, such as that found among *P. barbatus* genotypes.

Although RAPD is thought to be a low reproducibility technique, when correctly used and compared to other molecular techniques, it is efficient in genetic variability studies. Besides, it is the fastest, simplest technique, requires less DNA and is relatively low-cost (UPADHYAY et al., 2004).

This research has confirmed the applicability of the RAPD technique in identifying specific molecular markers in order to discriminate existing synonymy cases among boldo species, in view of the difficulty in characterizing them through morpho-phenological markers. Because the *Plectranthus* genus has several species that are empirically used in popular medicine, the scientific orientation as to their correct differentiation will contribute to studies that aim at a better exploitation of those plants with a higher potential for drug production. Furthermore, like RAPD, other molecular marker techniques that facilitate correct genotype identification will be extremely useful for germoplasm correct use. The genetic variability evaluation in this article is the first step towards mapping characteristics of interest for future genetic improvement research.

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