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Characterizing *Polygala* L. (Polygalaceae) Species in Southern Brazil Using ISSR

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Abstract The genus *Polygala* is one of the seven Polygalaceae genera that occur in the Brazilian flora, covering approximately 110 species. During the taxonomic review of Polygalaceae in Southern Brazil, difficulties were found when classifying species with very similar morphology, and morphological data alone could not clarify these interspecific relationships. In this context, inter-simple sequence repeat (ISSR) molecular markers were used in an attempt to characterize the genetic diversity and relationships among *Polygala* species. Nine *Polygala* species were analyzed using six selected ISSR primers that generated a total of 75 bands (100% polymorphic). The relationships were evaluated by dendrograms using the unweighted pair group method using arithmetic averages algorithm. The use of ISSR to solve the taxonomic problems was very useful for the Brazilian *Polygala* species. This is the first report of a molecular characterization of the Brazilian *Polygala* species to successfully group the different species. The ISSR results are in agreement with the morphological evidence of a new *Polygala* species from Southern Brazil.

Keywords Interspecific relationships · ISSR · *Polygala* · Polygalaceae · Southern Brazil

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Introduction

Polygalaceae encompasses 19 genera covering approximately 1,000 species widely distributed, especially in the tropical regions, with abundant distribution in Central and South America, Africa, and Asia (Paiva 1998). The genus *Polygala* L. is the largest of the family Polygalaceae, comprising 725 cosmopolitan species. In the Brazilian flora, *Polygala* is represented by 110 species with 30 varieties (Marques and Peixoto 2007) and can be distinguished from other Polygalaceae genera by the dehiscent capsule with two seeds.

Many taxonomic reviews of the genus *Polygala* have been conducted over the last 20 years in Brazil, such as the studies by Marques (1984a, b, 1988), Lüdtke and Miotto (2004), Aguiar et al. (2008), and Marques and Peixoto (2007). Besides these taxonomic studies, other important contributions for Polygalaceae were accomplished in the past years, such as studies about the family's floral anatomy and morphology (Milby 1976; Eriksen 1993a; Westerkamp and Weber 1999; Prenner 2004; Weekley and Brothers 2006; Bello et al. 2007) as well as a phylogeny based on morphological data (Eriksen 1993b) and on plastidial DNA regions (Persson 2001; Forest et al. 2007).

During the taxonomic review of Polygalaceae in Southern Brazil, 40 widely distributed *Polygala* species were recognized as occurring in the most diverse environments.

Difficulties were found when classifying species with very similar morphology. Since morphological data alone could not clarify these interspecific relationships, molecular markers were used with a view to obtaining a more accurate classification of these species.

Inter-simple sequence repeat (ISSR) markers are highly sensitive to detect polymorphisms and offer a great potential to determine intra- and interspecific levels of variation (Wu et al. 1994; Zietkiewics et al. 1994). ISSR have been widely used in genetic diversity studies (Li and Ge 2001; Brantestam et al. 2004; Wu et al. 2004, 2007; Alexander et al. 2004; Ge et al. 2005; Bao et al. 2006), DNA fingerprinting (Moreno et al. 1998; Blair et al. 1999; Bornet and Branchard 2001; Mattioni et al. 2002), phylogenetic studies (Joshi et al. 2000; Xu and Sun 2001; Yockteng et al. 2003; Vanderpoorten et al. 2003), population genetic studies (Esselman et al. 1999; Camacho and Liston 2001; Wróblewska et al. 2003; Alexander et al. 2004; McRoberts et al. 2005; Kochieva et al. 2006; Mehes et al. 2007), and studies with interspecific genetic relationships (Leroy et al. 2000; Ajibade et al. 2000; Reddy et al. 2002; Fineschi et al. 2004; Agostini et al. 2008).

The aims of this study were to determine whether a set of ISSR primers could be successfully used to characterize the interspecific relationships among Brazilian species of *Polygala*.

Material and Methods

Plant Material

Nine *Polygala* species (*Polygala cyparissias*, *Polygala aspalatha*, *Polygala pulchella*, *Polygala riograndensis*, *Polygala linoides*, *Polygala* sp., *Polygala campestris, Polygala extraaxillaris*, and *Polygala brasiliensis*) with three or four samples from each species, totaling 30 samples, were collected at different locations in the Rio Grande do Sul and Santa Catarina states (Brazil; Table 1). Morphologically, *P. brasiliensis* is quite distinct from other representatives of the subgenus *Polygala*, and *P. extraaxillaris* is a representative of another subgenus (*Hebeclada*). These species were used as outgroups in this analysis. Samples were deposited in the ICN Herbarium, Instituto de Biociências, Universidade Federal do Rio Grande do Sul (Brazil).

DNA Isolation

Equal amounts (0.3 g) of leaf tissue from each sample were prepared. Leave material dried in silica gel was placed in porcelain mortars refrigerated with liquid nitrogen and to a fine powder ground with pestle. Total genomic DNA was extracted by the CTAB-modified method described by Doyle and Doyle (1987). DNA samples were quantified by comparison with known molecular weight markers (*Low* molecular weight, Amersham Biosciences) on a GelRed-stained agarose gel.

ISSR–PCR Amplification

Five anchored oligonucleotide primers, $(GA)_8T$, $(CTC)_4RC$, $(CT)_8G$, $(AG)_8YC$, and $(AG)_8A$, and one nonanchored primer, $(GACA)_4$, were used to amplify all DNA samples. These primers were selected from a set of 20 ISSR primers based on the number of amplification products and on the quality of the profiles obtained using random samples.

PCRs were performed in a total volume of 25 µl containing 12 µl sterile Milli-Q purified water, 0.2 µl of Taq DNA Polymerase (5 U/ul), 2.3 µl of MgCl2 (25 mM), 2.5 µl of 10× buffer, 1 µl of each primer (10 pmol), 1 µl of 40 mM dNTP mixture (10 mM each dNTP), 1 µl of DMSO (2%), and 5 µl of DNA (total 30–50 ng). DNA amplifications were performed using a TONAGEN PALM thermal cycler. The amplification conditions were one initial 5-min step at 92°C followed by 35 cycles at 94°C (1 min), 45 s at the annealing temperature (Primer's Tm), and 72°C (2 min); the reactions were completed by a final extension step of 5 min (72°C) and 5 min at 4°C.

The ISSR amplification products were separated by electrophoresis in a 1.5% agarose gel in 1× TBE buffer (50 mM Tris, 50 mM boric acid, 2.5 mM EDTA, pH 8.3). Gels were stained with GelRed (5 ng/mL) and photographed under UV light. The size of the amplified products was determined by comparison with a 100-bp molecular weight ladder (CENBIOT).

Data Analyses

Bands were scored as a binary value, (1) for presence and (0) for absence. The binary matrix (1/0) was used to calculate the similarity by the Dice's coefficient among each pair of samples. All data for the three samples from each species were combined to calculate the Dice's similarities. Among the various similarity indices, the Dice's coefficient was chosen because it is one of the most appropriate for dominant markers, like ISSR and RAPD, since it does not attribute any genetic meaning to the coincidence of absence of bands. The Dice's coefficient was calculated using the formula: $2N_{AB}/(2N_{AB} + N_A + N_B)$, where N_{AB} is the number of bands shared by samples, N_A represents amplified fragments in sample A, and N_B represents fragments in sample B.

The relationships among species were evaluated using dendrograms constructed by unweighted pair group method using arithmetic averages (UPGMA) assisted by the

Table 1 Plant samples from each species of *Polygala* collected at different locations in Rio Grande do Sul and Santa Catarina states

Species	Collector Number	Voucher (Herbarium)	Geographical location (Brazilian state–city–local)
Polygala cyparissias A. St.-Hil. & Moq.	Lüdtke, 185	ICN	Rio Grande do Sul, Torres, Praia de Itapeva
Polygala cyparissias A. St.-Hil. & Moq.	Lüdtke, 186	ICN	Rio Grande do Sul, Balneário Pinhal
Polygala cyparissias A. St.-Hil. & Moq.	Kinupp et al., 2617	ICN	Rio Grande do Sul, Cidreira, Praia de Salinas
Polygala aspalatha L.	Malme, 364	S	Rio Grande do Sul, Rio Grande, Ilha dos Marinheiros
Polygala aspalatha L.	Rambo, 48843	PACA	Rio Grande do Sul, Viamão, Toca do Tigre
Polygala aspalatha L.	Lindeman et al., s.n.	ICN 8541	Rio Grande do Sul, Santana do Livramento, Cerro Palomas
Polygala aspalatha L.	Bordignon et al.,1435	HERULBRA	Rio Grande do Sul, Caçapava do Sul, Guaritas
Polygala pulchella A. St.-Hil. & Moq.	Lüdtke, 241	ICN	Santa Catarina, Bom Jardim da Serra, 28°24′04.0″ S, 49°33′04.6″ W
Polygala pulchella A. St.-Hil. & Moq.	Lüdtke, 315	ICN	Santa Catarina, Urubici, 28°03′53.5″ S, 49°22′58.6″ W
Polygala pulchella A. St.-Hil. & Moq.	Lüdtke, 224	ICN	Rio Grande do Sul, São Francisco de Paula, RS 020
Polygala pulchella A. St.-Hil. & Moq.	Lüdtke, 198	ICN	Rio Grande do Sul, Caseiros, BR 285, km 223
Polygala riograndensis A. St.-Hil. & Moq.	Lüdtke, 82	ICN	Rio Grande do Sul, Bagé, BR 153, km 632
Polygala riograndensis A. St.-Hil. & Moq.	Lüdtke, 77	ICN	Rio Grande do Sul, Bagé, BR 153, km 628
Polygala linoides Poir.	Lüdtke, 226	ICN	Rio Grande do Sul, São Francisco de Paula, RS 020, km 28
Polygala linoides Poir.	Lüdtke, 316	ICN	Santa Catarina, Urubici, 28°03′14.3″ S, 49°22′34.8″ W
Polygala linoides Poir.	Lüdtke, 240	ICN	Santa Catarina, Bom Jardim da Serra, 28°24′04,0″ S, 49°33′04,6″ W
Polygala campestris Gard.	Molz, s.n.	ICN 125355	Rio Grande do Sul, São José dos Ausentes
Polygala campestris Gard.	Lüdtke, 255	ICN	Santa Catarina, Urubici, Morro da Igreja
Polygala campestris Gard.	Lüdtke, 163	ICN	Rio Grande do Sul, Cambará do Sul, Cânion Fortaleza
Polygala campestris Gard.	Lüdtke, 314	ICN	Santa Catarina, Urubici, 28°03′53.5″ S, 49°22′58.6″ W
Polygala sp.	Soares, 135	ICN	Rio Grande do Sul, RS 324, km 148
Polygala sp.	Lüdtke, 244	ICN	Santa Catarina, Bom Jardim da Serra, SC 438
Polygala sp.	Lüdtke, 196	ICN	Rio Grande do Sul, Campestre da Serra, BR 166, km 63
Polygala sp.	Lüdtke, 199	ICN	Rio Grande do Sul, Caseiros, BR 285, km 232
Polygala extraaxillaris Chod.	Lüdtke, 202	ICN	Rio Grande do Sul, Coronel Bicaco, BR 468, km 31
Polygala extraaxillaris Chod.	Lüdtke, 139	ICN	Rio Grande do Sul, Santana do Livramento, Cerro Palomas
Polygala extraaxillaris Chod.	Lüdtke, 328	ICN	Santa Catarina, Curitibanos, 27°18′29.9″ S, 50°38′41.5″ W
Polygala brasiliensis L.	Lüdtke, 13	ICN	Rio Grande do Sul, São Francisco de Paula, RS 020, km102
Polygala brasiliensis L.	Lüdtke, 125	ICN	Rio Grande do Sul, Canguçú, BR 392, km107
Polygala brasiliensis L.	Lüdtke, 111	ICN	Rio Grande do Sul, Vacaria, BR 116

NTSYS Package (Rohlf 2001). The permutation analysis of Bootstrap (1,000 permutations) was performed using WinBoot (Yap and Nelson 1996) and the analysis of molecular variance (AMOVA) was performed with GenAlEx (Peakall and Smouse 2001) software.

The discrimination potential of each primer was expressed by the Simpson's coefficient $\left(h_j = \Sigma(1 - \Sigma p_i^2)/n\right)$, where p_i is the frequency of the *i*th allele, and *n* corresponds to the number of loci detected by each primer (Hunter and Gaston 1988; Valk et al. 2005). A value of 1.0 indicates that the

primer is able to discriminate between all samples, and a value of 0.0 indicates that all samples are identical.

Results and Discussion

Twenty primers were tested for ISSR amplification and six produced highly reproducible ISSR bands and were selected to evaluate the relationships of *Polygala* species. For all six primers, a Simpson's value was calculated (Table 2), revealing a good mean discriminatory value (0.789), justifying the use of a small number of primers in this work.

The PCR protocol with the six selected primers used to amplify the DNA of the 30 *Polygala* samples (three to four samples for each of the nine species) amplified in total 75 fragments (12.5 fragments per primer, on average). The size of the amplified products ranged from 250 to 1,600 bp. The number of amplified products per primer varied from nine [$(AG)_8$A and $(CTC)_4$RC] to 15 [$(AG)_8$YC]. Considering the species of interest (*P. cyparissias*, *P. aspalatha*, *P. pulchella*, *P. riograndensis*, *P. linoides*, *Polygala* sp., and *P. campestris*), the percentage of polymorphic bands was 82.6%; however, when the species *P. extraaxillaris* and *P. brasiliensis* were included as outgroup, 100% of the bands showed polymorphism. None of the ISSR primers tested produced species-specific bands. The percentage of polymorphism observed among *Polygala* species was similar to those previously reported by other authors in several families, like Lamiaceae (Fracaro et al. 2005; Agostini et al. 2008) and Fabaceae (Ajibade et al. 2000).

The inter- and intraspecific genetic similarities calculated using AMOVA were on average 36% and 64%, respectively. The high values obtained within species suggest that each species is genetically defined, with no gene flow occurring among different species. The highest similarity value calculated using the Dice's index among species was obtained for the comparison between *P. cyparissias*/*P. aspalatha* (0.88), *P. campestris*/*Polygala* sp. (0.82), and *P. pulchella*/*P. riograndensis* (0.78), while the lowest similarity values were obtained between *P. cyparissias*/*P. linoides* (0.63).

The ISSR-based UPGMA dendrogram (Fig. 1) separated the seven species of interest from the outgroup. The cluster formed by *Polygala* species was divided into two groups. The first one was composed by two subgroups: (1) *P. cyparissias*, *P. aspalatha*, and *P. pulchella*; and (2) *P. riograndensis*, *Polygala* sp., and *P. linoides*. The second group was formed only by *P. campestris*.

P. cyparissias and *P. aspalatha* are confirmed as two distinct but closely related species based on morphological and molecular data, with bootstrap value of 64.8. They share high similarity concerning the morphological data, and it is very difficult to differentiate them only using flowers or fruits measures. The different geographical distribution and the consistence of the leaves are the essential features that differentiate these two species. Based on ISSR patterns, both taxa correspond to distinct species—though *P. pulchella* is grouped with these two species with a close morphological relationship, despite the fact that no morphological data confirmed this group. The lowest Dice's similarity values obtained between *P. cyparissias* and *P. linoides* are consistent with the morphological data (Fig. 2).

The ISSR results are in agreement with the morphological evidence of a new *Polygala* species from Southern Brazil. In order to characterize this new species, a morphological and nomenclatural study was conducted by experts in this genus, which has been submitted for publication. *Polygala* sp. was identified in the Herbarium collections as *P. campestris*, due to the resemblance in habit and because of the similar geographical distribution, both occurring in Brazilian high fields of Santa Catarina and Rio Grande do Sul states. However, *Polygala* sp. is generally cespitosus and presents dense racemes, while *P. campestris* exhibits a prostate habit and the racemes are lax. Another difference between these two species is the margin of the bracts, which in *P. campestris* is ciliated, while in *Polygala* sp. these structures have glabrous margin.

ISSR results showed that *Polygala* sp. is more closely related to *P. linoides* than *P. campestris*, which confirmed the findings obtained by morphological data. The resemblances are noticed in the length of the floral whorls, but *P. linoides* keeps an erect habit, and its leaves are scanty. In *Polygala* sp., the leaves are abundant, and the habit is cespitosus.

This is the first report of a molecular characterization using ISSR markers for Brazilian species of the genus

Table 2 ISSR primers, number of polymorphic bands, and Simpson Index

Primers sequence	Annealing temperature (°C)	Number of bands	Polymorphic bands (%)	Simpson's Index for Polygala
$(GA)_8$T	50	14	100	0.8
$(CTC)_4$RC	48	9	100	0.633
$(CT)_8$G	48	14	100	0.844
$(AG)_8$YC	50	15	100	0.841
$(AG)_8$ A	50	9	100	0.764
$(GACA)_4$	48	14	100	0.854
		75	100	0.789

R A,G; *Y* G,T

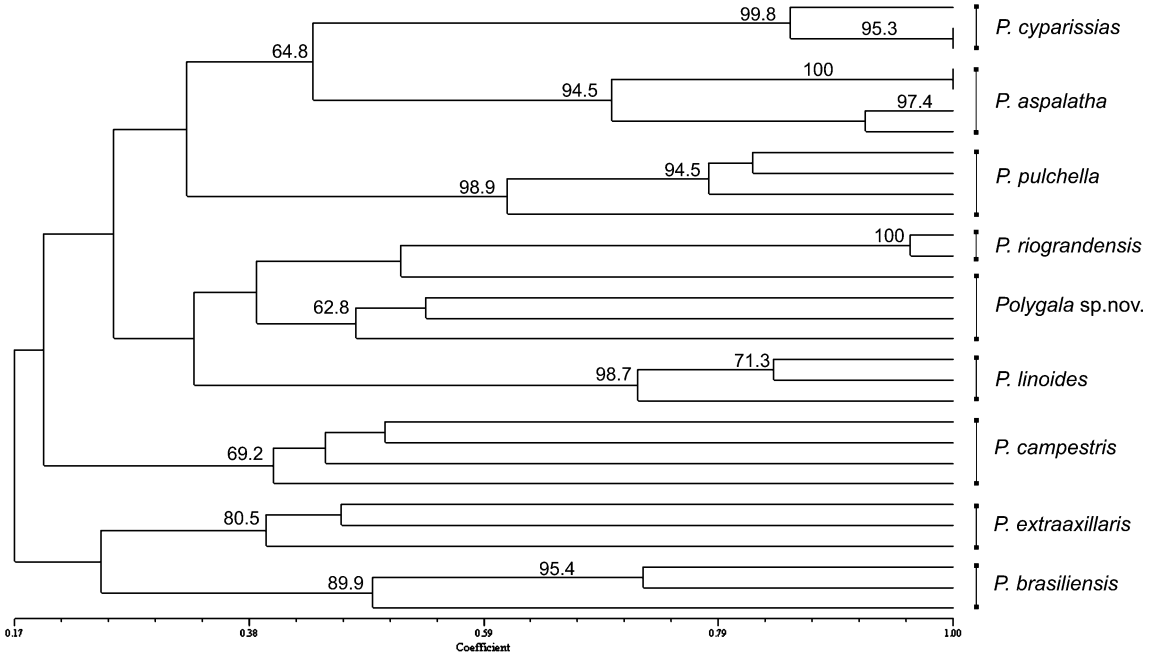

Fig. 1 UPGMA dendrogram of genetic similarity (Jaccard coefficient) among *Polygala* species based on ISSR markers. The bootstrap values (>60%) are shown on the branches (1,000 permutations)

Polygala. The data allow a better knowledge of the relationships among some species of *Polygala* occurring on Southern Brazil, and it was possible to confirm the existence of a novel *Polygala* species using other evidence besides morphological data. Combined studies using ISSR and other molecular markers will be important to increase the knowledge about the diversity of this genus in the future.

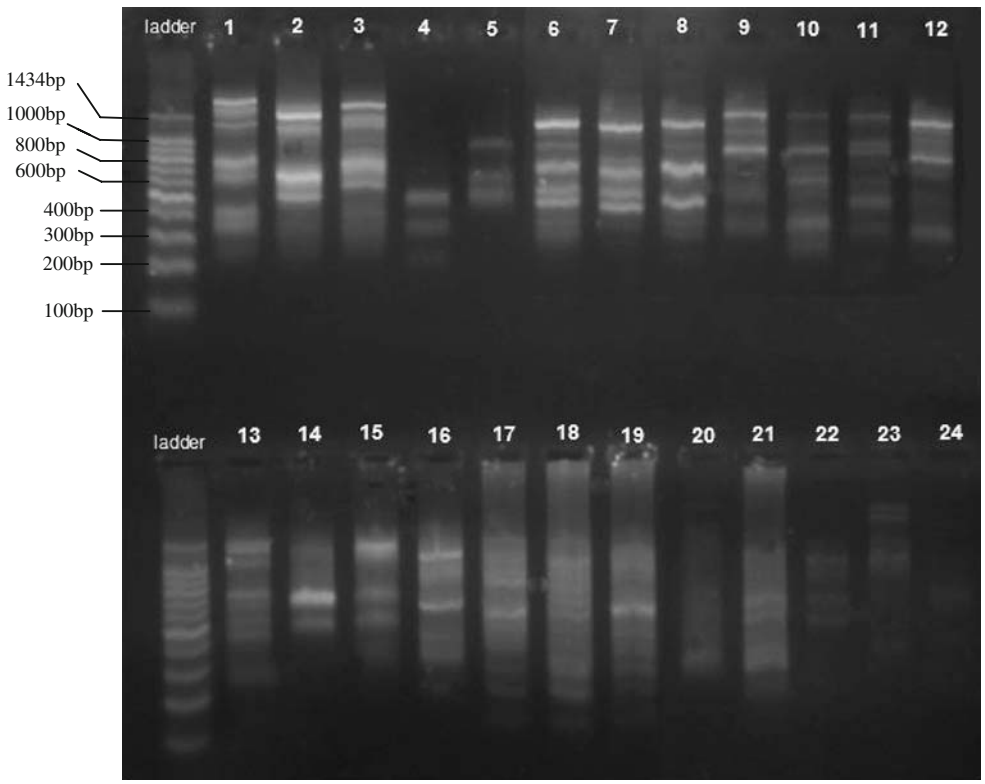

Fig. 2 ISSR fingerprint of nine *Polygala* species obtained with primer $(AG)_8A$: *1*, *2*, *3 P. cyparissias*; *4*, *5 P. aspalatha*; *6*, *7*, *8 P. brasiliensis*; *9*, *10*, *11*, *12 P. pulchella*; *13*, *14*, *15 P. linoides*; *16*, *17*, *18 Polygala densiracemosa*; *19 P. campestris*; *20*, *21 P. riograndensis*; *22*, *23*, *24 P. extraaxillaris*

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