



# Genetic Diversity and Chemical Profile of *Rhodophiala bifida* Populations from Brazil

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## Abstract

*Rhodophiala bifida* (Herb.) Traub, Amaryllidaceae, is a species native to South America known to produce alkaloids with potential pharmacological uses such as montanine, which has anti-inflammatory potential. *R. bifida* could be applied as a natural source of montanine. It is important to understand the genetic diversity of this species in order to assess the sustainable use of this plant. The aim of this study was to evaluate the genetic diversity and chemical profiles of the two known natural populations of *R. bifida* in Brazil. This report is the first population genetic study of *R. bifida*. We studied 93 individuals with six Inter-Simple Sequence Repeats (ISSR) primers. A total of 79 loci were amplified. Our results showed high-population structure ( $F_{st} = 0.16$ ), with greatest genetic variation at the intrapopulation level. Genetic analyses separated the individuals of *R. bifida* into two clusters that corresponded to each of the natural populations. Chemical profile evaluation was carried out on dried bulbs, leaves, flowers, and flower scapes by liquid chromatography and mass spectrometry. Montanine and nangustine were the main metabolites identified in both populations. These alkaloids concentrations differed by population and by plant part.

**Keywords** Amaryllidaceae alkaloids · Chemical profile · Genetic diversity · ISSR markers · Montanine

## Introduction

*Rhodophiala* C. Presl, Amaryllidaceae, is a plant genus that phylogenetically belongs to the tribe Hippeastreae, subtribe Hippeastrinae (Meerow et al. 2000). *Rhodophiala* plants have

a tunicate bulb 4–6 cm in diameter, which is set 20–30 cm underground (Olate and Bridgen 2005). The species of this genus are perennial geophytes with linear leaves less than 1 cm wide and one to seven zygomorphic funnelform flowers arranged in a scapose pseudo-umbellate inflorescence, with thin pedicels and short-tubed perianth (Meerow and Snijman 1998). Furthermore, these plants present ornamental potential because of their showy flowers (Muñoz et al. 2006).

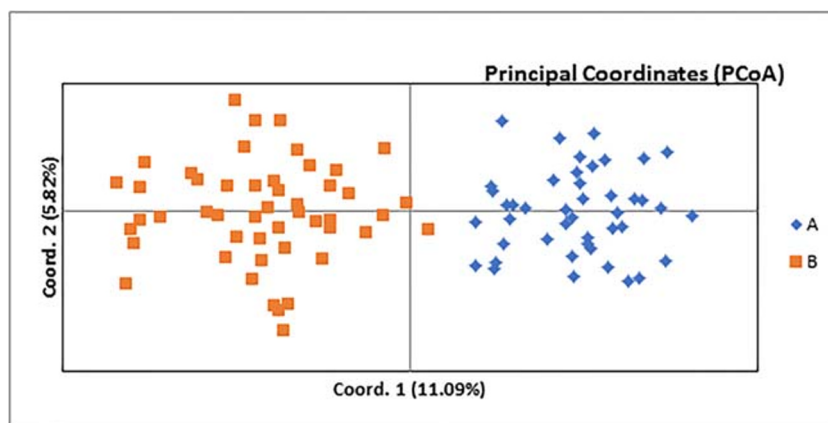
The species *Rhodophiala bifida* (Herb.) Traub, Amaryllidaceae, is distributed from Northeast Argentina to Uruguay and Southern Brazil (Olate and Bridgen 2005). In Brazil, only two populations are known, which are in Rio Grande do Sul, the southernmost state of the country (Reflora 2019). This species is known to produce the alkaloid montanine (Wildmann and Brown 1968), a compound with important biological effects such as antimicrobial activity (Castilhos et al. 2007); tumor growth inhibition (Silva et al. 2008); antidepressant, anxiolytic, and anticonvulsant properties; (Silva et al. 2006) and acetylcholinesterase enzyme inhibition (Pagliosa et al. 2010). In the past few years, our research group revealed the biological potential of montanine in autoimmune diseases, such as rheumatoid arthritis (Farinson et al. 2017). In 2015, we obtained a licensed patent regarding the

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**Fig. 1** Principal coordinate analysis (PCoA) made from ISSR data for POP1 and POP2 from *Rhodophiala bifida*



anti-inflammatory potential of montanine and its extraction process from *R. bifida* (De Oliveira et al. 2015). Recently, we also initiated the molecular characterization of two *R. bifida* genes involved in Amaryllidaceae alkaloid biosynthesis and provided new tools to improve montanine production in this species (Reis et al. 2019).

Taking into consideration both the ornamental and medicinal potential of *R. bifida*, the aim of this work was to evaluate the genetic diversity and chemical profile of this species in order to characterize the genetic and chemical profiles of the two known populations of the species in Brazil.

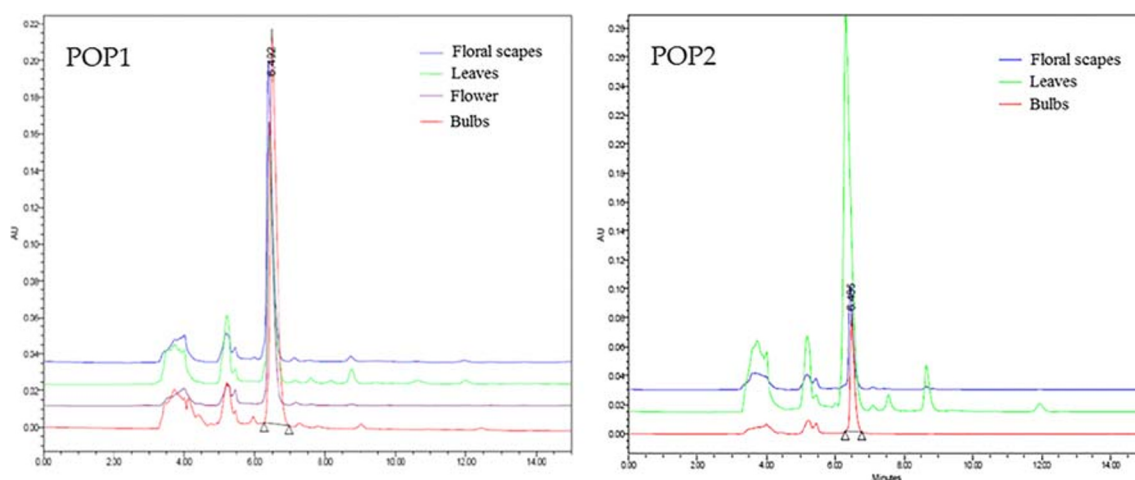
## Materials and methods

Samples of entire plants of *Rhodophiala bifida* (Herb.) Traub, Amaryllidaceae, were collected in Pelotas, Brazil, and each plant was manually separated into bulbs, leaves, and floral scapes. Voucher specimens of complete individuals from each population (POP1 and POP2) were

deposited at ICN Herbarium (Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil), with voucher numbers 192333 (POP1) and 184676 (POP2).

A total of 93 individuals were collected: 44 from POP1 and 49 from POP2. Floral scapes were fast-dried in silica gel for DNA isolation. Approximately 50 mg of dried floral scape samples was homogenized using TissueLyser (Qiagen) for 3 min at 3000 rpm. Total genomic DNA extraction was performed following the CTAB (cetyl hexadecyl trimethyl ammonium bromide) method according to Doyle and Doyle (1987), adapted to microcentrifuge tubes. DNA extraction details are described in the Supplementary Material. Six ISSR primers were selected for amplification (see Table S1 in Supplementary Material). Statistical analyses are described in Supplementary Material.

In order to obtain the chemical profile of these samples, all parts of the plant were dried at room temperature and reduced to powder with a knife mill. The alkaloid extracts of these samples were obtained according to the method proposed by Georgieva and co-authors (2007)



**Fig. 2** Chromatogram superposition profile of different organs extracts obtained from POP1 and POP2 by UPLC-DAD at 254 nm. Montanine was identified at 6.492 and 6.495 min in POP1 and POP2, respectively

with modifications. The alkaloid extraction process is reported in the Supplementary Material.

The extracts were evaluated by high-performance liquid chromatography (HPLC), Waters Alliance 2695 system, equipped with a diode array detector (DAD), Waters 996. The chromatographic conditions are given in the Supplementary Material. The main alkaloids present in these samples were collected from the HPLC system. The isolated alkaloids were analyzed by Ultra-Performance Liquid Chromatography (UPLC) equipped with a DAD coupled with electrospray ionization (ESI) quadrupole time-of-flight (Q-Tof) mass spectrometry (MS), UPLC-ESI-QTOF-MS (Waters Acquity, USA), operating in positive mode and using 4 kV as impact energy.

## Results and discussion

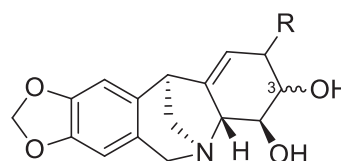
Amplified ISSR fragments generated an average of 13.1 bands per primer, and the number of amplified bands per primer ranged from six (SP3) to 19 (F13). Fragment sizes varied between 500 and 2080 bp. All six primers resulted in a total of 79 easily distinguishable fragments, for which the percentage of polymorphic loci ranged from 80 to 100%, with a polymorphism mean of 93.3% (see Table S1 in Supplementary Material). Other genetic studies in the family are scarce but in general reported a lower mean percentage of polymorphism assessed by ISSR 72.5% in *Phycella australis* Ravenna, assessed by ISSR (Flores et al. 2013), and 84.7% in *Narcissus* spp. (Jiménez et al. 2009).

We found significant genetic variation ( $p < 0.001$ ) between populations based on Analysis of Molecular Variance (AMOVA). The majority of the total variation (84.01%) was found between individuals within the same population, and only 15.99% of variation was found between populations. This result is consistent with other studies in Amaryllidaceae (e.g., *Phycella australis*, with 83% intrapopulation variation, Flores et al. 2013). Population structure (Fst) in *R. bifida* was 0.16 (see Table S2 in Supplementary Material), indicating a high-population structure despite the relatively short geographic distance between the populations (1 km), which were located at 31° 40' 48.5" S, 52° 26' 44.6" W (POP1) and 31° 40' 33.4" S, 52° 27' 1.3" W (POP2). This result shows that gene flow between these populations is relatively low and that gamete exchange preferably occurs between individuals from the same population. The population structure (Fst) of *R. bifida* is higher than that of other Amaryllidaceae species like *Lycoris longituba*, with a value of 0.12 (Deng et al. 2006), but it is slightly lower than that of *Phycella australis* (Fst = 0.17) (Georgieva et al. 2007).

Genetic diversity was slightly higher at POP2 in terms of number of bands, private bands, allele number, and Shannon's information index (see Table S3 in Supplementary Material).

The principal coordinate analysis (PCoA) showed individuals clustered into two different groups that corresponded to the two populations collected (Fig. 1). The first principal component explains 11% of the observed genetic variation, whereas the second principal component explains almost 6% of the variation (Fig. 1). Based on the Bayesian analysis provided by STRUCTURE, the most likely number of genetic clusters is  $k = 2$  (see Fig. S1A in the Supplementary Material). These two groups agree for the most part across the two collected populations (see Fig. S1B in the Supplementary Material or Fig. 2). Genetic variation values found in *R. bifida* suggest that this species uses cross-pollination as its breeding system. Although most of the individuals were assigned to a group corresponding to each geographic population, there were a few individuals, especially in POP2, that were assigned to the other population. This result suggests past and/or current gene flow between the populations, which can be explained by the proximity of the collection sites.

The chemical profiles of the POP1 and POP2 samples were evaluated by HPLC-DAD and UPLC-QTOF-ESI-MS (Fig. 2). Nangustine (1) and montanine (2) were the major peaks observed in all chromatograms. Both peaks were isolated and analyzed by mass spectrometry (see Figs. S2 and S3 in the Supplementary Material).



1 3 $\alpha$ -OH; R=H  
2 3 $\beta$ -OH; R=OCH<sub>3</sub>

Compound 1 showed a molecular ion at  $m/z$  [M + H]<sup>+</sup> 288.1210 (calculated for C<sub>16</sub>H<sub>18</sub>NO<sub>4</sub> 288.1199) and fragments  $m/z$  270, 241, 223, and 185, characteristic of nangustine alkaloid (Kokas et al. 2008). Compound 2 showed a molecular ion at  $m/z$  [M + H]<sup>+</sup> 302.1434 (calculated for C<sub>17</sub>H<sub>20</sub>NO<sub>4</sub> 302.1348) and fragments  $m/z$  270, 252, 223, 185, 174, and 165, characteristic of montanine alkaloid (Wildmann and Brown 1968; Castilhos et al.

**Table 1** Peak area averages of montanine obtained in POP1 and POP2 by UPLC-DAD, 254 nm

	POP1	POP2
Floral scapes	1817917	786275
Leaves	1556238	3857751
Flower	1365727	–
Bulbs	2389455	740847
Total	7129337	5384873

2007). Compound 2 presented a heterogenic distribution between the plant organs evaluated. According to the information described in Table 1, POP1 presented the highest concentration of compound 2 in bulbs (34%), followed by floral scapes (25%), leaves (22%), and flowers (19%), while in POP2, the majority of this alkaloid was detected in leaves (72%), followed by bulbs (14%) and floral scapes (14%). The quantity found on POP2 flowers was insufficient for extract preparation or an alkaloid determination profile. The high amount of compound 2 found in these extracts is consistent with preliminary investigations carried out by our research group on this species (Castilhos et al. 2007). The presence of this alkaloid, which presents anti-inflammatory activities regarding which pharmaceutical companies have an interest, suggests that *R. bifida* could play an important role in the development of new drugs, increasing its potential market.

Montanine-type alkaloids also were detected in four different *Rhodophiala* species collected in Chile: *R. araucana* (Phil.) Traub, *R. montana* (Phil.) Traub, *R. pratensis* (Poepp.) Traub, and *R. splendens* (Renj.) Traub. Among these, the highest level of montanine alkaloid was found in *R. pratensis* extracts (Tallini et al. 2018). A recent publication also described the detection of montanine-type alkaloids in *R. pratensis* (Poepp.) Traub, *R. bagnoldii* (Herb.) Traub, and *R. volckmannii* Phil. (Lizama-Bizama et al. 2018). The results obtained in this work and the information described in the literature corroborate the finding that montanine-type alkaloids could be characteristic of the *Rhodophiala* genus. Taking these results into consideration, *R. bifida* could be an important source of bioactive compounds, including montanine. Our results could become useful for conservation strategies for the sustainable commercial use of this species.

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**Authors' Contributions** JASZ and TTS-C conceived of and designed the experiments; MBS performed the experiments and analyzed the data; MS, JB, LRT, and NHO analyzed the data and wrote the paper.

## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflicts of interest.

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