

Development of SSR markers in *Valeriana clarionifolia* Phil. and their transferability to other *Valeriana* spp

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Research Article

Keywords: Medicinal plants, SSR, Transferability, Valeriana

Posted Date: November 8th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-3552969/v1>

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Additional Declarations: No competing interests reported.

Abstract

Background:

Several species from valeriana genus are valuable for their medicinal properties. Due to their economic potential, natural populations are threatened by an indiscriminate harvesting. A profitable plan for conservation and rational use must start with accurate identification and genetic diversity evaluation and tools for these purposes need development for valerian species.

In this study, we designed Simple Sequence Repeats (SSR) markers based on transcriptome data for *Valeriana clarionifolia* Phil. We investigated the polymorphism of these markers and the transferability to five Valeriana species, and we check the applicability in diversity analysis.

Methods and Results:

We designed 691 markers successfully from 2216 putative simple sequence repeat loci. We isolated 11 SSR markers from 60 selected marker loci and tested their polymorphism in *V. carnos*a Sm., *V. clarionifolia* Phil, *V. gamainensis* N. Nagh., *V. moyanoi* Speg, *V. philippiana* Briq. and *V. lapatifolia* Vahl. The number of alleles per locus ranged from two to eight and the Polymorphism Information Content varied between 0.29 and 0.77. The loci analysed were successfully cross-amplified in the five congeneric species.

Conclusions:

This is the first genomic study in native valerians from Patagonia that can be extended to others worldwide *Valeriana* species. These tools contribute to the identification, conservation, and material selection. In this way, we can attend a severe problem of sustainability production, environmentally respectful to and product quality. Native medicinal plants could turn out in innovative crops for agriculture diversification and a promising alternative for small regional economies.

1. INTRODUCTION

The genus *Valeriana* L (Caprifoliaceae) comprises ca. 250 species distributed in the temperate northern hemisphere, Africa, and South America, except Australia and New Zealand, being South America an important center of secondary diversification^{[1][2]}.

The underground organ of certain *Valeriana* species contains numerous compounds consumed worldwide for its sedative and relaxing properties, allowing the reduction of nervousness, stress-associated agitation, and antidepressant-like activity^[3]. This genus has been widely studied, with an special focus on its anxiolytic properties^[4].

In Argentina it is represented by approximately 50 species, of which 21 are distributed in Patagonia region^{[5][2][6]}. The Andean Region of Patagonia has a rich diversity of *Valeriana*, and Patagonian indigenous people and rural societies extensively used these plants since ancient times for healing various human ailments. Studies suggest that the secondary metabolites of *V. carnos*a Sm. are mainly valeriopatriates, flavonoids, phenolic acids, essential oils, among others^{[7][8][9][10]}. *Valeriana clarionifolia* Phil., known as “steppe ñankulawen”, also recognized as an ethno medicinal plant in Argentinean Patagonia with the same applications as *V. carnos*a Sm.^[11].

Due to their economic potential, efforts on the development of an efficient methodology for vegetative multiplication and *in situ* cultivation are under way^[12] to prevent the indiscriminate harvesting from natural populations. A profitable plan for conservation and rational use must start with the evaluation of the population diversity, so that tools to evaluate the available diversity and to assure the identity of the selected materials are necessary. The evaluation of traceability will support a novel sustainable profitable development in Argentinean Patagonia and molecular markers are good tools to reach this purpose.

The use of molecular markers has begun to take importance in studies of diversity and identification in medicinal species^[13]. Existing papers use universal markers, such as AFLPs (Amplified Fragment Length Polymorphism)^[14], RAPDs (Random Amplified Polymorphic DNA)^[15], inter-gene spacers (ITS1 (Internal Transcribed Spacer)^[16] or ribosomal genes^[17], mainly for phylogenetic

studies. An important application of molecular techniques is the authentication of medicinal plants^[18]. The essential role of characterization and documentation of plant with pharmaceutical value is well-documented.^[19]

The development of EST (Expressed Sequence Tag) based SSR markers and the study of cross-species transferability to other medicinal plants was made in other species like *Catharanthus roseus* (L.) G. Don^[20]. Transcriptomes of 75 non-model plants that produce compounds of interest for biotechnological applications were analysed using a data-mining approach.^[21]

In the genus *Valeriana*, there are precedents of the use of AFLPs and cpSSR (Chloroplast simple sequence repeats). In *Valeriana wallrothii* Kreyer,^[22] used them to estimate the risk of genetic erosion in response to anthropic pressure and for diversity evaluation. Based on a cladistic analysis, sequences of one plastid (*matK*) and one nuclear (ITS) molecular markers were used for phylogeny and generic delineation of the Valerianaceae family^[23]^[24]. The genetic diversity of *V. jatamansi* Jones have been evaluated using ISSR (Inter simple sequence repeats), RAPD and AFLP markers. Diversity of natural populations of *V. jatamansi* from Himalayal Pradesh was evaluated with AFLPs^[25]. Other authors analysed the genetic diversity of *V. jatamansi* with SSR markers together with other characterizations to identify promising germplasm sources and to develop conservation and management strategies^[26].

Lately, genetic diversity across habitat types and an altitudinal ranges were evaluated with nuclear (nuSSR) and chloroplast (*cpSSR*) SSR^[27]. Diversity Array Technology sequencing (DArT-seq) technique was used to analyse Polish accessions that originated from wild populations and cultivars of *V. officinalis* L.^[28].

Transcriptome analysis based on next-generation sequencing was done in *V. officinalis*^[21] and *V. fauriei* Briq.^[29], on New Word species, especially which grow in Patagonian Region Because of the importance of local species of *Valeriana*, we decided to develop SSRs from transcriptomic data, for they use in population characterization and germplasm identification, which provides robust and complementary tools to biochemical and morphological descriptors. Once the SSRs were developed, we tested cross transferability to other phylogenetically close valerians from Patagonia.

2. MATERIALS AND METHODS

2.1. Plant material

The material for RNA isolation was obtained from healthy plants of a wild population of *V. clarionifolia* located in northwest Chubut province, Argentina (-42.89 S and - 71.31 W), at 808 m a.s.l.

Transferability of the developed markers was studied by cross-species amplification of five Patagonian *Valeriana* species: *V. carnosa*, *V. gaimanensis* N. Nagh, *V. moyanoi* Speg., *V. philippiana* Briq. and *V. lapatifolia* Vahl.

For genetic diversity evaluation, we used DNA from eight individuals of *V. carnosa* from seven origins: Epulauquen (36°50' 40" S 71° 01' 43" W, Neuquén), Piltriquitrón (41°59'00" S 71°27'00" W, Rio Negro), Talagapa (42°11'41" S 68°20'29" W, Chubut), El Tero (49°42'0" S, 68°16'0" W, Chubut), Perito Moreno (46°36'0" S 70°55'60" W, Santa Cruz), Puerto Deseado (47° 44' 53" S 65° 53' 36" W, Santa Cruz) and Las Violetas (52°51'29" S 69°27'17" W, Tierra del Fuego, Antártida e Islas del Atlántico Sur). This sampling covers most of the natural distribution area of this species in the Argentinean Patagonia (Fig. 1).

2.2. RNA extraction

RNA was extracted from a pool of root tissue from four plants of the wild population of *V. clarinifolia*. Extraction was performed using TRIzol® Reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the protocol recommended by the manufacturer.

2.3. Genomic DNA extraction

Genomic DNA was isolated from leaves of plants using the CTAB method with slight modifications (Nagahama pers. comm.). The DNA concentration was quantified using Nanodrop® and quality was checked by 0.8% agarose gel electrophoresis with UV trans illumination.

2.4. Generation and assembly of transcript reads

The construction of the library and sequencing was carried out through the Genomics and Bioinformatics Platform of the Rosario Agro-biotechnology Institute (INDEAR, <http://webservices.indear.com/>) using the 454 Technology (Roche). The Newbler v2.6 assemblers (Roche™) was used for transcriptome assembly. The data is deposited in NCBI Sequence Read Archive BioProject ID PRJNA950387.

2.5. Detection of SSR-containing sequences

SSR containing sequences were identified by a Perl script based program, MISA software (MicroSatellite identification tool <http://misaweb.ipk-gatersleben.de/misa/>^[30]).

The possible functions of the unigenes were predicted using the Blast2Go program (<http://www.blast2go.de/>; ^[31]). The Evidence Code (EC) and Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.genome.jp/>) annotations were made. Sequence similarity was determined using the BLASTX method (translated query) against Genbank's (nr) protein database, and the results of the BLASTX similarity analysis were used to obtain Gene Ontology (GO, <http://www.geneontology.org>) annotations.

2.6. Primer design and amplification conditions

We selected SSRs with simple motives and used Primer3 ^[32] software for primer design, on their flanking sequences. Amplification reactions were carried out in a 20 µl reaction mixture. Each reaction contained 10 µM of each primer, 1.5 mM MgCl₂, 0.200 µM dNTPs, 0.5U Taq polymerase, and 30 ng of genomic DNA as template. Amplifications were performed with the following protocol: 94°C for 1 min, as an initial denaturing, followed by 35 cycles at 94°C for 30 s, annealing temperature (depending on the primer, between 50 and 61°C) for 30 s, 72°C for 1 min, and a final extension step at 72°C for 10 min.

PCR amplification products were separated on a 6% (w/v) polyacrylamide gel run in 0.5X/1X TBE at 50 W for 2–3 h (Life Technologies-Gibco BRL™ S2 Model) and visualized with silver staining (Silver Sequence Promega™ Biotech, Madison, WI). The size of each band was estimated simultaneously by means of a 10 bp (base pair) DNA Ladder.

2.7. Usefulness of SSRs marker for diversity analysis.

To assess the potential of the novel SSRs for genetic diversity analysis, we used DNA of eight individuals from seven wild populations of *V. carnososa* from their natural distribution area. This sampling covers most of the natural distribution area of this species in Argentinean Patagonia.

Diversity genetic parameters

Na: No. of Different Alleles

Ne: No. of Effective Alleles = $1 / \sum [p_i]^2$

I: Information Index = $-\sum p_i \ln p_i$

Ho: Observed Heterozygosity = No. of Hets / N

He: Expected Heterozygosity = $1 - \sum p_i^2$

uHe: Unbiased Expected Heterozygosity = $2N / ((2N-1)) * He$

F: Fixation Index = $He - Ho / He = 1 - (Ho / He)$

were calculated running the GenAlEx 6.5 program ^[33]. Also, we reported the Informativeness (I) which measures the amount of information that a marker provides for estimating relatedness or distinguish between two randomly selected individuals in a population.

$$I = - \sum_i^n p_i * \ln(p_i)$$

The Polymorphism Information Content (PIC) was estimated using the formula of [34]. $PIC = 1 - \sum_i^n p_i^2$

Where p_i is the frequency of the i^{th} allele for the population.

Allelic frequencies per population were used to construct the genetic distance matrix according to Prevosti formula [35]. The PCoA approach based on a genetic distance matrix is shown in Fig. 2.

3. RESULTS

3.1. SSR development

Using MisaSSR software [30], we detected 2216 SSRs, considering 864 in contigs, 317 in singletons and 1035 in isotigs (Table 1). The vast majority contained one SSR (1,994 sequences) and only 185 sequences contained more than one SSR motif motives distributed as 20,1% Di-, 70,8% Tri-, 3,5% Tetra-, 1,6% Penta- y 4,0% Hexa-nucleotides). Trinucleotide motive was the most frequent SSRs. This fact coincides with similar results found for eucots, monocots, other higher plants, and lower plants [36].

Table 1
Amount of sequences examined and SSR

	contigs	singletons	isotigs	total
Total number of sequences examined:	24,207	13,813	20,929	58,949
Total size of examined sequences (bp):	14,415,506	4,534,388	17,705,404	36,655,298
Total number of identified SSRs:	864	317	1,035	2,216
Number of SSR containing sequences:	778	290	926	1,994
Number of sequences containing more than 1 SSR:	72	22	91	185
Number of SSRs present in compound formation:	50	17	56	123

Only 123 SSR showed composite motives. Di- tri- and tetra-nucleotides SSR presented predominant motives: AT, TCA and TATG, respectively. Penta- and hexa-nucleotides type present more than one private motif.

We designed 691 primers from 778 containing SSR contigs and randomly selected 60 sequences. The sequences and characteristics of selected primers are shown in Table S1 Supplementary. Annotation data, blast results, top hit and similarity mean percentage are shown in table S2 Supplementary.

3.2. Transferability of the microsatellite markers

Based on the clear pattern of bands and their polymorphism, we selected 12 SSR markers among the 60 primers screened. The selected primers were amplified satisfactory in five species of *Valeriana*. We obtained fragments within the range (in base pairs) of the expected size (Table 2).

Table 2

Cross-amplification of 12 SSR markers isolated from *V. clarionifolia* across five species of valeriana. (+) amplification, (-) no amplification

<i>Species</i>	valcla 19778	valcla 4800	valcla 10517	valcla 9765	Valcla 9287	valcla 9255	valcla 4356	valcla 4770	valcla 4879	valcla 7794	valcla 7841	valcla 4033
<i>V. clarionifolia</i>	+	+	+	+	+	+	+	+	+	+	+	+
<i>V. carnosa</i>	+	+	+	+	+	+	+	+	+	+	+	+
<i>V. gaimanensis</i>	+	+	+	+	+	+	+	+	+	+	+	+
<i>V. lapatifolia</i>	-	+	+	+	+	+	+	+	+	+	+	+
<i>V. moyanoi</i>	-	+	+	+	+	+	+	+	+	+	+	+
<i>V. philippiana</i>	-	+	-	-	+	+	+	+	+	+	+	+

3.3. Diversity analysis

We tested the selected SSR markers in seven natural populations of *V. carnosa*. PIC values range from 0.81 to 0.29, with an average of 0.56. Nine loci of the eleven tested presented private alleles (private alleles defined as number of alleles unique to a single population) (Table 3).

Table 3. Polymorphism Information Content and Private alleles of the selected SSR

CHB: Chubut, NQ: Neuquén, RN: Rio Negro, SC: Santa Cruz, TF: Tierra del Fuego.

(+) presence (-) absence

Location			Pilquitron (RN)	El Tero (CHB)	Perito Moreno (SC)	Las Violetas (TF)	Talegapa (RN)	Epulauquen (NQ)	Puerto Deseado (SC)
SSR locus	PIC	# private alleles							
valcla10517	0.47	2	+	-	-	+	-	-	-
valcla9765	0.29	1	-	-	-	-	+	-	-
valcla9287	0.63	1	-	+	-	-	-	-	-
valcla9255	0.77	3	-	-	+	+	-	-	-
valcla4356	0.44	2	-	-	+	-	-	-	-
valcla4770	0.42	2	-	-	-	-	+	-	-
valcla19866	0.76	1	-	-	+	-	-	-	-
valcla4879	0.65	0	-	-	-	-	-	-	-
valcla7794	0.36	1	+	-	-	-	-	-	-
valcla7841	0.40	0	-	-	-	-	-	-	-
valcla4033	0.51	1	-	-	+	-	-	-	-

We identified 46 allelic variants across 11 loci ranging from (valcla9255) to 2 (valcla7841) with an average of 4 alleles per locus. The average of the effective alleles was 2.8.

The average information index (*I*) was 0.96. As the value of *I* gets closer to 1 a higher level of diversity is detected. This index is useful to choose the most informative SSR loci for their use in further genetic studies. All SSR loci were polymorphic showing variability across loci with Heterozygosity (*H*) values which ranged from 0.26 (valcla9765) to 0.77 (valcla9255). Only two loci (valcla9287 and valcla4879) present an excess of homozygotes (inbreeding; Table 4).

Table 4
Number of different alleles (*N_a*), Number of Effective Alleles (*N_e*), Information Index (*I*), Observed Heterozygosity (*H_o*), Expected Heterozygosity (*H_e*), Unbiased Expected Heterozygosity (*uH_e*), Fixation Index (*F*) of the 11 SSR selected for diversity analysis.

SSR ID	N	<i>N_a</i>	<i>N_e</i>	<i>I</i>	<i>H_o</i>	<i>H_e</i>	<i>uH_e</i>	<i>F</i>
valcla10517	56	4	1.90	0.85	0.00	0.47	0.48	1.00
valcla9765	55	3	1.36	0.47	0.20	0.26	0.27	0.24
valcla9287	56	5	2.74	1.16	0.96	0.63	0.64	-0.52
valcla9255	55	8	4.34	1.67	0.38	0.77	0.78	0.50
valcla4356	52	5	1.80	0.79	0.21	0.44	0.45	0.52
valcla4770	50	4	1.73	0.81	0.00	0.42	0.43	1.00
valcla19866	49	7	4.10	1.58	0.31	0.76	0.76	0.60
valcla4879	52	3	2.86	1.07	0.75	0.65	0.66	-0.15
valcla7794	56	4	1.57	0.73	0.18	0.36	0.37	0.51
valcla7841	54	2	1.67	0.59	0.37	0.40	0.40	0.08
valcla4033	53	3	2.06	0.81	0.00	0.51	0.52	1.00
Mean	53.45	4.36	2.38	0.96	0.31	0.52	0.52	0.43
SE±	0.74	0.54	0.31	0.12	0.09	0.05	0.05	0.15

Finally, the chosen SSRs were effective to discriminate the seven population of *V. carnos* and to characterize their diversity (see Fig. 2).

4. DISCUSSION

This study in *V. clarionifolia* provides valuable tools and information, based on simple sequence repeats derived from the transcriptome, which could be extended to other valerians, and possibly more ones not included in this study.

The SSRs developed from transcriptomic libraries have a high probability to be transferred to other species of the genus, as they correspond to highly conserved regions. This feature enhances its application to species that remained molecularly unexplored.

Different levels of polymorphism present in different species can be attributed to mating system type, age of distributions, dispersal and adaptive strategies, domestication grade or natural population status. High variability can be due to the size of the data set, SSR search criteria, and mining tool used for the SSR search [37]. The proportion of transcriptome containing SSRs varies in different plant species. In an exhaustive analysis of microsatellite patterns, The distribution across eukaryotic genomes remarked the distinct patterns of microsatellite, presence and abundance in the different taxonomic groups that represent their establishment in a common ancient founding member of the group [38]. In a comprehensive analysis of SSR from 112 plant species from different plant phyla [36], the amount of SSR in Valeriana was similar founded in eucots, monocots and other higher plants (including, Gymnospermae, Gnetophyta, Bryophyta, Pteridophyta). The largest amount of SSR found in lower plants (Chlorophyta) authors attributed the need of this plants to adapt to early extreme environments.

Transferability of SSR technique has been successfully applied to agriculture crops and wild relatives. *Helianthus annuus* L. and rare sunflower species were compared using EST-derived SSRs [39]. In this last, found that these markers were more than 3 times as transferable across species as compared with anonymous SSRs (73% vs. 21%, respectively). Transferability of rice SSR was tested across the genera [40], including wheat, oat, pearl millet, soybean, sunflower and chickpea and high value for specific genomic studies like genetic diversity studies or QTL mapping and marker-trait association were proved. Microsatellite marker transferability have also been successful from *Prunus* across rosaceous crops almond, peach, apricot, Japanese plum, European plum, cherry, apple, pear, and strawberry [41].

In the genus *Valeriana*, expressed sequence tags (ESTs) and nuclear sequence information was used in *V. jatamansi* [27]. Those tools were integrated with other attributes to harbor maximum genetic variation within the species, to identify several populations that could be used as a promising stock for mass multiplication and can be informative for conservation purposes [42].

In our study, all SSR loci were also useful for diversity analysis. Genetic variability detected in the studied populations suggest a good chance of success on a larger scale. Even the microsatellite with lowest PIC value had private alleles which aids to discriminate populations.

The success of cross-species markers and the application in compared studies of ecology [43] were extensively reviewed. Both, the proportion of amplified and polymorphic values represent a measure of the success of the transferability. Even if they do not focus on differential mutation rates, homoplasy or evolution, the potential of cross-species transfer of these markers is high.

Since their discovery, microsatellites or SSR benefits as a molecular marker over other marker systems were recognized. SSR takes advantage of RFLP with its greater polymorphism rates, higher reproducibility compares with RADP by its high annealing temperatures and easier use over AFLP as a PCR based technique. Additionally, they can be automated, and genotype costs can be lowered combining a set of markers in a single reaction.

High costs because of laborious and time-consuming process restrained de novo development of SSR markers in the past, as point it as the main disadvantage [44]. Nowadays, the evolution of mass sequencing techniques and the availability of expression and genomic libraries databases overcome this problem. Thus, SSR appears more favourable and accessible to conduct genetic experiments in non-model plant species, as SNP requires sophisticated instrumentation for their detection and validation.

This is the first development of molecular markers based on transcriptomic data in native valerians from Patagonia. The development of genomic tools will allow us the authentication, selection, and the tracking in multiplication cycles to produce selected “top” clones. Also, will contribute to the selection and maintenance of specific genotypes/chemo types.

In this way, we can attend a severe problem of sustainability production, environmentally respectful to and product quality. Native medicinal plants could turn out in innovative crops for agriculture diversification and a promising alternative for regional economies. The use of these markers can extend to population genetic analysis and gene flow estimation of *Valeriana* and other related taxa.

Declarations

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

FUNDING

This research was financially supported by funding from the National Institute of Agricultural Technology (INTA) Argentina Project PNBIO-1131044: “Genomics applied to studies of molecular ecology and genetic diversity” National Program of Biotechnology and PNHFA-1106094: “Technological and commercial platforms, for cultivated and native aromatic and medicinal species” National Program of Vegetables, Flowers Aromatic and Medicinal species.

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The first draft of the manuscript was written by Manifesto Maria Marcela, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures



Figure 1

Geographical location of the populations of *Valeriana carnososa* assessed in the present study

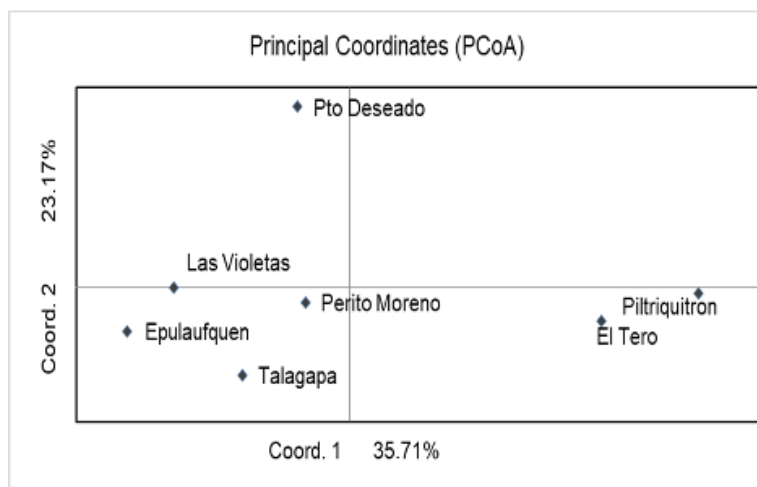


Figure 2

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