Journal of Biodiversity, Bioprospecting and Development

Verma et al., J Biodivers Biopros Dev 2016, 3:2
DOI: 10.4172/2376-0214.1000159

Research Article Open Access

Genetic Diversity Analysis among Accessions of *Desmodium gangeticum* (L) DL with Simple Sequence Repeat (SSR) and Internal Transcribed Spacer (ITS) Regions for Species Conservation

Ahmad Haji R F[1], Tiwari S[2], Gandhi S G[3], Kumar A[4], Brindavanam N B[4], Verma V[1*]

[1]*Department of Biotechnology, Shri Mata Vaishno Devi University, Sub-Post Office, Katra, Jammu and Kashmir, India*

[2]*Department of Biotechnology, School of Environmental Biology A.P.S University Rewa, Madhya Pradesh, India*

[3]*Plant Biotechnology Division, CSIR-Indian Institute of Integrative Medicine, Jammu and Kashmir, India*

[4]*Dabur Research and Development Centre, Dabur India Ltd, Ghaziabad, India*

[*]**Corresponding author:** Verma V, Department of Biotechnology, Shri Mata Vaishno Devi University, Sub-Post Office, Katra, Jammu and Kashmir, India, Tel: 91 1991285692; E-mail: v.verma@smvdu.ac.in

Rec date: March 12, 2016; **Acc date:** June 23, 2016; **Pub date:** July 1, 2016

Abstract

Desmodium gangeticum is one of the important species of ethno- medicinal plant. It finds its uses in numerous medicinal preparations including many industrial applications. Inspite of an important medicinal plant, till date little work have been conducted towards its molecular characterization and conservation. Therefore the present study was designed to bring out the relationships among different available accessions and study biodiversity conservation through simple sequence repeat (SSR) and inter transcribed spacer markers. The analysis revealed that intra-population mean genetic value (1.835) and Shannon (0.641) values vary among the accessions. However through AMOVA analysis, we observed that only a small variation exists among these accessions (*P*=1). The dendogram generated through distance matrix using the SIMQUAL-Dice Coefficient module of NTSYS pc ver matches to some extent with the PCA graph. In the barcode ITS marker analysis UPGMA shows the clustering of accessions into two groups. It is thus concluded that above based markers can be used for the population conservation and authentication of the above plant and could also be a lead for other medicinally important plants.

Keywords: *Desmodium gangeticum*; SSR; ITS; PCA; Dendogram

Abbreviations AFLP: Amplified Fragment Length Polymorphism; RFLP: Restriction Fragment Length Polymorphisms; SRAP: Sequence related Amplified Region; SNPs: Single Nucleotide Polymorphisms; EST: Expressed Sequence Tag ; AMOVA: Analysis of Molecular Variance; ISSR: Inter-simple Sequence Repeat; ITS: Internal Transcribed Spacer; PCoA: Principal Coordinate Analysis; PCR: Polymerase Chain Reaction; SAHN: Sequential Agglome rative Hierarchical Nested; SSR: Simple Sequence Repeat; UPGMA: Un-weighted Paired Group Method with Arithmetic Average; NCBI: National Center for Biotechnology Information; dNTPs: Deoxynucleotide Triphosphates

Introduction

Medicinal plants genetic resources play a vital role in the sustainable development of human therapeutic applications. The continuous and unchecked use of biodiversity resources however lead to severe constrain on the genetic diversity. Adequate and effective strategies thus needed to be developed towards conservation of these genetic resources. *Desmodium gangeticum* is a sub-tropical perennial herb, distributed throughout the warmer parts of India, tropics of Africa that grows with angular branches, simple leaves, and purplish flowers. The plant is of high medicinal value, with applications such as antioxidant, anti-inflammatory activities [1]. The roots were used for antileishmanial, immunomodulatory [2], curability in premature ejaculation [3] and also in treatment of ulcers [4]. Different types of alkaloids have been extracted from this plant i.e. gangetin, gangetinin, desmodin, and desmocarpin [5]. Phospholipids, sterols, and flavones glycosides have also been extracted [6-8] have various pharmaceutical applications. Being high in demand and extensive exploitation of this spp., stresses burdened on framing and adequate conservation strategy for the same. Otherwise the plant in future will be in the red list.

Molecular markers are used for understanding the basis of genetic diversity. Various researchers have used different markers for studies such as Random Amplified Polymorphic DNAs [9], Simple Sequence Repeats [10], Restriction fragment length polymorphisms RFLP [11], Amplified Fragment Length Polymorphisms AFLP [12-14], SRAP [15], Single Nucleotide Polymorphisms, SNPs, Simple sequence repeats (SSR) [16-19] etc. Simple sequence repeat markers are being used in genetic relatedness, diversity identification for the conservation and utilization of germplasm resources [20-23]. The specific characteristics of these markers include their simplicity, effectiveness, abundance, hypervariability, reproducibility, codominant inheritance, and extensive genomic coverage [24]. With the increase of EST sequences in the public database, a large number of available sequences provide opportunities to identify and validate usefulness of these microsatellites at a low cost and in an efficient manner.

There is another marker known as intern transcribed spacer (ITS) markers or it even called as barcode. In plant genomes the nuclear ribosomal RNA (rRNA) genes occur in thousands of copies and are organized in tandem arrays while the 5S rDNA locus encodes the 5S rRNA genes, the 45S rDNA locus contains genes for 18S, 5.8S and 26S rRNA, which are transcribed as a single unit and then spliced. The 18S, 5.8S and 26S rRNA genes are separated by internal transcribed spacers ITS1 and ITS2 or ITS4, and the 45S transcription units themselves are

separated from each other by non-transcribed intergenic spacers (IGS), extending from the 39 end of 26S rRNA to 59 end of the 18S rRNA coding regions [25]. The ITS marker has been used in genetic variation studies [26] in genera of compositae plants. In the recent times it has been used in different plants like Epimedium [27], Panax [28] and Asarum plant [29] etc. Seeking the importance of above markers in the diversity study the study was designed to assess of genetic variability and diversity among different accessions of *Desmodium gangeticum* using SSR and ITS. It is expected that the outcome will provide a genetic data and a theoretical basis for protection of the species which will contribute to monitoring of germplasm in future.

Materials and Methods

Plant material

In the present study, different accessions covering a wide range of the morphological variation were collected from different biodiversity regions of the India (Table 1). The collections were further maintained under in vitro and Green house conditions.

Plant code	Source	Date of Collection	Location
DG-1	Kukrail, Lucknow	07-04-2009	26°54'34.9"N80°59'01.7"E
DG-2	Rishikesh, Uttarakhand	07-03-2009	30°05'14.3"N78°15'57.5"E
DG-3	Rajpipla, Gujrat	31-08-2009	21°52'17.9"N73°30'10.2"E
DG-4	Umarpura, Madhya pradesh	30-08-2009	22°23'00.7"N75°25'36.1"E

Table 1: *Desmodium gangeticum* accessions collected from different geographical locations

DNA extraction

DNA was extracted with Plant Mini Kit (Qigen). Quantity and quality was assessed by electrophoresis in (0.8%) agarose gel stained with ethidium bromide (Amershco) using 0.5x TBE electrophoresis buffer at (95 V for 40 min). Quantitative estimates of sample DNA were made by nanodrop method (Thermo scientific).

Primer sequence

SSR primer sequences were available from the information available in NCBI. The sequences of SSR primers were selected on the basis of their proper properties of their broad coverage of the genome and their high levels of polymorphism. The inter transcribed spacer primers were chosen according to [26] to amplify the concerned region of the ribosomal DNA, which encompasses the 5.8S gene and both ITS-1 and ITS-4 regions. The primers were synthesised from Integrated DNA Technologies (USA).

SSR amplification

The PCR reaction mix up contained (30-45 ng) DNA, 1 unit Taq DNA polymerase (New England biolabs), 2Mm of dNTPs mix (NEB), 10 pmol primers and 10X buffer containing $MgCl_2$ (NEB) in a total mix volume of 10 µl reaction with following cycling conditions; 94°C for 5 min, 32 cycles of 93°C for 1 min, 50°C for 1 min, 72°C for 1 min, and a final extension temperature of 72°C for 5 min. The amplified products were separated on 1.8% (w/v) agarose gel at a constant voltage of 80v for 2 hours and were visualised in Bio-rad gel doc system

Internal transcribed spacer assay

The entire ITS region, comprising ITS1, 5.8S, and ITS4, was amplified from genomic DNA of all the accessions with primers ITS1 (5/ TCCGTAGGTGAACCTGCGG 3/) and ITS4 (5/ TCCTCCGCTTATTGATATGC 3/) in a 100 µl reaction in 10 times the reaction mix up used in SSR analysis. PCRs was performed with a Eppendorf PCR System using the conditions of 5 min at 95°C, followed by 32 cycles of 30 s at 94°C, 50 s at 55°C, and 1 min at 72°C, with a final extension step for 5 min at 72°C. PCR products were electrophoresed in 1.5% and purified with the Gel Extraction Kit (QIAGEN) according to the manufacturer's manual. The purified products were sequenced at http://www.scigenom.com/. Searches for similar sequences were done using the BLASTN and X algorithm available in the National Centre for Biotechnology Information (NCBI, http: //www.ncbi.nlm. nih.gov/BLAST/index.htm).

Data analysis

Simple Sequence Repeat (SSR): Most of the SSRs showed multiple bands which indicated that for a given SSR more than one allele was observed, which may reflect heterozygosity and/or segregation at the respective SSR marker. All the genotypes were scored for the presence and absence of the SSR bands. Some SSR were repeated twice and only the reproducible bands were scored and the data were entered into a binary matrix as discrete variables, 1 for presence and 0 for absence of the character and this data matrix was subjected to further analysis and imported in the form of excel file. POPGENE version 32 [30] was also used to estimate Gene diversity and Shannon's information index (I) for co-dominant data markers. Principal Coordinates Analysis (PCoA) was performed with the software NTSYS version 2.2 [31]. The allelic SSR matrix was used for the analysis of molecular variance (AMOVA) using GenAlEx 6.41 [32]. AMOVA was used to estimate the partition of the genetic variation within and among accessions. The significance of the variance components was determined with a permutation test (999 replicates). SAHN module plot of NTSYS version 2.2 [32] was used for plotting the dendrogram to observe genetic relatedness and the robustness of the tree was tested using bootstrap analysis (1000 iterations).

Intertranscribed spacer: BLAST (NCBI's web-based algorithm) was used to identify the query sequences. Each identification was made manually, taking E-value and maximum identity. Evolutionary analyses were conducted through MEGA6 [33].

Results and Discussions

There is growing concern about diminishing populations, loss of genetic diversity among medicinal plants. To some extinct DNA barcoding have been extensively used for species identification, diversity Barcode phylogeneny has been validated for conservation and identification purposes such as in Panax [28], Asarum plant [29] Epimedium [27] Gesneriaceae [34-37]. SSR based marker analysis used in the present study revealed small genetic divergence between different accessions. The 10 microsatellite primers (Table 2) were used in *D. gangeticum* and a total of 46 polymorphic bands sizes were generated.

S.no.	Marker	Position	Sequence (5′→3′)
1	SBT/2013/01	Forward	AGCAGGAGTACCCATGAAAGTCC
		Reverse	TATCACAGCACGAAGCGATAGATG
2	SBT/2013/02	Forward	CACAACTCCATCAGAGGACAGAGA
		Reverse	CTGCTACGACATACGCCAGGC
3	SBT/2013/03	Forward	CCGAAGATAACCAAACAATAATAGTAGG
		Reverse	ACTGTACGCCTCCCCTTCTC
4	SBT/2013/04	Forward	GCTCTATGTTATTCTTCAATCGGGC
		Reverse	GGTCGGTCGGTACTCTGCTCTA
5	SBT/2013/05	Forward	TGCCACCACAGCTTTCTCCTC
		Reverse	TATGAGAGAAGCGGTTGGCACG
6	SBT/2013/06	Forward	GGGAGGGTAGGGAAGCAGTG
		Reverse	GCGAACCACGTTCATGAATGA
7	SBT/2013/07	Forward	TTTACGCACCGCAGCACCAC
		Reverse	TGGACTCATAGAGGCGCAGAAAG
8	SBT/2013/08	Forward	ACCTAGAGCCTAATCCTTCTGCGT
		Reverse	GAATGTGAATATCAGAAAGCAAATGG
9	SBT/2013/09	Forward	GGGTAGTAAAGGAAAGAGAAGAAAGAG
		Reverse	CCACCTTCTCGTACTGTTCCATG
10	SBT/2013/10	Forward	GATGGACACCCTTCAATTTATGGT
		Reverse	TCCAAGTAT CAG GCACACCAG C

Table 2: Microsatellite Primers used to assess genetic diversity in *D. gangeticum*.

Some primes gave distinct polymorphic products were highlighted in bold in Table 2 and rest of markers could not be amplified or produce faint bands were observed. One reason for the lack of amplification could be rapid genome modification. Microsatellite fragments ranged from 150 bp to 750 bp in size (Figure 1).

Examination of intra-population mean genetic diversity revealed the value to be 1.8357 and mean Shannon to be 0.6411, (Table 3), Principal coordinates analysis (PCoA) and UPGMA cluster analysis (Figures 2 and 3) was also performed to display the spatial allocation and to examine relationships between different accessions in populations thus helps us in understanding the relationships between them. Based on AMOVA analysis, we did not noticed any variation among *Desmodium gangeticum* accessions, indicating statistically non-significant genetic differentiations among populations (P = 1), which may perhaps also be due to small number of sample size. Here we can also state that the marker SSR, used in the present study displayed a relatively weak genetic diversity among *Desmodium gangeticum* populations. Variability turned out to be in the same range in between the accessions which is probably the result of obligatory cross-pollination in this dioecious species. The genetic fragmentation of this species despite of its fragmented and isolated habitats did not happened yet.

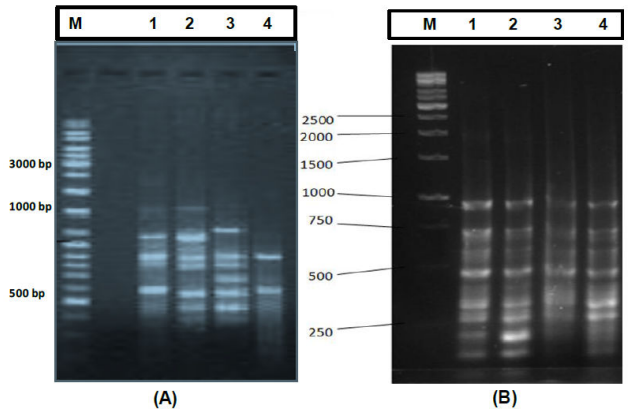

Figure 1: Gel pictures showing the genetic relatedness among *D. gangeticum* accessions using primers (A) SBT/2013/ 508 and (B) SBT/2013/510. M stands for marker and 1, 2, 3, 4 represent *D. gangeticum* accessions.

Marker	Gene Diversity	Shanon index (I*)
DG1	1.9737	0.6865
DG2	1.8648	0.6565
DG3	1.5515	0.5402
DG4	1.9538	0.6813
Mean	1.8357	0.6411

Table 3: Nei's Gene diversity and Shanon Index of SSR.

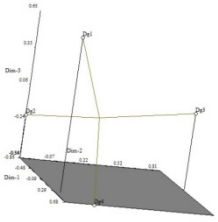

Figure 2: Three dimensional principal coordinates analysis (PCoA) of 4 accessions of *D. gangeticum*.

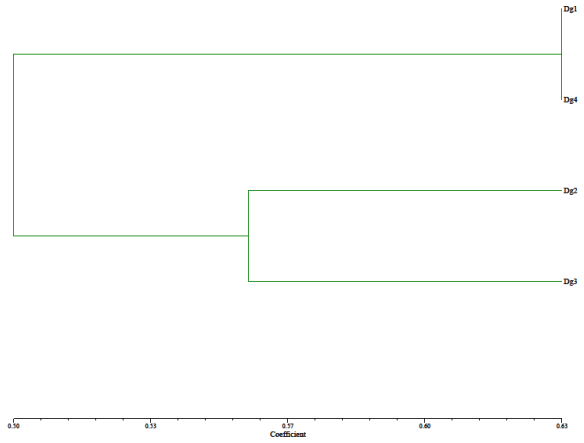

Figure 3: UPGMA dendogram analysis of accessions of *D. gangeticum* - Dg1, Dg2 and Dg3, Dg4 are clustered. This shows they are geographically less diverse.

In our study of *Desmodium gengaticum* species the phylogenetic tree (Figure 4) obtained with the Neighbor-Joining (NJ) algorithm, 4 accessions of *Desmodium* were differentiated into two clusters viz (Dg1 and 2) and (DG3 and 4) which is in accordance with the geographical locations. BLAST Results showed maximum E-value in DG2 and maximum identity in DG4 (Table 4). Therefore the above results of both markers (SSR and intertranscribed) may provide a lead and implications for the conservation of this threatened species.

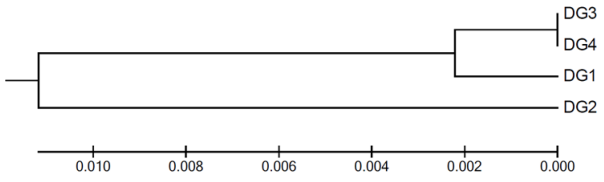

Figure 4: Sequence analysis and phylogenetic tree constructed from the ITS of *D. gangeticum* accessions. The four Dg1, Dg2 and Dg3, Dg4 accessions showed little diversity among themselves.

Marker	Acession	Species	E-value	Max Score
ITS	DG1	*Desmodium gangeticum*	0	1065
ITS	DG2	*Desmodium gangeticum*	8.00E-106	392
ITS	DG3	*Desmodium gangeticum*	0	395
ITS	DG4	*Desmodium gangeticum*	0	1101

Table 4: NCBI Blast results of the ITS sequences of *Desmodium gangeticum* accessions.

Conclusion

The plants are not necessarily rare, indeed, management measures for the conservation and restoration of the above species is required. Otherwise the plant spp. may quickly become critically endangered if they are harvested in an unsustainable way as is happening in current era. Therefore considering the medicinal importance of this spp. the above discussed markers will probably provide productive application to the cultivation, conservation of genetic resources, assessment of genetic relationships and sustainable utilization of this natural gifted species.

The authors declare that they have no competing interests.

Acknowledgement

The authors acknowledge to the Dabur Research Foundation New Delhi India for providing the financial assistance and SMVD University were the work was being carried in.

References

1. Govindarajan R, Vijayakumar M, Rao ChV, Shirwaikar A, Kumar S, et al. (2007) Antiinflammatory and antioxidant activities of Desmodium gangeticum fractions in carrageenanâ€induced inflamed rats. Phytotherapy research 21: 975-979.
2. Singh D, Govindarajan R, Rawat A (2008) High performance liquid chromatography as a tool for the chemical standardisation of Triphala an Ayurvedic formulation. Phytochemical analysis 19: 164-168.
3. Tabuti JR, Lye KA, Dhillion SS (2003) Traditional herbal drugs of Bulamogi, Uganda: plants, use and administration. J Ethnopharmacol 88: 19-44.
4. Kosalge SB, Fursule RA (2009) Investigation of ethnomedicinal claims of some plants used by tribals of Satpuda Hills in India. J Ethnopharmacol 121: 456-461.
5. Mishra PK, Singh N, Ahmad G, Dube A, Maurya R (2005) Glycolipids and other constituents from Desmodium gangeticum with antileishmanial and immunomodulatory activities. Bioorganic medicinal chemistry letters 15: 4543-4546.
6. Ghosal S, Banerjee PK (1969) Alkaloids of the roots of Desmodium gangeticum. Australian Journal of Chemistry 22: 2029-2031.
7. Yadava RN, Tripathi P (1998) A novel flavone glycoside from the stem of Desmodium gangeticum. Fitoterapia 69: 443-444.
8. Varaprasad MV, Balakrishna K, Sukumar E, Patra A (2009) Gangetial, a new pterocarpan from the roots of Desmodium gangeticum. Journal of the Indian Chemical Society 86: 654-656.
9. Rafalski JA, Tingey SV (1993) Genetic diagnostics in plant breeding: RAPDs, microsatellites and machines. Trends Genet 9: 275-280.
10. Levinson G, Gutman GA (1987) Slipped-strand mispairing: a major mechanism for DNA sequence evolution. Molecular biology and evolution 4: 203-221.
11. Becker J, Vos P, Kuiper M, Salamini F, Heun M (1995) Combined mapping of AFLP and RFLP markers in barley. Mol Gen Genet 249: 65-73.
12. Thomas CM, Vos P, Zabeau M, Jones DA, Norcott KA, et al. (1995) Identification of amplified restriction fragment polymorphism (AFLP) markers tightly linked to the tomato Cfâ€9 gene for resistance to Cladosporium fulvum. The Plant Journal 8: 785-794.
13. Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, et al. (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23: 4407-4414.
14. Zhu J, Gale M, Quarrie S, Jackson M, Bryan G (1998) AFLP markers for the study of rice biodiversity. Theoretical and Applied Genetics 96: 602-611.
15. Li G, Quiros CF (2001) Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its

application to mapping and gene tagging in Brassica. Theoretical and Applied Genetics 103: 455-461.

16. Vieux EF, Kwok PY, Miller RD (2002) Primer design for PCR and sequencing in high-throughput analysis of SNPs. Biotechniques 32: 28-30.
17. Varshney RK, Hoisington DA, Tyagi AK (2006) Advances in cereal genomics and applications in crop breeding. Trends Biotechnol 24: 490-499.
18. Kashi Y, King DG (2006) Simple sequence repeats as advantageous mutators in evolution. Trends Genet 22: 253-259.
19. Morgante M, Hanafey M, Powell W (2002) Microsatellites are preferentially associated with nonrepetitive DNA in plant genomes. Nature genetics 30: 194-200.
20. da Maia LC, de Souza VQ, Kopp MM, de Carvalho FI, de Oliveira AC (2009) Tandem repeat distribution of gene transcripts in three plant families. Genet Mol Biol 32: 822-833.
21. Homolka A, Berenyi M, Burg K, Kopecky D, Fluch S (2010) Microsatellite markers in the tree peony, Paeonia suffruticosa (Paeoniaceae). Am J Bot 97: e42-44.
22. Hou XG, Guo DL, Cheng SP, Zhang JY (2011) Development of thirty new polymorphic microsatellite primers for Paeonia suffruticosa. Biologia Plantarum 55: 708-710.
23. Wang S, Yang W, Shen H (2011) Genetic diversity in Apium graveolens and related species revealed by SRAP and SSR markers. Scientia Horticulturae 129: 1-8.
24. Powell W, Morgante M, Andre C, Hanafey M, Vogelet J, et al. (1996) The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Molecular breeding 2: 225-238.
25. Rogers SO, Bendich AJ (1987) Ribosomal RNA genes in plants: variability in copy number and in the intergenic spacer. Plant Mol Biol 9: 509-520.
26. Baldwin BG (1992) Phylogenetic utility of the internal transcribed spacers of nuclear ribosomal DNA in plants: an example from the Compositae. Molecular phylogenetics and evolution 1: 3-16.
27. Sun Y, Fung KP, Leung PC, Shi D, Shaw PC (2004) Characterization of medicinal Epimedium species by 5S rRNA gene spacer sequencing. Planta Med 70: 287-288.
28. Kim OT, Bang KH, In DS, Lee JW, Kim YC et al. (2007) Molecular authentication of ginseng cultivars by comparison of internal transcribed spacer and 5.8 S rDNA sequences. Plant Biotechnology Reports 1: 163-167.
29. Yamaji H, Fukuda T, Yokoyama J, Pak JH, Zhou CZ, et al. (2007) Reticulate evolution and phylogeography in Asarum sect. Asiasarum (Aristolochiaceae) documented in internal transcribed spacer sequences (ITS) of nuclear ribosomal DNA. Molecular phylogenetics and evolution 44: 863-884.
30. Yeh F, Yang R, Boyle T, Ye Z, Xiyan J (2000) PopGene32, Microsoft Windows-based freeware for population genetic analysis, version 1.32. Molecular Biology and Biotechnology Centre. University of Alberta, Edmonton, Alberta, Canada.
31. Rohlf F (2000) NTSYS-PC, numerical taxonomy system for the PC ExeterSoftware, Version 2.1. Applied Biostatistics Inc Setauket, USA.
32. Peakall R, Smouse PE (2006) GENALEX 6.5: genetic analysis in Excel. Population genetic software for teaching and research. Molecular ecology notes 6: 288-295.
33. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30: 2725-2729.
34. Moller M, Cronk Q (1997) Origin and relationships of Saintpaulia (Gesneriaceae) based on ribosomal DNA internal transcribed spacer (ITS) sequences. Am J Bot 84: 956.
35. Zimmer EA, Roalson EH, Skog LE, Boggan JK, Idnurm A (2002) Phylogenetic relationships in the Gesnerioideae (Gesneriaceae) based on nrDNA ITS and cpDNA trnL-F and trnE-T spacer region sequences. American journal of botany 89: 296-311.
36. Clark JL, Zimmer EA (2003) A preliminary phylogeny of Alloplectus (Gesneriaceae): implications for the evolution of flower resupination. Systematic botany 28: 365-375.
37. Roalson EH, Boggan JK, Skog LE, Zimmer EA (2005) Untangling Gloxinieae (Gesneriaceae). I. Phylogenetic patterns and generic boundaries inferred from nuclear, chloroplast, and morphological cladistic datasets. Taxon pp: 389-410.