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RESEARCH ARTICLE

DNA Barcoding Markers and Next Generation Sequence Analysis of Rbcl in Intraspecies Genetic Variations of Three Varieties of *Cissus quadrangularis* L.

Goli Penchala Prasad[1], G. P. Pratap[2], Srinivasan Marimuthu[1*], Shyam Baboo Prasad[1], Arun Manohar Gurav[1], Anupam Kumar Mangal[3] and N. Srikanth[3]

[1]Regional Ayurveda Research Institute for Fundamental Research, Kothrud, Pune – 411038, Maharashtra, India; srinimarimuthu@gmail.com
[2]Central Research Institute of Unani Medicine, Hyderabad – 500038, Telangana, India
[3]Central Council for Research in Ayurvedic Sciences, Janakpuri – 110058, New Delhi, India

Abstract

Cissus quadrangularis L. is a well-known plant used in Ayurveda. It is also called Asthisamharaka. The etymology of its name indicates its use in the healing of the fractured bone. It is a widely growing plant belonging to the family of vitaceae. *C. quadrangularis* (Family: Vitaceae) is commonly distributed throughout the hotter parts of India and Sri Lanka. *C. quadrangularis* is mentioned in classical literature like Bhava Prakash and Chakra Dutta. The present study design to compare three different varieties of *C. quadrangularis* which includes the species of quadrangular, round and flat types are used to study DNA barcoding marker and phylogenetic analysis. Whole-genome was isolated from the stem parts of three varieties of *C. quadrangularis*; quadrangular, round and flat. The PCR amplification of DNA barcoding markers (rbcl, mat k and ITS) was analyzed by DNA electrophoresis. The DNA barcoding markers and next-generation sequencing are able to identify the intraspecies genetic variations among these closely related plant varieties of *C. quadrangularis* of quadrangular, round and flat.

Keywords: Ayurveda, *Cissus quadrangularis* L., Mat k, Medicinal Plants, Next-Generation Sequencing, Rbcl, ITS4

1. Introduction

Asthisamharaka (*C. quadrangularis*) is a well known Ayurvedic drug for its effect on the reunion of a fractured bone. *C. quadrangularis* paste is also used in Vataja diseases (neuromuscular diseases), worm infestation, skin diseases, debility, arthritis, rheumatism etc[1]. Though in classical literature no varieties have been mentioned, in various geographical regions of Andhra Pradesh varieties of *C. quadrangularis* is classified to compare three different varieties of Cissus which includes the types of quadrangular, round and flat types are observed. Even studies denote that taxonomically quadrangular and flat variety do not have much difference[2]. Though there is a difference of opinions regarding the identification of flat and round varieties majority consider these as *C. quadrangularis* varieties and to be further confirmed and differentiated from *C. repens and C. repanda*[3]. The study is designed to evaluate the DNA barcoding markers of rbcl, mat k and ITS universal genes and next-generation sequencing to explore the intraspecies genetic variations among these closely related three varieties of *C. quadrangularis*.

*Author for correspondence

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2. Materials and Methods

2.1 Chemicals

The whole-genome kit was purchased from Favorogen, PCR master mix (2X) from Genetix Biotech Asia Pvt Ltd, New Delhi, India. Agarose, 6X gel loading dye, 100 bp, and 1 Kb DNA ladder were procured from Himedia Laboratories PVT, LTD, Mumbai, India, Primers mat k, rbcl, and ITS was synthesized by Eurofins Genomics India Pvt. Ltd, Bangalore, Karnataka, India. The chemicals and solvents were purchased from Himedia, India.

2.2 Plant Collection

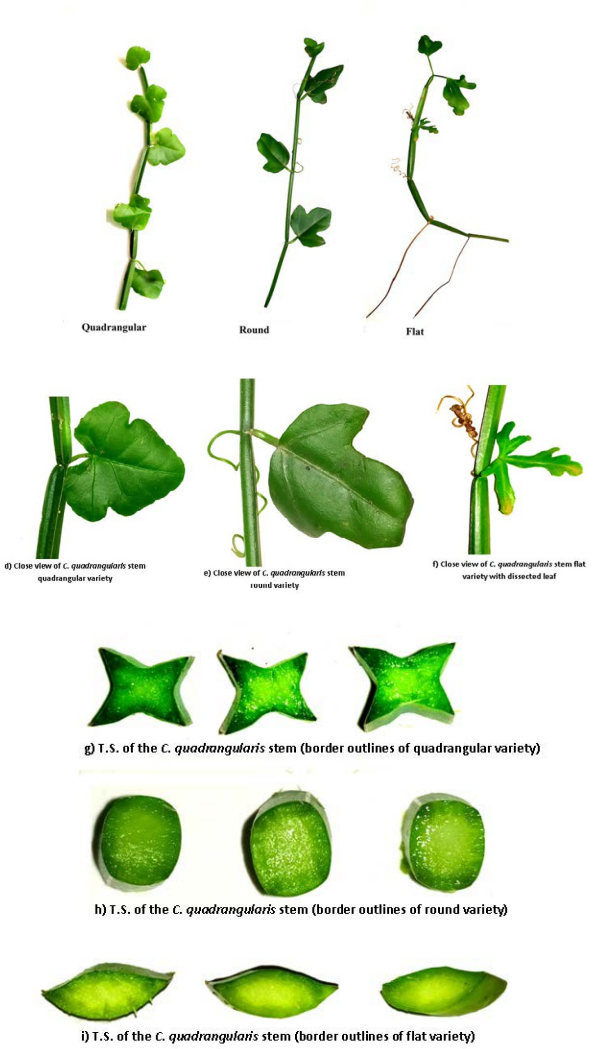

Figure 1. Morphological variations among the three varieties of *C. quadrangularis* (quadrangular, round and flat) (a,b,c) (d,e,f) (g,h,i).

Figure 1 (a,b,c) (c,d,e) (f,g,h) fresh stems of *C. quadrangularis* of quadrangular, round and flat varieties were collected from Medicinal plant garden at Regional Ayurveda Institute for Fundamental Research (RAIFR), Kothrud, Pune, Maharashtra, India. The plant identification was authenticated by Dr. Arun Manohar Gurav, Research officer (Botany) and the live plants are being maintained in the Arborium/Garden of RAIFR, Pune, Maharashtra, India with the registration number of stem parts of CqQu-01, CqRo-02 and CqFl-03 and leaf part of CqRL-01.

2.3 Extraction of Whole Genomic DNA

The stem parts from three varieties of *C. quadrangularis* viz quadrangular, round and flat, 100 mg each and leaf of round variety of *C. quadrangularis* were crushed with liquid nitrogen into a fine powder. Whole-genome was extracted from the plants using the Favorogen whole genomic isolation kit. The concentration of the genomic DNA was determined using a Nanodrop. The integrity of DNA was confirmed by visualization on 0.8% agarose gel using ethidium bromide staining.

2.4 PCR Amplification

The whole genome was used as a template in the PCR reaction. PCR amplification was conducted with PCR master mix (2X), 10 µM forward and reverse primer, nuclease-free water and 10 ng of template DNA (Table 1). The PCR conditions were initial denaturation at 94^0C for 5 minutes, 35 cycles of denaturation at 94^0C for 30-sec annealing at 50^0C for 1 minute, extension at 72^0C for 2 minutes followed by a final extension at 72^0C for 10 minutes. The amplified products were detected by agarose gel electrophoresis in Tris-Acetate-EDTA (TAE) buffer and visualized with gel documentation system.

Table 1. Primers for ITS and mat k and rbcl genomic regions

Primer name	Sequences (5′-3′)
ITS-F	5′-GGAAGTAAAAGTCGTAACAAGG-3′[4]
ITS-R	5′-TCCTCCGCTTATTGATATGC-3′
mat k-F	5′-TAATTTACGATCAATTCATTC-3′[5]
mat k-R	5′-CTTCCTCTGTAAAGAATTC-3′
rbcl-F	5′-ATGTCACCACAAACAGAGACTAAAGC-3′[6]
rbcl-R	5′-GTAAAATCAAGTCCACCRCG-3′

2.5 Amplification and Next Generation Sequence Analysis

PCR was performed using the rbcl gene region. The PCR was done in 25µL consisting of 2X PCR master mix, 0.25 µM forward and reverse primer and nuclease-free water and 10 ng of the whole genome. The PCR was incubated at 94^0C for 1 minute and amplification was performed with the following 35 thermal cycles; denaturation for the 30s at 94^0C, annealing for 40s at 53^0C, an extension for 40s at 72^0C, and the final extension for 5 min at 72^0C. The PCR products were detected by agarose gel electrophoresis and visualized with a gel documentation system. PCR products were precipitated using propanol and then washed with ethanol. The products were air-dried and resuspended in denaturing buffer. The sequencing was done using Applied Biosystems DNA sequencer following standard protocol. Sequence analyzing was outsourced to Eurofins Genomics India Pvt. Ltd, India and was analyzed by Next Generation Sequencing (NGS). The nucleotide sequence for rbcl gene sequence was subjected to sequence alignment using the Basic Local Alignment Tool (BLAST). The number of hits with homologous sequences is inferred based on similarity and alignment.

2.6 Phylogenetic tree

The phylogenetic analysis (MEGA) version 10, was used to construct a maximum likely hood tree for the obtained sequences to identify its intraspecies relationships.

3. Result

3.1 Genomic DNA quantification

The extracted plant DNA was analyzed using Nanodrop Eppendorf. The obtained DNA concentration from the stem of quadrangular variety was 20 ng/µl, the stem of round variety was 23 ng/µl, the stem of flat variety was 23 ng/µl and the leaf of round variety was 22 ng/µl respectively. Hence, due to the good quality of genomic DNA concentrations in all three varieties of *C. quadrangularis* was used as a DNA template for Rbcl, matk and ITS-4 and 5 for further molecular marker studies.

3.2 Internal Transcribed spacer (ITS) 4 and 5 gene amplification

ITS is situated between the small subunit rRNA and large subunit rRNA genes in the chromosomes. Figure 2 shows the changes in the ITS-4 and 5 universal gene amplification to generate the 670 base pairs (bp) in size of agarose gel electrophoresis. The gene was amplified successfully in *C. quadrangularis* of quadrangular, flat and round varieties.

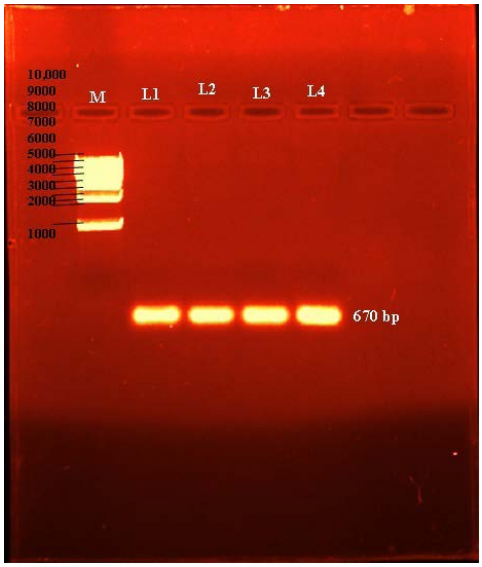

Figure-2

Figure 2. Changes in the expression of ITS in three different varieties of *C. quadrangularis*. M-represents marker 1 Kb DNA ladder and Lanes L1-*C. quadrangularis* of quadrangular of ITS, L2-*C. quadrangularis* of round of ITS, L3-*C. quadrangularis* flat of ITS and L4-*C. quadrangularis* round leaf.

3.3 Rbcl and Mat k Gene Amplification

Figure 3 shows the changes in the rbcl and mat k gene amplification to generate the PCR product of agarose gel electrophoresis. The results of DNA barcode markers of mat k and rbcl gene regions in the three varieties of *C. quadrangularis* i.e quadrangular, round and flat were also seen. The amplification of the gene was successful in rbcl gene region; the fragment sizes are about 550bp. Mat k gene expression was not amplified

when compared with rbcl gene. All three varieties of *C. quadrangularis* of rbcl gene amplification are similar in rbcl gene.

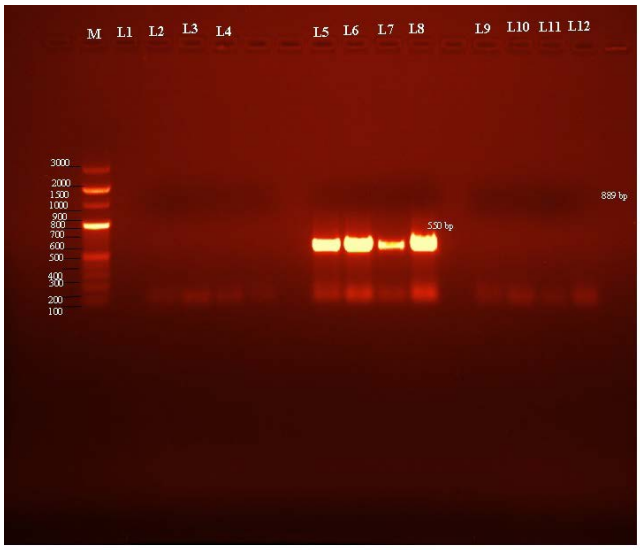

Figure 3. Changes in the expression of rbcl and mat k in three different varieties of *C. quadrangularis*. M represents marker 100 bp DNA ladder and Lanes L1- Forward primer control of rbcl gene, L2- Reverse primer control of rbcl gene, L3- Forward primer control of mat k gene, L4- Reverse primer control of mat k gene, L5- *C. quadrangularis* of quadrangular of rbcl gene, L6- *C. quadrangularis* round of rbcl gene, L7- *C. quadrangularis* flat of rbcl gene, L8- *C. quadrangularis* round leaf of rbcl gene, L9- *C. quadrangularis*of quadrangular of mat-k gene, L10- *C. quadrangularis* round of mat k gene, L11- *C. quadrangularis* of flat of mat k gene, L12- *C. quadrangularis* round leaf of mat k gene.

3.4 Next Generation Sequence Analysis

Homology of *C. quadrangularis* was detected using BLAST. The sequence length of *Cissus quadrangularis* isolate TMP 36 ribulose, *Cissus quadrangularis* isolate SBB 1201 was 609, 611 and 540 nucleotides match with *Cissus quadrangularis* of quadrangular, round and flat varieties respectively. rbcl sequence homology of all the three species was 99% identical (Table 2).

We observed that species substitution as marker nucleotides because they may be crucial for identifying each variety. In quadrangular variety there was a mismatch sequence that was observed at the position of 153(C,T), deletion and insertion were not observed. In *C. quadrangularis* round variety the mismatch sequences was observed at the position of 11(C,T), 527(G,T) and 531-532(TT,AC) nucleotide sequence and the insertion was observed at the position of 12-13(TG,--), 41481(T,-) and deletion was not observed. In *C. quadrangularis* of flat variety, we found that mismatch sequences at the position of 153(C,T) respectively and the insertion and deletion were not observed (Table 3).

3.5 Phylogenetic analysis

To study the phylogenetic relationships among the three species of *C. quadrangularis* i.e. quadrangular, round and flat. phylogenetic trees were constructed by applying the NJ method to the rbcl. As shown in the result of the phylogenetic tree.

Three DNA barcodes sequences were employed, *C. quadrangularis* round and *C. quadrangularis* quadrangular varieties were closer genetically when compared to *Cissus quadrangularis* flat (Figure 4). Therefore the rbcl barcode sequences provided

Table 2. BLAST Sequence homology of *C. quadrangularis* of quadrangular, round and leaf varieties with rbcl primers respectively

Species	Scientific name	Ref.ID	Best hit	Gene	Length	% Identification
Quadrangular	*C. quadrangularis*	KF381118.1	*Cissus quadrangularis* isolate TMP 36 ribulose	RBCL	609	99
Round	*C. quadrangularis*	KF381118.1	*Cissus quadrangularis* isolate TMP 36 ribulose	RBCL	611	99
Flat	*C. quadrangularis*	KJ667659.1	*Cissus quadrangularis* isolate SBB 1201	RBCL	540	99

Table 3. Identification of single gene mutation of three varieties of *C. quadrangularis* of quadrangular, round and flat

Species varieties	Scientific name	Mismatch	Insertion
Quadrangular	*C. quadrangularis*	153(C,T)	----
Round	*C. quadrangularis*	11(C,T);527(G,T);531-532(TT,AC)	12-13(TG,--);41481(T,-)
Flat	*C. quadrangularis*	153(C,T)	----

advanced resolution for the identification of the clusters that constitute clades within the same species. From these phylogenetic trees, it is confirmed that the identification of the three species can be achieved using rbcl gene.

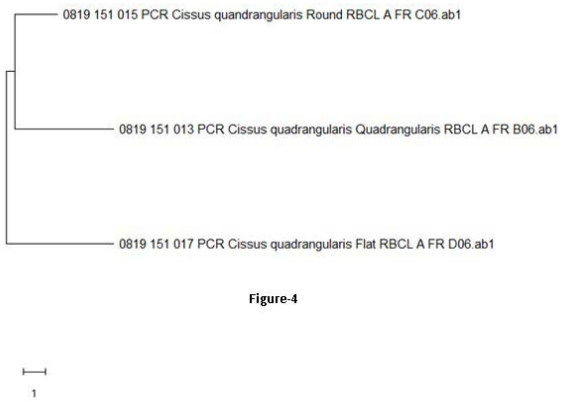

Figure 4. Phylogenetic tree showing the common linkage between *C. quadrangularis, C. quadrangularis* round and *C. quadrangularis* flat.

4. Discussion

C. quadrangularis is also called as Hadjod in Hindi and Asthisamharaka in Ayurveda[7]. It is used for the growth of bones and joints, bone mineral density; increases the rate of fracture healing and used to treat gout, internal bleeding, leucorrhea, and aphrodisiac action. It is used to improve men's stamina, vigor, and strength[8].

Many *Cissus* species are having medicinal values that are economically profitable; it is used as an ingredient of various Ayurvedic formulations and in fresh juice for bone fracture. There is a need for specific molecular markers to provide further rapid, automatable, and accurate species identification[9]. DNA barcodes can be used to verify accurate species identification. It is rapid and accurate identification of any plant species based on extracting short DNA sequences from a tiny sample of any organisms[10].

The plant working group of consortium for the barcode of Life's (CBOL) reported that DNA barcodes with mat k and rbcl gene loci are the use of two regions of plastid DNA as a standard protocol for the core barcoding of land plants[11]. In this study, we assessed the molecular markers of ITS-4 and 5, mat K and rbcL and next-generation sequences are used to identify the intraspecies genetic variations among these closely related three varieties of *Cissus quadrangularis* L.

Internal transcribed spacer (ITS) is a nonfunctional RNA situated between structural rRNA of a common precursor transcript[12]. ITS sequences were amplified from the genomic DNA of three varieties of Cissus species[13]. The amplified ITS region was approximately 670 bp in *C. quadrangularis*.

In our present study, we have found the amplification of the gene was successful in rbcl gene region, Mat K gene was not amplified when compared with rbcl gene. However, the mat k region has not been successful in the *Cissus* species studied and in the out-group[5]. In *Cissus* species, rbcL DNA barcode gene becomes suitable and can be used as a single region[14].

Next-generation sequencing and Sanger sequencing methods are of great importance in unraveling the complexity of genomes[15]. The present study amplified rbcl gene region and conserved sequence in three species of *C. quadrangularis*. Sequence homology of the amplified gene was detected using BLAST[16].

In our present study, we have identified the rbcl gene sequence homology of all the three varieties of *C. quadrangularis* i.e quadrangular, round and flat was 99% related to *Cissus quadrangularis* isolate TMP 36 ribulose. This shows that these three varieties are only of same species varieties i.e of *C. quadrangularis* In phylogenetic tree analysis we have found that *C. quadrangularis* round variety was closely related with that of quadrangular variety and distance relationship with flat.

5. Conclusion

Thus, from the results obtained it is observed that the DNA barcoding markers of ITS-4 and 5, mat k and rbcl gene amplification and sequencing are used to identify the intraspecies genetic variations among these closely related three varieties of *C. quadrangularis.* The quadrangular, round and flat varieties were closely related with *Cissus quadrangularis* isolate TMP 36 ribulose (99%). Rbcl barcode sequences provided higher resolution for the recognition of the clusters that constitute clades within the same varieties. These three quadrangular, round and flat variants can be considered as varieties of the same species i.e. *C. quadrangularis* Phylogenetic analyses confirm that the identification of the three species of *C. quadrangularis* can be achieved using rbcl gene sequence analysis.

6. Acknowledgement

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7. Conflict of Interest

The authors declare that there is no conflict of interest.

8. References

1. Nishteswar K, Hemadri K. Dravyaguna Vijnana. Chaukhambha Sanskrit Pratishthan, Delhi, India; 2013. p. 1–493.
2. Robert GW, Qing-Feng W, Yong W, You-Hao G. A taxonomic investigation of variation within *Cissus quadrangularis* L. (Vitaceae) in Kenya. Wuhan University Journal of Natural Sciences. 2001; 6(3):715–24. https://doi.org/10.1007/BF02830291
3. Sasidharan H, Varghese AP. Taxonomy of selected species of Cissus (Vitaceae) from Thrissur district. South Indian Journal of Biological Sciences 2016; 2(1):222–8. https://doi.org/10.22205/sijbs/2016/v2/i1/100403
4. Issaravanich S, Ruangrungsia N, Palanuvej C, Vipunngeun N, Rungsihirunrat K. Microscopic, molecular and scopolamine content evaluations of *Datura metel* L. var. metel and D. metel L. var. fastuosa in Thailand. Research Journal of Pharmaceutical Biological Chemical Sciences. 2013; 4:1009–21.
5. Sudmoon R, Chaveerach A, Tance T. Analysis of genetic and chemical relation compared to commonly used *Cissus quadrangularis* L. and barcode markers of some Thailand Cissus species. Pakistan Journal of Pharmaceutical Sciences. 2016; 29(1):65–75.
6. Hegde S, Saini A, Hegde HV, Kholkute SD, Roy S. Molecular identification of Saraca asoca from its substituents and adulterants. 3 Biotech; 2018; 8(3):161. https://doi.org/10.1007/s13205-018-1175-5. PMid:29527448 PMCid :PMC5835484
7. Sirasanagandla SR, Ranganath Pai KS, Potu BK, Bhat KM. Protective effect of *Cissus quadrangularis* Linn. on diabetes induced delayed fetal skeletal ossification. Journal of Ayurveda Integrated Medical Sciences. 2014; 5(1):25–32. https://doi.org/10.4103/0975-9476.128852. PMid:24812472 PMCid:PMC4012358
8. Brahmkshatriya HR, Shah KA, Ananthkumar GB, Brahmkshatriya MH. Clinical evaluation of *Cissus quadrangularis* as osteogenic agent in maxillofacial fracture: A pilot study. Ayurveda 2015; 36(2):169–73. https://doi.org/10.4103/0974-8520.175542. PMid:27011718 PMCid: PMC4784127
9. Srisook K, Palachot M, Mongkol N, Srisook E, Sarapusit S. Anti-inflammatory effect of ethyl acetate extract from *Cissus quadrangularis* Linn may be involved with induction of heme oxygenase-1 and suppression of NF-κB activation. Journal of Ethnopharmacology. 2011; 133(3):1008–14. https://doi.org/10.1016/j.jep.2010.11.029. PMid:21094244
10. Gao R, Zhang G. Potential of DNA barcoding for detecting quarantine fungi. Phytopathology. 2013; 103(11):1103–07. https://doi.org/10.1094/PHYTO-12-12-0321-R. PMid:23718836
11. Hollings worth PM, Forrest LL, Spouge JL. A DNA barcode for land plants. Proceedings of the National Academy of Sciences. 2009; 106(31):12794–7. https://doi.org/10.1073/pnas.0905845106. PMid:19666622 PMCid:PMC2722355
12. Hao DC, Xiao PG, Ma HY, Peng Y. He CN. Mining chemodiversity from biodiversity: Pharmacophylogeny of medicinal plants of Ranunculaceae. Chinese Journal

of Natural Medicines. 2015; 13(7):507–20. https://doi.org/10.1016/S1875-5364(15)30045-5

13. Ren FM, Wang YW, Xu ZC, Li Y, Xin TY, Zhou JG, et al. DNA barcoding of Corydalis, the most taxonomically complicated genus of Papaveraceae. Ecology and Evolution. 2019; 9(4):1934–45. https://doi.org/10.1002/ece3.4886. PMid:30847083 PMCid:PMC6392370
14. Nakagawa T, Doi M, Nishi K, Sugahara T, Nishimukai H, Asano M. A simple and versatile authenticity assay of coffee products by single-nucleotide polymorphism genotyping. Bioscience Biotechnology and Biochemistry. 2019; 83(10):1829–36. https://doi.org/10.1080/09168451.2019.1618697. PMid:31119972
15. Kim WJ, Ji Y, Choi G, Kang YM, Yang S, Moon BC. Molecular identification and phylogenetic analysis of important medicinal plant species in genus Paeonia based on rDNA-ITS, matk, and rbcl DNA barcode sequences. Genetics and Molecular Research. 2016; 15(3):1–11. https://doi.org/10.4238/gmr.15038472
16. Watto JI, Saleem MZ, Shahzad MS, Arif A, Hameed A, Saleem MA. DNA Barcoding: Amplification and sequence analysis of rbcl and matk genome regions in three divergent plant species. Advancements in Life Sciences. 2016; 4(1):3–7.