

ARTICLE

Open Access



# Development of a molecular marker based on chloroplast gene for specific identification of Korean Hibiscus (*Hibiscus syriacus* 'Simbaek')

Rongbo Wang<sup>1</sup>, Sang Yong Park<sup>2</sup>, Sul Woong Park<sup>2</sup>, Aditi Mitra Puja<sup>1</sup> and Yeon-Ju Kim<sup>1\*</sup>

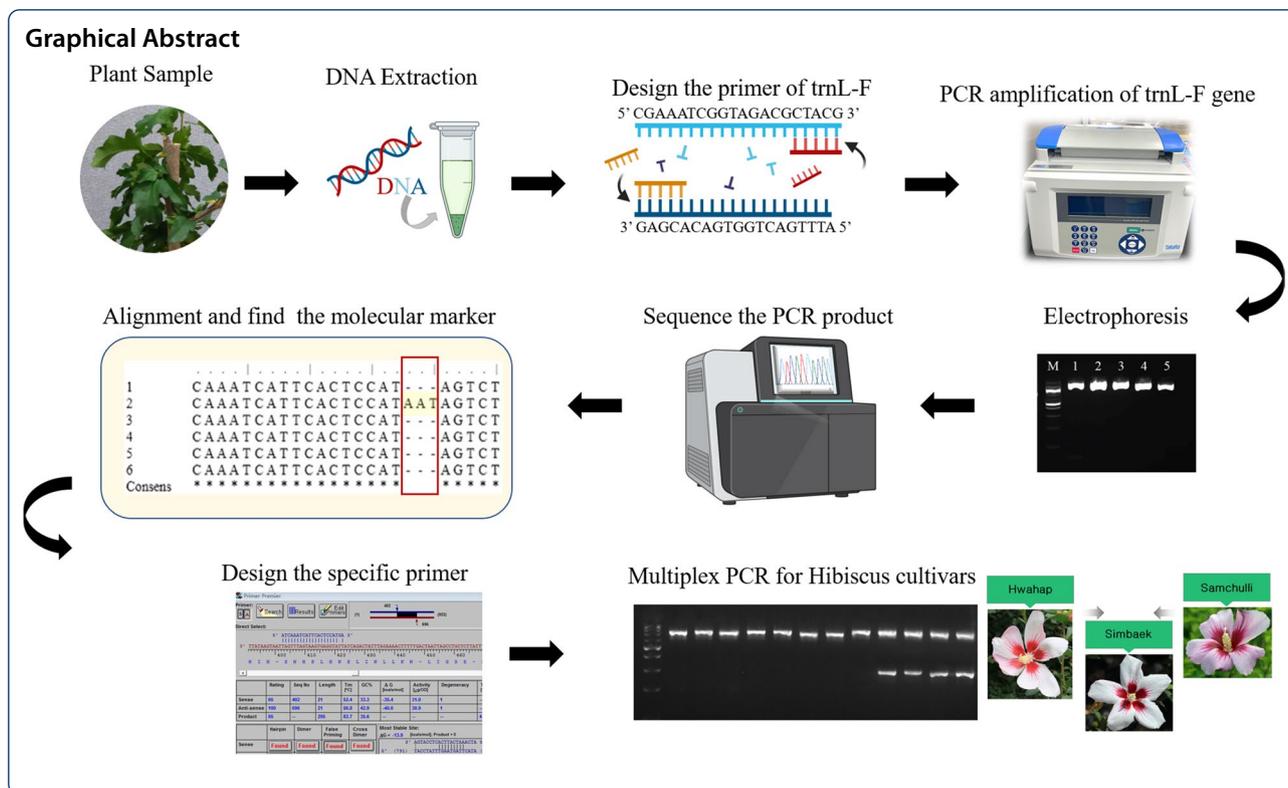
## Abstract

Due to the rise in substitution and adulteration of herbal products, as well as the lack of genetic information on *Hibiscus* plants, more molecular markers are needed to understand the genetic diversity and avoid their misidentification. There are many allelic variants of the functional genes in *Hibiscus* and other plants, which control their respective phenotypes and other characteristics. Identifying alleles of the desired trait by determining diversification through gene-typing allele-specific markers for authentication is, therefore, a potent strategy. The purpose of this study was to use insertion/deletion (InDel) markers to identify *Hibiscus syriacus* cultivars. We developed a novel InDel marker for Korean *Hibiscus* 'Simbaek', based on the trnL-F region of the chloroplast gene. Through this InDel site, a modified specific primer pair and a novel multiplex polymerase chain reaction (PCR) system were developed for specific identification of the Korean *Hibiscus* Simbaek cultivar. The molecular markers developed in this study were highly specific and accurately authenticated as Simbaek from the five main cultivars of *H. syriacus*. Taken together, the described method is a potential tool for the identification and selection of germplasm resource of Simbaek cultivar.

**Keywords:** *Hibiscus syriacus*, InDel, Multiplex PCR, Simbaek, trnL-F

\*Correspondence: yeonjukim@khu.ac.kr

<sup>1</sup> Graduate School of Biotechnology, and College of Life Science, Kyung Hee University, Yongin, Gyeonggi-do 17104, Republic of Korea  
Full list of author information is available at the end of the article



## Introduction

*Hibiscus syriacus* is a deciduous shrub belonging to the genus Malvaceae. *Hibiscus syriacus* originated in the Korean Peninsula and southern China, and later spread to the Indian subcontinent and middle-eastern regions. In temperate regions, *H. syriacus* is a common ornamental species with white, pink, red, or purple flowers [1]. In addition to its ornamental value, dried flowers and root bark of *H. syriacus* are known for their anticancer [2], antidepressant [3], and antioxidant [4] properties. *Hibiscus syriacus* is also mentioned in the Korean national anthem as the national flower. Since there is substantial history and culture associated with the flower, new varieties have been actively developed in Korea by natural and systematic selection. Each Korean hibiscus variety has unique and improved agronomic traits, such as appearance, water resistance, and disease resistance traits. The Korean hibiscus cultivar, Simbaek, was developed from two basic cultivar lines (Hwahap and Samchunli) and registered in 2014. Simbaek is used as a street-side landscaping plant because of the beautiful flowers and lush branches. It has also been found to have better anticancer effects than the other cultivars (Additional file 1: Fig. S1). In herbal markets, American *Hibiscus* (*Hibiscus sabdariffa*) is often regarded as an adulterant of Korean *Hibiscus* which also brings a lot of trouble to consumers. Traditionally, confirming the authenticity of species or cultivars

relies on morphological characteristics, such as growth environment, floral morphology, and other characteristics of the plant [5]. However, as majority of the herbal products are packaged in the form of powders that do not bear the original features of the plants, identification of the latter by morphological and histological techniques becomes difficult.

DNA molecular markers have several advantages over morphological characterization and chemical profiling for identifying the origin of different varieties, since the genetic composition is unique for each variety irrespective of its physical form and less affected by environmental factors as well as developmental stages [6–8]. The insertion/deletion markers (InDels), which are structural variations caused by polymerase slippage, transposons, and unequal exchange, usually include insertion/deletion and extension of base pairs. They are distributed in large numbers throughout the genome and are closely related to the gain/loss of biological functions [9–11]. InDels have been successfully used in rice [12], *Arabidopsis thaliana* [13], and *Panax ginseng* [14] germplasm genetics, in which genetic and association maps of different plants are constructed. In addition, genotyping using InDels through conventional gel electrophoresis does not produce non-specific bands. In some previous studies, InDels were found to be more polymorphic than microsatellite markers [15].

**Table 1** Information of the plant samples used in the study

<i>Hibiscus</i> samples	Registration number	Origin	GenBank accession number of trnL-F region
Samchunli	1481	Korea, Cheonan	MZ127173
Simbaek	5259	Korea, Cheonan	MZ127174
Jinsun	6335	Korea, Cheonan	MZ127175
Jamyung	5778	Korea, Cheonan	MZ127176
<i>H. sabdariffa</i>	–	USA	JQ625356.1

**Table 2** Primer sequences used in this study

Primer name	Direction	Nucleotide sequence (5'—3')
trnL	Forward	CGAAATCGGTAGACGCTACG
trnF	Reverse	ATTGAACTGGTGACACGAG
SimF	Forward	ATCAAATCATTCACTCCATGA
SimR	Reverse	AGGATACGCTGTAAAATGGC

Nevertheless, to the best of our knowledge, InDel markers have rarely been applied to the molecular genetics of *H. syriacus*. In this study, we report the first InDel marker of Simbaek cultivar developed using an easy and reproducible multiplex PCR method that amplifies the trnL-F region. This approach can be used for basic and applied research on *H. syriacus*, which as mentioned above, has potentially immense value.

## Materials and methods

### Plant and materials

This study was carried out in accordance with the guidelines provided by Kyung Hee University, Yongin-si, Gyeonggi-do, Republic of Korea. Four Korean cultivars of *H. syriacus* and *H. sabdariffa* were provided by Jangho Farm in Nonsan, Chungcheongnam-do, and the plant species were assessed by Dr. Kyung-gu Shim who has developed more than 100 new cultivars in Korea. All voucher specimens were morphologically identified and voucher specimen numbers are shown in Table 1.

### DNA extraction and PCR amplification of trnL-F genome

Leaves of the five different *Hibiscus* cultivars were placed in liquid nitrogen for 1 min and crushed into powder. Genomic DNA was isolated using the Exgene™ Plant SV mini kit (GeneAll Biotechnology Co., Seoul, Korea), according to the manufacturer's instructions. The primer pairs used for the amplification of trnL-F region were synthesized by Macrogen (Seoul, Korea); the sequences of the universal primers are listed in Table 2 [16]. The PCR reaction mixture consisted of 50 ng of DNA, 0.5 μM of primers, 10 μL of Taq polymerase (GeneAll

Biotechnology Co., Seoul, Korea), and 5 μL of distilled water. PCR products were analyzed by electrophoretic separation in a submarine horizontal agarose slab gel apparatus. For running the DNA samples, a gel strength of 2% agarose was dissolved in 0.5 X TAE buffer followed by heating in microwave oven. After cooling to approximately 55 °C, 0.05 μL/mL GelGreen® Nucleic Acid Gel Stain (Fremont, CA, USA) was added. Then, 300 mL of 0.5X TAE buffer was poured to sufficiently submerge the gel in the electrophoresis tank. Electrophoresis was performed at a constant voltage of 5 V/cm until the bromophenol dye reached almost the end of the gel. A UV-trans illuminator was used to visualize the DNA fragments of different sizes separated in the gel (Fig. 1).

### Sequencing and analysis of the PCR products

The PCR products were purified using a total fragment DNA purification kit (iNtRON Biotechnology, Seongnam, Korea), following the manufacturer's instructions. The purified products were sequenced by Genotech Co. (Daejeon, Korea) using the designated primers. The base sequence was decoded by cycle sequencing, using the BigDye Terminator Kit (Thermo Fisher Scientific, WA, MA, USA). On completion of the PCR, the purified product was dissolved in sterile water or HDF (Hi-Di Formamide) and mounted on the device (ABI 3730xl DNA analyzer) for electrophoresis using capillary method. The sequences of the trnL-F region were compiled using SeqMan software (SeqMan 2, DNASTAR, Inc., WI, USA), and BioEdit program (BioEdit 7.2.5, Ibis Biosciences, CA, USA) was applied [8]. ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2/>) was used to perform multiple sequence alignments.

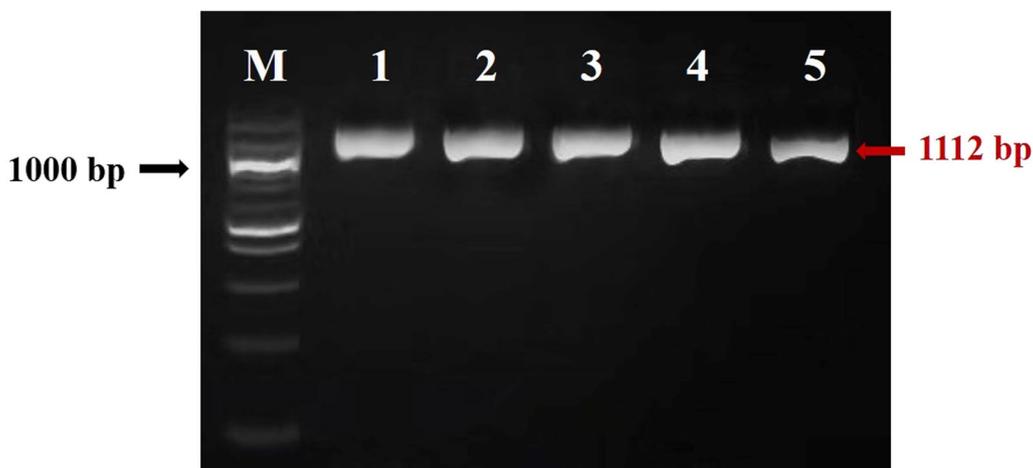
### Design and modification of specific primers

Specific primers, SimF and SimR, were designed according to the InDel site of Simbaek (Figs. 2, 3). A mismatch of the primer was incorporated into SimF by substituting A for G at the second base from the 3' end. Then, the corresponding reverse primer, SimR, was designed. The sequences and positions of the specific primers are listed in Table 1 and Fig. 3, respectively.

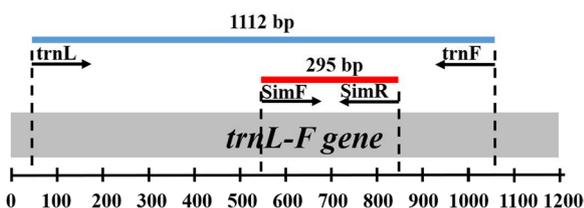
### Multiplex-specific PCR

Since the InDel site was found in the trnL-F sequences of the chloroplast gene of *H. syriacus*, the primer pair trnF and trnL was used as the universal primer set for the identification of all *Hibiscus* cultivars. SimF and SimR were designed and multiplex PCR was performed for the specific authentication of Simbaek from other *H. syriacus* cultivars and *H. sabdariffa*.

PCR was performed using 50 ng of DNA in a reaction volume of 20 μL that included 10 μL of Taq DNA



**Fig. 1** PCR products of five *Hibiscus* cultivars. Lane M: 100 kb DNA ladder; lane 1: samples of ‘Samchunli’ (*H. syriacus*); lane 2: samples of ‘Simbaek’ (*H. syriacus*); lane 3: samples of ‘Jinsun’; lane 4: samples of ‘Jamyung’ (*H. syriacus*); lane 5: samples of *H. sabdariffa*



**Fig. 2** Schematic diagrams of the PCR-amplified trnL-F region of *Hibiscus*. The PCR product amplified with universal primer sets, ‘trnL’ and ‘trnF’ and the PCR product amplified with Simbaek-specific primer, ‘SimF’ and ‘SimR’

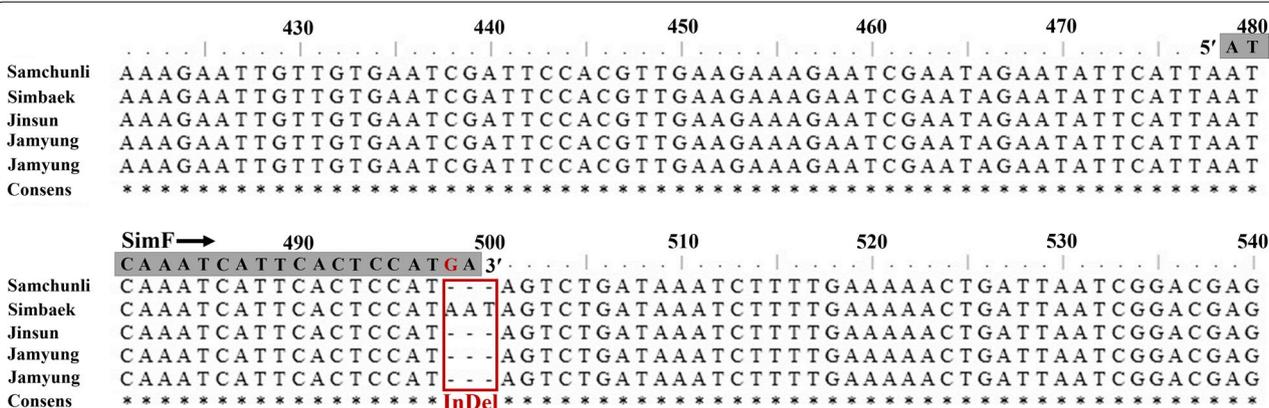
followed by 33 cycles of 30 s at 94 °C, 30 s at 56 °C, 40 s at 72 °C, and a final extension of 7 min at 72 °C.

### Results and discussion

#### Sequencing analysis of the PCR products

Since chloroplast genome sequences in plants evolve slowly and are highly conserved between species within a genus, they can be utilized for species identification and genetic diversity studies [17]. A trnL (UAA) intron and an intergenic spacer trnL (UAA)-trnF (GAA) are present in the trnL-F region of chloroplast DNA. Owing to its easy amplification, trnL-F has been proven to be a useful gene for the identification of many plants using primers homologous to its exons [17–19]. The trnL-F regions of the five cultivars, including 20 samples, were

polymerase (GeneAll Biotechnology Co., Seoul, Korea) and 0.5 μM of each primer (trnF, trnL, SimF, and SimR). The cycling conditions were as follows: 5 min at 94 °C,



**Fig. 3** Partial multiple alignments and primer binding sites of different *Hibiscus* cultivars. The arrow indicated the primer binding sites respective to each species and the box represents InDels

amplified and sequenced. The universal primer set for the trnL-F region produced amplified PCR products of approximately 1100 bp lengths (Fig. 1). The four trnL-F sequences have been deposited at NCBI with the accession numbers MZ127173 (Samchunli), MZ127174 (Simbaek), MZ127175 (Jinsun), and MZ127176 (Jamyung).

#### Using the InDel marker for *Hibiscus* cultivar identification

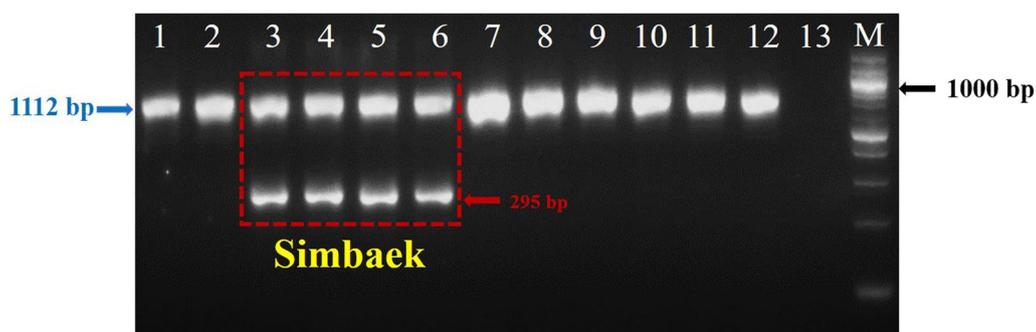
Although considerable development has been achieved in recent years, problems such as genetic pollution and variety degradation, in *Hibiscus* cultivation still persist, which have seriously affected the breeding and function-related studies of the plant. The accurate identification of different high-quality cultivars of *H. syriacus* is crucial for the cultivation of *Hibiscus* medicinal plants. DNA-based molecular markers, such as random amplified polymorphic DNA (RAPD) [5] and restriction fragment length polymorphism (RFLP) [20], have been used for the identification of plant and animal species. Although the complete chloroplast genome sequence of *H. syriacus* has been published [21], there are only a few reports on *Hibiscus* species-specific markers. Due to intra-species polymorphism between different cultivars of *Hibiscus*, developing the exact method to identify *Hibiscus* is essential for breeding and utilizing the plant resources. Recently, InDel, a new type of molecular marker regarded as an emerging technology for genomic identification, has been broadly applied in ecological studies [22]. InDel markers have characteristics, such as genetic inheritance and codominance, but to the best of our knowledge, there are no InDel markers developed for *Hibiscus*. Thus, to establish an economic and efficient method for the authentication of different *H. syriacus* cultivars, InDel genotyping using multiplex-specific PCR was performed. In view of the Simbaek cultivar-specific InDel sites explored in the trnL-F region, a pair of specific primers, SimF (5'-ATCAAATCATTCCTCCATGA-3') and

SimR (5'-AGGATACGCTTGTAATAATGGC-3'), were designed with specific mismatches (Fig. 2). As shown in Fig. 3, the substitution of G for A at the third base from the 3' terminus was deliberately introduced to ensure its absolute specificity and reliable discrimination between the alleles.

#### Authentication of Simbaek cultivar using multiplex-specific PCR

Specific primers, together with the universal primers of the trnL-F region, were used to differentiate the cultivars Samchunli, Simbaek, Jinseon, Jamyung, and *H. sabdariffa*, in multiple allele-specific PCR. As shown in Fig. 4, the DNA of different hibiscus cultivars cross-annealed with the specific and universal primers amplified into two fragment patterns. To determine the exact sequence and size of the multiplex PCR products, the amplicons were cloned and sequenced. A band at 295 bp was observed for Simbaek, indicating an insertion allele in the the trnL-F region from Simbaek, while only one band at 1112 bp was observed for the other cultivars. Two regions of the trnL-F gene from chloroplasts of different *H. syriacus* cultivars were amplified using specific and universal primers. Mismatches in the specific primers increased specificity of the PCR method and avoided non-specific amplification. Moreover, no cross-amplification or interference was observed. Taken together, the developed PCR assay ensured reliable authentication of Simbaek cultivar via simultaneous amplification of allele in a single reaction.

To ensure accuracy of results, the molecular identification system usually contains three conditions: primer versatility, sequence quality, and species distinction. However, for molecular marker research, the universality of primers is the most important prerequisite, because the region/site they bind to is unknown [23, 24]. This factor was also important in our research.



**Fig. 4** The PCR products in multiplex PCR using the primers, 'trnL', 'trnF', 'SimF', and 'SimR'. Lane M: 100 kb ladder, lanes 1–2: samples of 'Samchunli' (*H. syriacus*); lanes 3–6: four different samples of 'Simbaek' (*H. syriacus*); lanes 7–8: samples of 'Jinsun'; lanes 9–10: samples of 'Jamyung' (*H. syriacus*); lanes 11–12: samples of *H. sabdariffa*; lane 13: Negative control

For unknown samples, if the primers are not universal, PCR amplification may be affected because of the presence of plant secondary metabolites. Although our method provides a novel idea for the comprehensive exploration of the genetic information among different cultivars of *H. syriacus*, to understand the ecology, distribution, and cultivar evolution of *H. syriacus*, further studies focusing on the relationship between gametophyte and genetic traits are required. Further, the method developed in this study could precisely identify heterozygotes at the locus from homozygotes for either allele without requiring restriction enzyme digestion. In addition, the results of this method are consistent with those of allele assignments derived from direct sequencing of PCR products, thereby highlighting the simplicity and reliability of our method.

In conclusion, we developed a reliable and effective method to identify the Korean and American Hibiscus cultivars, using multiplex-specific PCR. By introducing mismatches in the specific primers, dimers, hairpins, and false-positive results due to faulty primers were successfully prevented. The method does not require sequence analysis of the PCR products; instead, the products can be conveniently evaluated using conventional gel electrophoresis. Furthermore, the molecular markers developed in this study can be widely applied in the identification of medicinal plants without being influenced by the growth stage and environmental conditions.

#### Abbreviations

InDel: Insertion/deletion; PCR: Polymerase chain reaction; RAPD: Random amplified polymorphic DNA; RFLP: Restriction fragment length polymorphism.

#### Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13765-021-00669-4>.

**Additional file 1: Fig. S1.** Cytotoxic effect of Simbaek callus extract against Human Colorectal Adenocarcinoma Cell Line (HT-29 cell line).

#### Acknowledgements

This work was supported by Gyeonggido Business & Science Accelerator (GBSA) Grant funded by the Gyeonggi provincial government, Republic of Korea.

#### Authors' contributions

RW: methodology, writing—original draft. SYP: sample preparation, SWP: funding acquisition. AMP: investigation. Y-JK: supervision, project administration. All authors read and approved the final manuscript.

#### Funding

No funding was received for the current study.

#### Availability of data and materials

Not applicable.

#### Declarations

##### Ethics approval and consent to participate

Not applicable.

##### Consent for publication

Not applicable.

##### Competing interests

The authors declare that there is no conflict of interest.

##### Author details

<sup>1</sup>Graduate School of Biotechnology, and College of Life Science, Kyung Hee University, Yongin, Gyeonggi-do 17104, Republic of Korea. <sup>2</sup>SD Biotechnologies Co., Ltd, Ganseo-gu, Seoul 07793, Republic of Korea.

Received: 6 October 2021 Accepted: 23 December 2021

Published online: 31 December 2021

#### References

- Hsu RJ, Hsu YC, Chen SP, Fu CL, Yu JC, Chang FW, Chen YH, Liu JM, Ho JY, Yu CP (2015) The triterpenoids of *Hibiscus syriacus* induce apoptosis and inhibit cell migration in breast cancer cells. *BMC Complement Altern Med* 15:1–9. <https://doi.org/10.1186/s12906-015-0592-9>
- Molagoda IMN, Lee KT, Choi YH, Kim GY (2020) Anthocyanins from *Hibiscus syriacus* L. inhibit oxidative stress-mediated apoptosis by activating the Nrf2/HO-1 signaling pathway. *Antioxidants* 9(1):42–60. <https://doi.org/10.3390/antiox9010042>
- Kim YH, Im A, Park BK, Paek SH, Choi G, Kim YR, Whang WK, Lee KH, Oh SE, Lee MY (2018) Antidepressant-like and neuroprotective effects of ethanol extract from the root bark of *Hibiscus syriacus* L. *Biomed Res Int*. <https://doi.org/10.1155/2018/7383869>
- Kwon SW, Hong SS, Kim JI, Ahn IH (2003) Antioxidant properties of heat-treated *Hibiscus syriacus*. *Biol Bull Russ Acad Sci* 30:15–16
- Barik S, Senapati SK, Aparajita S, Mohapatra A, Rout GR (2006) Identification and genetic variation among *Hibiscus* species (Malvaceae) using RAPD markers. *Zeitschrift für Naturforsch C* 61:123–128. <https://doi.org/10.1515/znc-2006-1-222>
- Mondini L, Noorani A, Pagnotta MA (2009) Assessing plant genetic diversity by molecular tools. *Diversity* 1:19–35. <https://doi.org/10.3390/d1010019>
- Newman TL, Tuzun E, Morrison VA, Hayden KE, Ventura M, McGrath SD, Rocchi M, Eichler EE (2005) A genome-wide survey of structural variation between human and chimpanzee. *Genome Res* 15:1344–1356. <https://doi.org/10.1101/gr.4338005>
- Mills RE, Luttig CT, Larkins CE, Beauchamp A, Tsui C, Pittard WS, Devine SE (2006) An initial map of insertion and deletion (INDEL) variation in the human genome. *Genome Res* 16:1182–1190. <https://doi.org/10.1101/gr.4565806>
- Lovett ST (2004) Encoded errors: mutations and rearrangements mediated by misalignment at repetitive DNA sequences. *Mol Microbiol* 52:1243–1253. <https://doi.org/10.1111/j.1365-2958.2004.04076.x>
- Mullaney JM, Mills RE, Pittard WS, Devine SE (2010) Small insertions and deletions (INDELs) in human genomes. *Hum Mol Genet* 19:R131–R136. <https://doi.org/10.1093/hmg/ddq400>
- Terakami S, Matsumura Y, Kurita K, Kanamori H, Katayose Y, Yamamoto T, Katayama H (2012) Complete sequence of the chloroplast genome from pear (*Pyrus pyrifolia*): genome structure and comparative analysis. *Tree Genet genomes* 8:841–854. <https://doi.org/10.1186/s40064-016-3702-3>
- Wu DH, Wu HP, Wang CS, Tseng HY, Hwu KK (2013) Genome-wide InDel marker system for application in rice breeding and mapping studies. *Euphytica* 192:131–143
- Păcurar DJ, Păcurar ML, Street N, Bussell JD, Pop TI, Gutierrez L, Bellini C (2012) A collection of INDEL markers for map-based cloning in seven *Arabidopsis* accessions. *J Exp Bot* 63:2491–2501. <https://doi.org/10.1093/jxb/err422>
- Tian XR, Lv SX, Tian HL, Wang RB, Wang HT (2020) Development of an accurate and reliable DNA method for botanical origin authentication of

- ginseng food products. *J Food Compos Anal*. <https://doi.org/10.1016/j.jfca.2020.103419>
15. Liu B, Wang Y, Zhai W, Deng J, Wang H, Cui Y, Cheng F, Wang X, Wu J (2013) Development of InDel markers for *Brassica rapa* based on whole-genome re-sequencing. *Theor Appl Genet* 126:231–239. <https://doi.org/10.1007/s00122-012-1976-6>
  16. Landegren U, Nilsson M, Kwok PY (1998) Reading bits of genetic information: methods for single-nucleotide polymorphism analysis. *Genome Res* 8(8):769–776. <https://doi.org/10.1101/gr.8.8.769>
  17. Androsiuk P, Jastrzębski JP, Pauksto Ł, Makowczenko K, Okorski A, Pszczółkowska A, Chwedorzewska KJ, Górecki R, Giełwanowska I (2020) Evolutionary dynamics of the chloroplast genome sequences of six *Colobanthus* species. *Sci Rep* 10:1–14. <https://doi.org/10.1038/s41598-020-68563-5>
  18. Aagesen L, Medan D, Kellermann J, Hilger HH (2005) Phylogeny of the tribe Colletieae (Rhamnaceae)—a sensitivity analysis of the plastid region trn L-trn F combined with morphology. *Plant Syst Evol* 250:197–214
  19. Lang P, Dane F, Kubisiak TL (2006) Phylogeny of *Castanea* (Fagaceae) based on chloroplast trn TLF sequence data. *Tree Genet Genomes* 2:132–139. <https://doi.org/10.1007/s11295-006-0036-2>
  20. Silva AJ, Hellberg RS (2020) DNA-based techniques for seafood species authentication. *Adv Food Nutr Res* 95:207–255. <https://doi.org/10.1016/bs.afnr.2020.09.001>
  21. Kwon HY, Kim JH, Kim SH, Park JM, Lee H (2016) The complete chloroplast genome sequence of *Hibiscus syriacus*. *Mitochondrial DNA A DNA Mapp Seq Anal* 27(5):3668–3669. <https://doi.org/10.3109/19401736.2015.1079847>
  22. Gull S, Haider Z, Gu H, Raza Khan RA, Miao J, Wenchen T, Uddin S, Ahmad I, Liang G (2019) InDel marker based estimation of multi-gene allele contribution and genetic variations for grain size and weight in rice (*Oryza sativa* L.). *Int J Mol Sci* 20(19):4824. <https://doi.org/10.3390/ijms20194824>
  23. Valentini A, Pompanon F, Taberlet P (2009) DNA barcoding for ecologists. *Trends Ecol Evol* 24:110–117. <https://doi.org/10.1016/j.tree.2008.09.011>
  24. Chariton A (2012) Short and informative DNA products to indirectly measure vascular plant biodiversity. *Mol Ecol* 21(15):3637–3639. <https://doi.org/10.1111/j.1365-294X.2012.05633.x>

### Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Submit your manuscript to a SpringerOpen<sup>®</sup> journal and benefit from:

- Convenient online submission
- Rigorous peer review
- Open access: articles freely available online
- High visibility within the field
- Retaining the copyright to your article

---

Submit your next manuscript at ► [springeropen.com](https://www.springeropen.com)

---