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일천궁과 토천궁의 구별을 위한 InDel 마커의 개발

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Development of Insertions and deletions (InDel) Markers for the Differentiation of *Cnidium officinale* Makino and *Ligusticum chuanxiong* Hort.

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ABSTRACT

Background: In Korea, the medicinal herb Cnidii Rhizoma is manufactured using *Cnidium officinale* Makino and *Ligusticum chuanxiong* Hort., with the former being the primary ingredient. *C. officinale* and *L. chuanxiong* have similar external appearances, and because their roots are dried for use as medicinal materials, it is difficult to distinguish between them. Therefore, there is a need to develop easy-to-use molecular markers to facilitate the classification of Cnidii Rhizoma varieties and the identification of medicinal materials.
Methods and Results: DNA was extracted from *C. officinale* and *L. chuanxiong* and analyzed using an Illumina HiSeq 2500 platform. After *de novo* assembly, the loci showing polymorphisms between *C. officinale* and *L. chuanxiong* were identified. Primers were designed for polymorphic loci, and genotyping was performed. Finally, three nuclear genome-based insertion and deletion (InDel) markers, CoInDel-01, CoInDel-02, and CoInDel-03, were developed. These markers produced a single band at 230, 369, and 245 bp, respectively, in *C. officinale*, whereas two bands were observed in *L. chuanxiong* at 230 and 264 bp, 304 and 369 bp, and 197 and 245 bp, respectively.
Conclusion: The three InDel markers developed in this study will be useful for differentiating between *C. officinale* and *L. chuanxiong*.

Key Words: *Cnidium officinale*, Genotyping, Insertion and Deletion, *Ligusticum chuanxiong*, Next Generation Sequencing

INTRODUCTION

Cnidium officinale Makino (Synonyms: *Ligusticum officinale* (Makino) Kitagawa, *Conioselinum officinale* (Makino) Ohashi & Ohashi) (Kitagawa, 1963; Ohashi and Ohashi, 2023), a perennial herb of Apiaceae, occurs in Korea, and has been widely cultivated in East Asia for a long time as medicinal plant. It was written as '川芎' in Chinese, the old East Asia character, and is called 'Cheongung' in Korean and 'Senkyu' in Japanese. In China, '川芎' is called Chuanxiong, which refers to *Ligusticum chuanxiong* (Yoon *et al*., 2023; Song *et al*., 2024).

According to the Korean Pharmacopoeia (MFDS, 2022), Cnidii Rhizoma is primarily made from the root stems of *C. officinale* or *L. chuanxiong* Hort. *ex Qiu, et al.*

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Cnidii Rhizoma have been used in the treatment of headaches, anemia, pain, and gynecological disorders, and their medicinal effects are being re-evaluated in modern medicine (Jeong *et al*., 2015). In particular, research on its antioxidant, anti-inflammatory, and antimicrobial effects is actively ongoing (Jeong *et al*., 2009; Um *et al*., 2017; Jeong *et al*., 2020; Lee *et al*., 2021). The major bioactive compounds found in Cnidii Rhizoma include cnidilide, ligustilide, and senkyunolide (Song *et al*., 2009).

Cnidii Rhizoma is referred to as *C. officinale* or *L. chuanxiong*, depending on the source plant (Song *et al*., 2009; Kim *et al*., 2020a). While *C. officinale* and *L. chuanxiong* are known to belong to the same lineage, there is controversy regarding the origin plant due to the indistinct differences between *C. officinale* and the others (Park, 1998; Song *et al*., 2009). *C. officinale* and *L. chuanxiong* have similar plant appearances, making it difficult to distinguish them by sight (Lee *et al*., 1999; Jung *et al*., 2019).

C. officinale cultivated in Korea, its stems are softer, with several small tubers fused together to form a circular shape, in contrast to *L. chuanxiong* (Kim *et al*., 2020b). *L. chuanxiong* is cultivated and distributed in Korea, but its botanical classification remains unclear, as it belongs to the genus *Angelica* (Suh *et al*., 2016; Kim *et al*., 2020b). Morphologically, it has rhizomes that develop into thin, fibrous root-like structures and grows larger than *C. officinale* (Jung *et al*., 2019; Kim *et al*., 2020a).

These two species have similar appearances, making accurate identification necessary. Furthermore, with the increasing demand for medicinal herbs, imports have risen, leading to confusion in the domestic distribution process. In the distribution process, issues have arisen due to unclear origins or similar names of medicinal plants (Han *et al*., 2015).

To address these issues, accurate identification, effective differentiation, and quality control procedures for medicinal plants are required. Recently, with the development of next generation sequencing (NGS) technology, Illumina, PacBio, Nanopore, etc., large amounts of data can be generated quickly and analyzed at a low cost. This enables the comparison of genetic information across different species, allowing for the study of the evolutionary relationships of plants (Han *et al*., 2015).

DNA molecular markers are used to identify or differentiate organisms at the molecular level based on their nucleotide sequences (Huh, 2015). DNA molecular markers include SNP (single nucleotide polymorphism), InDel (insertion and deletion), SSR (simple sequence repeat), RAPD (random amplified polymorphic DNA), RFLP (restriction fragment length polymorphism), among others (Al-Samarai *et al*, 2015). The appropriate marker is selected depending on the purpose of the research.

To date, molecular markers developed for Cnidii Rhizoma include studies on RAPD and RFLP markers, and an SNP marker has been developed in the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene (Ji *et al*, 2017). However, there are no reported cases of InDel marker development (Song *et al*., 2009; Kim *et al*., 2014). InDel markers can be easily detected through PCR (polymerase chain reaction), offering the advantage of simple analysis by comparing the size of the amplified products using gel electrophoresis (Kim *et al*., 2022; Kim *et al*., 2023).

This study aimed to clearly differentiate *C. officinale* and *L. chuanxiong* by developing InDel markers based on the sequences obtained using Illumina HiSeq 2500 platform from the plants cultivated in Korea.

Materials and Methods

1. Materials

In this experiment, young leaves from a total of 11 individuals, including 8 *C. officinale* and 3 *L. chuanxiong* cultivated in Korea, were collected and used (Table 1 and Fig. 1).

The leaves of *C. officinale* are lighter green and the mesophyll is thinner (Fig. 1A) than that of *L. chuanxiong* (Fig. 1B). The flower of *C. officinale* is a compound umbel and consists of about 10 to 13 inflorescences, with about 10 to 15 flowers per inflorescence (Fig. 1C), whereas the flower of *L. chuanxiong* is a compound umbel and consists of about 20 inflorescences, with about 25 to 30 flowers per inflorescence (Fig. 1D). The rhizome of *C. officinale* enlarges and forms a bulb (Fig. 1E), but that of *L. chuanxiong* develops thin and long (Fig. 1F).

2. DNA extraction

DNA extraction from the 11 individuals of *C. officinale* and *L. chuanxiong* was performed using the Biomedic® Plant gDNA Extraction Kit (Biomedic Co., Ltd., Bucheon, Korea) according to the manufacturer's instructions. DNA concentration was measured using NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and adjusted to 10 ng/$\mu\ell$ before storage at –20℃.

Table 1. List of 8 *C. officinale* and 3 *L. chuanxiong* genetic resources used in this study.

No	Sample name	Species	Collection date	Collection sites
1	Co2401_1	*C. officinale*	May 24, 2024	Chungbuk Eumseong[1)] 36°56'36"N 127°45'13"E
2	Co2402_1	*C. officinale*	May 24, 2024	Chungbuk Eumseong 36°56'36"N 127°45'13"E
3	Co2403_1	*C. officinale*	May 24, 2024	Chungbuk Eumseong 36°56'36"N 127°45'13"E
4	Co2405_1	*C. officinale*	May 24, 2024	Chungbuk Eumseong 36°56'36"N 127°45'13"E
5	Co2410_1	*C. officinale*	May 24, 2024	Chungbuk Eumseong 36°56'36"N 127°45'13"E
6	Co2411_1	*C. officinale*	May 24, 2024	Chungbuk Eumseong 36°56'36"N 127°45'13"E
7	Co2413_1	*C. officinale*	May 24, 2024	Chungbuk Eumseong 36°56'36"N 127°45'13"E
8	Co2414_1	*C. officinale*	May 24, 2024	Chungbuk Eumseong 36°56'36"N 127°45'13"E
9	Co2415_1	*L. chuanxiong*	May 24, 2024	Chungbuk Eumseong 36°56'36"N 127°45'13"E
10	Co2415_2	*L. chuanxiong*	May 24, 2024	Chungbuk Eumseong 36°56'36"N 127°45'13"E
11	Co2415_3	*L. chuanxiong*	May 24, 2024	Chungbuk Eumseong 36°56'36"N 127°45'13"E

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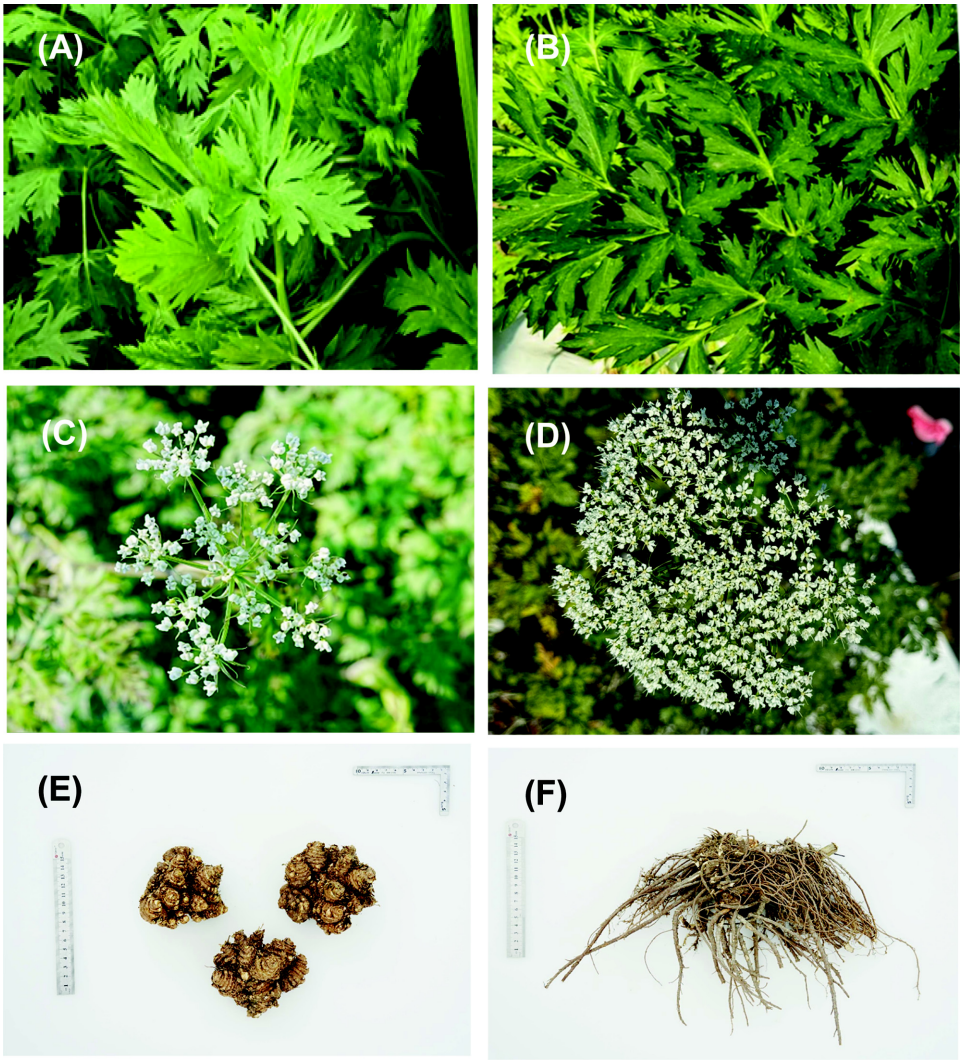

Fig. 1. Photograph of *C. officinale* (A, C, E) and *L. chuanxiong* (B, D, F). (A, B); Young leaves, (C, D); Flowers, (E, F); Rhizome.

3. Next-generation sequencing (NGS) analysis

Next-generation sequencing (NGS) analysis was performed on one individual of *C. officinale* (Co2401_1) and one individual of *L. chuanxiong* (Co2415_1), with paired-end libraries constructed using the TruSeq Nano DNA Sample Prep Kit according to the manufacturer's manual (Illumina, Inc., San Diego, CA, USA). Paired-end sequencing with a 151-bp read length conducted using the Illumina HiSeq 2500 platform (Illumina Inc., San Diego, CA, USA).

The raw data obtained from the NGS results were processed to improve quality, and the preprocessed reads were assembled *de novo* using the CLC Genomics Workbench ver. 20.0 (Qiagen, Aarhus, Denmark) software to obtain contigs.

4. InDel region identification and primer design

The contigs over than 10 kbp were selected from the assembled contigs of *C. officinale* were compared to the corresponding contigs of *L. chuanxiong* using alignment function of the CLC Genomics Workbench ver. 20.0 software to identify polymorphic InDel regions, and primers were designed for these regions using the same software.

The primer design conditions were as follows: length of 18 bp to 22 bp, annealing temperature of 48℃ to 60℃, GC content between 30% and 60%, and product size ranging from 150 bp to 450 bp.

5. Polymerase chain reaction (PCR) analysis

The composition of the PCR reaction mixture consisted of 1 ㎕ of gDNA, 1 ㎕ each of the forward and reverse primers, 10 ㎕ of 2 × Taq mix (Dongsheng Biotech Co, Ltd, Guangzhou, Guangdong, China) and 7 ㎕ of distilled water, for a total volume of 20 ㎕.

Amplification was carried out using a T100™ Thermal Cycler (Bio-Rad Inc., Hercules, CA, USA). The PCR conditions were as follows: pre-denaturation at 95℃ for 3 minutes, followed by 35 cycles of denaturation at 95℃ for 30 seconds, annealing at 54℃ or 55℃ for 30 seconds, and extension at 72℃ for 1 minute. A final extension was performed at 72℃ for 5 minutes. The PCR products were stored at –20℃.

6. Electrophoresis

Electrophoresis was performed to determine the genotypes of the PCR amplification products. The agarose gel used was prepared with 1 × TAE buffer and agarose at a concentration of 3%. DNA visualization was achieved using EtBr (ethidium bromide).

As a size marker, the 1 kb Ladder Plus (Dongsheongbio Company Ltd., Guangdong, China) was used to determine the size of the amplified products.

Electrophoresis was conducted for 30 minutes at 120 V using the Mini-sub Cell GT System (Bio-Rad Inc., Hercules, CA, USA), and the bands were visualized using the Gel Doc™ XR+ System (Bio-Rad Inc., Hercules, CA, USA).

7. Mapping of NGS data to the amplicons of three markers

The NGS data produced from *L. chuanxiong* were trimmed using the CLC Genomics Workbench ver. 20.0 (Qiagen, Aarhus, Denmark) software and then mapped to the nucleotide sequences amplified by each marker.

Mapping was performed using the following parameters (Match score = 1; Mismatch cost = 2; Cost of insertions and deletions = Linear gap cost; Insertion cost = 3; Deletion cost = 3; Length fraction = 0.8; Similarity fraction = 0.9; Non-specific match handling = Map randomly; Minimum seed length = 15).

RESULTS

1. NGS analysis and InDel region exploration results

According to the NGS analysis, the total read size of *C. officinale* (Co2401_1) was 68,358,861,794 bp, with a total of 452,707,694 reads, and the GC content was 36.4%. The total read size of *L. chuanxiong* (Co2415_1) was 51,704,152,808 bp, with a total of 342,411,608 reads, and the GC content was 36.8%.

A comparative analysis of the DNA sequences of *C. officinale* and *L. chuanxiong* was conducted to explore the regions exhibiting polymorphism. A total of 20 candidate InDel loci were selected, and primers were designed.

PCR analysis was performed to observe whether the designed primer sets amplified correctly and to assess the genotypes, and polymorphism was analyzed through gel electrophoresis. The electrophoresis genotyping results revealed the development of three InDel markers capable of distinguishing between *C. officinale* and *L. chuanxiong,* which were named CoInDel-01, CoInDel-02, and CoInDel-03 (Table 2).

2. Genotyping using developed InDel markers

Genotyping of a total of 11 individuals, including 8 *C. officinale* (Co2401-1, Co2402-1, Co2403-1, Co2405-1, Co2410-1, Co2411-1, Co2413-1, Co2414-1) and 3 *L. chuanxiong* (Co2415-

Table 2. Marker developed to distinguish between the *C. officinale* and *L. chuanxiong*.

No.	Marker name	Primer sequence (5´ → 3´)	Amplicon size (bp)	Annealing temperature (℃)
1	CoInDel-01	F : TAAAAACTCCCGTGCACT R : ATAGCATTCCCTTAGCCC	230, 269	55
2	CoInDel-02	F : TGAAGTGGTGAATTGGATAG R : GGGGCAGAAATAACAAAAAG	304, 369	54
3	CoInDel-03	F : ACTCTACTTTCCCAACATAC R : CCAATACTCTTCCGATTTCA	197, 245	55

1, Co2415-2, Co2415-3), was performed using the CoInDel-01 marker. The results showed a 230 bp band in all *C. officinale* individuals, while the *L. chuanxiong* individuals exhibited both a 230 bp band and an additional 269 bp band (Fig. 2A).

Genotyping using the CoInDel-02 marker showed that a single band appeared at 369 bp in all *C. officinale* individuals (Co2401-1, Co2402-1, Co2403-1, Co2405-1, Co2410-1, Co2411-1, Co2413-1, Co2414-1), while in the *L. chuanxiong* individuals (Co2415-1, Co2415-2, Co2415-3), an additional band was observed at 304 bp along with the 369 bp band (Fig. 2B).

Genotyping using the CoInDel-03 marker showed that a single band was amplified at 245 bp in all *C. officinale* individuals (Co2401-1, Co2402-1, Co2403-1, Co2405-1, Co2410-1, Co2411-1, Co2413-1, Co2414-1), while in the *L. chuanxiong* individuals (Co2415-1, Co2415-2, Co2415-3), two bands were amplified at 245 bp and 197 bp (Fig. 2C).

In all InDel markers, a single band (230 bp, 369 bp, 245 bp) was observed in *C. officinale,* while two bands (269 bp/230 bp, 369 bp/304 bp, 245 bp/197 bp) were observed in *L. chuanxiong*. Each analysis using the three InDel markers clearly distinguished between *C. officinale* and *L. chuanxiong.*

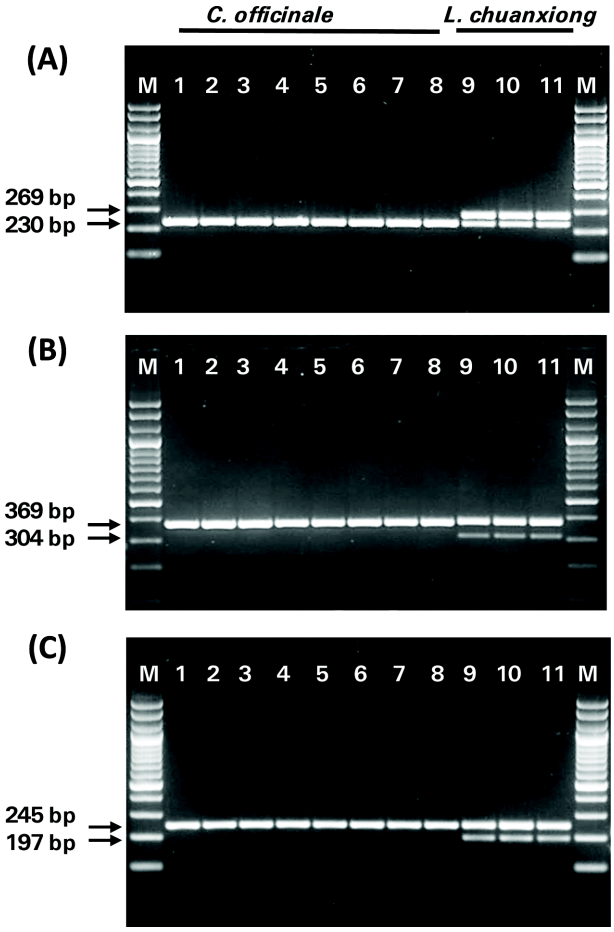

Fig. 2. Results of electrophoresis performed on 11 genetic resources using 3 InDel markers for the distinguishment of *C. officinale* and *L. chuanxiong*. (A); CoInDel-01, (B); CoInDel-02, and (C); CoInDel-03, Lane M; size marker GB-Ruler™ 100 bp Plus DNA Ladder (GeneBio Systems, Montreal, QC, Canada), Lane 1; Co2401-1, Lane 2; Co2402-1, Lane 3; Co2403-1, Lane 4; Co2405-1, Lane 5; Co2410-1. Lane 6; Co2411-1, Lane 7; Co2413-1, Lane 8; Co2414-1, Lane 9; Co2415-1, Lane 10; Co2415-2, Lane 11; Co2415-3.

DISCUSSION

Existing markers have been used to distinguish between *C. officinale* and *L. chuanxiong,* but there are limitations with these markers. For example, RAPD and RFLP markers suffer from reproducibility issues, and SNP markers developed in the ITS region have limited application issue (Ji *et al*, 2017). Therefore, the development of additional DNA molecular markers was necessary.

In this study, we developed InDel markers that can easily distinguish between *C. officinale* and *L. chuanxiong*. InDel markers can effectively differentiate plant species (Garcia-Lor *et al*. 2013; Yamaki *et al.* 2013; Cho *et al*. 2015; Kim *et al*. 2021). Examples of species or cultivar identification using InDel markers include the differentiation of *Schisandra* (Jeong *et al*., 2021), differentiation of domestic jujube varieties (Kim

Table 3. Read number and coverage of *L. chuanxiong* NGS data mapped to the amplicon sequences amplified by the markers.

Markers	Common amplicon			*L. chuanxiong* specific amplicon			Ratio[1)]
	Amplicon size (bp)	Read number	Coverage	Amplicon size (bp)	Read number	Coverage	
CoInDel-01	230	11	6.36	269	5	3.53	1.80
CoInDel-02	369	25	8.75	304	9	4.09	2.14
CoInDel-03	245	19	8.05	197	10	3.37	2.39

[1)]Ratio; coverage of common amplicon / coverage of *L. chuanxiong* specific amplicon.

et al., 2021), distinction between *Prunus mume*, *Prunus armeniaca* var. *ansu*, and their interspecific hybrids (Kim *et al*., 2022), differentiation between *Paeonia* (Lee *et al*., 2022), identification of *Codonopsis* species (Kim *et al*., 2023), and identification of plants in the genus *Broussonetia* (Lee *et al*., 2023).

In this study, 8 *C. officinale* individuals and 3 *L. chuanxiong* individuals were analyzed using InDel markers through PCR and gel electrophoresis. The results showed that all three markers clearly distinguished between *C. officinale* and *L. chuanxiong*.

Through additional analysis, the Illumina sequencing data of *L. chuanxiong* (Co2415-1) produced in this study were mapped to the sequences amplified by each marker. The ratio of the coverage of common amplicons to the coverage of *L. chuanxiong*-specific amplicons was calculated for the three markers developed in this study. The ratios for CoInDel-01, CoInDel-02, and CoInDel-03 were 1.80, 2.14, and 2.39, respectively (Table 3). This ratio can be seen as the coverage of the amplicon commonly found in *C. officinale* and *L. chuanxiong* being about twice the coverage of the amplicon appearing only in *L. chuanxiong*.

This suggests that the observed 2:1 ratio could be due to *L. chuanxiong* being a triploid with two chromosomes sharing the same sequence as *C. officinale* and one chromosome with a different sequence. A chi-square test for goodness of fit, based on the number of reads mapped to each locus, confirmed that the read distribution for all three markers followed a 2 : 1 ratio.

These results in line with previous report that *C. officinale* is a diploid plant (2n = 22) and *L. chuanxiong* is a triploid plant (2n = 33) (Song *et al*., 2021; Suh *et al*., 2016) and the assumption that *L. chuanxiong* may be a hybrid that includes the genome of *C. officinale* (Kondo *et al*., 1996).

Through the CoInDel-01, CoInDel-02, and CoInDel-03 markers developed in this study, it is now possible to genetically distinguish between *C. officinale* and *L. chuanxiong,* which were previously difficult to differentiate.

This will help prevent confusion in the market and provide a foundation for molecular marker-based research in origin studies. Furthermore, it is expected that these markers can be used in the development of superior varieties of *C. officinale* and *L. chuanxiong*.

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