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Review

The application of DNA molecular markers in the study of Codonopsis species genetic variation, a review

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Abstract: *Codonopsis* genus is comprised of species that are perennial plants primarily distributed across all east, southeast, and Central Asia. The most famous species of *Codonopsis* are C. *tangshen*, *C. lanceolate*, and *C. pilosula*. The records showed that they have a long story usage as traditional Chinese medicines, as they were alleged to be able to intensify the spleen and the lung as well as enriching blood and engendering liquid. Certain species have a culinary value in southern China and Southeast Asia, where they are considered as tea, wine, soup, plaster, and porridge. *Codonopsis* species were shown to be of great importance in medicine, due to their broad biological activity. Therefore, a clear understanding of their genetic diversity is needed. Adequate distinctions and descriptions of those species are necessary to preserve plant reservoir, investigations of genes associated with desirable traits, and understanding of evolutionary relationships. Subsequently, various molecular marker techniques such as Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeats (SSR), and Inter Simple Sequence Repeat (ISSR), Single Nucleotide Polymorphism (SNP), internal transcribed spacer (ITS), and Sequence-Characterized Amplified Region (SCAR) have been improved to provide detailed informations about genomes, that historically were not possible to obtain based on only phenotypic methods. This review represents the usage of DNA molecular markers for molecular diversity analysis of medically important species belonging to the genus *Codonopsis*.

Key words: *Codonopsis* species; DNA Molecular markers; Genetic diversity.

Introduction

The genus *Codonopsis,* is a dicotyledonous genus of herbaceous perennial plants belonging in *Campanulaceae* family, and it has 42 species commonly found in central, east, and south Asia; 40 *Codonopsis* species are located in China (1). It is one of the greatest Chinese medicinal plant widely used as a tonic agent (2). Pollen ultrastructure research has shown that *Campanulaceae* basal groups are *Platycodon, Codonopsis,* and *Cyananthus* (3). The genus of Codonopsis comprises three subgenera which are *Codonopsis*, *Pseudocodonopsis*, and *Obconicicapsula* (4). There are many different kinds of *Codonopsis* species, which are mainly cultivated in China (Shanxi, Gansu, Shaanxi, Sichuan, Hubei) and other provinces (5). In southern China and Southeast Asia, some species are also utilized as food items serving as tea, wine, soup, plaster, and porridge (6).

Importance of *Codonopsis* as a medicinal plant

Within the *Codonopsis* genus, *Codonopsis pilosula* (Franch.) *Nannf.*, *C. pilosula Nannf. var. modesta (Nannf.) L. D. Shen, C. tangshen Oliv.* and C. l*anceolata* (Sieb. et Zucc.) Benth. & Hook. f. ex Trautv. are the only species commonly utilized, in particular, their fresh or dried roots are generally considered as popular plant medicines and have been used in traditional medicine for centuries (7). *Codonopsis Radix* is recommended as the dried roots of *C. pilosula, C. pilosula var. modesta,* and C. *tangshen* in the Chinese Pharmacopoeia (8). It is known as "*Dangshen*" in Chinese and "*Tojin*" in Japanese. The researches have reported that the main phytochemicals compounds of *Codonopsis* species are triterpenoids, polysaccharides (9), phenylpropanoids, alkaloids, and polyacetylenes and they play a significant role in numerous bioactivity reactions (10). Hence, they are extensively utilized in traditional medicine and are regarded as having multiple medicinal characteristics (6). *Codonopsis radix* has long historical usage as traditional Chinese medicine (TCM) for regenerating qi deficiency, boosting the immune system, enhancing poor gastrointestinal mechanism, gastric ulcer, and appetite, reducing blood pressure, etc (11). It was frequently used as antitumor, antimicrobial, and antioxidant, and for cell immunity improvement (12). Three unique polyynes were extracted from the cultivated *C. pilosula*, and their

biological assessment indicated that they can suppress the expression of the squalene monooxygenase gene found in HepG2 cells, also these molecules might be implicated in lipid metabolism (13). *C. pilosula* major components are Sterol, triterpenes, glycosides, alkaloids, polysaccharides (14). A couple of hundred years ago, the effectiveness and safety of *C. pilosula* for the therapy of chronic obstructive lung disease have been successively evaluated (15), and these have led to its utilization for treating patients having that disease (16). Occasionally, its roots can be used as a replacement for ginseng therapy (2). Lately, a lot of studies concerning bioactivity of *C. pilosula* polysaccharides have shown that it has a potential role in cancer treatment and immunoregulation (14). Preliminary in vitro immunological studies showed that *C. pilosula* polysaccharides (CPPs) have a powerful stimulating impact of murine lymphocyte proliferation (17). Polysaccharides extracted from *C. pilosula* cultivated in Gansu province have been appeared to have a functional property of encouraging spleen cells to produce antibodies and to prevent the production of serum hemolysis in ordinary mice while restoring serum antibody concentrations and antibodies to spleen cells in immunosuppressed mice (12), and it could be a promising therapeutic candidate for Alzheimer's disease (18). Plant medicines play a significant role as a substitutes for manufactured pharmaceuticals, reaching $115 billion by 2020 (19). The supply for herbal medicines is growing daily because they have a greater safety margin than synthetic drugs, therefore, characterizing medicinal herbs is an essential issue (20). Most governmental organizations and pharmacopeias recommend various techniques such as macroscopic, microscopic and spectroscopic evaluation, unfortunately, these methods have limitations due to comparable morphological characteristics and cell types, but molecular markers are different because they usually referred to biochemical, phenotype and genotype components (21).

A great majority of molecular approaches were used to validate medicinal herbs depending on species-specific differences in the nuclear DNA and chloroplast sequences of various locations (22), and their advancement to detect and exploit polymorphism has a crucial role in plant breeding studies (23). The benefits of molecular authenticated herbal medicinal products are well recognized and were first included in the pharmacopeia of the people's republic of china (24). Several study institutes around the world are now associated with comprehensive studies on genomic molecular markers that are used effectively to identify and analyze herbal medicines (25).

Molecular markers techniques for studying the genetic heterogeneity of Codonopsis species

For a long time, the research on *Codonopsis* mainly focused on cultivation techniques, pharmacological effects, and physicochemical properties. While genetically, the diversity and the relationship of its germplasm resources were less studied. Presently, with the advancement of molecular biology especially the wide application of PCR technology, molecular marker technology has become an important means to study plant classification, genetic diversity, and their relationships (26). In this study, we review the molecular markers techniques for studying the genetic heterogeneity of Codonopsis species according to Fig. 1.

Definition of molecular markers

Molecular markers are genetic loci that can be easily tracked and quantified in a population and may be related to a specific gene or trait of interest (27). It can be defined as a variation that can be observed as a result of mutation or alteration in the genomic loci (28). A genetic marker might be a short sequence of DNA, such as a sequence of a single base-pair shift (single nucleotide polymorphism, SNP), or a long sequence, such as mini & microsatellites (29).

Categories of molecular markers

Molecular markers can be classified into three groups: (1) hybridization-based markers, such as restriction fragment length polymorphism (RFLP), (2) PCR-based markers such as (RAPD), (AFLP), and microsatellites or (SSR), (3) sequence-based markers, for instance (SNP) (30). Subsequently, these can be divided into three groups according to the throughput and detection technique: (1) low-throughput and hybridization are also known as first-generation markers such as RFLP, (2) medium-throughput and PCR-based also known as second-generation markers including RAPD, AFLP and SSR markers, and (3) high throughput and sequence-based markers also known as third-generation markers like SNP marker (31). These groups of markers can also be used to recognize dominance and co-dominance inside the genome.

Molecular marker-based on hybridization techniques

In hybridization techniques, DNA profiles are viewed by hybridizing the digested DNA by restriction enzymes to a designated sample, which can be a small part of DNA of familiar source or sequence. The first hybridization-based system was restriction fragment length polymorphism (RFLP), later on, some other markers including microsatellites, minisatellites and A sequence-tagged site (STS) were designed as markers based on hybridization, but with the development of simpler cloning and sequencing techniques and the availability of sequence databases, such markers have been transformed into PCR-based markers (32).

Restriction Fragment Length Polymorphism (RFLP)

RFLP was the first and the only hybridization-based marker system used in molecular biology (28), it has been used for plant genome mapping (23), also it is a type of southern blotting markers, called molecular markers of the first generation (30). Since the 1980s and 1990s RFLP marker was mainly used in plant genetic studies to identified polymorphisms that lead to modifications in nucleotide sequences in restriction enzyme recognition locations or from mutation occurrences of many nucleotides resulting in an apparent change in fragment

size (33). The hybridization-based like (RFLP) are not used for plant identification, but the microarray hybridization also known as the DNA chip or biochip can be used (20). Microarrays are valuable instruments not just for genome-wide transcript profiling, but also for the investigation of polymorphism and genotyping (34). A microarray of DNA is a multiplex technology used in medicine and molecular biology, which comprises a range of thousands of DNA oligonucleotide microscopic spots, and it was used in authentication of medicinal plants (22). RFLP was cheap but it is slow and complicated and needs a large amount of DNA (31, 35).

Molecular marker-based on PCR

PCR is a technique that is used in molecular biology to amplify one or many copies of DNA fragment, producing a considerable number of copies of a particular DNA sequence.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

PCR-RFLP study was introduced because less amount of DNA is needed and it is a quick technique, and much better to study traditional Chinese medicine (36). It utilizes endonucleases to digest PCR products from sequentially polymorphic areas (37). Medicinal *Codonopsis pilosula, C. tangshen, C. modesta,* and *C. nervosa var. macrantha* have been differentiated from two associated adulterants *Campanumoea javania* and *Platycodon grandifloras* by analyzing the DNA sequence of the internal transcribed spacer region (ITS) of 18s-26s nuclear ribosomal DNA (nrDNA) and PCR-RFLP. The results showed that the ITS rDNA sequences of the four *Codonopsis* are strongly related but not completely similar and differ considerably from those of the two adulterants (additive substances causing the original medicine to loose partially or completely its therapeutic capacity) (1, 38, 39). PCR and RFLP were also utilized to validate the existence of *Codonopsis tangshen oliver* (*Campanulaceae*) occasionally considered as a ginseng replacement (40). The nrDNA-RFLP application is not exclusive to *Codonopsis*. In reality, the authentication of other Chinese medicinal products can rely on that technique (41), as it was used to authenticate the species of *Panax, Fritillaria pallidiflora, Atractylodes*, and too many other species (21, 42, 43).

Non-coding nuclear DNA markers

Internal transcribed spacer regions of nuclear ribosomal DNA (ITS-nrDNA)

ITS is the inherited 18S–5.8S–26S nuclear ribosomal DNA internal transcribed spacer. The ITS is present in nearly all species as part of a nuclear ribosomal DNA (nrDNA) transcription system, except vertebrates, and its high copy number is one of the major reasons for the frequent usage in molecular systematics (44). ITS nuclear ribosomal DNA sequence is useful as a non-coding marker for inferring hybridization occurrences in *Codonopsis taxa* because it has a large degree of variability and elevated capacity to distinguish nearly associated species (45), it's sequencing were used to differentiate *C. pilosula, C. pilosula var. modesta,* and *C. tangshen* (42). Lately, the ITS of nrDNA sequences has been revealed as a molecular marker that could be efficiently and precisely identified in several *Codonopsis* species like *C. pilosula, C. pilosula var. modesta, C. tangshen*, and *C. lanceolata* and it can be used for *Codonopsis Radix* authentication (1). The ITS regions of five *Codonopsis* species (*C. pilosula, C. modesta, C. tangshen, C. javanica, C. kawakamii*) and also two adulterants associated with them (*C. lancifolia* and *P. grandiflorus*) have been amplified and sequenced (46).

Moreover, DNA sequence assessment (ITS) and (PCR RFLP) have been used in the differentiation of *C. pilosula C. tangshen, C. modesta*, and *C. nervosa var. macrantha*, from medicinal *Campanumoea javania* and *Platycodon grandifloras* species (41).

Microsatellite

Microsatellites are described as short fragments of DNA that contain homogeneous patterns repeated in tandem with two to six base pairs (47), the repeating size is less than or about 1 kb, and it could be found abundantly in non-coding genome sections such as introns, untranslated regions (UTRs), and intergenic areas, but also occur in exonic sequences coding (48). For the first time, ten microsatellite polymorphic loci were developed for *C. pilosula*, and the polymorphism of each locus was evaluated, using 27 individuals from four geographically distant populations of the 33 primers pairs, a total of 14 effectively amplified the target regions and 10 displayed polymorphic banding patterns (49). The PCR-based microsatellite amplification enables to create a distinctive pattern and a particular description for an individual, because of that it was used to identify and authenticate plants showing inter-specific variation (50).

Simple Sequence Repeats markers (SSRs)

SSR evaluation is mentioned as simple sequence length polymorphisms (SSLPs) also called microsatellite or short tandem repeats (STRs) (51). Presently, SSRs markers are broadly used in genetic studies and plant breeding (30), trinucleotide and dinucleotide repeats are predominant in most plants as expressed sequence tags SSRs. According to reports, it was indicated that *C. pilosula* transcriptome SSR is commonly used in China, as a total of 7327 SSRs were searched in the transcription group of *C. pilosula,* and by comparing the current frequency of *C. pilosula* SSR with other medicinal plants, *C. pilosula* frequency was lower than that of *Salvia miltiorrhiza* and higher than that of *Ginseng* (5). SSR markers were effectively identified and implemented to explore the genetic variation and the construction of the population in *C. tangshen* and *C. pilosula* (50). This technique was also used to study genomic variability in *C. lanceolate* (20).

SSR, EST-SSR, or SNP markers were suggested to be used to develop comprehensive genomics from plants without requiring genome reference (50). Consequently, DNA as a molecular marker has several benefits over classic phenotypic markers because the genetic structure is unique to each organism and is not influenced by time, physiological as well as ecological factors (20).

Inter Simple Sequence Repeats markers (ISSR)

ISSR is defined as a region between microsatellite loci within a genome. ISSR-PCR amplified sequences can be used for DNA fingerprints. The diversity of its sequences is lower than in SSR-PCR, since an ISSR may be a conserved or non-conserved region, yet it remains higher than in real gene sequences (22). The wild and cultivated population of *C. pilosula* maintains a comparatively elevated amount of genetic diversity by using ISSR analysis (52). ISSRs are commonly used in genetic diversity research since no previous data are required and low development costs and laboratory processes are readily transferable to any plant species (53). ISSR yields a multi-locus and highly polymorphic pattern (54). Referring to ISSR specific molecular markers to authenticate and differentiate different authentic species were established (55)

Random amplified polymorphic DNA (RAPD)

RAPD is PCR based technique cwhere a whole genomic DNA is amplified with a single, short nucleotide (~10 bp) base and a random primer (30), this method utilizes the PCR principle to extend DNA sequences randomly (56). RAPD marker can be used in quality control as a supplementary technique, to study the systematic relationship among related species, and to compare the genetic relationship between medicinal plants and their patterns of diversity (57). Arbitrarily primed polymerase chain reaction (AP-PCR) or RAPD distinguished *C. pilosula* samples from various areas of China (58). The genetic variability of *C. pilosula* showed a considerably higher level than the average perennial long-lived herbaceous and widespread species by using RAPD analysis (38). Molecular markers based on RAPD were discovered to help distinguish separate *Taxus wallichiana*, and *C. pilosula* accessions (59) as well as in comparing cultivated *Rheum tanguticum* and *C. pilosula* (39). The genetic variation between cultivated and wild type plants of *C. pilosula* was analyzed at the DNA level by RAPD molecular marker (60). The genetic relationship of *C. lanceolata* sampled from Baekdoo Mountain and Korea has been studied using RAPD. Also, the genetic relationship distinction between *Adenophorae tryphylla* and *C. Lanceolate* using the RAPD process was documented by Kim-Serim (50). And the effective development of ISSR and RAPD was usable successfully to *C. lanceolata* by Gao Jian-ping (61). The assessment of RAPD was appropriate for Korean and Chinese ginseng authentication. RAPD's main benefit is that a previous understanding of the DNA sequence is not required (37) and it is rapid and not expensive. But it has some limitations especially when DNA samples are degraded, because of that new longer and specific primers for the DNA sequence called sequence characterized amplified regions (SCAR) were designed (22). The change from RAPD to SCAR has considerably increased the reproducibility and reliability of PCR assays by creating longer, then more particular primers than RAPD sequences (62).

SCAR was used to differentiate the specimen by using particular primers designed by RAPD or ISSR fragments (20). And is a more effective marker than the common technique like RAPD, RFLP, and AFLP in differentiating many medicinal plant species from their alternatives or adulterants (63, 64). The SCAR technique is much more useful and less costly than the DNA barcoding technique (65). Yet, DNA barcoding is anticipated to be one of the biological taxonomy's most successful instruments, and it is regarded as an essential key barcode for *Codonopsis* barcoding at the species level (66).

Amplified Fragment Length Polymorphism (AFLP)

The AFLP method focuses on the specific PCR amplified restriction fragments from a whole genomic DNA, digested by the restriction enzyme. It consists of limiting genomic DNA followed by adapter ligation, selective amplification of digested fragments using primers comprising adapter sequences, and selective bases at the 3 ' terminals (67). AFLP combines the benefits of RFLP's reliability with PCR's strength. The number of polymorphisms may exceed RFLP or RAPD per response (20). At the level of the entire genome, AFLP has a high reproductive capacity, objective, and reactivity in contrast to other approaches, also it can amplify between 50 and 100 fragments at once (22). The method of complementary DNA cDNA-AFLP is strict and more reproducible than that of RNA Arbitrarily Primed-Polymerase Chain Reaction (RAP-PCR) (68). AFLP molecular marker and HPLC technique have been used to investigate the effect of the environment on the genetic structure and chemical ingredients of three different *Codonopsis* species (*C. pilosula, C .pilosula var. modesta*, and *C. tangshen*) introduced in Shanxi province from the different geographic area in China, and the results showed that the variation between them was caused by their inter-species genetic characteristic, while the similarities of genetic backgrounds in *Codonopsis* species were associated with the geographic space, and their chemical ingredients were easily affected by the cultivation environment (69).

Sequencing-based markers (SNP)

The DNA sequencing was used to identify species at the single nucleotide polymorphisms (SNP), genetic variation occurs widely. Usually, the identification depends on SNPs but other times it includes ITS, as ITS has proven to be a helpful sequence in many species for phylogenetic studies (59). SNP differences in a population's genome sequence (32) occur when a single nucleotide (A, T, G, or C) is distinct between species groups (70). SNP markers have recently had a huge interest in the development of dense genetic maps and genome-wide association research (71). In crop molecular genetics, SNP markers have become highly common owing to their genome-wide inexhaustible and amenability for platforms for high to extra-high-throughput

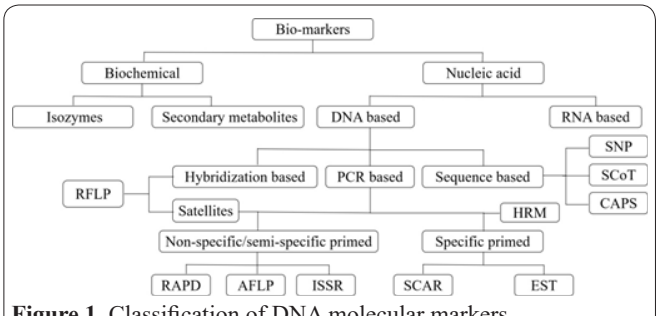

Figure 1. Classification of DNA molecular markers.

detection (72). They were used as molecular marker development in *C. lanceolata* and *Platycodon grandiflorus* species (73).

Other molecular markers were used to study the genetic diversity of medicinal plants other than *Codonopsis* species such as cleaved amplified polymorphic sequences (CAPS) marker, high-resolution melting (HRM) marker, inter-retrotransposon amplified polymorphism (IRAP) marker, retrotransposon-microsatellite amplified polymorphism (REMAP) marker, as well as Start Codon Targeted Polymorphism (SCoT) marker. To compare the chloroplast genomes of two *Glycyrrhiza* species (*G. glabra* and *G. lepidota*) the CAPS and HRM markers were developed based on SNP (74). Inter-retrotransposon amplified polymorphism (IRAP) and retrotransposon-microsatellite amplified polymorphism (REMAP) were used to study the genetic relationships among 34 varieties of *Lallemantia iberica*, which is considered in Iranian folk medicine as a treatment of various illnesses and as an expectorant cure.

The SCoT marker is a gene-targeted advantageous marker that targets the preserved region adjacent with the translation initiation start codon (ATG) of plant genes (75), and it became one of the important markers because it is more effective, informative cheaper (70), and even simpler to develop than SSR. SCoT is less expensive than AFLP and more reproducible than RAPD (76). It has been effective in evaluating genetic variation in plants such as *Andrographis paniculata* (77). According to our research, those molecular markers have not yet been used in *Codonopsis* species studies, but they can be considered in the future for that purpose.

Bioinformatic tools for analyzing genetic diversity studies of *Codonopsis* species

Bioinformatics is a science of interdisciplinary, it uses different informatics methods like computer science, statistics and mathematics to evaluate, arrange, and comprehend biological data in particular protein sequences and nucleotide (78). Bioinformatics provides a series of important methods for analyzing and interpreting large amounts of data produced using methods based on molecular biology (79). In bioinformatics, the basic local alignment search tool (BLAST) is used in GenBank to check the identity for each ITS sequence fragment. Lately, a set of operating guidelines has been suggested for ITS information (91). DNA barcodes of *Codonopsis* were aligned with Clustal X and modified by hand in BioEdit, then by using a sequence matrix to merge single-marker matrixes into multi-maker matrixes. The genetic distance of ITS and their combinations were systematically evaluated and compared (66). By comparing *C. pilosula, C. pilosula var. modesta*, and *C. tangshen* ITS sequences with the *Codonopsis* genus in GenBank online, high homology was discovered. the accession numbers were recorded as the pure line and the phylogenetic tree indicating that those species were strongly related. It has been found that the ITS marker alone is not enough to distinguish the three *Codonopsis taxa* therefore, other molecular markers are needed (11, 46). Phylogenetic trees of four species (*Adenophorae radix, Codonopsis Lanceolatae radix, Codonopsis pilosulae radix,* and *Glehniae radix*) were compared and the result showed that at the species levels *C. lanceolata* was related to *C. pilosula* in comparison with other species while *G. littoralis* was extremely different from the other species (65). Bioinformatics techniques were used to identify unigenes involved in *C. pilosula* polysaccharides biosynthesis (92). Bioinformatics instruments have been increasingly employed in the research of genomics, proteomics, transcriptomic, and medicinal plant metabolomics (93). Their advancements will enhance molecular marker detection with lower costs and more species identification (94). Molecular marker can be a valuable way of pharmacology in the medicinal plant, and bioinformatics methods can provide an important set of instruments for plant-based remedies to design effective and targeted studies (79).

Conclusion and prospects for further studies

Studies on the genetic variation among medicinal important *Codonopsis* species are crucial for permitting the validation of Chinese medicinal products because adulteration is a significant issue on the market for herbal drugs. The authentication helps ensure the safe and healthy usage of Chinese medicines, guaranteeing curative efficiency, diminish, unfair trade, and increase consumers' perception regard to them. It also plays a major role in their industrialization. Molecular markers are valuable tools for genetic variation study, in particular, DNA based markers are suitable for authentication of different medicinal plants (20). Although each technique has its advantages and limitations, a suitable choice of one marker and/or combination of different markers could be easily used to overcome these disadvantages by selecting an appropriate marker. Molecular markers with low price, comfort, quick, simple use, and automation are certainly useful instruments for molecular genetics differentiation (70). To our understanding, this is the first thorough and systematic report about the molecular marker application in *Codonopsis* species. And this report reveals that there is a significant genetic variation in cultivated *Codonopsis* compared to wild *Codonopsis* even though some species (*C. pilosula, C. pilosula var. modesta*, and *C. tangshen*) were strongly related. Studies of *C. pilosula* polysaccharides biosynthesis at the molecular level and the selection of accurate reference genes for a better knowledge of *C. pilosula* would be of good usage in the future (95). Furthermore, more studies on the establishment of other molecular markers for analysis of *Codonopsis* species are still urgently required. The molecular marks havebeen used in many researches (96-101).

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