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Current State and Prospects of DNA Barcoding and DNA Fingerprinting in the Analysis of the Quality of Plant Raw Materials and Plant-Derived Drugs

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Abstract—This paper reviews the use of molecular genetic methods (DNA barcoding and DNA fingerprinting) for the authentication of plant raw materials and herbal medicinal products. ITS2 is shown to be the most effective DNA barcode for plants. This marker allows researchers to identify even highly degraded DNA, which is especially important in the case of herbal preparations and biologically active additives. The main problem of traditional DNA barcoding via Sanger sequencing is that it is impossible to read a barcode without preliminary cloning of an amplificate when a plant mix includes a large amount of various adulterants and excipients of plant origin, since species-specific chromatograms are superimposed on each other. Thus, NGS sequencing of ITS2 amplicons is a preferable method for the DNA barcoding of plant materials. This method can be used to analyze highly degraded DNA in multicomponent plant mixes and can be used to identify all their ingredients. The review also covers such DNA fingerprinting techniques as RFLP, RFLP-PCR, RAPD, RAPD-SCAR, AFLP-PCR, and ISSR.

Keywords: DNA barcoding, DNA fingerprinting, DNA barcode, ITS2, Sanger sequencing, NGS, medicinal plant raw materials, herbal drugs

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INTRODUCTION

The use of traditional-medicine remedies is currently increasing worldwide, and there is a real boom in the industries of phytotherapy and biologically active additives (BAAs). The growing popularity of phytopreparations results in an increasing demand for plant raw material. Under such conditions, reliable identification of the initial material, its authenticity, and the absence of nondesirable adulterants are necessary requirements addressed by legislations of all developed countries (Parveen et al., 2016; Barnes et al., 2016; Sammons et al., 2016; Teng et al., 2016; Job et al., 2016; Enioutina et al., 2017; Simmler et al., 2017).

Modern pharmacognostic analysis includes several different methods to authenticate plant raw material. Traditional botanical methods of plant identification are used when possible, including the morphology of plants and their pollen; phytochemical identification is also possible with analytical chemistry, chromatography, and metabolomic analysis (Simmler et al., 2017). However, the discrimination of authentic material and adulterants or substitutes of raw herbal medicinal products by their chemical composition is very problematic, while the use of morphoanatomical characteristics for plant species identification is often very difficult or even impossible in the case of fine powders (a typical form for many plant raw materials). At the same time, the falsification of expensive components via their replacement with morphologically similar, but cheaper, type of plant material often occurs in pharmaceutical practice.

Let us give a recent example. Antidepressants such as Siberian ginseng (*Eleutherococcus senticosus*) and golden root (*Rhodiola rosea*) are very popular in Great Britain. Analysis of 25 commercial samples of Siberian ginseng and 10 samples of golden root delivered from China showed that all samples sold as Siberian ginseng contained plant material of this species, but nine samples also contained other *Eleutherococcus* species (*E. sessiliflorus*, *E. divaricatus*, or *E. seoulensis*). As for golden root, five samples contained only this species, one sample represented a mix of *R. rosea* and other species of this genus, and four samples did not contain *R. rosea* but included a mix of other *Rhodiola* species

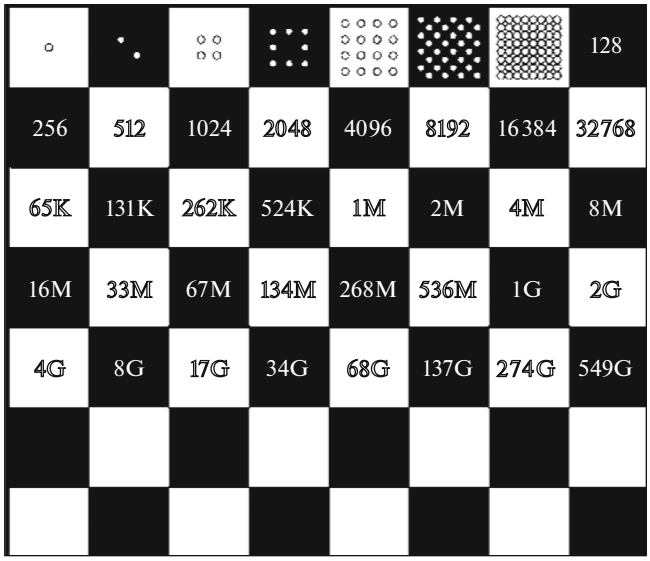

Fig. 1. PCR as an efficient method to obtain a preparative amount of DNA for species identification of plant material in herbal mixes.

(Ruhsam and Hollingsworth, 2018). This example is typical, and it clearly illustrates the existing problem of the market of herbal medicinal products.

In the described case, the presence or absence of contamination in commercial samples was determined by DNA barcoding, one of the most efficient methods of authentication of plant raw material (Povydysh et al., 2007; Simmler et al., 2017).

Like other modern molecular genetic methods used to determine the species composition of officinal plant mixes, DNA barcoding is based on polymerase chain reaction (PCR; Fig. 1). A peculiarity of the use of DNA barcoding in pharmacognosy is that suppliers of plant raw materials for the pharmaceutical industry do not plan to preserve DNA in samples for further analysis. Harvested plants undergo a preliminary technological treatment, such as drying (sometimes high-temperature drying), shredding, etc. At all stages, the raw material is subject to various damaging effects that lead to DNA degradation (UV radiation, light, high temperature and humidity, infestation with bacteria or fungi, etc.). Improper technology for plant harvesting and storage may result in cross-contamination with other samples, which may introduce errors into the results of DNA testing. The production of tinctures and extracts includes not only thermal treatment of a raw material but also filtration, extractive distillation, or supercritical fluid extraction; these methods may result in the destruction or even complete removal of DNA (Novak et al., 2007; Harnly et al., 2016). Moreover, plant secondary metabolites, such as polysaccharides, flavonoids, polyphenols, and terpene lactons, may hamper DNA extraction (Porebski et al., 1997; Barnwell et al., 1998; Khanuja et al., 1999; Sahu et al., 2012). The use of PCR makes it possible to analyze the remaining, relatively short DNA molecules via their amplification or sequencing or to study marker DNA, which store information about the composition of a plant mix, by other methods, such as AFLP, IRRS, SSR, etc.

METHODS BASED ON NUCLEOTIDE SEQUENCE DETERMINATION BY SANGER AND NGS SEQUENCING

The standard DNA barcode for animals, a 5'-fragment of the cytochrome *c* oxidase subunit 1 gene (CO1), cannot be used for plant DNA barcoding due to its very low variability, (Hollingworth et al., 2011), and it is not used for the DNA barcoding of plant raw material in pharmacognosy (De Boer et al., 2015; Parveen et al., 2016).

The Plant Working Group of the Consortium for the Barcode of Life (CBOL) recommended the use of several marker regions, such as *mat*K, *rbc*L, *psb*A–*trn*H, and ITS or ITS2 (CBOL, 2009). However, current studies show that the search for appropriate DNA regions for plant DNA barcoding must continue. Molecular biologists currently use several different loci or their combinations to identify plant material.

Approximately 150 studies dedicated to the DNA barcoding of officinal plants were published in 2005–2017. The authors of these papers used 17 DNA regions as barcodes: *mat*K, *rbc*L, ITS (ITS1, ITS2), *psb*A–*trn*H, *atp*F–*atp*H, *ycf*5, *psb*KI, *psb*M, *trn*D, *rps*16, *cox*I, *nad*1, *trn*L-F, *rpo*B, *rpo*C1, *atp*F–*atp*H, and *rps*16 (Kress and Erickson, 2007; Chen et al., 2010; Pang et al., 2012; Gere et al., 2013; Techen et al., 2014; Bolson et al., 2015; Parveen et al., 2016; Raclariu et al., 2018). Newmaster at al. (2006) proposed a new, multilevel approach, which included the use of the easily enhanced and aligned *rbc*L region as the basic level, as well as data from highly variable noncoding regions, such as ITS2 or *psb*A–*trn*H. With this multilevel approach, 75–80% of the tested plants were barcoded (Newmaster et al., 2006; Burgess et al., 2011). There was also another large-scale study on the DNA barcoding of officinal plants (Chen et al., 2010). In this study, seven DNA regions, *psbA–trn*H, *mat*K, *rbc*L, *rpo*C1, *ycf*5, ITS (ITS1 and ITS2), and ITS2 were evaluated as markers for the identification of >6600 samples (fresh leaves) of officinal plants and closely related species. The highest efficiency was observed for the ITS2 region, which allowed researchers to identify 92.7% of the studied species and was therefore proposed as the basic barcode for officinal plants (Chen et al., 2010). Different variants of plant DNA barcodes were later tested again in a joint study performed by 46 research laboratories in China. The researchers compared the results obtained for five main candidate barcodes, *rbc*L, *mat*K, *psb*A–*trn*H, ITS1–5.8S rDNA–ITS2, and ITS2 alone. These DNA regions were sequenced from 6286 plant samples

belonging to 1757 species of 75 different families (China Plant BOL Group, 2011). According to this comparison, the full-length ITS region was just slightly inferior to chloroplast markers in relation to the success of their amplification with universal primers (79 and 87–93%, respectively), but it surpasses others in its species discrimination potential (the discrimination strength of ITS, ITS2, and *rbc*L markers was 67.2, 54.6, and 26.4%, respectively). Among the most thoroughly studied genera (of which at least 50 species were included in the study) was the genus *Primula*; 88% of its species were discriminated by ITS, and only 42.5% were discriminated by *rbc*L. Tibetan and Mongolian medicine uses 22 species of the genus *Pedicularis* (Gammerman and Semichov, 1963; Khaidav et al., 1985). Within this genus, ITS and *rbc*L markers discriminated 86 and 46% of species, respectively. Traditional medicine uses 38 species of the genus *Rhododendron* (Popescu and Kopp, 2013). Within this genus, ITS and *rbc*L markers discriminated 15 and 10% of species, respectively (China Plant BOL Group, 2011).

Additional studies on different plant groups confirmed the efficiency of ITS2 for the identification of officinal plants; this marker reliably identified 24 species of the family Fabaceae used in Chinese medicine and 66 other species occurring as adulterants in medicinal herbal mixes. Analysis of 1507 DNA sequences of Fabaceae plants deposited in the GenBank from 1126 species showed that 80% of all species and 100% of genera differed in their ITS2 sequences, which was a very good result for plant barcoding (Gao et al., 2010).

A two-loci (ITS2 and *psb*A–*trn*H) approach was later used to create the Traditional Chinese Medicine Database, which contains the barcodes of more than 23000 species of officinal plants and known adulterants (Chen, 2011).

The ITS2 barcode was successfully used to identify 19 species of medicinal plant material belonging to the "bark" morphological group that were included in the Chinese pharmacopoeia (Sun and Chen, 2013). The ITS2 locus was also recommended for the identification of *Glehniae* roots, since it allowed the discrimination of *Glehniae* from possible adulterants.

A comparative analysis of the candidate barcodes for the species of the genus *Uncaria* was also carried out. Of the compared regions (ITS, ITS2, *mat*K, *rbc*L, *psb*A–*trn*H), the highest efficiency was shown for ITS2 (Zhang et al., 2015). The same markers were used to identify *Artemisia* species, and the same result confirming the potential use of ITS2 as a discriminating barcode was obtained (Wang et al., 2016). ITS2 as a potential barcode was used to discriminate rhubarb species *Rheum officinale* Baill., *Rh. palmatum* L., and *Rh. tanguticum* (Maxim. ex Regel) Maxim. ex Balf. harvested from the entire growing area from their adulterants (Zhou et al., 2017).

A study on the barcoding of a popular plant, *Boerhavia diffusa* (*Punarnava*), used in the Ayurveda (one of the branches of traditional Indian medicine) to treat liver diseases was carried out in India. Four regions, ITS1–5.8S rDNA–ITS2, ITS1, ITS2, and *psb*A–*trn*H, were considered as potential barcodes. The results showed that ITS and ITS1 can be used as candidate markers to identify *Boerhavia diffusa* and to discriminate this species from adulterants (Selvaraj et al., 2012). The API–RDBL (Ayurvedic Pharmacopoeia of India–Reference DNA Barcode Library) library of DNA barcodes, which contains high-quality, authentic *rbc*L barcodes, was created for the identification of officinal plants included in the Ayurvedic Pharmacopoeia of India (Vassou et al., 2016). An examination of medicinal plant material presented at Indian markets showed that only 79% of this material was authentic, while the remaining 21% were counterfeit. In addition, it was found that the falsification level was higher (~25%) if the raw material was triturated; the falsification level of the "seeds" morphological group was ~5% (Shanmughanandhan et al., 2016).

The main advantage of ITS2 as the barcode for the identification of plant material in medicinal mixes and raw material used for BAA production is its short length (200–230 bp on average), since the DNA in the majority of phytopreparations and raw materials for BAA is usually significantly destroyed, comprising <500-bp fragments. Thus, universal barcodes with a length of 600–800 bp are not suitable for the pharmacognosy and quality control of BAA raw material (Costa et al., 2015).

Another advantage of ITS2 as a target sequence for DNA barcoding is the fact that primers used for its amplification and sequencing also amplify the ITS2 region of endophytic fungi and mycorrhizal symbionts (Rodriguez et al., 2009; Ivanova et al., 2016). The fungal ITS2 region differs from that of plant DNA in both size and electrophoretic mobility, which allows researchers to reveal sample contamination by fungi via further Sanger sequencing even more efficiently than for contamination by other plant species, the detection of which requires a cloning procedure.

A peculiarity of the ITS2 and ITS1–5.8S rDNA–ITS2 regions with some theoretical implications is associated with the fact that the plant genome usually has several thousands of such sequences, which can be located on different chromosomes (Rodionov et al., 2016). Pyrosequencing shows that, for each moment in the genome of many species, there is a heterogeneous set of 35S rRNA genes (including different ITS regions) characterized by different degrees of similarity among themselves (Song et al., 2012). At the same time, the ITS sequences are somehow homogenized. It is believed that this occurs by conversion or, which is more probable, due to the "birth-and-death" mechanism of homogenization (Eickbush, T.H. and Eickbush, D.G., 2007). In most cases of Sanger sequenc-

ing of ITS regions, such homogenization of rDNA sequences, including ITS2, results in a lack of double peaks, which are comparable in height and indicate an intragenomic polymorphism (Rodionov et al., 2016). There can be some exceptions, such as peonies (Punina et al., 2012), but they are rather rare. Thus, despite the multiplicity of ITS sequences and the wide occurrence of hybridization and polyploidy, ITS sequences can be used as barcodes.

Sanger sequencing of DNA allows the determination of only the most common homogenized sequences (Sanger et al., 1977). As practice shows, from the point of view of classic taxonomists, phylogenetic hypotheses based on a comparison of such sequences usually appears to be very plausible for a comparison at the interspecies and intergenera levels or for the study of groups of organisms having higher taxonomic ranks (Shneyer, 2009; Hershkovitz and Zimmer, 1996; Song et al., 2012). In other words, though interspecific hybridization and polyploidy are common in plants, the divergence of ITS1 and ITS2 sequences, for unknown reasons, occurs in a way that correlates with the direction and rate of the morphological divergence of taxa; thus, ITS2 can be used as a DNA barcode in pharmacognosy.

Since medicinal herbal material often contains only short DNA fragments, it was recently proposed that so-called mini-barcodes be used to identify the plant ingredients in raw materials involved in the production of BAAs and medicinal herbal products. Mini-barcodes represent short (<200 bp) DNA sequences from relatively variable certain regions of *mat*K and *rbc*L genes. The successful use of these regions was proven for the identification of species composing some plant mixes represented on the North American pharmaceutical market; according to the technology of their production, they were composed of *Serenoa repens*, *Ginkgo biloba*, *Harpagophytum procumbens*, and *H. zeyheri* (Little and Jeanson, 2013; Little, 2014; Parveen et al., 2016).

The advantages of mini-barcodes include the ease of the search for DNA markers, even in processed materials, due to the short amplicon length, as well as the possibility to discriminate even closely related species due to their genus- or species-specificity (Parveen et al., 2016). Table 1 shows examples of the use of DNA barcoding to authenticate medicinal herbal products.

The basic barcoding techniques use universal primers for rapid plant identification (Kress et al., 2005; Lahaye et al., 2005; Burgess et al., 2011; Hollingsworth et al., 2011). Universal sets of primers recommended for plant barcoding are intended to amplify DNA from four regions of the nuclear (ITS (ITS2)) and chloroplast (*mat*K, *rbc*L and *psb*A–*trn*H) genomes (Table 2).

If a phytopreparation contains several plant species or some adjuvants, then coamplification of barcoding sequences occurs due to the universal character of the used primers (Parveen et al., 2016). If a preparation contains related plant species, it may cause the appearance of multiple or overlapping peaks during sequencing; as a result, the barcoding sequence of both the target and contaminating components of the plant mix cannot be determined. Poor-quality DNA decoding can be improved at the presequencing stages via amplicon coding; several (ten or more) clones are then sequenced to identify different DNA sequences (Aird et al., 2011).

A particular problem of DNA barcoding in pharmacognosy and the quality control of raw materials for BAA production is connected with the excipients used for plant mixes. The use of excipients obtained from wheat, rice, and soybean is common in the pharmaceutical industry and BAA production (Costa et al., 2015). The sequencing of DNA isolated from BAA raw material may result in multiple nucleotide sequences if the raw material contains more than one plant species, which is quite a common situation. A digital PCR approach was proposed and approved to resolve this situation. In digital PCR, samples of isolated DNA are diluted in a suitable buffer at a ratio of 1 : 5 to 1 : 50000, such that the final template DNA concentration is approximately one molecule per μL (Parveen et al., 2016).

The goal of such dilution is to dilute the DNA to the extent that a few samples contain only molecules of low-abundant DNA. The term "low-abundant DNA" refers to DNA that originated either from medicinal-plant material containing a large amount of DNA from an excipient material (e.g., rice flour) or DNA obtained from small amounts of adulterating plant material. The performance of several PCRs with the use of single DNA molecules as templates improves the chances that low-abundant DNA molecules will be amplified and, therefore, detected. The method was used to identify *Ginkgo biloba* in BAAs (Little, 2014).

Next-generation sequencing (NGS) was used to overcome the limitations of Sanger sequencing in the DNA barcoding of multicomponent samples (Kircher and Kelso, 2010). NGS technology allows the user to perform parallel sequencing of several DNA fragments from different DNA regions in one reaction (Kircher and Kelso, 2010). This method makes it possible to generate up to one million DNA sequences with lengths of up to 700 bp per sequencing session; the base length may significantly vary depending on the NGS platform or technology used. The method includes independent amplification of each individual DNA sequence in a mixture that excludes any superposition of the peaks typical for Sanger sequencing and, therefore, facilitates the decoding of DNA barcodes and the authentication of ingredients of multicomponent phytopreparations (Ivanova et al., 2016; Sarwat and Yamdagni, 2016). Table 3 shows examples of NGS application for DNA barcoding.

Table 1. Use of DNA barcoding for the identification of medicinal plant raw material

Medicinal plant	Used part of the plant	Medicinal use	Admixture/marker region	Reference
Sida cordifolia L.	Herbage and leaves	Antioxidant, anti-inflammatory, and antidiabetic drug	*Sida spinosa*, *S. alnifolia*, *S. scabrida*, *S. ravii*, *Abutilon* sp., *Ixonanthes* sp., *Terminalia* sp., *Fagonia* sp., *Tephrosia* sp./*psb*A–*trn*H and ITS2	Vassou et al., 2015
Indirubin (*Isatis tinctoria*, *Polygonum tinctorium*, *Strobilanthes cusia*)	Leaves	Treatment of chronic myeloid leukemia	*Polygonum hydropiper*, *P. chinense*, *Clerodendrum cyrtophyllum*, *Indigofera tinctoria*, *S. dimorphotricha*, roots of producing plant/ITS2	Hu et al., 2015
Peucedanum praeruptorum L.	Herbage	Expectorant	*Anthricus sylvestris* (L.) Hoffm./ITS	Zhou et al., 2014
Ginkgo biloba L.	Leaves	Treatment of chronic degenerative brain diseases and dementia; remedy delaying the development of the Parkinson's and Alzheimer's diseases	No/*mat*K	Little, 2014
Phoenix dactylifera L.	Fruits	Antimutagenic and antioxidant drug	No/*mat*K and *pro*C1	Enan and Ahamed, 2014
Piper nigrum L.	Fruits	Antibacterial, antioxidant, anti-inflammatory, and antitoxic drug	*Carica papaya* L./*psb*A–*trn*H, *rbc*L, *rpo*C1	Parvathy et al., 2014
Lonicera japonica	Leaves and flowers	Antipyretic, detoxicating, and anti-inflammatory drug	*L. japonica* var. *chinensis*, *L. similis*, *L. acuminate*/*psb*A–*trn*H and ITS2	Hou et al., 2013
Gentiana scabra, *G. triflora*, *G. manshurica*, *G. rigescens*	Leaves and roots	Treatment of liver diseases and hepatoprotective effect on acute liver injuries caused by paracetamol intake	*Gentiana rhodantha* and *Podophyllum hexandrum*/*mat*K and *rbc*L	Wong et al., 2013
Croton bonplandianum Baill.	Seeds	Treatment of jaundice, ascites, and internal abscesses	No/*mat*K	Chandramohan et al., 2013
Asparagus racemosus Willd.	Roots	Treatment of dyspepsia; lactogenic, antidiarrheal, antiseptic, and diuretic drug	*Asparagus gonoclados* Baker/ITS2	Rai et al., 2012
Scutellaria baicalensis	Roots	Treatment of hepatitis, jaundice, diarrhea, and inflammations	*S. amoena*, *S. rehderiana*, *S. viscidula*/*mat*K, *rbc*L, *psb*A–*trn*H	Guo et al., 2011
Ruta graveolens L.	Leaves, stems, and flowers	Fertility regulation; treatment of dysmenorrhea, ear-ache, headache, and nosebleeds; repellent action	*Euphorbia dracunculoides* Lam./ITS	Al-Qurainy et al., 2011
Taxillus chinensis	Branches and leaves	Improvement of renal function, tendons, and bones; relief of rheumatic symptoms; miscarriage prevention	*Thuja sutchuenensis*, *Scurrula parasitica*, *Scurrula parasitica* var. *graciliflora*/*psb*A–*trn*H	Li et al., 2010
Cinnamomum osmophloeum	Leaves	Antidiabetic, anti-inflammatory, astringent, and diuretic drug	No/ITS2	Lee et al., 2010

Table 2. Universal primers and PCR conditions of candidate barcode sequences

Marker	Primer	Sequence (5'–3')	PCR conditions
ITS2	S2F S3R	ATGCGATACTTGGTGTGAAT GACGCTTCTCCAGACTACAAT	(1) 94°C 5 min; (2) 94°C 30 s, 56°C 30 s, 72°C 45 s, 40 cycles; (3) 72°C 10 min
*rbc*L	1f 724r	ATGTCACCACAAACAGAAAC TCGCATGTACCTGCAGTAGC	(1) 95°C 2 min; (2) 94°C 1 min, 55°C 30 s, 72°C 1 min, 34 cycles; (3) 72°C 7 min
*psb*A–*trn*H	fwd PA rev TH	GTTATGCATGAACGTAATGCTC CGCGCATGGTGGATTCACAATCC	(1) 94°C 5 min; (2) 94°C 1 min, 55°C 1 min, 72°C 1.5 min, 30 cycles; (3) 72°C 7 min
*mat*K	390F 1326R	CGATCTATTCATTCAATATTTC TCTAGCACACGAAAGTCGAAGT	(1) 94°C 5 min; (2) 94°C 1 min, 48°C 30 s, 72°C 1 min, 26 cycles; (3) 72°C 7 min

Table 3. Use of NGS in studies of medicinal plants

Officinal plant	Activity	NGS platform used	Reference
Ocimum sanctum	Treatment of cough, cold, and bronchitis; expectorant	Illumina HiSeq2000	Rastogi et al., 2015
Beta vulgaris	Treatment of fever, constipation, upper respiratory tract diseases and infections	Illumina HiSeq2000	Dhom et al., 2014
Panax ginseng	Immunostimulating, antitumor, and cholesterol-lowering drug	Illumina HiSeq	Jayakodi et al., 2014
Elaeis guineensis	Laxative and diuretic drug; treatment of gonorrhoea, menorrhagia, and bronchitis	Roche/454	Singh et al., 2013
Curcuma longa	Antimalarial, anti-inflammatory, and antitumor drug	Illumina GAIIx	Annadurai et al., 2013
Catharanthus roseus	Antitumor drug	Illumina HiSeq2000	van Moerkercke et al., 2013
Withania somnifera	Restorative and antistress remedy; auxiliary drug for mental diseases; aphrodisiac	454-GS FLX sequencing (454 Life Sciences, Roche, USA)	Gupta et al., 2013
Azadirachta indica	Sedative, anaesthetic, antiepileptic, and hypertensive drug	Pyro-sequencing	Krishnan et al., 2012
Cannabis sativa	Hallucinogenic, somnifacient, sedative, anaesthetic, and anti-inflammatory drug	Illumina, Roche	van Bakel et al., 2011
Populus trichocarpa	Antipyretic, anaesthetic, and anti-inflammatory drug	Expressed sequence tag based methods	Tuskan et al., 2006

Validation is an important stage for the reliability of results obtained by any analytic method. Therefore, such a procedure should be developed for DNA barcoding in pharmacognosy. It is necessary to evaluate the level of variation of the used DNA sequence (barcode) in an officinal plant species, as well as the possibility of its detection in an herbal mixture. Since DNA barcoding should make it possible to discriminate officinal herbs from adulterating herbs and to determine the limit of their detection, it is necessary to prepare mixes of a target species with the known amounts of all possible admixtures. Note that DNA barcoding does not allow identification of the analyzed plant part (i.e., the morphological group of a raw material). The

validation process becomes more difficult in the case of the analysis of ready products. DNA barcoding methods are usually developed with the use of fresh or dried untreated plant material to determine whether it is possible to amplify a target DNA region and differentiate it from the DNA of related species (Newmaster et al., 2013; Palhares et al., 2015). Therefore, DNA barcoding of phytopreparations should be carried out with consideration of the following: (a) the treatment methods for a raw material may change or even exclude DNA, (b) DNA extraction and amplification processes may be influenced by excipients and secondary metabolites, and (c) the presence of DNA of auxiliary components may result in false results (Parveen et al., 2016).

In the case of phytopreparations, the stage at which DNA barcoding should be carried out is very important. For example, in the case of processed plant raw material, molecular analysis is more successful at the initial stage, i.e., harvesting of the plants used in phytopreparation production (Parveen et al., 2016).

In light of the limitations of and prospects for the use of DNA barcoding to authenticate medicinal plant material, one should note that DNA barcoding does not allow the user to discriminate different tissues or organs of the same plant species. For example, ginseng (*Panax ginseng*) roots are often mixed or even completely replaced by leaves. Both roots and leaves are characterized by a high panaxoside content, but they have different chemical profiles. DNA barcoding is not able to reveal such falsification. Moreover, DNA barcoding is not able to reveal "exhausted material" (the raw material from which active components were extracted or removed), as well as raw material harvested during an inappropriate season, which reduces the content of active compounds and, thus, the pharmacological activity of the final preparation. To overcome these limitations of DNA analysis, the identification of plant products requires the use of chemical profiling, as well as a macro- and microscopic analysis. Another limitation of DNA barcoding is the affinity of universal primers to the DNA of excipients and falsifying or replacement plant species; moreover, since excipients are usually added after the completion of the treatment of the plant material, their DNA remains intact. Therefore, primers will amplify mainly excipient DNA, which may cause false-negative result. This problem can be overcome by NGS, digital PCR, or cloning. In addition, the development of mini-barcodes is more preferable. One of the main problems of DNA barcoding for the identification of officinal plant raw material and medicinal phytopreparations is the lack of a reference library of mini-barcodes or barcodes containing all authentic standard barcodes associated with the corresponding taxonomically confirmed herbarium vouchers (Mishra et al., 2016).

USE OF METHODS BASED ON FRAGMENT DNA ANALYSIS (DNA FINGERPRINTING) TO IDENTIFY PLANT MATERIAL IN PLANT MIXES

The most popular of this group of methods is restriction fragment length polymorphism (RFLP), which refers to the treatment of DNA isolated from a definitively authentic sample with a restriction enzyme. The obtained fragments are separated by agarose gel electrophoresis and then transferred onto a nitrocellulose membrane with the same positions of the separated fragments (Southern blotting). Nucleic acid hybridization is performed with microsatellites as a radioactively labeled probe. The membrane is then placed on X-ray film, and the resulting radioautograph is used to determine the size of DNA fragments containing sequences homologous to the probe. At the next stage, restriction patterns are compared for plants of the target species that differ as much as possible in their origin. Unequal lengths of DNA fragments show differences at the level of DNA sequences, i.e., dynamic mutations connected with changes in the repeatability of microsatellite sequences, insertions or deletions in the genome regions between the neighboring restriction sites, mutations at the restriction sites, or deletions covering restriction sites (Povydysh et al., 2007; Matveeva et al., 2011). The selection of appropriate DNA probes providing species-specific combinations of fragments on radioautographs makes it possible to discriminate the tested object from related species and counterfeits. The fragment size in reference and tested samples is then compared. DNA probes can be labeled either radioactively, or fluorescently.

The drawbacks of the method are the following: (1) the large number of stages (restriction, electrophoresis, membrane transfer, hybridization, exposition, and the development of X-ray photographs), (2) the impossibility of automation, and (3) the large amount of DNA needed for analysis (2–5 μg). The advantages of the method include the relative ease of result interpretation (Ganie et al., 2015). RFLP was used to study species-specific DNA markers for *Capsicum annuum*.

Today, this method is commonly used in combination with PCR-based methods (Povydysh et al., 2007). For example, six species of officinal plants (*Desmodium gangeticum*, *Aegle marmelos*, *Solanum xanthocarpum*, *Solanum indicum*, *Tribulus terresteris*, and *Oroxylum indicum*) were identified by PCR-RFLP with the ITS region chosen for amplification with the ITS1 (F) and ITS4 (R) primers (Biswas, K. and Biswas, R., 2013). The same method was used to identify *Boerhavia diffusa* L. A 700-bp ITS region was amplified by PCR and then treated with a MspI restriction enzyme. The resulting four unique fragments enabled discrimination of the target species from *Trianthema portulacastrum* and *T. monogynya* (Biswas et al., 2013). This method was also proposed for the identification of *Angelica sinensis* and its discrimination from seven

Table 4. Use of RFLP and PCR-RFLP methods for the identification of medicinal plant products

Officinal plant	Plant part used	Medicinal use	Adulterant	Reference
Dracocephalum moldavica L.	Whole plant	Treatment of cardiovascular diseases; anaesthetic drug	*Melissa officinalis* L., *Nepeta cataria* L.	Horn et al., 2014
Maytenus ilicifolia Mart. ex Reissek	Leaves	Treatment of stomach ulcer, gastritis, and digestion disorders	*Sorocea bonplandii* (Baill.) Burger, Lanj. & Wess. Boer	Nakamura et al., 2013
M. aquifolia Mart.	Leaves and flowers			
Paris polyphylla Smith var. *yunnanensis*	Tubers	Antitumor, anaesthetic, anti-inflammatory, and antifungal drug	No	Liu and Ji, 2012
Fallopia multiflora (Thunb.) Haraldson	Roots	Treatment of carbuncles; drug preventing toxicity development; drug relaxing internal unstriated muscles	*Cynanchum auriculatum* Royle ex Wight	Zheng et al., 2012
Eleutherococcus senticosus (Rupr. & Maxim.) Maxim.	Roots, stems, and leaves	Adaptogen; treatment of many cancer types	*Periploca sepium* Bunge	Zhu et al., 2011

other adulterating species (Feng et al., 2010). It is important that this method is usually used with short-cutting restricting enzymes and makes it possible to reveal contamination with phylogenetically distant plants; closely related species or genera are usually not discriminated. Table 4 shows other examples of the use of RFLP and PCR-RGLP methods for the analysis of plant raw material.

PCR-BASED METHODS OF DNA FRAGMENT ANALYSIS

The most frequent methods used to analyze plant material are RAPD-PCR (random amplified polymorphic DNA) and AFLP-PCR (amplified fragment length polymorphism; Povydysh et al., 2007). RAPD-PCR is often used to reveal the genetic variability of plants within species and between related species. RAPD analysis is based on the PCR amplification of certain DNA regions with short (~10 bp), arbitrary primers. The amplification products are separated by agarose gel electrophoresis and stained with fluorochromes, so a whole range of amplified fragments can be visualized and photographed (Matveeva et al., 2011; Ganie et al., 2015).

The method is quite simple, requires a small amount of DNA (15–30 ng), and does not require any labeling procedures. These advantages are especially important for the identification of dried plant material, since it usually contains a small amount of DNA, which complicates the sequencing process. The method is used to differentiate such plant raw material as ginseng plants from different genera that differ in commercial value (*Panax ginseng* C.A. Mey, *P. quinquefolius* L., *P. notoginseng* (Burkill) F.N. Chen ex C.Y. Wu & K.M. Feng) and to discriminate them from adulterants. In addition, RAPD analysis is used to authenticate the raw material of *Juniperus communis* L., *Melissa officinalis* L., *Capsicum annuum* L., officinal species from the genera *Glycyrrhiza*, *Echinacea*, *Curcuma*, *Scutellaria*, and some other plants used in traditional Chinese and Indian medicine.

Despite of the popularity of the method, it has some drawbacks related to the dependence of the results on the PCR conditions (Povydysh et al., 2007). Because of insufficient reproducibility, it is difficult to use the RAPD method in interlaboratory studies. Therefore, the significance of the results and their interpretation may be in doubt (Ganie et al., 2015). A new type of marker, sequence-characterized amplified region (SCAR), was developed with the use of random primers and the corresponding RAPD bands, their separation, extraction, cloning, and sequencing, followed by the design of specific markers. The development and use of SCAR markers have grown exponentially since the beginning of 1990s. For example, specific SCAR markers enabling the discrimination of certain molecular phenotypes of different species of the same genus were developed. These markers are used in the taxonomy of ecotype discrimination and the determination of unique, somaclonal variants and molecular events associated with somaclonal variability (Matveeva et al., 2011). The RAPD-SCAR method is also used to identify officinal plant raw material (Table 5). V.M. Bayeva made a detailed review of the possible use of RAPD analysis to examine plant mixes (2009). In Japan the article "Examination of the Purity of Medicinal Raw Material Based on Genetic

Table 5. Use of RAPD and RAPD-SCAR methods for the identification of medicinal plant products

Officinal plant	Plant part used	Medicinal use	Adulterant	Reference
Litchi chinensis Sonn.	Fruits	Antitumor, antifungal, antiviral, antioxidant, antiaggregant, and antidiabetic drug	No	Cheng et al., 2015
Akebia quinata	Stems	Anti-inflammatory, diuretic, and anaesthetic drug	*Akebia trifoliata, Aristolochia manshuriensis, Clematis armandii*	Moon et al., 2015
Cissampelos pereira	Whole plant	Treatment of stomach pains, fever, skin diseases, and heart pain	*Cyclea peltata*, *Stephania japonica*	Vijayan et al., 2013
Podophyllum hexandrum Royle	Herbage and rhizomes	Antitumor drug	*Podophyllum peltatum* L.	Al-Shaqha et al., 2014
Fritillariae cirrhosae	Tubers	Antitussive and expectorative drug	Bulbus *Fritillariae pallidiflorae* (Yibeimu), B. *Fritillariae thunbergii* (Zhebeimu), B. *Fritillariae hupehensis* (Hubeibeimu), B. *Fritillariae ussuriensis* (Pingbeimu)	Xin et al., 2014
Angelica acutiloba Kitagawa var. *acutiloba* Kitagawa	Herbage	Treatment of female disorders	*A. acutiloba* Kitagawa var. *sugiyamae* Hikino	Matsubara et al., 2013
Knema andamanica	Whole plant	Antibacterial drug	No	Sheeja et al., 2013
Schisandra chinensis	Fruits	Astringent and anti-diarrheal drug; remedy preventing hyperhidrosis	*S. sphenanthera*	Lee et al., 2013
Shankhpushpi (*Convolvulus pluricaulis*)	Whole plant	Memory-enhancing drug	*Cansicora decussata*, *Clitoria ternatea*, *Evolvulus alsinoides*	Ganie et al., 2012
Ipomoea mauritiana	Roots	Afrodisiac; cardiotonic, sedative, diuretic, antipyretic, and lactogenic drug	*Pueraria tuberosa* (Roxb. ex Willd.) DC, *Adenia hondala* (Gaertn.) de Wilde, pith of *Cycas circinalis* L.	Devaiah et al., 2011

Table 6. Use of the AFLP-PCR method for the identification of medicinal plant products

Officinal plant	Plant part used	Medicinal use	Adulterant	Reference
Zanthoxylum acanthopodium, *Z. oxyphyllum*	Whole plant	Treatment of stomach diseases; remedy for blood purification and reduction of leukoderma manifestations	No	Gupta and Mandi, 2014
Zingiber officinale	Rhizome	Antioxidant, antitumor, and anti-inflammatory drug	*Z. montanum*, *Z. zerumbet*	Ghosh et al., 2011
Swertia chirayita	Seeds	Treatment of asthma and liver diseases	*Andrographis paniculata, Exacum tetragonum*, *E. pedunculatum*, *Slevolgia orientalis*, *S. alata*, *S. angustifolia*, *S. bimaculata*, *S. ciliata*, *S. densifolia*, *S. elegans*, *S. lawii*, *S. minor*, *S. paniculata*, *S. multiflora, S. cordata*	Misra et al., 2010a
Aconitum heterophyllum	Roots and stems	Treatment of stomach pains and fever	*Cyperus rotundus*	Misra et al., 2010b

Table 7. Use of ISSR markers for the identification of medicinal plant products

Officinal plant	Plant part used	Medicinal use	Adulterant	Reference
Scrophularia ningpoensis	Roots	Treatment of inflammations, laryngitis, tonsillitis, abscesses, and carbuncles	No	Chen et al., 2011
Rheum officinale Baill., *R. palmatum* L., *R. tanguticum* Maxim. ex Balf.	Rhizomes and roots	Laxative, anti-inflammatory, antibacterial, antipyretic, antitumor, and antimutagenic drug; treatment of renal diseases	*R. hotaoense*, *R. compactum*, *R. undulatum*, *R. emodi*	Wang, 2011

Information" was included in the pharmacopoeia starting from its XV edition (*The Japanese Pharmacopoeia*, 2006).

The use of the AFLP-PCR method allows researchers to avoid, to a great extent, the drawbacks of RAPD analysis. The method is based on the use of two specific restricting enzymes, followed by the ligation of restriction products with certain nucleotide sequences (adapters), further selective amplification with labeled primers, and analysis of the electrophoretic mobility of PCR products as compared to the reference sample of a raw material (Table 6).

ISSR (inter simple sequence repeats) markers were developed as an alternative to RAPD. This approach is based on the amplification of sequences bordered by two microsatellite repeats in the presence of a primer complementary to the sequence of this microsatellite (4–12 repeat units) that carry a sequence of 2–4 arbitrary nucleotides (so called "anchor") at one end (Matveeva et al., 2011). Such primers enable the amplification of DNA fragments located between two closely spaced microsatellite sequences; these fragments usually contain unique DNA sequences. As a result, a large number of fragments is amplified; the electrophoregram of these fragments represents a set of discrete bands (ISSR fingerprinting). The obtained patterns of PCR products are mainly species-specific and are significantly more reliable than RAPD markers (Table 7).

In addition to the methods described above, the derived methods including DAF (DNA amplification fingerprinting) and AP-PCR (arbitrary primed PCR) are also used (Povydysh et al., 2007).

CONCLUSIONS

This review demonstrates the great demand and relevance for the application of molecular genetic methods in the analysis of officinal plant raw material

and medicinal plant preparations. Sequence-based methods, especially NGS, seem to be the most promising. The DNA sequencing technologies used to identify officinal plant species in plant products and BAAs are very reliable and promising, but only under certain conditions, including the proper stage of analysis at which DNA can be detected; proper primer affinity; and a lack of DNA contamination. The use of the most appropriate method for the efficient detection and identification of the analyzed raw material or processed material is very important. Nevertheless, the limitations typical of DNA barcoding methods (impossibility of the determination of the analyzed plant part or the harvesting phase) do not allow its use as an independent instrument to identify officinal plant raw material and plant-derived medicinal preparations (Parveen et al., 2016). The combined use of molecular genetic methods and existing methods of pharmacognostic analysis is considered to be more appropriate.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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