New Aspects of DNA-based Authentication of Chinese Medicinal Plants by Molecular Biological Techniques

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Abstract
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DNA technology provides a powerful tool to complement chemical analyses for authentication of Chinese medicinal plants and to ensure that herbal materials are not contaminated with ineffective or potentially harmful substitutes or adulterants. In the last two decades molecular biotechnology has provided sophisticated molecular techniques for authentication of botanical materials at the DNA level. This review provides an account of the most commonly used DNA-based technologies (RAPD, RFLP, ARMS, CAPS, AFLP, DAF, ISSR, SSR, sequencing, hybridization and microarrays) including suitable examples of Chinese medical plants. A critical evaluation of all methods is presented concerning sensitivity, reliability, reproducibility, and running costs. Recent achievements in the field of DNA barcoding and DNA chip technology that offer great potentials for screening of DNA and emerging new developments for future identification of species are briefly outlined.

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Introduction
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China's plant diversity is exceptionally rich. The flora of China contains approximately 31 500 species of vascular plants representing nearly one-eighth of the world's total plant species [1, 2]. It is estimated that 10 000 species are endemic and that more than 5000 Chinese species are used for therapy in traditional Chinese medicine [3, 4]. Chinese medicinal herbs have been used for millennia and are becoming increasingly popular in the western world. Consequently, there is an enormous demand for medicinal plants.

A severe problem on the global market is that many erroneous substitutes and adulterants are traded due to their lower costs or misidentification of species with similar morphological features. There are several cases of Chinese herbs for which substitutes have been documented and reports that some of the adulterants or substitutes caused serious intoxications and even deaths [5–10]. Consequently, the authentication of Chinese medicinal plants depending on the correct identification of the species is an essential prerequisite to ensure safety, herbal drug quality, and therapeutic efficacy [11]. Identification of herbal materials, which commonly consist of dried or processed parts, is generally difficult. This is particularly true for several species which have the same name or similar looking herbal materials that can often vary remarkably in their medicinal properties [12–15]. In practice, the identification of medicinal plants relies mainly on morphological and chemical analyses. Many pharmacopoeias [16–18] refer to macroscopic and microscopic evaluation (morphology, histology) and chemical profiling (TLC-, HPLC-, and GC-fingerprinting) for quality control and standardization of raw and processed herbs [19, 20]. However, chemical variability within the plant material often hinders the confirmation of its botanical identity as the chemical composition is affected by growth and storage conditions as well as by the harvesting process. Otherwise microscopic examination of drugs requires botanical expertise for unequivocal authentication as related species often possess similar features.

With the improvements in molecular biotechnology and plant genetics in the past decades, genetic tools are considered to provide more reliability for the authentication of herbal materials at the DNA level. Thus various DNA-based molecular marker techniques are meanwhile applied in many fields and their application is remarkably increasing for species characterization in medicinal plants [21–24]. This is especially useful in case of those taxa that are frequently substituted or adulterated with other species or varieties that

are morphologically and/or phytochemically almost indistinguishable.

Benefiting in the first place from PCR techniques, DNA markers have become a powerful tool for identification and authentication of plant and animal species [25–27]. Contrary to chemical fingerprinting which is strongly influenced by age, physiological conditions, environmental factors, cultivation area, harvesting period, drying and storage conditions, DNA is an extremely stable macromolecule that is not affected by external factors and therefore can be recovered from fresh, dried and even processed biological material. Additionally, the markers are not tissue-specific and thus can be detected at any stage of organism development. Moreover, only a small amount of sample is sufficient for analysis.

Especially DNA-barcoding (an initiative by CBOL = Consortium for the Barcode of Life) uses standard DNA markers from plastidal, mitochondrial, and nuclear regions to facilitate a correct taxonomic identification of species and has become a basic tool for DNA chip technology [28,29].

This review provides an account on DNA-based technologies and most commonly used assays with emphasis on those based on DNA hybridization, restriction enzymes, random PCR amplifications, species-specific PCR primers, and DNA sequencing. A critical evaluation of all methods is presented focusing on their discriminatory power, sensitivity, reproducibility, user-friendliness, and costs.

Types of DNA Methods and Markers Used in Plant Genome Analysis
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There are various types of DNA-based molecular techniques that are used to evaluate DNA polymorphism for authentication of plant taxa [23,24,26,27]. These are hybridization-based methods, polymerase chain reaction (PCR)-based methods, and sequencing-based methods (◉ **Table 1**). In recent times the use of multilocus sequence analysis (MLSA), as commonly used for phylogenetic studies, has proven its discriminatory power. Additionally, DNA microarrays that contain thousands of probes are promising new developments for sensitive and high-throughput taxon identification [30,31].

Non-PCR-based methods

Hybridization-based methods (RFLP): DNA hybridization is a process in which two single stranded DNA fragments anneal into a double-stranded nucleic acid. Restriction fragment length polymorphism (RFLP) implies that a single restriction enzyme produces fragments of different lengths from the DNA marker of different strains of a species or from different related species. In a first step, genomic (or alternatively plastidal) DNA is digested with one or two selected restriction enzymes, and the fragments are separated through electrophoresis on an agarose gel. The resulting differential DNA fragment profile is then transferred to a matrix (e.g., nitrocellulose or nylon membranes) and hybridized with a chemically labelled DNA probe under conditions favoring DNA-DNA hybridization. The fragments to which the probe has hybridized are fluorescent labelled or sometimes linked with enzymes that catalyze a color reaction (digoxigenin – alkaline phosphatase) [32]. Polymorphisms are detected by the presence or absence of bands. The RFLP markers are relatively polymorphic, codominantly inherited, and highly reproducible. The method also provides an opportunity to simultaneously screen numerous samples. DNA blots can be analyzed repeatedly by stripping and reprobing with different RFLP probes. The technique is time-consuming, labor-intensive and requires a large quantity of high amounts of good quality or undegraded DNA.

RFLP combined with DNA hybridization has been mainly used for phylogenetic studies in the past, e.g., in *Lupinus* [33], *Hedysarum* [34], *Triticum* [35], *Musa* [36] and for detection of *Dendrobium* [37] and *Fritillaria* species [38].

PCR-based methods

PCR-based markers involve amplification of particular DNA loci, with the help of specific or arbitrary oligonucleotide primers and a thermostable DNA polymerase enzyme. The major advantages of PCR techniques are that mainly only a small amount of DNA is required, no prior sequence information is needed, and many genetic markers can be generated within a short time. Depending on the primers used for amplification, the PCR-based techniques can be grouped into 1) arbitrary or semi-arbitrary primed PCR techniques that need no prior sequence information (e.g., AP-PCR, DAF, RAPD, AFLP, ISSR); and 2) site-targeted PCR techniques that are developed from known DNA sequences (e.g., CAPS, SSR, SCAR, STS).

RAPD (random amplified polymorphic DNA): The RAPD technology utilizes low-stringency polymerase chain reaction (PCR) amplification with single, short, and arbitrary synthetic oligonucleotide primers (usually 10 bp length) to generate a high number of anonymous DNA fragments. DNA polymorphism can be due to mismatches at the primer site, appearance of a new primer site, and the length of the amplified region between primer sites. Amplification products are generally separated on agarose gels and stained with ethidium bromide. Decamer primer kits are commercially available from various sources. RAPDs are inherited as dominant-recessive characters which means that homozygotes and heterozygotes cannot be distinguished. Low expense, efficiency in developing large number of DNA markers in a short time, and requirement of basic molecular equipment has made the RAPD technique valuable although band reproducibility, problems of comigration, and scoring errors are a major problem.

The technique has been applied in many plant groups such as *Glycyrrhiza* [42], *Atractylodes* [43,44], *Astragalus* [45], *Amomun* [46], *Scutellaria* [47], *Panax* [48–51], *Aconitum* [52], *Ginkgo* [53], *Anectochilus* [54], *Lycium* [55], *Angelica* [56], *Bupleurum* [57], *Dendrobium* [58], *Magnolia* [59], *Asarum* [60], *Apocynum* [61], *Trollius* [62], *Phyllanthus* [63], *Indigofera* [64], *Coptis* [65], *Codonopsis* [66], *Taraxacum* [67], *Elephantopus* [68], and *Rehmannia* [69].

AP-PCR (arbitrary polymerase chain reaction): AP-PCR (or arbitrarily chosen primers ACP-PCR) is a special case of RAPD using single primers approximately 10–50 bp in length [39]. In AP-PCR the amplification is in three parts. In the first two cycles annealing is under nonstringent conditions. Higher primer concentrations are used in the first cycle. Often primers of variable length are used, and products are mostly analyzed on polyacrylamide gels. AP-PCR has been applied in various groups for identification of species and analysis of genetic variation [40,41].

DAF (DNA amplification fingerprinting): As with arbitrarily primed polymerase chain reaction (AP-PCR), DNA amplification fingerprinting (DAF) is also an independently developed methodology, which is a variant of RAPD. For PCR amplification, very short primers of 5–8 nucleotides are used that produce a very complex banding pattern [70,71]. DAF requires careful optimization of parameters and only two temperature cycles are required.

Table 1 Quick overview of DNA based methods or technologies, investigated TCM plant taxa (genus), and reference.

DNA method	Gene	TCM plant Systematic name–genus	References
Hybridization-RFLP		Lupinus	[33]
		Hedysarum	[34]
		Triticum	[35]
		Musa	[36]
		Dendrobium	[37]
		Fritillaria	[38]
RAPD		Glycyrrhiza	[42]
		Atractylodes	[43, 44]
		Astragalus	[45]
		Amomum	[46]
		Scutellaria	[47]
		Panax	[48–51]
		Aconitum	[52]
		Ginkgo	[53]
		Anectochilus	[54]
		Lycium	[55]
		Angelica	[56]
		Bupleurum	[57]
		Dendrobium	[58]
		Magnolia	[59]
		Asarum	[60]
		Apocynum	[61]
		Trollius	[62]
		Phyllanthus	[63]
		Indigofera	[64]
		Coptis	[65]
		Codonopsis	[66]
		Taraxacum	[67]
		Elephantopus	[68]
		Rehmannia	[69]
ISSR		Dendrobium	[75]
		Cistanche	[76]
		Fritillaria	[77]
		Salvia	[78]
		Vitex	[79]
		Cannabis	[80]
		Rhodiola	[81]
		Houttunya	[82]
AFLP		Panax	[87, 88]
		Actaea	[89]
		Plectranthus	[90]
		Caladium	[91]
		Cannabis	[92]
		Rehmannia	[93]
RAMPO		Phoenix	[95]
		Ficus	[96]
Sequencing	ITS	Panax	[101, 102]
		Asarum	[103–105]
		Astragalus	[106, 107, 171]
		Dendrobium	[108–111]
		Fritillaria	[112]
		Leonurus	[113]
		Perilla	[114]
		Rehmannia	[115]
		Salvia	[116]
		Swertia	[117]
		Plantago	[118]
		Bupleurum	[119]
		Euphorbia	[120, 121]
		Hedysarum	[171]

Table 1 Quick overview of DNA based methods or technologies, investigated TCM plant taxa (genus), and reference. *(continued)*

DNA method	Gene	TCM plant Systematic name–genus	References
	5S rDNA	Adenophora	[122]
		Aconitum	[123]
		Angelica	[124]
		Astragalus	[125, 126]
		Curcuma	[127]
		Epimedium	[128]
		Fritillaria	[129]
		Crocus	[130]
		Ligularia	[131]
		Pueraria	[132]
		Saussurea	[133]
	18S rDNA	Dioscorea	[134]
		Pinellia	[135]
		Panax	[136]
	26S rDNA	Fritillaria	[137]
	atpB-rbcL	Phyllanthus	[138]
	trnC-trnD	Panax	[139]
	trnL-trnF	Pueraria	[140]
		Rheum	[141]
		Ephedra	[142]
		Artemisia	[163]
	rpl16	Swertia	[143]
	rpl16-rpl14	Scutellaria	[144]
	atpF-atpA	Angelica	[145]
	trnD-trnT	Dyosma	[146]
	trnK	Actinidia	[147]
		Atractylodes	[148]
		Curcuma	[149]
	matK	Agastache	[150]
		Panax	[151]
	rbcL	Dryopteris	[152]
		Cnidium	[153]
		Pinellia	[154]
CAPS		Alisma	[157]
		Angelica	[158]
		Sinopodophyllum	[159]
		Dyosma	[159]
		Ephedra	[160]
		Fritillaria	[161, 162]
		Artemisia	[163]
		Panax	[164–167]
		Actinidia	[168]
		Atractylodes	[169]
		Glehnia	[170]
		Astragalus	[171]
		Dendrobium	[172]
		Duboisia	[173]
		Codonopsis	[174]
DALP		Stephania	[176]
		Panax	[177]
ARMS		Alisma	[179]
		Panax	[180, 181]
		Rheum	[182]
		Dendrobium	[183, 184]
		Curcuma	[185]
SCAR		Panax	[190, 191]
		Artemisia	[192]
		Phyllanthus	[193, 194]
		Pueraria	[195]
		Sinocalycanthus	[196]
		Embelia	[197]
		Lycium	[198]

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Table 1 Quick overview of DNA based methods or technologies, investigated TCM plant taxa (genus), and reference.   *(continued)*

DNA method	TCM plant	References
Gene	**Systematic name–genus**	
SSR	*Panax*	[202, 203]
	Acanthopanax	[204]
	Dendrobium	[205]
	Cymbopogon	[206]
	Bupleurum	[207]
	Schisandra	[208]
SAMPL	*Cicer*	[213]
	Lactuca	[214]
	Tribulus	[215]
DAMD	*Oryza*	[219]
	Panax	[220]
	Capsicum	[221]
	Salvia	[222]
	Morus	[223]
Microarrays	*Fritillaria*	[228]
	Dendrobium	[229, 230]
	Bupleurum	[231]
	Panax	[232]

DAF products are routinely separated by polyacrylamide gels and detected by silver staining [72].

Although DAF and AP-PCR are different with respect to the length of the random primers, amplification conditions, and visualization methods, they all differ from the standard PCR condition in that only a single oligonucleotide of the random sequence is employed and no prior knowledge of the genome is required.

ISSR (inter-simple sequence repeat): ISSR markers are more and more in demand, because they are known to be abundant, very reproducible, highly polymorphic, informative, and quick to use [73, 74]. Neither sequence information nor prior genetic studies are required. ISSR uses the presence throughout the genome of simple sequence repeats (SSRs) which are ubiquitous, abundant, and highly polymorphic tandem repeat motifs composed of 1 to 7 nucleotides. Inter-simple sequence repeat (ISSR) permits detection of polymorphisms in inter-microsatellite loci. The primers used can be 5′ or 3′ anchored by 1–3 selective nucleotides to prevent internal priming and to amplify only a subset of the targeted inter-repeat regions.

It has been used in the authentication of *Dendrobium* [75], *Cistanche* [76], *Fritillaria* [77], *Salvia* [78], *Vitex* [79], *Cannabis* [80], *Rhodiola* [81], and *Houttuynia* [82].

AFLP (amplified fragment length polymorphism): The AFLP technique is a powerful DNA fingerprinting technology applicable to any organism without the need for prior sequence knowledge. It is a multilocus approach combining restriction fragment analysis with selective PCR amplification [83, 84]. In a first step, total genomic DNA is digested with two or more restriction enzymes (e.g., *Eco*RI; *Mse*I) producing stick ends, and the fragments are ligated to specific adapters (oligonucleotide 10–30 bp length). The ligated DNA fragments are then amplified twice under highly stringent conditions by PCR using radioactive or fluorescence-labelled primers complementary to the adapter and restriction site sequence. Using selective primers which include additional nucleotides at their 3′ end, the complexity of the mixture of fragments can be reduced. The amplicons are separated on a polyacrylamide gel, followed by visualization of the banding pattern [85]. The AFLP technique is a reliable and robust molecular

marker assay that permits the simultaneous screening of different loci randomly distributed throughout the genome [86]. It is very efficient in revealing polymorphisms even between closely related individuals. However, degraded DNA can lead to wrong banding patterns. It has been used for recognition of individuals (paternity analysis, selfing rates, identification of cultivars, clones, etc.) and studying genetic diversity of Chinese medicinal plants. AFLP-based techniques were used in *Panax* [87, 88], *Actaea* [89], *Plectranthus* [90], *Caladium* [91], *Cannabis* [92], and *Rehmannia* [93].

RAMPO (random amplified microsatellite polymorphisms): This method, also termed RAHM (random amplified hybridization microsatellite) or RAMS (randomly amplified microsatellites), combines arbitrarily primed PCR (RAPD) with microsatellite hybridization to produce polymorphic genetic fingerprints [94]. No prior sequence information is needed. Genomic DNA is first amplified with a single arbitrary or microsatellite-complementary PCR primer. After electrophoretic separation and staining of the PCR products, the gel is either dried or blotted onto a nylon membrane, and subsequently hybridized to a labelled, microsatellite-complementary oligonucleotide probe (e.g., [GT]8 or [GA]8). The method is mainly used for identification and discrimination of genotypes within and among populations, cultivates, germplasm, etc. [95, 96].

PCR and DNA sequencing-based methods

DNA sequencing is a powerful tool to characterize species, analyze phylogenetic relationships, population genetics, and evolutionary processes [97]. DNA polymorphisms are revealed by determining the nucleotide sequence in a defined region of the genome and aligning the sequence with homologous regions of related organisms [98].

By choosing appropriate regions of the nuclear, plastidal, or mitochondrial genome this approach provides a highly reproducible analysis at various taxonomic ranks to differentiate TCM plants from its substitutes or adulterants.

In order to provide security for a correct species identification based on DNA sequence data, it is necessary to have a herbarium specimen for verification or a reliable database that guarantees that the reference specimen was correctly identified by a taxonomic expert. Additionally, the sequence should be obtained in independent studies including related taxa. A common way to assign a particular sequence to a taxon is to perform a BLAST search (basic local alignment search tool) in the GenBank database of NCBI. However, care must be taken when assigning the questioned sequence to the species with the highest similarity, because several gaps and false sequences are known to be present in these databases.

There are many studies concerning the application of DNA sequence-based markers to differentiate medicinal taxa used in TCM from its substitutes or adulterants. The ITS region of 18S-26S rDNA has proven to be an important and useful gene locus for DNA barcoding applications [99, 100]. Especially the ITS2 locus was revealed to discriminate among 4800 species with a probability of 92.7% [99, 100].

Sequencing analyses based on this nuclear marker have been applied to *Panax* [101, 102], *Asarum* [103–105], *Astragalus* [106, 107], *Dendrobium* [108–111], *Fritillaria* [112], *Leonurus* [113], *Perilla* [114], *Phyllanthus* [115], *Rehmannia* [116], *Salvia* [117], *Swertia* [118], *Plantago* [119], *Bupleurum* [120], and *Euphorbia* [121].

Another frequently used marker is the nuclear 5S rDNA intergenic spacer used for authentication of *Adenophora* [122], *Aconitum* [123], *Angelica* [124], *Astragalus* [125,126], *Curcuma* [127], *Epimedium* [128], *Fritillaria* [129], *Crocus* [130], *Ligularia* [131], *Pueraria* [132], and *Saussurea* [133].

From nuclear DNA also 18S rDNA has been tested in *Dioscorea* [134], *Pinellia* [135] and *Panax* [136], the 26S rDNA marker in *Fritillaria* [137].

From chloroplast DNA, a couple of markers including genes, intergenic spacers, or introns are applied. The atpB-rbcL region was used for differentiation of *Phyllanthus* [138], trnC-trnD in *Panax* [139], trnL-F in *Pueraria* [140], *Rheum* [141] and *Ephedra* [142], rpl16 in *Swertia* [143], rpl16-rpl14 spacer in *Scutellaria* [144], atpF-atpA in *Angelica* [145], trnD-trnT in *Dyosma* [146], trnK in *Actinidia* [147], *Atractylodes* [148] and *Curcuma* [149], matK in *Agastache* [150], *Panax* [151], rbcL in *Dryopteris* [152], *Cnidium* [153], and *Pinellia* [154].

CAPS or PCR-RFLP (cleaved amplified polymorphic sequence): CAPS, originally named PCR-RFLP is a combination of PCR of target DNA and subsequent digestion with a restriction enzyme [155,156]. CAPS markers are generated in two steps. In the first step, a defined sequence is amplified using specific primers. In the second step, the PCR product is digested with a restriction enzyme usually with a four-base recognition specifity. The digested fragments are separated on agarose gels. However, the ability of CAPS to detect DNA polymorphism is not as high as SSRs or AFLPs because nucleotide changes affecting restriction sites are essential for the detection of DNA polymorphism by CAPS. Furthermore, the development of CAPS markers is only possible where mutations disrupt or create a restriction enzyme recognition site. PCR-RFLP has been used for authentication of *Alisma* [157], *Angelica* [158], *Sinopodophyllum* and *Dysosma* [159], *Ephedra* [160], *Fritillaria* [161,162], *Artemisia* [163], *Panax* [164–167], *Actinidia* [168], *Atractylodes* [169], *Glehnia* [170], *Astragalus* [171], *Dendrobium* [172], *Duboisia* [173], and *Codonopsis* [174].

DALP (direct amplification of length polymorphisms): This method uses an arbitrarily primed PCR (AP-PCR) to produce genomic fingerprints and to enable sequencing of DNA polymorphisms in virtually any species [175]. For PCR higher stringency is necessary.
The uniqueness of DALP relies upon the specific design of primer pairs. It uses a selective forward primer containing a 5′ core sequence of the universal M13 sequencing primer plus additional bases (usually 2-5) at the 3′ end, and a common reverse M13 primer. Any of the bands generated by PCR can be excised from the gel and sequenced directly using forward or reverse primers. After sequencing the polymorphic bands among the samples, species specific primers can be designed. DALP has been used to detect polymorphisms between species [175] and to authenticate *Stephania yunnanensis*, [176], *Panax ginseng*, and *Panax quinquefolius* [177].

ARMS and Multiplex-ARMS (amplification refractory mutation system): ARMS, also known as allele-specific polymerase chain reaction (ASPCR) is a simple, timesaving, and effective method for detecting any mutation involving single base changes (SNPs) or small deletions. It has become a standard technique that allows the discrimination of alleles [178]. The basis of ARMS is that oligonucleotides with a mismatched 3′-residue will not function as primers in the PCR. ARMS allows amplification of test DNA only when the target allele is contained within the sample and will not amplify the nontarget allele. Following an ARMS reaction, the presence or absence of a PCR product is diagnostic for the presence or absence of the target allele. A main advantage of ARMS

is that the amplification step and the authentication step are combined, in that the presence or absence of a PCR product is diagnostic for the presence or absence of the target allele. The method provides a quick screening assay that does not require any form of labelling as the amplified products are visualized simply by agarose gel electrophoresis and ethidium bromide staining. Multiplex ARMS or MARMS is a similar approach but there are several primer combinations to be optimized simultaneously, which increases the complexity of the procedure. This technique has been applied in the authentication of *Alisma* [179], *Panax* [180,181], *Rheum* [182], *Dendrobium* [183,184], and *Curcuma* [185].

SCAR (sequence characterized amplified region): A SCAR can be used to rapidly amplify a diagnostic nucleic acid from herbal materials using a pair of specific oligonucleotide primers designed from polymorphic RAPD [186,187] or ISSR [188] fragments. Polymorphic regions from RAPDs or ISSR are selected among amplified fingerprints. After cloning and sequencing for the selected polymorphic regions, pairs of internal primers are designed to amplify a unique and specific sequence designed as a SCAR marker. PCR results in a positive or negative amplification in target-containing and nontarget-containing samples respectively or amplification products of different sizes in the case of closely related samples. SCARs are advantageous over RAPD markers as they detect only a single locus, their amplification is less sensitive to reaction conditions, and they can potentially be converted into codominant markers [189]. Prior sequence information (i.e., sequencing the polymorphic fragments) is required for designing the primers flanking the polymorphic region. As PCR inhibitory effects of ingredients can lead to false negative results, amplification of a control fragment using the same DNA template should be performed to ensure that the quality of sample DNA is suitable for PCR.
The SCAR technique has been used for authentication of *Panax* [190,191] and for discrimination of species of *Artemisia* [192], *Phyllanthus* [193,194], *Pueraria* [195], *Sinocalycanthus* [196], *Embelia* [197], and *Lycium* [198].

Microsatellites (SSR – simple sequence repeats): Hypervariable repetitive DNA sequences such as microsatellites, minisatellites or midisatellites, and satellites can be of great value in assessing a high level of polymorphism as they are distributed throughout the genomes.
Microsatellites are also known as simple sequence repeats (SSRs), short tandem repeats (STRs), or simple sequence length polymorphisms (SSLPs) are the smallest class of simple repetitive DNA sequences [199,200]. They are highly polymorphic and abundant sequences that are dispersed throughout most eukaryotic genomes. These molecular markers are widely used for DNA fingerprinting, paternity testing, linkage map construction, and population genetic studies but of less importance for species identification [201]. Based on tandem repeats of short (2 – 6 bp) DNA sequences, these markers are highly polymorphic due to variation in the number of repeat units. The repeat length at specific SSR loci is easily assayed by PCR using primers specific to conserved regions flanking the repeat array. PCR fragments are usually separated on polyacrylamide gels in combination with fluorescent detection systems. The hypervariability and codominance of SSRs, their dispersion throughout genomes and suitability for automatization are the principle reasons for their wide utility. A major limitation of SSRs is the time and high development cost required to isolate and characterize each locus when a preexisting DNA sequence is not available. Typically, this process requires

the construction and screening of a genomic library of size-selected DNA fragments with SSR-specific probes, followed by DNA sequencing of isolated positive clones, PCR primer synthesis, and testing.

Microsatellites have been applied in *Panax* [202, 203], *Acanthopanax* [204], *Dendrobium* [205], *Cymbopogon* [206], *Bupleurum* [207], and *Schisandra* [208].

SAMPL (selective amplification of microsatellite polymorphic loci): The SAMPL technique is an SSR-based modification of the amplified fragment length polymorphism (AFLP) procedure [209], but it differs from AFLP by using primers with compound microsatellite motifs in combination with oligonucleotides complementary to the end-ligated adapters for the selective amplification step [210]. In brief, genomic DNA is digested with restriction enzymes (commonly *EcoRI* and *MseI),* and the resulting fragments are ligated to adapters that contain sticky ends to the restriction sites for the enzymes at the genome fragments, and a preamplification reaction for all ligated DNA fragments is carried out with primers annealing to the adapters. These preamplified products, adequately diluted, are then used as templates for a selective SAMPL-polymerase chain reaction (PCR) that uses the adapter-primer *(EcoRI oligo-1)* in combination with an end-labelled microsatellite-based 15-mer oligonucleotide [211] to amplify a group of fragments from those that were restricted, ligated, and preamplified. This multiplexing genome profiling technique has not adequately been used in plant genomics, although a few reports have already documented its potential to detect polymorphisms [212]. This method was used for analysis of genetic diversity in *Cicer* [213], *Lactuca* [214] and *Tribulus* [215].

DAMD (directed amplification of minisatellite-region DNA): DAMD is a DNA fingerprinting method based on amplification of the regions rich in minisatellites at relatively high stringencies by using previously found VNTR core sequences as primers [216, 217]. Minisatellites, also known as variable number of tandem repeats (VNTR) or hypervariable repeats (HVR), are similar to microsatellites (SSR) except that the tandem repeat DNA sequences are longer and generally consist of 10–60-bp motifs. Extreme variations in the tandem repeat copy number of minisatellite loci are responsible for the polymorphism observed. By using the VNTR core sequence as primers, the directed amplification of minisatellite-region DNA (DAMD) with PCR is capable of producing RAPD-like results for the identification of species [218]. It is also used to generate highly variable probes for DNA fingerprinting. This method is more reproducible than RAPD due to the longer primers used.

Recently, DAMD-PCR has been successfully applied for genotyping of wheat cultivars and rice species [219]. The method has been used for authentication of *Panax* [220], *Capsicum* [221], *Salvia* [222], and *Morus* [223].

DNA-microarrays (DNA chip technology)

DNA microarrays or genechips are a high throughput technology for simultaneous analysis of multiple genes in many taxa or samples. To apply this technique for identification and authentication of herbal material it is necessary to identify a distinct DNA sequence that is unique to each species [224]. Based on the gained DNA sequences, corresponding probes are synthesized for many samples. These immobilized DNA fragments are arranged in a regular pattern on a microarray by fixation on glass slides, silicon or nylon membranes [225, 226].

DNA extracted from the target sample and labelled with a specific fluorescent molecule is then hybridized to the microarray DNA. A positive hybridization is detected and visualized with fluorescence scanning or imaging equipment. The microarray is scanned to obtain a complete hybridization pattern generated by the release of a fluorescent, chemiluminescent, or colorimetric signal associated with the binding of the probe to the target DNA. A number of terms such as DNA arrays, gene chips, and biochips are often used to describe these devices [227].

Recently this technique has been applied for the identification of various species of *Fritillaria* [228], *Dendrobium* [229, 230], and *Bupleurum* [231]. In addition, microarray technology has also been used to authenticate *Panax ginseng* [232] and toxic traditional Chinese medicinal materials [233] such as *Aconitum, Strychnos*, and *Datura*.

The results demonstrated that DNA microarray based technology can provide a rapid, high-throughput tool for correct botanical identification, authentication of crude plant materials, standardization and quality control, testing simultaneously hundreds of samples [226].

Future Developments

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New innovative automated assays and specific tools for DNA analysis are emerging and will contribute to the next generation of technologies. These are minisequencing [234, 235], nanoscale DNA sequencing [236] or microsphere-based suspension arrays [237]. Further promising developments are the nanopore technology for identification of DNA bases with very high confidence, and the arrayed primer extension reaction (APEX) which is an enzymatic genotyping method to analyze hundreds to thousands of variations in the genome simultaneously in a single multiplexed reaction [238, 239]. Another upcoming method for large-scale multiplex analysis of nucleic acid sequences is the multiplex oligonucleotide ligation assay (OLA), which can be applied for allelic discrimination in highly polymorphic genes [240].

These techniques have a high multiplexing capacity and great potentials for genotyping and future taxon identification [241].

Limitation of DNA methods

Molecular authentication methods have several advantages which make them suitable for the identification of herbs used in TCM, as compared to macroscopic, microscopic, and phytochemical analyses. The DNA-based techniques are not affected by environmental factors, independent from the physical form of the plant material, and only a low amount of material is required.

Although DNA analysis is currently considered to be cutting-edge technology, it has certain limitations.

▸ The applicability of a DNA-based method depends generally on the quality and quantity of the DNA, which might be a problem for dried or processed materials. Important drug-processing conditions, for example, temperature and pH, may lead to degradation (fragmentation) of the DNA, rendering PCR analysis impossible. However, depending on the degree of degradation of DNA some methods can still be used in processed materials. For these, it is necessary to develop very short amplicons to have a certain probability of successful application [242].

▸ High concentrations of secondary plant compounds (polysaccharides, tannins, essential oils, phenolics, alkaloids, etc.) may influence DNA extraction or PCR reaction. In tissues of medicinal plants, secondary compounds generally get accumulated and the problem becomes severe as the material gets older. Polysaccharide contaminations are particularly problematic as

they can inhibit the activity of many commonly used enzymes, such as polymerases, ligases, and restriction endonucleases. Polyphenol contamination of DNA makes it resistant to restriction enzymes and interacts irreversibly with proteins and nucleic acids. Choosing the most suitable DNA extraction procedure may help to eliminate the PCR inhibitors.

▸ Sometimes plant materials are contaminated with endophytic fungi, which might influence DNA sequencing and can be eliminated with a plant-specific primer design.

▸ DNA related methods can generally not be applied when the herb is processed to an extract.

▸ DNA markers, such as the internal transcribed spacer (ITS) region of the 18S-5.8S-26S nuclear ribosomal cistron, sometimes show intraspecific sequence variation due to nonfunctional paralogous sequences (pseudogenes). For DNA barcoding as a practical molecular method to identify species, only orthologous DNA sequences can be used. Consequently cloning of PCR products is sometimes inevitable.

▸ In order to establish a marker for identification of a particular species, DNA analysis of closely related species and/or varieties and common botanical contaminants and adulterants is necessary, which is a costly and time-consuming process.

Conclusion
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DNA technologies are reliable and powerful tools for identification of taxa at various taxonomic levels (e.g., species, subspecies, variety, strain) as they provide consistent results irrespective of age, tissue origin, physiological conditions, environmental factors, harvest, storage, and processing of samples. With the increasing demand of high-quality herbs, also the need for DNA authentication will accelerate for ensuring the therapeutic effectiveness, a fair trade of drugs and raising consumers' confidence. However, for the modernization of TCM it is inevitable in the future to compile a comprehensive database including DNA data for all investigated medicinal taxa with reference information on nomenclature, phylogenetic relationships, macroscopic and microscopic features, chemical constituents and profiling, toxicity, and voucher specimens in herbaria or museums.

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