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LEADING ARTICLE

DNA Barcoding and Pharmacovigilance of Herbal Medicines

Hugo J. de Boer[1,2] · **Mihael C. Ichim**[3] · **Steven G. Newmaster**[4]

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Abstract Pharmacovigilance of herbal medicines relies on the product label information regarding the ingredients and the adherence to good manufacturing practices along the commercialisation chain. Several studies have shown that substitution of plant species occurs in herbal medicines, and this in turn poses a challenge to herbal pharmacovigilance as adverse reactions might be due to adulterated or added ingredients. Authentication of constituents in herbal medicines using analytical chemistry methods can help detect contaminants and toxins, but are often limited or incapable of detecting the source of the contamination. Recent developments in molecular plant identification using DNA sequence data enable accurate identification of plant species from herbal medicines using defined DNA markers. Identification of multiple constituent species from compound herbal medicines using amplicon metabarcoding enables verification of labelled ingredients and detection of substituted, adulterated and added species. DNA barcoding is proving to be a powerful method to assess species composition in herbal medicines and has the potential to be used as a standard method in herbal pharmacovigilance research of adverse reactions to specific products.

Key Points

DNA barcoding provides a reliable and inexpensive tool for species authentication and monitoring in herbal products.

DNA barcoding can be used to determine plant species used in herbal medicines with adverse drug reactions.

1 Introduction

1.1 General Pharmacovigilance

Pharmacovigilance has been defined as "the study of the safety of marketed drugs under the practical conditions of clinical usage in large communities" [1]. It involves monitoring drug safety and identifying adverse drug reactions (ADRs) in humans, assessing risks and benefits, and responding to and communicating drug safety concerns [2]. The WHO Programme for International Drug Monitoring was set up in 1968 with the aim to ensure that early signs of previously unknown medicine-related safety problems are seen [3]. The safety problems would be identified from pooled data, and information about them shared and acted upon by the national pharmacovigilance centres participating in the programme. The WHO programme's signal detection process is based on data stored in the WHO global individual case safety report database, VigiBase, managed by the WHO Collaborating Centre for International Drug Monitoring, Uppsala Monitoring Centre (UMC). With more than 100 countries contributing data on

✉ Hugo J. de Boer
hugo.deboer@ebc.uu.se

1 The Natural History Museum, University of Oslo, Oslo, Norway

2 Department of Organismal Biology, Evolutionary Biology Centre, Uppsala University, Norbyvägen 18D, 75236 Uppsala, Sweden

3 NIRDBS/"Stejarul" Research Centre for Biological Sciences, Piatra Neamt, Romania

4 Centre for Biodiversity Genomics, Biodiversity Institute of Ontario (BIO), University of Guelph, Guelph, ON, Canada

a regular basis, VigiBase has a unique global coverage enabling international signal detection and assessment, as well as analysis of inter-country or inter-regional reporting patterns [4].

1.2 Pharmacovigilance of Herbal Medicines

Pharmacovigilance should embrace all preparations used medicinally regardless of their regulatory status, pharmaceutical composition, cultural use and philosophical framework [5]. Hence, the same aims and activities of pharmacovigilance apply to herbal medicines. However, pharmacovigilance activities have largely been focused on conventional medicines, and the current model of pharmacovigilance and its science and processes have developed in relation to synthetic drugs. Applying the existing model and its tools to monitoring the safety of herbal medicines presents unique challenges [5], and to that end the WHO has produced draft guidelines for pharmacovigilance practices for herbal medicines [6].

Herbal medicinal products are any medicinal product, exclusively containing as active substances one or more herbal substances or one or more herbal preparations [7], i.e. they contain as putative active ingredients only crude and/or processed plants and/or plant parts. The term 'herbal medicines' encompasses crude preparations, such as dried herbals in entire or powdered form and herbal tinctures, and manufactured or processed herbal medicinal products, formulated as tablets, extracts or capsules. Herbal medicines are commercialised without prescription through herbal medicine practitioners, herbal drug stores and increasingly common through online web shops.

Herbal medicines differ in many aspects from conventional medicines, and several of these aspects pose a variety of challenges to their pharmacovigilance. There are four main challenges:

1. *Substitution and adulteration*. The former concerns the substitution of the intended or labelled species for another species, and the latter the addition of other species than the intended or labelled species. These processes are partly driven by the growing demand for herbals and the limited supply of many species that are harvested from the wild. When the demand for a specific herb is greater than the supply, then there is an increased likelihood that adulterants and poor-quality material will be used. A lack of adequate enforcement of current regulations and quality control along the supply and manufacturing chain exacerbates the risk of not intended species ending up in products on the shelf [8, 9].
2. *Nomenclature of herbals and ingredients of plant origin*. The nomenclature of herbals is complex and can be a problem in pharmacovigilance and in medicine in general, especially when trying to collate data about the adverse responses to a species or product. Plant ingredient nomenclature lacks uniformity and does not follow standard scientific binomial nomenclature. Various types of names are currently in use: pharmaceutical names, scientific generic names, scientific binomials, obsolete scientific names (i.e. synonyms) and vernacular names. For review, see Barnes et al. [10]. This can become a problem if confusion arises from ambiguous vernacular and pharmaceutical names, scientific synonyms and the incorrect use of scientific names.
3. *Lack of monitoring*. Most herbal medicines, like over-the-counter drugs, can be obtained without a prescription from various outlets, not only pharmacies. The absence of a prescriber and registration of use add to the difficulty of monitoring ADRs of herbal medicines.
4. *Standardization* (for reviews see Barnes et al. [5, 10]). Plants are complex mixtures of chemicals comprising several hundreds of constituents. The chemical constituents of many herbal medicines are unknown [11], and the profile of constituents is not uniform throughout a plant or among individual plants grown in different environments [12, 13]. Moreover, different batches of herbal starting materials are likely to vary both qualitatively and quantitatively because of one or more of the following factors: inter- or intra-species variation in constituents; environmental factors, such as climate and growing conditions; time of harvesting; and post-harvesting factors, such as storage conditions and drying [14, 15].

Molecular biology has a significant potential in pharmacovigilance, and DNA barcoding can improve herbal pharmacovigilance related to the challenges discussed above. DNA barcodes are stable across species, including intra-specific variation, and whereas chemical variation among populations is a problem for analytical chemistry methods such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and mass spectrometry (MS), it is not for molecular methods for DNA barcoding using a tiered approach. Chemotaxonomy has been used to identify chemicals for a long time, but it has been known for a long time that it is not a good way to identify species [16, 17].

2 DNA Barcoding

2.1 Development of DNA Barcoding

Recent advances in molecular biology have made it feasible and cost effective to use DNA sequences for species

identification. The method of identifying living organisms to species level using DNA sequences has been coined DNA barcoding and advocated by Hebert et al. [18]. It makes use of short, agreed-upon regions of the genome (a 'barcode') that evolve fast enough to differ between closely related species [19]. When a barcode sequence has been retrieved from an unknown sample, an algorithm is used to compare it with a reference database containing barcodes from identified reference specimens, such as herbarium collections, thus enabling it to be identified. In other words, DNA barcodes function as molecular identifiers for individual species, in the same way as the machine-readable black and white barcodes are used in the retail industry to identify products [21].

Morphology-based identifications have several significant limitations [18]: (1) phenotypic plasticity and genetic variability in the characters employed for species recognition can lead to incorrect identifications; (2) morphologically cryptic taxa, which are common in many groups, can be overlooked [20–23]; (3) morphological keys are often effective only for a particular life stage or sex, and many individuals cannot be identified; (4) modern interactive keys represent a major advance, but the use of keys often demands such a high level of expertise that misdiagnoses are common; and (5) a taxonomic impediment due to a shortage of taxonomic knowledge, and, more problematically, a severe scarcity of trained taxonomists who can professionally identify plants [24, 25]. Identifications based on DNA sequence data have the additional specific advantage that DNA is present in all plant parts and that it is relatively stable. DNA for species identification can be extracted and sequenced from highly processed substrates such as ancient sediments [26, 27], sub-fossil remains such as permafrost preserved dung and stomach contents [28, 29], dried and powdered herbal preparations [32, 33] and, in some cases, even herbal tablets, pills and tinctures [30, 31].

2.2 Molecular Markers for DNA Barcoding

Hebert et al. [18] proposed the use of the mitochondrial gene *CO1* as the standard barcode for species identification in all animals. Assessments have since shown that *CO1* can be used to distinguish over 90 % of species in most animal groups [32, 33]. Barcoding of plants has taken longer to develop, and it has been used less in applied research so far [31, 34–38]. The mitochondrial genome in plants evolves far too slowly to allow it to distinguish between species [39], and various genes and non-coding regions of the plastid genome have been put forward as alternatives [19, 40–46].

In addition to being sufficiently fast evolving, a molecular barcode must also be flanked by conserved regions that can function as universal primer binding sites for polymerase chain reactions (PCRs) [19]. The ideal plant barcode needs to be amplifiable with only a small set of primers, so that it can be efficiently retrieved from any of the 350,000–400,000 species of plants [47, 48]. A single barcoding locus combining these two traits has not been found for plants, and the focus has shifted to a combination of two or more loci to approach a satisfactory level of species discrimination and universality [40]. The two-locus solution posed a problem in the alignment of highly variable or non-coding regions such as the *trnH-psbA* spacer. This issue is overcome by the tiered approach, which is based on the use of a common, easily amplified and aligned region such as *rbcL* that can act as a scaffold on which to place data from a highly variable non-coding region such as *trnH-psbA* or *ITS2* [49]. Using this approach, most species (approximately 75–85 %) can be barcoded, and the addition of surrogate regions can increase barcoding success to over 90 % in some floras [46, 50–53]. However, barcoding is challenging for some plants within taxonomically difficult groups (TDGs) due to frequent hybridisation and the relatively young age of many lineages among other issues [40, 42].

2.3 Species Delimitation and Inter- and Intra-Specific Variation

Choosing the correct additional barcode regions for plants in a TDG requires specific knowledge of marker specificity, species discrimination per marker, hybridisation and species polyploidy [19, 40, 44, 45]. Most species concepts agree on species being evolving metapopulation lineages, but delimiting species is often more problematic [54]. The important role of hybridisation in plant speciation makes species delimitation in plants much more complicated than in animals [55]. Polyploid speciation, in which the entire genome is duplicated, is particularly frequent in plants, and polyploidy accounts for up to 15 % of the speciation events in angiosperms [56, 57]. Species delimitation based on molecular data is being developed but requires many accessions as well as many loci [58]. The most reliable approach to DNA barcoding of plants is the creation of high-quality specific sequence reference databases for the identification of targeted species [31, 37, 59]. Such databases should include reference to a specific taxonomic voucher that has been determined by a professional taxonomist. It should also include sequence data and vouchers for all putative species that might be detected, e.g. an authentication database for ginseng should include sequences for all species that are known to be used as possible substitutes for ginseng. Not expected species, i.e. novel substitutes, can be detected by comparing sequences against a reliable database.

2.4 Availability of Data

Identification and authentication by DNA barcoding ultimately relies on the availability of reference sequence data, and the coverage of genetic variation within these data. The sequence data that are essential for this process are deposited and maintained in publicly funded databases and can be accessed without cost. The most important resources are as follows:

1. BOLD Systems (Barcode of Life Data Systems) (http://www.boldsystems.org) [60]: an informatics workbench aiding the acquisition, storage, analysis and publication of DNA barcode records. It contains over 370,000 plant barcodes representing over 58,510 species of plants. All of these records include DNA barcodes, vouchers, images, maps, collection coordinates and more ancillary collection data. Barcode sequence data of unknown species can be rapidly and accurately compared using a large suite of online database tools for the collection and management of specimen, distributional and molecular data as well as analytical tools to support their identification/validation. Partners in BOLD are iBOL [International Barcode of Life (http://www.ibol.org)], CBOL [Consortium for the Barcode of Life (http://www.barcodeoflife.org)], GBIF [Global Biodiversity Information Facility (http://www.gbif.org)] and NCBI GenBank [National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov)]. The BOLD database is currently missing many medicinal plant species and does not have complete coverage of various barcode regions such as *ITS2* for many of the medicinal species in the database.
2. NCBI GenBank [61]: a database that contains publicly available nucleotide sequences for over 300,000 formally described species. In October 2014, GenBank exceeded 1 Terabase (10^{12}) of assembled sequence data. Sequence data of unknown species can be rapidly and accurately compared to that of verified species using the BLAST (basic local alignment search tool) algorithm [62]. Critical reviews have suggested caution regarding the use of GenBank to identify unknown plant materials [63, 64]. This claim is supported by several facts, including (1) unreliable taxonomy (errors in specimen record keeping); (2) most GenBank sequences do not specify a corresponding taxonomic specimen; and (3) GenBank data are particularly unsuited to studying barcodes based on more than one marker because the sequences from different markers cannot be associated with a single specimen.
3. MMDBD (Medicinal Materials DNA Barcode Database) (http://137.189.42.34/mherbsdb/index.php) [65]: an integrated DNA barcode multimedia information platform that can be used for DNA sequence identification and data retrieval. MMDBD contains over 15,375 sequences representing 1660 species of medicinal materials listed in the Chinese Pharmacopoeia and American Herbal Pharmacopoeia combined. The database contains sequences for multiple regions including four nuclear regions, four mitochondrial regions and seven chloroplast regions. MMDBD also contains resources on adulterant information, medical parts, photographs, primers used for obtaining sequences and key references.

3 Plant Barcoding and Herbal Medicines

Various other molecular markers have also been developed for molecular plant identification, and some of these have been successfully applied to the identification of medicinal plants and herbal medicines. An extensive review [66] of the most common DNA-based methods examines random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplification refractory mutation system (ARMS), cleaved amplified polymorphic sequence (CAPS), amplified fragment length polymorphism (AFLP), DNA amplification fingerprinting (DAF), inter-simple sequence repeat (ISSR), simple sequence repeat (SSR), sequencing, hybridization and microarrays. The three methods most relevant to herbal pharmacovigilance are reviewed here with examples of their application to illustrate their use: Sanger sequencing of standard DNA barcoding markers, high-throughput metabarcoding and Bar-HRM (high resolution melting).

3.1 Sanger DNA Sequencing

Techen et al. [67] provide an overview of studies using DNA barcoding for medicinal plant identification in their review paper published in 2014.

The most comprehensive study of DNA barcoding of medicinal plants was performed by Chen et al. [35] who compared PCR amplification efficiency, differential intra- and inter-specific divergences, and the DNA barcoding gap of seven candidate DNA barcodes (*psbA-trnH*, *matK*, *rbcL*, *rpoC1*, *ycf5*, *ITS2* and *ITS*) from medicinal plant species. Their data suggested that the nuclear ribosomal DNA marker *ITS2* is the most suitable region for DNA barcoding applications. Testing the discrimination ability of this marker in more than 6600 plant samples belonging to 4800 species from 753 distinct genera found that the rate of successful identification with the *ITS2* was 92.7 % at the species level. The objective of their paper was to find the most universal marker for medicinal plant identification,

which is different from some of the following examples where barcoding was used to identify selected species from complexes of intended species and their substitutes. Recent research on *Cassia* [68], *Ginkgo* [69], *Hypericum* [70] and many other species [71] has shown great utility in using DNA barcodes for the authentication of ingredients in herbal medicines and related natural health products (NHPs).

Both Wallace et al. [71] and Zuo et al. [72] studied barcoding in ginsengs (*Panax*, Araliaceae), a well-known group of medicinal plants. Several ginseng species are endangered due to over-exploitation of natural resources, and both adulteration and substitution are rife due to the high prices of the raw herbals. Monitoring rare species and authentication of the true product could possibly mitigate some of these threats. Wallace et al. [71] tested 41 ginseng samples and found that the core barcodes *matK* and *rbcL* required additional data from *ITS* for successful species identification. Zuo et al. [72] used 95 ginseng samples, representing all of the species in the genus *Panax*, and found that the combination of *psbA-trnH* and *ITS* is sufficient for identifying all of the species and clusters in the genus. A limitation of these studies is the specific focus on ginseng from the genus *Panax*, and they do not yield sequences for identification of ginsengs from other genera, such as *Eleutherococcus* spp. (frequently referred to as 'Siberian ginseng') or *Pseudostellaria heterophylla* (Crown Prince ginseng). However, the biggest problem with *Panax ginseng* root adulteration is the admixture, or substitution with material, of *P. ginseng* leaves (which contain relatively high levels of ginsenosides, but in a different profile than found in the root). This type of adulteration is easily detected by chemical means, but cannot be detected using DNA-based methods. DNA barcoding does not address adulteration issues concerning different morphological parts from the same species. For example, some herbal species possess unique chemicals within different parts of the plant such as flowers, leaves, stems and roots; DNA barcoding cannot differentiate leaves from stems roots or flowers within the same species.

Vassou et al. [73] studied market samples of *Sida cordifolia* to quantify substitution using a reference library of 13 species of *Sida* and four molecular markers (*rbcL*, *matK*, *psbA-trnH* and *ITS2*). They showed that none of the market samples belonged to the authentic species. Of the market samples, 76 % belonged to other species of *Sida*. The authors conclude that such substitutions may not only fail to give the expected therapeutic effect, but may also give undesirable effects as in case of *Sida acuta*, which contains a sixfold higher amount of ephedrine than the roots of *S. cordifolia*. A similar study by Seethapathy et al. [74] on authentication of laxatives made from wild harvested *Cassia*, *Senna* and *Chamaecrista* species using four DNA barcoding markers (*ITS*, *matK*, *rbcL* and *psbA-trnH*) revealed considerable adulteration of herbal products and confirmed the suspicion that there is rampant herbal product adulteration in Indian markets.

Studies aiming to identify plant species traded as herbal medicines without defining a specific product, complex or taxonomic group are fewer. Kool et al. [37] used molecular identification to study plant roots from the Moroccan herbal pharmacopoeia traded by herbalists in marketplaces in southern Morocco. Roots such as these are challenging to identify using morphology alone and are known to be difficult to work with using molecular biology methods due to DNA contamination and degradation during processing. Combining *rpoC1*, *psbA-trnH* and *ITS* allowed the majority of the market root samples to be identified to genus level, and for a minority of the samples, the barcoding identification differed significantly from previous hypotheses based on the vernacular names. They also conclude that adulteration of roots is common, and suggest that this may indicate that the products are becoming locally endangered. Their study also highlights that samples of unknown taxonomic affiliation are more difficult to identify than earlier suggested, especially if the reference sequences were obtained from different populations.

A study that distinctly shows the potential of DNA barcoding for monitoring herbal products was performed by Newmaster et al. [31]. Their research aimed to investigate herbal product authenticity using DNA barcoding and they conducted a blind test of 44 herbal products representing 12 companies and 30 different species of herbs sold in North America. Using a tiered approach (*rbcL* + *ITS2*) they attained 95 % species resolution, and found that most of the products tested contained DNA barcodes from plant species not listed on the labels. Nearly half (48 %) of the products contained the intended species reported on the label, but the other samples were adulterated with species not listed on the label and/or fillers. Product substitution occurred in 32 % of the products tested and only two of 12 companies had products without any substitution, contamination or fillers. As this study considered fillers, the level of adulteration was inflated in comparison with other studies that did not consider fillers such as wheat and rice. Other studies of adulteration in NHPs have found similar estimates, including a recent paper, which revealed that the level of substitutions may be as high as 71% [102]. Supportive research has documented adulteration many herbal products including 50 % in ginseng and 25 % in black cohosh [71, 75], 33 % in herbal teas [76] and 16 % in *Ginkgo* products [69]. Estimates of adulteration are also similar in India, where they range from 37 % in *Senna* to 50 % for *Cassia* products [74]. All of these authors conclude that there is considerable product substitution, contamination and use of fillers in NHPs, and they suggest that the herbal industry should embrace DNA

barcoding for authenticating herbal products through testing of raw materials used in manufacturing products. In a 2014 US Pharmacopeial Convention (USP) workshop sponsored by the USP and United States Department of Agriculture (USDA) that was focused on "DNA for Quality Control of Botanical Products" (see http://www.usp.org), there were claims that 25 % of routine samples tested are adulterated and that the actual level of adulteration may be more than 50 %. More marketplace studies need to be published in scientific journals in order to have accurate estimates of NHP adulteration, which may provide a list of herbal species that are subject to higher rates of adulteration.

3.2 Metabarcoding and High-Throughput Sequencing

In the decade or so that DNA barcoding for species identification has been advocated and used, great advances have been made in methodology, cost and reference collections. Methodological advances in high-throughput DNA sequencing have made it possible to sequence DNA from processed and mixed samples. Metabarcoding combines two sequencing methods: DNA barcoding and high-throughput DNA sequencing [77]. It uses universal PCR primers to mass-amplify barcodes from DNA extracted from environmental, sediment, ancient or processed samples. The amplicons are then sequenced using high-throughput sequencing and the resulting DNA reads provide information that can be used to determine from what plant species the DNA originated. Metabarcoding has been used for species identification from highly processed substrates such as ancient sediments in cores from afroalpine lake sediments [26] and Greenlandic and Scandinavian ice age refugia [27, 78], sub fossil remains such as permafrost preserved dung and stomach contents [28, 29], processed foods [77, 79, 80] and even herbal tablets, pills and tinctures [30].

An example to demonstrate metabarcoding is the study by Coghlan et al. [30], who used it for detecting species from highly processed material. They used high-throughput next-generation sequencing to screen 15 complex traditional Chinese medicines (TCM) samples, and generated over 49,000 sequence reads. These reads were clustered into operational taxonomic units (OTUs) that were identified using BLAST sequence similarity matching [62] and belonged to 68 plant families, including two genera containing possibly toxic species. Their screening revealed that some of the TCM samples contained traces of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora)-listed animal and plant genera, such as the Asiatic black bear (*Ursus thibetanus*), the Saiga antelope (*Saiga tatarica*) and a wild ginseng species (*P. ginseng*).

3.3 Bar- High Resolution Melting

Bar-HRM is an emerging method that combines DNA barcoding with HRM [81]. The denaturation thermodynamics of individual double-stranded DNA to single strands are based on the binding affinities of individual nucleotide pairs, and the melting pattern will vary due to indels, mutations and methylation. Even a single base change between samples can be readily detected and identified [82, 83].

HRM does not require sequencing and is relatively low cost. The first study reporting the use of Bar-HRM to study herbal medicine substitution has recently been reported in a study on species substitution among three medicinal species of Acanthaceae [84]. Bar-HRM has also been used in a number of comparable methodologies [85], such as for authentication of an EU Protected Designation of Origin product made from *Lathyrus clymenum* [86], olive oil and adulterants [87], subspecies cultivar identification in eggplant [88], identification of closely related species relevant to TDG [89, 90], species distinction in Mediterranean pines [91], detection of allergenic hazelnut contamination [92], and processed bean crops [93–95].

Bar-HRM is quickly gaining popularity in application due to its low direct and indirect costs and high accuracy of detection, and it is very suitable for authentication of single-ingredient herbal products. This and other related techniques such as isothermal amplification [96] offer cheap, fast methods that can be easily deployed into the quality assurance and control systems of herbal product manufacturers and distributors.

3.4 Limitations of DNA Barcoding in Authentication of Herbal Products

The main limitations to DNA barcoding are related to the quality of the template DNA, the affinity of the primers, the effects of the PCR and the sequencing method. DNA barcoding relies on the availability of DNA, and plant DNA can be removed or degrade during the manufacturing process of herbal products: extensive heat treatment, irradiation, ultraviolet light exposure, filtration, extractive distillation or supercritical fluid extraction [97]. Processed products from which DNA is completely absent are not suitable for DNA barcoding. The level of DNA degradation at which useful sequences for identification can still be generated varies depending on the sequencing method, i.e. high-throughput sequencing approaches can work with fragment sizes of 50–400 base pairs (bp), whereas the PCR needed to amplify DNA for Sanger sequencing requires the fragments to be at least the size of the amplified fragment (>500 bp), but less fragmented total DNA yields much higher chances of amplification of target barcode markers. In addition, the

processes by which tinctures and extracts are made degrade or remove plant DNA from raw materials, thus making DNA barcoding unfeasible. PCR-based Sanger sequencing and amplicon barcoding are sensitive to PCR-bias in which primer affinity differences result in biased amplification. At each cycle of the PCR reaction the target DNA is doubled, and after a standard 35 cycles a single DNA target will have been amplified to more than 10 billion copies. If the product that needs to be authenticated contains DNA from another plant species, and if that DNA has a higher binding affinity for the barcoding primers, then during the PCR the DNA of that species could be preferentially amplified, which in turn would yield a false negative for the putative species during Sanger sequencing. PCR-bias and amplification from mixtures of plant DNA using Sanger sequencing can be partly overcome by doing multiple parallel PCRs (cf. Newmaster et al. [31]) or cloning specific PCR products into vectors to be able to PCR a purified target. High-throughput amplicon sequencing sequences all present DNA post-amplification, and this includes chimeral PCR products. PCR chimeras are believed to arise from incomplete elongation. During subsequent cycles of PCR, a partially extended strand can bind to a template derived from a different but similar sequence. This then acts as a primer that is extended to form a chimeric sequence [98]. The effect of chimeric sequences can be reduced by filtering out rare amplicons during downstream clustering of reads into OTUs [99]. Different sequencing methods also have different limitations. Sanger sequencing requires a pure PCR product for accurate base calling, i.e. PCR products cannot be mixtures of different sequences from the same or different organisms. High-throughput sequencing approaches have varying sequencing error rates depending on the platform: $\sim$0.1 % for Ion-Torrent™ Personal Genome Machine® (PGM), $\sim$1 % for Illumina platforms and $\sim$15 % for Pacific Biosciences. Sequencing errors appear to be similar to DNA mutations and can complicate accurate species discrimination. Limitations are inherent to every method of authentication, and the limitations of DNA barcoding do not outweigh its unprecedented ability to identify species that are processed beyond identification using morphology alone. We recommend the creation of short (<150 bp) mini barcode sequence libraries for herbal species as we have found that they considerably increase the success of attaining good-quality sequences from highly processed NHPs.

4 Barcoding and Pharmacovigilance of Herbal Medicine

Authentication of herbal medicines using other methods is already a regulatory requirement in many countries. In the USA, under the Food and Drug Administration (FDA) current Good Manufacturing Practice (cGMP) regulations for dietary supplements, it is the responsibility of any company that manufactures, packages, labels or holds dietary supplements to conduct at least one appropriate test or examination to verify the identity of any component that is a dietary ingredient [100]. DNA barcoding is not considered to be inappropriate by the FDA, but it currently does not yet use DNA barcoding for authentication of botanical extracts [101]. According to the World Health Organization (WHO), the use of incorrect species is a threat to consumer safety and a recent large study [102] of NHP adulteration has concluded that DNA barcoding should be used in a complementary manner for species identification alongside chemical analyses to detect and quantify the required chemical compounds, thus improving the quality of NHPs. In Europe, the European Medicines Agency (EMA) has issued numerous guidelines concerning the quality and testing of herbal substances and herbal medicinal products [7]. Identification tests should be specific for the herbal substance and are usually a combination of three or more of the following: macroscopical characters, microscopical characters, chromatographic procedures and chemical reactions. The EMA guidelines specifically encourage the use of evolving technologies if these are considered to offer additional assurance of quality [7].

The different examples of DNA barcoding of herbal products all reveal widespread adulteration and substitution in herbal medicines. These findings raise the important challenge in herbal pharmacovigilance that ADRs to herbal medicine could easily be caused by undeclared species or constituents. Signal detection in pharmacovigilance is performed by analysis of reported ADRs to specific products, medicines and ingredients, but herbal pharmacovigilance needs an additional focus on post-marketing analysis of herbal products using DNA barcoding to establish species constituents and as a highly reliable tool in the arsenal of analytical methods when appropriately applied to various types of herbal products.

5 Conclusion

DNA barcoding is a highly reliable tool for detection of species constituents in various types of herbal products, and should be advocated as an addition to the arsenal of current analytical methods. Routine DNA barcoding authentication of herbal medicines as part of the manufacturing process could raise quality, authenticity and accountability in the herbal industry, and facilitate pharmacovigilance monitoring and signal detection.

In the meantime, DNA barcoding to establish species composition in batches of herbal preparations that have led to suspected ADRs could aid in refining putative causes of

effect. Close collaboration between national medical product agencies and academic or commercial institutes skilled in plant DNA barcoding should be encouraged to pilot such DNA barcoding-based herbal pharmacovigilance.

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