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Review article

DNA barcoding: an efficient tool to overcome authentication challenges in the herbal market

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Summary

The past couple of decades have witnessed global resurgence of herbal-based health care. As a result, the trade of raw drugs has surged globally. Accurate and fast scientific identification of the plant(s) is the key to success for the herbal drug industry. The conventional approach is to engage an expert taxonomist, who uses a mix of traditional and modern techniques for precise plant identification. However, for bulk identification at industrial scale, the process is protracted and time-consuming. DNA barcoding, on the other hand, offers an alternative and feasible taxonomic tool box for rapid and robust species identification. For the success of DNA barcode, the barcode loci must have sufficient information to differentiate unambiguously between closely related plant species and discover new cryptic species. For herbal plant identification, *matK*, *rbcL*, *trnH-psbA*, *ITS*, *trnL-F*, *5S-rRNA* and *18S-rRNA* have been used as successful DNA barcodes. Emerging advances in DNA barcoding coupled with next-generation sequencing and high-resolution melting curve analysis have paved the way for successful species-level resolution recovered from finished herbal products. Further, development of multilocus strategy and its application has provided new vistas to the DNA barcode-based plant identification for herbal drug industry. For successful and acceptable identification of herbal ingredients and a holistic quality control of the drug, DNA barcoding needs to work harmoniously with other components of the systems biology approach. We suggest that for effectively resolving authentication challenges associated with the herbal market, DNA barcoding must be used in conjunction with metabolomics along with need-based transcriptomics and proteomics.

Introduction

Global resurgence in traditional health systems is expanding the herbal commodity market towards sustenance of healthy life. India is a mega hot spot of biodiversity, and medicinal plants are one of the fastest-growing segments of the alternative medicine market in India. India's traditional systems of medicine (Ayurveda, Yoga, naturopathy, Unani, Siddha and Homeopathy) are time-tested and benefitting mankind even today. According to a WHO report, about 80% of the world population relies on the plant-based systems of medicine for their primary healthcare needs (WHO e-link). Medicinal plants contribute 80% of the raw materials used in the preparation of traditional drugs. The efficacy of these drugs mainly depends upon the proper use and sustained availability of genuine raw materials. The dramatic increase in exports of medicinal plants in the past decade testifies to the worldwide interest in these products as well as in traditional health systems (Marichamy *et al.*, 2014).

Indian herbal medicine is part of a system of medical thought and practice that is distinctly different from that of Western medicine. Globalization of trade is expanding the herbal product market. However, there has also been an increase of unscrupulous commercial practices, whereby the authentic herb is substituted and contaminated with less effective and often deleterious herbs and unlabelled fillers. At present, there is no standard practice available/in place for identifying the plant species used in herbal products and the industry suffers from fraud and unethical practices (Newmaster *et al.*, 2013). It is worth mentioning that the consumer faith in herbal drugs is on the decline due to the prevailing trend of adulteration and substitution (Poornima, 2010). Substitution of main herbal ingredients by some other species and the presence of unlabelled fillers used in herbal products result in reduced therapeutic potential of the original drug, posing a serious risk to the health of the consumers (Newmaster *et al.*, 2013). The diagnostic morphological features of the plant species on which the traditional taxonomic system is based cannot typically be used for identifying powdered or otherwise processed plant materials. Thus, there is an urgent need to have in place broadly acceptable commercial tools for detecting substitution and authentication of herbs used industrially.

The broadly available commercial technologies used for authentication of plant-based commodities include physical methods, chemical/biochemical methods, immunoassays and the most recent DNA-based molecular tools. The classical approaches of plant identification involving the organoleptic method, micro- and macroscopic characters and chemical profil-

ing did not evolve successfully. While the former methods require trained personnel for taxonomical examinations, the latter may be affected by physiological and storage conditions. Still, sometimes there are differences of opinion among scientists regarding the exact taxonomic annotation of a species according to traditional taxonomy. Advancement in molecular techniques has enabled researchers to use simple and cost-effective rapid DNA analysis as a universally acceptable platform. In the last decade, several genome-based methods have been developed for identification and authentication of a large number of organisms, but there is no single universally applicable approach that provides a comprehensive solution for many of the problems concerning plant identification. Many a time various tools have been found to complement each other for specific applications. These techniques differ in their resolving power to detect genetic differences, the type of data generated and their applicability to particular taxonomic levels (Kumar *et al.*, 2009). Authentication at the DNA level provides more reliability because, in contrast to other macromolecules (proteins and RNA), DNA is more stable, is not affected by external factors and is found in all tissues. So, there is a potential need for the development of robust DNA-based markers for plant identification and authentication at commercial level (Sucher and Carles, 2008).

Among the prevailing genome-based approaches to overcome the difficulties of traditional taxonomy, DNA barcoding proposed by Hebert *et al.* (2003a) has been found to be successful in the identification of existing species and the discovery of unknown species. It is a recent and widely used molecular and computational-based identification system that aims to identify biological specimens and to assign them to a given species. It can be considered the core of an integrated taxonomic system. It is a technique in biodiversity research, wherein we use a standardized region of DNA for identifying a species or a taxon. The region used for identification is termed as a DNA barcode, which constitutes a small part (<1000 bp) of the genome and can be easily obtained. DNA barcoding is an oversimplified solution to a complex problem, which provides a way to confirm the authentication of raw plant material and establish a level of quality assurance within the market place (Li *et al.*, 2011b). The 5′ end of cytochrome *c* oxidase 1 (*CO1*) from the mitochondrial genome was considered a universal barcode marker in animals, but a similar general barcode for plants has remained elusive (Hebert *et al.*, 2003b; Kress and Erickson, 2008). In plants, it cannot be used preferably, due to its slow evolution and limited divergence. Therefore, the search for plant barcode region shifted towards chloroplast and nuclear genomes, which contain higher rates of substitution (Hollingsworth *et al.*, 2011). Species discrimination in plants is even more difficult due to greater levels of gene tree paraphyly (Fazekas *et al.*, 2009). During the last decade, major individual candidate regions *matK*, *rbcL*, *trnH-psbA*, *ITS, trnL-F*, *5S-rRNA* and *18S-rRNA* have been tested for use in plants with respect to their discrimination capacity. Due to differences in their efficiency, it was concluded that no single-locus plant barcode exists and the search for multilocus combinations was suggested and is still being sought. Two international initiatives working towards the development of DNA barcodes include the Consortium for the Barcode of Life (CBOL) and the International Barcode of Life (iBOL). The CBOL was established in 2004 for working towards the development of DNA barcoding as a global method for identification of flora and fauna constituting the earth's biodiversity. It suggested the combination of *matK* and *rbcL* as the potential plant barcoding region based on its universality and discriminating ability. The iBOL is the largest biodiversity genomics initiative ever undertaken, which maintains barcode reference library Barcode of Life Data systems (BOLD) and works through its constituent nodes comprising several nations clustered into separate working groups.

DNA barcoding of medicinal plants can be quite challenging, both in generating barcodes and in analysing the data to determine discrimination power (Cowan and Fay, 2012). Despite these challenges, the number of barcoding studies in plants is on the rise owing to its utility in instant identification of unknown samples (Figure 1). Apart from authentication of traded medicinal plants, DNA barcoding also finds application in biodiversity monitoring, conservation impact assessment, monitoring of illegal trading, forensic botany, etc. (Ferri *et al.*, 2015; Nithaniyal *et al.*, 2014; Verma and Goswami, 2014).

Here, we suggest DNA barcoding as the path ahead in the postgenomic era for the identification/authentication of medicinal plants used in trade. However, it will be better to complement it with metabolomics along with need-based transcriptomics and proteomics analyses. The reason being that contamination in a herbal product may not always be at the plant species level. It could also be due to replacement of the traditionally prescribed plant part by a non-prescribed one of the same plant species or by a traditionally prescribed part of the right plant species collected in the wrong season. In both cases, the content of the active metabolites will be suboptimal (leading to compromised thera-

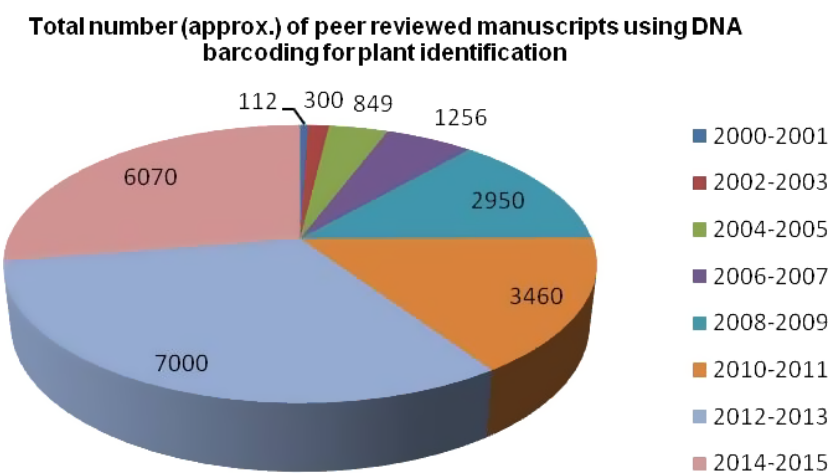

Figure 1 Total number (approximately) of peer reviewed manuscripts using DNA barcoding for plant identification. The chart is based on literature search performed with Google Scholar and SCI Finder on 3 March 2015.

peutic activity) and it could be detected only through metabolite analysis. Thus, the success of the approach for authentication will depend on how DNA barcoding is combined with other tools depending on the nature of the problem being tackled.

Bulk herb trade: the global scenario

Herbal medicinal products have become a subject of increasing global importance, for their health benefits and economic considerations. India holds 7%–8% of global biodiversity with enormous resources of medicinal plant species (approximately 45,500). Out of these, more than 8000 species of both higher and lower plant groups are of medicinal value and 960 species of medicinal plants are estimated to be in trade, of which 178 species have annual consumption levels in excess of 100 metric tons (Aneesh *et al.*, 2009; Efferth and Greten, 2012; www.cbd.int). The industrial demand for the medicinal plant resources has been on the rise due to worldwide growth in the herbal sector (Ved and Goraya, 2007). The Indian market is a hub of herbs with estimated trade of $140 million per year. According to the Medicinal Plants and Extracts report published in the Market News Service, December 2011 bulletin, the botanical and natural ingredient export was approximately $33 billion during 2010 (Figure 2) and by 2015 it is expected to reach $93 billion. The published estimates of international export of Indian medicinal plants and their products account for $0.2 billion. In addition to the international trade, there is a substantial volume of internal trade in medicinal plants in India with turnover of $1.6–$1.8 billion (Marichamy *et al.*, 2014).

Total global herbal market is of the size of 60 billion dollars annually with India's contribution being a meagre 2.5%, which shows that in spite of having a rich heritage of Ayurvedic literature and a wide range of medicinal plant species, India is still not able to tap the potential market demand available in this sector of foreign trade. Improvement of quality control, standardization, scientific methods of production and evaluation of commercial products is an immediate need for improving India's share of the global herbal market. The overall market potential of herbals can be increased only through the development of standardized herbal products that are tested using scientifically validated methods. This approach would not only maintain the quality and efficacy of the herbals, but will also provide a competing edge *vis-a-vis* modern medicine (Agarwal *et al.*, 2013).

Prevalent false advertising/labelling malpractices in the herbal industry

Adulteration and substitution of raw drugs have become a widespread problem in the herbal industry due to deforestation and extinction of many species as well as incorrect identification of many plants. The term adulteration specifies a number of conditions, which may be intentional or accidental. The crude drugs are substituted with inferior material or unlabelled fillers. This reduces the efficacy and therapeutic potential of original drugs, which in turn leads to loss of consumer faith. Unlabelled plant fillers found in herbal products sometimes pose potential health risks to consumers too (Newmaster *et al.*, 2013; Poornima, 2010). For safe and effective use, consistency in composition and biological activity is essential. However, the difficulties in plant identification and lack of information about active pharmacological principles, as well as variation in the process of cultivation/collection, extraction and growth conditions along with genetic variability, lead to failure of herbal drug standards.

Another major problem concerning the quality of herbal drugs is the report of heavy metals in plant materials (Ernst, 2002; Ernst and Thompson, 2001). DNA-based analysis also helps in the identification of phytochemically indistinguishable genuine drug from a substitute drug (Lazarowych and Pekos, 1998), where the substitute is unacceptable due to a known tendency to accumulate higher level of heavy metal. Some plant fillers also happen to come from species that have high heavy metal content. A comprehensive study carried out by Saper *et al.* (2004) on herbal medicinal products concluded that samples collected in India contained significant amounts of heavy metals (64% had mercury, 41% had arsenic, and 9% had cadmium). Even the traditional medicines from China, Malaysia, Mexico, Africa and the Middle East have also been shown to contain heavy metals (Ang *et al.*, 2003; Baer *et al.*, 1998; Ko, 1998; Lekouch *et al.*, 2001). Such contamination can lead to serious harm to patients taking such remedies and could also interfere with the assessment of safety in a clinical trial.

Various case studies on substitution and adulteration of popular Ayurvedic drugs have been carried out in the past (Madhavan *et al.*, 2010; Prakash *et al.*, 2013; Rai *et al.*, 2012). Such cases of deliberate adulteration of coveted ingredients are often difficult to distinguish from cases of misidentification. There is a need for high-quality studies to evaluate the value of traditional drugs as compared to modern medicines.

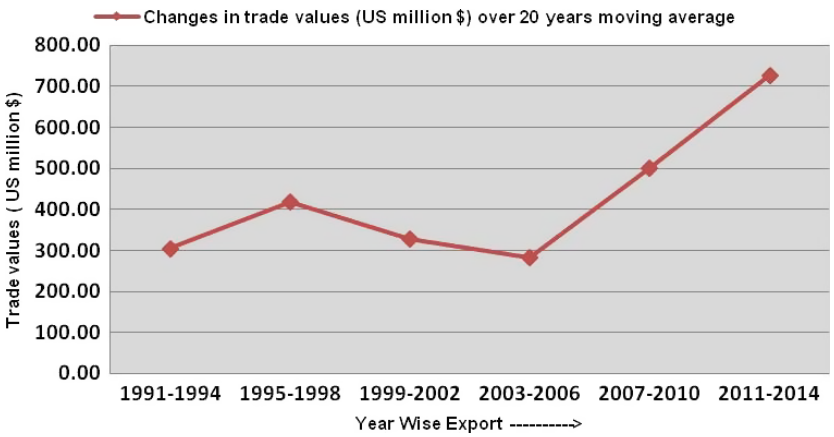

Figure 2 Export scenario of Indian medicinal plants over 20 years moving average (Kumar and Janagam, 2011; Marichamy *et al.*, 2014).

Based on the published resources with proper experimental evidence, we have made our best possible efforts to tabulate the existing substitutes/adulterants for highly traded and in demand medicinal plant species (Table 1). It is cautionary to note here that the Ayurvedic literature, although extensive, provides detailed information only on a limited number of species. Although Ayurvedic literature does not forbid substitutes, their regulations are often not wide enough for one to cover all that is currently available in the market.

Conventional approaches for plant authentication

Prior to the advent of modern molecular tools, morphological and anatomical descriptions were the only primary means of plant identification, which usually involve description of variation for morphological traits by expert taxonomist and trained technicians through experience. This routine species identification is declining due to certain limitations such as misdiagnosis caused due to lack of high level expertise, overlook of morphologically cryptic taxa and lack of effectiveness due to incomplete morphological keys for particular life stages (Vohra and Khera, 2013). Pharmacognostical study involving physical, chemical and sensory characters of raw drugs, both in whole state and in powder form paved the way for quality sourcing of crude medicinal materials. Asserting the physiochemical properties of individual drug or proprietary medicines by comparing with the standard values of Indian pharmacopoeia was another means to the authentication. The Ayurvedic pharmacopoeia of India published in two different parts comprising 7 and 3 volumes, respectively, reflects the overall monographs on herbal drugs including their microscopic characterization and their medicinal effects.

The botanical and descriptive aspects of pharmacognosy were supplemented by medicinal and pharmaceutical chemistry for drug quality assurance testing. Phytochemical analysis methods, such as Fourier transform infrared spectroscopy, high-performance liquid chromatography, mass spectrometry, nuclear magnetic resonance spectroscopy and thin-layer chromatography, are generally used in the authentication of plant materials (Harborne, 1998; Meena Devi *et al.*, 2010). The phytochemical profiles sometimes vary within the same species due to external factors such as temperature, light, humidity, soil composition and pH, storage conditions. This can be misleading if the samples are deliberately adulterated with a marker compound.

Besides, the traditional taxonomic practices are insufficient on their own to cope with the growing need for accurate identification. Trait expression is often subject to environmental variation and may be difficult to measure.

Modern approaches for plant authentication

Quality control has always been a key issue in the development and authentication of herbal medicines traded as bulk product. Many advanced countries are now widely accepting the powerful tool of genomic fingerprinting for quality control of multicomponent herbal medicines and their finished products. Different

Table 1 List of experimental evidence-based existing substitutes/adulterants for highly important medicinal plants

S.No	Plant species	Substituent/adulterant	References
1	*Aconitum heterophyllum*	*Cyperus rotundus*	Prakash *et al.* (2013)
2	*Alpinia calcarata*	*Alpinia galanga/officinarum*	Zhao *et al.* (2001)
3	*Asparagus racemosus*	*Asparagus* gonoclados	Singh et al. (2013)
4	*Baliospermum montanum*	*Plumbago zeylanica*	Prakash *et al.* (2013)
5	*Boswellia serrata*	*Boswellia carteri/Garunga pinnata*	Alam (2008)
6	*Catharanthus roseus*	*Solanum melogena/Lycospermum esculentum*	Srivastava and Srivastava (1988)
7	*Centella asiatica*	*Bacopa monnieri*	Jamil *et al.* (2007)
8	*Chlorophytum tuberosum*	*Chlorophytum arundinaceum/Chlorophytum borivilianum*	Katoch *et al.* (2010)
9	*Cinnamomum sulphuratum*	*Cinnamomum* species	Dhanya and Sasikumar (2010)
10	*Commiphora wightii*	*Boswellia serata*	Siddiqui (2011)
11	*Decalepis hamiltonni*	*Hemidesmus indicus*	Padmalatha *et al.* (2012)
12	*Garcinia indica*	*Garcinia gummi-gutta*	Prakash *et al.* (2013)
13	*Glycyrrhiza glabra*	*Glycyrrhiza uralensis/Abrus precatorius*	Khan *et al.* (2009)
14	*Holarrhena pubescens*	*Wrightia tomentosa/ Wrightia tinctoria*	Gahlaut *et al.* (2013)
15	*Illicium verum*	*Illicium anisatum*	Dhanya and Sasikumar (2010)
16	*Mesua ferrea*	*Nelumbo nucifera*	Prakash *et al.* (2013)
17	*Ocimum basilicum*	*Ocimum sanctum*	Prakash *et al.* (2013)
18	*Piper nigrum*	*Lantana camara*, *Embelia ribes*	Dhanya and Sasikumar (2010)
19	*Papavar somniferum*	*Amaranthus paniculatas*	Dhanya and Sasikumar (2010)
20	*Santalum album*	*Erythroxylum monogynum*	Chembath *et al.* (2012)
21	*Saraca asoca*	*Polyanthia longifolia*	Gahlaut *et al.* (2013)
22	*Saussurea lappa*	*Aristolochia debilis*	Chen *et al.* (2008)
23	*Sida rhombifolia*	*Sida acuta/Sida cordifolia*	Vassou *et al.* (2015)
24	*Swertia chirayta*	*Andrographis paniculata/Rubia cordifolia/Swertia* species	Joshi and Dhawan (2005)
25	*Valeriana wallichii*	*Saussrea lappa*	Prakash *et al.* (2013)
26	*Cuscuta reflexa*	*Cuscuta chinensis*	Khan *et al.* (2010)
27	*Echinacea purpurea*	*Echinacea angustifolia*, *Parthenium integrifolium*	Laasonen *et al.* (2002)
28	*Myristica fragrans*	*Myristica argentea*, *Myristica malabarica*	Dhanya and Sasikumar (2010)
29	*Pimenta dioica*	*Myrtus tobacco*, *Lindera benzoin*	Dhanya and Sasikumar (2010)

types of molecular techniques have been discovered and optimized and are now prevailing in the scientific arena for taxonomic identification as both taxonomy and quality control of herbal drugs go hand in glove. Besides taximetrics, chemotaxonomy is also employed many a times in the pharmaceutical industry. The inherent limitations in the information provided by the morphological characters led to the development of biochemical and molecular techniques for plant identification. Recently, the world is focusing on plant identification more precisely using molecular markers (DNA or protein based) (Buriani *et al.*, 2012; Ishtiaq *et al.*, 2010). Species-specific variations (polymorphisms) in the nucleotide sequence that are spread randomly over the entire genome and result in characteristic DNA fingerprints have been exploited through use of polymerase chain reaction (PCR) and its variants. With the advent of PCR in 1983 by Kary Mullis, a range of new technologies (molecular methods) have been developed, which vary in their capability to resolve genetic differences, the type of data generated, the taxonomic levels at which they are appropriately applied, and their technical and financial requirements. Properties desirable for ideal DNA markers include highly polymorphic nature, codominant inheritance, frequent occurrence within the genome, ease of access, easy and fast assay, high reproducibility, and easy exchange of data across laboratories (Joshi *et al.*, 1999). Many reviews have been written in the past regarding the basic principles of molecular genomics and the application of these techniques to study the extent of variation in species gene pool and gene banks (Arif *et al.*, 2010; Karp *et al.*, 1996; Kumar *et al.*, 2009). Table 2 summarizes an overview of the various techniques used so far for genome-based authentication of medicinal plants with their potential applications. Most of these techniques are based on approaches that determine the nucleotide sequence of one or more genetic loci in the plants of interest and identify nucleotide sequences that are characteristic of a given species.

Sequencing-based technologies with automated DNA sequencers made considerable reduction in the cost of gel-based fingerprints. Sequences contain a comprehensive record of their own history and are indeed the only appropriate method for taxonomic studies, but molecular data suffers the same problems (viz. problems of homology) as morphological data (Karp *et al.*, 1997). Sequencing allows the determination of relatedness of gene sequences within the samples and hence determines which genes share a most recent common ancestor. DNA sequence data can be deposited as simple text strings in electronic databases such as GenBank and mined easily using text-based bioinformatics tools in contrast to gel-based fingerprints, which require more complicated image analysis software. The enormous progress of massively parallel and clonal sequencing platforms to gain sequence information from single molecules within a complex source has added another dimension for species resolution and can be covered under an umbrella term of next-generation sequencing (NGS). (Mardis, 2013; Metzker, 2010).

High-density miniatured microarrays have revolutionized the traditional way of one gene per experiment for the genome studies. High throughput, sensitivity, accuracy, specificity, and reproducibility of transcriptomics applications have allowed DNA microarray to become a popular tool for authenticating herbal medicine (Chavan *et al.*, 2006; Lo *et al.*, 2012). Although DNA sequence-based markers have found extensive application in differentiating herbal medicinal products from their substitutes or adulterants (Beyrouthy and Abi-Rizk, 2013; Feng and Liu, 2010; Sahare and Srinivasu, 2012; Srivastava and Mishra, 2009; Techen *et al.*, 2014), it is better to have a holistic approach based on various components of systems biology for medicinal plant authentication, whereby metabolomics and need-based transcriptomics and proteomics data supplement the genomic data.

DNA barcoding: a genomics-based modern tool for plant authentication

Advances in the molecular genetics over the last few years have provided workers with a range of new techniques for easy and reliable identification of plant species (Figure 3). This growing progress in biotechnology and taxonomy played a major role in creation of another robust technology. A new tool called 'DNA barcoding', proposed by Hebert *et al.* (2003a), is a valuable addition to the taxonomic tool box. They advocated the use of short DNA sequences from the specified region of genome termed as DNA barcode for biological identification. It implies sequencing of a standard DNA locus as a tool for identifying species. An ideal DNA barcode should be easily retrievable with a single primer pair, be amenable to bidirectional sequencing and effectively provide high discrimination among species. This innovation gave rise to many controversial questions about the nature and purpose of systematics and various subdisciplines (DeSalle, 2006; Lipscomb *et al.*, 2003; Rubinoff *et al.*, 2006). In the space of a few years, DNA barcoding has moved from fantasy to reality (Frezal and Leblois, 2008). Various regions of DNA are used as markers in the DNA barcoding process, which characterizes its universality and high resolution. For efficient discriminatory power, a marker should necessarily show high inter- and low intra-specific variability. This difference between inter- and intraspecific distances is known as the 'DNA barcoding gap'. For the last several years, CBOL has focused on the identification of a universally informative plant barcode. The revolution introduced by DNA barcoding resides in the molecularization, computerization and standardization of taxonomic approach (Casiraghi *et al.*, 2010). Many authors have proposed DNA barcoding as an integrated approach with classical taxonomy for species identification and authentication in the postgenomics era (Kane and Cronk, 2008; Newmaster *et al.*, 2009; Sahare and Srinivasu, 2012; Vohra and Khera, 2013). In the case of plants, successful PCR of barcoding regions is often inhibited by the presence of secondary metabolites. However, modifications in extraction methods, primer sequences and the use of an engineered polymerase can usually overcome such problems. The combining of barcodes from multiple loci has also been used successfully. These approaches have been discussed below to familiarize the researchers with the current trends adopted for overcoming the challenges of DNA barcoding in plants.

Single-locus approach

Molecular information generated from *matK* has been used to resolve phylogenetic relationships ranging from shallow to deep taxonomic levels. *matK* is one of the most rapidly evolving coding regions of the plastid genome and is hypothetically the closest plant analogue to the mitochondrial gene *cytochrome oxidase 1* (*COI*) used as the animal barcode (Hollingsworth *et al.*, 2011). Among all the plastid regions used in plant systematics, *matK* stands out due to its higher rate of evolution (Barthet and Hilu, 2007; Hilu *et al.*, 2003; Wicke and Quandt, 2009). Lahaye *et al.* (2008) analysed 1084 plant species (nearly 96% of orchid species) and showed that a portion of the plastid *matK* gene could be a universal DNA barcode for flowering plants. According to the

Table 2 An overview of molecular techniques with their potential applications

S.No	Molecular techniques	Potential application	References
1	Multiplex PCR	Specific PCR primers are designed and amplification carried out both in individual pairs and in combinations of many primers, under a single set of reaction conditions	Chiang *et al.* (2012), Jigden *et al.* (2010a, 2010b)
2	PCR–short tandem repeats (STR)	Microchip electrophoresis method coupled with the PCR–short tandem repeats (STR) technique is developed	Qin *et al.* (2005)
3	Restriction fragment length polymorphism (RFLP)	PCR-amplified product of specific targeted gene is subjected to restriction digestion with different restriction enzymes. Resulting DNA fragments are separated on agarose gel electrophoresis and transferred to membrane via blotting procedure followed by detection on X-ray film with the labelled probes	Biswas *et al.* (2013), Lin *et al.* (2012), Watthanachaiyingcharoen *et al.* (2010)
4	Amplified fragment length polymorphism PCR (AFLP)	Genomic DNA is digested with pair of restriction enzyme followed by the ligation of adapters to the sticky end of the restriction fragments. Selective amplification of some of these fragments is carried out with two PCR primers that have corresponding adaptor and restriction site-specific sequences	Gowda *et al.* (2010), Passinho-Soares *et al.* (2006)
5	Random amplified polymorphic DNA (RAPD) and sequenced characterized amplified region marker (SCAR)	Specific marker-defined polymorphic termini are sequenced, and primers are designed for specific amplification of a particular locus in targeted species	Cao *et al.* (2010), Devaiah and Venkatasubramanian (2008), Ghosh *et al.* (2011), Gupta and Mandi (2013), Ruzicka *et al.* (2009), Wang *et al.* (2001)
6	Intersimple sequence repeat (ISSR) and simple sequence repeats (SSR)	ISSR amplification is carried out using PCR primers complementary to two neighbouring microsatellites. SSR are developed through the constructed genomic library enriched with repeated motifs. Isolation and sequencing of microsatellite containing clones are performed followed by designing of primer and PCR amplification using the designed primer pair	Sharma *et al.* (2008), Su *et al.* (2008), Tamhankar *et al.* (2009)
7	Arbitrarily primed (AP)-PCR and the direct amplification of length polymorphism (DALP)	The nucleotide sequences of one or more genetic loci (genes) are determined and the sequences that are characteristic of a given species are identified and sequenced	Cao *et al.* (1996), Desmarais *et al.* (1998), Ha *et al.* (2001)
8	Amplification refractory mutation system (ARMS) and multiplex ARMS (MARMS)	Allele-specific primer pairs were designed based on detected mutation site within sequence data of the target species and identification carried out using MARMS	Chiang *et al.* (2012), Diao *et al.* (2009), Qian *et al.* (2008), Wang *et al.* (2011)
9	Quantitative real-time PCR (Q-PCR/qPCR)	Specific genomic locus of choice is employed with the different analysis applications of real-time PCR to exhibit differentiation	Xue *et al.* (2009), Xue and Xue (2008)
10	Loop-mediated isothermal amplification (LAMP)	Amplification carried out under isothermal reaction conditions using allele-specific primers designed, based on the 18S ribosomal RNA gene sequence	Sasaki *et al.* (2008)
11	DNA sequencing	Involves thermostable DNA polymerase and dideoxynucleotide triphosphates to generate chain-termination sequence with a temperature-cycling format	Kretz *et al.* (1994)
12	DNA microarray	Species-specific oligonucleotide probes were designed from 5S ribosomal RNA gene sequence and immobilized on silicon chip. Target sequences were amplified and fluorescently labelled by asymmetric PCR	Carles *et al.* (2005), Schena *et al.* (1998)
13	Specific expression subset analysis (SESA)	SESA carried out on cDNA populations derived from the active part (therapeutically useful) and other major tissues of the target plant species, which serve as tester and driver, respectively. The ESTs obtained were further subjected to computational analysis	Shukla *et al.* (2013)

work of Johnson and Soltis (1994) and Olmstead and Palmer (1994), the rate of substitution in *matK* is three times higher at the nucleotide level and is six times higher at the amino acid level than that of *rbcL* due to almost even distribution of substitution rates among the three codon positions compared with most protein-coding genes where the rates are skewed towards the third

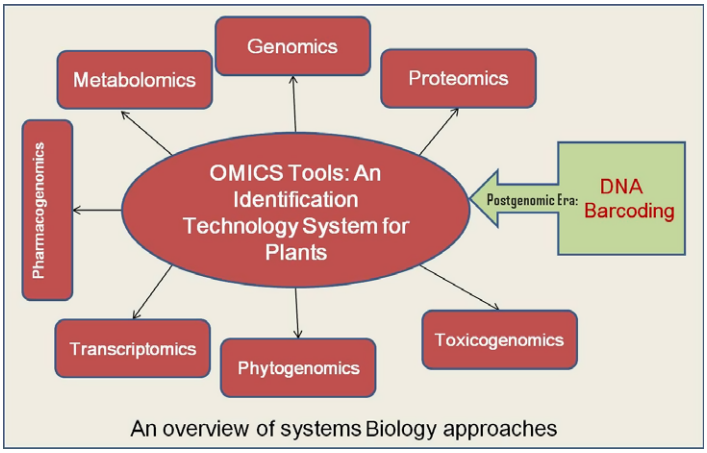

Figure 3 An overview of systems biology approaches to plant identification.

codon position (Soltis and Soltis, 2004). Despite several successful attempts, the unavailability of universal primer sets for all taxa along with the difficulties it poses during PCR leading to low PCR amplification success, especially in nonangiosperms and rapid rate of substitution, along with the rare presence of frameshift indels and a few cases of premature stop codons, prompted some researchers to suggest that *matK* may not be functional in some taxa (CBOL, 2009; Hidalgo *et al.*, 2004; Kugita *et al.*, 2003).

In contrast to *matK*, the highly conserved chloroplast gene *rbcL*, which encodes the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*RUBISCO*), has been widely sequenced from numerous plant taxa across various levels (Bousquet *et al.*, 1992a; Gaut *et al.*, 1992; Morgan and Soltis, 1993 and references therein). This single copy gene is approximately 1430 base pairs in length, is free from length mutations except at the far 3′ end and has a fairly conservative rate of evolution. However, it is apparent that the ability of *rbc*L to resolve phylogenetic relationships below the family level is often poor (Doebley *et al.*, 1990). Thus, interest exists in finding other useful DNA regions that evolve faster than does *rbc*L to facilitate lower-level phylogenetic reconstruction. The *mat*K gene is a promising gene in this regard.

Other than *rbcL* and *matK*, the chloroplast intergenic *trnH-psbA* spacer (approximately 450 bp) has recently become a popular tool in plant DNA-barcoding studies at low taxonomic level (Pang *et al.*, 2012; Yao *et al.*, 2009). This intergenic region consists of two evolutionarily distinct parts, that is the *psbA* 3′UTR, which is vital for post-transcriptional regulation of *psbA* gene expression, and the *psbA-trnH* intergenic spacer (IGS), which is highly variable and easily employed along a wide range of land plants (CBOL, 2009; Kress *et al.*, 2005; Liu *et al.*, 2012a). Main concern associated with the locus includes high frequency of mononucleotide repeats leading to unidirectional reads, thus hampering the recovery of bidirectional sequences and the presence of micro-inversion (Devey *et al.*, 2009; Whitlock *et al.*, 2010). Thus, the complex architecture of *trnH-psbA* makes it difficult to use as an individual barcode (Hao *et al.*, 2010a; Štorchová and Olson, 2007). However, the comprehensive evaluation of the utility of *trnH-psbA* and its combinations has been summarized by Pang *et al.* (2012).

Among the regions of the nuclear ribosomal cistron (18S-5.8S-26S), the internal transcribed spacer (*ITS*) region is the most commonly sequenced region across the plants with the most clearly defined barcode gap between inter- and intra-specific variations. The two spacers of this region *ITS1* and *ITS2* (each <300 bp) adjoining the 5.8S locus have a higher degree of variation than the rRNA genes, which they separate and contain enough phylogenetic signal for discrimination of both plants and animals (Baldwin *et al.*, 1995; Chen *et al.*, 2010; Yao *et al.*, 2010). The *ITS2* in comparison with *ITS1* is considered suitable for amplification and sequencing owing to its shorter length of the target region referred to as a mini-barcode (Gao *et al.*, 2010a; Han *et al.*, 2013). Li *et al.* (2011a) opined for the use of nuclear internal transcribed spacer (*ITS*) region as the standard barcode, and it was thus proposed to be incorporated as one of the core barcodes for seed plants. One of the major delimitations associated with the region is the problem of paralogy due to the occurrence of divergent copies within the individuals, which can lead to misidentification of samples (Bailey *et al.*, 2003). However, Hollingsworth (2011) in their study concluded that the presence of paralogous copies did not compromise the identification ability of the region, compared to other markers. The fungal *ITS* sequences share significant similarity with their plant counterparts. Thus, the primers used for amplification and sequencing of both are almost similar such that the fungal DNA is amplified by chance in many a cases especially those plants, which contain fungal endophytes. This can result in misidentification of samples. Therefore, regardless of the number of primer sets available for this particular barcode region, amplification and sequencing have been troublesome for diverse samples (Gonzalez *et al.*, 2009).

Apart from the other successful loci, the *5S rDNA* IGS (a variable region), plastid protein coding (*rpoB*, *rpoC1*), plastid IGSs (*atpF-H*, *psbK-I*) and low copy number genes, which are being tested for their identification success in different families, have also been reported to be useful for identification of plant material (Chen *et al.*, 2008; Law *et al.*, 2010; Pillon *et al.*, 2013; Yu *et al.*, 2008).

Multilocus/tiered approach

In view of the unsatisfactory performance by individual loci following initial *in silico* and laboratory-based assessment, the effective strategy of using a combination of barcodes emerged as a latest approach. The Plant Working Group of the CBOL examined the suitability of different leading candidate markers and proposed the two-locus combination of *matK* and *rbcL* as the core plant barcode (CBOL, 2009; Fazekas *et al.*, 2008; Newmaster *et al.*, 2008). But the lack of discriminatory power of *rbcL* and primer universality for *matK* subjects it to further improvement (Hollingsworth *et al.*, 2011). Other combinations particularly *rbcL* and *trnH–*

psbA, the internal transcribed spacers of nuclear ribosomal DNA (*nrITS/nrITS2*) have also been evaluated for their potential (Ferri *et al.*, 2008; Kress and Erickson, 2007; Kress *et al.*, 2005; Pang *et al.*, 2012; Tripathi *et al.*, 2013). It has been observed that in case of two loci, the conserved coding locus will align well with the taxa of a community sample to establish deep phylogenetic branches, while the hypervariable region of the DNA barcode will align with ease in the subclades of closely related species. Using a combination of three-loci did not improve discrimination beyond the best performing two-loci barcodes in a few cases and also to avoid the expenses of using a three-loci combination for large data sets, the two-loci barcode was announced as the standard barcode for land plants.

Due to hybridization and introgression observed in certain groups of plants, Newmaster *et al.* (2006) proposed the adoption of a tiered approach, wherein highly variable loci are nested under the core barcoding gene. They advocated the use of a first-tier coding region common in plants for differentiation at a certain taxon level followed by a more variable second-tier coding or noncoding region at the species level. This would reduce the difficulty of aligning noncoding regions from highly divergent genera at the second tier. Depending upon the group of interest, various and multiple noncoding regions can be used. A tiered approach will thus overcome the issue of alignment with noncoding regions, while providing the most variability from two-barcode regions for identifying closely related taxa (Newmaster *et al.*, 2013; Nithaniyal *et al.*, 2014; Purushothaman *et al.*, 2014).

After evaluation by Newmaster *et al.* (2006), *rbcL* emerged as a potential standard first-tier core coding region on the basis of being the most characterized plastid coding region in gene bank. It was analysed to resolve approximately 85% cases in congeneric species, given its universality and ease of amplification and alignment. The noncoding plastid region evaluated by Shaw *et al.* (2005) or *trnH-psbA* proposed by Kress *et al.* (2005) appears promising as a second-tier locus. The recent work of Xiang *et al.* (2011) supported the approach and suggested *matK* as the prior-tier DNA region at generic level. Thus, it was emphasised that the second-tier locus at species level should have enough stable variable characters for discrimination and thus need more attention. The tiered approach, in case of medicinal plants, is based on the use of a common, easily amplifiable and aligned region such as *rbcL* that can act as a scaffold for placing data from a highly variable region such as *ITS2*. *ITS2* is a preferred second-tier candidate for medicinal plants due to its high species resolution, its presence in the nuclear genome (that evolves at a different rate than the plastid genome) and its shorter sequence that enables higher recovery from processed plant materials found within herbal products (Newmaster *et al.*, 2013).

Next-generation sequencing and DNA barcoding

Next-generation sequencing (NGS) ignited a revolution in genomics and triggered numerous ground-breaking discoveries in the genome, transcriptome and epigenome of many organisms. This high-throughput technology platform enables researchers to move quickly from an idea to full data sets in a matter of hours or days, with a number of algorithms existing to address the needs of each application. In parallel with many algorithmic advances through *de novo* sequencing, targeted resequencing, RNA interface to metagenomics, the technique is now encompassing the Sanger sequencing platform in DNA barcoding (especially metabarcoding) with additional advantages of longer average sequence read lengths from Roche's 454 sequencing platform (Kircher and Kelso, 2010). The enormous number of reads generated by NGS enabled the sequencing of entire genomes at an unprecedented speed, which have proved to be very useful for phylogenetics at deeper level and genome evolution analysis, which in turn greatly helps in authenticating the useful medicinal plants for herbal drug preparations (Sarwat and Yamdagni, 2014). Amplicon sequencing using ion torrent or 454 technologies could potentially recover all of the filler plant species. Whole-chloroplast genome sequence of *Ceratophyllum demersum* obtained using Roche's 454 platforms by Moore *et al.* (2007) provided strong support to data obtained through traditional taxonomy. Parks *et al.* (2009) were able to assemble nearly complete plastomes for 37 *Pinus* species based on multiplex Illumina sequencing platform. The primary application of DNA barcodes will continue to be the identification of unknown samples. The adoption of NGS technologies for DNA barcoding will lead to tremendous growth in available sequence data, which will become a powerful molecular tool for species discovery, evolution and the conservation of biodiversity (Sucher *et al.*, 2012).

Real-time DNA barcode-based high-resolution melting curve analysis (Bar-HRM)

High-resolution melting (HRM) analysis coupled with DNA barcoding termed as Bar-HRM has widely been used for detection of contamination in herbal mixtures. It is a novel DNA-based method that allows genotyping and fingerprinting by discriminating DNA sequence variants based on the characteristics of thermal denaturation of the amplicons without sequencing or hybridization procedures (Wittwer, 2009). The amplicon is analysed by fluorescence monitoring of the melting curve of the dsDNA caused by the release of intercalating dye SYBR Green I in a real-time PCR system. HRM analysis requires no manual post-PCR processing, is performed in a closed-tube system and has a low reaction cost relative to other methods used to study genetic variation. These advantages make it widely used in pathogenic identification, food authenticity and biological diagnostics. Recently, Jiang *et al.* (2014) successfully reported the application of a barcoding melting curve analysis (Bar-MCA) method using chloroplast region *trnH-psbA* to identify adulterants in traded saffron by obtaining melting curves for saffron and its adulterants having significantly different specific peak locations or shapes. Another alternative application of HRM in species identification has been successfully described by Kalivas *et al.* (2014), whereby authentication and taxonomic identification of seven Greek *Sideritis* taxa was carried out on the basis of the nuclear *ITS2* DNA barcoding sequence. Thus, the approach could be applicable to a wide range of plants of medicinal importance especially within closely related species employed in the herbal sector. Besides, HRM conjugated with specific barcode regions such as mitochondrial DNA, *16S rDNA* regions and microsatellite markers has also been shown to be capable of accurately identifying products in the food industry (Bosmali *et al.*, 2012; Ganopoulos *et al.*, 2011, 2013).

Successful DNA barcoding of herbal products

The majority of the plant materials used in herbal medicine is procured from the markets in the form of dried or powdered plant parts. DNA barcoding has been found successful in identification of plants from the finished herbal products. A number of loci from different genomic regions including *rbcL,*

matK, ITS, ITS2 and psbA-trnH have been tested with different degrees of success (Table 3). Published estimates of successful and unsuccessful species resolution using these loci may be inflated depending upon the distance of the related species within the target genera. In the case of herbals, the dried plants as well as the herbal supplements/products can be barcoded using the universal protocol described for DNA barcoding. In recent years, considerable effort has gone into the search for suitable DNA barcodes for specific herbal products. This provides insight into substitutions or adulterations occurring in the commercial market. An overview of work has been presented in Table 4 which collates information from the published studies.

Future challenges for DNA barcoding as a plant authentication tool for herbal industry

Several studies carried out to date have highlighted many major challenges, including lack of reference libraries, unavailability of the vouchers to professionally identified specimens archived in a herbarium corresponding to the reference DNA sequences in the GenBank (consequently a GenBank reference sequence may be from an incorrectly identified plant species with no way to verify its specific origin) and variable rate of evolution corresponding to different loci (Newmaster *et al.*, 2013). Herbal industry needs to be provided with authentic standards of plant material for fulfilling the goal of Herb-Bol (barcode of life) research programme in the coming years. Development of a DNA herbal barcode reference library termed as biological reference material (BRM) would provide users with a universal platform for reference sequence database at species level to ultimately catalogue and provide high authenticity of the plant components used in the herbal industry. This would consist of taxonomically validated herbarium vouchers of known provenance. The barcode of an unidentified specimen or the species identities obtained from the commercial medicinal plant products can be compared with the reference barcodes to find the matching species. The use of BRM herbal barcode library for testing bulk materials could provide a method for good manufacturing practices (GMP) of herbal products.

Another major problem concerning the barcoding of herbal products is the use of only plastid barcode regions due to insufficient nucleotide sequence variability to distinguish among closely related species. Although the multilocus approach of combining different barcode regions has proven successful in certain cases in terms of species discrimination, the differentiation of closely related complex groups is still laden with uncertainty. Another major problem concerning the barcoding of herbal products containing mixture of multiple species arises due to varied PCR success of the selected gene in samples with potentially degraded DNA due to varied gene copy number and PCR bias (Fazekas *et al.*, 2009). Further studies are therefore needed for protocol improvement/development, particularly designing of novel universal primers with the development of BRM herbal barcode library to extend the barcoding for a broader coverage of plant species.

Table 3 Preferred/best loci for family level identification

S.No.	Family	Loci	References
1	Arecaceae	*matK* + *rbcL* + *trnH-psbA*	Yang *et al.* (2012)
2	Asteraceae	*ITS2*	Gao *et al.* (2010b)
3	Fabaceae	*ITS2*	Gao *et al.* (2010a)
4	Juglandaceae	*matK*	Xiang *et al.* (2011)
5	Lamiaceae	*matK* and *trnH-psbA*	Theodoridis *et al.* (2012)
6	Lauraceae	*psbA-trnH*	Liu *et al.* (2012c)
7	Leguminaceae	*trnL* and *ITS2*	Madesis *et al.* (2012)
8	Lemnaceae	*atpF-atpH*	Wang *et al.* (2010)
9	Nyssaceae	*ITS*	Wang *et al.* (2012)
10	Polygonaceae	*trnH-psbA*	Song *et al.* (2009)
11	Rosaceae	*ITS2*	Pang *et al.* (2011)
12	Rutaceae	*ITS2*	Luo *et al.* (2010)
13	Zingiberaceae	*ITS2; matK; rbcL*	Shi *et al.* (2011), Vinitha *et al.* (2014)
14	Araliaceaes	*ITS2*	Liu *et al.* (2012b)
15	Orchidaceae	*atpF-atp* + *psbK-psbI* + *trnH-psbA*	Kim *et al.* (2014)
16	Apiaceae	*ITS/ITS2* + *psbA-trnH, ITS* and *ITS2*	Liu *et al.* (2014)
17	Combretaceae	*rbc*La + *mat*K + *trnH-psbA*	Gere *et al.* (2013)
18	Angiosperms	*rbcL* + *matK*	CBOL (2009)
19	Nonflowering seed plants	*trnH-psbA, matK; rbcL, rpoB, rpoC1, ITS/ITS2*	Pang *et al.* (2012)

Table 4 Successful resolution of species barcodes recovered from herbal products

Locus/Loci	Herbal material	Success rate	References
psbA-trnH + *ITS2*	*Sida cordifolia* raw drug	Best two-marker combination	Vassou *et al.* (2015)
ITS2, *matK*, *rbcL*, and *psbA-trnH*	*Senna* herbal products	100% (ITS2), *matK*, *rbcL*, and *psbA-trnH* (<10%)	Seethapathy *et al.* (2014)
rbcL and *matK*	Saw palmetto	81%	Little and Jeanson (2013)
matK	Herbal juices	99%	Mahadani and Ghosh (2013)
rbcL + *ITS2*	North American herbal products	95%	Newmaster *et al.* (2013)
matK	Black cohosh	75%	Baker *et al.* (2012)
rbcL and *matK*	Commercial tea	90%	Stoeckle *et al.* (2011)
psbA-trnH	*Phyllanthus* raw drug	Highly effective	Srirama *et al.* (2010)

Concluding remarks

The advent of DNA barcoding to identify plant species in herbal medicine appears to be promising but remains to be fully exploited. DNA barcoding aims to find a single sequence to identify all species. Barcoding is generating a global, open access library of reference barcode sequences, which enables nontaxonomists to identify specimens. Due to certain limiting factors such as low PCR efficiency, gene deletion and inadequate variation, no single-locus barcode exists as a universal DNA barcode for plants. If a good discrimination success is needed, opting for a multilocus approach is a far better idea and is being

accepted as an effective strategy for barcoding land plants. By combining the universality, discriminatory power and amplification success of each locus, high discrimination oriented results can be obtained. The newer trends being followed in plant DNA barcoding with the approaches of NGS and HRM are proving to be immensely helpful in authenticating the useful medicinal plants for herbal drug preparations. These analyses are widely being used in many researches for detection of contamination in herbal mixtures.

Like all other living organisms, plants are influenced by both, their genome and their environment. Plant metabolism (mainly secondary metabolism, which is mainly responsible for the medicinal properties) is dependent on its environment (Briskin, 2000). Thus, merely relying only on the genome-based authentication will be insufficient for quality control of herbal products. Characterization for morphological and biochemical traits will also continue to play its parallel role in identification and assessment of medicinal plants in herbal industry (DeSalle, 2006).

Another aspect that needs attention is that contamination in herbal products may not only be at the plant species level. In traditional systems of medicine such as Ayurveda, specific plant parts/tissues collected in a season-specific manner have been prescribed to be used for therapeutic purpose (Shukla *et al.*, 2013). If the prescribed plant part is replaced by a non-prescribed part or by a prescribed part collected in the wrong season, then the therapeutic activity of the product might be compromised and DNA barcoding will fail to identify the adulteration responsible for lowering the quality of the product. Thus, DNA barcoding needs to go hand in glove with other suitable molecular biology and analytical chemistry tools, if it is to be successfully adopted for authentication purpose by the herbal industry. This will imply use of systems biology components encompassing genomics (DNA barcoding) and metabolomics (for active secondary metabolites) in a major way and supplemented with need-based use of transcriptomics [specific expression subset analysis (SESA)] and proteomics (specific proteome) tools.

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Conflict of interest

The authors declare that they have no conflict of interest.

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