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Authentication of medicinal plants by DNA markers

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ABSTRACT

Medicinal plants have been used worldwide for centuries to maintain health and to treat diseases, more so chronic diseases. However, adulteration and use of spurious materials as substitutes have become a major concern for users and industry for reasons of safety and efficacy. Therefore, authentication of medicinal plants is of utmost importance. Morphological, anatomical, chemical and DNA markers solve the problem by differentiating the genuine material from the adulterants, substitutes and spurious drugs. DNA markers use nucleotide sequences to identify species; it takes preference over the other two markers being not age dependent, tissue specific and having a higher discriminating power. Therefore, characterization of plants with such markers is an ideal approach for identification of medicinal plant species and populations/varieties of the same species. Availability of certified taxonomic specimens in herbaria is certainly required for unambiguous confirmation through final visual comparison and analysis.

Contents

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1. Introduction

Medicinal plant species are known to be in use since time immemorial for the treatment and cure of human and animal ailments. Though their use and practice registered a decline with the advent of antibiotics and sulfa-drugs, the toxicity and harmful effects associated with synthetic drugs and antibiotics have brought the herbal systems of medicine again to the forefront of healthcare system. According to WHO and other reports (WHO 2002; Willcox and Bodeker 2004), around 80% of the global population still relies on botanical drugs and there is speculation that more than two billion people may be heavily reliant on medicinal plants (Lambert et al. 1997). This system plays prominent role in the strategy to contain and treat severe acute respiratory syndrome (Wen et al., 2003). Vinblastin and Vincristine of *Catharanthus roseus* G. are potent anti-cancer agents and are the first agents to advance into clinical use for the treatment of cancer (Cragg and Newman 2005). The discovery of paclitaxel derived from *Taxus brevifolia* Nutt. (Taxaceae), is another evidence of the success in natural product drug discovery. Two most important anti-malarial drugs quinine and artemisinin are derived from traditional medical knowledge in Peru and China respectively. Survey reports showed that 78% of patients living with HIV/AIDS in the USA use medicinal herbs (WHO 2002) and similar patterns have been reported in other countries. Other systematic studies on efficacy are slowly emerging suggesting antiretroviral, immunomodulatory and opportunistic infection reducing effects of traditional management methods (Liu 2007). Apart from general healers, traditional orthopedic practitioners, birth attendants, poison healers, spiritual therapists, mental health providers, healers specialized in eye, pediatric conditions, skin diseases etc., are some of the specialty areas (Payyappallimana 2010).

The international trade of herbal products is one of the major forces in the global economy and the demand is increasing in both developed and developing countries. Survey of literature reveals that more than 1000 companies are engaged in the production of herbal products with the annual revenues in excess of US$60 billion (Newmaster et al. 2013). In North America, herbal medicinal market is considered to constitute the mostly rapidly growing segment (Gutierrez et al. 2004), with over 29,000 herbal substances (Astin et al. 1998; Kaye et al. 2000) generating billions of dollars in trade. These statistics are the direct indication of the rapid growth (approximately 15% per year) in the market place from natural plant products and broadening consumer base which show interest in herbal products from different countries including India. The value of botanical related trade in India is about US$10 billion per annum with the annual export of US$1.1 billion (Singh et al. 2003). China's annual herb drug production is worth US$48 billion with annual export of US$3.6 billion (Handa 2004). India is ranked third in the herbal medicine category with less than 2% global market share. The Indian market is growing at 15–20% per annum- Rs 7000 million or $150 million (www.indianmedicine.nic.in). Therefore, massive demand of herbal medicinal system at both national and international level has resulted in renewed interest of biologists in this field to maintain the quality and purity of herbal raw material and finished products.

The prime cause of the problems associated with the standardization of medicinal plants is due to reasons of complex composition of drugs used in the form of whole plants, plant parts or extracts obtained there from. Therefore, first step is to make reproducibility and minimal batch-to-batch variation a desirable quality of any herbal remedy, and side-by-side initiating steps towards use of authentic starting material. In ancient days, the activity of herb procurement, storage, preparation of drugs and its distribution remained mainly the responsibility of local physicians (Vaidyas and Hakims). They were very well versed with the identity of medicinal plants as they had very close contact with nature. But gradually in the process of urbanization this contact with nature was lost and, consequently, the knowledge about the identification of medicinal plants has also deteriorated to a great extent.

A perusal of literature available on medicinal plants (Sivarajan and Balachandran 1994) reveals that different workers have interpreted ancient texts differently resulting in coining of same Unani or Ayurvedic name for more than one plant and different names for the same drug plant. This way a number of medicinal plants have controversial botanical identity due to the existence of homonyms (same name for different drug plants) and synonyms (different names for same drug plants). Such situation has also been compounded by linguistic diversity, and prevalence of local dialects. For example, different botanically identified plants are being used for the same traditional drug and vice-versa in different parts of India. Thus, there exists at present, a chaotic state of botanical and vernacular nomenclature with regards to medicinal plants. Due to ambiguity of plant names, over-lapping of data occur in chemical or pharmacological information where the botanical identity of market samples of crude drugs taken up for research is not ascertained. This has put many drug plants in controversial position and their identity has become doubtful. In addition to ambiguity in nomenclature, the crude drugs sold in the market are adulterated or substituted by quite unrelated plant materials. Many researchers have examined a number of market samples of crude drugs used in Indian systems of medicine (Afaq 1999) and observed that the prevalence of adulteration is such that quite unrelated plants are being sold in the crude drug markets in place of genuine ones. For example, *Anemone* flowers are sold as 'Banafsa' (*Viola* spp.); paper colored, scented and cut into small pieces as 'Saffron' (*Crocus sativa*); *Malva rotundifolia* as 'Brahmi' (*Centella asiatica*); *Parthenium hysterophorus*, an obnoxious weed, as Shahtara '(*Fumaria indica*); culm of different grasses as 'Chirayata' (*Swertia* spp.); *Leea alata* as 'Manjith' (*Rubia cordifolia*), and so on. It is invariably found that the adverse effects/reports are not due to the intended herb, but rather due to the presence of an unintended herb (De Smet et al., 1992). The credibility of any system of medicine depends not only on the skill of the physician but also on the quality of medicine administered to the patients. Correct identification of plants forming the drug is a prerequisite and fundamental to whole realm of medicine and science. Thus, authentication of botanical source of plant taken up for research or medicinal use is a necessity to achieve satisfactory results and also to maintain efficacy and therapeutic property of the preparations in which these plants are used. We have therefore, tried to present a comprehensive review on strategies related to identification of medicinal plants.

1.1. Authentication of medicinal plants by molecular markers

DNA- based markers that are based on analysis of the unique genetic structure are undoubtedly higher level markers and have the upper hand over other marker systems because these are not affected by age, environmental factors and physiological conditions. Moreover, these markers are not tissue specific and thus can be detected at any stage of plant development. Compared with phenotypic and chemical markers, DNA- based technology can provide an efficient, accurate and cheaper means of testing the authenticity of hundreds of samples simultaneously as these can be amenable to automation. DNA based authentication of medicinal plants can be useful as a tool for quality control and safety monitoring of herbal pharmaceuticals and neutraceuticals and

Table 1
Comparison of different DNA markers used in biological sciences.

	RFLP	RAPD	AFLP	ISSR	SSR	SCAR	LAMP	DNA barcoding
Genomic abundance	High	Very high	Very high	Medium	medium	High	High	high
Genomic DNA required	2–5 μg	15–30 ng	200–300 ng	15–30 ng	30–50 ng	30–50 ng	10–20 ng	30–50 ng
inheritance	Co-dominant	Dominant	Dominant	Dominant	Co dominant	Co dominant	Co dominant	NA
Primers/probes used	Specific	Random	Specific to adapter sequence	Specific to repeats	Specific	specific	Specific	Specific
Type of polymorphism	Nucleotide base change that affect specificity of restriction endo-nucleases	Nucleotide base change that affect specificity of binding sequences	Nucleotide base changes that affect specificity of restriction endonucleases and presence/absence of nucleotide complementary to selective nucleotides	Nucleotide base change at primer binding sequences	Complete presence/absence of DNA fragment	Complete presence/absence of DNA fragment	Complete presence/absence of DNA fragment	Nucleotide changes in universal genes.
Reproducibility	Very high	Very low	High	Medium	Very high	Very high	Very high	Very high
Applicability in Plant authentication	Yes	Yes, but should not be used (It is genetic diversity marker)	Yes, but should not be used (It is a perfect genetic diversity marker)	Yes, but should not be used (It is genetic diversity marker)	Yes	Yes	Yes	Yes
Cloning/sequencing	Yes	No	No	No	Yes	Yes	Yes	Yes
Use of radioactivity	Yes, but not in PCR-RFLP	No	Yes	No	No	No (required in AFLP based SCAR)	No (required in AFLP based LAMP)	No
Detection of alleles	Yes	Generally no	Generally no	Generally no	Yes	Yes	Yes	Yes
Cost	High	low	High	Medium	medium	high	Medium	medium
Ease of use of automation	Not easy (PCR based AFLP is easy)	Very easy	difficult if radioactivity is used (Licor system is easy)	Easy	Very easy	Easy	Easy (However, primer designing sometimes becomes difficult)	Easy

will significantly add to the medical potential and commercial profitability of herbal products.

A genetic marker may be defined as gene or a nucleotide sequence on a chromosome that has the potential to differentiate cells, individuals or species. As the DNA sequences are highly specific, they can be identified with the help of the known molecular markers which can find out a particular sequence of DNA from a group of unknown.

1.1.1. Types of molecular markers.

A vast numbers of molecular markers (Table 1) are available of which the more common are Restriction Fragment Length Polymorphisms (RFLPs), Amplified Fragment Length Polymorphisms (AFLPs), Randomly Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSRs), Inter Simple Sequence Repeats (ISSR), Sequence Characterized Amplified Regions (SCAR), Loop Mediated Isothermal Amplification (LAMP), Single Nucleotide Polymorphisms (SNPs) and others. DNA barcoding, microarrays based markers and Next Generation Sequencing (NGS) based markers are of relatively recent development. Each of the techniques are targeted towards a particular component of the genome or is completely arbitrary, face unique methodological, technical and material challenges; thus no DNA marker can be considered ideal. The use of a particular marker is therefore, dependent on objectives of the researcher.

2. Restriction fragment length polymorphisms (RFLP)

RFLPs (Fig. 1) are considered as one of the first developments in the field of genetic markers, responsible for initiating the field of molecular genetics. The technique is based on the principle of variation that is present due to occurrence of mutations in restriction enzyme binding and cleavage sites; additionally, any re-organization in the genomic region flanked by restriction sites that also disrupts their distribution and thus causes polymorphism also contributes to RFLP. The digested fragments vary in size, have to be separated using Southern blot analysis and accordingly visualized by hybridization to specific probes which could be homologous or heterologous in nature. Alternatives of Southern analysis in RFLPs are polymerase chain reactions. When the flanking regions of nucleotide sequence are known, the region meant for RFLPs could be amplified through polymerase chain reaction. However, it must be kept in mind that If the length polymorphism is caused by a relatively large (> approx. 100 bp depending on the size of the undigested PCR product) deletion or insertion, gel electrophoresis of the PCR products should reveal the size difference and when the length polymorphism is caused by base substitution at a restriction site, PCR products must be digested with a restriction enzyme to reveal the RFLP. The positive point of this marker is its co-dominant nature. Like other DNA based markers, there is ample literature (mainly PCR-RFLP) that suggests use of this technique in the identification of medicinal plants. For example, Six plant species — *Desmodium giganicum*, *Aegle marmelos*, *Solanum xanthocarpum*, *Solanum indicum*, *Tribulus terresteris*, *Oroxylum indicum* were identified through polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and the regions amplified were Internal transcribed spacer (*ITS*) with the aid of *ITS1* (F) and *ITS4* (R) primers (Biswas and Biswas 2013). The same technique has been applied on *Boerhavia diffusa* L. in which 700 bp of *ITS* region was obtained via polymerase chain reaction and when this region was restricted with *Msp* I, provided four unique fragments that differentiated this specie from *T. portulacastrum* and *T. monogyna* (Biswas et al. 2013). Feng et al. (2010) differentiated *Angelica sinensis* from its seven different adulterant *Angelica* species; here a pair of primers specific for *ITS* region were designed which amplified 520 bp from the adulterants and no products was amplified with the DNA of *A. sinensis*.

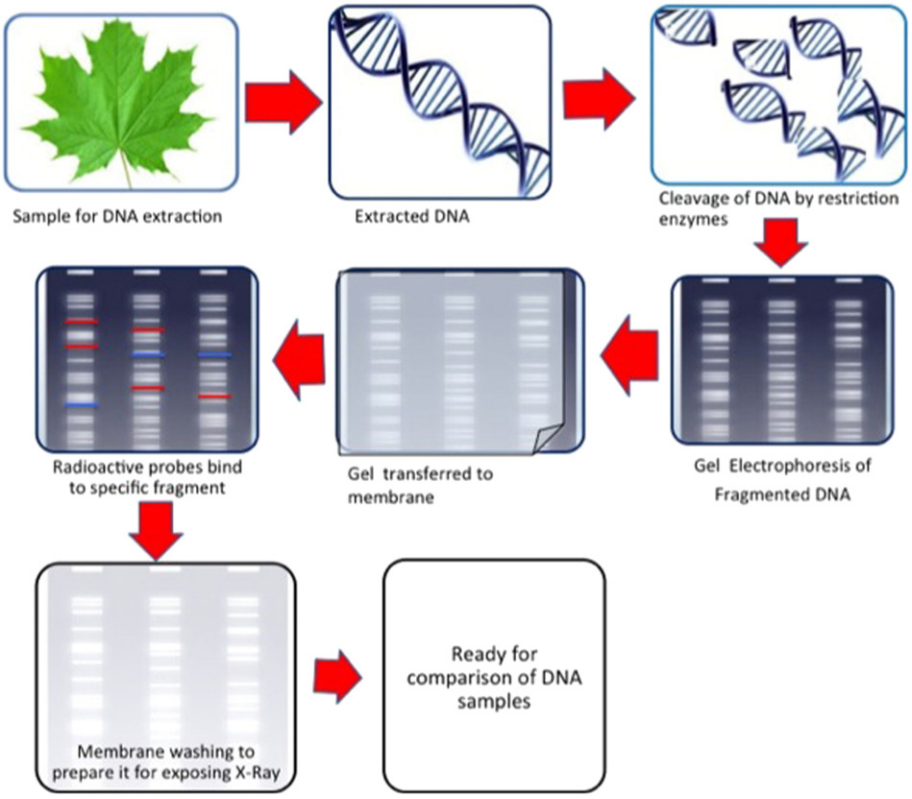

Fig. 1. Pictorial view methodology of RFLP.

2.1. Limitation of RFLPs

- The limited sensitivity of detection associated with RFLPs is the serious problem; because it is very difficult to get valid profiles from trace biological evidence or from samples that are too aged or have significantly compromised environmental insults.
- RFLP is time consuming, involves radioactivity, and is laborious and difficult to automate. However, this can be overcome by PCR-based DNA typing systems.

3. Simple sequence repeats (SSR)

Simple Sequence Repeats (Fig. 2) are comprised of 2–5 bp DNA monomeric units that are repeated multiple times at a specific locus. Such markers are completely co-dominant; used for fingerprinting, marker assisted selection, kinship, breeding behavior such as selfing and outcrossing, establish population structure etc. Microsatellites are locally tandemly duplicated, and are also found dispersed throughout the genome. Thus it becomes important to amplify a specific microsatellite in a locus specific manner using locus-specific primers. The primer designing involves identifying microsatellites, either is manual examination in case the sequences are small or of limited length, or using automated tools for locating microsatellite such as microsatellite finders. However, depending upon the research objectives, either unique flanking regions can be used for primer designing where the products are co-dominant, or microsatellites can themselves be used as primers where the products generated act as dominant markers. Microsatellite discovery also involves cloning microsatellite-enriched library and sequence analysis. The clones containing repeats can also be identified through hybridization of fluorescent labeled probe with the repeats. DNA segment containing the microsatellites is then sequence verified and employed for primer designing. Microsatellite markers are valued for their hyper-variability which occurs because these regions can add or delete one or two nucleotides because of the slippage during replication.

Research reports suggest involvement of microsatellites in authentication and identification of medicinal plants. Hon et al. (2003) applied 16 microsatellites to analyze 150 and 40 American ginseng and Oriental ginseng roots respectively. Of the 16 microsatellites, 9 could differentiate Chinese samples from American ginseng. *Capsicum* species which are commonly used as spices have been analyzed through microsatellites; the study involves 800 lines collected from different locations of Central and South America. SSR primers (5751 pairs) were designed and similarity search with tomato genome resulted successful mapping of 2245 *C. annuum* markers onto the tomato genome. Ninety six were found to span entire tomato genome and selected for further analysis. Sixty markers showed polymorphism in *Capsicum* lines and based on this, the 192 lines were grouped into five clusters. Additionally, plastid genes, *matK* and *rbcL*, divided the *Capsicum* lines into 3 main groups; over all, 19 marker loci reveal genotype specificity to species and clusters (Shirasawa et al., 2013).

3.1. Limitation of SSR

- It requires much time and cost to isolate and characterize each SSR locus when the DNA sequence of a plant species is not available.
- Another drawback is the occurrence of null alleles. This may be due to the poor primer annealing because of nucleotide sequence divergence, inconsistent DNA quality or low DNA quantity (Ellegren 2004) or it might be due to mutations in the primer binding site. This can cause difficulty in the determination of allelic and genotypic

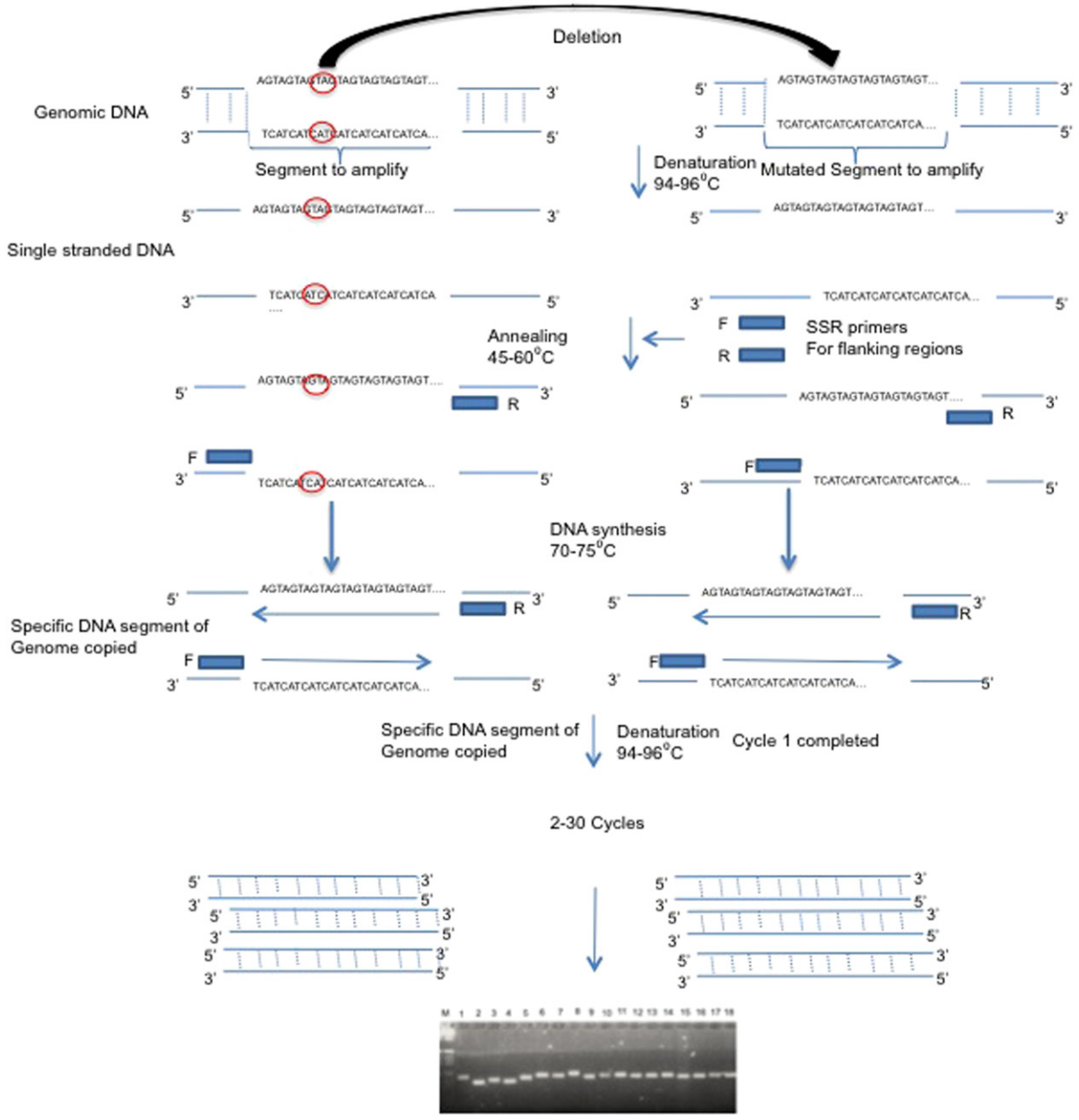

Fig. 2. Pictorial view methodology of SSR.

frequencies and an underestimation of heterozygosity (Kumar et al. 2009).

4. Random amplification of polymorphic DNA (RAPD):

In RAPD technology (Fig. 3), random short synthetic oligonucleotide primers (10–12 base pairs) are used to amplify the genomic DNA through polymerase chain reaction under low annealing temperature. The amplicons generated are separated on agarose gels based on sizes. As stated that primer size is short, therefore annealing temperature range is 28–38 °C. At this temperature range, primers anneal wherever they find complementary sequences from the genome and the profile of amplified DNA vary in size that depends on nucleotide sequence homology and the primer at the end of each amplified product. As it is obligatory that primers for RAPD are arbitrarily chosen, therefore, a minimum of 40% GC and the absence of palindrome sequence is a prerequisite for RAPDs.

Majority of the RAPD fragments are due to the amplification of one locus, furnishing two types of polymorphisms — the band could be present (1) or absent (0). The bands obtained may vary in intensity which could be due to the differences in copy number or relative sequence abundance; therefore, may work for distinguishing homozygote dominant from heterozygote because more bright bands are expected for the former. However, some workers (Thormann et al. 1994) have found no correlation between copy number and band intensity. The possible occurrence of fainter bands could be due to the varying degree of primer mismatch. The other drawback associated with RAPD markers is the low reproducibility. However, this problem could be overcome through the choice of an appropriate DNA extraction protocol to remove any contaminants, by optimizing the polymerase chain reaction parameters, by testing several oligonucleotide primers and most importantly scoring only reproducible DNA fragments.

RAPDs have been widely used for authentication of plant species of medicinal importance. Two varieties of *S. marianum* which are very difficult to distinguish in dried conditions could be differentiated with

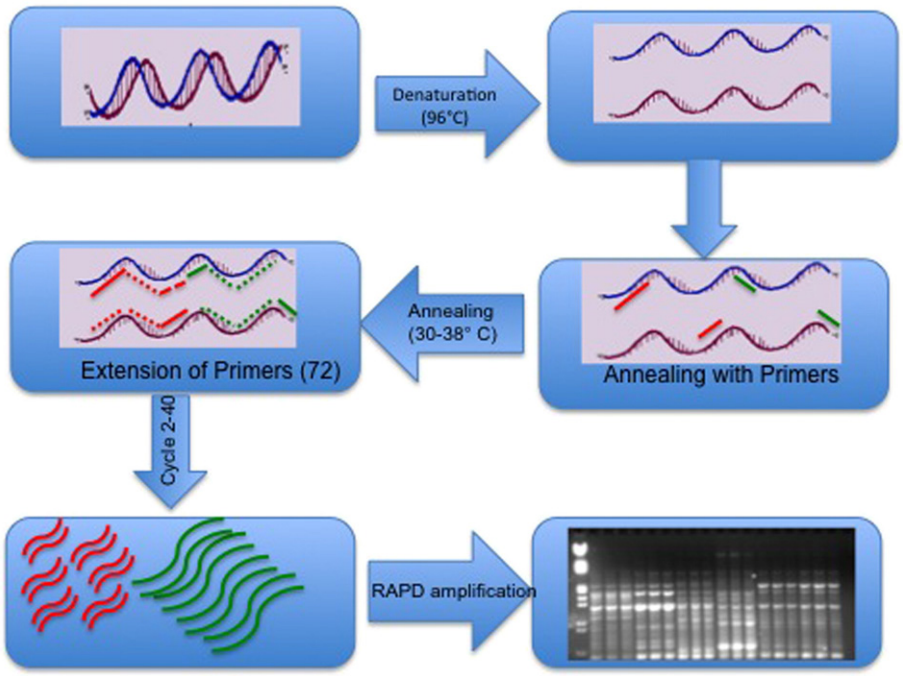

Fig. 3. Pictorial view methodology of RAPD.

RAPD. The banding pattern amplified with primer OPP-10 contained two (600 and 1000 bp) characteristic bands; primers OPG-03, OPC-17 generated two unique bands (1000 and 300 bp). This profile specific for *S. marianum* var. album could differentiate it from *S. marianum* var. purple (Abouzid 2014). Similarly, two species of the parasite *Cuscuta* (*C. reflexa* and *C. chinensis*) have been distinguished with primers OPC-1, OPC-02, OPC-03, OPC-04, OPC-05, OPC-06, OPC-07 and OPC-08 (Khan et al. 2010a). Ali et al. (2013), while characterizing *Clitoria ternatea* at inter-zonal level identified complete monomorphism with primer OPN-02, therefore, the identification of this herb with OPN-02 is the good choice. Molecular characterization of *Convolvulus pluricaulis* revealed that primer OPN-09 is the specie specific primer for the herb (Ganie et al., 2015). A common band of 2.2 kb amplified by Primer OPN-05 was found when different accessions of *Evolvulus alsinoides* were studied through RAPD analysis (Ganie and Sharma 2014).

Laboratories with limited budget prefer RAPDs as the entire process is only dependent on thermal cycler and gel electrophoresis unit. RAPDs can efficiently differentiate taxa below the species level (Choo et al. 2009), because it reflects both coding and non-coding regions of the genome.

4.1. Limitations of RAPDs

- RAPD is a less reproducible marker. Because the annealing temperature for such marker is low (28–38°C) therefore, there are chances of wrong annealing.
- It is a dominant marker

5. Amplified fragment length polymorphism (AFLP)

This technique (Vos et al., 1995) which is a combination of both RFLP and RAPD is based on the detection of restriction fragments by PCR amplification and can be used for DNA of any origin or complexity, and quality (Fig. 4). The fingerprints are produced without any prior knowledge of sequence using a limited set of primers. Fragments are generated with two different end sequences which form template for adapter ligation. Such adapters then form the basis for PCR which is performed using end-labeled nested primers with selective nucleotides. The amplified products are electrophoresed in a 6% denaturing polyacrylamide gel and autoradiographed or detected through fluroscent detection. AFLPs are generally considered highly valued marker for analyzing the genetic diversity for both animal and plant systems compared to authentication reports. Seven specific fragments identified with the primer combinations *E*-ATC + *M*- AG and *E*-GCA + *M*-GT could serve as the genetic markers for *Aloe vera* (Tripathi et al. 2011). Although the three species of the genus *Echinacea* could easily be distinguishable in fresh and intact conditions, but it becomes very tough to differentiate them in commercial preparations particularly ground, dry plant parts of *E. purpurea* (valuable species for chemotherapeutic properties) mixed with the other two species. Therefore, Russi et al. (2009) used fresh material collected from cultivated *Echinacea* spp. for the discrimination of these species by AFLP. *E* + CAC/*M* + AAT and *E* + CAC/*M* + AGC generated 13, 9, and 4 or 7, 5, and 5 specific fragments for *E. purpurea*, *E. angustifolia*, and *E. pallida*, respectively. Passinho-Soares et al. (2006) identified specie specific bands of different species of the genus *Plectranthus*. During the study 2, 2, 1, unique bands were confirmed for *P. barbatus*, *P. grandis* and *P. ornatus* respectively with the primer combination *EcoR* 1-CAC + *Msc* 1-GCA. Applying AFLP technique, Gowda et al. (2010) using *P* + GC/*M* + CTA combination, found three bands of the range 500–700 bp in *Embelica ribes* and could be considered unique to this species. In the same study, *P* + CA/*M* + CTG combination yielded two unique bands between 500 and 517 bp for *E. tsjeriam-cottam*.

5.1. Limitations of AFLPs

- Like RAPD markers AFLPs are also dominant.
- The method needs purified and high molecular weight DNA. Also, the procedure involves harmful radioactive materials; however, this can be overcome by using fluorescent tags.

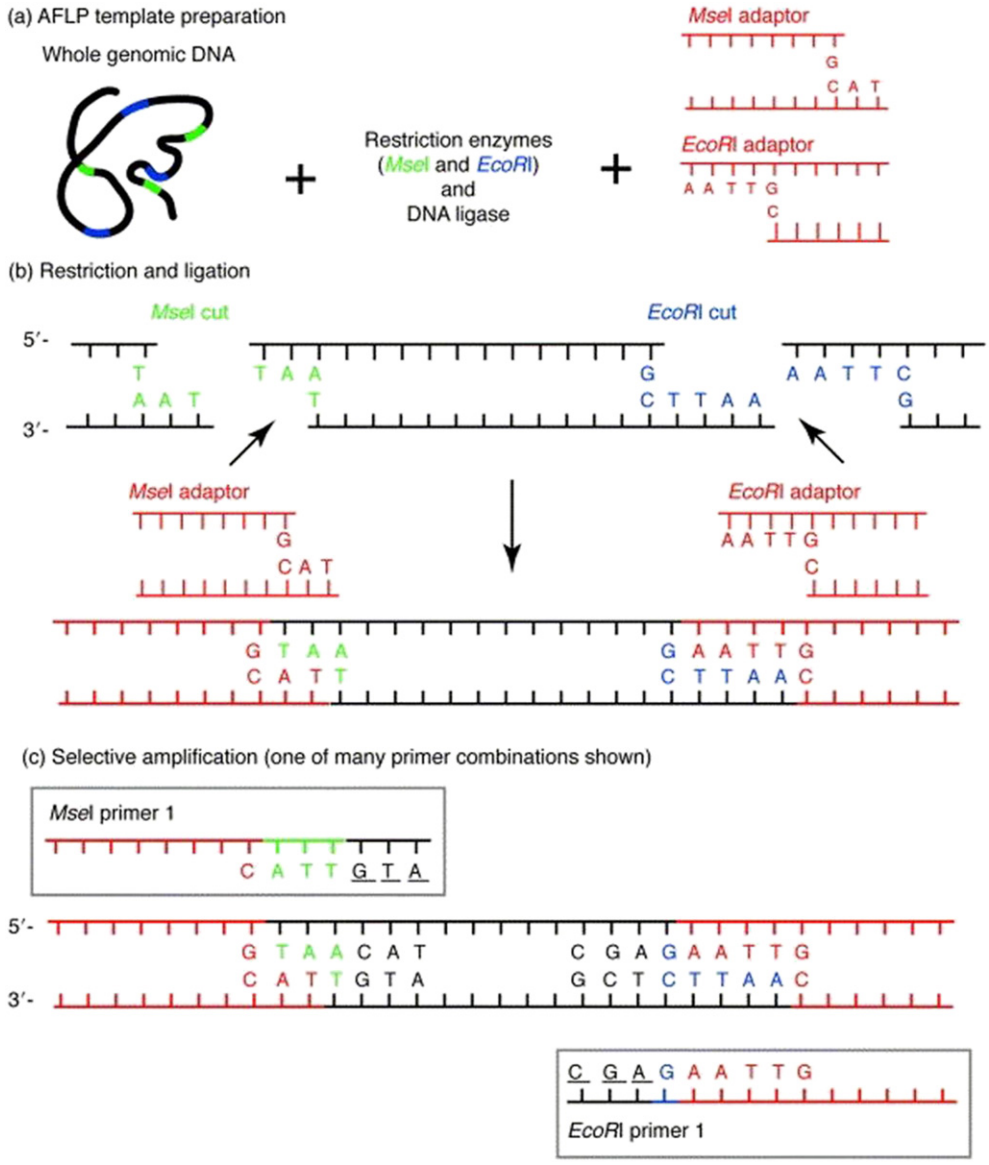

Fig. 4. Pictorial view methodology of AFLP. DNA digestion with restriction enzymes (a), adaptor ligation (b) Selective amplification (c); [Prior to selective amplification, pre amplification is carried out with a single nucleotide extension, followed by selective amplification using three 3-bp extension] (Mueller and Wolfenbarger 1999).

6. Inter-simple sequence repeats (ISSR)

ISSR (Fig. 5) is one of dominant markers involved in amplification of DNA segment present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction. This technique relies on the principle of using microsatellite as primers which target multiple genomic loci to amplify inter simple sequence repeats of the genomic DNA of different sizes. Microsatellite used as primers for ISSRs are di-penta nucleotide. The primers for the ISSRs could either be unanchored or anchored at 3`-5` end having 1–4 degenerate bases extended in to the flanking sequences. Compared with RAPDs the size of the primers in ISSRs are much longer (18–25 mers), which permit the annealing of the primers at higher temperatures leading to higher stringency. The DNA fragments obtained are generally 0.5–2.0 kb long and could be detected both by agarose and polyacrylamide gel electrophoresis. Survey of literature reveals that ISSR markers show high reproducibility than RAPDs (Kojima et al. 1998) although it varies with the detection method used. Fang and Roose (1997) reported greater than 99% reproducibility level after performing repeatedly tests for ISSR markers with the DNA samples of the same cultivar grown in different locations, DNA extracted from different aged leaves of the same individual, and by performing separate PCR runs. However, in other studies, the reproducibility of ISSRs amplification products are in the range of 86–94%, with the maximum when the priority is given to polyacrylamide gel electrophoresis and $AgNO_3$ staining over agarose gel electrophoresis and faint bands are totally excluded while scoring the bands.

The species of *Rheum* viz. — *Rheum officinale* Baill., *Rheum palmatum* L., and *Rheum tanguticum* Maxim. ex Balf are very difficult to distinguish on the basis of arial morphological and anatomical studies. Though root and rhizome is prescribed to have medicinal importance, however to distinguish these plant species, the aerial parts leave doubt regarding the differentiation of the three species. Wang (2011) used ISSRs to authenticate these *Rheum* species with different ISSR primers. A successful attempt by Tamhankar et al. (2009) was also made for authentication of samples of Chirayat complex (*Swertia angustifolia* Ham. ex D. Don Nauni, *S. chirayita* (Roxb. ex Fleming) Karsten, *S. cordata* Wall, *S. densifolia* Griseb, *S. lurida* Clarke, *S. ciliate*, *S. paniculata* Wall, *S. alata* Clark Mirik, *S. bimaculata*, Hook f. & Thoms, *Andrographis paniculata* Nees, *Enicostemma axillare*, *Exacum tetragonum* Roxb). The DNA of the

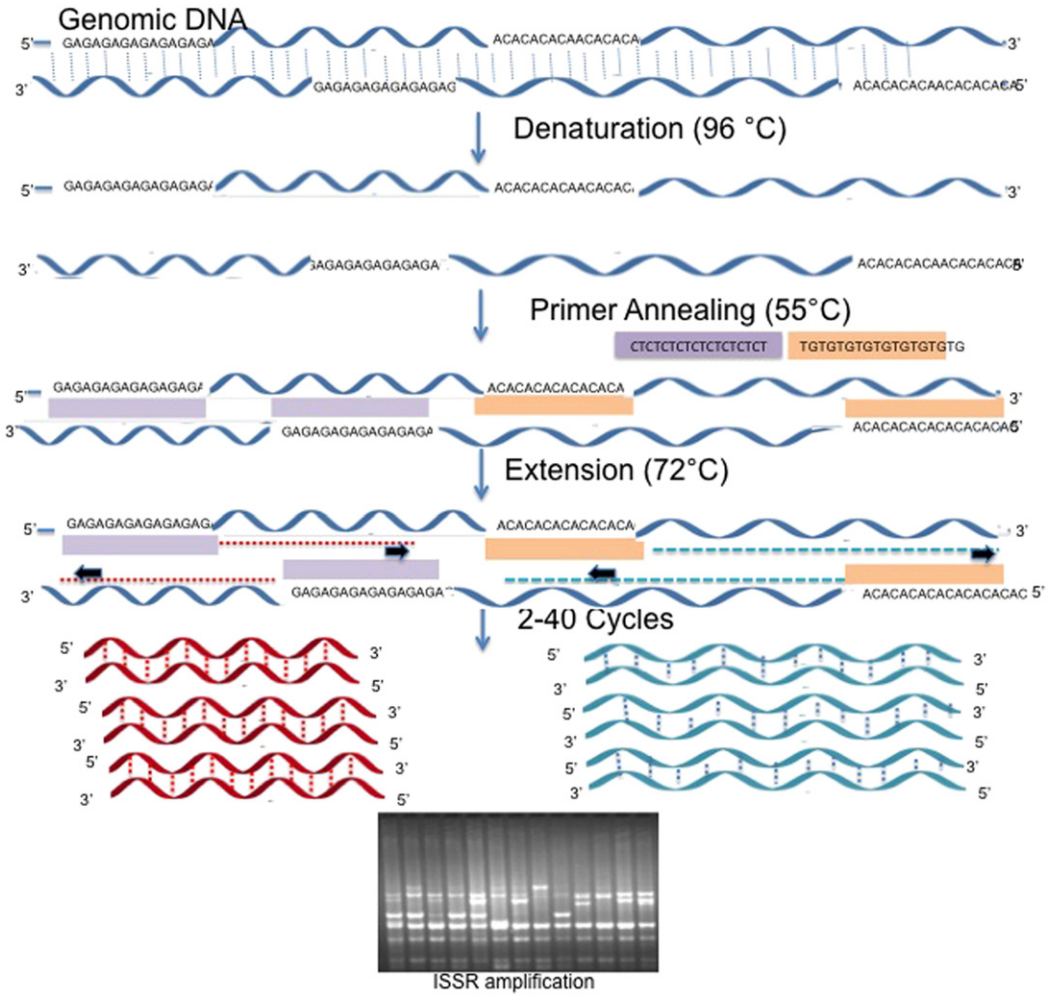

Fig. 5. Pictorial view methodology of ISSR.

market samples and the three authentic species was amplified using ISSR primers and the profile obtained was compared and the differentiations were obtained with primers 808 and 809. The roots of *Cissampelos pareira* L. var. *hirsuta* (Buch.-Ham. ex DC.) generally named as Patha in India is substituted with two other species, viz., *Cyclea peltata* (Lam.) Hook.f. & Thomson and *Stephania japonica* (Thunb.) Miers. ISSR profiles (Vijayan et al. 2014) distinguished genuine raw drug of 'Patha' from its substitutes/adulterants to guarantee the quality and legitimacy of this drug in the market.

6.1. Limitations of ISSRs

- ISSR markers do have more value compared to RAPDs; however, the marker has the reproducibility issues.
- This marker is also dominant.

7. Sequence characterization of amplified regions (SCAR)

The most important marker for authentication of medicinal plants is SCAR (Fig. 6). It is sequence-based mono-locus and a co-dominant marker in which forward and reverse primers are designed from the particular region of a cloned AFLP, RAPD and ISSR DNA fragment linked to a trait of interest. SCAR may be a specific gene or random DNA fragment in the genome of an organism and the primers for amplification are located at any suitable position within or flanking the unique AFLP, RAPD, ISSR amplicon. SCAR is fast, reliable and highly reproducible marker used in molecular biology. The designed primers are used to identify the target species from the pool of related species by the presence of a single, distinct and bright band in the desired sample (Kiran et al. 2010). The length and GC content is also concern for SCAR markers and generally 20–25 oligonucleotide bases are sequence specific (Kiran et al. 2010). SCAR markers developed from AFLP and SSR though is more reproducible but simultaneously such markers are costly, time consuming and more difficult (particularly in AFLP where silver staining is required for the elution of DNA fragment from polyacrylamide gels). To convert a selected unique RAPD, AFLP, ISSR or SSR band to a SCAR marker, each unique band is eluted, cloned and sequence verified. The nucleotide sequence of the unique DNA band is analyzed for uniqueness by comparing with the known DNA sequences available at various databases for synthesizing specific SCAR primers. These markers have the advantage of being co-dominant and are highly reproducible.

Molecular biologists prefer these markers and the most recent data shows that these markers are actively used to authenticate medicinal plants. Yadav et al. (2012) confirmed a 589 bp species specific band with RAPD primer OPAA-3 in *Bacopa monnieri* accessions and not found in other adulterant candidates. For further processing a pair of SCAR primers (between 406 bp of 589 bp sequence of RAPD amplicon) was designed. The PCR confirmation resulted a distinct band from *Bacopa monnieri* and not in the adulterants. Seethapathy et al. (2014) authenticate Ativisha (*Aconitum heterophyllum*) and Musta (*Cyperus rotundus*) using nrDNA *ITS* sequence based SCAR markers and when market samples were examined it was found that SCAR primers (Cyr-

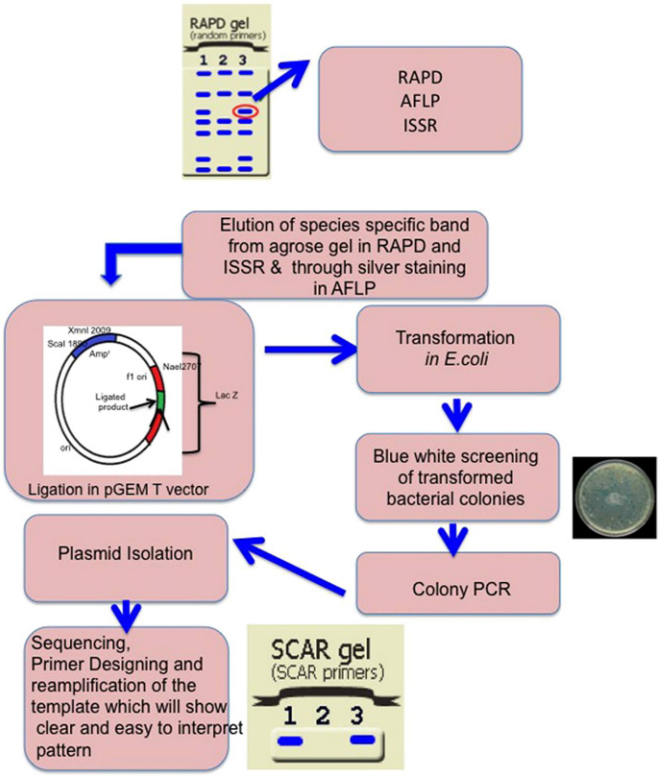

Fig. 6. Pictorial view methodology of SCAR.

FP and Cyr-RP) could identify tissue sample containing 750 μg to 4.76 mg/100 mg of Musta in complex mixtures of DNA extracted from commercial herbal drugs and it was also found Ativisha was not be identified through SCAR markers confirming that this authentic species is not used to prepare herbal drugs despite its being labeled as one of the ingredients in formulations. Abdin (2013) aimed to develop RAPD based SCAR markers for the genuine herbs, *C. angustifolia* and *C. acutifolia* and their adulterants, *C. sophera* and *C. tora*. Analysis of results confirmed that these samples were more than 50% adulterated. Fu et al. (2013) studied *Lonicera japonica*, a traditionally used medicinal plant by an improved random amplified polymorphic DNA (RAPD) analysis and SCAR markers developed differentiated the different accessions/populations. Similarly Li and Park (2012) while using SCAR markers "SA06 and SB05" differentiated *Ophiopogon japonicas* with an amplicon of 460 and 553-bp respectively from *Liriope platyphylla*; the marker SA12 amplified a 485-bp fragment specific to *Liriope platyphylla*. Another case is the *Phyllanthus amarus* Schum. & Thonn used as antipyretic, diuretic, to treat liver diseases and viral infections. Theerakulpisut et al. (2008) efficiently differentiated this specie from the less effective species which include *P. debilis* L. and *P. urinaria* Klein ex Willd.

7.1. Limitations of SCAR markers

- The need for sequence data to design the PCR primers is prerequisite for SCAR markers.

8. DNA barcoding:

DNA barcoding may be defined as use of short nuclear or organelle DNA sequences for the identification of organisms. DNA barcoding has a clear goal to identify an unknown sample utilizing pre-existing classification and not to determine patterns of relationship. This technique is over now a decade old when Hebert et al. (2003) proposed that the mitochondrial enzyme *CO* 1 (Cytochrome oxidase 1) coding DNA could be applied to generate DNA barcode in animals. As mitochondrial genes in plant systems are slowly evolving with very low substitution rates, therefore these are not considered suitable for barcoding (Techen et al. 2014). An alternative is the use of chloroplast/nuclear genomes which have high substitution rates. Many chloroplast genomic regions (*rbcL*, *matK*, *trnH-psbA*, *trnL-F*, *rpl36-rps8*, *ITS* and *5S rRNA*) have been evaluated in plant systems by- "The Consortium for the Barcode of Life Plant Working Group (CBOL)" (CBOL 2009), of which *rbcl* is considered as universal but is having low species resolution and reverse is the case with *matK*; combination of these two markers could show better results. Cameron and Chase (1999) showed less specie discriminating ability with *rbcl* and *matK* when very close taxa are concerned. Hence, Li et al. (2011) included nucleur *ITS* to the combination *matK* + *rbcL* with the aim to have better discriminating ability in closely related species. In DNA barcoding the main focus is to find out a universal DNA sequence that must a balance of conserved sequence as well as harbor enough diversity in order to differentiate organisms. Primers are therefore designed which are complementary to the conserved sequences and flank the variable barcode sites. This process becomes problematic when the primer binding sequences have also accumulated sufficient sequence divergence over evolutionary time. It is therefore; quite difficult to identify universal primer sets that satisfy all taxonomic hierarchies. To overcome this dilemma, barcode primers should accommodate sequence variation, or degeneracy, at one or several nucleotide positions. As is obvious that there are enough chloroplast genomes in a single plant cell, so, amplification of barcode regions *of rbcL* and *COI* are higher than nuclear loci, thus minute material provides ample template to get higher quantities of product simultaneously lowering contaminant concentration that could otherwise affect PCR. The DNA sequence of chosen DNA amplicon (e.g. *rbcL*, *COI* or *ITS*) is required in both orientation through bi-directional sequencing to obtain a reliable DNA barcode.

Several excellent reports have appeared towards authentication of medicinal plants through DNA barcoding. A very good example is of *Crocus sativus*, one of the most important and expensive medicinal spice products in the world. Because of its high market value, this herb is often adulterated with other spurious materials- *Carthamus tinctorius* L. and *Calendula officinalis* L. flowers, *Hemerocallis* L. petals, *Daucus carota* L. fleshy root, *Curcuma longa* L. rhizomes, *Zea mays* L., and *Nelumbo nucifera* Gaertn. stigmas (Jiang et al. 2014). Jiang et al. (2014) developed accurate detection of these adulterants in traded saffron with the application of barcoding melting curve analysis method (Bar-MCA). Another example is the discrimination of *Schisandra chinensis* (Li et al. 2013) at the species and population levels with internal transcribed spacer 2 (*ITS2*). Li et al. (2013) observed C → A substitution at site 86-bp in wild populations when compared with cultivated populations. Further, *ITS 2* region clearly differentiated *S. chinensis* from *S. sphenanthera*. Other study was aimed to identify *Astragalus* (Gao et al. 2009), many of its species share morphological similarity. During this study four coding (*trnH-psbA*, *rpoC1*, *rbcL*, *matK*) and two non-coding regions (*ITS*, *ITS2*) were compared among 319 species; *ITS2* and *ITS* barcodes were more productive towards discriminating the species. Moving forward is the case in which *Peucedanum praeruptorum* has been discriminated by DNA barcoding. Zhou et al. (2014) used *ITS* and nrDNA barcodes to distinguish it from common substitutes and adulterants. Further study about the applications of DNA markers is mentioned in Table 2.

8.1. Limitations of DNA barcoding

- It has been found that DNA barcoding fail to distinguish recently diverged species (Kerr et al. 2007).
- It is also to mention that use of barcoding depends to a large extent over the amplification of degraded DNA. This is one of the most difficult aspects because sequences characterized are longer than 500 bp

Table 2
Molecular markers used for authentication of medicinal plant species.

Name of the authentic drug	Part used	Medicinal uses	Substituent/adulterants if any	Technique used	Study	References
Litchi chinensis Sonn.	Fruit	Fruit and its secondary metabolic products have been reported to have anticancer, anti-inflammatory, antifungal, antiviral, antioxidant, antiplatelet and anticoagulant, and antidiabetic activities	None	RAPD-SCAR	Development and significance of RAPD-SCAR markers for the identification of *Litchi chinensis* Sonn. by improved RAPD amplification and molecular cloning	Cheng et al. (2015)
Akebia quinata	Stem	Antiphlogistic, diuretic, analgesic	*Akebia trifoliata*, *Aristolochia manshuriensis*, and *Clematis armandii.*	RAPD-SCAR	Authentication of *Akebia quinata* D_{ECNE} from its common adulterant medicinal plant species based on the RAPD-derived SCAR markers and multiplex-PCR	Moon et al. (2015)
Sida cordifolia	Root, leaves	Anti-oxidant, anti-inflammatory, anti-diabetic	*Abuliton indicum*, *Sida rhombifolia*	DNA barcoding	DNA barcoding for species identification from dried and powdered plant parts: a case study with authentication of the raw drug market samples of Sida cordifolia.	Vassou et al. (2015)
Zanthoxylum acanthopodium and *Zantho xylum oxyphyllum*	Whole plant	Useful against stomach trouble, blood purifier, reducing the incidence of leucoderma	None	AFLP	Species Specific AFLP Markers for authentication of *Zanthoxylum acanthopodium* & *Zanthoxylum oxyphyllum*	Gupta and Mandi (2014)
Cissampelos Pereira	whole plant	Stomach pain, fever, skin conditions, cardiac pain	*Cyclea peltata*, *Stephania japonica*	RAPD	Developing RAPD markers for identification of three source plants of Ayurvedic raw drug 'Patha.	Vijayan et al. (2013)
Dracocephalum oldavica L.	Whole plant	Coronary diseases, pain relief,	*M. officinalis*, *N. Cataria* L.	RFLP	Genetic authentication by RFLP versus ARMS? The case of Moldavian dragonhead (*Dracocephalum moldavica* L.)	Horn et al. (2014)
Podophyllum hexandrum Royle	Root, rhizome	Anti-cancer	*Podophyllum peltatum* L	RAPD-SCAR	SCAR Molecular Markers for identification and authentication of medicinal plants *Podophyllum hexandrum* Royle	Al-Shaqha et al. (2014)
Bulbus Fritillariae Cirrhosae	Bulb	Antitussive and expectorant	*Bulbus Fritillariae Pallidiflorae* (*Yibeimu*), *Bulbus Fritillariae Thunbergii* (*Zhebeimu*), *Bulbus Fritillariae Hupehensis* (*Hubeibeimu*); and *Bulbus Fritillariae Ussuriensis* (*Pingbeimu*).	RAPD-SCAR	Authentication of Bulbus *Fritillariae cirrhosae* by RAPD-Derived DNA Markers	Xin et al. (2014)
Peucedanum praeruptorum L.	Roots	Expectorant	*Anthricus sylvestris*	DNA-barcoding	Molecular authentication of the traditional medicinal plant *Peucedanum praeruptorum*and its substitutes and adulterants by dna - barcoding technique	Zhou et al. (2014)
Ginko biloba	Leaves	Used for treatment of degenerative diseases of the brain, dementia, and for slowing down the progression of Parkinson's disease and Alzheimer's disease	None	DNA-barcoding	Authentication of *Ginkgo biloba* herbal dietary supplements using DNA barcoding	Little (2014)
Phoenix dactylifera L	Fruits	Fruits are antimutagenic and anti-oxidants	None	DNA-barcoding	DNA barcoding based on plastid matK and RNA polymerase for assessing the genetic identity of date (*Phoenix dactylifera* L.) cultivars	Enan and Ahamed (2014)
Piper nigrum L.	Fruit	Antimicrobial, antioxidant, antiinflammatory and antitoxic	*Carica papaya* L.	DNA-barcoding	DNA barcoding to detect chili adulteration in traded black pepper powder	Parvathy et al. (2014)
Indirubin (*Isatis tinctoria*, *Polygonum tinctorium*, and *Strobilanthes cusia*)	Leaves	Treatment of chronic myelocytic leukemia	*P. hydropiper*, *P. chinense*, *Clerodendrum cyrtophyllum*, *Indigofera tinctoria*, and *S. dimorphotricha* and roots of the geniuine drug.	DNA-barcoding	Rapid Identification and Verification of Indirubin-Containing Medicinal Plants	Hu et al. (2014)
Angelica acutiloba Kitagawa var. acutiloba Kitagawa, *A. acutiloba* Kitagawa *var.* sugiyamae Hikino	Root	To treat gynecological diseases	*A. acutiloba Kitagawa* var. sugiyamae Hikino	RAPD	Authentication and Genetic Origin of Medicinal *Angelica acutiloba* Using Random Amplified Polymorphic DNA Analysis.	Matsubara et al. (2013)

M. ilicifolia and *M. Aquifolia*	*M. ilicifolia*- leaf, *M. Aquifolia*- leaf, flower	Treatment of ulcer, gastritis and indigestion	*Sorocea bonplandii*	PCR-RFLP	Molecular authentication of *Maytenus* sp. by PCR-RFLP	Nakamura et al. (2013)
Knema andamanica	Whole plant	Antimicrobial and therapeutic	None	RAPD-SCAR	RAPD, SCAR and conserved 18S rDNA markers for a red-listed and endemic medicinal plant species, *Knema andamanica* (Myristicaceae)	Sheeja et al. (2013)
Schisandra chinensis	Fruit	Astringent, treat diarrhea, arrest excessive sweating	*S. sphenanthera*	RAPD-SCAR	Development of RAPD-Derived SCAR Markers and Multiplex-PCR for Authentication of the *Schisandrae fructus*	Lee et al. (2013)
Lonicera japonica	Leaves, flowers	Possess h heat-clearing, detoxifying, and anti-inflammatory effects	*L. japonica* var. *chinensis*, *L. similis*, and *L. acuminate*	DNA-barcoding	Stability and Accuracy Assessment of Identification of Traditional Chinese Materia Medica Using DNA Barcoding: A Case Study on Flos *Lonicerae Japonicae*	Hou et al. (2013)
Gentiana scabra, *Gentiana triflora*, *Gentiana manshurica* and *Gentiana rigescens*	Leaves, roots	Treating liver diseases and hepatoprotective against acetaminopheninduced acute toxicity	*Gentiana rhodantha* and *Podophyllum hexandrum*	DNA-barcoding	Evaluation of seven DNA barcodes for differentiating closely related medicinal *Gentiana* species and their adulterants	Wong et al. (2013)
Croton bonplandianum Baill	Seeds	Treatment of jaundice, acute constipation abdominal dropsy and internal abscesses	None	DNA-barcoding	MATK gene based molecular characterization of medicinal plant — *Croton bonplandianum* Baill	Chandramohan et al. (2013)
Shankhpushpi (*Convolvulus pluricaulis*)	Whole plant	Memory enhancer	*Cansicora decussata*, *Clitoria ternatea*, *Evolvulus alsinoides*	RAPD	Authentication of shankhpushpi by RAPD markers	Ganie et al. (2012)
Paris polyphylla Smith var. yunnanensis	Tuber	Anti-tumor, analgesia, anti-inflammatory, and Antifungal	None	PCR-RFLP	Molecular authentication of the medicinal plant *Paris polyphylla* Smith var. yunnanensis (Melanthiaceae) and its related species by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP)	Liu and Ji (2012)
Fallopia multiflora	Root	Counteracting toxicity, cures carbuncles, and relaxes the bowels	*C. auriculatum*	PCR-RFLP	Molecular authentication *of Fallopia multiflora* by PCR-RFLP based on the trnL-trnF analysis	Zheng et al. (2012)
Catharanthus roseus L. Don.	whole plant	Anti-cancer	*Solanum melongena*, *Lycopersicon esculentum*	RT-SCAR	Real time sequence characterized amplified region (RT-SCAR) marker: Development and its application for authentication and quantification of *Catharanthus roseus* L. Don.	Chaudhary et al. (2013)
Asparagus racemosus Willd.	Root	Used for dyspepsia, lactogogue, anti-diarrhoeal, anti-septic, diuretic etc.	*Asparagus gonoclados* Baker	DNA-barcoding	DNA barcoding of authentic and substitute samples of herb of the family *Asparagaceae* and *Asclepiadaceae* based on the *ITS2* region	Rai et al. (2012)
Eleutherococcus senticosus	Root, stem and leaf	Adaptogen used to cure many cancers	*Periploca sepium*	PCR-RFLP	Genetic and chemical diversity of *Eleutherococcus senticosus* and molecular identification of Siberian ginseng by PCR-RFLP analysis based on chloroplast trnK intron Sequence	Zhu et al. (2011)
Zingiber Officinale	Rhizome	Antioxidant, antitumor, and anti-inflammatory	*Z. montanum*, *Z. zerumbet*	AFLP	Species-specific AFLP markers for identification of *Zingiber officinale*, *Z. montanum* and *Z. zerumbet* (Zingiberaceae)	Ghosh et al. (2011)
Ipomoea mauritiana	Roots, leaves	Aphrodisiac, cardiotonic, demulcent, diuretic, refrigerant and galactogogue	*Pueraria tuberosa* (Roxb. ex Willd.) DC (Fabaceae), *Adenia hondala* (Gaertn.) de Wilde (Passifloraceae) and pith of *Cycas circinalis* L.	RAPD-SCAR	Development of Randomly Amplified Polymorphic DNA Based SCAR Marker for Identification of *Ipomoea mauritiana* Jacq (Convolvulaceae)	Devaiah et al. (2011)
Scrophularia ningpoensis	Root	To treat inflammation, laryngitis, tonsillitis, abscesses of carbuncles	None	ISSR-SCAR	Molecular authentication of geo-authentic *Scrophularia ningpoensis*	Chen et al. (2011)
Scutellaria baicalensis	Root	Treatment of hepatitis, jaundice, diarrhea, and inflammatory diseases.	*S. amoena*, *S. rehderiana*, and *S. viscidula*	DNA-barcoding	DNA barcodes for discriminating the medicinal plant *Scutellaria baicalensis* (Lamiaceae) and its adulterants.	Guo et al. (2011)
Ruta graveolens L	leaves, stems, flowers	Fertility regulation, menstrual cramps, earache, headache, nose bleed, and insect repellent	*Euphorbia dracunculoides* Lam.	DNA-barcoding (*ITS*)	Authentication of *Ruta graveolens* and its adulterant using internal transcribed spacer (*ITS*) sequences of nuclear ribosomal DNA	Al-Qurainy (2010)
Pluricaria crispa, *Pluricaria undulata*	arial parts	Used against Gram positive bacteria	None	RAPD	Genetic relationship of two *Pluricaria* species and identification of their putative hybrids using RAPD markers	El-Kamali et al. (2010)

(continued on next page)

Table 2 (*continued*)

Name of the authentic drug	Part used	Medicinal uses	Substituent/adulterants if any	Technique used	Study	References
Piper nigrum	Fruits	Anti-bacterial, anti-inflammatory, antioxidant	*Carica papaya*	RAPD	Development of RAPD markers for authentication of *Piper nigrum* (L.)	Khan et al. (2010b)
Swertia chirayita	Seeds	Treating asthma and liver disorders	*Andrographis paniculata*, *Exacum tetragonum*, *E. pedunculatum*, *Slevolgia orientalis*, *S. alata*, *S. angustifolia*, *S. bimaculata*, *S. ciliata*, *S. densifolia*, *S. elegans*, *S. lawii*, *S. minor*, *S. paniculata*, *S. multiflora*, *S. cordata*	AFLP	AFLP markers for identification of *Swertia* species (Gentianaceae)	Misra et al. (2010a)
Aconitum heterophyllum	Root, stem	Used against stomach ache and fever	*Cyperus rotundus*	AFLP	AFLP markers for the identification of *Aconitum* species	Misra et al. (2010b)
Curcuma comosa	Rhizome	anti-inflammatory	*Curcuma latifolia*	AFLP	Identification and characterization of *Curcuma comosa* Roxb., phytoestrogens-producing plant, using AFLP markers and morphological characteristics	Keeratinijakal et al. (2010)
Rheum officinale Baill., *Rheum palmatum L.*, and *Rheum tanguticum* Maxim. ex Balf.	roots, rhizome	Purgative, anti-inflammatory, antibacterial, purging heat, curing renal disorders, antitumor and antimutagenicity	*R. hotaoense*, *R. compactum*, *R. undulatum* and *R. emodi*	ISSR	Inter-simple sequence repeats (ISSR) molecular fingerprinting markers for authenticating the genuine species of rhubarb	Wang (2011)
Cynanchum wilfordii, *Cynanchum auriculatum*, and *Polygonum multiflorum*	Roots	*Cynanchum wilfordii* tonic to promote renal function, *Cynanchum auriculatum*-antidepressant, Polygonum multiflorum-protects against free radical damage induced by ultraviolet B irradiation of the skin	*Cynanchum* auriculatum is the substitute of *Cynanchum wilfordii* and *Cynanchum wilfordii*, *Cynanchum auriculatum* are the substitutes of *Polygonum multiflorum*	RAPD-SCAR	Rapid molecular authentication of three medicinal plant species, *Cynanchum wilfordii*, *Cynanchum auriculatum*, and *Polygonum multiflorum* (*Fallopia multiflorum*), by the development of RAPD-derived SCAR markers and multiplex-PCR	Moon et al. (2010)
Taxillus chinensis	Branches, leaves	Kidney reinforcement, tendons and bones strengthening, relief for rheumatic conditions and abortion prevention	*Thuja*, *sutchuenensis*, *Scurrula parasitica*, and *Scurrula parasitica* var. *graciliflora*	DNA-barcoding	Authentication of *Taxillus chinensis* using DNA barcoding technique	Li et al. (2010)
Cinnamomum osmophloeum Kaneh.	Leaves	Anti-diabetic, anti-inflammatory, astringent and diuretic	None	DNA-barcoding	DNA Barcoding *Cinnamomum osmophloeum* Kaneh. Based on the Partial Non-Coding *ITS2* Region of Ribosomal Genes	Lee et al. (2010)

and therefore, create problem in amplification (Min and Hickey 2007). Hence, DNA barcoding shall not be considered universal like other conventional markers.

9. Loop mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is a molecular biology technique (Notomi et al. 2000) that relies on auto-cycling strand displacement DNA synthesis with *Bst* DNA polymerase. Unlike other DNA markers, here two primer pairs (inner and outer) are required to amplify the target gene. It has very high specificity, efficiency and rapidity and the molecular biology reports have shown these markers could amplify a specific gene from the whole genome discriminating a single nucleotide difference (Parida et al. 2008). DNA synthesis occurs within 1 h at a single temperature which is in range of 60–66°C. Further, this technique does not demand thermo-cycler as simple incubators and block heaters are good enough to furnish the temperature for successful amplification. Gel electrophoresis is also not mandatory since LAMP products can be detected by the turbidity that come to light due to a large amount of by-product, pyrophosphate ion, being produced, yielding an insoluble white precipitate of magnesium pyrophosphate in the reaction mixture (Mori et al. 2001). The applications of LAMP markers for medicinal plant identification, although is scarce; however, there are reports which confirm its utilization in plant authentication studies. Ganie et al. (2013) developed RAPD based LAMP markers to develop molecular ID for *Nigella sativa*. *Taraxacum formosanum* is substituted by many plant species which include *Taraxacum officinale*, *Ixeridium laevigatum*, *Youngia japonica*, *Ixeris chinensis* and *Emilia sonchifolia* var. *javanica*. Lai et al. (2015) designed four specific LAMP primers based on the nucleotide sequence of the internal transcribed spacer 2 (*ITS2*) and nuclear ribosomal DNA (nrDNA). LAMP amplicons were amplified and detected; here amplification of authentic plant species (*Taraxacum formosanum*) occurred and no such amplification in non-targeted adulterant plant DNA was observed. Twelve samples of *Zingiber officinale* from different regions of India were collected and screened with RAPD. A prominent specie specific DNA fragment of 780 bp was cloned and sequence verified with LAMP primers (Chaudhary et al. 2014). Other examples that support application of LAMP markers in plant medicinal biology include *Curcuma longa* (Sasaki and Nagumo 2007), *Panex ginseng* (Sasaki et al. 2008), *Catharanthus roseus* (Chaudhary et al., 2012).

9.1. Limitations of LAMP markers

- Primer designing in LAMP technology is complex; a minimum of two primer pairs is required to identify six different regions of target gene/DNA sequence.

10. Next generation sequencing (NGS)

The advent of reversible chain- termination reaction in sequencing coupled with high resolution detection, popularly termed as Next Generation Sequencing or NGS allows concurrent sequencing of a large number of molecules therefore generating vast amount of sequence data simultaneously. The technique (Fig. 7) is based on the principle that DNA templates are first fragmented and thereafter immobilized on a solid support. These fragments are to be amplified and sequenced. Three main technologies/strategies that are in practice for NGS are commercialized by Roche/454 Life Sciences (Indianapolis, IN), Illumina/Solexa Genome Analyzer (San Diego, CA) and Applied iosystems/SOLiD System (orange county, CA). All of them retain their distinctive enzyme systems, sequencing chemistry, hardware and software engineering (Mardis 2008; Shendure and Ji 2008; Metzker 2010); also, the sequencing reads obtained with these technologies varies in total sequencing output. Among these Illumina/Solexa and Applied

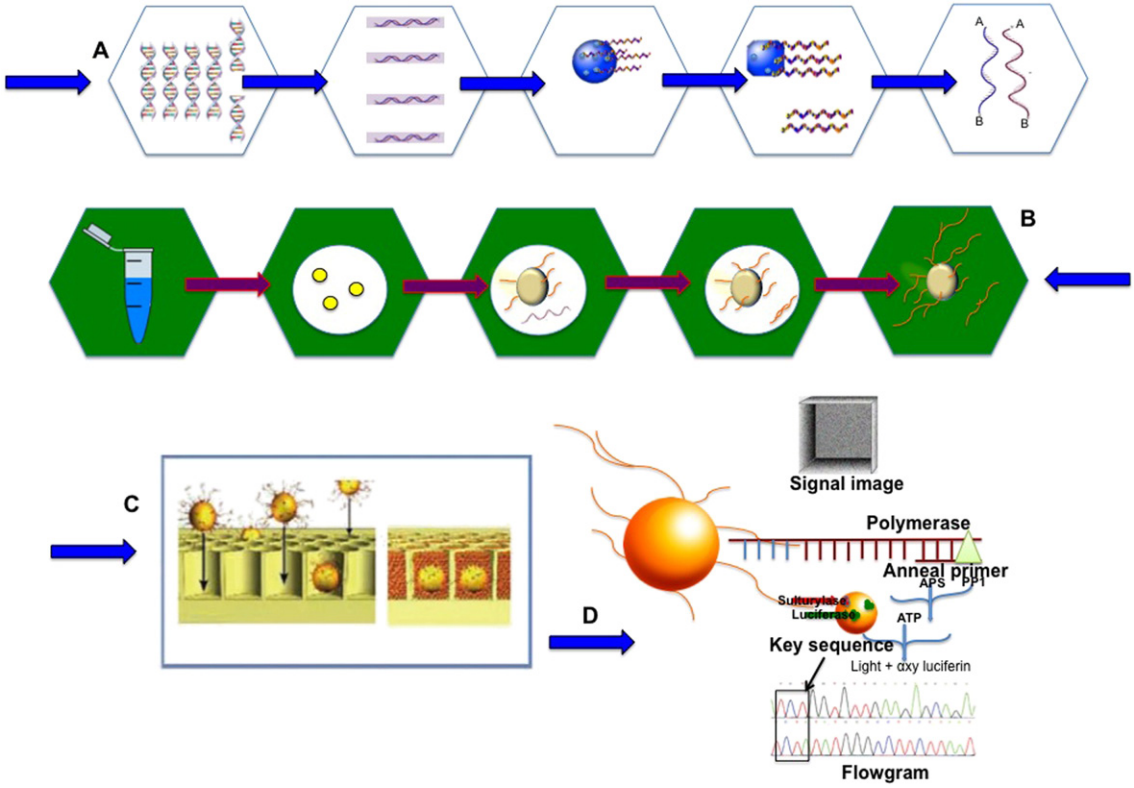

Fig. 7. Outline of the Roche/454 sequencer workflow (A) Single-strand template DNA library preparation (B) Emulsion-based clonal amplification (C) Depositing DNA beads into the PicoTiter Plate device (D) Sequencing by synthesis. [Next Generation Sequencing and Whole Genome Selection in Aquaculture, Ed. Zhanjiang (John) Liu (2011) Blackwell Publishing Ltd.]

Biosystems/SOLiD platforms have the ability to generate tens of millions of short reads (25–35 bp) per run; whereas Roche/454, might generate a few hundred thousand to 1 million reads of 400–500 bp DNA fragments per run (Sarwat and Yamdagni 2015). However, it must be kept in mind that Roche/454 is more advantageous regarding the identification of species because this system is more rapid and produces longer read lengths (Metzker 2010). The technology uses pyrosequencing in which pyrophosphate ions released are detected during the addition of nucleotides by DNA polymerase (Ronaghi 2001). The release of these ions starts off a series of reactions of which end product is to be detected by the formation of light by fire-fly luciferase.

NGS technology relies extensively on use of complex algorithms for filtering, assembly and sequence analysis. Most of the commercially available tools such as DNAStar, CLCBio, GeneSpring are now quiet capable of analyzing NGS data. The advent of such rapid and in-expensive method of data generation has now allowed analysis of transcriptomes of medicinal plant species through the sequencing of their cDNA (RNA sequencing) rather to enter whole genome profiling. Transcriptome data could be helpful to provide the details of plant's response to developmental cues and the related environment. With the aid of RNA-sequencing methodologies it is possible to address questions encompassing cell type-specific transcriptomics, transcript secondary structure and gene mapping (Sangwan 2014). Transcriptome analyses in medicinal plants furnish enormous data to screen the entire proposed metabolic pathways with massive flexibility to examine the data.

Next Generation Sequencing has been employed for identifying molecular signatures in the transcriptome related to physiological functions of plant tissues. For example the leaf transcriptome of *Costus pictus* was sequenced applying Illumina reversible dye terminator sequencing technology and using combination of bioinformatics tools, transcripts related to anti-diabetic properties of this plant species was identified (Annadurai et al. 2012). Yun et al. (2015) applied NGS (Roche 454 GS-FLX Titanium Platform) and developed 9 microsatellite markers for *Aconitum austrokoreense*, an endangered medicinal plant. The same platform was applied to find out the prime components of the biosynthetic pathway in *Dendrobium officinale* through the formation of ESTs (Guo et al. 2013). Luo et al. (2010) identified the genes and their transcriptome regulation meant for the biosynthetic pathway in *Huperzia serrata* and *Phlegmariurus carinatus* with the aid of Roche/454 Titanium platform. Two sesquiterpenes (VoTPS1, VoTPS2) have been identified from one million transcript reads in the roots of *Valeriana officinalis* (Pyle et al. 2012). Ghangal et al. (2013) identified 13,299 SSRs of the 10,980 (12.4%) seabuckthorn (*Hippophae rhamnoides* L.) transcripts of which the mononucleotide SSRs represented the largest fraction (56.4%) followed by di-nucleotide repeats (21.5%) and the tri nucleotide repeats constitute the least (18.9%). It is mandatory to mention that traditional full length sequencing is a better choice when the aim is to obtain full length barcodes; however, for the degraded (Shokralla et al. 2009) mixed species samples (Ivanova et al. 2009), species phylogeny (Liu et al. 2013) Roche/454 pyrosequencing or Illumina NGS is the best option. Further reports are mentioned in Table 3.

11. Issues of DNA markers towards authentication

DNA based technology though is superior has certain drawbacks. One requirement is of high quality DNA while analyzing samples, which might be a problem for dried or processed materials. During drug-processing, there could be a change in temperature and pH that may lead to degradation (fragmentation) of the DNA, rendering PCR analysis difficult. However, depending on the degree of degradation of DNA some methods can still be used in processed materials. Again, even low content of secondary metabolites (polysaccharides, tannins, essential oils, phenolics, alkaloids, etc.) may inhibit PCR or might affect DNA isolation. As secondary metabolite content increases with the age and this becomes severe as the material gets older. Such contaminations are problematic, either they stop or minimize the activity of many enzymes, such as polymerases, ligases, and restriction endonucleases. The market samples might be contaminated with endophytic fungi that could shatter the results of dominant markers like RAPD, AFLP and ISSR and might also influence DNA sequencing; however, this can

Table 3
Applications of next generation sequencing in medicinal plant research.

S. no.	Plant	Biological uses	Platform used in NGS	Study	Reference
1	*Ocimum sanctum*	Used for cough, Cold, bronchitis, expectorant.	Illumina HiSeq2000	Unraveling the genome of Holy basil: an “incomparable” “elixir of life” of traditional Indian medicine	Rastogi et al. (2015)
2	*Beta vulgaris*	For the treatment of fevers and constipation, disorders of the respiratory tract, fevers, and infections	Illumina HiSeq2000	The genome of the recently domesticated crop plant sugar beet (*Beta vulgaris*)	Dhom et al. (2014)
3	*Panax ginseng*	To stimulate immune system, anticancer, and anti-hyperlipidemic	Illumina HiSeq	Transcriptome profiling and comparative analysis of Panax ginseng adventitious roots	Jayakodi et al. (2014)
4	*Elaeis guineensis*	Used as laxative and diuretic, as a poison antidote, as a cure for gonorrhea, menorrhagia, and bronchitis	Roche/454	“Oil palm genome sequence reveals divergence of interfertile species in Old and New worlds.”	Singh et al. (2013),
5	*Curcuma longa*	Anti-malarial, anti-inflammatory and antitumor	Illumina GAIIx	De Novo Transcriptome Assembly (NGS) of Curcuma longa L. Rhizome Reveals Novel Transcripts Related to Anticancer and Antimalarial Terpenoids	Annadurai et al. (2013)
6	*Catharanthus roseus*	Anti-cancer	Illumina HiSeq2000	CathaCyc, a Metabolic Pathway Database Built from *Catharanthus roseus* RNA-Seq Data	Van Moerkercke et al. (2013)
7	*Withania somnifera*	Restorative tonic, stress, nerve disorder, aphrodiasiac.	454-GS FLX sequencing (454 Life Sciences, Roche, USA)	De Novo Assembly, Functional Annotation and Comparative Analysis of *Withania somnifera* Leaf and Root Transcriptomes to Identify Putative Genes Involved in the Withanolides Biosynthesis	Gupta et al. (2013)
8	*Azadirachta indica*	Sedative, analgesic, epilepsy, hypertensive.	Pyro-sequencing	A draft of the genome and four transcriptomes of a medicinal and pesticidal angiosperm *Azadirachta indica*	Krishnan et al. (2012)
9	*Cannabis sativa*	Hallucinogenic, hypnotic, sedative, analgesic, and anti-inflammatory agent	Illumina, Roche	“The draft genome and transcriptome of *Cannabis sativa*”	van Bakel et al. (2011)
10	*Populus trichocarpa*	Antipyretic, analgesic and to control inflammation	Expressed sequence tag based methods	“The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray)”.	Tuskan et al. (2006)

be overcome with a plant-specific primer design. DNA related methods totally fail when the herb is in capsule form or an extract. Many markers, like *ITS* region of the *18S*, *5.8S*, and *26S* nuclear ribosomal cistron, might show intra-specific sequence variation because of non functional paralogous sequences (pseudogenes) and for DNA barcoding, only orthologous DNA sequences is the choice. Consequently cloning of PCR products is sometimes inevitable. In order to develop a marker for identification of taxa, 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.

12. Conclusions

The genetic profile of the medicinal plant species will find applications in the quality control of the drugs obtained from these species not only by the pharmaceutical industries, but also Govt, agencies responsible for monitoring their quality. DNA markers which fill the maximum gap in solving the problem of identification, off-course is a boom to the molecular biology but is dependent over other two markers because you cannot find the efficiency of an authentic drug without chemical analysis. DNA markers are not tissue specific, is an advantage but, the same statement creates a problem when adulterants of the same plant are sold (Sometimes the flower of the plant species has the medicinal properties but instead of flowers some other part of the same plant e.g. roots, leaves etc. are sold), in such cases you simply cannot judge it by DNA markers because it will provide the same profile to all the different tissues of the same plant. In our viewpoint RAPD, AFLP, SAMPL and ISSR should not be allowed for authentication unless and until these markers are converted either into LAMP or SCAR. These markers can determine efficiently the genetic diversity of plant species. SCAR, LAMP and DNA bar-coding are ideal for medicinal plant authentication besides morphological, anatomical and chemical markers. In future, DNA markers can be used to build a reference library of traditional medicine (such as DNA sequences and fingerprints), in order to get complete rid of adulterants and spurious materials that has ruined this medicine.

Conflict of interest

The authors declare that they have no conflict of interest.

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