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Development of *Insilico* Based Molecular Marker Systems in Medicinal Plants of India: Future Prospects

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Abstract:

India has nine different Biodiversity zones, including more than 7000 medicinal plants. In several ancient books like Vedas, Ramayana and Mahabharat, the roles of medicinal plants have been described in details. The demand for medicinal plants in the Global markets is increasing day by day because of their broad spectrum like anti-microbial, anti-fungal and anti-cancer activity without any side effects as compared to synthetic drugs. Due to these advantages herbal drugs have a great potential in the developing and developed countries. Several traditional and modern technologies have been used for the identification and characterization of medicinal plants. Conventional DNA-based molecular markers including RAPD, RFLP, ISSR, AFLP, and Microsatellite are widely accepted by many research institutes all over the world. However, the limitation of these molecular markers restricts scientist and researchers to further investigations of medicinal

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plants at large scale. Insilico based molecular markers like EST-SSR, ILP, SNP have a great amplification, gene based information, and transferability properties. These specific properties of Insilico based molecular markers, may increase the herbal drug analysis and the authentication of plant species of medicinal importance.

Key words: *Insilico* Based Molecular Markers, medicinal plants, India, herbal drugs.

Introduction

Since the beginning of human civilization, medicinal plants are playing a very vital role in drug development, nature conservation, biodiversity and economy of the country. Even today, the millions of people from all over the worlds depend upon these herbal medicines, developed from individual plants and combination of plants (WHO 2005). The Indian subcontinent specially Himalaya region is a natural resource of herbal plants because it represents an extremely unique eco-system that is favorable for cultivation of medicinal plant associated with several ancient treatment systems of Ayurveda, Folk medicines, Homeopathy, Siddha and Amchi (Tibetan).

In India more than 8000 medicinal plants species are available among them 90% are belonging to forest habitats. Apart from India, several other hot spots have been identified and around 70,000 plant species are used in folk medicine globally (Table 1; Farnsworth and Soejarto 1991).

S. No.	Forest Zones of India	Area comes under these Zones	No. of known medicinal plants
1	Himalayan zone 1	Tibetan area	700
2	Himalayan zone 2	(i) North West Himalaya (ii) Western Himalaya (iii) Central Himalaya (iv) Eastern Himalaya	2900
3	Desert zones	Kutch and Thar	500

4	Semi-arid zone	Punjab Guiarat-Rajwara	1,000
5	Western Ghats	(i) Western Ghats mountains (ii) Malabar coasts	2,000
6	Deccan Peninsula	(i) Deccan Plateau south (ii) Central Plateau (iii) Eastern Plateau (iv) Chhota Nagpur (v) central Highlands	3,000
7	Gangetic Plains	(i) Upper Gangetic Plains (ii) Lower Gangetic Plains	1,000
8	North East India	(i) Brahmaputra valley (ii) Assam hills	2000
9	Islands	(i) Andaman islands (ii) Nicobar islands (iii) Lakshdeep islands	1,000
10	Coasts.	(i) West coasts (ii) East coasts	500
	Total no. of plants		14600

Table 1. Geographical distribution of medicinal plants under different zones in India

Now herbal drugs are widely accepted worldwide because of their specific biological activity without any side effect and cheaper in compare to synthetic drugs. According to Kate and Laird (1999), out of 150 top prescription drugs available in world 18% are developed from plant resources. Because of these medicinal properties cultivation and global demand for medicinal plant-based raw materials is growing day to day worldwide. According to WHO (2003) study, global market size for herbal and medicinal plants is projected to reach US$ 5 trillion by 2050.

Several new technologies now have been developed and successfully applied for large scale production of secondary metabolites and cultivation of herbal plants and extracts (Ferreira 1997; Veronese *et al.* 2002). Several countries now adopted breeding and biotechnological approaches to improve the quality and quantity of medicinal plants. Previously, traditional breeding methods like conventional breeding were

successfully utilized to improve the plant growth, high yield and improve the quality of secondary metabolites of medicinal plants. However, conventional breeding programme consisting of several steps like crossing and selection of the superior recombinants segregating populations. Indeed, such a procedure is laborious, long generation time and dependence on environmental conditions may make it further difficult to achieve the desired objective. After the discovery of DNA structure, molecular marker techniques greatly changed the scenario of crop and non crop plant genome research and breeding. These DNA based molecular markers like Random amplification of polymorphic DNA (RAPD), Restriction fragment length polymorphism (RFLP), Amplified fragment length polymorphism (AFLP), Simple sequence repeat (SSR) and *insilico* based markers EST-SSR have acted as very useful tools in several plant systems like medicago, pea, capsicum and wheat (Thoquet *et al.* 2002; Gupta *et al.* 2003; Portis *et al.* 2007; Mishra *et al.* 2009, 2012). DNA based molecular markers have several applications in plants including gene mapping, mapping, QTL analysis, evaluation of the seed purity, genetic diagnostics, characterization of transformants, study of genome organization and phylogenetic analysis (Jain *et al.* 2002). The molecular marker like RFLP, RAPD and AFLP are widely accepted for several diversity, mapping, and testing related work in mid 1990-2000, however now they are considered as 1st and 2nd generation of markers because of development of informative and highly polymorphic sequence based molecular markers.

Now scientists have developed *insilico* based molecular markers system that are more reliable, easy in process, time saving, more conserve between species and cheap in development. Extensive research is carried out by several research institute/seed company/pharmaceutical from all over the world to utilize these sequence based molecular markers systems in breeding, pharmacognostic, identification and

characterization of medicinally important trees/plant species. These DNA markers have proved their utility in various commercially important medicinal plants like *Catharanthus* and *Artemisia* (Mishra *et al.* 2011; Graham *et al.* 2010). Although several research and review papers were published on application of molecular markers in medicinal plants however very few studies are carried out on *Insilico* based molecular markers in medicinal plants. In this paper, we review the future developments and prospects for improving quality and quantity of herbal plants using advanced and *insilico* molecular marker technology.

Types of DNA markers used in plant genome analysis

Many molecular marker systems have been developed in last the three decades. Generally, they are divided into three types based on function (a) morphological markers (b) biochemical (c) molecular (DNA) markers. Presently molecular markers are most accepted and widely used in several plants systems, because of their abundance, polymorphism, reliability, reproducibility and free from environmental factors. In principle, these DNA based molecular markers can reveal or explored genetic differences between two plants that are called polymorphisms and these are described as co-dominant or dominant markers. Based on these features now molecular markers are becomes the most reliable techniques to identification of germplasm and information on genetic diversity at the DNA level (Raddova *et al.* 2003). Gupta *et al.* 2001, classified DNA markers into three classes according to their development in last three decades:

(a) First Generation: RFLPs, RAPDs and their modifications
(b) Second Generation: SSRs, AFLPs and their modified forms,
(c) The third generation molecular: ESTs and SNPs.

(d) Fourth generation: miRNA based (New marker system)

Further these molecular markers can be classified as hybridization based, PCR based and Sequencing-based markers. Hybridization based polymorphisms include RFLP (Restriction Fragment Length Polymorphism) (Sambrooke *et al*. 1989) and VNTR (Variable Number of Tandem Repeats) loci (Rogstad 1994, 1996; Weising *et al*. 1992), where random genomic clones, cDNA clones, microsatellite and minisatellite sequence were used as a probes for hybridization of digested DNA (Kumar 1999). In PCR-based methods, single primer or a primer pairs uses for DNA amplification reaction. PCR based methods are random amplified polymorphic DNA (RAPDs), Inter simple sequence repeats (ISSR), simple sequence repeats (SSRs), sequence-tagged sites (STS). Recent advances in sequencing technology open a new approach for large-scale development of Insilco based molecular markers in plants.

Available Marker Systems

Molecular marker based techniques have been widely accepted for characterization and authentication of different medicinal important plants/herbs. This technique is extremely useful in some specific cases where different plants varieties have similar phenotypes/phytochemical characters. Phenotypic/morphological markers were also used in several crop and non crop plants species for diversity and mapping study however they have some limitations since they are highly dependent on environmental factors and laborious and time consuming, need large space and money. In case of molecular markers, the polymorphisms are based on alteration of DNA sequence, are independent of external environmental factors. RFLP, AFLP and CAPS molecular markers classified as Hybridization-based assay however AFLP and CAPS are combination of PCR based technique and digestion with

restriction enzymes. The basic of hybridization based method was detecting digested DNA fragments by hybridization with various radio-labeled probes such as random genomic clones and cDNA clones. Various types of mutations like point, insertion, deletion, and inversion are responsible for RFLP polymorphism in DNA. These mutations can lead to create or abolished or rearrange of restriction sites in DNA. Because of this property RFLP marker was an important tool for genetic fingerprinting, genome mapping, gene identification and determination of risk for disease (Srivatsava and Mishra, 2009). The AFLP technology was based on combination of RFLPs and PCR-based methods. Development of large number of bands, highly polymorphism, reproducibility and no need of prior information related to DNA sequence make AFLP an attractive technique for several studies including constructing a linkage map from a segregating population, genetic variation, measuring población genetic structure (Shan *et al*. 2005; Mauro *et al*. 2009; Peters *et al*. 2009). AFLP markers successfully used in several medicinal plants like mentha (Shasany *et al*. 2005) and neem (Singh *et al*. 2002), *Glycyrrhiza uralensis* (Zhang *et al*. 2010).

DNA based research and marker-assisted applications in began to flourish in the late 1980s after the development of polymerase chain reaction (PCR; PCR; Mullis and Faloona 1987). Among the different types of PCR based markers, random amplified polymorphic DNA (RAPD) markers are free from any prior information of genotypes and more applicable for the assessment of genetic diversity among rare species (William *et al.* 1990). Because of their simplicity, high speed and low costs of application, RAPD markers became more popular as compared to other molecular markers. RAPD molecular markers successfully applied in several plants like neem (Farooqui *et al*. 1998), Allium subgenus *Melanocrommyum (*Friesen *et al*. 1997), *Allium schoenoprasum* L. (Friesen and Blattner, 2000), *Taxus allichiana (*Shasany *et al*. 1999),

*Codonopsis pilosula (*Fu *et al*. 1999), *Juniperus communis* L. (Adams *et al*. 2003), *Catharanthus roseus* (Gupta *et al*. 2007) for diversity study. In contrast to RAPD, ISSR primers are designed from SSR motifs and dispersed throughout the genome however both have similar practical approaches (Gupta *et al*. 1996; Buhulikar *et al*. 2004) and widely used in many respects such as the study of genetic diversity, gene mapping and molecular mapping in pea, *Cannabis sativa* (Kojoma *et al*. 2002), *Catharanthus* (Gupta *et al*. 2007, Mishra *et al*. 2009). Further these PCR based marker linked with trait of interest can be modified into more reliable form of marker like sequence characterized amplified region (SCAR) or sequence tagged site (STS) markers. The ability of modification of these markers have provided a new platform for the development of new marker system that are more appropriate for breeders of all commercial crops including medicinal plants (Jain *et al*. 2004; Jain *et al*. 2010; Ahn *et al*. 2011).

All these previous markers provided basic information for mapping and diversity study however they have some limitation like availability, reproducibility and time consumption. Further study of in this direction provide a new era of molecular markers with the development of simple sequence repeat markers (SSR; Gupta *et al*. 1996), also known as microsatellites. The microsatellite or genomic SSR are simple, highly polymorphic, reproducible, co-dominatant in nature and relatively inexpensive. These characters make them highly usable molecular markers in among the crops and non crops like pea (Mishra *et al*. 2009), chickpea (Chaudhary *et al*. 2006), capsicum (Lee *et al*. 2004), medicago (Chu *et al*. 2010), wheat (Marion *et al*. 1998)**.** Presently several number of genomic SSRs primer pairs are now developed in various medicinal plants like *Catharanthus* (Bhumika *et al*. 2010), *Artemisia* (Huang *et al*. 2010), *Isodon rubescens* (Harris and Klooster 2011), *Centella asiatica* (Rakotondralambo *et al*. 2012). However in comparison with other economical crop plants the

numbers of SSR markers are not very high.

Insilico based approach for development of Markers

After the discovery of advanced sequencing methods genome sequencing, transcriptome sequencing or gene expression projects were started worldwide in different plants system as well as medicinal plants also. These thousands of sequences in the form of BAC (bacterial artificial chromosome) clones, ESTs (expressed sequence tags), full length cDNA clones and genes are publically available for research purposes mainly into three databases: GenBank in the National Center for Biotechnology Information (NCBI; Sherry *et al*. 2001), the European Molecular Biology Laboratory (EMBL) nucleotide sequence database and the DNA database bank of Japan (DDBJ). Expressed Sequence Tags (ESTs) are a partial sequencing of cDNA libraries and can be used as a source of information for various types of research work. The cDNAs sequences used for EST developments are individual clones from a cDNA library. These EST are developed from coding region of DNA and can represent transcribed part of genes in different tissue or organ at different developmental stages. Because of these features, ESTs have become a reliable and valuable source for developing molecular markers (Gupta *et al*. 2003, Graham *et al*. 2010). Insilico based methods tries to derive information of sequence data with the help of various bioinformatics software. Amongst conventional methods of marker development, Insilco approaches are very useful, much faster, cheaper and informatics for the molecular maker development. Already a number of software programs have been developed and implemented in several plants and successfully identified molecular markers from sequence data. A list of software and pipeline provided in table can be used to detect SSRs and SNPs markers from genomic sequences as well as in ESTs (Table 2).

Name of Software	**Website address**
Sputnik	http://espressosoftware.com/pages/sputnik.jsp http://cbi.labri.fr/outils/Pise/sputnik.html)
Tandem Repeat Finder(TRF)	http://tandem.bu.edu/trf/trf.html
RepeatFinder	http://www.cbcb.umd.edu/software/RepeatFinder/
REPEATMASKER	http://www.repeatmasker.org.
SciRoKo (SSR Classification and Investigation by Robert Kofler)	www.kofler.or.at/Bioinformatics
SSRPrimer	http://hornbill.cspp.latrobe.edu.au http://acpfg.imb.uq.edu.au
SSR Indentification Tool (SSRIT)	www.gramene.org/db/searches/ssrtool
Tandem repeat Occurrence Locator (TROLL)	http://finder.sourceforge.net
SSR Poly	http://acpfg.imb.uq.edu.au/ssrpoly.php
MIcroSAtellite(MISA)	http://pgrc.ipk-gatersleben.de/misa/
Modified sputnik II	http://wheat.pw.usda.gov/ITMI/EST-SSR/LoRota
SSRSEARCH	ftp://ftp.gramene.org/pub/gramene/software/scripts/ssr.pl
Sorting intolerant from tolerant (SIFT;	http://blocks.fhcrc.org/sift/SIFT.htm
PolyBayes	http://bioinformatics.bc.edu/marthlab/PolyBayes
PolyPhred	http://droog.mbt.washington.edu
SNPDetector	http://lpg.nci.nih.gov/
NovoSNP	http://www.molgen.ua.ac.be/bioinfo/novosnp/
AutoSNP	http://acpfg.imb.uq.edu.au
Polymorphism phenotyping	(PolyPhen; http://genetics.bwh.harvard.edu/pph/;

MAPP tool (multivariate analysis of protein polymorphism)	http://mendel.stanford.edu/SidowLab/downloads/MAPP/index.html;
Prediction of amino acid replacement probability	Parepro; http://www.mobioinfor.cn/parepro/; Parepro
SNPs3D	(http://www.snps3d.org/)
large-scale human SNP annotated database	LS-SNP; http://modbase.compbio.ucsf.edu/LS-SNP/
SNPeffect	http://snpeffect.vib.be

Table 2: List of bioinformatics tools used for identification and analysis of SSR and SNP in plants and animals

(a) EST based SSR markers

Microsatellites or simple sequence repeats (SSRs) or short tandem repeats (STRs) are tandem repeats of 1-6 base pairs of DNA. In compare to other neutral regions of DNA the microsatellites are more variable because of high rate to mutations due to slipped strand mispairing (slippage) during DNA replication (Yamazaki *et al*. 1994). Due to their high variation nature, microsatellites are highly polymorphic, multi-allelic, co-dominant nature, relative abundance, transferable between different laboratory and reproducible (Chen *et al*. 1999; Kapteyn *et al*. 2002). Two types of methods are now available for development of SSRS (a) conventional methods (b) insilico based approach. Conventional method is based on construction of a genomic library from DNA and then screened these clones for the identification of SSR repeat motifs by hybridization (Shokeen *et al.* 2005). In contrast to conventional methods *Insilco* method is based on available EST database. Several software packages have been developed to screen these EST-databases for detection of SSR repeats motifs (Table 2). After the development of EST-SSR primer pairs, they are widely adapted for mapping and transferability studies in different

model plant systems (Gupta *et al.* 2003).

Presently thousands of EST database, developed from different genotypes of plants are freely available on public domain (Table 3).

S. No.	Genus	Species name	No. of ESTs available
1	*Acacia*	*caesia*	9110
		catechu	
		chundra	
		mangium	
		concinna	
		farnesiana	
		nilotica	
		sinuata	
2	*Adhatoda*	*beddomei*	1780
		vasica	
		zeylanica	
3	*Adiantum*	*capillus veneris*	30546
		lunulatum	
		philippense	
		venustum	
4	*Allium*	*ampeloprasum*	43376
		cepa _	
		sativum	
		stracheyi	
5	*Apium*	*graveolens*	2355
6	*Argemone*	*mexicana*	5488
7	*Aristolochia*	*bracteolata*	16451
		indica	
		rotunda	
		tagala	
8	*Artemisia*	*absinthium*	95021
		annua	
		maritima	
		nilagirica	
		pallens	
		parviflora	
9	*Berberis*	*aristata*	4978
		canadensis	
		asiatica	
		chitria	
		lycium	
		tinctoria	
		umbellata	

10	*Brassica*	*alba*	1107563
		juncea	
11	*Cajanus*	*cajan*	25314
12	*Cannabis*	*sativa*	12903
13	*Carica*	*papaya*	85961
14	*Carthamus*	*tinctorius*	41584
15	*Catharanthus*	*roseus*	19910
16	*Centella*	*asiatica*	4443
17	*Cichorium*	*endivia*	84,149
18	*Citrullus*	*lanatus*	12503
19	*Citrus*	*acida*	603955
		aurantifolia	
		aurantium	
		bergamia	
		limon	
		medica	
		reticulata	
20	*Clematis*	*gauriana*	5199
21	*Coffea*	*arabica*	174275
		travancorensis	
22	*Cucumis*	*melo*	130006
		sativus	
		trigonus	
23	*Cucurbita*	*maxima*	852
		moschata	
24	*Daucus*	*Daucus carota*	18137
25	*Dioscorea*	*alata*	44134
		bulbifera	
		deltoidea	
		oppositifolia	
		pentaphylla	
26	*Erythrina*	*indica*	726
		suberosa	
		variegata	
27	*Eucalyptus*	*citriodora*	168309
		globulus	
28	*Fumaria*	*indica*	4145
		officinalis	
		parviflora	
29	*Glycyrrhiza*	*uralensis*	50666
		glabra	
30	*Gossypium*	*arboreum* ----	467736
		herbaceum	
		hirsutum	

31	*Helianthus*	*annuus*	133698
32	*Hippophae*	*rhamnoides*	3412
33	*Hordeum*	*vulgare*	525999
34	*Ipomoea*	*batatas*	88199
		trifida	
		digitata	
		mauritiana	
		nil	
		obscura	
		pes-caprae	
		pes-tigridis	
		petaloidea	
		reptans	
		sepiaria	
		biloba	
35	*Jatropha*	*curcas*	46947
36	*Linum*	*usitatissimum*	286852
37	*Macrotyloma*	*uniflorum*	1025
38	*Mangifera*	*indica*	1665
39	*Medicago*	*sativa*	12371
40	*Mentha*	*aquatic*	1634
		arvensis	
		piperita	
		viridis	
41	*Nelumbo*	*nucifera*	2207
42	*Nicotiana*	*Tabacum*	334384
43	*Ocimum*	*americanum*	23260
		basilicum	
		gratissimum	
		kilimandscharicum	
		sanctum	
		tenuiflorum	
44	*Oldenlandia*	*corymbosa*	1117
		umbellata	
45	*Panax*	*ginseng*	11412
		pseudoginseng	
46	*Pandanus*	*fascicularis*	977
47	*Papaver*	*somniferum*	20918
48	*Phaseolus*	*mungo*	391150
		coccineus	123988
		vulgaris	
		radiatus	

49	*Pistacia*	*chinensis*	1299
		integerrima	
		lentiscus	
		vera	
50	*Plumbago*	*indica*	1780
		zeylanica	
51	*Prunus*	*armeniaca*	114096
		avium ----	
		cerasoides	
		dulcis	
		mahaleb	
52	*Pueraria*	*lobata*	6365
		tuberosa	
53	*Punica*	*granatum*	787
54	*Rheum*	*australe*	898
		emodi	
		moorcroftianum	
		palmatum	
		spiciforme	
		webbianum	
55	*Rhododendron*	*catawbiense*	1241
		campanulatum	
		lepidotum	
56	*Ricinus*	*communis*	62592
57	*Saccharum*	*munja*	283640
		officinarum	
		spontaneum	
58	*Salvia*	*aegyptiaca*	11973
		miltiorrhiza	
		fruticosa	
		haematodes	
		moorcroftiana	
		plebeia	
		sclarea	
59	*Saussurea*	*costus*	1897
		hypoleuca	
		lappa	
60	*Sesamum*	*indicum*	44722
		orientale	
61	*Setaria*	*italica*	66027
62	*Solanum*	*anguivi*	745876
		melongena	
		nigrum	
		surattense	

		torvum	
		trilobatum	
		violaceum	
		virginianum	
		xanthocarpum	
63	*Taraxacum*	*officinale*	41296
64	*Thalictrum*	*flavum*	4299
65	*Vigna*	*mungo –*	192440
		radiata	
		unguiculata	
		vexillata	
66	*Zingiber*	*officinal*	38115

Table 3: No. of ESTs available for medicinal plants on public domain

The availability of databases is providing rich resources for marker development. These data can be exploited for development of EST based SSR markers by using software based *In silico* analysis. The development method of SSR markers by Insilco analysis provides a better opportunity to understand the functional annotation of individual EST-sequence. This method is more preferable today because of several advantages (Scott *et al*., 2000; Temnykh *et al*., 2000) like they are co-dominant, highly transferable, computer based, need less time to development and more informative in comparison to Ist and 2nd generation markers (Zane *et al*., 2002). Also these EST-SSR markers provide an opportunity to identify or clone a gene when they show linkage with a trait of interest (Thiel *et al*., 2003).

ESTs are developed from transcribed or coding region of genome therefore they have more conserve region in compare to genomic SSRs. Because of this phenomena EST based SSR marker have high transferability between different cross-species (Portis *et al*. 2007; Varshney *et al*. 2005). As reported earlier, genomic microsatellites are more polymorphic when compared to EST-SSRs. However, owing to their low cost, easy development and greater information regarding gene discovery and cross-species transferability, make EST-SSRs more preferable than genomic microsatellites. All these unique

characters make EST-SSRs more suitable for diversity, transferability and breeding-related studies in several less-studied plants (Mishra *et al*. 2011). A large number of EST-derived SSR primers have been reported in several economically and commercial crops including wheat, barley, brassica, peanut, chickpea, horticulture crops like capsicum, lettuce (Gupta *et al*. 2003, Thiel *et al*. 2003, Saha *et al*. 2004, Portis *et al*. 2007, Varshney *et al*. 2009, Mishra *et al*. 2012) and medicinal plants like Catharanthsus, Artemisia, *Epimedium sagittatum* (Graham *et al*. 2010; Mishra *et al*. 2011; Shaohua *et al*. 2010).

(b) SNP markers

Single Nucleotide Polymorphisms (SNPs) are one of the most important and commonly used molecular markers for studying evolution, functional mapping and complex genetic traits (Syvanen *et al*. 2001). SNP is a variation in DNA sequence reported when any single nucleotide (A, T, C or G) differs between same members of species of genome. Because of this single nucleotide variation SNP is providing an opportunity for extremely fine genetic mapping. Distribution of SNP in coding and non-coding region of genomes is not homogenous. It is reported that SNPs present in coding sequences are used to study the functional traits (Grivet *et al*. 2003). Several reasoned have been identified for SNP variation in organism however the natural selection, recombination and mutations rate determine the density of SNP in genome. The polymorphism generated by SNP markers were based on variation in single nucleotide in DNA sequence, can be detected only by novel techniques and technology.

In contrast to previous molecular markers, SNPs cannot be resolved by conventional methods like agarose, metaphore and ploy acrylamide gel electrophoresis. Many PCR and hybridization based techniques are expensive, laborious and

time consuming, however they have great tendency for improvement. On the basis of this strategies, several novel techniques were developed and successfully using for identification and detection of novel SNPs including sequenced genomes and next-generation sequencing technologies (Martin *et al.* 2009) capillary electrophoresis (Drabovich *et al.* 2006); mass spectrometry (Griffin *et al.* 2000); single-strand conformation polymorphism (SSCP); electrochemical analysis; denaturizing HPLC and gel electrophoresis, restriction fragment length polymorphism; hybridization analysis and Insilico based analysis. Similar to SSR, development of SNPs with experimental methods in laboratory are time consuming and expensive process. In contrast, Insilico based approach to identify potential SNPs from publicly available database makes the development of SNP markers more easy, fast and inexpensive. Presently several number of Insilco based pipelines have been available/developing to automatically identify SNPs in available sequences (Table 2). The PHRED/PHRAP/PolyBayes system (Marth *et al.* 1999; Le *et al.* 2004) is the most popular among the available pipeline because it has ability to identify paralogs with SNP detection (Useche *et al.* 2001; Picoult-Newberg *et al.* 1999). The other type of pipeline can detect only SNPs, such as autoSNP (Wang *et al.* 2005) and SNiPpER (Kota *et al.* 2003).

(c) ILP markers

In eukaryotic genome, introns are non-coding sequence within a gene that is removed by RNA splicing during RNA maturation. Introns are widespread and constituted major part of eukaryotic (24% in human) Genomes. The number of introns depends on developmental complexity of the eukaryotic species in higher plants. Introns are not randomly scattered throughout the genomes but they are present on specific positions as a conserved region across the genome of same species, even in close related family. Previously it was assumed

that introns are non functional, unimportant sequences. However, now it is reported that Introns have little functional significance, encode specific proteins, may influence the level of gene expression, high variation in DNA sequences and more conserve nature. Because of these important features polymorphisms in introns can also be exploited as molecular markers.

Insilico based approach is a suitable method for analysis of polymorphism in Introns. For the detection of specific intron-based markers in ESTs sequences of crop and non-crop, model plant sequences use as a reference to predict intron positions and then designed a ILP primer pair from flanking exons using the PIP database (http://ibi.zju.edu.cn/pgl/pip/) (Yang *et al*. 2007). Intron length polymorphism markers successfully utilized in various plants system for genetic diversity, transferability and gene mapping (Coˆrte-Real *et al.* 1994; Wydner *et al*. 1994; Gupta *et al*. 2011). These molecular markers have several advantages over conventional methods such as AFLPs, SSR, RFLPs or their modifications. Similarly with EST-SSR an ILP marker also has the following advantages such as neutral in nature, co-dominant, stable, convenient, reliable and high transferability between plant interspecies that can be used for various types of study.

(d) MiRNA markers

In recent years, scientists have focused their studies on small molecules RNA (MiRNA) because of their specific mode of action and participation in post-transcriptional gene regulation. MicroRNAs (miRNAs) are small endogenous, non-coding, single-stranded RNA molecules encoded in the genomes of all eukaryotes (Liu and Chen 2010). These small stretches of RNA sequences play key roles in biological processes like regulation of root and shoot development, leaf and stem development, signal transduction, floral differentiation, timing and development (Jones-Rhoades *et al.* 2006; Chuck *et al*. 2009).

These miRNA are also playing a major role in physiological process like defense against biotic and abiotic stress in palnst system. In recent studies, it was observed that these Plant miRNAs are usually evolutionary conserved, inherited stably and remain active in the progeny. Because of these unique features a miRNAs can be applicable to the breeding of hybrid crops (Warthmann *et al*. 2008; Qunig and Yue-Quin 2010). Recently in japonica and indica rice, agronomically applications of miRNA have been reported (Warthmann *et al*. 2008). This approach can play an important role in validation of the functions of putative genes that are responsible for advantageous agronomical traits. Consequently, with the development of miRNAs as molecular markers may be more appropriate promising methods for the identification of genes of interest in crops/non crops.

Conclusion

After the discovery of several new advanced genomics tools in plant biology, our knowledge have enhanced on the genetics and production of secondary metabolites in medicinal important plants. In this direction one of the most important developments was molecular markers in plant breeding. Molecular markers not only improved our knowledge of the basic genetics that is important for secondary metabolites but also helped to development of genetic mapping, gene tagging, map based cloning and QTLs mapping. However several marker system are now available but progress remains slow due to high cost, labor intensive, time consuming and low availability of a number of molecular markers. These reasons are key factors for development of high quality map and improvement of quality and quantity of medicinal plants. However, after development of more advanced sequencing techniques, insilico based markers are now replacing conventional markers system. Approximately 65.9 million ESTs

are now available in public database covering several crops or non-crops. Developments of molecular markers from available resources are informative, polymorphic, low cost, and can be used in entire family because of high transferability. These databases are a rich source for markers development and increasing day by day with high throughout put sequencing technology. Sequencing based EST-SSR, SNP, ILP and miRNA markers can be developed within a timeframe with the help of available Insilco based tools. In several plant systems, Insilco based molecular markers like EST-SSR and SNP have been developed and succefully used however in case of medicinal plants very few studies are now reported. With the expansion of database and development of next generation sequencing technologies, there will be a rapid growth in markers for medicinal important plants and the use of these markers for diverse applications from crop breeding to predicting human disease risks, impacting both food production and human health for future generations.

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