Chapter 15

Spices

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1. Introduction

The past few decades have witnessed introduction of an array of marker technologies like DNA sequencing and high throughput genotyping methods. Molecular markers have established their position as one of the most powerful and sought-after tools over morphological data, because the bulk of variation at the nucleotide level is not visible at the phenotypic level. Utilization in studies on synteny, genome arrangement within taxa, germplasm analysis, trait mapping, and marker assisted selection, genome mapping *etc.* have led to incorporation of marker technologies to complement and validate phenotypic data.

Molecular tools have been used to help elucidate some aspects of genetic diversity in aromatic species, the genetic relationships between different cultivars and comparisons of molecular marker analysis to the chemical composition of plants. An overview of some important techniques involving molecular markers in spices, understanding what controls flavour and aroma production in different plants and its diverse forms, host-pathogen interactions in deciphering the disease progression pathway and identifying important genes in important genotypes, is presented in this chapter.

The route from genomics to proteomics is not well documented; however, Trindade (2010) has addressed the question of the synthesis of volatiles, with two different approaches first, the biochemical and genetic approach; and second, approaches involving functional genomics and finally, a brief survey of bioinformatics resources. The underlying fact useful for molecular systematics is that different genes accumulate mutations at different rates. This difference depends on how much change a gene can tolerate without losing its function. For example, histone molecules may become non-functional if some of its amino acids are replaced with different ones. On the other hand, ITS of ribosomal RNA can still fold properly even if many of its nucleotides are changed. Thus, ITS can accumulate mutations more rapidly than histones, reflecting the different functional constraints on their gene product. Ribosomal RNA is considered as one of the best target for studying phylogenetic relationship because it is universal and is composed of highly conserved as well as variable domains. Marker-assisted gene pyramiding (MAGP) is one of the most important applications of DNA markers to plant breeding. Gene pyramiding has been proposed and applied to enhance resistance to disease and insects by selecting for two or more genes at a time. For example, such pyramids have been developed in rice against bacterial blight and blast (Huang *et al.*, 1997; Luo *et al.*, 2012)

The advantages of using molecular data is obvious - molecular data are more numerous than fossil records and easier to obtain. There is no sampling bias involved, which helps to correct the gaps in real fossil records. A clearer and robust phylogenetic tree can be constructed with the help of molecular data. When variation in morphological data become insufficient to distinguish two organisms at different levels *viz.*, phyla, class, order, family *etc.*, the analysis of the biomolecules are considered, which are large in number and occur in various forms in species. Therefore, biomolecular markers have become a favourite and sometimes the only reliable information available for researchers to reconstruct evolutionary history.

2. Commonly Utilized Molecular Markers in Spices

Molecular markers are features determined by allelic forms of genes or genetic loci and can be transmitted from one generation to another, thus are being used to tag target genes. Classical markers and DNA markers are used in breeding programmes, however classical markers (including morphological markers, cytological markers and biochemical markers) are not associated with important economic traits always, but DNA markers have developed into many systems based on different polymorphism-detecting techniques or methods (Southern blotting, nuclear acid hybridization, PCR and DNA sequencing), such as RFLP, AFLP, RAPD, SSR, SNP, *etc.*

2.1. Molecular Characterization of Germplasm

2.1.1. Genus Piper

Black pepper, derived from *Piper nigrum* L., is the most important spice of the world. India is the native home of black pepper and is its major producer and exporter. Conserving and fingerprinting the genetic diversity in pepper and development of resistant varieties will increase the production and productivity of black pepper. Use of molecular markers to augment breeding through marker assisted selection is a recent development in black pepper. Most of the studies are confined to molecular profiling of germplasm and indexing genetic fidelity. For the molecular characterization of black pepper germplasm, RAPD, ISSR and SSR markers were used. Pradeep Kumar *et al.* (2001, 2003) characterized 24 black pepper (*Piper nigrum* L.) accessions using RAPD markers and cultivar specific bands were developed except for Panniyur 3. Nirmal Babu *et al.* (2003b). used RAPD markers for studying genetic inter-relationships among fourteen major cultivars and ten released varieties of black pepper and depicted distinct differences between most of the cultivars and varieties. Sreedevi *et al.* (2005) characterized seven high yielding black pepper cultivars using RAPD markers. Nazeem *et al.* (2005) analyzed the variability and relatedness among 49 cultivars/accessions of black pepper using RAPD and AFLP markers. Joy *et al.* (2007) studied the genetic relationships among popular and agronomically important cultivars of black pepper using AFLP analysis. The dendrogram grouped the 49 accessions into three major clusters and four diverse cultivars with only 30 per cent similarity. Menezes *et al.* (2009) developed nine SSR markers from microsatellite enriched library of black pepper. These SSR markers were used to study the genetic diversity of 20 black pepper varieties of germplasm collection in Brazil. Joy *et al.* (2011) developed seven microsatellite markers for black pepper of which four polymorphic markers generated 62 alleles with an average of 15.5 alleles over 4 loci.

The genus *Piper* shows extreme reduction of floral characters which are key factors for phylogenetic analysis. Hence molecular approaches have been used to gain better insights. Sebastian *et al.* (1996) used isoenzyme variation in 11 *Piper* species to study the inter relationships. Gaia *et al.* (2003) studied the genetic diversity in 78 clones of *Piper nigrum* from South America using eight isoenzymes and concluded that most of the clones of *P. nigrum* L. were quite similar and homogeneous. Lebot *et al.* (1991) studied 300 accessions of *P. methysticum* and *P. wichmannii* from Polynesia, Micronesia and Melanesia for isoenzyme variation and concluded that the genetic base of *P. methysticum* was narrow and most of the morphotypes and chemotypes apparently originated through human selection and preservation of somatic mutations in a small number of original clones. He suggested that *P. wichmannii* is the wild progenitor of *P. methysticum*. Molecular data was used to further corroborate taxonomic and morphometric information for better understanding of their origins and species inter-relationships in the genus *Piper*. Jaramillo and Manos (2001) used phylogenetic analysis of sequences of the Internal Transcribed Spacers (ITS) of nuclear ribosomal DNA based on a worldwide sample of the genus *Piper*. Sequences from a 51 species of *Piper* were aligned to yield 257 phylogenetically informative sites. A single un-rooted parsimony network suggested that taxa representing major geographic areas could potentially form three monophyletic groups: Asia, the South Pacific, and the Neo tropics.

Inter- and intra-specific relationships among different species were studied by Chaveerach *et al.* (2002) who demonstrated a closer relation between *P. retrofractum* and *P. kudsura* than between *P. chaba* and *P. retrofractum* using RAPD profiles. Johnson *et al.* (2003) reported that ISSR-PCR along with RAPD was a valuable tool for genetic diversity analysis in *Piper* species. The ISSR markers were also used in identifying selected cultivars and hybrids of black pepper.

Nirmal Babu *et al.* (2003b). studied molecular inter-relationships between 24 Piper species using RAPD profiles. The phylogenetic trees grouped *P. longum, P. hapnium* and *P. mullesua* in one group and *P. attenuatum* and *P. argyrophyllum* in another group. *P. pseudonigrum, P. nigrum* and *P. galeatum* are clustered together. The study helped in identifying the core collections of holotypes of *Piper* species. Nirmal Babu (2003) studied RAPD and AFLP polymorphism among nine species of *Piper*. The exotic species *P. colubrinum* and *P. arboreum* were found to be highly distinct. *P. argyrophyllum*, *P. attenuatum*, *P. bababudani* and *P. nigrum* were found to be closely related. *P. betle*, *P. chaba*, and *P. longum* L. stood independently. Specific marker probes for each species were developed and were and successfully hybridized with corresponding genomic DNA. Wadt *et al.* (2004), who studied 49 genotypes belonging to three species of *Piper viz.*, *Piper hispidinervum*, *Piper aduncum*, and *Piper hispidum*, used RAPD markers to prove the demarcation of *P. hispidinervum* and *P. aduncum* as two separate species. Liao *et al.* (2009) reported the isolation and characterization of eleven polymorphic microsatellites loci from an endemic species, *Piper polysyphonum* from China. Sen *et al.* (2010) evaluated genetic diversity of eight *Piper* species *viz.*, *P. nigrum*, *P. longum*, *P. betle*, *P. chaba*, *P. argyrophyllum*, *P. trichostachyon*, *P. galeatum*, and *P. hymenophyllum* using RAPD markers. Jiang and Liu (2011) used RAPD and SRAP (Sequence Related amplified polymorphism) to study genetic diversity among 74 *Piper* spp. in Hainan Island and SRAP technique could efficiently distinguish all *Piper* spp from each other. Patra *et al.* (2011) used RAPD and ISSR markers to analyse the genetic diversity among 15 cultivars of betel vine. Sheeja *et al.* (2013) reported the genetic diversity analysis of 27 *Piper* species using ISSR markers and generated 35 species specific bands for 19 different *Piper* species. The genetic diversity analysis of six different species of *Piper* from North East region of India using RAPD marker was reported by Chowdhury *et al.* (2014). Yoshida *et al.* (2014) developed and characterized nine microsatellite loci for natural populations of *Piper solmsianum*, a potential source of bioactive secondary metabolites. Anupama *et al.* (2015) evaluated six microsatellite markers, developed from black pepper, for cross-species amplification and genetic diversity analysis in 23 *Piper* species from different location in India which included Western Ghats, North East and Exotic regions. The dendrogram generated could discriminate the 23 *Piper* species evaluated into eight clusters; three of the clusters could be further divided into sub clusters. The black pepper-specific microsatellite markers were found to clearly demarcate diversity among Indian and exotic species (Figure 15.1).

2.1.2. Zingiberales

A phylogenetic analysis of the tribe *Zingibereae* (Zingiberaceae) was performed by Ngamriabsakul *et al.* (2003) using nuclear ribosomal DNA (ITS1, 5.8S and ITS2) and chloroplast DNA [*trn*L (UAA) 5′exon to *trn*F (GAA)]; based on the results obtained, it was suggested that the tribe *Zingibereae*, as well as the genus *Curcuma*, are monophyletic. Chase (2004) attempted to have an overview on the phylogeny and relationships in monocots based on analysis of DNA sequence data of seven genes representing all three genomes and reported high bootstrap support to the clades which included Zingiberales. Monocots have been shown in molecular clock studies to be at least 140 million years old, and all major clades and most families date to well before the end of the Cretaceous. Kress *et al.* (2002) studied the phylogeny of the ginger (Zingiberaceae) based on DNA sequences of the nuclear internal transcribed spacer (ITS) and plastid *matK* regions and proposed a new classification of the

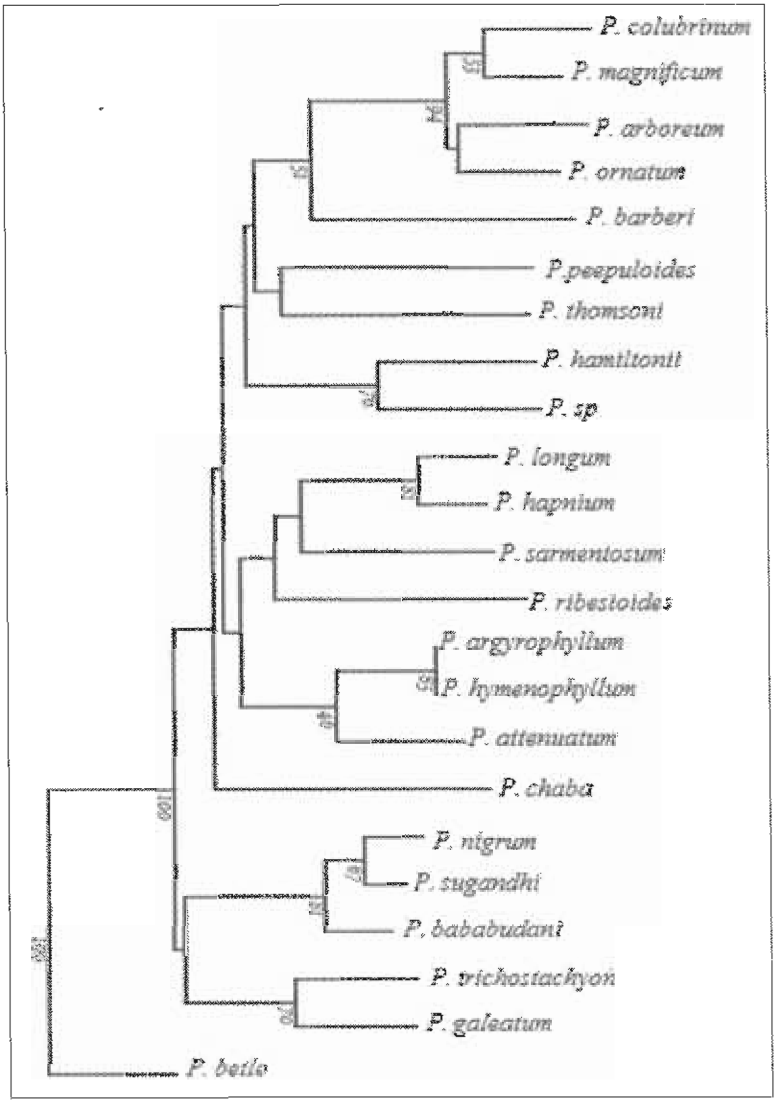

Figure 15.1: Dendogram Based on Six Polymorphic Microsatellite Markers to Study Genetic Relationship among 23 *Piper* Species Constructed by Neighbor Joining Method. Bootstrap values (based on 1000 permutations) are indicated in each node of the phylogram. (Source: Anupama *et al.*, 2015).

Zingiberaceae that recognizes four subfamilies and four tribes: Siphonochiloideae, Tamijioideae, Alpinioideae and Zingiberoideae (Zingibereae, Globbeae).

Jayakumar *et al.* (2005) studied about 11 species representing five major tribes *viz., Amomum, Aframomum, Alpinia, Hedychium* and *Elettaria* and collections of 96 cardamom genotypes of wide genetic variability using for RAPD, PCR-

RFLP and ISSR polymorphism. The collections of cardamom germplasm, which include released varieties, promising lines and local cultivars of cardamom, were characterized using 50 RAPD primers, six ISSR primers and two PCR-RFLP primers to study their relationships and diversity. The study indicated a clear divergence in Kerala and Karnataka collections, the two main regions of cardamom diversity and comparatively less divergence within the population is due to open pollinated seed origin (siblings). The collections and hybrids from Indian Cardamom Research Institute (ICRI), Myladumpara, Kerala, India formed one cluster and they are well separated from rest of the genotypes. The results indicated that controlled breeding, rather than selection from open pollinated progeny, is a preferred approach in cardamom to genrate more genetic variability in germplasm (Nirmal Babu *et al.*, 2005). *De novo* isolation, characterisation and utilization of microsatellite markers for the genetic diversity analysis of small cardamom has been recently reproted by Anu *et al.* (2016).

2.1.2.1 Curcuma Species

Genetic diversity of *Curcuma alismatifolia* Gagnep. Populations, from both cultivated and wild habitats, were studied by Paisooksantivatana *et al.* (2001). Cao *et al.* (2003) used trn K nucleotide sequencing, for identification of six medicinal *Curcuma viz., C. longa, C. phaeocaulis, C. sichuanensis, C. chuanyujin, C. chuanhuangjiang* and *C. chuanezhu* found in Sichuan, China. Hybrid detection and characterization of *Curcuma* spp. from Thailand using sequence characterized amplified (SCAR) DNA markers was done by Anuntalabochai *et al.* (2007). In the study, the tropical cut flower, *C. alismatifolia* variety 'Patumma', was analysed was using 11 RAPD primers. A robust sequence characterized amplified region with 600 bp in length was present in all 'Patumma' varieties and hybrids, which did not amplify in an additional series of 24 distinct *Curcuma* species used as an independent test. The molecular marker developed was useful for cost effective, morphologically independent characterization of *Curcuma* hybrids. Five species of *Curcuma* namely *C. longa, C. zedoaria, C. aromatica, C. amada and C. caesia* were characterized based on the RAPD profiles of DNA isolated from fresh rhizomes by Sreeja (2002). Eleven polymorphic bands were produced in the five species studied using three RAPD markers.

Kress *et al.* (2002) suggested that *Curcuma* is paraphyletic with *Hitchenia, Stahlianthus* and *Smithatris*. Molecular genetic fingerprints of 15 *Curcuma* species were developed using Inter Sinple Sequence Repeats (ISSR) and Random Amplified Polymorphic DNA (RAPD) markers to elucidate the genetic diversity/relatedness among the species (Syamkumar, 2008). Siju *et al.* (2010) developed 18 genomic microsatellite markers for turmeric (*Curcuma longa* L.). These markers when used to evaluate 20 turmeric accessions generated 103 alleles with an average of 5.7 alleles per locus. The 18 EST SSR markers developed by Siju *et al.* (2010) in turmeric (*Curcuma longa* L.) revealed 100 per cent cross species transferability among the 13 related species.

Nayak *et al.* (2006) carried out 4C nuclear DNA content and RAPD analysis of seventeen promising cultivars of turmeric (*C. longa*) from India. RAPD analysis clearly showed the genetic variation among the seventeen cultivars using twenty random decamer primers. The inter-cultivar polymorphism ranged from 35.6 per cent to 98.6 per cent among the seventeen cultivars studied and the amplification fragments per primer ranged from four to seventeen with fragment size ranging from 0.4 kb to 3 kb. Genetic diversity analysis of twenty accessions of C. *longa* from different parts of Brazil using RAPD markers produced forty-five polymorphic loci and the dendrogram produced by UPGMA grouping using Jaccard's Index of similarity formed two groups. Among the groups, 44. 4 per cent genetic variability was observed and most part of the variation was found within the groups (Pinheiro *et al.*, 2003). ISSR profiles were generated for seven released varieties of turmeric (Figure 15.2) by Nirmal Babu *et al.* (2016a).

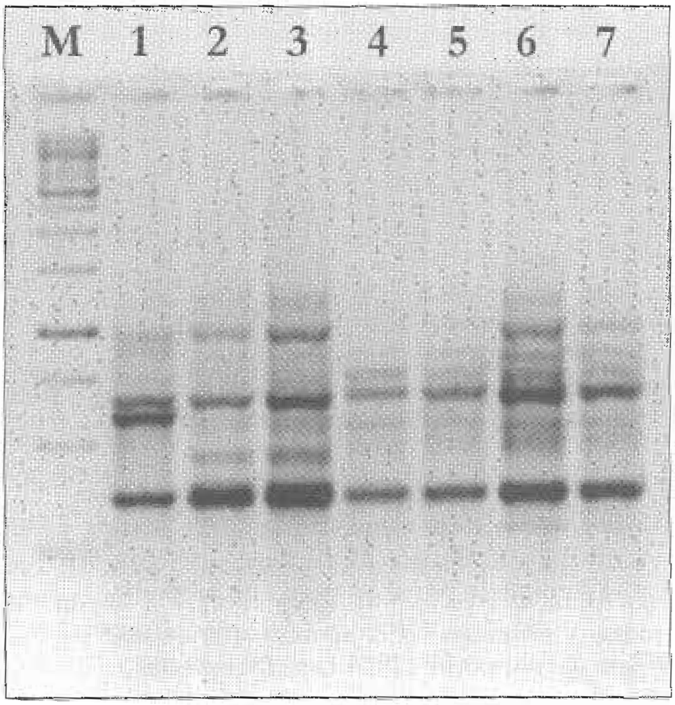

Figure 15.2: ISSR Profiling of Released Varieties of Turmeric using the Primer UBC 834a. Lanes 1–7: Suvarna, Suguna, Sudharsana, Prabha, Prathiba, Alleppey Supreme and Kedaram. M: Molecualr weight ladder.

2.1.2.2. Ginger

Wahyuni *et al.* (2003) studied genetic relationships among ginger accessions based on AFLP markers. Kavitha and Thomas (2008) reported *Zingiber zerumbet* (L) Smith, a wild species related to the cultivated ginger is a potential resistance donor for soft rot disease in ginger caused by *Pythium aphanidermatum* (Edson) Fitzp. They studied the genetic diversity and *P. aphanidermatum* resistance of 74 *Z. zerumbet* accessions belonging to 15 populations. Lee *et al.* (2007) reported isolation and characterization of eight polymorphic microsatellite markers for *Zingiber officinale*

Rosc. (Ginger) from a microsatellite enriched library. These were used to detect a total of 34 alleles across the 20 accessions with an average of 4.3 alleles per locus. The data generated indicated moderate level of genetic diversity among the ginger accessions genotyped with eight markers. ISSR profiles were generated for eight high yielding varieties of ginger (Figure 15.3) by Nirmal Babu *et al.* (2016b).

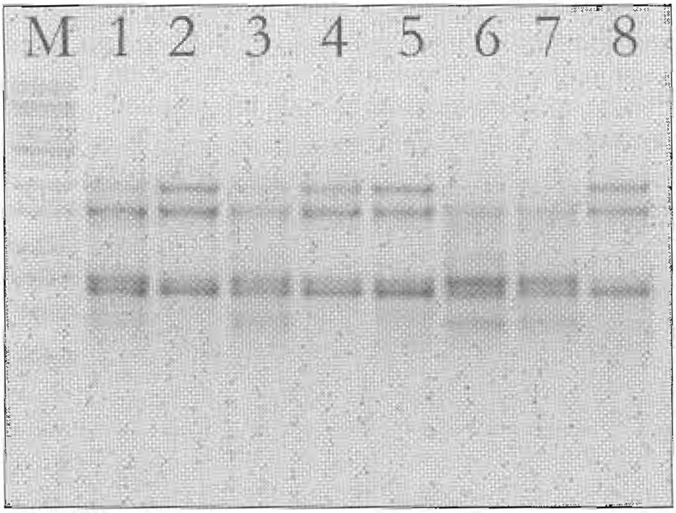

Figure 15.3: ISSR Profiling of High-yielding Varieties of Ginger using Primer UBC 810. Lanes 1-8: Varada, Rejatha, Mahima, Suprabha, Suruchi, Athira, Karthika and OCP 1222. M: Molecualr weight ladder (Source: Nirmal Babu *et al.*, 2016b)

2.1.2.3. Tree Spices

Perennial tree crops face problems like long pre-bearing period, dioecy in nutmeg and narrow genetic base in clove; hence, priority need to be given to characterization of germplasm based on molecular markers.

Genetic identification among cinnamon species was studied analyzing nucleotide sequences of chloroplast DNA from four species (*Cinnamomum cassia, C. zeylanicum, C. burmannii* and *C. sieboldii*). The two regions studied were the intergenic spacer region between the *trnL* 3′exon and *trnF* exon (*trnL -trnF* IGS) and the *trnL* intron region. We found nucleotide variation at one site in the *trnL-trnF* IGS, and at three sites in the *trnL* intron. With the sequence data from analysis of these regions, the four *Cinnamomum* species used in this study were correctly identified. Furthermore, single-strand conformation polymorphism (SSCP) analysis of PCR products from the *trnL-trnF* IGS and the *trnL* intron resulted in different SSCP band patterns among *C. cassia, C. zeylanicum* and *C. burmannii*. Judy (IISR, 2005) conducted a preliminary RAPD study on eleven species of cinnamon *viz. C. verum, C. cassia, C. malabatrum, C. riparium, C. macrocarpum, C. perottitii, C. weightii, C. citronella, C. tamala, C. sulphuratum, C. glaucens and C. camphora* using three primers (OPA 1, OPB 5 and OPC 15). The dendrogram indicated that *C. malabatrum* is closely related to *C. verum*. The species like *C. cassia, C. weightii* and *C. citronella, C. camphora* are grouped together. *Exotic* species like *C. sulphuratum, C. glaucens and C. camphora* were also placed in single group indicating similarities between them.

Molecular fingerprinting of selected elite accessions of *Myristica fragrans* by RAPD, ISSR and rDNA-RFLP markers was done by Sheeja *et al.* (2006). High yielding elite accessions, with high sabinene and low myristicin contents revealed good deal of polymorphism in RAPD but no polymorphism was detected in ISSR. An average genetic distance of 25.5 per cent was observed among the accessions due to the high degree of relatedness among them. Unique bands were identified in some of the rare accessions like A9/4, a very high yielding epicotyl graft with plagiotropic shoots, A9/150, possessing very thick mace and apple shaped bold fruits and A4/22 with unique character of high number of erect shoots for their unequivocal identification. All the accessions showed polymorphism and could be distinguished from each other through RAPD markers and qualified to be included in a core collection.

Garcinia species are distributed widely throughout the old world especially Asia and Africa. *Garcinia* belongs to the family Guttiferae. RAPD polymorphism was used to study species inter relationships between six *Garcinia* species namely *G. indica, G. gummi-gutta, G. cowa, G. mangostana, G.* tinctoria and *G. hombroniana* (Pulla Rao (2003).

In tamarind (*Tamarindus indica* L), it was observed that all the characters varied significantly, except thickness of pod, seed to pod ratio, number of seeds per pod, seed weight per pod, stamen length, filament length, and style length. The variability observed was narrow at molecular level compared to the variability at phenotypic level. Four elite tamarind genotypes were identified which are productive and regular bearing.

2.1.2.4. Vanilla

Continuous clonal propagation has resulted in very little variability for crop improvement programmes in vanilla. An attempt was made to increase the spectrum of variation by interspecific hybridization with *Vanilla aphylla*, an Indian species which is tolerant to *Fusarium*. Interspecific hybrids were successfully produced and morphological characters and molecular profiles revealed the true hybridity of the progenies. Seedling progenies of *V. planifolia*, and interspecific hybrids were evaluated and random amplified polymorphic DNAs (RAPDs) loci were marked (Figure 15.4). The profiles indicate similarity between the parents, selfed progenies and interspecific hybrids and that all the progenies tested were variable when compared to each other, which can be exploited for crop improvement in vanilla (Minoo *et al.*, 2006, 2016) (Figure 15.5).

Use of microsatellites allowed the first molecular based estimation of heterozygosity levels in vanilla, which was not possible with dominant markers like RAPD and AFLP. Bory *et al.* (2008) developed 14 microsatellite primers form di-nucleotide CT/GT enriched genomic library of *Vanilla planifolia*. These markers were monomorphic within cultivated accessions. The 14 SSR markers developed in cultivated Vanilla (*Vanilla planifolia* L.) were transferable to *Vanilla tahitensis* however, 11 loci were found to be polymorphic between these two species.

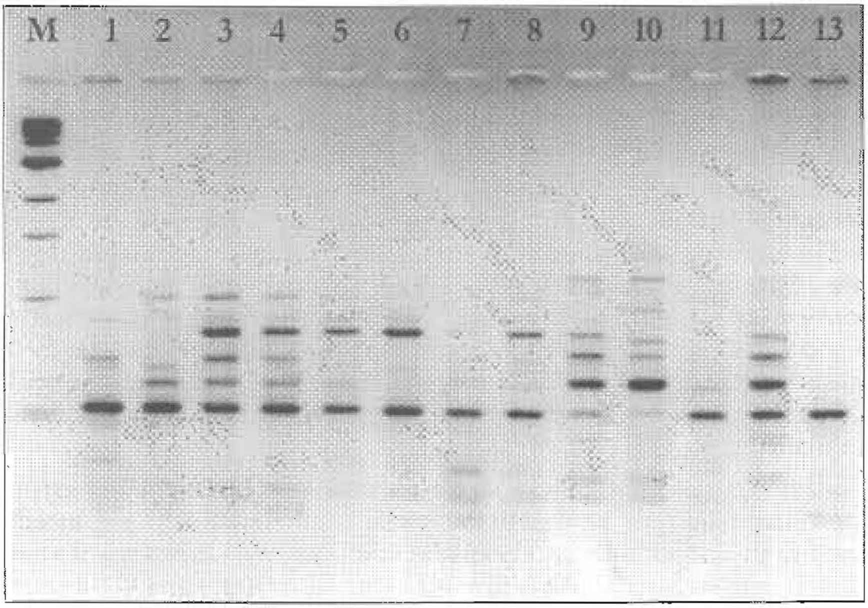

Figure 15.4: RAPD Profiles of Inter Specific Hybrids of Vanilla using OPERON primer OPB20 (Source: Minoo *et al.*, 2016).

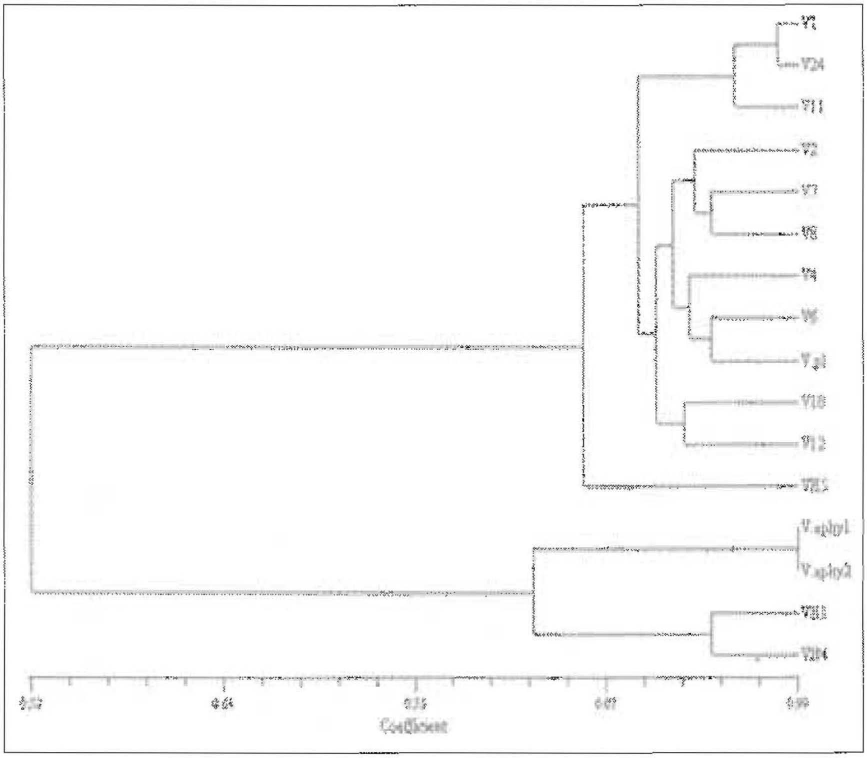

Figure 15.5: Dendrogram Showing Linkage Groups between Selfed Progenies and Interspecific Hybrids based on by RAPD Markers (Source: Minoo *et al.*, 2016).

Sequencing of nuclear genes was used for reconstructing the evolutionary history of Vanilloid orchids (Cameron, 2004). Nuclear (ITS) and plastid (*rbcL* gene) DNA sequences were also used for unraveling the origin of Tahitian Vanilla (Lubinsky *et al.*, 2008). The length polymorphism of neutral caffeic acid O-methyl transferase gene was used to analyse 20 vanilla species and confirmed the strong differentiation of Old World *vs* New World species in the genus (Besse *et al.*, 2009). Cameron (2004) has utilized plastid *psaB* gene sequences for investigating intrafamilial relationships within Orchidaceae.

Minoo *et al.* (2008) have utilized RAPD polymorphism to assess the levels of genetic diversity and interrelationships among different collections of *Vanilla planifolia* Andr. and some related species, which included leafless and leafy types such as *V. tahitensis* J.W.Moore, *V. andamanica*, Rolfe, *V. pilifera* Holtt. and *V. aphylla* Blume (Figure 15.6). Studies revealed the existence of limited variations within collections of *V. planifolia*, indicating its narrow genetic base. Furthermore, *V. tahitensis* was found to be the closest relative of nearest to *V. planifolia*.

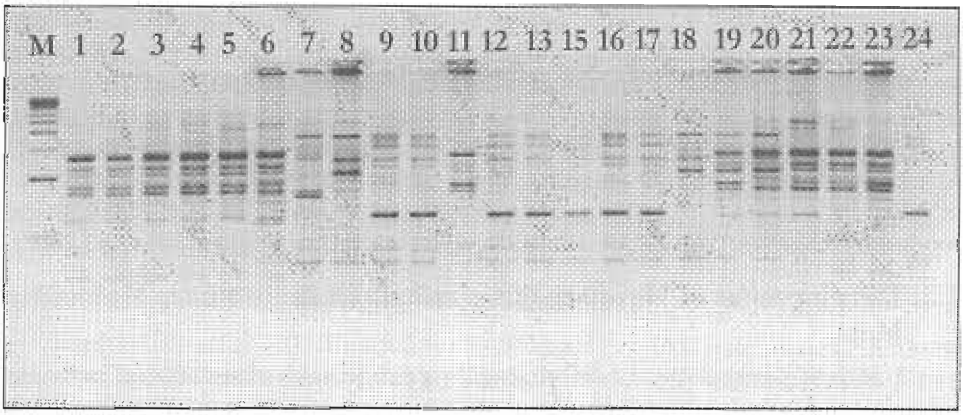

Figure 15.6: RAPD Polymorphism Observed in *Vanilla planifolia, V. tahitensis, V. andamanica*, *V. pilifera* and *V. aphylla* using OPERON Primer OPB14 (Source: Minoo *et al.*, 2008).

Scientists at the Fujian Agriculture and Forestry University and National Orchid Conservation Centre of China have colloborated to produce the world's first orchid genetic map. The collaboration have yielded and have complete genome sequence of *Vanilla shenzhenica*, in 2015, giving way to the possibility of altering vanilla properties and pathways through transgenic technology in the future.

2.2. Estimation of Genetic Fidelity of Micropropagated Plants using RAPDs

Morphological characters coupled with RAPD profiles have been used by Nirmal Babu *et al.* (2003a). to estimate the genetic fidelity of micropropagated plants pepper where the profiles indicated that the clones were genetically stable and that the micropropagation technology could be used for commercial cloning of black pepper. Shahanas *et al*, (2003) reported intra clonal fidelity of rooted cuttings of cv. Sreekara, derived from bamboo nursery using RAPD. Genetic fidelity of micropropagated *Piper longum* using RAPD profiling was reported by Ajith *et al.* (1997) and occurrence of somaclonal variation was observed.

In turmeric, Nirmal Babu *et al.* (2003a). studied morphological and molecular variations among micropropagated and callus regenerated plants and found variations in both but with higher percentage of variation in callus regenerated somaclones. *In vitro* plants developed through microrhizome exhibited least amount of variations. They inferred that this is due to the accumulated vegetative mutations (mosaic) in turmeric. The genetic fidelity studies of turmeric germplasm conserved in *in vitro* genebank using RAPD profiling showed their genetic integrity (Geetha, 2002; Ravindran *et al.*, 2004).

RAPD profiling, within the replicates of *in vitro* conserved and cryopreserved lines of ginger using operon random (RAPD) primers was studied, but it did not detect any polymorphism between the conserved lines in any of the primers tested, indicating the genetic stability (Geetha, 2002; Peter *et al.*, 2002).

Micropropagated multiple shoots of *Vanilla planifolia* developed from axillary bud explants and established in culture for 10 years, have been used to determine somaclonal variation using random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats markers (ISSR). No difference was observed in banding patterns of any of the samples for a particular primer, indicating the absence of variation among the micropropagated plants, concluding that the micropropagation protocol used for *in vitro* proliferation of vanilla plantlets could be applied for the production of clonal plants over a considerable period of time (Sreedhar *et al.*, 2007).

2.3. Development of Mapping Population and Preliminary Molecular Map

A mapping population of 200 segregating progenies was developed between Subhakara X Panniyur 1 for preparation of preliminary genetic map of black pepper (Nirmal Babu *et al.*, 2003b). RAPD and ISSR profiling of 96 progenies of first mapping population Subhakara X Panniyur 1 was done along with their parents and over 200 polymorphic markers segregating in the population were scored. Preparation of frame work molecular map is in progress using pseudo test cross approach.

2.4. Identification of Markers Linked to Important Characters

2.4.1. Sex Specific Markers

Banerjee *et al.* (1999) studied molecular basis of genotypic differentiation between the male and female plants of dioecious *P. longum* L. and identified two RAPD markers specific to male plants. Philip *et al.* (2000) used RAPD profiles to differentiate three female varieties of *P. longum*. Manoj *et al.* (2004) studied the molecular basis of differentiation between male and female plants of *Piper longum* using RAPD.

Shibu *et al.* (2000) identified sex specific DNA markers for identifying the productive female trees in nutmeg. Ganeshaiah *et al.* (2000) attempted to identify sex specific DNA markers that could potentially be used to determine the sex of

Myristica fragrans seedlings. Out of 60 random primers screened using RAPD-PCR, one primerm OPE II, gave a female sex specific DNA amplification product. Sheeja *et al.* (2006), reported that male seedlings could be characterized by presence of specific bands of 1300 and 1000 bp, during their study

2.4.2. Identification of Hybrids

Isoenzymes were utilized to prove hybrid nature of plants derived from inter specific crosses. Sasikumar *et al.* (1999) used isozyme analysis to characterize inter specific hybrids between *Piper nigrum.* x *P. attenuatum* and *P. nigrum* x *P. barberi* to identify the true hybrids by the presence of hybrid specific as well as male parent specific bands. Johnson *et al.* (2005) used male parent-specific RAPD markers for identification of hybrids in black pepper (*Piper nigrum* L.).

In other spices like *Thymus caespititius*, a low correlation was found between essential oil composition and RAPD analysis; however, molecular data clustered plants according to their geographic origin (Trindade *et al.*, 2008), suggesting that molecular tools should be explored in order to fully understand the inuence of both environmental and genetic factors on volatiles composition. RAPDs have also been used in understanding the genetic relationships and as reliable tools for the discrimination of the two parental taxa and the putative hybrid in natural populations of *Origanum* × *intercedens*, a hybrid between *O. onites* and *O. vulgare*. The results were compared to those obtained with the essential oil composition and morphological characteristics. It was found that DNA fingerprinting and general morphology placed the hybrid closer to *O. onites*, while its essential oil composition showed the hybrid to be more similar to *O. vulgare* (Gounaris *et al.*, 2002).

2.4.3. Markers Linked to Phytophthora Resistance

Nirmal Babu *et al.* (2003b). used for RAPD profiling to identify RAPD markers linked to *Phytophthora* resistance, among eleven lines each of *Phytophthora* susceptible and tolerant cultivars. The study indicated that in general the tolerant lines formed a cluster of their own and a few suscepts were grouped with tolerant lines. A marker at 700 bp region was found to be present in tolerant lines and absent in suscepts. Sheji *et al.* (2006) identified a RAPD marker associated with *Phytophthora* resistance and converted it in to SCAR marker (Figure 15.7). RAPD profiling of disease tolerant and susceptible lines of black pepper was done. A DNA fragment representing a RAPD marker linked to resistance was cloned, sequenced and converted into a SCAR marker. SCAR primers to the resistant allele were developed based upon a deletion region between susceptible and resistant plants. A unique band 360 base pair appeared in all the *Phytophthora* resistant lines but was absent in the susceptible lines, and this marker can be used for screening black pepper germplasm.

2.4.4. Identification of Adulterants

Dhanya *et al.* (2007) reported simple protocol for characterizing and checking adulteration in traded black pepper. Syamkumar *et al.* (2005) reported standardization of protocol for the isolation of amplifiable genomic DNA from dried capsules of traded cardamom. This protocol will help in the PCR-based characterization of different grades of commercially traded cardamom and to identify adulterants if any.

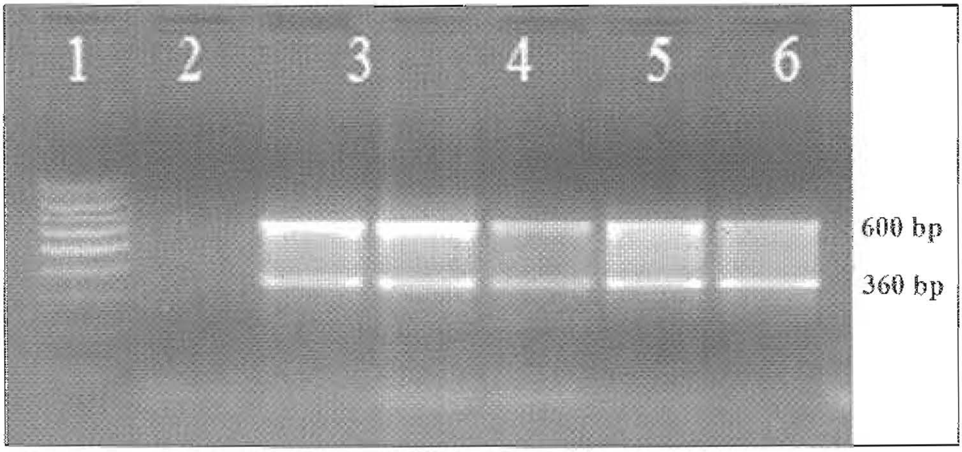

Figure 15.7: Differential Amplification of SCAR Markers among Phytophthora Susceptible and Resistant Lines of Black Pepper (Source: Sheji Chandran *et al.*, 2006).

Detection of extraneous *Curcuma* sp. contamination of powdered samples of turmeric using random amplified polymorphic DNA was reported by Sasikumar *et al.* (2004). The analysis of three market samples of turmeric powder from Kerala, and India, revealed the prevalence of *C. zedoaria* (wild species) powder over the *C. longa* (the common culinary turmeric) powder, although the curcumin levels of the samples were in accordance with the quality standards prescribed for the commodity. The other studies on studies on genetic purity and checking adulteration are those of Cao *et al.* (2001) and Sasaki *et al.* (2002, 2004) who used sequence analysis of Chinese and Japanese *Curcuma* drugs on the 18S rRNA gene and *trnK* gene and the application of amplification-refractory mutation system analysis for their authentication. Application of single nucleotide polymorphism analysis based on species specific nucleotide sequence was developed by Sasaki *et al.* (2004) to identify the plants and drugs derived from *C. longa, C. phaeocaulis, C. zedoaria* and *C. aromatica*. Based on the difference in the nucleotide positions at 177, 645, 724 and a 4 base indel on the *trn*K gene obtained using three different lengths of (26 mer, 30 mer and 34 mer) reverse primers helped to identify the four *Curcuma* species studied. The SNP analysis method developed become a useful method for the identification of botanical origins of *Curcuma* drugs such as 'Ezhu' used in Chinese medicine, which was difficult to identify morphologically and phytochemically.

Xia *et al.* (2005) used molecular (5S-rRNA spacer domains) and chemical fingerprints for quality control and authentication of *Rhizoma Curcumae*, a traditional Chinese medicine used in removing blood stasis and alleviating pain.

Genetic profiling of traded ginger from India and China using 20 RAPD primers and 15 ISSR primers gave consistent amplification pattern. Significant variation was observed between the produces from the two countries (un published, IISR 2008). Jiang *et al.* (2006) used metabolic profiling and phylogenetic analysis for authentication of ginger. They used these tools to investigate the diversity within the ginger species and between ginger and closely related species in the genus

Zingiber. Phylogenetic analysis demonstrated that all *Zingiber officinale* samples from different geographical origins were genetically indistinguishable. In contrast, other *Zingiber* species were significantly divergent, allowing all species to be clearly distinguished using this analysis. In the metabolic profiling analysis, the *Z. officinale* samples derived from different origins showed no qualitative differences in major volatile compounds, although they did show some significant quantitative differences in non-volatile composition, particularly regarding the content of [6]-, [8]-, and [10]-gingerols, the most active anti-inflammatory components in this species. The metabolic profiles of other *Zingiber* species were very different, both qualitatively and quantitatively, when compared to *Z. officinale* and to each other. Comparative DNA sequence/chemotaxonomic phylogenetic trees showed that the chemical characters of the investigated species were able to generate essentially the same phylogenetic relationships as the DNA sequences. This supports the contention that chemical characters can be used effectively to identify relationships between plant species.

2.4.5 Tagging and Isolation of Candidate Genes

2.4.5.1. Black Pepper

Isolation of genes responsible for agronomically important characters, especially for biotic and abiotic stresses is an area of high importance. Johnson *et al.* (2005) reported a method for isolation and reverse transcription of high quality RNA from *Piper* species. They reported amplification, isolation and sequencing of putative β-1,3-glucanase gene from *Piper colubrinum* was achieved using glucanase specific primer. Jebakumar *et al,* (2001) has reported differential induction of various PR proteins like Phenly alanine ammonia lyase (PAL), β-1,3- glucanase in *Phytophthora* tolerant black pepper upon inoculation with *P. capsici.*

In an attempt to isolate and cloning of a cDNA fragments encoding the defense related protein β-1,3-glucanase in black pepper (*P. nigrum* L.) and methyl glutaryl CoA reductase in *Piper colubrinum* were also reported (Girija *et al.*, 2005a, b). A PCR-based suppression subtractive hybridization (SSH) was used by Dicto and Manjusha (2005) to identify *P. colubrinum* resistance genes that are differentially expressed in response to the signaling molecule, salicylic acid (SA). A subtracted library of SA-Induced genes was synthesized and one of the clones showed sequence homology to osmotin, a member of Class-V group of pathogenesis-related (PR) gene family. Northern blot analysis revealed that osmotin is dominantly expressed in SA/ethylene-treated tissue. This indicates that SSH can be used to identify and clone PR genes in *P. colubrinum*. Bioprospecting of novel genes form black pepper was attempted by Sujatha *et al.* (2005). They used heterologus probes to identify the presence of pea lectin genes and tomato protease inhibitor genes in black pepper. Nazeem *et al.* (2008) also reported the role of ß-1, 3 glucanase and related enzymes in the defense mechanism in foot rot tolerant black pepper variety and in resistant genotype *P. colubrinum*. Varma *et al.* (2009) has explained differential induction of chitinase in *Piper colubrinum* in response to inoculation with *P. capsici*. Cloning and sequence characterization of two isoforms of *osmotin*, an antifungal *PR-5* gene homologue, from a salicylic acid-induced subtracted cDNA library was generated in *Piper colubrinum* (Mani and Manjula, 2010).

Piperine is the main bioactive compound among black pepper alkaloids, which performs unique physiological functions. However, the mechanisms of piperine synthesis were poorly understood and studies by Hu *et al.* (2015) were the first to describe the fruit transcriptome of black pepper by sequencing on Illumina HiSeq 2000 platform. A total of 56,281,710 raw reads were obtained and assembled, from which 44,061 unigenes with an average length of 1,345 nt were generated. During functional annotation, 40,537 unigenes were annotated in Gene Ontology categories, Kyoto Encyclopedia of Genes and Genomes pathways, Swiss-Prot database, and Nucleotide Collection (NR/NT) database. In addition, 8,196 simple sequence repeats (SSRs) were detected. In a detailed analysis of the transcriptome, housekeeping genes for quantitative polymerase chain reaction internal control, polymorphic SSRs, and lysine/ornithine metabolism-related genes were identified which could provide useful data for further research on piperine synthesis.

2.4.5.2. Cardamom

Development of structured populations for tagging of Katte and Rhizome rot resistance are in the process of field planting (IISR unpublished 2008). One putative RAPD marker was also identified associated with Katte Resistance (IISR 2003).

2.4.5.3. Ginger

Swetha and Subramanian (2008) reported isolation and molecular analysis of R-gene in resistant *Zingiber officinale* (ginger) varieties against *Fusarium oxysporum.* They observed that the R-gene is present only resistant varieties. These cloned R-genes provide a new resource of molecular markers for marker assisted selection (MAS) and rapid identification of *Fusarium* yellows resistant ginger varieties. Kavitha and Thomas (2006) reported *Zingiber zerumbet,* a close relative of ginger, as a potential donor for soft-rot resistance in Ginger. They employed AFLP markers and mRNA differential display to identify genes whose expression was altered in a soft rot-resistant accession of *Zingiber zerumbet* before and after inoculating it with. *Pythium aphanidermatum,* which is the principal causative agent of soft-rot disease in ginger A few differentially expressed transcript-derived fragments (TDFs) were isolated, cloned and sequenced. Homology searches and functional categorization of some of these clones revealed the presence of defence/stress/signalling group which are homologous to genes known to be actively involved in various pathogenesis-related functions in other plant species. They found *Z. zerumbet* shows adequate variability both at DNA level and in response to *Pythium.* (Kavitha and Thomas, 2006; 2008). Nair *et al.* (2010) identified a member of the pathogenesis-related protein group 5 (*PR5*) gene family in *Z. zerumbet* that is expressed constitutively but upregulated in response to infection by *P. aphanidermatum.* Nair and Thomas (2013) isolated the full length sequence of *ZzR1* resistance gene from *Zingiber zerumbet* with potential for imparting resistance to soft rot in ginger. Isolation of resistance genes from such related genera will help in ginger improvement via transgenic approaches.

2.4.5.4. Turmeric

Joshi *et al.* (2010) have reported isolation and characterization of resistance gene candidates in *Curcuma longa* cv.surama. R gene conferring resistance to

Pythium aphanidermatum was characterized in *Curcuma zedoaria* (Basudeba *et al.*, 2013). Annadurai *et al.* (2013) reported the presence of novel transcripts related to anti-cancer and anti-malarial terpenoides in the transcriptome of *Curcuma longa.*

2.4.5.5. Vanilla

Large gene sequence datasets from vanilla pods at different times of development, and representing different tissue types, including the seeds, hairs, placental and mesocarp tissues, were generated using next-generation sequencing technologies, for interrogation of pathways of vanillin and C-lignin biosynthesis in the pod and seed, respectively. The combined 454/Illumina RNA-seq platforms provide both deep sequence coverage and high quality *de novo* transcriptome assembly for this non-model crop species. The annotated sequence data provided a foundation for understanding multiple aspects of the biochemistry and development of the vanilla bean, as exemplified by the identification of candidate genes involved in lignin biosynthesis. Transcriptome data indicated that C-lignin formation in the seed coat involves coordinate expression of monolignol biosynthetic genes with the exception of those encoding the caffeoyl coenzyme A 3-*O*-methyltransferase for conversion of caffeoyl to feruloyl moieties. This database provides a general resource for further studies on this important flavor species (Xiaolan Rao *et al.*, 2014).

Gallage *et al.* (2014) reported that a single hydratase/lyase type enzyme designated vanillin synthase (*Vp*VAN) catalyses direct conversion of ferulic acid and its glucoside into vanillin and its glucoside, respectively. The enzyme shows high sequence similarity to cysteine proteinases and is specific to the substitution pattern at the aromatic ring and does not metabolize caffeic acid and *p*-coumaric acid as demonstrated by coupled transcription/translation assays. *Vp*VAN localizes to the inner part of the vanilla pod and high transcript levels are found in single cells located a few cell layers from the inner epidermis. Transient expression of *VpVAN* in tobacco and stable expression in barley in combination with the action of endogenous alcohol dehydrogenases and UDP-glucosyltransferases result in vanillyl alcohol glucoside formation from endogenous ferulic acid. A gene encoding an enzyme showing 71 per cent sequence identity to *Vp*VAN was identified in another vanillin-producing plant species *Glechoma hederacea* and was also shown to be a vanillin synthase as demonstrated by transient expression in tobacco.

Recently *de novo* biosynthesis of vanillin in *V. planifolia* was found to be catalysed by a single enzyme, vanillin synthase that catalyses ferulic acid and its glucoside to produce vanillin and vanillin glucoside. This promises transgenic plants with high vanillin synthase activity for use as vanillin production sources.

2.4.6 Comparative Genomics for Gene Tagging

2.4.6.1. Black Pepper

In perennial polyploids like black pepper, conventional mapping strategies are laborious. Hence genomics approach and using information from other sources, like comparisons with heterologus genomes or genes, could provide the necessary leads for tagging. Candidate genes responsible for pathogenesis can also be identified from sequence information available on *R* genes and information available from

Arabidopsis genome (Aarts *et al.*, 1998). With the advent of next generation sequencing techniques, genome wide analysis of NBS-LRR-encoding genes in *Arabidopsis* (Meyers *et al.*, 1999; Shen *et al.*, 1998) reported that the resistance gene candidates identified by PCR with degenerate oligonucleotide primers map to clusters of resistance genes in lettuce. These form an important approach for isolating R genes using candidate gene approach.

A PCR-based approach for isolating pathogen resistance genes from potato with potential for wide application in plants was reported by Leister *et al.* (1996). The genomic approach was used to isolate R genes in Black pepper. Degenerate primers were designed from the conserved motifs (Ploop and GLPL) of NBS region of known R genes and these were used to amplify similar sequences from genomic DNA of black pepper. A 500 bp fragment was amplified consistently from black pepper variety P 24 using degenerate primers from P LOOP GG – GLPLA3. This fragment was cloned in PCR XL TOPO vector and a library of 48 clones was developed by Nirmal Babu *et al.* (2003b).

Similarly information available on mapping of heterologous loci for example, *Ph-2* locus controlling partial resistance to *Phytophthora infestans* in tomato (Philippe *et al.*, 1998), genetic and physical mapping of molecular markers linked to the *Phytophthora* resistants gene *Rps 1*-k in soybean (Takao *et al.*,1997) can also be used to tag *Phytophthora* resistance in black pepper. Intraspecific comparative genomics to identity avirulence genes from *Phytophthora* was reported by Bos *et al.* (2003). Comparative genomics has already made much headway for solanaceous crops to which *Capsicum* belongs (Tanksley *et al.*, 1988; Livingstone *et al.*, 1999) and where much information on *Phytophthora* resistance is available.

RNA-Seq technology was employed, for the first time (Gordo *et al.*, 2012), to describe the root transcriptome of black pepper. The root transcriptome was sequenced by the NGS SOLiD platform and assembled using the multiple-k method. Blast-2-GO and orthoMCL methods were used to annotate 10338 unigenes. The 4472 predicted proteins showed about 52 per cent homology with the *Arabidopsis* proteome. Two root proteomes identified 615 proteins, which seem to define the plant's root pattern. This dataset of 10,338 unigenes will be important for the biotechnological breeding of black pepper.

2.4.6.2. Ginger

Aswati and Thomas (2006) reported isolation, characterization and expression of resistance gene candidates (RGCs) using degenerate primers based on conserved motifs from the NBS domains of plant resistance (R) genes were used to isolate analogous sequences or resistance gene candidates (RGCs) from cultivated and wild *Zingiber* species. Kavitha and Thomas (2006, 2008) employed mRNA differential display was employed to identify genes whose expression was altered in a soft rot-resistant accession of *Zingiber zerumbet*. A few differentially expressed transcript-derived fragments (TDFs) were isolated, cloned from *Pythium aphanidermatum*, which is the principal causative agent of soft-rot disease in ginger. Sequence and functional categorization of these clones revealed the presence of defence/stress/signalling group which are homologous to genes known to be actively involved in various pathogenesis-related functions in other plant species. Swetha and Subramanian (2008) reported isolation and molecular analysis of R-gene in resistant *Zingiber officinale* (ginger) varieties against *Fusarium oxysporum*. They observed that the R-gene is present only resistant varieties. Thus the cloned R-genes provide a new resource of molecular markers for marker assisted selection (MAS) and rapid identification of fusarium yellows resistant ginger varieties.

Violaxanthin de-epoxidase (VDE) as the key enzyme of xanthophyll cycle plays an important role in protecting photosynthesis apparatus from the damage of excessive light. Huang *et al.* (2007 reported molecular cloning and characterization of violaxanthin de-epoxidase (VDE) in ginger. A full length (2000 bp) cDNA encoding *violaxanthin deepoxidase* (GVDE) (GenBank accession no. AY876286) was cloned from ginger using RT-PCR and 50, 30 rapid amplification of cDNA ends (RACE). The expression patterns of GVDE in response to light were characterized. GVDE has a 1431 bp open reading frame and the predicted polypeptide contains 476 amino acids with the molecular mass of 53.7 kDa. Northern blot analysis showed that the GVDE was mainly expressed in leaves.

2.4.6.4. Vanilla

Two new Caffeoyl CoA *O*-methyltransferases (OMTs) - caffeoyl CoA OMT-like genes was identified by Widiez *et al.* (2011). by screening a cDNA library from specialized hair cells of pods of the orchid *Vanilla planifolia*. Characterization of the corresponding two enzymes, designated Vp-OMT4 and Vp-OMT5, revealed that both enzymes preferred as a substrate the flavone tricetin *in vitro*, yet their sequences and phylogenetic relationships to other enzymes were distinct from each other. Quantitative analysis of gene expression indicated a dramatic tissue-specific expression pattern for *Vp-OMT4*, which was highly expressed in the hair cells of the developing pod, the likely location of vanillin biosynthesis. Although Vp-OMT4 had a lower activity with the proposed vanillin precursor, 3, 4-dihydroxybenzaldehyde, than with tricetin, the tissue specificity of expression suggests it may be a candidate for an enzyme involved in vanillin biosynthesis. In contrast, the *Vp-OMT5* gene was mainly expressed in leaf tissue and only marginally expressed in pod hair cells. Phylogenetic analysis suggests Vp-OMT5 evolved from a cyanobacterial enzyme and it clustered within a clade in which the sequences from eukaryotic species had predicted chloroplast transit peptides. Transient expression of a GFP-fusion in tobacco demonstrated that Vp-OMT5 was localized in the plastids. This is the first flavonoid OMT demonstrated to be targeted to the plastids.

The Vanilla Sustainability Project, an international initiative involving vanilla scientists and researchers (France, Madagascar, Mexico, USA and others), has been actively engaged in the development of genomic resources for *Vanilla planifolia*, with the main objectives of: 1) the development of an annotated reference base for *Vanilla planifolia* using RNA-Seq that functions as a comprehensive reference transcriptome of expressed genes for multiple lines of inquiry including analyses of gene regulation, expression, structure, and number; 2) the analysis of fruit development to understand developmental mechanisms, biosynthetic and metabolic pathways important to fruit quality characteristics (*e.g.*, vanillin content, aroma, yield, non-dehiscence, abiotic stress tolerance) and transcriptional regulation of these important fruit processes; and 3) the analysis of *Vanilla* root responses and root structure to understand the innate defense response to pathogen infection. The factors chosen are critical for survival of the vanilla industry, with the threat of a *Fusarium* pandemic that is destroying vanilla, and the accelerated threat of global warming that has already affected the timing of *Vanilla planifolia* flowering and impacted successful fertilization, thus the database provides a general resource for further studies (Xiaolan Rao *et al.*, 2014).

2.7 Molecular Characterization and Detection of Pathogens

Gosh and Purkauastha (2003) used polyclonal antibodies and antigens of host and pathogen for early diagnosis of rhizome rot disease of ginger caused by *Pythium aphanidermatum* and *Pythium aphanidermatum*, was detected in ginger rhizome after eight weeks of inoculation by agar gel double diffusion and immuoelectrophoretic tests, but only one week after inoculation by indirect ELISA. Kumar and Anandaraj (2006) developed an efficient DNA isolation protocol and PCR based detection of bacterial pathogen in soil. This PCR based method using universal *Ralstonia solanacearum* specific primer offer a rapid method for unambiguous detection of this pathogen at a concentration of 10^3-10^4 cells per gram of soil.

During 2009 and 2010 in Papantla, the greatest vanilla-producing region of Mexico, *Fusarium* was isolated from vanilla roots and stems that showed symptoms of the disease. From 189 isolates 11 morphologically different colonies were selected to verify the species by amplifying and sequencing their ITS regions. The detected species corresponded to *F. proliferatum*, *Fusarium* sp., *F. oxysporum* f. sp. and *vanillae* (which was the most numerous and most pathogenic to vanilla stems and leaves), an undetermined species of *Fusarium* and *F. proliferatum*, which showed no evidence of producing disease symptoms by Adame-Garcia *et al.* (2015).

3. Future Prospects

Uses of molecular markers in the phylogenetic studies of various organisms have become increasingly important in recent times. Availability of fast DNA sequencing techniques along with the development of robust statistical analysis methods provides a new momentum to this field. In this context, utility of different nuclear encoded genes (like 16S rRNA, 5S rRNA, 28S and rRNA) mitochondrial (cytochrome oxidase, mitochondrial 12S, cytochrome b and control region) and few chloroplast encoded genes (like *rbcL*, *matK* and *rpl16*) will reveal important characters of functional value in spices. Though molecular markers are not free entirely of flaws, they can complement the traditional morphology based method for phylogenetic studies. This combinational approach will strengthen the basis of relationships of organisms to a great extent.

Crop specific strategies utilizing biotechnology, thus ensures conservation of resources unaffected by climatic changes, availability of an alternate set of genotypes that could be multiplied in large numbers, engineered with specific genes, and maintained safe from the threats of natural disasters for posterity. Understanding the molecular interactions between the host and pathogens and developing disease diagnostic tools, is important for developing resistant cultivars by biotechnological breeding.

Secondary metabolites impart lot of value to spices and their products; however secondary metabolism is species-specific, which makes the genome sequence of model plants such as *Arabidopsis* of only limited value. Related species can have different secondary metabolite profiles, however, initial metabolic pathways are similar in most plants, and thus homology between genes can be used for strategies to clone genes from other plants. The use of ESTs in combination with functional expression is another approach that could bring important advances in the future. The growing number of plant gene sequences with a known function could result in accumulating data that will result in exponential growth of similar genes identified in other plants. The area of functional genomics faces a major bottleneck due to the unknown biochemical pathways involved, involving assays for the enzymes involved in secondary metabolism. It would also be interesting to examine the molecular processes that bring about the variability in secondary metabolism, at the level of gene regulation, posttranscriptional regulation or protein evolution.

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