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Molecular Analyses of *Vanilla planifolia* Cultivated in India using RAPD and ISSR Markers

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ABSTRACT

The natural "vanilla flavour" obtained from the pods of *Vanilla planifolia* is the largest flavouring ingredient in food industry. In order to evaluate the usefulness of genetic markers such as RAPD and ISSR for assessing the diversity among clones of *V. planifolia* and to create a database for the available germplasm, we investigated the existing population cultivated in India. The genetic diversity among 25 accessions collected from 13 major locations was studied. Forty random amplified polymorphic DNA (RAPD) and 11 inter-simple sequence repeats (ISSR) primers resulted in 326 scorable bands ranging in size from 200 bp to 2800 bp and 83 scorable bands from 200 bp to 2500 bp, respectively. Banding pattern among the different samples collected within accessions was similar indicating that the morphological difference observed within accession had no genetic background. On the other hand, molecular analysis among different accessions from different locations also yielded identical PCR band profiles in both RAPD and ISSR analysis. These results clearly indicate that *V. planifolia* cultivated in India is likely to share the same genetic background and therefore, the genetic diversity is extremely low. Therefore we propose other biotechnological approaches to induce genetic variations to improve the agronomical traits.

Keywords: clonal propagation, DNA polymorphism, genetic diversity, orchid, vanilla flavour
Abbreviations: ISSR, Inter-Simple Sequence Repeats; **PCR**, Polymerase Chain Reaction; **RAPD**, Random Amplified Polymorphic DNA

INTRODUCTION

Vanilla planifolia (syn. *V. fragrans*), *V. tahitensis* and *V. pompona* of the family Orchidaceae are the commercially cultivated species for the production of natural vanilla flavour where *V. planifolia* is the most preferred one. There are over 170 flavouring compounds identified in the extract of cured vanilla beans out of which the major ones are vanillin, vanillic acid, para-hydroxybenzoic acid and para-hydroxybenzaldehyde. The differences in flavour profiles among the commercial clones cultivated in India have not been recorded, though there are reports on vanilla indicating the existence of differences in flavour profiles depending upon the agro-climatic conditions apart from the genetic factors (Ranadive 2006). Vanilla is cultivated in an area of 37,525 ha with a production of 4403 tonnes globally whereas the world demand for vanilla is around 32,000 tonnes (Kumar 2004). Natural vanilla flavour, extracted from cured vanilla beans, is one of the most important and universally used aromatic flavours in food, pharmaceutical, beverage and cosmetic industries. Vanilla was indigenous to Mexico and was introduced to Europe by the Spanish Conquistadores in 1520 (Dignum *et al* 2001). Vanilla cultivation was initiated in India, through the East India Company, nearly 250 years back in the spice garden at Kurtallam in Tamil Nadu (George 2005). Its organized cultivation started in 2001-2002 in 1600 ha yielding 60 tonnes of cured vanilla beans and steadily gained importance doubling its cultivation to 3427 ha in 2003-2004 resulting in the production of 131 tonnes (http://www.foodindianews.com). In India, only a few cultivars have been recognized from the species *V. planifolia*. In the countries where vanilla has been introduced, variability is likely to be highly limited as the species is propagated only vegetatively. However, seed germination is also reported for vanilla (Havkin-Frenkel and Dorn 1997) indicating the possibility of bird dispersal leading to the chance for variations in the populations. Therefore, it is necessary to analyze the extent of variations in *V. planifolia* plants collected from various locations in India.

Molecular markers have been successfully used to study introduced species and proven to be more useful tools in identifying the source of introduction and variability due to a new environment. PCR-based random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) have been widely used to survey genetic structure of populations. Among various molecular markers, the RAPD technique is simple, rapid, and requires only a few nanograms of DNA, has no requirement of prior information of the DNA sequence and has feasibility of automation with higher frequency of polymorphism, which makes it suitable for routine application for the analysis of genetic diversity (Babu *et al*. 2007). It is also proven to be quite efficient in detecting genetic variations, even in closely related organisms like two near isogenic lines of tomato (Martin *et al*. 1991). For the use of ISSRs, primers are not proprietary as in Microsatellites or Simple Sequence Repeats (SSRs) and can be synthesized by anyone and also allow the production of a high number of reproducible polymorphic bands. ISSR is found to be very simple, quick, cost-effective, highly discriminative and most reliable method which combines most of the advantages of SSRs and Amplified Fragment Length Polymorphism (AFLP) to the universality of RAPD (Reddy *et al*. 2002). ISSRs though considered mostly as dominant markers; they are shown to segregate co-dominantly in some cases (Sankar and Moore 2001) thus enabling distinction between homozygotes and heterozygotes. They are found to be more useful and reproducible than isozymes, RAPD and RFLP (Fang *et al*. 1997) and are also known to give more polymorphism than any other assay procedure (Virk *et al*. 2000). Detection of additional polymorphism could be done by the use of RAPD in combination with ISSRs (Joshi *et al*. 2000). Martins *et al*. (2004) suggest the use of a combination of two types of markers that amplify

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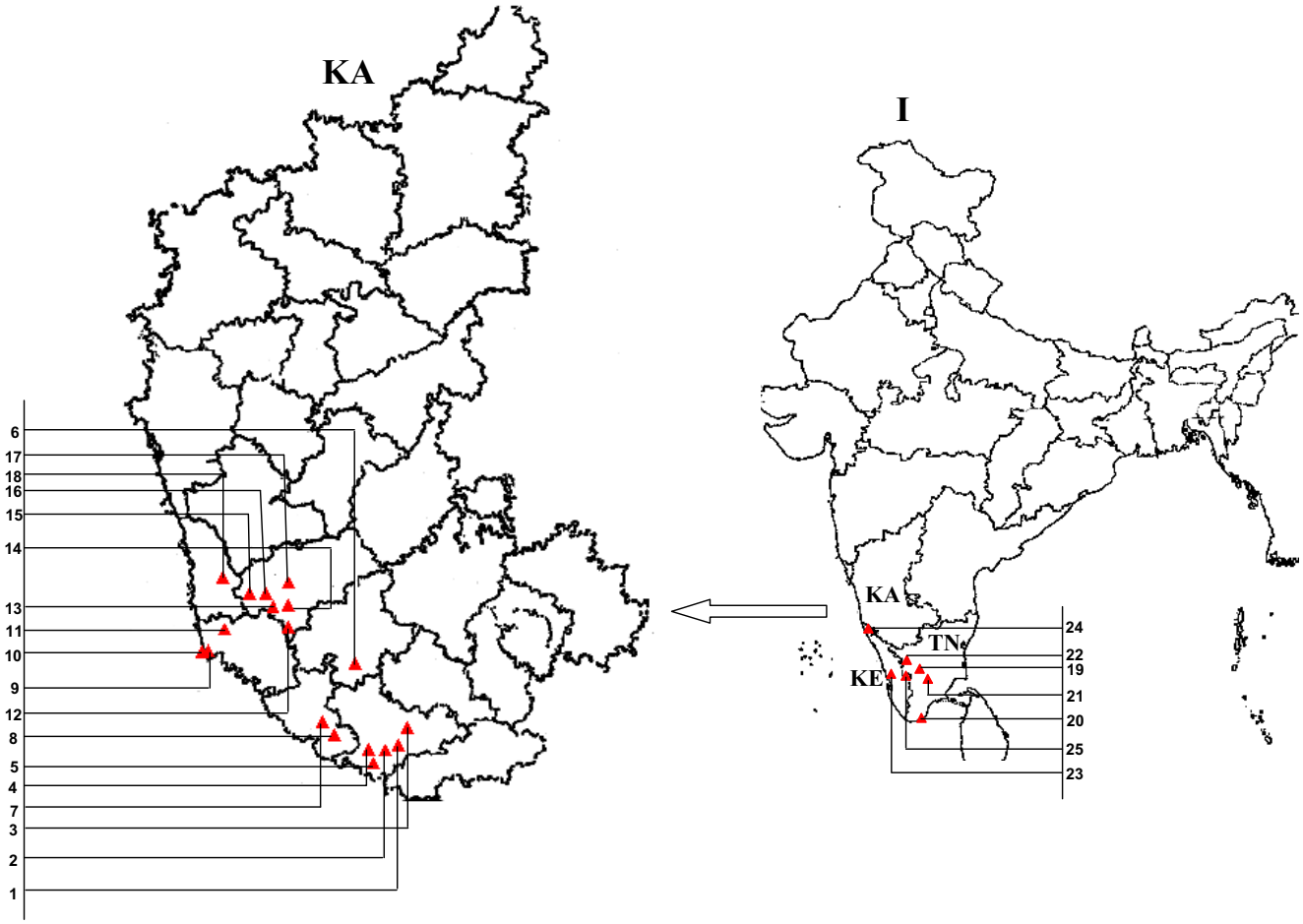

Fig. 1 Map of India (I) showing sampling locations (filled triangles) in Karnataka (KA), Tamil Nadu (TN) and Kerala (KE).

different regions of the genome and hence a better analysis of genetic variation can be made.

Molecular markers have been successfully used for various studies in orchids. For instance, Pillon *et al*. (2006) studied genetic diversity in the genus *Dactylorhiza* using molecular markers and concluded that the diversity was greatest in the Mediterranean basin and the Caucasus, which were then considered to be the major targets for conservation based on their results. They also showed that the results of phylogenetic analyses and genetic data obtained with molecular tools could offer an alternative measure of biodiversity that is not sensitive to taxonomic inflation. Study of genetic diversity and phylogenetic relationships among and within species of *Cymbidiums* using RAPD analysis showed full agreement with the groups identified by morphological, physiological and ecological characteristics. RAPD markers have also been successfully employed to reveal relationships and classifications in *Cymbidiums* at cultivar levels (Obara-Okeyo and Kako 1998; Ok *et al*. 2004). Chung *et al*. (2006) successfully differentiated *Paphiopedilum* and *Phragmipedium* using RAPD which were in good agreement with morphologically-based classification. A population genetic study of *Goodyera procera* with allozyme and RAPD markers supported that RAPD can detect higher levels of genetic variation than allozyme (Wong and Sun 1999).

In the present study, both the PCR based techniques, RAPD and ISSR, were adopted for the evaluation of genetic variation in *V. planifolia*. Therefore, the objectives of the present study were to assess the usefulness of genetic markers for assessing the diversity among clones of vanilla cultivated in India and to create a database for the available germplasm.

MATERIALS AND METHODS

Plant sampling

Since commercial cultivation of vanilla in India is mainly concentrated in the states of Karnataka, Kerala and Tamil Nadu, leaf samples were collected from plantations of these states (**Fig 1; Table 1**). Standard procedures were followed for the collection of samples (Li *et al*. 2006) and preparation of DNA extract. Briefly, leaf samples from six different vines per accession were collected by looking at the morphological variations. Samples were stored in plastic bags, which were kept at -20°C until DNA was extracted.

Table 1 List of accessions of *V. planifolia* and their geographical origin used in this study.

Sl. №	Population localities	Accession no.*
1	Mysore, Karnataka	1, 2, 3, 4, 5
2	Hassan, Karnataka	6
3	Madikere, Karnataka	7, 8
4	Dakshina Kannada, Karnataka	9, 10, 11
5	Shimoga, Karnataka	12, 13, 14, 15, 16, 17
6	Udupi, Karnataka	18
7	Coimbatore, Tamil Nadu	19
8	Kanyakumari, Tamil Nadu	20
9	Dindigul, Tamil Nadu	21
10	Ooty, Tamil Nadu	22
11	Mallapuram, Kerala	23
12	Kasargud, Kerala	24
13	Pallakad, Kerala	25

* Refer **Fig. 1** for location of accessions.

DNA extraction and quantification

Approximately 100 mg of young leaf tissue was ground into fine powder in liquid nitrogen and total genomic DNA was extracted using the GenElute^{TM} Plant Genomic DNA Mini prep Kit (Sigma

Aldrich, India). Quality and quantity of DNA preparations were checked by standard spectrophotometry and the samples were diluted to 25 ng/µl in TE buffer and stored at 4°C.

Primer selection

Various RAPD and ISSR primers were selected, based on specific relevance to family Orchidaceae to which vanilla belongs, from the studies of Besse *et al*. (2004), Tsai *et al*. (2002) and the NCBI-database. Others were those for monocots that were successfully used in our earlier study in banana (Venkatachalam *et al*. 2007). Out of the 60 RAPD 10-mer primers and 20 ISSR primers, 40 RAPD and 11 ISSR primers were selected depending on their consistency in amplification (**Table 2**).

Table 2 List of selected RAPD and ISSR primers.

RAPD primers	
Kit OPA	A-03; A-04; A-11; A-14; A-20
Kit OPC	C-01; C-02; C-04; C-05; C-06; C-07; C-08; C-09; C-10; C-12
Kit OPD	D-04; D-11; D-16
Kit OPF	F-12
Kit OPJ	J-07; J-08; J-09; J-10; J-11; J-12; J-13; J-15; J-16; J-17; J-18; J-19
Kit OPM	M-16; M-18; M-20
Kit OPN	N-03; N-04; N-06; N-09; N-10; N-14
ISSR primers	
Kit UBC	809; 810; 811; 813; 823; 824; 826; 834; 836; 840; 848

Table 3 List of selected primers used in RAPD analysis and number of scorable bands.

Sl. №	Primer	Primer sequence (5′–3′)	$MgCl_2$ concentration (mM)	№ of scorable bands
1	OPA 03	AGTCAGCCAC	0	11
2	OPA 04	AATCGGGCTG	1	5
3	OPA 11	CAATCGCCGT	0	10
4	OPA 14	CTCGTGCTGG	1	7
5	OPA 20	GTTGCGATCC	0	8
6	OPC 01	TTCGAGCCAG	0	7
7	OPC 02	GTGAGGGCTC	2	6
8	OPC 04	CCGCATCTAC	1	8
9	OPC 05	GATGACCGCC	1	8
10	OPC 06	GAACGGACTC	0	6
11	OPC 07	GTCCCGACGA	0	8
12	OPC 08	TGGACCGGTG	1	7
13	OPC 09	CTCACCGTCC	0	9
14	OPC 10	TCTCTGGGTG	0	3
15	OPC 12	TCTCATCCCC	1	7
16	OPD 04	TCTGGTGAGG	1	6
17	OPD 11	AGCGCCATTG	0	9
18	OPD 16	AGGGCGTAAG	0	8
19	OPF 12	ACGGTACCAG	1	8
20	OPJ 07	CCTCTCGACA	0	9
21	OPJ 08	CATACCGTGG	2	4
22	OPJ 09	TGAGCCTCAC	1	7
23	OPJ 10	AAGCCCGAGG	2	8
24	OPJ 11	ACTCCTGCGA	1	5
25	OPJ 12	GTCCCGTGGT	0	7
26	OPJ 13	CCACACTACC	1	6
27	OPJ 15	TGTAGCAGGG	0	5
28	OPJ 16	CTGCTTAGGG	1	8
29	OPJ 17	ACGCCAGTTC	2	11
30	OPJ 18	TGGTCGCAGA	2	6
31	OPJ 19	GGACACCACT	1	10
32	OPM 16	GTAACCAGCC	0	15
33	OPM 18	CACCATCCGC	2	6
34	OPM 20	AGGTCTTGGG	2	10
35	OPN 03	GGTACTCCCC	0	12
36	OPN 04	GACCGACCCA	1	10
37	OPN 06	GAGACGCACA	0	11
38	OPN 09	TGCCGGCTTG	2	11
39	OPN 10	ACAACTGGGG	1	14
40	OPN 14	TCGTGCGGGT	1	10

Table 4 List of selected primers used in ISSR analysis and number of scorable bands.

Sl. №	Primer name	Primer sequence (5′–3′)	$MgCl_2$ concentration (mM)	№ of scorable bands
1	UBC 809	$(AG)_8G$	0	4
2	UBC 810	$(GA)_8T$	0	9
3	UBC 811	$(GA)_8C$	0	10
4	UBC 813	$(CT)_8T$	1	5
5	UBC 823	$(TC)_8C$	0	4
6	UBC 824	$(TC)_8G$	0	6
7	UBC 826	$(AC)_8C$	1	15
8	UBC 834	$(AG)_8YT$	0	8
9	UBC 836	$(AG)_8YA$	0	5
10	UBC 840	$(GA)_8YT$	1	11
11	UBC 848	$(CA)_8RG$	0	6

Y = C or T

DNA amplification

Optimum PCR conditions for both RAPD and ISSR were standardized with different quantities of template DNA (12.5, 25 and 50 ng), dNTPs (100, 200 and 300µM) and $MgCl_2$ (1, 2 and 3 mM). Based on the results, RAPD amplifications were performed routinely using a PCR mixture (25 µl) which contained 25 ng of genomic DNA as template, 1X PCR buffer (Fermentas GMBH, Germany), 200 µM dNTPs (Fermentas GMBH, Germany), 1 unit (U) of *Taq* DNA polymerase (Bangalore Genei, India), 0.5 µM of each primer (Operon Technologies, Alameda, California, USA) with varied concentration of $MgCl_2$ (Fermentas GMBH, Germany) depending on the primer (**Table 3**). PCR was performed at initial denaturation at 93°C for 4 min followed by 36 cycles of 1 min denaturation at 94°C, 1 min annealing at 36°C and 2 min extension at 72°C with a final extension of 72°C for 10 min using a thermal cycler (Eppendorf thermal cycler 5332).

For ISSR primers, optimal annealing temperature was found to vary according to the base compositions of the primers. PCR mixture (25 µl) contained 25 ng of genomic DNA as template, 1X PCR buffer, 200 µM dNTPs, 1 unit (U) of *Taq* DNA polymerase, 0.5 µM of each primer with varied concentration of $MgCl_2$ depending on the primer (**Table 4**). PCR was performed at initial denaturation at 94°C for 4 min followed by 40 cycles of 1 min denaturation at 94°C, 1 min at 2°C lower than the specified annealing temperature for each primer and 2 min extension at 72°C with a final extension also at 72°C for 10 min using a thermal cycler.

The PCR products obtained were separated by gel electrophoresis on 2% agarose gel (ICN, USA), in 0.5X TBE buffer and stained with ethidium bromide (0.001%) and visualized by image analysis software documented in a gel documentation system (Lab works software, version 3.00,UVP Hero-Lab GMBH, Germany). The size of the amplification products was estimated from 100 bp DNA ladder (Fermentas GMBH, Germany).

Data analysis

Well-resolved and consistently reproducible fragments ranging form 200 bp-2.8 kb were scored as present or absent for both RAPD and ISSR markers for each sample. Comparison of RAPD and ISSR profiles of all the 25 DNA samples was done for the analysis. Genetic distances could not be established since there was not a single band differing from others in RAPD and ISSR reactions.

RESULTS

Standardization of conditions for PCR amplification such as concentrations of magnesium chloride, *Taq*-polymerase, template DNA and annealing temperature were initially varied to arrive at a most effective combination to obtain highly intense distinctly separated bands without smearing effects. Forty RAPD primers (**Table 2**) were selected from 60 arbitrary primers (having 60-70% GC content) based on their amplification products. Each RAPD primer generated a unique set of amplification products ranging in size from 200 bp to 2800 bp where the number of bands for each

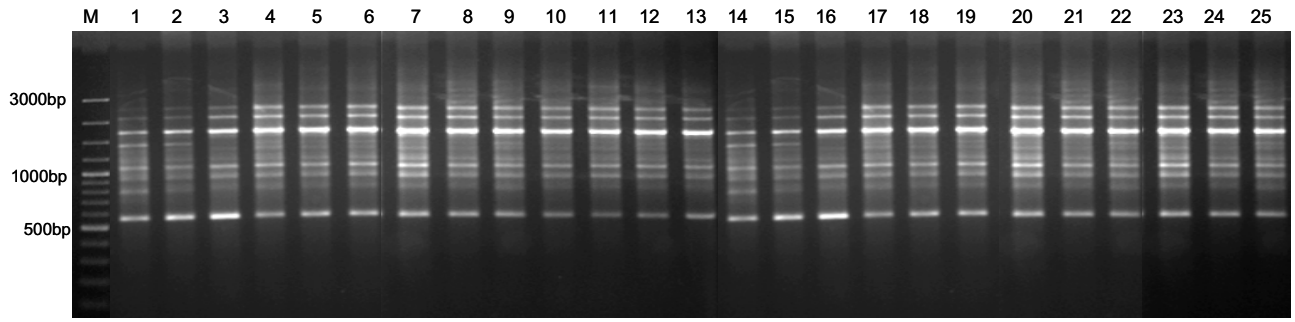

Fig. 2 Randomly Amplified Polymorphic DNA (RAPD) banding pattern generated by primer OPJ 10. Lanes 1-25 designate accessions. Lane M: GeneRuler™ 100 bp DNA Ladder Plus.

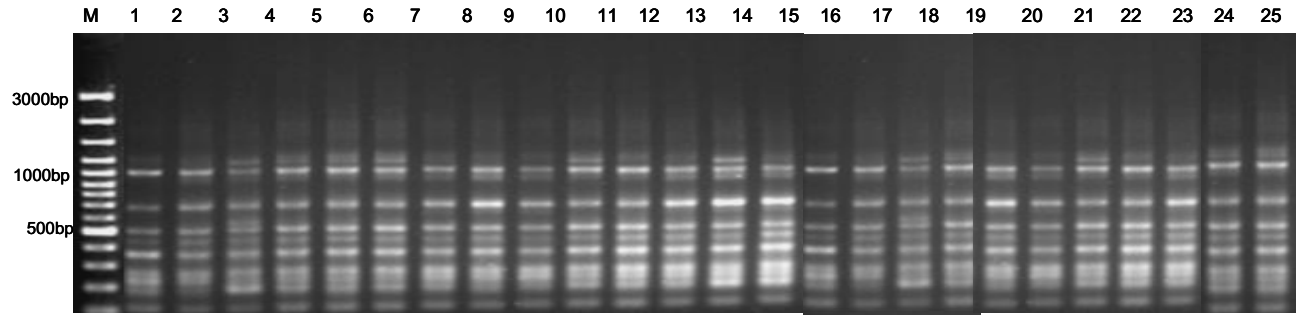

Fig. 3 Inter Simple Sequence Repeats (ISSR) of DNA showing amplification pattern generated by primer UBC 810. Lanes 1-25 designate accessions. Lane M: GeneRuler™ 100 bp DNA Ladder Plus.

primer varied from 4 in OPJ 08 and 15 in OPM 16 (**Table 3**). The 40 primers used in this analysis yielded 326 scorable bands with an average of 8.15 bands per primer. Screening with the 20 ISSR primers generated 83 scorable bands in 11 primers (**Table 2**) ranging from 200 bp to 2500 bp. An average of 7.54 bands per ISSR primer was obtained ranging from 4 to 15 (**Table 4**). Banding pattern among the different samples collected within an accession was similar indicating that the morphological difference observed within accessions had no genetic background. On the other hand, molecular analysis among different accessions from different locations also yielded an identical PCR band profile in both RAPD and ISSR analysis (**Figs. 2, 3**).

DISCUSSION

The present study involved two types of efficient genetic markers involving a large number of primers for marker-based genetic analyses of 25 accessions collected from different locations of India (**Fig. 1**; **Table 1**). This study has clearly showed the absence of genetic variation within and among *V. planifolia* populations. A very low level of genetic diversity was detected in *V. planifolia* in geographical areas such as Mexico (Soto and Arenas 1996; Cibrian 1999), Reunion Island (Besse *et al*. 2004) and Polynesia (Pacific Ocean) (Besse *et al*. 2004), which is in accordance with the vegetative mode of dispersion as stem cuttings and the history (introduced plant species) of recent introduction in these regions. A thorough analysis of different species (such as *V. planifolia, V. tahitensis* and *V. pompona*) and clones within the species of Vanilla cultivated in Reunion and Central America (Besse *et al*. 2004) showed no variation in the introduced locations.

It is well known that population genetic variation is influenced by factors such as historical events, genetic drift, breeding systems and natural selection (Barrett 1992). History of introduction determines the genetic variation within and among populations to some extent. Introductions usually consist of a small number of founders, resulting in lower genetic variability in introduced populations (Nei *et al*. 1975). Multiple introduced populations have higher genetic diversity than those that have been introduced only a few times (Li *et al*. 2006). Low genetic variation may also be a result of a few well-adapted genotypes that might have established successfully and expanded rapidly even in the case of multiple introductions. During the time of the introduction, a super aggressive genotype among the introduced ones might have been fixed by some biotic and abiotic factors (Li *et al*. 2006). The introduced plant need not accumulate enough genetic variation to adapt to its newer environment in its spread phase if its phenotypic plasticity is stronger and could buffer against the selection pressure (Weber and Schmid 1998). The degree to which introductions are accompanied by genetic bottlenecks depends on the species breeding systems and is expected to be lowest in highly selfing species or those that reproduce vegetatively (Nei *et al*. 1975).

Vanilla, being an introduced crop in most countries where it has been cultivated, much of the planting material is originated from limited clonal propagation, which largely limits the genetic variability in the crop (Divakaran 2006b). Vanilla might have been introduced to India through a "stepping-stone" process resulting in all cultivars sharing the same genetic background. Vanilla cultivation, having begun in India by the East India Company nearly 250 years back in the spice garden at Kurtallam in Tamil Nadu indicates that a few vines belonging to same mother plant or plantation might have been introduced and further expansion to all other parts is from these plants, which are of a similar genetic background. Reports indicate that plantations of Reunion, Mauritius, Seychelles and Malagasy Republic can all be traced back to a single clone (Madhusoodanan *et al*. 2003).

The development of strong adaptability of a plant species to its current environments is more important for its survival than the accumulation of rich genetic diversity, which usually takes a long time to achieve (Xu *et al*. 2003). Obviously, the shortcomings of low genetic diversity in a plant species can be highly compensated by the development of its strong adaptability, at least for a temporal period of time. However, it is difficult to predict the long-term effect caused by the low genetic variation of the clonal species. Through rapid and massive expansion, a few successful clones with favorable genotypes might be the essential

component in all individuals in its new colonies, although overall genetic variability in these clones might appear to be low.

This preliminary investigation has determined the absence of genetic variations in introduced and then comercially cultivated *V. planifolia* in India indicating a threat of extinction due to pest and environment vagaries. These observations indicate the need to increase the number of introductions and broaden the gene pool of cultivated vanilla in India to reduce its vulnerability to diseases and insect pests apart from its genetic improvement for other attributes. Genetic variability is required to permit an adaptive response of the introduced species to the new selective regime imposed by the environment. A recent study by Divakaran *et al*. (2006a) involved inter-specific hybridization in vanilla and molecular characterization of hybrids and selfed progenies using RAPD and AFLP markers indicated the possibility of taking up breeding work in vanilla. In order to broaden the genetic base of vanilla cultivars, further efforts such as seed germination, mutation breeding, genetic engineering and induction of somaclonal variants are to be urgently pursued.

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REFERENCES

Babu BK, Senthil N, Gomez SM, Biji KR, Rajendraprasad NS, Sateesh Kumar S, Chandra Babu R (2007) Assessment of genetic diversity among finger millet (*Eleusine coracana* (L.) Gaertn.) accessions using molecular markers. *Genetic Resources and Crop Evolution* **54**, 399-404

Barrett SEH (1992) Genetics of weed invasions. In: Jain SK, Botsford LW (Eds) *Applied Population Biology*, Kluwer Academic Publishers, Dordercht, The Netherlands, pp 91-119

Besse P, da Silva D, Bory S, Grisoni M, Le Bellec F, Duval M-F (2004) RAPD genetic diversity in cultivated vanilla: *Vanilla planifolia*, and relationships with *V. tahitensis* and *V. pompona*. *Plant Science* **167**, 379-385

Chung SY, Choi SH, Kim MJ, Yoon KE, Lee GP, Lee JS, Ryu KH (2006) Genetic relationship and differentiation of *Paphiopedilum* and *Phragmepedium* based on RAPD analysis. *Scientia Horticulturae* **109**, 153-159

Cibrian (1999) Variacion genética de *Vanilla planifolia* en Mexico. PhD Thesis, UNAM, Mexico City, 105 pp

Dignum MJW, Josef K, Verpoorte R (2001) Vanilla production: Technological, chemical and biosynthetic aspects. *Food Reviews International* **17**, 199-219

Divakaran M, Babu NK, Ravindran PN, Peter KV (2006a) Interspecific hybridization in *Vanilla* and molecular characterization of hybrids and selfed progenies using RAPD and AFLP markers. *Scientia Horticlturae* **180**, 414-422

Divakaran M, Babu NK, Ravindran PN, Peter KV (2006b) Conservation of *Vanilla* species, *in vitro*. *Scientia Horticlturae* **110**, 175-180

Fang DQ, Roose ML, Krueger RR, Federici CT (1997) Fingerprinting trifoliate orange germplasm accessions with isozymes, RFLPs and inter-simple sequence repeat markers. *Theoretical and Applied Genetics* **95**, 211-219

George CK (2005) My encounter with Vanilla. *Spice India* **May**, 21-25

Havkin-Frenkel D, Dorn R (1997) In: Risch SJ, Ho C-T (Ed) *Spices: Flavor Chemistry and Antioxidant Properties*, ACS Symposium Series Vol. 660, American Chemical Society, Washington DC, pp 29-40

Joshi SP, Gupta VS, Aggarwal RK, Ranjekar PK, Brar DS (2000) Genetic diversity and phylogenic relationship as revealed by inter-simple sequence repeat (ISSR) polymorphism in the genus *Oryza*. *Theoretical and Applied Genetics* **100**, 1311-1320

Kumar AAS (2004) Vanilla. In: *Kerala Calling*, Director of Information and Public Relations, Government of Kerala, Thiruvananthapuram, pp 26-30

Li W-G, Wang B-R, Wang J-B (2006) Lack of genetic variation of an invasive clonal plant *Eichhornia crassipes* in China as revealed by RAPD and ISSR markers. *Aquatic Botany* **84**, 176-180

Madhusoodanan KJ, Radhakrishnan VV, Sudharshan MR (2003) Botany of *Vanilla*. *Spice India* **June**, 14-18

Martin GB, Williams JGK, Tanskley SD (1991) Rapid identification of markers linked to a *Pseudomonas* resistance gene in tomato by using random primers and near isogenic lines. *Proceedings of the National Academy of Sciences USA* **88**, 2336-2340

Martins M, Sarmento D, Oliveira MM (2004) Genetic stability of micropropagated almond plantlets as assessed by RAPD and ISSR markers. *Plant Cell Reports* **23**, 492-496

Nei M, Maruyama T, Chakraborty R (1975) Bottleneck effect and genetic-variability in populations. *Evolution* **29**, 1-10

Obara-Okeyo P, Kako S (1998) Genetic diversity and identification of *Cymbidium* cultivars as measured by random amplified polymorphic DNA (RAPD) markers. *Euphytica* **99**, 95-101

Ok SH, Jeung JU, Kang HM, Jung KW, Lee JS, Shin JS (2004) Development and utilization of sequence tagged site-polymorphic sequence markers for classification of oriental *Cymbidium* cultivars and variegates. *Journal of the Korean Society for Horticultural Science* **45**, 216-221

Pillon Y, Fay MF, Shipunov AB, Chase MW (2006) Species diversity versus phylogenetic diversity: A practical study in the taxonomically difficult genus *Dactylorhiza* (Orchidaceae). *Biological Conservation* **129**, 4-13

Ranadive A (2006) Chemistry and Biochemistry of Vanilla Flavor. *Perfumer and Flavorist* **31**, 38-44

Reddy PM, Saral N, Siddiq EA (2002) Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. *Euphytica* **128**, 9-17

Sankar AA, Moore GA (2001) Evaluation of inter-simple sequence repeat analysis for mapping in *Citrus* and extension of genetic linkage map. *Theoretical and Applied Genetics* **102**, 206-214

Soto MA, Arenas (1996) A monograph of *Vanilla* (Orchidaceae). PhD Thesis, UNAM University, Mexico City, 110 pp

Tsai CC, Huang SC, Huang PL, Chen YS, Chou CH (2002) Phenetic relationship and identification of subtribe Oncidiinae genotypes by random amplified polymorphic DNA (RAPD) markers. *Scientia Horticulturae* **96**, 303-312

Venkatachalam L, Sreedhar RV, Bhagyalakshmi N (2007) Molecular analysis of genetic stability in long-term micropropagated shoots of banana using RAPD and ISSR markers. *Electronic Journal of Biotechnology* **10**, 1-8

Virk PS, Zhu J, Newbury HJ, Bryan GJ, Jackson MT, Ford-Lloyd BV (2000) Effectiveness of different classes of molecular markers for classifying and revealing variation in rice (*Oryza sativa*) germplasm. *Euphytica* **112**, 275-284

Weber E, Schmid B (1998) Latitudinal population differentiation in two species of *Solidago* (Asteraceae) introduced into Europe. *American Journal of Botany* **85**, 1110-1121

Wong KC, Sun M (1999) Reproductive biology and conservation genetics of *Goodyera procera* (Orchidaceae). *American Journal of Botany* **86**, 1406-1413

Xu C-Y, Zhang W-J, Fu C-A, Lu B-R (2003) Genetic diversity of alligator weed in China by RAPD analysis. *Biodiversity and Conservation* **12**, 637-645