KFRI Research Report No. 482
(Final Report of the Project KFRI 526/07)

EARLY SELECTION AND MASS MULTIPLICATION OF *EUCALYPTUS* INTERSPECIFIC HYBRID CROSSES

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May 2014

Abstract of the Project Proposal

1. Project No. : KFRI 526/07

2. Title : Early selection and mass multiplication of *Eucalyptus* interspecific hybrid crosses

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6. Objectives :

   i. Development of molecular marker technology for early identification of potential hybrid clones of eucalypt at seedlings stage

   ii. Multiplication of selected hybrid clones through mini-cuttings technique

   iii. Clonal testing of hybrid clones and mass production of selected clones

7. Duration : 3 Years

8. Funding Agency : KFRI Plan Fund

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ABSTRACT

Clonal eucalypt plantations are the major source of raw materials for paper and pulpwood industries. Clonal forestry has to be vibrant with an efficient research support for the continuous addition of new clones as existing clones become unsustainable due to changing climatic conditions and emerging pest and disease problems. Quicker development of eucalypt clones from heterotic interspecific hybrids using modern biotechnological tools of molecular markers and mini-cutting technology are the emerging methods capable of maintaining a dynamic clonal forestry programme. This project report describes standardization of methods for the development of eucalypt interspecific hybrid clones using microsatellite markers and mini-cutting technique.

Interspecific hybrids occur through natural pollination among different *Eucalyptus* spp. planted in KFRI provenance trial plots. These plots were established during 1990-1993 and maintained thereafter at Kottappara in Malayattur Forest Division and Vallakkadavu in Thekkady Wildlife Sanctuary. Seedlings raised from seeds of *E. tereticornis, E. pellita, E. urophylla* and *E. grandis* provenance trees provided putative heterotic natural hybrids also, showing morphological features of other eucalypt species besides true to type seedlings. These putative hybrid seedlings showed a mixture of desirable and undesirable characters of economic importance. Through a selection process, fast growing, disease and pest resistant putative hybrids were identified through field planting in clonal testing area at Kottappara. The best trees were felled when they were about 18 month-old and coppice shoots arising from stumps were utilized for standardizing the mass propagation technique using mini-cuttings. Mini-cutting technique is adopted for production of plantlets through rooting of second generation cuttings produced from axillary sprouts of first generation rooted cuttings of coppice shoots origin.

Large genetic variations occurred among the hybrids in respect of growth, adaptability, stem form and pest and disease susceptibility. Hence, intensive selection with combinations of as many desirable characters as possible was carried out for development

of heterotic interspecific hybrid clones. Generally, putative hybrid plants of *E. pellita* origin which showed some morphological features of *E. urophylla* or *E. tereticornis,* and *E. urophylla* origin plants which showed *E. grandis* features also were superior hybrids. Microsatellite markers were developed for DNA fingerprinting of interspecific hybrids and all potential pollen parents for quick identification of pollen parent of interspecific hybrids at seedling stage itself. This is achieved through species specific microsatellite markers and comparison of DNA fingerprints of mother plant and the hybrids with those of all the possible pollen parents. Knowledge of the pollen parent at the seedling stage itself gives an indication of the desirability of the genotype and an opportunity to eliminate undesirable ones.

A quicker method for mass clonal multiplication enhances the speed of developing new clones. This is made possible through adoption of mini-cutting technique of mass clonal multiplication. Mini-cuttings from five potential hybrid plants, two each belonging to *E. pellita* and *E. urophylla* and one belonging to *E. tereticornis* were compared with mini-cuttings from five popular KFRI eucalypt clones simultaneously in mist chamber at KFRI clonal nursery at Kottappara. While the percentage of success in sprouting and establishment of mini-cuttings varied from 13-92% for putative hybrids, the percentage of success for popular KFRI clones ranged between 2-43% only. The highest percentage of success was shown by *E. tereticornis* origin putative hybrids. The study showed the potential of the combination of DNA marker technology and mini-cutting technique for quicker development of superior eucalypt hybrid clones.

1. INTRODUCTTION

Eucalypts planting in India was dominated by Mysore Gum, supposed to be an open pollinated hybrid derivative of *E. tereticornis* and *E. camaldulensis* (Bowden, 1964). In Kerala, it was planted in low lands till 1997. Mysore Gum was highly susceptible to various kinds of diseases especially in high rainfall areas of Kerala. Subsequently, seeds of new provenances of Eucalypts especially those of *E. tereticornis, E. pellita* and *E. grandis* were brought from Australia by Kerala Forest Department and Kerala Forest Development Corporation for planting. *E. tereticornis* and *E. pellita* were planted in low lands and *E. grandis* in high ranges. Seedlings were raised in root trainers and compost was used as potting medium. Technical support was provided by KFRI for raising seedlings in root trainers.

KFRI had established Eucalypts provenance trial plots in 1990, 1992 and 1993 at Kottappara (15 ha) in Kodanad Range of Malayattur Division, at Vallakkadavu near Thekkady, and Muthanga in Wayanad. *E. tereticornis, E. camaldulensis, E. urophylla* and *E. pellita* were the predominant species planted in Kodanad range while *E. grandis* and *E. urophylla* were planted in high ranges. Plus trees were selected from these provenance trees from 1996 onwards and they were felled to induce coppice shoots production from stumps. These plus trees were mass multiplied by rooting of coppice shoot cuttings. KFRI has produced about 125 clones of 5 species of *Eucalyptus*. Since 1999, more than 1.4 lakh ramets of about 35 fast growing, disease resistant clones of eucalypts have been supplied to Kerala Forest Department (KFD) and Hindustan Newsprint Ltd. (HNL) for raising clonal plantations and for establishment of clonal multiplication area (CMA) (Balasundaran and Maria Florence, 2007). Genetic diversity among 15 popular clones were assessed using RAPD fingerprinting for suggesting precautions to be taken during field deployment of clones (Balasundaran and Maria Florence, 2005). Recently some of these clones have been affected by new pests and diseases and the yield has come down drastically. Hence, modern methods have to be developed for quick production of new fast growing disease resistant clones.

The significance of Eucalypt hybrids in tropical and subtropical plantation forestry has been stressed by Potts and Dungey (2004) particularly where clonal propagation is adopted in a large scale (Gwaze *et al.*, 2000). It has been reported that eucalypt clones performing better than their parent trees in terms of productivity, pulp quality and disease resistance can be identified among their inter-specific hybrids (Eldridge *et al.*, 1993). The promising hybrids can be mass multiplied vegetatively for raising superior clonal plantations. But it takes several years to identify fast growing disease resistant interspecific clones, mass multiply and field plant them in order to evaluate those clones through multi-location trials.

Luxuriantly growing hybrid seedlings of eucalypts occur among normal seedlings raised using seeds collected from individual trees of multi-species provenance trial of eucalypts. These interspecific hybrids are formed through natural crossing by wind-borne or insect-borne pollen. The seedlings can be identified not only through their vigorous growth but also through their morphological features such as leaf characters. The pollen parent of the seedlings can be identified adopting DNA marker technique through comparison of the DNA fingerprints of the hybrid seedling and the mother parent with that of the potential pollen donors (Kirst *et. al.*, 2005).

DNA markers are generally used as a tool for locating genes of interest in a genome for utilizing them for genetic engineering and for obtaining information on population structure, mating systems and pedigree confirmation. Among the DNA markers, microsatellites are commonly used for such studies of plant genome (Brondani *et al.*, 1998) due to their wide occurrence, high level of polymorphism and repeatability. Microsatellites are short tandemly repeated motifs of 1–6 bases, also known as simple sequence repeats (SSR). Because of their co-dominant nature, they are widely used in genetic improvement of crop plants through hybridization and marker assisted selection of desirable genotypes.

The identification of the pollen parent may give indication of the potentiality of the hybrid seedling as a promising future clone. Some of the hybrid clones with *E. camaldulensis* as either of the parents are often susceptible to diseases in moist tropics such as Kerala. But hybrids between *E. tereticornis, E. grandis, E. urophylla and E. pellita* are

reported to perform well in tropical countries (Grattapaglia *et al.,* 2004). Such hybrid seedlings can be multiplied through rooting of coppice shoot cuttings. In mist chamber, these cuttings produce a few axillary sprouts. A large number of ramets can be produced through rooting of axillary sprouts arising from rooted cuttings. These rooted cuttings are otherwise called mini cuttings (Assis, 2001; Evans and Turnbull, 2004). Sprouts arising from such cuttings can be again rooted to produce next generation mini rooted cuttings. Availability of a sizable number of such plantlets within a few months enables multi-location clonal testing earlier than a time frame actually needed when conventional technique is adopted.

This project report describes how fast-growing disease resistant hybrid clones of *E. tereticornis, E. urophylla, E. pellita* and *E. grandis* can be produced within a short time using marker assisted selection and mass multiplication through mini cuttings.

2. MATERIALS AND METHODS

Seed source and seedlings

Seeds of *E. tereticornis, E. urophylla and E. pellita* were collected from large trees maintained at Eucalypt provenance trial plots established at Kottappara in Kodadad range (Malayattoor Forest division) and *E. grandis* and *E. urophylla* from Vallakkadavu (Periyar Wildlife sanctuary) (Balasundaran and Maria Florence, 2007). The quality of mother trees in respect of height, girth, tree form, branching pattern and disease resistance were ensured before seed collection.

Seeds were sown on raised beds and in polythene bags at Kottappara and in KFRI Central Nursery at KFRI campus, Peechi during February 2008. Seedling growth was monitored continuously, especially for disease occurrence. Girth at collar region and height of seedlings were measured at the end of three months. Seedlings which showed apparently hybrid characters in leaf morphology, branching pattern, colour of twigs, petiole, etc. among progenies of each mother tree were tagged. Putative hybrid seedlings growing in polythene bags and transplanted from nursery bed were transported to KFRI provenance trial plot at Kottappara and field planted in 2m x 2m spacing. A maximum of 25 seedlings were selected from each mother tree. These seedlings were selected based on their height, girth at collar region and seedling vigour. The field-planted seedlings were monitored for occurrence of pink disease, leaf blight and gall formation every month, and girth and height measured every six months. Twenty five seedlings selected at random from apparently non-hybrid seedlings from each mother tree were also planted in the trial plot along with the putative hybrid seedlings.

A few of the putative hybrid seedlings raised in polythene bags and such seedlings growing in nursery beds at KFRI central nursery, Peechi were retained for molecular studies. The plants raised in polythene bags were transferred to large pots and pruned. The putative hybrid seedlings maintained on seedling beds were also coppiced retaining a stump of 20 cm height. The coppice shoots arising from pruned stumps were used for DNA extraction.

Development of microsatellite markers for parental identification

Development of microsatellite markers (SSR markers) is expensive as the process includes construction of genomic libraries, library screening, sequencing of clones, sequence analysis, and design of primers. Hence, initially, six EMBRA primers (Table 1) reported by Brondani *et al.* (1998) were used for amplification of microsatellites from the genome of the five species of Eucalypts. But the PCR amplification of target fragment did not give satisfactory result. As an alternative method, Inter Simple sequence Repeat-PCR (ISSR-PCR) amplification was done, which took advantage of the ubiquitously distributed SSRs in the eucalypt genomes (Van Der Nest *et al.*, 2000).

Juvenile leaf samples of mother trees of eucalypt species namely *E. pellita, E. urophylla, E. teriticornis* and *E. camaldulensis* were collected from KFRI eucalypt provenance trial plot established at Kottappara in Kodanad Range in Malayattur Division and *E. grandis* and *E. urophylla* from Vallakkadavu in Periyar Tiger Reserve. Genomic DNA was isolated from the juvenile leaf samples following the protocol of Doyle and Doyle (1990) with slight modifications. The purified DNA pellets were dissolved in double distilled water and the concentration of DNA was estimated using calf thymus DNA as standard and checked for purity on agarose gels. Six ISSR primers (Table 2) were employed for amplification of DNA samples from mother trees. All the PCR amplification reactions were performed in 25µl reaction volume.

Amplified products were visualized by horizontal gel electrophoresis on wide mini-sub-cell GT (Biorad, USA) in 1.5% agarose gel with 5µl aliquots of the PCR product and stained in an aqueous solution of ethidium bromide (10mg/ml). Primer 2, $((AG)_8CA)$ amplified a 700 bp band uniformly from DNA samples from all the species. These amplified DNA fragments were eluted out of agarose gel and purified for sequencing and microsatellite development at Xcelris Labs Ltd, Ahmadabad. The sequencing reactions were performed using BigDye terminator v3.1 cycle sequencing kit containing AmpliTac DNA Polymerase (Applied Biosystems). From the sequence data, 10 primer pairs capable of amplifying microsatellite regions in the five *Eucalyptus* species were constructed. Two sets of repeat motifs were

detected for each sequence and primers were designed to flank these microsatellite motifs, and product sizes ranging between 150-250bp regions were amplified.

Table 1. SSR locus and the primer sequence used for the amplification of SSR loci in *E. pellita* (Brondani *et al.*, 1998)

Sl. No.	Loci	Sequence
1	EMBRA 2-F	5'-CGT GAC ACC AGG ACA TTA C-3'
	EMBRA 2-R	5'-ACAAAT GCA AAT TCA AAT GA-3'
2	EMBRA 5-F	5'-ATGCTGGTCCAACTAAGATT-3'
	EMBRA 5-R	5'-TGAGCCTAAAAAGCCCAAC-3'
3	EMBRA 8-F	5'-CAC AAC TAA AAA TCA AAA CCC-3'
	EMBRA 8-R	5'-AAA GAG CAG ATT ATT ACA GAA GC-3'
4	EMBRA 10-F	5'-GTA AAG ACA TAG TGA AGA CAT TCC-3'
	EMBRA 10-R	5'-AGA CAG TAC GTT CTC TAG CTC-3'
5	EMBRA 13-F	5'-ATT TCC CTA GGT TTG ACA TG-3'
	EMBRA 13-R	5'-TCC AAC ATC TTA CTC AAC CA-3'
6	EMBRA 17-F	5'-AGG ATA CTC GTG AGA GAA GC-3'
	EMBRA 17-R	5'-GTA GAT CTG TTC TGC ATG TTG-3'

Table 2. List of ISSR primers used

Sl.No.	Sequence	
1	5'-CACACACACACACACAG-3'	$(CA)_8G$
2	5'-AGAGAGAGAGAGAGAGCA-3'	$(AG)_8CA$
3	5'-GAGAGAGAGAGAGAGATC-3'	$(GA)_8TC$
4	5'-AGAGAGAGAGAGAGAGT-3'	$(AG)_8T$
5	5'-CACACACACACACACAAG-3'	$(CA)_8AG$
6	5'-CTCTCTCTCTCTCTCTGC-3'	$(CT)_8GC$

All the ten SSR primers were tested for microsatellite amplification of genomic DNA samples from mother trees, putative hybrids and all the eucalypt trees growing near the mother trees for identification of pollen parents. PCR amplification reactions were performed using Finnzyme High Fidelity PCR Kit. PCR products also separated

electrophoretically on 12% denaturing polyacrylamide gel and bands were visualized using silver staining.

Multiplication of selected hybrid clones through mini-cuttings technique

Coppice shoots arising from stumps of putative hybrid seedlings of *E. pellita, E. urophylla* and *E. tereticornis* along with fast growing *E. tereticornis* clones maintained at KFRI clonal nursery at Kottappara were used for production of mini-cuttings. Forty five-days-old coppice shoots were made into single-noded cuttings, leaves pruned and the bottom stem tip treated with 4000 ppm IBA. The hormone-treated cuttings were planted in root trainers filled with vermiculite. The root trainers were kept in mist chamber used for mass production of rooted and sprouted cuttings. After 21 days, 10-12cm long sprouts arising from the rooted cuttings were carefully excised and the stem pieced into 3-6cm long mini-cuttings. After hormonal treatment, these cuttings were again planted in root trainers filled with vermiculite and kept for next generation rooting. Initially, 100 mini-cuttings were prepared from each of the KFRI clones and the putative inter-specific hybrid clones totaling 1000 cuttings. When these mini-cuttings produced sprouts of 8-10 cm height, they were removed from vermiculite and used for preparing new set of mini-cuttings. This cycle was repeated. Portions of sprouted mini-cuttings after 21 days growth in the mist chamber were transferred to hardening chamber. Hardened plants were subsequently field-planted for clonal testing.

3. RESULTS AND DISCUSSION

It has been reported that interspecific hybrids of *Eucalyptus* spp. provide tremendous scope for selection of potential hybrid plants among them for clone development. The selection has to be appropriate so that the clone should be fast growing, disease and pest resistant and locally adapted. Conventional technique of hybridization, screening F_1 plants through field trial, selection of fast growing, disease resistant F_1 hybrid plants and their mass multiplication through rooting of coppice shoot cuttings are labour intensive and time consuming. Hence, utilization of hybrid plants occurring through natural hybridization of *Eucalyptus* spp. and early selection of promising hybrid plants of appropriate parental combination through DNA marker-assisted selection have been reported to be a quicker, alternative method. In this project, natural hybrid plants available from the seeds collected from KFRI Eucalypt provenance trial plots maintained at Kodanad, in Malayattur Division and Vallakkadavu in Thekkady Wildlife sanctuary are utilized for clone development. Microsatellite markers have been developed for identification of pollen parent. Micro-cutting technology has been standardized for quicker mass multiplication of appropriate hybrids for field screening and development of clones.

Collection of seeds

Seeds were collected from provenance plantations established during 1990-1993. While seeds of *E. pellita, E. tereticornis, E. camaldulensis* and *E. urophylla* were collected from KFRI eucalypts provenance trial plot at Kottappara, *E. grandis* and *E. urophylla* seeds were collected from Provenance trial plot established at Vallkkadavu in Periyar Wildlife sanctuary (Table 3).

Growth of plants

Disease and pest incidence, and height of seedlings and field-transplanted plants of putative hybrids (Fig. 1-2) and control plants were measured periodically. (It was not

Table 3. Eucalypt species and the number of trees from which seeds were collected

Species	No. of mother trees
E. pellita	6
E. tereticornis	6
E. urophylla	7
E. grandis	2

possible to take timely observations and measurements of a few of the field-planted trees at 18th month due to continuous wild elephant attack in the plot at KFRI clonal nursery and experimental field established at Kottappara in Kodanad Range. It was for the first time in the last 20 years after the establishment of clonal nursery at Kottappara, elephant herd invaded the plot from distant natural forest). Mean height of seedlings, and height and girth of field-planted trees are provided in Table 4 & 5 and Fig. 3-5. Measurements showed that putative hybrid seedlings of all the species showed more height and girth than those of normal seedlings. Field planted putative hybrid plants of *E. urophylla* showed highest percentage increase (9.6-62.8%) in girth and height measurements over normal plants.

Disease incidence

Seedlings and field-transplanted plants of all the species were subjected to scoring for the occurrence of pink disease caused by *Corticium salmonicolor,* leaf blight caused by *Cylindrocladium* spp. and gall formation caused by *Leptocybe invasa* (Fig.6). Naturally uninfected putative hybrid plants were artificially inoculated (Fig.7) with *C. salmonicolor* isolated from infected plants of *E. tereticornis* in order to confirm the resistance of the hybrids against pink disease so that such resistant and fast growing interspecific natural hybrids can be used for cloning (Fig.6 c). Normal seedlings of *E. camaldulensis* as well as putative hybrid seedlings of *E. pellita, E. urophylla, E. grandis* and *E. tereticornis* showing *E. camaldulensis* morphological features (as pollen parent) showed severe incidence of CLB. Besides CLB, serious incidence of pink disease was also observed in such field-planted seedlings after one year. Hence, *E. camaldulensis* and all the putative hybrid seedlings having *E. camaldulensis* leaf and stem characters were excluded from the study.

a b

Fig. 1. a, *E. pellita* seedlings in the nursery; b, *E. pellita* seedlings showing hybrid characters (in pots).

Fig.2. Field-planted *E. pellita* and *E. urophylla* plants including putative interspecific hybrids

Table 4. Mean height and girth of all seedlings and putative hybrid seedlings upto 10 month growth

Species	Provenance name	Tree No.	Mean height (cm) and standard error of all seedlings in KFRI nursery during 5th month	Mean height (m) and standard error of all seedlings in field trial during 10th month	Mean height (m) and standard error of Inter-specific hybrids in field trial during 10th month
E. pellita	Between GGOE-Kiriwa, PNG	P1	0.32±0.06	0.81±0.08	2.58±0.07
		P2	0.33±0.36	0.86±0.13	2.45±0.07
		P3	0.29±0.04	0.82±0.12	2.49±0.08
		P4	0.30±0.08	0.94±0.12	2.13±0.06
		P5	0.32±0.06	0.99±0.16	2.28±0.07
		P6			*
E. tereticornis	Cardwell, QLD	T1		1.75±0.08	*
	40K N of Gladstone, QLD	T2		1.84±0.11	*
	40K N of Gladstone, QLD	T3		1.87±0.18	*
	Kennedy CK Penden RD, QLD	T4		1.25±0.14	*
	Palmer River, QLD	T5			*
	S.of Helenvale, QLD	T6			*
E. urophylla	Mt. Lewotobi Flores, IND	U1		1.95±0.42	2.93±0.18
	Mt.Wuko Flores Is, IND	U2		2.05±0.22	*
	Mandiri Flores, IND	U3		2.11±0.21	*
	Mount Mutis	U4		2.28±0.28	*
	S. of Hato Bulico, IND	U5			*
	Unknown	U6			*
	Unknown	U7			*

Table 5. Mean height and girth of all seedlings and putative hybrid seedlings from 10-18 months growth

Species	Provenance name	Tree No.	Mean height (m) and standard error of all seedlings in field trial during 18th month	Mean height (m) of inter-specific hybrids in field trial during 18th month and % increase over mean height of all seedlings in parenthesis	Mean girth (cm) and standard error in field trial of all seedlings during 18th month	Mean girth(cm) of inter-specific hybrids in field trial during 18th month and % increase over mean girth of all seedlings in parenthesis
E. pellita	Between GGOE-Kiriwa, PNG	P1	2.72±0.18	*	6.8±0.51	*
		P2	2.48±0.05	3.23(30.2)	6.36±0.50	8.5(33.6)
		P3	2.68±0.17	3.28(22.4)	7.07±0.55	7.2(1.8)
		P4	2.91±0.16	3.51(20.6)	6.97±0.50	7.6(9.4)
		P5	3.00±0.15	3.41(13.7)	7.61±0.50	9.0(18.3)
		P6	2.91±0.05	*	5.67±0.04	*
E. tereticornis	Cardwell, QLD	T1	3.43±0.18	3.62(5.5)	7.33±0.58	9.1(24.1)
	40K N of Gladstone, QLD	T2	3.24±0.19	*	7.30±0.88	*
	40K N of Gladstone, QLD	T3	2.99±0.20	4.57(52.8)	6.11±0.47	9.3(52.2)
	Kennedy CK Penden RD, QLD	T4	3.53±0.14	*	7.61±0.47	*
	Palmer River, QLD	T5	2.37±0.15	*	6.7±0.34	*
	S.of Helenvale, QLD	T6	1.95±0.10	*	4.2±0.43	*
E. urophylla	Mt. Lewotobi Flores, IND	U1	3.73±0.21	4.47(19.8)	8.76±0.56	10.7(22.1)
	Mt.Wuko Flores Is, IND	U2	3.15±0.12	3.76(19.4)	8.12±0.29	8.9(9.6)
	Mandiri Flores, IND	U3	2.28±0.17	3.44(50.9)	5.92±0.49	8.0(35.1)
	Mount Mutis	U4	2.77±0.22	4.60(39.8)	7.37±0.58	12.0(62.8)
	S. of Hato Bulico, IND	U5	2.08±0.11	*	4.17±0.36	*
	Unknown	U6	1.68±0.09	2.33(38.7)	4.62±0.42	6.2(34.2)
	Unknown	U7	1.92±0.13	2.77(44.3)	4.95±0.44	7.8(57.6)

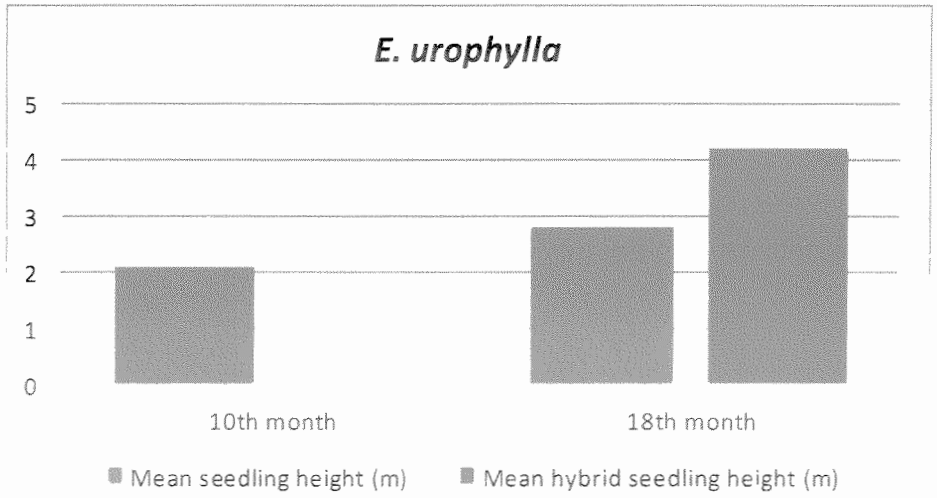


Fig.3. Mean height of *E. urophylla* seedlings and putative hybrid seedlings

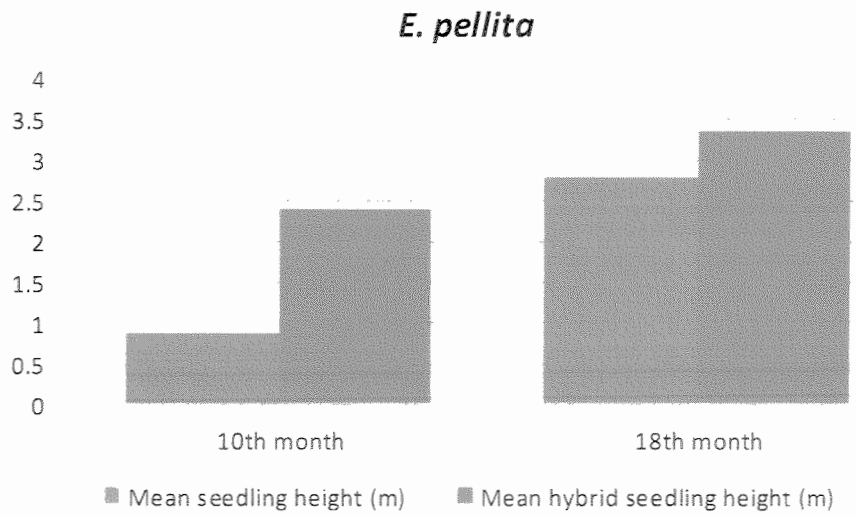


Fig.4. Mean height of *E. pellita* seedlings and putative hybrid seedlings

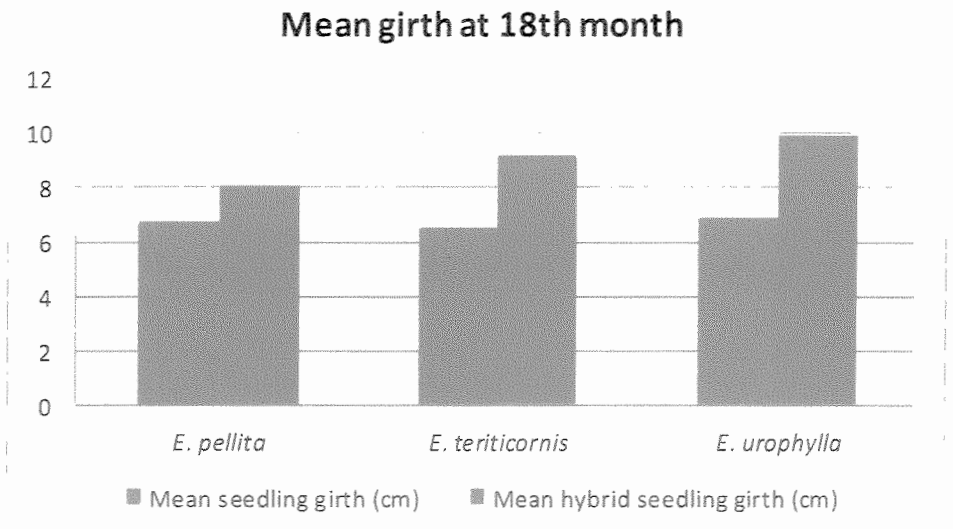
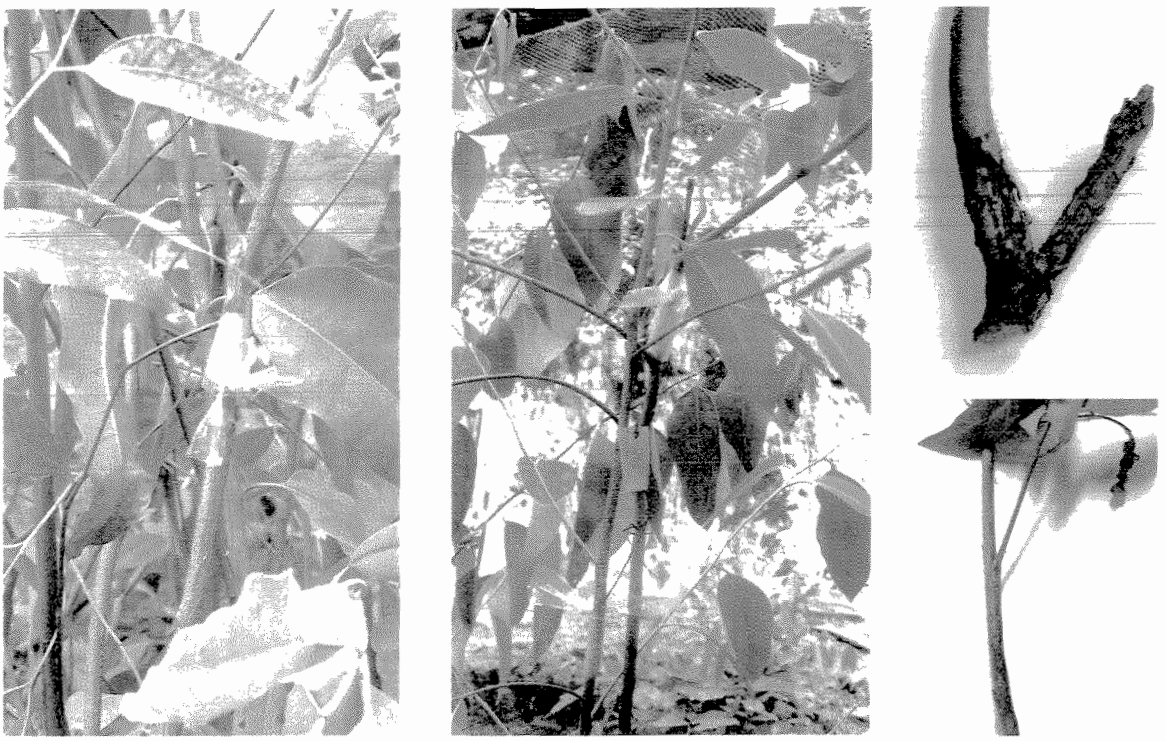


Fig.5. Mean girth of *E. pellita, E. tereticornis* and *E. urophylla* seedlings and putative hybrids at 18 months

a b c

Fig. 6. a, Pink disease affected (natural) *E. urophylla* putative hybrid; b, *Leptocybe invasa* gall-infected *E. tereticornis* hybrid; c, Disease-free *E. pellita* hybrid stump with coppice shoot initials



a b c

Fig. 7. Putative hybrid plants artificially inoculated with *C. salmonicolor* for testing disease resistance. a, *E. pellita ;* b, *E. urophylla;* c, *E. tereticornis* (infected branches)

Development of microsatellite markers for identification of pollen parents

Initially, the following EMBRA primers (Brondani *et al.*, 1998) were used for amplification of microsatellites from the genome of *E. pellita, E. tereticornis, E. urophylla, E. grandis* and *E. camaldulensis* (Table 1). But appropriate marker for differentiating the five eucalypt species, especially *E. pellita* was unavailable. Hence, new Simple Sequence Repeat (SSR) markers are developed after designing efficient primers from sequences of PCR-amplified Inter-simple sequence repeat (ISSR) fragments. The sequence of the ISSR markers are given in Table 2.

Development of new microsatellites from ISSR amplified DNA

All the six ISSR primers (Table 2) amplified a variety of fragments ranging from 100 to 10,000 bp in size when DNA from each of the four species of Eucalypts was used for PCR-amplification. The DNA banding profile for each primer was also different. Among the 6 primers, the most suitable primer which produced banding in all the species of mother plants was primer 2 ($(AG)_8CA$) and it was selected as the best for PCR-amplification. The amplified fragment was a 700 bp DNA band (Fig. 8), uniformly present when all Eucalypt DNA samples were amplified. This fragment was used for nucleotide sequencing and microsatellite development.

The single band was eluted out of agarose gel, purified and sequenced. The sequencing was done at Xcelris Labs Ltd (Ahmadabad). The sequencing reactions were performed using BigDye terminator v3.1 cycle sequencing kit containing AmpliTac DNA Polymerase (Applied Biosystems). From the sequence data, 10 SSR primer pairs capable of amplifying microsatellite regions in five *Eucalyptus* species were constructed (Table 6). Two sets of repeat motifs were designed for each sequence and primers were designed to flank these microsatellite motifs. The amplified product sizes ranged between 150-250bp regions.

All the 10 amplified DNA products showed SSR motifs when sequenced. They included dinucleotide repeats (GA)n, (TC)n, (AC)n; trinucleotide repeats (ACC)n, (TCC)n, (CTC)n and polynucleotide repeats (CCGG)n, (CTTC)n. Primer EP1 amplified a perfect $(TC)_8$

dinucleotide repeat and $(CCGG)_4$ tetra repeat, whereas EP2 amplified a perfect $(ACC)_6$ trinucleotide repeat along with many other repeats in *Eucalyptus pellita*. Primer EG1 amplified a perfect $(TC)_7$ dinucleotide repeat and $(CAC)_6$ and $(CTC)_4$ trinucleotide repeats while EG2 amplified a perfect $(TC)_6$ dinucleotide repeat and $(ACC)_7$ trinucleotide repeat and plenty more in *Eucalyptus grandis*. Primer EU1 amplified a perfect $(CTTC)_6$ tetra repeat when EU2 amplified a $(CCGG)_8$ tetra repeat and $(ACC)_9$ trinucleotide repeat with several other repeats in *Eucalyptus urophylla*. Primer ET1 amplified a $(CTTC)_7$ tetra repeat and $(GA)_6$ dinucleotide repeat where ET2 amplified a $(CT)_8$ dinucleotide repeat and $(CCGG)_5$ along with various others in *Eucalyptus tereticornis*. Primer EC1 amplified a perfect $(GCG)_9$ trinucleotide and $(AG)_8$ dinucleotide repeat along with lots of other repeats in *Eucalyptus camaldulensis*. Mononucleotide repeats (A)n, (T)n were also amplified by some primers. All the ten primer pairs cross amplified all the five *Eucalyptus* species included in the study. This result could be of great economic importance as it lowered the difficulty of creating new species-specific SSR primers for each *Eucalyptus* species through cloning.

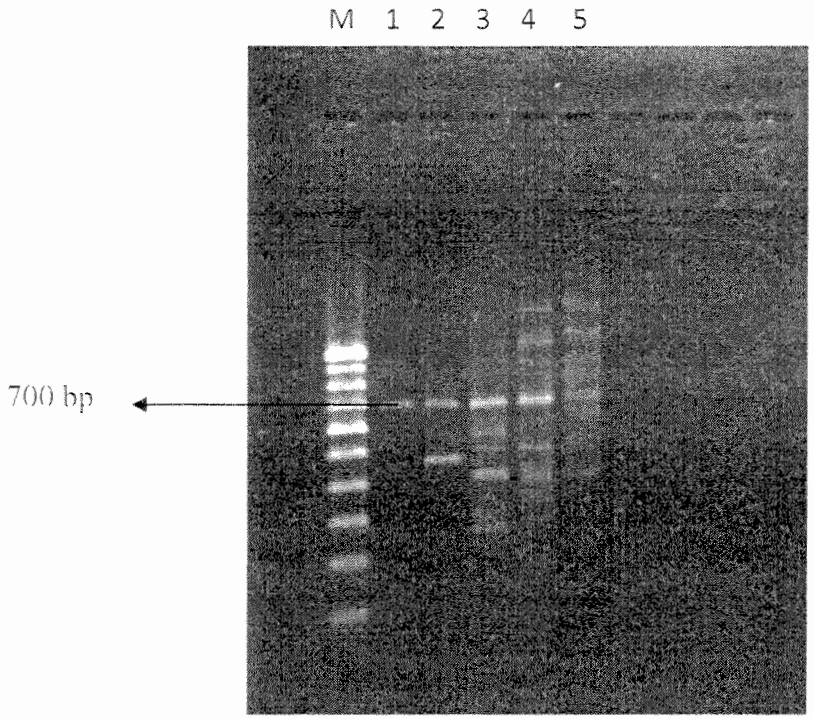


Fig. 8: Amplification and selection of 700bp band of ISSR-PCR products using primer 2 ($(AG)_8CA$) for sequencing. M: 100bp marker, 1: *Eucalyptus pellita*, 2: *Eucalyptus grandis*, 3: *Eucalyptus urophylla*, 4: *Eucalyptus teriticornis*, 5: *Eucalyptus camaldulensis*

Table 6. Microsatellite primers developed for identification of pollen parent of putative hybrid clones

Sl. No	Primer Type	Primer Sequence (5' to 3')
EP1	Forward	TGCTTCAGCACCACCTCATA
	Reverse	ATCCATTGTTGCCCCTGAG
EP2	Forward	GGCTTCGTCACATCCAAATG
	Reverse	CCTCATCCCTGCTCAATCTC
EG1	Forward	GTTTGCCAATCCTGCCTCT
	Reverse	ATGTCTGGCGTCCTTCTTTG
EG2	Forward	CCGAGACCACACTCACCAC
	Reverse	GCGAAACCCGAGTAGAGAAT
EU1	Forward	TCTGCTCCCTCACCAATTCT
	Reverse	ATGTCTGGCGTCCTTCTTTG
EU2	Forward	CCGAGACCACACTCACCAC
	Reverse	CAAGAGCGAAACCCGAGTAG
ET1	Forward	CTTCGTCCATCCAAATGCTC
	Reverse	CAGGGCGAGCAAGAAACA
ET2	Forward	CCACCTCATACACCCTCTCC
	Reverse	GAATCCAAAGATCCATTCTTGC
EC1	Forward	GGCTTCGTCCATCCAGAG
	Reverse	CGCATCCCTGCTCAATCT
EC2	Forward	CTCGGCCTCCTCTGTTTCTT
	Reverse	GGCGAAACCCGAGTAGAGA

Standardization of mini-cutting technique

For standardization of mini-cutting technique, four popular *E. tereticornis* clones and one *E. camaldulensis* clone were compared with two putative hybrid plants each of *E. pellita* and *E. urophylla* and one of *E. teretiornis*. These putative hybrids were fast growing and

disease resistant. The mini-cuttings were prepared from 10-12 cm long sprouts arising from the rooted coppice shoot cuttings. The sprouted mini-cuttings showed profuse root system (Fig.9). Such mini-cuttings from rooted cuttings of putative hybrid plants showed higher percentage of success and establishment in the field. While the percentage of success in sprouting and establishment of first generation mini-cuttings varied from 13-92% for putative hybrids, the percentage of success for popular KFRI clones ranged between 2-43% only (Fig.10 and 11). This showed the superior efficiency of hybrid clones in mass multiplication through the mini-cutting technique. However, there was wide variation in the percentage of success. While the *E. tereticornis* putative hybrid clone showed the highest (92%) percentage of success, *E. pellita* and *E. urophylla* hybrids showed 12-51% success only. This shows that the technique needs further standardization and testing of more number of putative hybrid clones to improve the success percentage. Commercial hybrid clones should give more than 90% success with mini-cuttings.

a b

Fig. 9. a, A mini-cutting of *E. tereticornis* interspecific hybrid planted in vermiculite showing new stem. b, close up of the mini-cutting with elongating stem and profuse root system.

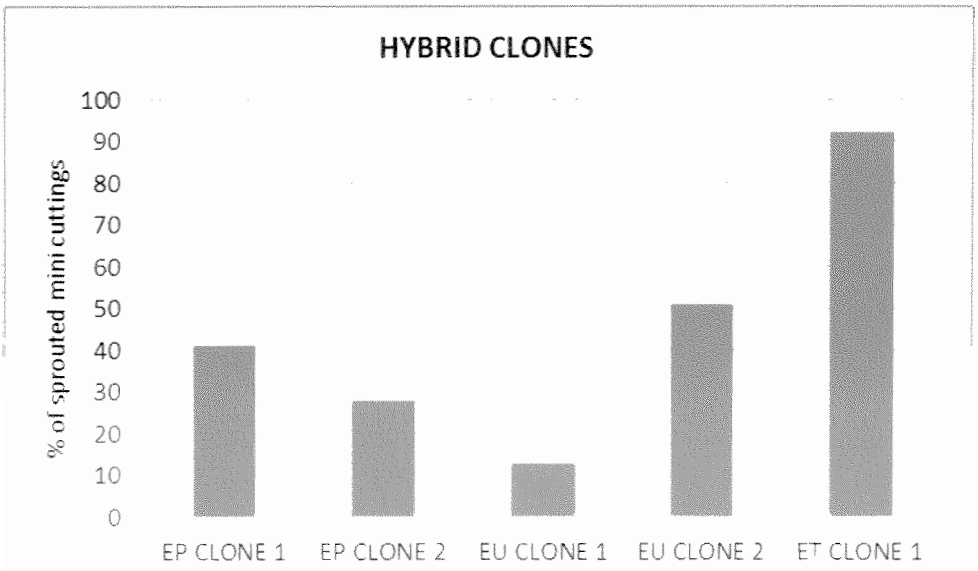


Fig.10. Percentage of success of mini cutting technique of mass clonal multiplication for three species of putative hybrid plants

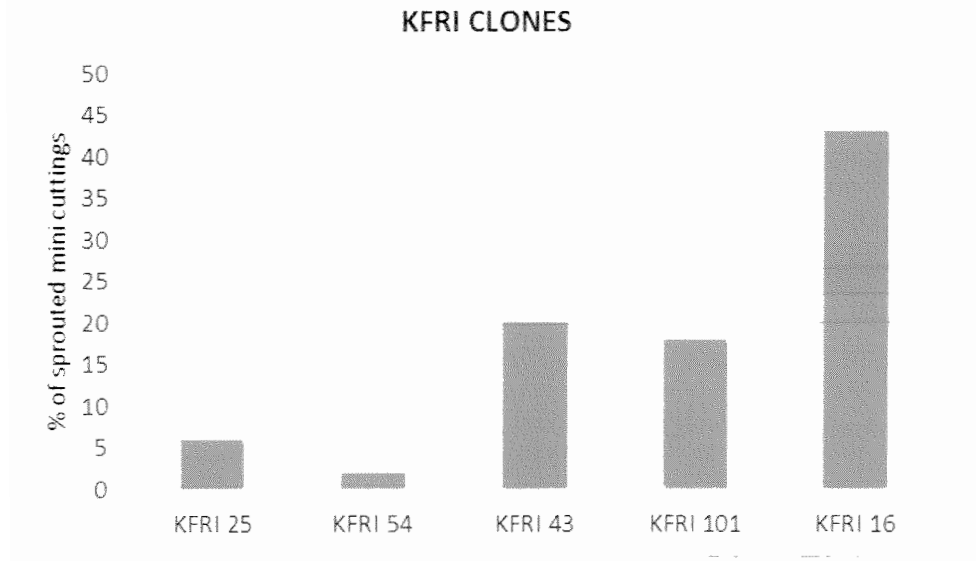


Fig.11. Percentage of success of mini cutting technique of mass clonal multiplication for four KFRI clones

4. CONCLUSIONS

Interspecific hybrids occurring through natural pollination among different *Eucalyptus* spp. planted in provenance trial plots are a mixture of desirable and undesirable genotypes. Large genetic variations occur among the hybrids in respect of growth, adaptability, stem form and pest and disease susceptibility. Hence, intensive selection with combinations of as many desirable characters as possible has to be carried out for identifying a heterotic hybrid genotype for development as an interspecific hybrid clone. Quick identification of the pollen parent of natural interspecific hybrid is now possible at seedling stage itself with DNA marker studies, especially through microsatellite markers. This is achieved through comparison of DNA fingerprints of mother plant and the hybrid with those of all the possible pollen parents using appropriate population genetics computer package. Knowledge of the pollen parent at the seedling stage itself gives an indication of the desirability of the hybrid genotype and provides an opportunity to eliminate undesirable ones. A quicker method for mass clonal multiplication enhances the speed of developing new clones from such interspecific hybrids. This is possible through adoption of mini-cutting technique of clonal multiplication of hybrids. However, the percentage of rooting and sprouting of hybrid clones depend upon the genotype. Hence, another round of selection based on success percentage in clonal multiplication is necessary for achieving mass multiplication of interspecific eucalypt hybrids for raising large scale industrial plantations. The present study showed rooting superiority of *E. tereticornis* hybrids. But *E. tereticornis* hybrids are more susceptible to pest and diseases than *E. pellita* hybrids. Therefore, a large number of *E. pellita, E. urophylla* and *E. grandis* hybrids have to be screened at seedling stage itself through the microsatellite marker studies for identification of best parental combinations. Mini-cuttings technique can be adopted for mass clonal multiplication of such hybrids for commercial planting.

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