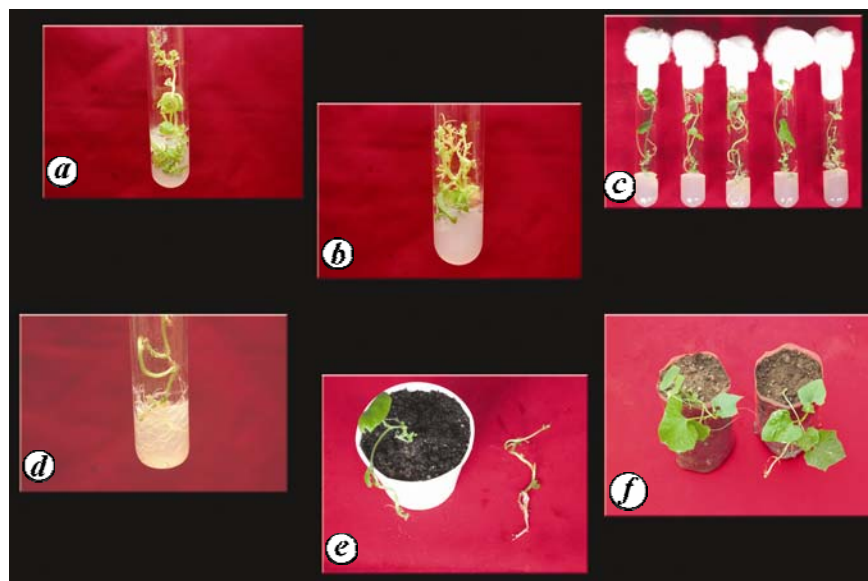

Figure 2. ***a***, Regeneration of male sterile plant from the field in establishment medium, MS + IAA 1.5 mg l^{-1} + BAP 2 mg l^{-1}; ***b***, Multiple shoot formation of male sterile line; ***c***, ***d***, *In vitro* rooting and shoot growth of male sterile line; ***e***, ***f***, *Ex vitro* establishment of male sterile line.

field. But the rate of survival during hardening was found to be low (40%). Different rooting media were thus tried to induce healthier roots among which hormone-free medium with ½MS salts alone was found to give the best results (Figure 2 *d*). There were numerous healthy and strong roots in the basal medium which helped the plants to establish efficiently during the hardening process (Figure 2 *e*). The rooted plants were transferred to the mist house in polybags where they were kept for a month before transplanting to the main field (Figure 2 *f*). Tissue culture plants took 50 days after transplanting into the field for flowering and were all male sterile in nature. This protocol can be used for maintenance and multiplication of male sterile ridge gourd plants.

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Assessment of *Pongamia pinnata* (L.) – a biodiesel producing tree species using ISSR markers

Pongamia (Indian beech) is a non-edible oil-producing tree legume (Syn: *Pongamia glabra* Vent.), that has recently gained importance for its oil utility as biodiesel[1]. Various parts of Pongamia are used in medicinal and other applications. To date, there is little information regarding the germplasm of this tree. *In vitro* regeneration protocols have been standardized for clonal propagation[2,3] and for application in gene-transfer technologies towards its improvement. However, for commercialization of the protocol, the genetic fidelity of the *in vitro*-raised clones needs to be assessed using molecular markers. Moreover, the wide range of variability among accessions of Pongamia in oil content, seed and pod morphology, *in vitro* response, etc. necessitates the understanding of its germplasm using molecular markers. Hence the present work was undertaken to study the fidelity of the *in vitro*-raised clones and the variability among the selected trees using ISSR markers.

Among the propagules raised *in vitro* from mature plant-derived axillary buds[2,4], 12, 13 and 12 propagules from three mother trees identified as P1, P2 and P3 respectively, were tested for fidelity analysis. Ten trees of the same height (~ 12 ft) from the natural population were identified randomly as source of explants in our earlier study[2]. Of these, six trees that were from the population at location-A (Range hills) and four trees from the population at location-B (Pashan), were tested for variability studies using ISSR markers.

Total DNA from fresh leaves was isolated from samples for both studies using miniprep method. All 100 UBC–ISSR primers were screened with the samples for amplification products. ISSR–PCR reaction was performed with 20 ng template DNA; *Taq* buffer with 1.5 mM $MgCl_2$; dNTPs (1 mM/μl); *Taq* polymerase (3 units/μl); ISSR primer (1.5 pm/μl); Spermidine (20 mM) and reaction volume of 25 ml. Amplifications were performed on a Stratagene Robo-

Cycler for 44 cycles with annealing at 50°C. Among the primers that gave reproducible and precise amplification products, three were chosen for fidelity analysis, and five were chosen for variability studies. Presence/absence of each scorable fragment was recorded in a binary data matrix, and the percentage of polymorphism was determined. Similarity matrix and dendrogram were generated from the data.

Characters like seed oil content, pod and seed morphology were also studied for correlation with marker studies. A simplified method using hexane was used for quantification of oil. The oil content was expressed in grams per 10 g of seeds and the percentage of oil content was calculated. Morphological characters like shape and size of the pods, number of seeds per pod, and colour and shape of the seeds were noted. Pods were collected from the base of the inflorescence, as they were the most mature ones. These were pooled from several inflorescences of the same tree.

Pongamia species show significant variability in phenotype as well as chemotype and are not well documented for unique marker profiles at the DNA level. The results of ISSR primer screening in Pongamia suggest that dinucleotide repeat motifs occur at high frequencies along the genome similar to crops like bean, rice, maize and soybean. Thus, the three di-nucleotide ISSR primers (UBC set # 9-807, 808, 809) having AG, GA repeats were chosen for the fidelity analysis. Polymorphism was not detected among the *in vitro*-raised plantlets that gave rise to 107 amplification products. Similar observations were noted in *Swertia chirayita*[5] and gerbera[6].

However, genetic variability between the three mother plants tested during fidelity studies indicated the possibility of genetic differences among the plants, which responded differently in culture. Thus, the variability study was carried out in ten trees using ISSR markers with three di-nucleotide repeat motifs (UBC set # 9-807, 808 and 809). Since it produced more polymorphic bands (80%), the study was extended with a di-nucleotide primer (UBC 890) having degenerate primer sequence and penta-nucleotide repeat primer (UBC 881) to resolve the relative frequency of occurrence in the Pongamia genome (Figure 1 *a* and *b*). UBC 890 did not produce a distinct band pattern, probably due to the degeneracy of the bases. Generally, the pentanucleotide primers are less frequent and their use in ISSRs is less. But in Pongamia, they produced maximum (25) scorable bands (see Table 1), which showed their relative abundance in this genome, and produced the largest amplification fragment of 2.5 kb.

The similarity matrix was calculated based on Jaccard's similarity coefficient which ranged from 0.38 to 1.00. Plants selected from the population of location-B were more closely related than those selected from location-A. Tree-10 and tree-8 were genetically similar. Tree-5 was closely related to tree-3 from the same location. This could be due to population similarity. Cluster analysis also produced similar results (Figure 1 *c*). Overall, high level of polymorphism was obtained in Pongamia (76%), as reported in other species – 62% in sweet potato[7] and 65.2% in Juglans[8].

Knowledge of genetic variability and its association with oil content, seed and pod traits is mandatory for genetic improvement of Pongamia[9]. In the present

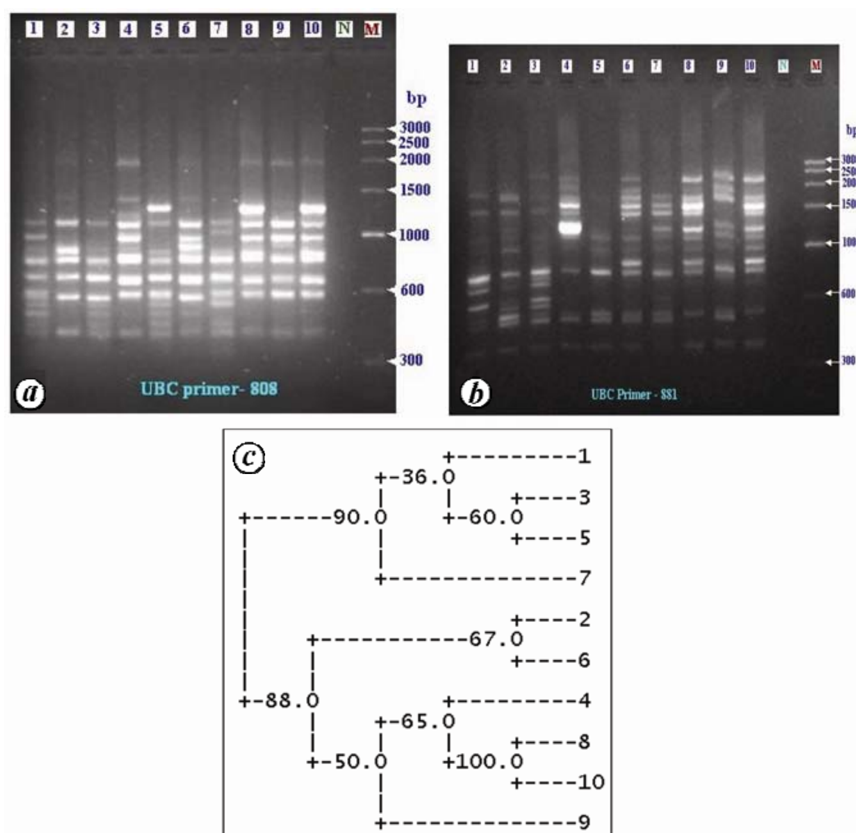

Figure 1. ***a***, ***b***, Amplification products obtained in ten trees using UBC 808 and UBC 881 primers respectively. Lane 1–10; Trees in order; lane M; Low-range DNA ruler (3 kb) and lane N; Negative control. ***c***, Dendrogram showing genetic relationship among ten trees of Pongamia generated using ISSR data.

Table 1. ISSR analysis of the ten *ex vitro* grown trees for variability studies

Primer ISSR	Total no. of bands	Number of bands scored	Percentage of polymorphic bands	Range of amplified bands (bp)
UBC-807	22	14	85.7	366–2000
UBC-808	23	15	73.3	433–1850
UBC-809	18	10	80	360–1410
UBC-881	32	25	76	480–2250
UBC-890	16	11	63.6	475–2500
Total	111	75	76% (average)	

Table 2. Oil content of Pongamia seeds of ten different trees collected from two locations

Location	Tree	Oil content (g/10 g) Mean ± SD	Percentage of oil (w/w)
A	1	2.40 ± 0.08	24.0
	2	2.20 ± 0.16	22.0
	3	2.11 ± 0.06	21.1
	4	1.25 ± 0.03	12.5
	5	1.84 ± 0.10	18.4
	6	2.00 ± 0.17	20.0
B	7	2.68 ± 0.11	26.8
	8	2.33 ± 0.02	23.3
	9	2.80 ± 0.06	28.0
	10	2.48 ± 0.33	24.8
ANOVA		S 1%	
		CD at 5% – 0.238	

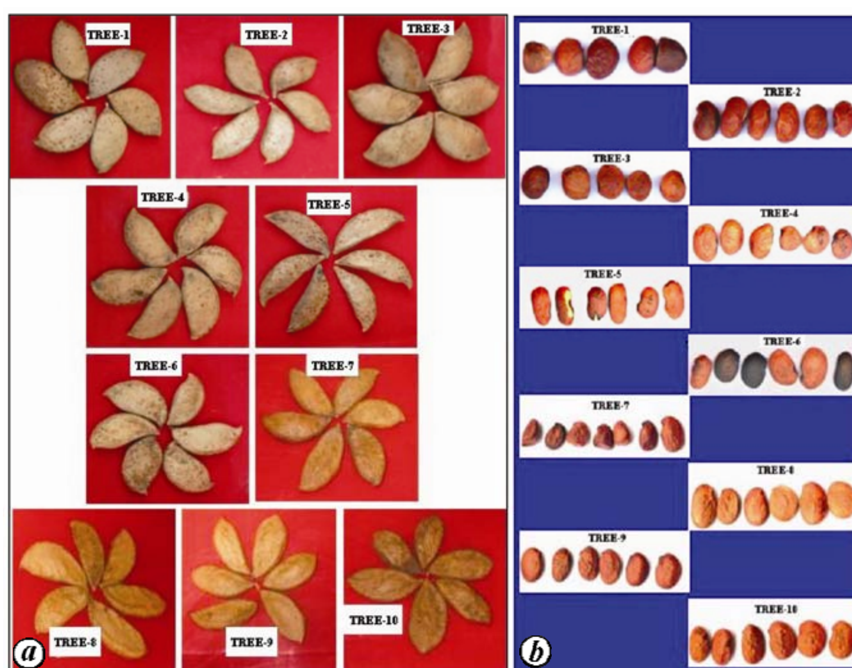

Figure 2. Pods and seeds collected from ten trees.

study, the oil content recovered from 10 g of seeds ranged from 1.25 to 2.8 g (Table 2). The high oil (28%)-bearing tree-9 did not show any distinguishable banding pattern with the primers tested. Tree-4, which had an oil content of 12.5%, demonstrated a noticeable doublet fragment of ~1250 bp with pentanucleotide primer 881. Morphologically, the pods and seeds of the selected trees growing in close proximity in either location appeared different. Similar observations were reported by Naresh *et al.*[10]. The seed colour ranged from light reddish-brown to dark brown. Seeds were more rounded in tree-3 and were long in tree-5. Tree-5 having elongated pod and seed showed no amplified fragment above 1500 bp (Figure 2 *a* and *b*). The seeds of tree-5, tree-8 and tree-10 were elongated and these samples showed distinct amplification fragment of ~1300 bp with the primer 808.

In conclusion, fidelity studies undertaken on the *in vitro*-raised Pongamia plantlets produced no polymorphic band patterns. This confirmed the uniformity of the clones and validated the reliability of the protocol developed for clonal propagation of this species. On the contrary, high degree of variability (76%) among the selected trees in variation analysis suggests applicability of the tissue culture techniques for multiplication of high oil-yielding Pongamia trees and exploration of desired wild germplasm characteristics. The present work advocates further study on the extent of variability existing in the characters like oil content, seed or nature of the pod with detailed molecular profiling.

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