PROGENY TESTING AND GENETIC DIVERSITY ANALYSIS IN PLUS TREES OF *Melia dubia* Cav.

by

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(2019-17-009)

THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"PROGENY TESTING AND GENETIC DIVERSITY ANALYSIS IN PLUS TREES OF *Melia dubia* Cav."** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis entitled **"PROGENY TESTING AND GENETIC DIVERSITY ANALYSIS IN PLUS TREES OF *Melia dubia* Cav."** is a record of research work done independently by Mr. Shifin S. Ravuther under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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We, the undersigned members of the advisory committee of **Mr. Shifin S. Ravuther,** a candidate for the degree of **Master of Science** in Forestry with major in forest biology and tree improvement, agree that the thesis entitled **"PROGENY TESTING AND GENETIC DIVERSITY ANALYSIS IN PLUS TREES OF *Melia dubia* Cav."** may be submitted by Mr. Shifin S. Ravuther, in partial fulfilment of the requirement for the degree.

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1. INTRODUCTION

Melia dubia Cav. also known as Malabar neem, is a fast-growing tropical moist deciduous species coming under the family Meliaceae. It is naturally distributed in Sikkim, Himalayas, the upper part of northeastern states, Khasi Hills, Odisha, Deccan region and the Western Ghats at an altitude of 1400 – 1900 m (Shrivastava *et al.,* 2017). It is an excellent raw material for wood-based industries like plywood, matchwood and paper for its high strength and pulp recovery. The low lignin content in the wood makes the species much in demand in the paper industry for getting high-quality paper. In agroforestry systems, the tree had become an important component owing to its potential role as an alternative to exotics and fodder production (Bijalwan and Dobriyal, 2015). Being a fast-growing crop with a shorter rotation period, the *M. dubia* timber gives a higher rate of return in terms of both pulp as well as money compared to other fast-growing timber species in the timber market like eucalyptus and casuarina . These factors had attracted large plantation fields to get crowded with this species. A 40 cm girth tree (two-year-old) can be sold at the timber market at a minimum rate of Rs. 2000 per ton for the pulp and veneer making industry (Parthibhan *et al.,* 2009). Wood is also widely used for making furniture, agricultural implements, packing cases, ceiling planks, pencils and musical instruments due to its moderately hard, light, easy to saw and durable nature (Chauhan and Kumar, 2014).

Industrially the tree has also been put into various medicine and pesticide component preparation. Each part of this plant is observed to be efficient for the preparation of traditional herbal medicines that can treat leprosy, eczema, asthma, malaria, fever, venereal diseases (Govindachari, 1992), cholelithiasis, acariasis and body pain (Kokwaro, 1976). They also possess anthelmintic, antiviral, carminative, antineoplastic properties (Vijayan *et al.*, 2004; Susheela *et al.*, 2008). The potential of *M. dubia* as an effective biopesticidal and pharmacological agent is due to the presence of phytochemicals like linolenic acid, palmitic acid, caryophyllene, humulene, aromadendrene, probucol, germacrene-d, phthalic acid and 6-ethyl-3-octyl. The leaf oil is reported to exhibit bacteriostatic, fungistatic

and antifeedant activity (Goswami *et al.,* 2020). Among the phytochemical caryophyllene plays a crucial role in providing the plant medicinal as well as insecticidal property.

Being a versatile species in nature, *M. dubia* has a wide diversity at its genetic level, which is expressed through its morphological and physiological traits. Studying these can help researchers use this information as a source for improved tree breeding and improvement programmes, which will further help in the development of improved tree varieties and enhance the genetic gain for several useful traits. This kind of studies ensures the vitality of trees as a whole by their capacity to withstand diverse biotic and abiotic stressors under changing and unpredictable environmental conditions. Progeny studies in nursery conditions revealed that there exists variation in growth and physiological traits among plus tree seed progenies from different natural population of *M. dubia* distributed in Kerala (Binu and Santhoshkumar, 2019). However, their performance in the field was not evaluated. Little information is available on *M. dubia* germplasm diversity in Kerala. Hence, it becomes necessary to catalog the natural diversity of the species to raise good quality planting stock and preserve the valuable species for long term use. In this context, the documentation of genetic variation in different populations of *M. dubia* would facilitate screening genetically diverse and productive parents in individual populations of different geographical origin and help in crop improvement, conservation, sustainable management and utilisation. There is also a need to study the variation of a phytochemical, caryophyllene among the individual plus tree seed progenies, for better documentation of its phytochemical character with respect to different location in kerala and there by individual with higher caryophyllene be identified. With this background, a study was conducted with the following objectives.

1. To evaluate the field performance of plus tree seed progenies of *Melia dubia*.
2. To assess the molecular genetic diversity in *Melia dubia* plus tree seed progenies.
3. To quantify caryophyllene in plus tree seed progenies.

2.REVIEW OF LITERATURE

The review of literature on progeny testing and genetic diversity analysis in plus trees of *Melia dubia* Cav. are described in this chapter.

2.1. Growth and general description

Melia dubia also known as Malabar neem belongs to the family Meliaceae, is an industrially and economically important fast-growing multipurpose tree species found across India and other countries like Southeast Asia, Australia (Kumar and Aiswarya, 2017). For its higher productivity return from the plantations and agroforestry farms the species has been recognized widely across the global timber (Goswami *et al.,* 2020). The plant can adapt to a wide range of environments and can thrive in areas with a mean annual temperature of 23°C-27°C and rainfall of 350-2000 mm. In India, the tree can be found across Very Moist Teak Forests (3B/C1a), Secondary Moist Deciduous Forests (3C/2S1), and Dry Mixed Moist Deciduous Forests (5B/C2). In its natural habitat, it is seen at a height of 600 - 1,800 m in the Himalayan parts of Sikkim, Northern Bengal, Assam, the Khasi Hills, the Deccan Plateau, the hilly regions of Orissa, and the Western Ghats (Gamble, 1992). In Kerala, it is found across semi-evergreen and moist deciduous forests (Nair, 1991).

In its natural habitat the can trees grow up to a maximum height of 25 m and girth of 1.5 m at its breast height. The bark is smooth, greenish when it is young, and turns dark brown with fissures on maturation. The leaves are compound, which are bipinnate in nature. The stalks of the leaves elongate up to 90 cm. The leaves start shedding during December and new young leaves start sprouting from February and March. Flowering occurs at the upper axil of the terminal panicle from February and March. A large number of flowers with star-shaped hairy petals of greenish and white colour are formed during this month. Fruiting begins between October and February. The fruits are drupaceous with an ellipsoid shape along with smooth and shiny

outer parts. When immature, the fruits are green in colour, and become yellow on maturation. The seeds are usually solitary with pointed ends and smooth surfaces (Troup, 1921). From a single flowering, an average weight of 15-20 kg fruits are produced by a tree. The flowers undergo self-fertilization in most of the conditions (Johar *et al.,* 2015). Kumar *et al.* (2013) had observed that the coppicing ability of the Meliaceae members are very high compared to any other family, as it was observed in the family members that they produce new shoots at the earliest where injured. This pollarding ability helps the tree to form numerous coppice shoots from the stem and branches.

As the tree can be harvested on a short-rotation basis, it has been rapidly emerging as a potential timber species for the production of plywood and pulpwood on a commercial scale (Uday *et al.,* 2011). The timber is moderately hard, light in weight, easy to saw, and durable, which has attracted the plywood industries. The brown colour of the wood has ready acceptance as a face veneer. The ply thus made from the wood is found to have higher glue-shear strength which is up to 200 kg/inch and also hold a good threshold level of 100 kg/inch (Uday *et al.,* 2011). The tree is gifted with special geometry of architecture which is free from knots as well as crooking, which has made it a suitable choice of species in the timber industry also. The decorative appearance of the wood makes it a suitable choice of species for furniture applications. The paper industries have started to use more timber for pulp because of its increased pulp recovery, exceptional strength, and anti-termite property (Parthiban *et al.,* 2009). Suresh and Devakumar (2017) reported a 50 percent pulp recovery from the wood, which was found to be higher than that of acacia and eucalyptus, as well as a Kappa number (used to assess bleachability) of less than 20, which is of high quality when compared to other traditional raw materials (Saini *et al.,* 2007). The resulting bleached papers from *M. dubia* had a brightness of nearly 82 percent (Parthiban *et al.,* 2009). The species can also be utilised for bio-energy applications due to its high calorific value (5.04-5.08 cal), specifically for biomass gasification for

the production of producer gas which can be further used in limekilns to replace fuel oil (Chinnaraj *et al.,* 2011). The wood is also used for making cigar boxes, agricultural implements, pencils, matchboxes, splints, kattamarams, ceiling planks, building and construction materials, packing cases, musical instruments, tea boxes, and plyboard. In Ceylon, the boat outriggers are constructed with the woods of *M. dubia*. The multi-purpose benefits obtained from *M. dubia* had made it a great deal of allurement and uplifted demand in the commercial markets around the tropical nations (Chinnaraj *et al.,* 2011).

Other than its industrial and commercial importance, the plant has proficient medicinal properties. Various extracts have been identified from the plant from its different part that is known to have different pharmacological importance which includes, anthelmintic nature, antidiabetic, antileprosy properties, antineoplastic, antiviral, antifungal and antibacterial property (Susheela *et al.,* 2008; Sharma and Arya, 2011). It is also used for the preparation of some of the important traditional medicines that are used for treating patients with asthma, malaria, leprosy, eczema, fevers, and venereal disease (Govindachari, 1992), as well as acariasis, cholelithiasis, and pain (Kokwaro, 1976). In Indian folk medicine, it is used as an important biocontrol against insect pests (Kaul *et al.,* 2000). The leaves of the trees are used as excellent fodder for human ruminants because of their rich source of mineral elements, crude proteins, and vitamins (Leela *et al.,* 2016).

The tree is suitable in the agroforestry systems due to its non-allelopathic effect and as a potential alternative to the exotic timber for the farmers in rainfed ecosystems of arid and semi-arid countries. The industrial, as well as ecological importance of the species, has emboldened the farmers to take up block plantations on a large scale (Parthiban *et al.,* 2009). The timber from a well-established plantation can earn money for the farmers within 7 to 8 years after planting. In well-irrigated conditions, a four-year-old

tree can attain an average top height of 14 m with 10 m of clear bole and girth of 67 cm, while in the natural rainfed conditions, it can attain an average height of 12 m with a clear bole of 7 m and 50 cm girth (Thakur *et al.,* 2018). The tree may indeed be a feasible option for restoring degraded ravine lands into profitable and sustainable land by implementing modified planting techniques. Through its large carbon sequestration capability, and other beneficial features, it can be used for several natural resource conservation and reclamation of damaged soils (Jinger *et al.,* 2021). The increase in demand for its timber in the market had attracted a large number of agriculture farmers to establish and raise large-scale plantations of the tree in different parts of India along with their agricultural crops as intercrops. The tree is also known for its resistance to insect and termite attacks which guarantee better longevity of the finished products made out of it and avoid the fear of crop loss during its growth stage to the farmers (Chinnaraj *et al.,* 2011).

2.2. Tree variability

The primary necessity for a tree breeding programmes is its genetic variability. More variability, better the tree improvement programme can be applied to the crop of interest. The tree breeders make use of this variability through various breeding methods and screen preferred genotypes through various selection methods. Evaluating the germplasm to establish its range of variation, as well as its systematic exploitation in tree improvement, is critical in any tree improvement programmes. It is studied that the large genetic variance and heritability of various traits are considered to be controlled by additive gene interactions. The existence of additive genes underlines the relevance of phenotypic performance in plant selection (Ghosh and Gulati, 2001). Understanding the additive gene will help the breeder to know about the heritability of the species (Dorman, 1976).

2.3. Progeny testing

One of the most important factors to be considered while selecting a breeding programme is the identification of plant's phenotypic or genotypic traits which are necessary for plant domestication and the parents' ability to pass these superior traits of importance (for which they were initially selected) to their progeny. The data acquired from this type of study can be utilised to identify superior parents with higher general combining ability value and hence best parents can be mass propagated vegetative to meet the needs of the planting purpose, and can be used for future breeding programmes.

Borem and Milach (1997) defines progeny testing as "the evaluation of progeny genotypes based on the phenotypes of their offspring." Progenies are examined in trials with rigorous experimental designs, resulting in more precise measurements. Selection with progeny testing is more efficient than mass selection because it provides a more accurate evaluation of the plants selected. Progeny testing is a kind of reverse selection method for the selection of the best parent. The genetic selections are made through phenotypic assessment of the individual offspring. The phenotypic selections are made through the performance of an individual for traits that have high heritability. However, as the environmental component of phenotypic variation becomes significant, assessing an individual's breeding value based only on its phenotype becomes problematic. More importantly, phenotypic assessment of an individual's worth is difficult, such as for sex-limited features or qualities that need the individual's loss for assessment. By evaluating several offspring from a tested individual, progeny testing solves this obstacle.

2.3.1. Concepts of progeny test analysis in forest breeding

The forest tree breeders carried out progeny testing to determine the breeding value of the parent population based on the performance of its progeny. To make selections, the breeder ranks parents according to their projected breeding values (or a function of their predicted breeding values). Progeny trials are repeated across different planting locations to study the performance of progeny with respect to the chaging environmental conditions.

2.3.2. Evaluation of progeny of selected plus trees

Another important question in a selection is whether it should be based on the phenotypic characteristics of the tree or based on the performance of the progenies. The results of the assessment of progenies of the plus trees can be used not only to evaluate the progeny themselves, but also the ability of the parents to pass on to their progeny, the good characteristics for which the tree was originally selected. The information gathered from this type of study can be used for selecting exceptionally good parents, which can be used over and over again in the future for the establishment of seed orchards and development of hybridization programme.

Schmutterer (1995) reported the existence of variability in growth parameters due to difference in genotypes and their variations in soil and climatic conditions. Study conducted to find the genetic variations among the half-sib families of 20 selected plus tree progenies in *M. dubia,* has observed consistent superiority for height, basal diameter and volume index which was mainly due to the presence of genetic variation (Kumar *et al.*, 2013). The existence of significant differences and the superiority of a few seed sources among different open-pollinated families and provenances was reported for *Lagerstroemia* spp. (Jamaludheen *et al.,* 1995). *Acacia nilotica* (Ginwal *et al.*, 1995), *Acacia catechu* (Mohapatra, 1996; Gera, 2006), *Prosopis cineraria* (Manga and Sen, 1998), *M. azaderach* (Thakur and Thakur, 2015).

The causes of variation be assessed by partitioning the total variability into phenotypic and genotypic variability, which is heritable and can be exploited for future use. The above results indicate, there exists an environmental effect on these characters. It was reported earlier in a study done on neem (Pandey *et al.,* 2018). In *M. dubia,* Kumar *et al.* (2013) had observed higher phenotypic coefficient of variation (PCV) and genotypic coefficient of variation (GCV) values for volume followed by plant height and collar diameter. High GCV for the vigour index had been reported in *Tectona grandis* (Arun, 1996) and low GCV for height in *Eucalyptus tereticornis* (Sundararaju *et al.,* 1995). In *Bambusa pallida*, low GCV and PCV were reported for height and collar diameter (Singh and Beniwal, 1993).

Parthibhan *et al.* (2017) observed moderate heritability for *M. dubia* girth at breast height (DBH), followed by its height and volume, which was assumed to be due to the complexity of the quantitative trait, which is prone to high environmental effect. Arun (1996) observed high heritability accompanied by moderate to high genetic advance for growth parameters in *Tectona grandis*. Solanki *et al.* (1984) in *Prosopis cineraria,* Gera *et al.* (2001) in *Dalbergia sissoo,* Dhillon *et al.* (2003) in *Azadirachta indica* also made similar observation. In *Eucalyptus globulus* low heritability for diameter at breast height was reported during the field study of the eight sub-races (Apiolaza *et al.,* 2005). Similarly, low to moderate heritability was recorded in *Eucalyptus globulus* and *E. nitens* (Raymond, 2002), for different genetic parameters and low to moderate heritability for height and tree volume in *E. grandis* and it was also observed that the heritability varied with changing environment and age (Devagiri *et al.,* 1997).

A progeny test was conducted by Siregar *et al.,*(2021) in *Falcataria moluccana,* in a randomised complete block design (RCBD). Growth parameters such as germination (percentage), height (m), and diameter (mm) was measured and they showed significant variation. Moderate heritability

observed for height and diameter variables but low heritability was observed for the germination percentage.

Stejskal *et al.,* (2021) studied the breeding values and response to selection in forest tree breeding using stochastic simulations under standard pedigree (BLUP) and genomic-based evaluation methods (GBLUP). The latter gives genomic selection a methodological foundation. The effect of clonal replication in progeny testing on the response to selection achieved in seed orchards was investigated. They discovered that clonal replication improved selection accuracy in progeny trials, resulting in greater genetic benefits under BLUP. While GBLUP showed a similar pattern, the additional gains did not outweigh those seen with BLUP. As a result, breeding operations that use extensive progeny testing and clonal replication may not benefit from genomic information deployment.

In a progeny test, on 2413 Christmas trees (*Araucaria columnaris*) for its resistance/susceptibility, against *N. neomacrospora* it was observed that there was pronounced additive genetic variation in sensitivity to *N. neomacrospora* and Christmas tree traits. Susceptibility to *N. neomacrospora* had a narrow-sense heritability of 0.63. For all attributes, significant variations between selfing and outcrossed trees were observed (Xu *et al.,* 2021).

In shortleaf pine, a progeny test of 84 accessions was conducted to investigate the impact of genetic and genetic environment variance on performance traits (diameter at breast height, tree height, and survival). For both girth and height, family variations were shown to be significant ($p > 0.01$), but not for survival ($p>0.05$). For girth, seed sources were considerably different ($p>0.05$), with more pronounced latitudinal differences. Individual tree and full-sibling family mean heritability showed relatively strong genetic to environmental variation (Hossain *et al.,* 2021).

The progeny study by Danu *et al.* (2021) in *M. azedarach* Linn. to understand growth performance and genetic parameters showed significant differences for its height and diameter, but the heritability for both stem diameter and tree height remained relatively low. They also observed a strong correlation between morphological traits such as height and girth.

Half sib progenies of twenty-four genotypes of *Toona ciliata* were studied for their juvenile growth performance. It performed exceptionally well in terms of growth indicators. For all parameters, the phenotypic coefficient of variation was found to be larger than the genotypic coefficient of variation. Branch angle and plant height showed moderate heritability and genetic gain. Plant height with collar diameter and stem straightness, as well as collar diameter with the number of branches per plant, showed highly significant and positive genotypic and phenotypic associations (Kundal *et al.*, 2020).

Using data from ten families of five-year-old *M. dubia* raised by seed sources, the phenotypic and genotypic correlations between tree growth traits (height, diameter, and biomass) and wood quality parameters such as basic density, cellulose content, lignin content, and fibre characteristics, viz. length, diameter, lumen diameter, double cell wall thickness, and fibre content, were calculated (CPTs). The findings revealed that there were moderate to high genetic and phenotypic relationships within and between the growth traits and wood quality indicators investigated. The biomass of trees was found to have a direct positive strong relationship with their height and diameter. Similarly, cellulose content, fibre length, and fibre content were phenotypically and genotypically associated with wood basic density; additionally, all of these parameters were negatively associated with lignin content, fibre diameter, lumen diameter, and double cell wall thickness. The height and diameter of trees had a favourable influence on wood quality indicators such as basic density, cellulose content, fibre length, and fibre content, according to genetic correlation (Dhaka *et al.*, 2020).

2.3.3. Physiological studies of the progenies of plus trees

Among the several physiological, biochemical, and molecular processes that drive plant growth and development, photosynthesis is the fundamental mechanism which, provides the organic blocks to the plant and thereby contributes significantly to plant growth and development (Rapparini and Penuelas, 2014). It is a crucial process for plant development as well as its survival. Photosynthesis is influenced by a complex process of interaction between the genetic factors of plants and environmental factors around them (Yamori *et al.,* 2016).

Another important plant physiological activity is stomatal conductance. It is the measurement of the degree of stomatal opening during plant transpiration. Understanding the stomatal conductance will help one to determine the water status of a plant (Carmen *et al.,* 2013). It is critical for both desiccation avoidance and CO_2 acquisition in the cycling and balancing of water, CO_2, and energy between plants and the atmosphere. The early plant response to water deficiency has been recognised as stomatal regulation of water loss, which leads to a limitation in carbon uptake by the leaves (Chaves, 1991). Plants having a higher stomatal conductance have been linked to higher leaf water content in studies (Auge *et al.*, 2015). Stomatal closure is frequently thought to be an early physiological response to water deficiency, resulting in reduced photosynthesis due to low carbon dioxide availability in the mesophyll cells (Cornic and Massacci, 2000). It has been found that net photosynthetic efficiency and stomatal conductance are frequently linked (Salisbury and Ross, 1992), also that when stomata open, net photosynthetic rates rise, and when they close, they fall (Bunce, 1988). As a result, variations in stomatal conductance may result in variations in photosynthetic rates (Meng and Arp, 1993). Variations in net photosynthesis, stomatal size, and density are also reported to be key factors of plant productivity (Wang *et al.*, 1995). It is commonly found that there is variation in photosynthetic rate and stomatal conductance between and within species. In a study conducted by

Gago *et al.* (2016) using data sets of 17 published studies, the net photosynthetic rate was found to be related to each other as a plant with a higher photosynthetic rate showed higher stomatal conductance.

In higher plants, the chlorophyll content in its leaves was found to be a useful metric for assessing plant photosynthetic efficiency and stress (Zhu *et al.*, 2012). Under stress conditions, the plant loses its chlorophyll content, and it can cause significant damage to photosynthetic pigments as well as the breakdown of the thylakoid membrane. As a result, such plants exposed to stress will suffer a decrease in photosynthetic activity.

The studies on the variance of photosynthetic factors between plants (clones) and their interaction with growth traits will be valuable information for the tree improvement programmes. The insights gathered will aid in the development of high-yielding, homogeneous clones that may be employed in large-scale plantations. Based on this idea, a study was conducted on *Populus nigra* at various places and revealed that the gas exchange and chlorophyll parameters were related to the species' growth, and it was recorded that the light use efficiency was high in species that were studied from Siberia, making the species a suitable candidate for future breeding (Chu *et al.*, 2010). In *T. grandis*, it was found that the photosynthetic parameters are substantially regulated by genetic variables and have a high narrow sense of heritability. Photosynthetic parameters and growth traits in clones from different provenances exhibited a lot of genetic heterogeneity, according to the study. The seedlings' net photosynthetic rate and growth characteristics were shown to be highly connected. Some teak clones exhibited a better photosynthetic rate than others, which could be used as a valuable resource for future breeding programmes (Huang *et al.*, 2019).

In neem seedlings study from the selected trees, significant differences in net photosynthesis, stomatal conductance, stomatal density, total guard cell length, leaf area, and whole-plant dry weight were observed (Kundu and

Tigerstedt, 2012). Mebrahtu and Hanover (1991) found similar results for black locusts (*Robinia pseudoacacia* L.) and black spruce (*Picea mariana* Mill.). In a study done on the effect of chlorophyll concentration on stomatal conductance in five and ten-year-old *Quercus serrata*, researchers discovered that a decrease in chlorophyll concentration induces a drop in stomatal conductance (Matsumoto *et al.,* 2005).

Studies on heritability in plant seedlings and clones of *Tectona grandis* (Mckown *et al.,* 2014), *Populus nigra* (Chu *et al.,* 2010), *Dalbergia sissoo* (Sharma and Bakshi, 2014) it was observed that the photosynthetic rate, chlorophyll content, and stomatal conductance of the plant showed high heritability. An important conclusion made from this study was that the net photosynthetic rate shows a strong positive connection with seedling height and individual volume. The findings also showed that plus tree seedlings with a high photosynthetic rate produce fast-growing plants. It was found out that species from Serbia had a strong link with growth, gas exchange, and chlorophyll fluorescence characteristics in *P. nigra*. Highly associated germplasm was also exploited as a future breeding resource with high light-use efficiency (Chu *et al.,* 2010). For the above-mentioned features, high heritability and genetic progress were seen in *Populus niagra*, *D. sissoo* and *P. trichocarpa* (McKown *et al.,* 2014).

Most of the earlier studies give importance to the effect of ecophysiological aspects such as stress, light intensity (Zhang *et al.,* 2002) and CO_2 concentration (Su *et al.,* 2003) in plant photosynthetic response. However, studies on the physiological factors such as photosynthetic gas exchange rate and chlorophyll parameters and their correlation studies were very few especially in *M. dubia*.

2.4. Genetic study in *Melia dubia* Cav.

This section aims to provide key insights into genetic diversity assessment research, including the rationale, major assessment approaches, and the use of molecular markers in general, and ISSR in particular. The assessment of genetic variation is a major concern in various tree improvement programmes for a variety of reasons, including distinguishing different genotypes of importance, which are then used for breeding programmes and population-genetic analysis, estimating the amount of diversity within and between genotypes for predicting potential genetic gain in a breeding programme, and setting up appropriate cross-breeding approaches.

The heritable variety within and between populations of organisms, or a pool of genetic variation within the inter-mating population, is referred to as genetic diversity (Rao and Hodgkin, 2002). It is a key indicator of biodiversity and a quantitative measure of population variability, indicating a balance between a mutation (creation of new variation) and genetic variation loss (Leffler *et al.*, 2012).

2.4.1. The value of genetic diversity

Genetic diversity among and within genera, species, subspecies, populations, and elite breeding materials is equally of interest in plant genetics and breeding. While taxonomists and germplasm banks are primarily interested in the higher levels of this hierarchy. The plant breeders are mainly concerned with the diversity among and within breeding populations and elite germplasm, for the reason that it largely determines the prospects of success in breeding programs and foundation for any form of domestication effort. The genetic variability in wild populations has come from years of natural selection, gene flow, genetic drift, and mutation. As their numbers fall, there will be a loss of genetic resources in the near future. Even now also a majority of genetic resources are underutilized and are left to die in "gene morgues"

(Hobbelink, 1991). Hence, studying the genetic diversity of species in the present scenario has huge importance in the field of conservation.

To efficiently exploit and conserve plant genetic resources, genetic diversity and relatedness between or among different species, groups, and individuals must be investigated. It is well established that plant genetic diversity changes across time and space. The quantity and distribution of genetic variability in a plant species are determined by its evolution, breeding system, ecological, geographical conditions, historical bottlenecks, and, various human actions (Rao and Hodgkin, 2002).

2.4.2. Techniques for assessment of genetic diversity

Prior to 1970, usual methods for measuring genetic diversity between taxonomic units have relied on pedigree analysis and morphological, physiological or cytological markers. In the successive two decades, it became propitiously used in large numbers of taxonomic and evolutionary studies (Hamrick and Godt, 1997); however, they often failed in the classification of elite breeding materials due to the limited number of marker loci available and the low level of polymorphism. The evolution of molecular markers such as RFLPs, RAPDs, AFLPs, and SSRs in recent years has dislodged most of the drawbacks associated with isozymes and other previous markers. Meanwhile, dense marker linkage maps have been developed in all major crop species in and around the world. The use of new marker systems reveals differences at the level of DNA, and thus they provide an extremely powerful tool for the assessment of genetic diversity in cultivated and wild plant species (Westmann and Kresovich, 1997).

The markers are of mainly two types which are classical (morphological, anatomical, cytological) and molecular (biochemical and molecular genetic markers). Since ancient times, humans have successfully used several morphological markers to analyse plant diversity for use in crop improvement programmes and these markers can be used to visually discern

important plant characteristics which require no specialised knowledge (Kresovich *et al.,* 1992). However, the successive use of morphological markers was found to be a tried-and-true technique for distinguishing genotypes (Sumathi and Balamurugan, 2014). The limited availability of easily scorable markers, difficulty in scoring homozygous from heterozygous individuals, the influence of environment, and difficulty in comparing phenotypes with genotypes are its major limitations to use. When it comes to molecular markers the biochemical markers can uncover minute differences in the gene product as these markers study the products of gene expression, which is the protein. The molecular genetic markers or the DNA based markers look for differences in an individual's constitution at the genetic level. These markers can be identified within the whole genome at specific locations, considering this property these markers are used for chromosome mapping and used to flag the position of a particular gene. On the contrary, molecular markers offer several advantages as these are more adaptable, exhibit abundant polymorphism, display no pleiotropic effect, are less affected by the environment and are easily detectable. Molecular markers are valuable tools for analysing genetic diversity both within and between species (Nadeem *et al.,* 2018). The use of genetic markers was not a novel concept in modern science as they were used in several crop improvement as well as breeding programmes, such as genetic diversity studies, DNA fingerprinting, genetic mapping, purity assessment, and enhanced variety identification, among others (Semagn *et al.,* 2006).

DNA-based markers are the best choice for screening tissue culture-induced mutations as they are unaffected by environmental variables and produce more consistent and accurate results. There are mainly two types of molecular markers which are utilized to evaluate DNA polymorphism, one is the PCR based markers and the other is hybridization-based markers. The PCR based markers involve in vitro amplification of a specific DNA sequence with the help of specifically chosen oligonucleotide primers whereas the

hybridization-based markers analyse DNA profiles by hybridizing the restriction enzyme digested DNA to a specific labelled probe, which is a DNA fragment of known origin or sequence. The molecular markers like RAPD, RFLP, AFLP, SNP, and microsatellite or SSRs have evolved as key tools for analysing genetic variability within and between populations/clones, varieties, species, and genera, as well as for evaluating the genetic fidelity of propagated plants and developing genomic libraries. It was based on RFLP, AFLP, microsatellite, and isoenzyme markers, Lespinsse *et al.,* (2000) created the first genetic linkage map of rubber trees (Hevea spp.).

Molecular markers have been utilised by many researchers to assess genetic fidelity of micro propagated plants and genetic stability in several species, including molecular markers have been used by various workers to assess the genetic fidelity of micro propagated plants such as *Panax notoginseng, Eucalyptus tereticornis, E. camaldulensis* (Rani and Raina, 1998), *Santalum album*, *Jatropha curcas*, and *Azadirachta indica* (Arora *et al.,* 2011). The reliability of a DNA marker approach is necessary for genetic mapping and long-term goal setting.

2.4.3. Genetic diversity assessment using molecular markers

The development of a large number of hybrids and variants in crops with a small genetic base has been facilitated by the advancement of contemporary breeding methods. As they have little phenotypic diversity, morphological and biochemical markers are insufficient for identifying and distinguishing the crops, making them challenging to employ. However, the development in molecular technologies has enabled the effective study of genotypic variance for unambiguous identification by identifying sequence polymorphisms (Semagn *et al.,* 2006). The discovery of Polymerase Chain Reaction by Kary Mullis in the year 1998 had made a huge development in the field of DNA-based molecular marker identification through the amplification of particular DNA sequences using primers multiple times and

thereby reducing the demand for large amounts of pure DNA for studying. The most important PCR-based markers used for fine-scale genetic characteristics are Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeats (SSR), Inter Simple Sequence Repeats (ISSR), and so on.

In forest tree species microsatellite markers were found to be the most precise tools for assessing the genetic diversity as these markers are abundant, reproducible, co-dominant, multiallelic, highly polymorphic and only a very minute amount of DNA is required for analysis (Tibihika *et al.,* 2019). The development of different types of microsatellite markers has been made useful for studying the diversity of a large number of tree species.

2.4.4. Inter-Simple Sequence Repeats for genetic diversity assessment.

After the discovery of the PCR in 1983, new PCR-based DNA marker systems were continuously being developed. In the early 1990s, the development of inter-simple sequence repeat (ISSR) markers was independently reported by different research groups (Meyer *et al.,* 1993; Gupta *et al.,* 1994; Zietkiewicz *et al.,* 1994). ISSR markers are segments of DNA that are flanked at both ends by short DNA motifs or microsatellite sequences (usually 2-5 nucleotides long) which are repeated multiple times in a row (e.g., ACACACACACAC). Using arbitrarily designed primers that contain repetitive sequences complementary to microsatellite regions in the genome (ISSR primers), random DNA segments in the genome can be PCR-amplified and used as markers for genetic variation studies (Ng and Tan, 2015).

ISSR markers have come out as an alternative system with reliability and advantages of microsatellites (SSR). The method involves amplification of genomic segments which are flanked by inversely oriented and closely spaced microsatellite sequences by a single primer or a pair of primers based on SSRs anchored 5' or 3' with 1-4 purine or pyrimidine residues.

The sequences of repeats and anchor nucleates are randomly chosen. ISSR amplification can reveal a much larger number of fragments per primer than RAPD. As the ISSR markers are inherited in mendelian mode and segregated as dominant markers, this technique provides a rapid, reliable, and highly informative system for DNA fingerprinting. This technique has been used widely in the studies of cultivar identification, genetic mapping, gene tagging, genetic diversity, evolution and molecular ecology (Wang, 2002).

When sequence information is unavailable, ISSR markers are the best option for analysing population genetic diversity. These markers are composed of highly variable microsatellite sequences that are broadly distributed across the genome, have higher repeatability, and are less labour and money intensive than AFLPs. Many plants use ISSR molecular markers for population genetic study, and they create more uniform and reproducible bands than RAPD (Dai *et al.,* 2010). It is effective in researching plant genetic variety (Etminan *et al.,* 2016). Scientists have used ISSR molecular primers to investigate genetic diversity and polymorphism in plant species such as *Salvadora persica* (Monfared *et al.,* 2018), Lotus species (Ducar *et al.,* 2018), *Capsicum species* (Costa *et al.,* 2006). They are beneficial in determining the level of polymorphism and diversity in a variety of forest plant species. The ISSR markers have some flaws also possible, that fragments with similar mobility come from non-homologous regions, skewing genetic similarity estimates and the molecular nature of the polymorphism can only be determined by sequencing the fragments recovered from the gel (Reddy *et al.,* 2002). The use of an ISSR marker assay to detect genetic homogeneity has been successfully demonstrated in micro propagated plants of *Prunus dulcis*, (Guo *et al.,* 2006), Musa spp. *Swertia chirayita* (Joshi and Dhawan, 2007), *Anoectochilus formosanus* (Zhang *et al.,* 2010). Using ISSR primers, Leroy *et al.* (2001) evaluated the genetic fidelity of cauliflower and discovered genetic instability at an early stage, but RAPD markers were unable to detect changes in the repetitive region of the genome in micro propagated kiwi fruit

plants, whereas ISSR markers were able to detect genetic variation (Palombi and Damiano, 2002).

A study was conducted in eleven natural populations and seven plantations of *M. dubia*, spanning eight districts in Karnataka, to investigate the genetic diversity of the population using 15 ISSR markers, totalling 232 samples. Estimates of genetic diversity at the species level were found to be high, including percent polymorphism (94.6), percentage of polymorphic loci (PPL) (98.8), the observed number of alleles (Na=1.98), effective number of alleles (Ne=1.59), Nei's gene diversity (H) (0.340.15), and Shannon's information index (I) (0.510.19). According to molecular variance analysis, the majority of genetic variation (68 percent) was found within populations rather than between them. The dendrogram created using the unweighted pair group approach with arithmetic average did not demonstrate geographical subgrouping of genetic variance, except for a few populations. Based on the genetic variability observed, superior seed sources can be identified, and tree improvement programmes can be devised for the conservation and continuous advancement of the species (Rawat *et al.,* 2018).

ISSR primers were successfully used to evaluate and analyse genetic relationships within and among Alhagi populations in a study on *Alhagi graecorum* species by El-Hak and Hassan (2019). The study observed that ISSR is a valuable indicator in genetic diversity evaluation. Another study on the genetic diversity and population structure of 23 *Chukrasia* (Meliaceae) subpopulations from all over Asia's geographical distribution using ISSR markers had observed variable differences between and within the population. The DNA results agreed with the morphological differentiation and were consistent with the two known taxa (Wu *et al.,* 2014).

The genetic diversity of Pan-Indian accessions of the medicinal plant *Lepidium sativum* from 19 Indian states was examined using ten ISSR primers. Because 139 of the 172 scoreable amplified DNA bands obtained

were polymorphic, UPGMA was utilised to divide the 94 accessions into three groups (Kumar and Yadav, 2019). Using 15 ISSR primers that produced 191 polymorphic amplified DNA bands, the genetic diversity of ten populations of the medicinal plant *Hypericum perforatum* growing in different agro-climatic regions of Iran was investigated, revealing that the germplasm could be classified into four distinct groups almost following geographical trends (Morshedloo *et al.*, 2014). Gupta *et al.* (2014) examined the genetic diversity of 45 individuals from eight populations of *Commiphora wightii* in the Indian Thar desert using ten RAPD and ISSR primers, generating 155 scorable, amplified DNA bands with 86.72% polymorphism.

The genetic variation study in 150 *Argania spinosa* trees (critically endangered) was measured using inter-simple sequence repeats by Mouhaddab *et al.*, (2017) using nine ISSR primers. At the population level, they observed high polymorphism ranging between 75.58 and 82.56 percent. Even though the polymorphism was higher the genetic diversity was observed to be lower (Nei's genetic diversity index (H) was 0.30 to 0.34, and Shannon's information index (I) was 0.44 to 0.49).

To develop a novel microsatellite marker for *M. dubia* using genomic data, a study was conducted by (Annapoorna *et al.*, 2021) using a direct seq-to-SSR approach. For validation, 151 markers were chosen, 50 of which were genomic markers picked at random and 101 of which were genic markers discovered using BLAST2GO. All loci showed amplification, with 81.4 percent producing high-quality, repeatable amplicons of the expected size. The genetic diversity of 75 genotypes from three populations was assessed using ten highly polymorphic markers. There were 114 alleles found, with moderate diversity and a positive fixation index. For the diversity assessment of 24 genotypes, 29 genic markers representing 13 enzymes with polymorphism for wood stiffness were chosen. The genome was covered by-products ranging in size from 87 to 279, which covered the majority of the

genome. Based on the trait, cluster and structural analysis separated about 80% of the genotypes.

An important study done by Rawat *et al.,* (2021) for understanding the genetic diversity of the *M. dubia* population in Karnataka, used six SSR markers and they observed 99 percent variation within the populations, which is consistent with the variation displayed by outcrossing plant species, as opposed to selfing plant species, which had on average 50% variation among populations. They had a moderate amount of genetic diversity.

Random amplified polymorphic DNA (RAPD) molecular markers were employed to examine the genetic diversity in populations of *M. dubia* Cav (Family: Meliaceae) (Burma dek) from different agro-climatic zones of India. Thirteen primers produced polymorphic banding patterns, out of a total of 38. Overall, 105 distinct bands could be reliably acquired, with 69 (65.7%) of them being polymorphic. The similarity values ranged from 0.80 to 0.91, showing that the genetic base of *M. dubia* germplasm in India is somewhat limited (Johar *et al.,* 2017). As the genetic base of *M. dubia* in India is very narrow and hence there is a need for conservation of these resources by studying their genetic variability in a natural population spread across Indian forests.

2.5. Phytochemical studies and medicinal properties

Tropical plants produce a large number of secondary metabolites, consisting of a wide spectrum of phytochemicals that occur naturally (Sinha and Dogra, 1985). These secondary metabolites aid the plants to thrive in harsh environmental conditions such as extreme temperature and render protection from large herbivores and infectious agents through antimicrobial activity through inhibition and killing (Tariq and Reyaz, 2013). These phytochemicals also function as effective pollinator guides enabling successful pollination in plants. Phytochemicals provide different colours to plants and a wide variety of flavours which can be either pleasant or

unpleasant for consumption (Christena *et al.,* 2017). Other important functions of phytochemicals include regulating the vegetation pattern, dormancy period and production of allelochemicals. In the case of human health benefits, phytochemicals play a major role, when they are consumed, many of them can provide nutrients essential for optimal health (Bohn, 2014). The secretion of these phytochemicals in terms of their quantity can vary from species to species or between plants of the same species to their geographical distribution (Tariq and Reyaz, 2013). Phytochemicals are specific to plant and plant parts and their abundance increase mostly in response to stress events They are classified as polyphenols, terpenoids, alkaloids, phytosterols, and organosulfur compounds (Somani *et al.,* 2015). Among these classes, we can see that a major number of studies have been done on polyphenols.

M. dubia is well known for its medicinal and insect-pest repellent properties (Murugesan *et al.,* 2013). The phytochemicals extracted from the members of Meliaceae have good insect pest management properties which can be used without causing any damage or harm to the environment. The plant is one of its kind from the Meliaceae family whose entire parts are used in the traditional herbal treatments for varied ailments including leprosy, eczema, asthma, malaria, cholelithiasis, fevers, and other venereal disorders (Govindachari, 1992). Studies on the plant fruits for their medicinal value has found their effectiveness against colic disease, skin disorders, and parasitic worm infection (Purushothaman, 1984). The plant is well-known for being a good source of bioactive limonoids, that exhibit a wide array of bioactivities like antibacterial, anti-inflammatory, antimalarial, insect antifeedant, antifungal and cytotoxic effects (Awang *et al.,* 2007). The pharmacological activities of the plant have paved the way for the treatment of various chronic diseases in humans which has been in society for a long (Kaul *et al.,* 2004).

Based on the studies in *M. dubia* leaf essential oil for quantifying the phytochemical constituent, it was reported that the oil is primarily composed of monoterpene, hydrocarbons and oxygenated monoterpenes, with lower

amounts of alkanes, sesquiterpene hydrocarbons, and phenylpropanoids (Nagalakshmi *et al.,* 2001). Thus, it can be concluded that the pest management and its control property of the plant are mainly owing to the presence of phytochemicals such as tetranortriterpenoids (Purushothaman *et al.*, 1984) and monoterpenes (Nagalakshmi *et al.*, 2001) as the main ingredients. These phytochemical extracts were found to be effective against harmful pests such as *Spodoptera litura* and *Helicoverpa armigera*, a major boll worm in cultivated crops (Opender *et al.*, 2002) and teak defoliator *Hyblaea puera* (Senthilkumar *et al.*, 2012). *M. dubia* phytochemical extracts inhibit the growth of insect pests by creating a toxic effect in the stomach of insects and thereby creating moulting abnormalities and morphogenetic defects (Bhuiyan *et al.*, 2001). These studies show the fact that the plant is an important choice as an antifeedant for the farmers in their agricultural feeds to prevent crop loss due to pest attacks. In humans, the leaf extract was found to be an efficient probucol, a hydrophobic antioxidant medication that decreases the plasma cholesterol and may slow the course of atherosclerosis and post angioplasty restenosis.

Murugesan *et al.* (2013) identified 42 phytochemical constituents in the leaf extract of *M. dubia* using GC-MS-MS and they listed out the major phytochemical compounds present in the extract, which were Octadecanoic acid (15.71%), Hexadecanoic acid (11.10%), Humulene (3.24%), Caryophyllene (6.07%), Aromadendrene (3.53%) and Germacrene-D (2.89%. Further studies on the pesticide action of each phytochemical compound documented and mentioned the importance of the sesquiterpene (caryophyllene) as an important factor that contributes to the pesticide property of *M. dubia*.

2.5.1. Sesquiterpene

Sesquiterpenes are secondary metabolites found in the essential oils of many plants. Farnesol, humulene, caryophyllene, vetivazulene, guaiazulene, copacetic are the major sesquiterpene compounds studied in plants. They belong to the C15 terpenoids family. It is produced in plants *via* the mevalonate pathway, an important biochemical cyclic pathway in the plant. The presence of unsaturated lactone ring creates a structural diversity among the compound and there by causes a wide spectrum of biological actions in the plants which are responsible for their establishment and survival. Studies on sesquiterpene isolated from various plant sources have reported its anti-cancer activity (Mateji *et al.,* 2010) and showed promise in its ability to fight against cancer cells (Beer *et al.,* 2019). Many herbivores and insects are repulsed or poisoned by the presence of sesquiterpene in plants and thus it operates as a natural defence for plants. Among the different derivatives of sesquiterpene, caryophyllene and humulene are the most important ones having different pharmacological effects as well as insect pest control properties.

2.5.1.1. Beta-caryophyllene (BCP)

Beta-caryophyllene (BCP) is a natural bicyclic sesquiterpene consisting of fifteen carbon chain $C_{15}H_{24}$ which is found in a wide range of plants belonging to the cannabinoid family members around the world. It is a pale-yellow oily liquid with an odour of clove and turpentine. It is best known for its anti-inflammatory properties and anti-cancerous cell property. One of the most important properties of caryophyllene is its ability to bind to the cannabinoid receptors 2 (CB2) in the living cells of organisms. This property has been exploited widely to make medicines for the treatment of cancer as a pain reliever (morphine). It is the first cannabis-derived compound other than THC, CBD, and CBN which can bind directly to endocannabinoid receptors (Gertsch, 2008). Apart from the above benefit, it is a potent anti-

inflammatory, antimicrobial, antibacterial, and antioxidant agent. β-caryophyllene has been known as the first known "dietary cannabinoid," which is a common component of food having GRAS (Generally Recognized as Safe) status and is permitted by the Food and Drug Administration. to use in food as a flavouring agent. It is also found in the essential oils of spices. In plants, caryophyllene is mostly found in larger plant species such as *Syzygium aromaticum* (Ghelardini *et al.,* 2001), *Piper nigrum* where the caryophyllene provide spiciness (Jirovetz *et al.,* 2002), *Cannabis sativa* (Gertscch *et al.,* 2008), and *Cinnamomum zeylanicum* (Kaul *et al.,* 2003).

Caryophyllene is a phyto cannabinoid that was identified as a plant product of *Cannabis sativa* L. Natural and synthetic cannabinoids can both activate the cannabinoid receptors (CB1 and CB2), but caryophyllene, which is found in high concentrations in the essential oil of *C. sativa* (up to 37%) (Mediavilla and Steinemann, 1997) can activate only CB2 and has no affinity for CB1. This shows that caryophyllene that occurs naturally is free of psychotropic side effects associated with CB1 activation and that it may have medical applications. As medicine caryophyllene is widely used as an anti-depressant, as an anti-inflammatory, for neuroprotective treatment for anti-viral, anti-oxidant, anti-cancer and against ischemia (Yokubaitis *et al.,* 2021). Caryophyllene is also used to treat people with cocaine addiction-related behaviour by the activation of PPARα and PPARγ receptors in the human body cell (Galaj *et al.,* 2021).

Tung *et al.* (2008) investigated the anti-inflammatory properties of *Cinnamon osmophloeum* essential oil and found out that caryophyllene is the reason behind this phenomenon owing to its ability to suppress NO gas. Studies in the essential oil of *Psidium guajava* Linn leaves have found that its major oil compound, caryophyllene (24%) is more effective against fungi such as *Rhizoctonia solani* and *Helminthosporium oryzae*, having ED50 values of 450 and 510 μg/ml (Jassal *et al.,* 2021), confirming the antifungal property of caryophyllene. Caryophyllene and its oxides, which are derived

from the leaves of *Calocedrus formosuna*, have anti-termite and anti-fungal properties against the fungus *Laetiporus sulphurous*. The anti-proliferative activity of caryophyllene against human renal adenocarcinoma and amelanotic melanoma cells was discovered by Loizzo *et al.* (2008). Caryophyllene improves the anti-cancer efficacy of alpha humulene, isocaryophyllene, and paclitaxel against tumour cell lines (Legault and Pichette, 2007).

The glutathione-S-transferase (GST) enzyme is a key detoxifying enzyme found in mice's liver and small intestine. Zhang *et al.*, (2011) discovered three substances that greatly increase GST enzyme activity and found out that caryophyllene was the major chemical that stimulates the action of detoxification enzymes.

Lemon balm oil, an important grass family member used for a different type of medical treatment of ageing, was found to have caryophyllene as its major oil component, which is about 13 ± 6 % by GC peak area measurement (Meyer and Gerhard, 1996).

Scientists have found out that the beta-caryophyllene, a CB2 receptor for the selective natural dietary cannabinoid, may be a candidate for targeting the trinity of infection, immunity, and inflammation, due to COVID-19 due to its unique functional receptor selectivity, wide availability and accessibility, dietary bioavailability, nonpsychoactive, and low toxicity, as well as drugable features such as excellent pharmacokinetic and physicochemical properties of caryophyllene (Jha *et al.*, 2021).

Using an excito-repellency test technique, the contact irritating and non-contact repellent actions of caryophyllene oxide were compared to the synthetic repellent DEET against laboratory strains of female *Anopheles minimus*, a significant malaria parasite vector. DEET and -caryophyllene oxide were evaluated at different concentrations. In contact and non-contact trials, *Anopheles minimus* was found to be more sensitive to -caryophyllene

oxide and showed high avoidance reaction rates. DEET, on the other hand, had lower irritancy and repellences capacities than caryophyllene oxide at the same concentrations (Sukkanon *et al.,* 2019)

In a pilot study done in Arabidopsis plant for the insect repellent property, it was demonstrated that transgenic plants with an intrinsic propensity to emit caryophyllene could be exploited to protect trees from Huanglongbing (HLB), a disease that has no cure as of yet and which is threatening the production of citrus, the world's most important fruit tree crop (Alquezar *et al.,* 2017). Studies on the bioactivities of *Peucedanum terebinthaceum* (Fisch.), it was found that the sesquiterpenoid caryophyllene was an important volatile compound in its essential oil that acted as an insect repellent (Sun *et al.,* 2020). The essential oils of *Varronia curassavica* (caryophyllene and humulene) were studied for their deadly and sub-lethal effects on the ant *Dorymyrmex thoracicus*, which is a pest in urban areas. In partially treated arenas, bioassays of fumigation toxicity and locomotor activity were conducted. The fatal concentrations of EOs to kill 50% of the *D. thoracicus* population ranged from 0.69 to 2.48 L/L, whereas the (E)-caryophyllene and humulene compounds ranged from 3.75 to 1.49 L/L (De Oliveira *et al.,* 2019.

In a study by Rifel *et al.,* (2021) it was found that when sugarcane borer-genera plants were exposed to volatile caryophyllene, they enhanced the parasitoid (*C. flavipes*) recruitment, which acts as a biological pest control.

The presence of -caryophyllene has facilitated the development of lateral roots and enhanced plant resilience to microorganisms (Ditengou *et al.,* 2015; Wenig *et al.,* 2019). Studies on *Arabidopsis thaliana* with *Pseudomonas syringae* have shown that plant signalling networks consist of isoprene and caryophyllene, which induce resistance to microbial diseases in neighbouring plants, which can be used in a variety of crop management applications (Frank *et al.,* 2020).

3.MATERIAL AND METHODS

The present research work titled “Progeny testing and genetic diversity analysis in plus trees of *M. dubia* Cav.” was devised to observe the field performance of selected *M. dubia* plus tree progenies from different parts of Kerala, exploring their genetic diversity and quantifying one of its major phytochemical caryophyllenes. In this chapter, the details regarding the study material, experimental design, methodology, observations taken and the analyses conducted to achieve each of these objectives are mentioned.

3.1. Study area

The study was conducted at the College of Forestry, Kerala Agricultural University, Vellanikkara, Thrissur, in the department of Forest Biology and Tree Improvement from 2020 to 2021. The progeny testing field is located inside the college campus at 40 m above sea level at latitude and longitude of 10º31'N and 76º26'E. The area experiences a warm, humid climate with a distinct rainy season. The annual rainfall during 2020-2021 was less than 3000 mm. The soil in this area is porous and well-drained. The climatic data during the observation period are given in Appendix I. The data was collected from the agrometeorological observatory, COH, Vellanikkara.

3.2. Experimental material

All the samples used for the entire work was from the *M. dubia* gene bank established at the College of Forestry, Kerala Agricultural University, Vellanikkara, Thrissur. This gene bank has one-year-old (as of 2020 May) *M. dubia* plus tree seed progenies collected and maintained from 25 plus tree populations across the natural distribution of *M. dubia* in Kerala (Fig. 1). The gene bank consists of 150 plus tree seed progeny, planted in a compact family block design from which regular morphological and physiological observations were made. Leaf samples for genetic as well as biochemical were taken from 25 different accession seed progenies in the gene bank.

Table 1. Details of the twenty-five selected plus tree mother plant of *Melia dubia.*

Sl. No.	Location	Range/WLS	Accession No.	Location
1	Thirunelly	Begur	FCV-MD-01	11°54'16.9"
				75.59°'59.77"
2			FCV-MD-02	11°54'37.68"
				76°00'02.40"
3	Tholpetty	Tholpetty WLS	FCV-MD-03	11°47'46.90"
				76°05'04.10"
4			FCV-MD-04	11°53'13.20"
				76°04'34.70"
5	Dasanakara	Chedelthu	FCV-MD-05	11°52'53.10"
				76°04'39.20"
6	Neykavala	Chedelthu	FCV-MD-06	11°25'32.10"
				76°06'02.30"
7	Dhoni	Olavakode	FCV-MD-07	10°27'02.10"
				76°12'24.30"
8	Pothundy	Nelliampathy	FCV-MD-08	10°31'00.92"
				76°37'00.28"
9	Attappady	Agali	FCV-MD-09	11°05'30.50"
				76°43'23.50"
10	Thiruvazhiyadu	Nelliampathy	FCV-MD-10	10°31'15.20"
				76°36'18.70"
11			FCV-MD-11	10°31'01.90"
				76°36'30.40"
12	Walayar	Walayar	FCV-MD-12	10°51'35.90"
				76°37'15.70"
13			FCV-MD-13	10°51'33.50"
				76°37'27.10"
14	Parambikulam	Sungam	FCV-MD-14	10°22'56.90"
				76°45'45.01"
15			FCV-MD-15	10°26'41.3"
				76°49'35.90"
16			FCV-MD-16	10°24'40.40"
				76°49'35.30"
17	Chinnar	Chinnar WLS	FCV-MD-17	10°21'16.20"
				77°11'32.20"
18			FCV-MD-18	10°21'16.20"
				77°11'29.60"
19			FCV-MD-19	10°21'13.50"
				77°11'38.20"
20	Peechi Thrissur	Peechi WLS	FCV-MD-20	10°31'56.20"
				76°22'26.20"
21			FCV-MD-21	10°29'01.00"
				76°22'00.01"
22	Akamala	Machad	FCV-MD-22	10°40'46.10"
				76°18'10.30"
23	Kulathupuzha	Kulathupuzha	FCV-MD-23	08°50'36.80"
				77°02'53.2"
24	Aryankavu	Thenmala	FCV-MD-24	08°51'22.3"
				77°08'48.60"
25			FCV-MD-25	08°55'55.40"
				77°08'38.70"

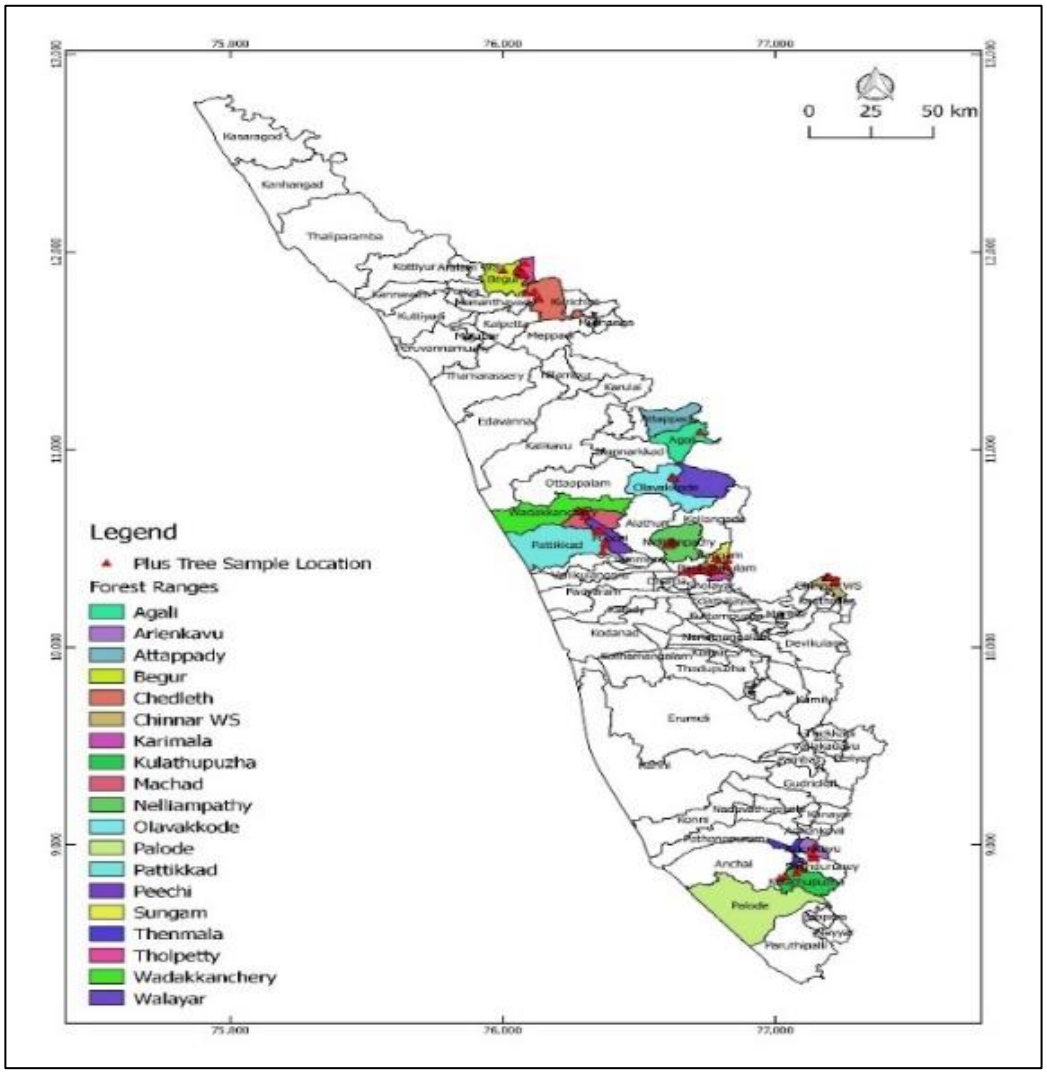


Fig. 1. Distribution of *Melia dubia* plus tree seed progeny parent population in Kerala

3.3. Laboratory chemicals, glass wares and plasticware

Analytical Reagent (AR) grade and plastic were from, Merck India Ltd., HIMEDIA SISCO research laboratories and Tarson India Ltd were used for the analysis. ISSR (Inter-Simple Sequence Repeats) primers and the master mix from Sigma Aldrich Chemicals Pvt. Ltd., Bangalore.

3.4. Equipment

The lab facilities and equipment available in the Department of Forest Biology and Tree Improvement, College of Forestry and Department of Plant physiology, College of Agriculture, Vellanikkara, KAU was utilized for the molecular biology and biochemical work. Centrifugation was performed using a high speed refrigerated micro centrifuge (Eppendorf Centrifuge 5418R). The quality and quantity of extracted DNA were estimated using Nano drop spectrophotometer Jenway-Genova Nano and Eppendorf Bio-Spectrophotometer. DNA PCR amplification was done using Nexus gradient Thermal cycler (Eppendorf Master Cycler) and Horizontal gel electrophoresis unit BIO-RAD (USA) was used for agarose gel electrophoresis. Documentation of gel was done in the molecular imager gel doc^{TM} XR+ imaging system (BIO-RAD).

3.5. Evaluation of the seed progenies

The progeny test was done on the seedlings of twenty-five half-sib seed progeny already raised in the field at College of Forestry, Kerala Agricultural University, Vellanikkara, Thrissur. The layout out of the planting of 150 seedlings was done in a compact family block design consisting of two replications of 25 accessions (25 x 2), with each accession consisting of three seedlings (25 x 3 x 2). The progeny was studied for both morphological and physiological traits. Observations were taken at an interval of three months for one year.

3.5.1. Biometric observations of progenies

3.5.1.1. Plant height

The height of the plant was measured from the collar to the terminal bud with aluminium telescopic levelling staff and expressed in meters (Plate 1).

3.5.1.2. Collar girth

The collar girth of the plant was measured at the bottom of the plant just above the ground using a measuring tape and expressed in meters (Plate 1).

3.5.1.3. Crown width and number of leaves

The length of the longest and shortest diameter of the crown was measured and the two values were averaged. The measurement was done using a meter scale and expressed in meters.

3.5.1.4. Volume

Calculated using quarter girth formula after applying 0.7 as the form factor.

$$\text{Volume} = \frac{\text{Plant height (m) x girth}^2\text{(m) x 0.7}}{4\pi}$$

3.5.1.5. Absolute growth rate

The absolute growth rate is the total gain in height by a plant within a specific time interval. It is generally expressed as cm/day.

$$\text{Absolute Growth Rate} = \frac{h_2 - h_1}{t_2 - t_1}$$

h_1- Plant height at time (t_1)

h_2- Plant height at time (t_2)

3.5.2. Physiological Observations

3.5.2.1. Chlorophyll Content

The chlorophyll content of the plant was measured using a chlorophyll meter (SPAD-502, Minolta). The measurement was made from three different heights of the plant leaf and the resultant value was averaged for the final data (Plate 1).

3.5.2.2. Photosynthetic Rate

The photosynthetic rates of plants were recorded using Infra-red gas analyser (IRGA) (LI 6400 m portable photosynthesis system, LI-COR) model (Plate 1). The light intensity was fixed to the ambient light conditions (1000 - 1500 lux) and the amount of CO_2 expressed in µmole CO_2 m^2 s^{-1} (McDermitt *et al.,* 1989).

3.5.2.3. Transpiration Rate

The transpiration rates of plants were recorded using an Infra-red gas analyser (IRGA). The light intensity was fixed to the ambient light conditions (1000 - 1500 lux) and the amount of H_2O expressed in m. mole H_2O m^2 s^{-1} (McDermitt *et al.,* 1989).

3.5.2.4. Leaf Temperature

The leaf temperature of the plant was recorded using a thermocouple attached to the IRGA and expressed in ^oC (McDermitt *et al.,* 1989).

3.5.2.5. Stomatal Conductance

The stomatal resistance of the seedlings was recorded using an Infra-red gas analyser (IRGA) and expressed in mol H_2O m^2 s^{-1} (McDermitt *et al.,* 1989).

(a)

(b)

(c)

(d)

Plate 1. Measurement of morphological and physiological trait. (a) height using Aluminum telescopic levelling staff. (b) collar girth using measuring tape. (c) photosynthetic rate, transpiration rate, stomatal conductance using IRGA. (d) Chlorophyll content using SPAD

3.5.2.6. Relative Water Content

To estimate the relative water content, twenty small discs of leaf were cut, and fresh weight was taken. Immediately it was put in water for 4 hours. After removing from the distilled water, the leaf samples were dried using blotting paper and turgid weight was measured. These samples were then dried in a hot air oven set at a temperature of 70^{o}C to constant weight and dry weight was taken after 3 days. The RWC was calculated based on the formula.

$$\text{Relative water content} = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Turgid weight} - \text{Dry weight}} \text{ X } 100$$

All the physiological data were taken for the period 30 MAP and 36 MAP.

3.5.3. Estimation of genetic parameters

3.5.3.1. Variability Studies

These parameters were estimated as per the method described by Johnson *et al*. (1955).

3.5.3.1.1. Genotypic Variance (G.V.)

$$GV = \frac{\text{Genotypic mean square} - \text{Error mean square}}{\text{replication}}$$

3.5.3.1.2. Phenotypic Variance (P.V.)

$$PV = \sigma^2_g + \sigma^2_e$$

σ^2_g = Genotypic variance

σ^2_e = Environmental variance

3.5.3.1.3. Phenotypic (PCV) and genotypic (GCV)

PCV and GCV coefficients of variability were computed using the following equations suggested by Burton and De-Vane (1953).

Phenotypic co-efficient of variability

Phenotypic co-efficient of variability was calculated using the formula as given below

$$PCV\ (\%) = \left(\frac{\sqrt{\sigma^2_p}}{\mu}\right) \times 100$$

μ = Population mean for each trait

Genotypic co-efficient of variability

Genotypic co-efficient of variability was calculated using the formula as given below

$$GCV\ (\%) = \left(\frac{\sqrt{\sigma^2 g}}{\mu}\right) \times 100$$

3.5.3.1.4. Environmental coefficient of variation (ECV)

It is a measure of the total environmental variation existing for a character.

$$GCV\ (\%) = \left(\frac{\sqrt{\sigma^2 g}}{\mu}\right) \times 100$$

3.5.3.1.5. Heritability

Broad Sense heritability (H^2)

Broad sense heritability (H^2), which is a measure of the amount of phenotypic variance contributed by genetic factor was calculated according to Lush (1940).

$$H^2 = \frac{\sigma^2 g}{\sigma^2 p}$$

$$\text{Heritability percentage} = H^2 \times 100$$

3.5.3.1.6. Genetic advance

Genetic advance is the expected increase in the magnitude of a specific character when a selection pressure of chosen intensity is applied. The expected genetic advance at 5 percent selection intensity was worked out as suggested by Johnson *et al.,* (1955). The genetic advance was calculated as:

$$\text{Genetic advance (GA)} = iH^2\sigma_P$$

Where, selection intensity (i) was assumed to be 2.06, which is the expectation in the case of 5 percent selection in large samples from a normally distributed population (Allard, 1960). Genetic advance as a percentage of mean was calculated using the formula given by Johnson *et al.,* (1955).

Genetic advance as a percentage of mean = (GA* 100)/Grand mean

3.6. Analysis of genetic variation using ISSR markers

Molecular analysis was done for 25 plus tree seed progenies of *M. dubia* raised at College of Forestry, Kerala Agricultural University, Vellanikkara, Thrissur. These 25 plus tree seed progenies are having their parent location at 25 different locations in Kerala. The genetic variation was analysed using 15 ISSR primers.

3.6.1. Genomic DNA extraction

Fresh mature green leaf samples were collected from the 25 plus tree seed progeny. The leaves were cleaned and kept in a zip lock cover containing silica gel for the absorption of moisture. After dehydration, the dry leaves were taken from silica gel stored at room temperature for twenty-four hours. The dried leaf samples were used for extracting DNA using a modified CTAB method given by Rawat *et al.,* (2016).

3.6.1.1. DNA extraction

The dried mature leaves were wiped with 70% alcohol for removing the foreign particles if present. After cleaning the leaflets, 200 mg of the leaf lamina (excluding the large veins) was taken. These laminas were grounded to a very fine powder using liquid nitrogen along with five to eight milligrams of polyvinylpyrrolidone (PVP) in a sterilized ceramic mortar using a pestle. To the powdered leaf sample, 2ml of prewarmed sucrose extraction buffer (Appendix II) was added. The sample was transferred immediately to a 2ml centrifuge tube and 50 µL of β-mercaptoethanol was added to it and mixed well by gently inverting it 3 to 4 times. The tubes were incubated in a water bath at 65°C for 60 minutes and gently mixed at an interval of fifteen minutes. After incubation, the tubes were centrifuged at 10,000 rpm for 10 minutes at 4°C and the supernatant was carefully pipetted out to a new microcentrifuge tube. To this supernatant equal volume of CTAB extraction buffer II (Appendix II) was added and mixed well incubated in the water bath at 60°C for 30 minutes. After incubation an equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1, v/v) was added and mixed vigorously by inverting it fifteen to twenty times. The tube containing the samples were then centrifuged at 12,000 rpm for 10 minutes at 4°C. The top aqueous layer was carefully pipetted out to a new microcentrifuge tube. To this solution, 25 µl of RNase (20 mg/ml) was added and incubated in a water bath for 60 minutes at 37°C. After incubation the top aqueous layer was pipetted out into a new tube which was then washed with an equal volume of Chloroform: Isoamyl alcohol (24:1, v/v) and centrifuged at 12,000 rpm for 10 minutes and the clear top layer supernatant was taken out. The Chloroform: Isoamyl alcohol (24:1, v/v) wash was done until a clear aqueous phase was obtained. An equal volume of chilled isopropanol was added to the clear aqueous phase through the side of the tube and was inverted gently and kept −20˚C overnight. The next day the samples were centrifuged at 12,000 rpm for 10 minutes. DNA pellets were given 70% (v/v) ethanol washed two times, then air-dried at room

temperature for 30 to 60 minutes and dissolved in 50 µl of TE buffer (Appendix III) and was stored at -20˚C until further use.

3.6.1.2. Quantification of DNA

The quality and quantity of DNA extracted were estimated using the Nanodrop spectrophotometer (Jenway- Genova Nano) OD at 260 and OD 280 taken and OD 260/ OD 280 were calculated.

3.6.1.3. Agarose gel electrophoresis

DNA samples were electrophoresed in 0.8% agarose gel in 1x TAE buffer at 70 V for 3 hr (Plate 5). Reagents (Appendix III).

3.6.2. Molecular markers used for the study

Fifteen ISSR (Inter-Simple Sequence Repeats) primers (Rawat *et al.*, 2018) were used to analyse the genetic diversity among the 25 *M. dubia* plus tree seed progenies. All the reported ISSR primers were selected for the study (Table 2).

3.6.2.1. Amplification using ISSR primer

Good quality DNA (150-1000 ng/µL) isolated from plus tree seed progenies of *M. dubia* was used for ISSR analysis. The template DNA was diluted to a concentration of 100 ng/µL. Selected primers were bought from Sigma Aldridge. Emerald master mix (Takara) was used in this study instead of a separate PCR. The PCR amplification was carried out in a 20µl reaction mixture, which contained the Genomic (template) DNA (100ng/µl) of 2µl, Emerald Mastermix (Takara)(2x) of 10µl, Nuclease free water of 6µl and Primer (100ng/µl) of 2µl.

PCR was carried out using Nexus gradient Thermal cycler (Eppendorf Master Cycler) under the following conditions. The initial cycle of the denaturation was done at 94°C for three minutes, followed by 39 cycles of

denaturation at 94°C for thirty seconds, annealing at 30-58 °C (depending upon the Tm of primer used (Table 2) for thirty seconds, extension at 72°C for one minute and final extension at 72°C for ten minutes (Rawat *et al.,* 2018). The amplified products along with control were run for 4 hours at 45 V in a 2% agarose gel in a 1XTAE buffer (Appendix III). The gel profile was viewed under UV Transilluminator and was documented using the molecular imager gel Doc™ XR+ imaging system (BIO-RAD). The documented ISSR marker profile was evaluated in detail for the amplification. Robust and unambiguous bands were evaluated. The number of polymorphic and monomorphic bands in the profile were recorded for further analysis.

Table 2. Annealing temperature (T_a) and Melting temperature ™ of ISSR primers which amplified DNA for genetic diversity assessment of *Melia dubia*.

SL No.	Primer	Sequence	Annealing temperature (Ta^oC)	Melting temperature (Tm^oC)
1	UBC 809	AGAGAGAGAGAGAGAGG	43.2	46.6
2	UBC 810	GAGAGAGAGAGAGAGAT	40.0	42.6
3	UBC 813	CTCTCTCTCTCTCTCTT	39.5	43.5
4	UBC 816	CACACACACACACACAT	47.5	51.2
5	UBC 823	TCTCTCTCTCTCTCTCC	45.1	47.5
6	UBC 847	CACACACACACACACARC	44.5	46.0
7	UBC 864	ATGATGATGATGATGATG	49.0	51.3
8	UBC 884	HBHAGAGAGAGAGAGAG	32.4	35.0
9	UBC 888	BDBCACACACACACACA	40.5	44.0
10	UBC 890	VHVTGTGTGTGTGTGTG	47.2	51.8
11	UBC 891	VHVGTGTGTGTGTGTGT	48.0	51.2
12	L1	AGAGAGAGAGAGAGAGGT	40.3	48.3
13	L3	CCAGTGGTGGTGGTG	55.0	57.4
14	L4	DBDCACACACACACA	33.6	36.5
15	L9	GAGAGAGAGAGAGAGAGAC	40.3	36.5

3.6.2.4. Diversity analysis using ISSR markers

15 ISSR primers were selected for ISSR analysis to evaluate the genetic diversity among the accessions of *M. dubia*.

3.6.2.4.1. Scoring of bands and data analysis

Scoring of bands agarose gel was done for robust and unambiguous bands based on the presence and absence of the band. The bands were encoded using the binary method of Wendell and Weeden (1989) for presence (1) or absence (0) of bands respectively. Based on this hierarchical cluster analysis was done. Only well distinct and resolved DNA bands were scored for analysis. Similarity coefficients were computed as Jaccard's coefficient through the SIMQUAL routine and clustering was done using the SAHN routine of the package. A dendrogram was constructed based on Jaccard's similarity coefficient and Nei's gene diversity coefficient generated using UPGMA (Unweighted Pair Group Method with Arithmetic mean) in NTSYSpc ver. 2 software. Association between the accessions was found out from the dendrogram.

$$\text{Jaccard's similarity coefficient} = \frac{a}{a+b+c}$$

Where,

a = number of bands present in both genotypes in a pair

b = number of bands present in the first genotype but not in other

c = number of bands present in the second genotype but not in other

3.7. Phytochemical characterization of *Melia dubia* progeny

This experiment tries to quantify an important phytochemical (caryophyllene) in *M. dubia* using HPLC (Plate 2).

3.7.1. Collection of plant materials

Fresh leaves of *M. dubia* were collected from the 25 plus tree seed progeny in College of Forestry, Kerala Agricultural University, Vellanikkara, Thrissur.

3.7.2. Preparation of plant material

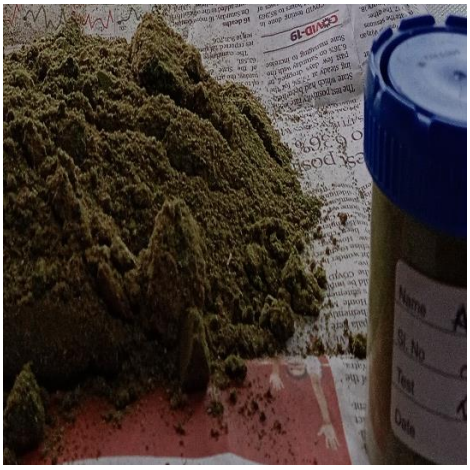
The collected plant materials were shade dried for 10 days and ground into a uniform powder. 10g of dry powder of plant sample was extracted with solvent acetone using 500 ml Soxhlet apparatus (Plate 2) for six hours. The extract was then filtered over anhydrous sodium sulphate followed by concentrated using a rotary evaporator (Plate2) was suspended with methanol at the concentration of 100 mg/ml (w/v) followed by filtration through Varian Bond Elute C_{18} solid-phase extraction to remove the impurities. 20 µl of this solution was employed for HPLC analysis.

3.7.3. HPLC analysis for caryophyllene

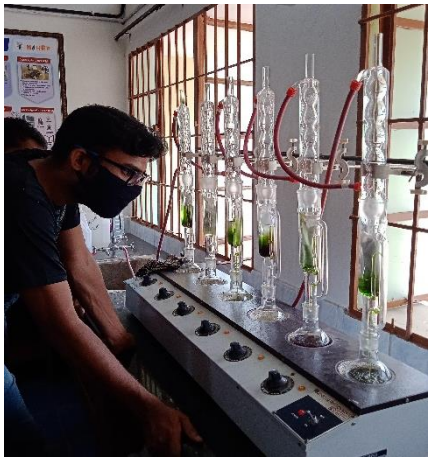
A High-Performance Liquid Chromatography technique was employed to quantify the caryophyllene present in the leaves of 25 plus tree seed progenies.

3.7.3.1 . Preparation of caryophyllene standard

The caryophyllene standard was prepared using acetonitrile from the 2 ml (2000 ppm) stock solution. 12.5 ppm, 25 ppm, 50 ppm, 100 ppm, 200 ppm working solutions were prepared. 20 µL working solutions from each concentration were injected into the HPLC system calibration curve for each concentration was prepared against the respective peak areas to get a regression curve. Based on the regression curve obtained, caryophyllene in the samples was estimated.



(a)



(b)

(c)

(d)

Plate 2. HPLC Quantification (a) powdered dry leaves (b) Soxhlet apparatus (c) Rotary evaporator (d) HPLC

3.7.3.2. Procedure

The HPLC columns were cleaned and run with methanol as control. The samples were then injected one by one and then loaded to get the peaks in the Post Run Analysis software. The standard of caryophyllene was run and the peak was compared to the peaks of the samples. The areas of the peaks were calculated using the Post Run Analysis software. Based on the peak areas, the % caryophyllene was estimated using the standard formula:

% Caryophyllene = (Sample Area/Standard Area) × (Standard Weight/Standard Dilution) × (Sample Dilution Weight of the sample) × Purity of Standard

3.7.3.3. HPLC instrument conditions

The HPLC conditions used for drawing the calibration curve for caryophyllene were column: RP-C18, 250 x 4.6 mm, 5 um sizes (Phenomenex), detector: SPD-M 20A photodiode array detector (PDA), Wavelength: 254 nm, Flow rate: 15 mL min, Injection volume: 20 µL, Mobile phase Pump A (25% acetonitrile), Pump B (75% ammonium acetate) in an isocratic mode. The solvents in the mobile phase were filtered and sonicated before use.

3.8.1. Statistical analysis

The compact family block design analyses were done using ‘Agricole’ inbuilt packages of R v. 4.0.3.

4.RESULTS

The results obtained in the present investigation titled "Progeny testing and genetic diversity analysis in plus trees of *M. dubia* Cav.)" are presented in this chapter.

4. Evaluation of the seed progenies

4.1. Morphological traits

4.1.1. Plant height

The height of the plus tree seed progenies observed at definite intervals is given in Table 3. It was observed that at 24 MAP, the accession FCV-MD-03 (Tholpetty) recorded the maximum mean value for height (3.65 m). The value was were however on par with the accessions FCV-MD-01, FCV-MD-02, FCV-MD-04, FCV-MD-6, FCV-MD-8, FCV-MD-11, FCV-MD-13, FCV-MD-16, FCV-MD-18, FCV-MD-19, FCV-MD-21, FCV-MD-24, FCV-MD-25. The least mean value of height was observed for the plus tree seed progeny collected from the accession FCV-MD-17 of Chinnar and was found to be on par with FCV-MD-05, FCV-MD-07, FCV-MD-09, FCV-MD-10, FCV-MD-14, FCV-MD-15, FCV-MD-17, FCV-MD-20, FCV-MD-22, and FCV-MD-23. At 27 MAP a significant difference in the mean height of the seedlings was also observed. The plus tree progenies from the accession FCV-MD-01, FCV-MD-02, FCV-MD-03, FCV-MD-04, FCV-MD-6, FCV-MD-8, FCV-MD-16, and FCV-MD-18 were observed to be on par and the seedling of FCV-MD-03 (Tholpetty) accession showed the highest mean value, 3.96 m. The lowest was observed for the plus tree seed progeny FCV-MD-17 of Chinnar (2.24 m). At 30 MAP similar trend was observed with the seedlings from accessions FCV-MD-01, FCV-MD-02, FCV-MD-03, FCV-MD-04, FCV-MD-6, FCV-MD-13, FCV-MD-16, FCV- FCV-MD-25 with higher mean value were on par and the plus tree seed progeny FCV-MD-03 (Tholpetty) again topped among them with a mean value of 4.64 m. At 33 MAP it was observed that the seedlings from the plus tree seed progeny accession FCV-MD-01, FCV-MD-02, FCV-MD-03, FCV-MD-04,

FCV-MD-6, FCV-MD-8, FCV-MD-13, FCV-MD-16, FCV-MD-18, FCV-MD-22, FCV-MD-25 were on par and the plus tree seed progeny FCV-MD-03 (Tholpetty) showed the highest value (4.97 m). The lowest value (2.93 m) was observed for the plus tree seed progeny from the accession FCV-MD-17 (Parambikulam). At the end of the experiment (36 MAP), it was observed that the seedlings of the plus tree seed progeny accession FCV-MD-01, FCV-MD-02, FCV-MD-03, FCV-MD-05, FCV-MD-6, FCV-MD-8, FCV-MD-16, FCV-MD-22, FCV-MD-25 were on par for their higher mean value of height and the plus tree seed progeny FCV-MD-03 from Tholpetty again showed the highest mean value (5.61 m). The lowest value (3.28 m) was observed for the plus tree seed progeny from the accession FCV-MD-17 (Parambikulam). It can be concluded from the above results that the plus tree progenies (FCV-MD-03) selected from Tholpetty in Wayanad showed the highest mean value consistently throughout the field trial experiment whereas the lowest value was observed for the plus tree seed progeny (FCV-MD-17) from Chinnar for the first three observations. However, for the last observation, the least performance was observed in the plus tree seed progeny (FCV-MD-15) from the Parambikulam region.

Table 3. Height (m) of *Melia dubia* plus tree seed progenies at three months intervals.

Accession no.	24 MAP	27 MAP	30 MAP	33 MAP	36 MAP
FCV-MD-01	3.35^{abc}	3.66^{ab}	4.08^{ab}	4.50^{abcd}	5.12^{abc}
FCV-MD-02	3.22^{abc}	3.55^{ab}	4.02^{abc}	4.41^{abcde}	5.06^{abc}
FCV-MD-03	3.65^{a}	3.96^{a}	4.64^{a}	4.97^{a}	5.61^{a}
FCV-MD-04	2.93^{abcdef}	3.24^{abcd}	3.85^{abcd}	4.11^{abcdef}	4.48^{bcd}
FCV-MD-05	2.83^{bcdefg}	3.03^{bcdefg}	3.64^{bcdef}	3.92^{cdefgh}	4.67^{abcd}
FCV-MD-06	3.00^{abcde}	3.25^{abcd}	3.98^{abc}	4.38^{abcde}	5.26^{abc}
FCV-MD-07	2.29^{efg}	2.48^{efgh}	2.88^{fgh}	3.28^{fghi}	3.81^{def}
FCV-MD-08	3.06^{abcde}	3.23^{abcd}	3.73^{bcde}	4.08^{abcdefg}	4.68^{abcd}
FCV-MD-09	2.28^{efg}	2.62^{cdefgh}	3.08^{defgh}	3.51^{efghi}	3.98^{def}
FCV-MD-10	2.15^{fg}	2.42^{gh}	2.75^{gh}	2.93^{i}	3.46^{ef}
FCV-MD-11	2.95^{abcdef}	3.16^{bcdef}	3.53^{bcdefg}	3.83^{defghi}	4.48^{bcd}
FCV-MD-12	2.79^{bcdefg}	2.97^{bcdefgh}	3.40^{bcdefgh}	3.85^{defgh}	4.35^{bcde}
FCV-MD-13	3.09^{abcde}	3.22^{bcd}	3.60^{bcdef}	4.06^{abcdefg}	4.40^{bcde}
FCV-MD-14	2.56^{cdefg}	2.75^{cdefgh}	3.21^{cdefgh}	3.51^{efghi}	3.93^{def}
FCV-MD-15	2.18^{fg}	2.43^{fgh}	2.67^{h}	2.93^{i}	3.28^{f}
FCV-MD-16	3.29^{abc}	3.55^{ab}	4.11^{ab}	4.80^{abc}	5.31^{abc}
FCV-MD-17	2.10^{g}	2.24^{h}	2.67^{h}	3.10^{hi}	3.80^{def}
FCV-MD-18	3.08a^{bcde}	3.31^{abc}	3.79^{bcde}	4.07^{abcdefg}	4.65^{abcd}
FCV-MD-19	3.17^{abcd}	2.96^{bcdefgh}	3.43^{bcdefgh}	3.73^{defghi}	4.33^{cde}
FCV-MD-20	2.40^{defg}	2.61^{cdefgh}	3.03^{efgh}	3.40^{fghi}	3.98^{def}
FCV-MD-21	3.03^{abcde}	3.19^{bcde}	3.56^{bcdef}	3.95^{bcdefgh}	4.58^{bcd}
FCV-MD-22	2.74^{bcdefg}	2.97^{bcdefgh}	3.73^{bcde}	4.07^{abcdefg}	4.65^{abcd}
FCV-MD-23	2.34^{efg}	2.57^{defgh}	2.85^{fgh}	3.20^{ghi}	3.71^{def}
FCV-MD-24	3.01^{abcde}	3.22^{bcde}	3.70^{bcde}	4.05^{bcdefg}	4.55^{bcd}
FCV-MD-25	3.41^{ab}	3.60^{ab}	4.21^{ab}	4.83^{ab}	5.35^{ab}
SEM	0.22	0.21	0.27	0.31	0.39
CD	0.96	0.94	1.07	1.14	1.28
F value	1.99*	1.81*	1.76*	1.70*	1.85*

* indicates 5 percent level of significance ($p<0.05$)

4.1.2. Absolute growth rate

Table 4. Absolute growth rate (cm day^{-1}) of *Melia dubia* plus tree seed progenies at three months intervals.

Accession No.	27 MAP	30 MAP	33 MAP	36 MAP
FCV-MD-01	0.34	0.47	0.46	0.98
FCV-MD-02	0.37	0.51	0.44	0.83
FCV-MD-03	0.34	0.75	0.36	0.77
FCV-MD-04	0.34	0.67	0.29	0.72
FCV-MD-05	0.24	0.68	0.3	0.72
FCV-MD-06	0.27	0.82	0.44	0.72
FCV-MD-07	0.21	0.44	0.44	0.71
FCV-MD-08	0.32	0.55	0.38	0.68
FCV-MD-09	0.37	0.51	0.53	0.68
FCV-MD-10	0.36	0.37	0.22	0.66
FCV-MD-11	0.24	0.4	0.33	0.66
FCV-MD-12	0.28	0.47	0.5	0.64
FCV-MD-13	0.14	0.41	0.51	0.64
FCV-MD-14	0.21	0.51	0.33	0.64
FCV-MD-15	0.27	0.26	0.29	0.59
FCV-MD-16	0.29	0.61	0.76	0.59
FCV-MD-17	0.16	0.47	0.47	0.57
FCV-MD-18	0.26	0.53	0.3	0.57
FCV-MD-19	0.57	0.52	0.33	0.57
FCV-MD-20	0.23	0.47	0.4	0.55
FCV-MD-21	0.27	0.56	0.33	0.46
FCV-MD-22	0.32	0.85	0.37	0.46
FCV-MD-23	0.21	0.35	0.38	0.45
FCV-MD-24	0.22	0.53	0.38	0.38
FCV-MD-25	0.21	0.66	0.69	0.37
MSE	0.0004	0.0005	0.0003	0.0008

The absolute growth rate of the plus trees seed progenies calculated are given in Table (4). It was observed that the values were non-significant for the observations. A maximum mean growth rate (0.62 cm^{-1}) was observed between 33 MAP and 36 MAP. The lowest mean growth rate (0.27 cm^{-1}) was observed between the month of 24 MAP and 37 MAP. FCV-MD-02 (Thirunelly) showed the highest absolute growth rate throughout the field trial experiment of the plus tree progenies.

4.1.3. Plant girth

The collar girth of the plus tree seed progenies recorded for the progeny test is given in Table (5). The maximum mean value for the collar girth (0.26m) at 24 MAP, was observed in the accession FCV-MD-02 (Thirunelly) and was found to be on par with FCV-MD-03 (Tholpetty) with a girth of 0.24 m. The least mean girth was observed in FCV-MD-17 of Chinnar (0.09 m) and FCV-MD-23 of Kulathupuzha (0.086 m). At 27 MAP a significant difference in the girth of the seedlings was observed. The plus tree progenies from the accession FCV-MD-01, FCV-MD-02, FCV-MD-03 were on par. It was observed that the seedlings of the plus trees FCV-MD-03 (Tholpetty) showed the highest value, 0.291 m. The lowest girth was observed for the plus tree seed progeny of FCV-MD-17 of Chinnar (0.10) and FCV-MD-23 of Kulathupuzha (0.10). At 30 MAP it was observed that the plus tree seed progeny FCV-MD-03 showed the highest value (0.33 m), from Tholpetty. At 33 MAP FCV-MD-03 showed the highest value (0.37 m), from Tholpetty. The lowest value (0.16 m) was observed for the accession FCV-MD-10 (Thiruvazhiyadu). At the end of the experiment 36 MAP, it was observed that the seedlings from the plus tree seed progeny accession FCV-MD-01, FCV-MD-02, FCV-MD-03, FCV-MD-04, FCV-MD-6, FCV-MD-11, FCV-MD-16, FCV-MD-18, FCV-MD-21, and FCV-MD-25 were on par. The plus tree seed progeny FCV-MD-03 from Tholpetty showed the highest value (0.42 m). The lowest value (0.02 m) was observed for the accession FCV-MD-10 from Thiruvazhiyadu. The results showed that the plus tree seed progeny (FCV-MD-02) selected from Thirunelly in Wayanad showed the highest girth for the first observation and later FCV-MD-03 selected from Tholpetty in Wayanad, which showed the highest value throughout the observation period. The lowest value was observed for the plus tree seed progeny (FCV-MD-17) from Chinnar and FCV-MD-23 from Kulathupuzha during 24 MAP and 27 MAP, and later FCV-MD-10 from Thiruvazhiyadu

showed the lowest value consistently for the remaining observation (30 MAP, 33 MAP, 36MAP).

Table 5. Girth (m) of *Melia dubia* plus tree seed progenies at three months intervals.

Accession No.	24 MAP	27.MAP	30 MAP	33 MAP	36 MAP
FCV-MD-01	0.206^{bc}	0.25^{abc}	0.29^{abc}	0.33^{abc}	0.37^{abcd}
FCV-MD-02	0.25^{a}	0.27^{ab}	0.31^{ab}	0.37^{a}	0.41^{ab}
FCV-MD-03	0.23^{ab}	0.29^{a}	0.33^{a}	0.37^{a}	0.42^{a}
FCV-MD-04	0.15^{def}	0.18^{def}	0.22^{cdefg}	0.29^{bcdef}	0.33^{abcdef}
FCV-MD-05	0.15^{def}	0.18^{def}	0.22^{defgh}	0.26^{cdefgh}	0.29^{defg}
FCV-MD-06	0.17^{cde}	0.22^{bcd}	0.27^{abcd}	0.31^{abcd}	0.37^{abcd}
FCV-MD-07	0.19^{hij}	0.12^{hi}	0.16^{hij}	0.23^{efghi}	0.28^{defg}
FCV-MD-08	0.16^{cdef}	0.18^{defg}	0.22^{defgh}	0.25^{defgh}	0.31^{bcdefg}
FCV-MD-09	0.11^{fghij}	0.14^{fghi}	0.18^{ghij}	0.23^{efghi}	0.27^{6defg}
FCV-MD-10	0.09^{ij}	0.11^{hi}	0.13^{j}	0.16^{i}	0.22^{g}
FCV-MD-11	0.12^{efghij}	0.14^{efghi}	0.21^{defgh}	0.27^{cdefg}	0.34^{abcdef}
FCV-MD-12	0.13^{defghij}	0.15^{efghi}	0.20^{efghi}	0.23^{efghi}	0.29^{defg}
FCV-MD-13	0.15^{defg}	0.17^{efgh}	0.21^{defgh}	0.26^{cefgh}	0.30^{defg}
FCV-MD-14	0.12^{fghij}	0.14^{fghi}	0.18^{ghij}	0.22^{fghi}	0.24^{fg}
FCV-MD-15	0.10^{ghij}	0.12^{ghi}	0.16^{hij}	0.19^{hi}	0.23^{g}
FCV-MD-16	0.17^{cd}	0.20^{cde}	0.25^{bcdef}	0.31^{abcd}	0.32^{abcde}f
FCV-MD-17	0.08^{j}	0.10^{i}	0.14^{ij}	0.20^{ghi}	0.26^{efg}
FCV-MD-18	0.15^{defg}	0.18^{defg}	0.23^{cdefg}	0.30^{bcdef}	0.35a^{bcde}
FCV-MD-19	0.16^{cdef}	0.16^{defg}	0.20^{fghi}	0.25^{efghi}	0.31^{cdefg}
FCV-MD-20	0.11^{fghij}	0.13^{fghi}	0.17^{ghij}	0.22^{fghi}	0.28^{defg}
FCV-MD-21	0.14^{defgh}	0.15^{efghi}	0.23^{cdefg}	0.29^{bcdef}	0.37^{abcd}
FCV-MD-22	0.13^{defgh}	0.15^{efghi}	0.22^{defgh}	0.27^{cdef}	0.32^{bcdefg}
FCV-MD-23	0.08^{j}	0.10^{i}	0.17^{ghij}	0.23^{efghi}	0.26^{efg}
FCV-MD-24	0.14^{defg}	0.16^{efgh}	0.21^{defgh}	0.28^{bcdef}	0.30^{defg}
FCV-MD-25	0.17^{cd}	0.20^{cde}	0.26^{bcde}	0.35^{ab}	0.41^{abc}
SEM	0.001	0.002	0.002	0.007	0.003
CD	0.082	0.101	0.109	0.181	0.127
F Value	1.76*	2.21*	3.23*	1.87*	2.20*

* indicates 1 percent level of significance ($p<0.01$)

4.1.4. Crown diameter and number of leaves

The crown diameter and the number of leaves of the plus tree seed progeny are given in Table (6). At 24 MAP the number of leaves was counted and it was observed that FCV-MD-19 of Chinnar has got the highest mean number of leaves (32.67) and was identified to be the best compared to all others and it was on par with FCV-MD-01, FCV-MD-03, FCV-MD-05, FCV-MD-6, FCV-MD-8, FCV-MD-09, FCV-MD-11, FCV-MD-18, FCV-MD-20, FCV-MD-21, FCV-MD-24 FCV-MD-25. The least number of leaves mean observed were for FCV-MD-13 from Walayar and FCV-MD-23 from Kulathupuzha. At the 27th MAP it was found that FCV-MD-01, FCV-MD-03, FCV-MD-05, FCV-MD-6, FCV-MD-8, FCV-MD-09, FCV-MD-11, FCV-MD-12, FCV-MD-13, FCV-MD-17, FCV-MD-18, FCV-MD-19, FCV-MD-20, FCV-MD-21, FCV-MD-22, FCV-MD-24 FCV-MD-25. The plus tree seed progeny FCV-MD-19 from Chinnar has got the highest mean number of leaves (36.16). At 30 MAP the crown diameter was measured as it was found difficult to count the number of leaves. During this month it was observed that the accession FCV-MD-25 (Aryankavu) in Kollam showed the highest value and lowest by the accession FCV-MD-15 (Parambikulam).

Due to the deciduous nature of the tree, it was difficult to measure the crown diameter at 33 MAP and hence the data was not recorded. At the end of the experiment (36 MAP), data recorded showed that FCV-MD-01, FCV-MD-02, FCV-MD-03, FCV-MD-04, FCV-MD-06, FCV-MD-13, FCV-MD-16, FCV-MD-22, FCV-MD-24 FCV-MD-25. The highest value was observed for plus tree seed progeny from the accession FCV-MD-25 (Aryankavu) with a mean diameter of 4.05 m. The lowest crown diameter was observed for FCV-MD-15 (Parambikulam).

Table 6. Crown diameter (m)/Number of leaves of *Melia dubia* plus tree seed progenies at three months intervals.

Accession No.	24 MAP No. of leaves	27 MAP No. of leaves	30 MAP Crown diameter (m)	36 MAP Crown diameter (m)
FCV-MD-01	28.50^{abc}	32.50^{ab}	3.15^{abc}	3.60^{abc}
FCV-MD-02	19.00^{bcdef}	21.67^{bcd}	2.90^{abcde}	3.15^{abcde}
FCV-MD-03	25.00^{abcde}	27.83^{abcd}	2.95^{abcd}	3.26^{abcd}
FCV-MD-04	17.33^{cdef}	20.00^{bcd}	2.85^{abcde}	3.15^{abcde}
FCV-MD-05	23.00^{abcdef}	28.17^{abcd}	2.5^{bcdef}	2.82^{bcdef}
FCV-MD-06	23.10^{abcdef}	28.67^{abcd}	3.30^{ab}	3.70^{ab}
FCV-MD-07	17.16^{cdef}	19.83^{bcd}	1.90^{efg}	2.10^{efg}
FCV-MD-08	22.67^{abcdef}	26.67^{abcd}	2.15^{cdefg}	2.55^{cdefg}
FCV-MD-09	20.50^{abcdef}	24.33^{abcd}	1.85^{defg}	2.20^{defg}
FCV-MD-10	13.00^{ef}	16.50^{cd}	1.2^{g}	1.60^{g}
FCV-MD-11	25.67^{abcd}	29.33^{abc}	2.34^{bcdef}	2.78^{bcdef}
FCV-MD-12	19.00^{bcdef}	22.69^{abcd}	2.30^{bcdef}	2.75^{bcdef}
FCV-MD-13	20.00^{bcdef}	23.67^{abcd}	2.70^{abcde}	3.10^{abcdef}
FCV-MD-14	15.67^{def}	19.17^{bcd}	1.78^{efg}	2.10^{efg}
FCV-MD-15	12.33^{f}	15.16^{d}	1.10^{g}	1.45^{g}
FCV-MD-16	16.83^{cdef}	19.67^{bcd}	3.25^{ab}	3.45^{ab}
FCV-MD-17	19.41^{bcdef}	22.33^{abcd}	1.65^{fg}	2.00^{fg}
FCV-MD-18	31.06^{ab}	29.50^{abc}	2.15^{cdefg}	2.55^{cdefg}
FCV-MD-19	32.67^{a}	36.16^{a}	2.28^{cdefg}	2.56^{cdefg}
FCV-MD-20	22.83^{abcdef}	25.69^{abcd}	2.05^{defg}	2.40^{defg}
FCV-MD-21	24.16^{abcdef}	23.32^{abcd}	2.48^{bcdef}	2.85^{bcdef}
FCV-MD-22	20.00^{bcdef}	23.00^{abcd}	2.95^{abcde}	3.22^{abcde}
FCV-MD-23	12.33^{f}	16.00^{cd}	2.0^{defg}	2.25^{defg}
FCV-MD-24	23.50^{abcdef}	26.33^{abcd}	2.65^{abcde}	2.92^{abcdef}
FCV-MD-25	23.60^{abcdef}	26.25^{abcd}	3.65^{a}	4.05^{a}
SEM	58.86	28.17	0.41	0.47
CD	15.79	15.74	1.35	1.42
F Value	1.79*	2.32*	2.15*	1.89*

* indicates 1 percent level of significance ($p<0.01$)

4.1.5. Volume

The volume of the plus trees seed progeny is given in Table (7). Significant variations were observed during the field study. At 24th MAP plus tree seed progeny from the accession FCV-MD-02 (Thirunelly) and FCV-MD-03 (Tholpetty) showed the maximum mean value and was found to be on par with FCV-MD-024, FCV-MD-25 of Aryankavu. The least value was observed in FCV-MD-16 (Parambikulam). At 27 MAP a significant difference in the mean value volume of the seedlings was observed. The plus tree progenies FCV-MD-01, FCV-MD-02, FCV-MD-03, and FCV-MD-16 were found to be on par in the plus tree progeny FCV-MD-03 (Tholpetty) showed the highest value, 0.0046 m^3. The lowest volume was observed for the plus tree seed progeny of FCV-MD-17 of Chinnar (0.00064 m^3). At 30 MAP it was observed that the plus tree seed progeny from the accession FCV-MD-01, FCV-MD-02, FCV-MD-03, and FCV-MD-23 were on par. The plus tree seed progeny FCV-MD-03 (Tholpetty) showed the highest value (0.0062 m^3). At 33 MAP the plus tree seed progeny FCV-MD-03 (Tholpetty) showed the maximum value (0.0081 m^3). The lowest value was observed in FCV-MD-17 of Chinnar. At the end of the experiment (36 MAP), it was observed that the plus tree seed progeny from the accession FCV-MD-01, FCV-MD-02, FCV-MD-03, FCV-MD-6, FCV-MD-16, FCV-MD-25 were on par and FCV-MD-03 (Tholpetty) showed the highest mean value for volume (0.0110 m^3). The lowest value was observed for the accession FCV-MD-15 from Parambikulam. The results showed that the plus tree seed progeny FCV-MD-03 selected from Thirunelly and FCV-MD-03 selected from Tholpetty in Wayanad recorded maximum mean value for the first observation at 24 MAP, and later FCV-MD-03 selected from Tholpetty in Wayanad, showed the highest value for the remaining period. The lowest value was observed in FCV-MD-16 from Parambikulam at 24 MAP, however for the remaining observation it changed to FCV-MD-10 (Thiruvazhiyadu), and FCV-MD-17 (Chinnar) showed the lowest value (0.26 m^3).

Table 7. Volume (m^3) of *Melia dubia* plus tree seed progenies at three months intervals.

Accession No.	24 MAP	27 MAP	30 MAP	33 MAP	36 MAP
FCV-MD-01	0.0024^{ab}	0.0036^{abc}	0.0046^{abc}	0.0060^{abcd}	0.0075^{abcde}
FCV-MD-02	0.0035^{a}	0.0041^{ab}	0.0055^{ab}	0.0077^{abc}	0.0094^{abc}
FCV-MD-03	0.0030^{a}	0.0046 ^{a}	0.0062^{a}	0.0081^{a}	0.0170^{a}
FCV-MD-04	0.0013^{bcdefgh}	0.0020cd^{ef}	0.0029^{bcdef}	0.0047^{fgh}	0.0062^{bcdefgh}
FCV-MD-05	0.0013^{bcdefgh}	0.0019^{defg}	0.0028^{cdefg}	0.0040^{defgh}	0.0050^{cdefghi}
FCV-MD-06	0.0017^{bcde}	0.0035^{bcd}	0.0043^{bc}	0.0056^{abcde}	0.0079^{abcd}
FCV-MD-07	0.0005^{fghi}	0.0008^{fgh}	0.0014^{fgh}	0.0029^{fgh}	0.0044^{fghi}
FCV-MD-08	0.0009^{bcdef}	0.0017^{defg}	0.0026^{cdefg}	0.0035^{defgh}	0.0053^{cdefghi}
FCV-MD-09	0.0010^{efghi}	0.0011^{fgh}	0.0018^{efgh}	0.0030^{efgh}	0.0041^{fghi}
FCV-MD-10	0.0013^{ghi}	0.0007^{fgh}	0.0011^{h}	0.0016^{h}	0.0029^{hi}
FCV-MD-11	0.0008^{cdefghi}	0.0012^{defgh}	0.0027^{cdefgh}	0.0042^{defgh}	0.0071^{abcdefg}
FCV-MD-12	0.0006^{cdefghi}	0.0013^{defgh}	0.0022^{defgh}	0.0029^{efgh}	0.0047^{defghi}
FCV-MD-13	0.0017^{bcdefg}	0.0016^{defgh}	0.0025^{cdefgh}	0.0037^{defgh}	0.0049^{defghi}
FCV-MD-14	0.0004^{fghi}	0.0011^{efgh}	0.0019^{efgh}	0.0028^{defgh}	0.0033^{ghi}
FCV-MD-15	0.0012^{abcd}	0.0008^{fgh}	0.0015^{gh}	0.0020^{gh}	0.0030^{i}
FCV-MD-16	0.0014^{i}	0.00225^{ab}	0.0035^{bcd}	0.0052^{abcd}	0.0059^{abcdef}
FCV-MD-17	0.0007^{bcdefg}	0.00064^{h}	0.0011^{h}	0.0022^{gh}	0.0038^{fghi}
FCV-MD-18	0.0012^{bcde}	0.00178^{defg}	0.0029^{bcdef}	0.0049^{bcdef}	0.007^{abcdefg}
FCV-MD-19	0.0014^{efghi}	0.0015^{defgh}	0.0022^{defgh}	0.0034^{defgh}	0.0052^{defghi}
FCV-MD-20	0.0007^{bcdefg}	0.0010^{fgh}	0.0017^{efgh}	0.0027^{fgh}	0.0044^{efghi}
FCV-MD-21	0.0012^{cdefghi}	0.0014^{defgh}	0.0028^{bcde}	0.0042^{cdef}	0.0076^{abcdef}
FCV-MD-22	0.0009^{hi}	0.0013^{defgh}	0.0026 ^{cdefg}	0.0042^{cdefg}	0.0057^{cdefghi}
FCV-MD-23	0.0004^{cdefghi}	0.0006^{ab}	0.0016^{efgh}	0.0029^{fgh}	0.0041^{ghi}
FCV-MD-24	0.0011^{abc}	0.0015^{defgh}	0.0025^{cdefgh}	0.0043^{cdefg}	0.0049^{defghi}
FCV-MD-25	0.0017^{ab}	0.0023^{ab}	0.0045^{abc}	0.0071^{ab}	0.0094^{ab}
SEM	0.026	0.028	0.063	0.206	0.117
CD	0.332	0.3453	0.518	0.453	0.705
F value	1.78**	2.08*	2.32*	1.90**	2.31*

* indicates 1 percent level of significance ($p<0.01$)

4.2. Genetic parameters for morphological and growth traits

4.2.1. Plant height

The genetic parameters for morphological and growth traits at 36 MAP (Table 8) showed that the broad-sense heritability for plant height was 0.683. It was observed that the PCV, GCV and ECV were 27.55, 23.789 and 15.7 respectively. The value for the genetic advance (%) of mean was 37.

4.2.2. Plant girth

For the plant girth, the broad-sense heritability was observed to be 0.75. The PCV, GCV and ECV values were 37.9, 33.1 and 18.6 respectively. The value for the genetic advance (%) of the mean was 50 (Table 8).

4.2.3. Crown diameter

Broad sense heritability for the crown diameter was observed to be 0.62. The values for PCV, GCV and ECV were observed to be 44.82, 35.51 and 27 respectively. The value for the genetic advance (%) of the mean was 73 (Table 8).

4.2.4. Volume

The broad-sense heritability for this trait was 0.65. The values for PCV, GCV and ECV were observed to be 66.8, 35.51 and 39 respectively. The value for the genetic advance (%) of the mean was 78 (Table 8).

Table 8. Estimated genetic parameters of morphological traits of *Melia dubia*

Parameters	**Phenotypic variance**	**Genotypic variance**	**H^2**	**PCV**	**CV**	**CV**	**Genetic advance (%) of mean**
Height	1.1	0.8	0.68	27.5	23.7	15	37
Girth	0.01	0.01	0.75	37.9	33.1	18	50
Crown diameter	1.5	0.94	0.62	44.8	35.5	27	73
Volume	0.2	0.18	0.65	66.8	53.9	39	78

4.3. Path co-efficient analysis

The direct and indirect effects of morphometric traits on volume are presented in Table (9). Since the residual value was found to be 0.0082 we can say both physiological and morphological traits were having both direct and indirect effects over the volume. The morphological traits such as plant height (0.43), collar girth (0.63), absolute growth rate (0.14), and physiological trait stomatal conductance (0.01) exercised a positive direct effect on volume. The maximum indirect effect was caused by the girth on to the volume through height.

Table 9. Path co-efficient analysis of direct and indirect effect on volume.

Parameters	H	G	AGR	CD	CC	PR	SC	TR	RWC
Height (H)	0.434	0.388	0.094	-0.396	-0.094	-0.079	0.149	-0.163	0.132
Girth (G)	0.565	0.632	0.135	-0.522	-0.093	-0.139	0.256	-0.260	0.198
Absolute growth rate (AGR)	0.003	0.003	0.014	-0.004	-0.005	-0.006	0.001	0.007	0.008
Crown diameter (CD)	0.049	0.044	0.005	-0.058	-0.006	-0.007	0.013	-0.015	0.016
Chlorophyll content (CC)	0.009	0.005	0.001	-0.004	-0.003	-0.009	0.001	-0.001	-0.001
Photosynthetic rate (PR)	0.002	0.003	0.006	-0.002	-0.004	-0.014	0.013	-0.012	0.009
Stomatal conductance (SC)	0.001	0.002	0.001	-0.001	-0.001	-0.004	0.005	0.004	0.003
Transpiration rate (TR)	0.007	0.007	-0.001	-0.005	-0.008	-0.005	-0.001	-0.001	0.003
Relative water content (RWC)	0.002	0.003	0.005	-0.003	-0.003	-0.006	-0.006	-0.006	-0.009

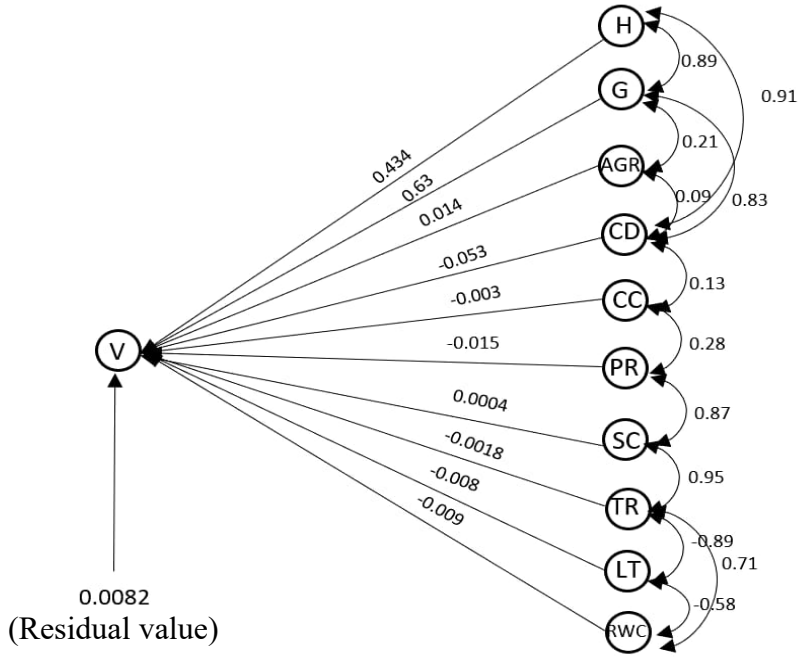


Fig 2. Path diagram

4.4. Physiological parameters

At 27 MAP it was observed that only chlorophyll content and relative water content were having significant variation (Table 10). The physiological parameters showed a significant difference for the last observation (36 MAP). Except for the relative water content value, all other parameters showed higher values than its previous data recorded.

4.4.1. Chlorophyll content

As it was found significant, post hoc analysis was carried out for the observations (27 MAP and 36 MAP). From the Table (10) on 27 MAP it was observed that FCV-MD-01, FCV-MD-03, FCV-MD-04, FCV-MD-05, FCV-MD-08, FCV-MD-09, FCV-MD-14, FCV-MD-16, FCV-MD-17, FCV-MD-19, FCV-MD-20, FCV-MD-21, FCV-MD-22 were on par and FCV-MD-03 from Tholpetty shows the highest mean chlorophyll content of 61.93. The lowest value for chlorophyll content among the plus tree seed progeny was shown by FCV-MD-11 (44.13) from Thiruvazhiyadu. At

36 MAP FCV-MD-03 (Tholpetty) showed the highest mean chlorophyll content of 50.6. The lowest value for chlorophyll content among the plus tree seed progeny was shown by FCV-MD-11 (34.30) from Thiruvazhiyadu. It was observed that both duration plus tree seed progeny from Tholpetty showed better performance than all other progenies.

4.4.2. Photosynthetic rate (µmol CO_2 m^2 s^{-1})

At 27 MAP the photosynthetic rate was found to be non-significant for the plus tree progenies. The highest value (0.5095 µmol CO_2 m^2 s^{-1}) was shown by FCV-MD-10 (from Thiruvazhiyadu which was on par with remaining plus tree progenies. At 36 MAP the progenies were found significant and progeny FCV-MD-04 (Tholpetty) showed the highest mean photosynthetic rate of 3.09 µmole CO_2 m^2 s^{-1}. The lowest value for photosynthetic rate among the plus tree seed progeny was shown by FCV-MD-14 (0.33 µmole CO_2 m^2 s^{-1}) from Parambikulam (Table 10).

4.4.3. Stomatal conductance (mol H_2O m^2 s^{-1})

On 27 MAP the plus tree progenies were found non-significant for their stomatal conductance (Table 10). The highest value was shown by FCV-MD-06 (0.0993 mol H_2O m^2 s^{-1}) from Neykavala which was on par with remaining plus tree progenies. At 36 MAP the progenies were found significant and it was observed that the plus tree seed progeny from the accession FCV-MD-02, FCV-MD-03, FCV-MD-04, FCV-MD-23 were on par and FCV-MD-03 (Tholpetty) showed the highest mean stomatal conductance of 3.59 mole H_2O m^2 s^{-1}. The lowest value for stomatal conductance rate among the plus tree seed progeny was shown by FCV-MD-11 (0.11 mole H_2O m^2 s^{-1}) from Thiruvazhiyadu and FCV-MD-12 (0.11 mole H_2O m^2 s^{-1}) from Walayar.

4.4.4. Transpiration rate (m mole H_2O m^2 s^{-1})

On 27 MAP the plus tree progenies were found non-significant for their transpiration rate. The highest value was shown by FCV-MD-20

(1.05 m mole H_2O m^2 s^{-1}) from Peechi in Thrissur which was on par with remaining plus tree progenies. At 36 MAP the progenies were found significant and on post hoc analysis it was observed that the plus tree seed progeny from the accession FCV-MD-02, FCV-MD-03, FCV-MD-04, FCV-MD-05, FCV-MD-20, FCV-MD-22 were on par and FCV-MD-03 (Tholpetty) showed the highest mean transpiration rate of 3.53 m mole H_2O m^2 s^{-1}. The lowest value for transpiration rate among the plus tree seed progeny was shown by FCV-MD-10 and FCV-MD-11 from Thiruvazhiyadu with a mean value of 0.37 m mole H_2O m^2 s^{-1}.

4.4.5. Leaf temperature (ºC)

The parameter for leaf temperature was found non-significant in both 27 MAP and 36 MAP and hence post hoc analysis was not carried out. At 27 MAP the plus tree seed progeny FCV-MD-10 from Thiruvazhiyadu showed a higher value which was found to be on par with the other plus tree progenies. AT 36 MAP the plus tree seed progeny FCV-MD-11 from Thiruvazhiyadu showed higher value compared to other progenies but was found to be on par.

4.4.6. Relative water content (%)

Both the observation from the experimental plot of the plus tree seed progenies at 27 MAP and 36 MAP were found significant and hence the data was used for the post hoc analysis. At 27 MAP it was observed that FCV-MD-03, FCV-MD-04, FCV-MD-22 were on par and FCV-MD-04 from Tholpetty shows the highest relative water content **(**95%). The lowest value for relative water content among the plus tree seed progeny was shown by FCV-MD-08 (76%) from Pothundy. At 36 MAP it was observed that the plus tree seed progeny from the accession, FCV-MD-03, FCV-MD-21, FCV-MD-22 were on par and FCV-MD-03 (Tholpetty) showed the highest relative water content The lowest value was shown by FCV-MD-09 (Attapady) and FCV-MD-11 (Thiruvazhiyadu).

Table 10. Physiological parameters of *Melia dubia* plus tree progenies at 27 MAP and 36 MAP

Accession No.	Chlorophyll content		Photosynthetic rate (µmole CO_2 m^2 s^{-1})		Stomatal conductance (mole H_2O m^2 s^{-1})		Transpiration rate (m mole H_2O m^2 s^{-1})		Leaf temperature (ºC)		Relative water content (%)	
	27MAP	36MAP	27MAP	36MAP	27MAP	36MAP	27MAP	36MAP	27MAP	36MAP	27MAP	36MAP
FCV-01	56.1^{abcde}	48.1^{abc}	0.38	2.09^{bcdefg}	0.094	0.121^{cdefgh}	1.02	1.85^{bcde}	30.1	32.6	86^{cdefgh}	81 ^{bcde}
FCV-02	50.5^{cdefgh}	43.6^{abcdef}	0.22	2.69^{abc}	0.091	0.230^{abc}	1.01	2.84^{abc}	30.3	31.0	82^{ghij}	82 ^{cdef}
FCV-03	61.9^{a}	50.6^{a}	0.37	2.85^{ab}	0.074	0.301^{a}	0.84	3.53^{a}	31.5	31.0	93^{ab}	88 ^{a}
FCV-04	60.8^{ab}	48.4^{ab}	0.46	3.09^{a}	0.077	0.255^{ab}	0.78	3.0^{ab}	30.3	31.5	94^{a}	87 ^{ab}
FCV-05	53.1^{abcdefg}	47.0^{abcde}	0.21	2.18^{bcdefg}	0.069	0.151^{bcde}	0.91	2.29^{abcd}	32.1	33.8	83^{defg}	80 ^{cdef}
FCV-06	52.7^{bcdefgh}	42.4^{bcdefgh}	0.52	0.85^{gh}	0.099	0.072^{efghi}	1.16	1.15^{defg}	31.4	34.3	85^{cdefg}	80 ^{cdef}
FCV-07	50.6^{cdefgh}	44.1^{abcdefgh}	0.39	0.77^{gh}	0.082	0.033^{ghi}	0.98	0.86^{efg}	30.8	35.0	84^{cdefg}	83 ^{bcd}
FCV-08	54.0^{abcdef}	47.3^{abcdef}	0.30	1.02^{efgh}	0.097	0.045^{fghi}	1.11	0.93^{efg}	31.5	35.1	75^{j}	73 ^{hi}
FCV-09	54.0 ^{abcdef}	49.5^{abcd}	0.27	0.89^{efgh}	0.083	0.021^{hi}	0.85	0.58^{fg}	29.2	35.6	76^{hij}	72 ^{i}
FCV-10	51.4^{cdefgh}	38.7^{bcdefgh}	0.50	0.84^{fgh}	0.078	0.022h^{i}	0.81	0.54f^{g}	30.1	36.8	83^{efghi}	78 ^{defgh}
FCV-11	44.1^{g}	34.3^{h}	0.41	0.85^{fgh}	0.085	0.011^{i}	1.04	0.37^{g}	32.1	37.1	75^{ij}	72 ^{i}
FCV-12	49.2^{defgh}	41.8^{bcdefgh}	0.24	0.89^{fgh}	0.075	0.011^{i}	0.85	0.37^{g}	31.1	37.0	88^{bcd}	82 ^{bcde}
FCV-13	44.8^{fg}	36.7^{gh}	0.43	0.33^{h}	0.089	0.020h^{i}	0.98	0.50f^{g}	30.7	36.7	75^{ij}	74 ^{ghi}
FCV-14	58.6^{abc}	48.4^{ab}	0.40	2.55^{abc}	0.086	0.111^{defghi}	0.94	1.76^{bcdef}	31.0	31.8	84^{cdefg}	81 ^{cde}
FCV-15	51.4^{cdefgh}	38.0^{defgh}	0.36	2.49^{abcd}	0.073	0.143^{bcdef}	0.79	1.95^{bcde}	30.1	31.2	82^{defgh}	80 ^{cdef}
FCV-16	57.6 ^{abcd}	48.8^{ab}	0.37	1.85^{bcdefg}	0.088	0.143^{bcdef}	0.96	2.09^{bcde}	31.0	32.1	87^{bcdef}	81 ^{bcde}
FCV-17	56.3 ^{abcde}	42.9^{abcdef}	0.31	1.19^{defgh}	0.093	0.112^{defghi}	1.00	2.11^{bcde}	30.1	33.3	88^{bcde}	79 ^{defg}
FCV-18	47.7^{efgh}	40.0^{bcdefgh}	0.65	1.75^{bcdefgh}	0.081	0.132^{cdefg}	0.96	1.85^{bcde}	32.0	32.1	82^{fghij}	77^{efghi}
FCV-19	55.6 ^{abcde}	45.4^{abcdef}	0.49	1.15^{cdefgh}	0.073	0.091^{defghij}	0.86	1.63^{cdefg}	31.6	32.4	85^{cdefg}	79 ^{cdefg}
FCV-20	54.6 ^{abcdef}	47.1^{abcde}	0.28	2.11^{bcdefg}	0.083	0.142^{bcdef}	1.05	2.30^{abcd}	32.3	31.9	86^{cdefg}	79 ^{cde}
FCV-21	57.4^{abcd}	47.6^{abcd}	0.41	1.51^{bcdefgh}	0.079	0.121^{cdefg}	0.89	2.28^{abcd}	30.9	32.4	88^{bcd}	81 ^{abcd}
FCV-22	58.2^{abcd}	48.7^{abcde}	0.63	1.46^{bcdefgh}	0.077	0.132^{cdefg}	0.81	2.38^{abcd}	30.0	31.8	90^{abc}	85 ^{abc}
FCV-23	50.2^{cdefgh}	37.2^{efgh}	0.31	2.24^{abcdef}	0.075	0.182 ^{abcd}	0.90	1.90^{bcde}	31.3	31.9	84^{cdefg}	78^{defh}
FCV-24	48.3^{efgh}	38.2^{cdefgh}	0.25	2.37^{abcde}	0.084	0.103^{defghi}	0.96	2.15^{bcde}	30.9	32.9	87^{bcdefg}	81^{cde}
FCV-25	46.0^{efg}	36.6^{fgh}	0.32	1.95^{bcdefg}	0.087	0.103^{defghi}	0.92	1.98^{bcde}	30.8	33.8	85^{cdefg}	81 ^{cde}
*MSE	1.37	0.78	0.01	0.52	0.0001	0.003	0.01	0.08	1.5	1.9	4.2	3.3

4.5. Genetic parameters for physiological traits

4.5.1. Chlorophyll content

The genetic parameters for the physiological traits of plus tree progenies were measured using the data from 36 MAP (Table 11). It was observed that the broad-sense heritability for chlorophyll content was 0.86. The PCV, GCV and ECV are 9.01, 8.67, and 2.40 respectively. The genetic advance (%) was 18.

4.5.2. Photosynthetic rate

The genetic parameters for the physiological trait (photosynthetic rate) of plus tree progenies were measured using the data from 36 MAP (Table 11). It was observed that the broad-sense heritability for the photosynthetic rate was 0.75. The PCV, GCV and ECV are 43.74, 37.53, and 21.90 respectively. The genetic advance (%) was 33.

4.5.3. Stomatal conductance

The genetic parameters for the physiological trait (Stomatal conductance) of plus tree progenies were measured using the data from 36 MAP (Table 11). It was observed that the broad-sense heritability for stomatal conductance was 0.82. The PCV, GCV and ECV are 65.28, 58.50, and 26. 75 respectively. The genetic advance (%) was 13.

4.5.4. Transpiration rate

The genetic parameters for the physiological trait (Transpiration rate) of plus tree progenies were measured using the data from 36 MAP (Table 11). It was observed that the broad-sense heritability for transpiration was 0.91. The PCV, GCV and ECV are 31.41, 30.03, and 9.19 respectively. The genetic advance (%) was 20.2.

4.5.5. Relative water content

The genetic parameters for the physiological trait (Relative water content) of plus tree progenies were measured using the data from 36 MAP (Table 11). It was observed that the broad-sense heritability for relative water content was 0.79. The PCV, GCV and ECV are 5.06, 4.52, 2.28.75 respectively. The genetic advance (%) was 8.

Table 11. Estimated genetic parameters of physiological traits of *Melia dubia*

Parameters	PV	GV	H^2	PCV	GCV	ECV	G A (%) of mean
Chlorophyll content	60	52	0.86	9	8.67	2.4	18%
Photosynthetic rate	89	67	0.75	43	37.5	21.9	33
Stomatal conductance	84	69	0.82	65	58.5	26.7	13%
Transpiration	1.0	0.9	0.91	31	30.0	9.1	20%
RWC	16	13	0.79	5	4.5	2.2	8%

* PV: Phenotypic variation, GV: Genotypic variation, H^2: Heritability, PCV: Phenotypic coefficient of variation, GCV: Genotypic coefficient of variation, GA: Genetic Advance

4.6. Correlation study of the morphological and physiological characters of plus tree seed progenies

Correlation studies for the morphological and physiological traits of 25 plus tree progenies are given in Table 12. It was observed that among the morphological traits, height had a significant ($P<0.01$) positive relationship with other morphological traits such as girth (0.89), crown diameter (0.91) and volume (0.95), however, height did not show a significant relationship with plant absolute growth rate even though the relationship was positively correlated. The correlation of the height with the plant physiological traits like chlorophyll content (0.27), photosynthetic rate (0.18), stomatal conductance (0.34), transpiration rate (0.36) and relative water content (0.30) of the seedlings were all correlated positively but with no significant correlation. The height of the plant had a non-significant negative relationship with leaf temperature. The girth of the plus tree seedling showed a positive significant ($P<0.01$) correlation with the growth trait *viz.* volume (0.98), crown diameter (0.82) and non-significant positive relation with the plant absolute growth rate (0.21). The girth relation with physiological traits was found positively significant ($P<0.05$) with stomatal conductance (0.40) and transpiration rate (0.41) and non-significant positive correlation to the chlorophyll content (0.14),

photosynthetic rate (0.22), and relative water content (0.31). Absolute growth rate (AGR) was found not to have any significant relation with morphological as well as physiological parameters. The volume of the seedlings were significantly correlated with the height, girth, crown diameter (0.86) and transpiration rate (0.42). It showed a non-significant positive relation with chlorophyll content (0.17), photosynthetic rate (0.21), stomatal conductance (0.39), and relative water content (0.31). The chlorophyll content of the leaves of plus tree progenies was non-significantly correlated to the morphological traits studied. It was non-significantly correlated to photosynthetic (0.27), stomatal conductance (0.37) and significantly correlated ($P<0.01$) to transpiration rate (0.42), relative water content (0.39). The chlorophyll content was found negatively correlated ($P<0.01$) to leaf temperature (-0.39). The relation of photosynthetic rate with stomatal conductance (0.874), transpiration rate (0.84) and relative water content (0.60) was positively correlated and was significant at a 0.01 percent level. However, a significantly ($P<0.01$) negative correlation was observed with leaf temperature (0.80). The stomatal conductance was found positively correlated ($P<0.01$) to transpiration rate (0.94) and relative water content (0.67), and it was negatively correlated with leaf temperature (-0.85). A significant positive correlation ($P<0.01$) was observed for transpiration rate with the relative water content (0.70). The leaf temperature was found to have a negative relation with all the morphological as well as physiological traits of the plus tree seed progeny. The relative water content showed a significant positive relationship with all the physiological traits of the plant studied.

Table 12. Correlation study of the morphological and physiological characters of plus tree seed progeny.

	H	G	AGR	V	CD	CC	PR	SC	TR	T	WC
H	1										
G	.893**	1									
AGR	.218	.214	1								
V	.954**	.980**	.232	1							
CD	.912**	.827**	.095	.868**	1						
CC	.271	.147	.370	.177	.129						
PR	.183	.221	.041	.213	.144	.275					
SC	.343	.405*	.089	.395	.245	.373	.874**				
TR	.369	.411*	-.053	.402*	.287	.422*	.840**	.946**			
LT	-.185	-.231	.103	-.207	-.107	.399*	-.802**	-.856**	-.895**		
RWC	.304	.314	.058	.311	.306	.397*	.609**	.674**	.708**	.587**	

*H: Height, G: Girth, AGR: Absolute growth rate, V: Volume, CD: Crown diameter

CC: Chlorophyll content, PR: Photosynthetic rate, SC: Stomatal conductance,

TR: Transpiration rate, LT: Leaf temperature, RWC: Relative water content

4.7. Analysis of genetic variation using ISSR markers

Genetic diversity of 25 plus tree seed progeny was analysed at the molecular level using ISSR marker for 15 parent population *viz.* Thirunelly, Tholpetty, Dasanakara, Neykavala, Dhoni, Pothundy, Attapady, Thiruvazhiyadu, Walayar, Parambikulam, Chinnar, Peechi, Akamala, Kulathupuzha and Aryankavu. The mature leaf samples were collected from the plus tree seed progeny gene bank present in the College of Forestry, Kerala Agricultural University, Thrissur.

4.7.1. DNA quantification

The amount of DNA extracted from all the 25 plus tree seed progeny, as quantified by the Nanodrop spectrophotometer is presented in Table (13). The DNA extricated was pure, having an $OD_{260/280}$ value of 1.8-2.0. The quantity of DNA ranged from 141.94 µg mL^{-1} and 1126 µg mL^{-1}.

4.7.2. Standardization of ISSR-PCR conditions

The plus tree seed progeny FCV-MD-02 (Thirunelly), DNA sample isolated through standardized DNA extraction protocol was used as a template for ISSR-PCR and it showed very good and clear amplification with the primer UBC 890. The optimum annealing temperature for the primers selected for study was obtained after running in gradient PCR and the temperature was found to be 3°C to 4°C higher than the melting temperature (Table 2). The amplified products showed a very clear band in two percentage agarose gel.

4.7.3. Primer screening

A total number of 18 ISSR primers were screened, from which 15 primers were selected for amplification. Molecular genetic diversity assessment was carried out with 15 ISSR primers *viz.* UBC 809, UBC 810, UBC 813, UBC 816, UBC 823, UBC 847, UBC 864, UBC 884, UBC 888, UBC 890, UBC 891, L1, L3, L4, and L9.

Table 13. Amount of DNA extracted as quantified by Nanodrop spectrophotometer.

Location	OD $_{260/280}$ ratio	Quantity of DNA (µg $mL^{-1)}$
Thirunelly	1.89	1126
Thirunelly	1.93	750
Tholpetty	1.85	551.56
Tholpetty	1.90	1213
Dasanakara	2.09	421.88
Neykavala	2.09	920.35
Dhoni	1.89	141.94
Pothundy	1.94	420.87
Attapady	1.97	745.99
Thiruvazhiyadu	1.93	427.6
Thiruvazhiyadu	1.99	309.89
Walayar	1.92	877.02
Walayar	1.80	889.07
Parambikulam	1.84	852.35
Parambikulam	1.98	440.21
Parambikulam	1.93	580.32
Chinnar	1.84	719.16
Chinnar	1.81	437.70
Chinnar	1.99	720.54
Peechi Thrissur	1.85	942.21
Peechi Thrissur	1.86	420.23
Akamala	1.81	880.15
Kulathupuzha	1.84	593.45
Aryankavu	1.95	605.49
Aryankavu	1.97	470.92

4.7.4. Genetic diversity among *Melia dubia* plus tree seed progenies

The 15 primers selected for studying the genetic diversity produced a total number of 164 reproducible amplicons of which 113 (68%) bands were polymorphic and 51 bands were monomorphic. The number of reproducible bands produced by the ISSR primers varied from seven (UBC 847) to fifteen (L9). The number of polymorphic bands observed ranged between three and thirteen. UBC 847 produced the maximum number of polymorphic bands and L4 produced the least number of bands. Primer UBC 847 showed the maximum percentage of polymorphism of 86.6 % followed by the primer UBC 809 (85.7%) and the least percentage of polymorphism was shown by the primer L4 (24.2%). The total number of amplicons produced by each primer and the detail of polymorphism are depicted in Table 14.

Table 14. Total number of bands and polymorphism exhibited by different primers in *M. dubia* plus tree seed progenies

Primer	Total bands	Polymorphic bands	Monomorphic bands	Percentage polymorphism
UBC 809	7	6	1	85.7%
UBC 810	8	6	2	75.0%
UBC 813	10	8	2	80.0%
UBC 816	11	9	2	81.2%
UBC 823	9	6	3	66.6%
UBC 847	15	13	2	86.6 %
UBC 864	12	10	2	83.3%
UBC 884	10	6	4	60.0%
UBC 888	14	6	8	42.0%
UBC 890	10	7	3	70.0%
UBC 891	12	9	3	75.0%
L1	8	6	2	75.0%
L3	12	8	4	66.6%
L4	11	3	8	27.2%
L9	15	10	5	66.6%
Total	164	113	51	

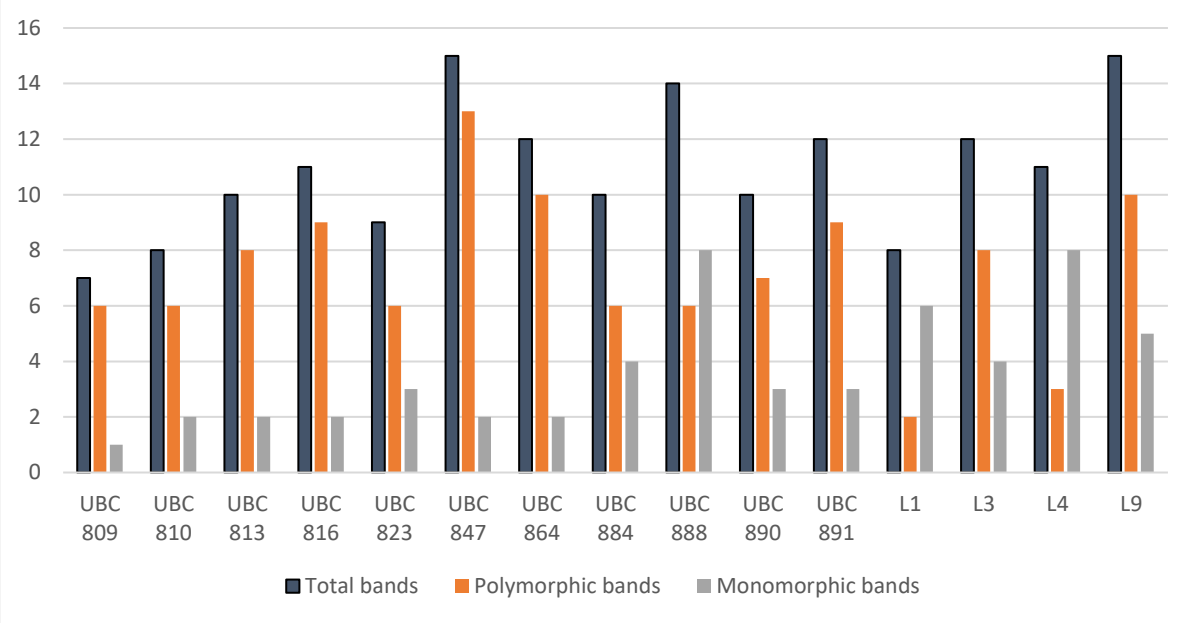


Fig. 3. Number of total amplicons and polymorphic bands produced by ISSR primer

UBC 809

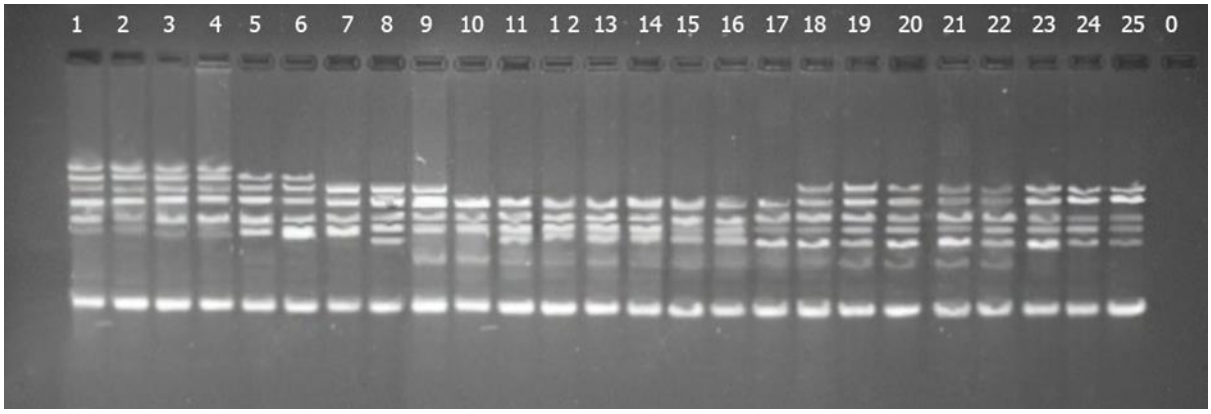
A total of 9 amplicons were obtained using the primer UBC 809 after polymerase chain reaction. The amplification pattern is shown in Plate 3. The primer showed 85.7 % polymorphism. Six bands were polymorphic while 1 was a monomorphic band. This primer could differentiate among the progeny populations from Wayanad (FCV-MD-1 to 6) to the rest of the population in Kerala Western Ghats. This primer could distinguish the subpopulation in Tholpetty (FCV-MD-3, 4) and Thirunelly (FCV-MD-1, 2) from the rest of the population in Wayanad. Absence of unique band in the population Thiruvazhiyadu (FCV-MD-10, 11), Walayar (FCV-MD-12, 13), Parambikulam (14, 15, 16), Chinnar (FCV-MD-17). Specific bands were obtained for populations in Attapady (9), Thiruvazhiyadu (FCV-MD-10, 11), Walayar (FCV-MD-12, 13), Parambikulam (FCV-MD-14, 15, 16), Chinnar (FCV-MD-17, 18, 19), Peechi (FCV-MD-20, 21), Akamala (FCV-MD-22). This primer can be used as an effective marker in distinguishing the population in northern, central and southern parts of Western Ghats.

UBC 810

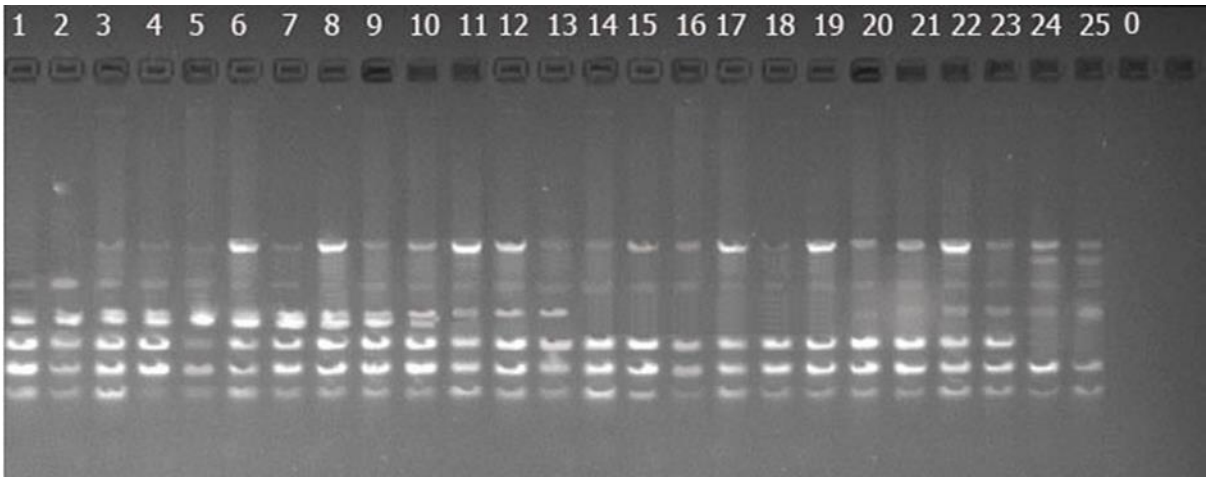
A total of 7 amplicons were obtained using the primer UBC 810 after polymerase chain reaction (Plate 3). 3 polymorphic bands and 4 monomorphic bands were observed. Characteristic bands were observed in the population of Kulathupuzha (FCV-MD-23) and Aryankavu (FCV-MD-24, 25). Specific bands were also observed in the population from Thirunelly (FCV-MD-1, 2), Tholpetty (FCV-MD-3, 4), Dasanakara (FCV-MD-5), Neykavala (FCV-MD-6), Dhoni (FCV-MD-7), Pothundy (FCV-MD-8), Attapady (FCV-MD-9), and Thiruvazhiyadu (FCV-MD-10). Specific bands were absent in Thirunelly (FCV-MD-1, 2) population and its presence in the remaining population make this primer an effective marker for distinguishing the population.

UBC 813

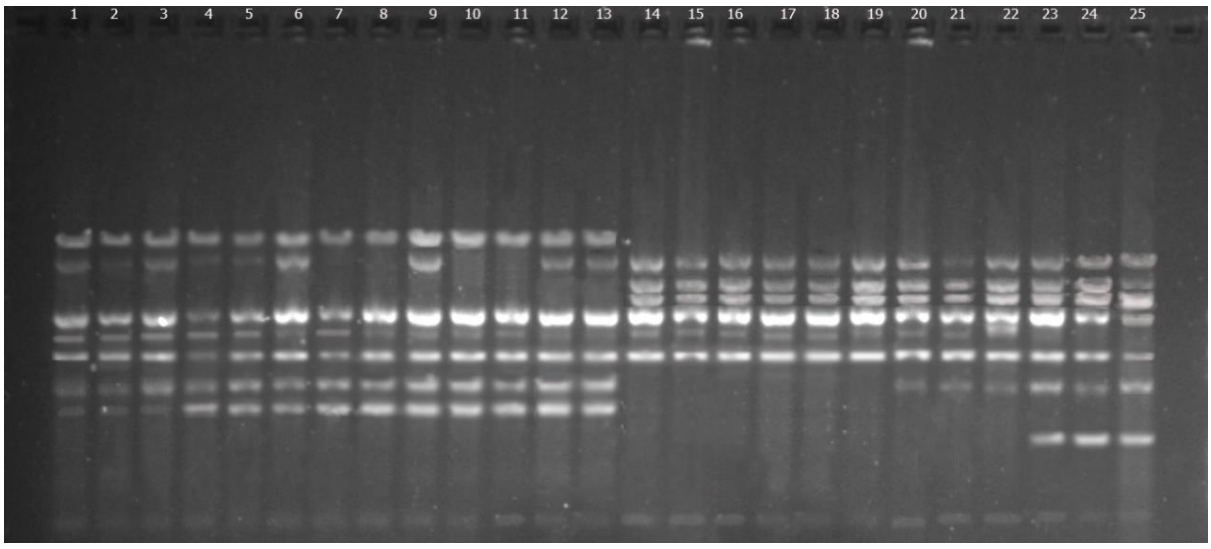
10 amplicons were obtained using the primer UBC 813 after polymerase chain reaction. The amplification pattern is shown in Plate 3. The primer showed 80% polymorphism. There were 8 polymorphic bands and 2 monomorphic bands. This primer showed the presence of a characteristic band for the Wayanad population (FCV-MD-1, 2, 3, 4, 5, and 6). The presence of the same number band in Wayanad and Thrissur (FCV-MD-20, 21, 22) show the characteristic relation between the populations the presence and absence of the band. Presence of characteristic band in the population of Thirunelly (FCV-MD-1, 2), Tholpetty (FCV-MD-3, 4), Dasanakara (FCV-MD-5), Neykavala (FCV-MD-6), Dhoni (FCV-MD-7), Pothundy (FCV-MD-8), Attapady (FCV-MD-9), Thiruvazhiyadu (FCV-MD-10, 11), Walayar (FCV-MD-12, 13) which is absent in other population. Specific bands can also be observed in the population of Parambikulam (FCV-MD-14, 15, 16), Chinnar (FCV-MD-17, 18, 19), Thrissur (FCV-MD-20, 21, 22), Kulathupuzha (FCV-MD-23), Aryankavu (FCV-MD-24, 25). A specific band was absent in the population of Kulathupuzha (FCV-MD-23) and Aryankavu (FCV-MD-24, 25) but a separate band was also observed in Kulathupuzha (FCV-MD-23) and Aryankavu (FCV-MD-24, 25) making this primer an



(a)



(b)



(c)

Plate 3. Amplification profile gel images of ISSR primers (a) UBC 809 (b) UBC 810 (c) UBC 813.

effective marker for identifying population in Kulathupuzha (FCV-MD-23) and Aryankavu (FCV-MD-24, 25).

UBC 816

A total of 11 amplicons were obtained using the primer UBC 816 after polymerase chain reaction. The amplification pattern is shown in Plate 4. The primer showed 81.2 % polymorphism. Nine polymorphic bands were obtained. This primer could distinguish between Neykavala (6) and Dasanakara (FCV-MD-5) plus tree seed progeny from Wayanad. Presence of polymorphic band in the population of Akamala (FCV-MD-22), Kulathupuzha (FCV-MD-23) and Aryankavu (FCV-MD-24, 25). Presence of characteristic band in the population of Thirunelly (FCV-MD-1, 2), Tholpetty (FCV-MD-3, 4), Dasanakara (FCV-MD-5), Neykavala (FCV-MD-6), Dhoni (FCV-MD-7), Pothundy (FCV-MD-8), Attapady (FCV-MD-9) within these populations the presence of the separate band was found in Thirunelly (FCV-MD-1, 2), Tholpetty (FCV-MD-3, 4), Dasanakara (FCV-MD-5), Neykavala (FCV-MD-6). This primer can be used as an effective marker in identifying the population Chinnar (FCV-MD-17, 18, 19), Thrissur (FCV-MD-20, 21, 22), Kulathupuzha (FCV-MD-23), Aryankavu (FCV-MD-24, 25) from other populations in Kerala. The absence of a certain band in the population of Kulathupuzha (FCV-MD-23), Aryankavu (FCV-MD-24, 25) separate these populations from other populations. This primer could be used for finding the genetic diversity among the *M. dubia* population in Kerala.

UBC 823

A total of nine amplicons were obtained using the primer UBC 823 after polymerase chain reaction. The amplification pattern is shown in Plate 4. The primer showed 66.6% polymorphism. There was a total of six polymorphic bands and three monomorphic bands. This primer could distinguish between plus tree seed progeny of Wayanad (FCV-MD-1, 2, 3, 4, 5, 6) and Attapady (FCV-MD-9) to the rest of the population in Kerala. Separate bands were observed in Thirunelly (FCV-MD-1, 2),

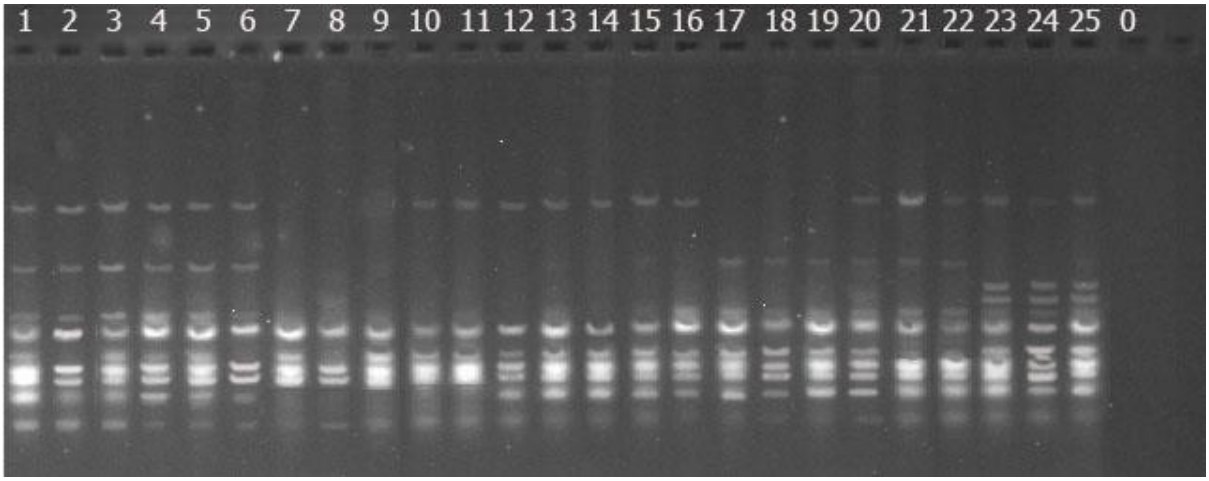
Tholpetty (FCV-MD-3) but absent in Tholpetty (FCV-MD-4) which make this primer an important marker to distinguish the population within Tholpetty of Wayanad population. Hence from the above observation, it can be concluded that this primer could be used to distinguish the population within Wayanad.

UBC 847

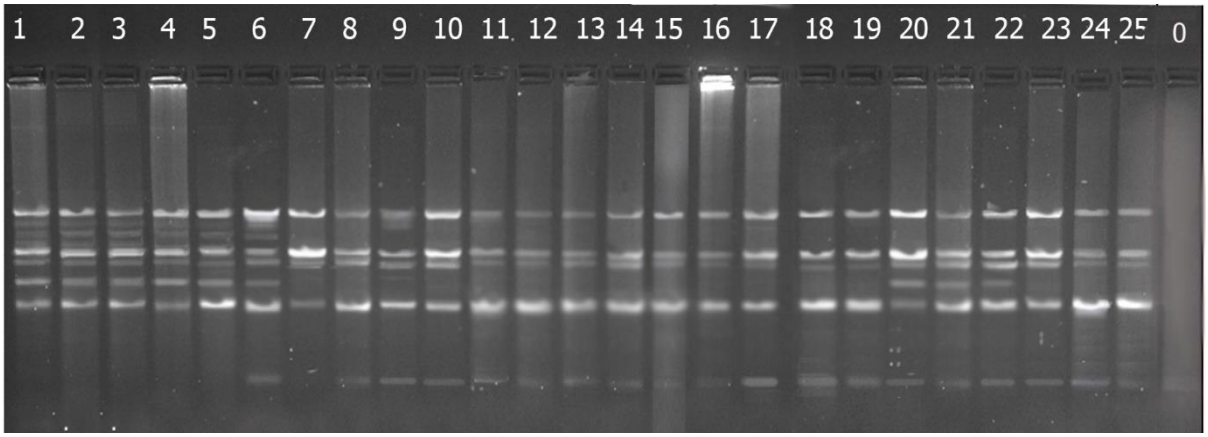
A total of 13 amplicons were obtained using the primer UBC 847 after polymerase chain reaction. The amplification pattern is shown in Plate 4. The primer showed 86.6% polymorphism with the greatest number of polymorphic bands and hence this primer can be effectively used to distinguish genetically the different populations of *M. dubia* in Kerala. The total number of polymorphic bands obtained was 13 and monomorphic was two. This primer was able to distinguish Thiruvazhiyadu (FCV-MD-10, 11) and Walayar (FCV-MD-12, 13) plus tree seed progeny of Palghat. It can also separate the population of the southern Kerala population of Kulathupuzha (FCV-MD-23), Aryankavu (FCV-MD-24, 25) from other populations in Kerala due to the presence of a separate band. Specific bands were also observed for the population of Parambikulam (FCV-MD-14, 15, 16), Chinnar (FCV-MD-17, 18, 19), Thrissur (FCV-MD-20, 21, 21). Characteristic bands were also observed for the Wayanad (FCV-MD-1, 2, 3, 4, 5, 6) population. This primer was found to be good at distinguishing the *M. dubia* population in Kerala.

UBC 864

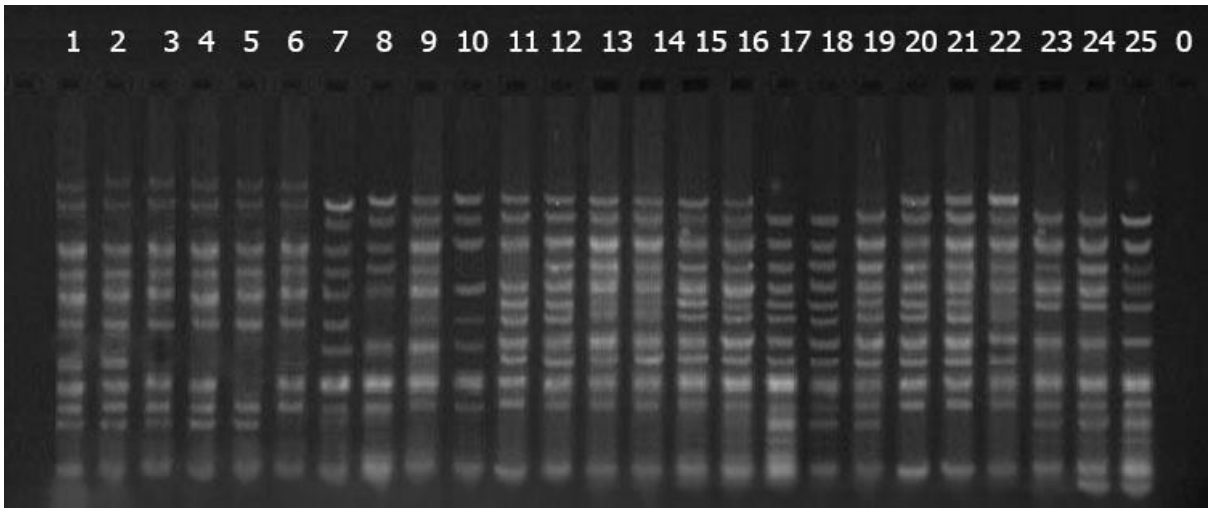
A total of 12 amplicons were obtained using the primer UBC 864 after polymerase chain reaction. The amplification pattern is shown in Plate 5. The primer showed 83.3 % polymorphism. A total number of 10 polymorphic bands and two monomorphic were observed. This primer was able to distinguish the different populations of *M. dubia* in Kerala. The characteristic band were observed for the populations of Kulathupuzha (FCV-MD-23), Aryankavu (FCV-MD-24,25), then for Wayanad (FCV-MD-1,2,3,4,5,6) populations, followed by populations of Dhoni



(a)



(b)



(c)

Plate 4. Amplification profile gel images of ISSR primers (a) UBC 816 (b) UBC 823 (c) UBC 847.

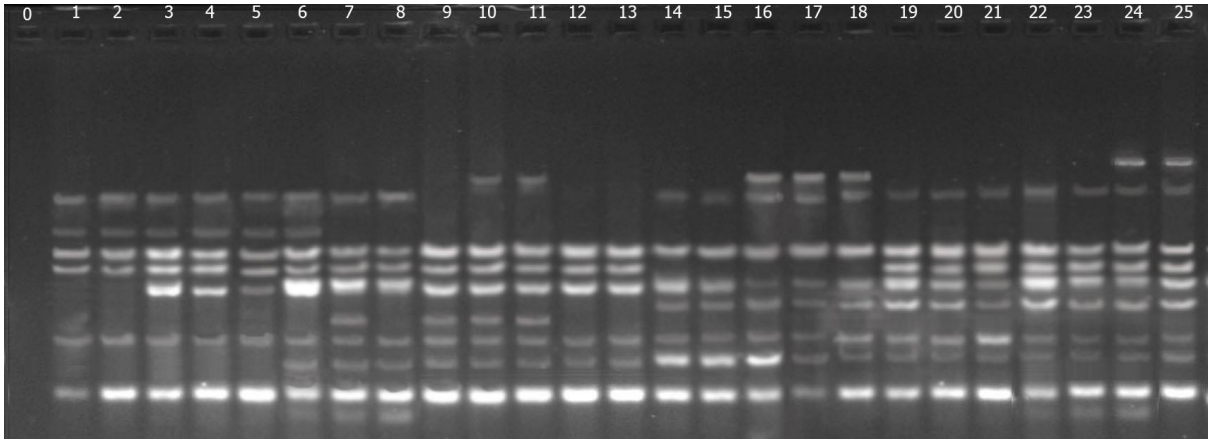
(FCV-MD-7), Pothundy (FCV-MD-8), Attapady (FCV-MD-9), Thiruvazhiyadu (FCV-MD-10, 11) and population of Thiruvazhiyadu (FCV-MD-10, 11), Chinnar (FCV-MD-17, 18, 19). This primer was able to distinguish the populations of the middle part of Kerala from other populations. Through this primer, it was observed that there is wide diversity between the population than within the populations of *M. dubia* in Kerala.

UBC 884

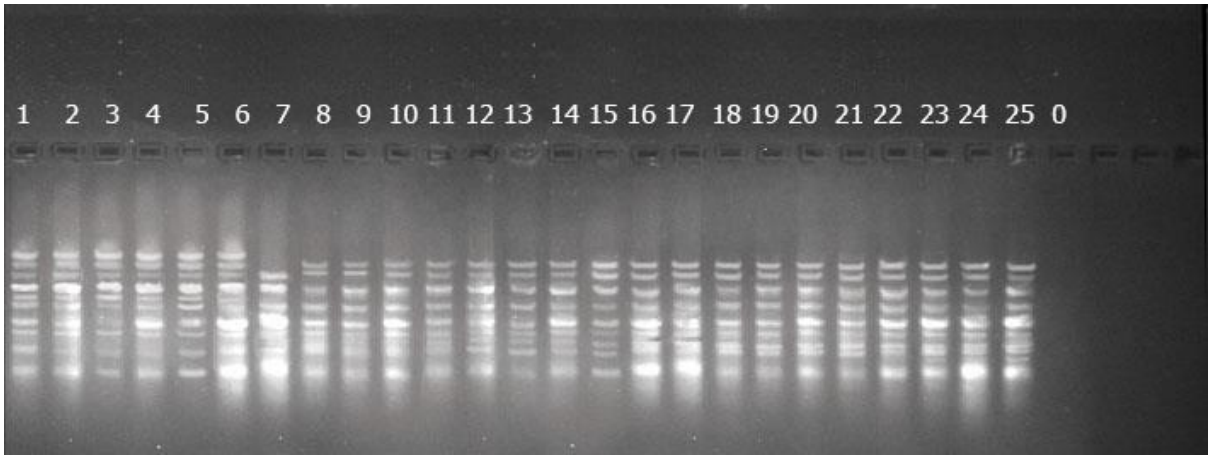
A total of 10 amplicons were obtained using the primer UBC 884 after polymerase chain reaction. The amplification pattern is shown in Plate 5. The primer showed 60% polymorphism. The total number of polymorphic bands obtained was 6 and monomorphic was 4. Even though the amount of polymorphism was less compared to other primers, it was able to differentiate the population of Wayanad (FCV-MD-1, 2, 3, 4, 5, and 6) to the rest of the population (FCV-MD-7-25) due to the presence and absence of the polymorphic band. Within the population from FCV-MD-7 to 25, the primer could separate the population of Kulathupuzha (FCV-MD-23), Aryankavu (FCV-MD-24, 25) due to the presence of a separate band.

UBC 888

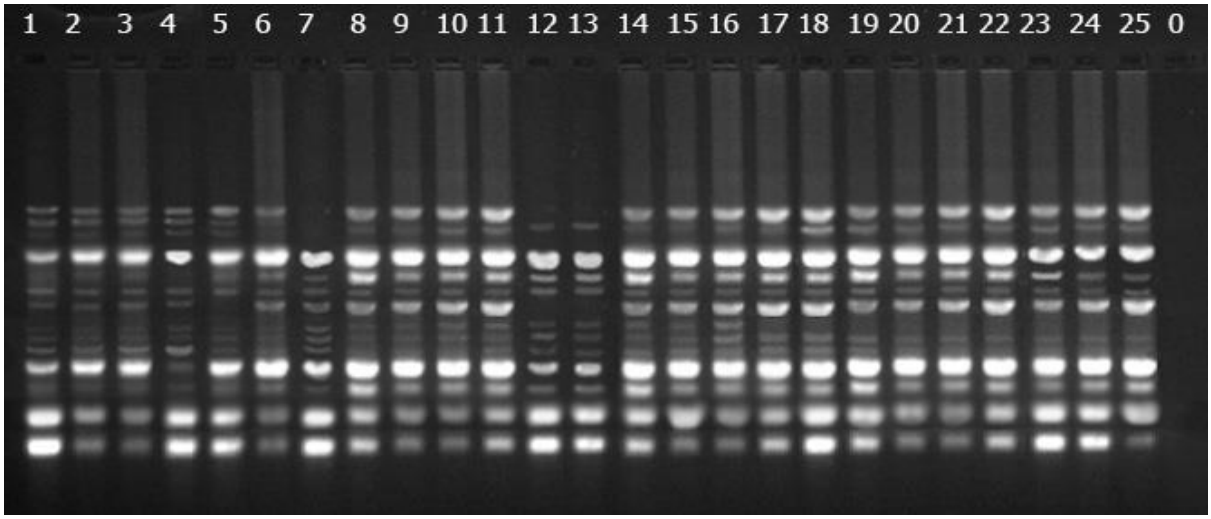
A total of 14 amplicons were obtained using the primer UBC 888 after polymerase chain reaction. The amplification pattern is shown in Plate 5. The primer showed 40% polymorphism. Six polymorphic bands and eight monomorphic bands were obtained. This primer showed a more monomorphic band compared to other primers used for diversity study. separate bands were observed for the population of seed progenies from Wayanad (FCV-MD-1, 2, 3, 4, 5, 6) and population of Dhoni (FCV-MD-7), Pothundy (FCV-MD-8), Attapady (FCV-MD-9), Thiruvazhiyadu (FCV-MD-10, 11) and population of Thiruvazhiyadu (FCV-MD-10, 11), Walayar (FCV-MD-12, 13) Parambikulam (FCV-MD-14, 15, 16). This primer can be used as a



(a)



(b)



(c)

Plate 5. Amplification profile gel images of ISSR primers (a) UBC 864 (b) UBC 884 (c) UBC 888.

good marker to distinguish the population of the northern part of Kerala from the central and southern sides.

UBC 890

A total of 10 amplicons were obtained using the primer UBC 890 after polymerase chain reaction. The amplification pattern is shown in Plate 6. The primer showed 70% polymorphism. The total number of polymorphic bands obtained was seven and monomorphic was three. This primer was found to be suitable for distinguishing the population from different accession in Kerala. Using this primer unique band was observed for the population from Walayar (FCV-MD-12, 13) which was not observed using any other primer and hence this primer is a suitable choice as a marker to distinguish the population of Walayar. Another important observation observed using this primer was the presence of separate band for the Thirunelly population in Wayanad along with the population of Neykavala (FCV-MD-6), Dhoni (FCV-MD-7), Pothundy (FCV-MD-8), Attapady (FCV-MD-9), Thiruvazhiyadu (FCV-MD-10, 11) and population of Thiruvazhiyadu (FCV-MD-10, 11), Walayar (FCV-MD-12, 13). Separate bands were observed in the population of Attapady (FCV-MD-9), Thiruvazhiyadu (FCV-MD-10, 11) and population of Thiruvazhiyadu (FCV-MD-10, 11), Walayar (FCV-MD-12, 13) Parambikulam (FCV-MD-14, 15, 16), Chinnar (FCV-MD-17, 18, 19), Thrissur (FCV-MD-20, 21, 22), Kulathupuzha (FCV-MD-23), Aryankavu (FCV-MD-24, 25).

UBC 891

A total of 10 amplicons were obtained using the primer UBC 891 after polymerase chain reaction. The amplification pattern is shown in Plate 6. The primer showed 75% polymorphism. The total number of polymorphic bands obtained was nine and monomorphic was three. The unique feature of this primer is that it can distinguish the population of Chinnar (FCV-MD-17) from other plus tree seed progeny populations. Another important observation was the observation of a unique band in

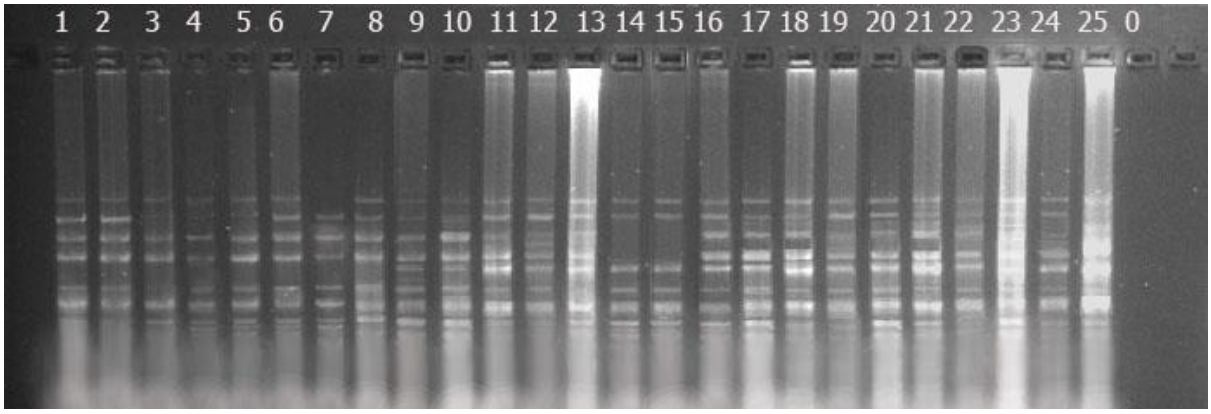
Thiruvazhiyadu (FCV-MD-10, 11), making this primer an important marker for identifying the populations of Thiruvazhiyadu in Kerala. This primer could also give a separate band for the population of Wayanad (FCV-MD-1 to 6) and Dhoni (FCV-MD-7) from other populations. Characteristic bands were also observed for the plus tree seed progeny population of Dhoni (FCV-MD-7), Pothundy (FCV-MD-8), Attapady (FCV-MD-9), Thiruvazhiyadu (FCV-MD-10, 11), Thiruvazhiyadu (FCV-MD-10, 11), Walayar (FCV-MD-12, 13). This primer was found to be suitable for distinguishing the population from the central and northern parts of Kerala to the southern side.

L1

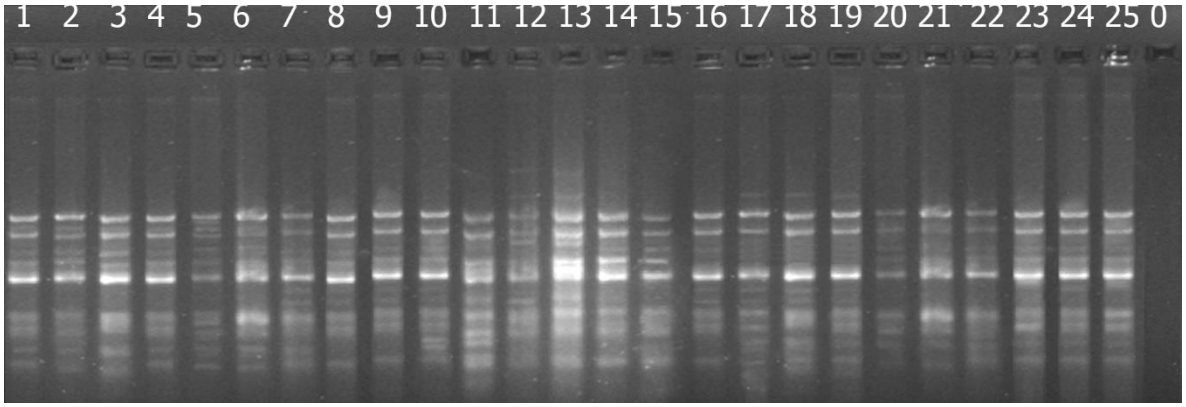
A total of 8 amplicons were obtained using the primer L1 after polymerase chain reaction. The amplification pattern is shown in Plate 6. The primer showed 75% polymorphism which was very less due to its greater number of monomorphic bands. The total number of polymorphic bands obtained was six and monomorphic was two. A characteristic band was observed in the Wayanad population except for the population from Dasanakara (FCV-MD-5) in Wayanad. Unique bands were also observed in the population of Dasanakara (FCV-MD-5), Neykavala (FCV-MD-6), Dhoni (FCV-MD-7), Pothundy (FCV-MD-8), Attapady (FCV-MD-9), Thiruvazhiyadu (FCV-MD-10, 11) and population of Thiruvazhiyadu (FCV-MD-10, 11), Walayar (FCV-MD-12, 13), Parambikulam (FCV-MD-14, 15, 16), Chinnar (FCV-MD-17, 18, 19), Thrissur (FCV-MD-20, 21, 22), Kulathupuzha (FCV-MD-23), Aryankavu (FCV-MD-24, 25). This primer was not able to distinguish most of the population in Kerala except for the population of Wayanad and Palghat.

L3

A total of 12 amplicons were obtained using the primer L3 after polymerase chain reaction. The amplification pattern is shown in Plate 7. The primer showed 66.6% polymorphism. The total number of polymorphic bands obtained were eight and monomorphic was four. Unique bands presence was observed using this primer in the



(a)



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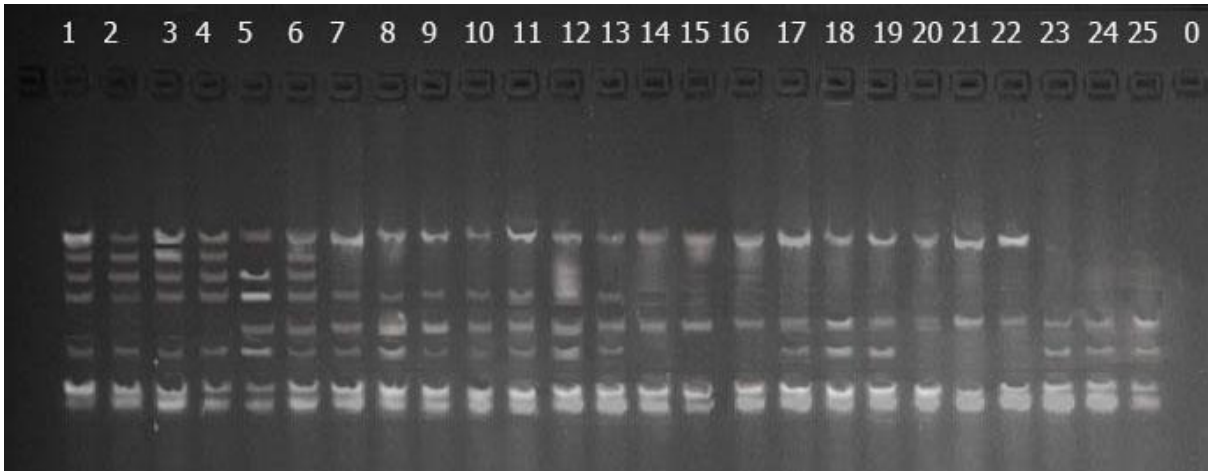


(c)

Plate 6. Amplification profile gel images of ISSR primers (a) UBC 890 (b) UBC 891 (c) L1

population of Aryankavu (24, 25) which was not observed using any other primer. The presence of a unique band in Aryankavu shows how much the population of the southern side are diverse. Unique bands were also observed for the population of Wayanad (FCV-MD-1 to 6). This primer was found to be suitable in differentiating population between the Palghat gap due to the presence of a unique band in the population of the northern side of the Palghat gap which includes the Dhoni (FCV-MD-7), Attapady (FCV-MD-9), Thiruvazhiyadu (FCV-MD-10, 11) as these unique bands were absent in southern Palghat gap population in Palghat. Unique bands were also observed in the population from Thiruvazhiyadu (FCV-MD-10, 11), Walayar (FCV-MD-12, 13), Parambikulam (FCV-MD-14, 15, 16), Chinnar (FCV-MD-17, 18, 19), Thrissur (FCV-MD-20, 21, 22), Aryankavu (FCV-MD-24) which was absent in Kulathupuzha (FCV-MD-23), Aryankavu (FCV-MD-25) which tells how much the population in the southern side are diverse.

L4

A total of 11 amplicons were obtained using the primer L4 after polymerase chain reaction. The amplification pattern is shown in Plate 7. The primer showed 27.2 polymorphism which was very little for understanding the genetic diversity. Only three polymorphic bands were observed. Characteristic bands were observed in the Wayanad (FCV-MD-1, 2, 3, 4, 5, and 6) plus tree seed progeny population. The presence of a unique band in Dasanakara (FCV-MD-5), Neykavala (FCV-MD-6) of Wayanad to other populations in Kerala show how much these populations are related and how much they are different from the population in Wayanad (FCV-MD-1, 2, 3, 4). This primer was found to be less choice for differentiating the populations of *M. dubia* in Kerala.

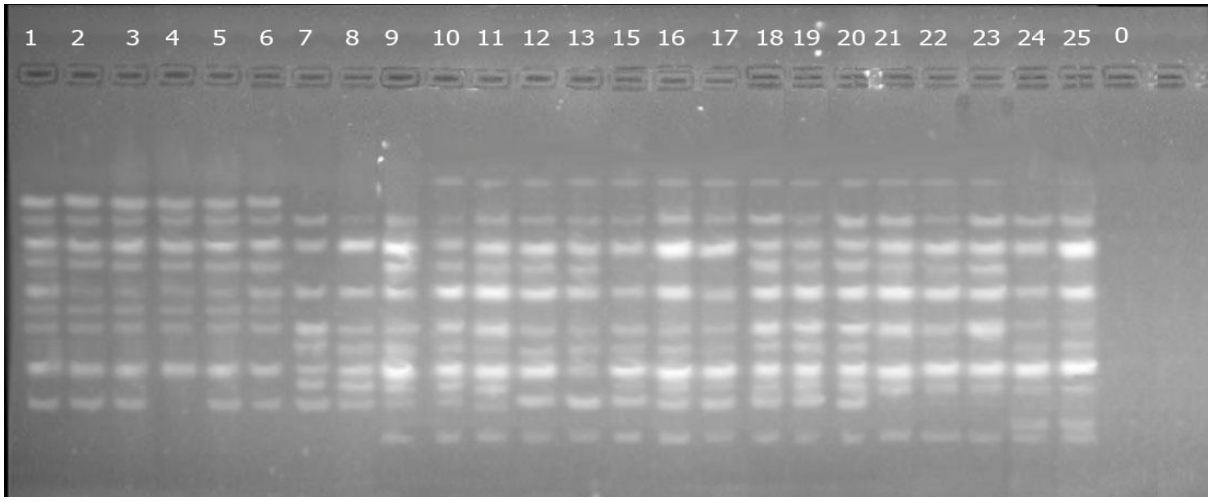
L9

A total of 15 amplicons were obtained using the primer L9 after polymerase chain reaction. The amplification pattern is shown in Plate 7. The primer showed 66.6% polymorphism. The total number of polymorphic bands showed was 10 in number which was of great significance in distinguishing the plus tree seed progeny population from different accession in Kerala. Specific bands were observed for the population of Thirunelly (FCV-MD-1, 2). Unique bands were also observed for the population of the southern side of Kerala including Kulathupuzha (FCV-MD-23), Aryankavu (FCV-MD-24,25) and hence this primer can be used as an effective marker to distinguish the population FCV-MD-1, 2 and FCV-MD-23, 24, 25. The polymorphic band was observed for the population of Pothundy (FCV-MD-8), Attapady (FCV-MD-9), Thiruvazhiyadu (FCV-MD-10, 11) and population of Thiruvazhiyadu (FCV-MD-10, 11), Walayar (FCV-MD-12, 13), Parambikulam (FCV-MD-14, 15, 16), Chinnar (FCV-MD-17, 18, 19), Thrissur (FCV-MD-20, 21, 22), Kulathupuzha (FCV-MD-23), Aryankavu (FCV-MD-24, 25). This primer can be used as an important marker for identifying the populations of *M. dubia* in Kerala.

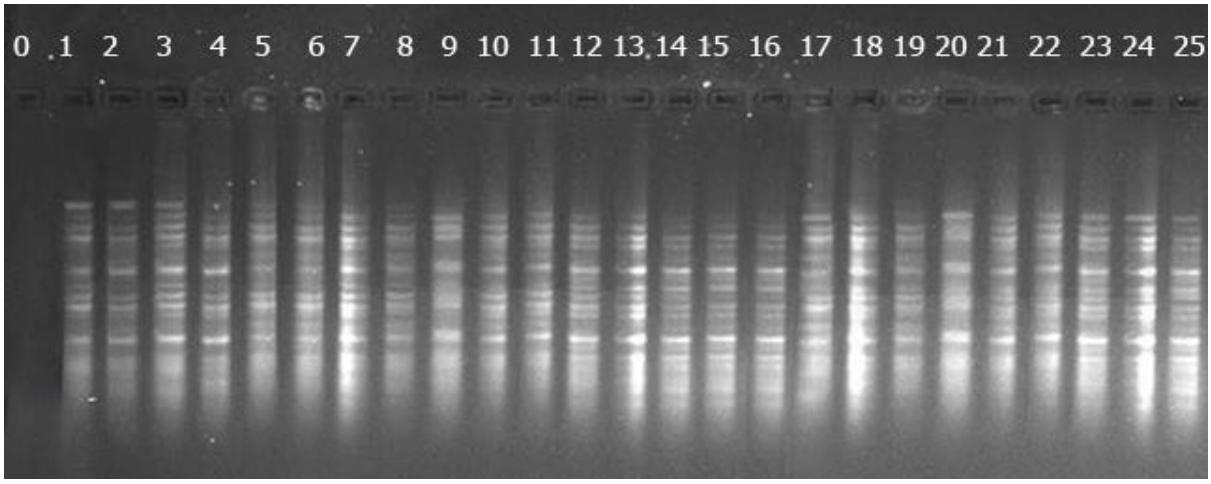
Table 15. Primers with specific accession

Location/Accession	Primer
Peechi, Akamala (20,21)	UBC 890 (p)
Thirunelly (1,2)	UBC 847 (p)
Tholpetty (3)	UBC 890 (a)
Thiruvazhiyadu (10,11)	UBC 891 (p)
Walayar (12,13)	UBC 888 (a)
Parambikulam (14, 15, 16)	UBC 890 (a)
Chinnar (17, 18, 19)	UBC 891 (a)
Aryankavu (24, 25)	810 (p), 864 (p), L9 (p)

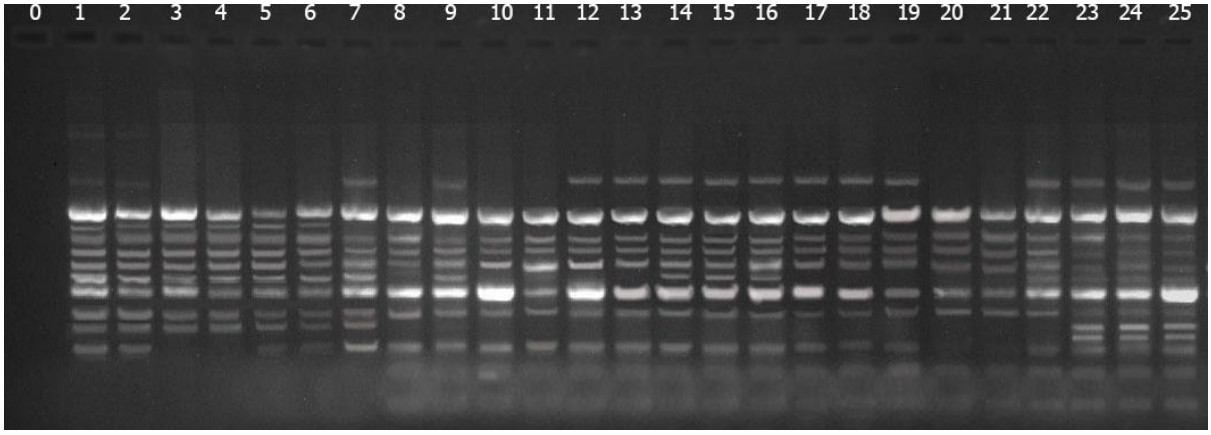
a- absence
p- presence



(a)



(b)



(c)

Plate 7. Amplification profile gel images of ISSR primers (a) L3 (b) L4 (c) L9

4.7.4. Polymorphic Information Content

The PIC value of the 15 primers was calculated by taking the average number of bands produced by the primer. Its value ranged between 0.05 and 0.30 (Fig. 4). The least value was recorded for the primer L4 and the highest value was for UBC 813.

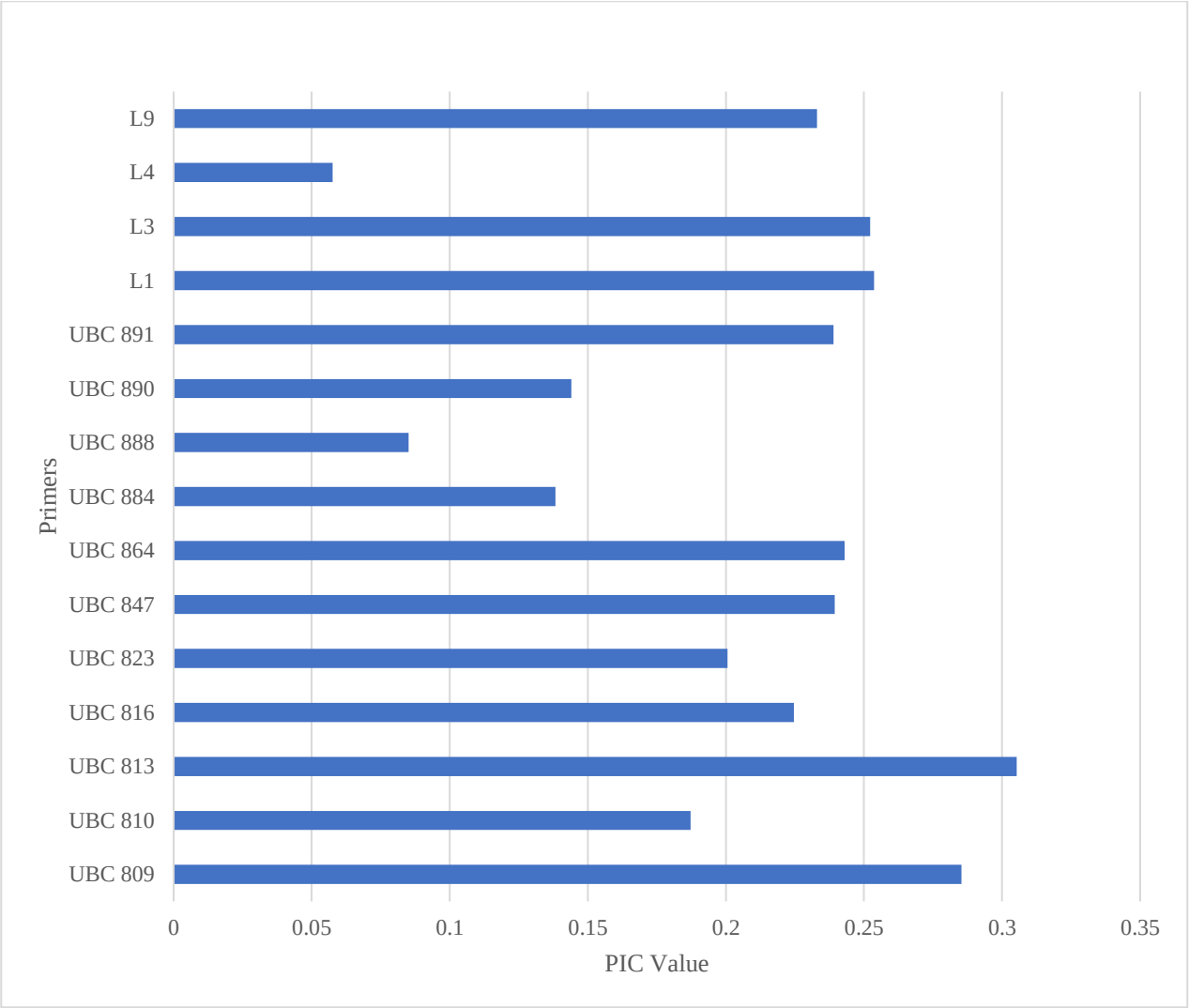


Fig 4. PIC value of 15 ISSR primer

4.7.5. Clustering

From the gel doc image of DNA, scorable and highly reproducible amplicons were converted into binary character matrices (Wandell and Weeden, 1989) and were scored as 0 for the absence of DNA bands and 1 for the presence of bands at each locus of the sample. Based on this binary data, a dendrogram was generated using cluster analysis based on the unweighted pair group method with arithmetic mean (UPGMA) using DARwin software. Factorial analysis was carried out using DARwin software to classify the population into the cluster (Fig.5). The entire population was classified into four clusters (Table 16).

Table 16. Classification of *Melia dubia* plus tree seed progeny into the cluster.

Cluster	Accession
I	FCV-MD- 07
II	FCV-MD- 01, 02, 03, 04, 05, 06
III	FCV-MD- 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25
IV	FCV-MD- 08, 09, 10, 11, 12, 13

Classification of *M. dubia* plus tree seed progeny based on factorial analysis using DARwin software is presented in Table (19). The entire population was classified into 4 clusters (Fig. 5). Cluster 1 consist plus tree seed progeny from the accession Dhoni (FCV-MD-7) in Palghat. Cluster II consist of the Wayanad population (FCV-MD-1-6). Cluster III consist of the population from Parambikulam (FCV-MD-14, 15, 16), Chinnar (FCV-MD-17, 18, 19), Peechi (FCV-MD-20, 21), Akamala (FCV-MD-22), Kulathupuzha (FCV-MD-23), Aryankavu (FCV-MD-24, 25). Cluster IV consists of the population from Pothundy (FCV-MD-8), Attapady (FCV-MD-9), Thiruvazhiyadu (FCV-MD-10, 11), and Walayar (FCV-MD-12, 13).

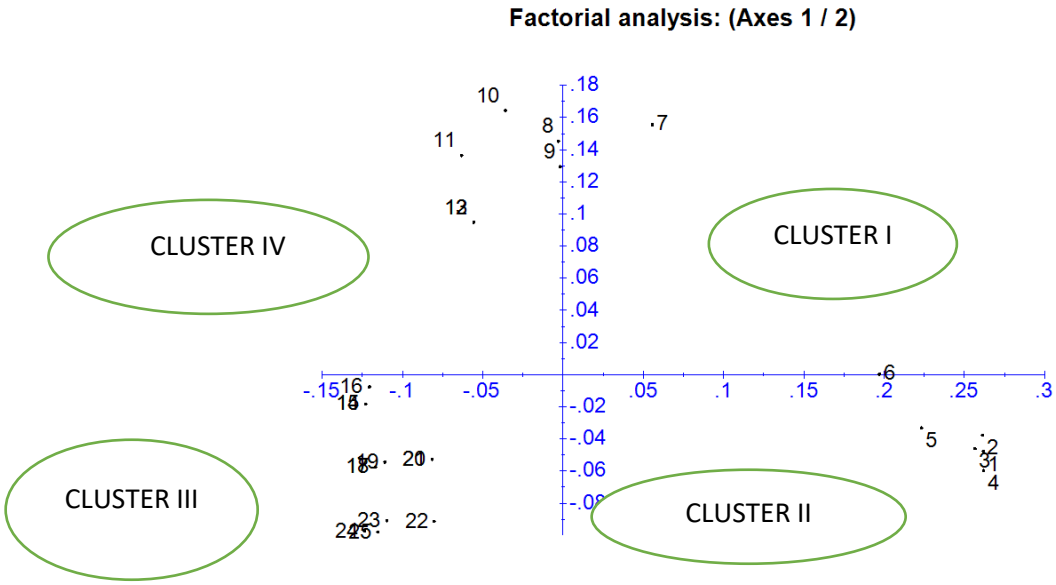


Fig. 5. Factorial analysis based on ISSR primer amplicons.

4.7.8. ISSR analysis

Genetic similarity computed from the ISSR profile is presented in Table 17. The value ranged from 0.604 to 1.000 for the different accessions of *M. dubia* plus tree seed progeny. The maximum similarity was observed between accessions from the Wayanad population in northern Kerala. The least similarity (0.604) was observed between the accession from Parambikulam (FCV-MD-14) and Thirunelly (FCV-MD-1). Jaccard's similarity coefficient values were used for the clustering of the plus tree seed progeny accession for constructing the dendrogram (Fig. 6).

4.7.7. Hierarchical dendrogram

The dendrogram obtained using NTSYSpc software had classified the entire accession plus tree seed progeny of *M. dubia* into two main clusters based on Jaccard's similarity coefficient (Fig. 6). Cluster I consist of the population from the Wayanad region (FCV-MD-1,2,3,4,5,6) of Kerala whereas cluster II mainly consist of the population from Palghat, Thrissur, Idukki, Kollam and Thiruvananthapuram. Cluster I produced two subpopulations. Cluster II produced two subpopulations for the plus tree seed progeny accession. It forms a sub-cluster for the populations which comes under the south of Palghat (FCV-MD-10-13) and for populations under the north of Palghat (FCV-MD-7-9). It formed a sub-cluster for the population from the south of Western Ghats (FCV-MD-23, 24, 25). The sub cluster obtained from the south of Weatern Ghats was further subdivided into two new sub clusters, consisting of accession from the southern side Aryankavu pass (Kulathupuzha FCV-MD-23) and the other sub cluster with two accession from the northern side of Aryankavu pass (FCV-MD-22, 23) in kollam. Cluster I and Cluster II populations are showing a similarity of 64%. The similarity of each accession is presented in Table (17). Another dendrogram was obtained based on the diversity of the population using Nei's gene diversity coefficient (Fig 7). The maximum diversity was shown by the Wayanad population (FCV-MD-1-6) when compared to the remaining population of *M. dubia* in Kerala (0.31). The highest gene diversity was obtained between the Thirunelly (FCV-MD-1) and Aryankavu (FCV-MD-24). Nei's gene diversity coefficient of 25 plus tree progenies are presented in Table (18).

Table 17. Similarity between progeny using Jaccard's similarity coefficient.

	1	2	3	4	5	6	7	8
1	1.0000							
2	0.9921	1.0000						
3	0.9308	0.9231	1.0000					
4	0.9225	0.9147	0.9600	1.0000				
5	0.8797	0.8722	0.9000	0.9213	1.0000			
6	0.8889	0.8955	0.9091	0.9008	0.9308	1.0000		
7	0.6738	0.6786	0.6763	0.6667	0.7059	0.7426	1.0000	
8	0.6458	0.6503	0.6596	0.6500	0.6884	0.7372	0.8559	1.0000
9	0.6735	0.6781	0.6759	0.6667	0.7042	0.7518	0.8537	0.8926
10	0.6225	0.6267	0.6351	0.6259	0.6621	0.7083	0.8016	0.8537
11	0.6144	0.6184	0.6267	0.6174	0.6531	0.6986	0.7752	0.8400
12	0.6443	0.6486	0.6463	0.6370	0.6736	0.7203	0.7874	0.8240
13	0.6443	0.6486	0.6463	0.6370	0.6736	0.7203	0.7874	0.8240
14	0.6040	0.6081	0.6054	0.5959	0.6319	0.6667	0.6870	0.7480
15	0.6045	0.6081	0.6054	0.5959	0.6319	0.6667	0.6870	0.7480
16	0.6093	0.6133	0.6107	0.6014	0.6370	0.6712	0.7045	0.7656
17	0.6159	0.6093	0.6174	0.6081	0.6438	0.6554	0.6618	0.7197
18	0.6159	0.6093	0.6174	0.6081	0.6438	0.6554	0.6618	0.7197
19	0.6225	0.6159	0.6242	0.6149	0.6507	0.6622	0.6691	0.7273
20	0.6443	0.6376	0.6575	0.6597	0.6853	0.7083	0.6815	0.7538
21	0.6443	0.6376	0.6575	0.6597	0.6853	0.7083	0.6815	0.7538
22	0.6486	0.6419	0.6507	0.6528	0.6783	0.7014	0.6866	0.7197
23	0.5909	0.5948	0.6026	0.6040	0.6284	0.6622	0.6940	0.7143
24	0.5769	0.5806	0.5882	0.5894	0.6133	0.6467	0.6765	0.6963
25	0.5806	0.5844	0.5921	0.5933	0.6174	0.6510	0.6815	0.7015

	9	10	11	12	13	14	15	16
9	1.0000							
10	0.8960	1.0000						
11	0.8672	0.9669	1.0000					
12	0.8661	0.8730	0.9040	1.0000				
13	0.8661	0.8730	0.9040	1.0000	1.0000			
14	0.7769	0.7557	0.7846	0.8254	0.8254	1.0000		
15	0.7769	0.7557	0.7846	0.8254	0.8254	1.0000	1.0000	

16 |	0.7939	0.7727	0.8015	0.8425	0.8425	0.9655	0.9655	1.0000
17 |	0.7481	0.7537	0.7820	0.7939	0.7939	0.8770	0.8770	0.8790
18 |	0.7481	0.7537	0.7820	0.7939	0.7939	0.8770	0.8770	0.8790
19 |	0.7556	0.7612	0.7895	0.8015	0.8015	0.8699	0.8699	0.8720
20 |	0.7687	0.7744	0.8030	0.8295	0.8295	0.8852	0.8852	0.8871
21 |	0.7687	0.7744	0.8030	0.8295	0.8295	0.8852	0.8852	0.8871
22 |	0.7481	0.7279	0.7556	0.7939	0.7939	0.8618	0.8618	0.8640
23 |	0.7299	0.6857	0.7122	0.7481	0.7481	0.7829	0.7829	0.8000
24 |	0.7122	0.6809	0.7071	0.7426	0.7426	0.7769	0.7769	0.7939
25 |	0.7174	0.6738	0.7000	0.7353	0.7353	0.7692	0.7692	0.7863

	17	18	19	20	21	22	23	24
17 |	1.0000							
18 |	1.0000	1.0000						
19 |	0.9915	0.9915	1.0000					
20 |	0.8952	0.8952	0.9032	1.0000				
21 |	0.8952	0.8952	0.9032	1.0000	1.0000			
22 |	0.8720	0.8720	0.8800	0.9421	0.9421	1.0000		
23 |	0.7939	0.7939	0.8015	0.8154	0.8154	0.8504	1.0000	
24 |	0.7879	0.7879	0.7955	0.8092	0.8092	0.8438	0.9750	1.0000
25 |	0.7803	0.7803	0.7879	0.8015	0.8015	0.8359	0.9832	0.9916

*1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,2324,25 –

different accessions of plus tree seed progeny.

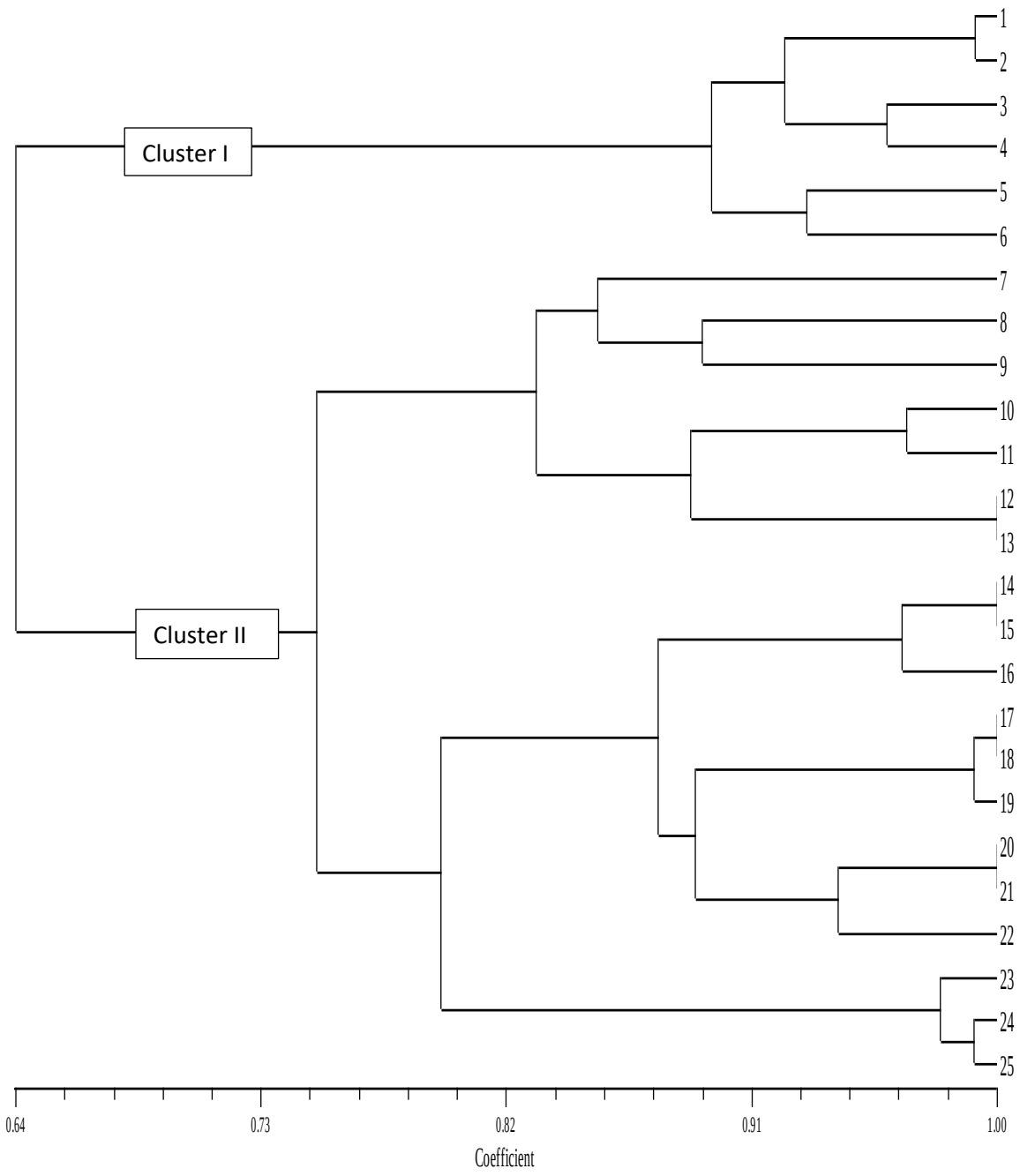


Fig. 6. Hierarchical dendrogram of 25 plus tree progenies based on Jaccard's similarity coefficient.

Table 18. Nei's gene diversity coefficient of 25 plus tree progenies

	1	2	3	4	5	6	7	8
1	0.0000							
2	0.0040	0.0000						
3	0.0364	0.0408	0.0000					
4	0.0409	0.0454	0.0205	0.0000				
5	0.0660	0.0706	0.0541	0.0418	0.0000			
6	0.0606	0.0567	0.0487	0.0532	0.0363	0.0000		
7	0.2139	0.2099	0.2125	0.2218	0.1874	0.1566	0.0000	
8	0.2397	0.2358	0.2278	0.2373	0.2025	0.1611	0.0808	0.0000
9	0.2165	0.2126	0.2147	0.2231	0.1905	0.1521	0.0813	0.0577
10	0.2641	0.2602	0.2522	0.2614	0.2271	0.1864	0.1159	0.0816
11	0.2725	0.2686	0.2606	0.2699	0.2355	0.1948	0.1342	0.0900
12	0.2431	0.2391	0.2416	0.2506	0.2167	0.1766	0.1258	0.1009
13	0.2431	0.2391	0.2416	0.2506	0.2167	0.1766	0.1258	0.1009
14	0.2815	0.2776	0.2808	0.2912	0.2545	0.2209	0.2051	0.1556
15	0.2815	0.2776	0.2808	0.2912	0.2545	0.2209	0.2051	0.1556
16	0.2771	0.2731	0.2761	0.2861	0.2503	0.2178	0.1899	0.1421
17	0.2706	0.2774	0.2694	0.2792	0.2439	0.2324	0.2270	0.1775
18	0.2706	0.2774	0.2694	0.2792	0.2439	0.2324	0.2270	0.1775
19	0.2641	0.2709	0.2629	0.2724	0.2376	0.2264	0.2202	0.1712
20	0.2431	0.2496	0.2311	0.2294	0.2065	0.1864	0.2092	0.1506
21	0.2431	0.2496	0.2311	0.2294	0.2065	0.1864	0.2092	0.1506
22	0.2388	0.2454	0.2374	0.2357	0.2125	0.1920	0.2050	0.1775
23	0.2966	0.2926	0.2846	0.2834	0.2588	0.2264	0.1984	0.1817
24	0.3118	0.3079	0.2999	0.2988	0.2739	0.2409	0.2134	0.1965
25	0.3076	0.3037	0.2957	0.2946	0.2696	0.2366	0.2092	0.1923

	9	10	11	12	13	14	15	16
9	0.0000							
10	0.0564	0.0000						
11	0.0738	0.0169	0.0000					
12	0.0744	0.0702	0.0517	0.0000				
13	0.0744	0.0702	0.0517	0.0000	0.0000			
14	0.1337	0.1495	0.1280	0.1002	0.1002	0.0000		
15	0.1337	0.1495	0.1280	0.1002	0.1002	0.0000	0.0000	

16 |	0.1220	0.1372	0.1166	0.0893	0.0893	0.0175	0.0175	0.0000
17 |	0.1555	0.1513	0.1304	0.1220	0.1220	0.0675	0.0675	0.0665
18 |	0.1555	0.1513	0.1304	0.1220	0.1220	0.0675	0.0675	0.0665
19 |	0.1499	0.1457	0.1251	0.1167	0.1167	0.0718	0.0718	0.0708
20 |	0.1402	0.1360	0.1156	0.0979	0.0979	0.0625	0.0625	0.0617
21 |	0.1402	0.1360	0.1156	0.0979	0.0979	0.0625	0.0625	0.0617
22 |	0.1555	0.1713	0.1499	0.1220	0.1220	0.0769	0.0769	0.0758
23 |	0.1697	0.2063	0.1840	0.1556	0.1556	0.1295	0.1295	0.1177
24 |	0.1840	0.2106	0.1882	0.1598	0.1598	0.1337	0.1337	0.1220
25 |	0.1798	0.2168	0.1941	0.1655	0.1655	0.1394	0.1394	0.1274

	17	18	19	20	21	22	23	24
17 |	0.0000							
18 |	0.0000	0.0000						
19 |	0.0043	0.0043	0.0000					
20 |	0.0569	0.0569	0.0522	0.0000				
21 |	0.0569	0.0569	0.0522	0.0000	0.0000			
22 |	0.0708	0.0708	0.0659	0.0302	0.0302	0.0000		
23 |	0.1220	0.1220	0.1167	0.1072	0.1072	0.0843	0.0000	
24 |	0.1263	0.1263	0.1209	0.1115	0.1115	0.0885	0.0127	0.0000
25 |	0.1317	.1317	0.1263	0.1167	0.1167	0.0936	0.0085	0.0042

*1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,2324,25-

Different accessions of plus tree seed progeny.

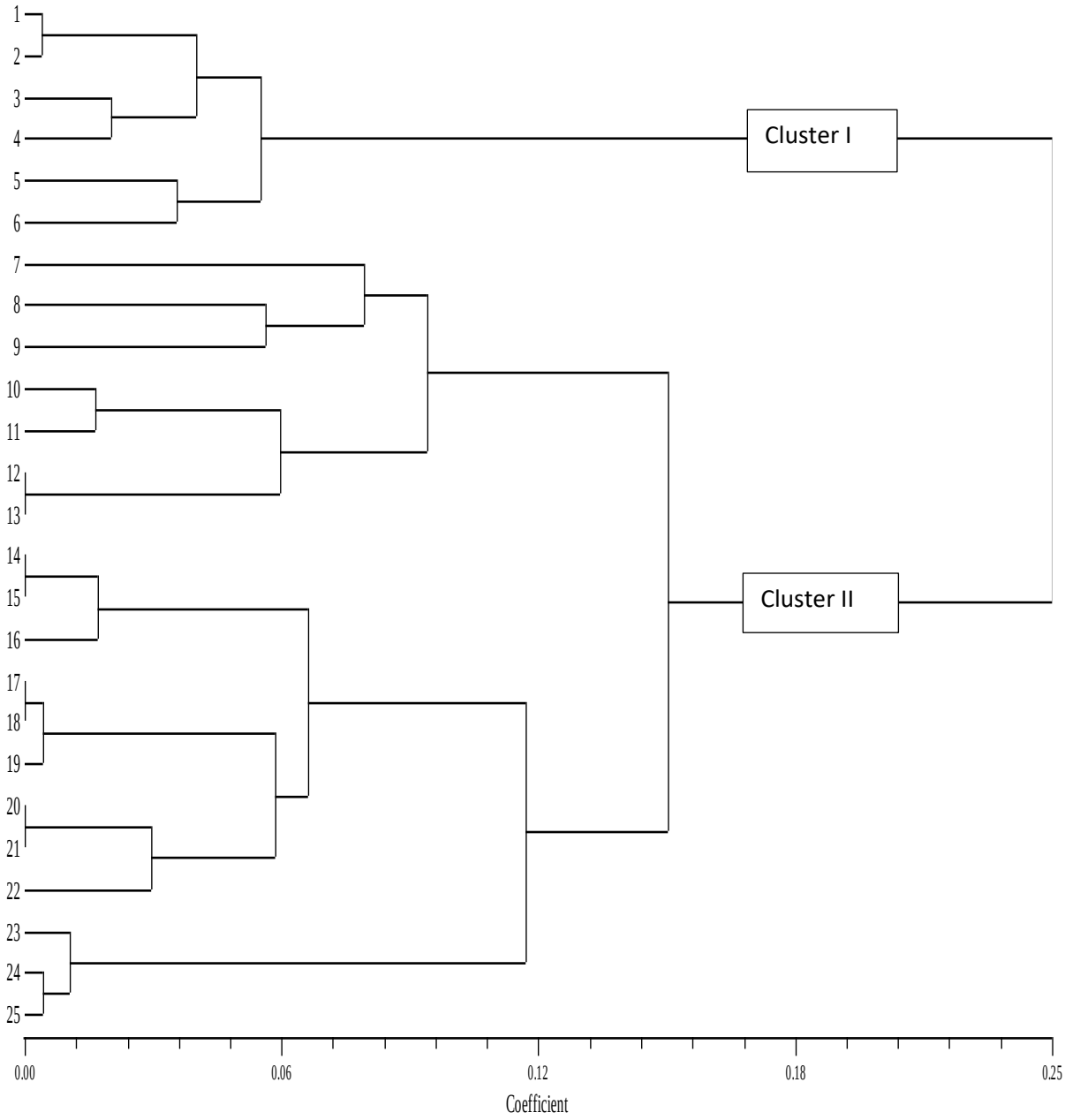


Fig. 7. Hierarchical dendrogram of 25 plus tree progenies based on Nei's gene diversity coefficient

4.8. Variation in caryophyllene yield among plus tree seed progenies of *Melia dubia* Cav.

Caryophyllene is an important bioactive chemical present in *M. dubia* which contributes to the phytochemical property of the plant. Owing to its importance, the quantification of this chemical was done in plus tree seed progenies using HPLC and its variation among different progenies were analysed.

4.8.1 Caryophyllene standardization

Linearity was tested using 12.5 ppm, 25 ppm, 50 ppm, 100 ppm, 200 ppm concentrations of caryophyllene working standards. Calibration curves for each concentration were prepared for respective peak areas to get a regression curve ($y = 388415x + 32176$; $R^2 = 0.99988a$) as shown in Fig. 8.

4.8.2 Caryophyllene quantification in HPLC

The study observed that the quantity of caryophyllene varied significantly among the plus tree seed progenies. The retention time of this chemical was 5.63 min in HPLC. The percentage of caryophyllene leaf extract is shown in Table 19. The highest concentration of caryophyllene was observed in the plus tree seed progeny FCV-MD-8 (Pothundy) followed by progeny FCV-MD-3 (Tholpetty) and FCV-MD-10 (Thiruvazhiyadu). The lowest concentration was observed in FCV-MD-12 (Walayar). The average value of the entire progenies was 2.3 percent. A typical chromatogram obtained on analysis is presented in Fig. 10

Table 19. Percentage of caryophyllene in *Melia dubia* Cav. plus tree seed progenies

Accession No.	% Caryophyllene
FCV-MD- 1	0.07
FCV-MD- 2	0.3
FCV-MD- 3	9.6
FCV-MD- 4	1.1
FCV-MD- 5	1.6
FCV-MD- 6	2.6
FCV-MD- 7	0.04
FCV-MD- 8	16.4
FCV-MD- 9	3.5
FCV-MD- 10	6.6
FCV-MD- 11	1.4
FCV-MD- 12	0.7
FCV-MD- 13	0.04
FCV-MD- 14	1.0
FCV-MD- 15	0.04
FCV-MD- 16	1.0
FCV-MD- 17	2.3
FCV-MD- 18	0.05
FCV-MD- 19	1.0
FCV-MD- 20	0.6
FCV-MD- 21	1.0
FCV-MD- 22	1.6
FCV-MD- 23	0.9
FCV-MD- 24	0.15
FCV-MD- 25	4.2

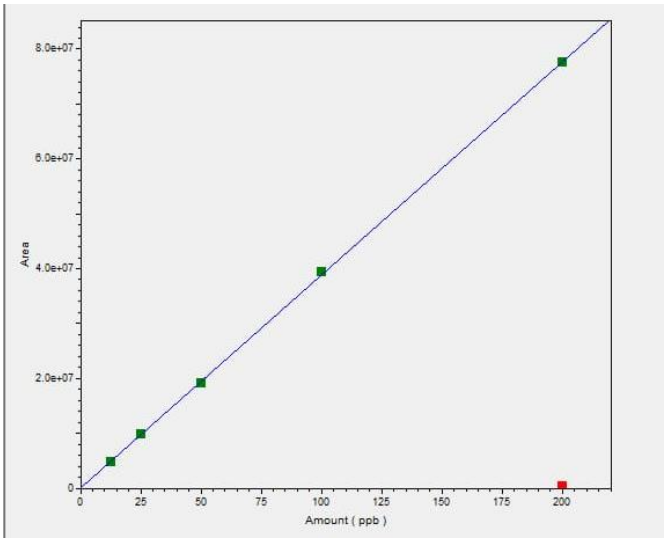


Fig. 8. Calibration curves for each concentration of caryophyllene

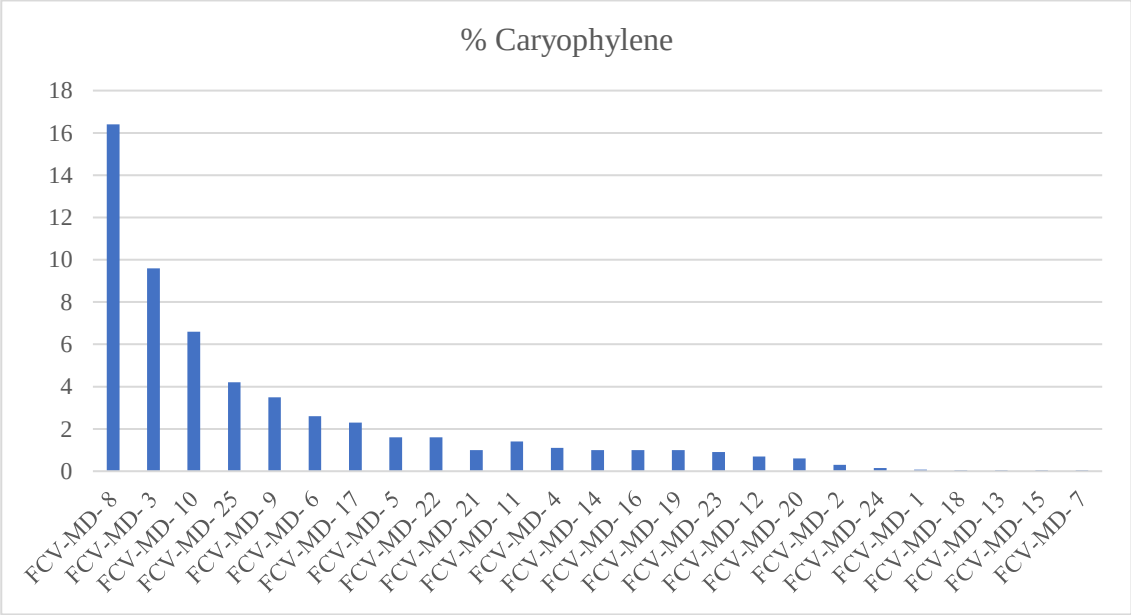


Fig. 9. Variation of caryophyllene (percentage) different in plus tree seed progenies

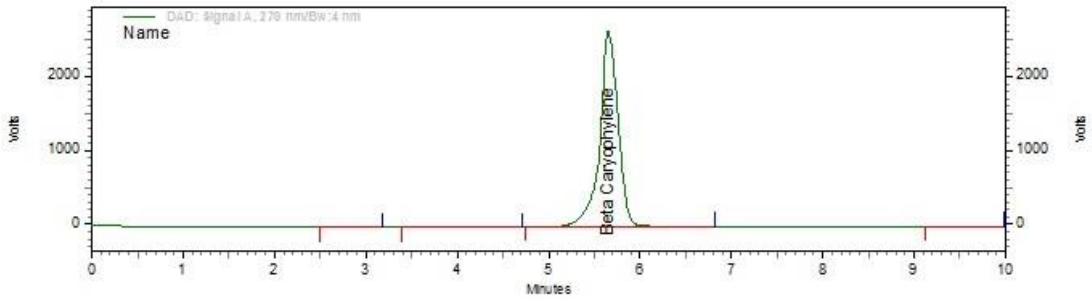


Fig. 10. A typical chromatogram obtained on analysis

5.DISCUSSION

5.1. Evaluation of selected plus tree progenies

In the tree breeding programme, the progeny test plays a very important part in studying the parent through their offspring, as the progeny from a given parent provide better information to those hereditary properties of the parent which are of more economic value than does the parent itself. Hence, progeny trials were carried out by tree breeders around the world to understand the genetic worthiness of the parent based on its offspring's growth performance in the environment (White and Hodge, 1989). The positive outcomes studied through a progeny evaluation trial can be used for further genetic improvement as well as breeding programmes of the parent plant for improved productivity, yield, quality of produce and profitability. Keeping these objectives in mind, we carried out a progeny evaluation trial in one-year-old plus tree seed progenies of *M. dubia* planted in a compact family block design (CFBD). This design helped us to homogenise the environmental error that occurs during the continuous field trial and study the variation caused only because of genetic factors. Understanding the amount of genetic variation only in a plant can help a tree breeder for better future tree improvement programme (Zobel, 1971).

For the 25 progenies evaluated, we observed variations significant among each other for their morphological (plant height, collar girth, number of leaves, crown diameter, and volume) and physiological traits (photosynthetic rate, transpiration rate, stomatal conductance, chlorophyll content and relative water content). Among the progenies evaluated, the superiority of FCV-MD-02 (Thirunelly) and FCV-MD-03 (Tholpetty) was evidenced consistently in all the four growth periods observed. Earlier a was study done by Binu and Santhoshkumar (2019) for the 25 plus tree seed progenies of melia in nursery for six months. The result showed a significant variations in the morphological as well as physiological traits and further it was observed that the the assessions FCV-MD-02 (Thirunelly) and FCV-MD-03 (Tholpetty) performed

consistently better. In our field study also the performance of FCV-MD-02 (Thirunelly) and FCV-MD-03 (Tholpetty) assesions form Wayanad region showed better performance for both the morphological and physiological trait, which give more strength to our current study and better authenticity to the present plus tree progeny trials under the field condition. These morphological, as well as physiological trait variations, can be inferred because of its varying environmental as well as genetic factors (Parthiban *et al.*, 2017). Even though in our study the 25 plus tree progenies were planted in a compact family design to homogenise the environmental effect, the progenies showed significant differences for its growth and physiological. Thus, it can be inferred that the plant growth and physiological traits had very little environmental influence and the majority of the variations in the trait are caused by the genetic makeup of the plant. Similar observations were made by Parthiban *et al.,* (2017) for their studies on *M. dubia*, *Leucaena leucocephala*, and *Nolamarckia cadamba* which give more credibility to the current inference made in this study. A superfluity of workers reported the existence of significant differences and superiority of a few seed sources, progenies and provenance in several tree species like *Santalum album* (Bagchi and Sindhu veerendra, 1991), *Lagerstroemia* spp. (Jamaludheen *et al.,* 1995), *Dalbergia sissoo* (Rawat and Nautiyal, 2007), *Pinus elliotti* var *elhatta* (Vergara *et al.,* 2007), *Ailanthus excelsa* (Daneva *et al.,* 2018), *M. azaderach* (Thakur and Thakur, 2015), and *M. dubia* (Kumar *et al.,* 2013) which thus give support to the present findings in *M. dubia*.

5.1.1. Path analysis

Path analysis gives a comprehensive idea about the intricate relation between different traits in a biological system as these traits are affected by a wide range of associated characters. In our study, it was observed that the morphological trait *viz*., plant height, collar girth exerts positive direct as well as indirect effect on the volume of the tree whereas among the physiological trait only stomatal conductance was showing a direct effect. As the collar girth of the plant had a maximum direct and indirect effect on volume, it can be concluded that the high and positive association of

a direct effect of plant height and collar girth on volume could be used as a valuable and reliable measure of selection index for *M. dubia* tree improvement programme. Similar positive correlation and positive direct effect were shown by height and girth on the volume of plants *viz*. *Simarouba glauca* (Kumaran *et al.,* 2010) and *Terminalia arjuna* (Srivastava *et al.,* 1993). On the contrary in some studies negative association between the height, diameter, and volume was also observed for *M. dubia* (Parthiban *et al.,* 2017).

5.2. Genetic diversity analysis

5.2.1. Morphological traits

The assessment of the genetic variability of a plant is a crucial step for any tree improvement as well as a breeding programme for developing new strategies. The magnitude of this variability in a plant is understood by measuring the genotypic coefficient of variation (GCV) and phenotypic coefficient of variation (PCV) value which gives the relative amount of variation in different plant traits (Divakara *et al.,* 2012), as these coefficients of variations being independent of the scale of the unit of measurement, for different mean values. It can be conveniently used for comparison between different populations. Thus, heritable variations observed can be exploited for future tree improvement and breeding programmes. In the present study, it was observed that the PCV and GCV values recorded for volume were higher (66.8, 53.9) followed by crown diameter (44.82, 35.51), then plant collar girth (37.9, 33.1) and the plant height (27.55, 23.7). The observed higher PCV and GCV value in the current study indicates the amount of variability present in *M. dubia*. Parthibhan *et al.,* (2017) had observed higher GCV and PCV values for *M. dubia* while doing progeny evaluation which gives a good validation to our observations. They also observed higher PCV and GCV values in *Leucaena leucocephala, Neolamarckia cadamba.* Similar observations were made in teak where higher GCV value for volume was observed (Arun, 1996), low GCV for height in the neem was reported (Pandey *et al.,*

2018). Similarly, low GCV and PCV for height and collar diameter were also reported in *Bambusa pallida* (Singh and Beniwal, 1993) and low GCV and PCV for plant height in *M. dubia* (Kumar *et al.*, 2013). The genotypic and phenotypic coefficient of variation gives an idea of the quantum of genetic variability in traits like height, collar girth and other morphological as well as physiological traits. The observed genotypic variabilitythrough estimating the GCV, PCV, heritability and genetic advance in the current study are near to the findings which had been observed earlier in tree species like *Azadirachta indica* (Dhillon, *et al.*, 2003), *Eucalyptus grandis* (Subramanian *et al.*, 1995) and in progenies of *Dalbergia sissoo* (Dogra *et al.*, 2005) which underpin the findings of the current study in *M. dubia.*

5.2.2. Heritability and Genetic advance

Heritability has an important role in the tree improvement programme as it gives an index of the relative strength of heredity versus environment. It is useful for ranking important traits for a crossbreeding programme. Estimation of broad-sense heritability for different characters showed moderate to good heritability for collar girth (0.75), height (0.68), volume (0.65) and crown length (0.62) which can be due to the complexity of traits prone to high environmental influences. The higher heritability observed for collar diameter (0.75) in the present study indicated the predominance of additive gene action for this character as reported in teak (Kumar *et al.,* 1997). As the collar diameter, plant height, crown diameter and volume showed moderate heritability percentage, the selection for these traits would be very effective in the breeding programme.

Although heritability in a broad sense indicates the relative value of selection in the material at hand, to give a more authentic conclusion, heritability and genetic advances have to be considered together. Johnson *et al.,* (1955) had mentioned that heritability estimates along with genetic advances are more useful in predicting its resultant effect for selecting the best individuals from a population. Heritability

estimates in a broad sense will be effective when it is accompanied by high genetic advance (Burton and Devane, 1953). Similar observations were made by Wilcox *et al.,* (1967) on *Salix erocephala*, and on *Bambusa pallida*, *Bambusa balcooa* (Singh and Beniwal, 1993) on. In the current study it was observed that the plant volume was having higher genetic advance (Table 12) which was followed by plant crown diameter (73%), then collar girth (50%) and height (37%). These findings indicate a broad scope of genetic improvement possibility in the species due to its high Genetic Advance ratio which is influenced by additive gene effect. These findings of the current study are in line with those of Ramachandra (1996) in *Acacia catechu*. Other researchers have also reported similar results in *Terminalia arjuna* (Srivastava *et al.,* 1993) and also in *Eucalyptus grandis* (Subramanian *et al.,* 1995).

It is understood that better gain can be achieved for the characteristics that are strongly under genetic control and that have a broad range of variability. The characters with high heritability together with higher genetic gain can act as a dependable indicator as observed in salix (Singh *et al.,* 2012). Hence, the high heritability along with high genetic gain for tree volume exhibited in the current study shows that these characteristics are highly under the influence of genetic control and can be made use for future tree improvement programmes.

5.3. Physiological variation of the progenies and correlation with the growth characters

The physiological traits such as chlorophyll content, photosynthetic rate, stomatal conductance, transpiration rate, leaf temperature and relative water content of the plus tree progenies were determined for two periods, 27 MAP and 36 MAP. From the recorded data it was observed that the mean value for the physiological parameters was higher on 36 MAP except in the case of chlorophyll content and relative water content. Throughout data collection, FCV-MD-03 from Tholpetty showed the highest value for the physiological parameters. Similar results were also observed by Binu and

Santhosh (2019) in *M. dubia* plus tree progeny studies in nursery. During the entire experiment physiological parameters like chlorophyll content, photosynthetic rate, stomatal conductance, transpiration rate, leaf and relative water content were significant except for the leaf temperature. Significant provenance variations in net photosynthesis, stomatal conductance was observed in neem seedlings (Kundu and Tigerstedt, 2012). The results are in agreement with the studies carried out by Mebrahtu and Hanover (1991) for black locust (*Robinia pseudoacacia* L.) and black spruce (*Picea mariana* Mill.) by Johnsen and Major (1995).

In the current study, it was observed that the PCV and GCV for the physiological traits were higher for the stomatal conductance (65.28, 58.50) followed by photosynthetic rate (43.74, 37.53), transpiration rate (31.41, 30.03), chlorophyll content (9.01, 8.67) and relative water content (5.06, 4.52). Throughout the observation of physiological parameters, it was found out that PCV was higher than GCV imparting the influence of the environment on the plant physiology. The values of heritability for all the physiological observations were found to be moderately higher. Its value varied from 0.91 (transpiration) followed by 0.82 (chlorophyll content), then 0.82 (stomatal conductance), 0.79 (relative water content) and 0.75 (photosynthesis). The values were found to be more than fifty percent. Similar observations for its high heritability in chlorophyll content, photosynthetic rate and stomatal conductance was observed in teak (Arun, 1996). In *Populus nigra* (Chu *et al.,* 2010), *Dalbergia sissoo* (Sharma and Bakshi, 2014) and *P. trichocarpa* (Mckown *et al.,* 2014) similarly, high heritability and genetic advance were observed for its physiological traits. These higher values indicates that there is a high probability of genetic trait to be transferred to the next generation.

The photosynthetic rate in the plant is determined by both environmental as well as genetic factors and through its complex process of interaction. Stomatal conductance is an indicator of plant water status. It has been identified as an early plant response to

water deficit, which limits the uptake of carbon thereby influencing the rate of photosynthesis in the plant leaves. Higher the stomatal conductance, higher will be the leaf water content (Auge *et al.,* 2015). Researchers have also reported that plant productivity is directly dependent on net photosynthesis, stomatal conductance (Wang *et al.,* 1995). From the morphological data of the current study, it was observed that FCV-MD-03 (Tholpetty) was showing better growth performance when compared to other accessions and its rate of photosynthesis and stomatal conductance were found to be higher compared to other plus tree progenies. From the study, it can be concluded that physiological traits such as photosynthetic rate, stomatal conductance can be used to find morphologically better performing plants as these physiological traits are highly heritable (Wang *et al.,* 1995).

5.4. Correlation study

In our study, it was observed that the selected plus trees had a significant positive correlation for the morphological and physiological traits. The photosynthetic rate was found to be positively correlated to the stomatal conductance, transpiration and relative water content. In a study where a multispecies meta-analysis was done from the data sets of 17 published studies, a significant positive correlation was observed between photosynthetic rate and stomatal conductance (Gago, *et al.,* 2016) which give strength to our current observation. The results of our experiment showed that seedlings from different plus trees have different photosynthetic rates. The seedlings of plus trees FCV-MD-03 (Tholpetty) and FCV-MD-04 (Tholpetty) had high photosynthetic rates. It was observed that net photosynthetic rate showed a significantly positive correlation with seedling height, individual volume, which was an interesting finding of this study. The results indicate that the seedlings of plus trees with a high photosynthetic rate result in fast-growing plants. The above result was generally inconsistent with the studies done in different clones of *Tectona grandis*, where high heritability was observed for the photosynthetic rate, chlorophyll content and stomatal conductance for some of the

clones studied. These traits were positively correlated with seedlings height and volume of the clones. Their study further revealed that this parameter can be regarded as a key resource for future breeding and germplasm resource management (Huang, 2019). In *Populus nigra*, it was observed that species originating in Serbia had a high correlation with the growth, the gas exchange also with chlorophyll fluorescence parameters. Highly correlated germplasm was further used as a resource with high light-use efficiency for future breeding (Chu *et al.,* 2010). High heritability and genetic advance for the above-mentioned traits were observed for *Populus niagra* (Chu *et al.,* 2010); in *Dalbergia sissoo* (Sharma and Bakshi, 2014); in *Populus trichocarpa* (Mckown *et al.,* 2014).

Based on the earlier studies, it can be concluded that the net photosynthetic rate, stomatal conductance and transpiration rate as a variable has a high empirical significance which can be used for improving the efficiency for better tree breeding programmes in *M. dubia*. Apart from this the chlorophyll content and relative water content was found to be useful as an important parameter for the selection and improvement of *M. dubia* for various plant breeding programme (Auge *et al.,* 2015).

5.4. Analysis of genetic variation using ISSR markers

The molecular markers have been proved to be a fundamental and reliable tool for fingerprinting varieties, establishing the fidelity of progenies etc. The advent of automated PCR technology made a new set of markers available to scientists interested in comparing organisms at the molecular level. ISSR markers were used for studying the genetic diversity and polymorphism owing to its reproducible, and dominant nature (Reddy *et al.,* 2002). ISSR markers were found to be efficient as they can bridle the limitations of other primers based on PCR like AFLP, RAPD, SSR markers. In general, it was observed the primers with AG, GA, TC, AC, and CA repeats show higher polymorphism than primers with other di, tri or tetranucleotide repeats. Though studies had reported that the genome of a plant contains more (AT) rich repeats (Akagi *et al.,* 1997), primers with (AT) rich repeats failed to produce any amplification due to self-

annealing of the primers. Blair *et al.* (1999) had reported that a more common dinucleotide pattern was more responsible for ISSR analysis than the more uncommon tri, tetra and Penta nucleotide motifs. As the ISSR markers are cheaper and easier marker systems with high efficiency in producing polymorphism among the closely related species, they would play a major role in plant genome analysis in the future.

5.4.1. Standardisation of DNA isolation

The most important step for any molecular study is the isolation of pure, intact and high-quality DNA. However, the presence of secondary metabolites inhibits this extraction process making it very difficult. *M. dubia* plant leaves consist of a large number of secondary metabolites thus making the process of DNA extraction a difficult task, using an ordinary CTAB protocol. Hence for the extraction of pure DNA from *M. dubia* modified CTAB protocol (Rawat *et al.*, 2016) was followed. A large quantity of pure DNA was obtained using this protocol (OD260/280 value of 1.8-2.0).

The stage of development and maturity of the plant has a crucial role in determining the quality of DNA obtained. The extraction of high-quality DNA from young leaves were highly affected by its large amount of polyphenolic content. The DNA thus obtained from young leaves showed smear, which was due to the presence of excess polyphenols. However, DNA from mature dry leaves having less polyphenolic content was found to give a good quantity and quality of DNA without smear after gel electrophoresis. 300 mg of leaf sample were enough to give an ample quantity of DNA.

The homogenisation, pulverisation and uniformity of grinding of plant tissue were found to be essential during the DNA extraction. For the homogenisation of the leaf tissue excess liquid nitrogen was used. Liquid nitrogen helps to freeze the leaf tissue, prevent nucleic acid degradation and the effect of secondary metabolites and provide a better mechanical breaking of the leaf tissue. The yield of DNA and its purity varied with different accession of the plus tree seed progeny. The yield ranged from

141.94 µg mL^{-1} (Dhoni) to 1213 µg mL^{-1} (Tholpetty). The purity (A260/ A280) ranged from 1.80 (Walayar) to 2.09 (Dasanakara, Neykavala). This variation in value could be from the interference of various compounds in the plant tissue during the extraction procedure.

5.4.2. ISSR ASSAY

The good quality genomic DNA isolated from the leaf samples of the *M. dubia* plus tree progeny were subjected to ISSR analysis as per the protocol reported by Rawat *et al.,* (2016). The PCR condition standardised for ISSR by Rawat *et al.,* (2016) was used. Venkatachalam *et al.* (2008) and Chandrika and Rai (2009) have used different ISSR series primers for their ISSR marked based genetic studies.

5.4.3 Amplification with selected ISSR primer

In the present study, a total of 25 *M. dubia* plus tree progenies of 15 natural populations in Kerala were analysed. Fifteen selected dominant ISSR primers were used for PCR. These primers had produced a different number of bands depending upon their sequence and variation in the sample used for studying the genetic diversity. In the current research, it can be observed that most of the primers that were showing high polymorphism contain dinucleotide repeats viz. CA, CT, AG, GA, GT, TC, AC and tri-nucleotide repeat ATG indicating the plethora of these repeating sequences in *M. dubia* genomic sequences. Similarly, the richness of di-nucleotide repeats has been reported by Rawat *et al.,* (2018) in their study on the genetic diversity of *M. dubia* using ISSR markers in Karnataka. Familiar observations were also made in species like *Populus cathayana* (Ranjbarfard *et al.,* 2014), *Curculigo latifolia* (Lu *et al.,* 2006), *Medicago species* (Mahalakshmi *et al.,* 2002), an abundance of tri-nucleotide repeats in neem (Ranade and Farooqi, 2002) and tri and tetra repeats in Teak (Ansari *et al.,* 2012).

M. dubia plus tree seed progeny possessed a high level of polymorphism (113 polymorphic bands out of the 164 amplicons detected by 15 primers). The number of

amplicons produced by the primer varied from seven (UBC 809) to fifteen (L9 and UBC 847). Studies have inferred that the natural populations of *M. dubia* is having high polymorphism within the population and one of the major reasons for it is due to its major mode of seed dispersal which is through herbivores as they feed on the fruits (Neginhal, 2004). High variation within populations has been reported in other Meliaceae family members also like *Swetinia macrophyla*, *M. azedarach* (Naik *et al.,* 2009) and in other species like *Abies nephrolepis* (Woo *et al.,* 2008), Teak (Ansari *et al.,* 2012) and *Prosopis cinerania* (Sharma *et al.,* 2011).

The primers used for the current studies showed a polymorphism range of 27.2 % to 86.6 %. The maximum polymorphism was observed for the primer UBC 809 and the lowest for the L4 primer. A mean value of 70 % polymorphism was produced by the primers (Table 18). Studies on *M. dubia* using ISSR primer among 232 populations in Karnataka showed an average polymorphism of 94.6 % (Rawat *et al.,* 2018). To understand the efficiency of molecular markers used, polymorphic information content (PIC) was calculated. From the result obtained it was observed that primer UBC 813 showed the highest value (0.305) and lowest by the primer L4 (0.0576) which implicate the primer UBC 813 as the best one for polymorphism study in the *M. dubia* plant. Similar observations were also made by Rawat *et al.,* (2018) in *M. dubia* from the Karnataka region. Accession specific bands were observed using the primer 891 (Chinnar 17). Population-specific bands were produced by primer 890 (Walayar 12, 13), 891 (Thiruvazhiyadu 10, 11), L9 (Thirunelly 1, 2) and hence these primers can be used effectively to identify the specific plus tree progeny.

5.4.4 Data analysis

The value of each of the 15 ISSR markers was used to find the PIC value. The value ranged between 0.0576 (UBC 813) and 0.305 (L4). Rawat *et al.,* (2018) reported the PIC value in their studies on *M. dubia* as 0.24.70 to 0.83 using ISSR UBC primer.

Polymorphism in a population is frequently caused by the presence of genetic variants, which are represented by the number of alleles at a locus and their frequency of distribution in a population. Heterozygosity is the chance that two alleles drawn at random from a population can be distinguished by the marker in question. As a result, a convenient quantitative estimate of marker use and polymorphism identification can be provided in terms of the nei's genetic diversity. The ISSR data observed nei's genetic diversity for all the 25 plus tree seed progeny were analysed using 15 ISSR primers and its value was found as 31 %. Rawat *et al.,* (2018) reported the nei's genetic diversity of 232 accessions using 15 primers as 35 %. Both the result from the current study and other previous study shows that the diversity of *M. dubia* in Kerala natural populations are very low.

Using the results obtained from the software Dendrogram NTSYSpc using Jaccard's similarity coefficient and nei's gene diversity coefficient the dendrogram of the 25 plus tree seed progeny accessions were constructed. It categorized the plus tree seed progeny accession into two main clusters. One of the clusters contained the population from Wayanad accession alone and the other cluster contained the population from Palghat, Thrissur, Idukki, and Kollam. Most of the progeny from different accession of the same population showed more than 95 % similarity. It can be observed that the two-cluster population are separated by the Palghat gap of Western Ghats and it could be one of the reasons for the wide genetic variation between the populations from Wayanad to the rest of the population in the Western Ghats of Kerala.

A dendrogram was obtained for the entire accession of plus tree seedlings of *M. dubia* using NTSYSpc software using Jaccard's similarity coefficient and nei's gene diversity coefficient which categorized the plus tree seed progeny accession into two main clusters. One of the clusters contained the population from Wayanad accession alone and the other cluster contained the population from Palghat, Thrissur, Idukki, and Kollam. Most of the progeny from different accession of the same population showed more than 95 % similarity.

Molecular markers are scattered all over the genome and their association with various morphological as well as physiological traits are influenced by each other. More studies on morphological and physiological traits with a greater number of reliable markers like SSR, SNP will help confirm the present study. Information on the degree of relationship between these progenies will be important for the germplasm collection, *in situ* conservation Melia breeding programmes. The result of the current study will be useful in the *M. dubia* fingerprinting and in determining the genetic diversity among the plus tree seed progeny accession.

5.5. Variation in caryophyllene yield among plus tree seed progenies of *Melia dubia* Cav.

Caryophyllene is an important phytochemical present in *M. dubia* which play an important role in providing various medicinal property to the plant. This chemical was reported to be present in all parts of the plant with the highest concentration in leaves (more than 28 %) of *Copaifera langsdorffi* (Maffei, 2020). Owing to the importance of the chemical in the plant world, the chemical was quantified in *M. dubia* plus tree seed progenies from Kerala for the current study.

Studies have shown that caryophyllene was never quantified using HPLC in *M. dubia* as it is necessary to know the accurate amount of important biochemical in a plant. Most of the previous studies on *M. dubia* for biochemical was carried out only using GC-MS. In the present study, we standardised an HPLC method for the quantification of caryophyllene in *M. dubia*. The regression curve obtained for the standard was 0.999885, thus validating the HPLC quantification. De Almeida Borges *et al*., (2013) standardised HPLC quantification of caryophyllene in copaiba oleoresin where the regression curve obtained for the standard was greater than 0.98.

The present study observed that the quantity of caryophyllene varies among the plus tree seed progenies in leaves with an average value of 2.3 percent. The highest concentration was observed in FCV-MD-8 (Pothundy) and lowest in FCV-MD-12 (Walayar). Murugesan *et al.* (2013) had observed 6 percent of caryophyllene in

M. dubia leaves using GC-MS. Maffei (2020) has reported the presence of more than twenty-eight percent of caryophyllene in Meliaceae family members. From table 18, it could be observed that the presence of chemicals was not uniform across the different progenies which were raised in a homogeneous field. This variation of caryophyllene concentration among the plus tree seed progenies shows that the accumulation of this chemical in the plant is genetically controlled. Maffei (2020) had reported that the yield of caryophyllene in plants depends upon several factors like genetic variability and phenotypic plasticity. From these observations, it was concluded that *M. dubia* from Pothundy can be selected for its higher phytochemical property and can be raised to meet the pharmacological requirements.

6.SUMMARY

Melia dubia is a fast-growing indigenous species amenable for plantation and agroforestry due to its multifarious domestic and industrial utility. The present study entitled "Progeny testing and genetic diversity analysis in plus trees of *M. dubia* Cav.*"* was carried out during the period from 2020 to 2021 at the Department of Forest Biology and Tree Improvement, Kerala Agricultural University. The objective of the study was to do progeny testing in *M. dubia* plus tree seed progeny, to explore the genetic diversity among these plus tree seed progenies and analyse the phytochemical "caryophyllene". The salient feature of this study is:

- Significant variation among plus tree seed progenies was observed for the morphological traits and physiological traits.
- Plus, tree seed progeny FCV-MD-02 and FCV-MD-03 from Thirunelly and Tholpetty accession showed the highest growth consistently throughout the experiment for the morphological traits such as collar height, collar girth, number of leaves and volume. FCV-MD-19 of Chinnar showed a higher growth rate for its crown diameter. The lowest value for the plant height was observed for FCV-MD-15 from Parambikulam, while a lower value for the collar girth and crown diameter was observed for the progeny FCV-MD-10 of Thiruvazhiyadu. The least value for volume was observed for the plus tree seed progeny FCV-MD-15 from Parambikulam.
- The data collected from this research work can be used to select the elite parent with the best growth character and use them for further propagation and establish a seed orchard.
- The values for the physiological traits such as chlorophyll content, photosynthetic rate, stomatal conductance and relative water content showed significant variation for 36 MAPS. The progenies FCV-MD-03 and

FCV-MD-04 of Tholpetty were found to be superior in terms of their physiological traits.

- Heritability for the various morphological and physiological traits showed moderate to high heritability. The highest heritability was shown by girth in morphological traits and transpiration in physiological traits. The high values of heritability help the breeder in a better selection programme.

- A significant positive correlation was observed for the morphological trait and physiological trait. Except for the leaf temperature, all other traits were found to be positively correlated. It was observed that net transpiration rate and stomatal conductance showed a significantly positive correlation with plant girth and volume. The net photosynthetic rate was also found to be positively correlated to the morphological traits and hence we can conclude that the physiological parameters such as photosynthetic rate, transpiration rate and stomatal conductance has high practical significance and can be effectively used for improving the efficiency of *M. dubia* breeding which can provide a means of rapid evaluation of *M. dubia* germplasm, for introduction, utilization, and improvement of *M. dubia* resources for the future breeding and improvement programs.

- Fifteen good amplifying Inter Simple Sequence Repeats (ISSR) primers were screened and selected for ISSR profiling of 25 plus seed tree progeny accession of *M. dubia*

- A total of 164 ISSR amplicons were generated of which 113 amplicons were polymorphic, thus giving an average of 4 polymorphic bands per ISSR primer. The average polymorphism was 68 percent.

- Accession specific bands were observed using the primer 891 (Chinnar 17). Population-specific bands were produced by primer 890 (Walayar 12, 13), 891

(Thiruvazhiyadu 10, 11), L9 (Thirunelly 1, 2) and hence these primers can be used effectively to identify the specific plus tree progeny.

- The primer 813 had the highest PIC value of 0.30.
- The scored data based on ISSR banding was used to construct a dendrogram using NTSYSpc - 2.021 software. Hierarchical clustering analysis (UPGMA) classified the 25 plus tree seed progeny accession into two distinct classes based on the amplicon profiles of the ISSR primers considered for the study. The second cluster was the largest cluster having 19 accessions. The first cluster included only six accessions of plus tree seed progeny from the Wayanad population. The Thirunelly and Tholpetty accession in Wayanad showed the most similar accession using Jaccard's similarity coefficient.
- The ISSR assay confirmed the existence of substantial variation at the DNA level in the plus tree seed progeny. This genetic diversity among the accession must be tapped for future breeding programmes and domestication.
- Standardization of HPLC protocol for caryophyllene quantification present in *M. dubia* was developed.
- The quantification of caryophyllene phytochemical was quantified in 25 plus tree seed progeny accession. The highest concentration was observed in FCV-MD- 8 of Poothundy in Palghat with 16.4% followed by FCV-MD- 3 of Tholpetty in Wayanad with 9.6%. The lowest concentration was observed in FCV-MD- 7 of Dhoni in Palghat with 0.040%.

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PROGENY TESTING AND GENETIC DIVERSITY ANALYSIS IN PLUS TREES OF *Melia dubia* Cav.

by

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ABSTARCT

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8.ABSTRACT

The present study titled “Progeny testing and genetic diversity analysis in plus trees of *Melia dubia* Cav.” was carried out from 2020 to 2021 with the objective of progeny evaluation in 25 half sib plus trees identified from different part of Kerala, by studying the growth performance of its seed progeny planted at college of forestry germplasm field. The study also includes exploring the genetic diversity of these 25 plus tree seed progenies along with analysing the caryophyllene content, one of the economically important phytochemical compounds present in *M. dubia*.

The plus tree seed progeny differed significantly in morphological traits like height, collar girth, volume and physiological parameters like photosynthetic and transpiration rate, stomatal conductance, chlorophyll content and relative water content. The progeny FCV-MD-03 of Tholpetty in Wayanad district performed better in growth parameters *viz.*, height (5.61 m), collar girth (0.42 m) and tree volume of (0.017 m^3). The variability study indicated that the morphological trait (volume) and physiological trait (stomatal conductance) exhibited higher PCV and GCV. From the heritability estimation it was observed that the most heritable morphological trait was collar girth followed by the plant height. For the physiological parameter, transpiration rate (0.91) was the most heritable trait followed by the chlorophyll content. These morphological and physiological traits were positively correlated implicating the significance of physiological parameters as source to identify better progeny. Path analysis on morphological trait has showed that the collar girth had the highest positive direct effect on the plant volume.

Genetic variation study in 25 plus tree progenies using ISSR primer produced 164 amplicons which estimates *viz.,* percentage of polymorphism (68%), polymorphism information content (0.70 to 0.83), Nei’s gene diversity (31%). The dendrogram obtained by using UPGMA classified the 25 accession of *M. dubia* into 2 distinct clusters. Cluster I constitute population from Wayanad and the rest of the

population in cluster II. Based on the genetic variation observed, superior seed sources can be identified and tree improvement programme could be developed for the conservation and further development of *M. dubia*.

Standardization of HPLC for the quantification of caryophyllene phytochemical in *M dubia* was studied. Significant variation was observed for the total caryophyllene content for the different accession of *M dubia* seed progeny. The maximum quantity was observed in FCV-MD-08 (16.4 %) of Pothundy in Palghat district followed by FCV-MD-3 of Tholpetty in Wayanad.

9.APPENDICES

APPENDIX I

Mean monthly weather parameters during May 2020 – May 2021 of the study area recorded by Department of Agricultural Meteorology, College of Agriculture, Vellanikara, Kerala

Months	2020			
	Maximum Temperature (ºC)	**Minimum Temperature (ºC)**	**Rainfall (mm)**	**Relative Humidity (%)**
May	31.10	23.70	427.20	85.00
June	30.50	23.20	563.00	87.00
July	30.20	23.10	607.70	87.00
August	30.00	22.40	587.60	88.00
September	31.00	21.50	310.30	82.00
October	33.00	22.00	56.10	70.00
November	32.00	21.90	7.70	65.00
December	31.10	23.70	427.20	85.00

Months	2021			
	Maximum Temperature (ºC)	**Minimum Temperature (ºC)**	**Rainfall (mm)**	**Relative Humidity (%)**
January	32.00	21.90	7.70	65.00
February	32.30	21.30	45.70	64.00
March	34.60	21.60	0.00	54.00
April	36.80	23.00	31.80	59.00
May	34.90	23.60	72.40	73.00

APPENDIX II

1. **Sucrose extraction buffer I – 100 ml**
    - 0.5 M Sucrose
    - 120 mM Tris HCl
    - 50 mM EDTA
    - 1.7 M NaCl
2. **CTAB extraction buffer II - 100 ml**
    - 2% CTAB
    - 100 mM Tris HCl
    - 20 mM EDTA
    - 1.7 M NaCl
3. Polyvinylpyrrolidone (PVP)
4. β-mercaptoethanol
5. Phenol: Chloroform: Isoamyl alcohol (25:24:1, v/v)
6. Chloroform: Isoamyl alcohol (24:1, v/v)
7. Chilled Isopropanol (100%)
8. Ethanol (70%)
9. RNase A (20mg/ mL)
10. TE buffer

APPENDIX III

1. **TE Buffer stock**
    - 10 mM Tris·Cl, pH 8.0
    - 1 mM EDTA
2. **TAE Buffer 5x Stock**
    - 24.2 g tris base in double-distilled H_2O
    - 5.71 ml glacial acetic acid
    - 10 ml 0.5 M EDTA solution (pH 8.0)

    Make up into 100 ml
3. **Agarose gel electrophoresis**

    Reagents used
    - Agarose (0.8%) in 1X TAE buffer
    - 50X TAE buffer (pH 8.0)
    - Tracking/loading dye (6X)
    - Ethidium bromide (stock 10 mg/ ml, working concentration 0.5 µg/ mL)