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Original Research Paper

Survey and assessment of genetic diversity for yellow leaf disease, and DNA fingerprinting of Arecanut (*Areca catechu* L.) genotypes using molecular markers

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ABSTRACT

Areca nut traditionally known for its stimulant properties, has gained recognition for its medicinal uses in treating schizophrenia and glaucoma, while also serving as a mild stimulant and digestive aid. Its production faces significant challenges, primarily from biotic stresses such as yellow leaf disease (YLD), particularly endemic in the Malnad region of Karnataka, India. Yellow leaf disease poses a substantial threat, causing heavy yield loss with no current cure; therefore, breeding for resistance is imperative. Disease-free palms identified in heavily affected regions may harbor unique gene families. This study utilized three marker systems *viz*., random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR), and resistant gene-based markers (RGP) to differentiate YLD-resistant areca palms from susceptible ones. The resistant gene primer (RGP) demonstrated the highest polymorphism, with a polymorphic information content (PIC) ranging from 0.45 to 0.83 and an average PIC of 0.72 among the arecanut genotypes. Other markers, OPAF 06, UBC 351, and RGP1, also exhibited significant polymorphism. The markers effectively differentiated susceptible and resistant genotypes into distinct clusters. The outcomes provide valuable insights for map-based cloning of YLD resistance genes. This molecular characterization lays the foundation for developing targeted treatments and diagnostics for YLD, emphasizing the importance of genetic approaches in securing areca nut production against this devastating disease.

Keywords: Arecanut, ISSR, PIC, RAPD, RGP, Western Ghats, yellow leaf disease

INTRODUCTION

Areca nut (*Areca catechu* L.), a tropical palm extensively cultivated in Asian regions and certain parts of Africa (Heatubun et al., 2012), serves as a prominent cash crop in the Malnad and coastal regions of India, commonly known as betel nut (Hiremata et al., 2022). Indigenous to the Malay Peninsula and Cochin China, also recognized as a native plant in Indonesia.

India holds the global lead in arecanut production, with Myanmar, Bangladesh, China, and Indonesia following suit. India's productivity stands at 1676 kg per ha, resulting in the production of 8.33 lakh tonnes of arecanut from 4.97 lakh ha (Indiastats, 2023). Karnataka, Kerala, and Assam contribute significantly, accounting for 80% of both the area and production. Primarily utilized for chewing alongside betel leaves or as *Tambula*, arecanut holds cultural and religious significance in India, playing a vital role in various social events (Hiremata et al., 2020a). Moreover, it finds applications in Ayurveda and Veterinary medicines. Approximately 90% of the areca harvest is processed into various commercial preparations, with around 150 trade types differing in maturity, processing conditions, and taste.

The cultivation of areca faces significant challenges from various diseases and pests throughout its growth stages. Key threats include koleroga (fruit rot), crown rot, crown choking, Anaberoga (foot rot), leaf spot, yellow leaf disease, and inflorescence dieback. Among these, yellow leaf disease (YLD) stands out as a particularly recent and impactful ailment affecting areca palms in the Indian states of Kerala and Karnataka. Initially reported in the Central Kerala regions of Muvattupuzha, Meenachil, and Chalakudi,

YLD detrimentally affects the typical growth and vigor of the palms (Liu., 2010). The most conspicuous symptom is foliar yellowing, beginning in the inner whorl and gradually spreading outward. Leaves in the affected whorl exhibit chlorosis from the edges of individual leaflets to the mid-rib region. Concurrently, the conducting strands in the palm stems break down, rendering them spongy and friable. In advanced stages, the stem's top breaks off, and root rot becomes evident. This progression results in smaller nuts with black kernels.

The phytoplasmal etiology of arecanut YLD in India has been demonstrated using electron microscopy (Nampoothiri et al., 2000) and straight forward staining techniques. Furthermore, *Proutista moesta* was identified as the disease vector by Ponnamma et al. (1991). Molecular techniques have identified the phytoplasma associated with YLD as 16s rDNA group XI (Manimekalai et al., 2010; Yang et al., 2020). Given the phytoplasma nature of the governing pathogen, achieving efficient management practices and complete eradication of yellow leaf disease (YLD) proves challenging. The identification of field-tolerant or resistant varieties from natural palm populations emerges as a viable long-term solution. YLD's presence has been confirmed in all six major areca-growing districts of Karnataka (Rawther., 2000; Hiremata et al., 2020b).

The intricate and lengthy life cycles of areca palms necessitate considerable time and effort for the selection and characterization of resistant and susceptible varieties using conventional methods. Molecular markers, with their unlimited number, rapid assay, and high accuracy, emerge as preferable over other morphological markers for genetic variability analysis (Garg et al., 2006). Both random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers prove equally effective in detecting polymorphism.

Disease resistance often results from the presence of a specific resistance gene (R-gene) in the plant, coupled with a corresponding avirulence gene (avr) in the pathogen. Plant genomes commonly contain complex clusters of R-genes (Mutlu et al., 2006). Clones of disease R-genes have been identified in various plant species. Polymerase chain reaction (PCR) analysis with gene-specific primers enables the monitoring of gene expression in different tissues and developmental stages (Michelle et al., 2002). In a study by Panwar et al. (2011), the use of resistant gene primers allowed the clear distinction between resistant and susceptible bulks. The application of these primers facilitated the precise clustering of resistant genotypes separately from susceptible genotypes.

In other plants, successful genetic variability studies between resistant and susceptible varieties using molecular markers such as ISSR, RAPD, AFLP and SSR have been reported (Qi et al., 2025). To discern the molecular diversity between Yellow Leaf Disease (YLD) resistant and susceptible areca palms, we employed two dominant markers, ISSR and RAPD, along with a gene-specific marker. Notably, there have been no reports on molecular-based genetic variability in areca palms before this study. Given the inefficacy of current YLD management practices, an alternative approach involves developing resistant plants by identifying natural escape routes as the most effective means of eradicating this disease. Within the areca nut breeding program for YLD resistance, field-resistant palms have been identified in disease hotspots. Therefore, recognizing the crop's significance and the aforementioned facts, there is a crucial need to generate information on the degree and pattern of morphological and molecular diversity to identify natural escapes.

MATERIALS AND METHODS

Survey for resistant and susceptible plants

A roving survey was conducted in September 2018 to evaluate the prevalence of YLD in the two main arecanut growing talukas of Chikkamagaluru district in Malnad, Karnataka. At each location, a minimum of five fields were chosen at random. Seven villages in Sringeri taluk (Honnavalli, Bandlapur, Melnemmar, Muruvinakombe, Addagadde, Benkikudige, and Doddahonne) and seven villages in Koppa taluk (Kachkal, Hosakoppa, Totadakoppa, Makkimane, Hoskeri, Talamakki and Gunavante) were selected for the survey. The objective of the experiment was to study the visual characteristics of yellowing affected arecanut palms to fix up indices for a scoring system to assess the intensity of yellowing. The extent of yellowing (Y), necrosis (N) and crown size reduction (R) were considered to quantify the intensity of yellowing was given by George et al. (1980).

$$\textbf{Yellowing index (I)} = \{(Y+N)/L + R\} \times 10$$

The disease incidence percentage was calculated using the following formula (PDI).

Per cent disease incidence = Number of infected plants/total number of plants examined × 100

DNA extraction

Tender and soft textured leaf samples (1 g) were cut into small pieces and macerated into fine powder using liquid nitrogen. DNA was extracted by using the standardized protocol (Rajesh et al., 2007). The DNA purity and intactness were checked on 0.8 per cent agarose gel stained with ethidium bromide following the standard protocol Genomic DNA was quantified using the Nanodrop instrument and finally the samples were diluted to a concentration of 50 ng µL. After extraction of genomic DNA, equal quantities of DNA from four samples representing, each accession, were pooled together and used for analysis.

Molecular markers (RAPD, ISSR and RGP) analysis

For conducting the polymerase chain reaction (PCR), 6 RAPD, 6 ISSR and 8 RGP primers were tested. Resistant gene primers for were developed using different classes of "R" genes (Ramaswamy et al., 2013). PCR amplification was carried out in 20 µl PCR mixture containing 20 ng DNA, 200 µM each dNTPs, 20 picomoles primers, 1X Taq buffer, Taq polymerase (0.5 units) and 2.5 mM $MgCl_2$. The thermocycler (VWR Peqlab thermocycler) was programmed for 39 cycles of 1 min denaturation at 94°C, 1 min annealing at 42°C, and 1 min and 30 seconds of primer extension at 72°C before finishing with a 10 min extension at 72°C for ISSR and RGP. However, annealing was continued for 45 seconds at 38°C for RAPD primers. With the use of 1X TBE buffer (pH 8.0), the amplified products were separated by electrophoresis on 1.5% agarose gel containing ethidium bromide. The application of steady voltage at 10 V/cm for two hours was used to separate the materials. The sizes of amplified fragments were measured using a conventional DNA ladder mixture (1 kb and 100 bp). A gel documentation system (UPV) was used to take pictures of the gel. Amplification profiles were used to compare genotypes, and bands of DNA fragments were graded as present (one) or missing (zero).

Statistical analysis

The NTSYS PC package was used to do a cluster analysis based on the Jaccard's similarity coefficient (Exeter, New York). Using the NTSYS program of UPGMA (unweighted pair group method with arithmetic average) method, a dendrogram was created using similarity coefficients. The average polymorphic information content (PIC) was calculated by applying the formula given by Powell et al. (1996). $PIC = 1-\Sigma fi^2$, where i=1 ton and f is the frequency of the i^{th} allele. The number of alleles refers to the number of scored bands. The frequency of an allele was obtained by dividing the number of accessions where it was found, by the total number of accessions. The polymorphic information content value provides an estimate of the discriminating power of a marker.

RESULTS AND DISCUSSION

Based on a comprehensive survey on arecanut yellow leaf disease in the Sringeri and Koppa talukas of the Chikkamagaluru district, the disease index varied from 88.56% to 95.34%, while the incidence ranged from 87.28% to 96.20%. Sringeri exhibited the highest mean disease incidence and disease index compared to Koppa taluka. The extensive cultivation of arecanut in these vast areas is likely a contributing factor to the elevated disease incidence. However, it has been observed that the prevalence of plant hoppers and the cultivation of specific varieties or hybrids also influence yellow leaf disease occurrence. This observation can be attributed to the genetic makeup of the palms and the variations in their adaptive responses to disease infection.

Individual villages were assessed for the number of natural escapes and disease incidences. Honnavalli village (05) stood out with a higher count of natural escapes and a lower disease incidence at 87.28%. Conversely, Muruvinakombe village in Sringeri taluk exhibited the highest disease incidence and disease index at 96.20% and 95%, respectively, coupled with a comparatively lower number of natural escapes (Table 1). The elevated incidences of yellow leaf disease (YLD) in certain areas can be attributed to climatic conditions favoring the disease, including high rainfall, high humidity, low temperature, and a higher vector population. Additionally, the varietal behavior of the plants plays a significant role in these higher disease incidences (Hiremata et al., 2020b).

Table 1 : YLD incidence in areca nut plantations in Chikkamagaluru district Taluka Village Disease

Taluka	Village	Disease incidence (%)	Disease index	Natural escapes	Mean disease incidence (%)	Mean Disease index (%)
Sringeri	Honnavalli	87.28	88	5	90.7	92.31
	Doddahonne	89.02	91	4		
	Addagadde	90.27	93	3		
	Muruvinakombe	96.20	95	2		
	MelNemmar	91.34	92	4		
	Benkikudige	92.36	93	3		
	Bandlapur	88.49	90	5		
Koppa	Kachkal	89.21	91	5	89.42	91.96
	Talamakki	94.89	95	3		
	Hosakeri	92.20	94	3		
	Gunavante	86.59	91	4		
	Hosakoppa	87.29	90	5		
	Makkimane	88.50	89	3		

RAPD amplification

Distinct fingerprints generated by each marker approach can offer insights into the genetic differences among resistant and susceptible individuals specific to the location. However, due to each marker system targeting a different region of the genome, variations in resolution were identified. Among the six RAPD markers, a lower degree of polymorphism was observed in this study (54.13%) compared to reports by Rajesh et al. (2007), Purushotham et al. (2008), Goswami & Tripathi (2010), Ramaswamy et al. (2013), Bharath et al. (2015) showing 78% polymorphism. The RAPD marker produced a total of 109 bands, of which 59 were polymorphic. Notably, the primer OPAH18 yielded the highest number of bands (29), with OPAF06 (83.33%) exhibiting the highest polymorphism, while OPAH18 (41.38%) showed the lowest (Fig. 1).

The major allele frequency ranged from 74% to 84%, with OPE13 having the highest (83%) and OPAH18 and OPAF15 the lowest (74%), averaging 77.55%. polymorphic information content (PIC) values ranged

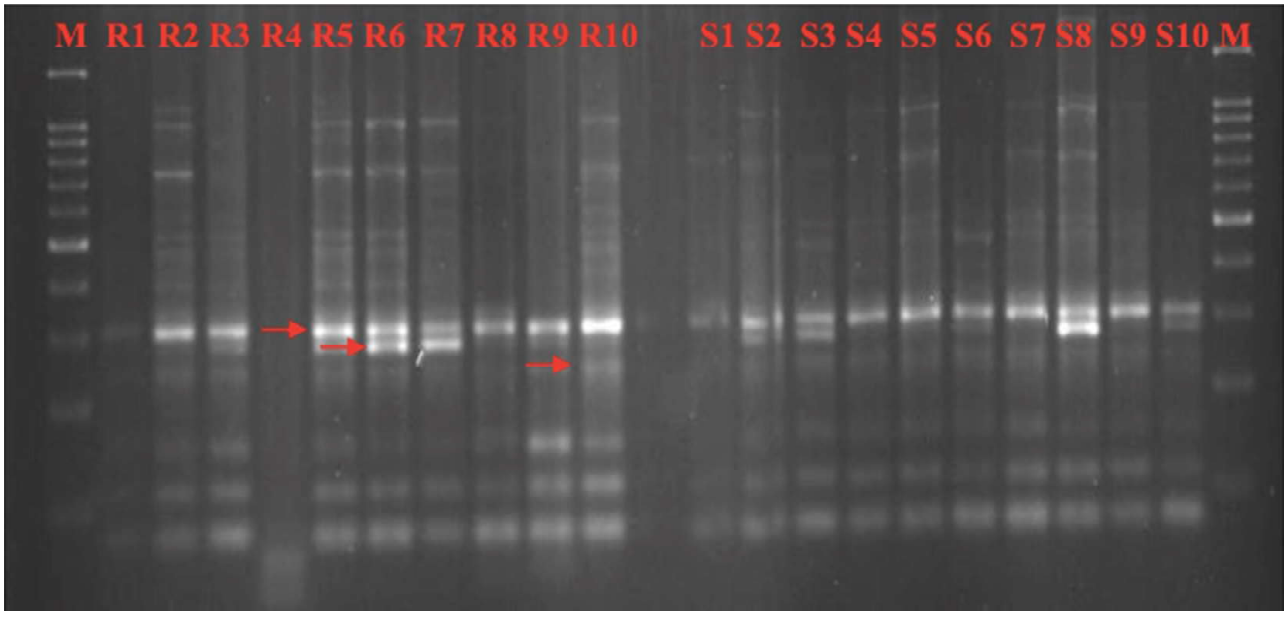

Fig. 1 : Molecular diversity of YLD resistant and susceptible in arecanut using RAPD marker (OPE13)

Table 2 : Details of RAPD primers used for screening YLD resistant and susceptible Arecanut palms

Marker	Nucleotide Sequence	Total no. of bands	No. of polymorphic bands	Polymorphisim (%)	PIC Value	Major allele frequency (%)
OPAF 06	5'CCGCAGTCTG3'	12	10	83.33	0.80	82
OPE 13	5'CCCGATTCGA3'	26	13	50.00	0.68	83
OPAH01	5'TCCGCAACCA3	11	8	72.73	0.75	75
OPAH18	5'GGGCTAGTCA3'	29	12	41.38	0.62	74
OPAF 15	5'CACGAACCDC3'	9	6	66.67	0.66	74
OPBA 20	5'GAGCGCTACC3'	22	10	45.45	0.73	77
Total		109	59	-	-	-
Average		18.16	9.83	54.13	0.71	77.55

from 0.62 to 0.80, with OPAF06 having the highest (0.80) and OPAH18 the lowest (0.62), averaging 0.71 (Table 2). The results of this investigation effectively differentiated and characterized samples based on their gene response to yellow leaf disease (YLD), indicating the suitability of the RAPD marker system for analyzing YLD-resistant and susceptible arecanut germplasm accessions.

Acknowledging the reproducibility challenges of RAPD markers, primarily attributed to mismatch annealing, the annealing temperature was raised to 42°C for improved reproducibility. Experiments were conducted three times to confirm the banding patterns.

ISSR amplification

Six ISSR markers were assessed, and all six (64.74%) displayed distinct and receptive polymorphic bands in the analysis of 20 arecanut genotypes. The previous reports on coconut population studies, such as the 40.2% reported by Manimekalai & Nagarajan (2006) and the 78% reported by Ramaswamy et al. (2013) for arecanut and 88% in pointed guard by Goswami & Tripathi (2010). ISSR markers generated a total of 160 bands, with 103 being polymorphic. UBC2 produced the highest number of bands, while UBC52, UBC351 and UBC321 produced the highest number of polymorphic bands, and UBC72 had the lowest polymorphic bands (Fig. 2).

Among the ISSR markers, UBC351 exhibited the highest major allele frequency at 83%, while UBC84 displayed the lowest at 73%, with an average of 78.66%. The polymorphic information content (PIC) values ranged from 0.68 to 0.83 with UBC52 having the highest PIC value of 0.83 and UBC84 the lowest at 0.68 (Table 3). These results illustrate the variability among arecanut genotypes concerning yellow leaf disease (YLD) resistance, demonstrating the utility of ISSR markers for analyzing YLD-resistant and

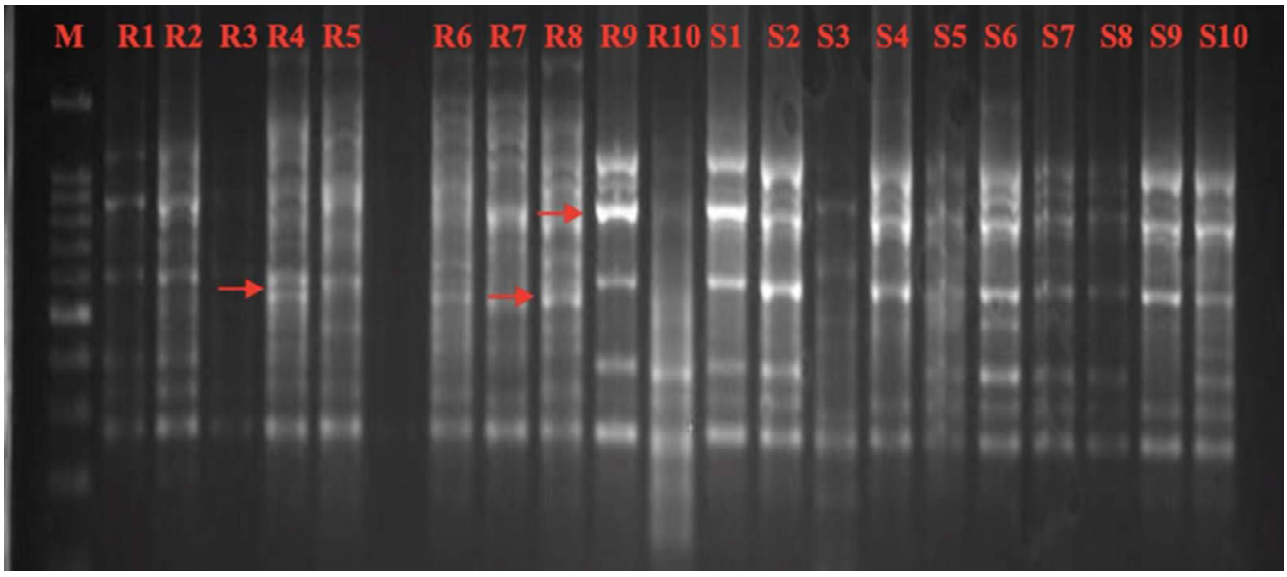

Fig. 2 : Molecular diversity of YLD resistant and susceptible in arecanut using ISSR markers (UBC321)

Table 3 : Details of ISSR primers used for screening YLD resistant and susceptible Arecanut palm

Marker	Nucleotide Sequence	Total no. of bands	No. of polymorphic bands	Polymorphisim (%)	PIC Value	Major allele frequency (%)
UBC 2	5'CCT GGG CTT A3'	29	17	58.62	0.77	77
UBC 72	5'CAG CAC GGG A3'	27	15	55.56	0.75	79
UBC 52	5'TTC CCG GAG C 3'	24	18	75.00	0.83	78
UBC 84	5'GGG CGC GAG T3'	27	17	62.96	0.68	73
UBC 321	5'ATC TAG GGA C3'	28	18	64.29	0.69	82
UBC 351	5'CTC CCG GTG G3	25	18	72.00	0.81	83
Total		160	103.00	-	-	-
Average		26.66	17.16	64.74	0.76	78.66

susceptible arecanut germplasm accessions differentiated by UBC351 at 450bp.

Resistant gene primer

Eight RGP markers were examined, and all markers (62.45%) demonstrated distinct polymorphic bands in the analysis of 20 arecanut genotypes. The observed level of polymorphism was lower compared to a report by Ramaswamy et al. (2013), who reported 78% polymorphism. RGP markers generated a total of 106 bands, of which 69 were polymorphic. RGP5 (26) produced the highest number of bands, while the lowest polymorphic bands were produced by RGP7 (3). Notably, RGP1 exhibited the highest polymorphism (72.73%), followed by RGP2 and RGP4 (71.43%).

Among the RGP markers, RGP1 and RGP4 displayed the highest major allele frequency at 84%, while RGP2 had the lowest at 67%, with an average of 79%. The Polymorphic information content (PIC) values ranged from 0.45 to 0.78, with RGP1 having the highest PIC value of 0.78 and RGP7 the lowest at 0.45 (Table 4 & Fig. 3). These results underscore the genetic variability among arecanut genotypes concerning yellow leaf disease (YLD) resistance, emphasizing the utility of RGP markers for analyzing YLD-resistant and susceptible arecanut germplasm accessions.

Table 4 : Details of RGP primers used for screeing YLD resistant and susceptible Arecanut palms

Res. gene primer	Forward sequence	Reverse sequence	T_m	Total no. of bands	No. of polymorphic bands	Polymor-phism (%)	PIC Value	Major allele frequency (%)
RGP 1	5'GCATTGGAAC AAGGTGAA3'	5'AGGGGGACC ACCACGTAG3'	45.0	11.00	8.00	72.73	0.78	84
RGP 2	5'TAGTTCGGAC GTTTACAT3'	5'AGTGTCTTGT AGGGTATC3'	45.4	14.00	10.00	71.43	0.69	67
RGP 3	5'ACAGAACTGC ATCAGCATCG3'	5'AGGCAGTCT CACCATGATCC3'	45.4	15.00	9.00	60.00	0.58	73
RGP 4	5'TGCAAAGCAG GTGCAGTATC3'	5'GTTCTTGCG GACGTCTTCTC3'	45.0	14.00	10.00	71.43	0.67	76
RGP 5	5'TGCGAGCAGC TACAGACACT3'	5'GGGAGGCCA GAAGCATAAAT3'	47.9	26.00	16.00	61.54	0.66	72
RGP 6	5'GTTGGGAAGA CAACGTTGC3'	5'CAACTCAAC ATTCAACCGAGG3'	46.7	15.00	10.00	66.67	0.63	71
RGP 7	5'TGGGTGGAGT TGGTAAGACC3'	5'TGGTGAGGA GAGAGGCAAGT3'	46.7	3.00	1.00	33.33	0.45	70
RGP 8	5'GGTGGGGTTG GGAAGACAACG3'	5'CCACGCTAG TGGACCTCC3'	46.7	8.00	5.00	62.50	0.68	72
Total				106	69	-	-	-
Average				13.25	8.63	62.45	0.68	73

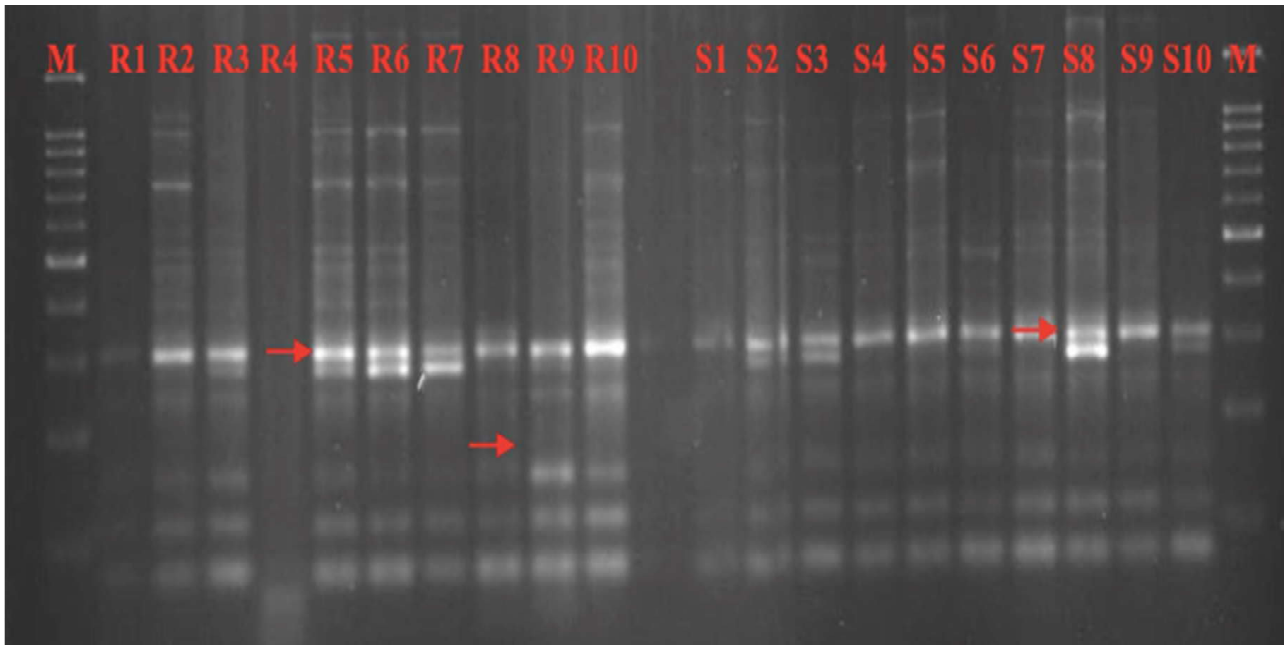

Fig. 3: Molecular diversity of YLD resistant and susceptible in arecanut using RGP markers (RGP5)

Comparison between the RAPD, ISSR and RGP markers

ISSR markers exhibited the highest number of bands (160), followed by RAPD (109) and RGP (106) markers. The ISSR markers also displayed the highest number of polymorphic bands (103) and a high average number of bands per primer (23.93). In terms of percentage of polymorphism, RAPD markers showed a considerably lower value (54.13%) compared to ISSR (64.74%) and RGP (62.45%). The average number of polymorphic bands per primer was the lowest in RGP markers (8.63) and the highest in ISSR (17.16), indicating a preference for ISSR markers in future studies (Table 5). All three markers effectively clustered resistant and susceptible genotypes into separate groups.

The genetic characterization of genotypes represents the initial step towards more effective conservation, maintenance, and utilization of existing genetic diversity. When subjected to cluster analysis, the dendrogram resulting from the combination of three sets of marker data (Fig. 4) revealed that the majority of resistant genotypes clustered together, exhibiting a high degree of genetic similarity among themselves, just as the susceptible genotypes formed a distinct cluster. Two main clusters were identified, with the resistant group comprising 10 candidates (R1, R2, R3, R4, R5, R6, R7, R8, R9, and R10). The susceptible genotypes (S1, S2, S3, S4, S5, S6, S7, S8, S9, and S10) grouped together in the cluster with the least divergence (Table 6). The dendrogram effectively illustrates the genetic similarity among susceptible candidates by placing them in the same or close to the convergence area. These two clusters are distinctive as they exclusively include susceptible candidates. The resistant and susceptible variants were clearly separated into two parts in a 3D clustering representation among the 20 areca nut palms, highlighting the precision of this classification.

Table 5 : Comparison between RAPD, ISSR and RGP markers

Particular	RAPD	ISSR	RGP
Number of primers used	6	6	8
Total number of polymorphic bands	59	103	69
Total number of bands	109	160	106
Polymorphism (%)	54.13	64.74	62.45
Average number of bands/ primer	18.16	23.93	13.25
Average number of polymorphic bands/primer	9.83	17.16	8.63

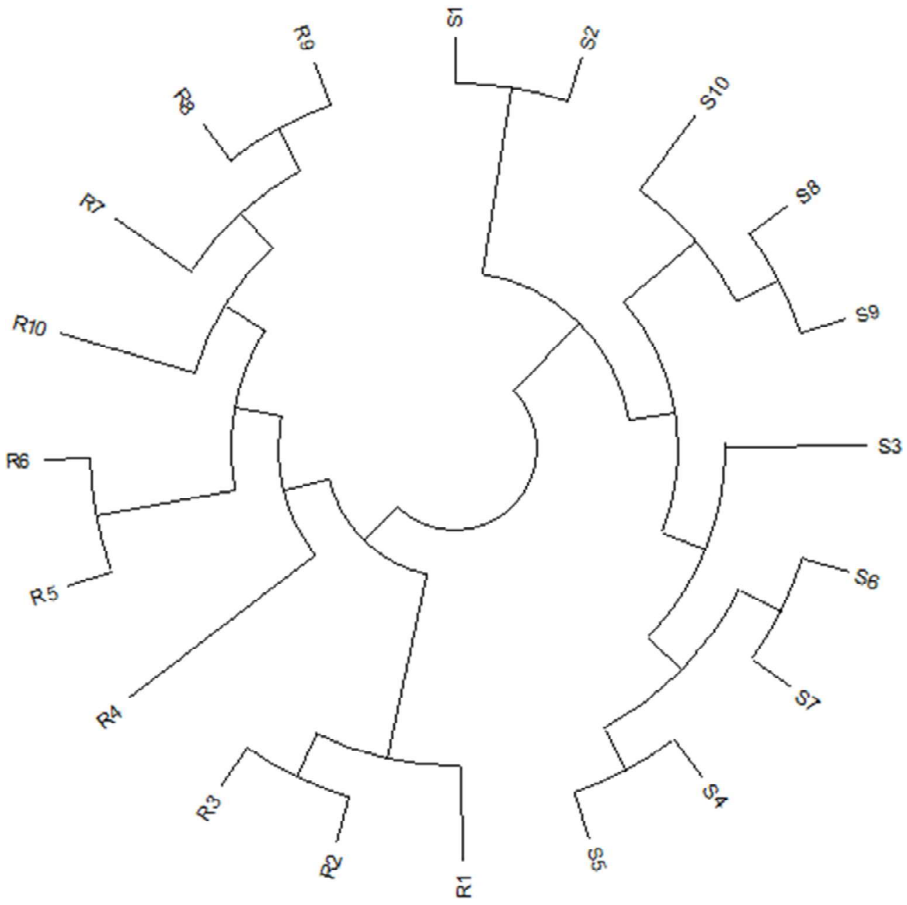

Fig. 4 : Dendrogram of molecular diversity for YLD resistant and susceptible plants

S: susceptible to YLD (Totadakoppa^{s}, Muruvinakombe^{s}, Doddahonne^{s}, Hosakeri^{s}, Makkimane^{s}, Kachkal^{s} Hosakoppa^{s}, Benkikudige^{s}, Melnemmar$_{s}$ and Huluve^{s})
R: resistance to YLD (Totadakoppa^{R}, Muruvinakombe^{R}, Doddahonne^{R}, Hosakeri^{R}, Makkimane^{R}, Kachkal^{R}, Hosakoppa^{R}, Benkikudige^{R}, Melnemmar^{R} and Huluve^{R})

To characterize the genomic region linked to resistance genes, the polymorphic segments obtained can be sequenced. A UPGMA cluster analysis demonstrated that closely related cultivars from the same geographic region can be distinguished from one another, establishing their genetic differences clearly. Overall, the genetic diversity study found fewer genetic similarities between resistant and vulnerable palm species, with certain palms exhibiting outliers. Although the genetic basis of YLD-resistant and susceptible palms is the same, they have congregated based on disease manifestation. Further investigation of resistant palms may involve sequencing the polymorphic region to create specific primers for the isolation and cloning of resistance genes.

Table 6 : Clustering pattern of YLD resistant and susceptible Arecanut palms based on RAPD, ISSR and RGP marker analysis

Cluster	No. of Genotype	Genotype
I	10	Totadakoppa^{s}, Muruvinakombe^{s}, Doddahonne^{s}, Hosakeri^{s}, Makkimane^{s}, Kachkal^{s} Hosakoppa^{s}, Benkikudige^{s}, Melnemmar^{s} and Huluve^{s}
II	10	Totadakoppa^{R}, Muruvinakombe^{R}, Doddahonne^{R}, Hosakeri^{R}, Makkimane^{R}, Kachkal^{R} Hosakoppa^{R}, Benkikudige^{R}, Melnemmar^{R} and Huluve^{R}

CONCLUSION

The study establishes yellow leaf disease (YLD) as an endemic issue in Sringeri and Koppa districts, evident from elevated disease incidence values. At the molecular level, the genetic divergence of YLD-resistant and susceptible genotypes of areca nut was characterized using ISSR, RAPD, and RGP markers. The findings revealed a high degree of polymorphism between the resistant and susceptible genotypes for all markers. ISSR markers, with the maximum number of polymorphic bands and a higher percentage of polymorphism, demonstrated significant importance for future studies. Additionally, both RGP and ISSR markers effectively differentiated susceptible and resistant genotypes into distinct clusters. The polymorphic markers identified in this study hold significant importance for the diagnosis and management of YLD. The results will contribute to the development of region-specific markers for enhanced genetic discrimination among genotypes, facilitating the cloning and sequencing of distinct alleles. This research paves the way for future investigations relying on a combination of markers for comprehensive discrimination in resistant and susceptible diversity assays. Furthermore, it lays the groundwork for marker-assisted selection aimed at breeding disease-resistant areca nut varieties.

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