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# F-ISSR Marker Based Genotyping of Wild Tuber Population of *Dioscorea* Exclusive to Tribal Utility in Kerala, India

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**A B S T R A C T**

*Dioscorea* is a staple food crop and one of the top tuber crops in terms of economic gain, is cultivated in many parts of the world and is mostly grown in tropical countries owing to its subterranean tubers. *Dioscorea* samples collected from tribal regions of Kerala state, India. Using fluorescently tagged ISSR primers (F-ISSR), genetic diversity analysis of *Dioscorea* samples was carried out. Out of 38 primers employed, 8 primers displayed polymorphism, with varying numbers of polymorphic loci observed for each primer. The genetic distance within the population was determined using Nei's similarity - distance matrix, maximum distance was observed between *D. tomentosa* and *D. rotundata* (0.5379). The unweighted pair-group approach with arithmetic mean analysis was used to assess the comparative data, resulting in the construction of adendrogram with 8 clusters. The bands assessed were ranged in size from 80-600 bp were aligned with the size standard bands. *D. alata* (0.2686) and *D. oppositifolia* (0.2892) showed the highest genetic diversity within the population. Compared with other species in the population, *D. esculenta* and *D. pentaphylla* exhibited the highest degree of resemblance. *D. rotundata*, an exotic variety, formed a distinctive cluster from the other members of the population. Molecular marker-assisted genetic diversity studies on wild varieties of *Dioscorea* contribute to resolving disputes related to folk systematics and provide advancement in propagation and conservation techniques.

## Introduction

The genus *Dioscorea* comprises approximately 600 species worldwide (Mukherjee *et al.*, 2013), only six are edible or food yams, 50 species of this genus are considered medicinal (Adewumi *et al.*, 2021) and 50 species in India. Members of this genus contain a pharmacologically important component called diosgenin, a steroidal saponin that functions as phytoestrogen (Salehi *et al.*, 2019). Therefore, tubers of this genus have been used in food and medicine in many cultures worldwide. *D. alata* is the most cultivated species among the tribal communities of Kerala. However, several wild tubers of other species of *Dioscorea* collected from the forest were also used by the tribes. The documentation of these wild *Dioscorea* landraces is vital for conserving and transferring the ethnic

knowledge to future generations. One of the benefits in the genetic evaluation of the collected *Dioscorea* samples is that the confusion in the folk systematics (different names for the same landraces used among the tribes in different locations) can be solved. For example, the species *D. oppositifolia* have three names in three tribal communities in Kerala. It is called as '*thettam*' among the Mannan community of Idukki, '*kavala*' is the name used by Kani community in Thiruvananthapuram and '*kanjiravallikizhangu*' by Kuruma community in Wayanad district. Therefore, an interdisciplinary approach using scientific documentation and genetic analysis will substantiate the data recorded from the tribes.

Balakrishnan *et al.,* (2003), reported that taxonomy of quite a few species in the genus *Dioscorea* is considered problematic, which is attributed to the highly continuous variability of morphological characters, especially of aerial parts such as leaves and bulbils (Arackal, 2015). To avoid confusion among genotypes and improper selection of parents for breeding programs, molecular markers can be used to characterize and classify them appropriately.

Genetic relationships between wild and cultivated yams have been studied using molecular markers such as RAPD, DS-PCR analyses (Mignouna *et al.,* 2005), and AFLP (Malapa *et al.,* 2005). The biggest advantage of molecular markers is their environmentally independent nature, irrespective of the time of development; they are not influenced by epistatic interactions and can target any part of the genome.

Different molecular markers can target different regions of the genome, thus providing more detailed and diverse information on the actual diversity present within genotypes (Kumar *et al.,* 2019). Some studies have used RAPD (Rao *et al.,* 2020), SSR (Cao *et al.,* 2021), ISSR (Nudin *et al.,* 2017; Rao *et al.,* 2020), and AFLP (Amponsah *et al.,* 2023) markers to study the genetic diversity of *Dioscorea* sp. The consistent use of molecular markers such as ISSR for genetic diversity studies in any crop requires the selection of primers that will provide a reliable and distinct band needed to study the divergence that occurs within the species (Snehalatha, *et al.,* 2022).

ISSR genotyping is an experimental procedure that identifies differences in DNA sequences between individuals or populations. (Mondel *et al.,* 2018; Amom and Nongdam, 2017). An amplifiable distance between two identical microsatellite repeats or SSR regions that are orientated in opposing directions may be precisely determined using the PCR-based ISSR approach. In this approach, SSRs are employed as primers to amplify mostly intersimple sequence repeat regions. Owing to their high resolution and outstanding data repeatability, fluorescent ISSR markers have been shown to be more effective and sensitive than regular or non-fluorescent ISSR markers (Obručová *et al.,* 2016). These approaches tag PCR products using fluorescent dyes. In contrast to fluorescent ISSR, which produces 20–30 times more bands overall and resolves them exclusively by peaks with varying heights depending on DNA fragment length, the number of bands acquired in standard ISSR gels is quite low (Kawar *et al.,* 2009). In this study 44 different landraces of *Dioscorea* classified under 8 species were used to analyse the genetic similarity and distance among them using fluorescent labelled markers through fragment analysis.

## Materials and Methods

### Plant Materials

Tender leaves of *Dioscorea* sp. were collected from different districts of Kerala (Wayanad, Idukki, and Thiruvanathapuram) used by the tribal communities and their common names were documented. A total of 44 accessions belonging to eight species of *Dioscorea* were used for this study. The eight *Dioscorea* species were identified using morphological parameters as per Flora of Presidency of Madras, *D. alata*, *D. esculenta*, *D. bulbifera*, *D. oppositifolia*, *D. rotundata*, *D. tomentosa*, *D. wallichi* and *D. pentaphylla* were used for genetic

analysis. Each landrace was given specific accessions based on the place from which they were collected. The landraces of *Dioscorea* spp. collected from different tribal areas are listed below:

## DNA isolation

Initially, the isolation procedure was standardized. Higher phenolic interference was evident throughout the isolation procedure. The collected tender leaf samples (200 g) were ground in liquid nitrogen, and genomic DNA was isolated using the modified C-TAB method (Doyle and Doyle method, 1987). Total DNA was electrophoresed on a 1% agarose gel to determine its consistency and purity. A quality check using a Nanodrop was performed, and the observed 260/280 absorbance value was 1.8.

## Polymerase chain reaction

The stock DNA sample was diluted with sterile TE buffer to prepare a working solution at a uniform concentration of 100 ng/µL. PCR was done by using 6-FAM (carboxyfluorescein)- labelled ISSR markers as primers. Eight ISSR primers (Table 1), synthesized by Sigma-Aldrich, were used for the analysis out of a total of 38 primers checked for polymorphism in the *Dioscorea* population. The amplification was performed on a total reaction volume of 10 µL with 10 pM primer, 5 µL Applied Biosystems® AmpliTaq Gold® 360 master mix, 0.2 µl enhancer, 100ng template DNA, and nuclease-free water to make up to 10 µL. The PCR program was as follows: initial denaturation at 95°C for 5 min, denaturation at $95^0$C for 30 s, annealing with temperature based on the specific ISSR primer used, extension at 72°C for 60 s of 35 cycles, final extension at 72°C for 7 min, and later to 4°C was done using an Applied Biosystem Veriti 96 well thermal cycler.

## Fragment analysis

Fragment analysis was performed on an Applied Biosystems 3730xl DNA analyzer using POP7 polymer. Each well with total reaction of 10 µl volume prepared by 2µl of PCR product, 8.8µl of formamide, and 0.2µl of liz600 (internal size standard) (Figure 1). The samples were denatured at 95°C for 5 min, and automated capillary electrophoresis was performed on an analyzer.

## Data analysis

Genemapper 6 software was used to analyze the fragment data. Scoring was performed based on the presence or absence of fragments with size between 80bp and 600bp. The resultant data were in the form of binary scores, with 1 for presence and 0 for absence, since the ISSR primers were dominant molecular markers. Only clear and sharp bands obtained from the fragment analysis were considered for scoring. The sample size varied from 2 to 17 accessions for each species, with a total of 44 accessions. PopGene 32 software was used for genetic variation studies. Based on Nei's genetic identity, the distance matrix was analyzed based on the phylogenetic tree obtained.

## Results and Discussion

44 morphologically distinct landraces of *Dioscorea* were documented and used for our genetic diversity study. The genomic DNA was isolated and given accession number for present study and future references. The most cultivated species among tribes, *D. alata*, is native to Asia (Ngo *et al.*, 2015). *D. rotundata* is a native African variety that is exotic to Kerala (Noorman *et al.*, 2012). According to tribal farmers in Kerala, yields are often poor despite their significance because of insect infestations, illnesses, and proliferation (vegetative multiplication). The present study on different landraces of *Dioscorea* collected from tribal areas of Kerala showed high genetic diversity. We calculated five important parameters including observed number of alleles (Na), effective number of alleles (Ne), Shannon's information index (I), Gene diversity (h), Percentage of polymorphic loci (P%)on genetic diversity using data analysed from Popgene software and based on which a dendrogram was also obtained.

Polymorphism information content (PIC) was calculated from the binary presence/absence matrix. The only bands that scored, those that were > 80 bp in length according to the size standard bands and those that were reproducible (Figure 1). In the present study, the number of polymorphic loci detected per primer combination varied according to the primer.

Among the 38 primers used, 8 showed polymorphic bands in the *Dioscorea* population. The maximum polymorphic band was shown by primer 3 ((GA) 6CC), a total of six bands, and the minimum number of bands was shown by primer 8 ((CA)6AC), two bands. A total of 36 polymorphic bands were obtained by screening eight primers on eight populations of *Dioscorea*, with an average of 4.5 polymorphic bands.

The percentage of polymorphic loci varies between 26-99% (Table 2). *D. oppositifola* showed high genetic diversity among the population with a value of 0.2892, which significantly supports the study on the *Dioscorea* population in China by analyzing 26 phenotypic traits (Cao *et al.*, 2021).

The maximum gene diversity within the population was shown by *D, alata* (0.2686) and *D. oppositifolia* (0.2892). The most cultivated species of *Dioscorea*, that is, *D. alata*, showed the highest percentage of polymorphism (99%) as compared to others (Table 2).

*Dioscorea tomentosa*, the species that is consumed by tribes after undertaking different preparative methods, such as repeated boiling to remove higher content of anti-nutritional factors, was obtained as a different cluster from all other species of *Dioscorea* population collected from tribal area. Our study on Nei's identity and the distance matrix, *D. tomentosa* and *D. rotundata* (the exotic species) showed the highest distances from all other clusters.

According to previous reports, wild yam species have a higher alkaloid content than farmed ones (Padhan *et al.,* 2020) which might be related to the variation of these species (*D. tomentosa and D. rotundata*) from other cultivated varieties. *D. tomentosa* contains a small amount of these anti-nutritional factors; thus, the tribal people utilize additional preparative methods (proper boiling for the long term). This species also contains diosgenin, which possesses medicinal properties.

The similarity matrix value ranged between 0.5839 to 0.9774 (Table 2). Among the eight species under study, *D. oppositifolia* and *D. wallichi* belonged to the same cluster, and *D. esculenta* and *D. pentaphylla* belonged to the same cluster (Figure 2). *D. esculenta* and *D. pentaphylla* showed maximum similarity (0.9774) as compared to others in the population. The maximum distance was calculated between *D. tomentosa* and *D. rotundata* (0.5379).

The current study aligns with previous study on genetic diversity validation of *Dioscorea* species (Barman *et al.*, 2018). To strengthen the crops of these wild species, yam genetics and genomics must be addressed (Ngo *et al.*, 2015) and our study using fragment analysis technique was comparatively easy and reproducible.

Future studies are required to gain a deeper understanding of the phenotypic traits of wild species and investigate their latent genetic potential for managing biodiversity for long-term sustainability and germplasm preservation. Therefore, ISSR-assisted phylogenetic studies may contribute to molecular marker identification, which ultimately benefits crop protection and improvement.

Genetic research plays a crucial role in preserving traditional knowledge and understanding the diversity, distribution, and development of wild tubers such as *Dioscorea*. Molecular markers enable the examination of genetic composition, identifying unique qualities that can be preserved and utilized in breeding programs, including traits like disease resistance and nutritional value. By preserving these important traits, local communities' cultural practices and historical values can be safeguarded.

**Table.1**

| Sl. No. | Land races | Accession code |
|---|---|---|
| ***Dioscorea alata* land races** | | |
| **1** | Sugandhakachil (Wayanad) | WD2 |
| **2** | Undakachil (Wayanad) | WD4 |
| **3** | Neendikachil (Wayanad) | WD5 |
| **4** | Mattukachil (Wayanad) | WD6 |
| **5** | Kaduvakayyankachil (Wayanad) | WD7 |
| **6** | Kappakachil (Wayanad) | WD11 |
| **7** | Injikachil (Wayanad) | WD12 |
| **8** | Chorakachil (Wayanad) | WD13 |
| **9** | Quintalkachil (Wayanad) | WD18 |
| **10** | Thoonankachil (Wayanad) | WD27 |
| **11** | Neelakachil (Idukki) | ID6 |
| **12** | Kachil (common variety) (Trivandrum) | TD2 |
| **13** | Thamburankachil (Trivandrum) | TD5 |
| **14** | Neendikachil (Idukki) | ID37 |
| **15** | Quintalkachil (Idukki) | ID39 |
| **16** | Vellakkachil (Trivandrum) | TD7 |
| **17** | Neelakizhangu(Trivandrum) | TD9 |
| ***Dioscorea esculenta* land races** | | |
| **18** | Mukkizhangu (Wayanad) | WD10 |
| **19** | Cherukizhangu (small) (Wayanad) | WD18 |
| **20** | Cherukizhangu (large) (Wayanad) | WD23 |
| **21** | Vankizhangu (Wayanad) | WD32 |
| **22** | Mullankizhangu (Idukki) | ID9 |
| **23** | Cherukizhangu (Idukki) | ID12 |
| **24** | Nanakizhangu (Wayanad) | WD22 |
| **25** | Nanakizhangu (Trivandrum) | TD1 |
| **26** | Mukkizhangu (Trivandum) | TD8 |
| ***Dioscorea bulbifera* land races** | | |
| **27** | Erachikachil (Wayanad) | WD1 |
| **28** | Adathappu (Wayanad) | WD29 |
| **29** | Pattikachil (Wayanad) | WD30 |
| **30** | Kattuadathappu (Idukki) | ID13 |
| ***Dioscorea oppositifolia* land races** | | |
| **31** | Kanjiravallikizhangu (Wayanad) | WD20 |
| **32** | Naarakizhangu (Wayanad) | WD32 |
| **33** | Thettam (Idukki) | ID7 |
| **34** | Kavalakkizhangu (Trivandrum) | TD11 |
| ***Dioscorea rotundatal* and races (exotic variety)** | | |
| **35** | Nigeriankachil (White) | WD8 |

| | | |
|---|---|---|
| **36** | Nigeriankachil (Blue) | WD9 |
| ***Dioscorea wallichi*land races** | | |
| **37** | Mogappan (Wayanad) | WD28 |
| **38** | Kattukizhangu (Idukki) | ID11 |
| **39** | Neduvankizhangu (Trivandrum) | TD3 |
| ***Dioscorea pentaphylla* land races** | | |
| **40** | Nooran (Wayand) | WD3 |
| **41** | Nooran (Trivandrum) | TD4 |
| **42** | Nooran (Idukki) | ID10 |
| ***Dioscorea tomentosa* land races** | | |
| **43** | Chaakon (Idukki) | ID8 |
| **44** | Nooli (Trivandrum) | T10 |

**Table.2** List of F-ISSR molecular markers and sequences.

| Sl. No. | Primer (ISSR markers) | Annealing temperature (°C) |
|---|---|---|
| **1** | AGAGAGAGAGAGAGAGAGAGAGT- (AG)11T | 55 |
| **2** | CTCTCTCTCTCTCTCTCTCTT- (CT)10T | 55 |
| **3** | GAGAGAGAGAGACC- (GA)6CC | 50 |
| **4** | CACACACACACACAG- (CA)7G | 50 |
| **5** | GAGAGAGAGAGAGAGAGACG- (GA)8CG | 50 |
| **6** | ACACACACACACACACT- (AC)8T | 50 |
| **7** | GTGTGTGTGTGTCC- (GT)6CC | 50 |
| **8** | CACACACACACAC- (CA)6AC | 50 |

**Table.3** Genetic diversity parameters of *Dioscorea spp.*

| SI. No. | Population | N | Na | Ne | I | H | P% |
|---|---|---|---|---|---|---|---|
| **1** | *D. alata* | 17 | 1.909 | 1.1286 | 0.4394 | 0.2686 | 99 |
| **2** | *D. esculenta* | 9 | 1.8571 | 1.4040 | 0.3914 | 0.1078 | 85.71 |
| **3** | *D. bulbifera* | 4 | 1.4286 | 1.1295 | 0.1688 | 0.2321 | 42.86 |
| **4** | *D. oppositifolia* | 4 | 1.8571 | 1.3022 | 0.3939 | 0.2892 | 84.57 |
| **5** | *D. rotundata* | 4 | 1.5223 | 1.3030 | 0.2592 | 0.1775 | 43.02 |
| **6** | *D. wallichi* | 3 | 1.2857 | 1.1223 | 0.1362 | 0.0856 | 28.57 |
| **7** | *D. pentaphylla* | 3 | 1.7143 | 1.4007 | 0.3989 | 0.2679 | 71.43 |
| **8** | *D. tomentosa* | 2 | 1.100 | 1.111 | 0.1262 | 0.0875 | 26.3 |

N – Sample size; Na – Observed number of alleles; Ne – Effective number of alleles; I – Shannon's information index; h – Gene diversity; P% - Percentage of polymorphic loci.

**Table.4** Similarity and distance matrix expressed as Nei's coefficient among the eight genotypes of *Dioscorea* as revealed by eight ISSR primers.

| | ***D. alata*** | ***D. esculenta*** | ***D. bulbifera*** | ***D. oppositifolia*** | ***D. rotundata*** | ***D. wallichi*** | ***D. pentaphylla*** | ***D. tomentosa*** |
|---|---|---|---|---|---|---|---|---|
| ***D.alata*** | **** | 0.9604 | 0.9426 | 0.845 | 0.8087 | 0.8855 | 0.9501 | **0.6943** |
| ***D.esculenta*** | 0.0405 | **** | 0.9259 | 0.9 | 0.7776 | 0.9705 | 0.9774 | **0.8057** |
| ***D.bulbifera*** | 0.0592 | 0.077 | **** | 0.8086 | 0.8539 | 0.889 | 0.8781 | **0.6922** |
| ***D.oppositifolia*** | 0.1684 | 0.1053 | 0.2124 | **** | 0.713 | 0.9395 | 0.8478 | **0.8639** |
| ***D.rotundata*** | 0.2123 | 0.2515 | 0.1579 | 0.3383 | **** | 0.7394 | 0.6883 | **0.5839** |
| ***D.wallichi*** | 0.1216 | 0.0299 | 0.1176 | 0.0624 | 0.302 | **** | 0.9145 | **0.8415** |
| ***D.pentaphylla*** | 0.0512 | 0.0229 | 0.13 | 0.1652 | 0.3736 | 0.0894 | **** | **0.8042** |
| ***D.tomentosa*** | **0.3648** | **0.216** | **0.3679** | **0.1463** | **0.5379** | **0.1725** | **0.218** | **** |

**Fig.1** Figure showing fragment (blue bands) generated using ISSR primers and the co-migrated size standard (orange peaks)

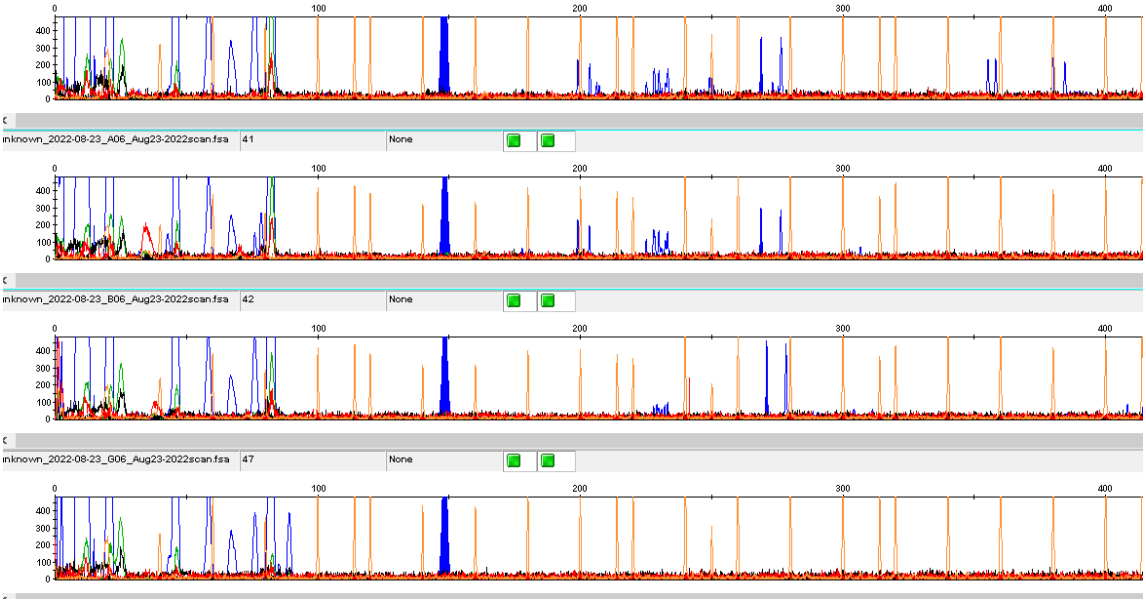
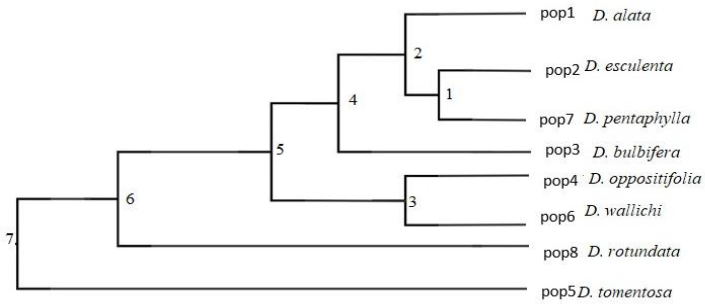


**Fig.2** Dendrogram Based Nei's (1972) Genetic distance, method UPGMA modified from neighbor procedure of PHYLIP Version 3.5



Furthermore, genetic research supports the establishment of seed banks and conservation initiatives, ensuring the protection of genetic diversity. These measures are essential for maintaining the availability of genetic resources for future generations, preventing the loss of valuable genetic traits. In the specific context of Kerala, our study focused on genetic diversity analysis of 44 land races belonging to 8 *Dioscorea* species used by tribal communities. This molecular screening and characterization of genotypes contribute to the conservation and preservation of native species.

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## Conflict of interest

The authors declare that they have no coflicts of interest.

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