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# Analysis of Fluorescently Labeled ISSR Markers to Reveal the Genetic Diversity of Cassava Varieties (*Manihot esculenta* Crantz) in the Kerala's Tribal Area of India

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***Authors' contributions***

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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## ABSTRACT

The study Inter Simple Sequence Repeat (ISSR) molecular markers to genotype distinct cassava (*Manihot esculenta Crantz*) cultivars within tribal communities in Kerala, India. Fluorescent ISSR primers were labelled with 6-carboxyfluorescein and used for PCR amplification. The total number of bands produced was 72, of which 49 were found to be polymorphic markers; an average of 17 polymorphic bands was obtained per primer. The manuscript is a scientific validation of the

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traditional knowledge of tribal people regarding the diversity and wealth of cassava crops in the region, and this validation can therefore help in protecting the farmers right on a particular landrace that they cultivate. The technique used in the diversity analysis is a time-saving process as compared to conventional breeding programs for crop improvement. The data in the manuscript also supports similarity and differences of each variety to one another, which is analysed statistically with the molecular marker-based data generated. By examining genetic diversity at a high resolution, ISSR markers can provide valuable insights into the evolutionary dynamics of these cassava populations. This research contributes to the broader goal of enhancing agricultural sustainability and food security in tribal communities.

*Keywords: Cassava; ISSR; molecular markers; genotyping; genetic diversity.*

## 1. INTRODUCTION

Cassava is considered the most important staple food for about 800 million people in the tropics and subtropics. Cassava is a widely grown, drought-tolerant crop that can produce a high yield in suitable growing conditions. Its storage roots are a major source of food for humans and animals in tropical regions [1]. Its roots are a good source of carbohydrates and starch form it is used in various industrial applications [2]. It, also known as tapioca, is a widely grown, drought-tolerant crop that can produce a high yield in suitable growing conditions. Its storage roots are a major source of food in tropical regions [1]. Cassava thrives appreciably well in poor soils with low fertility, producing higher yields than any other tuber crop [3]. Understanding the genetic diversity of cassava [4] varieties is crucial for sustainable farming and food security. Molecular markers are useful for breeding purposes, genetic diversity studies, and knowing relatedness among varieties [5,6,7]. Markers like RAPD, AFLP, SSRs [8], microsatellites [9], and ISSRs are commonly used for various studies. The PCR-based technique, ISSR, has better sensitivity and reproducibility [10]. Genetic variation detection in plants facilitates the identification of useful genetic divergence for population improvement [11]. The use of Inter Simple Sequence Repeat (ISSR) markers provides a powerful technique to genotype unique cassava cultivars in tribal communities in Kerala, India. By examining genetic diversity at a high resolution, ISSR markers can provide valuable insights into the evolutionary dynamics of these cassava populations. Additionally, morphological analysis helps to connect genotype with observable plant properties, aiding in the understanding of adaptation mechanisms. Integrating both genotypic and morphological data not only helps to preserve genetic resources but also has implications for agricultural innovations, such as breeding programs to develop resilient and region-specific cassava varieties. Comprehending the genetic variety of cassava varieties is crucial for pursuing sustainable farming methods and improving food security.

ISSR markers are co-dominant markers on occasion, although they are dominant markers overall. The ISSR marker approach creates multilocus products from inter-sequence regions (Fig. 1) by PCR using simple sequence repeat primers. There are reports that ISSR primers were successful in generating reproducible and reliable amplicons from four cassava genotypes that were identified [12].

The present work uses Inter Simple Sequence Repeat (ISSR) markers as a potent technique to genotype cassava cultivars that are unique to particular tribal communities in the Idukki district of Kerala, India. The ISSR markers were selected based on the previous reports on cassava [13]. ISSR-PCR uses a single fluorescently labelled primer to target the region between identical microsatellites. Fluorescent ISSR primers were labelled with 6-carboxyfluorescein and consist of di-nucleotide motifs [14]. Furthermore, morphological research will supplement the genotypic analysis and offer a comprehensive view of the distinct characteristics displayed by these wild cassava populations. Experiments were conducted to determine the selection of morphological descriptors in cassava crops by multivariate techniques with the aim of optimizing the use of this technique in order to provide reliable, useful information for the program of genetic improvement and conservation of the species [15].

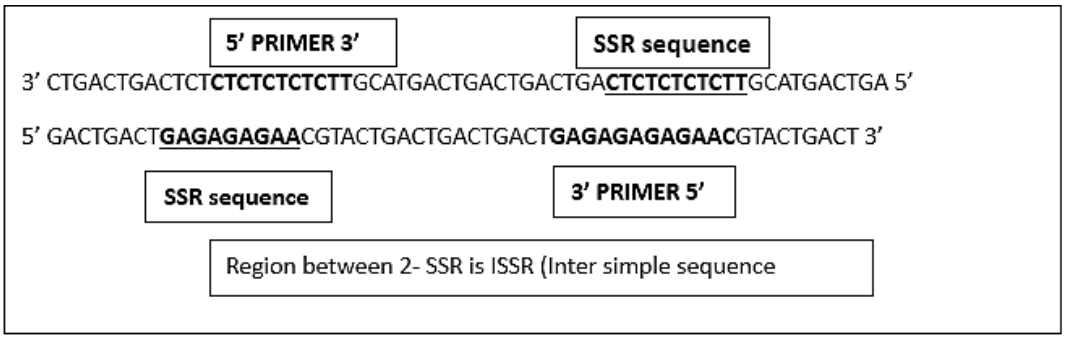



**Fig. 1. Diagrammatic representation showing fragment amplification between two SSR, which is ISSR (Inter Simple Sequence Repeat) (Courtesy: Mondal A et al. [14])**

The ability of cassava to adapt to a wide range of environmental conditions is well recognized, and this ability is frequently mirrored in the genetic composition of the plant. By using ISSR markers, genetic diversity may be examined at high resolution, providing important information about the evolutionary dynamics of these wild cassava populations. Because ISSR markers are based on polymorphic areas found in microsatellite sequences, they offer a repeatable and affordable way to identify genetic variants. This makes them especially useful for studying non-model organisms, such as wild cassava varieties.

A morphological investigation will also be carried out in parallel to describe the phenotypic characteristics of these cassava populations. Morphological attributes provide a concrete connection between the genotype and the observable properties of the plant, such as plant height, tuber characteristics, and leaf shape. A thorough understanding of the adaptation mechanisms used by these wild cassava varieties in response to their particular ecological niches can be obtained by integrating morphological and genotypic data.

The thorough morphological and genotypic characterization of wild cassava varieties yields vital data for the preservation of these exceptional genetic resources. Cassava conservation strategies need to be context-specific, taking into account the special adaptations that have developed within these tribal regions [16]. The whole range of genetic variation found using ISSR markers must be preserved in order to guarantee the preservation of desirable features that might be essential to the long-term viability of cassava farming in the area.

Promising implications for agricultural innovation stem from the physical characteristics and genetic markers that have been uncovered [17]. It is possible to improve breeding programs and produce better cassava varieties by utilizing the adaptive features seen in wild cassava populations. Breeding efforts can incorporate traits like higher production potential, tolerance to environmental stressors, and resistance to particular pests or diseases to generate varieties that answer the particular issues faced by farmers in tribal communities [18]. In addition to aiding in the preservation of these rare genetic resources, the combination of morphological analysis with ISSR markers has implications for agricultural innovation. Finding certain genetic markers linked to desired characteristics, such as disease or pest resistance, can help breeding programs create cassava varieties that are not only resilient but also specifically suited to the needs of regional agricultural systems.

## 2. MATERIALS AND METHODS

### 2.1 Morphological Study of Farmer Varieties

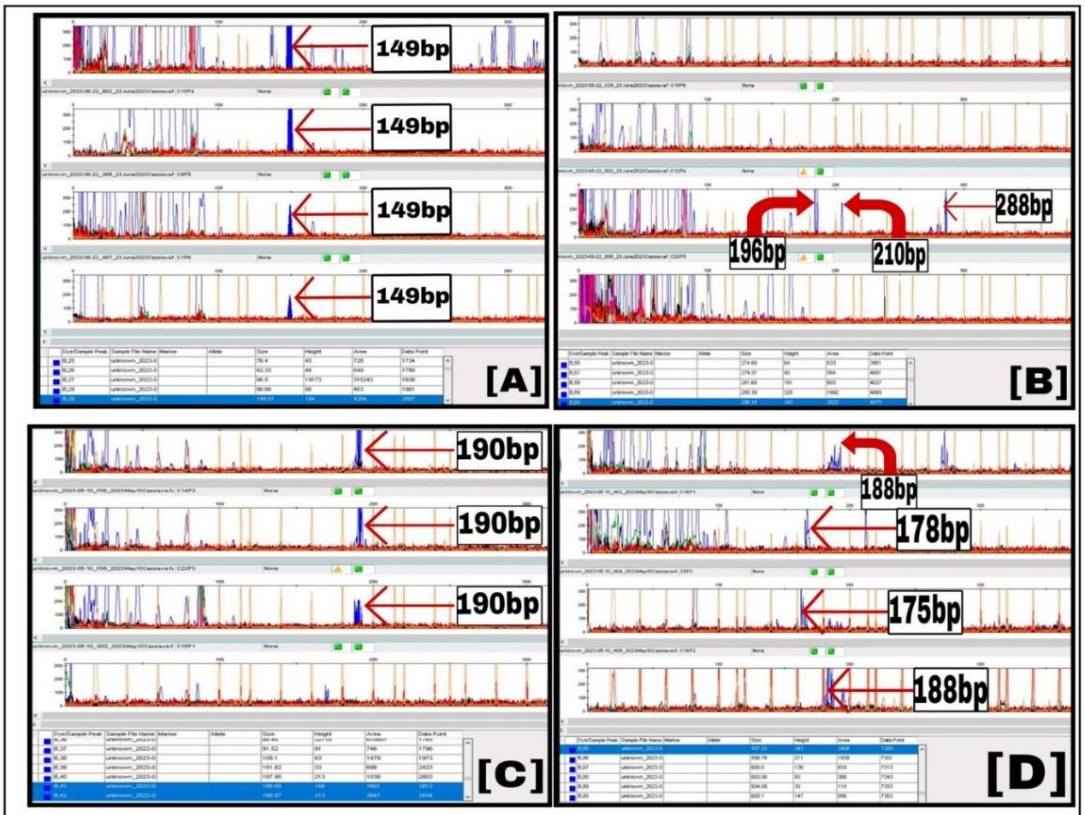
Morphological characterization of 23 farmer varieties of cassava (Table 1) was carried out. The morphological characters used for comparison are pubescence on apical leaves, colour of the first fully expanded leaf, predominant shape of the central leaf lobe, petiole colour, mature leaf colour, predominant number of leaf lobes, length of central leaf lobe (cm), width of central leaf lobe (in cm, at max. width), leaf lobe margin, petiole length (cm), leaf vein colour, young stem colour (at top 20 cm of plant), prominence of foliage scars, colour of mature stem: exterior, stem: distance between leaf scars, growth habit of stem, plant type, plant height (cm), and plant branching habit.

**Table 1. List of cassava landraces used for genetic analysis using ISSR markers**

| No | *Cassava* samples | Accession code | Variety |
|---|---|---|---|
| 1 | Ceylonkappa | IT1 | Farmer's variety |
| 2 | Akashavani | IT2 | Farmer's variety |
| 3 | Dany z kappa | IT3 | Farmer's variety |
| 4 | League kappa | IT4 | Farmer's variety |
| 5 | Nehamya | IT5 | Farmer's variety |
| 6 | Kariveppu kappa | IT14 | Traditional variety |
| 7 | Ambakkadan kappa | IT15 | Traditional variety |
| 8 | Ceylon kappa | IT16 | Farmers variety |
| 9 | Pathinettu kappa | IT17 | Traditional variety |
| 10 | NDA mixer kappa | IT18 | Farmer's variety |
| 11 | Jaivasree kappa | IT19 | Farmer's variety |
| 12 | Sthothram kappa | IT20 | Farmer's variety |
| 13 | Indian kappa | IT21 | Farmer's variety |
| 14 | Powervision kappa | IT22 | Farmer's variety |
| 15 | Keralathala kappa | IT23 | Farmer's variety |
| 16 | Mathrubhumi kappa | IT24 | Farmer's variety |
| 17 | Issac kappa | IT25 | Farmer's variety |
| 18 | Venmony chulli kappa | IT26 | Farmer's variety |
| 19 | Abelrose kappa | IT27 | Farmer's variety |
| 20 | Idukki kappa | IT40 | Farmer's variety |
| 21 | Etha kappa | IT41 | Traditional variety |
| 22 | Deshabhimani kappa | IT42 | Farmer's variety |
| 23 | Deepika kappa | IT43 | Farmer's variety |

**Table 2. List of primers used for the study**

| Al No. | Primer (ISSR markers) | Primer sequence | Annealing Temperature (ºC) |
|---|---|---|---|
| 1 | UBC 807 | AGAGAGAGAGAGAGAGT | 56 |
| 2 | UBC 808 | AGAGAGAGAGAGAGAGC | 56 |
| 3 | UBC 810 | GAGAGAGAGAGAGAGAT | 56 |
| 4 | UBC 811 | GAGAGAGAGAGAGAGAC | 56 |
| 5 | ISSR815 | CTCTCTCTCTCTCTCT8G | 56 |
| 6 | UBC 817 | CACACACACACACACAA | 56 |
| 7 | ISSR818 | CACACACACACACACAG | 56 |
| 8 | UBC 820 | GTGTGTGTGTGTGTGTC | 56 |
| 9 | ISSR822 | TCTCTCTCTCTCTCTCA | 56 |
| 10 | UBC 826 | ACACACACACACACACC | 56 |
| 11 | ISSR827 | ACACACACACACACACG | 56 |
| 12 | UBC 836 | AGAGAGAGAGAGAGAGYA | 56 |
| 13 | UBC 845 | CTCTCTCTCTCTCTCTRG | 56 |
| 14 | ISSR857 | ACACACACACACACACCG | 55 |
| 15 | (AG)11T | AGAGAGAGAGAGAGAGAGAGAGT | 55 |
| 16 | (CT)10T | CTCTCTCTCTCTCTCTCTCTT | 56 |
| 17 | (GA)6CC | GAGAGAGAGAGACC | 55 |
| 18 | (CA)7G | CACACACACACACAG | 56 |
| 19 | (GA)8CG | GAGAGAGAGAGAGAGAGACG | 56 |
| 20 | (AC)8T | ACACACACACACACACT | 55 |
| 21 | (GT)6CC | GTGTGTGTGTGTCC | 56 |
| 22 | (CA)6AC | CACACACACACAC | 56 |




[A] Four cassava accessions showing similar fragments of length 149bp, [B] One of the cassava accession showing additional fragments of length 196bp, 210bp, 288bp, [C] Three of the cassava accessions showing similar fragment of length 190bp and in one of the accession it is dissimilar, [D] All four accessions of cassava showing dissimilar fragments of variable length (273bp,178bp,175bp,188bp).

**Fig. 2. Capillary gel electrophorogram showing polymorphism of the PCR products amplified using fluorescently labeled primers (5' FAM labeled), which is showing difference in profiles. Orange coloured products are size standard**

## 2.2 Genetic Analysis

Tender leaves of Cassava sp. were collected from the tribal areas of Idukki district, Kerala, India, and used for phylogenetic analysis. Genetic analysis is done at Rajiv Gandhi centre for Biotechnology, Thiruvananthapuram, Kerala, for which, 200 g for each sample was weighed and grounded in liquid nitrogen. A modified ce-TAB method [19] was used for isolating the genomic DNA. The total DNA was run on a 1% agarose gel to determine consistency and purity. A quality check using Nanodrop was done, and the observed 260/280 absorbance value was 1.8. The stock DNA sample was diluted with sterile TE buffer to make a working solution with a uniform concentration of100 ng/µl. PCR was done by using 6-FAM (carboxyfluorescein)-labelled ISSR markers as primers. 22 ISSR primers (Table 2), synthesized by Sigma Aldrich, were used for analysis. Fragment analysis was done on a DNA analyzer using 2µl of PCR product, 8.8µl of formamide, and 0.2µl of Liz 600 (size standard). Genemapper 6 software was used for the analysis data (Fig. 2). Scoring was done based on the presence or absence of fragments of size between 80 bp and 600 bp. The resultant data was in the form of binary scores, with '1' scored for presence and '0' scored for absence, since the ISSR primers were dominant molecular markers. The sample size varies from 2 to 17 numbers of each variety of cassava, for a total of 23 accessions. PopGene32 software was used for the genetic variation studies. Based on Nei's genetic identity, the distance matrix (Table. 3) was analysed based on the phylogenetic tree that was obtained.

**Table 3. Similarity (above ***) /Distance (below****) index matrix of 23 Cassava land races**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | **** | 0.9876 | 0.8376 | 0.9753 | 0.9758 | 0.9666 | 0.8977 | 0.9492 | 0.9805 | 0.9285 | 0.939 | 0.939 | 0.9868 | 0.9784 | 0.9785 | 0.9575 | 0.9467 | 0.9782 |
| 2 | 0.0124 | **** | 0.9003 | 0.9905 | 0.9707 | 0.9776 | 0.9469 | 0.973 | 0.966 | 0.9736 | 0.9703 | 0.9703 | 0.9939 | 0.9854 | 0.9574 | 0.9324 | 0.9221 | 0.9527 |
| 3 | 0.1772 | 0.105 | **** | 0.9197 | 0.8544 | 0.9214 | 0.987 | 0.9617 | 0.833 | 0.968 | 0.9729 | 0.9729 | 0.9078 | 0.9203 | 0.7596 | 0.6994 | 0.7586 | 0.752 |
| 4 | 0.025 | 0.0095 | 0.0837 | **** | 0.9635 | 0.9643 | 0.9671 | 0.9767 | 0.9511 | 0.9786 | 0.9804 | 0.9804 | 0.9966 | 0.994 | 0.9512 | 0.9148 | 0.9325 | 0.9455 |
| 5 | 0.0245 | 0.0298 | 0.1574 | 0.0372 | **** | 0.9682 | 0.908 | 0.9479 | 0.9955 | 0.923 | 0.9453 | 0.9453 | 0.9668 | 0.9642 | 0.9613 | 0.8927 | 0.8712 | 0.9225 |
| 10 | 0.034 | 0.0227 | 0.0819 | 0.0364 | 0.0323 | **** | 0.9429 | 0.989 | 0.9726 | 0.9563 | 0.9788 | 0.9788 | 0.9754 | 0.9776 | 0.9039 | 0.8585 | 0.8668 | 0.8966 |
| 11 | 0.1079 | 0.0545 | 0.0131 | 0.0334 | 0.0966 | 0.0588 | **** | 0.9774 | 0.8847 | 0.9906 | 0.9901 | 0.9901 | 0.9539 | 0.9601 | 0.847 | 0.7909 | 0.8335 | 0.8347 |
| 12 | 0.0521 | 0.0274 | 0.0391 | 0.0236 | 0.0535 | 0.0111 | 0.0228 | **** | 0.9435 | 0.9782 | 0.997 | 0.997 | 0.9802 | 0.9868 | 0.8846 | 0.838 | 0.8754 | 0.8838 |
| 13 | 0.0197 | 0.0346 | 0.1828 | 0.0502 | 0.0045 | 0.0278 | 0.1225 | 0.0582 | **** | 0.9048 | 0.9335 | 0.9335 | 0.9621 | 0.9596 | 0.9566 | 0.8974 | 0.8759 | 0.9273 |
| 14 | 0.0741 | 0.0268 | 0.0325 | 0.0216 | 0.0801 | 0.0447 | 0.0095 | 0.022 | 0.1001 | **** | 0.9867 | 0.9867 | 0.9699 | 0.9671 | 0.8828 | 0.8475 | 0.8619 | 0.8763 |
| 15 | 0.0629 | 0.0301 | 0.0274 | 0.0198 | 0.0563 | 0.0214 | 0.01 | 0.003 | 0.0688 | 0.0133 | **** | 1 | 0.9775 | 0.984 | 0.8825 | 0.8285 | 0.8657 | 0.874 |
| 16 | 0.0629 | 0.0301 | 0.0274 | 0.0198 | 0.0563 | 0.0214 | 0.01 | 0.003 | 0.0688 | 0.0133 | 0 | **** | 0.9775 | 0.984 | 0.8825 | 0.8285 | 0.8657 | 0.874 |
| 17 | 0.0133 | 0.0061 | 0.0967 | 0.0034 | 0.0338 | 0.0249 | 0.0472 | 0.02 | 0.0386 | 0.0306 | 0.0228 | 0.0228 | **** | 0.9974 | 0.9543 | 0.9258 | 0.9436 | 0.9568 |
| 18 | 0.0219 | 0.0147 | 0.0831 | 0.006 | 0.0364 | 0.0226 | 0.0407 | 0.0133 | 0.0412 | 0.0334 | 0.0161 | 0.0161 | 0.0026 | **** | 0.9391 | 0.9017 | 0.9357 | 0.9415 |
| 19 | 0.0218 | 0.0435 | 0.2749 | 0.05 | 0.0395 | 0.1011 | 0.166 | 0.1227 | 0.0444 | 0.1247 | 0.125 | 0.125 | 0.0468 | 0.0628 | **** | 0.9738 | 0.9388 | 0.9819 |
| 20 | 0.0434 | 0.07 | 0.3575 | 0.0891 | 0.1135 | 0.1526 | 0.2345 | 0.1768 | 0.1082 | 0.1655 | 0.1881 | 0.1881 | 0.0771 | 0.1035 | 0.0265 | **** | 0.9612 | 0.9918 |
| 22 | 0.0547 | 0.0811 | 0.2763 | 0.0699 | 0.1378 | 0.143 | 0.1822 | 0.133 | 0.1325 | 0.1486 | 0.1442 | 0.1442 | 0.058 | 0.0664 | 0.0631 | 0.0396 | **** | 0.9819 |
| 23 | 0.022 | 0.0485 | 0.285 | 0.056 | 0.0806 | 0.1092 | 0.1807 | 0.1236 | 0.0755 | 0.1321 | 0.1347 | 0.1347 | 0.0442 | 0.0603 | 0.0183 | 0.0083 | 0.0183 | **** |

## 3. RESULTS AND DISCUSSION

Based on the ISSR marker analysis, the results showed a considerable degree of genetic variation among the cassava types under study. Clustering analysis provided insights into the relationships and distinctiveness of these varieties, reflecting the complex evolutionary history of the tubers and adaptive strategies within tribal farming communities. The result significantly clustered out five traditional varieties exclusive to these areas, namely Kariveppu kappa, Ambakkadan kappa, Ceylon kappa, Pathinettu kappa, and Etha kappa (Fig. 3). The overall polymorphic percentage varied between 43-89%, and a grouped population analysis of 23 varieties specifies a value of gene diversity of 47%.

A comprehensive study of the genetic landscape of wild cassava populations has been made possible by the use of ISSR markers. The significant genetic diversity found in these groups is indicated by the high polymorphism revealed by ISSR analysis in line with Asha et al. [13]. The conservation of these genetic resources greatly benefits from the identification of distinct alleles and polymorphic areas. With the help of ISSR markers, population structure may be clearly defined, displaying complex interactions and gene flow between several wild cassava populations. This discovered genetic diversity is probably a result of the unique ecological niches found in Kerala's tribal regions. These niches help cassava populations adapt and evolve because of variables like soil composition, climate fluctuations, and local biotic interactions [12]. Developing successful conservation measures to preserve these exceptional genetic resources for future studies and crop improvement requires an understanding of genetic diversity and population structure.

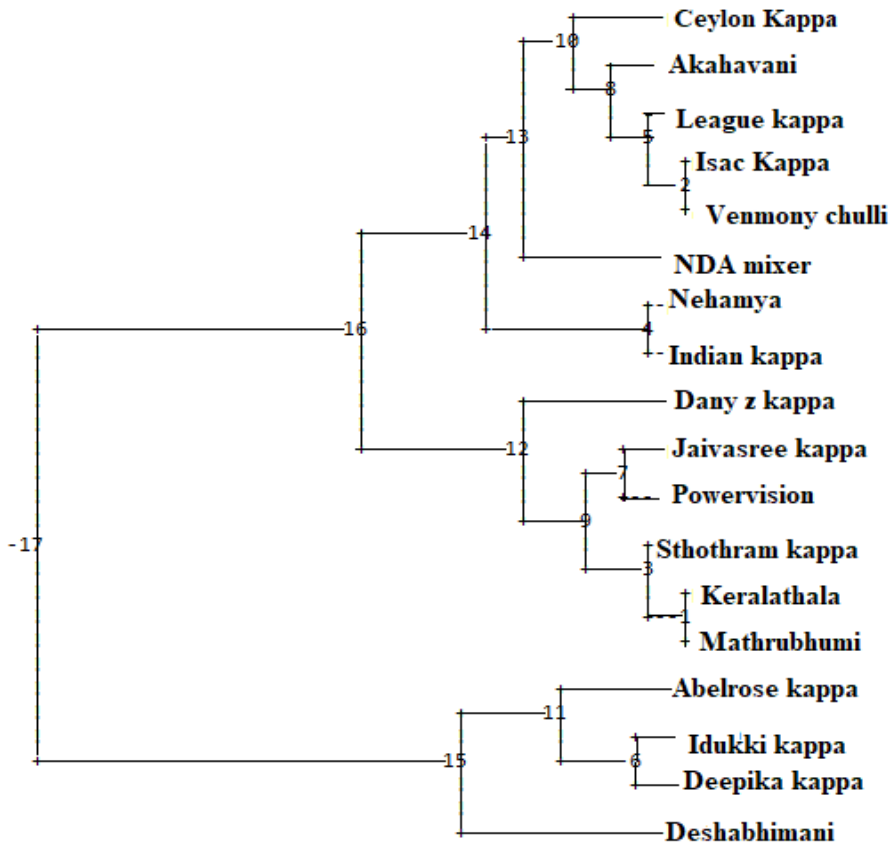



**Fig. 3. The dendrogram showing 17 farmers' varieties and one traditional variety (Ceylon) being grouped in 5 clusters (3 major + 2 minor clusters) based on the similarity / distance matrix data**

Our comprehension of the phenotypic expressions associated with the observed genetic diversity is improved by the incorporation of morphological data. There is a clear link between the genotype and the external features of the plants when important morphological factors are recorded, such as petiole structure, tuber properties, and leaf morphology [20]. The morphological analysis has revealed distinguishing characteristics that could provide these wild cassava varieties with adaptation advantages in their particular settings. For example, certain physical traits might be linked to disease or insect resistance in the area, while other traits might support increased resilience under harsh environmental circumstances. As per our observation, the NDA mixer variety in cluster I show distinctness, while Nehamya and Indian kappa are shown as distinct in their cluster. Dany Z. Kappa is shown as distinct in cluster II, and Able Rose shows its distinct presence in cluster III. The relationship between morphological features and genotypic diversity provides the basis for focused breeding efforts that aim to create strong cassava varieties that are also adapted to the distinct agro ecological conditions of Kerala's tribal districts [21].

Further investigation is necessary to fully grasp the functional genomics of the detected markers and to learn more about the adaptive importance of particular morphological features. Participatory breeding efforts with indigenous communities also guarantee that the created varieties meet the needs and preferences of tribal area farmers.

The research conducted on wild cassava varieties in Kerala's tribal areas, along with genotyping using ISSR markers, has significantly contributed to our understanding of genetic diversity and population dynamics. This information is crucial for preserving genetic resources and developing resilient cassava varieties that are well suited to the region's agro ecological conditions. By emphasizing the importance of maintaining and utilizing genetic diversity in crop improvement efforts, these findings contribute to the broader discussion on sustainable agriculture and food security. It is exciting to see how this research can positively impact agricultural practices and help ensure a more sustainable future.

The integration of morphological data with genetic analysis enhances the understanding of the relationship between genotype and phenotype in wild cassava varieties. There is significant link between genotype and observable characteristics, like mixer variety in cluster I, shows unique traits showing advantages in adaptation to local conditions. There are varieties in each cluster, show specific characteristics that may confer benefits such as disease resistance or drought tolerance. Distinct traits observed in Dany Z. Kappa from cluster II and Able Rose from cluster III underscore the importance of morphological diversity in tailoring cassava varieties to different ecological niches. These physical traits could be indicative of the plants' ability to withstand environmental stresses or resist local pests, which are crucial for the successful cultivation of cassava in Kerala's diverse agro-ecological zones.

The link between morphological features and genetic diversity provides a robust foundation for targeted breeding programs. Breeders can develop cassava varieties that are not only high-yielding but also well-suited to the specific conditions of Kerala's tribal districts. This targeted approach aligns with the broader goals of agricultural improvement and sustainability. Investigating the functional genomics of the identified markers will deepen our understanding of how specific traits influence plant performance and adaptation. Additionally, engaging with indigenous communities in participatory breeding programs is vital. Such collaborations ensure that the developed varieties meet the practical needs and preferences of local farmers, promoting the adoption and success of these new cultivars.

## 4. CONCLUSION

The contribution of this research extends beyond academic knowledge; it plays a critical role in preserving genetic resources and advancing crop improvement strategies. By emphasizing genetic diversity and its implications for sustainable agriculture, our findings support broader efforts to enhance food security and agricultural sustainability. The potential impacts of this research are far-reaching, with the promise of improving agricultural practices and ensuring a more resilient and sustainable future for cassava cultivation in the region. In summary, this study underscores the importance of integrating morphological and genetic data to advance our understanding of cassava diversity and adaptation. It paves the way for innovative breeding strategies and highlights the need for continued research and community engagement to address the challenges of sustainable agriculture.

## DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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