

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

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 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.

Key words: 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 (Howeler, 2013). Its roots are a good source of carbohydrates and starch from it is used in various industrial applications (Sanchez et al., 2009). 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 (Howeler R et al., 2013). Cassava thrives appreciably well in poor soils with low fertility, producing higher yields than any other tuber crop (Temegne et al., 2015). Understanding the genetic diversity of cassava (Balyejusa Kizito E et al 2007) varieties is crucial for sustainable farming and food security. Molecular markers are useful for breeding purposes, genetic diversity studies, and knowing relatedness among varieties (Marico and Mark, 2004; Wang and Bughrara, 2003; Beeching and Marmey, 1993). Markers like RAPD, AFLP, SSRs (Emmy et al, 2014), microsatellites (Elias M, 2004), and ISSRs are commonly used for various studies. The PCR-based technique, ISSR, has better sensitivity and reproducibility (Obrucova et al., 2016). Genetic variation detection in plants facilitates the identification of useful genetic divergence for population improvement (Andrade et al., 2017). 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 (Zayed et al., 2012).

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 (Asha et al, 2019). 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 (Mondal, et al., 2018). 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 (Afonso et al., 2014)

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 (Tiago et al., 2016). 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 (Vidal et al., 2015). 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 (Carrasco et al., 2016). 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. Material and Methods

2.1 Morphological study of Farmer varieties

Morphological characterization of 23 farmer varieties of cassava (Table. I) 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	Jaiwasree 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

2.3 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 (Doyle and Doyle method of 1987) 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 of 100 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 2. List of primers used for the study

Al No.	Primer (ISSR markers)	Primer sequence	Annealing Temperature (°C)
1	UBC 807	AGAGAGAGAGAGAGAGT	56
2	UBC 808	AGAGAGAGAGAGAGAGC	56
3	UBC 810	GAGAGAGAGAGAGAGAT	56
4	UBC 811	GAGAGAGAGAGAGAGAC	56
5	ISSR815	CTCTCTCTCTCTCT8G	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	CTCTCTCTCTCTCTTRG	56
14	ISSR857	ACACACACACACACACCG	55
15	(AG)11T	AGAGAGAGAGAGAGAGAGAGT	55
16	(CT)10T	CTCTCTCTCTCTCTCTCTT	56
17	(GA)6CC	GAGAGAGAGAGAGACC	55
18	(CA)7G	CACACACACACACAG	56

19	(GA)8CG	GAGAGAGAGAGAGAGACG	56
20	(AC)8T	ACACACACACACACT	55
21	(GT)6CC	GTGTGTGTGTGCC	56
22	(CA)6AC	CACACACACAC	56

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

Pop id	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	0.9390	0.9390	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	0.9730	0.9660	0.9736	0.9703	0.9703	0.9939	0.9854	0.9574	0.9324	0.9221	0.9527
3	0.1772	0.1050	****	0.9197	0.8544	0.9214	0.9870	0.9617	0.8330	0.9680	0.9729	0.9729	0.9078	0.9203	0.7596	0.6994	0.7586	0.7520
4	0.0250	0.0095	0.0837	****	0.9635	0.9643	0.9671	0.9767	0.9511	0.9786	0.9804	0.9804	0.9966	0.9940	0.9512	0.9148	0.9325	0.9455
5	0.0245	0.0298	0.1574	0.0372	****	0.9682	0.9080	0.9479	0.9955	0.9230	0.9453	0.9453	0.9668	0.9642	0.9613	0.8927	0.8712	0.9225
10	0.0340	0.0227	0.0819	0.0364	0.0323	****	0.9429	0.9890	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	0.8470	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	0.9970	0.9970	0.9802	0.9868	0.8846	0.8380	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	0.0220	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	0.0100	0.0030	0.0688	0.0133	****	1.0000	0.9775	0.9840	0.8825	0.8285	0.8657	0.8740
16	0.0629	0.0301	0.0274	0.0198	0.0563	0.0214	0.0100	0.0030	0.0688	0.0133	0.0000	****	0.9775	0.9840	0.8825	0.8285	0.8657	0.8740
17	0.0133	0.0061	0.0967	0.0034	0.0338	0.0249	0.0472	0.0200	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	0.0060	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	0.0500	0.0395	0.1011	0.1660	0.1227	0.0444	0.1247	0.1250	0.1250	0.0468	0.0628	****	0.9738	0.9388	0.9819
20	0.0434	0.0700	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	0.1430	0.1822	0.1330	0.1325	0.1486	0.1442	0.1442	0.0580	0.0664	0.0631	0.0396	****	0.9819
23	0.0220	0.0485	0.2850	0.0560	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. Result 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., (2019). 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 (Zayed et al., 2013). 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.

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 (Moreira et al., 2019). 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 (Rabbi et al., 2015).

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.

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)

Option 1:

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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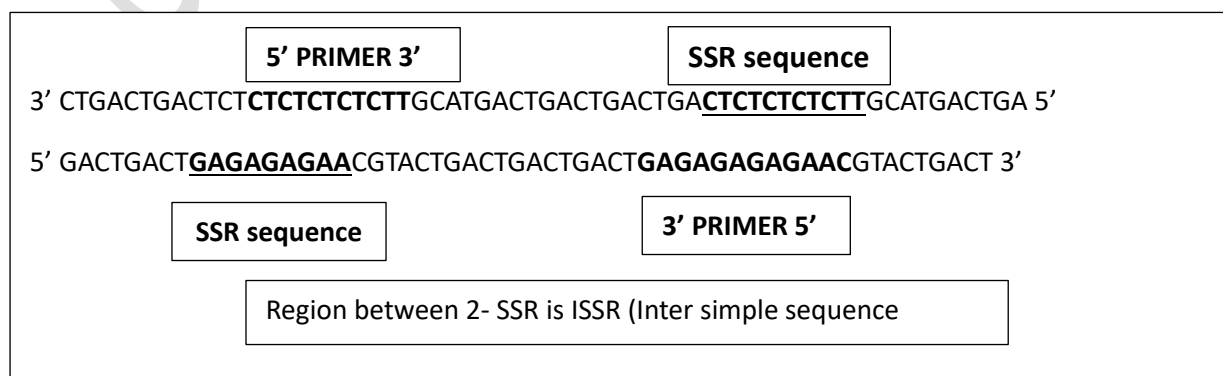


Figure 1. Diagrammatic representation showing fragment amplification between two SSR, which is ISSR (Inter Simple Sequence Repeat) (Courtesy: Mondal A *et al.*, 2018).

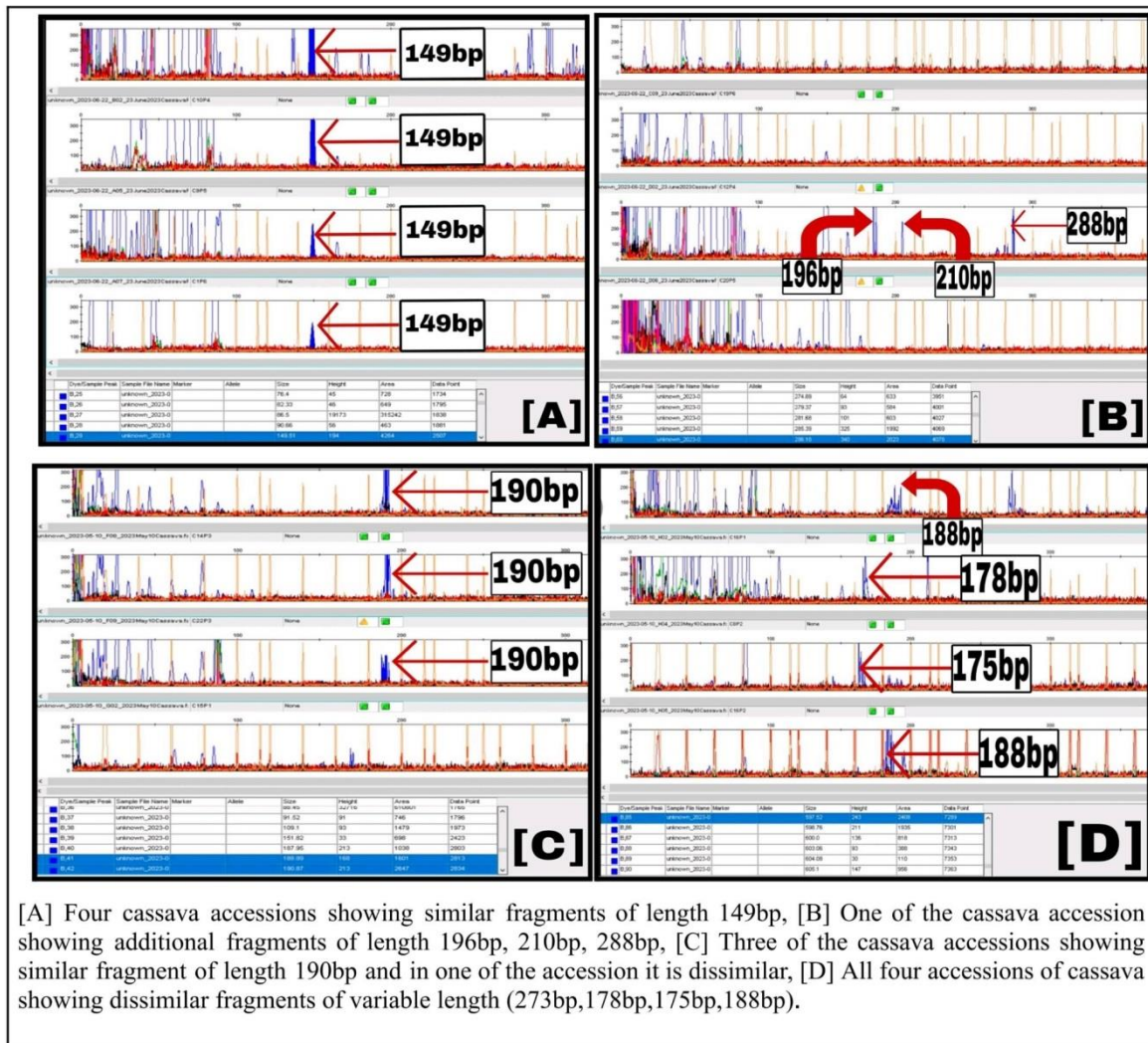


Figure 2. Capillary gel electropherogram showing polymorphism of the PCR products amplified using fluorescently labeled primers (5' FAM labeled), which is showing difference in profiles. Orange coloured products are size standard.

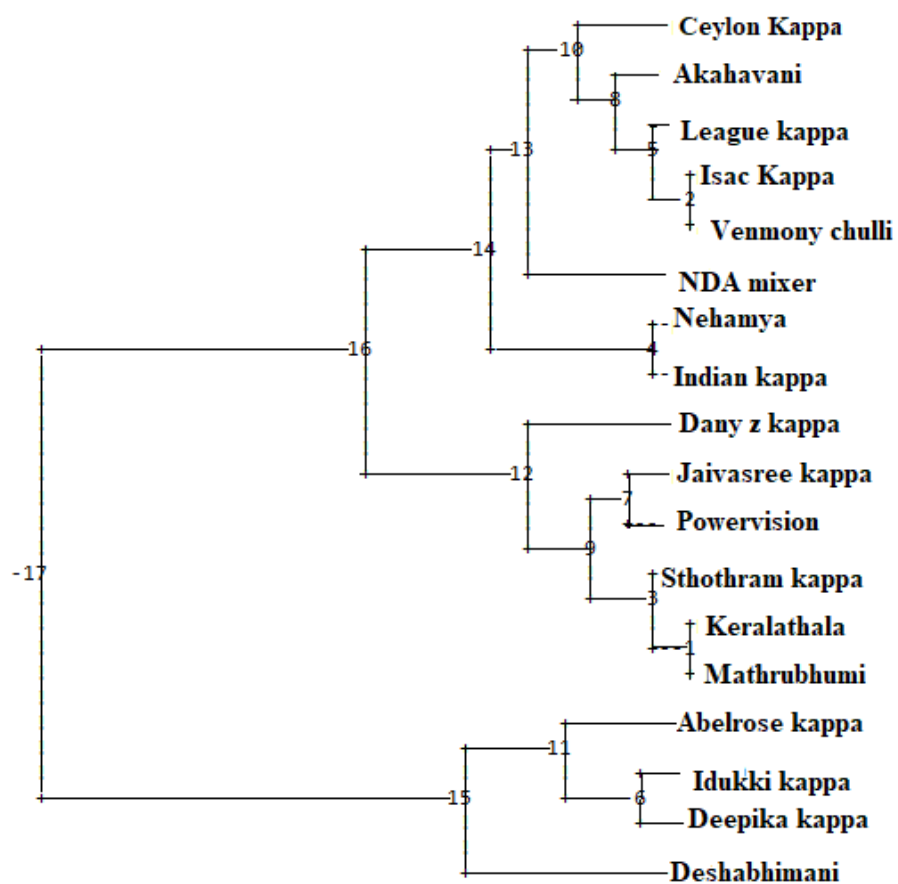


Figure 3: The dendrogram showing 17 farmers' varieties and one traditional variety (Ceylon) being grouped in 5 clusters (3 major + 2 minor clusters) based on the similarity / distance matrix data