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Research Article

# GENETIC DIVERSITY ANALYSIS OF *FICUS RACEMOSA* USING ISSR MARKERS FROM THREE LANDSCAPE ELEMENTS OF DRY DECIDUOUS FOREST BELTS IN KODAGU, KARNATAKA, INDIA

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**Abstract:** Genetic estimation of population sizes has been critical for monitoring Ficus species. However, population estimates do not inherently reveal the permanence or stability of the population under study. Thus, it is important to monitor not only the number of individuals in a population, but also how they are associated in groups and how those groups are distributed across the landscape. The additional challenge was to estimate the genetic diversity exists in *Ficus racemosa* individuals from natural forests, sacred groves and coffee plantations of Kodagu, Karnataka. The leaf samples were collected from each landscape and analysed for its genetic diversity as per standard procedure. The diversity computed based Nei's formulae and Shannon's information index showed consistent results. Natural populations recorded higher diversity (0.2948 and 0.4456, respectively) and comparable diversity was observed in sacred groves and coffee plantations population (0.2049 and 0.3110: 0.2352 and 0.3583, respectively). The relative differentiation among sub populations (GST) value for *Ficus racemosa* populations was 0.5272 and the variation for the total population was 0.3052 growing in different landscape elements of dry deciduous forest belts. Whereas, the variation into the diversity among subpopulation within a zone was low (0.3196). Analysis of Molecular Variance which indicated that within the population had higher genetic variability (97%) and only 3% of the total variation was portioned between population among different landscape element. The genetic diversity exists in natural population and sacred groves help in capturing the genes for further conservation of the species and establishing *Circa situm* forest gene bank in coffee plantations.

**Keywords:** *Circa situm, Conservation, Ficus racemosa, Genetic diversity, Landscape*

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## Introduction

Ficus (Moraceae) constitutes one of the largest genera of angiosperms consisting of about 1000 species from tropical and subtropical regions [1]. Many species of ficus are from both indoor ornamental and ecoservices in nature [2] and formed a distinctive monophyletic clade within the family [3]. Ficus species are considered the pre-eminent group of keystone plant resources for fruit consumers in Southeast Asia. The associated round-the-year flowering makes them a keystone resource for forest fruit dispersers. Indeed, the fruits of Ficus spp. form a large part of the diet for several important frugivores including hornbills, bats, squirrels and primates [4].

Survival and future evolution of Ficus spp. are thus important not only for their diverse associated micro-hymenopteran communities but also for the many vertebrates relying on fig fruits as a food resource and effective seed dispersal. Ficus are known for low population density [5] and long distance pollen dispersal [6]. Long distance pollen dispersal could compensate for low population density, and limit genetic drift, and thus homogenize inter-population genetic diversity while maintaining high genetic diversity within populations.

Many DNA based markers are available to identify the varieties / species. These markers can be effectively used to answer the phylogenetic relationship between ficus varieties/species [7]. Genetic diversity within ficus species is highly dependent on species-specific traits such as life form, seed dispersal mechanisms, pollinators and geographical distribution range [8] and opined that tropical forest trees are predominantly outcrossed which have extensive gene flow and maintain high levels of genetic diversity.

Despite their importance in tropical forests as keystone species, most genetic studies on the genus Ficus have been largely restricted to the domesticated dioeceious fig (*Ficus carica*). Considering the importance of ficus species, the genetic diversity studies related to *Ficus racemosa* was undertaken with ISSR molecular markers in quantifying the genetic variation exists within and between the populations of natural forests, sacred groves and coffee plantations of dry deciduous forest belt of Kodagu, Karnataka, India.

## Materials and Methods

The study was conducted in forest-coffee agroforest landscape mosaics of Kodagu district which lies in the Central Western Ghats region, Southern India, geographically stretched between 11º 56' to12º 52' N and 75º 22' to76º 12' E, covering an area of 4106 km$^2$ of which about 38 percent of area is under natural forests and tree plantations. Three landscape elements such as natural forests (NF), sacred groves (SG) and coffee plantations (CFP) were selected in dry deciduous forest belt of Kodagu, Karnataka, India.

## Sampling

Leaf material was collected from adult individuals (>10 cm dbh) in 24 accessions of *Ficus racemose* in adjoining natural forests, sacred groves and coffee plantations [Fig-1]. Collection of leaf material in continuous forest was restricted to plots of approximately 1 ha and to individuals at least 500 m apart. All the leaves were stored in individual zip lock plastic covers with labelling and shade dried in the laboratory before the DNA was extracted.

Details of the leaf material used for genetic diversity in selected tree species

| SN | Landscape element | Sample code | Name of the place | Latitude (N) | Longitude (E) | Altitude (m) |
|---|---|---|---|---|---|---|
| 1 | Natural forests, sacred grove sand coffee plantations | MUK | Mukkodlu | 12º 26'13.18" | 75º 48'9.11" | 982 |
| 2 | | PLB | Polibetta | 12º 24'9.76"l | 75º 14'48.66" | 772 |
| 3 | | THM | Thitimathi | 12º 5'25.27" | 75º 2'14.68" | 799 |
| 4 | | ALU | Alur | 12º 54'12.08" | 75º 54'53.02" | 979 |

**DNA extraction and PCR amplifications**

DNA was extracted from leaf material (100 mg) using cetyl trimethyl ammonium bromide (CTAB) technique [9] and was purified using DNA easy Plant Mini kit (Qiagen,USA). The quantity and quality of the genomic DNA were assessed using Nanodrop2000 (Thermo Fisher Scientific, USA), Qubit (Thermo Fisher Scientific, USA) and agarose gel electrophoresis. Eighteen 100-200 bp primers were tested for the process and only those primers that produced high intensity and reproducible bands were used for the remainder of the analyses.

Amplification was conducted in an eppendorf master cycler with a heated lid. Amplification was initiated for 3min at 94.0°C, a total of 35 cycles of the following: denaturation at 94°C for 30 sec, annealing at 45°C for 1 min, and elongation at 72°C for 30 sec. An additional extension at 72°C for 7 min was used to ensure that all amplified products completed their elongation. Amplification products were resolved electrophoretically on a 2 % agarose gel at a constant voltage of 75 V for 3 h with a 19 TAE buffer stained with ethidium-bromide. The bands were visualized with ethidium bromide fluorescence. Samples were assigned randomly to lanes and all gels included lanes containing DNA ladders to facilitate standardization. Gels were digitally photographed and the images of multiple gels were standardized using Alpha imager, J.H. Bio software.

ISSR Primers used for PCR amplification

| ISSR Primer | Sequence | Annealing Temperature (°C) |
|---|---|---|
| UBC873 | $(GACA)_4$ | 50 |
| ISSR3 | DBDA$(CA)_7$ | 45 |
| ISSR4 | HVH$(CA)_7$ | 45 |
| ISSR6 | $(CA)_8$RY | 55 |

**Data scoring and analysis**

Data were scored as 1 for the presence and 0 for the absence of a DNA band for each locus across the 24 individuals in *Ficus racemosa*. Using population genetics computer programs, genetic diversity within population was analyzed.

**Effective number of alleles (ne)**

The effective number of alleles was calculated by using the equation [10]

Ne=(NK-1) / (K-1+V/R)

Where, K is the mean number of loci, V variation in number of loci/allele,

N Number of Loci/bands

**Polymorphism Information Content (PIC)**

The level of within population genetic diversity was assed using the percentage of polymorphic loci (threshold level at 95%) of each locus was determined using the formula as described [11]

$PIC=1-\sum Pij^2$

Where, Pi is the frequency of the $i^{th}$ allele in the genotype

**Nei's Gene Diversity (h)**

Average expected gene diversity was calculated using the formula given by [12]

$h_j = h_1+h_2$ / *Total Number of Loci*

Where, $h_1$, $h_2$ represents intralocus gene diversity (*i.e.*, $h_j=(1-p^2-q^2)$

**Shannon's Information Index (I)**

The genetic variation was assessed by using Shannon's Information Index [13]

$I=\sum Pi \log Pi$

Where, pi the frequency of the allele $i^{th}$ in the population.

**Clustering and Principal coordinates analysis**

Unweighted Pair Group Method with Arithmetic mean dendrogram or phenogram was constructed using set of variable data using distance based method as suggested by [14] and neighbor joining (NJ) [15]. The clustering and principal coordinate analysis (PCoA) of 24 populations was performed using DARwin version 6 software and PCoA relates the relationship between distance matrix elements based on their first two principal coordinates.

**Genetic differentiation**

**At the one level of population**

Coefficient of gene differentiation for one level of structure for the total population (GST) was measured by using the formula as given GST

$GST = H_T - H_S / H_T$

Where, GST is measure of the relative differentiation among subpopulation

$H_T$ is heterozygosity in the total population

$H_S$ is the average heterozygosity in subpopulation

**More than one level of population**

Coefficient of gene differentiation for more than one level of structure for the total population (FSR and FST) was measured by using the formulae as given [16]

Partition the variation into the diversity among subpopulation within a dry deciduous forest belt

$FSR=H_R-H_S/H_R$

$FSR=H_T-H_R/H_T$

Where, HR is the Mean allelic frequency within each group

Fixation index (FST) is a measures or values that could help to understand the degree of population differentiation within species. It is developed as a special case of Wright's F-statistics as the most commonly used statistics in population genetics studies.

**Analysis of Molecular Variance**

Analysis of Molecular Variance (AMOVA) was used to detect population differentiation utilizing molecular markers and calculated using the software GenAlEx [17, 18].

**Results**

A total of 24 ISSR markers were used for this study, but only 19 ISSR primers were successfully amplified for 8 samples [Table-1]. Only bands that were consistently reproduced across amplifications were considered for the analysis. Bands with the same mobility were considered as identical fragments, receiving equal values, regardless of their staining intensity [Fig-2]. Nineteen primers produced a total of 100 bands among the *Ficus racemosa* populations. The size of the amplified products ranged from 100 bp to 200 bp. The number of scorable bands produced per primer ranged from 1 to 43. Of the 100 amplified fragments, 43 were polymorphic with the average number of bands per primer and average polymorphic bands per primer to be 5.26 and 2.26, respectively. The total number of polymorphic bands and the percentage of polymorphism ranged from 12 to 17 and 63.16 % to 89.47 % respectively. The observed number of alleles were highest in natural forest (1.8947) followed by coffee plantations (1.7368) and least in sacred grove (1.6316). Whereas effective number of alleles per locus was highest in natural forest (1.5044) and comparable with the sacred grove (1.3449) as compared to populations of coffee plantations (0.3857). The diversity computed based Nei's formulae and Shannon's information index showed consistent results. Sampled populations from all the three landscapes showed relatively lower diversity. While the natural populations recorded higher diversity (0.2948 and 0.4456, respectively) and comparable diversity was observed in sacred groves and coffee plantations population (0.2049 and 0.3110: 0.2352 and 0.3583, respectively). Using ISSR primers, the reproducible bands were in the range of 100-200 bp for *Ficus racemosa* [Table-2]. The total polymorphic bands were more (43) and similarly total number of monomorphic bands was high (57) with total number of bands (100).

Table-1 *Genetic diversity of Ficus racemosa populations within different landscape elements of dry deciduous forest belt based on ISSR markers (Sample size: 8 in each landscape element).*

| Population | Number of Polymorphic loci (NPL) | Percentage of polymorphic loci (PPL) | Observed number of Alleles (na) | Effective number of Alleles (ne) | Nei's genetic diversity (h) | Shannon's Information Index (I) |
|---|---|---|---|---|---|---|
| Natural forests | 17 | 89.47 | 1.8947 (±0.3153)* | 1.5044 (±0.3689)* | 0.2948(±0.1766)* | 0.4456 (±0.2319)* |
| Sacred groves | 12 | 63.16 | 1.6316 (±0.4956) | 1.3449 (±0.3716) | 0.2049 (±0.1990) | 0.3110 (0.2820) |
| Coffeeplantations | 14 | 73.68 | 1.7368 (±0.4524) | 0.3857 (±0.2352) | 0.2352 (±0.1935) | 0.3583 (±0.2678) |
| Mean | 13.667 | 75.43 | 1.9762 | 1.594 | 0.3474 | 0.5190 |
| t-value | 41.00 | 9.866 | 83.034 | 29.692 | 13.988 | 16.38 |
| p-value | 0.0005 | 0.0101 | 0.0001 | 0.0011 | 0.0050 | 0.0037 |

*t- value is based on one sample analysis, P- values < 0.05 is significant at 95% confident interval, *values in parentheses indicate standard deviation from mean value*

Table-2 *A list of genetic diversity of selected tree species using ISSR primers*

| SN | Parameters | *Ficus racemosa* |
|---|---|---|
| 1 | Numbers of primer used | 19 |
| 2 | Amplified product range (bp) | 100-200 |
| 3 | Total number of polymorphic bands | 43 |
| 4 | Total number of monomorphic bands | 57 |
| 5 | Total number of bands | 100 |
| 6 | Average percentage of polymorphic (%) | 75.43 |
| 7 | Average number of bands/primer | 5.26 |
| 8 | Average number of polymorphic bands/primer | 2.26 |

Table-3 *Analysis of molecular variance (AMOVA) for 24 individuals sampled from natural forests, sacred groves and coffee plantations in dry deciduous forest belt using ISSR markers*

| Source | df | SS | MS | Estimated Variance | % Variation | $F_{ST}$ | P value |
|---|---|---|---|---|---|---|---|
| Among populations | 2 | 6.917 | 3.458 | 0.081 | 3% | 0.028 | 0.258 |
| Within populations | 21 | 59.000 | 2.810 | 2.810 | 97% | | |
| Total | 23 | 65.917 | | 2.891 | 100% | | |

*df: Degrees of freedom, SS: Sum of square, MS: Mean sum of square, F'ST: F statistics, P: Probability*

Table-4 *Coefficient of gene differentiation for one level and more than one level of structure for the total population in three landscape elements of dry deciduous forest belt studied based on Nei's genetic diversity using ISSR markers.*

| Tree species | $h_S$ | $h_T$ | $h_S/h_T$ | $GS_T$ | $F_{ST}$ | $F_{SR}$ |
|---|---|---|---|---|---|---|
| *Ficus racemosa* | 0.3474 | 0.7349 | 0.4727 | 0.5272 | 0.3052 | 0.3196 |

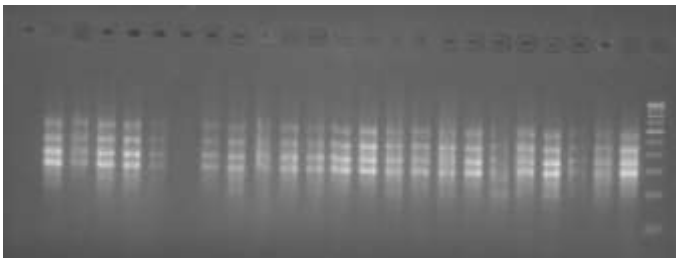
*Where hS: Average heterozygosity in sub populations, hT: Heterozygosity in the total population, GST: Relative differentiation among sub population, FST: Partition of the variation for the total population and FSR: Partition of the variation into the diversity among subpopulation within a zone*

**Population genetic structure**

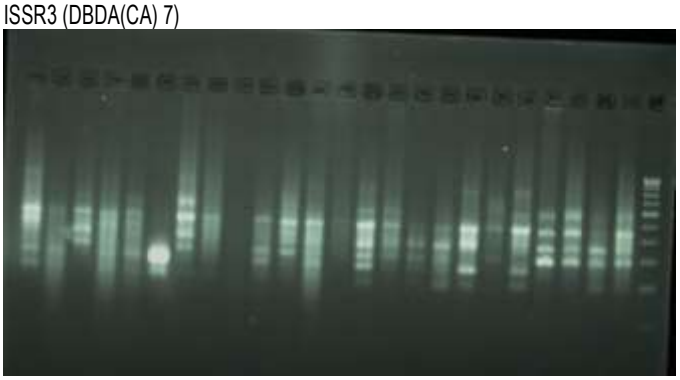
In population genetics, gene differentiation based on GST value is classified as low (<0.05), medium (0.05 -0.15 and high (>0.15) [Table-3]. The relative differentiation among sub populations (GST) value for *Ficus racemosa* populations was 0.5272. The variation for the total population of *Ficus racemosa* (0.3052) growing in different landscape elements of dry deciduous forest belts. Whereas, the variation into the diversity among subpopulation within a zone was low in *Ficus racemosa* (0.3196).

The calculated GST value for the three species was supported by AMOVA *i.e.*, Analysis of Molecular Variance which indicated that within the population had higher genetic variability (97%) and only 3% of the total variation was portioned between population among different landscape element [Table-4]. In addition to analyses of different population differentiation, the data generated during the study was further analyzed using cluster analysis and principal co-ordinate analysis. The clustering of the populations was clearly separated from each other forming their own clusters.

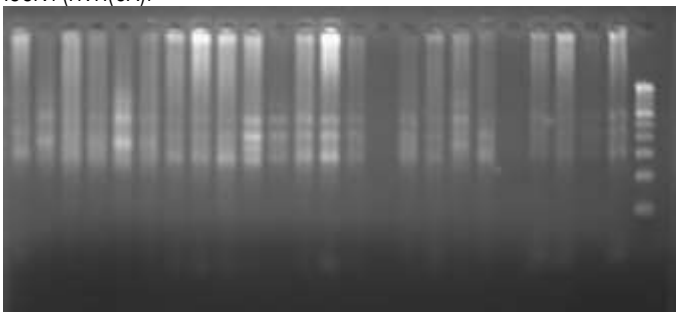
Fig-1 Collection of leaf samples of *Ficus racemosa* in coffee plantations for genetic diversity studies



ISSR3 (DBDA(CA) 7)



ISSR4 (HVH(CA)7



ISSR6 ((CA)8RY)

Fig-2 Bands obtained using ISSR primers for *Ficus racemosa* populations

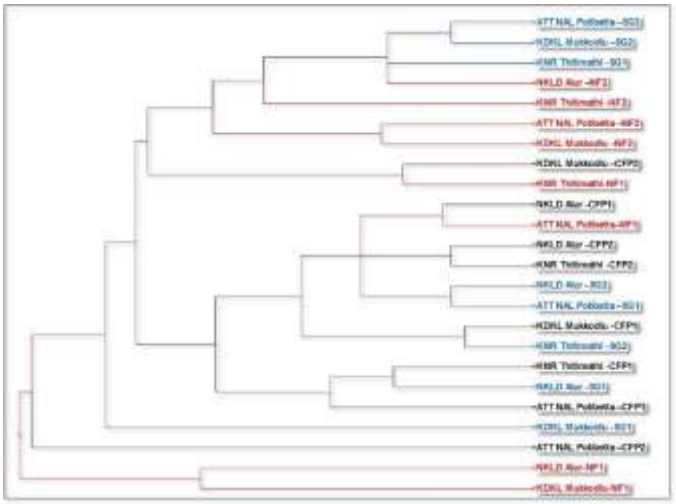


Fig-3 Hierarchical clustering of *Ficus racemosa* populations in landscape elements of dry deciduous forest belt

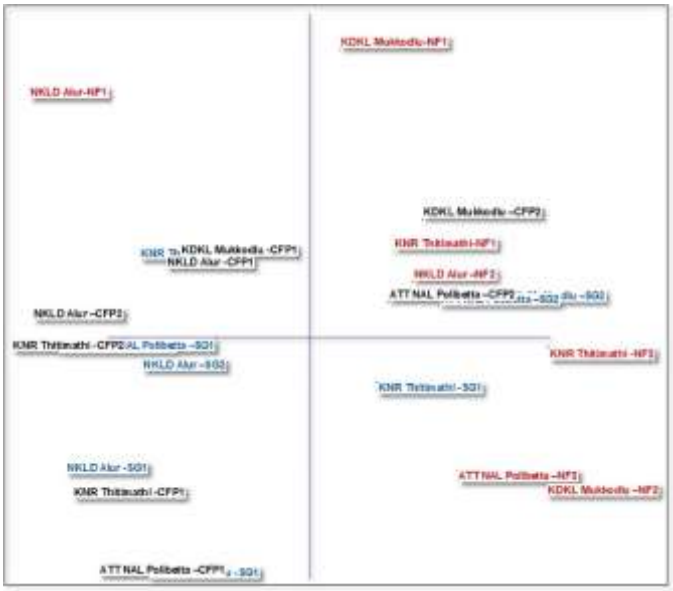



Fig-4 Principal co-ordinate analysis of *Ficus racemosa* populations in landscape elements of dry deciduous forest belt

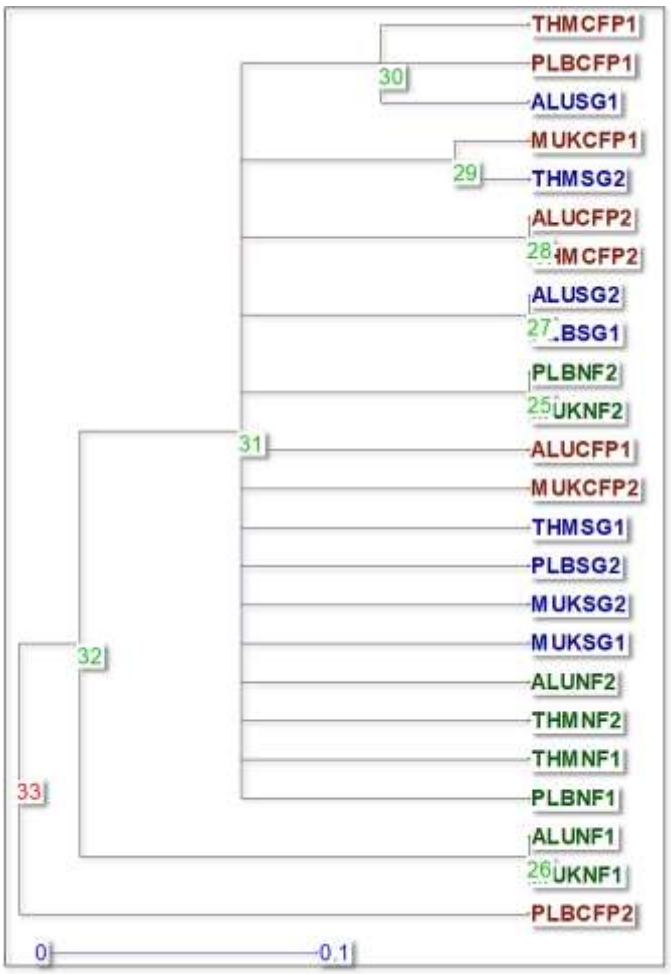



Fig-5 UPGMA phenogram based on Jaccard's dissimilarity coefficient for 24 populations of *Ficus racemosa* based on Nei's minimum genetic distance (Hierarchical joining)

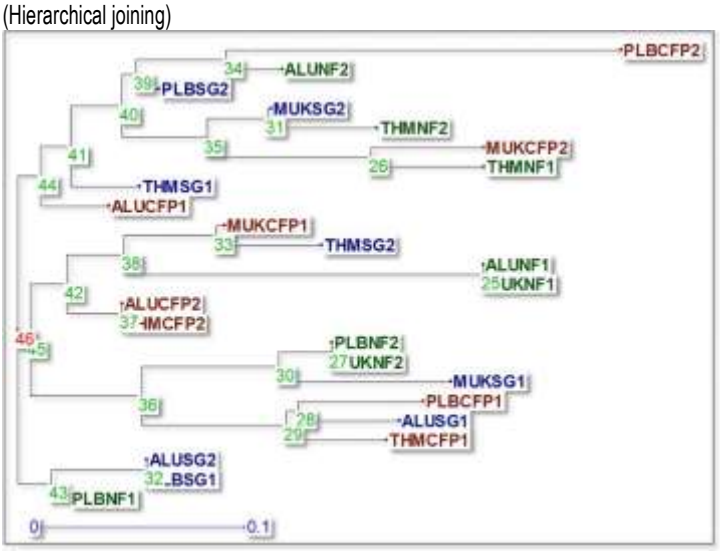



Fig-6 Neighbour joining tree for 24 populations of *Ficus racemosa* based on Nei's standard genetic distance

**Discussion**

Ficus species are considered as keystone species in an ecosystem and assessing their level of genetic diversity is important. However, till date several studies have focused on domesticated Ficus species and population genetic diversity studies using molecular markers are quite limited in this genus [3]. Highest percentage of polymorphism was recorded in populations from natural forests (89.47) and coffee plantations (73.68). The level of genetic diversity based on Nei's formulae and Shannon's information index showed higher in natural forests (0.2948 and 0.4456 respectively) and comparable diversity was observed in coffee plantation population (0.2352 and 0.3583, respectively). The results of the study are highly in reflection with studies by [16] where wild populations of a dioeceious species *F. pumila* expected heterozygosity values (0.53) that were higher than the mean value obtained in our studies for Neis genetic diversity and Shannon's information index (0.3474 and 0.5190 respectively). The probable reason for the higher genetic diversity in natural forests are due to highly outcrossed by insects, high gene flow and more aggregation of species confined to dry deciduous forest patches as it aids in moisture conservation. In Kodagu, most of the coffee farms have maximum number of *Ficus racemosa* which helps in the pollination of coffee flowers apart from its various ecosystem services.

The coefficient of gene differentiation at one level of population was moderate (0.5272). Variation for the total population at more than one level of structure, coefficient of gene differentiations was less.

The low value of gene differentiation may be due to the genetic makeup of trees as it sometimes behaves like monoeceious or dioeceious and these species are mainly propagated through vegetative cuttings. Analysis of molecular variance indicated that within the populations genetic variation was highest (97%) as compared to among the populations. The higher variations in this study could be due to higher activities of insect population within the area.

The population of *Ficus racemosa* growing in natural forests of KDKL Mukkodlu, NKLD Alur, sacred groves of KDKL Mukkodlu and coffee plantations of ATT NAL Polibetta formed separate cluster [Fig-3 and 4].

UPGMA phenogram based on Jaccard's dissimilarity coefficient for 24 populations of *Ficus racemosa* based on Nei's minimum genetic distance suggests that *Ficus racemosa* growing in coffee plantation of PLB, natural forests of ALR, MUK had a higher dissimilarity as compared to other populations [Fig-5 and 6].

**Conclusion**

In general, considering three landscapes, it can be concluded that coffee-based agroforestry regions do harbor higher level of diversity, mostly comparable to natural forests and sometimes better than that of sacred groves. This is perhaps the only study which has brought out this unique conservation value of coffee landscapes. Further, coffee plantations are in continuity with the natural forests, gene flow between them always encourages a better regeneration. However, as the habitats are reduced, population sizes plummet and become increasingly isolated and the gene flow between them can become restricted. This leads to genetic bottlenecks, increased random genetic drift, and inbreeding depression, can ultimately result in a loss of genetic variation and increased genetic differentiation between remnant populations.

**Application of research:** The finding of the research helps in genetic conservation of tree species in farmlands, plantations of coffee or tea planters so that its genetic erosion will be controlled.

**Research Category:** *Circa situm* conservation, Genetic conservation

**Abbreviations:**

ISSR: Inter Simple Sequence Repeats
DNA: Deoxy ribo Nucleic Acid, Bp: Base pairs
AMoVA: Analysis of Molecular Variance
PoCA: Principal Component Analysis
UPGMA: Unweighted Pair Geomtric Mean Arithmetic

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University: University of Agricultural Sciences, Dharwad, Karnataka, India
Research project name or number: PhD Thesis

**Author Contributions:** All authors equally contributed

**Author statement:** All authors read, reviewed, agreed and approved the final manuscript. Note-All authors agreed that- Written informed consent was obtained from all participants prior to publish / enrolment

**Study area / Sample Collection:** Kodagu district, Karnataka, India

**Cultivar / Variety / Breed name:** *Ficus racemosa*

**Conflict of Interest:** None declared

**Ethical approval:** This article does not contain any studies with human participants or animals performed by any of the authors.
Ethical Committee Approval Number: Nil

**Ethical Committee Approval Number:** Institutional Animal Ethics Committee (IAEC) -if the project involves field trails/experiments/exchange of specimens, human & animal materials etc.

**References**

[1] Frodin D.G. (2004) *Taxon*, 53,753-776.
[2] Wagner W.L., Herbst D.R. and Sohmer S.H. (1999) *"Manual of the Flowering Plants of Hawai'I". 2 vol. Bishop Museum Special Publication 83, University of Hawai'i and Bishop Museum Press, Honolulu, HI.*
[3] Judd W. S., Cambell C. S., Kellogg E. and Stevens P. F. (1999) *"Plant Systematics, a phylogenetic approach". Sinauer Associates. Inc.* pp.72, 73, 290, 291, 302, 304.
[4] Muscarella R T. and Fleming H. (2007) *Biological Review*, 82, 573-590.
[5] Wang R., Al B., Gao B.Q., Yu S., Li Y.Y. and Chen X.Y. (2009) *Population Ecology*, 51, 307-315.
[6] Ahmed S., Compton S.G., Butlin R. K. and Gilmartin M. (2009) *Proc. Natl. Acad. Sci. USA* 106, 20342-20347.
[7] Chatti K., Salhi-Hannachi A., Mars M., Marrakchi M. and Trifi M. (2003) *Biology*, 2, 1- 4.
[8] Dick C.W., Hardy O.J., Jones F.A. and Petit R.J. (2008) *Tropical Plant Biology*, 1, 20-33.
[9] Doyle J.J.and Doyle J. (1987) *Phytochemical Buletin*, 19, 11-15.
[10] Kimura M., Crow J.F. (1963) *Evolution*, 17, 279-288.
[11] Weir B.S. (1990) *Genetic data analysis-Methods for discrete genetic data. Sinauer Associates Inc Sunderland.*
[12] Nei M. (1973) *Academia Science*, 70, 3321-3323.
[13] Lewontin R.C. (1972) Evolutionary Biology, 6, 381-398.
[14] Sneath P.H.A., Sokal R.R. (1973) *Numerical taxonomy. W. H. Freeman and Company, San Francisco*.
[15] Saitou N., Nei M. (1987) *Molecular Biology and Evolution*, 4, 406-425.
[16] Weir B.S., Cockerham C. (1984) *Evolution*, 38(6), 1358-1370.
[17] Peakall R., Smouse P. E. (2006) *Ecological Notes*, 6, 288-295.
[18] Guasmi F., Ferchichi A., Fares K. and Touil L. (2006) *African Journal of Biotechnology*, 5(15), 1370-1374.