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RESEARCH ARTICLE

Genetic diversity analysis of endemic *Cycas* species using ISSR and SSR markers

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Abstract

Cycas nayagarhensis is a recently discovered cycad species in Odisha, India. Only a few populations were located in different regions of Nayagarh district. Due to the small population size, the species is under severe threat. A study was conducted to determine the genetic status of the population using various molecular markers. Inter-simple sequence repeat (ISSR) and simple sequence repeat (SSR) were utilised in the study. A total of 20 ISSR markers amplified 132 loci and 10 SSRs amplified 29 loci. The combined data were analysed through STRUCTURE software and revealed seven distinct populations (K = 7). Based on the combined data, the dendrogram segregated the samples into two distinct clusters. The clusters have high bootstrap values and cophenetic correlation, indicating the stability of the groups. A high degree of genetic similarity and less polymorphism showed the alarming conditions of the species. Average similarity among different samples was 0.78, with maximum and minimum similarity being 0.98 and 0.52 respectively. Among the different populations, maximum diversity was observed for Dasapalla (*Population 02*). Since there are few individuals in these populations, all the populations may be given special attention to conserve the species. This study will help formulate a conservation strategy for the endangered *Cycas* species.

Keywords: AMOVA; *Cycas*; genetic diversity; ISSR; SSR; structure analysis

Introduction

Cycads are one of the primitive groups of plants distributed throughout the world. These plants are under severe threat due to multiple reasons, such as habitat degradation and extensive use and exploitation for medicinal, food and decorative purposes. These species are listed among the families that are under the threatened category in the IUCN red list of plants (1). The *Cycas* population of the Indochina region was reviewed and the status of the different *Cycas* populations of the region was reported (1). Among different *Cycas* species, *Cycas nayagarhensis* is critically endangered (2). This species was discovered in the Nayagarh district of Odisha, India, located at a Latitude of 19^0 54' to 200 32' N and a Longitude of 84^0 29' to 850 27' E, as shown in Fig. 1 (1). The species is observed in very few populations in the Nayagarh district of Odisha, India. The habitat of *C. nayagarhensis* is severely affected due to urbanisation and unsustainable harvesting of seeds, leaves and male cones. Ovules, seeds and leaves are consumed in different forms. The leaves are also used for decorative purposes. Sometimes the entire plant is harvested and used for decoration of marriage or puja mandaps. Male cones are used as repellents for insects and bugs in Odisha and the north-eastern states of India. Decoction of leaves is very useful in curing cystolithiasis and stomach ache (1). The ever-increasing human population is the most important reason for the establishment of unauthorised human settlements near the *Cycas* population, causing forest clearing. The transformation of the forest into cultivated land and unhealthy cultivation practices, like shift cultivation, resulted in the depletion of the species. Rukhi, Balram and Gamein are the locations where only a few plants were reported (1). Presence of small population size and few mature trees; it may be listed as Critically Endangered, B2ab (iii, v) [IUCN Red List Categories and Criteria (Ver. 9, 2011)]. An elaborate study on the species *C. nayagarhensis* and its various causes of depletion has been previously reported (1).

Another observation made was that the pith of the plant is harvested for the extraction of the starch. This starch is profusely used in the making of Chyawanprash (3). For the above purpose, the plant is cut from the base, so there is almost no chance for the restoration of the plant. As this gymnosperm rarely forms branches, the chances of rejuvenation of the plant are very rare. The major drawback with the cycas plant is its slow rate of growth and germination from seeds takes a very long time (4). There is insufficient data about the plant, its genetic diversity and the status of the species. Similarly, there is no formal initiative for conservation purposes from any government or non-governmental organisation for either in situ or ex situ conservation. There is a lack of any study related to genetic resources and genetic diversity of the species. Besides their study, a thorough molecular study is essential to understand the genetic status of the plants. Population genetics plays a vital role in developing effective conservation methods (4, 5). So, in the present investigation, molecular characterisation was conducted to find out a potential population for conservation purposes.

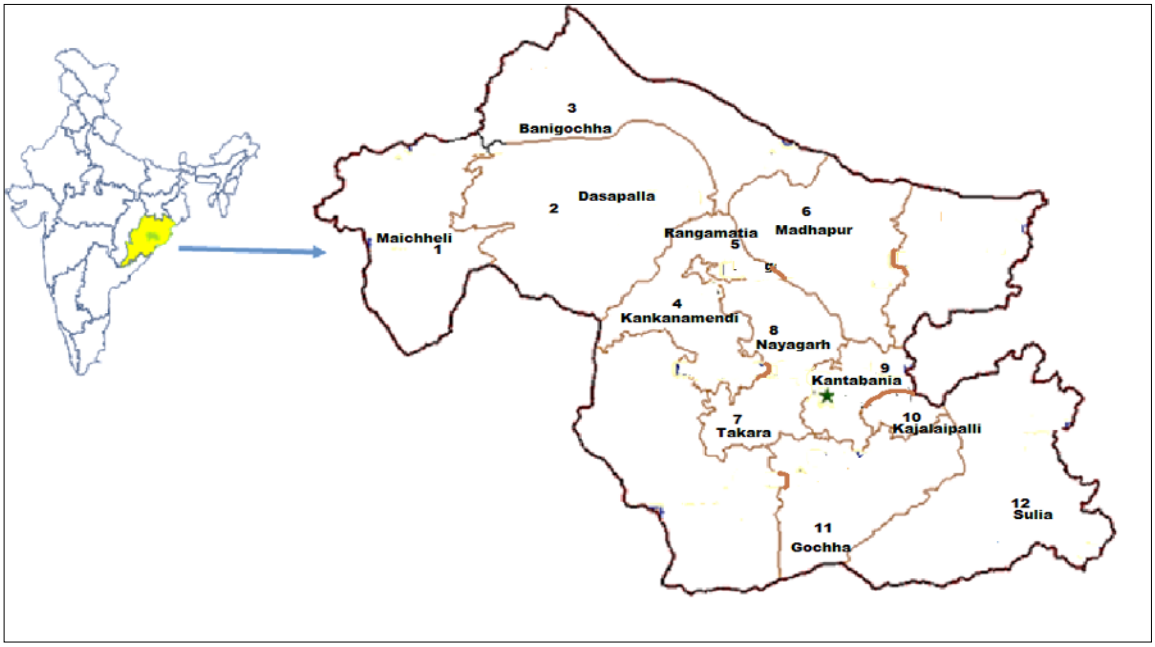


Fig. 1. Sample collection sites for *C. nayagarhensis.*

Materials and Methods

A total of 12 populations were identified and samples were collected in an ice box (Fig. 2 and Table 1). DNA was isolated from the fresh leaves following the standard methods (6). Twenty-four samples were taken from different populations. DNA was isolated from at least 10 plants from each population and pulled to two samples for each individual population. After isolation, purification and quantification, the DNA was diluted to 25 ng/uL for the Polymerase chain reaction. PCR was conducted as per established protocol (7). The PCR cycle consisted of three steps: denaturation, primer annealing and amplification at 92 °C for 1 min. Primer-specific annealing temp (Table 2 and 3) for 1 min. and 72 °C for 2 min. respectively. There was initial denaturation at 94 °C for 4 min. and final extension for 7 min. A total of 18 SSRs were searched from the EST data set available for *Cycas rumphii*. For this purpose MISA was used to detect the SSRs. Primers were designed using primer 3 plus software. Primers were procured from IDT, USA. Primers were dissolved as per manufacturers' instructions. PCR was performed following standard protocol. The amplified product was resolved in 2 % agarose gel stained with ethidium bromide. Gel running was done at 60V for 2 hr. The gels were documented through BioRad gel doc. DNA bands in gel images were scored as 1 and 0 for the presence and absence of a band.

Data analysis

Data were analysed using NTSYS-PC, POPGENE, Structure and GenAlex software (8–11). Structure analysis was done with the parameters such as burn-in period of 100000, MMC length of 100000, number of iterations to 20 per K with K value ranging from 1–10. Admixture and correlated allele frequency models were used with the Evanno ΔK method. The bands amplified from SSRs and ISSRs were scored as '1' and '0' for presence and absence of band, respectively. Both monomorphic and polymorphic loci were used for similarity calculation to avoid the bias of overestimation of distance (1). The Jaccards' similarity coefficient and a dendrogram were constructed on the basis of the similarity coefficient through the unweighted pair group method using arithmetic averages (UPGMA) and SHAN clustering. The resolving power (Rp) of the ISSR and SSR was observed (12–14). Resolving power is:

$$Rp=\Sigma IB \text{ (Band informativeness is } IB = 1-2*(0.5-P)] \quad \text{(Eq.1)}$$

Where P is the proportion of the samples containing the band.

We have calculated the Primer index (PI) from the polymorphic index.

A polymorphic index (PIC) was calculated as

$$PIC = 1-\Sigma P_i^2 \quad \text{(Eq.2)}$$

Where P_i is the band frequency of the i^{th} allele (9, 15). In the case of ISSRs, the PIC was considered to be $1-p^2-q^2$, where p is the band frequency and q is the no-band frequency (16). PIC value was then used to calculate the primer index. The primer index is the sum of the PIC of all the markers amplified by the same primer. Principal coordinate analysis (PCA) was used to retrieve information about the clustering pattern of the analysed species.

Table 1. Sample collection location of *C. nayagarhensis*

Location	Latitude	Longitude
Maichheli	20°10' 43.27'' N	85°00' 23.15''E
Dasapalla	20°19' 23.07'' N	84°50'28.87'' E
Banigochha	20°23' 41.81'' N	84°36'10.06'' E
Kankanamendi	20°23' 01.53'' N	84°31'37.65'' E
Rangamatia	20°21' 51.57'' N	84°47'32.43'' E
Madhapur	20°20' 43.01'' N	85°13'51.57'' E
Takara	20°22' 54.09'' N	84°46'06.24'' E
Nayagarh	20°08' 26.64'' N	85°06'17.51'' E
Kantabania	20°03' 53.24'' N	85°06'54.48'' E
Kajalaipalli	19°59' 46.47'' N	85°06'53.01'' E
Gochha	19°57' 29.77'' N	85°05'57.81'' E
Sulia	20°20' 11.14'' N	85°10'08.33'' E

Table 2. Banding pattern analysis for ISSR marker in *C. nayagarhensis*

ISSR	Primer	Annealing temp. (°C)	Total no of fragments	Range of amplicon (bp)	Resolving power	Primer index
1	(GACA)4	43	8	2500-100	8.5833	2.2604
2	(AGG)6	55	7	2000-100	6.5853	3.1146
3	(GTG)5	45	4	1500-500	3.4167	1.6215
4	(GA)9T	51	6	3000-500	8.25	1.5799
5	T(GA)9	51	8	3000-100	7.5833	3.191
6	(CAA)5	37	8	3000-150	7.4167	8.3438
7	(GTGC)4	51	3	3000-400	3.8333	1.3542
8	(AG)8G	47	4	3000-1000	4.25	1.2257
9	(AC)9G	53	3	3000-500	2.6667	1.3611
10	(AC)9T	51	5	3000-500	6.5	2.158
11	(TC)8G	45	8	3000-500	12.5	1.9514
12	(CGC)5	55	11	3000-50	12.5	4.5486
13	(CTC)6	55	10	3000-50	13.75	4.684
14	(GCT)5	45	6	3000-200	6.5	2.7222
15	(AGC)5CG	53	7	3000-100	7.833	3.9931
16	T(AC)9	51	5	3000-400	6.333	2.3819
17	G (AC)9	53	4	3000-1000	3.25	1.7882
18	(CA)8G	47	6	3000-100	8.75	3.2604
19	(CA)8AT	47	3	3000-2000	8.75	1.9826
20	(CA)6GG	47	5	3000-100	14.667	5.9722
21	(GAC)5	45	7	3000-100	6.917	3.1007
22	(CAA)5G	39	4	3000-100	4.9167	1.71
Total			132			

Table 3. Banding pattern analysis for SSR marker in *C. nayagarhensis*

SSR	Forward primer	Reverse primer	Repeat motif	No of allele	Annealing temp. (°C)	PIC
GM13	GAAGCAGAAGATTGCGAAGAA	AGCTTTCTGGGCCTGAGTTT	(AGA)4	5	55	0.638
GM14	CAATTTGTGGATTGCTGGTG	GCCTTGCCTTCCACATTTTA	(ACT)4	2	49	0.062
GM15	CAAGCGCTTATTGGAAGAGG	TAATCTCGTAAACCGGGACG	(CTT)4	2	52	0.492
GM16	TTGTTGGCAAAAAGGAGGAC	TGCTCTTGGAATGTCACTGC	(TAATT)3	3	53	0.478
GM17	GTATTCAGCTGTGGATCGCC	ATACTTGCGCTTCTCCCAAA	(AGA)4	2	49	0.363
GM18	GCAAAGCTCGATGAGCTACC	GTGAACCTCGAAATCCCAAA	(TAAT)3	3	57	0.258
GM19	GCCACCAGTAATGCCTGAAT	CATGACAGCCGTGGCTACTA	(TAGT)3	2	53	0.5
GM20	TCCATGTGCTTTGGAATCAA	GCAGACTTCTTTTCAACCCG	(AT)7	4	49	0.346
GM21	GAAGCACATCTTACCTCGCC	TTCCTTCGTTCTCCAGTGCT	(GCA)4(ACA)4	3	52	0.598
GM22	TGCTTTTGAAGAATTGCCCT	AGGGTCTCTTCGAACCGAAT	(AATT)3	3	55	0.478
Total				29		



Fig. 2. *Cycas nayagarhensis* at the site of collection.

Results

Inter-simple sequence repeat

Twenty-two ISSR primers resulted in the amplification of 132 loci. All the loci were amplified in the range of 3000 to 100 bps. Most of the fragments were monomorphic, while a few polymorphic bands were found for some primers. Among all the primers, (CGC)5 showed amplification for 7 loci and the primer (CTC)6 showed 10 loci (Fig. 3). The minimum amplifications (3 loci) were observed for (GTGC)4, (AC)9G and (CA)8AT. Among the primers, the (CA)6GG showed the highest resolving power (14.667). The least resolving power was recorded for the primer (AC)9G (2.666). The primer with the highest resolving power will be able to differentiate the populations well. Similarly, the primer index was also calculated for the different primers used. In this study, it was found that the maximum and minimum primer indices were 8.343 and 1.225 for the primers (CAA)5 and (AG)8G, respectively (Table 2). Jaccard's similarity was calculated between any two populations or samples. The maximum similarity was obtained between Cn20 and Cn22 (Jaccard's similarity 1.00), while the minimum similarity was observed for Cn22 and Cn3. The average similarity was found to be 0.495 between any two samples. The similarity by chance was observed to be 6.269×10^{-6}. The value indicated that the similarity as revealed from the molecular marker study is authentic but not due to mere chance. High bootstrap and cophenetic correlation values indicated the degrading status of the population as '5'. A dendrogram constructed with Jaccard's similarity segregated the samples into three clusters. Cn5 was separated into a distinct group, while the rest of the 23 accessions formed two groups. All the clusters have high bootstrap values, indicating the stability of the dendrogram.

Simple sequence repeat

A simple sequence repeat searched from *C. rumphii* helped in characterising the indigenous and endemic populations. A total of 1299 EST sequences were examined for SSR identification. This resulted in 118 EST sequences containing 138 SSRs. Out of these SSRs, 14, 63, 44, 14 and 3 are di-nucleotide, tri-nucleotide, tetra-nucleotide, penta-nucleotide and hexa-nucleotide repeat units, respectively (Fig. 4). The SSRs were tested and the 10 best SSRs in terms of the repeating unit and length of the amplicons were selected for the present study. All the samples showed an average of three alleles. A maximum of five alleles were observed for the GM 13 SSR (Fig. 5 & Table 3). The SSRs ' polymorphic information content was found to be 0.638 for the repeat motif (AGA)4 and the least informative SSR was 0.062 for the repeat unit (ACT)4. Jaccard's similarity was calculated for different samples of different populations on the basis of the SSR data. The maximum similarity was obtained for samples Cn22 and Cn21. The samples Cn19 and Cn10 were most distantly placed with a similarity index of 0.640. All the samples are related to each other by an average similarity of 0.799. The similarity by chance between the two samples is 0.1210. The high similarity by chance indicates that there is a narrow genetic diversity among the samples and population of *C. nayagarhensis*. All the SSRs are good in their PIC value. One dendrogram was constructed using Jaccard's similarity coefficient. All the samples were segregated into seven clusters. Samples sharing the highest similarity are found in cluster '1'. Fair cophenetic correlation and bootstrap value indicated that the dendrogram is very stable and robust. The maximum and minimum cophenetic correlations were 0.488915 and 0.922078 between several samples and Cn17 & Cn18, respectively. All the samples have an average cophenetic correlation of 0.799 and the cophenetic correlation by chance between any two samples is 0.011. Satisfactory amplifications were obtained with 13 primers. Details of the banding pattern and other information are presented in Table 3.

Structure analysis

The ISSR and SSR genotyping data were utilised to analyse the population structure using the STRUCTURE programme for the accessions under an admixed model (10). The estimated likelihood (LnP (D)) was observed to be highest when K = 7, indicating that the accessions used in the present investigation contained seven clusters (Fig. 6). None of the clusters was distinctly separated from each other; rather, all were admixtures. There is no clear-cut genetic divergence between the different populations; rather, the individuals share ancestry from multiple groups. This indicates that there is extensive gene flow due to migration and mixing events or interbreeding. There is unclear genetic separation between the populations. So, it is difficult to distinguish separate genetic groups within the samples**.** The members of these populations were seen as intermingled with each other from the bar plot of the STRUCTURE programme (Fig. 7). Though all the samples were collected from 12 populations, the data, when analysed, showed only seven distinct populations.

POPGENE analysis

All the populations were analysed to find out different characteristics of the populations. We found the maximum number of alleles observed (1.565) and allele expected (1.4484) for the 7th population. But this was minimum for population 3 for both observed number of alleles (1.35) and expected number of alleles (1.28). Similar observations were obtained when the Neis' diversity and Shannon's diversity indices were calculated. Both Neis' diversity and Shannon's diversity were maximum for the 7th population and minimum for the 3rd population (Table 4).

AMOVA analysis

Analysis of molecular variance showed maximum similarity among the different populations taken for the study. Similar findings were observed with ISSR, SSR and combined data. However, there exists a huge difference within a population (Fig. 8). With the ISSR marker system, the variation among populations was 2 % and the molecular variance within the population was 98 %. But in SSR, the variation among and within the population was 84 % and 16 %, respectively. The AMOVA results indicated that the genetic distinction and informational diversity are maintained within individual populations but not between different populations. However, the Shannon index indicated high genetic diversity among populations. The low level of molecular differentiation among populations indicates limited genetic variation at the molecular level. So, each population has a broad genetic base and as per Shannon's information, there is strong genetic connectivity with potential and ecological or adaptive divergence. These are important for conservation to maintain diversity within populations. However, when data for both marker systems were combined, it was seen that the overall diversity among populations was 6 % and the diversity within the population was 94 %. This indicates that there is not much variation among different populations of the *C. nayagarhensis* species. So, all the populations are equally important and need to be protected, giving equal weightage.

Table 4. Popgene analysis for the collected sample of *C. nayagarhensis*

	Na	Ne	H	I	PA	PPA	Nm
Pop1	1.5223	1.4178	0.2321	0.3324	82	52.23	0.2321
	0.5011	0.4009	0.2227	0.319			0.0496
Pop2	1.535	1.428	0.2378	0.3406	84	53.5	0.2378
	0.5004	0.4003	0.2224	0.3184			0.0495
Pop3	1.3567	1.2854	0.1585	0.227	56	35.67	0.1585
	0.4806	0.3844	0.2136	0.3059			0.0456
Pop4	1.5287	1.4229	0.235	0.3365	83	52.87	0.235
	0.5008	0.4006	0.2226	0.3188			0.0495
Pop5	1.3949	1.3159	0.1755	0.2514	62	39.49	0.1755
	0.4904	0.3923	0.218	0.3121			0.0475
Pop6	1.4076	1.3261	0.1812	0.2595	64	40.76	0.1812
	0.493	0.3944	0.2191	0.3138			0.048
Pop7	1.4586	1.3669	0.2038	0.2919	72	45.86	0.2038
	0.4999	0.3999	0.2222	0.3182			0.0494
Pop8	1.5605	1.4484	0.2491	0.3568	88	56.05	0.2481
	0.4979	0.3984	0.2213	0.3169			0.049
Pop9	1.3497	1.2854	0.1585	0.227	56	35.67	0.1585
	0.4806	0.3844	0.2136	0.3059			0.0456
Pop10	1.5285	1.4229	0.235	0.3365	83	52.87	0.235
	0.5008	0.4006	0.2226	0.3188			0.0495
Pop11	1.3959	1.3159	0.1755	0.2514	62	39.49	0.1755
	0.4894	0.3923	0.218	0.3121			0.0475
Pop12	1.4176	1.3261	0.1812	0.2595	64	40.76	0.1812
	0.493	0.3944	0.2191	0.3138			0.048

Na	Ne	H	I	Ht	Hs	Gst	PA	PPA	Nm
1.9554 ±0.027	1.7202 ±0.285	0.3977 ±0.1298	0.575 ±0.168	0.3977	0.291	0.4742	150	95.54	0.3977 +0.01

Na: Observed number of alleles; Ne: Effective number of alleles; Ht: Heterozygosity; H: Neis' gene diversity; I: Shannons' Information index; PA: Polymorphic amplicons; PPA: Percentage of Polymorphic amplicons.

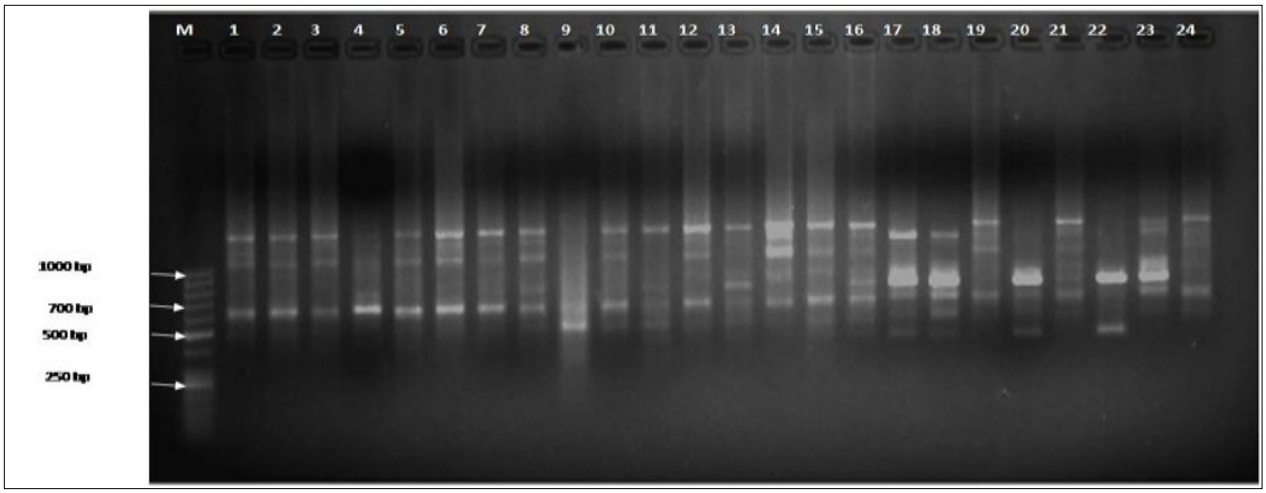


Fig. 3. ISSR Banding pattern in *C. nayagarhensis.*

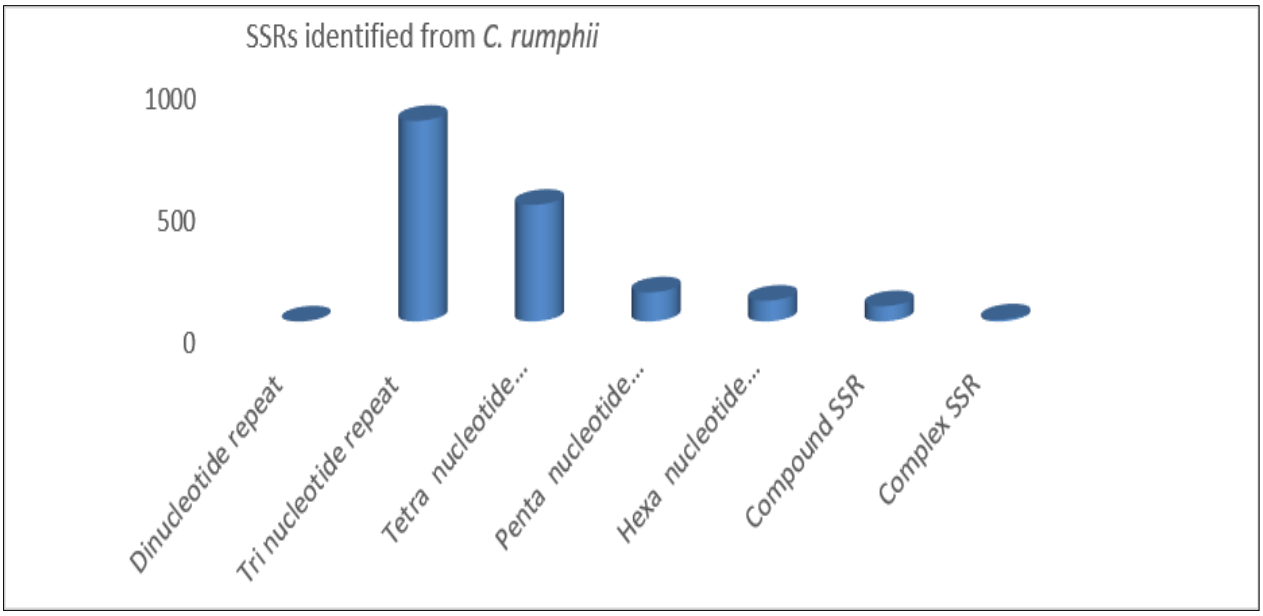


Fig. 4. Distribution of SSR from *C.rumphii* available from NCBI data.

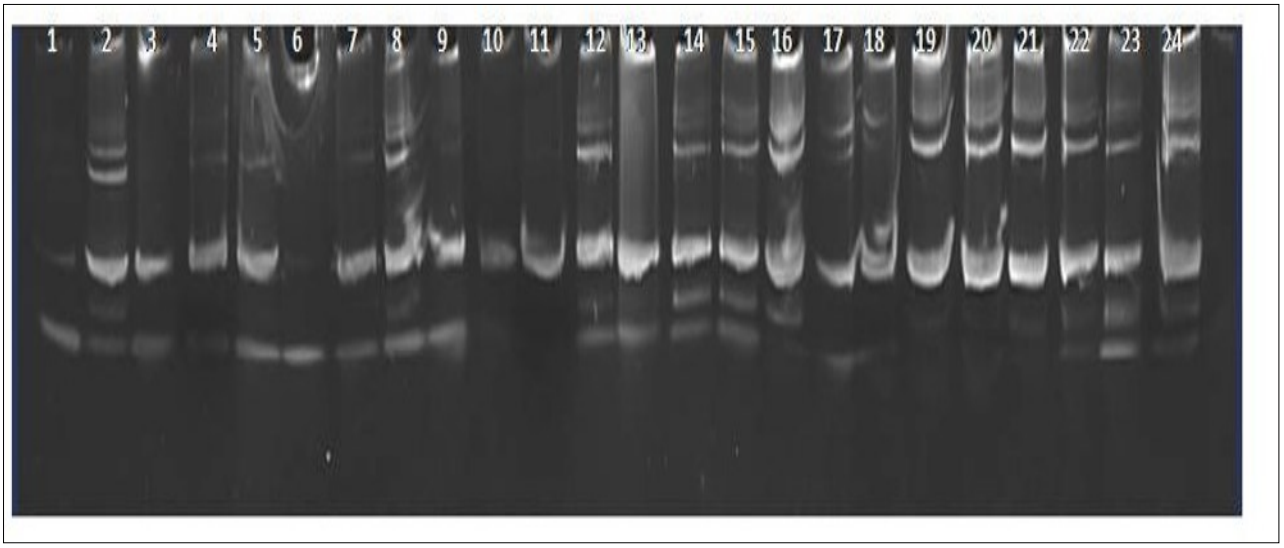


Fig. 5. SSR banding pattern in *C. nayagarhensis*.

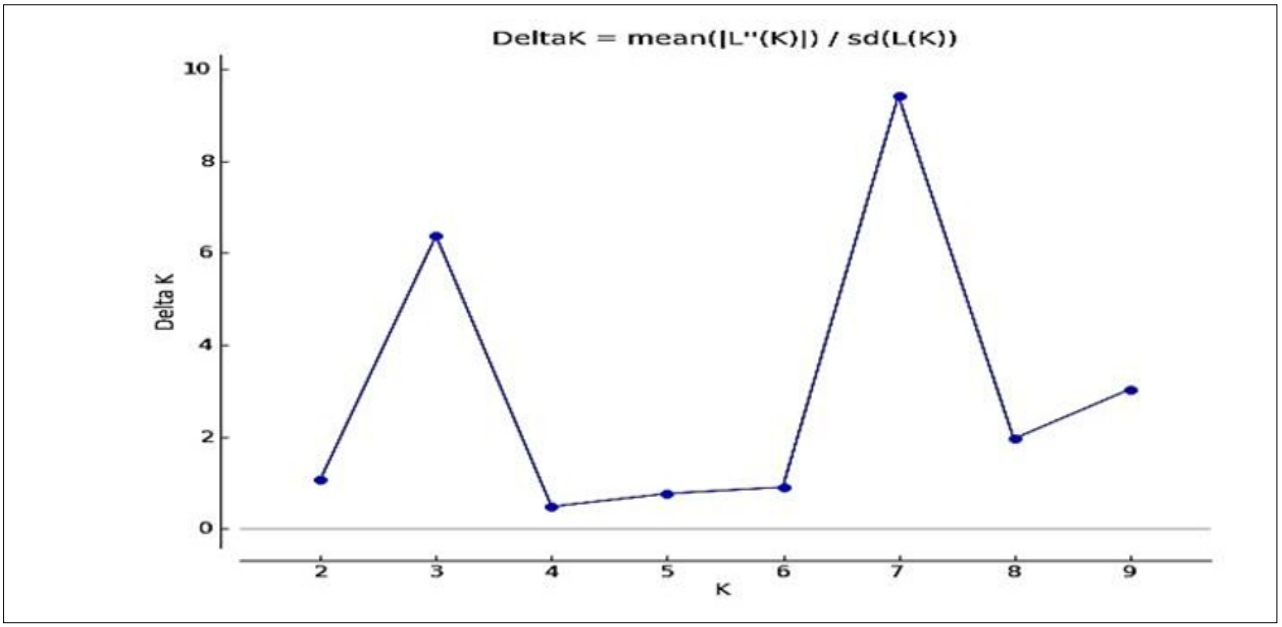


Fig. 6. Populations as revealed from structure.

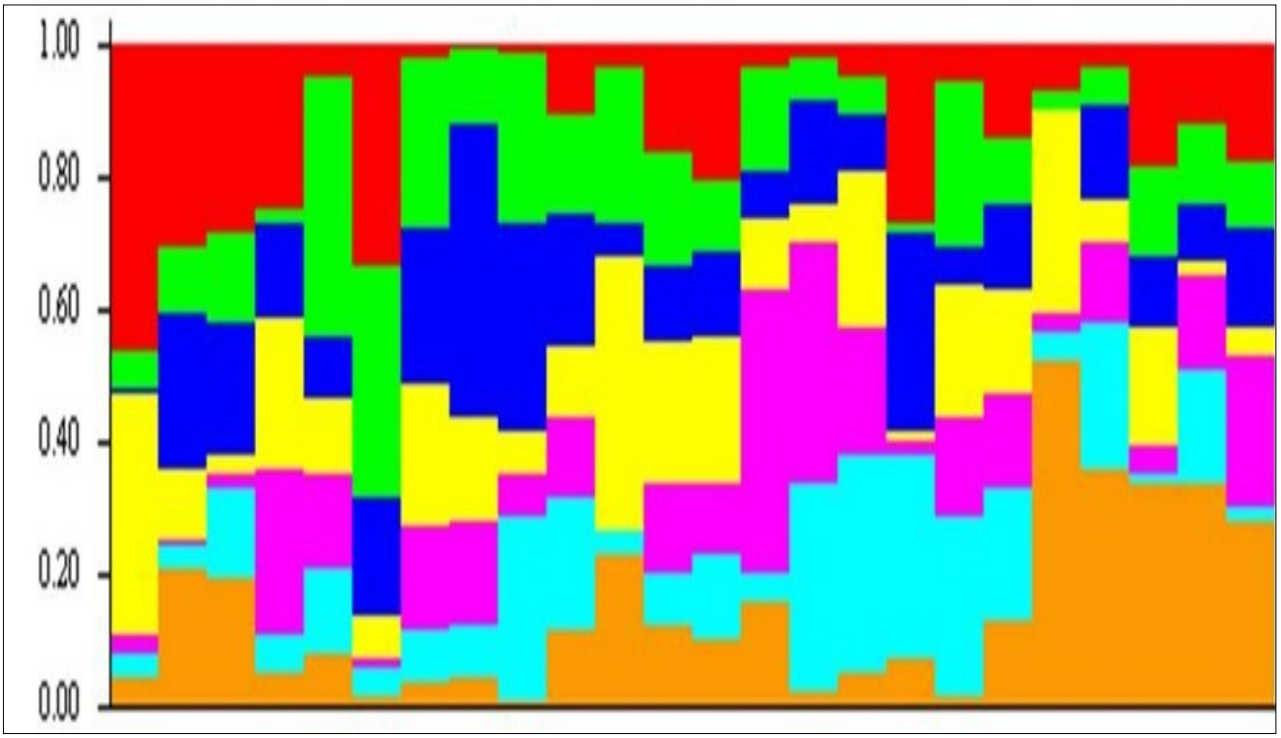


Fig. 7. Population distribution as shown through structure harvest.

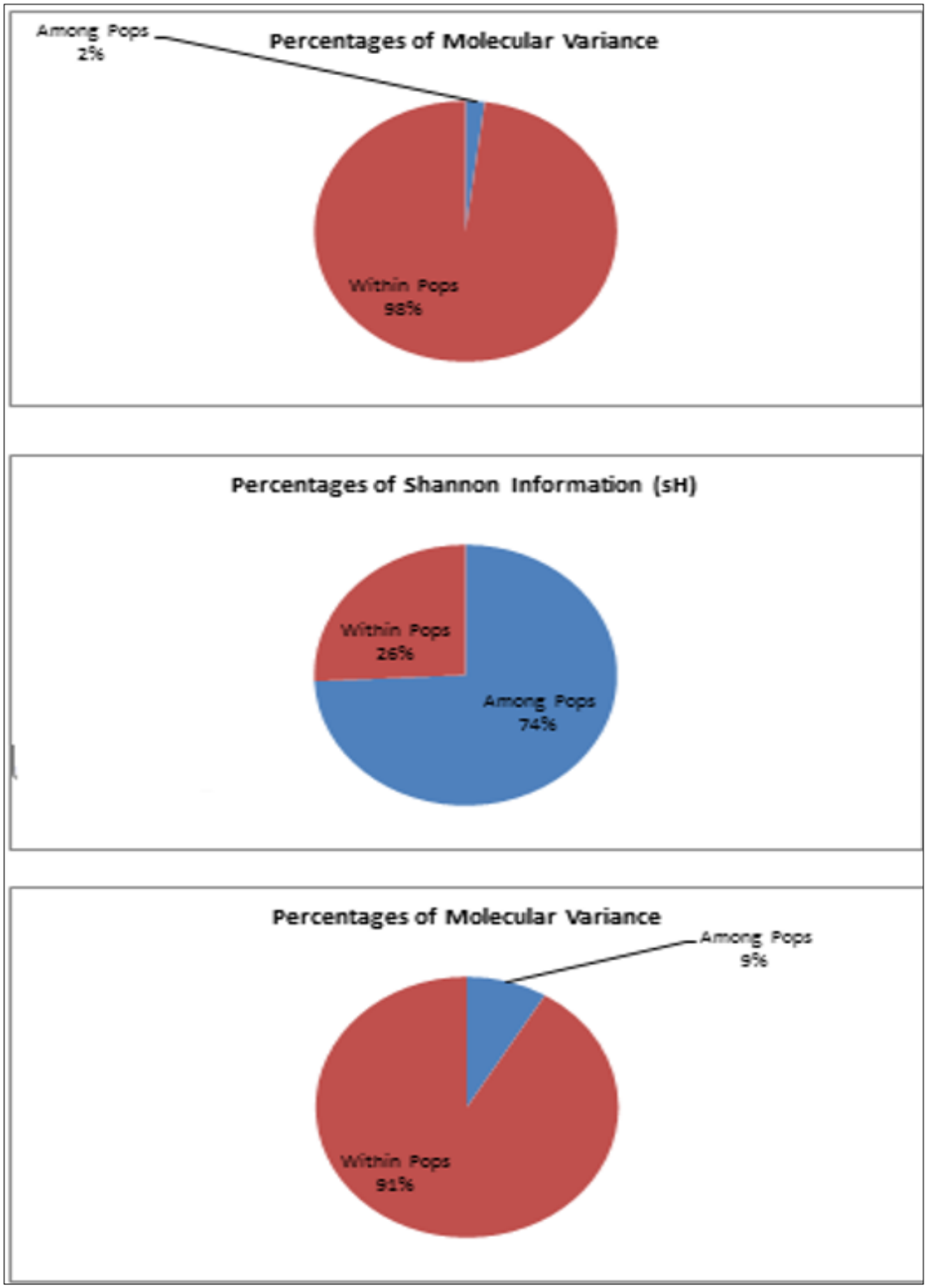


Fig. 8. AMOVA analysis of the populations.

Dendrogram constructed for combined data

A dendrogram was constructed on the basis of combined information revealed from SSR and ISSR data. Jaccard's similarity was calculated from the pooled data. It was quite interesting to see that there are certain samples for which Jaccard's similarity was fairly high, indicating the sample might represent a common origin. But some populations are quite distantly placed from each other. The highest similarity (1.00) was observed between Cn20 and Cn22. The samples Cn5 and Cn22 are most widely separated. All the samples were related to one another with an average similarity of 0.78. The samples are related due to co- incidence was very remote, as similarity by chance is 0.000145. A dendrogram was constructed and all the samples were segregated into 7 clusters. Not all the samples were grouped with other samples of the same population in some cases. The cophenetic correlation for the dendrogram and the bootstrap value for the clusters were calculated. High bootstrap value and cophenetic correlation indicated that the clustering pattern will normally not change with the addition of primers (Fig. 9).

Principal coordinate analysis

Principal coordinate analysis showed that all the members of the populations were seen as mixed and distributed along all the coordinates (Fig. 10). Though seven groups can be seen in the diagram, different samples of unrelated populations were seen together. It has been found that samples of the same population are grouped along with other populations. There are very small groups, containing only one sample. So, with a greater number of alleles, the grouping may change. But one thing can be said: the

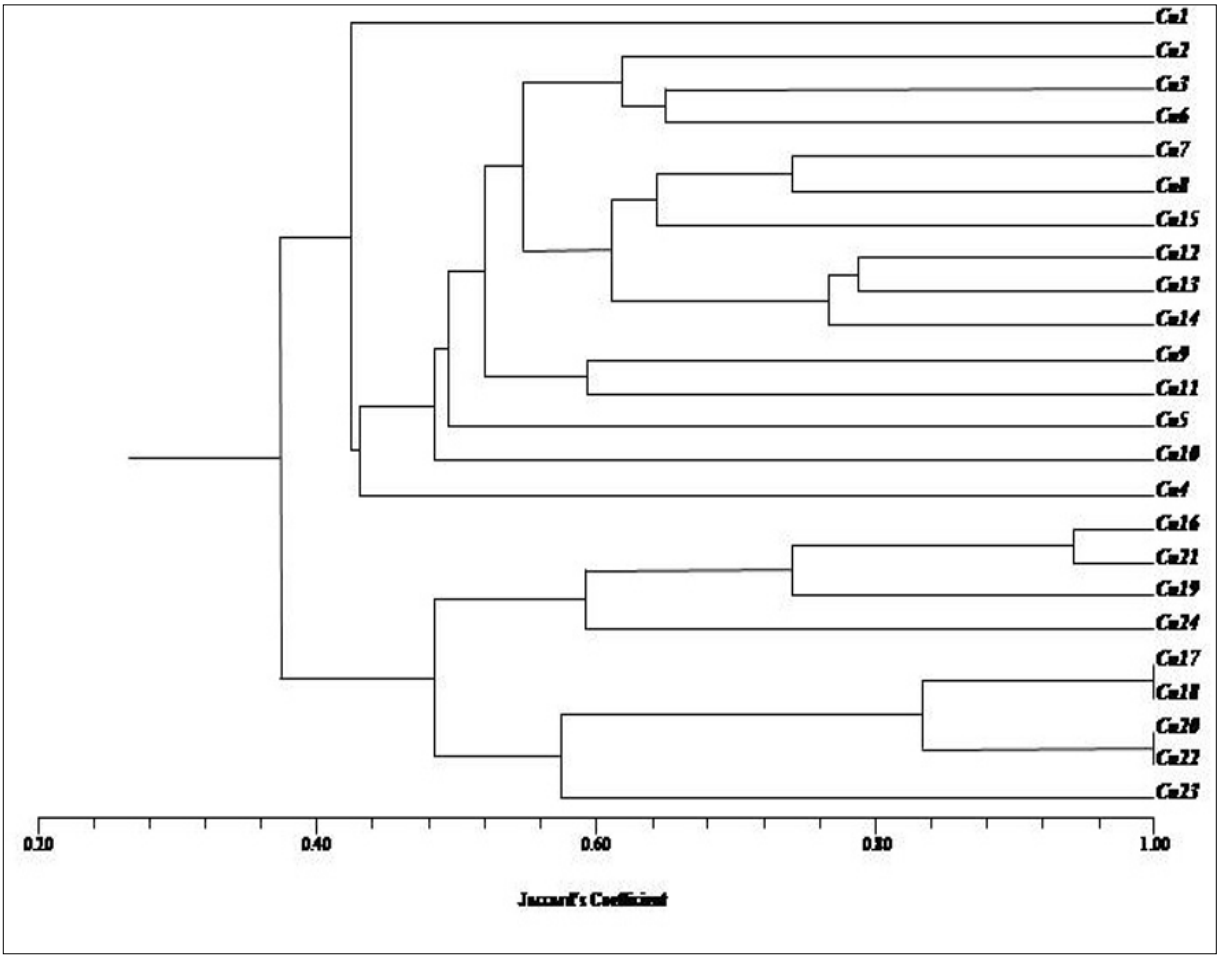


Fig. 9. Dendrogram among different samples collected from different locations.

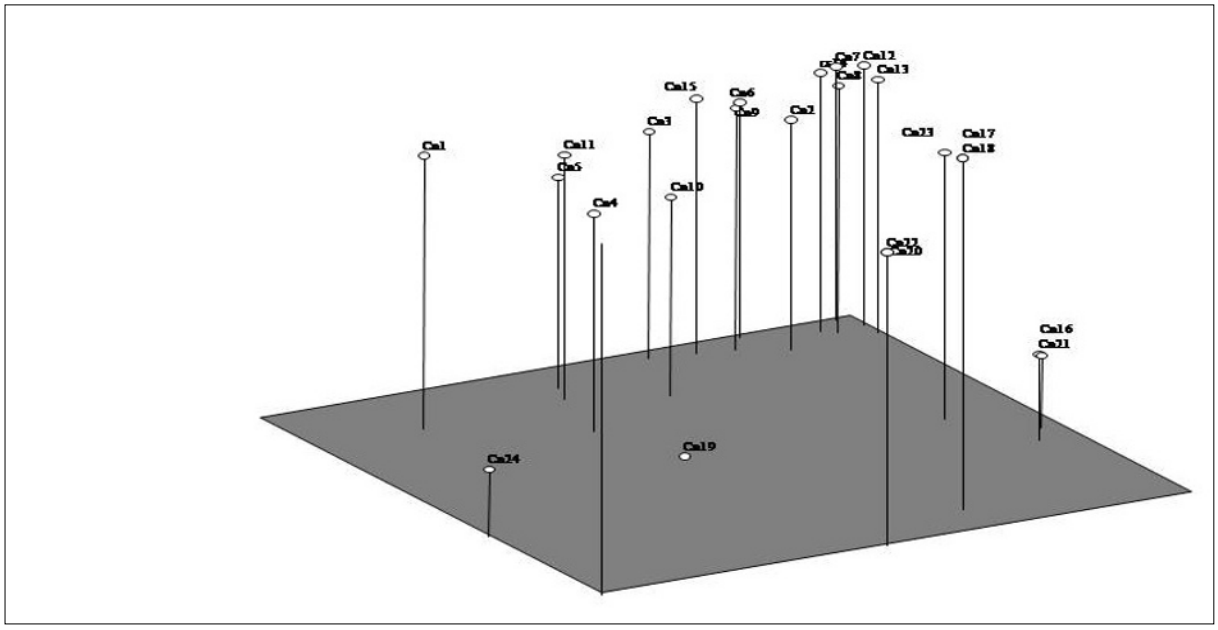


Fig. 10. Principal component analysis of samples collected from different locations.

populations were very closely related. This indicates that they are very close to each other without any distinct genetic variation. All the individuals of different populations were not segregated into distinct groups; rather found intermingled with each other. This may be an indication of a fragmented population.

Discussion

A high degree of correlation (0.85) was observed between the ISSR and SSR markers. Similarly, the latter was more strongly correlated with the combined data (0.93) than the former marker, which had a correlation of 0.85 with the pulled marker data. Significant correlations among the marker systems indicated the robustness and complementary characteristics of these systems in deciphering genetic diversity in *C. nayagarhensis*. These results were consistent with earlier studies on rare species characterised by combinations of marker systems (17, 18). A fair correlation among the marker systems demonstrated the efficacy and reliability of the genetic pattern independent of a particular marker system.

Genetic diversity and population structure

Across all populations, homozygosity was observed, which is a significant conservation concern. Homozygosity in small populations indicated the prevalence of inbreeding depression (19, 20). A higher proportion of monomorphic than polymorphic loci was observed with the ISSR marker system, indicating genetic erosion in *C. nayagarehnsis*. Exactly similar patterns were observed in other endangered cycads, such as *Cycas calcicola* in Australia (21). The species has low to moderate gene diversity (uHE=0.037-0.135) and a high inbreeding level (FIS=0.49). Interestingly, a similar observation was made in *Cycas guizhouensis*, with low genetic diversity at both the population and species levels, which may be due to a limited number of individuals and gene flow (22). All individuals in the present investigation showed high to moderate relatedness, as indicated by Jaccard's similarity coefficient. The mean similarity for ISSR and SSR was 0.495 to 0.799. Similar results were obtained for the threatened cycad species *Cycas seemannii** from Fiji, where similarity ranged from 0.52 to 0.81 (23). The maximum similarity observed between Cn20 and Cn22 with ISSR and between Cn21 and Cn22 with SSR may be due to clonal propagation, localised seed dispersal, or population bottlenecks. Samples were collected from 12 sites, but structure analysis identified seven populations. The result may be due to genetic admixture and population connectivity. No clear-cut genetic groups were indicated; ancestral sharing of genetic material across multiple populations and historical gene flow. Different marker systems target different regions of the genome. So, the SSR and ISSR systems showed differences in similarity levels. The high similarity in SSR and low similarity in ISSR reveal a higher resolution of recent demographic events (24).

The AMOVA results revealed the population structure among different *C. Nayagrhensis* populations exhibiting 2 % and 16 % genetic variation, as determined from ISSR and SSR data, respectively. So, most genetic variation occurs at the individual rather than the population level. This pattern, in which within-population variation accounts for 94 % to 97 % of total diversity, has been reported in other tree species, such as *Castanopsis fargesii** and indicates that populations are not genetically separated (25). The combined data, which showed 6 % genetic variation among populations and 94 % within populations, provide an ultimately reliable estimate of genetic diversity. Contrasting results were obtained between ISSR and SSR markers with respect to population differentiation (2 % and 16 %). Results were identical to those reported for *Lavandula stoechas**, where ISSR markers revealed 35 % variation among populations, whereas EST-SSRs showed distinct patterns (26). It may be due to differences in the targeting loci in the genome by the ISSR marker system, which may be under different selective pressures or evolutionary constraints compared to the genic regions targeted by SSRs (27). So, the importance and implications of the multiple marker system are essential for characterising populations. The diversity observed within the population was higher than the differentiation among populations. It may indicate that *C. nayagarhensis* populations were historically connected and that substantial gene flow occurred. So, the current population fragmentation may not be in the distant past, but relatively recent. The genetic signatures of this disintegration may not be complete due to the generational lag effect (28). Even if populations stabilised demographically, there is a chance of further genetic error in subsequent generations (29).

Genetic load and inbreeding depression

Low polymorphism and higher homozygosity were observed in the present investigation, suggesting the accumulation of genetic load in *C. nayagarhensis*. Genetic load reduces fitness by accumulating deleterious alleles in the gene pool (30). When populations are small, they are vulnerable to the fixation of deleterious genes through genetic drift (31). Deleterious mutations accumulate faster than selection when the population size is below the critical level, pushing the population towards extinction (23). The high homozygosity observed in **C. nayagarhensis* may be due to substantial conversion of masked load into realised load through inbreeding, potentially reducing population fitness. This conversion occurs when the small population size increases drift and inbreeding. Inbreeding allows recessive deleterious alleles to become homozygous and expressed (32). Hence, the fitness consequences of such realised load can manifest as reduced seed set, lower germination rates and decreased low-seeding survival. After several generations, due to genomic impacts, population decline lags behind demographic changes (33). So, the current genetic character reflects the population's size in the recent past, not its current condition, leading to genetic erosion, even as populations stabilise demographically. This genetic and demographic disconnect complicates the conservation strategy and underscores the importance of proactive genetic management (34).

Comparison with other cycad studies

The results obtained in the present investigation are in agreement with specific findings, while contrasting with others. In *C. segmentifida*, researchers identified two genetic groups confined to northwest and southwest China (35). However, the plant shows relatively low genetic vulnerability to climate change rather than a potential evolutionary response (35). Exactly similar observations were made in *C. calcicola*, with low genetic diversity (0.37-0.135), limited gene flow and high inbreeding. In other cycad species, such as *Encephalartos* species in South Africa, high genetic differentiation was observed. The genetic differentiation is attributed to limited seed dispersal and habitat fragmentation (36). However, moderate genetic differentiation and historical gene flow were observed among Zamia species in the Caribbean. Habitat fragmentation recently impacted genetic structure (37). In *C. nayagarhensis*, limited differentiation exists among populations, unlike in *C. taitungensis*, which showed significant differentiation due to geographic isolation (38). However, in the present study, there is no geographic isolation resulting in low genetic differentiation. *Cycas fairylakea* in China exhibited pronounced population structure corresponding to the locations of reported watershed boundaries (39). The population structure is very weak, the geographic range is restricted and continuous population fragmentation is recent, leading to the accumulation of genetic differentiation.

Shannons' diversity index and population viability

Maximum observed and expected alleles were obtained for population 7, while minimum values were obtained for population 3. Similarly, both Shanons' and Neis' diversity indices were highest in population 7 and lowest in population 3. So, population 7 is a potential genetic reservoir and priority must be given to its conservation. Similarly, population 3 is at higher risk and genetic augmentation is required to restore the population. Shannons' diversity index values in this study are comparable to those

reported earlier for other threatened cycads. In *Cycas szechuanensis*, Shannons' diversity ranged from 0.214 to 0.387 across populations, while *Cycas balansae* showed values of 0.198 to 0.312 (40, 41). The values obtained for *C. nayagarhensis* fall within this range, confirming that the species exhibits genetic diversity typical of threatened cycads, but concerning from a conservation perspective. In conservation biology, there is an established relationship between genetic diversity and population viability (42). Populations with low genetic diversity are at increased risk of extinction and inbreeding depression (43). The high level of diversity in *C. nayagarhensis*, coupled with its small population size, suggests a higher risk. Long-term population viability will depend on maintaining and, if possible, increasing diversity through habitat protection and population expansion (44).

Population fragmentation and demographic concerns

Representative samples from different populations are seen intermingled in the principal coordinate analysis. The samples are not clustered into distinct geographic regions, suggesting a fragmented yet historically connected metapopulation structure. The high bootstrap values (indicating dendrogram stability) and cophenetic correlations (0.488915 to 0.922078 for SSR data) confirm that these genetic patterns are robust and not due to sampling or marker preference. The major threats to *C. nayagarhensis* are habitat degradation and population fragmentation. The rate of loss of alleles is inversely related to adequate population size and in small populations, genetic erosion is rapid (45). Genetic erosion reduces genetic diversity and increases extinction risk under a changing environment (46).

Conservation implications and recommendations

All populations are worthy of conservation and protection. The AMOVA results indicated low genetic differentiation. To capture the entire genetic breadth of the species, all populations may need to be protected. Population 7 is the best population due to its higher Neis' and Shannons' indices and it has the highest chance of sustainability. Given the high diversity of the population, the population size must be maximised to conserve the species. In larger populations, diversity is less likely to be maintained due to genetic drift (44). The effects of fragmentation can be reduced through habitat restoration and the establishment of corridors for gene flow. The risk of homozygosity can be addressed through genetic monitoring and consideration of genetic rescue through managed gene flow between populations. Genetic rescue, the introduction of individuals from genetically distinct populations to reduce inbreeding and increase fitness, has proven effective in numerous species (47).

Along with *in-situ* conservation, ex-situ conservation may also be preferred and must include representatives of all seven distinct populations as identified by structure analysis. Priority should be given to population 5 for its higher genetic diversity and sustainable properties. Attention must be paid to pollination ecology, reproductive biology and seed dispersal mechanisms to maintain and increase genetic diversity. Early germination protocols and propagation methods will allow for sustaining and increasing the population size of *C. nayagarhensis*.

The genetic characteristics of *C. nayagarhensis* indicate the species' vulnerability. In particular, low genetic diversity, homozygosity and small population size are the major factors underlying the species' current status. This study indicates the plants' fragile nature. So, the species needs special attention for its conservation, including increasing diversity and protecting its habitat.

Conclusion

The present study focused on the evaluation of the genetic diversity of different populations of *Cycas nayagarhensis* to understand their conservation potential. It was observed that there is a maximum of seven populations and each of them has a similar genetic structure. So, all the populations should be given due attention for conservation. Since habitat loss is a major concern, care must be taken to restore the habitat and prevent its utilisation for other purposes. Similarly, it is important to stop the cutting of mature plants for commercial utilisation and ex-situ conservation methods must be adopted to conserve the species before it becomes extinct in the wild.

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Authors' contributions

GM conducted the experiments and wrote the original draft. LA contributed to writing, review and editing, supervision and conceptualisation. Both authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

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