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Using RAPD Analysis for *in situ* and *ex situ* conservation of *Conocarpus erectus* in the Central Region of Ghana

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Abstract: ***Conocarpus erectus*** **is a woody shrub belonging to Combretaceae family. The species provides important ecological and socio-economic benefits to many organisms at coastal areas of Ghana. However, *C. erectus* is threatened to extinction from wildfires, sand winning, wood collections, farming, building, road construction and illegal mining, leaving just a few fragmented populations that require immediate protection. Little efforts by local communities, Ghana Forestry Commission, and non-governmental organizations to conserve *C. erectus* through *in situ* and *ex situ* methods have been challenging as they relied on general seed collections from fragmented populations without any genetic investigation about these populations. To propose scientific and effective *in situ* and *ex situ* conservation of *C. erectus* in the Central region of Ghana, investigating genetic diversity of the fragmented populations is needed in selecting best genotypes for seed collection. Random Amplified Polymorphic DNA (RAPD) analysis was used. Fifteen primers were screened to ten which were used to produce clearly polymorphic bands with average number of 85.1 per primer. Analysis of Molecular Variance (AMOVA) identified 90% and 10% variance between and within populations respectively. An average number of 10.2 bands per primer was recorded with amplification products ranging between 200 and 2000 bp. PHIst of 0.90 implies significant degree of variability in *C. erectus* populations, therefore seeds should be collected from all fragmented populations to preserve more of the species' unique characters for *in situ* and *ex situ* conservation of C. *erectus*.**

Keywords*: Conservation; Conocarpus; Fragmented Populations; Genotypes; Seed Collection.*

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I. INTRODUCTION

In Ghana, about 23.54 km (3.7%) of the total coastal stretch of 636.24 km between Eastern and Western collidors are covered by mangroves, mostly found near lagoons and lower delta of River Volta of which *Conocarpus erectus* receives the least recognition and protection among the four mangrove types (Bunting et al., 2022; Nunoo and Agyekumhene, 2022). *Conocarpus erectus* is an evergreen woody shrub belonging to the family Combretaceae (Bashir et al., 2015). According to Aye et al. (2019), Das and Crépin (2013) and other researchers including Sandilyan and Kathiresan (2012), and Das et al. (2022), C. *erectus* serves as nursery habitats for young fishes, crabs, birds, and many organisms along the coast, serves as wind and wave breakers, traps solid pollutants including used polyethene bags, and controls soil erosion. Traditionally, *C. erectus* is used for treatment of ailments including catarrh, diabetes, fevers, conjunctivitis, headache, anemia, diarrhea, wounds gonorrhea, syphilis, tumors, and hemorrhoids (Nascimento et al., 2016; Raza et al., 2018; Santos et al., 2018; Khurm, 2023). *Conocarpus erectus* contains anticancer and antioxidant materials used in pharmaceutical industries (Bashir et al., 2015; Raza et al., 2018; Namjoyan et al., 2020). *Conocarpus erectus* suffer threats from over-exploitation for firewood and charcoal production, farming activities, building, road construction and illegal gold miming (Olowokudejo and Ozioma, 2020; Nunoo and Agyekumhene, 2022) leaving just a few fragmented populations along the coasts. However, these few extant populations face threats to extinction therefore require immediate protection. Efforts to safeguard the species are very slow and have relied on non-governmental organizations (NGOs), Ghana Forestry Commission (GFC) and local communities.

Successful restoration of *C. erectus* through *in situ* and *ex situ* conservation methods could largely depend on genetic diversity of the few remaining fragmented populations to identify trees with best genotypes to serve as seed source for nursery establishment. According to Ivetić and Devetaković (2017) and Oluwajuwon (2022), gathering information on genetic diversity of tree species for restoration activities is essential to combat changing climatic conditions because adequate genetic variations within populations have improved fitness, productivity, resilience, and fecundity. Again, Nonić and Šijačić-Nikolić (2021) also found from their study that there is a positive relationship between levels of genetic diversity and productivity in restored forests. However, there is limited information on the levels and trends of diversity among the few remaining fragmented *C. erectus* populations. Just a few molecular studies of the species have been carried out as compared with other mangrove species in Ghana (Nunoo and Agyekumhene, 2022). To recommend best and effective *in situ* and *ex situ* conservation methods to protect the extant populations from extinction, this study used Random Amplified Polymorphic DNA (RAPD) marker to uncover genetic variability within and between the few remaining fragmented populations of *C. erectus* in the Central region of Ghana.

II. METHODOLOGY

A. Collection of Sample

Young, fresh, and healthy leaf samples of *Conocarpus erectus* were collected from Apam, Winneba, Saltpond, Cape Coast, and Elmina coastal communities from the Central Region of Ghana as showed in figure 1. The study area is located within latitudes 5.3511 and 5.0847, and longitudes -0.6231 and -1.3509 covering about 90 km stretch along the coast. Collection of leaf samples were done in February 2024 and kept in labelled zip-lock plastic bags containing silica gel for rapid drying of the leaves until extraction in the laboratory. At least five individuals were randomly selected from each site, tagged and about four young leaf samples were harvested from each tagged tree.

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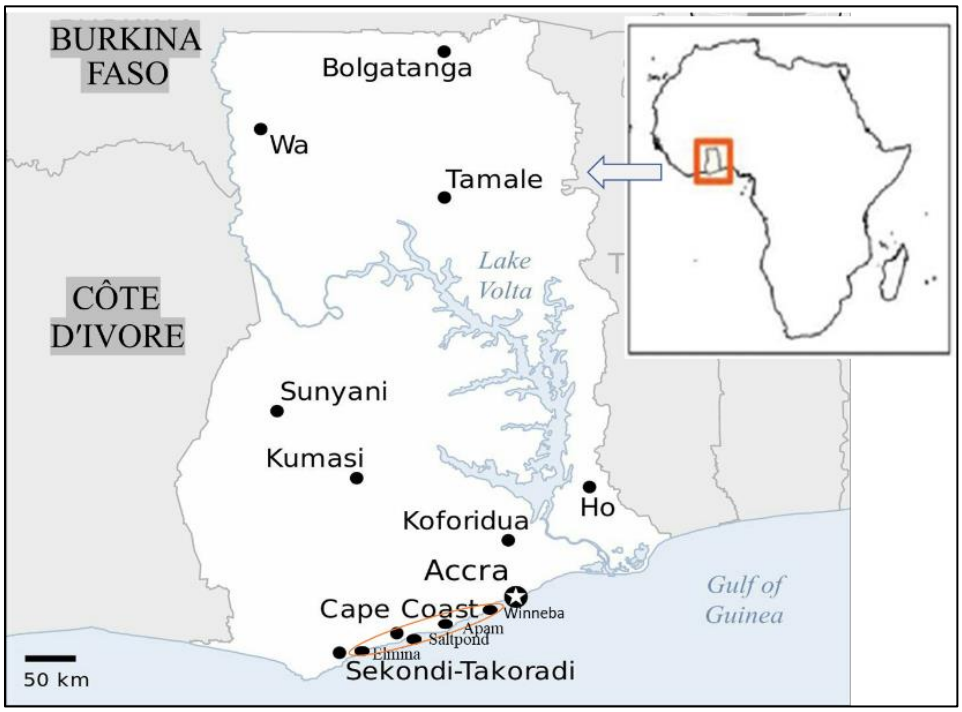

Fig 1 Map of Ghana showing study sites at Cape Coast, Elmina, Saltpond, Apam and Winneba in the Central Region of Ghana

B. DNA Extraction and Amplification

RAPD marker was used to characterize *C. erectus*. Reason for its selection includes its readily availability from manufacturers, less expensive, straightforward procedure which does not require any prior information about the target genome and requires just a small quantity of the leaf sample. Mortar and pestle which had been chilled (prior to use) were used to pound one gram of dried leaf samples into very smooth powder in liquid nitrogen and kept in centrifuge tubes. About 20 mg of the leaf samples were weighed for genomic DNA extraction using DNeasy plant mini kit (QIAGEN, UK.LTD.), at CSIR-Crops Research Institute Molecular Laboratory at Fumesua, Kumasi, Ghana following the steps in the manufacturer's protocol.

The first step involved addition of lyses buffer followed by RNase to the weighed leaf sample before incubating it at 65ºC for 10 min in a water bath. This step activated RNase to digests RNA present in the leaf sample. The lysate was spun for 10 min at 13,000 rpm to remove polysaccharides, proteins, detergents, and salt precipitates. This step was repeated twice to further remove any remaining polysaccharides, polyphenols, and secondary metabolites of *C. erectus* that may hinder efficient extraction of pure DNA.

This step was followed by transfer of the collected supernatant to a QIAshredder spin column before spinning at 13000 rpm for 2 min to remove non-pelleted precipitates and cell debris. The next step involved addition of binding buffer solution to which a specified amount of ethanol had been added, to the collected lysate to promote binding of DNA to the DNeasy membrane in a new tube. The mixture was transferred to a DNeasy mini spin column before spinning at 8000 rpm for 1 min for binding of DNA to the membrane. This step was followed by addition of buffer AP3 to which ethanol was added prior to use, to facilitate efficient binding of DNA to the membrane. The remaining contaminants including proteins and polysaccharides, were further removed by washing with buffer AW twice. Spinning was done for 1 min at 8000 rpm in the first wash, followed by repeated spinning for 2 min in the second wash to dry the membrane and removed any remaining ethanol likely to interfere with the remaining reactions in the subsequent step. Transfer of the DNeasy mini column to a new storage tube was done to elute the pure genomic DNA by using a 100 µl volume of low salt suspension buffer and spinning at 8000 rpm for 1 min. This step was repeated with another 50µl.

The DNA extracts were quantified by using spectrophotometer (UV/Vis Spectrophotometer U-2001, Hitachi Instruments Inc.). Absorbance at 260 nm (A_{260}) was measured before use. Purity of the extracted genomic DNA was checked using the ratio of absorbance at 260 nm to 280 nm and 230 nm. Polyphenol and polysaccharide contaminations were checked using the ratio A_{260}/A_{230}. Testing quality of the extracted DNA was done by running the DNA samples for 1 hour 30 mins at 80 volts on a 1% agarose gel which had been stained with ethidium bromide, using 1×TBE buffer solution. UV trans-illuminator was used to visualize the agarose gel. Gel Doc (Bio Rad) was used to take photograph.

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C. Primer Screening, Amplification of PCR Products and, Running of DNA on Agarose Gels

Randomly testing of primers were done for their polymorphism in C*onocarpus erectus* for selection of best primers to differentiate the populations. Fifteen primers were selected for screening for their quality to amplify PCR products and reproducibility. Screening was repeated twice, and ten best primers were selected (Table 1). Amplification reactions of DNA were performed in volumes of 25µl per sample in each run that contained $MgCl_2$, nucleotides (DNTPs), 1 x enzyme buffer, a reaction mixture of DNA suspended in buffer solution, primer, and Taq DNA polymerase enzyme. A negative control, which targeted DNA was replaced by deionized water was included in each amplification test. Thermocycler (PCR Express, Hybaid, UK., Ltd.) was used to conduct thermocycling of 36 cycles for 2 min at 94^{o}C to denature the double stranded DNA. Primer annealed to the DNA when temperature was lowered to 45^{o}C for 1min. Extension of the DNA strand was followed when the temperature was raised to 72^{o}C for 2min. This cycle was followed by a final extension of DNA for 7 min at 72^{o}C shortly after completion of all cycles. This ensured that the DNA strand was completely extended. The products of the PCR samples were stored at 4^{o}C prior to electrophoresis. Running of the PCR products were done on a 1 % agarose gel (stained with ethidium bromide to facilitate visualization) using 1×TBE buffer solution for 80 minutes at 100 volts. The DNA amplified fragments obtained were visualized under ultraviolet transluminator at 302nm wavelength recording the pattern by a camera linked with a computer. The DNA bands were observed in a gel documentation system (Alpha Innotech). Photograph was taken by using Gel Doc (Bio Rad).

D. Data Analysis

The DNA amplified bands from the photograph of the agarose gel were scored for presence (1), or absence (0) of specific PCR bands generated by a primer. Unclear and low visual intensity DNA bands were not scored. Bands scoring data were used for different analyses which included Jaccard's coefficient of similarity (Jaccard, 1908), measured to generate a dendrogram based on similarity coefficients that was generated by the un-weighted pair group method (UPGMA) using arithmetic averages. Euclidean distance which is a measure of dissimilarity between samples was done using PCORD 5.0 (McCune and Mefford, 1999) software programme. The result was used in cluster analysis for construction of a dendrogram which was used in describing genetic relationships among populations using Ward's sorting (McCune and Mefford, 1999). PCORD 5.0 was used to perform Correspondence Analysis to provide scatter plot of similarity between individual tagged trees from the fragmented populations from the five communities. A pairwise Euclidean distance matrix was generated to describe the genetic structure and variability among populations using AMOVA-PREP (M. P. Miller: web site hppt://herb.bio.nau.edu/~miller/amovaprp.htm). AMOVA-PREP was used to perform Analysis of Molecular Variance (AMOVA) on these distances to divide the variation into within and between population components (Excoffier et al., 1992) and Principal co-ordinate analysis (PCA) was used to confirm grouping of the accessions.

III. RESULTS

Though all the fifteen RAPD screened primers produced scorable bands, ten primers that produced better and more clearly polymorphic bands to determine genetic diversity among the *C. erectus* fragmented populations were used. The A_{260}/A_{280} ratio lied in the range of 1.68 to 1.74 with A_{260}/A_{230} ratio >2 which implied that the extracted DNA was good and pure, and free of proteins, polyphenolics, and polysaccharides. The DNA concentration ranged from 6.8 to 7.6µg/mL Average number of bands obtained per primer was 10.2 and amplification products ranged from 200 bp to 2000 bp. An average number of 85.1 polymorphic bands was obtained per primer with primers GEN I-60A, GEN I-60I and GEN I-60J recording highest number of polymorphisms and primers GEN I-60C and GEN I-60E recorded lowest (Table 1).

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Table 1 RAPD Profiling of *C. erectus* Showing Levels of Polymorphism from 10 Primers

Primer code	Nucleotide sequence (5'-3')	Total no. of bands	Polymorphic bands		Molecular size range (bp)
			Number	%	
GEN I-60A	CGCAGTACTC	11	10	90.9	200-2000
GEN I-60B	GTCCTACTCG	10	9	90.0	200-1300
GEN I-60C	CTACACAGGC	7	6	85.7	200-1300
GEN I-60D	GTCCTTAGCG	10	8	80.0	200-2000
GEN I-60E	GTCCTCAACG	9	7	77.8	200-1200
GEN I-60F	GAGTCACTCG	11	9	81.8	200-2200
GEN I-60F	GTCCTCAGTG	10	8	80.0	200-2000
GEN I-60H	CGTCGTTACC	11	9	81.9	200-2000
GEN I-60I	GCAGACTGAG	12	11	91.7	200-1500
GEN I-60J	GCAGACTGAG	11	10	90.9	200-2000
Average		**10.2**	**8.7**	**85.1**	

The 10 RAPD primers used detected adequate amount of genetic diversity between and within the fragmented populations of *C. erectus.* Within and between population variability estimated by percentage of polymorphic loci in the *C. erectus* populations from the five communities are shown in Table 2. AMOVA identified 90% and 10% variance between populations and within populations respectively. A coefficient of population differentiation (PHIst) value of 0.90 showed very high population differentiation between populations, low gene exchange between the fragmented populations of *C. erectus* from the five communities but high gene flow among individuals within the various populations.

Table 2 AMOVA Structure and Results from 26 Populations of *C. erectus* on RAPD Analysis.

Sources of Variation	Sums of Squares	Degrees of Freedom	Mean Sums of Squares	Variance Component	% Total Variation
Between populations	22	3.629	0.165	0.165	90.0
Within populations	4	1.038	0.260	0.018	10.0

* PHIst=0.90; evaluated by 1000 permutations

All the ten primers selected showed high degrees of polymorphism within and between populations of the C. *erectus* samples run on agarose gel (Fig. 2). Banding patterns within populations showed few variations. Some populations also showed population-specific bands.

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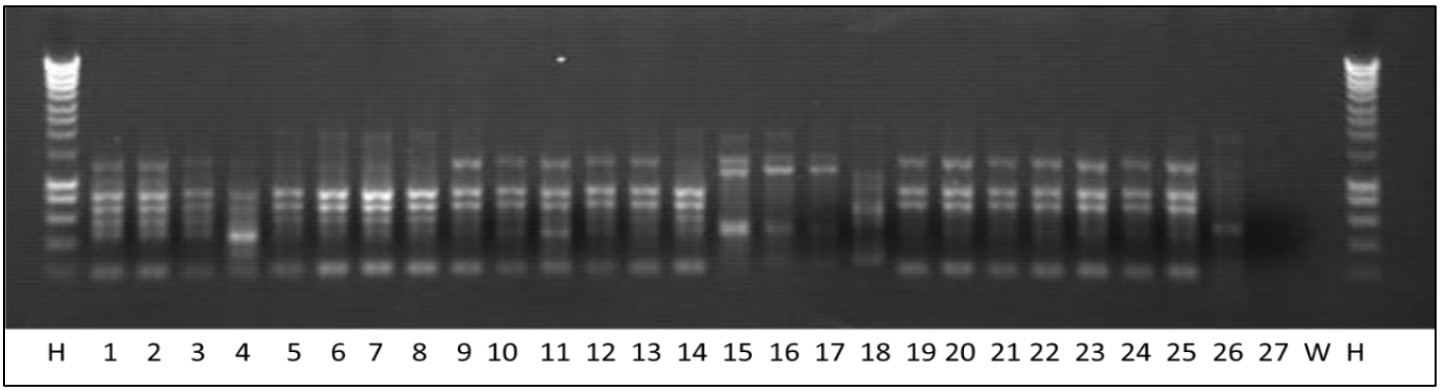

Fig. 2 Gel Image Showing PCR Product Amplifications of *Conocarpus erectus* Using Primer GEN I-60I. H=hyperladder; lanes 1-5=Winneba; lanes 6-10=Apam; lanes 11-15=Saltpond; lanes 16-20= Cape Coast; lanes 21-26= Elmina; lane 27=positive control (*Rhizophora mangle*); W=negative control (water)

Most of the fragmented populations of *C. erectus* from the five communities clustered together indicating their genetic relatedness. The dendrogram based on the cluster analysis grouped *C. erectus* genotypes into four main groups. One cluster consisted of two genotypes from Apam and Winneba. A second group consisted of mixed populations from Winneba, Apam, Saltpond and Elmina. Genotypes from Cape Coast got separated from the remaining *C. erectus* populations to form third and fourth groups although they shared some commonalities with just some few genotypes from Elmina and Saltpond. Populations from Winneba, Apam, Saltpond and Elmina shared common genes together but segregated themselves from genotypes from Cape Coast and Saltpond (Fig. 3), however, *Rhizophora mangle* used as positive control were separated from *C. erectus* accessions and was extreme in the dendrogram. The Principal Co-ordinate Analysis (PCoA) also separated *C. erectus* accessions into four groups along the first coordinate axis, segregating *C. erectus* populations from *Rhizophora mangle* which was used as positive control.

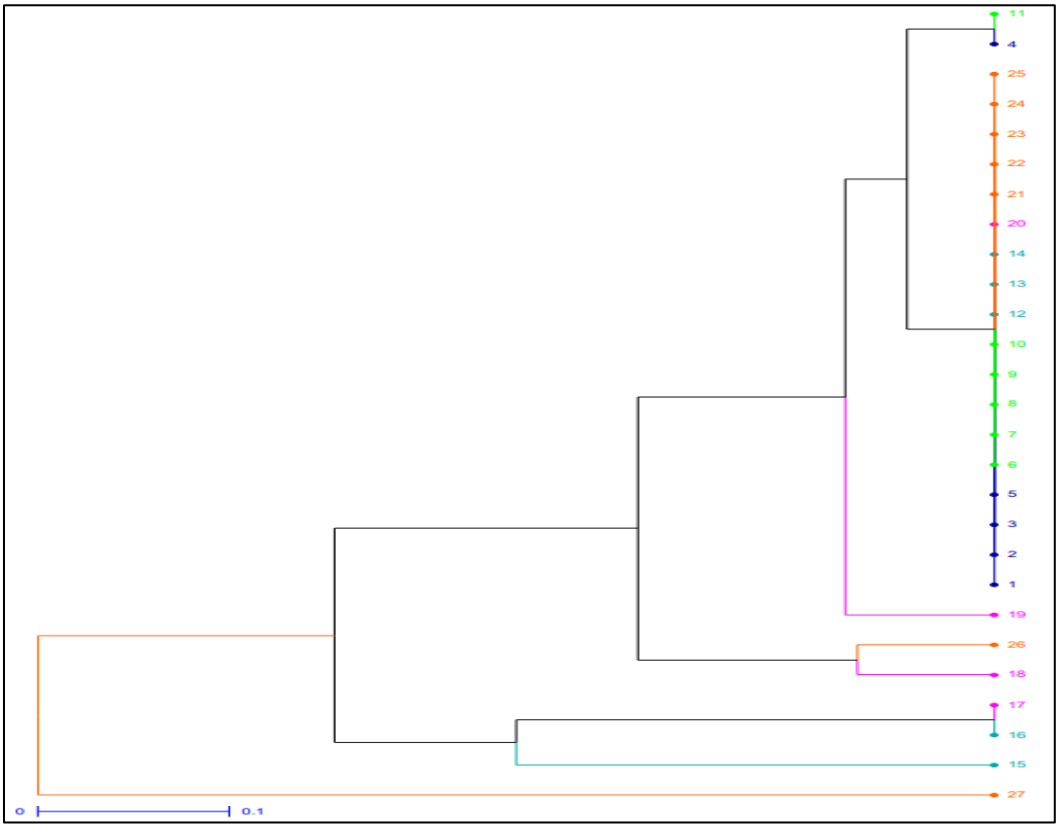

Fig. 3 Genomic Relationship Among 26 genotypess of *Conocarpus erectus* using RAPD marker. 1-5=Winneba; 6-10=Apam; 11-15=Saltpond; 16-20=Cape Coast; 21-26=Elmina; 27=positive control (*Rhizophora mangle*); W=negative control (water)

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IV. DISCUSSION

The present study represents the use of molecular (DNA) markers in estimating genetic diversity in *Conocarpus erectus* population. The results showed that RAPD is very useful and informative in assessing genetic diversity in *C. erectus*, which conforms with research by Hadrys et al. (1992), Williams and Clair (1993), Tewari et al. (2022) and Dompreh et al. (2026) that RAPDs is very useful marker in assessing genetic diversity among plant populations. Twenty-six accessions of *C. erectus* were studied using ten primers, and DNA amplification fingerprinting technique clearly differentiated between the fragmented populations of *C. erectus* from the five communities in the Central Region of Ghana. Most variation was identified between fragmented populations of *C. erectus* from Winneba, Apam, Saltpond, Cape Coast and Elmina. High PHIst value of 0.90 recorded indicated high level of genetic differentiation between the fragmented populations implying low gene flow between individuals. However, some of the genotypes of *C. erectus* clustered together to show their genetic relatedness irrespective of low gene flow between populations. The *Rhizophora mangle* used as positive control got separated from the *C erectus* populations indicating its genetic difference. This is confirmed by their morphological differences in some characteristics including leaf structure, flower type and absence of pneumatophores. However, they share some common morphological characters that justify their taxonomic alienation. AMOVA identified 10% variance within populations giving indications of high gene exchange between individuals within populations but poor gene exchange between populations making them differentiated from each other as 90% variance between *C. erectus* populations were recorded. Similarly, Dompreh et al. (2011) recorded weak genetic variation within but strong between fragmented plant populations of their study of greater than 30 km apart.

Distances between some *C. erectus* populations greater than 10 km serve observed may serve as geographical barriers that prevent free flow of genes among the populations leading to genetic isolation and inbreeding depression within populations. This results support findings of others including Frankham et al. (2002), Allendorf et al. (2013), Binks (2019) and Salgotra (2023) that free gene flow between individuals trees within and between populations helped maintain and improved genetic diversity of the species. Similarly, Wu et al. (2023) concluded in their plant populations study that habitat fragmentation, human interference, restricted gene flow and biological characteristics among plant populations are some of the main drivers of genetic diversity and structure of plant populations. This study also is in line with findings of other studies including Carugati et al. (2018) that anthropogenic activities and climate change are some of the contributing factors of population fragmentation and deterioration of mangroves. However, Reynolds et al. (2012), revealed that maintenance of genetic diversity of extant and restored mangrove populations offers opportunities for natural selection occurrence in response to changing environmental conditions. The distance between some fragmented populations is less than 200 m and this may have contributed to low variation within populations. According to Lowenfeld (1992), mangrove populations with less than 200 m apart have limited genetic structure that can facilitate high self-pollination rates among individuals. This calls for urgent need to maintain genetic diversity and preserve the few fragmented populations from extinction. *In situ* and *ex situ* conservation of *C. erectus* in the coastal regions of Ghana using seeds from trees with best genotypes will ensure high percentage germination, survival rate, and growth performance of the species. Planting seedlings with best genotypes in populations with low genetic variability will help improve the species' fitness to combat the changing climatic conditions as suggested by Hoffmann et al. (2021). Provenance mixing experiments conducted by Pretzsch (2021) showed better improvement in genetic diversity, tree growth and stand productivity when different provenances of one tree species were mixed. Lack of education, law enforcement and community participation affect conservation of the species. As suggested by Nonić and Šijačić-Nikolić (2021), education, law enforcement and community involvement will help protect threatened species. Conservation of *C. erectus* in the coastal areas of Ghana will help improve mangrove populations, ecosystem services, protect coastal areas against storms, and support fish population in the coastal communities.

V. CONCLUSIONS AND RECOMMENDATIONS

RAPD marker was effective in assessing and delineating genetic variability within and between fragmented populations of *C. erectus.* Genetic diversity of *C. erectus* suggests efficient sampling strategies to be considered in deciding on which populations should be targeted for seed collection and its implications for conservation of the species. Limited gene flow between *C. erectus* populations may cause more deterioration of genetic diversity within populations, influence inbreeding, and lower population differentiation at individual levels. High population differentiation between populations was revealed. All fragmented populations of *C. erectus* from the five communities should be considered important. Trees with best genotypes from each population should be tagged for seeds collection for nursery establishment to preserve more of the species unique characters from all populations. Exchange of seedlings among populations in *in situ* and *ex situ* methods will facilitate gene exchange and improve species' fitness to withstand changing climatic conditions in a long term. Education, law enforcement and community involvement will help protect the few remaining fragmented *C. erectus* populations from extinction in Ghana.

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