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Genetic diversity and population structure of the antimalarial plant *Cryptolepis sanguinolenta* in Ghana

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Cryptolepis sanguinolenta is an important medicinal plant used in the treatment of malaria in Ghana. Overharvesting, destruction of entire plant populations and poor seed viability have resulted in a substantial decrease in wild populations thereby threatening its long-term potential and survivability. In this study, fifteen polymorphic microsatellite loci were used to evaluate the genetic diversity and population structure of 179 *C. sanguinolenta* individuals among eight subpopulations in Ghana. The subpopulations were separated by a distance of 8.3 − 233.3 km. Our results indicated relatively high levels of genetic diversity (Ho= 0.41; He=0.61) and high gene flow (Nm=7.06), an indication of greater stability and adaptability within the ecosystem, limited genetic differentiation (mean F_{ST}=0.05; highest F_{ST}=0.1), which suggested insignificant differentiation among the subpopulations. The high levels of gene flow resulting from the wind-dispersed seeds might have contributed to the limited genetic differentiation among the subpopulations. The Bayesian cluster analysis revealed the presence of a population structure (K=2). A lack of isolation by distance (r=0.012; P=0.34) indicated an increase in the genetic similarity among the subpopulations as the geographic distance between them decreased. This study described the genetic diversity and population structure in the current *C. sanguinolenta* accessions and laid a foundation for future breeding efforts.

KEYWORDS

anti-malarial, indigenous plant, genetic diversity, gene flow, medicinal plants

1 Introduction

According to a World Health Organization (WHO) 2021 report, 241 million cases of malaria were reported in 2020 (WHO, 2021). Most of these cases occurred in the African region (228 million), followed by the Eastern Mediterranean (5.75 million), and the South-East Asian region (5 million). Globally, malaria deaths reached 627,000, out of which, 96% are accounted for in sub-Saharan Africa (WHO, 2021). In addition, children under five accounted for 80% of the malaria deaths in Africa (WHO, 2021). Although efforts have been made towards reducing the global burden of malaria, drug resistance, accessibility, and the affordability of antimalarial medications continue to threaten this effort. To mitigate these issues, alternative approaches to using traditional medicine could provide more sustainable and cost-effective treatment options.

An estimated 80% of the world's population almost exclusively depends on local medicinal plants for the prevention and treatment of various diseases because they are readily accessible and affordable (Kirby, 1996; WHO, 2002). Several efficacious medicines such as aspirin (an analgesic) derived from *Salix alba* L., Digoxin (cadiotonic) from *Digitalis lantana* Ehrh., Artemisinin (anti-malarial) from *Artemisia annua* L., and Quinine from *Cinchona ledgeriana* Moens ex Trimen. are used for the of malaria treatment (Luo et al., 1998) and originate from plants (Licciardi and Underwood, 2011; Veeresham, 2012). In the fight against malaria, medicinal plants with a history of use as potential antimalarials in resource-poor communities should be considered in the development of intervention approaches (Abebe and Garedew, 2019). In that capacity, steps to conserve and promote sustainable, resilient, and climate-smart agro-ecosystems must be encouraged while documenting and preserving traditional medicinal plants knowledge limited to local healers and elders in these communities. Abebe and Garedew (2019) identified 25 plant species from 22 families that had been used in traditional treatments of malaria in Ethiopia with *Cyperus* spp., *Allium sativum* L., *Lepidium sativum* L., and *Echinops kebericho* Mesfin. are utilized most extensively.

With an increase in anthropogenic activities, there is an urgent need to prioritize the protection of wild populations and the cultivation of useful medicinal plants to ensure future availability for healthcare needs. *Cryptolepis sanguinolenta*, locally known in Ghana as 'Nibima' in Twi, 'Gangnamau' in Hausa, or 'Kadze' in Ewe, is a perennial vine belonging to the Apocynaceae family with a history of use in Africa (Luo et al., 1998; Osafo et al., 2017). It grows in rainforests, deciduous forest belts, and secondary forest clearings along the west coast of Africa from Nigeria through Ghana to Senegal (Dokosi, 1998). In Ghana, however, it is located along the slopes of the Akwapim and Kwahu mountain ranges (Addae-

Kyereme, 2004) with a long-standing history of use in the treatment of malaria.

Roots, the portion of the plant of commercial and medicinal value, contain the highest concentration of indoloquinoline and cryptolepine, which have the most potent antiplasmodial activity (Dwuma-Badu et al., 1978; Tackie et al., 1991; Cimanga et al., 1997).

Minor alkaloids including hydroquinone, quindoline, cryptoquindoline, cryptoheptine and 11-hydroxy derivatives of cryptolepine with biological/pharmacological activities have also been isolated (Dokosi, 1998). The combined presence of these alkaloids accounts for the biological and/or pharmacological activity of *C. sanguinolenta*. The effectiveness of its extracts is comparable to chloroquine when taken orally (Boye, 1989). Apart from its antimalarial properties (Ansah et al., 2005; Tempesta, 2010; Osei-Djarbeng et al., 2015), it is recognized for its anticancer (Ansah and Mensah, 2013), antihyperglycemic (WHO, 2002), antimicrobial (Sawer et al., 2005) and antimycrobacterial (Gibbons et al., 2003) potential. Preparations from *C. sanguinolenta* were approved in February 2021 for clinical trials for the treatment of COVID 19 by the Food and Drugs Authority of Ghana (FDA, 2021) and has been shown to inhibit hepatitis B virus replication (Domfeh et al., 2021).

In Ghana, *C. sanguinolenta* harvesting is done by smallholder farmers (plant collectors) who use the roots to make antimalarial decoctions. "Nibima" is one of the popular decoctions sold in local markets and is produced by the Centre for Plant Medicine Research (CPMR) (https://www.cpmr.org.gh/product-category/decoctions). Overtime, the plants have been overharvested and these plant collectors must travel further into the forest, often with overnight stays, to gather *C. sanguinolenta* (Amissah, personal communication 2021). Destructive harvesting of the entire plant, along with its root system, is not sustainable in the long term and has already resulted in a substantial decrease in wild populations (Jansen and Schmelzer, 2010; Amissah et al., 2016a).

Over collection and habitat loss poses a significant threat to the conservation efforts of endangered plant species, especially those used for medicinal purposes (Kala, 2000; Reed and Frankham, 2003). Human activities, such as illegal poaching or disruptive harvesting of endemic, medicinal plants, can negatively impact biodiversity at the gene, species, community and ecosystem levels (Meffe and Carroll, 1997; Templeton et al., 2001). This can result in habitat fragmentation, erosion of natural and adaptive genetic diversity, reduction in effective population size, lower evolutionary potential and ultimately contribute to species extinction (Templeton et al., 2001; Soares et al., 2019). Understanding the population genetics of a plant species provides insight into the effects of natural selection, genetic drift, mutation and gene flow that can be used to develop suitable conservation methods and assist with future breeding

programmes for plant species on the verge of extinction. Population genetics studies have played a significant role in the conservation of several species. For example, genetic diversity and inbreeding were significantly influenced by the size of the population of *Chersophilus duponti*, a threatened lark in the Alaudidae (Meffe and Carroll, 1997) and *Hypericum cumulicola*, a rare species of flowering plant found in small and isolated populations (Oakley, 2015).
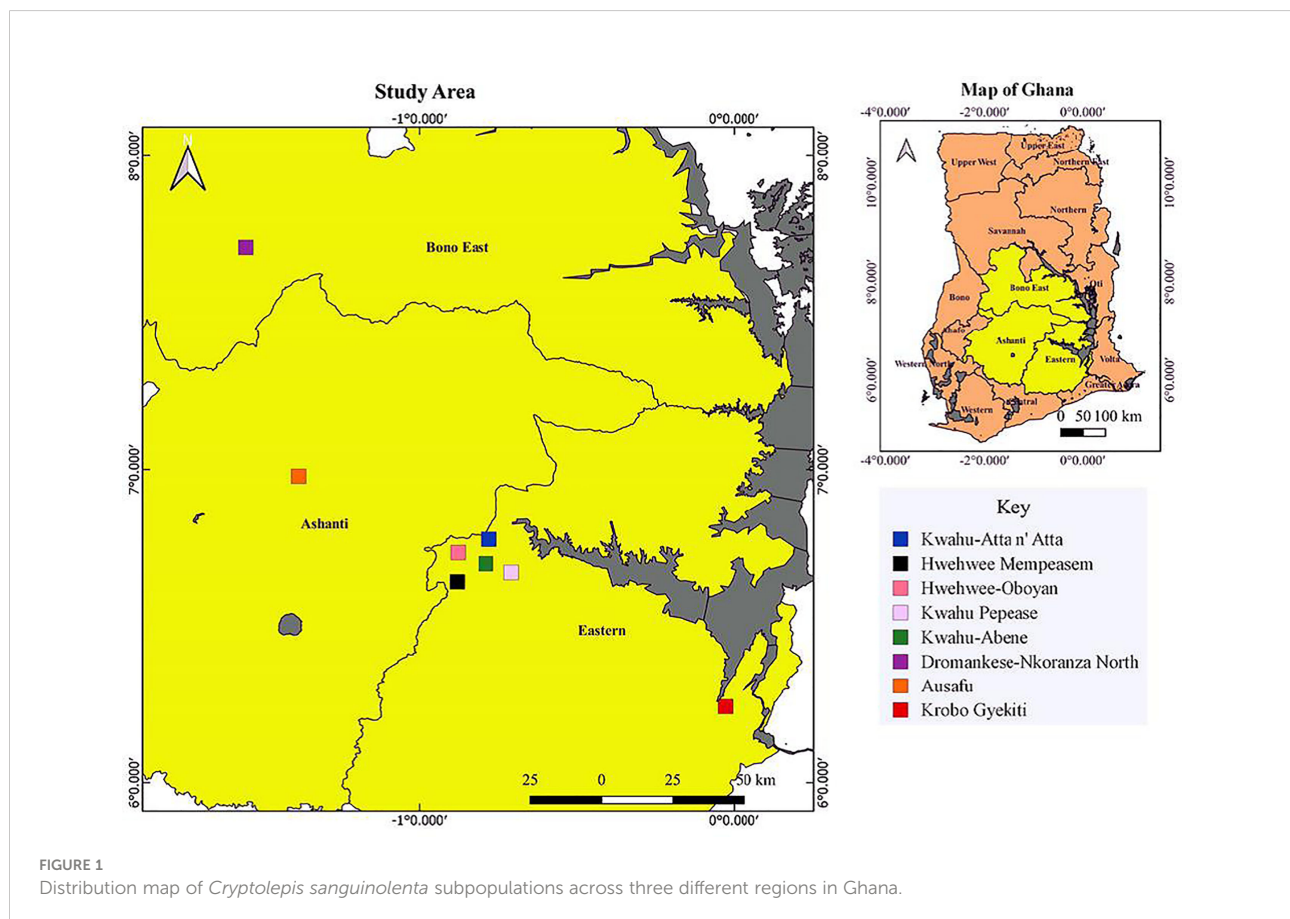
As is the practice with several important medicinal plants, there is very little effort devoted to the conservation of *C. sanguinolenta*. There is an urgent need for establishing a conservation strategy for medicinal plant species in general and specifically for *C. sanguinolenta*. Currently, there is very limited knowledge of the population dynamics of *C. sanguinolenta*. As part of research efforts geared at conserving this species, our group has developed protocols for the cultivation of *C. sanguinolenta* (Jansen and Schmelzer, 2010), in addition, we have developed microsatellite loci for future genetic diversity studies (Amissah et al., 2016b). To address these knowledge gaps, this study seeks to: i) measure the genetic diversity of *C. sanguinolenta* across eight subpopulations from natural habitats within three regions of Ghana; and ii) describe the spatial structure of these subpopulations.

2 Materials and methods

2.1 Sampling localities, DNA extraction, isolation and PCR amplification

One hundred and eighty-six plants of *C. sanguinolenta* were collected and assigned to eight subpopulations of which each was represented by 17 to 29 individuals located within the Ashanti (Asuafo-Sekyere West), Eastern (Hwehwee-Ata n' Ata, Hwehwee-Yaw Gyeni, Kwahu Abene, Kwahu Pepease, OgenyaKrobo/Krobo Gyekiti), and Brong Ahafo (Dromankese Nkoranza North) region in the period between April and December 2015 (Figure 1) .

The identification of plants collected from each location was based on morphological characteristics of the species and verified using herbarium samples from an earlier study. Samples were maintained and grown at the Department of Crop Science of the University of Ghana under field conditions. The site falls within the Coastal Savannah agroecological zone of Ghana with a total annual rainfall of approximately 748 mm and a temperature of between 24 - 32°C. The soil at the site is sandy loam and classified as the Adenta series by the Soil Survey Staff (1999).

FIGURE 1
Distribution map of *Cryptolepis sanguinolenta* subpopulations across three different regions in Ghana.

2.2 Genotyping

Genomic DNA was isolated from fresh *C. sanguinolenta* leaf samples using a CTAB protocol (Porebski et al., 1997). The concentration and purity of DNA samples were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) before analysis. Polymerase chain reaction (PCR) amplification was performed in 10 µl reaction mixtures containing 1 µl DNA template (4 ng/µl), 1 µl of GeneAmp 10x PCR Buffer II (Applied Biosystems, Branchburg, NJ, USA), 1 µl of 25 mM MgCl$_2$, 1 µl of each 2 mM of dNTPs, 1 µl of 2.5 µM specific polymorphic microsatellite primers, 0.08 µl of 5 U AmpliTaq Gold$^®$ DNA polymerase (Applied Biosystems), and 4.92 µl of sterile nanopure water. Nine polymorphic microsatellite primer pairs, developed from a previous study (Amissah et al., 2016b), as well as six additional polymorphic microsatellite primer pairs (Table 1) were selected and used for the population studies.

Amplification reactions were temperature cycled in 96-well plates using an Eppendorf Mastecycler Thermocycler (Eppendorf AG, Hamburg, Germany) with the following program: 94°C for 3 min, followed by 35 cycles of 94°C for 40 seconds, 58°C for 40 seconds, 72°C for 30 seconds, and a final extension at 72°C for 4 min. PCR products were analyzed with the QIAxcelCapillary Electrophoresis System (Qiagen, Valencia, CA, USA) using an internal 25-bp DNA size ladder. Data were automatically recorded and exported using QIAxcel ScreenGel Software,which provided both a gel view and an electropherogram of the separated PCR products (alleles).

TABLE 1 Microsatellite loci used to analyze 179 *Cryptolepis sanguinolenta* samples collected from eight locations in Ghana.

GenBank accession No.	Repeat motif	Locus	Primer sequences (5'-3')	Size range (bp)
KU361063	(AAAT)$_4$	Cry007*	F: GACATAGCTTTGGAAGGGTAG	265-286
			R: TCAGGATCAATTTCCACTTG	
KU746826	(TTA)$_3$	Cry011	F: TTTGGATTGACTCTAGCACTGG	125-155
			R: GTGCCTCTCCAATCACTTCCT	
KU746827	(CTT)$_3$	Cry015	F: TGCGAAGACTCACTGAATCAA	155-196
			R: CCCAACTCCTTGGTAATGAGC	
KU746828	(AAT)$_3$	Cry018	F: GTACTGTGCTCGCAGTTCGTT	190-216
			R: CGAGCTCCATTAAGAATCAGG	
KU361064	(GAT)$_3$	Cry021*	F: GGTGACCCTCAATCTGATGAA	179-213
			R: GCTCCTGACCCCATATCAGTT	
KU746829	(AAAT)$_4$	Cry023	F: TCCTCTTCCTGAAAGGTAACAAG	226-237
			R: GGTGATCCAGCAGCGTAGTTA	
KU361066	(AT)$_2$	Cry030*	F: GGCATTGGCCATATATACTCCT	144-159
			R: CATGTTCCAGGGCAGTAGAAA	
KU361067	(TAA)$_3$	Cry031*	F: AGGATACGTGGCTCCAAGATT	136-147
			R: AGGATGCCAATGAAAGGATCT	
KU361068	(TTTA)$_4$	Cry032*	F: GGAAGCATCCATAAGGAGGAG	107-123
			R: TGGATATTCTAGTTGCCTTGTGG	
KU361071	(TAT)$_3$	Cry036*	F: GCGCAATTAAGAAATGCGTA	244-265
			R: CAGCGCATTTCCAACAATAA	
KU361072	(ATT)$_3$	Cry037*	F: GCGCAATTAAGAAATGCGTA	209-262
			R: CAGCGCATTTCCAACAATAA	
KU361074	(AAC)$_3$	Cry044*	F: CGGTTCCCCTTCTACAATTTC	238-257
			R: CCCATCAACTAACGAAAAAGG	
KU361075	(TTCC)$_4$	Cry047*	F: TAGGGCTTCTTTCATGCGTTT	134-154
			R:CAGATTCAAGTGGACGAAACC	
KU746830	(TTAT)$_4$	Cry058	F: TCCAAGGGGAATGAAGAAAA	237-250
			R: TATTACGGGTGGCATTTTGTG	
KU746831	(TA)$_2$	Cry073	F: TACGGACTGCAGATCCAGAAG	147-168
			R: CCATGTGCCAATGTAGTCATC	

*Primer sequences from (Amissah et al., 2016b).

2.3 Data analyses

2.3.1 Loci polymorphism and population diversity

The 186 individuals were analyzed using 15 species-specific polymorphic microsatellite primers. DNA of seven individual samples failed to amplify across several loci and therefore were excluded from further analyses resulting in 179 individuals in the study. The program FLEXIBIN (Amos et al., 2007) was used to assign alleles into appropriate classes. To achieve the best allelic class fit, the program uses a simple algorithm through a series of steps and phases to find the best binning combinations.

Standard measures of genetic diversity among 179 individuals of *C. sanguinolenta* were determined using Nei's gene diversity (Amos et al., 2007) in FSTAT version 2.9.3.2 (Goudet, 2001; Goudet, 2005) and program R version 4.1.2 using packages poppr (Kamvar et al., 2014; Kamvar et al., 2015) and hierfstat (Goudet, 2005). Analyses included the number of alleles observed at each locus (N) and across subpopulations (Na), number of effective alleles (Ne), and allelic richness (A_R), a measure of the number of alleles independent of sample size. Allelic richness, calculated using a rarefaction method based on a minimum sample size of 15 diploid individuals or 30 genes, is used as an estimate of the long-term evolutionary potential of given individual(s) to adapt and persist in a population (El Mousadik and Petit, 1996). We also estimated observed and expected heterozygosity (gene diversity) under Hardy Weinberg equilibrium (Ho and He, respectively), linkage disequilibrium (rbard) (non-random association of alleles between loci), and Shannon-Weiner diversity index (H), which considers both allele richness and evenness of the allelic distribution. Further analyses included the number of unique private alleles across loci and subpopulations. F-statistics estimates included inbreeding coefficient (F_{IS}) and fixation index (F_{ST}). In addition, gene flow (Nm) estimates were calculated using GenAlEx version 6.502 (Peakall and Smouse, 2006; Peakall and Smouse, 2012).

2.3.2 Population structure and historical demography

Genetic structure analysis of *C. sanguinolenta* was performed using Bayesian cluster analysis (Peakall and Smouse, 2012) in program STRUCTURE version 2.3.4 (Pritchard and Donnelly, 2001) with a series of complementary approaches including analysis of molecular variance (AMOVA) (Excoffier and Lischer, 2010), and Mantel test for isolation by distance. The program STRUCTURE was used with 20 independent runs for each k value between one and ten at 500,000 MCMC (Markov Chain Monte Carlo) repetitions and a burn-in period of 500,000 iterations using an admixture model with correlated allele frequencies (without prior assumption of population origin). The admixture model assumes that individuals with admixed ancestry can inherit a portion of their genome from ancestors in population k (Porras-Hurtado et al., 2013). STRUCTURE HARVESTER (Earl and VonHoldt, 2012), a web-based program for obtaining K values and summarizing output data from STRUCTURE, was used to estimate the optimum value of genetic clusters using Evanno's method (Evanno et al., 2005). Data was visualized using program web-based program POPHELPER (Francis, 2017). Because the STRUCTURE-like approach assumes panmictic populations and unlinked markers (Pritchard et al., 2000), alternatively, model-free approaches that utilize multivariate K-means clustering such as Discriminant Analysis of Principal Components (DAPC) are often used. Here, we used DAPC to further investigate the underlying structure among *C. sanguinolenta* populations using R package *adegenet* (Jombart, 2008; Jombart and Ahmed, 2011). The initial selection includes a principal component analysis (PCA) identification, followed by the selection of appropriate PCA vectors that can explain the majority of variance while minimizing over-fit of the DAPC, and finally using a selected number of PCAs that could reveal differences between groups and minimize within-group variations (Ony et al., 2021). We also performed pairwise F_{ST} analysis for all pairs of subpopulations using 10,000 permutations in GenAlEx. Significant F_{ST} values were calculated after sequential Bonferonni corrections (Rice, 1989; Bergamaschi and Lama, 2015).

The hierarchical distribution of genetic variation of the *C. sanguinolenta* subpopulations was measured using Arlequin version 3.5.1.2 (Excoffier and Lischer, 2010). AMOVA was used to partition the total variance into hierarchical components (among subpopulations and within subpopulations). Although AMOVA findings can complement STRUCTURE results, these outcomes should not be taken as independent confirmation of implied hierarchical structure. AMOVA analyses for *C. sanguinolenta* subpopulations were partitioned based on all sampling localities clustered as one hierarchal group and clusters identified by STRUCTURE. For STRUCTURE partitioning, each individual was clustered into one of the two groups as determined by the program.

To determine whether or not geographical structure is best explained by hierarchical clustering, the correlation between pairwise genetic and geographic distance was estimated using *mass* package (Venables and Ripley, 2002) in program R. Statistical significance was evaluated using the Mantel test using 9,999 permutations. R program was also utilized for the construction of UPGMA (unweighted pair group method of arithmetic averages) dendrogram based on Bruvo's distance with 1,000 bootstrap replicates and threshold set at 50% using package *poppr* (Kamvar et al., 2014; Kamvar et al., 2015). Lastly, *C. sanguinolenta* populations were tested for evidence of bottlenecks using the software BOTTLENECK version 1.2 (Piry et al., 1999). The program evaluates the presence of population bottleneck as an excess (recent population bottleneck) or deficit (recent

population expansion) of genetic diversity, relative to the number of alleles present in the *C. sanguinolenta*.

3 Results

3.1 Population diversity

One hundred and one alleles were identified using 15 polymorphic microsatellite loci across the three regions in Ghana where *C. sanguinolenta* plants are found (Table 2).

The mean allelic richness (A_R), as assessed with 15 microsatellites was 4.43 and the number of alleles varied from 5 (Cry015) to 10 (Cry036) with a total number of 101 alleles (Table 2). Overall, subpopulations differentiation was low (F_{ST}=0.05) and indicated little genetic differentiation, high gene flow (Nm=7.06), significant inbreeding (F_{IS}=0.36) and structure among the *C. sanguinolenta* subpopulations (Ho<He) (Table 2). Expected heterozygosity across all loci ranged from 0.43 to 0.76 with a mean of 0.60. Twelve of the fifteen tested microsatellite loci yielded private alleles and resulted in the discovery of 26 unique alleles (Table 2).

The effective number of alleles (Ne) ranged from 2.49 to 2.72 with an average of 2.60 across all subpopulations. Shannon-Weiner diversity index (H) and allelic richness (A_R) values were high across all subpopulations (H=3.10, A_R=4.15) but generally higher for subpopulations from the Eastern region (H=3.12, A_R=4.21 for six Eastern subpopulations) when compared to Ashanti (H=3.09, A_R=3.89) and Brong-Ahafo (H=3.0, A_R=4.02) regions (Table 3).

Mean observed (Ho) and expected heterozygosity, or gene diversity (He), were 0.41 and 0.57, respectively, indicating the presence of population structure (Table 3). Weak but significant linkage disequilibrium was detected across all eight subpopulations (rbard = 0.08, P<0.01) (Table 3).

Private alleles (total P_A=26) were identified from all eight subpopulations of *C. sanguinolenta*. (Table 3). Krobo Gyekiti (P_A=7), Kwahu-Atta n' Atta (P_A=5), Kwahu Pepease (P_A=3), and Hwehwee-Oboyan (P_A=3) subpopulations from the eastern region had the highest number of private alleles per subpopulations (Table 3). The highest average number of alleles was found in the eastern region (Hwehwee-Mempeasem, Kwahu Pepease and Krobo Gyekiti subpopulations) compared to Ashanti and Brong-Ahafo regions (Asuafu-Sekyere and Dromankese-Nkoranza North subpopulations) (Table 3).

3.2 Population structure and historical demography

Subpopulations of *C. sanguinolenta* demonstrated very low levels of population differentiation (F_{ST}=0.05) (Table 2) despite the distribution of the plant across three different regions in Ghana. Comparisons among Asuafu-Sekyere West and Dromankese-Nkoranza North subpopulations resulted in non-significant pairwise F_{ST} values (Table 4). However, significant values were obtained for the Hwehwee-Mempeasem subpopulation from the east when compared to the other seven *C. sanguinolenta* subpopulations in Ghana (Table 4).

TABLE 2   Diversity indices and gene flow estimates for fifteen microsatellite loci over *Cryptolepis sanguinolenta* populations.

Locus	N	A_R	Ho	He	F_{IS}	F_{ST}	Nm	Pa
Cry007	6	3.70	0.15	0.43	0.60	0.10	2.14	0
Cry011	6	4.06	0.37	0.66	0.40	0.07	3.08	2
Cry015	5	3.46	0.04	0.58	0.94	0.10	2.27	2
Cry018	6	4.36	0.20	0.70	0.71	0.04	6.12	1
Cry021	7	4.28	0.83	0.67	-0.24	0.01	22.75	4
Cry023	6	4.09	0.45	0.47	0.04	0.05	4.85	1
Cry030	7	3.67	0.11	0.45	0.77	0.06	4.22	2
Cry031	6	4.84	0.89	0.68	-0.31	0.02	12.15	0
Cry032	7	4.99	0.97	0.75	-0.33	0.04	6.71	2
Cry036	10	4.01	0.13	0.58	0.79	0.03	7.15	4
Cry037	6	3.60	0.26	0.46	0.45	0.07	3.58	2
Cry044	8	5.32	0.91	0.76	-0.21	0.03	9.14	2
Cry047	6	4.34	0.14	0.46	0.70	0.02	12.30	2
Cry058	6	5.32	0.58	0.71	0.19	0.05	4.62	0
Cry073	9	6.35	0.07	0.67	0.89	0.05	4.85	2
Mean/Total	101	4.43	0.41	0.60	0.36	0.05	7.06	26

N, number of alleles observed at each locus; A_R, Allelic richness corrected for sample size; Ho, observed portion of heterozygotes; He, expected heterozygosity (gene diversity); F_{IS}, coefficient of inbreeding; F_{ST}, genetic differentiation coefficient, independent of the number of samples; Nm, gene flow; Pa, number of private alleles.

TABLE 3 Genetic diversity estimates for sampled *Cryptolepis sanguinolenta* individuals averaged across eight subpopulations and 15 microsatellite loci.

Subpopulations	Region	MLG	Na	Ne	Ho	He	H	A_R	rbard	P value (rbard)	P	P_A
Asuafu-Sekyere West	Ashanti	22	4.07	2.49	0.40	0.54	3.09	3.89	0.12	<0.001	100	2
Dromankese-Nkoranza North	Brong-Ahafo	20	4.13	2.66	0.38	0.59	3.00	4.02	0.11	<0.001	100	3
Hwehwee-Mempeasem	Eastern	23	4.60	2.64	0.45	0.57	3.14	4.30	0.08	<0.001	100	2
Hwehwee-Oboyan	Eastern	24	4.20	2.52	0.40	0.54	3.18	4.00	0.11	<0.001	100	3
Kwahu Pepease	Eastern	23	4.60	2.67	0.40	0.58	3.14	4.25	0.11	<0.001	100	3
Kwahu-Abene	Eastern	17	4.47	2.72	0.47	0.6	2.83	4.41	0.12	<0.001	100	1
Kwahu-Atta n' Atta	Eastern	21	4.27	2.53	0.38	0.58	3.04	4.10	0.09	<0.001	100	5
Krobo Gyekiti	Eastern	29	4.67	2.56	0.36	0.56	3.37	4.21	0.07	<0.001	100	7
Mean/Total		22	4.38	2.60	0.41	0.57	3.10	4.15	0.08	<0.001	100	26

MLG, multilocus genotypes; Na, the average number of alleles; Ne, the average number of effective alleles; Ho, observed heterozygosity; He, expected heterozygosity (gene diversity); H, Shannon–Wiener index; A_R, allelic richness; rbard, linkage disequilibrium; P, the percentage of polymorphic loci; P_A, number of private alleles.

Analyses of all other subpopulations resulted in non-significant F_{ST} values (Table 4) except for Kwahu-Abene and Krobo Gyekiti subpopulation from the eastern region.

The greatest pairwise genetic differentiation was among Asuafu-Sekyere West and Hwehwee-Mempeasem (F_{ST}=0.061; P<0.001) and Hwehwee-Mempeasem and Krobo Gyekiti subpopulations (F_{ST}=0.060; P<0.001) (Table 4), which could be biased by differences in sample size among the regions (Table 3; Table 4). Population structuring is best explained by the presence of two genetic clusters (K=2), even though we examined delta k for the presence of three and four genetic clusters (Figure 2).

All cluster groups had representatives of individuals from the eight subpopulations present, with high levels of admixture among the individuals in each subpopulation, and no evidence of geographical structuring (Figure 2). Consistent with the lack of isolation by distance (P=0.34; r=0.012; Figure 3), our findings are further supported by the Discriminant Analysis of Principal Components (DAPC) plot that indicated the presence of two distinct groups with mixed individuals from different subpopulation (Figure 4).

To test whether Isolation by Distance (IBD) occurs on a smaller scale, *C. sanguinolenta* individuals were clustered based on geographical subpopulation assignment and analyzed separately. Three subpopulations from the eastern region indicated significant evidence of isolation by distance - Kwahu Pepease, Kwahu-Atta n' Atta and Krobo Gyekiti (P<0.05). When *C. sanguinolenta* individuals were grouped based on STRUCTURE findings, the two clusters did not have significant correlation between genetic and geographic distance (P=0.06). The UPGMA dendrogram revealed a similar pattern of two groups and a lack of geographical structuring among the examined subpopulations (Figure 5).

Two different analyses of molecular variance (AMOVA) were conducted. The results of the first AMOVA (one hierarchical grouping), showed that the variation within subpopulations was higher (96.95%), than among subpopulations (3.05%) (Table 5). A similar trend of high individual variation (74.56%) and relatively lower variation among subpopulations (25.44%) was obtained after data was partitioned based on the STRUCTURE results (Table 5).

TABLE 4 Pairwise F_{ST} values between eight *Cryptolepis sanguinolenta* populations.

Subpopulation Region	ASW Ashanti	DNN Brong-Ahafo	HM Eastern	HO Eastern	KP Eastern	KA Eastern	KAA Eastern	KG Eastern
ASW	0							
DNN	0.021	0						
HM	**0.061**	**0.046**	0					
HO	0.011	0.016	**0.053**	0				
KP	0.022	0.013	**0.045**	0.017	0			
KA	**0.035**	0.026	**0.032**	0.023	0.026	0		
KAA	0.020	0.018	**0.041**	0.016	0.025	0.014	0	
KG	0.015	0.019	**0.060**	0.013	0.019	**0.032**	0.022	0

ASW, Asuafu-Sekyere West; DNN, Dromankese-Nkoranza North; HM, Hwehwee-Mempeasem; HO, Hwehwee- Oboyan; KP, Kwahu Pepease; KA, Kwahu-Abene; KAA, Kwahu-Atta n' Atta; KG, Krobo Gyekiti. Significant F_{ST} values based on 10,000 permutations are bolded.
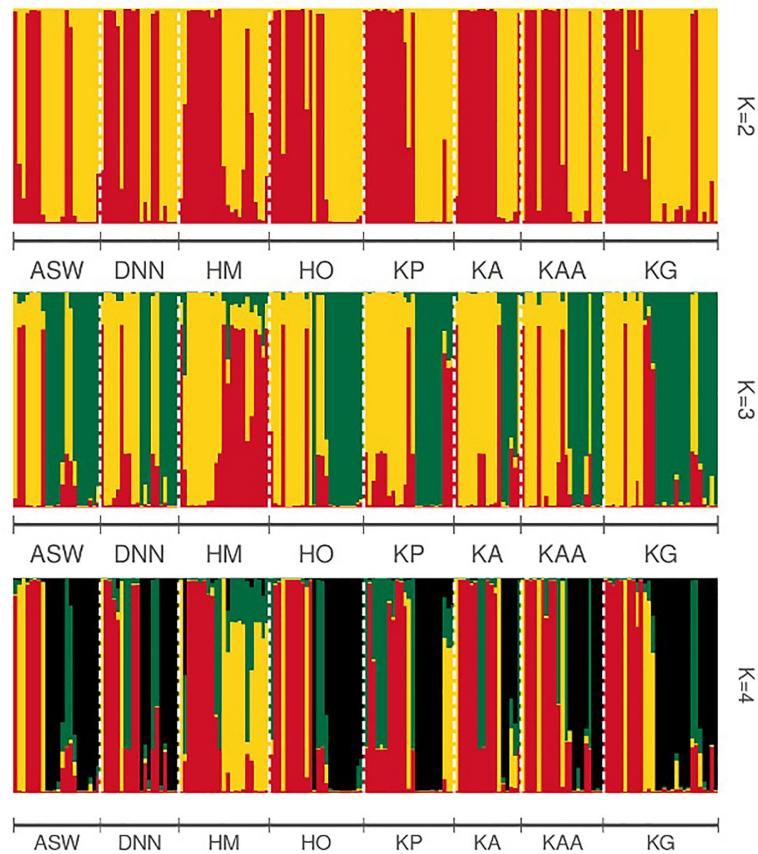
FIGURE 2
STRUCTURE bar plot for eight *Cryptolepis sanguinolenta* subpopulations showing results for (K=2-4). Each bar indicates the individual assignment probability of that individual belonging to one of the clusters indicated by different colors, based on twenty iterations. Evanno's method indicated that two genetic clusters (K = 2) best explain the population structure in this species. Population designations are ASW, Asuafu-Sekyere West; DNN, Dromankese-Nkoranza North; HM, Hwehwee-Mempeasem; HO, Hwehwee-Oboyan; KP, Kwahu Pepease; KA, Kwahu-Abene; KAA, Kwahu-Atta n' Atta; KG, Krobo Gyekiti.

4 Discussion

The large-scale use of artemisinin since its initial discovery in the early 1970s, has resulted in worldwide drug resistance prompting a switch in strategy to artemisinin-based combination therapy (ACT) in 2005 (Tu, 2011; WHO, 2020). The problem is further compounded by the increased resistance of malaria vectors to many insecticides currently available (Balkew et al., 2012; Massebo et al., 2013), which are cost-prohibitive on a large-scale and unsustainable in the long term. According to reports from WHO (WHO, 2015), artemisinin-based combination therapy (ACTs) had been adopted as a first-line treatment policy in several countries but may not be readily available in rural, remote and poverty-stricken communities of Western Africa, including Ghana. As a result, in Ghana, Mali, Zambia and Nigeria, traditional medicine approach is practiced with 60% of children who reported high fever resulting from malaria infection (Abdullahi, 2011).

People living in developing countries have limited access to modern medicine and sophisticated treatments (Reed and Frankham, 2003), thus often relying on medicinal plants that offer an alternative, cost-effective, and freely available traditional approach for malaria control (Turkson et al., 2019). In some areas, the traditional medicine approach is the only source of medical care available (Romero-Daza, 2002; Abdullahi, 2011). Based on African Health Monitor Report in 2003, there is one healer for every 500 individuals vs. one medical doctor for every 40,000 patients in Africa; for Ghana, there is one healer for every 200 individuals vs. one medical doctor for every 20,000 patients (Chatora, 2003; Abdullahi, 2011; WHO, 2014). These staggering numbers, combined with the fact that the majority of doctors reside in urban areas, stress the importance of traditional healers and indigenous plants in treatments of communities in rural Africa. The healers, also known as traditional doctors, approach the patients holistically, treating not only the given condition but restoring the social, emotional, and spiritual equilibrium of their
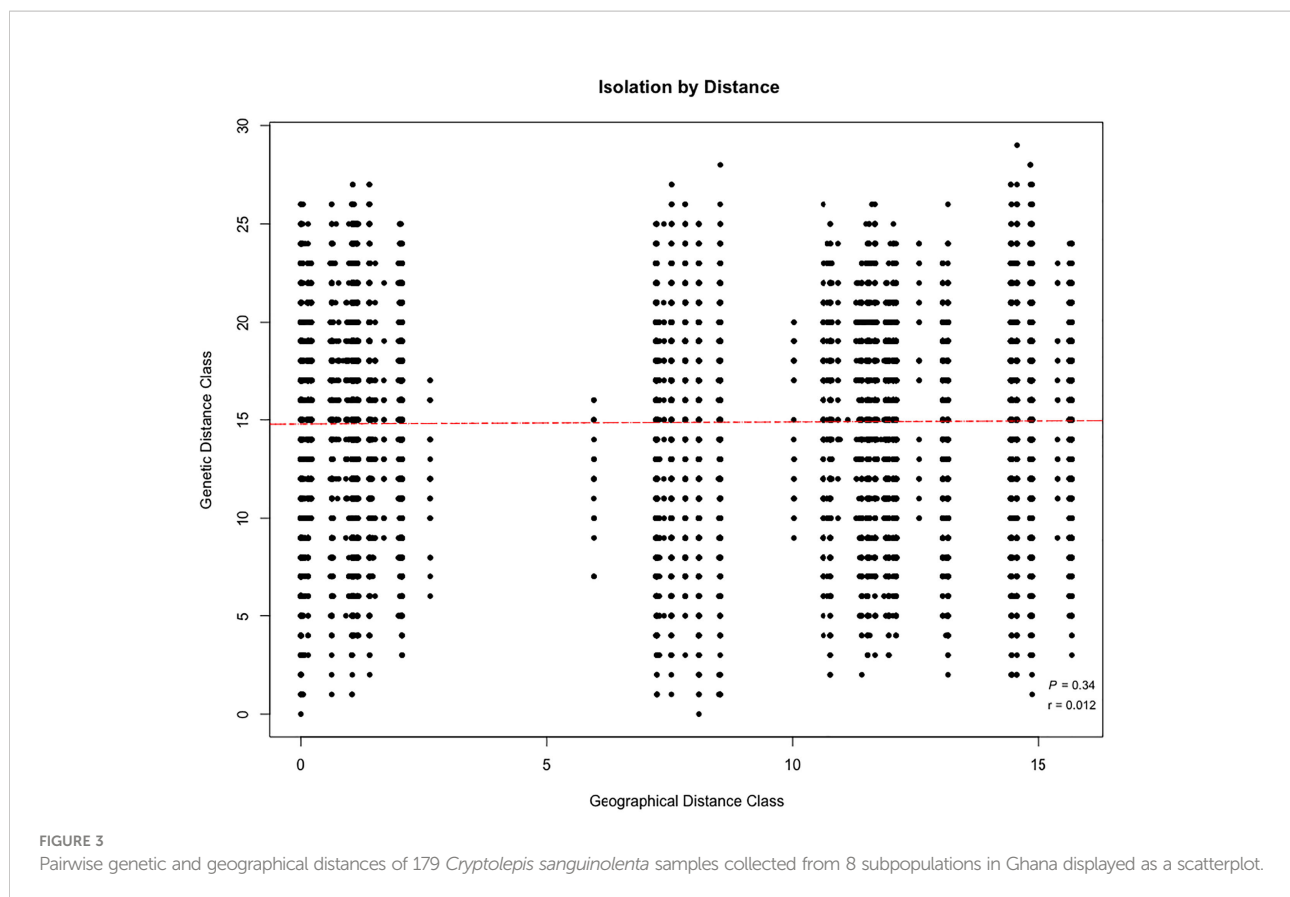
FIGURE 3
Pairwise genetic and geographical distances of 179 *Cryptolepis sanguinolenta* samples collected from 8 subpopulations in Ghana displayed as a scatterplot.

patients based on community history and customs (Hillenbrand, 2006; Abdullahi, 2011).

Traditional African knowledge of indigenous plants have been utilized for healing and treating various ailments, including malaria, for centuries. Preservation and conservation of these nearly lost species is imperative, and documentation of alternative medicine applications and approaches used by traditional healers as an ancient and culture-bound method of healing for centuries should be prioritized (Abdullahi, 2011; Veeresham, 2012). These plants are largely understudied, and very limited financial and technological resources are available for research related to drug development in these countries (Reed and Frankham, 2003). Indigenous *C. sanguinolenta* contains not only antimalarial, anti-cancer, antihyperglycemic, antimicrobial, and antimycobacterial properties, but can be utilized to promote sustainable and climate-smart agro-ecosystems across West Africa. This, in turn, would allow sustainable commercialization of this plant as a cash crop across its native distribution, thus providing farmers with additional income while preventing destructive collection.

Despite overharvesting and near extinction of these indigenous plants, our findings provided evidence of high genetic diversity among *C. sanguinolenta* subpopulations across Ghana. In declining and isolated populations unable to respond to a variety of selection pressures, genetic variation can be reduced rapidly as a result of the limited number of individuals and inbreeding within the populations that are often driven by random genetic drift (Ellstrand and Elam, 1993; Hawley et al., 2006; Luan et al., 2006). This can further decrease species fitness, prospects for adaptive change, ultimately causing species extinction (Fischer and Matthies, 1998; Severns, 2003; Hawley et al., 2006; Hadziabdic, 2010). However, this is the first comprehensive study focusing on the population genetic and spatial structure of this medicinal plant, thus we can expect these individuals to be somewhat resistant to apparent population bottleneck and genetic drift effects, at least in the short term. Long term monitoring, better propagation systems, and selective breeding processes are necessary for better informed conservation efforts.

Our results indicated the presence of a weak population structure low genetic differentiation, and high gene flow among wild *C. sanguinolenta* subpopulations. Findings from this study can be attributed to the specialized wind dispersal mechanism of *C. sanguinolenta* seeds over long distances, as the main factor affecting variation in gene flow and population structure similarly reported in *Cecropia obtusifolia* (Epperson and Alvarez-Buylla, 1997), and some woody plant species (Hamrick et al., 1992), and thus providing long-term potential for the conservation of *C. sanguinolenta*. Gene flow is a directional transfer of genetic material across populations
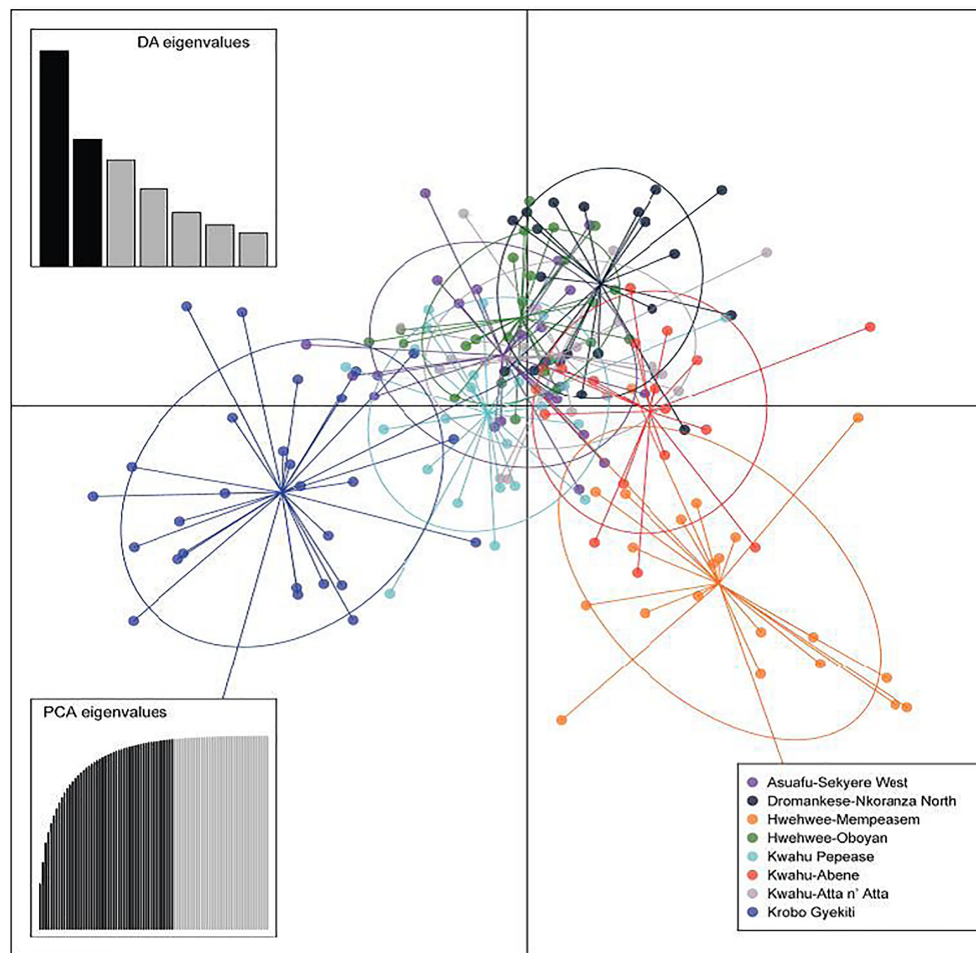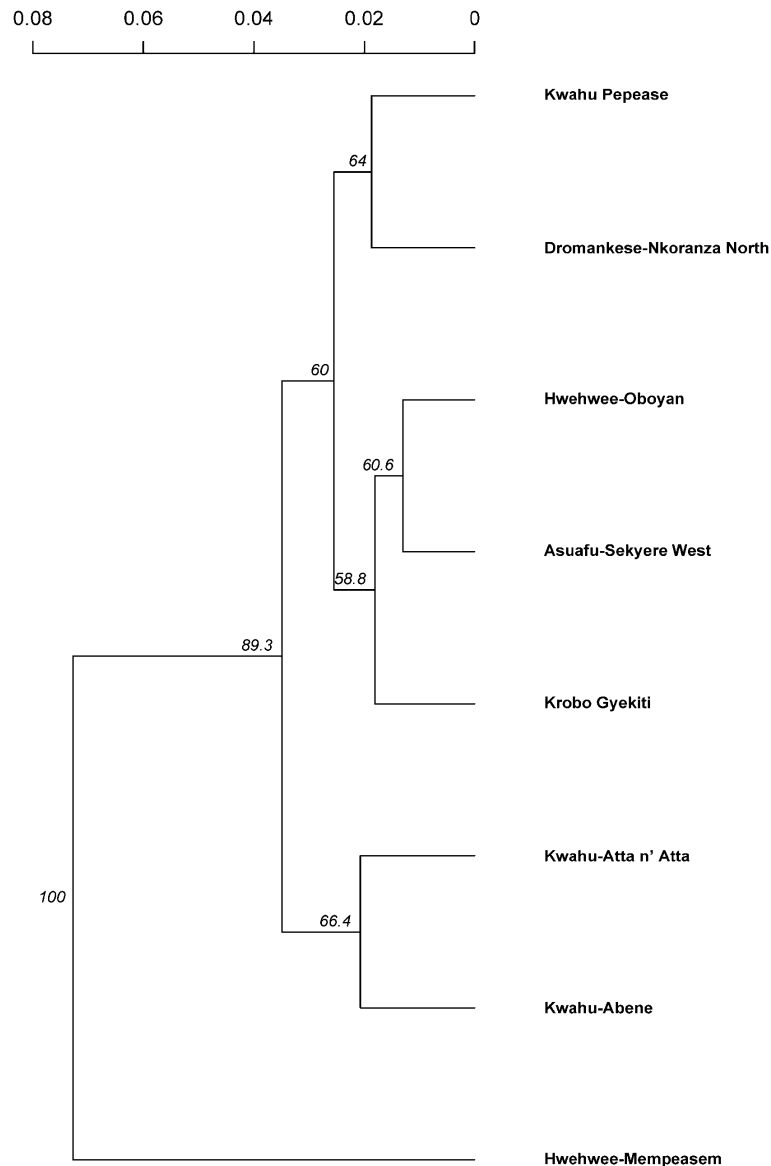
FIGURE 4
Discriminant Analysis of Principal Components (DAPC) plot of *Cryptolepis sanguinolenta* individuals across eight subpopulations in Ghana. Data was constructed using 1,000 permutations. Discriminant Analysis eigenvalues are also presented.

as a result of seed dispersal over a period of time and in some tree and shrub species, it is positively correlated with the direction of wind flows and rates (Kling and Ackerly, 2021). The signal of a weak population structure could be due to high gene flow in the species as reported by Waples (1998), since gene flow is known to be the predominant evolutionary force for the genetic structure (García-Verdugo et al., 2010). The unlimited gene flow and low magnitude of genetic differentiation observedcould be attributed to the relatively larger genetic neighbourhood and sampling size. According to Franceschinelli and Kesseli (1999), the relative magnitude of genetic differentiation is directly influenced by the size of the sampling area such that genetic differentiation increased with decreased sample plots in *Helicteres brevispira*.

In a large genetic neighbourhood, proposed by Wright (Wright, 1943; Wright, 1946), uniform selection pressures may limit differentiation among individuals. Alternatively, local differences and a relatively slow dispersal process of new alleles in these smaller geographical areas may increase differentiation between the groups (Wright, 1943; Wright, 1946). The presence of a contiguous genetic neighbourhood in *C. sanguinolenta* subpopulations in the present study is supported by STRUCTURE findings and indicated the presence of two distinct genetic clusters and extensive admixture as a result of gene flow. The high gene flow found in our study indicates that the effective population size of current *C. sanguinolenta* wild plants may be sufficient to offset the impact of population decline and the effect it has on genetic diversity. Similar to findings by Ratnaningrum et al. (2017), larger gene flow was found to be the main emphasis for natural genetical processes in *Santalum album* population.

The Mantel test revealed no correlation between Bruvo's distance and geographic distance suggesting that genetic

FIGURE 5
An unweighted pair group method with arithmetic mean (UPGMA) dendrogram of *Cryptolepis sanguinolenta* populations using 15 polymorphic microsatellite primers based on Bruvo's genetic distance. Numbers indicate the percentage of bootstrap support using 1,000 replications (threshold set at 50%).

differentiation in the 8 subpopulations is not caused by geographic isolation due to distance. A possible explanation for the absence of the influence of isolation distance on genetic diversity could be due to high gene flow, sexual reproduction, and wind dispersal of seeds in *C. sanguinolenta*. The results of the Mantel test are similar to findings in Miscanthus, *M. sinensis* (Nie et al., 2014; Zhao et al., 2017).

These findings have practical implications for the conservation of *C. sanguinolenta*. First, the absence of

geographic distance on genetic differentiation in *C. sanguinolenta* subpopulations, suggests that sampling from one geographic area could help cover a significant proportion of the genetic diversity in *C. sanguinolenta*. We recommend a protection strategy as follows: 1) protection of the plants in the Kwahu-Abene area, and 2) scaling-up the sampling to other *C. sanguinolenta* populations with high genetic diversity such as Hwehwee-Mempeasem, Hwehwee-Oboyan, Kwahu Pepease, and Asuafu-Sekyere West. In addition, *ex situ* conservation by

TABLE 5 Analysis of molecular variance (AMOVA) for *Cryptolepis sanguinolenta* subpopulations using 15 microsatellite loci.

Groups*	Source of variation	d.f.	Sum of squares	Variance component	% of variation	P value	F-statistics
One hierarchical group	Among subpopulations	7	61.06	0.11 Va	3.05	<0.001	F_{ST}=0.03
	Within subpopulations	350	1269.68	3.63 Vb	96.95	<0.001	
	Total	357	1330.74	4.20			
Two STRUCTURE clusters	Among subpopulations	1	197.31	1.09 Va	25.44	<0.001	F_{ST}=0.25
	Within subpopulations	356	1133.43	3.18 Vb	74.56	<0.001	
	Total	357	1330.74	4.27			

*Significance test was obtained after 50,000 permutations.

Lastly, bottleneck results indicated normal L-shaped distribution among C. sanguinolenta subpopulations and suggested that populations have not undergone a recent population bottleneck or expansion.

encouraging the cultivation of *C. sanguinolenta* among smallholder farmers should be explored to ensure its availability in the needed quantities, thus contributing to a decrease in the harvest volumes from the wild, as a way of conserving the species *in situ*.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Author contributions

Conceptualization JNA, RNT, and DH; methodology JNA and SLB; validation JNA and SLB; formal analysis JNA and DH; investigation, JNA; resources JNA, DH, and RNT; data curation JNA and DH; writing—original draft preparation JNA and DH; writing—review and editing all authors; visualization JNA; supervision JNA; project administration JNA; funding acquisition JNA. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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