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# Thonningia sanguinea Vahl. (Balanophoraceae) in Southern Nigeria: II. Patterns of Genetic Diversity and Population Structure within and between Populations

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## Cover Page Footnote

The authors would like to acknowledge the staff members of various national parks and forest reserves for their assistance in the course of sample collections. We would also like to express our gratitude to the management of the International Institute of Tropical Agriculture for providing a laboratory space (Bioscience Laboratory) for this work.

# *Thonningia sanguinea* **Vahl. (Balanophoraceae) in Southern Nigeria: II. Patterns of Genetic Diversity and Population Structure within and between Populations**

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#### **Abstract**

Studies have yet to assess the genetic variability in *Thonningia sanguinea* populations in Southern Nigeria*.* Hence, this study was conducted to elucidate the pattern of genetic variability and population structure among *T. sanguinea* populations in Southern Nigeria. Genomic DNA was extracted from 31 individuals in 15 populations and tested using random amplified polymorphic DNA (RAPD) primers. Several genetic diversity parameters were examined using GenALEx Ver. 6.5. Reproducible RAPD markers indicated that all the sampled populations were composed of individuals with a high genetic variability. The populations were grouped into four distinct clusters. The populations from the Okour community forest had high gene diversity and Shannon index. Conversely, the populations from Cross River National Park had the lowest gene diversity and Shannon index. Genetic variability did not correlate with geographic distances. Analysis of molecular variance revealed that most (82.3%) of the diversities could be explained by allelic variations within the population. An indirect estimate of gene flow yielded gave an N<sub>m</sub> of 1.09, indicating a low migration level among populations. Results demonstrated that *T. sanguinea* populations in Southern Nigeria exhibited outcrossing strategy expected of the sexual exchange of gametes by different individuals within a local population.

*Keywords: parasitic plants, RAPD, AMOVA, thonningia sanguinea, balanophoraceae*

## **Introduction**

A high degree of forest fragmentation has prompted researchers to assess the genetic variability and population structure of highly specialized species with a threatened conservation status. Genetic variability assessment provides an estimate of species fitness and the likelihood of a continuous adaptation to fluctuating environmental conditions [1]. Hence, thus procedure is an important prerequisite for the prediction of evolutionary responses [2]. Information on the genetic distinctiveness of plant species has been largely exploited to prioritize and make informed decisions on which species should be protected to conserve a large portion of the overall genetic diversity [3].

Genetic variability in parasitic plants slightly differs from that of other free-living plant species. In addition to the established agents of genetic variations, such as reproduction systems, genetic drift, and gene flow, the interactions of parasitic plants with their host plants exert a form of selective force that can lead to genetic variation and subsequent speciation [4-8]. This speciation

type occurs as a result of genetic modification that favors parasitic development on a particular host [9]. The high selection pressure associated with such host-parasite interactions can affect the morphological and physiological characteristics of a parasite in new directions, thereby leading to a phenomenon known as host-specific speciation [8].

*Thonningia sanguinea* Vahl (Balanophoraceae) is an endemic species in the tropical region of Africa [10]. As an obligate root parasite of a wide range of host species [11], it lacks true roots, stems, and leaves [12]. An array of *Thonningia* spp. includes *T. sanguinea* Vahl., *T. elegans* Hemsl., *T. dubia* Hemsl., *T. angolensis* Hemsl., *T. ugandensis* Hemsl., *T*. *sessilis* Lecomte., *T. malagasica* Fawcett. [13]. However, *Thonningia* is monotypic and other previously recognized species are regarded as synonyms [14,15]. The controversy regarding the monotypic nature of this species has yet to be resolved because of the simplified nature of its morphological characteristics, and previous attempts to assess the variability in *T. sanguinea* used mostly morphological and anatomic characteristics [14,16,17].

Advancements in accurate methods, such as molecular markers, offer a better understanding of systematic relationships within closely related species [18-21]. Among numerous molecular marker options available, AFLP, random amplified polymorphic DNA (RAPD), and SSR markers have been recommended for the delimitation of closely related species [22]. In RAPD based on polymerase chain reaction, small amounts of plant tissue are utilized and previous knowledge of the genome of the target species is not required [2,23]. Although,the RAPD marker usage is limited by experimental reproducibility, relatively low-resolution power, laboratory dependence, and difficulty in interpreting results. The use of a neutral marker that does not require previous knowledge of the genome of the target species and offers high genome coverage, to ensure that many loci are sampled for each individual per reaction justifies RAPD as a suitable start marker for *T. sanguinea*.

Therefore, this study was conducted to (i) elucidate the genetic variability and pattern of relatedness among *T. sanguinea* populations in Southern Nigeria. (ii) investigate the level of gene flow and test if the genetic distances among populations corresponded to geographic distances, and (iii) quantify the distribution pattern of genetic variation within and among populations.

## **Materials and Methods**

**Sample Collection.** Thirty-one individuals representing 15 populations of *T. sanguinea* were investigated and

two to three individuals were sampled per population (Table 1).

**DNA Isolation.** The bracts of the inflorescence were carefully excised and small roots and other attached debris were cleaned. They were surface sterilized with sterile distilled water and then with 80% ethanol. Genomic DNA was extracted in accordance with the CTAB method with slight modifications. In brief, 600 µL of freshly prepared CTAB extraction buffer was used for initial incubation. Afterward, 600 µL of 100% cold isopropanol (2-propanol; stored at -20  $^{\circ}$ C) was added to the resultant pure aqueous layer and mixed gently for about 5 min or gently inverted 50 times to allow the nucleic acid to precipitate. DNA pellets were washed twice with 400 µL of 70% ethanol. Then, 3 µL of RNAse A was added to degrade RNA.

**Quantification and Purification Assessment of DNA.** DNA quality and purity were assessed using a UV spectrophotometer. The ratio of  $OD_{260}/OD_{280}$  was determined to assess the purity of the sample. Then, 1 ml of Tris-EDTA (TE) buffer was placed in a cuvette, and the spectrophotometer was calibrated at 260 and 280 nm. 10 µL of each extracted and purified genomic DNA samples was added to 900 µL of TE buffer and mix well.  $OD_{260}$  and  $OD_{280}$  were recorded on a spectrophotometer, and TE buffer was used as a blank in the other cuvette. Data were collected for calculations by using the  $OD<sub>260</sub>/OD<sub>280</sub>$  ratio.

<b>Sampling location (State)</b>		Sample code Number of samples	<b>Coordinates</b>	<b>Host species</b>	
Cross River National Park (Cross River)	<b>CRNPI</b>	$\overline{2}$ 05°21.863″ 008°26.438″ E		Unidentified	
	<b>CRNP II</b>	$\overline{2}$	05°22.172″ 008°26.182″ E	Lophira alata	
	<b>CRNP III</b>	$\overline{2}$	05°21.863″ 008°26.438″ E	Musanga cecropiodes	
	<b>CRNP IV</b>	3	05°21.925″ 008°26.350″ E	Lophira alata	
Okomu National Park (Edo)	<b>ONP 55</b>	$\overline{2}$	06°21.656″ 005°21.587″ E	Ricinodendron heudelotii	
	ONP <sub>61</sub>	$\overline{2}$	06°20.764″ 005°20.697″ E	Musanga cecropiodes	
	<b>ONP 53</b>	$\overline{2}$	06°20.135″ 005°20.470″ E	Myrianthus arboreus	
	ONP <sub>33</sub>	$\overline{2}$	06°24.113″ 005°19.44″ E	Theobroma cacao	
Idanre Forest Reserve (Ondo)	Idanre FR	$\overline{c}$	07°01.954″ 005°09.868″ E	Theobroma cacao	
Ofosu Forest Reserve (Ondo)	Ofosu FR	$\overline{2}$	06°43.278″ 005°07.852″ E	Musanga cecropiodes	
Oba hills Forest Reserve (Osun)	Oban hills FR	$\overline{c}$	07°45.275″ 004°07.752″ E	Unidentified	
<b>IITA Forest Reserve (Oyo)</b>	<b>IITA FR</b>	$\overline{2}$	07°29.820″ 003°53.530″ E	Unidentified	
Community forests (Edo)	Iyanomo FR	$\overline{c}$	06°09.746" 005°34.898" E	Hevea brasiliensis	
	Okour F	$\overline{2}$	06°11.962″ 006°04.928″ E	Hevea brasiliensis	
	Okokhuo F	2	06°34.909″005°36.415″E	Hevea brasiliensis	

**Table 1. Sample Collection, Location and Georeference of** *Thonningia* **across Southern Nigeria**

**Random Amplified Polymorphic DNA procedure.** Eight (8) RAPD primers (Thermo Scientific, Lithuania) were prescreened to select the best markers for further RAPD analysis. Genomic DNA was subjected to the following PCR cocktail mix and conditions. A reaction volume of 10 µL, containing the following was used: 1.0  $\mu$ L of 10X PCR buffer, 0.4  $\mu$ L of 50 mM MgCl<sub>2</sub>, 0.1 µL of 5µ/µL *Taq* DNA polymerase, 0.8 µL of DMSO, 0.8 µL of 10 mM dNTPs (2.5 mM each of dATP, dCTP, dGTP, and dTTP), 0.5 µL of 5 µM primer (forward and reverse primers), 3.0  $\mu$ L of 100 ng  $\mu$ L<sup>1</sup> template DNA, and 2.9 µL of distilled water. Thin-walled 0.2 mL PCR tubes were used for PCR carried out using a thermocycler. Amplification conditions were as follows: initial denaturation at 94 °C for 5 min; followed by 40 cycles of denaturation at 94 °C for 30 sec., annealing at 37 °C for 30 s, and elongation at 72 °C for 1 min; final extension at 72 °C for 7 min; and a hold period at 10 °C. RAPD products were separated on 1.5% agarose gel electrophoresis through 1*×* TBE buffer stained with GR Green and visualized on a gel documentation system. The molecular size of the amplified products was estimated using a 50 bp ladder from Thermo Scientific.

**Data Analysis.** A binary matrix of the amplified fragments was created by visually scoring with (1) and (0) indicating the presence and absence of bands respectively. The scoring procedure was replicated by up to three times, to avoid scoring errors, and only distinct clear bands were noted. The presence - absence matrices of RAPD bands were analyzed using a neighbor-joining agglomerative clustering technique (Paleontological Statistics version 3.23) to illustrate the relatedness among individuals and populations and check the patterns of host-derived speciation. The neighbor-joining method permits different branch lengths, indicating the evolutionary time or amount of evolutionary changes along a branch [24]. Genetic divergence among populations was estimated by calculating Nei's genetic distance (D) and identity (I) for all population pairs [25]. Other standard measures of the genetic diversity of each population, including the number of different alleles, the number of effective alleles, Shannon's information index, gene diversity, unbiased diversity, and percentage of polymorphic loci, were also calculated. Analysis of molecular variance (AMOVA) was conducted to show the variance components and their significance variation levels within and among *T. sanguinea* populations by using GenALEx software ver. 6.5. A mantel test was carried out to examine whether the matrices of genetic distances between populations were significantly correlated with the matrices of geographic distances (9999 permutations; Paleontological Statistics; version 3.23). Under the assumptions of the wright island model, gene flow (the number of migrants per generation =  $N_m$ ) was estimated through AMOVA  $\varnothing$  statistics which is analogous to Fstatistics, as expressed in the following equation:  $Nm =$ 

 $1/4[(1/\mathcal{O}st) - 1]$ , where, N<sub>e</sub> = is the effective population size, and  $M =$  is the migration rate [26].

#### **Results**

**Nei's Genetic Variability Statistics for all Loci per Population of** *T. sangunea***.** Eight primers were optimized to determine the best polymorphic primers to be used for assessing the variability pattern in *T. sanguinea.* They included OPT –01, OPH –02, OPT –04, OPH –05, OPH  $-06$ , OPT  $-07$ , OPB  $-10$  and OPT  $-20$ . Of these primers, OPH-05 and OPH-07 primers were selected on the basis of polymorphism effectiveness.

The Nei genetic variability statistics of all loci per population revealed that the observed number of alleles (Na) ranged from 1.00 to 1.57 with an average of 1.25 allele per population. The effective number of alleles (Ne) ranged between 0.57 and 1.43 with an average of 0.92 per population. Shannon's information index (I) ranged between 0.09 and 0.39 with an average of 0.17 per population. Gene diversity (h) ranged between 0.07 and 0.29 with an average of 0.12 per population. The unbiased diversity was highest in *T. sanguinea* populations from Okour Forest and the lowest in *T. sanguinea* populations from CRNP I (Table 2).

The genetic variability statistics of *Thonningia* showed that populations from Okuor community forest had the highest Shannon diversity index and gene diversity value of 0.396 and 0.286, respectively. These results indicated the composition of *T. sanguinea* individuals with high genetic heterogeneity. However, the populations from Cross River National Park had the lowest, Shannon's information index, gene diversity and percentage polymorphic loci.

**Genetic Distance and Identity of the selected populations of** *T. sanguinea* **Populations from Southern Nigeria**. The genetic distance and identity of various populations of *T. sanguinea* from Southern Nigeria are presented in Table 3. The upper matrix value between the two populations revealed the genetic distance between them. The populations from Okomu National Park (ONP 31) and CRNP III were the most genetically distinct of all other population with a genetic distance of 0.773. Geographical distance did not affect genetic distance. Geographically close populations were relatively expected to have a low genetic distance. However, the data observed showed a high value. For example *T. sanguinea* populations from Cross River National Park (Location 1) were geographically close with Cross River National Park (Location III), and both had a genetic distance of 0.77. Conversely, a geographically distant *T. sanguinea* population pair, namely, CRNP I and Iyanomo population had a low genetic distance value of 0.204 apart. Genetic identities, a measure of the proportion of genes that are identical in two populations, follow the same sequence with a genetic distance but with a reverse interpretation. The populations with a wide genetic distance would have a low genetic identity value. Hence, the population pairs of CRNP III and Iyanomo had a low genetic distance and a high genetic identity of 0.262 and 0.769, respectively.



**Figure 1. Parts of Southern Nigeria where** *T. sanguinea* **Samples were Collected for DNA analysis**





Legend: Na = No. of Different Alleles; Ne = No. of Effective Alleles; I = Shannon's Information Index; h = Gene Diversity; uh = Unbiased Diversity; PPI= Polymorphic loci.





Legend: **CRNP-** Cross River National Park**; IITA -** International Institute for Tropical Agriculture; **ONP -** Okomu National Park**; FR -** Forest Reserve



**Figure 2. Zoom in Image of** *T. sanguinea***. (A) Male Inflorescence Head (B) Female Inflorescence Head**

**Phenogram Genetic Distance among** *T. sanguinea* **Population from the selected Sites in Southern Nigeria**. In Figure 3, the phenogram genetic distance among *T. sanguinea* populations constructed from RAPD data by using the neigbour-joining algorithm depicted four major clusters; cluster (1) populations from the Okour community forest; cluster (2) populations from the Okokhuo community forest and a geographically distant Cross River National Park (CRNP III) population; cluster (3) populations from the Okomu National Park, and the Okokhuo community forest; and cluster (4) geographically separated populations, which include those

in Cross River National Park (I), Cross River National Park (II) and Cross River National Park (IV), IITA Forest Reserve, Ofosu Forest Reserve, Iyanomo Forest Reserves and Oban Hills Forest Reserve.

**The Pattern of Host-Derived Speciation in** *T. sanguinea***.** One case of close relationship between the populations Id/Theobroma from Idanre Forest Reserve and ONP/Theobroma from Okomu National Park from a common *Theobroma* host source in different geographic locations was recorded (Figure 4).



**Figure 3. RAPD PCR Band Pattern of** *Thonningia sanguinea* **Population with (A) HO5 and (B) HO7 Primers**

Lane 1: 50 bp ladder; Lanes 2 and 3: Iyanomo FR; Lane 4 & 5: Okour F; Lanes 6 and 7: Okokhuo F; Lanes 8 and 9: ONP 55; Lanes 10 and 11:ONP 61; Lanes 12 and 13: ONP 55; Lanes 14 and 15: ONP 33; Lanes 16 and 17: CRNP I; Lanes 18 and 19: CRNP II; Lanes 20 and 21: CRNP III; Lanes 22 - 24: CRNP IV; Lanes 25 and 26: Idanre FR; Lanes 27 and 28: Ofosu FR; Lanes 29 and 30: Oban Hill FR; Lanes 31 and 32: IITA FR.



**Figure 4. Phenogram Constructed from RAPD data by Using Neigbor-joining Method and Showing the Genetic Distance among** *T. sanguinea* **Populations**



**Figure 5. Neighbor-joining Tree Generated from RAPD DNA Data Showing Patterns of the Host-derived Speciation of** *T. sanguinea*



**Figure 6. Relationship between Pairwise Geographic and Pairwise Genetic Distances among 15** *T. sanguinea* **Populations. Correlation R = 0.0412;**  $p = 0.286$ 

**Table 4. Population Genetic Structure and Estimated Gene Flow Value of** *T. sanguinea* **in Southern Nigeria**

<b>Source of variation</b>	$d.f^*$	SS.	MS.		Variance % of total variance	$Fst(\emptyset)$	$N_{\rm em}$
Among population	14	16.898 1.207		0.186	18.6		
Within population	16	13.167 0.823		0.823	82.3	0.186	1.09
Total	30	30.065	1.00	1.009	100.0		
. ___							

\*d.f: degree of freedom

SS: sum of square

Ms: mean sum of square Vc: estimate of variance component

Fst: Fixation index

Nem: average number of migrant exchange per generation

% of total variance: the percentage of total variance gives the contribution of each component to the total variation. The difference between values is highly significant ( $P < 0.0001$ ).

#### **AMOVA**

AMOVA revealed the percentage of the total variance that outlined the contribution of each component (among population and between populations) to the total genetic variability of the population. Table 4 shows that a large proportion (82%) of the genetic variation could be explained by allelic variation within the population, whereas the variation among the populations was 17.7%.

**Correlation of Geographical and Genetic Distance.** Figure 5, the mantel test revealed that the relationship between the straight geographic distance and Nei's genetic distances was not statistically significant ( $p = 0.286$ ; R = 0.0412; Permutation =  $9999$ ). Thus, geographic distance

did not explain genetic distance among *T. sanguinea* populations in Southern Nigeria.

#### **Discussion**

The genetic variability within and among *T. sanguinea* populations was problematic because of the use of morphological and anatomic characters, which were highly limited and plastic, consequently resulting in the low detection of genetic differences among populations. This study applied molecular characterization through RAPD techniques, neighbor-joining clustering method, and analysis of molecular variance, to give a clearer picture of its relationship, genetic variability, and population structure of this species.

The genetic variability pattern of *T. sanguinea* populations in Southern Nigeria comprised four distinct clusters. The first cluster was composed of populations from the Okuor community forest. The uniqueness of this population was further validated by the possession of a high Shannon diversity index and gene diversity value, and this population consequently represented the most genetically diverse of all *Thonningia* populations analyzed in this study. The second cluster included populations from Cross River National Park (CRNP III) and Okokhuo community forest. This finding showed the genetic relatedness of two geographically distant populations of over 585 km apart. The third cluster constituted a rather complex aggregation of populations from Okomu National Park, Idanre Forest Reserve, and Okokhuo community forest. However, if populations from Idanre Forest Reserve were left out, the close geographic distance between populations of the Okomu National Park and Okokhuo forest was an indication of a relatively recent separating population that was initially one. The fourth clade comprised populations from Cross River National Park, IITA Forest Reserve, Ofosu Forest Reserve and Oba hill Forest Reserve. Although we recognized the disadvantages in the utilization of the RAPD technique in systematics, the neutrality of the marker was consistent with the fact that it requires no prior knowledge of the genome of the target species, and this finding justifies its use in the study. Other studies have supported and reiterated confidence in the use of RAPD as a suitable method for the studies on genetic variation among populations or species [20,27-30].

The populations from Okuor community forest had the highest Shannon diversity index and gene diversity of 0.396 and 0.286, respectively, so they contained individuals with a high genetic heterogeneity. The population-CRNP I from Cross River National Park had low generic variability indices as indicated by Shannon's information index (0.00), gene diversity (0.00), and percentage polymorphic loci (0.00). The low genetic variability index recorded for the populations from Cross River National Park could be best explained by the geographic separation from other populations in Southern Nigeria. The distribution of *T. sanguinea* in Southern Nigeria indicated a form of the vicariance pattern of distribution, and it was attributed to the depleted nature of the forests of Enugu, Imo, Anambra, and Ebonyi. Thus, a partial savannah-like environment inhabitable for *T. sanguinea* was created. The implication of such a vicariance pattern of distribution could have resulted in the formation of a founder population as exemplified in the CRNP I populations.

The phenomenon of host-specific speciation in a parasite - host relationship has yet to be elucidated. Although, a majority of studies have shown that host specificity does not appear to be the primary factor shaping the population structure of parasitic plants [31,32]. However, host specificity is observed in *Orobanche minor* that genetically and physiologically adapts to their hosts [8]. In the present study, we recorded one case of a close clustering pattern between the populations Id/Theobroma from Idanre forest reserve and ONP/Theobroma from the Okomu National Park using a *Theobroma* host source in different geographic locations. However, other populations did not form clusters following the type of host species.

The relationship between pairwise geographic and pairwise genetic distance among the 15 populations of *T. sanguinea* was not statistically significant ( $p = 0.286$ ). This finding indicated that the genetic relationship among *T. sanguinea* populations was not correlated with geographic distances. Consequently, the population pairs of relatively close geographic distances had a wide genetic distance and a corresponding low genetic identity. This finding corroborates with those from similar genetic diversity studies on different plant species [29,30,33,34]. Although different molecular markers, such as isozyme, AFLP, and RAPD are used in genetic diversity analysis in plants, it is believed that this occurrence is not affected by molecular maker selection and that it is not a sampling artifact [30].

AMOVA revealed that the genetic variation in *T. sanguinea* is partitioned within individuals. Most (82.3%) of the diversity could be explained by allelic variation within the population, whereas the variation among the population was 17.7%. This finding demonstrated that *T. sanguinea* exhibited an outcrossing strategy expected of the sexual exchange of gametes by different individuals within a local population. The genetic variation among the populations precludes the possible occurrence of genetic drift, which ultimately led to speciation. The high frequency of insect-inflorescence association might be largely responsible for inducing pollination within individuals of close geographic proximities.

An indirect estimate of gene flow yielded an N*m* of 1.09, which indicated a low migration level of propagules among *T. sanguinea* populations. The high N*m* again corroborated the low level of population genetic differentiation (Fst) among the populations. Although the high genetic variability within individuals of a local population may suggest the sexual outcrossing among individuals in such a local deme, indirectly estimated gene flow among demes was low. The ecological characteristics of this plant are likely to explain the dispersal pattern. The spatial geographical distribution of *T. sanguinea* is highly dependent on the distribution of host species and habitat preference. These factors create forms of isolated populations, separated over a considerable distance which precludes the likelihood of genetic variation by hampering the movement of propagules such as seeds and pollens among populations. Longdistance dispersal agents might be deficient. In addition to insects that serve as potential pollinators, slender mongoose (*Galerella sanguinea*) and duiker *Cephalophus* sp*.* regularly feed on inflorescence. However, the mechanism of dispersal by these agents has not been properly established. Therefore a low gene flow was recorded, suggesting that different *T. sanguinea* populations in Southern Nigeria are not too genetically distinct from one another. In this case, the monotypic nature as previously perceived remains.

## **Conclusion**

The evaluation of the genetic variability of *T. sanguinea* in Southern Nigeria showed that this species exist as cryptic taxa in which an individual could be observed genetically, but no morphological distinction could be found. The genetic relationship among *T. sanguinea* populations in Southern Nigeria was also clarified and the populations were separated into four distinct groups. In terms of population genetic structure, most  $(82.3\%)$ of the genetic variabilities were found within populations rather than among populations in different locations. This finding suggested that *Thonningia* could be an outbreeder, and the high frequency of insect inflorescence association might be responsible for the high within-population genetic variability observed in local *T. sanguinea* populations of the plant.

## **Acknowledgments**

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