



## PHYLOGENETIC STUDY OF WEST AFRICAN MANATEE (*TRICHECHUS SENEGALENSIS* LINK (1708) ALONG RIVER BENUE, NIGERIA

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### ABSTRACT

*The Phylogenetic study of West African Manatee was carried out to determine the relatedness of the same species at two distant locations Lokoja and Makurdi along River Benue in Nigeria. DNA was extracted and sequenced according to standard protocols. The result indicated that transition/transversion rate /ratios are  $k_1 = 2.245$  (purines) and  $k_2 = 0.00$  (pyrimidines). The estimated transition/transversion bias  $R = 0.701$ . The average population coefficient of evolutionary differentiation ( $D=0.013\pm0.004$ ) was 38.5% less than the intra-population value ( $D=0.018\pm0.006$ ). K2P distance for nucleotide diversity shows that the Lokoja A (LA) sample is close to the Makurdi B (MB) sample while MB is closer to Lokoja B (LB). In terms of the phylogenetic tree the result indicates that the species Lokoja A is more closely related to Makurdi than it is to Lokoja B. It is either that Lokoja A migrated to Lokoja from Makurdi or the sample was taken there by the fishermen.*

**Keywords:** phylogenetic, west African manatee, Benue

### INTRODUCTION

Conservation genetics is an interdisciplinary subfield of Population Genetics that aims to understand the dynamics of genes in populations principally to avoid extinction. Genetic data have been instrumental in the determination of listing status for several species. The data from genetics, coupled with other demographic sources of information on life history parameters, abundance, distribution and habitat are necessary for determining which population units or stock will benefit from applied management decisions.

Several authors have reported on the genetic studies of manatees (Contanhede *et al.*, 2005, Tucker *et al.*, 2013, Hunter, 2013 and Anne *et al.*, 2017). Most of these studies are on the Florida manatee as well as the Amazonian manatee and their subspecies. Hunter *et al.*, (2010) reported on the low genetic variation and evidence of limited dispersal in the regionally important Belize Manatee. A low genetic diversity was detected ( $H_E = 0.455$ ;  $N_A = 3.4$ ) corresponding to the small

population size and long term exploitation. The manatees from the Belize city Cayes and Southern Lagoon system were genetically different with microsatellite and mitochondrial  $F_{st}$  values of 0.229 and 0.078 respectively ( $P \leq 0.05$ ).

Contanhede *et al.*, (2005) reported on the phylogeography and population genetics of the endangered Amazonian Manatee (*Trichechus Inunguis*). Nested Clade analysis indicated restricted gene flow or dispersal. A study on the low genetic diversity and minimal population substructure in the endangered Florida Manatee was reported by Tucker *et al.*, (2013). The work used microsatellite markers to genotype 362 Florida Manatees and reported that they observed and expected heterozygosity and average number of alleles are  $0.455 \pm 0.04$ ,  $0.479 \pm 0.04$ , and  $4.77 \pm 0.51$ , respectively. The overall estimate of differentiation was low, though significantly greater than zero and analysis of molecular variance indicated that over 95% of the total

variance was among individuals within predefined management units or among individuals along the coastal sub-population, with only minor portions of variance explained by between group variance. Hunter (2013) reported that the Antillean Manatee has a low genetic diversity than the Florida Manatee. Pause (2000) reported on the conservation genetics of the Florida Manatee (*Trichechus Manatus latirostis*) using eleven new microsatellite loci and seven previously used loci as markers, thereby developing a manatee DNA individual genetic identification system (MIGS). The system was developed as a model to be used in the future for capture-recapture population modeling application. Secondly, the report indicated that population genetic analysis suggests that there is subtle, but statistically significant population structure that corresponds to the previously designated management unit.

Genetic studies on the African manatee (*Trichechus senegalensis*) have not been reported much in literature. Keith-Diagne (2014) reported on the phylogenetic and feeding ecology of the African Manatee. The report found fourteen new control regions and nine haplotypes for the species. The new haplotypes were characterized by high haplotypes diversity, low nucleotide diversity, indicating expansion after a period of low effective population size. Divergence analyses estimates that the African manatee diverged from the West Indian species 3.49Mya (CI 95%) further analyses indicated a separation of African manatee north and south regional clades at 1.51 Mya (CI 95%). While there are no *Trichechus senegalensis* subspecies, the existing genetic information indicates high genetic diversity (Vianna *et al* 2005) low nucleotide diversity and the existence of two clades that align geographically. Anne *et al.*, (2017) also reported low genetic diversity of African manatee in the Congo River. Vianna *et a.,l* (2006) reported on the phylogeography of African manatees. The report indicated that there were eighty polymorphic sites for the mt DNA control region including 74 transitions and 11 transversions among *Trichechus manatus*, *Trichechus inunguis* and *Trichechus senegalensis*. Both transitions and transversions were detected at positions 192,267, 328,331 and 332. The African species displayed the highest haplotype diversity ( $H = 0.933$   $N = 6$ ) following by *Trichechus inunguis* ( $H = 0.8772$ ,  $n = 92$ ) and *Trichechus manatus* ( $h = 0.08544$ ,  $N = 2227$  excluding the likely hybrid. However, the

Amazonian manatee had the lowest nucleotide diversity ( $TT = 0.005353$ ), while the West Indian Manatee had the highest nucleotide diversity ( $TT = 0.038648$ ) followed by African manatee ( $TT = 0.019581$ ).

The phylogenetic relationship among the three species suggest that *Trichechus inunguis* and *Trichechus manatus* form a monophyletic clade, with *Trichechus inunguis* more related to the cluster of *Trichechus manatus*. This means *Trichechus manatus* is a paraphyletic species. In another study on Antillean Manatee in Northern Brazil, Luna *et al.*, (2012) reported that mitochondrial DNA analyses identified low haplotype ( $h = 0.08$ ) and nucleotide ( $ST = 0.0026$ ), genetic diversity in the three closely related haplotypes. All three haplotypes, (MO1, MO3, and a previously unidentified haplotype MO4) were found in the Northern portion of the regions, while only a single haplotype (MO1) was represented in the south. This suggested the presence of two genetic groups with a central mixing zone. They concluded that the small population size coupled with low genetic diversity indicated that the north-east Brazil manatee population is susceptible to inbreeding depression and possible local extinction. This work tries to answer the question of whether there exist differences in genetic structure of the same species of manatees at two distant locations along river Benue.

## MATERIALS AND METHODS

### Study Area

The study covered a section of the River Benue system stretching a total of 462 km in length from Gbajimba town, the Headquarters of Guma Local Government Area of Benue State to Lokoja the capital of Kogi State.

The area lies within latitude  $07^{\circ} 49'N$  and  $07^{\circ} 52'N$  and longitude  $08^{\circ} 36'E$  and  $08^{\circ} 40'E$ . The major tributaries in the River Benue along this area include Rivers Guma, Katsina-Ala, Mu, and Gwer. Several small fishing settlements are found all along this area, the major ones being, Gbajimba, Makurdi and Abinsi.

River Benue takes its origin from the Adamawa highlands in the Western Cameroon and it is the largest tributary to the River Niger which its confluences with at Lokoja, Kogi State. River Benue enters Nigeria a few kilometers East of Yola in Adamawa State, flowing westwards for a

distance of about 780 km before joining River Niger at Lokoja. It flows through Adamawa, Taraba, Benue and Kogi State and has several tributaries. Unlike other major African Rivers, the Benue flows free of rapids and waterfalls (Udoh, 1981)

### Data Collection and Analysis

Four (4) samples of Manatee carcass, the bone and penile organ (2) were collected from fishermen in Lokoja while the bones and skin (2) was obtained from Makurdi-Gbajimba axis along River Benue. The collected samples were sun dried and using pestle and mortar pound into powder, sealed in clean labeled bottles and sent to DNALAB Kaduna for DNA extraction, PCR and sequencing using Trichechid primers obtained from NCBI. The DNA sequenced data was analyzed using the MEGA 6 programme after alignment. Sequenced data was converted to FASTA format in order to be identified in MEGA 6. The sequenced files were open and saved in MEGA format.

To obtain the Nucleotide composition, the statistic option on the sequenced data explorer was used. This option computed the nucleotide frequencies and nucleotide composition. Mutations (Transition/Transversion). To get an idea on mutations, the transition/transversion rates ratios was used. To obtain this the model Tab on the main MEGA6 window was used. The Maximum composite likelihood (MCL) Transition/Transversion bias was selected and results obtained. The model used in this regard was the Tamura-Nei model.

### Diversity

To obtain estimate of diversity, the distance diversity Tab on the main MEGA6 window was used. To obtain the mean diversity within subpopulation the Distance Tab was used. This also used the maximum composite likelihood method with the assumption that uniform rates exist within the subpopulation. To achieve the inter-population diversity, the between group mean distance option on the Distance Tab was selected. To obtain the entire population diversity, the overall mean distance option of the Distance Tab option was selected.

### Pairwise Kimura 2 parameter

To determine the Pairwise Kimura2 parameter distances of the three manatee samples, the Pair distance option was selected.

### Relative synonymous codon usage (RSCU)

To derive the Relative synonymous codon usage, between manatee populations in Lokoja and Makurdi, the Lokoja and Makurdi groups were each selected and analyzed separately using the statistic menu on the sequenced data explorer window. Result were obtained on Microsoft excel. Nucleotide sequences for *T. senegalensis*, *T. manatus* and *T. inunguis* were obtained from the NCBI website (<https://www.ncbi.nlm.nih.gov/>) in FASTA format before being aligned alongside the local specimen nucleotide sequence for phylogenetic relationship or evolutionary history analysis.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates (is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates are collapsed. The evolutionary distances were computed using the Kimura 2-parameter method adopted by Kimura (1980) and were in the units of the number of transitional substitutions per site. The analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 1039 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 according to Tamura *et al.* (2013).

## RESULTS

### Mutations (Transition/Transversion)

The transition/transversion rate ratios (Table1) are  $k_1 = 2.245$  (purines) and  $k_2 = 0.00$  (pyrimidines). The estimated transition/transversion bias  $R = 0.701$ . Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in *italics*.  $[R = (A * G * k_1 + T * C * k_2) / ((A + G) * (T + C))]$

**Table 1: Mitochondrial Cytochrome oxidase b (CYTB) Nucleotide Composition** (Average nucleotide frequencies (%))

Location	Nucleic acids			
	T	C	A	G
Lokoja_A	26.8	17.0	25.6	30.7
Lokoja_B	26.4	17.3	25.5	30.8
Makurdi_B	26.2	16.7	25.4	31.7
Avg.	26.5	17.0	25.5	31.1

T= Thymine; C = cytosine; A = Adenine; G = Guanine

**Table 2: Maximum Composite Likelihood Estimate of the pattern of Nucleotide Substitution**

	A	T	C	G
A	-	8.11	5.38	21.46
T	7.64	-	0	9.56
C	7.64	0	-	9.56
G	17.16	8.11	5.38	-

**Transition/Transversion (ti/tv) rate ratios**

Purines (A-G) $k_1$	2.245
Pyrimidines (C-T) $k_2$	0.000
Overall ti/tv bias (R)	0.707

**Key:** A=Adenine, T=Thymine, C=Cytosine, G=Guanine,

**Diversity: Cytochrome C Oxidase Subunit I (COI) divergence**

The average population coefficient of evolutionary differentiation ( $D=0.013\pm0.004$ ) was 38.5% less than the intra-population value ( $D=0.018\pm0.006$ ) (Table3. K2P distance for

nucleotide diversity (Table 3) shows that the Lokoja A (LA) sample is close to the Makurdi B (MB) sample while MB is closer to Lokoja B (LB). Surprisingly, LB and LA have the highest distance between them.

**Table 3: Estimate of the Coefficient Of Evolutionary Differentiation (SE = Standard error)**

	Mean diversity within sub population*		Mean diversity Inter population diversity		Mean diversity of entire population	
	D	SE	D	SE	D	SE
Diversity	0.018	0.006	0.010	0.004	0.013	0.005

\* Estimate is for one sub-population alone (i.e. Lokoja)

**Table 4: Pairwise Kimura-2 Parameter (K2P) Distances of Three Manatee Strains.**

Inter-strain distances are on lower diagonal while standard errors are on upper diagonal

Location	LA	LB	MB
LA		0.00619	0.003805
LB	0.018280		0.005432
MB	0.006803	0.013672	

Inter-strain distances are on lower diagonal while standard errors are on upper diagonal

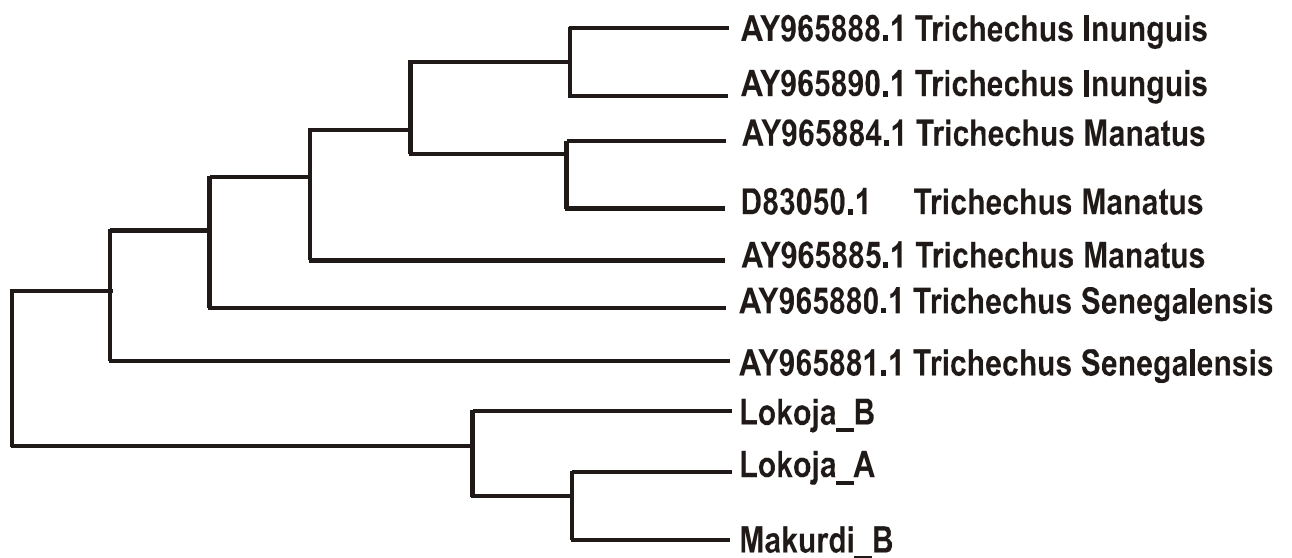
**Key:** LA=Lokoja A, LB=Lokoja B , MB=Makurdi B

**Table 5: Relative Synonymous Codon Usage (RSCU) between Manatee Populations in Lokoja and Makurdi**

Location	Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU
Makurdi	UUU(F)	3	2	UCU(S)	5	2.73	UAU(Y)	2	2	UGU(C)	3	2
	UUC(F)	0	0	UCC(S)	0	0	UAC(Y)	0	0	UGC(C)	0	0
	UUA(L)	0	0	UCA(S)	0	0	UAA(*)	0	0	UGA(W)	1	0.4
	UUG(L)	6	6	UCG(S)	3	1.64	UAG(*)	8	1.33	UGG(W)	4	1.6
Lokoja	UUU(F)	1	0.67	UCU(S)	2.5	1.3	UAU(Y)	0	0	UGU(C)	6	1.71
	UUC(F)	2	1.33	UCC(S)	2	1.04	UAC(Y)	0	0	UGC(C)	1	0.29
	UUA(L)	3	0.73	UCA(S)	0.5	0.26	UAA(*)	1	0.42	UGA(W)	7	0.93
	UUG(L)	4	0.98	UCG(S)	0.5	0.26	UAG(*)	1.5	0.63	UGG(W)	8	1.07
Makurdi	CUU(L)	0	0	CCU(P)	6	1.6	CAU(H)	3	2	CGU(R)	1	1
	CUC(L)	0	0	CCC(P)	1	0.27	CAC(H)	0	0	CGC(R)	0	0
	CUA(L)	0	0	CCA(P)	5	1.33	CAA(Q)	2	0.8	CGA(R)	1	1
	CUG(L)	0	0	CCG(P)	3	0.8	CAG(Q)	3	1.2	CGG(R)	2	2
Lokoja	CUU(L)	1	0.24	CCU(P)	0	0	CAU(H)	1	1	CGU(R)	5.5	1.52
	CUC(L)	5	1.22	CCC(P)	3	4	CAC(H)	1	1	CGC(R)	0	0
	CUA(L)	6.5	1.59	CCA(P)	0	0	CAA(Q)	2	1.33	CGA(R)	4	1.1
	CUG(L)	5	1.22	CCG(P)	0	0	CAG(Q)	1	0.67	CGG(R)	5	1.38
Makurdi	AUU(I)	4	1.14	ACU(T)	5	1.82	AAU(N)	9	1.8	AGU(S)	2	1.09
	AUC(I)	3	0.86	ACC(T)	0	0	AAC(N)	1	0.2	AGC(S)	1	0.55
	AUA(M)	1	0.18	ACA(T)	1	0.36	AAA(K)	4	1.33	AGA(*)	4	0.67
	AUG(M)	10	1.82	ACG(T)	5	1.82	AAG(K)	2	0.67	AGG(*)	12	2
Lokoja	AUU(I)	2	0.8	ACU(T)	1	1.33	AAU(N)	2.5	1.43	AGU(S)	4	2.09
	AUC(I)	3	1.2	ACC(T)	1	1.33	AAC(N)	1	0.57	AGC(S)	2	1.04
	AUA(M)	6	1.26	ACA(T)	0	0	AAA(K)	2.5	1.11	AGA(*)	4	1.68
	AUG(M)	3.5	0.74	ACG(T)	1	1.33	AAG(K)	2	0.89	AGG(*)	3	1.26
Makurdi	GUU(V)	4	0.94	GCU(A)	7	2	GAU(D)	5	1.67	GGU(G)	1	0.8
	GUC(V)	2	0.47	GCC(A)	1	0.29	GAC(D)	1	0.33	GGC(G)	0	0
	GUA(V)	1	0.24	GCA(A)	1	0.29	GAA(E)	2	0.67	GGA(G)	1	0.8
	GUG(V)	10	2.35	GCG(A)	5	1.43	GAG(E)	4	1.33	GGG(G)	3	2.4
Lokoja	GUU(V)	0.5	0.29	GCU(A)	1.5	3	GAU(D)	2.5	1.43	GGU(G)	4	0.86
	GUC(V)	1.5	0.86	GCC(A)	0	0	GAC(D)	1	0.57	GGC(G)	2	0.43
	GUA(V)	4	2.29	GCA(A)	0	0	GAA(E)	4.5	2	GGA(G)	7.5	1.62
	GUG(V)	1	0.57	GCG(A)	0.5	1	GAG(E)	0	0	GGG(G)	5	1.08

**Legend**

	<b>Letter code</b>
Amino acid	
Alanine	A
Arginine	R
Asparagine	N
Aspartic acid	D
Cysteine	C
Glutamine	Q
Glutamic acid	E
Glycine	G
Histamine	H
Isoleusine	I
Leusine	L
Lysine	K
Metionine	M
Phenylalanine	F
Proline	P
Serine	S
Threomine	T
Tryptophan	Y
Tryptophan	V
Selenocyteine	U



**Figure 2: Phylogenetic Tree of three Species of Manatees in Relation to those of Makurdi and Lokoja**

## DISCUSSION

### Mitochondrial COI Nucleotide Composition

Overall nucleotide content across all samples of manatees evaluated shows that average nucleotide frequencies were 25.50% (A), 26.50% (T), 17.00% (C), and 31.10% (G). Guanine was high in all samples with a sample from Makurdi having the highest frequency of 31.10%. The frequency of cytosine is low in all samples compared to the frequencies of the other nucleic acids. The same sample from Makurdi that had the highest frequency of guanine has the lowest frequency of cytosine (16.70%) while the highest frequency of cytosine occurred in a sample from Lokoja (17.30%).

### Mutations (Transition/Transversion)

The transition/transversion rate ratios (Table 2) are  $k_1 = 2.245$  (purines) and  $k_2 = 0.00$  (pyrimidines). The estimated transition/transversion bias  $R = 0.701$ . Therefore average transitional pairs were more than transversional pairs. The rate of transitional substitution is higher between G and A and non-existent between C and T. On the other hand, the rate of transversional substitution is not neutral since values are not the same for each transversional pair of nucleic acids.

Diversity: Cytochrome c oxidase subunit I (COI) divergence

The average population coefficient of evolutionary differentiation ( $D=0.013\pm 0.004$ ) was 38.5% less than the intra-population value ( $D=0.018\pm 0.006$ ) (Table 3). K2P distance for nucleotide diversity shows that the Lokoja A (LA) sample is close to the Makurdi B (MB) sample while MB is closer to Lokoja B (LB). Surprisingly, Lokoja B and Lokoja A have the highest distance between them. This could be as a result of one of the samples coming from another location probably from the Niger Delta area or it could indicate positive mutation among the species in the area. Hunter (2013) reported that the Antillean Manatee has a low genetic diversity than the Florida Manatee. In another work Gomez-Corrasco *et al* (2018) on the genetic diversity and structure from Antillean Manatee (*Trichechus Manatus*) in the southern Gulf of Mexico in comparison to connected and isolated Island. The result indicated that landlocked manatees had lower genetic diversity when compared with the open populations. Estimate of the coefficient of

evolutionary differentiation-The coefficient of Evolutionary differentiation indicate a high mean diversity (0.018) within the Lokoja manatee samples, Makurdi had only one sample and so no within species analysis was carried out. The mean diversity between Lokoja and Makurdi(0.010) is lower than within Lokoja(0.018) and the mean diversity of the entire population(0.005). This result contrast with those of Anne *et al.* (2017) who reported that west African manatees in the Congo Basin had low genetic diversity. Keith-Diagne (2014) also discovered new haplotypes characterizes by high haplotype diversity, low nucleotide diversity which indicated an expansion after a low population size on that the West African manatee.

### Relative Synonymous Codon Usage (RSCU) between manatee populations in Lokoja and Makurdi.

Amino acids, the monomeric units of proteins are encoded by triplet of nucleotides called codons. Most of the amino acids have alternative codons which are known as synonymous codons. (Hoda *et al.*, 2014). Plotkin *et al.*, (2004) had reported that codon usage is tissue-specific. The phenomenon of codon usage bias, which can be interpreted as an essential mutational bias or translational selection, is an essential feature of most genomes across all the three domains of life(Duret,2002).Relative synonymous codon usage(Table 5) indicate that in Makurdi the codons UUU for the amino acid phenylalanine, UUG for Leucine, UCU for serine, UCG for serine, UAU for Tyrosine, CCU for proline, UGU for cysteine, UGG for Tryptophan, CCU for proline, CAU for Histidine, CGG for Arginine, AUU for Isoleucine, AAU for Asparagine GAU for Aspartic acid, GAG for Glutamic acid are preferentially coded as compared to Lokoja. In Lokoja, the codons preferred were UUC for phenylalanine, CUC for Leucine, CUA for leucine, CUF for Leucine, CCC for proline, CAA for Glutamine, AUC for Isoleucine, AUG for Methionine, AGU for serine, AGC for serine, GUA for Valine, GUA for Alanine, and GGA for Glycine. Codons with values above one indicate that these codons are highly used for a given amino acid. Conversely, the frequency of codon usage less than 1 is determined as low frequency codon, which is likely to affect the expression efficiency.

The differential codon usage bias of both Makurdi and Lokoja suggest that the two have the potential for genetic variability and hence the ability to survive the hazards of environmental changes. This potential agrees with United States Geological Survey USGS, (2018), who reported that, genetic diversity plays a critical role in the ability of a species to survive and that a diverse gene pool can increase a populations ability to adapt to disease, habitat modification, changes in the environment, such as altered hydrology and hurricane. In terms of the phylogenetic tree of the three existing manatee species in comparison to those of makurdi and Lokoja along River Benue the result indicates that the species Lokoja A is more closely related to Makurdi than it is to Lokoja B. It is either that Lokoja A migrated to Lokoja or that the carcass sample collected as Lokoja A may have been taken there by the fishermen. The three strains are more closely related to the DNA sequences of *Trichechus senegalensis* deposited with the National center for Biotechnology information (NCBI), they are also more related to *Trichechus manatus* than *Trichechus inunguis*. This result conforms with those of Keith-Diagne(2014) who reported similar relatedness among the three species of manatees with the west African Manatee.

## CONCLUSION

Manatees still exist along River Benue but they are threatened by mostly anthropogenic activities such as deliberate kills for their meat and fat used for various medicinal purposes as well as habitat destruction. The comparison of DNA samples from two locations 462km apart along the river

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indicates significant differences in the chromosomal sequences. This means that the manatees have innate ability to survive the natural disasters in their habitat. If protected from human induced threats, west African manatee are capable of surviving into the foreseeable future due to their positive ability for transversion mutations.

## RECOMMENDATION

In view of the heavy negative anthropogenic impact on West African Manatee along the River Benue, it is hereby recommended that:

1. Sensitization and Education of the Fishermen on the values of conserving the West African manatee in the river be carried out in all the fishing settlements along the river.
2. Aquaculture be introduced to the fishermen as additional source of livelihood to prevent them from killing manatee along the river.
3. The National Parks Commission should in collaboration with other Agencies make a section of the River Benue especially along Gbajimba an Aquatic Park. This would be the first of such protected zone in Nigeria.
4. Regular monitoring of water health and other parameters be carried out to gain more information on manatee habitat along the river. This will enhance West African Manatee management along the river.
5. More genetic study need to be undertaking to get a clear picture of manatees profile along the river.

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