SHST
Horticulture and Forestry Society of Transylvania

Iyiola OA *et al.* (2023)
Notulae Scientia Biologicae
Volume 15, Issue 4, Article number 11607
DOI:10.15835/nsb15411607
Research Article

NSB
Notulae Scientia Biologicae

DNA barcoding of *Tribolium castaneum* (Coleoptera: Tenebrionidae) from Selected states in Nigeria based on mitochondrial DNA sequences

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Abstract

Tribolium castaneum also known as red flour beetle is one of the most important pests of stored grain product with a cosmopolitan distribution in Nigeria and all over the world contributing to food spoilage. The aim of this study was to characterize the *T. castaneum* by morphometric and molecular analyses. Samples of yam flour with evidence of the red flour beetles present inside were obtained from four locations in Kwara, Kogi, Oyo and Ekiti states in Nigeria. Morphological and molecular identifications of *T. castaneum* were carried out using standard methods. A dissecting microscope was used to identify the beetles and measurements were taken using ImageJ. Genomic DNA was extracted and checked on 1.5% agarose gel to confirm the presence of DNA. Species-specific primers were used to amplify mitochondrial cytochrome oxidase I (COI) gene of *T. castaneum* and the PCR amplicon size was also checked on 1.5% agarose. Morphometric measurements showed that the highest mean number (33.00±4.24 mm) of *T. castaneum* larvae observed was recorded on day 61 in Ilorin and the lowest was in Iwo, Osun state (4.00±0.00 mm) on the same day. The mean of the total body length of larvae from sampling sites was (1.31±0.37 mm) with minimum and (1.63±1.14 mm) maximum lengths respectively. There was no significant difference ($p>0.05$) between the mean length of the larvae collected from the study locations. Aligned cytochrome oxidase subunit 1 (COI) sequences of 313bp were analyzed. Phylogenetic analysis inferred by maximum likelihood method showed that the *T. castaneum* sequences analyzed for this study and sequences obtained from GenBank formed a monophyletic group. The molecular and phylogenetic analyses confirmed the presence of a single species of *T. castaneum*. The results from this study showed low levels of genetic diversity and variability in the studied *T. castaneum* populations. The observed genetic similarity in *T. castaneum* could be due to the fact that they were probably from similar origin when compared with those in the GenBank database. However, further studies are needed with more samples to characterize *T. castaneum* species from stored food grains across Nigeria.

Received: 24 Jun 2023. Received in revised form: 27 Nov 2023. Accepted: 08 Dec 2023. Published online: 16 Dec 2023.
From Volume 13, Issue 1, 2021, Notulae Scientia Biologicae journal uses article numbers in place of the traditional method of continuous pagination through the volume. The journal will continue to appear quarterly, as before, with four annual numbers.

Keywords: COI gene; mitochondrial DNA; PCR; *Tribolium*

Abbreviations: COI (Cytochrome Oxidase I); PCR (Polymerase chain reaction); UPGMA (Unweighted Pair Group Method of Arithmetic mean); AMOVA (Analysis of Molecular Variance); EDTA-Ethylene diamine tetraacetic acid

Introduction

There is always primary competition for available human food from insect pests both on the field and in storage (Rizzo *et al*., 2021). There is a documented report which showed that one third of the world's food grains are lost yearly in storage due to insect infestation leading to reduction in useful nutritional content (Mestrovic *et al.,* 2006). Tropical and subtropical environments provide optimum conditions for pest activities and multiplication resulting in massive grain losses (Harein and Davis, 1992). Grain foods mostly made from wheat, oats, rice, rye, barley, millet and corn that are not properly stored may be susceptible to pest infestation and spoilage (Ogedegbe and Edoreh, 2014). Provisions of storage facilities have assisted in reducing food spoilage from pests thereby ensuring sustainable food productions. Global increases in trade and transportations have led to introductions of agriculturally important pest into entirely new geographical areas (Meurisse *et al.,* 2021).

T. castaneum (Herbst) commonly known as red flour beetle, is a recognized pest of food stored products-both raw and processed and is ranked among the most important pests ravaging stored food in storage facilities (Campbell *et al*., 2004; El-Aziz, 2011; Pointer *et al*., 2023). *T. castaneum* was first described by Herbst 1797 in one of the early attempts at a complete survey of the Order Coleoptera. It belongs to the order Coleoptera and family Tenebrionidae (Brown *et al*., 2009). *Tribolium* species has been adapted as a model organism because of its small size, short life cycle, and its association with human (Pointer *et al*., 2021). It has been used in genetic and ecological studies but in recent times, it has been employed for studies relating to evolution, general insect biology and developmental genetic system (Angelini and Jockusch, 2008). *T. castaneum* and *T. confusum,* two common species found in Nigeria, similar in appearance at their life stages and occupy similar ecological niches (Walter, 1990; Ming *et al*., 2014; Ogedegbe and Edoreh, 2014). *T. castaneum* is mostly found in tropical regions but it can survive under temperate conditions (Padin *et al*., 2013).

The red flour beetle is economically important because it reduces crop yield and value of stored food products with its droppings and fragments. These excrements from *T. casteneum* have been suggested to have devastating effects on human health through consumption of contaminated stored food (Zhang *et al*., 2016). Ogedegbe and Edoreh reported that different food substrates could contribute to different population growth of *Tribolium* species. *Tribolium* species are known to be destructive against stored food products thereby having negative impact on food availability especially in tropical countries of the world (Padin *et al*., 2013; Kayode *et al.,* 2014). Similarities in morphological characteristics of *Tribolium* species have rendered traditional methods insufficient for correct identification of individual species. *Tribolium* species are phenotypically similar in their adult and non-adult stages making morphological identification extremely difficult (Zhang *et al*., 2016). Zohry (2017) previously investigated *Tribolium species* morphologically using scanning electron microscope but morphological identification has proven to be unreliable, time consuming and confusing. Therefore, concerted effort is required for its management by agricultural experts and government agencies (Hodges *et al*., 1996) which is hinged on baseline information on its biology and genetic diversity (Demuth and Wade, 2007). This can only be achieved through correct, quick, and reliable methods of *Tribolium* species identification. Rapid and reliable approaches using molecular methods will assist the global effort to precisely detect *Tribolium species* with the overall aim to improve stored product integrated pest monitoring and management strategies and eradicate cereal pest responsible for infestation and contamination of wide variety of food items. In addition,

it is anticipated correct and precise identification of pest of stored product will help safeguard food poisoning. A combination polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) techniques have been previously used for species identification of many insect taxa (Szalanski *et al*., 2003; Thyssen *et al*., 2005; Rugman-Jones *et al*., 2006; Qin *et al*., 2008; Wang *et al*., 2009). Investigating molecular diversity and evolutionary relationship that exist among *Tribolium* species using different mitochondrial gene region has shown high rates of nucleotide base substitution and variations (Havill *et al*., 2007). Some molecular markers such as COI, ITS1 and ITS2, 28S rDNA have been explored in many studies for accurate identification of *Tribolium* species (Mestrovic *et al.,* 2006; Nowaczyk *et al*., 2009; Ming *et al*., 2014; Zhang *et al*., 2016). Ming *et al*. (2014) investigated interspecific variations between *T. castaneum* and *T. confusum* using PCR-RFLP marker of 28S rRNA gene region and the findings revealed seventy-six species-specific differences amongst *Tribolium* samples studied with no intra-specific variations observed.

Ming *et al.* (2014) also sequenced the Cyt b and COI regions of *Tribolium* species and reported that although *T. castaneum* and *T. confusum* are similar in size and form, they are genetically different. Ming *et al.* (2014) then suggested that *T. castaneum* is more closely related to *T. freemani* while *T. confusum* shares genetic similarities with *T. destructor*. However, there is paucity of documented information on *Tribolium* identification using DNA barcoding approach (Zhang *et al*., 2016). DNA barcoding identifies sequence similarity amongst organisms of a reference taxon. The barcoding region used is usually a fragment from the mitochondrial COI gene. This method is a simple molecular identification tool and considerably reliable and suitable for species delimitation. Its application is extensive across metazoan taxa (Virgilio *et al*., 2010). This method is advantageous because it requires small quantity of sample, can easily distinguish species that appear phenotypically similar and can be used for identification of all life cycle stages of animal species (Ajamma *et al.,* 2016). In a comparative study of the variation in the satellite DNA of the *Tribolium* species carried out by Ugarkovic *et al.* (1996), the findings revealed that sequence variations exist in the satellites DNA, genetic and cytogenetic characters of *Tribolium* species studied. Precise identification of pest of stored products is an important part of integrated pest management. Therefore, this study investigated molecular diversity existing among *T. castaneum* from grain food samples collected from selected states in Nigeria using DNA barcoding approach. This is required to provide baseline information on genetic diversity of *T. castaneum* and suggest DNA-based method as an appropriate alternative to conventional taxonomy. This study directly contributes to the development of a molecular reference library of *Tribolium* species from Nigeria which is essential for its correct identification and confirmation to support integrated pest management strategies and prevent pest infestation of stored food grains required for sustainable food production.

Materials and Methods

Sample collection

Infested yam flour samples were collected from four different locations namely Lokoja (Kogi), Ilorin (Kwara) states of Northcentral Nigeria and Iwo (Osun) and Ado-Ekiti (Ekiti) states in the Southwest Nigeria. The larvae, pupae and adults of *T. castaneum* were cultured under laboratory conditions and adults were separated according to sex and collections sites into labeled petri dishes. Morphological identifications were carried out using standard keys as previously described (Ho *et al*., 1960; Ho *et al*., 1967).

Morphometric analysis

Twenty gram of yam flour were transferred into four Petri dishes each and kept in a refrigerator for a period of 7 days for sterilization after which they were infested with two adult females and males each collected from each collection sites. *Tribolium* samples were reared under temperature range of 26 to 28 °C and humidity

range of 79-81%. The experimental procedures were replicated three times. Larvae count was done by carefully spreading yam flour samples on a sterile clean white paper and the number of larvae observed were counted and recorded at different days interval. Larva measurement was done by placing larva on a glass slide and mounted on a dissecting microscope (Model: Olympus) and images were taken and analyzed using ImageJ (Fiji Software). ImageJ is a Java-based image processing program was developed by Wayne Rasband at the National Institutes of Health, United States of America and can be used to analyse image, measure image distances and pixel value statistics.

Data analysis

Data generated from morphological identification were analyzed using Microsoft Office Excel 2016. Differences between means of the larvae counts and larvae length from different locations were compared using one-way analysis of variance (ANOVA) which was also used to compare variations in the different sampling locations. The differences in the means were separated using Duncan multiple range test (DMRT). Level of significance for statistical comparison of means was set at $p < 0.05$.

Genomic DNA Extraction

The DNA isolation was performed using Bioline Isolate II Genomic DNA Kit catalogue No: BIO-52067 from Meridian Bioscience, USA and as described by the manufacturer with some modifications (homogenization step of the lysis stage) to the manufacturer's protocol. The presence of genomic DNA (gDNA) was detected on 1% agarose gel electrophoresis. Genomic DNA concentration was quantified using Thermo Scientific™ NanoDrop™ 8000 Spectrophotometer manufactured by Thermo Fisher Scientific, USA and stored at -20 °C.

PCR amplification

A partial fragment of the mitochondrial cytochrome subunit 1 (CO1) was amplified using primers as shown in Table 1. The 25 µL polymerase chain reaction (PCR) mixture was prepared, and it contained 21.125 µL of sterilized ultrapure water, 3.0 µL of 109 PCR buffer (including $MgCl_2$), 1.5 µL of each primer (10 mmol/L), 1.5 µL of dNTPs (2.5 mmol/L each). PCR conditions used for this study are stated as follows: 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 50 s, annealing at 55 °C for 45 s and extension at 72 °C for 1 min, with a final extension for 10 min at 72 °C. The PCR amplicons was checked on 1.5% agarose gel with precast 5% (v/v) ethidium bromide staining and visualize under a UV transilluminator. NEB Quick-Load® 100 bp DNA ladder was used to estimate the molecular weight of the amplicons. The electrophoresis was run at 80 volts for 30 mins.

Table 1. Details of primers specific for the mitochondrial cytochrome oxidase subunit 1 (COI) used to detect *T. castaneum* polymorphism

S/N	Primer name	Nucleotide sequence	Tm (°C)	% GC content	Expected PCR product size
1	Forward	AGCAGGAACAGGGTGAACCC	62.07	60	313bp
2	Reverse	TCCCCCAGCAGGGTCAAAGA	63.00	60	313bp

COI sequencing and sequence analysis

The samples sequences were obtained by automated sequencer at Inqaba Biotec (Pretoria, South Africa). The PCR products were confirmed on agarose gel and gel extraction was carried out using Zymoclean Gel DNA Recovery Kit. Extracted fragments were sequenced bidirectionally using Nimagen, Brilliant Dye Terminator cycle sequencing kit V3.1, BRD3-100/1000 and they were purified using Zymo Research, ZR-96 DNA Sequencing clean up kit. The purified fragments were analyzed on ABI 3500xl Genetic Analyzer for each reaction of every sample sequenced. Although both forward and reverse sequencing were carried out, only the

forward sequences were analyzed. Eight (8) sequences were queried on the BLASTn feature on the NCBI database. Percentages of DNA sequence similarities were calculated and compared with the already submitted sequences in the GenBank and Barcode of Life Database (BOLD) databases. The COI similarity was inferred by aligning the sequences from this study and other homologous sequences from GenBank using the ClustalW multiple sequence alignment feature on BioEdit software. The evolutionary diversity (maximum likelihood analysis) and pairwise genetic distance amongst these aligned sequences were then inferred using MEGA7 and default parameters with 1000 bootstrap replications. *Tribolium confusum* was used as an outgroup. The DNA sequences were submitted to GenBank and accession numbers generated were MW898237.1-MW898244.1 (Table 3).

Results

Larvae count and measurement

The highest mean number of *T. castaneum* larvae observed in the yam flour on day 61 was 33.00±4.24 mm in Ilorin (Kwara state), 12.50±3.54 mm in Lokoja (Kogi state), 9.00±0.00 mm in Ado-Ekiti (Ekiti state), and the lowest was in Iwo (Osun state) with 4.00±0.00 mm (Table 2). There was a significant difference (p=0.0188) between the mean number of larvae observed in Ilorin and Iwo. It was also observed that between the columns with regards to the day's larvae counts were recorded, there was a significant relationship (p=0.0258, F (3, 16) = 4.036) between what was observed in *T. castaneum* collected from each of the sampling locations. It was observed that *T. castaneum* larvae from Iwo, Osun state, Nigeria exhibited the highest mean length (1.63±1.14 mm) followed by samples from Ado Ekiti (1.44±0.59 mm) and Ilorin (1.41±0.40 mm) and the lowest mean length measured was recorded in Kogi (1.31±0.37 mm). There was however no significant difference (p>0.05) between the mean length measurement of the larvae collected from study locations (Figure 1).

Table 2. Mean number of larvae from different locations collected from yam flour

Location	Day 30	Day 37	Day 44	Day 51	Day 61
Ilorin (Kwara)	3.50±0.7[a]	9.00±2.83[a]	15.00±0.00[a]	25.00±2.83[a]	33.00±4.24[a]
Lokoja (Kogi)	1.00±0.00[ab]	7.00±2.83[ab]	8.00±2.83[ab]	10.00±4.24[ab]	12.50±3.54[ab]
Iwo (Osun)	2.50±0.7[b]	2.50±0.7[b]	3.00±0.00[b]	4.00±0.00[b]	4.00±0.00[b]
Ado-Ekiti (Ekiti)	2.50±0.71[ab]	7.50±0.71[ab]	9.00±0.00[ab]	9.00±0.00[ab]	9.00±0.00[ab]

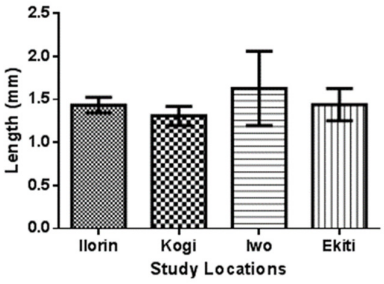


Figure 1. Mean length of larvae from different locations collected from yam flour
Figure across the column, mean numbers with different subscripts are significantly different (P<0.05)

This study investigated the biology (larvae count and measurement) and genetic diversity of *T. castaneum* from selected locations in Nigeria. It was observed that there were varying numbers of larvae counts observed in samples from different locations in Nigeria when reared in the same substrate under the same

condition. The larvae count of *T. castaneum* in yam flour at day 61 of culture was observed to be significantly higher in Ilorin samples compare to samples collected from Iwo, Osun state of Nigeria. Also, the samples with the longest larvae length were found in Iwo, Osun state. According to Hiruy and Getu (2018), some biological parameters of *T. castaneum* are influenced by grain textures. There was no evidence according to the findings from this study to show that rates of larvae growth and survival were influenced by temperature, substrate texture and collection sites because there was no significant difference ($p>0.05$) between the mean length measurement of the larvae collected from study locations.

Molecular analysis

Cytochrome oxidase I (COI) gene from the mitochondrial DNA region containing 313 bp long was amplified from eight samples of *T. castaneum*. Confirmation of amplification of the gene of interest was done using DNA agarose gel electrophoresis. PCR products were sequenced. The sequenced data were compared with ten additional nucleotide sequences retrieved from the GenBank database.

Analysis of the COI sequence similarity

The COI sequence results were queried on the BLASTn feature on the NCBI database. The COI sequences had percentage similarities ranging from 99.61-100% with other *T. castaneum* samples in the GenBank database (https://blast.ncbi.nlm.nih. gov/Blast.cgi) using Megablast search.The DNA sequences obtained from this study were also queried on the Barcode of Life Database (BOLD) at (*http://www.boldsystems.org/index.php/IDS_OpenIdEngine*) and they showed similarities with other submissions in the BOLD database ranging from 98.78-100% (Table 3). Search for sequence similarity of ≥ 99% was carried out in GenBank. The DNA nucleotide sequences from this study were uploaded to the GenBank database and assigned accession numbers MW898237.1- MW898244.1 (Table 3). Alignment of these sequences showed that out of the 677 sequences analyzed, 567 of them were conserved, 106 were variable and 10 of them were parsimony informative sites. The percentage of nucleotide composition were Adenine (A)= 28.9%, Thymine (T)= 31.79%, Cytosine (C)= 23.81% and Guanine (G)=15.52%. The sequences were AT rich with an A+T content of 60.69% and a G+C content of 39.33%. The estimate of the pattern of nucleotide substitution is as shown in Table 4. It shows that there is a larger probability of transition especially G to A transitions (24.63) and C to T (19.93). The highest probabilities of transversion can be observed in A to T and G to T.

Table 3. Percentage of query identities on BLAST and BOLD

S/N	Sequence ID	BLAST Percentage similarity (%)	BOLD Percentage similarity (%)	BIN number
1	*T. castaneum* (MW898237.1)	100	100	AAH8019
2	*T. castaneum* (MW898238.1)	100	100	AAH8019
3	*T. castaneum* (MW898239.1)	99.61	99.61	AAH8019
4	*T. castaneum* (MW898240.1)	100	100	AAH8019
5	*T. castaneum* (MW898241.1)	100	98.82	AAH8019
6	*T. castaneum* (MW898242.1)	100	98.78	AAH8019
7	*T. castaneum* (MW898243.1)	100	100	AAH8019
8	*T. castaneum* (MW898244.1)	100	98.78	AAH8019

Table 4. Maximum composite likelihood estimates of the pattern of nucleotide substitution

Nucleotide Base	A	T	C	G
A	-	*4.34*	*3.25*	**13.23**
T	*3.94*	-	**14.93**	*2.12*
C	*3.94*	**19.93**	-	*2.12*
G	**24.63**	*4.34*	*3.25*	-

*Each entry shows the probability of substitution of the bases in the rows with the bases in the column. The figures in **bold** represent the different nucleotide base transition substitutions while the ones in *italics* represent the different base transversion substitution mutation. As shown in this table, the transition/transversion bias is R= 2.544.

The agarose gel images of the extracted genomic DNA and PCR amplicons are shown in Figures 2A and 2B respectively. PCR amplicon shows a band size of 313 base pairs on the gel when compared with the 100 bp DNA ladder which served as a reference marker.

Multiple sequence alignment profile of the eight *T. castaneum* samples sequenced for this study together with ten sequences *T. castaneum* retrieved from GenBank and the *T. confusum* which was used as an outgroup for reference (Figure 3).

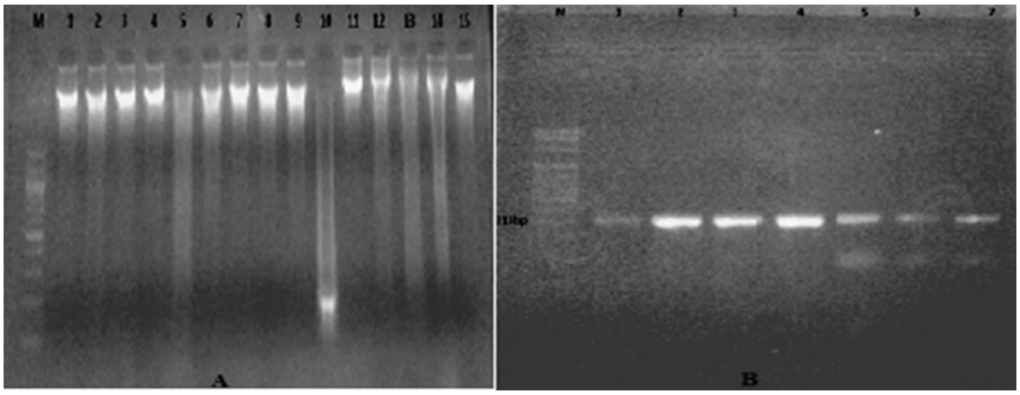
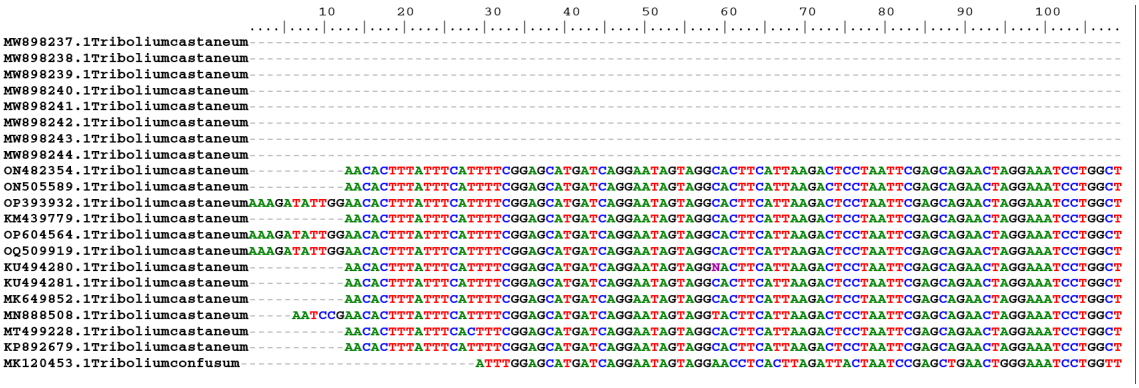


Figure 2. A) Extracted genomic DNA of *T. castaneum* samples collected from sampling locations in Nigeria. M- 100 bp marker. B) PCR Amplification profile of *T. castaneum* populations with mitochondrial cytochrome oxidase subunit 1 (COI) primers from some locations in Nigeria. M-DNA marker 100bp. PCR product size of 313 bp was generated



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                                          110       120       130       140       150       160       170       180       190       200
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MW898237.1Triboliumcastaneum     ------------------------------------------------------------------------------------------------------
MW898238.1Triboliumcastaneum     ------------------------------------------------------------------------------------------------------
MW898239.1Triboliumcastaneum     ------------------------------------------------------------------------------------------------------
MW898240.1Triboliumcastaneum     ------------------------------------------------------------------------------------------------------
MW898241.1Triboliumcastaneum     ------------------------------------------------------------------------------------------------------
MW898242.1Triboliumcastaneum     ------------------------------------------------------------------------------------------------------
MW898243.1Triboliumcastaneum     ------------------------------------------------------------------------------------------------------
MW898244.1Triboliumcastaneum     ------------------------------------------------------------------------------------------------------
ON482354.1Triboliumcastaneum     CCTTAATTGGAGATGACCAAATTTATAATGTTATTGTTACTGCCCACGCATTCATCATAATTTTCTTCATAGTAATACCAATCGTAATTGGGGGATTCGG
ON505589.1Triboliumcastaneum     CCTTAATTGGAGATGACCAAATTTATAATGTTATTGTTACTGCCCACGCATTCATCATAATTTTCTTCATAGTAATACCAATCGTAATTGGAGGATTCGG
OP393932.1Triboliumcastaneum     CCTTAATTGGAGATGACCAAATTTATAATGTTATTGTTACTGCCCACGCATTCATCATAATTTTCTTCATAGTAATACCAATCGTAATTGGAGGATTCGG
KM439779.1Triboliumcastaneum     CCTTAATTGGAGATGACCAAATTTATAATGTTATTGTTACTGCCCACGCATTCATCATAATTTTCTTCATAGTAATACCAATCGTAATTGGAGGATTCGG
OP604564.1Triboliumcastaneum     CCTTAATTGGAGATGACCAAATTTATAATGTTATTGTTACTGCCCACGCATTCATCATAATTTTCTTCATAGTAATACCAATCGTAATTGGAGGATTCGG
OQ509919.1Triboliumcastaneum     CCTTAATTGGAGATGACCAAATTTATAATGTTATTGTTACTGCCCACGCATTCATCATAATTTTCTTCATAGTAATACCAATCGTAATTGGAGGATTCGG
KU494280.1Triboliumcastaneum     CCTTAATTGGAGATGACCAAATTTATAATGTTATTGTTACTGCCCACGCATTCATCATAATTTTCTTCATAGTAATACCAATCGTAATTGGAGGATTCGG
KU494281.1Triboliumcastaneum     CCTTAATTGGAGATGACCAAATTTATAATGTTATTGTTACTGCCCACGCATTCATCATAATTTTCTTCATAGTAATACCAATCGTAATTGGAGGATTCGG
MK649852.1Triboliumcastaneum     CCTTAATTGGAGATGACCAAATTTATAATGTTATTGTTACTGCCCACGCATTCATCATAATTTTCTTCATAGTAATACCAATCGTAATTGGAGGATTCGG
MN888508.1Triboliumcastaneum     CCTTAATTGGAGATGACCAAATTTATAATGTTATTGTTACTGCCCACGCATTCATCATAATTTTCTTCATAGTAATACCAATCGTAATTGGAGGATTCGG
MT499228.1Triboliumcastaneum     CCTTAATTGGAGATGACCAAATTTATAATGTTATTGTTACTGCCCACGCATTCATCATAATTTTCTTCATAGTAATACCAATCGTAATTGGAGGATTCGG
KP892679.1Triboliumcastaneum     CCTTAATTGGAGATGACCAAATTTATAATGTTATTGTTACTGCCCACGCATTCATCATAATTTTCTTCATAGTAATACCAATCGTAATTGGAGGATTCGG
MK120453.1Triboliumconfusum      CACTAATTGGTGATGATCAAATCTATAACGTTATTGTTACAGCCCATGCATTTATCATAATTTTCTTCATAGTAATACCAATTATAATTGGAGGCTTTGG

                                          210       220       230       240       250       260       270       280       290       300
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MW898244.1Triboliumcastaneum     ------------------------------------------------------------------------------------------------------
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MK649852.1Triboliumcastaneum     AAACTGATTAGTCCCTTTAATACTAGGAGCCCCTGATATAGCATTTCCACGAATAAACAACATAAGATTCTGACTACTTCCCCCATCCCTTTCACTTCTT
MN888508.1Triboliumcastaneum     AAACTGATTAGTCCCTTTAATACTAGGAGCCCCTGATATAGCATTTCCACGAATAAACAACATAAGATTCTGACTACTTCCCCCATCCCTTTCACTTCTT
MT499228.1Triboliumcastaneum     AAACTGATTAGTCCCTTTAATACTAGGGGCCCCTGATATAGCATTTCCACGAATAAACAACATAAGATTCTGACTACTTCCCCCATCCCTTTCACTTCTT
KP892679.1Triboliumcastaneum     AAACTGATTAGTCCCTTTAATACTAGGGGCCCCTGATATAGCATTTCCACGAATAAACAACATAAGATTCTGACTACTTCCCCCATCCCTTTCACTTCTT
MK120453.1Triboliumconfusum      AAATTGATTAGTACCACTAATACTAGGAGCCCCTGATATAGCATTCCCCCGAATAAATAACATAAGATTCTGGCTCCTGCCACCCTCATTAAGACTTCTT

                                          310       320       330       340       350       360       370       380       390       400
                                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....
MW898237.1Triboliumcastaneum     ------------------------TAGCAGGAACAGGGTGAACCGTTTATCCACCCCTCTCATCAAACATTGCACATGGCGGATCCTCTGTTGATCTTG
MW898238.1Triboliumcastaneum     ------------------------------------------------------------------------------------------GTTGATCTTG
MW898239.1Triboliumcastaneum     ------------------------------------------------------------------------------------GGATCCTCTGTTGATCTTG
MW898240.1Triboliumcastaneum     ----------------------TTTAGCAGGAACAGGGTGAACCGTTTATCCACCCCTCTCATCAAACATTGCACATGGCGGATCCTCTGTTGATCTTG
MW898241.1Triboliumcastaneum     ------------------------------------------------------------------------------------GATCCTCTGTTGATCTTG
MW898242.1Triboliumcastaneum     ------------------------------------------------------------------------------------------------GATCTTG
MW898243.1Triboliumcastaneum     ------------------------------------------------------------------------------------CCTCTGTTGATCTTG
MW898244.1Triboliumcastaneum     ------------------------------------------------------------------------------------------------GATCTTG
ON482354.1Triboliumcastaneum     CTCATAAGAAGGATTGTTGAAAGAGGGGCAGGAACAGGGTGAACCGTTTACCCACCCCTCTCATCAAACATTGCACATGGCGGATCCTCTGTTGATCTTG
ON505589.1Triboliumcastaneum     CTCATAAGAAGAATTGTTGAAAGAGGGGCAGGAACAGGGTGAACCGTTTACCCACCCCTCTCATCAAACATTGCACATGGCGGATCCTCTGTTGATCTTG
OP393932.1Triboliumcastaneum     CTCATAAGAAGAATTGTTGAAAGAGGGGCAGGAACAGGGTGAACCGTTTATCCACCCCTCTCATCAAACATTGCACATGGCGGATCCTCTGTTGATCTTG
KM439779.1Triboliumcastaneum     CTCATAAGAAGAATTGTTGAAAGAGGGGCAGGAACAGGGTGAACCGTTTACCCACCCCTCTCATCAAACATTGCACATGGCGGATCCTCTGTTGATCTTG
OP604564.1Triboliumcastaneum     CTCATAAGAAGAATTGTTGAAAGAGGGGCAGGAACAGGGTGAACCGTTTACCCACCCCTCTCATCAAACATTGCACATGGCGGATCCTCTGTTGATCTTG
OQ509919.1Triboliumcastaneum     CTCATAAGAAGAATTGTTGAAAGAGGGGCAGGAACAGGGTGAACCGTTTACCCACCCCTCTCATCAAACATTGCACATGGCGGATCCTCTGTTGATCTTG
KU494280.1Triboliumcastaneum     CTCATAAGAAGAATTGTTGAAAGAGGAGCAGGAACAGGGTGAACCGTTTATCCACCCCTCTCATCAAACATTGCACATGGCGGATCCTCTGTTGATCTTG
KU494281.1Triboliumcastaneum     CTCATAAGAAGAATTGTTGAAAGAGGNGCAGGAACAGGGTGAACCGTTTATCCACCCCTCTCATCAAACATTGCACATGGCGGATCCTCTGTTGATCTTG
MK649852.1Triboliumcastaneum     CTCATAAGAAGAATTGTTGAAAGAGGAGCAGGAACAGGGTGAACCGTTTATCCACCCCTCTCATCAAACATTGCACATGGCGGATCCTCTGTTGATCTTG
MN888508.1Triboliumcastaneum     CTCATAAGAAGAATTGTTGAAAGAGGAGCAGGAACAGGGTGAACCGTTTATCCACCCCTCTCATCAAACATTGCACATGGCGGATCCTCTGTTGATCTTG
MT499228.1Triboliumcastaneum     CTCATAAGAAGAATTGTTGAAAGAGGGGCAGGAACAGGGTGAACCGTTTACCCACCCCTCTCATCAAACATTGCACATGGCGGATCCTCTGTTGATCTTG
KP892679.1Triboliumcastaneum     CTCATAAGAAGAATTGTTGAAAGAGGGGCAGGAACAGGGTGAACCGTTTACCCACCCCTCTCATCAAACATTACACATGGCGGATCCTCTGTTGATCTTG
MK120453.1Triboliumconfusum      ATCATAAGAAGAGTAGTAGAAAGAGGGGCAGGAACAGGATGAACTGTTTACCCCCCTTTATCATCTAATATCGCTCACGGAGGAGCTTCAGTTGACCTAG

                                          410       420       430       440       450       460       470       480       490       500
                                 ...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....
MW898237.1Triboliumcastaneum     CCATTTTCAGACTACACTTAGCAGGAATCTCATCAATTCTGGGAGCAGTTAATTTCATTACAACAGTAATCAATATACGACCCCAAGGAATAACCCTTGA
MW898238.1Triboliumcastaneum     CCATTTTCAGACTACACTTAGCAGGAATCTCATCAATTCTGGGAGCAGTTAATTTCATTACAACAGTAATCAATATACGACCCCAAGGAATAACCCTTGA
MW898239.1Triboliumcastaneum     CCATTTTCAGACTACACTTAGCAGGAATCTCATTAATTCTGGGAGCAGTTAATTTCATTACAACAGTAATCAATATACGACCCCAAGGAATAACCCTTGA
MW898240.1Triboliumcastaneum     CCATTTTCAGACTACACTTAGCAGGAATCTCATCAATTCTGGGAGCAGTTAATTTCATTACAACAGTAATCAATATACGACCCCAAGGAATAACCCTTGA
MW898241.1Triboliumcastaneum     CCATTTTCAGACTACACTTAGCAGGAATCTCATCAATTCTGGGAGCAGTTAATTTCATTACAACAGTAATCAATATACGACCCCAAGGAATAACCCTTGA
MW898242.1Triboliumcastaneum     CCATTTTCAGACTACACTTAGCAGGAATCTCATCAATTCTGGGAGCAGTTAATTTCATTACAACAGTAATCAATATACGACCCCAAGGAATAACCCTTGA
MW898243.1Triboliumcastaneum     CCATTTTCAGACTACACTTAGCAGGAATCTCATCAATTCTGGGAGCAGTTAATTTCATTACAACAGTAATCAATATACGACCCCAAGGAATAACCCTTGA
MW898244.1Triboliumcastaneum     CCATTTTCAGACTACACTTAGCAGGAATCTCATCAATTCTGGGAGCAGTTAATTTCATTACAACAGTAATCAATATACGACCCCAAGGAATAACCCTTGA
ON482354.1Triboliumcastaneum     CCATTTTCAGACTACACTTAGCAGGAATCTCATCAATTCTGGGAGCAGTTAATTTCATTACAACAGTAATCAATATACGACCCCAAGGAATAACCCTTGA
ON505589.1Triboliumcastaneum     CCATTTTCAGACTACACTTAGCAGGAATCTCATCAATTCTGGGAGCAGTTAATTTCATTACAACAGTAATCAATATACGACCCCAAGGAATAACCCTTGA
OP393932.1Triboliumcastaneum     CCATTTTCAGACTACACTTAGCAGGAATCTCATCAATTCTGGGAGCAGTTAATTTCATTACAACAGTAATCAATATACGACCCCAAGGAATAACCCTTGA
KM439779.1Triboliumcastaneum     CCATTTTCAGACTACACTTAGCAGGAATCTCATCAATTCTGGGAGCAGTTAATTTCATTACAACAGTAATCAATATACGACCCCAAGGAATAACCCTTGA
OP604564.1Triboliumcastaneum     CCATTTTCAGACTACACTTAGCAGGAATCTCATCAATTCTGGGAGCAGTTAATTTCATTACAACAGTAATCAATATACGACCCCAAGGAATAACCCTTGA
OQ509919.1Triboliumcastaneum     CCATTTTCAGACTACACTTAGCAGGAATCTCATCAATTCTGGGAGCAGTTAATTTCATTACAACAGTAATCAATATACGACCCCAAGGAATAACCCTTGA
KU494280.1Triboliumcastaneum     CCATTTTCAGACTACACTTAGCAGGAATCTCATCAATTCTGGGAGCAGTTAATTTCATTACAACAGTAATCAATATACGACCCCAAGGAATAACCCTTGA
KU494281.1Triboliumcastaneum     CCATTTTCAGACTACACTTAGCAGGAATCTCATCAATTCTGGGAGCAGTTAATTTCATTACAACAGTAATCAATATACGACCCCAAGGAATAACCCTTGA
MK649852.1Triboliumcastaneum     CCATTTTCAGACTACACTTAGCAGGAATCTCATCAATTCTGGGAGCAGTTAATTTCATTACAACAGTAATCAATATACGACCCCAAGGAATAACCCTTGA
MN888508.1Triboliumcastaneum     CCATTTTCAGACTACACTTAGCAGGAATCTCATCAATTCTGGGAGCAGTTAATTTCATTACAACAGTAATCAATATACGACCCCAAGGAATAACCCTTGA
MT499228.1Triboliumcastaneum     CCATTTTCAGACTACACTTAGCAGGAATCTCATCAATTCTGGGAGCAGTTAATTTCATTACAACAGTAATCAATATACGACCCCAAGGAATAACCCTTGA
KP892679.1Triboliumcastaneum     CCATTTTCAGACTACACTTAGCAGGAATCTCATCAATTCTGGGAGCAGTTAATTTCATTACAACAGTAATCAATATACGACCCCAAGGAATAACCCTTGA
MK120453.1Triboliumconfusum      CAATTTTTAGATTACACTTAGCTGGTATTTCTTCAATCTTAGGAGCTGTAAATTTTATTACTACAGTAATTAATATACGACCTACAGGAATATCATTTGA
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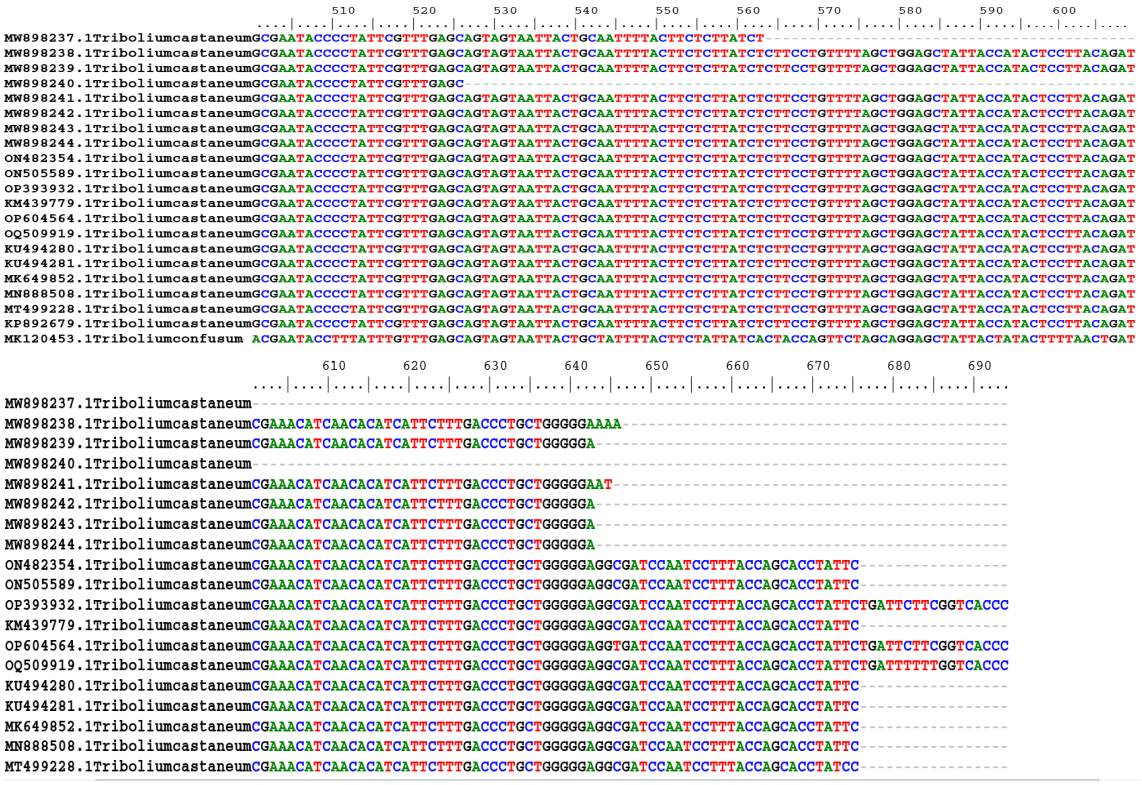

Figure 3. ClustalW multiple alignment of *T. castaneum* samples sequenced including sequences retrieved from GenBank. *T. confusum* was used as an outgroup

The evolutionary history was inferred by using the Maximum Likelihood method as described by Tamura-Nei *et al.* (2011). Evolutionary distance was inferred after 1000 bootstrap replicates. The *T. castaneum* sequenced for this study clustered together with others obtained from GenBank forming a monophyletic group (Figure 4). The calculated pairwise genetic distance among *T. castaneum* is extremely low (Table 5).

Figure 4. Molecular phylogenetic analysis by maximum likelihood method inferred by the Tamura-Nei model with 1000 bootstrap replicated. *Tribolium confusum* was used as an out-group

Despite similarities in the morphological characteristics of *T. castaneum* and *T. confusum,* it is not surprising that the two closely closely-related species differ molecularly as indicated in the genetic variations between *T. casteneum* DNA sequences and the *T. confusum* (27.795) used as the outgroup (Table 5). The pairwise genetic distance between the *T. castaneum* sequences and those from the GenBank ranged from 0.000-0.008 (Table 5). *T. castaneum* and *T. confusum* are genetically distinct at nucleotide and amino acid sequence levels despite morphological similarity as reported in previous study (Ming *et al*., 2015).

Evolutionary relationship between taxa as shown in Table 5 and phylogenetic results in Figure 3 revealed clear evidences that there were no distinct genetic lineages between populations in Nigeria and isolates from other countries (i.e. Angola, Thailand, India, Germany and Mexico). However, this study has demonstrated that there is a lack of phylogeographic relationship between the samples from this study and the samples from other countries. Further studies, with increased numbers of specimens sourced from wider biogeographic areas in Nigeria, and the incorporation of additional markers that are more variable, will be beneficial in helping to unravel the presence of additional haplotypes.

Table 5. Pairwise genetic distance of the mitochondrial COI sequences of *T. castaneum* samples from this study and from GenBank with *T. confusum* as an outgroup

Samples	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
MW898237.1_*T. castaneum_1*	-																				
MW898238.1_*T. castaneum_2*	0.000	-																			
MW898239.1_*T. castaneum_3*	0.008	0.008	-																		
MW898240.1_*T. castaneum_4*	0.000	0.000	0.008	-																	
MW898241.1_*T. castaneum_5*	0.000	0.000	0.008	0.000	-																
MW898242.1_*T. castaneum_6*	0.000	0.000	0.008	0.000	0.000	-															
MW898243.1_*T. castaneum_7*	0.000	0.000	0.008	0.000	0.000	0.000	-														
MW898244.1_*T. castaneum_8*	0.000	0.000	0.008	0.000	0.000	0.000	0.000	-													
ON482354.1_*T. castaneum* (Korea)_9	0.000	0.000	0.008	0.000	0.000	0.000	0.000	0.000	-												
ON505589.1_*T. castaneum* (Mexico)_10	0.000	0.000	0.008	0.000	0.000	0.000	0.000	0.000	0.000	-											
OP393932.1_*T. castaneum* (India)_11	0.000	0.000	0.008	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-										
KM439779.1_*T. castaneum* (Germany)_12	0.000	0.000	0.008	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-									
OP604564.1_*T. castaneum_* (India)_13	0.000	0.000	0.008	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-								
OQ509919.1_*T. castaneum_* (India)_14	0.000	0.000	0.008	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-							
KU494280.1_*T. castaneum_* (Angola)_15	0.000	0.000	0.008	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-						
KU494281.1_*T. castaneum* (Angola)_16	0.000	0.000	0.008	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-					
MK649852.1_*T. castaneum* (Thailand)_17	0.000	0.000	0.008	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-				
MN888508.1_*T. castaneum* (India)_18	0.000	0.000	0.008	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-			
MT499228.1_*T. castaneum* (India)_19	0.000	0.000	0.008	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-		
KP892679.1_*T. castaneum* (China)_20	0.000	0.000	0.008	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-	
MK120453.1_*T. confusum* (Bangladesh)_21	27.795	27.795	27.853	27.795	27.795	27.795	27.795	27.795	27.795	27.795	27.795	27.795	27.795	27.795	27.795	27.795	27.795	27.795	27.795	27.795	-

Discussion

There is a dearth of information on the genetic diversity of Nigerian *T. castaneum* and this information is vital for understanding the beetle's biology and the development of different pest management strategies. This study was conducted using *T. castaneum* from yam flour samples obtained from different locations in Nigeria. It was reasoned that *T. castaneum* samples collected from different locations might vary genetically as previously reported by Wool (1982). Morphologically similar *T. castaneum* may vary genetically due to environmental influences such as temperature and humidity of the environment under which they develop. In the present study effects of temperature and humidity on the rate of growth, species distribution and genetic diversity of *T. castaneum* from yam flour in Nigeria were investigated. Howe (1956) previously investigated

temperature and humidity effects on the rates of development and mortality of *T. castaneum*. It was observed that there was varying number of counts observed in samples from different locations in Nigeria when reared in the same substrate under same condition. The larvae count of *T. castaneum* in yam flour as at 61st day of culture was observed to be significantly higher in Ilorin samples compared to samples collected from Iwo, Osun state. It was observed that the sample with the longest larvae length was found in Iwo.

To the best of our knowledge, this is the first molecular study of *T. castaneum* in Nigeria. Species identification has taken an exciting turn since the emergence of molecular techniques. Comparing amplified DNA sequences of animals gives a broader means of identification than morphological characteristics (Nowaczyk *et al*., 2009; Ming *et al*., 2015). Our study characterized *T. castaneum* samples collected from selected locations in Nigeria. *T. castaneum* is a flour pest that reduces the quality of flour with the insect's wastes and excrements (Zhang *et al*., 2016). Almeida and Stouthamer (2015) identified *Trichogramma* species from South America using the ITS-2 region and they reported that this region is good for identification due to the low range of intraspecific variation. Nasir *et al*. (2012) described ITS2 are a highly conserved region within species and shows significant variation amongst species which makes it a suitable molecular marker for identification and analysis. ITS-2 has been used in taxonomy, phylogenetic development and investigating species evolution of insects (Ajamma *et al*., 2016).

This shows that there is low intraspecific variation due to minimal mutation and evolutionary changes among the *Tribolium* species used in this study. This result is not consistent with the previous study carried out (Ming *et al*., 2015) where evolutionary rate was reported to be slightly higher due to accumulation of mutations over a long period of time (Aslam *et al*., 2019). The low levels of genetic distances observed in this study can be interpreted as a function of inbreeding existing among the sampled populations with no evidence of evolutionary history. This may be because the *Tribolium* samples were sourced from markets, farms and storage facilities. It is possible they are sourced from the same location but find their way to different states of Nigeria with the same population re-infesting all year around. Previous study has suggested that when organisms from different environments may exhibit different phenotypes and genotypes (Falconer, 1952). The small population size used in this study and the observed lack of gene flow between *Tribolium* populations are possible explanations for the observed low genetic diversity within the populations. This study demonstrated that the *T. castaneum* used in this study is closely related with the *T. castaneum* found in other parts of the world.

Consistent with this observation, there is possibility that the global genetic diversity of *T. castaneum* may also be probably low. Phylogenetic analysis revealed that *T. castaneum* used in this study exhibited monophyletic group with no deviations. This is an indication of valuable insight into the genetic structure of the *T. castaneum.* The low genetic distance observed in this study suggests frequently active dispersal, consistent gene flow significantly mitigate founder effects and genetic drift among the *T. castaneum* populations sampled which may possibly be as a result of a recent population expansion. Another reason for low genetic distances observed within the population sampled may be due to relatively small population samples of *T. castenium* used in this study. The observed low genetic differentiation is consistent with previously published study which reported low genetic differentiation between beetles caught at storages and in fields (Ridley *et al*., 2011). This study demonstrated that the *T. castaneum* used in this study is closely related with the *T. castaneum* found in other parts of the world as indicated in the phylogenetic analysis. There is therefore, the possibility that the global genetic diversity of *T. castaneum* may be low.

Conclusions

This study concluded that DNA barcoding of COI gene region is reliable for precise identification and delimitation of *Tribolium* species. The COI sequences of *Tribolium* species generated from this study have already been deposited in the Genbank databases with unique accession numbers for public accessibility and reference library of *Tribolium* species in Nigeria. It is, therefore, recommended that future work should also focus on developing DNA barcodes library using COI gene region as reliable DNA marker for reliable identification of other insects of medical and economic importance. This study provided a basis for future comprehensive studies on *Tribolium* species identification in Nigeria. In summary, findings from this study have provided baseline information for further utilization of COI barcoding as a rapid and precise method for exploring molecular diversity of *Tribolium castaneum* in Nigeria which will assist in reliable pest identification required for effective pest control management strategies in Nigeria.

Authors' Contributions

OAI and AOO conceived and designed the experiments. OAI and AOO coordinated sample collections and preservations; OAI and RDA performed the experiments; All authors contributed to data interpretation and article preparation. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

Acknowledgements

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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