Uncovering the identity of the living laboratory: A combined approach of COI gene sequencing and phylogenetic analysis of the African giant pouched rats

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SUMMARY

The African giant pouched rats belongs to the genus *Cricetomys* and they are exploited to aid the detection of landmines in landmine affected areas in different countries, and for diagnosing pulmonary tuberculosis in health laboratories. These rats are usually captured within Morogoro Tanzania and trained at Sokoine University of Agriculture before deploymemt for actual action. However there is a dearth of information on the taxonomy and phylogeny of *Cricetomys species* occurring in Tanzania. This study used phenotypic appearance, polymerase chain reaction (PCR) amplification and sequensing of cytochrome oxidase subunit I (COI) gene from one hundred and twenty African giant pouched rats trapped from the wild to characterize and identify the phenotypes and identity of the rats. Two groups were identified based on colour of the pelage; the grey group 68% (n=82) and brown group 32% (n=38). The phylogeny and molecular evolutionary analyses revealed the maximum nucleotides homology of 100% and minimum of 97.3% with *Cricetomys gambianus* despite the differences in their pelage colour. This study identified the esistence of *Cricetomys gambianus* species as a dominant species in Morogoro Municipality.

Key words: Laboratory, Tuberculosis, *gambianus*, landmmines, *Cytochrome, subunit I*, Oxidase

INTRODUCTION

The African giant pouched rat (Genus *Cricetomys*) is a solitary rodent of the family *Cricetidae* (Rosevear, 1969). The Genus *Cricetomys* is commonly used as bush meat particularly in West Africa (Ajayi, 1977; Assogbadjo *et al*., 2005). Several approaches have been employed to identify different species of the genus *Cricetomys*, among those are morphological features such as snout width (Olayemi and Akinpelu, 2008); and colour of the pelage (Rosevear, 1969; Van der Straeten *et.al.* 2008). Species distinctions based on morphological features are rendered difficult and designated taxonomic statuses of all the *Cricetomys* species remain to be further explored (Musser & Carleton, 2005). Karyotyping technique was also employed by some authors interested in classification based on chromosomal structures (Granjon *et al*. 1992; Corti *et al*. 2005). Corti *et al*. (2005). Karyotyping technique to identify *C. ansorgei* from Morogoro, Tanzania has been reported by Olayemi *et al.* (2012). However Olayemi *et al*. (2012) combined molecular cytochrome b and multivariate craniometric approach which came up with three new species. Despite of this development, the exact number of species within the genus *Cricetomys* remains unclear. Genest-Villard (1967) have reported four species; namely *C. gambianus* , *C. emini*, *C. ansorgei* and *C. kivuensis*, whereas Olayemi *et al.* (2012) reported the existence of the same species identified by Genest-Villard (1967). In addition, Olayemi *et al.* (2012) identified *C.*

kivuensis and three new species which are referred to as *Cricetomys* sp.1, *Cricetomys* sp.2 and *Cricetomys* sp.3

The cytochrome oxidase subunit I (COI) gene controls respiratory process of the cell by producing cytochrome oxidase c enzyme (Garcia-Horsman *et al*. 1994). The gene is used in taxonomic method which utilizes short genetic marker for species identity (Stoeckle 2003) and it is considered as an accurate barcode identifier (Hebert 2004). Several researchers have utilized COI gene in specie identification including Chaves *et al.* (2008) and Moritz and Cicero (2004). The use of COI gene provides an accurate solution for species classification.

In Tanzania, the humantarian organisation called *Anti-Persoonmijnen Ontmjinende Product Ontwikelling* (APOPO) based at Sokoine University of Agriculture (SUA), Morogoro has been training *Cricetomys species* for scent-detection tasks specifically to detect landmines (Verhagen *et al*., 2003) and tuberculosis (TB) in human sputum samples (Weetjens *et al*., 2009). Until 2021 these rats have helped to clear 151,267 landmines in Angola, Cambodia and Mozambique covering 70,999,370 M^2 of land with more than 1,801,697 beneficries freed from the terror of landmines (APOPO annual report 2021). The rats have also helped to screen as many as 805,263 suspected TB samples and diagnosed 23,298 positive patients who were missed by their clinics in Tanzania and Mozambique (APOPO Annual Report, 2021). Thus, the operational use of these rats given the name 'Hero rats' for scent-detection purposes has been proven as a highly valuable and cost-effective tool for detection of landmines and TB infections. Despite their vital role, it still not clear which *Cricetomys species* is dominant in Morogoro, and used as Hero rats. The purpose of this study was therefore to identify to species level the Cricetomys found in Morogoro municipality.

MATERIALS AND METHODS

Blood sample collection

The study received relevant institutional approval (Approval No. 2019-46-NA-2019-41), and was conducted in accordance with the SUA institutional guidelines. A total of one hundred and twenty African giant pouched rats were captured using Havahart live animal traps (Havahart, Woodstram Corp, Lititz, PA, USA) at three main sites in Morogoro municipality, which are Modeko (Mazimbu ward), Mafiga ward and Vibandani (Mbuyuni ward) (Figure 1). The rats were captured from abandoned animal houses, market places, nearby human settlements, as well as in maize farms. Trapping was done during night time by using ripe banana as bait. Traped animals were collected early the next day in the morning and then transported to the laboratory. The animals were then grouped based on their pelage colour followed by blood collection for DNA extraction. Blood samples were drawn from the tail vein using a needle and EDTA vacutainer tube and the collected blood samples were stored at -20°C.

DNA extraction and species identification using COI gene

Total DNA from whole blood of each group was isolated by using the TIANamp Genomic DNA Kit (DP 130227, Tiangen Biotech Co. Ltd., Beijing, China) according to the manufacture's protocol. Briefly 200µl of the whole blood was mixed with 20µl of Proteinase K then the mixture was votexed and incubated at 56°C until the tissue completely lysed. Then 200µl of the buffer was added and again votexed and incubated at 70°C for 10minutes followed by addition of 200µl of absolute ethanol and the whole mixture was votexed for 15 seconds. The mixture was then centrifuged at 12,000rpm for 30 minutes followed by additional of buffer and then centrifuged again at the same conditions.

Finally the spin Column was transfered into new clean 1.5µl microcentrifuge tubes and 100µl of the buffer TE was added and incubated at room temperature for 5 minutes and centrifuged at 12,000rpm for 2 minutes. Finally 100µl of DNA was eluted. All the extracted DNA quantification was done using a Nanodrop (Thermo-Scientific, UK).

The extracted DNA were then preserved at -20°C until use. COI gene amplification was done using universal foward (VF1d) and reverse (VR1d) primers:: (TCTCAACCAACCACAARGAYATYGG) and: (TAGACTTCTGGGTGGCCRAARAAYCA) respectively as described by Ivanova *et al.* (2012).

PCR amplification was performed using AccuPower® PCR PreMix from Bioneer (Bioneer Corporation, 8-11 Munpyeongseo-ro, Daedeok-gu, Daejeon 306-220,Republic of Korea). The reaction mixture of 20µl consisted 2µl of extracted DNA, 1µl of forward primer (VF1d), 1µl of reverse primer (VR1d) and 16µl of nuclease free water in micro-tube containing AccuPower® PCR PreMix concentrate.

Cycling conditions consisted of initial denaturation at 94 °C for 2 min followed by 5 cycles of 94°C for 30 s, 50 °C for 40 s and 72 °C for 1 min; and followed by another 35 cycles of 94°C for 30 s, 55 °C for 40 s and 72 °C for 1 min.
Final extension at 72 °C for 10 min was performed to complete the extension, and electrophoresis to visualize the PCR products was done on 1.5% agarose gel stained with 4 µl of EZ-Vision® in 1 x Tris-acetate-EDTA (TAE) buffer for 40 minutes at the voltage of 100V.
For each sample, 4µl was loaded into each well of the gel and 4µl of the 100 bp long DNA ladder was loaded to the first well in order to indicate the size of any fragments. Archived DNA sample of a goat confirmed by COI gene PCR and sequencing form the Department of Physiology, Biochemistry and Pharmacology and nuclease free water were also run on each gel as positive and negative controls respectively.

Samples with good PCR nucleotides amplification were submitted to Macrogen Europe (Meibergdree 57, 1105 BA, Amsterdam the Netherland) for sequencing using Sanger method. The PCR products were purified and sequenced directly using BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and a genetic analyzer (ABI 3730xl System from Applied Biosystems).

Comperative sequence and phylogenic analysis

The raw sequence data were cleaned, edited and assembled by Geneious prime (version 2021.2.2) software to get consensus sequences (Kearse *et al.* 2012). The obtained nucleotide sequences were subjected to the Basic Local Alignment Search Tool (BLAST) to determine the identity of the study nucleotide sequences by comparing with other published rodent species available in the GenBank database.

Multiple alignment of COI gene partial nucleotide sequences from this study with other selected reference COI gene nucleotide sequences from GenBank was performed using Clustal W (Thompson *et al.* 1994) . Evolutionary relationships amongst sequences were estimated by constructing phylogenetic trees using maximum likelihood (ML) and the Kimura 2-parameter model with a bootstrap frequency of 1000 replicates, as implemented in MEGA XI software (Tamura *et al.* 2021).

The phylogenetic tree was constructed based on the partial nucleotide sequences of rodents COI gene from 60 samples and 11 nucleotide sequences from references strains obtained from the GenBank.

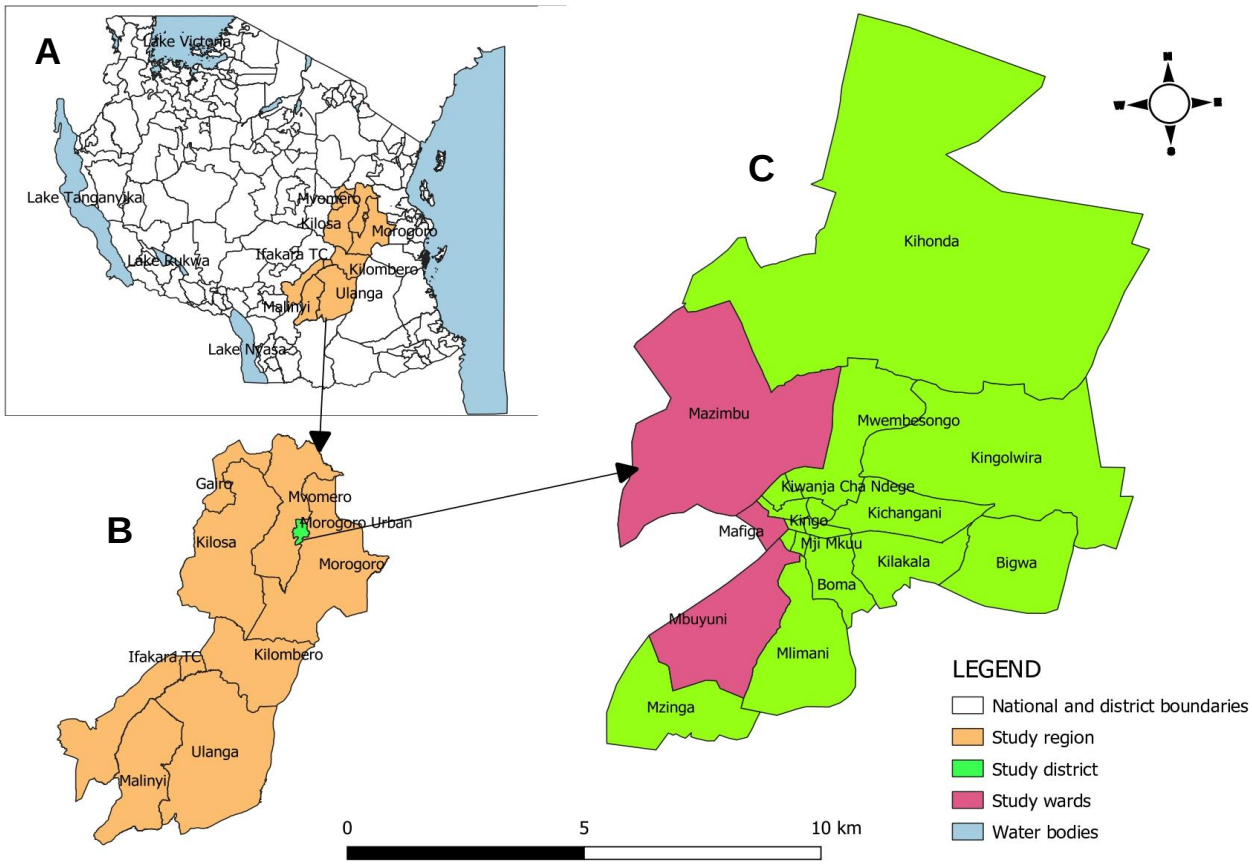

Figure 1
A map of Tanzania (A), Morogoro region (B) and Morogoro Municipality (C) showing sources of *Cricetomys gambianus* in this study (Mazimbu, Mafiga. and Mbuyuni wards).

RESULTS

Phenotypic appearence and nucleotide amplification

Phenotypically two groups of *Cricetomys* were identified based on the colour of the pelage; the grey pelage comprising eighty two individuals and the other group was formed by thirty eight rats of brown pelage. Rats with a mixture of both grey and brown pelages were grouped based on which colour predominated.The PCR amplification of the targeted COI gene from 60 samples generated the expected band size (750 bp) on gel-electrophoresis (Figure 2)

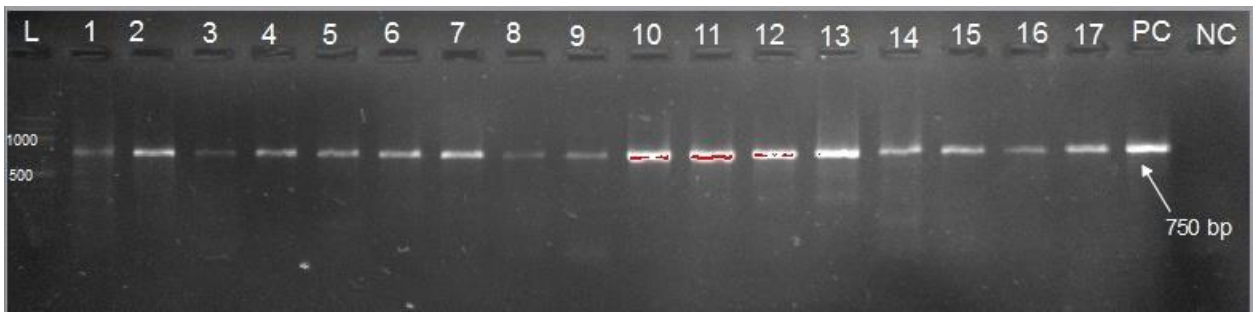

Figure 2: Gel-electrophoresis results of PCR amplification products of COI gene of the African giant pouched rats. The PCR amplicons indicate an expected band size of 750 bp as delineated by forward and reverse primers. L= PCR DNA Ladder, Numbers 1-17= Sample DNA (PCR products), PC= PCR positive control, NC= PCR negative control

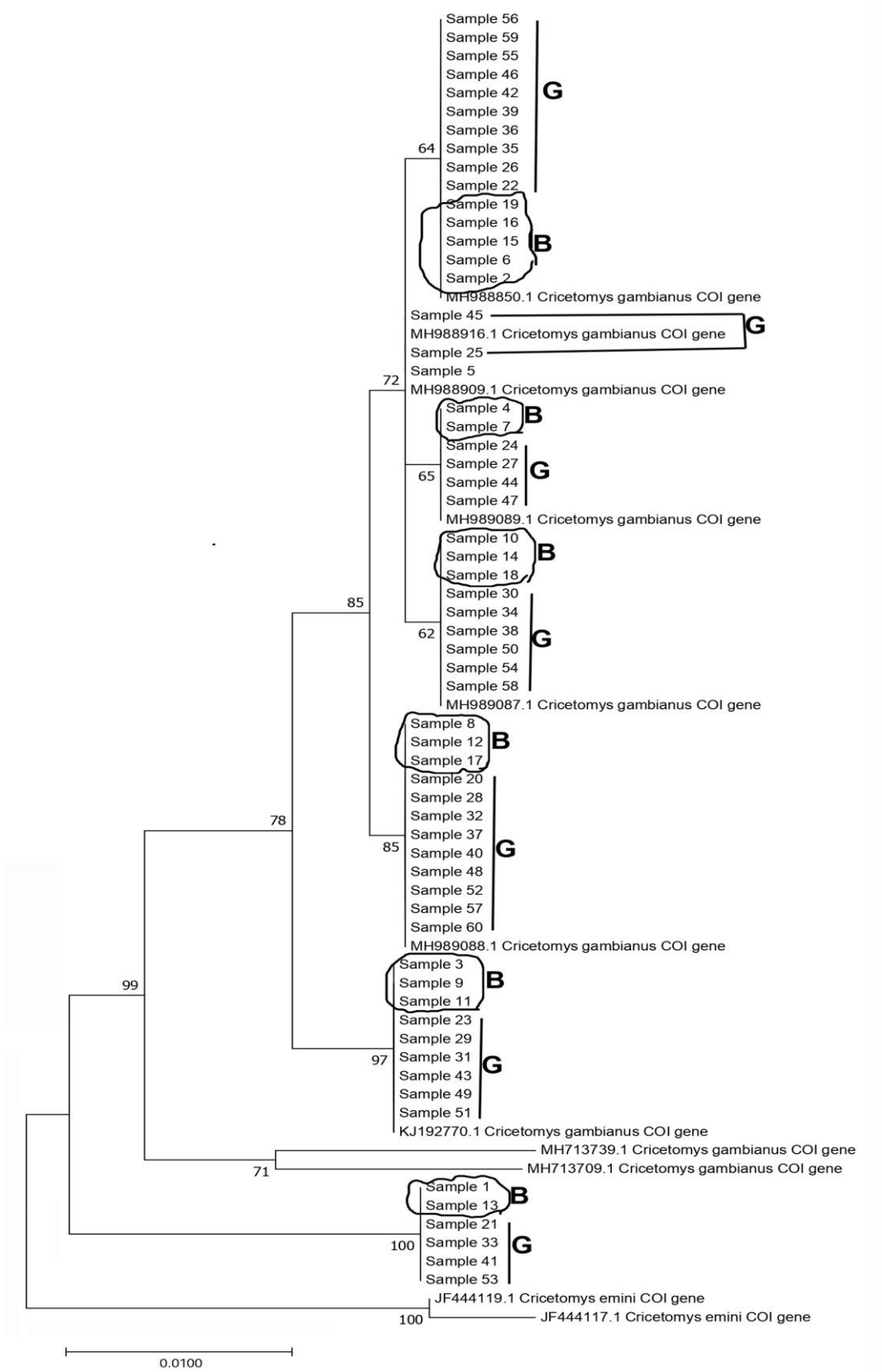

Figure 3: The phylogram indicating the relationship between published sequences of *Cricetomys gambianus* and sequences of the current study of the individuals with brown (B) and grey (G) pelage: Numbers on joining lines = bootstrap value

Phylogenetic analysis of African giant pouched rats

A BLAST search for COI gene nucleotide sequences from both groups revealed the maximum sequence identity of 100% with *Cricetomys gambianus* isolates (MH989088 and MH988909) both from South Sudan , 99.85% with *Cricetomys gambianus* isolates

(MH989089,MH989087.1 and MH988850) from South Sudan, 99.69% with *Cricetomys gambianus* isolate (MH989088.1) from Sudan, 99.08% with *Cricetomys gambianus* isolate (KJ192770.1) from Ghana and 97.39% with *Cricetomys gambianus* isolates (MH713739.1 and MH713709.1) from Sierra Leone . The sequences also showed less percentage identity with *Cricetomys emini* isolates 92.46% (JF444119.1) and 92.00% (JF444117.1) both from Cote d'voire (Table 1). The sequences showed good conformity with the *Cricetomys gambianus* COI gene sequences available on GenBank (Table 1). The phylogenetic tree revealed high assamblage of sequences of the current study to the sequences of *Cricetomys gambianus* from GeneBank (Figure 3).

A phylogenetic analysis of the sequences showed that the African giant pouched rats COI gene sequences obtained from Morogogoro Municipality clustered into only one species *Cricetomys gambianus* therefore, one of the best sequences out of sixty samples was selected for GenBank repository and phylogenetic analysis (accession number: OQ259530).

Table 1: BLAST search results for COI gene nucleotide sequences of *C. gambianus* from GeneBank and selected sequences from the current study used for comperative genomic analysis

Description	Max score	Total score	Query cover	Nucleotide identity	Accession	Country
Cricetomys gambianus	1216	1216	100%	100%	MH989088.1	South Sudan
Cricetomys gambianus	1203	1203	100%	100%	MH988909.1	South Sudan
Cricetomys gambianus	1197	1197	100%	99.85%	MH989089.1	South Sudan
Cricetomys gambianus	1197	1197	100%	99.85%	MH989087.1	South Sudan
Cricetomys gambianus	1197	1197	100%	99.85%	MH988850.1	South Sudan
Cricetomys gambianus	1192	1192	100%	99.69%	MH989088.1	Sudan
Cricetomys gambianus	1170	1170	100%	99.08%	KJ192770.1	Ghana
Cricetomys gambianus	1109	1109	100%	97.39%	MH713739.1	Sierra Leone
Cricetomys gambianus	1109	1109	100%	97.39%	MH713709.1	Sierra Leone
Cricetomys emini	929	929	99%	92.46%	JF444119.1	Cote d'voire
Cricetomys emini	929	929	99%	92.00%	JF444117.1	Cote d'voire

The African giant pouched rat from Morogoro municipal is more closely related to *Cricetomys gambianus* with the percentage similarity in base sequence of 97.39-100%.

DISCUSSION

Molecular dataset in this study confirms the existence of *Cricetomys gambianus* species in Morogoro Municipality, Tanzania. It was further revealed that majority pelage colour is not a suitable diagnostic phenotypic trait to classify African giant pouched rats species. The phylogenetic tree constructed based on the partial nucleotide sequences of rodents COI gene from sixty samples obtained from brown and grey African giant pouched rats and eleven nucletide sequences from reference strain obtained from the GeneBank clustered into *C. gambianus* with high nucleotide percentage similarity (97.39-100%) despite the differences in their pelage colour.

Currently six species of the genus *Cricetomys* have been recognised namely; *C. gambianus, C. ansorgei, C. emini, C. kivuensis, C. species 1 and C. species 2.* Interspecies resemblance in the genus *Cricetomys* is very complex and appears to follow the ecology of the specific species. *C. gambianus* which is the savannah dweller craniometrically resembles *C. ansorgei* which is also inhabiting the savannah than its sister taxon *C. Species 1* from the forest (Olayemi 2012). The same pattern was reported in *C. Species 2* from the forest which is more related to *C. emini* from the same ecological environment than its sister taxon *C. ansorgei* from savannah. This shows that craniophenotypic resemblance in *Cricetomy*s species is more influenced by ecology than phylogeny (Olayemi 2012) and complicates the use of morphometrical identification for identifying the various apecies in the genus *Cricetomy*s. This fact was confirmed in this study where the phylogenetic tree constructed based on the partial nucleotide sequences of rodents *COI* gene from brown and grey African giant pouched rats clustered into *C. gambianus* with high nucleotide percentage similarity (97.39-100%). Furthermore, *Cricetomys gambianus* in Morogoro appears to be more related to *C. gambianus* found in South Sudan with a 100% sequence identity compared to *Cricetomys* found in the West African region (Table 1).

The Tanzanian *C. gambianus* clade exhibits a pedigree of heterogeneity to *C. emini* found in Cote d'voire using nucleotide sequences obtained from the GeneBank. The two species phylogenically appears to be distantly related forming a separate cluster where they are related to one another by 92% sequence similarity, and form an independent branch in the phylogenic tree.

The finding that *C. gambianus* exists in Morogoro is in agreement with Corti *et. al* (2005) who reported *C. gambianus* species from Morogoro but contradict with Olayemi *et al* (2012) who reported *C. ansorgei* as the amin species. Although the findings further confirms the existence of *C. gambianus*, it should be noted that, this study was limited in terms of geographical coverage, therefore, it is possible that other species may exist in other parts of Morogoro beyond the studied area.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest

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