Genetic and haplotype diversity of redbreast tilapia (*Coptodon rendalli*) based on *cytochrome oxidase subunit I* and D-loop

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A B S T R A C T

The genetic diversity in Redbreast tilapia (*Coptodon rendalli*) is under threat from climate change, human activities, and natural hybridisation, prompting conservation concerns. To investigate the genetic characteristics of *C. rendalli*, genetic diversity, genetic structure, population differentiation, and historical demography were evaluated using mitochondrial DNA COI (*cytochrome oxidase subunit I*) and D-loop sequences from various countries. Phylogenetic and genetic diversity analysis based on the mtDNA sequences (COI H = 9, haplogroup = 3, and D-loop H = 5, haplogroup = 3) showed private haplotypes in some of the populations. The results showed significant differences in genetic diversity among the analysed populations (P < 0.001). Molecular variance analysis showed low variation levels among populations (COIF$_{ST}$ = 0.02176, D-loopF$_{ST}$ = 0.52), which was significant only for D-loop. The neutrality tests and mismatch distribution based on the mtDNA supported the hypothesis of under-purifying selection of *C. rendalli*. The results indicated that the population did not undergo expansion. The findings of this study revealed molecular divergence among the *C. rendalli* populations and may serve as pivot information for the genetic improvement of this species. In conclusion, this study has elucidated the utility of two reliable and cost-effective molecular markers for analysing genetic diversity. Notably, the mitochondrial D-loop region's sensitivity to reflecting genetic diversity in *C. rendalli* surpasses that of the COI gene.

1. Introduction

Coptodon rendalli is a native and important tilapia species used in aquaculture in the southern half of Africa. Total aquaculture production worldwide for *C. rendalli* was 22000 tons in 2022 (FAO, 2023). Redbreast tilapia has a wide and varied diet, from plankton for juveniles to leaves and stems of aquatic plants, as well as algae, vegetative detritus, insects, and crustaceans for adults (Lamboj, 2004). This fish is very adaptable to different conditions, temperatures (11-37℃), and salinities (Skelton, 2012). For aquaculture and weed control, this fish was introduced from Africa to Asia (1955 and 1981) and to America in 1957 (Devick, 1991). Redbreast tilapia in 1955 was introduced from Belgium to Thailand and Brazil (Lazzaro, 1991). This fish was introduced from D.R. Congo to Mauritania and Madagascar (Konings et al., 2019). Nevertheless, *C. rendalli* is classified on the IUCN Red List as vulnerable (Konings et al., 2019). Thus, it is essential to assess the genetic integrity of *C. rendalli* globally. Historical gene flow patterns significantly affect the genetic structure of *C. rendalli* populations

(Tibihika et al., 2020). The diversification, adaptation, and speciation of species are largely influenced by external and internal factors, which play a crucial role in structuring genetic variation (Schluter, 2000).

COI gene is a small, highly conserved, heme-containing bifunctional protein widely utilised in species identification and biodiversity research (Arai et al., 2023). On the other hand, the mitochondrial control region (CR) is the longest noncoding region in animal mitochondrial DNA (mtDNA) (Stoneking et al., 1991). The CR is advantageous for phylogenetic studies and genetic diversity due to its maternal inheritance, lack of recombination, exceptionally fast evolutionary rate (Meyer 1993), polymorphic nature (Ghatak et al., 2014) and presumed selective neutrality as a noncoding region (Rech et al., 2014). COI and D-loop gene is used as a genetic marker for phylogenetic analysis and genetic diversity of various animals, including fish species (Jamandre et al., 2014), such as *Coptodon zillii* (Soliman et al., 2017), wild Tilapia species in Japan (Fatsi et al., 2020).

This study aimed to determine the genetic diversity of *C. rendalli* globally. Moreover, the study examined phylogenetic relationships

among different countries' populations. This was done by analysing and utilising two mtDNA markers (COI and D-loop). This study investigates the genetic diversity of *C. rendalli* worldwide and analyses the genetic variation and differentiation among its populations utilising mitochondrial DNA (mtDNA). A universal study of the genetic diversity, haplotype diversity, and population differentiation of *C. rendalli* could lead to guidance for effectively managing this fish in aquaculture and inform conservation strategies.

2. Material and methods

2.1. Study area, DNA extraction, amplification, and sequencing

This study focused on the global perspective of *C. rendalli*. To determine the genetic structure of *C. rendalli* worldwide, all available COI and D-loop sequences from various countries were collected from GenBank (Table S1 and S2). To supplement the dataset, 20 specimens of *C. rendalli* were collected randomly in Doorndraai Dam (24°16′51.0 ″S 28°46'38.0″ E) Limpopo Province, South Africa.

Genomic DNA was extracted from fin clips using the NucleoSpin® Tissue kit (Macherey–Nagel, Düren, Germany). The DNA quality and quantity were assessed with a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., USA). PCR was performed on two regions of mitochondrial DNA: COI applying universal primers FishF1, 5′-TCAAC-CAACCACAAAGACATTGGCAC-3′ and FishR1, 5′-TAGACTTCTGGG TGGCCAAAGAATCA-3′ (target sequence length 655 bp) (Ward et al., 2005) and the D-loop (noncoding) control region using universal primers CR-A, 5′-TTCCACCTCTAACTCCCAAAGCTAG-3′and CR-E, 5′-CCTGAAGTAGGAACCAGATG-3′ (target sequence length 405 bp) (Lee et al., 1995). The PCR reaction comprised 30 μl total volume with eight ng/μl of genomic DNA, one μl each of forward and reverse primers, 12 μl of Taq Master Mix, and eight μl of nuclease-free water. Cycling conditions included an initial denaturation at 95 °C for 15 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing (55 °C for D-loop, 50 °C for COI) for 45 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. After electrophoresis on a 1.5 % agarose gel in 1 × TAE buffer for 30 min at 120 V, the products were visualised under UV light. The PCR products were purified and sequenced successfully at Inqaba Biotec (Pretoria, South Africa).

2.2. Genetic diversity and structure

The quality of all sequences generated from the current study (Accession no COI. PP352615- PP352627; Accession no D-loop. PP350705- PP350724) was checked in BioEdit (Hall, 1999). Sequence alignment was conducted by MEGA X (Kumar et al., 2018), and all COI and D-loop gene sequences were standardised to 581 bp and 390 bp, respectively, to terminate missing data by FaBox online toolbox (Villesen, 2007).

Genetic variation was assessed using parameters such as haplotype diversity (h), haplotype number (H), segregating sites (S), and nucleotide diversity (π) across populations and regions, utilising DnaSP version 6 (Rozas et al., 2017). Sequence data was exported for the TCS haplotype network (Clement et al., 2002) from DnaSP v6.12.03 to analyse haplotypes in the D-loop and COI loci of the *C. rendalli* population with the PopART program (Leigh and Bryant, 2015). Genetic diversity within and among populations was evaluated through analysis of molecular variance (AMOVA), and F-statistics (FST) were calculated with 10,000 permutations in Arlequin version 3.5 (Excoffier and Lischer, 2010), quantifying population differentiation through pairwise FST values (P < 0.05).

2.3. Phylogenetic analysis

Phylogenetic trees were constructed employing Bayesian inference in Mr. Bayes 3.1.2 (Ronquist and Huelsenbeck, 2003). The GTR + I + G

model was chosen with jModeltest 2.1.10 (Guindon and Gascuel, 2003) and executed for 1,000,000 generations. *Chetia flaviventris* (accession number COI: OL306332, D-loop: AY913914) served as the outgroup. Phylogenetic trees were visualised utilising FigTree v1.4.4 (Rambaut, 2018).

2.4. Neutrality test

Tajima (1989) and Fu's Fs (1997) assess the neutrality of mutations. Analyses were conducted utilising Arlequin v3.5 (Excoffier and Lischer, 2010) with 1000 simulations under a neutrality model. Population size changes were analysed using DnaSP v6.12.03.

3. Results

3.1. Genetic diversity

Sequence fragment lengths in *C. rendalli* were 581 bp of COI, and 390 bp of D-loop. TCS haplotype network spanned 9 and 5 haplotypes, obtained from genes COI and D-loop, respectively, representing the dispensation pattern of haplotypes from *C. rendalli* populations per country (Fig. 1). The most widespread haplotype was haplotype 1 in the COI marker (8 populations). Haplotype 1 was the same among South Africa, Namibia, Congo, Mauritania, Colombia, Madagascar, Mozambique, and Panama populations. Haplotype 4 was a shared haplotype between the Tanzania and Zimbabwe populations for the D-loop marker.

South African and Tanzania populations from gene COI and South African and Zimbabwe populations from gene D-loop have the highest number of haplotypes. The shared haplotypes represented 22.22 % and 20 % (COI and D-loop) of the entire data set, while private or singleton haplotypes represented 77.77 % and 80 % (COI and D-loop) (Fig. 2), which indicated the different levels of genetic diversity in all populations.

The average haplotype diversity (Hd) value for COI was 0.45, and for D-loop was 0.70. The average nucleotide diversity (π) for COI was 0.0083, and for D-loop was 0.0090 (Table 1). Haplotype diversities among all genetic markers ranged from 0.00 to 0.66. The COI indicated lower haplotype diversity (0.00–0.60) than D-loop (0.00–0.66) among all populations.

3.2. Genetic structure and population differentiation

Analysis of Molecular Variance (AMOVA) was applied to distinguish the genetic structure of populations in different countries, as shown in Table 2. The AMOVA grouped all localities as one population, indicating that the percentage of variation among populations is lower for COI (2.18 %) and slightly higher for the D-loop (51.86 %) marker than within populations (COI:97.82 %; D-loop: 48.14 %), with a significant structure for D-loop marker (COI: Fst = 0.02176, P = 0.262; D-loop: Fst = 0.5186, P < 0.001) (Table 2).

The F_{ST} values were utilised to determine differentiation in all populations, whether due to drift, inbreeding, or adaptive population structures. The results for COI marker showed a low and insignificant F_{ST} value (Fst = 0.02176, P = 0.262), indicating that advantageous alleles were not spread throughout the population, and the populations were more isolated (Table S3). On the other hand, the D-loop marker had a high and significant F_{ST} value (F_{ST} = 0.52; P < 0.001), indicating adaptive connectivity, where advantageous alleles spread throughout the population (Table S4).

3.3. Phylogenetic analysis

The phylogenetic analysis of COI revealed three major clades, including Clade I, which comprised haplotypes from South Africa, Namibia, D.R. Congo, Thailand, Mauritania, Colombia, Tanzania, Brazil,
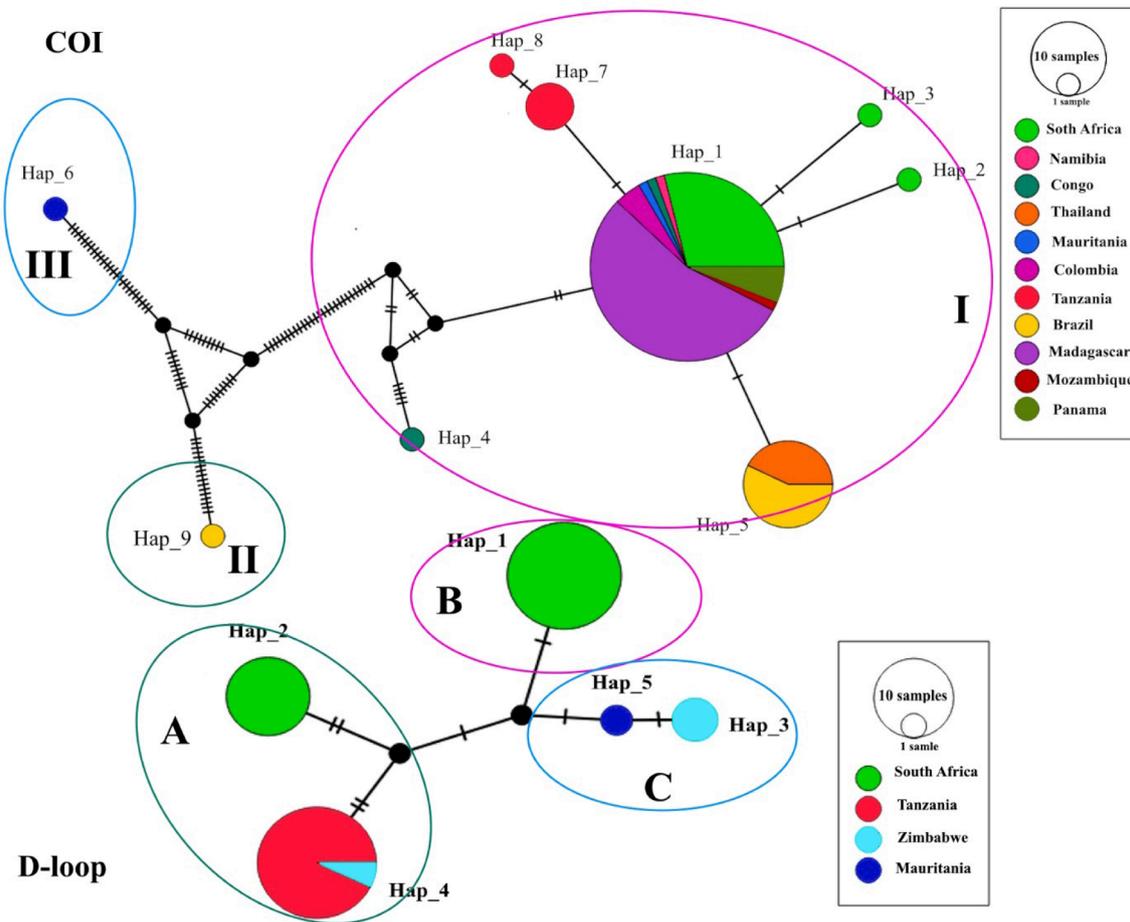
Fig. 1. TCS haplotype network inferred from mtDNA cytochrome oxidase subunit I (COI), and D-loop region. The size of the circle indicates the relative frequency of the corresponding haplotype, and the colors represent the corresponding population. The branches' black lines point to the mutational changes between two haplotypes.

Madagascar, Mozambique, and Panama; Clade II, which represented haplotypes from Brazil. Clade III represented haplotypes from Tanzania. The phylogenetic tree showed a high posterior probability value (Fig. 3).

The result of phylogenetic analysis for the D-loop indicated three clades, including Clade A, which represented haplotypes belonging to South Africa, Tanzania, and Zimbabwe. Clade B represented haplotypes belonging to South Africa. Clade C represented haplotypes belonging to Zimbabwe and Mauritania (Fig. 3).

3.4. Neutrality and population size change test

The results of the neutrality tests (Table 1) and the mismatch distribution (Fig. 4, Fig. S1) based on the COI gene provide support for the hypothesis of population expansion or under-purifying selection of *C. rendalli*. Tajima's D-test of the COI gene showed a negative value (−2.76), and Fu's FS-test reveal a positive value (1.88) with a significant level (total: $P < 0.001$). The mismatch distribution analysis, which was based on the COI gene, observed an unimodal pattern in all populations (raggedness index 0.13; $P > 0.05$) and per location (Figure S1). An unimodal mismatch distribution graph for COI showed the pairwise distribution for entire sequences, indicating a recent population expansion, which was not significant. However, Tajima's D (1.38) and Fu's FS (3.0) of the D-loop region were positive while not reaching a significant level ($P > 0.05$). The D-loop region revealed a bimodal distribution in all populations (Figure S1) during mismatch analyses (raggedness index 0.66; $P > 0.05$). The bimodal mismatch distribution graph implies an equilibrium in population structure. In addition, the result for the D-loop marker showed the

population had no expansion (Fig. 4).

4. Discussion

We analysed mtDNA markers to investigate the genetic diversity among global populations of *C. rendalli*. The analysis revealed a distinct genetic population structure globally in Redbreast tilapia, as determined by mtDNA markers. The mitochondrial genes of *C. rendalli* exhibited high genetic diversity for both markers in all the countries, which suggests that the current genetic structure arises from populations being geographically isolated. The results also showed that this fish has high genetic diversity due to its biological fertility and ecological adaptability in different countries (e.g. Tanzania and Zimbabwe). The wide geographical distribution of haplotype 1 indicates a shared haplotype (Leigh et al., 2019). The existence of common haplotypes in different countries indicates a common ancestral lineage. This finding demonstrates that a shared origin is linked to the introduction of fish for aquaculture. As a result, *C. rendalli* was cultured and widely introduced for mosquito control from Africa to Asia and America (Devick, 1991). Therefore, all countries to which this fish was introduced have a shared haplotype (Hap_1). Shared haplotype, seen as an ancestral lineage, expanded to other regions through mutation and recombination over time, resulting in the emergence of other haplotypes. However, it may take at least 60 years for exotic populations to develop genetic differences (Zidana et al. 2009), which could explain why some populations have only one haplotype (e.g., in Asia and America). Although the D-Loop's haplotype network revealed only one shared haplotype among two populations, this may be due to DNA super-diversity (Robalo et al.,
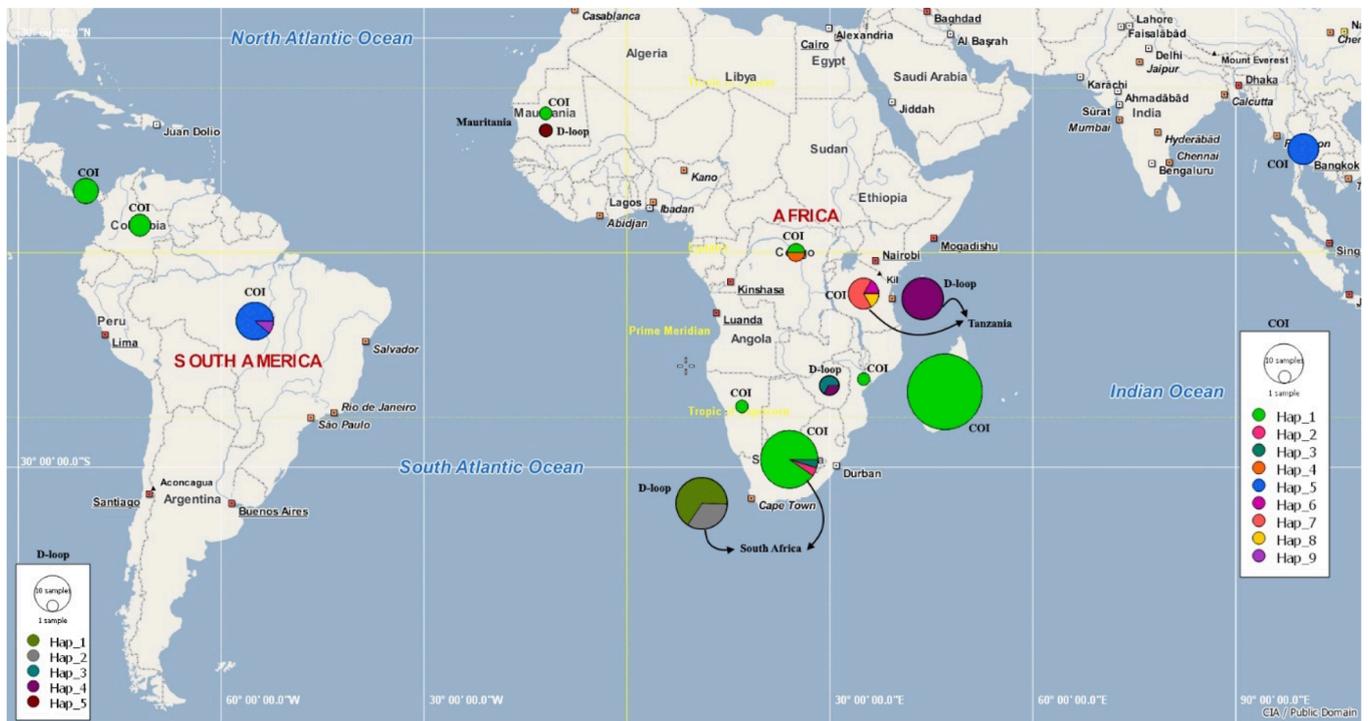
Fig. 2. Frequency of haplotypes in different countries. Colors show various haplotypes of COI and D-loop in the *Coptodon rendalli* population. The size of the circle is proportional to the number of specimens in different geographic regions.

Table 1

Genetic diversity values of *Coptodon rendalli* of mtDNA. N: sample size, h: Number of haplotypes, Hd: haplotype diversity, π: Nucleotide diversity, S: Number of segregating sites in different populations for COI and D-loop.

Countries	Genetic diversity					Neutrality test	
	N	h	H_d	π	S	Tajima (D)	Fu's Fs
COI							
Madagascar	36	1	0.00	0.00	0	NA	NA
South Africa	21	3	0.18	0.00046	2	−1.51	−1.92
Brazil	9	2	0.22	0.031	58	−2.03[**]	10.00[**]
Thailand	6	1	0.00	0.00	0	NA	NA
Tanzania	6	3	0.60	0.052	65	−1.54[**]	5.27[**]
Panama	4	1	0.00	0.00	0	NA	NA
Colombia	3	1	0.00	0.00	0	NA	NA
D.R. Congo	2	2	1	0.022	9	NA	NA
D-loop							
South Africa	20	2	0.47	0.006	4	2.02	5.15
Tanzania	6	1	0.00	0.00	0	NA	NA
Zimbabwe	3	2	0.66	0.010	5	NA	NA

NA = not applicable;

[**] significant (P < 0.001).

2020). This result may be clarified by combining small sample sizes and high mutation rates in D-loop sequences (Fourdrilis et al., 2019). Consequently, COI results might offer more reliable insights into spatial distribution and connectivity. Similar patterns of genetic differentiation have been detected in *C. zillii* in Egypt, as reported in a previous study by Soliman et al. (2017). The presence of *C. zillii* and hybridisation with *C. rendalli* (Mwanja, 2000) is the possible reason for the high genetic diversity in *C. rendalli* (D.R. Congo and Tanzania). On the other hand, the results indicated that genetic diversity was low in the introduced population (Thailand, Panama, and Colombia).

The study's results revealed that the total genetic diversity in the COI marker was lower than that of the D-loop despite the total number of COI sequences being higher than those of the D-loop. This discrepancy may be due to the varying evolutionary rates of the dissimilar molecular markers (Yan et al., 2009; Fang et al., 2022). There is no selection pressure for coding, resulting in a higher mutation and evolution rate in the D-loop region compared to the COI gene (Antoniou and Magoulas, 2014). The findings clearly indicate that both COI and D-loop markers are valuable for analysing the genetic variation of *C. rendalli*. Moreover, the nucleotide diversity (π) value of mitochondrial DNA is crucial for measuring the population's genetic diversity. A higher nucleotide diversity (π) value indicates greater polymorphism within the population. The results suggested that nucleotide diversity (π) of *C. rendalli* for the COI gene was lower than D-loop. These results indicate that the D-loop is more sensitive in reflecting the genetic diversity of *C. rendalli* than the COI gene. This also confirms that the evolution rate of the D-loop is higher than the COI gene. Several studies indicated that the D-loop

Table 2

AMOVA analysis was carried out using the Arlequin program for *Coptodon rendalli* based on COI and D-loop.

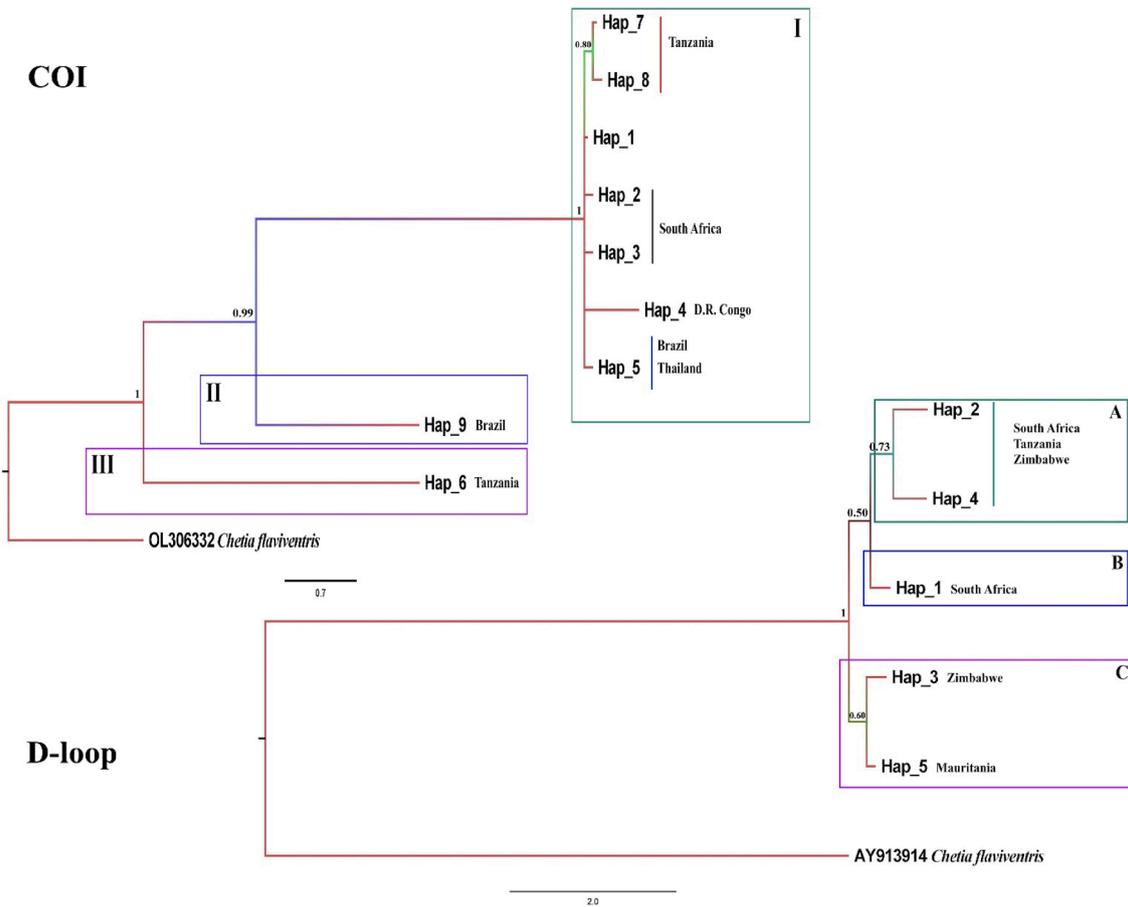| Source of variance	df	Sum of squares	Variance component	% Total of variance	Significance
COI					
Among populations	10	982.64	1.821	2.18	P = 0.262
Within populations	79	6468.22	81.87	97.82	P = 0.262
Total	89	7450.86	83.69	100	P = 0.262
D-loop					
Among populations	3	116.209	2.912	51.86	P < 0.001
Within populations	33	89.467	2.711	48.14	P < 0.001
Total	36	205.676	5.632	100	P < 0.001

COI

D-loop

Fig. 3. The Bayesian tree inferred for *Coptodon rendalli* using haplotypes. *Chetia flaviventris* (COI: OL306332; D-loop: AY913914) was used as out-group.
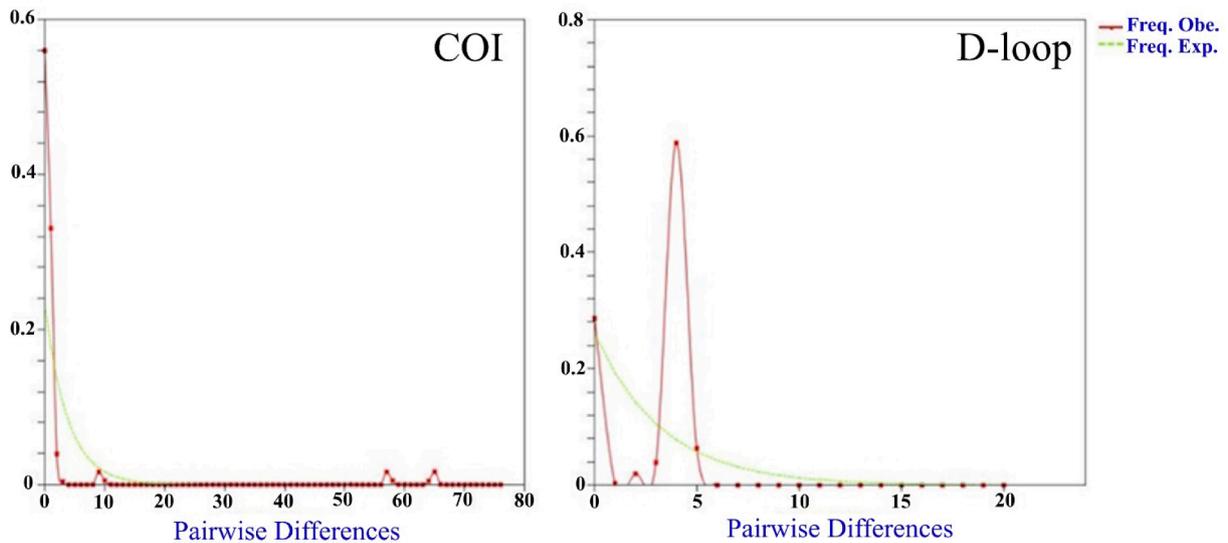

Fig. 4. Mismatch distributions from COI and D-loop sequences of all *Coptodon rendalli* populations. The pairwise distribution is shown in a Uni-modal mismatch distribution graph for COI and a multimodal mismatch distribution graph for D-loop, implying the equilibrium in population structure, regardless of location. X-axis (x): number of pairwise differences; Y-axis (y): frequency of mismatches; Freq. Exp.: frequency expected (green dashed line) and Freq. Obs: frequency observed (red solid line).

marker can detect genetic differentiation better than the COI marker (Soliman et al., 2017; Fang et al., 2022; Zhao et al., 2023). Thus, the D-loop region is more effective in detecting genetic variability within species and may help explain the discrepancies in genetic variability data obtained using different mtDNA markers (Butkauskas et al., 2023).

Haplotype network analysis revealed a complex pattern. Some haplotypes were separated by a several number of mutations. In contrast, others are separated by only a few, which is represented on the

phylogenetic tree. The result of phylogenetic analysis for COI revealed the clades I, II, and III were also inferred in the TCS Network for haplotype analysis (Fig. 1), supporting the topology of the phylogenetic tree (Fig. 3). Tanzania and D.R. Congo had a high value of haplotype diversity, and these populations in the phylogenetic tree were divided into two clades that confirmed the results of haplotype diversity. The phylogenetic analysis of the D-loop inferred clades A, B, and C. The TCS Network haplotypes (Fig. 1), support the topology of the phylogenetic tree (Fig. 3). Zimbabwe and South Africa had high values for haplotype diversity (0.66 and 0.47), and these populations in the phylogenetic tree were divided into two clades that confirmed the results of haplotype diversity. Our research revealed that the *C. rendalli* located in Tanzania and D.R. Congo may have a higher polymorphism.

Population genetic structure undergoes dynamic modifications due to historical and ongoing evolutionary processes (Wereszczuk et al., 2017). Identifying the population structure is crucial because it helps to understand the genetic processes between past and present species, which have shaped the natural selection of current species (Fatsi et al., 2020). Another significant discovery about the genetic structure of *C. rendalli* mainly happened within the population (Fst COI = 0.02176; P > 0.005), as indicated by the COI marker. Due to all sequences being from different countries with different ecological situations, we expected a significant genetic structure and differentiated genetic clusters in all populations (Table S3). The results showed that the COI marker could not show differentiated genetics in the *C. rendalli* populations. The results for the COI marker were also in compliance with the findings of *Collichthys lucidus* (Song et al., 2014), *Larimichthys crocea* (Han et al., 2015), *Gymnocypris przewalskii* (Fang et al., 2022), and *Ocypode ceratophthalmus* (Zhao et al., 2023). In contrast, AMOVA based on the D-loop marker was conducted in all populations, and the percentage of variation observed among populations was the highest, with significant differences (Fst D-loop = 0.5186, P < 0.005). This result indicates that *C. rendalli* has a different pattern in all populations (Table S4). The result of the analysis of the haplotype network was consistent with the AMOVA, and showed that there was a 51.86 % genetic structure among populations. D-loop marker clearly illustrates the genetic ancestry of all the populations (Fatsi et al., 2020). Likewise, D-loop marker results were in agreement with several studies (Barasa et al., 2016; Jiang et al., 2019; Fatsi et al., 2020). Fang et al. (2022) indicated that different molecular marker methods can show different genetic structures.

Tajima (D) and Fu's Fs results were obtained for only a single population (South Africa) in D-loop region. However, due to the slightly faster evolutionary rate in this region, it is not suitable for accurately analysing the population dynamics of *C. rendalli*. Therefore, we used the findings from the COI gene analysis only. Fu (1997) suggested that demographic expansion indices (Fu's Fs and Tajima's D) can identify populations that have recently expanded or are under purifying selection. The negative Tajima's D values and positive Fu's Fs values suggest that the actual polymorphism frequency is lower than expected (Zhang et al., 2021). These results indicate that *C. rendalli* populations have been notably affected by purifying or population expansion (Fatsi et al., 2020) as the number of polymorphic sites exceeds the average heterozygosity. In this study, we detected significant values of Tajima's D and Fu's Fs for COI. Additionally, *C. rendalli* can hybridise with *C. zillii* (Tanzania population). Therefore, purifying selection is likely a more influential force in shaping *C. rendalli* population demographics than a demographic expansion.

Based on mismatch analysis, the population of *C. rendalli* showed an unimodal pattern for all populations based on the COI gene. An unimodal mismatch distribution graph for COI showed the pairwise distribution for entire sequences, indicating a recent population expansion, which was not significant (Figure S1). However, all populations showed a bimodal distribution based on the D-loop (Figure S1). A bimodal mismatch distribution graph for the D-loop showed the pairwise distribution for entire sequences, implying an equilibrium in population structure. Bimodal distribution, which implies a stable population

(Barasa et al., 2016). The nonsignificant raggedness index showed a stable population size of *C. rendalli*. Several studies reported unimodal (COI marker) and bimodal (D-loop marker) distribution in fish taxa, including *G. przewalskii* (Fang et al., 2022), *Clarias gariepinus* (Barasa et al., 2016), *Schilbe intermedius* (Nneji et al., 2020), and *Labeo rohita* (Modeel et al., 2023). The analysis of the historical evolution of the *C. rendalli* population suggests that it has not experienced significant population expansion in recent times and has remained relatively stable. This stability is likely due to changes in environmental conditions not having a substantial impact on the population. However, further investigation is needed to confirm this finding.

Understanding genetic diversity is crucial for developing effective conservation and management plans for *C. rendalli* populations. This knowledge also guides the selection of fish species and their long-term genetic progress. We found the haplotype diversity of *C. rendalli* populations from Zimbabwe, Tanzania, and the Democratic Republic of Congo (D.R. Congo) was the highest, indicating that these populations are genetically diverse. This finding highlights spatially explicit management strategies. Such strategies should include reducing pollution, minimising habitat degradation and fragmentation, and decreasing fishing pressure to ensure the sustainable use of fish stocks.

5. Conclusion

The current study provides information on the global haplotype diversity of *C. rendalli* for the first time. It has also elucidated the utility of two reliable and cost-effective molecular markers for analysing genetic diversity. Notably, the mitochondrial D-loop region's sensitivity to reflecting genetic diversity in *C. rendalli* surpasses that of the COI gene, but COI results might offer more reliable insights into spatial distribution and connectivity. Considering the number of existing sequences in databases, studies about this fish are limited. Therefore, a study on mitochondrial sequences in different countries would be recommended. Further research is required, utilising additional molecular markers and samples to expand current knowledge.

CRediT authorship contribution statement

Mehrnoush Aminisarteshnizi: Writing – review & editing, Writing – original draft, Software, Data curation, Conceptualization. **Ngonidzashe A.G. Moyo:** Supervision. **Mokgadi E. Raphalo:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jksus.2024.103585.

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