Research Article

D-Loop Markers for the Conservation of the Miniature Fish *Paedocypris progenetica*

Nur Jasmin Hussin[1], Izzati Adilah Azmir[1]*, Yuzine Esa[2], Amirrudin Ahmad[3], Sri Novalina Amrizal[4]

[1]Faculty of Applied Sciences, Universiti Teknologi MARA (UiTM), Shah Alam, Selangor, Malaysia; [2]Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor Malaysia; [3]School of Marine and Environmental Sciences, Universiti Malaysia Terengganu, 21030, Kuala Nerus, Terengganu; [4]Faculty of Marine and Fisheries Sciences, Raja Ali Haji Maritime University, Tanjungpinang, Indonesia.

Abstract | *Paedocypris progenetica*, the smallest fish species in the world, is endemic to the blackwater peat swamps of Peninsular Malaysia. Its population is rapidly declining due to habitat degradation caused by illegal logging, industrialization, and urban expansion, increasing the risk of extinction. Despite its conservation importance, the lack of mitochondrial D-loop markers limits genetic monitoring of this species, particularly given its low population abundance and the difficulty of sampling within peat swamp ecosystems. This study aimed to develop species-specific mitochondrial markers to support genetic monitoring and conservation assessment of *P. progenetica* using publicly available whole mitochondrial genome sequences from Peninsular Malaysia. A total of 48 specimens collected from Selangor and Perak were sequenced. Amplification produced fragments of approximately 980 bp for Selangor populations and 1100 bp for Perak populations, suggesting population-level polymorphism within the mitochondrial control region. Marker validation through PCR amplification confirmed consistent amplification of the targeted D-loop region across the sampled individuals. The successful amplification of the mitochondrial D-loop region demonstrates its suitability as a genetic marker for species identification. This study provides a foundational molecular resource to support conservation monitoring, population genetic studies, and future biodiversity research on *P. progenetica* in Malaysia.

Keywords | *Paedocypris progenetica,* Molecular markers, Mitochondrial DNA, D-loop, Peninsular Malaysia, World smallest fish species

Received | February 23, 2026; **Accepted** | April 04, 2026; **Published** | May 15, 2026
***Correspondence** | Izzati Adilah Azmir, Faculty of Applied Sciences, Universiti Teknologi MARA (UiTM), Shah Alam, Selangor, Malaysia; **Email:** izzati_adilah@uitm.edu.my
Citation | Hussin NJ, Azmir IA, Esa Y, Ahmad A, Amrizal SN (2026). D-Loop Markers for the Conservation of the Miniature Fish *Paedocypris progenetica*. Adv. Anim. Vet. Sci., 14(5):1044-1050.
DOI | https://dx.doi.org/10.17582/journal.aavs/2026/14.5.1044.1050
ISSN (Online) | 2307-8316

INTRODUCTION

Paedocypris progenetica, one of the smallest vertebrates in the world, is a miniature cyprinid fish exhibiting larval-like morphology and specialized adaptation to acidic blackwater peat swamp ecosystems (Britz and Kottelat, 2008). This genus is endemic to Southeast Asia such as Pulau Singkep, Pontianak, Pulau Banka, Kalimantan Tengah (Wang *et al.*, 2014) and Malaysia (Sam *et al.*, 2021). Unfortunately, these peat swamp habitats are rapidly declining due to urbanization, industrialization, and agricultural expansion, which threaten the survival of this miniature fish. Molecular methods are widely used in biological research, particularly for population genetic studies (Casillas and Barbadilla, 2017). In many vertebrates, the number, order, and orientation of mtDNA components

are highly conserved in most animal species and exhibit typical maternal inheritance (Wang *et al*., 2014).

Recently, Hussin *et al*. (2022) reported mitogenomic differences among *Paedocypris progenetica*, *P. carbunculus*, and *P. micromegethes*. However, the limited availability of mitochondrial markers has constrained genetic monitoring and molecular identification within the genus *Paedocypris*. The mitochondrial D-loop, a non-coding control region responsible for initiating mtDNA replication and transcription, is characterized by elevated mutation and substitution rates, making it particularly useful for examining phylogenetic relationships and intraspecific population variation (Rianti *et al*., 2021). Although the mitochondrial cytochrome c oxidase subunit I (*COI*) gene is widely used as the standard DNA barcoding marker for species identification, the D-loop region provides greater sequence variability and therefore higher resolution for detecting population-level genetic variation. This is particularly advantageous for habitat-restricted species such as *Paedocypris progenetica*, which inhabits fragmented and vulnerable peat swamp ecosystems. Designing primers targeting this highly variable region is fundamental for molecular studies, as primers determine the specificity and efficiency of PCR amplification (Beyene, 2014). We hypothesized that the high variability of the mitochondrial D-loop region would reveal genetic differences between populations of *Paedocypris progenetica*, providing a useful molecular tool for conservation monitoring. Therefore, this study aimed to develop and validate mitochondrial D-loop markers for species identification and future genetic monitoring of *P. progenetica* populations.

MATERIALS AND METHODS

DNA SAMPLING AND ISOLATION

Samples of *Paedocypris progenetica* were collected from peat swamp habitats in Pondok Tanjung, Perak (n= 23) and North Selangor Peat Swamp Forest, Selangor (n= 25), Peninsular Malaysia, using handheld scoop nets with a mesh size of approximately 2 mm (Figure 1). Sampling was conducted across spatially separated microhabitats within each site, and captured individuals were immediately transferred into separate containers to prevent accidental resampling of the same fish. Genomic DNA was extracted from tissue samples using the ReliaPrep gDNA Tissue Miniprep System (Promega, Madison, WI, USA). The remaining voucher tissues are currently deposited at Universiti Putra Malaysia (UPM).

PRIMER DESIGN AND VALIDATION IN SILICO METHOD

Marker development was conducted using two publicly available mitochondrial genome sequences of *Paedocypris micromegethes* (NC_051487 and NC_051488) from Sarawak, Malaysia (Sam *et al*., 2021), together with two *Paedocypris progenetica* sequences (OK356905 and OK413207) obtained from Perak and Selangor, Peninsular Malaysia (Hussin *et al*., 2022) (Table 1, Figure 2). The sequences were aligned using the ClustalW algorithm implemented in MEGA X to identify conserved and variable regions across the mitochondrial genomes. Candidate regions within the mitochondrial D-loop were subsequently examined for primer development.

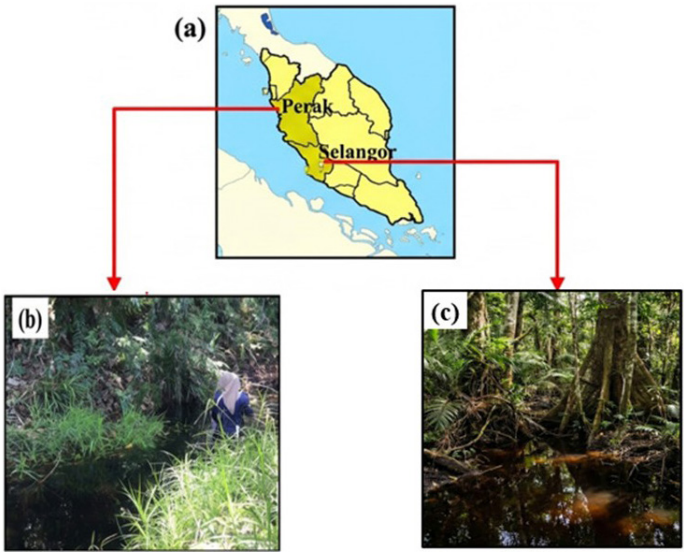

Figure 1: Geographic location of sampling sites for *Paedocypris progenetica* in Peninsular Malaysia showing (a) national overview, (b) sampling regions in Perak and Selangor, and (c) representative peat swamp habitat.

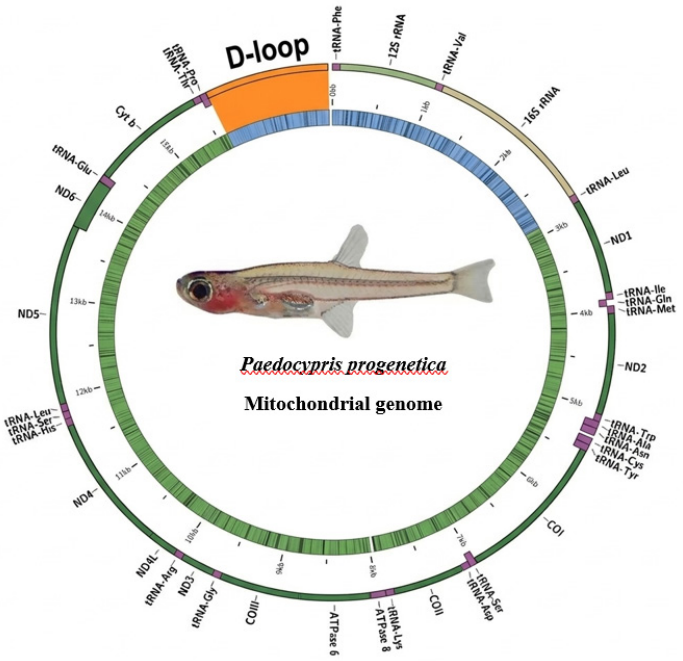

Figure 2: Circular map of the *Paedocypris progenetica* mitochondrial genome showing the location of the D-loop region (GenBank accessions OK356905 and OK413207). The inner ring indicates GC content.

Table 1: Mitochondrial genome sequences obtained from NCBI and used for D-loop marker development.

Species	GenBank accession number	Locality	Genome size
Paedocypris micromegethes	NC_051487	West Malaysia	17208 bp
	NC_051488		17208 bp
Paedocypris progenetica	OK356905	Peninsular Malaysia	16827 bp
	OK413207		16827 bp

Primer design was performed using the NCBI Primer-BLAST tool (Ozirmak *et al*., 2022) using OK356905 and OK413207 as reference templates. Primer efficiency and characteristics were evaluated using PCR Primer Stats (Stothard, 2000), including assessment of GC content, melting temperature (Tm), primer length, and the absence of potential secondary structures such as hairpins or primer–dimer formations. Primer design parameters were set as follows: PCR product size 70–1000 bp, primer melting temperature 50–60 °C, primer length 18–26 bp, and GC content 40–60% (Rianti *et al*., 2021), with minor modifications. Regions showing multiple nucleotide polymorphisms between *P. progenetica* and *P. micromegethes* were visually inspected in the alignment and considered highly variable regions suitable for primer design (Rianti *et al*., 2021). The sequences of the two successful primer pairs are provided in Supplementary Table S1.

PCR amplification

PCR amplification was performed in a total reaction volume of 25 µL, consisting of 12.5 µL of 2× PCR Master Mix, 1 µL of template DNA, 1 µL of forward primer (20 µM), 1 µL of reverse primer (20 µM), and 9.5 µL of nuclease-free water. PCR amplification was carried out using the following thermocycling conditions: initial denaturation at 95°C for 3 min; followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 54°C for 30 sec, and extension at 72°C for 1 min; with a final extension at 72°C for 5 min. The optimized conditions consistently produced clear amplification bands for the validated primer pairs.

RESULTS AND DISCUSSION

Primer design products

A pilot study was conducted to evaluate the functionality of the designed primers across 14 haplotypes of *Paedocypris progenetica*, based on sequences obtained from all examined specimens (GenBank accession numbers OQ388341–OQ388354). These haplotypes included various nucleotide repeat patterns and were used to assess primer performance across two distinct populations from Perak and Selangor. Four primer pairs were initially designed based on two mitochondrial genomes of *P. progenetica*, representing the Perak (OK356905) and Selangor (OK413207) populations. Experimental validation showed that two primer pairs (JASCRF3–JASCRR3 and JASCRF4–JASCRR4) successfully amplified the target region, whereas the remaining two primer pairs (JASCRF1–JASCRR1 and JASCRF2–JASCRR2) produced no detectable amplification. The failure of two primer pairs during experimental validation is not unexpected when working with highly variable mitochondrial regions such as the D-loop. The mitochondrial control region often contains repeat motifs, insertions, and deletions that can interfere with primer binding and amplification efficiency (Wang *et al*., 2014; Rianti *et al*., 2021). Primer design followed standard criteria for primer development, including GC content between 40–60%, appropriate primer length, and melting temperature parameters to ensure stable primer binding and efficient amplification (Rianti *et al*., 2021). Therefore, the observed primer failure likely reflects underlying sequence diversity within the mitochondrial control region of *P. progenetica*. These findings highlight the importance of empirical primer validation when developing molecular markers for species with high mitochondrial variability and suggest that future primer design could benefit from incorporating a broader range of mitochondrial sequences from multiple populations.

Notably, amplification of the mitochondrial D-loop region revealed a clear fragment size difference between the two populations, with Perak samples producing fragments of approximately 1100 bp and Selangor samples producing fragments of approximately 980 bp, ssuggesting population-level polymorphism which may reflect insertion–deletion variation commonly observed in mitochondrial control regions (Wang *et al*., 2014). The primer design process relied on a limited number of publicly available mitochondrial genomes for Paedocypris species, which may not fully represent the genetic diversity across populations. In addition, unassessed primer secondary structure effects, such as potential hairpin formation or primer–dimer interactions, may also have contributed to the lack of amplification.

Utility of the mitochondrial D-loop marker

Although mitochondrial cytochrome c oxidase subunit I (COI) is widely used for species identification, the mitochondrial D-loop region exhibits higher mutation rates and sequence variability. This makes it particularly suitable for detecting intraspecific variation and monitoring population-level genetic changes. For miniature and habitat-restricted species such as *Paedocypris progenetica*, which inhabit fragmented peat swamp ecosystems, this increased variability provides greater resolution for conservation genetics and long-term population monitoring (Casillas and Barbadilla, 2017; Sam *et al*., 2021).

PCR optimization

For the initial PCR optimization, the concentrations of the four primer pairs in the PCR mixture were kept constant at 10 µM and amplification was performed using representative samples from Perak (P1) and Selangor (S5). The first parameter tested was annealing temperature. Gradient PCR was conducted across a temperature range of 48–58 °C to determine suitable amplification conditions for the designed primers. An annealing temperature of 54 °C produced clear, distinct amplification bands for samples P1 and S5 (Figure 3) and was therefore selected for subsequent PCR reactions. Similar observations have been reported in previous PCR optimization studies, where clearer amplification bands are often obtained at relatively higher annealing temperatures (Aifat *et al*., 2016). After establishing the optimal annealing temperature, additional parameters such as primer concentration were further optimized. During the initial optimization stage, double bands were observed in several Selangor samples (Figure 3), suggesting non-specific amplification under certain conditions. These samples were subsequently subjected to further optimization through adjustment of primer concentration.

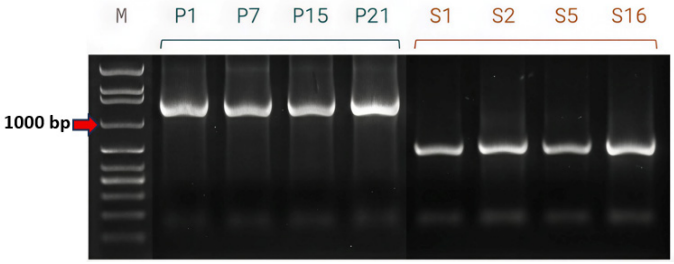

Figure 3: Gradient PCR amplification of the mitochondrial D-loop region in *Paedocypris progenetica* using primer pair JASCRF3–JASCRR3. Fragments of approximately 1100 bp (Perak) and 980 bp (Selangor) were observed. M indicates the DNA size marker.

Primer concentration can influence the specificity and efficiency of PCR amplification. Therefore, primer concentration was further optimized to determine suitable conditions for amplifying the target DNA template. Representative samples from Selangor (S2, S5, S16, S38, S51, S54) and Perak (P7, P15, P21, P4) (Supplementary Table S1) were used to evaluate amplification performance across a range of primer concentrations. Primer concentrations ranging from 10 µM to 20 µM were tested, and a concentration of 20 µM produced clear, distinct amplification bands in the agarose gel (Figure 4). The remaining samples were subsequently amplified using the optimized primer concentration, which consistently produced detectable amplification bands. Proper optimization of PCR mixture components is essential to obtain clear and reliable amplification results (Md-Zain *et al*., 2010).

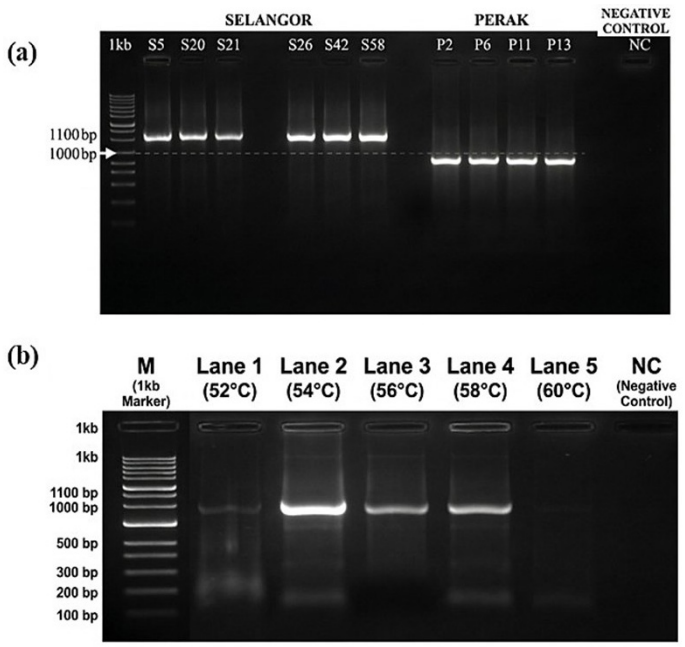

Figure 4: Agarose gel electrophoresis showing PCR amplification of the mitochondrial D-loop region in *Paedocypris progenetica*. (a) Amplification products from Selangor (S) and Perak (P) samples showing fragments of approximately 980 bp and 1100 bp, respectively. NC indicates the negative control. (b) Gradient PCR optimization showing amplification across annealing temperatures (52–60 °C). The clearest amplification was obtained at 54 °C. M indicates the 1 kb DNA marker.

Under these optimized conditions, two primer pairs successfully amplified the mitochondrial D-loop region. The primer pair JASCRF3–JASCRR3 (forward: 5′-CACCTCTGACTCCCAAAGCCA-3′; reverse: 5′-ACACCTTCAATACACTTTGTCAGG-3′) amplified fragments of approximately 1100 bp from Perak samples, whereas the primer pair JASCRF4–JASCRR4 (forward: 5′-ATTTGAACTCCCACCTCTGACTC-3′; reverse: 5′-CTTCCTTGGTTTCGGGGTTTGA-3′) amplified fragments of approximately 980 bp from Selangor samples. This size polymorphism between the two populations is clearly visible in the agarose gel electrophoresis results presented in Figure 4. The observed fragment length difference of approximately 120 bp between Perak and Selangor populations suggests structural variation within the mitochondrial control region. Length polymorphisms in the mitochondrial D-loop are commonly associated with insertions, deletions, or repeat expansions (Wang *et al*., 2014). Such variation can reflect population-level differentiation and may indicate restricted gene flow or independent evolutionary trajectories between geographically separated populations. In peat swamp ecosystems where habitats are naturally fragmented and vulnerable to disturbance, these types of mitochondrial polymorphisms may provide important insights into the genetic structure of populations. These

amplified fragments produced high-quality sequences with clear chromatogram peaks and minimal background noise (Abdul-Latiff *et al*., 2017), demonstrating their suitability for future population genetic studies. The successfully amplified sequences were deposited in GenBank, and the corresponding accession numbers together with sample IDs and haplotype designations are summarized in Table 2.

Table 2: GenBank accession numbers, sample IDs, and haplotypes of *Paedocypris progenetica* from Peninsular Malaysia.

Sample ID	GenBank accession number	Primers pair	Haplotype
P7	OQ388341	JASCRF3- JASCRR3	H1
P15	OQ388342	JASCRF3- JASCRR3	H2
P21	OQ388343	JASCRF3- JASCRR3	H3
P25	OQ388344	JASCRF3- JASCRR3	H4
P4	OQ388345	JASCRF3- JASCRR3	H5
P24	OQ388346	JASCRF3- JASCRR3	H6
S1	OQ388347	JASCRF4- JASCRR4	H7
S2	OQ388348	JASCRF4- JASCRR4	H8
S5	OQ388349	JASCRF4- JASCRR4	H9
S16	OQ388350	JASCRF4- JASCRR4	H10
S26	OQ388351	JASCRF4- JASCRR4	H11
S38	OQ388352	JASCRF4- JASCRR4	H12
S51	OQ388353	JASCRF4- JASCRR4	H13
S54	OQ388354	JASCRF4- JASCRR4	H14

The generation of high-quality mitochondrial sequences and validated D-loop markers provides a valuable genetic resource for future conservation studies of *Paedocypris progenetica*. These markers can be applied to assess genetic diversity and population structure across multiple peat swamp habitats, which is essential for identifying distinct management units and prioritizing conservation actions. In addition, the high variability of the mitochondrial control region makes these markers suitable for the development of environmental DNA (eDNA)-based detection methods, enabling non-invasive monitoring of this miniature fish in low-abundance or difficult-to-sample habitats (Thomsen and Willerslev, 2015). Such applications will be particularly important for detecting population decline, evaluating genetic bottlenecks, and monitoring the long-term genetic health of populations in rapidly degrading peat swamp ecosystems (Avise, 2000).

The detection of fragment length polymorphism further supports the use of the mitochondrial D-loop region as a suitable marker for population-level studies of *Paedocypris progenetica*. While the mitochondrial cytochrome c oxidase I (*COI*) gene is widely used for species identification, it generally exhibits lower sequence variability compared to the control region. The higher mutation rate and structural variability of the D-loop allow the detection of subtle genetic differences between populations that may not be detected using more conserved markers such as COI. Therefore, the markers developed in this study provide improved resolution for monitoring population structure and genetic diversity in this miniature fish species.

In addition to fragment length variation, the identification of 14 haplotypes among the sampled individuals indicates considerable mitochondrial diversity within the studied populations. Haplotype diversity is an important indicator of genetic variability and population history in freshwater fishes. Moderate to high haplotype diversity may reflect historical population connectivity, mutation accumulation within isolated habitats, or demographic processes such as population expansion and fragmentation. In peat swamp systems where environmental disturbances and habitat degradation are common, maintaining genetic diversity is critical for long-term population resilience and adaptation.

DNA CHROMATOGRAM

PCR products that produced clear amplification bands were selected for sequencing to evaluate the efficiency of the optimized PCR conditions. Sequence quality was assessed based on chromatogram peak clarity, signal intensity, and base-calling accuracy. All sequences obtained from the 48 samples exhibited well-resolved peaks with minimal background noise, indicating high-quality sequence data suitable for downstream analysis. Sequence editing and alignment were performed using BioEdit v7.2.3 (Hadj-Henni *et al*., 2015).

The development of species-specific mitochondrial D-loop markers provides an essential molecular resource for monitoring populations of *Paedocypris progenetica*, a miniature fish restricted to vulnerable peat swamp ecosystems. Given the rapid degradation and fragmentation of blackwater habitats across Peninsular Malaysia, reliable genetic markers are crucial for detecting population decline, assessing genetic variability, and supporting conservation management strategies. The high variability of the mitochondrial control region makes the developed primers particularly suitable for future applications including population genetic studies, non-invasive sampling, and environmental DNA (eDNA)-based detection in low-abundance systems. Consequently, these markers establish a foundational framework for long-term conservation genetics and biodiversity monitoring of peat swamp fishes. Alternative PCR optimization strategies such as touchdown PCR may further enhance amplification specificity and could be considered in future studies.

The most important next step in applying these validated D-loop markers is to expand sampling across a broader geographic range of *Paedocypris progenetica* populations in Peninsular Malaysia in order to assess haplotype diversity, population structure, and genetic connectivity among fragmented peat swamp habitats. This would provide the first population-level genetic baseline for the species and help determine whether the Perak and Selangor populations represent distinct management units. In the longer term, these markers may also support the development of environmental DNA (eDNA)-based detection approaches and the assessment of genetic bottlenecks in declining populations. Consolidating these future applications highlights the value of the present study as a foundational step toward long-term conservation genetics and monitoring of this threatened miniature fish.

CONCLUSION

In conclusion, this study addressed the limited availability of mitochondrial genetic markers for the threatened miniature fish *Paedocypris progenetica* by developing and validating mitochondrial D-loop primers suitable for genetic analysis. Two primer pairs successfully amplified the target region across 48 individuals from two geographically distinct populations in Peninsular Malaysia. Importantly, the amplified fragments revealed fragment length polymorphism and haplotype variation between populations, suggesting preliminary genetic differentiation within the species. These validated markers provide an important molecular resource for future studies on population genetics, phylogeography, and conservation monitoring of *P. progenetica*. The availability of these genetic tools will facilitate the generation of high-resolution genetic data that can support evidence-based conservation management and long-term monitoring of populations inhabiting vulnerable peat swamp ecosystems. Such genetic monitoring is particularly important for peat swamp habitats that are rapidly declining due to land-use change and habitat degradation.

ACKNOWLEDGMENT

The authors would like to express their gratitude to everyone from Universiti Putra Malaysia (UPM), Universiti Teknologi Mara (UiTM), Universiti Malaysia Terengganu (UMT), Selangor Forestry Department, and local communities who were involved in the sample collection. This study was fully funded by the Ministry of Higher Education, Malaysia, under the Fundamental Research Grant Scheme (Ref. No: RACER/1/2019/STG05/UITM//5) through UiTM (Ref No: 600-IRMI/FRGS-RACER 5/3 (058/2019). This manuscript has not been previously published or submitted to another journal.

NOVELTY STATEMENT

This study represents the first development and validation of mitochondrial D-loop markers for Paedocypris progenetica, the world's smallest fish species from the threatened peat swamp ecosystems of Peninsular Malaysia. The validated markers successfully revealed population-level fragment length polymorphism and haplotype variation between geographically distinct populations, providing a novel molecular resource for future conservation genetics, population monitoring, and environmental DNA (eDNA)-based studies.

AUTHOR'S CONTRIBUTION

Nur Jasmin Hussin: Writing, data curation, formal analysis, investigation. Izzati Adilah Azmir: Conceptualization, funding acquisition, project administration, methodology, data curation, supervision, writing, validation. Yuzine Esa: Conceptualization, verification, resources, writing. Amirrudin Ahmad: Conceptualization, data curation, supervision, validation, writing. Sri Novalina Amrizal: Writing.

Ethical statement

All sampling procedures followed institutional guidelines for field research and complied with local regulations for wildlife sampling. The sampling involved non-invasive collection of fish specimens and according to institutional guidelines, formal animal ethics approval was not required for this study. All efforts were made to minimize stress to the fish and to ensure compliance with local conservation regulations.

Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Abdul-Latiff M, Aifat N, Yaakop S, Md-Zain B (2017). A noninvasive molecular approach: Exploiting species-locus-specific PCR primers in defeating NUMTS and DNA cross-contamination of cercopithecidae. J. Anim. Plant Sci., 27(3).

Aifat NR, Yaakop S, Md-Zain BM (2016). Ancient DNA analyses of museum specimens from selected Presbytis (Primate: Colobinae) based on partial Cyt b sequences. AIP Conf. Proc., 1784(1): 060024. https://doi.org/10.1063/1.4966862

Avise GC (2000). Phylogeography: The History and Formation of Species. Harvard University Press. https://doi.org/10.2307/j.

ctv1nzfgj7
Beyene G (2014). Primer design methods and applications in molecular biology. J. Mol. Biol. Res., 4(1): 12–20.
Britz R, Kottelat M (2008). *Paedocypris carbunculus*, a new species of miniature fish from Borneo (Teleostei: Cypriniformes: Cyprinidae). Raffles Bull. Zool., 56: 415-422.
Casillas S, Barbadilla A (2017). Molecular population genetics. Genetics, 205(3): 1003-1035. https://doi.org/10.1534/genetics.116.196493
Hadj-Henni L, De Meulemeester T, Depaquit J, Noël P, Germain A, Helder R, Augot D (2015). Comparison of vertebrate cytochrome b and prepronociceptin for blood meal analyses in Culicoides. Front. Vet. Sci., 2(15). https://doi.org/10.3389/fvets.2015.00015
Hussin N, Azmir IA, Esa Y, Ahmad A, Salleh FM, Jahari PNS, Munian K, Gan HM (2022). Characterization of the first mitogenomes of the smallest fish in the world, *Paedocypris progenetica*, from peat swamp of Peninsular Malaysia, Selangor, and Perak. Genom. Inf., 20(1): e12. https://doi.org/10.5808/gi.21081
Md-Zain BM, Mohamad M, Ernie-Muneerah MA, Ampeng A, Jasmi A, Lakim M, Mahani MC (2010). Phylogenetic relationships of Malaysian monkeys, cercopithecidae, based on mitochondrial cytochrome c sequences. Genet. Mol. Res., 9: 1987-1996. https://doi.org/10.4238/vol9-4gmr942
Ozirmak LN, Gray SB, Bowen CM, Reyes-Uribe L, Dray BK, Deng N, Harris RA, Raveendran M, Benavides F, Hodo CL, Taggart MW, Maresso KC, Sinha KM, Rogers J, Vilar E (2022). Comparative molecular genomic analyses of a spontaneous rhesus macaque model of mismatch repair-deficient colorectal cancer. PLoS Genet., 18(4): e1010163. https://doi.org/10.1371/journal.pgen.1010163
Rianti P, Hutapea AL, Rahman DA, Santosa Y (2021). Primer design of D-loop region for wild population genetics of *Rusa timorensis* in Indonesia. IOP Conf. Ser. Earth Environ. Sci., 948(1): 012017. https://doi.org/10.1088/1755-1315/948/1/012017
Sam KK, Lau NS, Shu-Chien AC, Muchlisin ZA, Nugroho RA (2021). Complete mitochondrial genomes of *Paedocypris micromegethes* and *Paedocypris carbunculus* reveal conserved gene order and phylogenetic relationships of miniaturized cyprinids. Front. Ecol. Evol., 9: 662501. https://doi.org/10.3389/fevo.2021.662501
Stothard P (2000). The sequence manipulation suite: Java Script programs for analyzing and formatting protein and DNA sequences. Biotechniques, 28: 1102-1104. https://doi.org/10.2144/00286ir01
Thomsen PF, Willerslev E (2015). Environmental DNA– an emerging tool in conservation for monitoring past and present biodiversity. Biol. Conserv., 183: 4–18. https://doi.org/10.1016/j.biocon.2014.11.019
Wang J, Peng LY, You CP, Li QL, Wen M, Liu SJ, Hong YH (2014). Minifish mtDNA has abundance of repeat sequences and inefficient replication *in vitro*. Curr. Mol. Med., 14(10): 1299-1307. https://doi.org/10.2174/1566524014666141202163927

Supplementary Table 1: Specifications of primers designed for amplification of the mitochondrial D-loop region in *Paedocypris progenetica*.

Primer's Name	Sequence Gene	Optimal annealing temp. (°C)	Product length (bp)	PCR result
JASCRF1	5'-CTCCCCACAACGAGGACTAACTTTC-3'	54	1700	No amplification
JASCRR1	5'-GACCAAGCCTTTGTGCTAGTGGGACT-3'	54		
JASCRF2	5'-ATGCCAGTAGAACACCCGTTCATTA-3'	54	1678	No amplification
JASCRR2	5'-TGGCACGAGTTTTACCGGCCCTTTA-3'	54		
JASCRF3	5'-CACCTCTGACTCCCAAAGCCA-3'	54	1100 (Perak) / 980 (Selangor)	Successful
JASCRR3	5'-ACACCTTCAATACACTTTGTCAGG-3'	54		
JASCRF4	5'-ATTTGAACTCCCACCTCTGACTC-3'	54	958	Successful
JASCRR4	5'-CTTCCTTGGTTTCGGGGTTTGA-3'	54		

Note: Differences in fragment length (e.g., 1100/980 bp) reflect amplification results from samples collected from Perak and Selangor populations, respectively.