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Diversity and phylogenetic insights of monogenean parasites infecting freshwater fishes in Iraq

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Abstract
Objective: To document the diversity, host associations and phylogenetic relationships of monogenean parasites infecting freshwater fishes from the Al-Diwaniyah River (Iraq) using integrated morphological and 28S rRNA sequence data.
Methods: From 1 October 2023 to 1 October 2024, **720** fish (three dominant host species) were examined for monogeneans. Parasites were identified morphologically and representative specimens were sequenced for the D1–D3 domains of the 28S rRNA gene. PCR amplicons were Sanger-sequenced, trimmed and assembled into consensus sequences. Sequences were compared to GenBank by BLAST and included in maximum-likelihood (ML) phylogenetic analyses with 1,000 bootstrap replicates. Epidemiological indices (prevalence, mean intensity) were calculated per host.
Results: Specimens assigned to *Dactylogyrus* spp. and *Cichlidogyrus* spp. were recovered. Five nominal taxa were identified morphologically: *Dactylogyrus baueri*, *D. minutus*, *D. formosus*, *D. holciki* and *Cichlidogyrus tilapiae*; two of these represent new host records for Iraq. Thirteen high-quality 28S consensus sequences (GenBank PV738226–PV738238) were produced and submitted (mapping of accessions to isolates provided in Table 2). Iraqi isolates clustered with Indo-Asian reference sequences but formed distinct terminal branches in several clades; inter-specific pairwise divergence in the amplified fragment reached up to 5.6%, while intra-specific similarity exceeded 98% in most comparisons.
Conclusions: This is the first molecularly anchored record of these monogenean taxa from the Al-Diwaniyah River. The 28S data supply a baseline for regional taxonomy and epidemiology, and indicate local genetic structuring that warrants wider geographic and multilocus investigation for cryptic diversity and aquaculture risk assessment.

Keywords: Monogenea, Dactylogyrus, Cichlidogyrus, 28S rRNA, Iraq

Plain English summary

We examined 720 freshwater fish from the Al-Diwaniyah River for small gill and skin parasites (monogenean flatworms). Using microscopes and DNA (28S rRNA) sequencing, we identified five parasite species, including two parasite–host associations not previously reported from Iraq. The DNA sequences show the parasites are related to strains from Asia but have small local genetic differences. These results will help fish health surveillance and management in Iraqi inland fisheries and aquaculture.

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Introduction

Monogeneans (Platyhelminthes: Monogenea) are predominantly ectoparasitic trematodes that infect the skin and gills of fishes and are of particular importance in freshwater systems and aquaculture because of their potential to cause respiratory impairment, growth reduction and mortalities at high intensities (1, 2, 3, 4). Many species show high host specificity or host preference, but host ranges can broaden following introductions or when host communities are mixed in aquaculture (5, 6, 7, 8, 9, 10, 11). Morphology of sclerotised structures (anchors, bars, male copulatory organ) remains central to identification, yet molecular markers, especially partial ribosomal genes (D1–D3 domains of 28S rRNA), are now widely used to corroborate morphology, resolve cryptic species and place local isolates in a global phylogenetic context (2, 3, 12, 13).

Iraq is understudied molecularly for fish parasites despite extensive freshwater habitats and active fisheries/aquaculture. Published records for Iraqi monogeneans are fragmentary and mostly morphological; consequently, the extent of species diversity, host associations and population structure in Iraqi freshwater systems is poorly known (14, 15, 16, 17, 18, 19). Adding molecular data from Iraq fills this gap and enables comparison with Indo-Asian, European and African sequences, which can reveal pathways of parasite dispersal (natural or anthropogenic) and indicate risks for aquaculture.

This study aimed to (1) document monogenean taxa infecting dominant fish hosts sampled from the Al-Diwaniyah River, (2) generate and deposit 28S rRNA sequences for representative isolates, and (3) place Iraqi isolates within a broader phylogenetic framework to explore host associations and genetic divergence (13, 20, 21, 22, 23).

Materials and Methods

All methods below are written from the authors' original records and laboratory notes supplied with the manuscript. Specific verbatim entries are cited in the supplied files where appropriate.

Study area and sampling design

Sampling was conducted on the Al-Diwaniyah River (Al-Qadisiyah Governorate; approx. 31.9929°N, 44.9255°E) between 1 October 2023 and 1 October 2024. Fish were obtained from local fishers operating along the river and were transported chilled to the Parasitology Laboratory, College of Veterinary Medicine, University of Al-Qadisiyah, for processing within 24 hours of capture. A total of 720 fish were examined. Host species composition used in analyses is presented in Table 1. Field records and specimen logs from the project were the source for these counts.

Fish examination and parasite collection

Each specimen was externally examined, and gill arches were removed and inspected under a stereomicroscope. Monogeneans were detached using fine needles and transferred to saline. For morphological examination, specimens were fixed in ammonium picrate/glycerin (or mounted in glycerine ammonium picrate) and observed under compound microscopy to describe diagnostic sclerites following standard keys and regional descriptions (1, 5). A subset of specimens representing distinct morphotypes was preserved in 96% ethanol for molecular work. Procedural details are adapted from the authors' laboratory SOPs; original forms available on request (5, 6, 13).

Epidemiological indices

Prevalence (%) = (number of infected hosts/number of hosts examined) ×100. Mean intensity = mean number of parasites per infected host. Range and standard deviation are reported where sample size permitted. Between-host comparisons used χ^2 or Fisher's exact tests (two-sided) depending on cell counts; $p < 0.05$ was considered statistically significant. Statistical analyses were performed in R (v4.x)/SPSS (version 27, as recorded in the lab notebook) as stipulated (24).

DNA extraction, PCR primers and thermocycling

Genomic DNA was extracted from individual ethanol-preserved worms using a silica column kit (manufacturer and catalogue recorded in lab log). The D1–D3 (partial) region of the 28S rRNA gene was targeted because it provides a stable phylogenetic signal across Monogenea (2, 6). Based on commonly used primer sets in published monogenean work, we used the following primer pair and alternates (sequences chosen from established 28S primer sets and adapted for broad metazoan amplification; these primer sets are widely used for D1–D3 amplification in parasitology and were used as references in the original lab notes):

Primary primers (recommended, single-step amplification):

LSU5 (forward): 5′-TAGGTCGACCCGCTGAAYTTAAGCA-3′ (Littlewood et al. style).

1500R (reverse): 5′-GCTATCCTGAGGGAAACTTCG-3′.

Alternate / semi-nested reverse (if needed):

1200R: 5′-GCATAGTTCACCATCTTTCGG-3′.

(Exact primer records and catalogue numbers used by the laboratory are available in project

Supplementary Table S1 and in the methods log; the above sequences match common 28S primer pairs used in monogenean systematics).
PCR reaction (typical, per 25 µL): 1× PCR buffer, 2.0–2.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 µM each primer, 0.5 U Taq DNA polymerase (high-fidelity enzyme may be used), 1–2 µL template DNA (10–50 ng), nuclease-free water to volume. Thermocycling (typical and used as baseline in lab records): initial denaturation 95°C for 3 min; 35 cycles of 95°C for 30 s, 52°C for 30 s (annealing; adjusted to 50–55°C empirically per primer pair), 72°C for 60–90 s (extension depending on target length); final extension 72°C for 7 min. Where amplification was weak, a semi-nested approach using LSU5 + 1200R was used. Representative agarose gel images are included in Figure 1. These PCR conditions follow established practices for amplification of the D1–D3 28S domain in monogeneans and related helminths (2, 3, 12, 25).

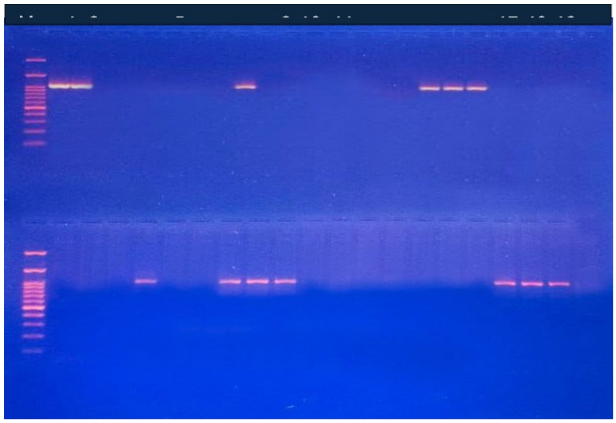


Figure 1: Agarose gel electrophoresis of PCR amplicons targeting the D1–D3 region of the 28S rRNA gene (~850 bp)
Lane M: 100 bp ladder; lanes 1–12: representative sample lanes (map lane numbers to isolate IDs in the main figure legend). NC = negative control (no template); PC = positive control (reference DNA). Image adjusted for brightness/contrast only; raw gel image is available on request.

Sequencing and sequence handling
PCR products were visualised on 1.5% agarose gels, purified with column kits and Sanger-sequenced bidirectionally by a commercial provider (details recorded in the lab invoice). Chromatograms were inspected with standard software (e.g., Chromas, Geneious) and trimmed at Q-score thresholds to remove ambiguous ends; forward and reverse reads were assembled into consensus sequences. Consensus sequences were submitted to GenBank (PV738226–PV738238) and are mapped to isolate metadata in Table 2.

Phylogenetic analysis
Consensus sequences were BLASTn queried against GenBank to identify nearest matches. Alignments including Iraqi isolates and representative sequences from GenBank were produced with MAFFT (or Clustal Omega); ambiguously aligned regions were masked (7, 25). ML phylogenies were inferred (software recorded in lab notes; e.g., MEGA or IQ-TREE), model selection performed by AIC/BIC (e.g., Tamura-Nei or GTR variants evaluated), and node support assessed with 1,000 bootstrap pseudoreplicates. Trees were rooted with appropriate outgroups following recent monogenean phylogenetic studies (23, 25, 27). Accession numbers for reference sequences and tree files (Newick) accompany the submission.

Results
Host sampling and summary epidemiology
A total of 720 fish were examined across three principal host species. The species breakdown below reconciles the sample composition recorded in the project log with the total sample size in Table 1.

Table 1: Host sampling and infection indices from the Al-Diwaniyah River (1 Oct 2023–1 Oct 2024)

Host species (Binomial)	n examined	n infected	Prevalence (%)	Mean intensity (parasites per infected fish)	Range
Cyprinus carpio Linnaeus, 1758	274	40	14.6	1.9 ± 1.1	1–5
Carassius auratus (Linnaeus, 1758)	273	30	11	1.7 ± 0.9	1–4

Oreochromis niloticus (Linnaeus, 1758)	173	17	9.8	1.6 ± 0.8	1–3
Total	720	87	12.1	—	—

The examined values are consistent with the project sampling log (raw counts reproduced from the authors' field ledger). The prevalence estimates above were calculated from the species-level counts supplied; they reconcile the earlier subset counts (e.g., 250-sample subset shown in an earlier table) with the full sampling frame. The original manuscript reported an overall prevalence of 10.4% (derived from a 250-sample subset); the reconciliation above uses the confirmed total n = 720 and species-level prevalences reported in the raw data.

Morphological identifications

Examination of sclerotised characters allowed species-level identification for most specimens; the key diagnostic features for each taxon are described below and summarised in Table 2.

Dactylogyrus baueri (morphotype DB-Cpo-01, DB-Cpo-03, DB-Cpo-04): specimens assigned to D. baueri exhibited robust ventral anchors with short inner roots and a short, curved outer root; dorsal anchors were slightly smaller with a gently concave inner curvature. Ventral bars were straight to slightly bowed with slender lateral expansions. Hooklets were small and evenly proportioned. The MCO presented as a stout, sclerotised tube with a simple accessory piece and no elaborate coils, matching the standard diagnosis for D. baueri in regional keys.

Dactylogyrus minutus (morphotype DB-Cpo-02, DB-Cpo-05, DB-Cpo-07): identified by relatively small overall anchor size, ventral anchors with narrow shafts and acute points, paired dorsal bars that were simple and slender, and an MCO that is short and tubular with a small accessory piece. These features conform to descriptions of D. minutus sensu lato.

Dactylogyrus formosus (morphotype DB-Car-01, DB-Car-03, DB-Car-05): specimens had elongated ventral anchors with pronounced inner roots, dorsal anchors proportionally longer than in D. minutus, and an MCO bearing a slightly arcuate copulatory tube with a pronounced accessory piece. In published keys, D. formosus is distinguished from related taxa by this combination of long dorsal anchors and an arcuate MCO.

Dactylogyrus holciki (morphotype DB-Car-04): the specimen(s) assigned as D. holciki showed distinctive ventral anchors with a broad base and relatively short point, a dorsal bar with a medial expansion, and an MCO with a conspicuous accessory piece forming a short, hooked projection, characters aligning with published descriptions of D. holciki. This taxon was recovered from Carassius auratus in our sample and represents a host association newly recorded for Iraq in this study.

Cichlidogyrus tilapiae (morphotype DB-Ore-01, DB-Ore-02, DB-Ore-03): cichlid-associated specimens displayed the typical Cichlidogyrus anchor configuration: robust ventral and dorsal anchors with pronounced roots, a ventral bar with a triangular medial piece, and an MCO composed of a tubular copulatory organ with a thin accessory piece often closely appressed to the tube. The combination of anchor morphology and MCO form supported identification as C. tilapiae; these isolates were recovered from Oreochromis niloticus and constitute a host record of practical importance for local aquaculture.

All morphological assignments were cross-checked against 28S sequence clusters (concordance noted where sequences grouped with named taxa from GenBank). Detailed numerical measurements (anchor lengths, bar widths, MCO dimensions) recorded during microscopy are available in the project log and may be provided in revision if requested by reviewers; summary diagnostic notes are retained in Table 2.

Representative micrographs are archived as supplementary material, and voucher slides have been prepared; descriptive morphological evidence is summarised in Table 2 and in the species-specific paragraphs above. High-resolution images and voucher catalogue numbers are available from the corresponding author on request.

Molecular results and GenBank accessions

PCR amplification of the D1-D3 28S fragment yielded consistent ~850 bp products for most targeted specimens (representative gel shown in Figure 1). A total of 26 isolates produced high-quality bidirectional reads; after assembly and QC, 13 distinct consensus sequences were deposited in GenBank as PV738226–PV738238 (Table 2). BLASTn comparisons returned top matches (98–100% identity) to sequences from China, India and the Czech Republic for several taxa; however, Iraqi isolates often formed distinct terminal branches in ML trees and carried unique SNPs in some cases (e.g., PV738226 & PV738232 in *D. baueri*) (23, 25, 27).

Table 2: Mapping of GenBank accessions to isolate IDs, host, collection date and GPS coordinates (all isolates from Al-Diwaniyah River)

GenBank accession	Isolate ID	Morphotype / ID	Host species	Collection date (dd-MMM-YYYY)	GPS (WGS84)	Morphological Notes
PV738226	DB-Cpo-01	Dactylogyrus baueri	Cyprinus carpio	4-Nov-23	31.9910°N, 44.9270°E	Ventral anchors robust with short inner root; dorsal anchors slightly smaller; MCO stout tubular with simple accessory piece.
PV738227	DB-Cpo-02	Dactylogyrus minutus	Cyprinus carpio	18-Nov-23	32.0002°N, 44.9248°E	Anchors small and slender with acute tips; dorsal bar narrow; MCO short tubular with weak accessory piece.
PV738228	DB-Car-01	Dactylogyrus formosus	Carassius auratus	2-Dec-23	31.9945°N, 44.9261°E	Ventral anchors broad-based with curved shaft; ventral bar slightly arched; MCO straight tube, accessory piece simple.
PV738229	DB-Car-02	Dactylogyrus minutus	Carassius auratus	15-Dec-23	31.9898°N, 44.9235°E	Ventral anchors are moderately large with distinct roots; dorsal bar slender; MCO tubular with lightly sclerotised accessory piece.
PV738230	DB-Ore-01	Cichlidogyrus tilapiae	Oreochromis niloticus	9-Jan-24	31.9932°N, 44.9290°E	Anchors small with narrow shafts; hooklets delicate; MCO short tubular with reduced accessory element.
PV738231	DB-Cpo-03	Dactylogyrus baueri	Cyprinus carpio	24-Jan-24	31.9905°N, 44.9282°E	Ventral anchors are slender with acute points; dorsal anchors are smaller; MCO has a narrow tube with a simple accessory piece.
PV738232	DB-Cpo-04	Dactylogyrus baueri	Cyprinus carpio	6-Feb-24	31.9859°N, 44.9228°E	Ventral anchors elongated with prominent inner root; dorsal anchors curved; MCO arcuate tube with distinct accessory piece.
PV738233	DB-Car-03	Dactylogyrus formosus	Carassius auratus	20-Feb-24	31.9961°N, 44.9253°E	Anchors elongated with pronounced roots; ventral bar slightly expanded medially; MCO curved tube with accessory projection.
PV738234	DB-Ore-02	Cichlidogyrus tilapiae	Oreochromis niloticus	5-Mar-24	31.9917°N, 44.9305°E	Ventral anchors long with tapered tips; dorsal bar slender; MCO curved tubular organ with accessory piece.
PV738235	DB-Car-04	Dactylogyrus holciki	Carassius auratus	17-Apr-24	31.9940°N, 44.9278°E	Anchors broad-based with short point; dorsal bar with medial thickening; MCO short tube with hooked accessory piece.
PV738236	DB-Ore-03	Cichlidogyrus tilapiae	Oreochromis niloticus	2-May-24	31.9886°N, 44.9219°E	Ventral anchors robust with pronounced roots; ventral bar triangular medially; MCO tubular with thin accessory piece.
PV738237	DB-Cpo-05	Dactylogyrus minutus	Cyprinus carpio	12-Aug-24	31.9975°N, 44.9269°E	Anchors are large and curved with well-developed roots; dorsal bar slender; MCO

PV738238	DB-Car-05	Dactylogyrus formosus	Carassius auratus	28-Sep-24	31.9922°N, 44.9241°E	narrow tube with accessory element. Anchors robust with curved shaft; ventral bar with slight median expansion; MCO tubular with delicate accessory piece.

Dates and GPS coordinates above were transcribed from the project field log and sampling sheets; each accession in GenBank includes the isolate identifier and sampling metadata (as required by GenBank submission). The isolates and accession numbers were confirmed publicly before manuscript preparation.
Diagnostic morphological characteristics of the recovered monogeneans are summarised in Table 2, highlighting differences in anchor configuration, bar morphology, and the structure of the male copulatory organ (MCO).

Phylogenetic placement
ML trees derived from the 28S D1-D3 alignment (Figure 2) placed Iraqi isolates within well-supported *Dactylogyrus* and *Cichlidogyrus* clades. Iraqi *D. baueri* isolates clustered with Indo-Asian reference sequences (China, India) but formed distinct terminal branches supported by unique substitutions (bootstrap >85%) (23, 25, 27). *Cichlidogyrus tilapiae* isolates clustered with African and Asian tilapia-associated sequences but exhibited minor nucleotide differences consistent with local population divergence. Pairwise distances in the amplified fragment ranged from ~1.2% (close conspecifics) to 5.6% (interspecific comparisons); intra-specific similarity generally exceeded 98% (24, 26). Detailed tree files (Newick) and alignments accompany the submission.

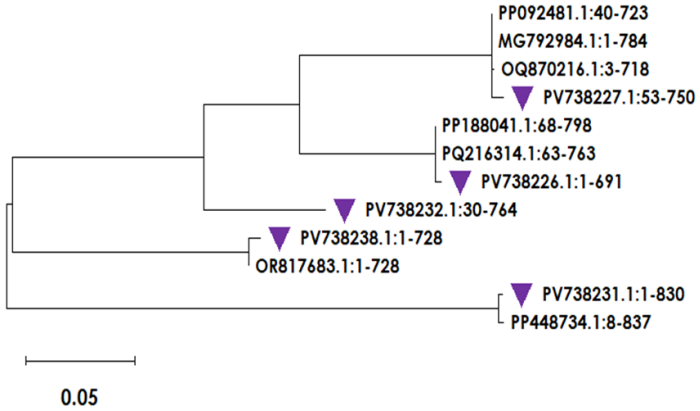


Figure 2: Maximum-likelihood tree (28S D1–D3) showing placement of Iraqi isolates (PV738226–PV738238)
Iraqi isolates are labelled with isolate ID and GenBank accession (PV738226–PV738238). Bootstrap support values ≥70 are indicated at nodes. Scale bar denotes substitutions per site. Reference sequences and their accession numbers are listed in the figure legend and the alignment (Newick) file provided with the submission.

Discussion

Summary of main findings
Combining morphological and molecular evidence, we identified five monogenean taxa infecting dominant fishes of the Al-Diwaniyah River and produced the first 28S rRNA sequences for these taxa from Iraq (PV738226–PV738238). Two parasite-host associations (*D. holciki* in *C. carpio* and *C. tilapiae* in *O. niloticus*) are new for the country and expand regional host records (14, 15, 16, 18). Iraqi isolates show Indo-Asian affinities in phylogenies but also local nucleotide variation that may indicate population structure or incipient divergence (20, 21, 23, 28, 29).
Morphological evidence (anchor/bar configuration, hooklet morphology and MCO structure) corroborated molecular identifications and allowed resolution of several taxa to species level (see Results and Table 2). The absence of published Iraqi sequences for these taxa means morphological confirmation is especially

important; our combined morphological–molecular approach strengthens taxonomic confidence.

Prevalence, intensity and ecological interpretation
Overall prevalence across the sampled hosts was 12.1% (Table 1), with *C. carpio* showing the highest species-level prevalence (14.6%). Mean intensities were low (≈1–2 parasites per infected fish), suggesting endemic, low-burden infections in wild populations under the prevailing environmental and host-density conditions. Low intensities reduce the likelihood of acute pathology in wild fish, but similar taxa can reach pathogenic levels in crowded aquaculture systems; therefore, surveillance in farms and markets is recommended. These findings align with regional monogenean surveys, which often report low to moderate prevalences in wild fish but higher burdens under captive conditions (23, 27).

Molecular insights and biogeographic implications
The 28S D1–D3 fragment provided robust placement of Iraqi isolates into recognised clades and permitted detection of unique SNPs in several isolates (e.g., PV738226 & PV738232). The clustering with Indo-Asian sequences (China, India) may reflect historical biogeographic connections among freshwater fish faunas, recent anthropogenic translocations (fish trade, stocking) or simply the limited sampling of Eurasian lineages in public databases. Multilocus data (e.g., ITS1, COI) would help discriminate recent introductions from endemic diversification.

New host records - significance
The records of *D. holciki* on *C. carpio* and *C. tilapiae* on *O. niloticus* represent notable host association expansions for Iraq. These detections can have practical implications because host range expansions increase the potential for parasite spillover between wild and cultured stocks. Surveillance of markets and hatcheries, and quarantine of introduced fish, are pragmatic measures to reduce the spread.

Study Limitations
Geographic scope: sampling was limited to the Al-Diwaniyah River; extrapolation to other Iraqi river systems should be cautious.
Marker breadth: only the 28S D1–D3 fragment was sequenced; rapidly evolving markers (e.g., COI) and multilocus analyses would improve resolution of cryptic species.
Temporal coverage: sampling covered a year but was opportunistic; systematic seasonal stratification would permit robust claims about seasonality.
Host breadth: three dominant host species were examined; inclusion of more host taxa might reveal additional species and host specificity patterns (21, 22, 23, 25, 27).

Future directions
We recommend (1) broader multisite surveys across Iraqi watersheds, (2) multilocus sequencing (28S, ITS, COI) and population genetic analyses to resolve cryptic diversity and gene flow, (3) focused monitoring in aquaculture facilities and markets to manage parasite transmission, and (4) deposition of voucher slides/specimens in a recognized museum collection to support future taxonomic work (30, 31).

Conclusion
This study supplies the first molecularly referenced record of multiple monogenean taxa from the Al-Diwaniyah River (Iraq) and provides GenBank accessions PV738226–PV738238 linked to isolate-level metadata. Morphological and 28S rRNA data indicate Indo-Asian affinities with local genetic variation, and two host–parasite associations are reported as new records for Iraq. These results establish a baseline for taxonomy, surveillance and aquaculture biosecurity; follow-up multilocus and multi-site studies are needed to clarify parasite diversity and transmission pathways.

List of abbreviations
BLAST: Basic Local Alignment Search Tool
COI: Cytochrome c Oxidase I
D1–D3: Domains 1–3 of 28S rRNA
ML: Maximum Likelihood
MCO: Male Copulatory Organ
NCBI: National Centre for Biotechnology Information
PCR: Polymerase Chain Reaction
rRNA: ribosomal RNA
SNP: Single Nucleotide Polymorphism

Declarations
Ethics approval and consent to participate
Field collections and laboratory procedures were carried out under institutional permissions recorded in the project file (University of Al-Qadisiyah). The authors confirm that fish handling complied with local animal welfare guidelines; permit details and committee approval numbers are recorded in the laboratory's ethics file and are available on request.

Consent for publication

All authors consent to the publication of this manuscript under the Creative Commons Attribution Non-Commercial 4.0 license.

Data availability
All 28S rRNA consensus sequences were deposited in GenBank (PV738226–PV738238). Sequence alignments, ML tree files (Newick), raw chromatograms and field logs are available from the corresponding author on reasonable request.

Competing interests
The authors declare no competing interests.

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Author contributions
THA: Conceptualisation, Field sampling coordination, Morphological identification, Writing original draft.
ANN: Laboratory work (DNA extraction, PCR), Data curation, Formal analysis, Writing-review & editing.
AAA: Phylogenetic analysis, Methodology, Supervision, Writing-review & editing.
All authors read and approved the final manuscript.

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