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Description of three new species of *Gyrodactylus* von Nordmann, 1832 (Monopisthocotylea: Gyrodactylidae) on *Triplophysa* species (Nemacheilidae) from Qinghai-Tibet Plateau

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Research Paper

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Abstract

Three new species of *Gyrodactylus* were identified from the body surface of the *Triplophysa* species from the Qinghai-Tibet Plateau, *Gyrodactylus triplorienchili* n. sp. on *Triplophysa orientalis* in northern Tibet, *G. yellochili* n. sp. on *T. sellaefer* and *T. scleroptera* and *G. triplsellachili* n. sp. on *T. sellaefer* and *T. robusta* in Lanzhou Reach of the Yellow River. The three newly identified species share the nemachili group species' characteristic of having inturning hamulus roots. *Gyrodactylus triplorienchili* n. sp. shared a quadrate sickle heel and a thin marginal hook sickle, two morphological traits that set them apart from *G. yellochili* n. sp. However, they may be identified by the distinct shapes of the sickle base and marginal hook sickle point. *Gyrodactylus triplsellachili* n. sp. had much larger opisthaptoral hard part size than the other two species. The three new species show relatively low interspecific differences of 2.9–5.3% p-distance for ITS1-5.85-ITS2 rDNA sequences. Phylogenetic analysis indicated that the three new species formed a well-supported monophyletic group (bp = 99) with the other nemachili group species.

Introduction

The monopisthocotylean ectoparasite *Gyrodactylus* von Nordmann, 1832 (Gyrodactylidae) is a species-diverse genus containing > 500 species found on 20 bony fish orders (Harris *et al.* 2004; Kritsky *et al.* 2013; Rahmouni *et al.* 2023). Owing to a direct life cycle and a relatively high host-specificity, infection and transmission of *Gyrodactylus* species are highly host-dependent (Bakke *et al.* 2007). Some species, such as *G. salaris* Malmberg, 1957 and *G. cichlidarum* Paperna, 1968, are harmful and are noted to cause substantial economic losses in aquaculture (Abdel-Latif and Khafaga 2020; Harris *et al.* 2011).

The Qinghai-Tibet Plateau (QTP) is widely recognized as the "roof of the world" because of its average altitude exceeding 4,000 m (Peng *et al.* 2006). *Schizothoracinae* (Cypriniformes: Cyprinidae) and *Triplophysa* species (Cypriniformes: Nemacheilidae) are the dominant species of fish fauna in this region (Wu and Tan 1991). The fish genus *Triplophysa* Rendahl, 1933 is one of the largest groups (with approximately 160 species) in the family Nemacheilidae, and 60% of the species are distributed in the rivers and lakes on the QTP and its adjacent regions of China (Sheraliev and Peng 2021; Zheng *et al.* 2009).

In neighboring Central Asian countries bordering the QTP, 12 species of *Gyrodactylus* have been described thus far on *Triplophysa* species (Ergens and Allamuratov 1972; Ergens and Karabekova 1980; Ergens and Kartunova 1991; Přikrylová *et al.* 2008; Pugachev *et al.* 2009), including: *G. parvus* Bychowsky, 1936, *G. kessleri* Gvosdev & Martechov, 1953, *G. luckyi* Ergens, 1970 and *G. incognitus* Ergens & Gusev, 1980 collected from *T. strauchi* Kessler, 1874; *G. afghanensis* Ergens, 1979 and *G. moraveci* Ergens, 1979 from *T. griffithi* Günther, 1868; *G. karatagensis* Ergens & Allamaturov, 1972 and *G. tibetanus* Dzhalilov, 1980 collected from *T. stoliczkae* Steindachner, 1866; *G. nemachili* Bychowsky, 1936, *G. paranemachili* Ergens & Bykhovsky, 1967, *G. pseudonemachili* Ergens & Bykhovsky, 1967 and *G. gvosdevi* Ergens & Kartunova, 1991 from *T. dorsalis* Kessler, 1872.

Species identification of *Gyrodactylus* in earlier times is mainly based on the morphological features of the haptoral hard parts, especially the shape of the marginal hooks, which has proven to be a valuable character to distinguish closely related *Gyrodactylus* species (Malmberg 1970; Ziętara and Lumme 2004). *Gyrodactylus* species on related hosts usually share marginal hooks resemblance (Huyse *et al.* 2003), such as rugiensis group species on marine gobies (Huyse and

Volckaert 2002). For well over 25 years, *Gyrodactylus* has been identified using the molecular markers nuclear internal transcribed spacer region of ribosomal DNA (ITS rDNA), which encompasses ITS1-5.8S-ITS2. This indicates that ITS rDNA is an excellent tool for taxonomy and interspecific relationship inference (Cunningham 1997; Lumme *et al.* 2017; Pinacho-Pinacho *et al.* 2021). Currently, there is no molecular data available for the description of *Gyrodactylus* species in *Triplophysa* hosts. In view of this, we applied morphometric and molecular methods to describe three new *Gyrodactylus* species on *Triplophysa* fish in Qinghai-Tibet Plateau, China.

Materials and Methods

Parasites collection and fixation

The fish *Triplophysa orientalis* Herzenstein, 1888 (one specimen) was collected from a wetland nearby Cuona Lake (Coordinates 32.14479N, 91.43786E; altitude 4553 m; temperature 3 °C), Anduo County, Naqu Prefecture, China on May 10, 2021. The first sampling site was in northern Tibet in the subcold monsoon climate zone, with an annual average temperature of –0.9 to –3.3 °C and a rainfall range of 100 to 200 mm (http://www.naqu.gov.cn/nqsrmzf/c100074/list_tt.shtml). *Triplophysa sellaefer* Fang, 1941 (10 specimens), *T. scleroptera* Herzenstein, 1888 (24 specimens) and *T. robusta* Kessler, 1876 (15 specimens) were collected from Lanzhou Reach of the Yellow River (Coordinates 36.42335N, 103.39658E; altitude about 2000 m; temperature 8 °C), Yongdeng County, Lanzhou City, China on October 14, 2021. The second sampling site was in the northeastern margin of QTP in the temperate continental climate zone, which has an annual average temperature of 10.9 °C and a rainfall of 300 mm (https://www.lanzhou.gov.cn/col/col5/index.html). The fish, upon collection, were anaesthetized with 0.02% MS-222, and their body surface were examined for gyrodactylids using a stereoscopic microscope Stemi SV6/AxioCam MRc5 (Zeiss, Oberkochen, Germany); worms collected using fine-pointed forceps. Gyrodactylids were fixed in 75% alcohol, haptors and bodies of some fixed specimens were cut using a sharp scalpel for morphological and molecular identification.

Morphological analyses

Specimens were fixed in glycerine and ammonium picrate (GAP) (Ergens 1969) and photographed using Axioplan 2 imaging and Axiophot 2 (Zeiss). The body and dorsal bar were measured according to Christison et al. (2005); the hamulus, ventral bar, and marginal sickle were measured according to Shinn et al. (2004); and the marginal hook filament loop was measured according to Malmberg (1970). All the measurements were given in micrometers (μm). The type-material was remounted in Canada balsam and deposited in the Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan City, Hubei province, China.

Principal component analysis was used to assess the morphological differences between the three new species using 11 haptoral sclerite morphological traits (hamulus total length, hamulus shaft length, hamulus point length, hamulus root length, hamulus aperture distance, marginal hook total length, marginal hook shaft length, marginal hook sickle length, marginal hook sickle proximal width, marginal hook sickle distal width and marginal hook aperture) (Shinn et al. 1996). The analyses were based on the covariance matrix and performed in the R package "vegan" using log-transformed data (R Core Team 2017).

DNA extraction, amplification, and sequencing

Genomic DNA was extracted from the worm body using a Tissue Cell Genome Kit (TIANGEN, Beijing, China) following the manufacturer's instructions. A partial sequence of the 18S rDNA gene was amplified using the forward primer PBS18SF (5′-CGC GCA ACT TAC CCA CTC TC-3′) and reverse primer PBS1863R (5′-CAA AGG GCA GGG ACG TAT TCA GCA CA-3′) (Gilmore *et al.* 2012). The region of rDNA spanning the 5′ end of partial sequences of the internal transcribed spacer 1 (ITS1), 5.8S rDNA, ITS2, and the 5′ end of partial sequence of the 28S rDNA gene was targeted using primers BD1 (5′-GTC GTA ACA AGG TTT CCG TA-3′) and BD2 (5′-TAT GCT TAARTT CAG CGG GT-3′) (Luton *et al.* 1992). The *cox1* was amplified with cox1F (5′-TAG CNG CDG GNA THA CHA TG-3′) and cox1R (5′-GGD TTA CCD CGH CGW GTW TG-3′) primers (Jin *et al.* 2022). Polymerase chain reaction (PCR) amplification was conducted using LA Taq polymerase (Sangon Biotech, Shanghai, China) as follows: 5 min at 94 °C as the initial step; then 35 cycles of 30 s at 94 °C, 30 s at 50 °C, 1 min at 72 °C, and the final step being 7 min at 72 °C. After purification by SanPrep Column PCR Product Purification Kit (Sangon Biotech), PCR products were sequenced with the primers described previously that were produced by Sangon Biotech and assembled manually with the help of DNAStar's SeqMan software (DNASTAR, Madison, WI).

Molecular analysis

The obtained sequences of 18S, ITS (ITS1-5.8S-ITS2), and *cox1* were deposited in GenBank and compared using BLAST on the NCBI database (https://www.ncbi.nlm.nih.gov/).

ITS sequences (Table 1) for molecular analysis were chosen based on similarity and hamulus resemblance, then imported into PhyloSuite (Xiang *et al.* 2023; Zhang *et al.* 2020) and aligned with other available *Gyrodactylus* spp. in GenBank by MAFFT 7.149 (Nakamura *et al.* 2018) using the "G-INS-I" strategy and normal alignment mode. Poorly aligned segments were trimmed using Gblocks (Talavera and Castresana 2007); parameters were set according to the previous research (Jin *et al.* 2022). The uncorrected p-distance among the three new species was calculated based on the aligned ITS sequences using Mega X (Kumar *et al.* 2018). *Gyrodactylus rugiensis* Gläser, 1974 and *G. rugiensoides* Huyse & Volckaert, 2002 from subgenus *G.* (*Paranephrotus*) were used as outgroups. Phylogenetic analyses based on the ITS sequences were performed using Maximum Likelihood methods. Based on the Akaike's information criterion, as implemented in ModelFinder (Kalyaanamoorthy *et al.* 2017), TVM+F+G4 was chosen as the best-fitting model for nucleotide evolution. Phylogenies were inferred using IQ-TREE (Minh *et al.* 2020).

Results

Taxonomic summary

Family Gyrodactylidae van Beneden and Hesse, 1864.
Genus *Gyrodactylus* von Nordmann, 1832.
Subgenus G. (*Limnonephrotus*) Malmberg, 1970.
Species group G. nemachili Prikrylova et al. 2008.

***Gyrodactylus triplorienchili* n. sp.** (Fig. 1, Table 2)

Description: Morphological measurements were conducted on 22 specimens. Body length 357.0 (260.0–471.4; $n = 15$), width 89.5 (65.1–113.1; $n = 15$). Head had a pair of cephalic lobes and

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Table 1. List of *Gyrodactylus* ITS rDNA sequences used in this study

Species	Host	Locality	GenBank ITS rDNA	Reference
G. ajime	*Niwaella delicata*	Kyoto, Japan	LC545570	Nitta 2021
G. banmae	*Danio rerio*	Guangzhou City, China	MW353802	Jin et al. 2022
G. cf. *konovalovi*	*Rhynchocypris lagowskii*	Vladivostok, Russia	OQ672252	Lebedeva et al. 2023
G. cf. *lagowskii*	*Rhynchocypris lagowskii*	Vladivostok, Russia	OQ672253	Lebedeva et al. 2023
G. cf. *mantshuricus*	*Rhynchocypris lagowskii*	Vladivostok, Russia	OQ672248	Lebedeva et al. 2023
G. gracilihamatus	*Gasterosteus aculeatus*	Baltic Sea, Finland	AF484532	Ziętara et al. 2008
G. gurleyi	*Carassius auratus*	Wuhan City, China	KC922453	Li et al. 2014
G. gymnodiptychi	*Gymnodiptychus dybowskii*	Yili River, China	MH445968	Zhang et al. 2023
G. hildae	*Oreochromis niloticus niloticus*	Ethiopia	FJ231869	García-Vásquez et al. 2011
G. jiroveci	*Barbatula barbatula*	Czech Republic	AM502860	Přikrylová et al. 2008
G. jussii	*Phoxinus phoxinus*	River Merenoja, Finland	AY061982	Ziętara and Lumme 2003
G. kobayashii	*Carassius auratus*	Wuhan City, China	KC922452	Li et al. 2014
G. longoacuminatus	*Carassius auratus*	Wuhan City, China	KC922451	Li et al. 2014
G. macronychus	*Phoxinus phoxinus*	River Merenoja, Finland	AY061981	Ziętara and Lumme 2003
G. mongolicus	*Oreoleuciscus humilis*	River Teysin, Mongolia	OQ641768	Lebedeva et al. 2023
G. nemachili	*Barbatula* sp.	Chono Kharaikh River, Mongolia	OQ641770	Lebedeva et al. 2023
G. papernai	*Barbatula barbatula*	Baltic Sea basin, Finland	AF484533	Ziętara et al. 2008
G. pseudonemachili	*Thymallus brevirostris*	Zavkhan river, Mongolia	OQ641755	Lebedeva et al. 2023
*G. pseudonemachili**	*Barbatula barbatula*	Czech Republic	AJ567674	Matejusová et al. 2003
G. triplsellachili n. sp.	*Triplophysa sellaefer*	Lanzhou City, China	OP793876	Present study
G. triplorienchili n. sp.	*Triplophysa orientalis*	Northern Tibet, China	MW353802	Present study
G. tayshirensis	*Barbatula conilobus*	Zavkhan River, Mongolia	OQ641774	Lebedeva et al. 2023
G. yellochili n. sp.	*Triplophysa scleroptera*	Lanzhou City, China	OP793878	Present study
G. zavkhanensis	*Thymallus brevirostris*,	Zavkhan River, Mongolia	OQ641773	Lebedeva et al. 2023
Outgroup				
G. micropsi	*Pomatoschistus microps*	North Sea basin, Belgium	AF328868	Ziętara et al. 2002
G. rugiensis	*Pomatoschistus microps*	North Sea, Belgium	AF328870	Ziętara et al. 2002

spike sensilla. Pharynx campaniform with anterior (smaller) and posterior pharyngeal bulb, total length 34.4 (32.2–35.5, n = 4), width 28.0 (23.7–33.3, n = 4). Male copulatory organ posterior to pharynx 18.1 (16.0–19.7, n = 5) in diameter, armed with a single large central spine with two medium spines on each side and eight smaller spines opposite to the central spine. Hamulus total length 40.5 (38.9–43.3; n = 18); hamulus shaft length 30.0 (25.0–32.2; n = 15), proximal shaft width 6.2 (5.5–6.9; n = 17), distal shaft width 3.8 (3.1–4.3; n = 16); hamulus point length 24.5 (22.1–26.6; n = 16); hamulus root heavily bent, length 11.0 (10.3–12.7; n = 16); hamulus aperture distance 14.1 (13.0–16.1; n = 18). Ventral bar width 26.5 (24.2–28.8; n = 14), total length 21.8 (19.2–23.6; n = 4); ventral bar process broad and triangular, length 2.8 (2.6–3.4; n = 8); process-to-mid length 2.7 (1.9–3.8; n = 6), median length 5.1 (4.0–5.9; n = 18); ventral bar membrane extended "V" shape, length 15.2 (14.3–16.0; n = 4). Dorsal bar straight without any decoration, total length 24.3 (20.2–27.5; n = 13), width 2.6 (2.0–3.1; n = 17). Marginal hook total length 25.3 (24.3–26.7; n = 11); shaft length 19.5 (18.3–20.5; n = 11); sickle length 6.3 (5.7–6.8; n = 16); sickle shaft slender, steeply sloping; sickle point exceeded the toe, slightly curved downward; sickle proximal width 4.3 (4.0–4.7; n = 17); distal width 3.7 (2.9–4.6; n = 16); sickle base flat with quadrate heel and long triangular toe, toe length 2.0 (1.9–2.3; n = 15), with spike on top; marginal hook aperture 5.4 (4.6–6.1; n = 16); filament loop length 10.7 (9.3–12.2; n = 10).

Type host: *Triplophysa orientalis* Herzenstein, 1888.

Type locality: A wetland near Cuona Lake (32.14479N, 91.43786E), Salween River, Anduo County, NaQu Prefecture at northern Tibet, China.

Infection site: Caudal fin.

Type material: The type materials were fixed in GAP, dehydrated in graded alcohol, and mounted in Canada balsam. Holotype (TO-GA 202101) and five paratypes (TO-GA 202102-202106) are

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Table 2. Morphometric parameters of *Gyrodactylus triplorienchili* n. sp., *G. yellochili* n. sp. and *G. triplsellachili* n. sp

Measurement	N_1	*G. triplorienchili* n. sp.	N_2	*G. yellochili* n. sp.	N_3	*G. triplsellachili* n. sp.
Body length	15	357.0 ± 53.2 (260.0–471.4)	3	412.0 ± 116.3 (317.5–541.9)	6	438.2 ± 33.6 (390.5–480.4)
Body width	15	89.5 ± 17.2 (65.1–134.7)	3	108.6 ± 25.0 (82.4–132.1)	6	131.5 ± 19.1 (108.6–155.4)
Dorsal bar total length	13	24.3 ± 2.1 (20.2–27.5)	21	22.8 ± 0.9 (20.8–23.7)	12	36.4 ± 1.7 (34.1–38.8)
Dorsal bar width	17	2.6 ± 0.3 (2–3.1)	23	3.0 ± 0.3 (2.6–3.6)	17	4.4 ± 0.5 (3.7–5.6)
Ventral bar length	4	21.8 ± 2.0 (19.2–23.6)	8	23.9 ± 2.2 (20.8–26.7)	4	26.9 ± 2.9 (24.0–30.7)
Ventral bar width	14	26.5 ± 1.7 (24.2–28.8)	13	24.8 ± 1.2 (22.8–26.7)	5	37.9 ± 3.2 (33.5–40.8)
Ventral bar process length	8	2.8 ± 0.3 (2.6–3.4)	6	1.7 ± 0.3 (1.4–2.1)	4	3.4 ± 0.6 (2.7–4.0)
Ventral bar membrane length	4	15.2 ± 0.8 (14.3–16.0)	7	16.0 ± 1.2 (14.7–17.6)	3	19.3 ± 4.3 (15.6v24.0)
Ventral bar median length	18	5.1 ± 0.6 (4.0–5.9)	13	5.9 ± 1.2 (4.2–7.8)	4	10.2 ± 2.0 (8.2–12.9)
Ventral bar process to mid-length	6	2.7 ± 0.8 (1.9–3.8)	4	2.2 ± 0.9 (1.4–3.5)	3	1.5 ± 0.6 (1.0–2.2)
Hamulus total length	18	40.5 ± 1.3 (38.9–43.3)	29	40.5 ± 0.9 (38.2–42.6)	19	57.0 ± 1.6 (54.8–61.7)
Hamulus shaft length	15	30.0 ± 1.6 (25.0–32.2)	19	31.1 ± 0.9 (29.5–32.7)	13	41.3 ± 1.6 (38.5–44.2)
Hamulus point length	16	24.5 ± 1.1 (22.1–26.6)	19	25.4 ± 0.6 (23.8–26.4)	13	29.4 ± 0.7 (27.8–30.7)
Hamulus root length	16	11.0 ± 0.6 (10.3–12.7)	19	8.3 ± 0.5 (7.4–9.1)	15	14.2 ± 0.8 (13.1–15.7)
Hamulus aperture distance	18	14.1 ± 1.4 (13.0–16.1)	23	14.5 ± 1.0 (12.8–16.5)	13	26.0 ± 1.9 (22.8–30.0)
Hamulus proximal shaft width	17	6.2 ± 0.5 (5.5–6.9)	26	6.6 ± 0.3 (5.9–7.1)	17	9.1 ± 0.5 (8.0–10.0)
Hamulus distal shaft width	16	3.8 ± 0.4 (3.1–4.3)	19	4.2 ± 0.4 (3.7–4.8)	8	5.9 ± 0.4 (5.4–6.5)
Marginal hook total length	11	25.3 ± 0.7 (24.3–26.7)	24	24.8 ± 0.6 (23.3–26.4)	17	39.5 ± 1.7 (37.0–43.2)
Marginal hook shaft length	11	19.5 ± 0.7 (18.3–20.5)	24	19.1 ± 0.6 (18.0–21.0)	17	32.5 ± 1.5 (30.2–35.7)
Marginal hook sickle length	16	6.3 ± 0.3 (5.7–6.8)	24	5.5 ± 0.2 (5.1–5.8)	20	6.8 ± 0.3 (6.3–7.1)
Marginal hook sickle distal width	16	3.7 ± 0.5 (2.9–4.6)	24	3.4 ± 0.3 (3.0–4.0)	20	5.5 ± 0.4 (4.8–6.0)
Marginal hook sickle proximal width	17	4.3 ± 0.2 (4.0–4.7)	24	3.9 ± 0.3 (3.2–4.4)	20	5.4 ± 0.2 (4.9–5.7)
Marginal hook aperture	16	5.4 ± 0.4 (4.6–6.1)	24	5.3 ± 0.2 (4.9–5.7)	20	6.3 ± 0.3 (5.6–7.0)
Marginal hook toe length	15	2.0 ± 0.1 (1.9–2.3)	24	1.8 ± 0.1 (1.6–2.1)	20	2.0 ± 0.2 (1.8–2.4)
Marginal hook filament loop length	10	10.7 ± 0.9 (9.3—12.2)	20	10.7 ± 1.0 (9.4–12.5)	18	14.6 ± 1.1 (13.0–16.7)

N, the number of gyrodactylid specimens measured.

deposited in the Museum of the Institute of Hydrobiology, Chinese Academy of Sciences, China.

Zoobank registration: urn:lsid:zoobank.org:act:DFF5B644-C63E-47A7-B9D5-D4F59CE4165D

Molecular marker: ITS rDNA (OP793877) comprises ITS1 (548 bp), 5.8S (157 bp) and ITS2 (390 bp). The partial 18S (OP793865) length 1200 bp. The partial *cox1* (OP787151) length 1257 bp, with 99.6–100% sequence identity. A BLASTn search of the 5.8S sequence in GenBank revealed 100% identity with *G. papernai* Ergens & Bychowsky, 1967 (AF484533), *G. nemachili* (OQ641770) and *G. tayshirensis* Lebedeva, Ziętara, Mendsaikhan, Ermolenko & Lumme, 2023 (OQ641774) from Nemacheilidae fish, *G. mongolicus* Ergens & Dulmaa, 1970 (OQ641768), *G.* cf. *lagowskii* (OQ672253) and *G.* cf. *mantshuricusi* (OQ672248) from Cyprinidae fish, *G. zavkhanensis* Lebedeva, Ziętara, Mendsaikhan, Ermolenko & Lumme, 2023 (OQ641773) from salmonids, and *G. gymnodiptychi* Zhang, Hao, Arken, Rong, Tian, Kadir & Yue, 2023 (MH445968) from Schizothoracinae fish. The ITS rDNA sequence exhibited 94.7% identity with *G. papernai*, and *cox1* showed 85.1% identity with *G. kobayashii* Hukuda, 1940 (NC_030050).

Etymology: The specific epithet was derived from the first five letters of the generic name and the first five letters of the species name of the type host, "*Triplophysa orientalis*", which ended with "chili" indicating the hamulus root inturning feature. The combined name was "triplorienchili".

Remarks: *Gyrodactylus triplorienchili* n. sp. was the first *Gyrodactylus* species reported on *Triplophysa* species in Tibet. The most distinctive feature of this species was hamulus root inturning, which was typically appeared on nemachili group species (Přikrylová *et al.* 2008) such as *G. incognitus*, *G. paranemachili*, *G. pseudonemachili*, *G. karatagensis*, *G. gvosdevi* and *G. nemacheili* that were collected from *Triplophysa* fish at high-altitude areas in central Asia (Ergens and Kartunova 1991), or *G. tayshirensis*, *G. mongolicus*, and *G. pavlovskyi* Ergens & Bychowsky, 1967 collected from *Barbatula* spp. in Mongolia; *G. papernai* Ergens & Bychowsky, 1967 and *G. jiroveci* Ergens & Bychowsky, 1967 collected from *Barbatula* spp. in Europe (Lebedeva *et al.* 2023; Přikrylová *et al.* 2008). The middle of the marginal hook sickles of *G. nemacheili*, *G. jiroveci*, *G. incognitus*, and *G. pavlovskyi* had a severe curvature, and the sickle point was comparatively longer than the shaft section. Whereas in *G. triplorienchili* n. sp., sickles were slender, which made it similar to *G. nemacheili*, *G. paranemachili*, *G. karatagensis* and *G. pseudonemachili*. However, *G. triplorienchili* n. sp. could be distinguished from those

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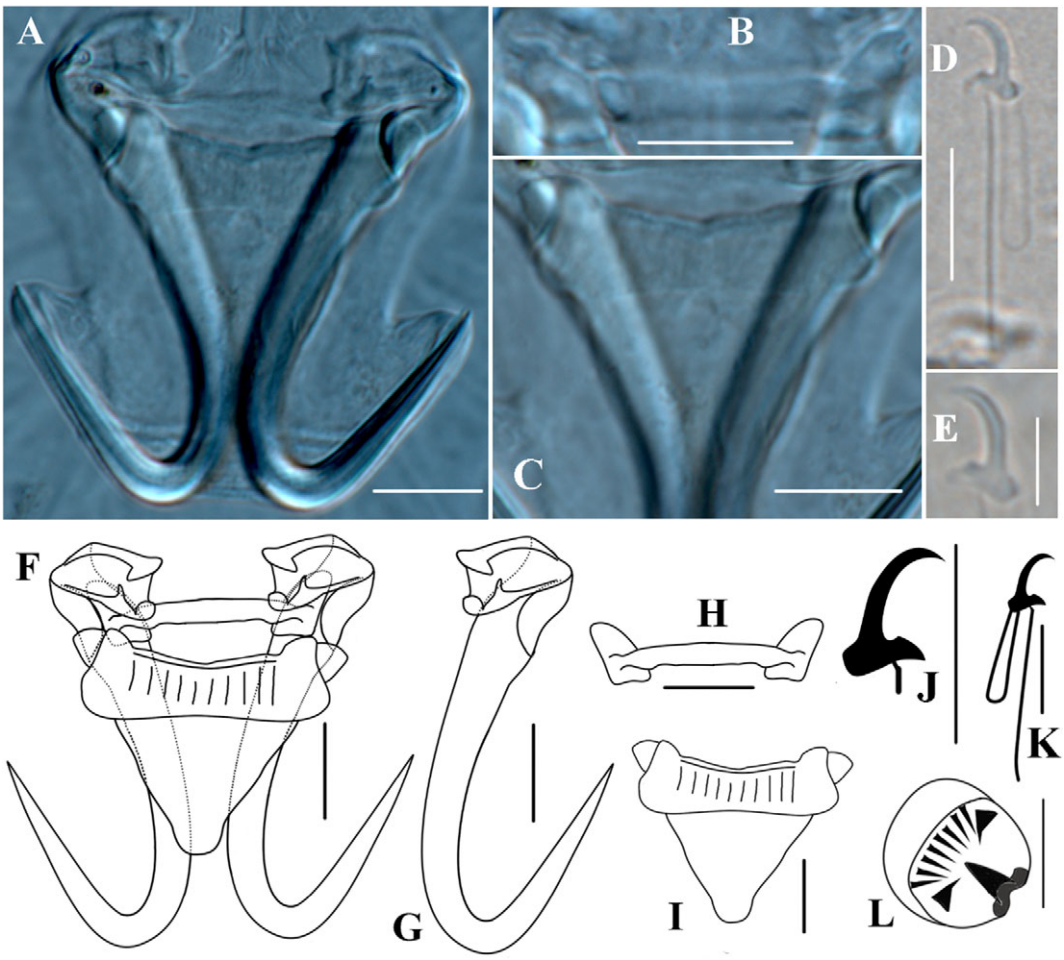

Figure 1. Light micrographs (A-E) and line drawings (F-L) of *Gyrodactylus triplorienchili* n. sp. A, central hook complex; B, ventral bar; C, dorsal bar; D-E, marginal hook; F, hamulus-bar complex; G, hamulus; H, dorsal bar; I, ventral bar; J-K, marginal hook; L, male copulatory organ. Scale bars: A-D, F-L 10 μm; E 5 μm.

species listed previously by different sickle base and toe shape. Hamulus total length of *G. triplorienchili* n. sp. (38.9–43.3) was longer than *G. nemacheili* (36–38) and *G. karatagensis* (32–34), similar with *G. paranemachili* (36–42). The marginal hook total length of *G. triplorienchili* n. sp. (24.3–26.7) was longer than that of the *G. nemacheili* (20–22) and *G. paranemachili* (17–18) but shorter than that of the *G. karatagensis* species (31–33) (Ergens and Allamuratov 1972; Přikrylová *et al.* 2008; Pugachev *et al.* 2009).

***Gyrodactylus yellochili* n. sp. (Fig. 2, Table 2)**

Description: Morphological measurements were conducted on 32 specimens. Body length 412.0 (317.5–541.9; n = 3), width 108.6 (82.4–132.1; n = 3). Male copulatory organ 17.5 (15.8–19.2, n = 2) in diameter and armed with a single large central spine, two medium spines on each side, and six smaller spines opposite the central spine. Hamulus total length 40.5 (38.2–42.6; n = 29); shaft length 31.1 (29.5–32.7; n = 19), proximal shaft width 6.6 (5.9–7.1; n = 26), distal shaft width 4.2 (3.7–4.8; n =19); hamulus point length 25.4 (23.8–26.4; n = 19); hamulus root heavily bent, length 8.3 (7.4–9.1; n = 19); hamulus aperture distance 14.5 (12.8–16.5; n = 23). Ventral bar width 24.8 (22.8–26.7; n = 13), total length 23.9 (20.8–26.7; n = 8); ventral bar process broad and triangular, length 1.7 (1.4–2.1; n = 6); process-to-mid length 2.7 (1.9–3.8; n = 6), median length 5.9 (4.2–7.8; n = 13); ventral bar membrane extended in a "U" shape, length 16.0 (14.7–17.6; n = 7). Dorsal bar straight without any decoration, total length of 22.8 (20.8–23.7; n = 21), width 3.0 (2.6–3.6; n = 23). Marginal hook total length 24.8 (23.3–26.4; n = 24); shaft length 19.1 (18.0–21.0; n = 24); sickle length 5.5 (5.1–5.8; n = 24); sickle shaft slender, steeply sloping; sickle point exceeded the toe, slightly curved downward; sickle proximal width 3.9 (3.2–4.4; n = 24); distal width 3.4 (3.0–4.0; n = 24); sickle base flat with quadrate heel and long triangular toe, toe length 1.8 (1.6–2.1; n = 24); instep with a spike; marginal hook aperture 5.3 (4.9–5.7; n = 24); filament loop length 10.7 (9.4–12.5; n = 20).

Type host: *Triplophysa scleroptera* Herzenstein, 1888.

Other hosts: *Triplophysa sellaefer* Fang, 1941.

Type locality: Lanzhou Reach of the Yellow River (36.42335N, 103.39658E), Lanzhou City, China.

Infection site: Fins and skin.

Type material: Type materials were fixed in GAP, dehydrated in graded alcohol, and mounted in Canada balsam. Holotype (GQ-GH-202201) and five paratypes (GQ-GH-202202-202206) are deposited in the Museum of the Institute of Hydrobiology, Chinese Academy of Sciences, China.

Zoobank registration: urn:lsid:zoobank.org:act:41077872-68FE-462E-AEFF-D7785EE56516

Molecular marker: ITS rDNA (OP793878) comprises ITS1 (570 bp), 5.8S (157 bp) and ITS2 (413 bp). The partial 18S

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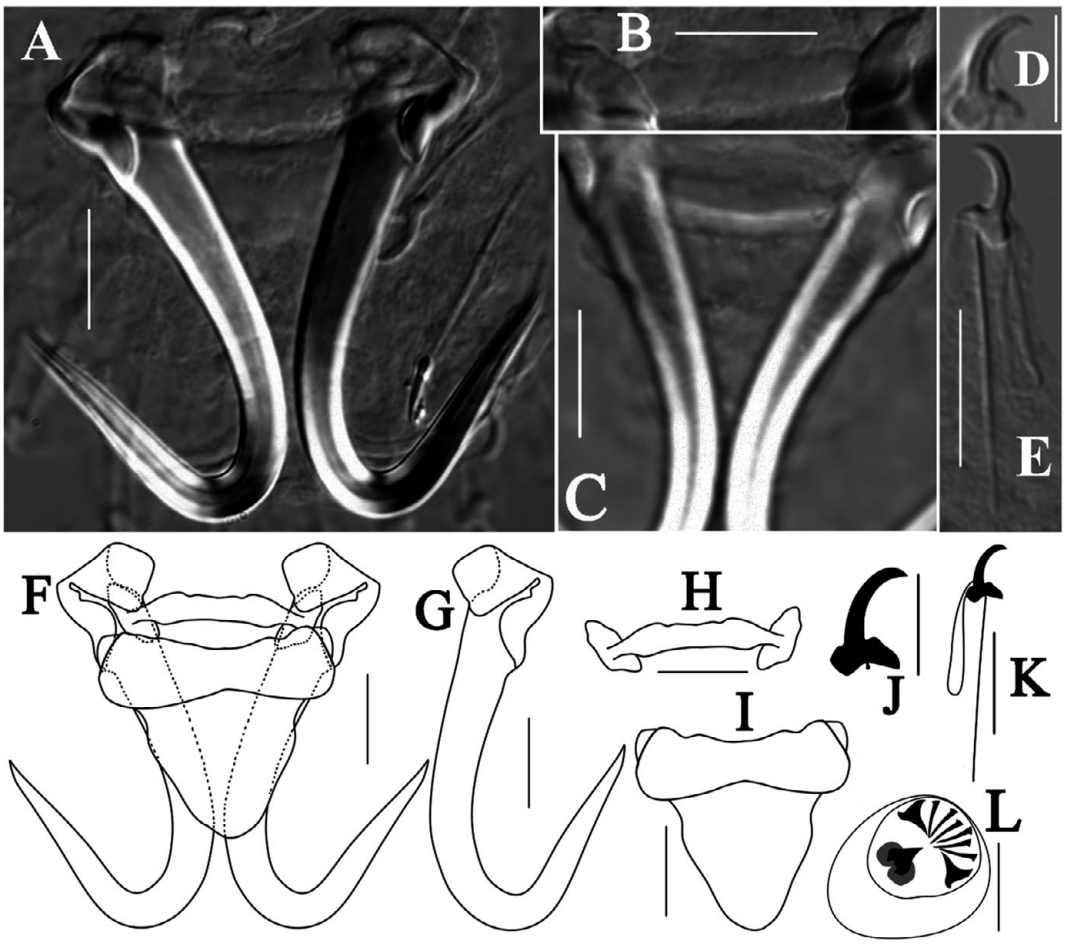

Figure 2. Light micrographs (A-E) and line drawings (F-L) of *Gyrodactylus yellochili* n. sp. A, central hook complex; B, ventral bar; C, dorsal bar; D-E, marginal hook; F, hamulus-bar complex; G, hamulus; H, dorsal bar; I, ventral bar; J-K, marginal hook; L, male copulatory organ. Scale bars: A-C, E-I, K-L 10 μm; D, J 5 μm.

(OP793867) length 1242 bp. The partial *cox1* (OP787152) length 1171 bp. The 5.8S sequence was identical to *G. triplorienchili* n. sp. and *G. triplsellachili* n. sp. A BLASTn search of the ITS rDNA and 18S sequence in GenBank revealed 96.9% and 99.9% identity with *G. triplorienchili* n. sp., respectively.

Etymology: The specific epithet was derived from the sampling location "Yellow river", then ended with "chili", which indicates the inturning hamulus root feature. Thus, the combined names were "yellochili".

Remarks: *Gyrodactylus yellochili* n. sp. was the second *Gyrodactylus* species reported on *Triplophysa* fish in China. The sampling location of *G. yellochili* n. sp. was 1200 km distant away from *G. triplorienchili* n. sp. The two species could be easily confused because both have a slender marginal hook sickle, quadrate sickle heel, and long triangular sickle toe. The hamulus and marginal hook size in *G. yellochili* n. sp. was also similar to *G. triplorienchili* n. sp. (hamulus total length, 38.2-42.6 vs. 38.9-43.3; marginal hook total length, 23.3–26.4 vs. 24.3–26.7). However, *G. yellochili* n. sp. was shorter than *G. triplorienchili* n. sp. in hamulus root (7.4–9.1 vs. 10.3–12.7), marginal hook sickle (5.1–5.8 vs. 5.7–6.8) and ventral bar process (1.4–2.1 vs. 2.6–3.4). The marginal hook sickle point in *G. yellochili* n. sp. was blunt and not exceeding the sickle base toe. In *G. triplorienchili* n. sp., sickle point was sharp and exceeded the sickle base toe. Middle part of the ventral bar was smooth in *G. yellochili* n. sp., but it was pleated in *G. triplorienchili* n. sp.

***Gyrodactylus triplsellachili* n. sp.** (Fig. 3, Table 2)

Description: Morphological measurements were conducted on 22 specimens. Body length 438.2 (390.5–480.4; n = 6), width 131.5 (108.6–155.4; n = 6). Male copulatory organ diameter 26.6 (n = 1), armed with a single large central spine, two medium spines on each side, and six smaller spines opposite the central spine. Hamulus total length 57.0 (54.8–61.7; n = 19); shaft length 41.3 (38.5–44.2; n = 13), proximal shaft width 9.1 (8.0–10.0; n = 17), distal shaft width 5.9 (5.4–6.5; n = 17); hamulus point length 29.4 (27.8–30.7; n = 13); hamulus root heavily inturning, inturning part warping on each side, length 14.2 (13.1–15.7; n = 15); hamulus aperture distance 26.0 (22.8–30.0; n = 13). Ventral bar dumpy, width 37.9 (33.5–40.8; n = 5), total length 26.9 (24.0–30.7; n = 4); ventral bar process broad and triangular, length 3.4 (2.7–4.0; n = 4); process-to-mid length 1.5 (1.0–2.2; n = 3), median length 10.2 (8.2–12.9; n = 4); ventral bar membrane extended in a "V" shape, length 19.3 (15.6–24.0; n = 3). Dorsal bar slightly curved in the middle part, without any decoration, total length 36.4 (34.1–38.8; n = 12), width 4.4 (3.7–5.6; n = 17). Marginal hook huge, total length 39.5 (37.0–43.2; n = 17); shaft length 32.5 (30.2–35.7; n = 17); sickle length 6.8 (6.3–7.1; n = 20); sickle shaft thick, proximal part approximately perpendicular to the base and middle part rapidly curved downward; sickle point far exceeding the sickle base toe; sickle proximal

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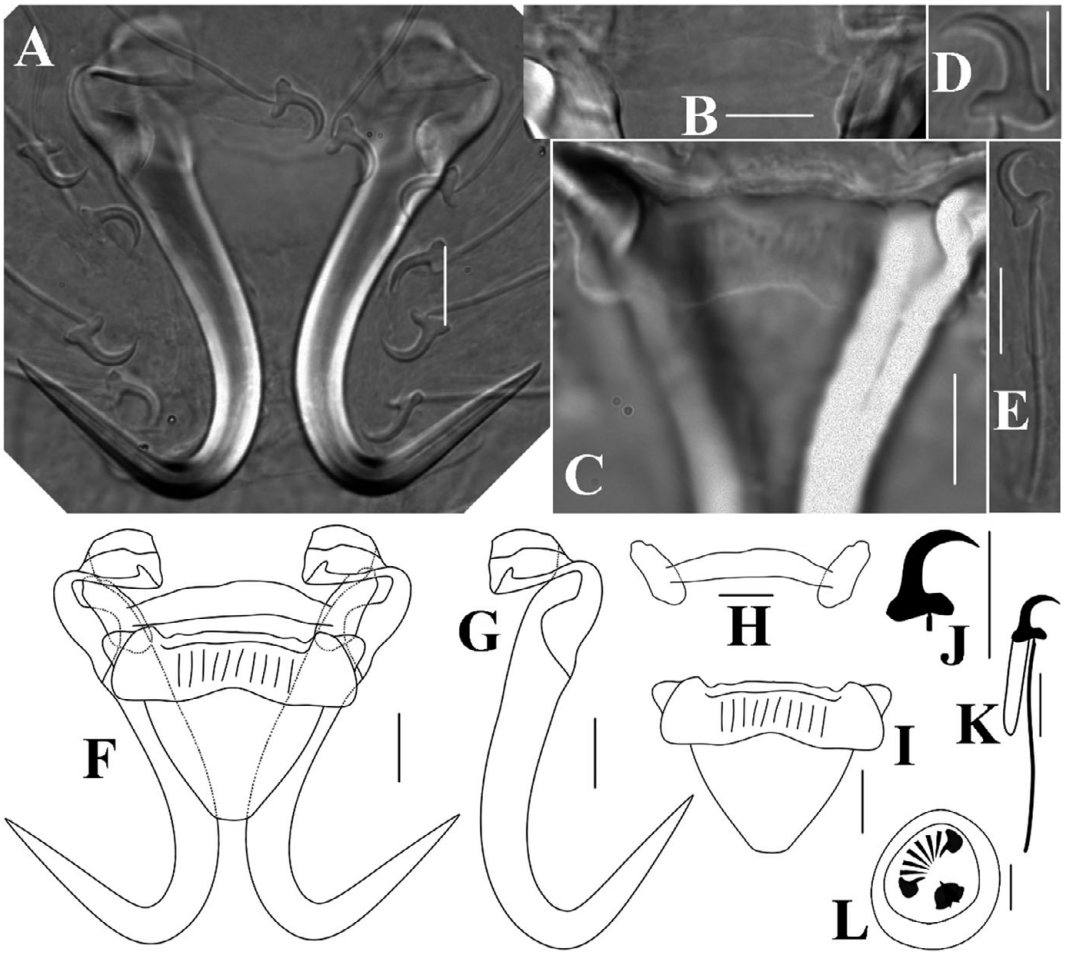

Figure 3. Light micrographs (A-E) and line drawings (F-L) of *Gyrodactylus triplsellachili* n. sp. A, central hook complex; B, ventral bar; C, dorsal bar; D-E, marginal hook; F, hamulus-bar complex; G, hamulus; H, dorsal bar; I, ventral bar; J-K, marginal hook; L, male copulatory organ. Scale bars: A-C, E-L 10 μm; D 5 μm.

width 5.4 (4.9–5.7; *n* = 20); distal width 5.5 (4.8–6.0; *n* = 20); sickle base flat with quadrate heel and trapeziform toe, toe length 2.0 (1.8–2.4; *n* = 20); marginal hook aperture 6.3 (5.6–7.0; *n* = 20); filament loop length 14.6 (13.0–16.7; *n* = 20).

Type host: *Triplophysa sellaefer* Fang, 1941.

Other hosts: *Triplophysa robusta* Kessler, 1876.

Type locality: Lanzhou Reach of the Yellow River (36.42335N, 103.39658E), Lanzhou City, China.

Infection site: Fins and skin.

Type material: Type materials were fixed in GAP, dehydrated in graded alcohol, and mounted in Canada balsam. Holotype (GQ-GC-202201) and five paratypes (GQ-GC-202202-202206) are deposited in the Museum of the Institute of Hydrobiology, Chinese Academy of Sciences, China.

Zoobank registration: urn:lsid:zoobank.org:act:FCE32B1F-BB26-4C00-946B-114F66BB7E7D

Molecular marker: ITS rDNA (OP793876) comprises ITS1 (571 bp), 5.8S (157) and ITS2 (409 bp). The partial 18S (OP793865) length was 1241 bp. The partial *cox1* (OP787150) length was 1165 bp. The 5.8S sequence was identical to *G. triplorienchili* n. sp. and *G. yellochili* n. sp. The ITS rDNA and 18S sequence exhibited 95.4% and 99.8% identity with *G. yellochili* n. sp., respectively.

Etymology: The specific epithet was derived from the first five letters of the generic name and the first five letters of the species name of the type host "Triplophysa sellaefer", then end with "chili", which indicates the bent hamulus root feature. The combined names became "triplsellachili".

Remarks: *Gyrodactylus triplsellachili* n. sp. was the third *Gyrodactylus* species collected from *Triplophysa* fish in China, the same location as *G. yellochili* n. sp. It could be easily distinguished from *G. yellochili* n. sp. by different opisthaptor size (hamulus total length, 54.8–61.7 *vs.* 38.2–42.6; marginal hook total length, 37.0–43.2 *vs.* 23.3–26.4). Both *G. triplsellachili* n. sp. and *G. papernai* had thick sickle shafts and elongated sickle point. Whereas in *G. triplsellachili* n. sp., sickle point smoothly curved and sickle base with quadrate heel and blunt toe. In *G. papernai*, sickle point sharply curved and sickle base with round heel and triangular toe (Fig. 4). Compared to other large opisthaptor size species in nemachili group such as *G. papernai*, *G. triplsellachili* n. sp. showed equal hamulus shaft length (38.5–44.2 vs. 38.0–43.0), but longer in marginal hook total length (37.0–43.2 vs. 26.5–31.5) (Přikrylová *et al.* 2008).

Morphometric analysis

Data for 11 morphometric features from 11 specimens of *G. triplorienchili* n. sp., 14 specimens of *G. yellochili* n. sp. and 10 specimens of *G. triplsellachili* n. sp. were included in the principal components analysis. The first principal component

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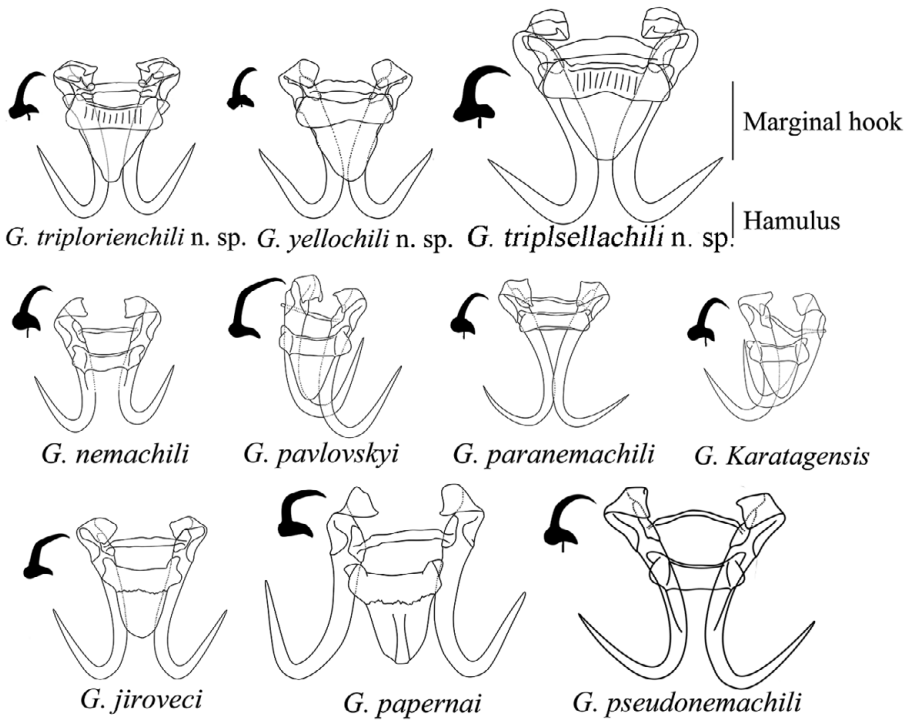

Figure 4. Comparison of marginal hook sickle and hamulus-bar complex between *Gyrodactylus triplorienchili* n. sp., *G. yellochili* n. sp. and *G. triplsellachili* n. sp. and the morphologically similar species; *G. nemacheili*, *G. pseudonemachili*, *G. pavlovskyi*, *G. paranemachili* and *G. karatagensis* from Ergens and Kartunova (1991); *G. jiroveci* and *G. papernai* and from Přikrylová et al. (2008). Scale bars: 10 μm.

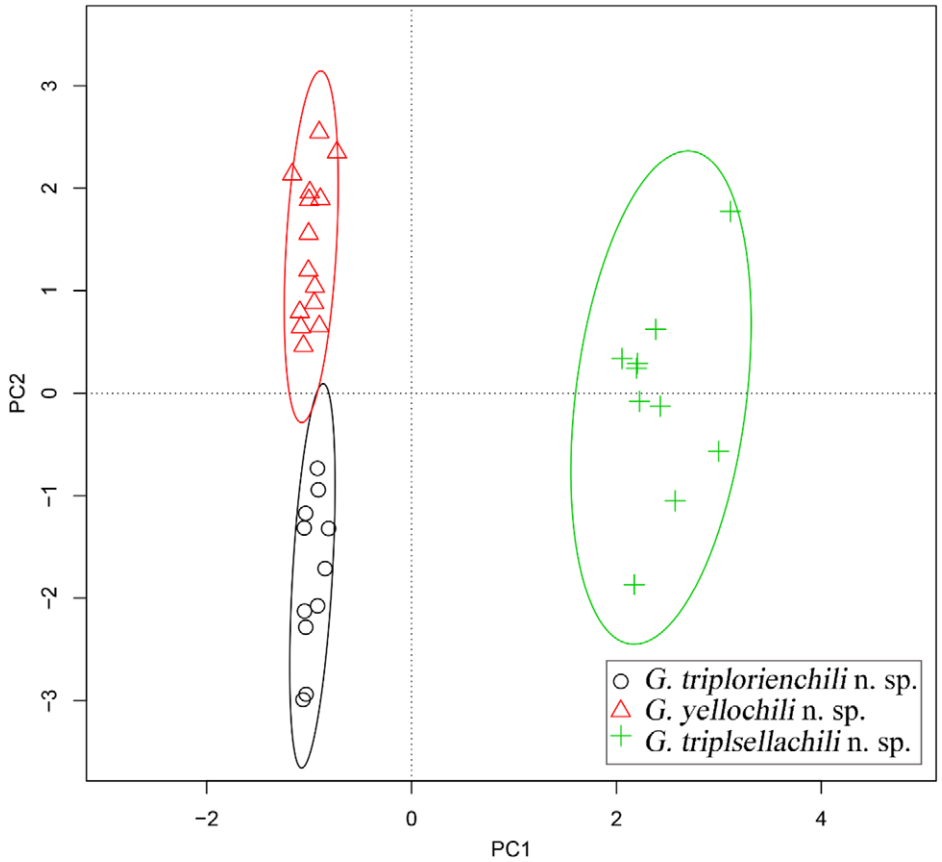

Figure 5. Principal component analysis plot of the 11 measurements on the opisthaptoral hard parts among *G. triplorienchili* n. sp., *G. yellochili* n. sp. and *G. triplsellachili* n. sp.

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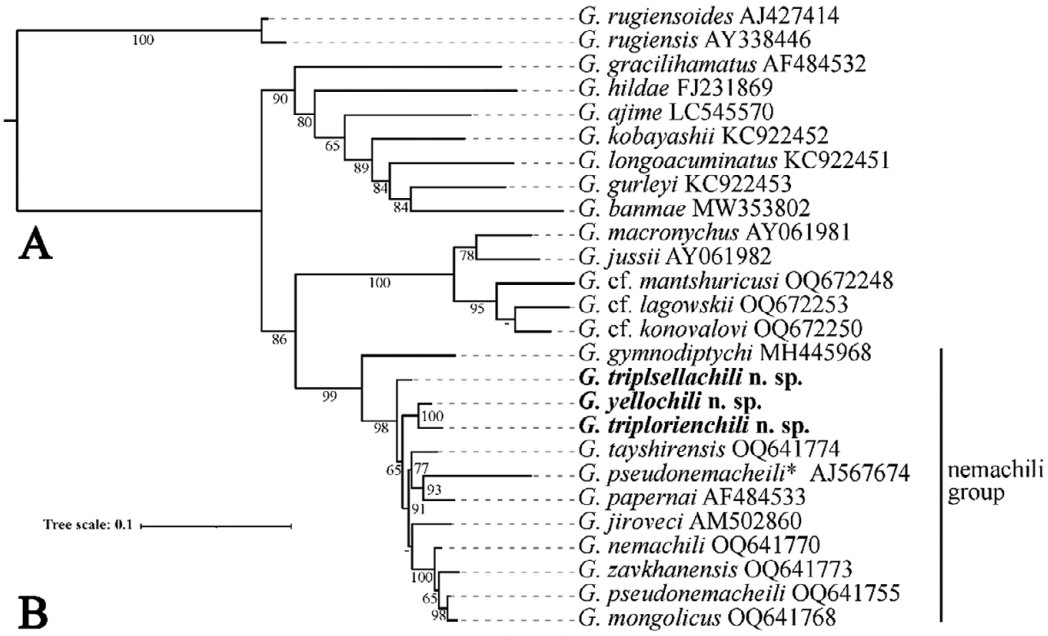

Location	Species	1	2	3	4	5	6	7	8	9	10	11
China-QTP	1. *G. triplsellachili* n. sp.		16.7	15.7				14.2		14.9		
	2. *G. triplorienchili* n. sp.	5.3		14.4				14.7		15.9		
	3. *G. yellochili* n. sp.	4.6	2.9					15.8		16.5		
Europe	4. *G. pseudonemachili* *	8.7	9.4	9.2								
	5. *G. jiroveci*	7.2	7.1	7.0	8.2							
	6. *G. papernai*	6.5	7.0	6.8	6.5	7.8						
Mongolia	7. *G. mongolicus*	7.3	8.6	6.7	11.0	9.0	8.1			10.3		
	8. *G. nemachili*	6.6	7.5	6.5	9.8	7.8	6.6	2.8				
	9. *G. pseudonemacheili*	7.1	7.9	6.2	10.4	8.6	7.6	1.1	2.3			
	10. *G. tayshirensis*	6.2	6.9	6.7	9.3	7.0	6.5	7.6	7.0	7.7		
	11. *G. zavkhanensis*	7.1	8.5	7.2	10.6	8.1	7.7	3.5	2.9	2.9	7.5	
China-XJ	12. *G. gymnodiptychi*	13.9	14.4	14.0	18.2	14.6	15.8	15.8	15.8	16.1	15.4	15.9

Figure 6. Molecular analysis of the nemachili group species, i.e., phylogenetic position inferred using the ITS rDNA sequences (A); and genetic distance inferred using the ITS rDNA sequences (lower left) and *cox*1 (upper right) (B). Node numbers represent the bootstrap values. China-QTP and China-XJ represent the location Qinghai-Tibet Plateau and Xinjiang Region in China, respectively. *Cox1* data for *Gyrodactylus mongolicus* (OQ661870) and *G. pseudonemachili* (OQ661866) sourced from Lebedeva et al. (2023).

explained 87% of the data variability, to which the second component contributed 1.4%. The first component was associated with the total length of the hamulus root, marginal hook sickle, and marginal hook shaft length and marginal hook aperture. Symbols in the principal component analysis diagram indicated that the three new species could be well distinguished (Fig. 5).

Molecular analysis

The three new species formed a monophyletic group (99 of bootstrap) with *G. papernai*, *G. jiroveci* and *G. pseudonemachili** (the asterisk represents that the name was previous attached to an ITS rDNA barcode AJ567674 but proved to be different from the type species *G. pseudonemachili*) from European *Barbatula barbatula*; *G. pseudonemachili*, *G. nemachili*, *G. tayshirensis*, *G. mongolicus* and *G. zavkhanensis* from Mongolian *Oreoleuciscus* spp. and *Barbatula* spp.; *G. gymnodiptychi* from Xinjiang region *Gymnodiptychus dybowskii*, all belonging to the nemachili group. *G. triplorienchili* n. sp. formed a sister clade (100 of bootstrap) with *G. yellochili* n. sp. and exhibited a relatively far phylogenetic relationship with *G. triplsellachili* n. sp. (Fig. 6A). The genetic distances among *Gyrodactylus triplorienchili* n. sp., *G. yellochili* n. sp. and *G. triplsellachili* n. sp. varied from 2.9 to 5.3% for ITS rDNA and 14.4 to 16.7% for *cox1* (Fig. 6B).

Discussion

Three new *Gyrodactylus* species, including *G. triplorienchili* n. sp., *G. yellochili* n. sp., and *G. triplsellachili* n. sp. have been described on *Triplophysa* fish in the Qinghai-Tibet Plateau, China. These new species possessed the notable feature of "hamulus root inturning", a typical characteristic in nemachili group species (Ergens and Bykhovsky 1967; Přikrylová *et al.* 2008). Although *G. yellochili* n. sp. and *G. triplsellachili* n. sp. were collected from the same location (Yellow River), nevertheless, the latter had smaller opisthaptoral hard parts. Interestingly, *G. yellochili* n. sp. morphologically resembled *G. triplorienchili* n. sp., which was collected at a considerably higher altitude: i.e., both had long straight hamulus points, thin marginal hook sickle, and long triangular sickle toe. Multivariate analysis based on hamulus and marginal hooks suggested that the three new species could be effectively separated. The hamulus root and marginal hook sickle in *G. triplorienchili* n. sp. were longer than that of *G. yellochili* n. sp. Previous studies on specific *Gyrodactylus* species (Geets *et al.* 1999; Mo 1991; 1993) had

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documented a tendency for longer hamulus roots in colder water, whereas the marginal hook sickle was stable. For the two similar species described, the former feature variation might reflect difference in geographical and climatic conditions of their hosts. The varied features of the latter indicated that they belong to two distinct species. Morphological similarities among the three new species were further confirmed by molecular data, with an almost identical 18S sequence (99.8–99.9% similarity) among them. Genetic distance (p-distance for ITS rDNA) between *G. triplorienchili* n. sp. and *G. yellochili* n. sp. (2.9%) is closer than that between *G. triplorienchili* n. sp. and *G. triplsellachili* n. sp. (5.3%) but exceeding the 1% ITS rDNA sequence difference, which was suggested as a threshold for *Gyrodactylus* species delimitation (Ziętara and Lumme 2002).

The three new species showed relatively low interspecific differences of 2.9–5.3% (p-distance for ITS rDNA), which is below the difference among other nemachili group members found in Europe on *Barbatula barbatula* L. 6.5–8.2%, or higher than the difference between *G. pseudonemachili* and *G. nemachili* found in Mongolia on *Barbatula* sp. 2.3% (Fig 6B). In other *Gyrodactylus* species, sequence variation (Kimura 2P distances) within the parasite genus ranged from 2.7 to 56% and 1.5 to 38.7% for ITS1 and ITS2, respectively (Matejusová *et al.* 2001), and species differences (HKY distances for ITS1-ITS2) of the *Gyrodactylus* within host genus *Pomatoschistus* varied from 2.5 to 16.5% (Huyse and Volckaert 2002). The low interspecific genetic differences reflect the tight phylogenetic relationship among nemachili group species, and their host stone loaches (including *Triplophysa* and *Barbatula* species) show a similar pattern, which include numerous morphologically and molecular-related species, with of the majority of them being concentrated in a plateau in Central Asia and adjacent regions (Li *et al.* 2017; Tang *et al.* 2006; Wang *et al.* 2016, 2020).

Compared to other species groups, such as wageneri group and rugiensis group (Huyse and Volckaert 2002; Malmberg 1970) defined mainly based on the shape of the marginal hook sickle, nemachili group species showed higher shape consistency in hamulus, but less shape consistency in marginal hooks (Fig. 4). Sickle distal was smoothly curved in *G. triplorienchili* n. sp., *G. yellochili* n. sp., *G. triplsellachili* n. sp., *G. nemachili*, *G. mongolicus*, *G. pseudonemachili*, *G. paranemachili* and *G. kataragensis*. This sickles pattern can also be found in *G. tokobaevi* Ergens & Karabekova, 1980, *G. aksuensis* Ergens & Karabekova, 1980, *G. llewellyni* Ergens & Dulmaa, 1967, *G. osoblahensis* Ergens, 1963 and *G. gracilihamatus* Malmberg, 1964 parasitic on Cyprinidae fish (Pugachev *et al.* 2009). Contrarily, the sickle point was sharply curved in *G. jiroveci*, *G. zavkhanensis*, *G. papernai* and *G. pavlovskyi*. Similarly, sickle pattern can also be found in the following eight species, parasitic on Cobitidae, Nemacheilidae, Cyprinidae, and Salmonidae: *G. ajime* Nitta, 2021, *G. asiaticus* Ergens, 1978, *G. cobitis* Bychowsky, 1933, *G. dzhalilovi* Ergens & Ashurova, 1984, *G. mantschuricus* Ergens & Yukhimenko, 1977, *G. pewzowi* Ergens, 1980 and *G. tincae* Malmberg, 1957 (Nitta 2021). Also, species within the nemachili group did not cluster based on their sickle shape; rather, they were scattered across the phylogenetic tree. Those molecularly closely related members such as *G. triplorienchili* n. sp. and *G. yellochili* n. sp., or *G. nemachili*, *G. tayshirensis*, *G. mongolicus* and *G. zavkhanensis* (fig. 6B) show greater morphology differences compared to the cryptic species such as *G. pakan* and *G. teken* (4.1–4.3% p-distance for ITS1-ITS2 rDNA) (Razo-Mendivil *et al.* 2016), and *G. sphinx* and *G. gerasevi* (1.3% p-distance for ITS rDNA) (Dmitrieva *et al.* 2022).

Most species within the nemachili group were found on stone loach (Přikrylová *et al.* 2008). Although some members, such as *G. mongolicus* and *G. pseudonemachili* were collected from *Oreoleuciscus humilis* Warpachowski, 1889 (Cyprinidae), *G. zavkhanensis* was collected from *Thymallus brevirostris* Kessler, 1879 (Salmonidae) in Mongolia (Lebedeva *et al.* 2023). *G. gymnodiptychi* was found on *Gymnodiptychus dybowskii* Kessler, 1874 (Cyprinidae) in Xinjiang, China (Zhang *et al.* 2023). Considering that there are geographical distribution overlaps between the aforementioned host species and stone loach, the appearance of nemachili group species on non-stone loach host is likely due to host switching (Ziętara and Lumme 2002). Genetically, *G. gymnodiptychi* is relatively far from other members (see Fig 6B), suggesting that host switching is an important trigger for diversification in *Gyrodactylus* (Boeger *et al.* 2003). In addition, the "inturning hamulus root" feature can also be found in many *Gyrodactylus* under saline conditions, such as *G. orecchiae* Paladini, Cable, Fioravanti, Faria, Di Cave & Shinn, 2009 on *Sparus aurata* Linnaeus, 1758 (Sparidae) (Paladini *et al.* 2009), *G. chileani* Ziętara, Lebedeva, Muñoz & Lumme, 2012 on *Helcogrammoides chilensis* Cancino, 1960 (Tripterygiidae) (Ziętara *et al.* 2012), and *G. amphibious* Lebedeva, Muñoz & Lumme, 2021, *G. scartichthi* Lebedeva, Muñoz & Lumme, 2021, *G. viridae* Lebedeva, Muñoz & Lumme, 2021 and *G. zietarae* Lebedeva, Muñoz & Lumme, 2021 on clingfsh *Sicyases* spp. (Gobiesocidae) (Lebedeva *et al.* 2021). They are not closely related to nemachili group species; therefore, it is possible that hamulus root inturning is a result of morphology convergence under extreme conditions.

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Ethical approval. The authors assert that the animal studies were conducted in accordance with the ethical standards and approved by the Animal Ethics Committee of the Institute of Hydrobiology, Chinese Academy of Sciences.

Declarations

Consent for participation. All authors have approved the final version of the manuscript and consent to participate in publishing this manuscript in this journal.

Consent for publication. The authors of this manuscript unequivocally state that this manuscript is original research that has not been published previously and is not under consideration for publication elsewhere, in whole or in part, and that all the authors consent to publishing this manuscript.

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