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Museum Fish Specimens and DNA Barcoding Reveal the Invasion History of the Zoonotic Yellow Grub Parasite (*Clinostomum sinensis*) in Taiwan's Rivers

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Clinostomum species are typical trematodes (or flatworms) and zoonotic parasites of humans, fish, and birds. These parasites require at least two definitive hosts, fish and birds, to complete their life cycle. Previous studies indicated that the yellow grub, identified as *C. complanatum*, first appeared in northern Taiwan around the 1990s, with uncertain origins. This study identified 65 of 2,181 museum fish specimens with leech-like metacercariae across four main river systems (Tamshui, Houlong, Tzengwen, and Xiuguluan Rivers) and documented new infection records in fishes from Beigang, Puzih, Kaoping, and Bie Rivers during subsequent field work. The parasite appears to have established in the Houlong and Tamshui Rivers before dispersing to southern and eastern

waterways. *COI* barcode analysis revealed that most metacercariae belong to *C. sinensis* with low nucleotide diversity (π = 0.00314353). The closely related haplotypes with insignificant Tajima's *D* (-1.89473 with *p*-value = 0.981839) suggest a gentle population expansion after their colonization to Taiwan. Additionally, yellow grub infections were more prevalent in carnivorous fishes (>60%) compared to omnivorous and algal-feeding fishes. The high infection rates documented in literature and museum specimens suggest that Jhonggang and Houlong rivers represent the primary (or earlier) infection areas from which the parasite subsequently spread throughout Taiwan, highlighting the need for enhanced regulations to protect endangered or cultivated species.

Keywords: Parasite, Yellow Grub, Metacercariae, Museum, *Clinostomum*, Infection

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BACKGROUND

Clinostomum species are parasitic flukes (class Trematoda), typical trematodes and zoonotic pa rasites that infect newt (Caffara et al. 2014), fish (Aghlmandi et al. 2018; Aohagi et al. 1993; Aohagi et al. 1992; de Lima et al. 2014; Dias et al. 2006; Locke et al. 20 19;Monnens et al. 2023; Shareef and Abidi 2012; Simsek et al. 2018; Sohn et al. 2019; Wang et al. 2017), bird (Monnens et al. 2023; Sereno-Uribe et al. 2013; Shamsi et al. 2013) and humans by ingestion of raw freshwater fish (Chung et al. 1995; Hara et al. 2014; Kim et al. 2023; Park et al. 2009vSong et al. 2018; Tiewchaloern et al. 1999). The life cycle of *Clinostomum* species has been well documented (Sutili et al. 2014): eggs are released from definitive hosts (such as egret) into freshwater, where molluscs (first intermediate hosts, such as *Radix swinhoei*) ingest them. The cercariae subsequently emerge from the molluscs and infect fish (second intermediate hosts) (Fig. 1). A recent study further

suggests that the distribution of *Clinostomum* species may be linked to migratory bird routes (Monnens et al. 2023).

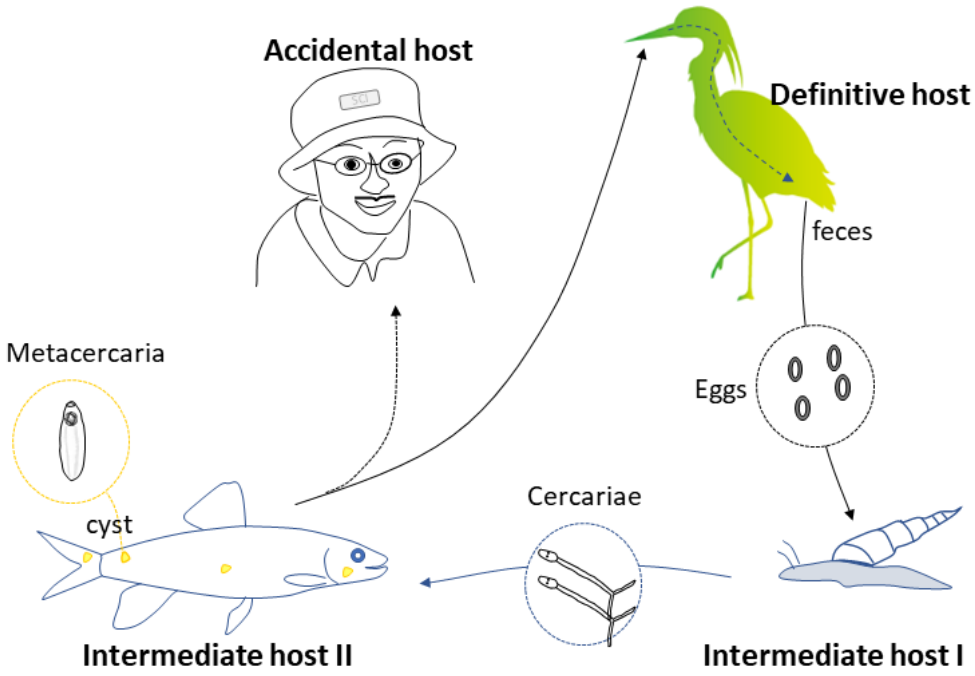

Fig. 1. Life cycle of *Clinostomum* species (adapted from Sutili et al., 2014). The adult parasite releases eggs with the definitive host's feces through the digestive tract into freshwater environments. A mollusc (first intermediate host, such as *Radix swinhoei*) ingests these eggs, allowing for larval development. Cercariae subsequently emerge from the mollusc and infect fish (second intermediate hosts), where they develop into metacercariae and form cysts in various tissues. When piscivorous birds consume infected fish, the parasites are released in the bird's digestive tract, mature into adults, and complete the cycle. Humans may become accidental hosts through consumption of raw or undercooked infected fish, resulting in halzoun syndrome.

The leech-like metacercariae, known as yellow grubs, represent the larval stage of *Clinostomum* species. These parasites infect fish and form cysts in their body cavity, visceral organs, and muscles (Hollis and Coker 1948; Li et al. 2018), with heavy infections potentially causing fish mortality (Lo et al. 1985; Shareef and Abidi 2012; Szalai and Dick 1988).

These metacercariae have been documented in many fish taxa serving as intermediate hosts, including cyprinids (Aohagi et al. 1993; Li et al. 2018; Sohn et al. 2019; Wang et al. 2017), cobitids

(Aghlmandi et al. 2018,Fedorcak et al. 2019), snakehead fish (Shareef and Abidi 2012), medaka (Gholami et al. 2011; Ngamniyom et al. 2012) and trout (Szalai and Dick 1988). The metacercariae may remain with their fish hosts from juvenile to adult stages for many years.

Several historical records document yellow grub infections in Taiwanese fishes. The first official record comes from cultured pond loach and ayu at the Chupei branch station of the Freshwater Aquaculture Research Center in northern Taiwan during 1977–1980 (Lo et al. 1981). A subsequent record documents infections in the Dahan River, a tributary of the Tamshui River in northern Taiwan during 2011–2013 (Wang et al. 2017). Wang et al. (2017) systematically investigated 5 families and 23 species of freshwater fishes, finding that 8 cyprinid species were particularly susceptible to yellow grub infection. While one study identified the Asian yellow grub as *Clinostomum sinensis*, distinct from its sister species *Clinostomum complanatum* (Locke et al. 2015), another study identified it as *C. complanatum* (Won et al. 2020).

Despite occasional reports of infection events in social media and by fishermen, no systematic study has documented the subsequent infection patterns in Taiwan's rivers. Therefore, this study examines freshwater fish specimens from two museums (BRCAS and NTU) to document infected fish species, analyze the *COI* barcode of yellow grubs, and reconstruct the historical pattern of infection events.

MATERIALS AND METHODS

Specimen survey from museum records

A comprehensive examination was conducted on 2,181 specimens representing 19 families of freshwater and brackish water fishes collected from 51 rivers in Taiwan between 1916 and 2024 (Table 1 and Table S1). These specimens, housed in the Biodiversity Research Center, Academia Sinica (BRCAS) and National Taiwan University (NTU) museums, were systematically inspected

for the presence of yellow grubs through both visual examination and microscopic analysis.

Table 1. Museum fish specimens used in this study

Family	Specimens number	infection number
Anguillidae	1	
Loricariidae	1	
Salmonidae	1	
Kuhliidae	2	
Osmeridae	2	
Siluridae	3	
Poeciliidae	4	
Rhyacichthyidae	4	
Adrianichthyidae	11	5
Channidae	6	
Mugilidae	21	
Bagridae	36	1
Cichlidae	39	
Cobitidae	42	
Ambassidae	46	
Balitoridae	91	
Eleotridae	121	
Gobiidae	378	10
Cyprinidae	1372	49
Total	2181	65

Based on museum records and historical reports, we conducted additional field investigations to collect contemporary samples from several key river systems: Tamshui River (including its tributaries Dahan and Keelung Rivers), Beigang River, Puzih River, Kaoping River, and Xiuguluan River (Table 2). All isolated yellow grubs and infected fish specimens were preserved in 95-100% ethanol and stored at -20°C prior to DNA extraction. We calculated the infection rate as (number of infected individuals ÷ total number of individuals) × 100%.

Table 2. 65 infected fish specimens in museum records and field sampling in this study

Species	Localities/Rivers in Taiwan	Sampling date	Numbers of infected fish	Catalog numbers	GPS position	*COI* code	Notes
1. Adrianichthyidae:							
Oryzias sinensis	Shuanglienpi restoration center, Lanyang River	2024/6/12	5	ASIZP0082263	24.754080, 121.635828		this study
2. Bagridae:							
Tachysurus sp.	Yujing, Tzengwen River	2014/9/1	1	ASIZP0075190	23.117404, 120.458390		formalin sample
3. Cyprinidae:							
Acrossocheilus paradoxus	upper reach of Houlong River	1997/3/15	1	ASIZP0062072	24.458407, 120.875027		formalin sample
	Pinglin, Tamsui River	2001/11/9	1	ASIZP0062933	24.934156, 121.711069		formalin sample
	Jhuci, Puzi River	2022/12/25	1	ASIZP0081491	23.518180, 120.593478	PAPC1	this study
	Fuli, Bie River, Xiuguluan River	2023/7/14	1	ASIZP0081721	23.169728, 121.244701	ASIZP0081721a, ASIZP0081721b	this study
Candidia barbata	middle reach of Laotianliao River, Houlong River	1997/3/15	2	ASIZP0062076	24.582391, 120.877173		formalin sample
	Yanshuikeng, Houlong River	1997/5/20	1	ASIZP0062092	24.489633, 120.899309		formalin sample
	Xizhi, Jingmei River, Tamsui River	2005/5/19	1	ASIZP0080872	25.086548, 121.630359		formalin sample
	Jhuci, Puzi River	2023/7/1	1	CbPT18, 19	23.518306, 120.593583	CbPT18	this study
	Daxi, Nanzaigou stream	2023/9/17	1	CbNZG5,7,11,16	24.8303217, 121.3033791	CbNZG5,7,11,16	this study
Metzia formosae	restored population, Keelung, Tamsui River	2021/12/29	1	ASIZP0081111	25.107780, 121.732679		this study
	restored population, Keelung, Tamsui River	2021/12/29	1	ASIZP0081113	25.107780, 121.732679		this study
	restored population, Keelung, Tamsui River	2021/12/29	1	ASIZP0081114	25.107780, 121.732679		this study
	restored population, Keelung, Tamsui River	2021/12/29	1	ASIZP0081115	25.107780, 121.732679		this study
	restored population, Keelung, Tamsui River	2021/12/29	1	ASIZP0081122	25.107780, 121.732679		this study

	restored population, Keelung, Tamsui River	2021/12/29	1	ASIZP0081123	25.107780, 121.732679		this study
	restored population, Keelung, Tamsui River	2021/12/29	1	ASIZP0081124	25.107780, 121.732679	ASIZP0081124	this study
	restored population, Keelung, Tamsui River	2021/12/29	1	ASIZP0081125	25.107780, 121.732679	ASIZP0081125	this study
	restored population, Keelung, Tamsui River	2021/12/29	1	ASIZP0081126	25.107780, 121.732679		this study
	restored population, Keelung, Tamsui River	2021/12/29	1	ASIZP0081127	25.107780, 121.732679	ASIZP0081127	this study
	restored population, Keelung, Tamsui River	2021/12/29	1	ASIZP0081128	25.107780, 121.732679	ASIZP0081128	this study
	restored population, Keelung, Tamsui River	2021/12/29	1	ASIZP0081129	25.107780, 121.732679		this study
	restored population, Keelung, Tamsui River	2021/12/29	1	ASIZP0081130	25.107780, 121.732679		this study
Onychostoma alticorpus	Fuli, Bie River, Xiuguluan River	2022	1	SOPC1, SOPC2	23.136833, 121.280917	SOPC1, SOPC2	this study
	Fuli, Bie River, Xiuguluan River	2015/8/20	1	ASIZP0078309	23.172622, 121.252765		formalin sample
	Fuli, Bie River, Xiuguluan River	2015/8/20	1	ASIZP0078310	23.172622, 121.252765		formalin sample
	Fuli, Bie River, Xiuguluan River	2015/10/16	1	ASIZP0078973	23.172622, 121.252765		formalin sample
Opsariichthys evolans	Yingge, Tamsui River	1963/4/9	3	NTUM02098	24.945005, 121.352803		formalin sample
	middle reach of Laotianliao River, Houlong River	1997/3/15	1	ASIZP0062075	24.582391, 120.877173		formalin sample
	Ruifang, Tamsui River	2018/1/24	6	OeRF1~6	25.106256, 121.808995	OeRF1~6	this study
	Daxi, Nanzaigou stream	2023/9/17	1	OeNZG1	24.8303217, 121.3033791	OeNZG1	this study
Opsariichthys pachycephalus	upper reach of Houlong River	1997/3/15	1	ASIZP0062073	24.458407, 120.875027		formalin sample
	lower reach of Laotianliao River, Houlong River	1997/5/20	1	ASIZP0062068	24.580461, 120.846260		formalin sample
	middle reach of Laotianliao River, Houlong River	1997/5/20	2	ASIZP0062066	24.582391, 120.877173		formalin sample
	Jhuci, Puzi River	2023/7/1	1	OpPT31	23.518306, 120.593583	OpPT31	this study

	Fuli, Bie River, Xiuguluan River	2023/7/14	1	ASIZP0081718	23.169728, 121.244701	ASIZP0081718a, ASIZP0081718b	this study
	Fuli, Bie River, Xiuguluan River	2023/7/14	1	ASIZP0081719	23.169728, 121.244701	ASIZP0081719a, ASIZP0081719b	this study
	Fuli, Bie River, Xiuguluan River	2023/7/14	1	ASIZP0081720	23.169728, 121.244701	ASIZP0081720a, ASIZP0081720b	this study
	Daxi, Nanzaigou stream	2023/9/17	1	OpNZG3	24.8303217, 121.3033791	OpNZG3	this study
Rhodeus ocellatus	Shuanglienpi, Lanyang River	2024/3/20	1	ASIZP0081884	24.750607, 121.634699		this study
Scaphesthes barbatula	Pingxi, Tamsui River	1998/6/10	1	ASIZP0060306	25.043634, 121.780406		formalin sample
4. Gobiidae:							
Rhinogobius candidianus	Jhuci, Puzi River	2022/12/25	1	ASIZP0081496	23.518180, 120.593478	PRCC1	this study
	Jhuci, Puzi River	2022/12/25	1	ASIZP0081497	23.518180, 120.593478	PRCC2	this study
	Jhuci, Puzi River	2022/12/25	1	ASIZP0081498	23.518180, 120.593478	ASIZP0081498a, ASIZP0081498b	this study
Rhinogobius rubromaculatus	Huben, Beigang River	2022	1	BRRC1, BRRC2	23.726499, 120.623439	BRRC1, BRRC2	this study
	Kaoping River	2022/7/14	1	GRRC1, GRRC2	22.755556, 120.449111	GRRC1, GRRC2	this study
	Huben, Beigang River	2023/10/7	2	ASIZP0082226	23.726499, 120.623439		this study
	Huben, Beigang River	2019/11/11-2020/1/30	3	Rru-cenB012,023,034	23.726499, 120.623439		this study

Morphological observation

Parasitic worms were dissected and immediately placed in near-boiling vertebrate saline (0.85% NaCl), then rapidly transferred to fresh, room-temperature saline and preserved in 10% formalin (Cribb and Bray 2010). Specimens were stained with acetocarmine solution (cat. A0050, Tokyo Chemical Industry Co., Ltd.) for 5 min and subsequently destained with double-distilled water (Chung et al. 1995). The stained specimens were examined and imaged under a light microscope (Carl Zeiss stereo Discovery V8).

Mitochondrial *COI* amplicon sequencing

At least three yellow grubs from each river system were randomly selected for genomic DNA extraction and *COI* amplicon sequencing. Genomic DNA was extracted using the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Specific primers targeting the parasite *COI* barcode region (approximately 700-bp) were CC_CO1/F (5'-GCCGGGATAGGTTGAACYTT-3') and CC_CO1/R (5'-TGAAAATGRGCAATCACAAA-3'), modified from (Won et al. 2020). Based on the mitogenomes of *Clinostomum* species (Chen et al. 2016; Locke et al. 2019; Monnens et al. 2023), we designed an additional primer pair (CC_CO1-F0: 5'-ATGAGTTGGTTGTTATCAT-3'; CC_CO1-R0: 5'-CACTAATATAAGTTCTATGATG-3') to amplify a longer 1.5-kb fragment for comparative analysis.

The *COI* amplicons were amplified in 50-μL reaction mixtures containing 200 μM dNTP, 0.3 μM primers, with 50 ng of genomic DNA, one unit of Taq™ DNA Polymerase with the manufacturer-supplied buffer. PCR amplification consisted of 30-35 cycles under the following conditions: denaturation at 95°C for 30 s, annealing at 50–55°C for 40 s, extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. The resulting *COI* amplicons were sequenced by Genomics Ltd (Taipei, Taiwan). All haplotype sequences were deposited in GenBank

under accession numbers PV540711–PV540747.

Mitochondrial *COI* phylogeny

COI sequences of *Clinostomum* species (Table S2) were obtained from NCBI GenBank based on previous phylogenetic studies (Chen et al. 2016; Locke et al. 2019; Locke et al. 2015; Monnens et al. 2023; Won et al. 2020) and combined with newly sequenced *COI* amplicons generated in this study. Phylogenetic analysis was conducted using MEGA X with default parameters (Kumar et al. 2018). Haplotype networks were constructed to visualize genetic relationships among samples: haplotype data were first processed using DnaSP ver. 6.12.03 (Rozas et al. 2017), and the minimum spanning network was subsequently generated and visualized using PopART ver. 1.7 (Leigh and Bryant 2015). In addition, since previous studies amplified different *COI* regions, we analyzed 215- and 694-bp fragments to achieve finer-scale resolution in the haplotype network analysis.

RESULTS

Morphological observation

Parasitic worms were dissected and isolated from a freshly collected *Rhinogobius rubromaculatus* specimen and subsequently stained and imaged under a light microscope (Fig. 2).

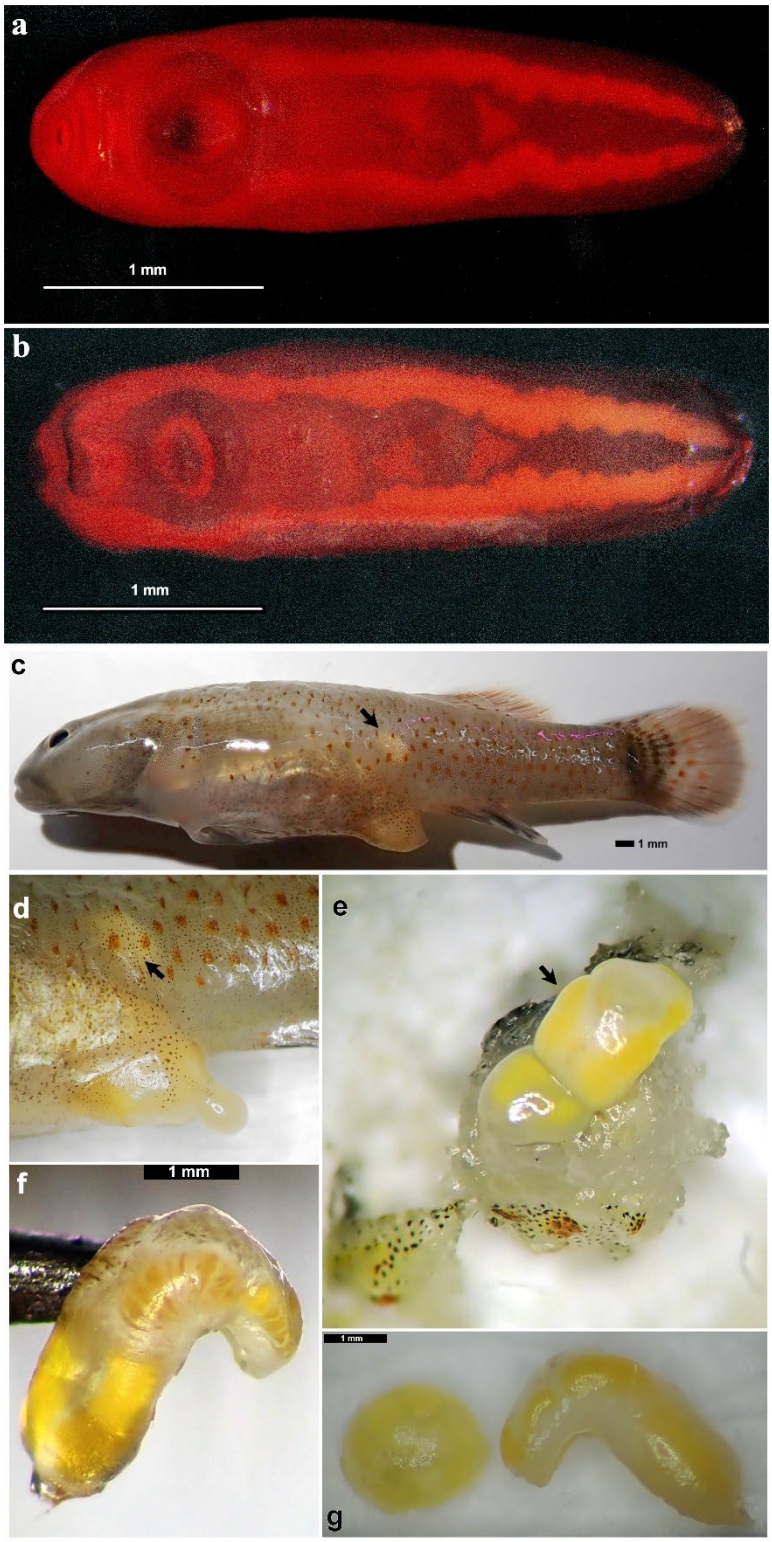

Fig. 2. Ventral (a) and dorsal (b) views of a freshly fixed specimen stained with acetocarmine, isolated from its host, the goby *Rhinogobius rubromaculatus* (c–e); (f) a freshly isolated yellow grub; (g) left, goby egg; right, yellow grub. Scale bar = 1 mm. Arrows indicate the position of the specimen.

Infection status of fish species

A total of 2,181 fish specimens representing 104 species from 19 families were examined for *Clinostomum* species infection across 51 rivers in Taiwan. The surveyed fish taxa included Cyprinidae, Bagridae, Balitoridae, Gobiidae, Cichlidae, and others (Table 1 and Table S1). The number of parasites per infected fish specimen was documented to reconstruct historical infection patterns (Table 2). Sixty-five infected fish specimens with metacercariae were identified, including eight species of Cyprinidae, one species of Bagridae, one species of Adrianichthyidae, and two species of Gobiidae (Table 2; Fig. 3). Six host species documented in this survey represent new infection records: *Oryzias sinensis, Onychostoma alticorps*, *Opsariichthys evalans*, *Metzia formosae*, *Rhinogobius rubromaculatus* and *Tachysurus* sp. Notably, nine infected specimens—three *Candidia barbata*, one *Acrossocheilus paradoxus*, and five *Opsariichthys pachycephalus*—were collected from the same river, the Houlong River, in 1997. Additionally, a high infection rate (20.7%) was observed in cultivated *Metzia formosae*, with 12 of 58 specimens infected during a 2021 investigation. These findings suggest that the parasite exhibits relatively low host preference.

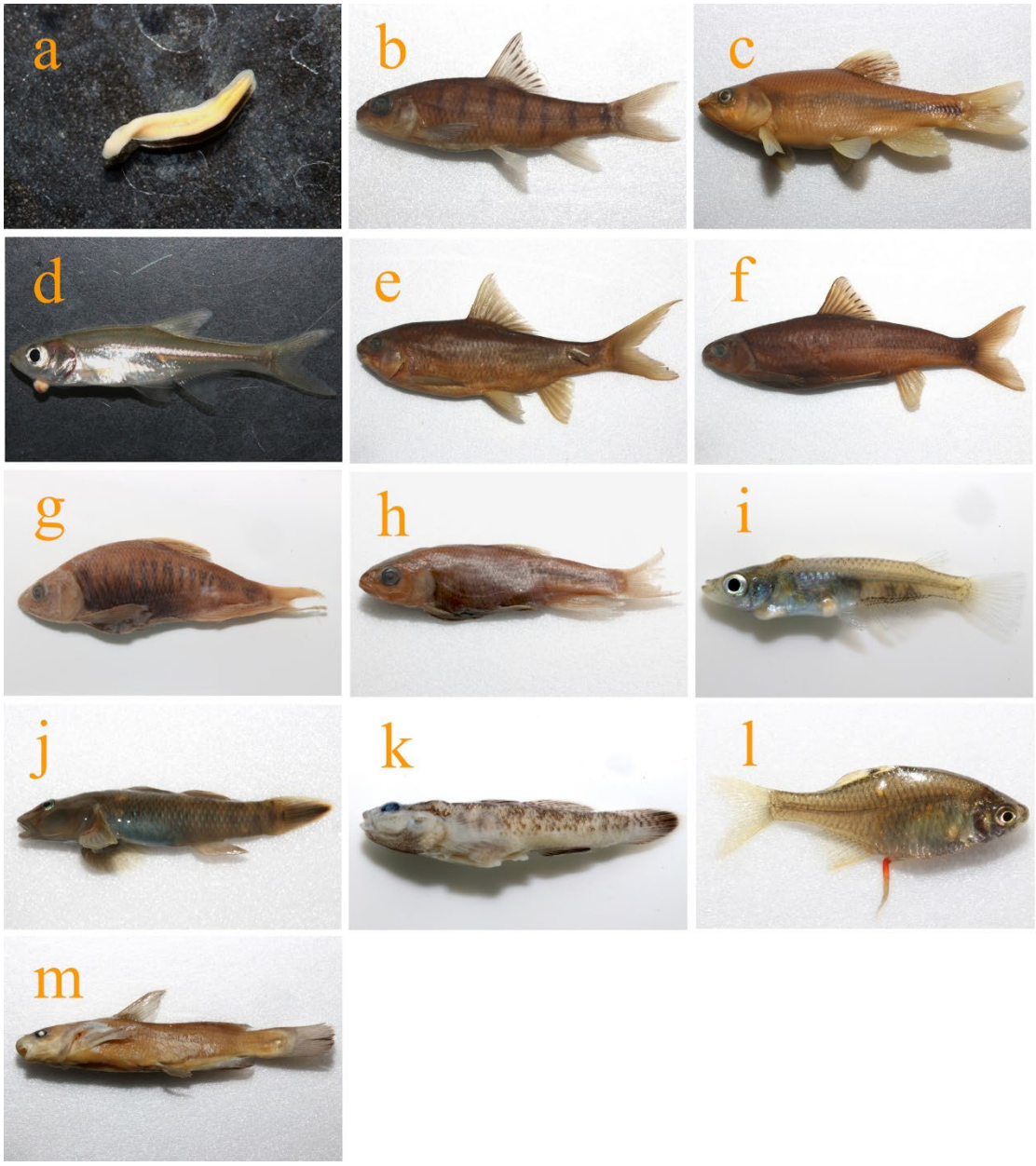

Fig. 3. *Clinostomum sinensis* metacercariae discovered in 12 species of freshwater fish from museum collections. Representative specimen photographs are shown as follows: (a) isolated metacercaria of *C. sinensis*, ASIZFZ002436, collected from ASIZP0081122; (b) *Acrossocheilus paradoxus*, ASIZP0062933; (c) *Candidia barbata*, ASIZP0080872; (d) *Metzia formosae*, ASIZP0081122; (e) *Onychostoma alticorpus*, ASIZP0078310; (f) *Scaphesthes barbatula*, ASIZP0060306; (g) *Opsariichthys evolans*, ASIZP0062075; (h) *Opsariichthys pachycephalus*, ASIZP0062073; (i) *Oryzias sinensis*, ASIZP0082263; (j) *Rhinogobius candidianus*, ASIZP0081497; (k) *Rhinogobius rubromaculatus*, ASIZP0082226; (l) *Rhodeus ocellatus*, ASIZP0081884; (m) *Tachysurus* sp., ASIZP0075190.

Infection location in the fish body

Analysis of parasite distribution within fish hosts revealed two primary infection sites: the

mandible muscle near the mouth (23.3%) and caudal fin base (24.0%) (Fig. 4). This distribution pattern implies that parasite cercariae in the water may enter through the fish's mouth and subsequently settle down to specific tissues where they develop into metacercariae. Additionally, our examination of chub eggs and larvae indicated that infection can occur during early developmental stages, with parasites establishing as metacercariae beneath the body skin or within the caudal peduncle musculature, where they may persist for several years throughout the host's life cycle. Additionally, Wang et al. (2017) reported that metacercariae are primarily located in the head region of fish, including the operculum, mandible, cranial muscles, and oral cavity (Fig. 4b).

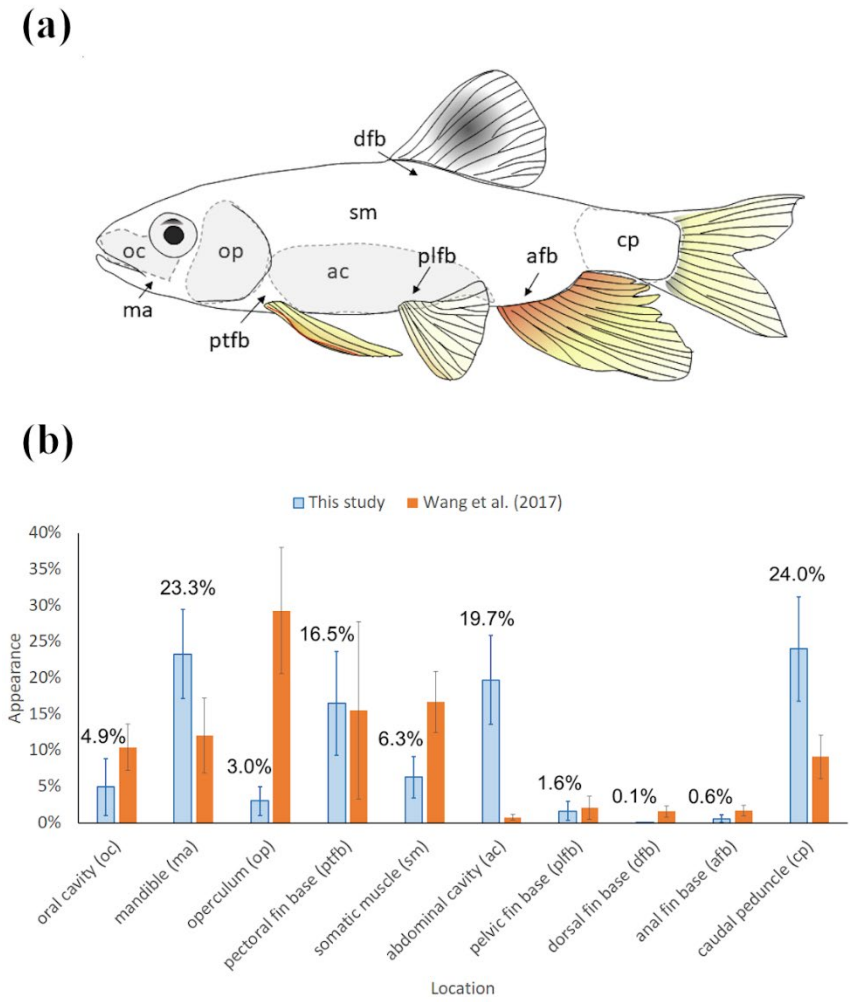

Fig. 4. Infection area of fish body surface. (a) definition area, adopt from Wang *et al.* (1997): oral cavity (oc), mandible muscle (ma), operculum (op), pectoral fin base (ptfb), somatic muscle (sm), abdominal cavity (ac), pelvic fin base (plfb), dorsal fin base (dfb), anal fin base (afb), caudal

peduncle (cp); (b) mean appearance in this study. The metacercariae were primarily found in front of fish head, such as oc, ma, op, and ptfb.

Historical infected event in museum records and literature

According to the infected specimens documented in Table 2, this study identified the earliest infection record in *Opsariichthys evolans* (NTUM02098) from Dahan River in 1963. A second wave of infections appears to have occurred in Houlong River around 1997 (Table 3). The third wave of infections was detected in southern and eastern Taiwan, specifically in the upstream region of southern Tzengwen River in 2014 and the upstream area of eastern Xiuguluan River in 2015. These findings indicate that yellow grub parasites have been widely distributed from northern to southern Taiwan since at least the 1960s (Fig. 5a).

Table 3. Comparison of infected cyprinids between Houlong River (this study) and Dahan River (Wang et al. 2017)

	Houlong River (1997)		Dahan River (2011-2013)	
Species	No. of examined fish (infected fish)	*P* [b] (%)	No. of examined fish (infected fish)	*P* [b] (%)
Acrossocheilus paradoxus	58 (1)	1.7%	154 (49)	31.8%
Candidia barbata	71 (3)	4.2%	344 (31)	9.0%
Opsariichthys evolans [a]	23 (2)	8.7%	205 (33)	16.1%
Opsariichthys pachycephalus	14 (3)	21.4%	107 (22)	20.6%
Scaphesthes barbatula	3 (0)	0.0%	239 (1)	0.4%
Hemibarbus labeo	-	-	25 (3)	12.0%
Carassius auratus	-	-	47 (1)	2.1%
Cyprinus carpio	-	-	9 (1)	11.1%

[a] Previous named as *Zacco platypus* in Wang et al. 2017. [b] Prevalence (infection rate).

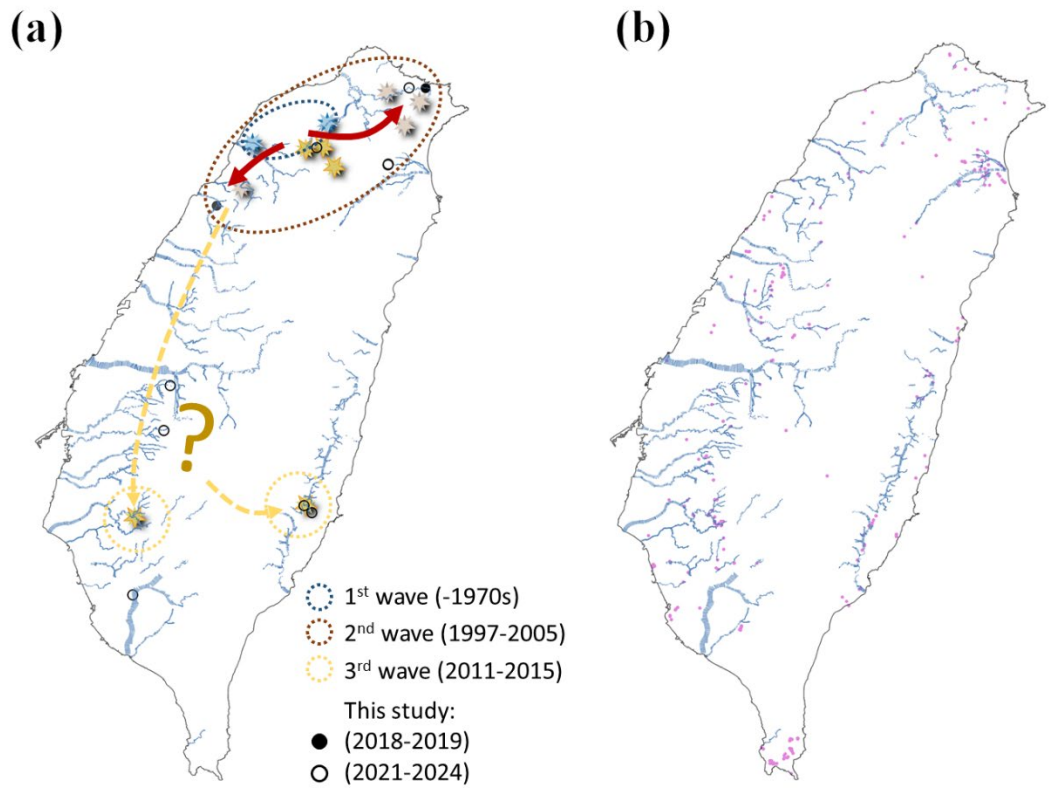

Fig. 5. Infection history base on museum records (a) and the distribution of freshwater snails (family Lymnaeidae; pink dots), the primary intermediate hosts (b). The distribution was referred from Taiwan Biodiversity Network (30 July 2025) TBN Occurrence Download: https://www.tbn.org.tw/dlpage/b8a1f889-f158-443b-b2e3-5afee3480215.

Molecular phylogeny of *COI* haplotypes

Based on the historical records identified above, 37 yellow grub individuals from 9 sampling sites across 6 main rivers were selected for *COI* barcode sequencing and 17 haplotypes were identified (Table 2 and Table S2). Analysis of these *COI* sequences revealed 6 single nucleotide polymorphism (SNP) sites that were used for phylogenetic classification (Fig. 6). The results showed 39-44 SNP differences between Asian *C. sinensis* and two European *C. complanatum*. The *COI* phylogeny (Fig. 6a) indicated that all metacercariae examined in this study belong to a single species, *C. sinensis*. The minimum spanning network analysis revealed a common haplotype shared among Korean, Chinese and at least 25% Taiwanese individuals (Fig. 6b, 6c and Table S2). Table 4 indicates low nucleotide diversity ($\pi = 0.00314353$), with most metacercariae sharing closely

related haplotypes (Fig. 6b, 6c). Using the longer *COI* fragment, Tajima's *D* was negative (-1.89473) with a non-significant *p*-value (0.981839), suggesting a recent population expansion or purifying selection. The magnitude of *D* does not reach the commonly cited threshold of −2 and the test is not statistically significant. Therefore, rather than indicating a population bottleneck or shrinkage, these results may more cautiously be interpreted as reflecting a gentle population expansion following the colonization in Taiwan.

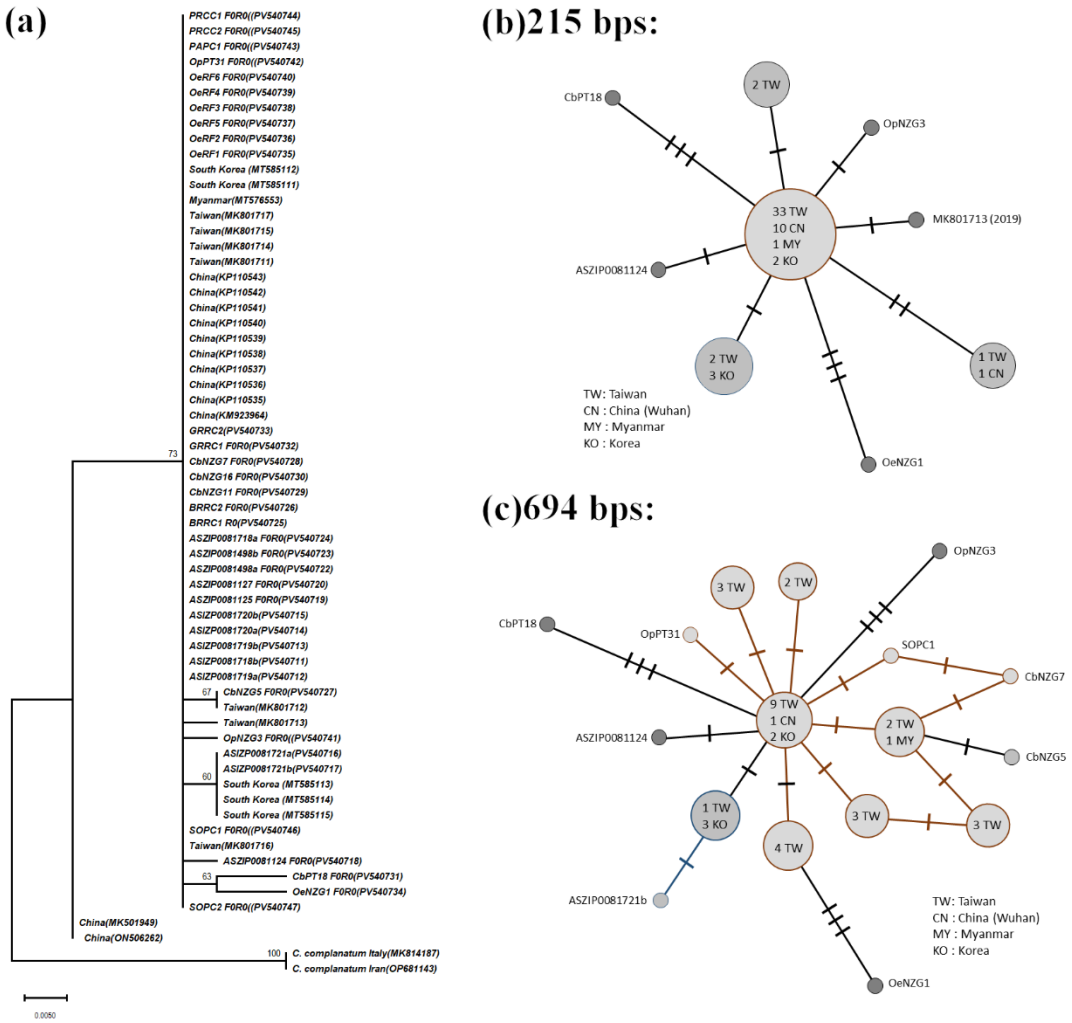

Fig. 6. *COI* phylogeny and haplotype network. (a) The ML tree was constructed with 61 Asian *C. sinensis* and two European *C. complanatum* sequences using HKY model with 100 bootstrapping replicates in MEGA X. Minimum spanning networks were constructed with (b) 58 and (c) 41 *C. sinensis COI* sequences, respectively.

Table 4. Nucleotide diversity and Tajima's *D* statistic from *COI* amplicons of Asian *C. sinensis* and two European *C. complanatum*

Amplicon primer pairs	CC_CO1/F x CC_CO1/R	CC_CO1-F0 x CC_CO1-R0
Individuals (n)	58	41
amplicon length (bp)	680	1500
aligned sequence length (bp)	212	680
Nucleotide diversity (π)	0.00641911	0.00953733
Number of segregating sites	17	55
Number of parsimony-informative sites	11	43
Tajima's D statistic	-1.97526 (*p*-value = 0.98708)	-1.89473 (*p*-value = 0.981839)

DISCUSSION

Relation between feeding habits and infection rate in fish

Based on museum records, 9 of 30 infected specimens originated from Houlong River, representing a relatively higher infection rate compared to other river systems. To better understand infection patterns, we compared infection rates between Houlong River (this study) and Dahan River (Wang et al. 2017). We found that carnivorous and insectivorous cyprinids (Shao 2025)—*Acrossocheilus paradoxus*, *Opsariichthys pachycephalus*, *Candidia barbata*, and *Zacco platypus*—were more susceptible to yellow grub infection than herbivorous and omnivorous fishes (Table 3). Furthermore, the anatomical distribution of parasites revealed that yellow grubs were commonly found in the mouth and inner surface of the gill cover (Fig. 4). This distribution pattern likely results from cercariae settling in these regions as water flows through the fish's mouth and gills. In addition, among all examined fish specimens, our results revealed that only freshwater-inhabiting fish species (a total of 12 species, see Fig. 3) were infected, all belonging to primary freshwater fish species (such as *Metzia formosae* and *Opsariichthys evolans*) or land-locked gobies (such as *Rhinogobius candidianus* and *Rhinogobius rubromaculatus*). In contrast, no brackish water fish

species (such as *Cryptocentrus yatsui*) were infected in this study.

Infections and the conservation implications of rare fish species

High infection rates have previously been documented in loach and ayu cultured in pond environments (Lo et al. 1981). Similarily, our study reveal substantial infection rates (prevalence) in artificially restored populations. In an artificial restored population of *Metzia formosae* maintained in an open pond in Keelung, 12 of 58 individuals (20.7%) were infected with yellow grubs. A comparable situation was observed in the Taiwan regional vulnerable freshwater fish *Oryzias sinensis* (listed as National vulnerable species, NVU in red lists of freshwater fishes of Taiwan, 2017), which was part of an artificially restored population cultured near the Shuanglienpi National Wildlife Refuge in Yilan.

These infections pose a significant threat to endangered species such as *Metzia formosae* (protected status: III) and *Oryzias sinensis*, potentially causing harm or mortality. Therefore, implementing robust monitoring protocols for parasite infections is crucial, particularly in small water systems like open breeding ponds, especially those serving as habitats for rare or protected fish species. Such monitoring would enable early detection and intervention to prevent parasite-induced population declines in vulnerable species.

Before stocking, the breeding pond must be thoroughly disinfected with pond-wide treatments (*e.g.*, quicklime or bleaching powder) to reduce resident pathogens (Lio-Po and Inui 2010; Sobsey et al. 2006). For example, quicklime (calcium oxide) can inactivate pathogens, intermediate hosts, pathogen-carrying animals and pests in the breeding pond, while also conditioning pond soils, increasing alkalinity, and promoting primary productivity, thereby improving water quality. Because farm tools often act as fomites for fish diseases, equipment should be dedicated to individual ponds and disinfected between uses. During culture, install physical barriers (e.g., netting or overhead lines) or bird-deterrent devices (*e.g.*, reflective tape) to prevent piscivorous birds from accessing ponds and defecating in the water. Trapping and removing snails is an additional measure to disrupt

parasite life cycles.

Implication of *COI* phylogeny

The *COI* phylogenetic analysis revealed that most sampling sites across Taiwan share several common and/or closely related haplotypes (Fig. 6). Moreover, two major phylogenetic groups included haplotypes from Myanmar, China, and Korea, suggesting regional connectivity. Table 4 demonstrates a low nucleotide diversity (π = 0. 00314353) in *COI* sequences, with most metacercariae exhibiting closely related haplotypes. The negative but statistically insignificant Tajima's *D* value (-1.89473 with *p*-value = 0.981839) suggests that the yellow grubs have experienced a gradual population expansion following colonization in Taiwan, though the signal is moderate.

Notably, the absence of geographically isolated haplotypes suggests that the parasites can readily spread throughout Taiwan. This genetic pattern provides valuable context for interpreting the historical invasion and spread of these parasites across Taiwan's river systems.

Historical infected events

Based on museum specimens and literature records, we have identified at least three waves of infection spread across Taiwan's rivers (Fig. 5a). The earliest documented infection was found in Dahan River (northern Taiwan) in 1963, which may be connected to the infection of cultured pond loach reported in 1977 (Table 2). The second wave appears to have spread southward to Houlong River (documented in this study) and Jhonggang River (Chen-Hsiung Yang; personal communication), as well as northward to other tributaries of the Tamshui River around 1997–2000s. Fish populations in these rivers subsequently experienced heavy infection rates during 2011–2013 (Wang et al. 2017), with yellow grub infections continuing to be reported by fishermen and in local news. The third wave of infections was detected in southern and eastern Taiwan during 2014–2015,

demonstrating the parasite's expansion throughout the island.

Yellow grub completes its life cycle in egrets, which serve as definitive hosts and acquire infection by consuming parasitized freshwater fish (Wang et al. 2017). The spatiotemporal distribution of infection records in Taiwan aligns with major egret migration routes, might suggest avian-mediated dispersal of the parasite. In addition, the freshwater snail, such as *R. swinhoei* (Family: Lymnaeidae), has been reported as an intermediate host with low infection prevalence, evidenced by cercarial shedding (Wang et al. 2017). Occurrence data from the Taiwan Biodiversity Network (n = 785) indicate that the freshwater snails (Lymnaeidae) could be found in documented infection areas (Fig. 5b). Together, these patterns underscore the need for targeted, longitudinal studies that integrate bird movement data with surveys of intermediate hosts to elucidate transmission dynamics and to forecast potential spread to additional watersheds.

CONCLUSIONS

This study definitively identifies the yellow grub parasites infecting Taiwanese freshwater fishes as *Clinostomum sinensis*, rather than *C. complanatum*. Molecular analysis of *COI* haplotypes reveals that Taiwanese parasite populations are closely related to those found elsewhere in Asia, suggesting regional connectivity. Through systematic examination of museum specimens spanning several decades, we have reconstructed the historical spread of this parasite, identifying at least three distinct waves of infection events across Taiwan's river systems since the 1960s. This historical perspective provides valuable insights into the invasion dynamics of this zoonotic parasite and establishes a foundation for monitoring its future spread and ecological impact.

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Supplementary materials

Table S1. The metadata of the examined 2181 individuals of fish specimens. (download)

Table S2. *COI* haplotypes and reference sequences obtained from NCBI. (download)