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Non-lethal detection of mtDNA from the monogenean *Gyrodactylus mutabilitas* which recorded for the first time from the gill mucus of *Cyprinus carpio*, Iraq

Detecção não letal de mtDNA do monogenético Gyrodactylus mutabilitas que registrou pela primeira vez no muco branquial de Cyprinus carpio, Iraque

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ABSTRACT

The monogenean species of the genus *Gyrodactylus* are harmful to the common carp *Cyprinus carpio*. In this study fish sampling were collected from al-Tarmiyahm fish farm, Baghdad Iraq. The occurrence of the monogenean *Gyrodactylus mutabilitas* is reported in the present study for the first time from gills of the common carp. Morphological characterization and measurements of this parasite are given. The distribution of this monogenean over the gill arches of the hosts showed a specific right-side preference, there were obvious preferences for the second gill arches was recorded by this monogenean. Usually, accurate diagnosis of the monogenean is relied on traditional methods by their morphology and it can only be achieved by fish post-mortem dissection. To support common carp conservation and management in the fish farm, a non-lethal method for pathogen detection is urgently needed. In this study, gill swabbing seems to provide a useful method for *Gyrodactylus mutabilitas* DNA samples. Fisheries managers may find this method be a useful tool to assess infection status which may prevent the spreading of any possible infection.

KEYWORDS: *Gyrodactylus mutabilitas;* common carp; non-lethal sampling; site preference; Iraq.

RESUMO

As espécies monogenéticas do gênero Gyrodactylus são nocivas à carpa comum Cyprinus carpio. Neste estudo, amostras de peixes foram coletadas na fazenda de peixes al-Tarmiyahm, em Bagdá, Iraque. A ocorrência do monogenético Gyrodacty-lus mutabilitas é relatada no presente estudo pela primeira vez em brânquias da carpa comum. São fornecidas caracterização morfológica e medidas deste parasita. A distribuição deste monogenético sobre os arcos branquiais dos hospedeiros mostrou uma preferência específica pelo lado direito, havia preferências óbvias para os segundos arcos branquiais registrados por este monogenético. Normalmente, o diagnóstico preciso do monogenético baseia-se em métodos tradicionais pela sua morfologia e só pode ser alcançado por dissecação post-mortem do peixe. Para apoiar a conservação e gestão da carpa comum na piscicultura, é urgentemente necessário um método não letal para a detecção de agentes patogénicos. Neste es-tudo, o esfregaço branquial parece fornecer um método útil para amostras de DNA de Gyrodactylus mutabilitas. Os gestores das pescas podem considerar este método uma ferramenta útil para avaliar o estado da infecção, o que pode impedir a propa-gação de qualquer possível infecção.

PALAVRAS-CHAVE: *Gyrodactylus mutabilitas;* carpa comum; amostragem não letal; preferência de site; Iraque.

INTRODUCTION

The common carp is an economically important teleost fish in the aquaculture industry with over 20 million metric tons of fish production worldwide approximately a total of 70% of global freshwater aquaculture production (XU et al. 2014). On the other hand, this considerable growth in aquaculture has led to some serious parasit-ic diseases, affecting the health and productivity of carp and other fish species (BONDAD-REANTASO & TAN 2005). Several studies have reported that common carp are commonly infected with the ectoparasitic monogenean *Gyrodactylus*. As a result of their viviparous reproduction these monogeneans have been described one of the most invasive fish parasites which attack especially the gills and fish skin by their 16

marginal hooklets and one pair of median hooks and feed on host dermal mucus, blood and cells debris (BAKER et al. 1998). Although, most species of *Gyrodactylus* consider to be almost less harmful to their hosts, heavy parasitic infection with *Gyrodactylus sp.* is leading to increase mucous secretion with severe injuries in the skin and gills lamellae especially in the case of juvenile fish which have been frequently shown high mortality rate (BUCHMANN 2012). *Gyrodactylus salaris*, for example has had recognized as a fatal lesion on a population of young Atlantic salmon that cause a huge economical loss in Norway (BAKKE et al. 2007).

The first *Gyrodactylus* species has been described in Iraq was G. elegans from gills of both *C. carpio* and *Planiliza abu* (which was reported as Liza abu) (ALI & SHAABAN 1984), since then, there are 58 gyrodactylid species to be reported from varied Iraqi fish.

The absence a notable clinical sign of disease on some of fish this may lead to easily misidentify *Gyrodactylus* infection particularly in the early stage of infection (PALADINI et al. 2014). This is precisely noticed by the study of (PALADINI et al. 2009) when an old sample of infected rainbow trout skin scrape dating back to 2000 that was preserved in formalin has been examined to indicate that *G. salaris* infection had been found in Italy at least nine years before its first discovery.

Currently, monogeneans detection is carried out by post-mortem gills examination, requiring lethal sampling of fish, therefore, morphological characterizations are fundamentally used to identify parasite genus or species. In the case of *Gyrodactylus*, the hard parts of the haptor, with the marginal hook and the reproductive organs have been frequently used for discrimination of very closely related species (MALMBERG 1970). Serological tests are usually used in parasite identifying, serum of the tilapia *Oreochromis niloticus* infected with the monogenean *Cichlidogyrus* spp was used to evaluate humoral immune response using enzyme-linked immuno-sorbent assay (ELISA) (SANDOVALGÍO et al. 2008). However, morphological and serological test are both lethal to the host, both require a high level of technical experience, furthermore presumptive parasitic diagnosis in a fish farm has an extra financial cost for treatment and management (EK-HUCHIM et al. 2012).

Non-lethal sampling is optimal for the species in danger of extinction or for fish that are frequently re-sampled. Using animals widely in research projects has shed light on the regulations and ethical implication (HENDERSON et al. 2016; FERREIRA et al. 2019). Recently, several studies have been evaluated non-lethal detection of DNA from varied fish pathogens by using different methods to examine fish from viral infection (COUTINHO et al. 2023); bacterial (TAVARES et al. 2016) and parasitic (KRKOŠEK et al. 2005; DA CUNHA et al. 2020; NORRIS et al. 2020; DUVAL et al. 2021; DE NOIA et al. 2022; HANSEN et al. 2024).

It is cleared that from previous studies there was no published work regarding *G. mutabilitas* in Iraq. So, this paper aims to investigate the gyrodactylid and successfully record this parasite for the first time in Iraq. The second aim is to investigate whether it is possible to detect *Gyrodactylus* infection in gill mucus of common carp and how non-invasive test could be used to examine low levels of parasitic infections.

MATERIALS AND METHODS

Fish sampling and examination for parasites

Between September and December 2020, a total of 35 common carp were collected from a fish farm at al- Tarmiyah region, Baghdad province by using the cast net with fish farm owners and workers assistance. Fish were transported alive to the laboratory and examined immediately for parasites. The gills were separately examined under a stereo microscope for any metazoan infection. Skin, fin, and gills smears were prepared to investigate *Gyrodactylus* sp. infection.

To examine if *G. mutabilitas* shows a preference in the distribution between right and left gills or in other body parts examined, the number of parasites on each gill arch or body part was counted. For species identification, parasites were prepared following the standard protocol CRIBB and BRAY (2010). Freshly prepared parasites were photographed and measured with a specific digital camera connected to the microscope. Drawings were made with the aid of a camera lucida. Identification of parasites was carried out according to keys by BYKHOVSKAYA-PAVLOVSKAYA et al. (1962) and GUSSEV (1985).

Mucus isolation from the gills of common carp and DNA extraction

A total of 25 common carp that were known to be infected with *G. mutabilitas* were collected, the gills were subsequently swabbed to collect mucus. Following the procedure by EK-HUCHIM et al. (2012) fish were then dissected and had their gills removed in order to determine the worm burden. Infected gills were removed and placed individually in Petri dishes containing 0.7% saline solution. For DNA extraction parasites were

counted and kept individually in Eppendorf tubes with 70% ethanol, for negative controls, mucus from 10 non-infected fish was collected following the same protocol for positive sample.

DNA extraction from gill swab samples and PCR assays

Briefly, fish were placed on a clean glass tray, with the aid of a technician who hold them tightly with his hand then they swabbed with a sterile cotton swab stick. To prevent causing severe tissue damage, the swab stick was carefully placed inside the operculum and rotated for five seconds on the external surface of the left and right-sides of the fish gills. After that, the swab was removed gently from the gills and returned to the sterile container and taken through the DNA extraction procedure immediately.

DNA extraction was completed using Geneaid, gSync ™ DNA Extraction Kit according to the manufacturer's protocol. Following swab collection, fish were dissected to asses worms' burden and to collect gills infected tissue samples for parasitic genomic DNA comparison. Mucus was obtained from 10 uninfected fish and used for the extraction of DNA of negative controls samples. The concertation of DNA was measured using Nanodrop which used to assess the purity and quantity of the DNA, which was then stored at -20 ^{o}C for future use. As a DNA barcode, the mtDNA cytochrome c oxidase subunit I (COI) gene was amplified using the polymerase chain reaction (PCR) method. PCR amplification was attempted using the primer pairs (Bioneer/Korea) the forward primer

(LCO1490) 5´- GGTCAACAAATCATAAAGATATTGG-3´ and the reverse primer (HC02198) was 5´-TAAACTTCAGGGTGACCAAAAAATCA-3´by FOLMER et al. (1994).

The polymerase chain reaction (PCR) mix for amplification of the *COI* gene was optimized as follows: primers performed in a total volume of 25 µl with 5µL of the mixture (Bioneer \Korea) mix PCR, 5 µL of DNA (30-50 ng/µl) 1 µL of each primer, 13 µL of the doubled distilled water. The PCR mixture mentioned above was placed in the thermal cycler (Bioneer/Korea). The PCR program used in this amplification was optimized as follows: initial denaturation at 95 °C for three min followed by 35 cycles of 40 s at 95 °C, annealing step one min at 45 °C; extension step of 45 s at 72 °C and a final extension for seven min at 72 °C.

PCR products were checked by using a standard 1.5% agarose gel dissolved in 1X TBE (Bioneer, Korea), 0.5 µl ethidium bromide was added and then poured into the gel former after which 2 µl of 100 bp DNA ladder (Bioneer, Korea) was added. PCR products were run at 75 volts for two hrs. The agarose gel was documented with a UV documentation system.

Statistical analysis

The spatial distribution of the monogenean *G. mutabilitas* between the left and right sides of the fish gills including gill arches which were numbered 1-4 from the first gill arch below the operculum to the posterior, ANOVA mixed effects model was used to test the correlation between the mean abundance of infection in both right and left/gill arches. All statistical analysis was carried out using Minitab18 statistical software.

RESULTS

Gyrodactylus mutabilitas were isolated from the gills of common carp. All the measurements were based on 11 specimens of parasites which have been described particularly in terms of size and morphological characters of the hamulus, the opisthaptor, the dorsal and ventral bars and other diagnostic variables. Four parasite specimens were used to get the measurements.

As illustrated in Figure 1, a brief description and measurements (in mm) of this parasite are given: Worms are small in size 0.37-0.45 (0.40), width 0.14-0.18 (0.16), opisthaptor length 0.1-0.15 (0.12), opisthaptor width 0.1-0.15 (0.12), dorsal bar 0.019-0.025 (0.021) x 0.0017- 0.0023-0.0028 (0.0026), hamulus length 0.06-0.075 (0.064), hamulus point length 0.02-0.026 (0.023), hamulus shaft length 0.021-0.026 (0.023), 0.038-0.043 (0.04), ventral bar 0.017-0.02 (0.018) x0.0038-0.0042 (0.004), length of membranoid extension 0.017-0.021 (0.019), length of marginal hooks 0.030-0.040 (0.035) and finally sickle 0.009-0.014 (0.011).

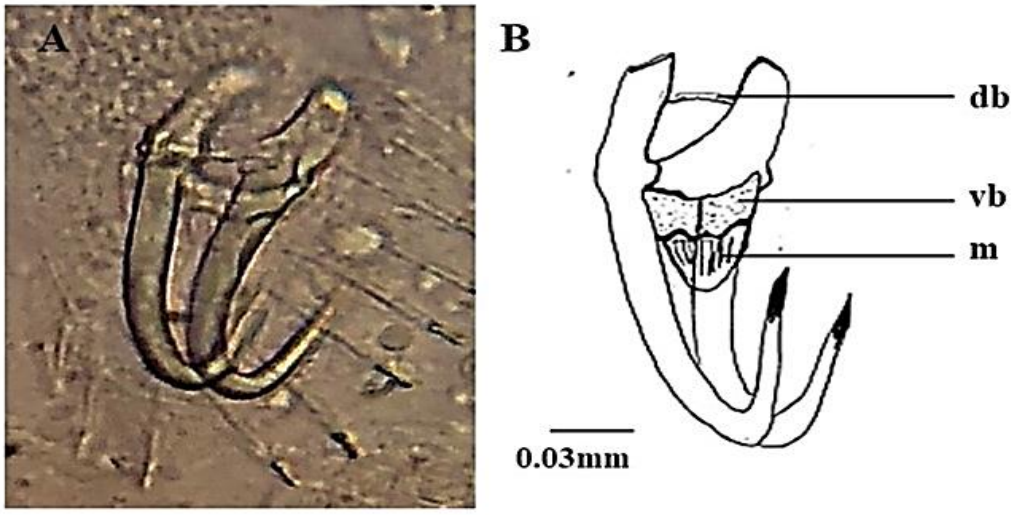

Figure 1. *Gyrodactylus mutabilitas* from the common carp. A- Light micrograph of the haptoral part, B- Camera Lucida drawing of the haptor. db= dorsal bar; vb= ventral bar; m= membranoid extension.

Site selection of *G. mutabilitas* within *C. carpio* gills

The data analysis did show significant differences in the mean intensity of infection over the months of the study between the left and right sets of gill arches when significantly a greater number of *G*. *mutabilitas* reported on the right-side of the host gill ($P< 0.0001$, Table 1, Figure 2).

Moreover, the gill arches infected with *G*. *mutabilitas* were varied significantly in parasites number, the analysis revealed that there were fewer parasites occurred on the first; third and fourth gill arches than on the second gill arches ($P< 0.002$, Figure 3).

Table 1. *G*. *mutabilitas* distribution (mean and SD) in the right and left sets of the gills of the common carp. Gill arches were numbered 1-4 from the first gill arch below the operculum to the posterior.

Side	Right gill	Left gill
Gill arches		
1	1.76 ±1.12	072 ±0.79
2	3.32 ±1.52	1.72 ±1.10
3	0.36 ±0.57	0.16 ±0.37
4	0.16 ±0.79	0.08 ±0.28

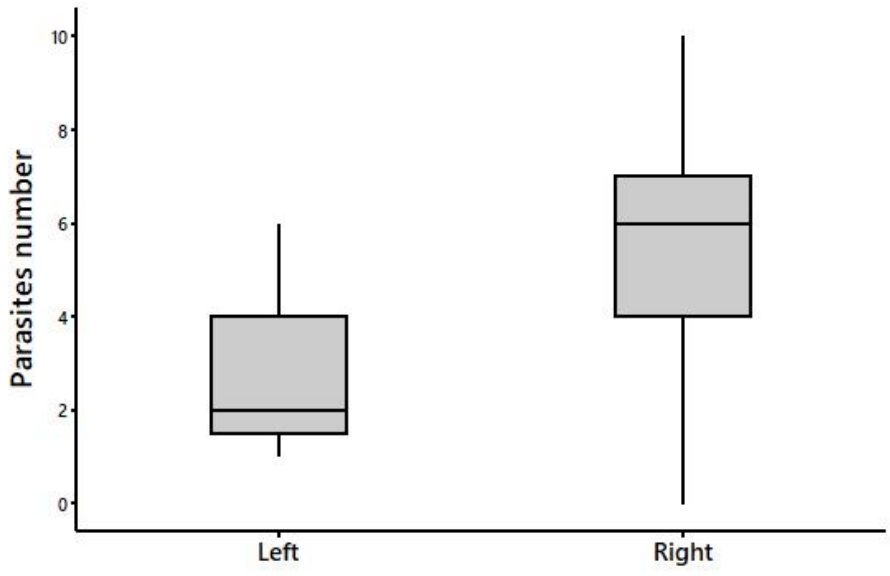

Figure 2. The number of *G*. *mutabilitas* were isolated from the gills of *C*. *carpio* presented by infection side.

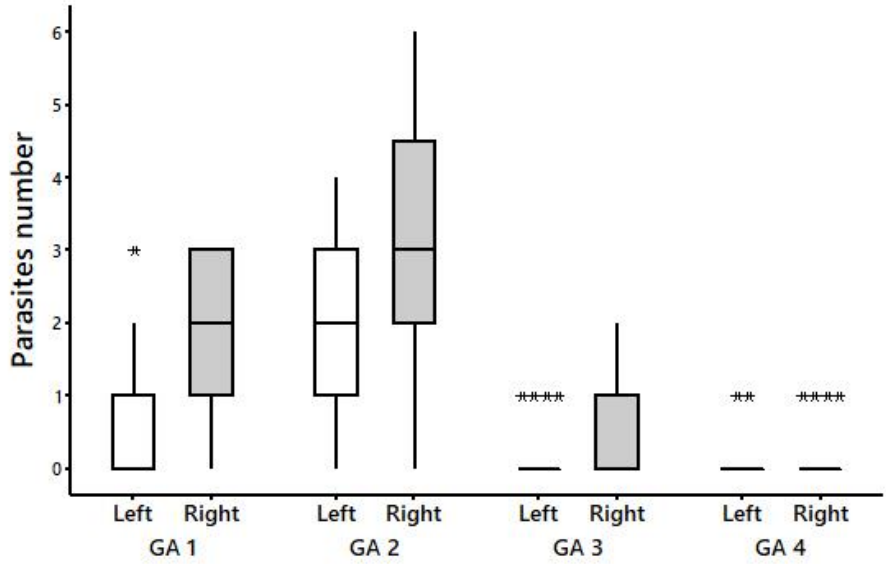

Figure 3. Boxplot showing distribution of *G*. *mutabilitas* on the gill arches GA 1-GA 4 between right and left gills.

Parasite mtDNA *COI* Gene amplification and PCR

The parasitic mtDNA *COI* gene was detected by using a PCR technique with two primers LCO1490 and HC02198. To examine the sensitivity of the test for subsequent usage with common carp mucus, DNA from collected parasites was used for PCR standardization. PCR positive reaction with the presence of the parasite's DNA was achieved when the primers successfully amplified two areas of mtDNA in the form of two fragments which was 709pb long.

One of the objectives of this study was to test whether the mucus DNA approach represented a reliable method to detect the parasite infection of fish. DNA concentration in mucus samples and the DNA from the collected parasites sample were strongly and positively correlated (Figure 4) indicating that both approaches yielded very similar estimates of DNA parasite concentrations. DNA amplification was not observed in the mucus of the 10 non-infected *C*. *carpio*. PCR tests amplified DNA fragments of the same size as for the G. *mutabilitas* from the 25 infected common carp.

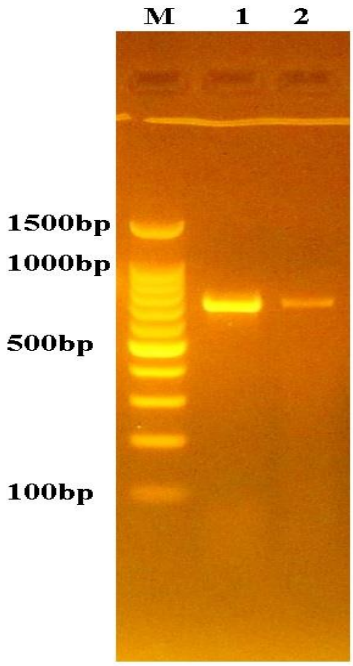

Figure 4. Electrophoresis agarose gel ***1***.5% amplified target *CO1* gene. (1) parasites sample, (2) gill mucus from infected *Cyprinus carpio*.

DISCUSSION

In this study the monogenean *G*. *mutabilitas* was reported for the first time in Iraq. According to the index-catalogue of parasites and disease agents of Iraqi fishes [Mhaisen, 2022 Index-catalogue of parasites and disease agents of fishes of Iraq (Unpublished: mhaisenft@yahoo.co.uk)] no previous record of this parasite from Iraqi fishes has been documented. Mhaisen (2022) data pointed that a total of 58 *Gyrodactylus* species have been recorded from different Iraqi fish, specifically 34 of them are described mainly from *C*. *carpio* so far.

Transport, establishment, and demographic growth are only a few of the variables that might affect whether a species becomes invasive in a new area (TORCHIN & MITCHELL 2004).

Gyrodactylus mutabilitas has been described formerly from varied Iranian freshwater fishes (BARZEGAR et al. 2018, MASHALY et al. 2019, MIRGHAED et al. 2018). Since Iran is a neighbouring country to Iraq, this might explain that this monogenean has been introduced into new habitat area.

Monogeneans have been a recurring concern for common carp for many years. Current detection methods which are widely used necessitate fatal sampling of farms fish, sometimes this method is ineffective and raises costs in local farms. In this study non-lethal method was used for ectoparasite infection detection, PCR-based testing was used to detect *G. mutabilitas* infection either by DNA isolated di-rectly from fresh collected parasites as well as parasite's DNA in mucus from *C. carpio*.

Because they are highly conserved and present in numerous copies, mitochondrial genes COI are the primary target genes in PCR-based detection techniques (PAOLETTI et al. 2018). Mitochondrial genes provide useful markers for diagnostics and taxonomy of a population studies. In *Gyrodactylus* populations, the COI gene is frequently utilized as a universal barcode for species identification (NITTA 2023; JIN et al. 2024).

Gyrodactylus has significant variety as shown by COI mtDNA sequences (HANSEN et al. 2007). In order to identify the presence of *Gyrodactylus* parasites in commercially and wild fish species, the mitochondrial gene *COI* has been considerably used for example (GILMORE et al. 2010, PLAISANCE et al. 2007, BUENO-SILVA & BOEGER 2014, KUUSELA et al. 2008, ONDRAČKOVÁ et al. 2020, PINACHO-PINACHO et al. 2021).

The test in this study relies on a simple PCR, not qPCR. Nonetheless, real time PCR has provided high efficiency and accuracy in detecting the presence of a parasite within\on its host. However, concerning their use in the detection of fish diseases, these methods' costs could be expensive, on the other hand the standard PCR uses inexpensive reagents and equipment.

Generally, monogenoids show a selective pattern of spatial distribution on their host body, when most species are found only on fish gills and they may have a preference for specific gill arches or even specific sectors of those arches (BAGGE & VALTONEN 1999). The selective-distribution of monogenoids on their hosts body has been linked to a variety of environmental and host biological parameters (PIE et al. 2006). Several studies have investigated the microhabitat distribution of mono-geneans on their host's gills (SIMKOVÁ et al. 2000; KADLEC et al. 2003; RUBIO-GODOY 2008; IANNACONE & ALVARIÑO 2012; KUMAR et al. 2017).

As there were a significant difference in the mean intensity of infection between the left and right gill when the right-side has the greater number of parasites reported on. This also agrees with earlier observations regarding two monogenean which found that *Dactylogyrus amphibothrium* has a right-side preference over the gill apparatus of the ruffe (WOOTTEN 1974) and the *Microcotyle mugilis* also has a left set preference on the gills of the striped mullet (EL HAFIDI et al. 1998).

This outcome is contrary to that of MASHALY et al. (2019) who found no preference was observed by *Gyrodactylus rysavyi* between the right and left set of gills of the Nile catfish *Clarias gariepinus*. The authors findings suggest that the fish hosts are symmetric, receiving equal amounts of water on both sides of their bodies.

According to CHAPMAN et al. (2000), the gill monogenean *Neodiplozoon polycotyleus* in the African cyprinid fish *Barbus neumayeri* may use the second gill arch to position itself in a region with the most laminar flow in the gill. This could indicate that some monogenean species are rheophilic, meaning they prefer lotic streams to lentic streams. The distribution of *G*. *mutabilitas* over the hosts' gill arches revealed a distinct right-side preference, as well as a clear predilection for the second gill arches, according to this monogenean. This finding is consistent with that of MASHALY et al. (2019) who reported that the second gill arch was the most favourable site of attachment for *G*. *rysavyi* over the others.

Differences in water circulation over different sections of the gill surface have been thought to play a role in parasite distribution on the gills (WOOTTEN 1974). The most powerful water stream runs through the

middle of the gill arches, providing ideal circumstances for parasite establishment. The amount of water going through the gills may alter the aerobic conditions in some sections of the gills, thus facilitating parasite attachment, but also reflecting the larger surface area accessible for parasite attachment on these gills (WOOTTEN 1974).

The main factors promoting monogenean invasion of the gills are morphology, particularly the degree of differentiation of the branchial apparatus, as well as the fine-scaled histological structure of the gills (IZJUMOVA 1956).

In current study, gill mucus DNA is used as a non-lethal method for detection of specific parasite of common carp. This unique method is non-invasive to the host and can identify parasitic DNA in from infected gill mucus may make it easier to employ testing to prevent financial losses on carp farms. To achieve the assessment of *C*. *carpio* stock in fish farm, low sample costs test sensitive and quick non-lethal techniques are urgently needed. In addition, frequent and rapid screening of fish farms might stop the spread of the pathogens which help avoid economic losses.

CONCLUSION

The monogenean *Gyrodactylus mutabilitas* was recorded for the first time from gills of the common carp in Iraq. Gill mucus DNA can be used as a non-lethal method to detect parasitic infection in fish. Such a non-lethal technique is urgently needed as it represents a frequent and rapid screening of fish farms may help to stop the spread of pathogens, thereby avoiding economic losses.

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