



Occurrence of dactylogyrid and gyrodactylid Monogenea on common carp, *Cyprinus carpio*, in the Southern Caspian Sea Basin

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ABSTRACT

In this study, we genetically characterised Monogenea found on common carp in Iran. In total, 5603 parasites were recovered from 112 fish. The parasites were first identified morphologically as *Dactylogyrus extensus*, *D. anchoratus*, *D. vastator*, *D. minutus*, *D. achmerowi* and *Gyrodactylus sprostonae*. Representative samples were then subjected to sequencing. This is one of the first studies which has provided both morphological and sequence data for *Dactylogyrus* spp. and *G. sprostonae*. Our findings provide a foundation for future research into the genetic make-up of these economically significant parasites and the establishment of effective strategies for their control and prevention in aquaculture systems.

Common carp (*Cyprinus carpio*) are widely cultured in most parts of Europe and Asia, and on a smaller scale in Africa and Latin America. According to the Food and Agriculture Organization (2016), the global aquaculture production for common carp was estimated at > 4.5 million tonnes. This species is one of the most popular farmed cyprinids in Iran. Carp farming in pond systems began in the northern regions of Iran during the 1960s and is today widely distributed across regional Iran.

Class Monogenea is a group of ectoparasites commonly found on the gills and the skin of fish where they feed on mucus, epithelial cells and sometimes, blood. Infection with Monogenea may lead to serious hyperplasia of the gill epithelium, which has been shown to impair respiratory function, negatively affecting growth and leading to high mortality, particularly in juvenile carp [1]. *Dactylogyrus* and *Gyrodactylus* are among the dominant genera of Monogenea. These are common ectoparasites which usually attach to the gills of freshwater fish of the family Cyprinidae. The fish heavily infected with *Dactylogyrus* spp. are susceptible to secondary infections. The pathogenicity of both parasite and secondary infection is significant and may result in considerable economic losses [2–4] in fish farming systems. In Iran, common carp from both pond and lake culture systems are known to be parasitised by at least five species of *Dactylogyrus* [5–8] and seven species of *Gyrodactylus* [9]. Studies of Monogenea which infect Iranian fish have commonly used the morphological method for parasite

identification which can be problematic and is often ambiguous for closely related species. Recently, molecular markers have provided a useful alternative for the identification and the validation of monogenean species [1]. Our knowledge of the genetic make-up of Iranian Monogenea is poor, and little is known about molecular aspects of these parasites [10–13]. In the present study, the monogenean species found on the gills of cultured common carp from the Caspian Region were genetically characterised on the basis of the sequences of a large sub-unit of rDNA for *Dactylogyrus* and 5.8S and ITS2 for the *Gyrodactylus* specimens.

The fish were collected from 10 fish farms located in Guilan Province, North Iran, in September 2015–August 2016. These fish farms are located in the southern part of the Caspian Sea Basin. The water supply is obtained from both local rivers and bore water. In all, 112 common carp (*Cyprinus carpio*) were collected. One summer-old fish were placed in plastic tanks filled with water obtained from the collection site and then transferred to the Parasitology Laboratory of National Inland Water Aquaculture Research Centre in Bandar Anzali. The fish were randomly distributed into several 100-L aquariums containing water from the fish farms and examined within 72 h to prevent/minimise the possibility of horizontal transmission in the tank. The number of fish in each tank was 12–15. The fish had an average total length of 10.9 cm ± 2.1 cm and an average weight of 19.6 g ± 6.6 g. The specimens were examined for infection with parasites according to

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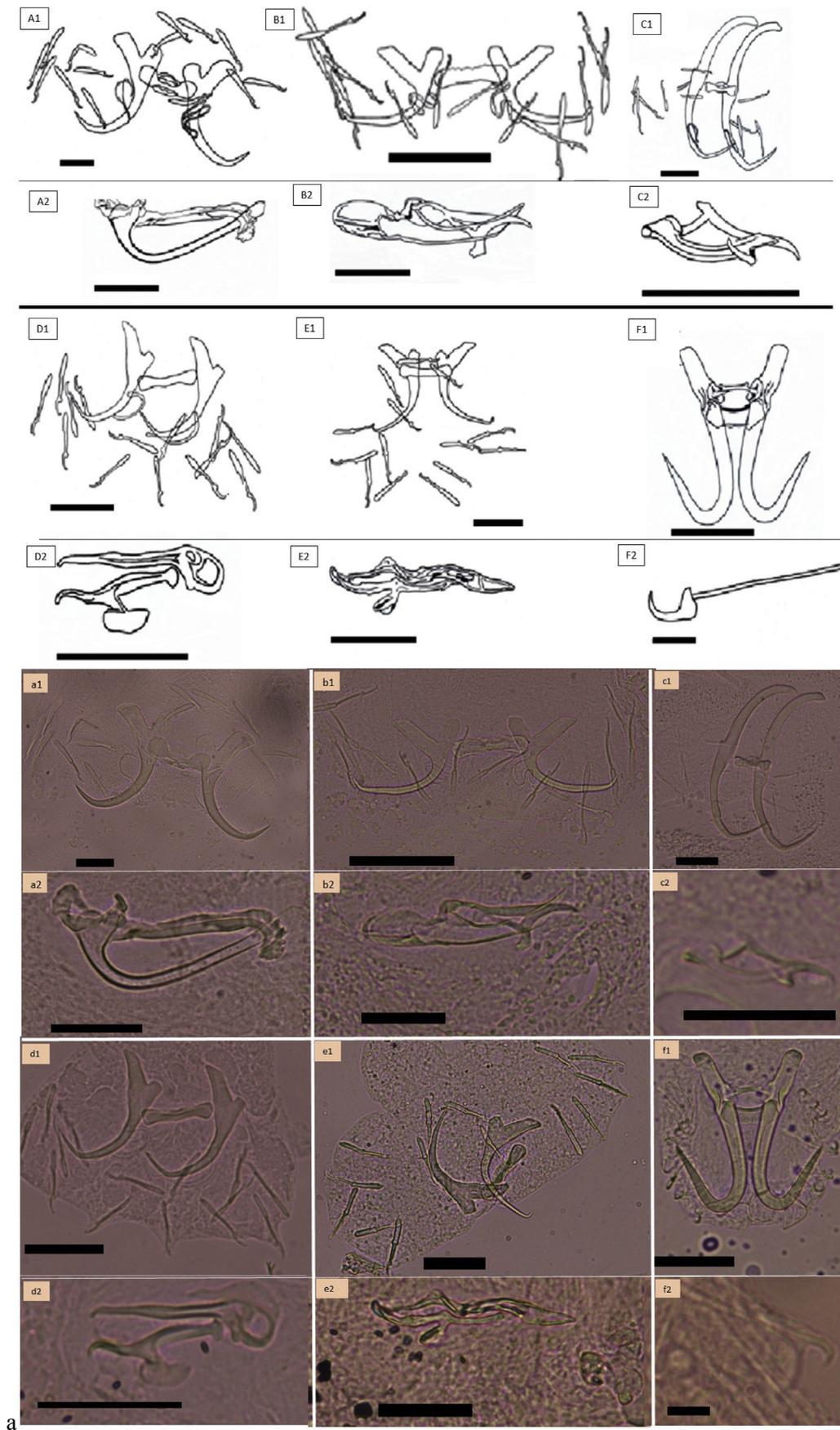


Fig. 1. Line drawings (images labelled with capital letter) and light microscopy (images labelled with lower case letter) of the opisthohaptor and copulatory organs of Monogenea found in the present study, including *D. extensus* (A1, A2, a1 & a2), *D. vastator* (B1, B2, b1 & b2), *D. anchoratus* (C1, C2, c1 & c2), *D. minutus* (D1, D2, d1 & d2), *D. achmerowi* (E1, E2, e1 & e2) and *G. sprostonae* (F1, F2, f1 & f2). Scale bars represent 25 μm, except in image F2 and f2 which represent 5 μm.

Table 1
Comparative morphometric (μm) of *Dactylogyrus* spp. in the present study and those previously described by Bykhovskaya-Pavlovskaya, 1962 (indicated as B-P 62).

Species characteristic	<i>D. extensus</i> (n = 13)		<i>D. anchoratus</i> (n = 5)		<i>D. vastator</i> (n = 10)		<i>D. minutus</i> (n = 8)		<i>D. achmerowi</i> (n = 4)		<i>G. sprostonae</i> (n = 5)	
	Present study	B-P 62	Present study	B-P 62	Present study	B-P 62	Present study	B-P 62	Present study	B-P 62	Present study	B-P 62
Body length	1692.04 \pm 408.73 (697.5–2196)	> 1500	334.3 \pm 45.7 (294–384)	> 500	1070.79 \pm 384.46 (290–1601.28)	> 1100	266.33 \pm 55.42 (206–315)	> 480	266.66 \pm 144.92 (177–430)	> 350	277.43 \pm 73.24 (173.85–346.32)	> 420
Body width	273.04 \pm 67.20 (150–395)	> 310	88 \pm 17.3 (64–105)	> 100	261.54 \pm 119.26 (108–413.01)	> 400	62.08 \pm 14.46 (360–793)	> 110	89.02 \pm 11.78 (79–109)	> 110	85.11 \pm 8.55 (77.61–97.33)	> 100
Length of marginal hooks	32.85 \pm 2.03 (31–36)	27–36	22.3 \pm 2.8 (18–27)	14–35	33.31 \pm 2.76 (29.00–37.00)	29–35	20.89 \pm 1.83 (19.00–23.60)	13–23	23.61 \pm 2.76 (20–27)	21–029	23.12 \pm 1.65 (20.56–24.77)	20–25
Length of median hooks	65.14 \pm 3.33 (60–69)	62–89	91.61 \pm 4.39 (86–97)	92–130	35.22 \pm 0.98 (34.21–37.10)	35–41	40.68 \pm 1.87 (39.0–42.9)	39–49	43.73 \pm 1.02 (42.6–44.6)	43–56	-	-
Connecting bar length	41.23 \pm 1.84 (39.5–44)	33–59	20.28 \pm 1.44 (18–22)	18–29	35.85 \pm 1.38 (33.72–37.02)	32–38	25.04 \pm 0.71 (23.70–25.80)	25–32	30.31 \pm 1.57 (29.2–32.60)	29–33	-	-
Connecting bar width	12.59 \pm 0.97 (11–13.32)	8–16	6.88 \pm 1.55 (5–8.4)	8	5.63 \pm 1.07 (4.04–6.76)	6	3.84 \pm 0.05 (3.80–3.90)	3–4	4.87 \pm 0.84 (4.06–5.7)	4–6	-	-
Total length of copulatory organ	77.3 \pm 2.59 (74.9–81)	72–82	28.16 \pm 2.92 (26–31.5)	20–32	52.13 \pm 5.70 (46.3–0-58.72)	44–58	35.4–2.89 (33.3–038.7)	28–45	56.16 \pm 0.72 (55.7–57.0)	52–58	-	-
Total length of anchor	-	-	-	-	-	-	-	-	-	-	51.01 \pm 2.10 (48.47–54.23)	40–51
Length of anchor root	-	-	-	-	-	-	-	-	-	-	16.56 \pm 1.89 (15.51–19.32)	-
Length of anchor shaft	-	-	-	-	-	-	-	-	-	-	39.77 \pm 1.72 (37.33–41.76)	-
Length of anchor point	-	-	-	-	-	-	-	-	-	-	22.76 \pm 0.90 (21.70–24.08)	-
Length of sickle	-	-	-	-	-	-	-	-	-	-	4.53 \pm 0.29 (4.11–4.88)	-
Length of handle	-	-	-	-	-	-	-	-	-	-	19.39 \pm 0.92 (18.12–20.67)	-
Width (distal) of sickle	-	-	-	-	-	-	-	-	-	-	3.09 \pm 0.46 (2.50–3.76)	-
Width (proximal) of sickle	-	-	-	-	-	-	-	-	-	-	3.19 \pm 0.26 (2.93–3.43)	-
Length of ventral bar	-	-	-	-	-	-	-	-	-	-	19.74 \pm 1.26 (18.03–21.25)	13–20
Width of ventral bar	-	-	-	-	-	-	-	-	-	-	3.42 \pm 0.38 (3.04–3.83)	3–4
length of ventral bar membrane	-	-	-	-	-	-	-	-	-	-	13.32 \pm 1.09 (12.26–14.81)	-
Total length of dorsal bar	-	-	-	-	-	-	-	-	-	-	18.23 \pm 1.21 (17.35–19.96)	13–20
Width of dorsal bar	-	-	-	-	-	-	-	-	-	-	0.99 \pm 0.13 (0.833–1.17)	> 1

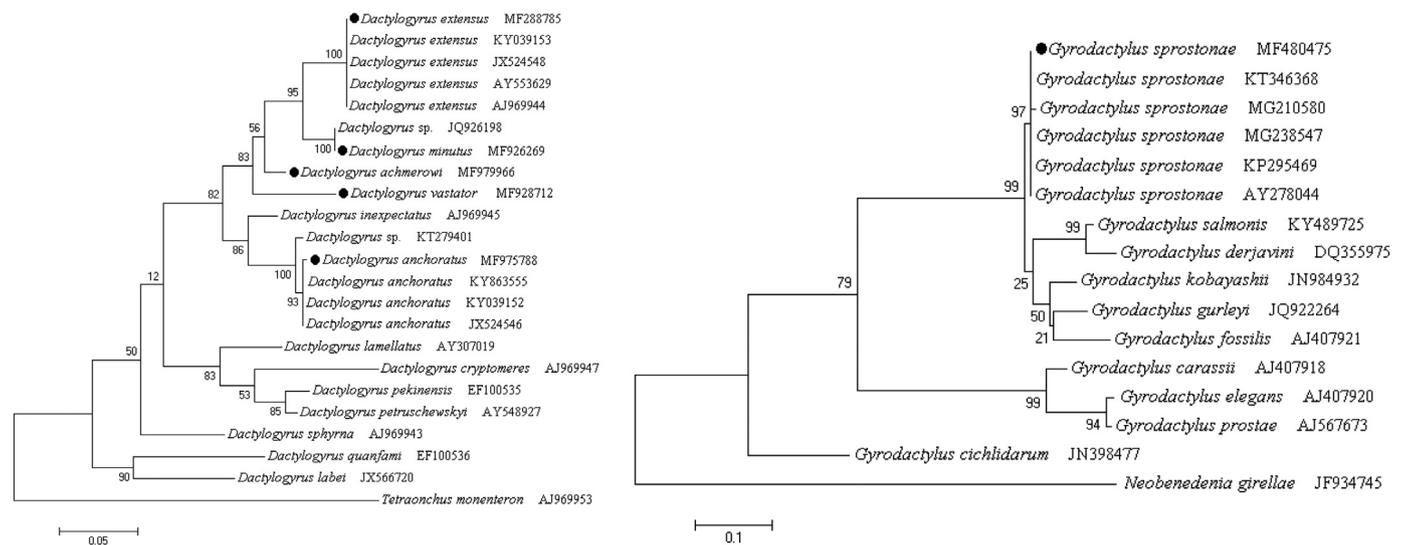


Fig. 2. Phylogenetic tree constructed by maximum likelihood analysis based on 28S rDNA sequences for selected species of *Dactylogyrus*, with *Tetraonchus monenteron* used as the outgroup (left) and phylogenetic tree constructed by maximum likelihood analysis based on partial 5.8S rDNA and ITS-2 for selected species of *Gyrodactylus*, with *Neobenedeniagirellae* used as the outgroup (right). Black circles indicate sequences obtained in the present study.

standard protocols [14,15]. In brief, both sets of gills were removed, separated and individually placed in a petri dish; each gill was thoroughly examined, with each filament observed under a stereomicroscope.

Live dactylogyrid parasites were collected and mounted on a slide in glycerine jelly and the members of the *Gyrodactylus* were mounted in ammonium picrate glycerine (APG). A representative sample from each parasitic group was preserved in 75% ethanol for molecular analyses. Monogenea were morphologically identified on the basis of the characteristics of their copulatory organ and opisthaptor, including anchors and bars [16]. Photographs of the mounted parasites were captured at magnifications of 20 \times and 40 \times with a Nikon Eclipse 50i compound microscope with the Nikon Digital Sight DS-L1 image analysis software and a Nikon Digital Sight DS-SM camera. For the morphometric analysis, in all, 7 and 16 point-to-point measurements were made on the *Dactylogyrus* and the *Gyrodactylus* photographs, respectively, by using the Image J software. Drawings were made with the aid of a drawing tube attached to the Nikon compound microscope. Finally, the specimens and their measurement data were compared using a parasite identification key [16,17].

The genomic DNA was extracted using the YTA Genomic DNA Extraction Mini Kit (Yekta Tajhiz Azma, Tehran, Iran) according to the manufacturer's manual. The 28S rDNA region of the *Dactylogyrus* specimens was amplified using the primer sets (5'-TCTAGTAACGGCGAG TGAACG-3') (forward primer) [18] and the modified reverse primer (5'-GTGGGAAGGTCTACCTCAGC-3') 5.8S and ITS2 region of the *Gyrodactylus* specimens was amplified using the primer sets forward primer (5'-CGATCATCGTCTCTCGAAC-3') [10] and modified reverse primer (5'-TAAAGGAAGAACCCTAGAG-3'). The amplification reactions (25 μ L) consisted of 12.5 μ L 2 \times Taq Mastermix, 1 μ L of each primer (Macrogen, South Korea) and 2 μ L of the genomic template DNA. The amplification was carried out in a thermocycler (BIO-RAD, USA). The PCR program for the *Dactylogyrus* specimens was as follows: 4 min at 94 $^{\circ}$ C followed by 35 cycles of 30 s at 94 $^{\circ}$ C, 30 s at 59 $^{\circ}$ C and 1 min at 72 $^{\circ}$ C, and a final elongation at 72 $^{\circ}$ C for 10 min. For the *Gyrodactylus* specimens, the initial denaturation started at 95 $^{\circ}$ C for 4 min and was followed by 35 cycles of 30 s at 94 $^{\circ}$ C, 30 s at 57 $^{\circ}$ C and 30 s at 72 $^{\circ}$ C, and a final elongation at 72 $^{\circ}$ C for 10 min. The PCR products were analysed on 1.5% agarose gel and visualised under a UV illuminator. Sequencing was carried out using the same primers as those used for PCR amplification (Macrogen, South Korea). The sequence data were submitted to a nucleotide BLAST search with the default parameter setting to

confirm as *Dactylogyrus* and *Gyrodactylus* in GenBank. In order to place the parasites considered in this study within the phylogeny of *Dactylogyrus*, 17 sequences from species representing the major groupings of the genus were retrieved and *Tetraonchus monenteron* was used as the outgroup because of it being less closely related to *Dactylogyrus* spp. and the availability of the comparable sequence in GenBank. For the *Gyrodactylus* specimens, 14 sequences were retrieved and *Neobenedeniagirellae* was used as the outgroup for the same reason as that mentioned above. Further, 28S rDNA of *Dactylogyrus* and partial 5.8S rDNA with ITS2 sequences of the *Gyrodactylus* specimens in the present study were aligned separately by using the Clustal W software and then manually adjusted to perform the phylogenetic analysis. Gaps and ambiguously aligned regions were removed. Next, phylogenetic trees were built using Mega 6.0 by using the maximum likelihood method.

The prevalence (P), mean intensity (MI), mean abundance (MA) and dominance (D) of the parasites were calculated according to the methods proposed by Bush, Lafferty, Lotz and Shostak [19].

The mean intensity and abundance in different seasons were compared using the Kruskal–Wallis (KW, multiple comparisons) and Mann–Whitney tests. Results were considered significant at the 95% level ($p < 0.05$). Computations were performed using the SPSS.15 programme.

In all, 5603 monogenean parasites belonging to two genera and six species were collected. On the basis of the morphological characteristics of the taxonomically important features (Fig. 1), they were identified as *D. extensus*, *D. anchoratus*, *D. vastator*, *D. achmerowi*, *D. minutus* and *Gyrodactylus sprostonae* (Table 1). Phylogenetic analyses supported the morphological distinction between the taxa found in the present study (Fig. 2). Of the fish examined, 98 (87.5%) fish were found to be infected with monogenean parasites of which 47 fish (42.0%) were infected with one monogenean species, 33 fish (29.4%) with two species, 12 fish (10.7%) with three species, 4 fish (3.6%) with four species and 2 fish (1.8%) with five species of monogenean parasites. In terms of the abundance, 64 fish (65.3%) were infected with < 20 monogenea; 15 fish (16.0%) had 20–50 parasites; 8 fish (8.5%) had 50–100 parasites; 5 fish (5.3%) had 100–200 parasites; 2 fish (2.0%) had 200–700 parasites; and the two fish (2.0%) infected with 1307 and 1630 parasites had the highest intensity of monogenean infection. The prevalence of infection with *Dactylogyrus* spp. and *Gyrodactylus* sp. was 81.2% and 21.4%, respectively. Tables 1 and 2 provide a summary of the monogenea belonging to different taxa found in this study, their morphometrics and abundance in various seasons (Mann–Whitney test,

Table 2

Details of the overall occurrence of the dactylogyrids found in the present study and the seasonal occurrence of monogenean parasites found in the present study; For seasons, numbers in each cell are referring to prevalence (%), mean ± Standard Error, range and total, for the particular parasite in the specified season, respectively.

Parasite Species	<i>D. anchoratus</i>	<i>D. extensus</i>	<i>D. vastator</i>	<i>D. minutus</i>	<i>D. achmerowi</i>	<i>G. sprostonae</i>
Number of parasites	4735	432	193	43	24	176
Prevalence (%)	23.21	55.35	25.89	11.60	9.82	21.42
Mean intensity ± SE	157.83 ± 67.82	6.96 ± 1.76	6.65 ± 1.60	3.30 ± 1.05	2.18 ± 0.39	7.33 ± 3.60
Range	1–1618	1–96	1–36	1–14	1–4	1–86
Mean abundance ± SE	42.27 ± 18.89	3.85 ± 1.02	1.72 ± 0.49	0.38 ± 0.15	0.21 ± 0.07	1.57 ± 0.81
Dominance (%)	84.50	7.71	3.44	0.76	0.43	3.14
Spring N = 18	16.66	83.33	16.66	–	–	33.33
	4.66 ± 0.89	7.26 ± 2.49	2 ± 0.40	–	–	3 ± 0.12
	2–9	1–42	1–4	–	–	1–7
	14	109	6	–	–	18
Summer N = 19	73.63	78.94	52.63	36.84	–	10.52
	261.16 ± 102.74	11.66 ± 5.55	11.5 ± 2.78	1.85 ± 0.28	–	1 ± 0
	19–1618	1–96	1–36	1–4	–	1
	4701	175	115	13	–	2
Autumn N = 71	12.67	43.66	21.12	8.45	15.49	22.53
	2.22 ± 0.32	4.54 ± 0.82	4.73 ± 0.61	5 ± 0.61	2.18 ± 0.17	9.75 ± 2.53
	1–9	1–37	1–18	1–14	1–4	1–86
	20	141	71	30	24	156
Winter N = 4	–	25.00	25.00	–	–	–
	–	7 ± 0	1 ± 0	–	–	–
	–	7	1	–	–	–
	–	7	1	–	–	–

N: number of examined fish; SE: Standard Error; Data with different letters in column are statistically significant at the p < 0.05 level.

p < 0.05). As shown in Table 1, there were differences in the size of some of the features between the conspecifics found in the present study and the previous studies, which might be attributed to various factors, including methods used to prepare the samples, environmental temperature affecting the growth of the parasites, or impact of the host size.

This is the first time that monogenean parasites, including *D. vastator*, *D. minutus*, *D. achmerowi* and *G. sprostonae* have been genetically characterised in Iran. Monogenea *D. extensus* and *D. anchoratus* were genetically characterised for the first time in Guilan Province. This is also the first study providing sequence data for the 28S rDNA region of *D. vastator*, *D. minutus*, and *D. achmerowi*. Phylogenetic trees built on the basis of the sequence data confirmed the specific identity of these taxa. Of these Monogenea, *D. minutus* found during the current study is considered a new locality record for Iran. This species has been previously described in the neighbouring countries of Iran, including Turkey, Iraq and Syria [20–22]. The phylogenetic tree (Fig. 2) showed that *D. extensus* found in the present study was genetically the same as that previously reported from other countries. It has been suggested by other authors that this species is widely distributed and has the ability to adapt itself to a broad range of environments [23]. It was also the most prevalent species in the present study (Table 2). The phylogenetic tree (Fig. 2) also showed that *D. anchoratus* from the present study is genetically identical to that previously characterised from other countries. *Dactylogyrus anchoratus* is an exotic monogenean which was introduced to the country by non-native fish. *D. anchoratus* requires special attention because of its low host specificity and high tolerance to a wide range of temperature and salinity [23]. This parasite attaches to the base of the primary filaments where osmoregulating chloride cells are located. Therefore, infection with the parasite could be pathogenic, particularly in fry-sized hatchery fish [8]. For the other taxa, there were no comparable sequences available in GenBank, but morphometric comparisons were in accordance with previous studies. In the present study, there were no new gyrodactylids identified to infect common carp. The isolate of *Gyrodactylus sprostonae* described here is the first gyrodactylid to be formally identified from *C. carpio* in Iran by using both morphological and molecular data. The phylogenetic tree (Fig. 2) revealed that *G. sprostonae* is genetically identical to that previously characterised from other hosts in Iran and other countries. Based on Table 2, the prevalence and the abundance of the *G. sprostonae* isolate were low. The infected fish did not show any of the obvious

pathogenic changes or mortality previously associated with a gyrodactylid infection in the farmed Iranian carp. Fishes living in the water systems of the Caspian Sea proved to be infected with monogeneans from European and Central Asian waters [24]. The northern part of Iran, including the study area, belongs to the Ponto–Aralo–Caspian zoogeographical region of the Palearctic where the composition of the fish fauna is approximately the same as that in Europe and in the neighbouring Central Asian region. Previous studies [24–26], based on the morphological identification of Monogenea, have concluded that monogenean parasites found in the northern part of Iran are the same as those in Europe and in the neighbouring Central Asian region. Our study provides the sequence data confirming previous findings [6–9] based on morphological data and is of significance in terms of understanding the distribution of these potentially harmful parasites in the broader region (Europe and Central Asian region) through introduced species.

The prevalence and the abundance (Table 2) of most monogenean species were significantly higher in summer than in other seasons (p < 0.05). In similar environments, it has also been reported [27] that monogenean parasites, although present on the fish throughout the year, were more abundant during the spring and summer months. This emphasises the importance of parasite prevention strategies in fish farms within the region to be developed in consideration with the parasite biology.

Guilan Province is the most significant carp farming region in the country, which has been adversely affected by Monogenea in recent years. The members of Dactylogyridae and Gyrodactylidae identified in the present study are the most reported monogenea in wild and cultured freshwater fish. They are also among the most pathogenic parasites for fish. Thus far, 12 monogenean parasites belonging to the families Dactylogyridae and Gyrodactylidae, including five species of *Dactylogyrus* and seven species of *Gyrodactylus* have been reported from common carp in Iran [6,8,9]. Here, we reported several of these previously reported parasites in a small geographical location, i.e. Guilan Province, which is indicative of an area suitable for the occurrence of these parasites, which is concerning because Guilan Province is home to several fish farms as well as home to several native fish inhabiting in the freshwater systems belonging to the Caspian Sea Basin. It is considered that an evaluation of the current preventative measures used for parasite control such as the timing of chemotherapies is advisable for

fish farms in the region.

In conclusion, this study provides further insights into the biodiversity and the taxonomic status of monogenean parasites in Iran, a country with various zoogeographical regions and a high diversity of monogenean parasites. Therefore, there is a huge potential for further work on the genetic make-up of monogenean fauna in the region to understand their relationship with those from the rest of the world.

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