

**Molecular phylogeny and histopathological studies of *Gotocotyla secundus* (Monogenea: Polyopisthocotylea) isolated from Narrow-barred Spanish mackerel, *Scomberomorous commerson* in Suez Governorate, Egypt**

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## ABSTRACT

This study investigated the prevalence, molecular typing, as well as histopathological changes associated with *Gotocotyla secundus* infection in *Scomberomorous commerson* collected from Suez Governorate, Egypt. Identification of *G. secundus* was performed microscopically and genetically using C-PCR assay. Moreover, phylogenetic tree was performed with MEGA X software analysis based on sequence analyses of the 28S rRNA gene using both neighbor – joining (NJ) and maximum-likelihood (ML) methods. The prevalence, abundance, and intensities of the parasite were calculated based on their collintary relationship with fish length and weight. Herein, *G. secundus* was mainly isolated from the gills and pseudobranchs of infested fish. Mostly, naturally infected fish have no pathognomonic lesions, except for a few cases that showed marbling of the gills. Histopathological studies revealed necrosis and fibrosis of the gill filaments with severe inflammatory reactions represented by the presence of lymphocytes and numerous giant cells.

## INTRODUCTION

The family Scombridae consists of 15 genera and 49 species of epipelagic marine fishes (mackerels, bonitos and tunas). These fish are of commercial and recreational importance throughout the world's fisheries (Muruges, 1995). The Egyptian Mediterranean waters have two species of this genus, *Scomberomorous tritor*, (Cuvier and Valenciennes, 1832) - (first record) a species native to the Atlantic, can be spotted in the Canary Islands, Senegal, Gulf of Guinea, and Angola . *Scomberomorous commerson* is a Red Sea immigrant from the Indo-West Pacific (Lester *et al.*, 2001; Pauly and Froese, 2016) migrated to the eastern Mediterranean Sea by the way of the Suez Canal. El Sayed (1994) and Rizkalla (1997) recorded this species in Egyptian waters. The gill apparatus of a mackerel, a common habitat for ectoparasites, is composed of five paired units: the pseudobranch, attached to the operculum and four regular gill arches. Pelagic fishes, such

as *Scomberomorus commerson*, are usually infected by fewer species of parasites than benthic coastal fishes and the infection levels are usually low (Helna *et al.*, 2016). Monogeneans, usually thrive mostly on the skin and gills of fish, are among the most numerous and abundant ectoparasites. Monogeneans are found in small numbers within their ecological hosts and inflict few, if any, host disturbances (Ramasamy and Ramalingam, 1989; Rohde, 1993); Monogeneans, on the other hand, have been identified as threatening agents that limiting aquaculture productivity in circumstances where the hosts are kept in culture, as they frequently induce mixed infections with other parasites and secondary bacterial diseases ( Cruz e Silva *et al.*, 1997; Antonelli *et al.*, 2010) . Monogeneans require a strong attachment mechanism to the host. This attachment is normally accomplished through a well-developed, highly complex adhesive organ known as a haptor (Brennan and Ramasamy, 1996), which was previously used to distinguish the two major groups of Monogeneans (Monopisthocotylea and Polyopisthocotylea) based on their mode of attachment to the host via a posterior organ haptor (or opisthaptor). In the Polyopisthocotylea (blood feeding), the posterior haptor consists of suckers or clamps, (Hayward, 2005) where in Monopisthocotylea (epithelium feeding), the haptor contains one or two pairs of hooks (anchors). Polyopisthocotylean monogeneans are distinctly host specific than other groups of parasites (Kearn, 1998; Whittington *et al.*, 2000). *Gotocotyla secundus* belongs to the Order Monogenea, Suborder Polyopisthocotylea which lead to a reduction in healthy of stock due to lethargy , hypoxia and severe anemia (Sitjà-Bobadilla *et al.*, 2006). Also lead to hemorrhages, necrosis and deletion of filaments due to the insertion points of hooks (Paperna *et al.*, 1984). Monogeneans have been extensively used as an appropriate model to explore the patterns and processes of parasite evolution due to their high species diversity and host specificity (Desdevises *et al.*, 2002; Šimková *et al.*, 2002; Šimková *et al.*, 2006). The evolutionary relationships between different families of Polyopisthocotylea have been previously resolved using morphological characters (Boeger and Kritsky, 2001). When compared to morphology-based methods, phylogeny molecular markers provide a great deal of trustworthy information on evolutionary relationships. (Scotland *et al.*, 2003). The importance of molecular analysis has grown in recent years in order to conduct a quick and efficient phylogenetic investigation of the parasite. For the phylogenetic investigation of monogeneans, the partial sequences of the 28S rRNA gene were employed (Mollaret *et al.*, 2000; Mendlová *et al.*, 2010) This gene was selected as it has a wide number of domains with various evolutionary rates that can be applied to reconstruct phylogenies (Pace *et al.*, 1986). Thus, the current study aimed to identify monogeneans *G. secundus* in *S. commerson* based on some morphological and molecular characteristics, study their total and seasonal prevalence, and determine histopathological changes associated with the natural infections.

## MATERIALS AND METHODS

### 2.1. Fish sampling:

A total number of 100 *Scomberomorus commerson* (Lacepède, 1800) with different body weights (100-700 g) and lengths (20- 40 cm) were collected randomly and seasonally from the Red Sea<sup>02</sup> at the Suez Governorate from December -2019 to October-2020. The collected samples were identified according to Langdon and Jones (2002) and then transferred alive to the laboratory of Fish Diseases, Suez Canal University for further parasitological examinations

### 2.2. Clinical examination:

Fish total length (TL in cm) and bodyweight (BW in grams) were measured. Specimens of gills, gill chambers, mouth, and skin were examined macroscopically for the presence of monogenean parasites according to Noga (2010). Following clinical investigation, some left or right gills and pseudobranchs were selected randomly and fixed in 10% buffered formalin for histopathological studies.

### 2.3 Morphological analysis of isolated parasite:

The isolated monogeneans were fixed in 70% ethyl alcohol, stained with Semichon's acetocarmine, washed in several changes of distilled water, dehydrated through a graded ethanol series (70%–100%), cleared in clove oil, xylene, and mounted in Canada balsam. Micrographs were made with an Olympus camera attached to a light microscope according to (Pritchard and Kruse, 1982).

### 2.4. Molecular detection and sequence analysis of *Gotocotyla secundus*:

The gDNA of parasite preserved in 70% ethanol was extracted and purified using a commercial QIAamp DNA Mini Kit (Qiagen) and stored in hydration solution at 4 °C until used. PCR amplification for the ribosomal 28S rRNA was performed using universal primers AP103 F: 5' AGAGCGCAGCCAACCTGTGTGA 3' as forward primer and AP103 R: 5' TGCCACGTCCTAGCATCAGCC 3' as reverse primer, as described by (Arya *et al.*, 2016). PCR was carried out using 50 µL of a total reaction volume (2X Red master mix1X: Forward primer 20 picomole, Reverse primer 20 picomole, DNA extract 100 ng, Nuclease free water Up to 50µL). Standard cycle conditions for PCR were set as initial denaturation for 5 min at 94°C, followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 38 s at 56°C, and extension at 72°C for 42 s, and a final extension at 72°C for 7 min. To confirm the targeted PCR amplification, 5 µL of the amplicons were separated by 1% agarose gel at constant 80V for 30 min. The amplified product was visualized as a single compact band of expected size (300 bp) under UV light and documented by Samsung smart phone. Subsequently, Amplified DNA products were purified with Solgent Co. Ltd (South Korea). DNA sequence files were visualized and checked for quality in Chromas 2.6.6 (Technelysium Pty Ltd, Queensland, Australia) then

analyzed using the standard nucleotide Basic Local Alignment Search Tool (BLAST) BLAST® (**Johnson *et al.*, 2008**). Consequently, the sequence data were aligned, and the phylogenetic tree was constructed with MEGA X (**Kumar *et al.*, 2018**) using both the neighbor – joining (NJ) and maximum-likelihood methods according to Tamura-Nei model (**Tamura and Nei, 1993**) with 1000 bootstrap replicates.

### 2.5. Histopathological examination:

Using an automatic tissue processor, samples fixed in formalin were rinsed in running tap water for 24 h prior to getting dehydrated through a graded alcohol series and cleaned by xylene before being paraffin-embedded according to standard techniques (**Roberts, 2001**). Sections of 5µm thickness were obtained with a microtome (RM2245, Leica Biosystems, Germany). Sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope (Nikon Eclipse 80i) and photographed.

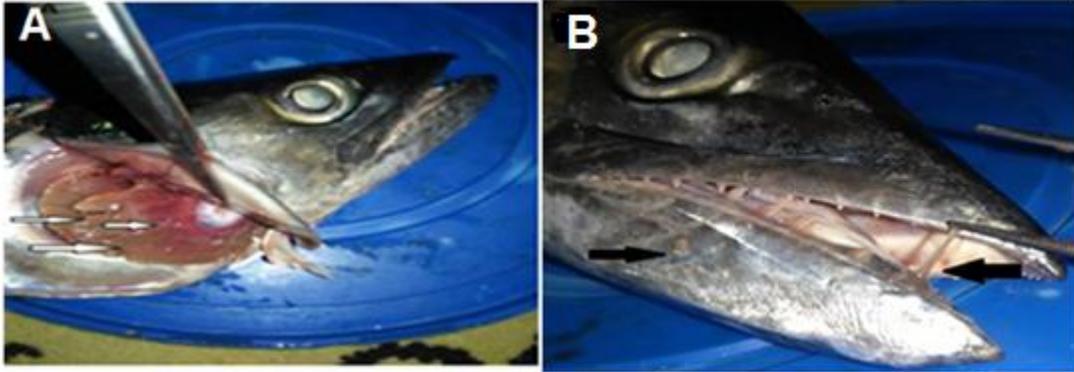
### 2.6 Statistical analysis of the parasite population

Prevalence (P%), abundance (A), and mean intensity (MI) were calculated according to **Bush *et al.* (1997)**. The effects of TL and BW on the parasite's prevalence and abundance as dependent variables were assessed using generalized linear models (GLMs). The collinearity between explanatory variables (TL and BW) was checked prior to the GLM analysis using the Spearman rank correlation test ( $\rho$ ). Because fish length and weight were positively correlated ( $(\rho = 0.980, p < 0.01)$ ), only BW was considered in the prevalence and abundance analyses. A binomial distribution with a logit link function was used to model the prevalence of infection (**Zuur *et al.*, 2009**). Because parasite abundance is a count data, various models such as the Poisson and negative binomial distributions were examined. The dispersion parameter ( $\emptyset$ ) was determined after constructing a Poisson GLM model. The Poisson distribution, corrected for over-dispersion, was used for ( $\emptyset$ )value  $>4$ , while the response variable was assumed to follow a negative binomial distribution for greater ( $\emptyset$ ) values (**Zuur *et al.*, 2009**). Both distributions were fitted using the log-link function. The GLM models were performed using IBM SPSS statistics Subscription software version.

## RESULTS

### 3.1. Clinical Examination of naturally Infected Fishes:

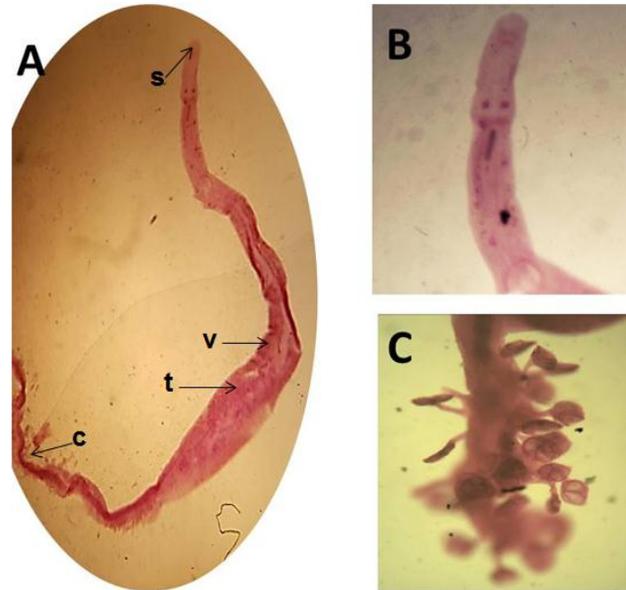
Most clinical signs in the naturally infected *Scomberomorous commerson* revealed no pathognomonic clinical abnormalities but in some cases, marbling appearance (areas of congestion and paleness) and necrosis (**Fig. 1A**). Other cases showed excess mucus and presence of parasites around the mouth area (**Fig.1B**)



**Fig. 1.** Naturally infected *Scomberomorus commerson* showed (A) necrosis and marbling appearance (areas of congestion and paler) in gills (B) excessive mucus secretion on the mouth of the infested fish with the presence of black spots around the mouth.

### 3.2. Morphology of isolated parasites:

The parasitological examination of all examined fish revealed the presence of monogenetic trematodes of family: Gotocotylidae (*Gotocotyla secundus*) which was isolated from the gills of *Scomberomorus commerson* with a range of 2-8/ fish. *G. secundus* was characterized by the body is flat, smooth and tapering from the level of middle testes to posterior end. The clamps are formed at the anterior end of opisthaptor. Anterior part of opisthaptor is with 2-3 rows and the posterior part is with 1 row of clamps along each side. One pair of hamuli present at the end of opisthaptor. Clamp is pedunculated, with a median sclerite that has winged ends, half of clamp is with smaller part of the median sclerite which has two submedian sclerites resting on winged end of the median sclerite and each supporting 1 transverse apical sclerite; Pre oral lobe is with two submedian fields of pre haptor gland openings. The mouth is subterminal, two buccal suckers are present, pharynx is large, esophagus is short and caeca are with medial and lateral diverticula except for most posterior parts. Numerous testes are found, in a median field from behind ovary to end of caecal diverticula there is a coiled vas deferens that enters long muscular cirrus pouch. There are germinal and terminal parts of ovary directed posteriorly, ovarian loop directed anteriorly, ootype, transverse yolk duct and seminal receptacle directed anteriorly to the germinal part of the ovary; uterus is median with a large round vaginal sucker, which is dorsal behind cirrus pouch (**Fig. 2**). It was identified based on **Tripathi (1956)**

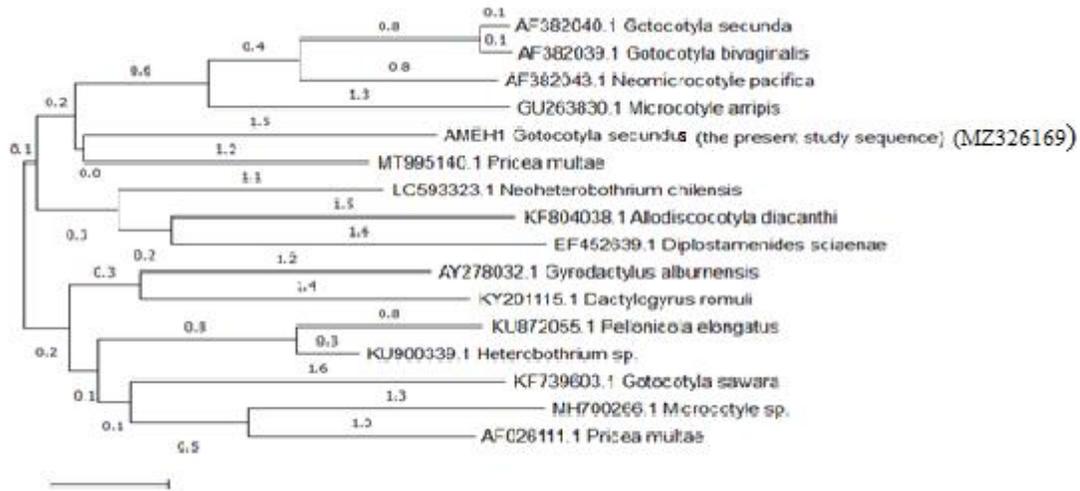


**Fig. 2.** Light photomicrograph of (A) *Gotocotyla secundus*; (B): anterior part; (C): posterior part; s.: sucker, t: testis , v: vitelline gland, c: clamps

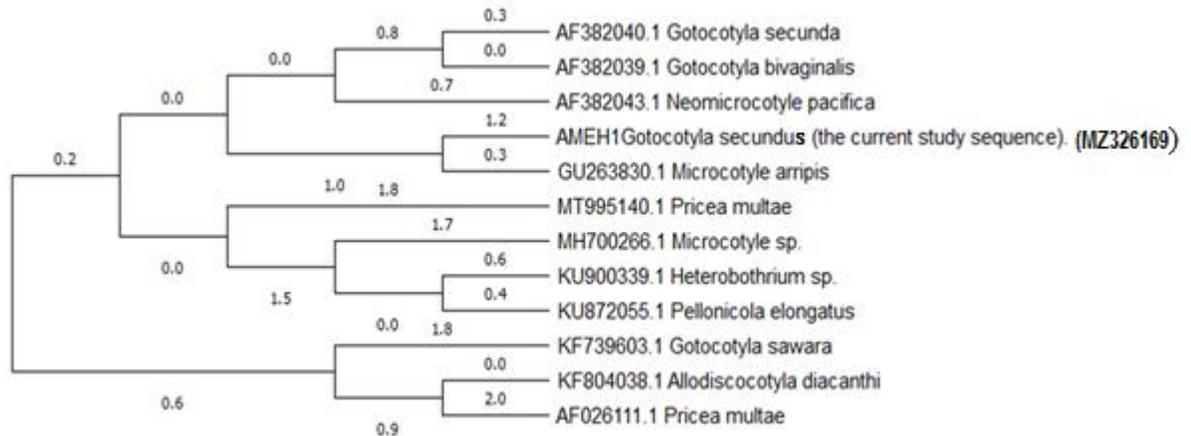
### 3.3. Molecular Identification of *Gotocotyla secundus*:

Based on the 28S rDNA sequences and the target sequence of AP103 primers, molecular identification of some of the collected monogenean species clearly visible bands surrounding the 300 bp on gel electrophoresis. The DNA sequences were blasted in GenBank and identified as *Genus Gotocotyla*.. The aligned sequence was submitted to the GeneBank database with accession No. MZ326169.

The dendrogram was constructed using both neighbor – joining (NJ) and the Maximum Likelihood methods (**Figs. 3& 4**), both NJ and ML methods clustered the sequences in two major groups with slightly different topology. In NJ method each group was divided in to two lineages and the analysis revealed that the current study sequences (*Gotocotyla secundus*) showed a close relationship between the species that were recorded from NCBI, *Gotocotyla secunda* (AF382040.1), *Gotocotyla bivaginalis* (AF382039.1), *Neomicrocotyle pacifica* (GU263830.1), *Microcotyle arripis* (AF382043.1) and *Pricea multae* (MT 995140.1), as they clustered together sharing the same ancestor, but ML method showed the same analysis except the species of *Pricea multae* (MT 995140.1) was found in a separate group.



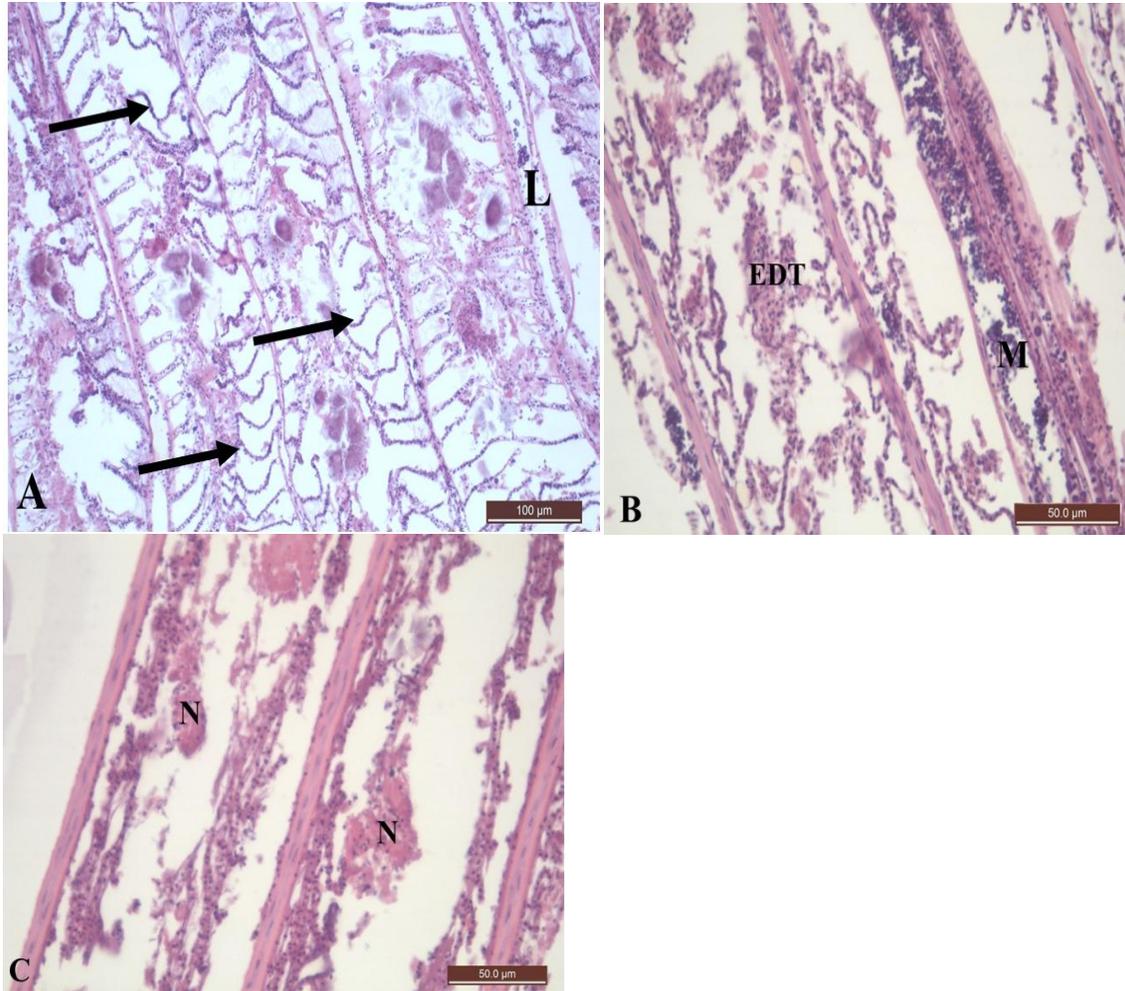
**Fig. 3 .** Phylogenetic analysis using neighbor – joining (NJ) methods, to construct the phylogenetic tree of some of Monogeneans species sequences from the GenBank and our sequence samples are included based on 28S rRNA sequences.



**Fig. 4.** Phylogenetic analysis using the Maximum Likelihood methods, to construct the phylogenetic tree of some of Monogeneans species sequences from the GenBank and our sequence samples are included based on 28S rRNA sequences.

### 3.4. Histopathological alterations:

The histopathological examination of the gills of *Scomberomorus commerson* revealed curling of secondary lamellae and leukocyte infiltration (**Fig. 5A**). Erosions, necrosis of the secondary lamellae with epithelial detachment (sluffing) and massive mononuclear cell infiltration at the base of gill arch and primary lamellae were observed (**Fig. 5B**). In addition, edema of the gill arches and secondary lamellar fusion along with atrophy and shortening of some lamellae were also present (**Fig. 5C**).



**Fig. 5. Photomicrographs of histopathological examination of *Scomberomorus commerson* infected with *Gotocotyla secundus*** **A)** Gills showing curling of secondary lamellae (black arrows) and leukocytic infiltration (L) H&E X 100. **B)** Gills showing necrosis of the secondary lamellae with epithelial detachment (EDT) and massive mononuclear cell infiltration (M) H&E X 400. **C)** Gills showing secondary lamellar fusion and necrosis (N) (H and E; X400).

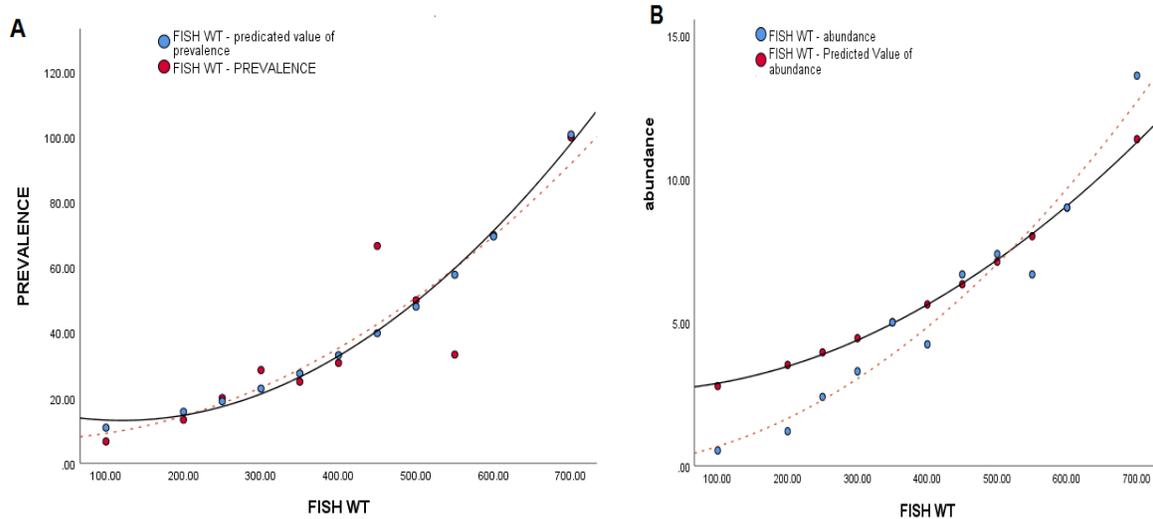
### 3.5. Statistical analysis of the parasite population:

Of the 100 host specimens analyzed, 37 (P% = 37%) were infected with *Gotocotyla secundus*. The seasonal prevalence was the highest in summer (13/37) (P% = 35.13%) followed by winter (10/37) (P% = 27.02%) then spring (8/37) (P% = 21.62%) and the lowest prevalence recorded in autumn (6/37) (P% = 16.21%) (**Table 1**). The lowest prevalence in relation to body weight was at >100 g. General linear model showed that the prevalence increased significantly with fish weight ((Intercept) B = 2.017, SE = 0.225,  $p = 0.00$ ; [WT] B = 0.004, SE = 0.004,  $p = 0.00$ ) (**Fig. 6A**). Predicted prevalence indicated a cumulative increase in the infection with fish growth. For specimens weighing 500 g TW, the model predicted a 50% infection rate and a 100% infection rate for those weighing 700 g and more. The abundance of parasite per infected fish ranged between

0.5 and 13.6. The mean abundance and mean intensity of parasites were 4.73 and 13.5, respectively. With an estimated dispersion parameter of 4.121, the Poisson GLM model fitted to parasite abundance showed over-dispersion; hence, a Poisson GLM corrected for over-dispersion was used. Fish weight had a significant effect on parasite abundance ([Intercept]  $B = 2.285$ ,  $SE = 0.869$ ,  $p = 0.009$  [TW],  $B = 0.03$ ,  $SE = 0.002$ ,  $p = 0.104$ ). The abundance of parasites increased with the increasing host weight (**Fig. 6B**).

**Table (1):** Variations in prevalence, mean intensity and abundance of infestation with *Gotocotyla secundus* in relation to fish weight

Fish (BW) g	No. Of Examined Fish	No. Of Infected Fish	Prevalence (%)	No. Of <i>Gotocotyla secundus</i>	Mean Intensity	Abundance
100-200	15	1	6.67	8	8.00	0.53
200-250	15	2	13.33	18	9.00	1.20
250-300	5	1	20.00	12	12.00	2.40
300-400	7	2	28.57	23	11.50	3.29
350-450	4	1	25.00	20	20.00	5.00
400-450	13	4	30.77	55	13.75	4.23
450-500	6	4	66.67	40	10.00	6.67
500-550	8	4	50.00	59	14.75	7.38
550-600	12	6	50	80	13.33	6.67
600-700	10	7	70.00	90	12.86	9.00
700-750	5	5	100.00	68	13.60	13.60
Total	100	37	37	473	13.51	4.73



**Fig. 6.** Relationship between *Scomberomorus commerson* body weight (BW in g) and prevalence (a) and abundance (b) of *Gotocotyla secundus*. The solid lines represent the best GLM models for characterising the relationships, while the dashed lines denote the 95 % confidence intervals.

## DISCUSSION

*Scomberomorus commerson* is a pelagic fish and it is like most pelagic fish that feed on planktonic crustaceans and small fish (Cardona *et al.*, 2012; Shawket *et al.*, 2017). Monogeneans are one variety of parasites found in fish. Regardless of the fact, that parasitic diseases are the most common infectious diseases in fish, they can also act as final, paratenic, or intermediate hosts in the parasite life cycle. (Roberts *et al.*, 2009). This study of monogeneans of *Scomberomorus commerson* in Suez governorate was based on the examination of gills and external body surface. *Gotocotyla secundus* were found in gills attaching to the host's surface by clamps (Justine *et al.*, 2013; Wong and Gorb, 2013) resulting in the mosaic appearance of the gills with sticking gill tips. This may be attributed to the destruction of the efferent vessels by such monogenean where the blood pressure is low and extensive hemorrhage cause very hard clotting of blood resulting in ischemia which in turn leads to necrosis in some areas due to the inflammation and congestion of some areas with progressive degeneration of other parts of the gill filaments giving the appearance of such phenomenon (Eissa *et al.*, 2017). Excessive mucus secretion may be to dilute the irritation caused by attachment, fixation and locomotion of monogenean parasites (Eissa *et al.*, 2016; Paladini *et al.*, 2017).

Species identification of monogenean parasites (2-6 mm in length) was based on the features of sclerotized parts of haptor and male copulatory organ (Ishii, 1936; LI, 2002). as *Gotocotyla secundus*. According to the criteria of Rohde (1976), the parasite described here is most likely from the *Gotocotyla* genus. (Tripathi, 1954). Some of the morphological characteristics revealed in this study were comparable to those previously reported for *Gotocotyla sawara* (Shi *et al.*, 2014) and *Gotocotyla acanthura* (Fernandes *et al.*, 2009; Pamplona-Basilio *et al.*, 2011).

Phylogenetic analyses based on morphological and molecular genetic data have played an important role in parasitological studies. Although the relevance of morphological analysis cannot be overstated, advances in sequencing technology have raised the importance of molecular analysis for phylogenetic studies as a more quick, efficient, and cost-effective tool (Perkins *et al.*, 2010). Many methods for constructing phylogenetic trees have been established, but none of them is consistently better than the others, and the product can be improved by combining methods (Guindon and Gascuel, 2003). Although the NJ method is regarded to be superior to the Maximum Parsimony method, it may produce the desired consequence if a correct distance measure is applied, which is dependent on the situation (Jin and Nei, 1990). In the current study both methods showed the same analysis, with slightly different sequence compositions, as one sequence species was in the same lineage with the current study in NJ method analysis, was found in a separate group with ML method analysis but still sharing the same ancestor of the major group with the current study sequence.

In the current study, the prevalence of infection of *Gotocotyla secundus* was 37%, which is lower than that reported by (Shawket *et al.*, 2018) which was 51.58%. The total

prevalence of *Gotocotyla* of Atlantic horse mackerel (*Trachurus trachurus*) in Moroccan North Atlantic and (Rohde, 1976) who reported *G. acanthura* in *Scomberomorus commersoni* (P% = 80.0) . Our results are higher than (Da Silva *et al.*, 2017) who found first record of *Gotocotyla acanthura* on the gills of *Katsuwonus pelamis* in the southwestern atlantic ocean with (11.76%). These differences in ratio may be due to different fish species and different localities. Here, the seasonal prevalence was higher in summer (35.13%) and winter seasons (27.02%). These results are similar to Da Silva *et al.* (2017) who found *Gotocotyla acanthura* in *Katsuwonus pelamis* mostly in winter (35.67%) and summer seasons (10.333%) . It was at its maximum in the summer because to the temperature-dependent acceleration of the free-swimming monogenean larvae life cycle (Etile and N'douba, 2018) . Bakke *et al.* (2007); Winger *et al.* (2008); Luo and Yang (2010) and Brazenor *et al.* (2018) confirmed that the high water temperature increased a short induction period, quick development and high egg production , confirming that high water temperature enhances monogenean infestations. Cool hypersaline environments, on the other hand, increased infection duration and success (Brazenor and Hutson, 2015). Furthermore, some monogenean species reproduce more quickly in warmer water temperatures, whereas others prefer cooler water temperatures (Öztürk and Altunel, 2006; Luo and Yang, 2010). Our results revealed that there was a collintary relationship between fish body length and weight and prevalence and abundance of the parasite with increasing the fish length and weight, the parasitic infestation of fishes was predicted to increase. This result was in agreement with Zelmer and Arai (1998); Polinas *et al.* (2018) . This could be attributable to the parasites accumulating over time, as well as the huge surface area of gills available for the parasites to settle down (Koskivaara *et al.*, 1992; Machado *et al.*, 2005; Šimková *et al.*, 2006). Also, water that passes through the gills increases the opportunities for the parasite larvae to invade the host (Buchmann and Uldal, 1997; El Madhi and Belghyti, 2006a, 2006b). The study of host-parasite relationships in natural ecosystems can help researchers better understand fish pathologies in confined habitats, where infestations are more prevalent. As a result, the findings of this study are still remarkable.

Concerning the histological findings, there were curling of secondary lamellae and leukocytic infiltration. Erosions, necrosis of the secondary lamellae with epithelial detachment (sluffing) and massive mononuclear cell infiltration at the base of gill arch and primary lamellae were observed. In addition, edema of the gill arches and secondary lamellar fusion along with atrophy and shortening of some lamellae were also present. These results were in agreement with Mahmoud *et al.* (2014); Hassan *et al.* (2015) and Gado *et al.* (2017) .

## CONCLUSION

We concluded that, there was no relationship between parasitic infestation and different seasons as parasitic infection occurred during warm and cool temperature with higher rates but there was a relative relationship between fish growth (increased weight

and length) with infection as higher infection rate occurred in the bigger fish. Molecular analyses and sequencing are the most efficient methods for identification of parasites.

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