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Identification of Gnathiid Isopods as Ectoparasites of the Brown-spotted Grouper *Epinephelus chlorostigma* from the Red Sea of Saudi Arabia

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ABSTRACT

Background: Gnathiids are considered excellent ectoparasites widely distributed along the fish body with a biphasic life cycle including parasitic larval stages and free-living adults. This study focused on gnathiids infecting the brown-spotted grouper fish and confirming the identity of the host species.

Methods: Twenty *Epinephelus chlorostigma* (Serranidae) were collected from the Red Sea Coast (especially off Jeddah, Saudi Arabia) and examined for gnathiid fish parasites. Species identification of the larvae was based on morphological criteria of Smit and Basson (2002). In contrast, the taxonomy of fish species was supported by amplification and sequencing of host DNA via the mitochondrial DNA genes (COI and 16S rRNA) using gnathiids' blood meals.

Result: Praniza larvae of a gnathiid isopod were collected from the gill chambers of the examined fish. The prevalence rate was 60% and mean intensity of the pranzia larvae was 16. The pranzia larva is described microscopically and distinguished by large body size (3.51-5.90 mm), the presence of 8th teeth in the mandible, maxilliped with 5-7th teeth on the first palp and the endite hooked, pleotelson with concave anterolateral margins and rami of uropods extend its apex. The recovered larvae could be identified as pranzia larvae of *Gnathia pantherina* Smit and Basson, 2002 due to the morphological resemblance. The host species identity was confirmed molecularly with COI and 16S rRNA genes and showed highly stringent criteria with the previously *E. chlorostigma* sequence data. The obtained host DNA sequences were deposited in NCBI database under accession numbers ON384530.1 for COI and ON384544.1 for 16S rRNA.

Key words: 16S rRNA gene, COI gene, Gnathiidae, Host specificity, Isopoda, Pranzia larva.

INTRODUCTION

Crustaceans establish one of the four recent subphyla of the phylum Arthropoda. They occur on a fish host on the outer body or fins, in the mouth, gill chambers, or nostrils, or occasionally in self-made pockets in the flesh of their hosts (Hoffman 1999). Three main parasitic crustaceans affecting commercially important fish species: Branchiura, Copepoda and Isopoda (Jithendran *et al.* 2008). Isopoda is a widespread crustacean order, including 17 families recognized as parasitic taxa and each showed distinct morphology and ecology (Sikkel and Welicky 2019). Gnathiidae Leach, 1814 has been recognized as a highly specialized family among Isopoda and includes 16 genera and about 294 species (WoRMS 2022).

Gnathiid isopods exhibit significant morphological differences between the larva, adult male and adult female (Chong *et al.* 2015) and undergo a biphasic life cycle involving parasitic larvae and non-feeding adults. The larvae (praniza and zuphea) actively swim to attach to infect teleosts and/or elasmobranch fish to suck their body fluid. In contrast, the adults are non-feeding and only reproduce in benthic substrata (Tinsley and Reilly 2002). The taxonomic descriptions of gnathiids have been based entirely on adult male morphology, making identifying of females and larvae difficult (Ferreira *et al.* 2009). Juveniles are also morphologically and ecologically similar across taxa, so there is little about their natural history to assist in classifying them (Smit and Davies 2004). These identification problems and infrequent detection have led to a relatively poor understanding of gnathiid host specificity (Jones *et al.* 2007).

DNA-based taxonomy and identification, a powerful tool incorporated into many current biomonitoring studies, has resulted in the discovery of new faunas (Baird and Sweeney 2011; Verma 2022). Using DNA barcoding for species identification is becoming more accessible, reliable and more widely adopted, supplanting traditional morphology-based species identification (Ali *et al.* 2014). Phylogeny hypotheses

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based on the evaluation of DNA sequences were proposed by Jones *et al*. (2007) reported that the specific vertebrate 12S and 16S rDNA mitochondrial gene regions from fed (praniza) gnathiids could help in the identification of the host specificity in some gnathiids of the genus *Gnathia* Leach, 1814. Nerlović *et al*. (2015) stated that the fish identity was confirmed by the partial analysis of mitochondrial cytochrome c oxidase subunit I (COI) and 16S ribosomal RNA (16S rRNA) gene sequences. Hendrick *et al*. (2019) and Cucalón *et al*. (2022) ensured successful host identification by using DNA-based methods.

Despite an extensive descriptive study of their biology early in the last century, little progress has been made in understanding aspects of their basic biologies, such as taxonomy and host specificity (Tanaka 2004). Therefore, the host preference of each gnathiids species is uncertain. However, literature on copepod parasites of the groupers is relatively rare. The present study aimed to study the prevalence and description of gnathiid isopods from the brown-spotted grouper *Epinephelus chlorostigma* from the Red Sea (Saudi Arabia). Additionally, identify the host that corresponding with gnathiids molecularly.

MATERIALS AND METHODS

Collection and examination of fish

Samples of the brown-spotted grouper *Epinephelus chlorostigma* (n=20) were collected from the boat landing sites of Jeddah (the Red Sea coast, Saudi Arabia) during the period of January-December 2021. Fish were transported immediately to the laboratory of Parasitology (Zoology Department, College of Science, King Saud University, Riyadh, Saudi Arabia) for further examination.

Parasitological examination

The four-gill arches were examined microscopically for the presence of ectoparasites with a stereomicroscope (Nikon SMZ18, NIS ELEMENTS software). Isopods were collected and fixed in 70% ethanol for morphological studies or 99% ethanol for molecular analysis. The preserved specimens (70% ethyl alcohol) were dehydrated with a glycerin ethanol series and then mounted as temporary preparations in lactophenol, according to Pritchard and Kruse (1982). Specimens were observed by a Leica DM 2500 microscope (NIS ELEMENTS software, version 3.8) and illustrations for body parts were done with a Camera Lucida. Measurements were taken from digitalized illustrations using Image J 1.53e software (Wayne Rasband and contributors, National Institute of Health, USA) and expressed in millimeters. Parasitological terms for prevalence and mean intensity were calculated according to Bush *et al*. (1997).

Molecular barcoding of fish

The preserved gnathiids were washed twice in 200 µl 1× TE buffer pH 8.0 (to remove ethanol) and then squashed (to expose blood meals) for digestion overnight in 10 µl proteinase K at 37°C. After lysis, 56 µl of 5 M sodium chloride and 40 µl CTAB buffer were added to the mixture and then incubated at 65°C for 1 hr, according to Hirose *et al*. (2009). DNA was extracted using a DNeasy Blood and Tissue Kit (QIAGEN, Germany) following the recommended steps. From the mitochondrial genome, two gene regions were targeted for the COI gene using primers of FISH-BCL (5′-TCA ACY AAT CAY AAA GAT ATY GGC AC -3′) and FISH-BCH (5′- ACT TCY GGG TGR CCR AAR AAT CA -3′), which designed by Baldwin *et al*. (2009) and the 16S rRNA gene using primers of 16Sar (5′- CGC CTG TTT ATC AAA AAC AT -3′) and 16Sbr (5′- CCG GTC TGA ACT CAG ATC ACG T -3′), which designed by Palumbi *et al*. (2002). PCR amplification under the following conditions: 95°C for 3 min, followed by 35 cycles at 95°C for 20 s, 55°C (COI) and 52°C (16S) for 20 s and 72°C for 40 s, with a final extension at 72°C for 10 min. PCR products were sequenced using ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). A search on BLASTn was performed to determine the most related sequences available in GenBank for COI and 16S rRNA datasets (Table 1 and 2). Analysis of the aligned DNA sequences of the partial COI and 16S rRNA gene regions was done by the maximum likelihood (ML) using MEGA7 (Tamura *et al*. 2013) with the best-fit substitution models. Statistical support for each node was evaluated using a non-parametric bootstrap test with 1000 replicates.

RESULTS AND DISCUSSION

Natural prevalence and mean intensity of parasitic infection

A parasitological study of the examined fish *E. chlorostigma* revealed the presence of one isopod species belonging to the family Gnathiidae. This parasite was naturally infected gill fish chambers. Only pranzia larval stage for *Gnathia* species was identified morphologically. These larvae were detected more in the first gill followed by the rest (Table 3).

Table 1: Gen bank accession numbers for COI sequences used in ML analysis.

Family	Species	Accession no.
Serranidae	*Epinephelus areolatus*	KU499599.1
Serranidae	*Epinephelus polylepis*	MF383178.1
Serranidae	*Epinephelus chlorostigma*	MN708876.1
Serranidae	*Epinephelus spilotoceps*	FJ237772.1
Serranidae	*Epinephelus japonicus*	LC554183.1
Serranidae	*Epinephelus flavocaeruleus*	MH707753.1
Serranidae	*Epinephelus multinotatus*	MH707783.1
Serranidae	*Epinephelus cyanopodus*	MH707749.1
Serranidae	*Epinephelus malabaricus*	MN708928.1
Serranidae	*Epinephelus costae*	MT231143.1
Scorpaenidae	*Scorpaena plumieri*	MT998289.1
Pomacentridae	*Neopomacentrus cyanomos*	MT178808.1
Lethrinidae	*Lethrinus lentjan*	MW737442.1
Holocentridae	*Ostichthys japonicus*	MN870599.1
Carcharhinidae	*Prionace glauca*	MW793454.1
Belonidae	*Belone belone*	KY176405.1

The prevalence and mean intensity of the collected parasite taxa are recorded as 65% (13/20) and 16, respectively.

Description for pranzia larva of *Gnathia* species

The body was 3.51-5.90 mm long. It is divided into three parts of cephalosome (including antennae and mouthparts), pereon (with six pairs of pereopods) and pleon (with five pairs of pleopods and telson with one pair of uropods).

Cephalosome sub-circular with straight posterior margins (Fig 1). Compound eyes are oval-shaped and located on lateral margins of cephalosome. Labrum was semicircular with the apical process. Antennule with three pedunculated articles; articles one and two with a pair of setae; article three longest with a couple of setae. Antenna with four pedunculated articles and the fourth is the largest. Mandible stout and the distal margin with eigth large teeth.

Table 2: Gen bank accession numbers for 16S rRNA sequences used in ML analysis.

Family	Species	Accession no.
Serranidae	*Epinephelus macrospilos*	AY731072.1
Serranidae	*Epinephelus sexfasciatus*	DQ067310.1
Serranidae	*Epinephelus bleekeri*	DQ088042.1
Serranidae	*Epinephelus flavocaeruleus*	EF503625.1
Serranidae	*Epinephelus awoara*	AY947558.1
Serranidae	*Epinephelus chlorostigma*	KM077973.1
Serranidae	*Epinephelus areolatus*	DQ088038.1
Serranidae	*Epinephelus cyanopodus*	KM077976.1
Serranidae	*Epinephelus malabaricus*	AJ496738.1
Serranidae	*Epinephelus costae*	KM077988.1
Labridae	*Scarus frenatus*	JX026486.1
Mullidae	*Pseudupeneus grandisquamis*	AY947853.1
Lutjanidae	*Lutjanus campechanus*	KX354246.1
Labridae	*Halichoeres marginatus*	JF457481.1
Gobiidae	*Eutaeniichthys gilli*	AB108578.1
Chaetodontidae	*Chaetodon fremblii*	DQ278888.1

Gnathopod 7-segmented; basis and ischium fused; merus rectangular; carpus triangular; propodus rectangular; and hooked dactylus. Maxilliped is cylindrical and composed of three articled palps. Article one with five teeth and article three with six setae. Maxillule long with seven teeth on the distal margin. Paragnaths were elongated and terminated at a sharp point.

The pereon was larger than the cephalosome (Fig 2). Pereonite 1 short and fused with cephalon. Pereonite two with anterior constriction separating it medially from first pereonite. Third pereonite is the largest one. Fourth pereonite with a rounded posterior margin stretching over fifth pereonite and lateral shields at the leg connection. Fifth pereonite with bulbous shields on lateral sides at leg connection. Sixth pereonite rectangular with concave posterior margin. Seventh pereonite is small with a rounded posterior margin overlapping the first pleonite.

Pereopods was six-segmented and larger than gnathopod. Base is larger than the others, with one seta. Ischium was three-quarters of a base length. Merus is equipped with an anterior bulbous protrusion and long setae. Carpus is similar to merus but without bulbous protrusion. Propodus is equipped with one to four setae. Dactylus is terminating in a sharp point with no setae.

Pleon with five pleonites covered with varying number of setae (Fig 3). Pleopods have exopod and endopod of similar size (endopod with six setae and exopod with nine setae). Pleotelson was triangular with two pairs of setae on the dorsal surface and the distal tip ending in a couple of setae. Uropods were biramous, endopod longer and broader than exopod, both with six simple setae.

Molecular identification of fish host

The partial sequences of the mt COI and 16S rRNA genes were amplified *via* PCR reaction generating 653 and 608 bp, respectively. The average base-pair proportions for COI were A(23.12% 151) | C(29.56% 193) | G(19.45% 127) | T(27.87% 182) and for 16S rRNA were A(28.95% 176) |

Table 3: Infection and site attachment of pranzia larvae of *Gnathia* species in the infected fish host.

Infected host number		No. larvae in each gill arch				Total number of pranzia larvae
		Gill 1	Gill 2	Gill 3	Gill 4	
Epinephelus chlorostigma	1	8	5	2	1	16
Epinephelus chlorostigma	2	11	2	1	-	14
Epinephelus chlorostigma	3	10	4	2	1	17
Epinephelus chlorostigma	4	10	5	3	-	18
Epinephelus chlorostigma	5	8	4	3	1	16
Epinephelus chlorostigma	6	11	5	2	-	18
Epinephelus chlorostigma	7	10	4	2	1	17
Epinephelus chlorostigma	8	7	3	2	0	12
Epinephelus chlorostigma	9	8	5	2	-	15
Epinephelus chlorostigma	10	11	3	2	1	17
Epinephelus chlorostigma	11	9	4	2	1	16
Epinephelus chlorostigma	12	10	6	2	1	19
Epinephelus chlorostigma	13	7	4	2	1	14

C(24.18% 147) | G(22.53% 137) | T(24.34% 148). PCR products were deposited in GenBank under the accession numbers ON384530.1 (for COI gene) and ON384544.1 (for 16S rRNA gene). One family (Serranidae) for the fish host was identified for the recovered praniza larva. All the GenBank entries that matched our COI sequence under the highly stringent criteria (92.70-99.69% identity, 96-100% query coverage and E-value 0.0) were assigned to the species of *Epinephelus*. The GenBank datasets that related to our 16S rRNA sequence data with homology 98.74-99.67% with 91-100% query coverage and E-value 0.0 were also recorded for *Epinephelus* species. In ML analyses (Fig 4), the taxa of *Epinephelus* were grouped in a distinct clade with high bootstrap values of 93 (COI) and 100 (16S rRNA). Dendrograms confirmed the association of our specimen with *Epinephelus* group, with special reference to *E. chlorostigma* for COI (MN708876.1) and 16S rRNA (KM077973.1).

Gnathiidae is a family of isopod crustaceans with a biphasic life cycle that includes larval and adult stages (Smit and Davies 2004; Chong *et al.* 2015). Gnathiid larvae are temporal ectoparasites of teleosts and elasmobranchs (Ota *et al.* 2012). The current study reported the natural occurrence of gnathiid larvae in gill chambers of the examined fish (*E. chlorostigma*), which agreed with Tanaka (2007) stated that gnathiids are generally found to infect various sites of the hosts' bodies such as gills, buccal cavity, nares, eyes, body surface and fins. Concerning the gnathiid prevalence (60%) and intensity (16), these results agreed

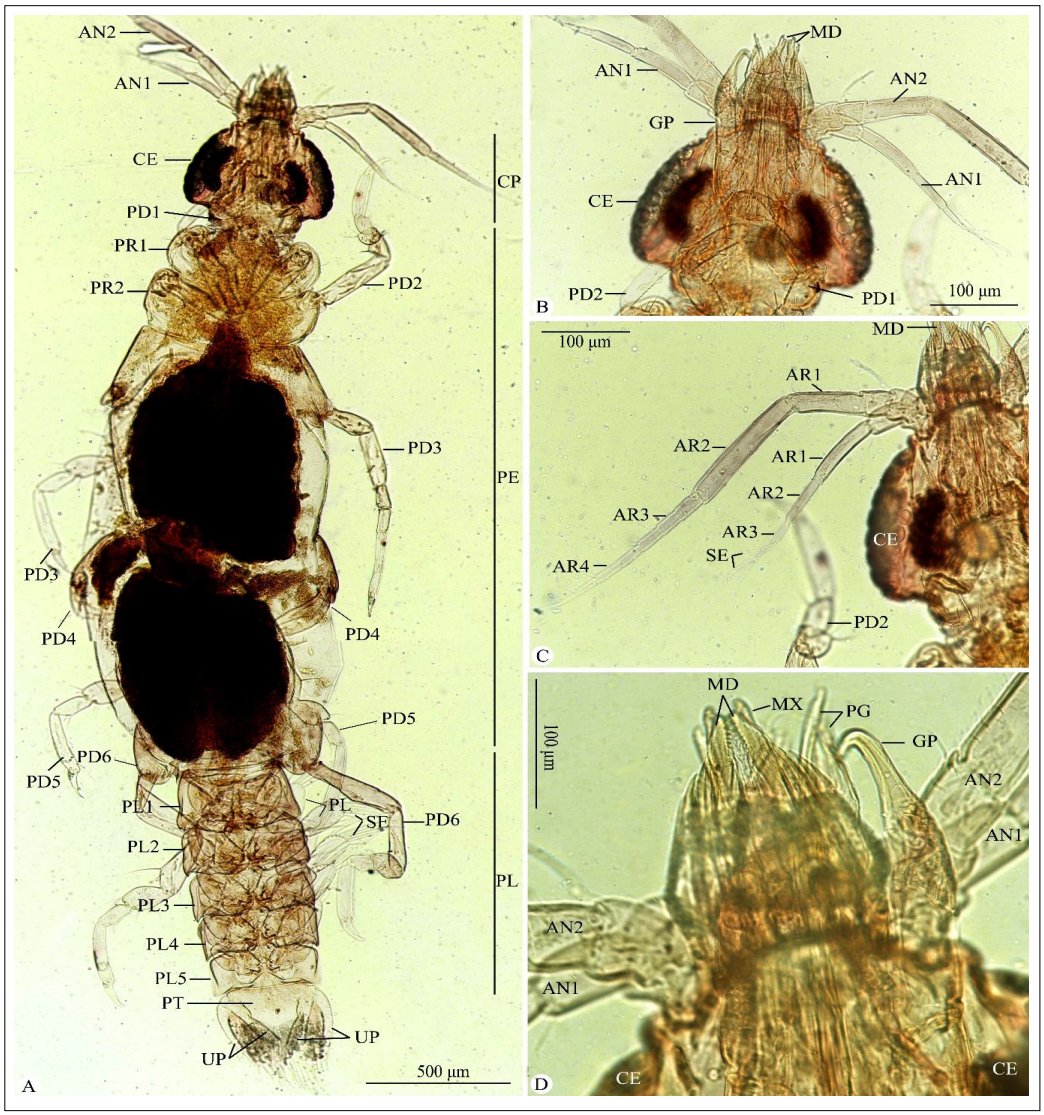

Fig 1: Light micrographs for praniza larva of *Gnathia* species infecting *E. chlorostigma*. (A) Whole mount preparation. (B-D) High magnification for cephalosome and its appendages. Note: CP: Cephalosome; PE: Pereon; PL: Pleon; AN1: Antennule; AN2: Antenna; PR: Pereonite; PD: Pereopod; PT: Pleotelson; UP: Uropod; GP: Gnathopod; PG: Paragnaths; CE: Compound eye; MD: Mandible: SE: Setae; AR: Article; MX: Maxillule; PL: Pleonite; PLO: Pleopod.

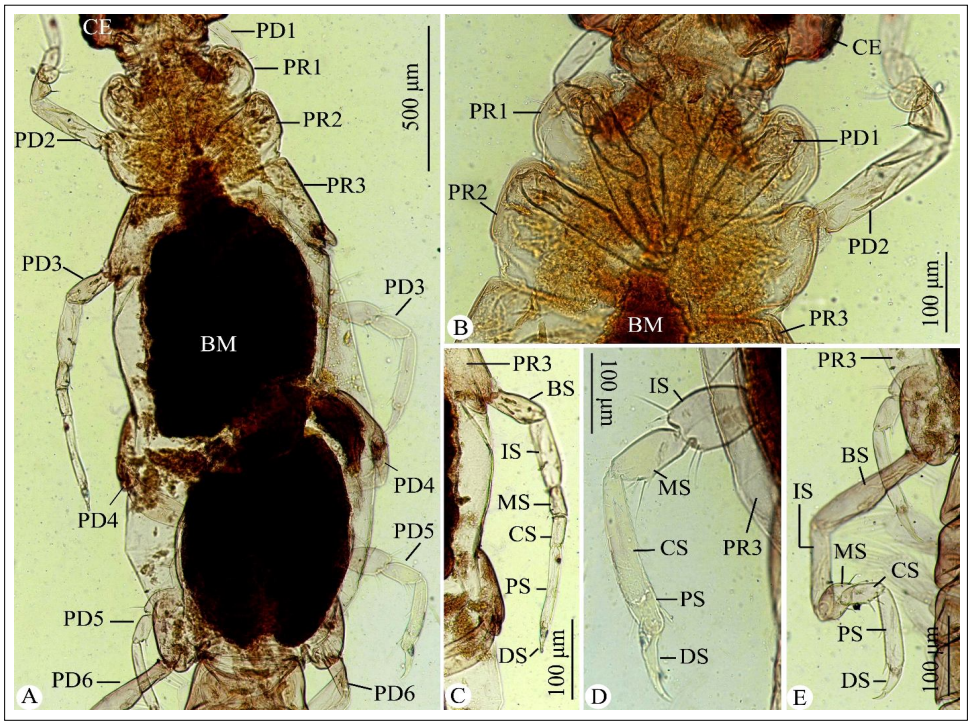

Fig 2: Light micrographs for praniza larva of *Gnathia* species infecting *E. chlorostigma*. (A) Pereon with its appendages. (B-E) High magnification for different pereon parts. (B) First and second pereonites and associated pereopods. (C) Third pereopod. (D) Fourth pereopod. (E) Fifth pereopod. Note: CE: Compound eye; PR: Pereonite; PD: Pereopod; BM: Blood meal; BS: Base; IS: Ischium; MS: Merus; CS: Carpus; PS: Propodus; DS: Dactylus.

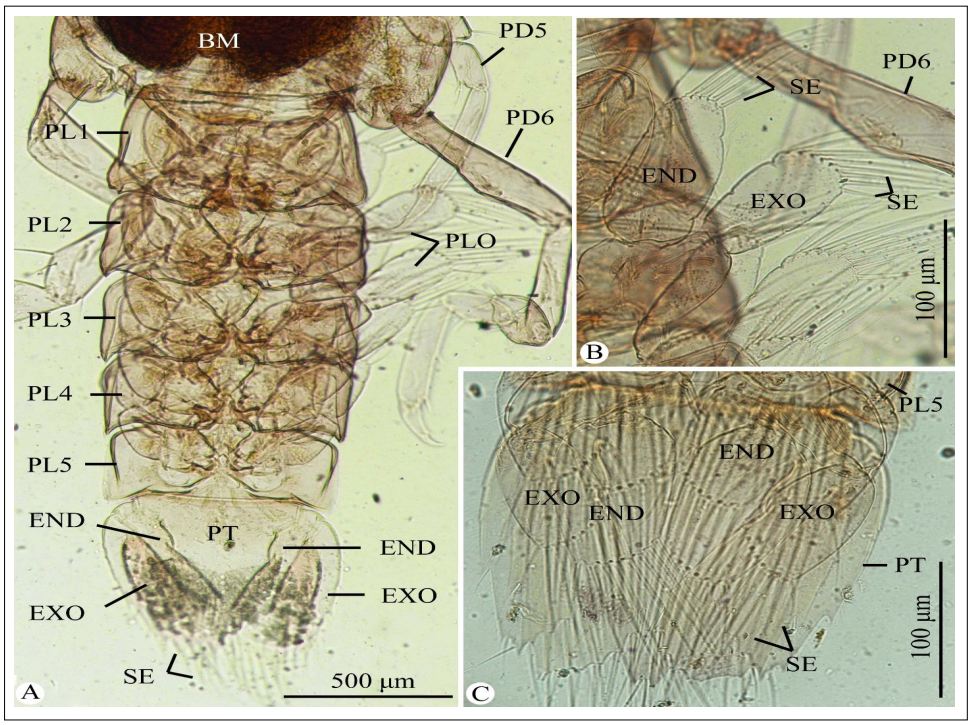

Fig 3: Light micrographs for praniza larva of *Gnathia* species infecting *E. chlorostigma*. (A) Pleon and its appendages. (B) (C). First pleopod with exo- and endopods provided with setae. (C) Pleotelson overlapped with last pair of pleopod and uropods. Note: BM: Blood meal; PD: Pereopod; PL: Pleonite; PLO: Pleopod; END: Endopod; EXO: Exopod; PT: Pleotelson; SE: Setae.

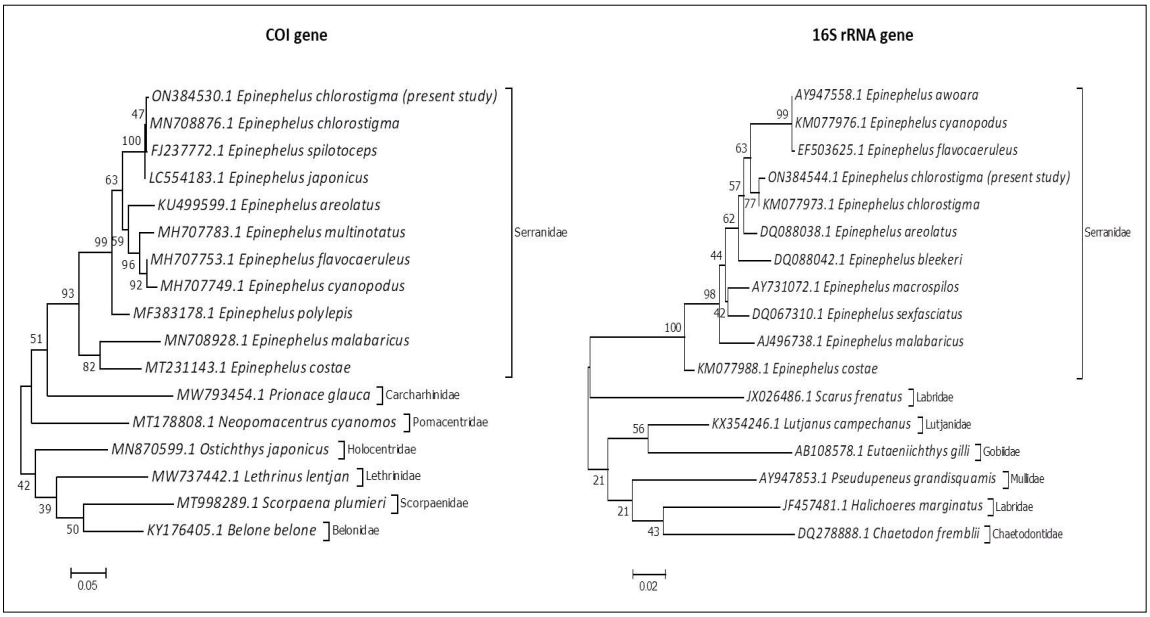

Fig 4: Molecular phylogenetic analysis was done by ML method for COI and 16S rRNA gene regions based on the Tamura-Nei model.

with data obtained by Bayoumy *et al*. (2013) for *Epinephelus tauvina* (Arabian Gulf, Saudi Arabia) and Tuan *et al*. (2015) for *Parupeneus hepatacanthus* (Cam Rahn Bay, Vietnam).

Gnathiid taxonomy is traditionally based on the morphology of adult males (Ota *et al.* 2016), but no males were collected in this study. Instead, the current investigation must consider the taxonomic position of the gnathiids from the Red Sea coast (Saudi Arabia) based on the praniza larvae. Smit and Basson (2002) reported the main diagnostic criteria for differentiation of praniza larvae to include the body length, pleotelson shape and the number of mandibular teeth. There are few reports of gnathiids on marine fish in Saudi Arabia (Bayoumy and Abu-Taweel 2012; Bayoumy *et al.* 2013; Bakhraibah 2018). Therefore, this study is focused on providing a detailed larval description. By revising the morphology of the previously described gnathiid larvae, the present larva is somewhat resemblance to those of *Gnathia pantherina* Smit and Basson, 2002, by having common features with special reference to the presence of eight teeth in the mandible, five to seven teeth in the first palp of maxilliped, the hooked maxilliped endite, concave anterior lateral margins of pleotelson and the uropodal rami extend beyond the apex of the pleotelson.

Gnathiids are considered excellent blood-sucking parasites due to the presence of well-developed mouthparts (Hadfield 2019). DNA from gnathiid blood meals can be sequenced to identify hosts (Hendrick *et al.* 2019). The mitochondrial DNA (mtDNA) has several advantages in understanding host specificity (Jones *et al.* 2007; Teletchea 2009; Nerloviæ *et al.* 2015). Herein, two mtDNA genes of COI and 16S rRNA were amplified and sequenced to identify the fish host, which is consistent with Armani *et al*. (2012) to be the most commonly used mitochondrial genes, due to a lower inter-species variability for 16S rRNA (Gerber *et al.* 2001) and low intra-species variation for COI (Xiong *et al.* 2016). By analyzing the COI and 16S rRNA sequences, the host species identity was confirmed via >99% matches with *E. chlorostigma* (Serranidae) sequence data recorded in the NCBI GenBank database. These results are agreed with Genc *et al*. (2005, 2011), Bunkley-Williams *et al*. (2006), Genc (2007), Chong *et al*. (2015), Cruz-Lacierda and Nagasawa (2017) reported that gnathiids appear to be much more host-specific to the Epinephelinae group, which related to the duration of attachment with the host between days to weeks (McKiernan *et al.* 2005).

CONCLUSION

The recovered larvae could be identified as the pranzia larva of *G. pantherina* with a new host species (*E. chlorostigma*) and geographical distribution (the Red Sea especially off Jeddah, Saudi Arabia). In addition, this study confirms the ability of DNA in blood meals of *Gnathia* species for host identification of gnathiids. Future studies are recommended to identify its pathogenicity and impact on *E. chlorostigma* fish.

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Compliance with ethical standards

Conflict of interest

The author(s) declare that they have no conflict of interest regarding the content of this article.

Data availability statement

All the datasets generated or analyzed during this study are included in this published article.

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