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First report on nematode parasite infection in the yellowbar angelfish *Pomacanthus maculosus* (Perciformes: Pomacanthidae) from the Iraqi coral reef, with description of a new species of *Cucullanus* (Nematoda: Ascaridida) using the integrated approaches

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ABSTRACT

The yellowbar angelfish *Pomacanthus maculosus* (Forsskål) (Perciformes: Pomacanthidae) is a beautiful, marine ornamental fish with very important commercial value. However, to date, no information is available on nematode parasite infection in *P. maculosus*. In the present study, the integrated approaches including light and scanning electron microscopy, and sequencing and analysing ribosomal [small ribosomal DNA (18S) and internal transcribed spacer (ITS)] and mitochondrial [cytochrome *c* oxidase subunit 1 (*cox*1)] target regions, respectively, were employed for the systematic evaluation of the nematode parasites firstly isolated from *P. maculosus* in the Iraqi coral reef. The results revealed that these nematodes represent a new species of *Cucullanus* (Ascaridida: Cucullanidae). The phylogenetic analyses based on 18S, ITS and *cox*1 sequences were constructed, respectively, to assess the phylogenetic relationships between the new species and the other cucullanids, and the monophyly of *Cucullanus*, as well as some its related genus-level taxa. The results supported *C. extraneus* n. sp. appear to be sister to *C. hainanensis*, and the genera *Cucullanus*, *Dichelyne* and *Truttaedacnitis* may be not monophyletic assemblages. This is the first report of the occurrence of nematode parasites in *P. maculosus*.

1. Introduction

The yellowbar angelfish *Pomacanthus maculosus* (Forsskål) (Perciformes: Pomacanthidae) is a beautiful, omnivorous, coral reef fish distributed throughout the Arabian Gulf, the northwestern Indian Ocean and the Red Sea, which mainly feeds on algae, sponges and encrusting invertebrates [1–4]. Owing to its striking colour patterns, *P. maculosus* is also a marine ornamental fish with very important commercial value [5]. However, to our knowledge, there have been no nematode parasites recorded from this fish. During a helminthological survey of the marine fishes in the Arabian Gulf, numbers of *Cucullanus* nematodes were collected from *P. maculosus* in the Iraqi coral reef. The accurate identification of parasites at any developmental stages is imperative to study parasite biology, resolve systematic problems and diagnose and control of parasite disease [6–8]. However, it is not easy to exactly recognise of *Cucullanus* species purely based on morphological methods because of their considerably uniform morphology in the species. Consequently, in the present study, the integrated approaches, including light and scanning electron microscopy, and sequencing and analysing the ribosomal [small ribosomal DNA (18S) and internal transcribed spacer (ITS)] and mitochondrial [cytochrome *c* oxidase subunit 1 (*cox*1)] target regions, respectively, were used to exactly identify these parasites to the species level. In addition, in order to assess the phylogenetic relationships between the *Cucullanus* nematodes obtained herein and the other cucullanids, and the monophyly of *Cucullanus*, as well as some its related genus-level taxa, the phylogenetic analyses based on different genetic markers were constructed, respectively.

2. Materials and methods

2.1. Light and scanning electron microscopy

Ten *P. maculosus* with body length 18.5–31.0 cm caught in the Iraqi coral reef, Arabian Gulf (29°25′N, 48°48′E), were examined for parasites. Nematodes recovered from the intestine of fish, were washed in

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physiological saline and fixed and stored in 80% ethanol until studied. For light microscopical studies, nematodes were cleared in lactophenol. Drawings were made with the aid of a Nikon microscope drawing attachment. For scanning electron microscopy (SEM), specimens were fixed in 4% formaldehyde solution, post-fixed in 1% OsO4, dehydrated *via* an ethanol series and acetone and critical point dried. The specimens were coated with gold and examined using a Hitachi S-4800 scanning electron microscope at an accelerating voltage of 20 kV. Measurements (the range, followed by the mean in parentheses) are given in micrometres unless otherwise stated. The type-specimens are deposited in College of Life Science, Hebei Normal University, Hebei Province, China.

2.2. Molecular data

One female and two males were randomly selected for molecular analysis. Genomic DNA from individual worms was extracted using a Column Genomic DNA Isolation Kit (Shanghai Sangon, China) according to the manufacturer's instructions. DNA was eluted in elution buffer and kept at −20 °C until use. The partial 18S rDNA was amplified by polymerase chain reaction (PCR) using the primers 18SF (forward: 5′-CGC GAA TRG CTC ATT ACA ACA GC-3′) and 18SR (reverse: 5′-GGG CGG TAT CTG ATC GCC-3′) [9]. The *cox*1 was amplified by PCR using the primers CO1F (forward: 5′-TTT TTT GGT CAT CCT GAG GTT TAT-3′) and CO1R (reverse: 5′-ACA TAA TGA AAA TGA CTA ACA AC-3′) [10]. The ITS region was amplified by PCR using the primers A (forward: 5′-GTC GAA TTC GTA GGT GAA CCT GCG GAA GGA TCA-3′) and B (reverse: 5′-GCC GGA TCC GAA TCC TGG TTA GTT TCT TTT CCT-3′) [11]. The cycling conditions were as described previously [12]. PCR products were checked on GoldView-stained 1.5% agarose gels and purified with Column PCR Product Purification Kit (Shanghai Sangon, China). Sequencing was carried out using a DyeDeoxyTerminator Cycle Sequencing Kit (v.2, Applied Biosystems, California, USA) and an automated sequencer (ABI-PRISM 377). Sequencing for each sample was carried out for both strands. Sequences were aligned using ClustalW2 [13] and adjusted manually. The newly-generated sequences were compared (using the algorithm BLASTn) with those available in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm. nih.gov).

2.3. Phylogenetic analyses

Phylogenetic trees were inferred using maximum likelihood (ML) and Bayesian inference (BI). *Zeldia punctata* (Rhabditida) and *Meloidogyne haplanaria* (Tylenchida) were chosen as the outgroup according to Choudhury & Nadler (2016) [14]. Sequences of 18S, ITS and *cox*1 were individually aligned using MUSCLE algorithm [15] in MEGA 7 [16] with default alignment parameters and then refined manually. We used MEGA 7 to select the best-fitting substitution model by BIC criterion for each alignment. The K2 + G + I model was identified as optimal for 18S and ITS and the HKY + G model was for *cox*1. We executed the analyses under Bayesian inference using MrBayes v3.2.6 [17,18] on XSEDE from the CIPRES Science Gateway [19], using each of the three alignments and models defined above, with default priors. We ran two simultaneous MCMC runs, each with four chains, for a total of 10 million generations and sampled every 1000 generations. We applied 25% burn in fraction and used the last 7500 trees to obtain a 50% majority-rule consensus tree and estimate the node posterior probabilities for each gene. The maximum likelihood inference with rapid bootstrap analysis (1000 replicates; GTR + Gamma model) was executed on each alignment using the RAxML v8 [20] on the XSEDE [19].

3. Results

3.1. Parasite description

Cucullanus extraneus n. sp. (Figs. 1, 2).

Medium-sized, whitish nematodes. Body elongate, cylindrical, maximum width at about region of middle body, tapering towards tail. Cuticle with fine transverse striations. Lateral alae absent. Oral aperture dorsoventrally elongate, slit-like, surrounded by distinct collarette, bearing row of small denticles on inner surface; 2 pairs of large, submedian cephalic papillae and 1 pair of prominent, lateral amphids present (Figs. 1A,C, 2A). 2 pairs of small, submedian inner labial papillae observed under SEM (Fig. 2A). Oesophagus muscular, slightly expanded at anterior end to form pseudobuccal capsule (oesophastome); posterior part remarkably expanded, distinctly wider than pseudobuccal capsule (Fig. 1A,B). Oesophagus opens posteriorly into intestine through valvular apparatus (Fig. 1A,B). Nerve-ring at about 2/5 of oesophageal length. Intestinal caecum absent (Fig. 1A,B). Deirids situated laterally, hooked, at anterior to oesophago-intestinal junction (Figs. 1B, 2B). Post-deirids not observed. Excretory pore far posterior to oesophago-intestinal junction (Fig. 1A). Tail in both sexes conical, with pointed tip (Figs. 1E,F, 2E,F).

Male [Based on 6 mature specimens] Body 11.8–16.4 (14.4) mm long, with maximum width 240–439 (366). Length of entire oesophagus 828–966 (901), representing 5.65–7.02 (6.32)% of body length, minimum width of oesophagus 72–98 (83.0), maximum width of posterior part 125–180 (148). Pseudobuccal capsule 169–250 × 70–82 (210 × 74.8). Nerve-ring, deirids and excretory pore at 280–375 (352), 670–887 (821) and 1.05–1.36 (1.22) mm from anterior extremity, respectively. Posterior end of body remarkably curved ventrally. Precloacal ventral sucker, distance from centre to cloaca 814–985 (889) (Figs. 1G, 2C). Ventral region of cloacal opening distinctly elevated (Figs. 1E, 2E). Spicules of almost equal length, alate, pointed at distal end, 1.23–1.90 (1.59) mm long, representing 10.3–11.6 (11.1)% of body length (Fig. 1G). Gubernaculum well sclerotised, boat-like in lateral view, 130–188 (156) long (Fig. 1H). Caudal papillae 10 pairs in total. Precloacal papillae 3 pairs arranged as follows: first pair at anterior to precloacal ventral sucker, second pair just posterior to ventral sucker, third pair approximately mid-way between second pair and cloacal opening (Figs. 1G, 2C). Paracloacal papillae 4 pairs: first and second pairs slightly anterior to cloacal aperture; third pair almost at level of cloacal opening, fourth pair lateral, just anterior to cloacal opening (Figs. 1E,G, 2C,E). Three postcloacal pairs arranged as: first and third pair subventral, at nearly mid-length of tail and near tail tip, respectively; second pair lateral, situated between first and third pairs (Figs. 1E,G, 2C,E). Single, medio-ventral precloacal papilla present on anterior cloacal lip (Figs. 1E, 2E). Small phasmids lateral, just anterior to first pair postcloacal papillae (Figs. 1E,G, 2E). Tail 239–288 (263) long.

Female [Based on 8 gravid specimens] Body 14.7–20.6 (17.9) mm long, maximum width of mid-body 294–562 (446). Length of entire oesophagus 0.82–1.07 (0.97) mm, representing 4.17–6.48 (5.45)% of body length, minimum width of oesophagus 72–118 (95.6), maximum width of posterior part 125–170 (147). Pseudobuccal capsule 157–250 × 72–100 (190 × 84.8). Nerve-ring, deirids and excretory pore at 294–408 (368), 771–913 (856) and 1.35–1.54 (1.44) mm from anterior extremity, respectively. Vulva slit-like, postequatorial, at 9.30–13.9 (11.3) mm from anterior extremity, 57.9–70.6 (63.3)% of body length; vulval lips distinctly elevated (Fig. 1D). Vagina muscular, directed anteriorly from vulva. Uteri amphidelphic. Eggs oval, thin-walled, 43–78 × 43–55 (66.4 × 48.5) ($n = 30$) (Fig. 1I). Tail 309–429 (392) long. Small lateral phasmids present (Figs. 1F, 2F).

Type host and type locality: Yellowbar angelfish *Pomacanthus maculosus* (Forsskål), (Perciformes: Pomacanthidae); Iraqi coral reef, Arabian Gulf (29°25′N, 48°48′E).

Site of infection: Intestine.

Prevalence and intensity of infection: 4 out of 10 *P. maculosus* were infected with intensity 1–13 (mean 5.0).

Type specimens: Holotype: male (HBNU–F15011L), allotype: female (HBNU–F15012L), paratypes: 5 males, 7 females (HBNU–F15013L).

Etymology: The specific epithet is derived from the Latin word *extraneus* (= strange), and refers to the particular morphology of the caudal papillae in male.

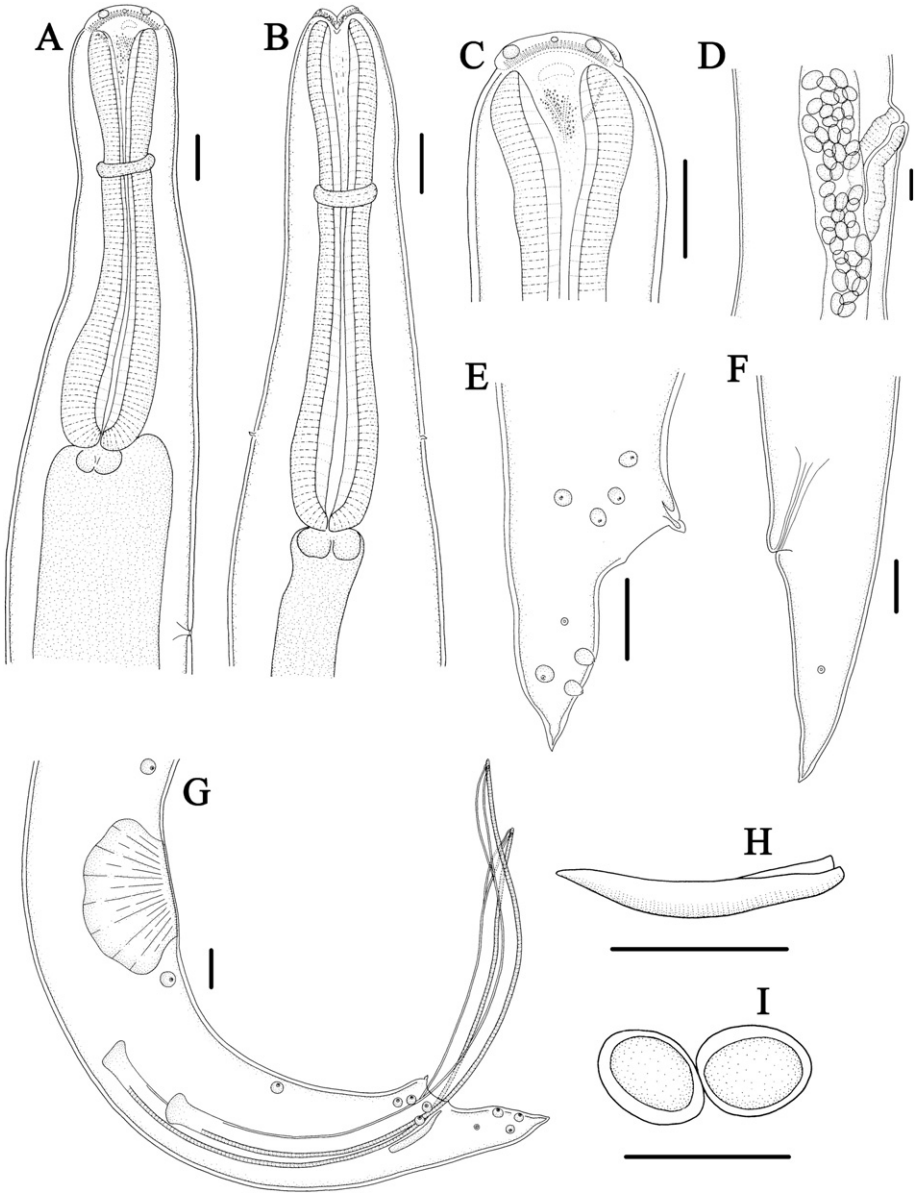

Fig. 1. *Cucullanus extraneus* n. sp. (holotype and allotype) from *Pomacanthus maculosus* (Forsskål) (Perciformes: Pomacanthidae) in the Arabian Gulf, Iraq. A, Anterior part of female, lateral view; B, Anterior part of male, dorsal view; C, Cephalic end of male, lateral view; D, Region of vulva, lateral view; E, Tail of male, lateral view; F, Posterior end of female, lateral view; G, Posterior end of male, lateral view; H, Gubernaculum, lateral view; I, Eggs. *Scale-bars*: A–I, 100 μm.

3.2. Molecular analysis

Three sequences for the 18S region of *Cucullanus extraneus* n. sp. obtained herein were all 884 bp long, which represented only one genotype. There are 18 cucullanid species with 18S sequences available on GenBank. A comparison of the 18S sequences of *C. extraneus* n. sp. with these cucullanid species showed 0.57% (KJ855210) to 8.48% (KP275683) nucleotide variability. Three sequences for the ITS region of *C. extraneus* n. sp. obtained herein were all 1150 bp long, which also represented only one genotype. There are 6 cucullanid species with ITS sequences available on GenBank. A comparison of the ITS sequences of *C. extraneus* n. sp. with these cucullanid species displayed 31.2% (KX302631) to 47.0% (KF484728) nucleotide variability. Three sequences for the *cox*1 region of *C. extraneus* n. sp. obtained herein were all 384 bp long, which represented three different genotypes. The three different genotypes exhibited 0.26–0.52% nucleotide differences. There are 4 cucullanid species with *cox*1 sequences available on GenBank. A comparison of the *cox*1 sequences of *C. extraneus* n. sp. with these cucullanid species displayed 11.2% (KX302632) to 18.9% (KM031789–KM031791) nucleotide variability. The 18S (KT203366–KT203368), ITS (KT192060–KT192062) and *cox*1 (KT260150–KT260152) sequences of *C. extraneus* n. sp. are deposited in the GenBank database (http://www.ncbi.nlm.nih.gov).

3.3. Phylogenetic analyses

The Bayesian tree based on the 18S sequences obtained herein slightly differs from ML tree in topology, but both displayed that the representatives of the genera *Cucullanus*, *Dichelyne* and *Truttaedacnitis* were mixed together (most branches with low support values in Bayesian tree and ML tree) (Fig. 3). The Bayesian tree and ML tree based on the ITS sequences obtained herein were similar in topology, and both revealed that the species of *Dichelyne* were mixed among *Cucullanus* (most branches with high support values in BI tree, but support values relatively low in ML tree) (Fig. 4). The Bayesian tree and ML tree based on the *cox*1 sequences obtained herein also showed the similar

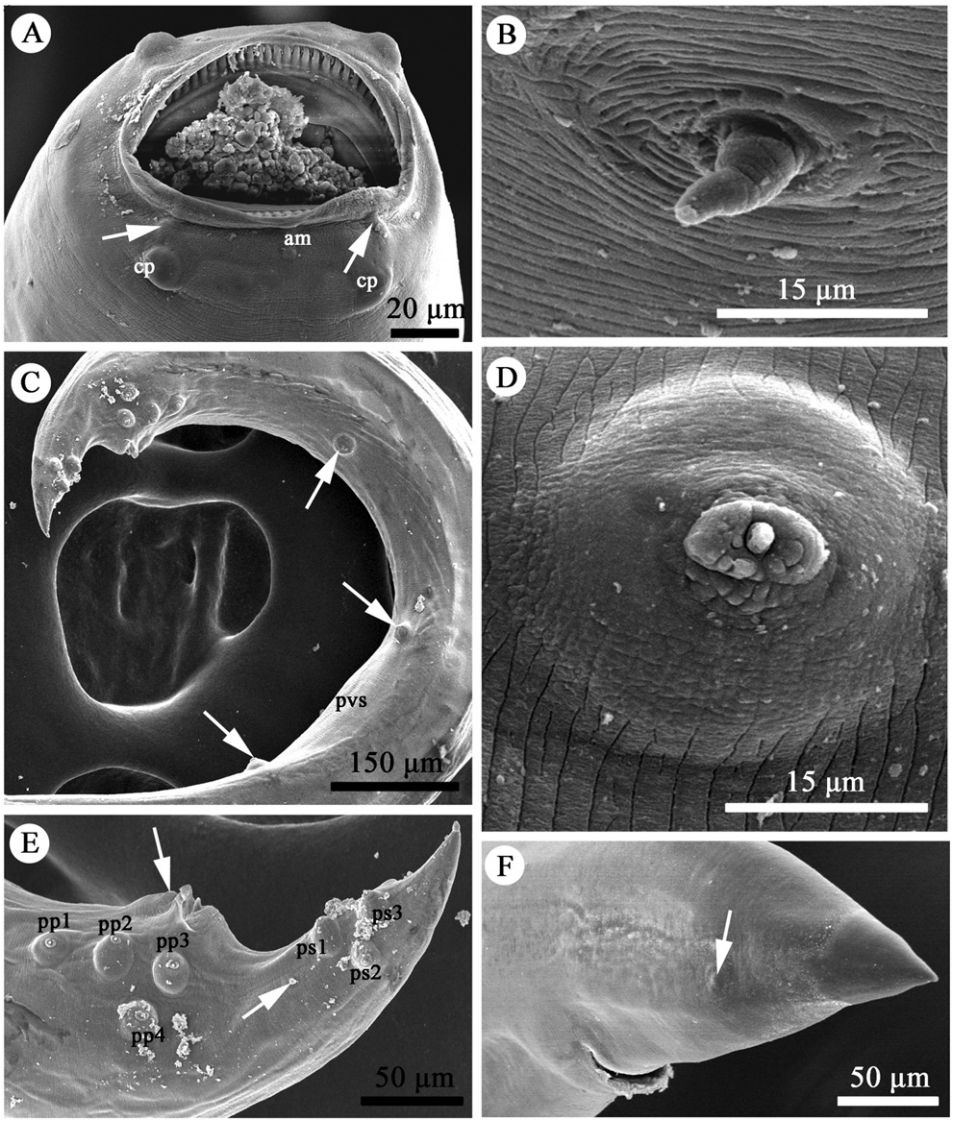

Fig. 2. Scanning electron micrographs of *Cucullanus extraneus* n. sp. from *Pomacanthus maculosus* (Forsskål) (Perciformes: Pomacanthidae) in the Arabian Gulf, Iraq. A, Cephalic end of male (submedian inner labial papillae indicated by arrows), apical view; B, Magnified image of deirid; C, Posterior end of male (precloacal papillae indicated by arrows), lateral view; D, Magnified image of precloacal papilla; E, Tail of male (medio-ventral precloacal papilla and phasmid indicated by arrows), lateral view; F, Tail of female (phasmid indicated by arrow), lateral view. *Abbreviations*: cp, submedian cephalic papillae; am, lateral amphids; pvs, precloacal ventral sucker; pp1–4, first to fourth pair paracloacal papillae; ps1–3, first to third pair postcloacal papillae.

results (Fig. 5). The present results of phylogenetic analyses based on the three different genes supported *Cucullanus extraneus* n. sp. appear to be sister to *C. hainanensis*, and the genera *Cucullanus, Dichelyne* and *Truttaedacnitis* may be not monophyletic assemblages.

4. Discussion

The genus *Cucullanus* currently includes over 100 nominal species, which commonly occur in the digestive tract of various teleost fishes and rarely in aquatic turtles [21–26]. Russell (1980) [27], Dunn et al. (1983) [28] and Rezaei et al. (2013) [29] proved that infection with the species of *Truttaedacnitis* and *Dichelyne* (Cucullanidae) can affect the growth rate and health of the fish hosts, make them more vulnerable to diseases and even result in mortalities. However, our knowledge of the pathogenicity of *Cucullanus* species to their hosts is completely lacking now. We speculated that *C. extraneus* n. sp. may have the similar pathological effects on the host *P. maculosus* as the species of *Truttaedacnitis* and *Dichelyne*. The life cycle of *Cucullanus* species is still imperfectly known, but several studies have revealed that the small aquatic crustaceans and cephalopods can serve as intermediate hosts for *Cucullanus* species [30–33]. Thus we considered that *P. maculosus* was likely infected with *C. extraneus* n. sp. through eating these marine invertebrates, which are in its diet [2,3].

According to Petter (1974) [21], Moravec et al. (1997, 2005, 2008) [23,25,34], Caspeta-Mandujano et al. (2000) [35], López-Caballero et al. (2009) [36] and Yooyen et al. (2011) [37], the host groups and zoogeographical regions are important criterion for differentiating and identifying of species of *Cucullanus*. However, as far as we are aware, there is no *Cucullanus* species found in marine perciform fishes of the family Pomacanthidae. To date, over 30 nominal species of *Cucullanus* were reported from perciform fishes worldwide [25,36–48]. *Cucullanus extraneus* n. sp. can be easily distinguished from the great majority of these above-mentioned species by having the relatively large body size (over 10.0 mm in male), the long spicules (over 1.0 mm) and the presence of precloacal ventral sucker. Among the *Cucullanus* spp. reported from perciform fishes, only *C. hians* (Dujardin, 1845), *C. himezi* Yamaguti, 1941 and *C. bourdini* Petter & Le Bel, 1992 agree with the three above-mentioned traits [36,38,40,49,50]. However, *C. extraneus* n. sp. is readily distinguished from them by the following differences: *C. himezi* has a distinctly longer oesophagus (1.32–1.82 mm in male),

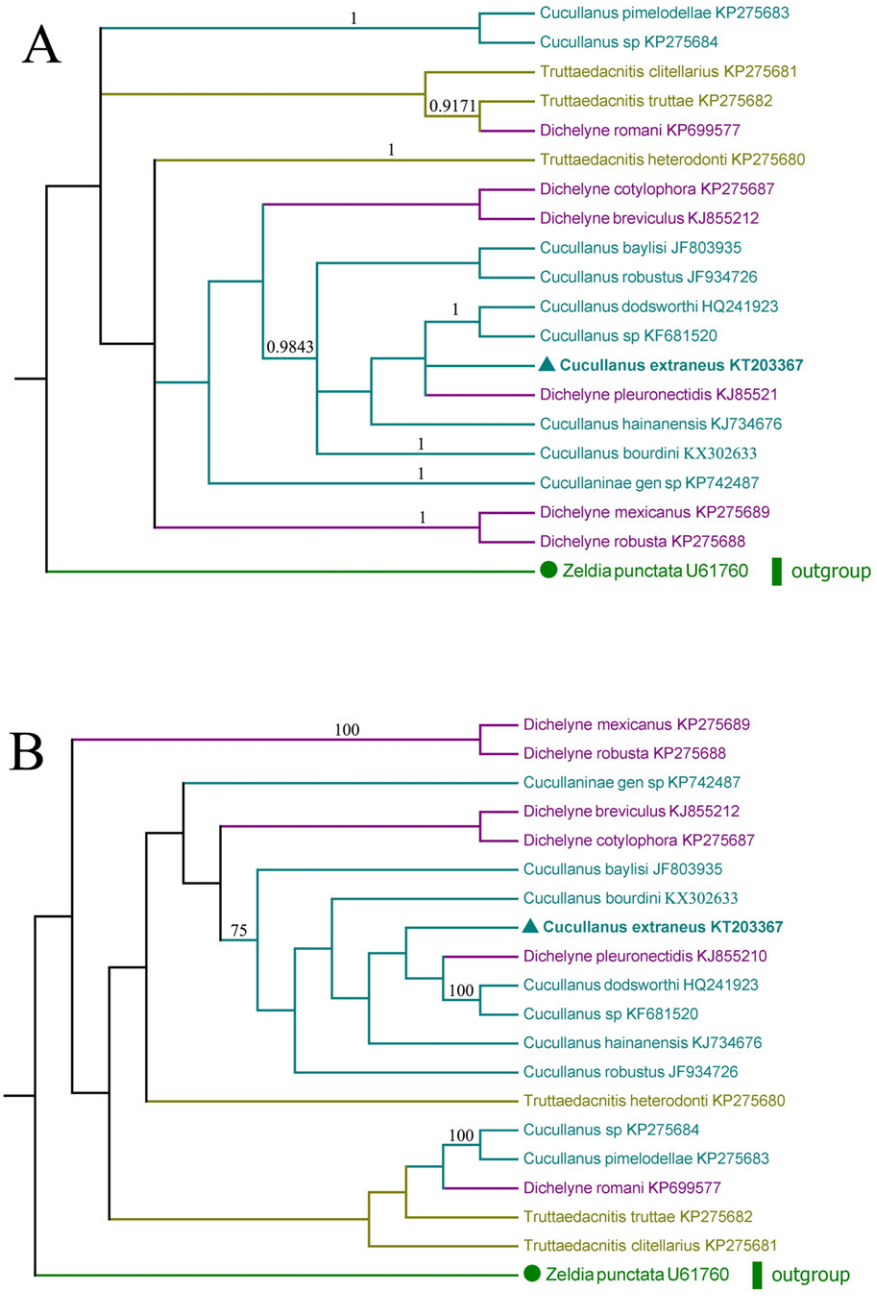

Fig. 3. Phylogenetic relationships between *Cucullanus extraneus* n. sp. isolated in the present study (shown in bold) and the other cucullanid species registered in GenBank based on 18S rDNA sequences. *Zeldia punctata* was chosen as the outgroup. Bootstrap values exceeding 70 in ML tree and BPP values exceeding 0.900 in Bayesian tree were displayed. A, Bayesian tree revealing the genetic relationships among cucullanid species; B, Maximum likelihood (ML) tree revealing the genetic relationships among cucullanid species.

much shorter spicules (0.7–1.16 mm), more anterior position of excretory pore (around oesophago-intestinal junction) and different arrangement of caudal papillae [38,46]; *C. bourdini* has a pseudobuccal capsule as wide as the posterior oesophageal inflation, the relatively longer oesophagus (representing 7.14–12.0% of body length in male), much shorter spicules (0.74–1.07 mm, representing 6.34–9.08% of body length) and different morphology of caudal papillae [49,50]; *C. hians* has a pseudobuccal capsule slightly wider than posterior part of oesophagus, an excretory pore slightly at anterior to oesophago-intestinal junction and shorter spicules (only 0.88–1.20 mm) [40].

At present, more than 40 nominal species of *Cucullanus* are known from a variety of freshwater, brackish-water and marine fishes in Asia [22,25,26,37–40,51–56]. Of them, with the exception of *C. himezi*, *C. bourdini* and *C. hians*, only *C. robustus* Yamaguti, 1935, *C. filiformis* Yamaguti, 1935, *C. arii* Yamaguti, 1954, and *C. spirocaudus* Li, 1984 possessing the relatively large body size (over 10.0 mm in male), the long spicules (over 1.0 mm) and the presence of precloacal ventral sucker, are similar to *C. extraneus* n. sp. [38,39,51,55,57]. However, *C. extraneus* n. sp. can be easily differentiated from them by the following differences: *C. arii* has distinctly longer oesophagus (1.2–1.3 mm) and male tail (0.37–0.39 mm), and slightly shorter gubernaculum (0.09–0.11 mm); *C. robustus* and *C. filiformis* have a pseudobuccal capsule almost as wide as or slightly wider than posterior part of oesophagus; moreover, *C. robustus* has distinctly longer oesophagus (1.43–1.51 mm, representing 9.1–10.3% of body length); *C. filiformis* has much shorter spicules (0.75–1.15 mm); *C. spirocaudus* has distinctly longer oesophagus (1.21–1.34 mm in the males) and different number and arrangement of caudal papillae.

Recently, molecular techniques, utilising the ribosomal (18S or ITS) or mitochondrial (*cox*1) regions as genetic markers, have proved to be

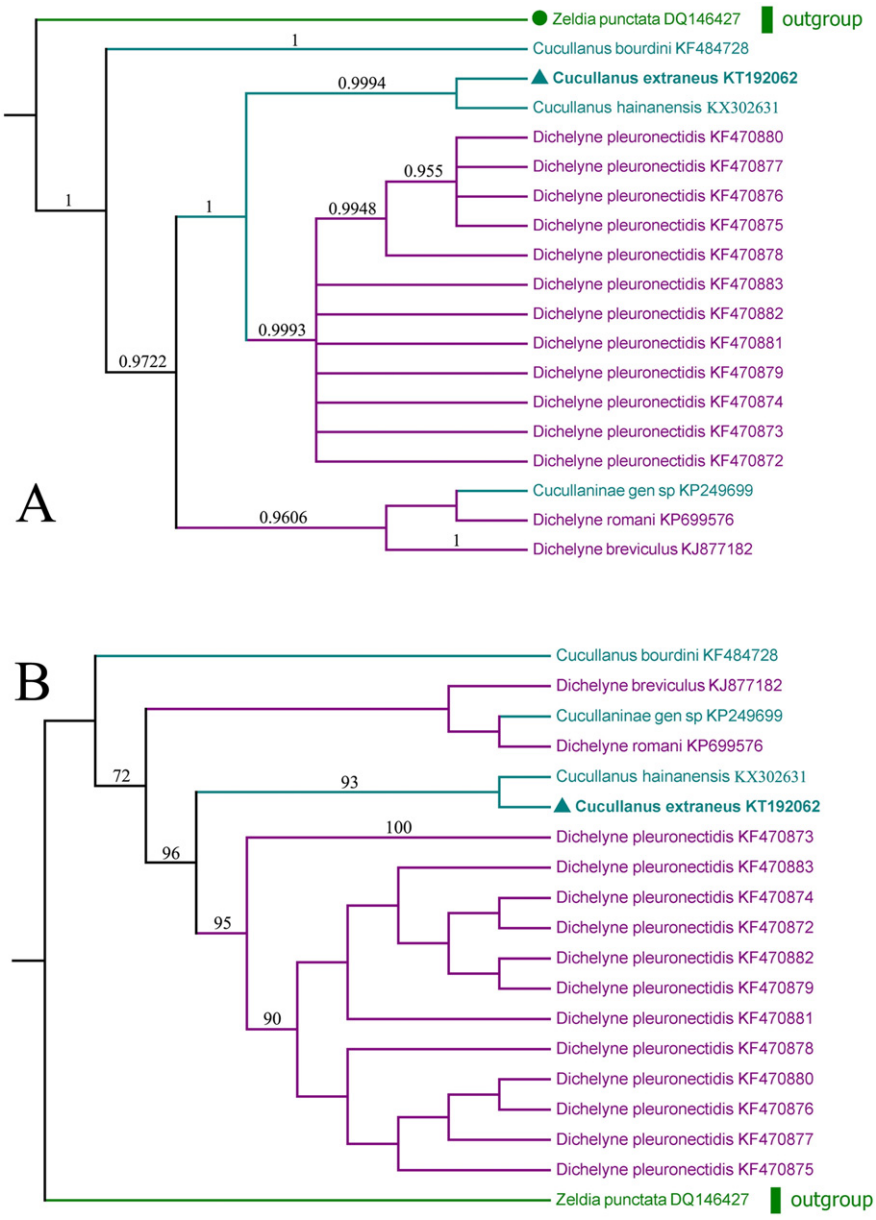

Fig. 4. Phylogenetic relationships between *Cucullanus extraneus* n. sp. isolated in the present study (shown in bold) and the other cucullanid species registered in GenBank based on ITS rDNA sequences. *Zeldia punctata* was chosen as the outgroup. Bootstrap values exceeding 70 in ML tree and BPP values exceeding 0.900 in Bayesian tree were displayed. A, Bayesian tree revealing the genetic relationships among cucullanid species; B, Maximum likelihood (ML) tree revealing the genetic relationships among cucullanid species.

particularly useful for the exact discrimination and identification of cucullanid nematodes at the species level [12,26,58,59]. In the present study, there was no nucleotide difference detected in the 18S and ITS rDNA among the different individuals of *C. extraneus* n. sp. examined herein. However, genetic comparison of the 18S and ITS sequences of *C. extraneus* n. sp. with its congeneric species showed 0.57–8.48% and 31.2–47.0% nucleotide variability, respectively. The results strongly supported that these nematode parasites collected from *P. maculosus* in the Arabian Gulf, represented a single species *C. extraneus* n. sp., which also agree well with the morphological observation (There is no remarkable morphological difference observed). To our knowledge, this is the first time to utilize the *cox*1 region as a genetic marker for the accurate identification of species of *Cucullanus*. The level of intraspecific nucleotide variability in the *cox*1 region among the different individuals is only 0.26–0.52%. Pairwise comparisons of the *cox*1 sequences of *C. extraneus* n. sp. with these cucullanid species registered in the GenBank displayed 11.2–18.9% nucleotide variability, which is remarkably higher than the level of the intraspecific nucleotide variation in *C. extraneus* n. sp. Thus the results from the present study demonstrate that the *cox*1 region should be also a suitable and useful genetic marker for distinguishing and identifying of *Cucullanus* species.

According to the classification of Cucullanidae provided by Petter (1974) and Chabaud (1978) [21,60], Cucullanidae is divided into six genera *Oceanicucullanus*, *Campanarougetia*, *Truttaedacnitis*, *Cucullanus*, *Neocucullanus* and *Dichelyne*. Of them, the relationship between *Cucullanus* and *Dichelyne* remains unclear. According to the traditional diagnostic characters and keys to the genera of the subfamily Cucullaninae provided by Petter (1974) and Chabaud (1978) [21,60], the absence or presence of intestinal caecum is a key morphological feature for distinguishing *Dichelyne* from *Cucullanus*, but Li et al. (2014) [59] considered this feature to be questionable and unreliable as the generic diagnostic criterion. The recent phylogenetic analyses also challenge the traditional classification. Although the results of phylogenetic analyses based on 18S rDNA, including very limited number of cucullanid spp., provided by Mejía-Madrid & Aguirre-Macedo (2011) [48], Laetsch et al. (2012) [61] and Choudhury & Nadler (2016)

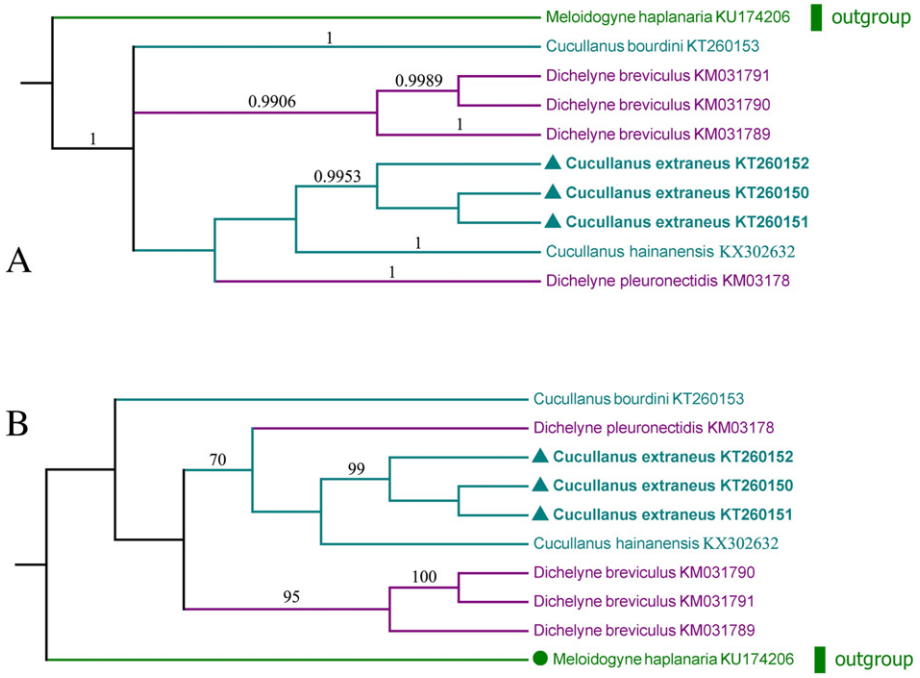

Fig. 5. Phylogenetic relationships between *Cucullanus extraneus* n. sp. isolated in the present study (shown in bold) and the other cucullanid species registered in GenBank based on cox1 sequences. *Meloidogyne haplanaria* was chosen as the outgroup. Bootstrap values exceeding 70 in ML tree and BPP values exceeding 0.900 in Bayesian tree were displayed. A, Bayesian tree revealing the genetic relationships among cucullanid species; B, Maximum likelihood (ML) tree revealing the genetic relationships among cucullanid species.

[14] supported Cucullanidae may be monophyletic; Choudhury & Nadler (2016) [14] revealed that the genera *Truttaedacnitis*, *Cucullanus* and *Dichelyne* appear to be not monophyletic. The present results of our phylogenetic analyses based on the 18S sequences agree well with Choudhury & Nadler's (2016) [14] study, and also supported *Truttaedacnitis*, *Cucullanus* and *Dichelyne* are not monophyletic assemblages. In addition, the phylogenetic trees were firstly inferred using maximum likelihood (ML) and Bayesian inference (BI) based on the ITS and *cox*1 sequences, respectively, and the results further confirmed *Cucullanus* and *Dichelyne* may be not monophyletic. However, a more rigorous study with broader representation of Cucullanidae is required to elucidate the phylogenetic relationship between *Cucullanus* and its related genus-level taxa.

Conflict of interest

The authors do not have any potential conflicts of interest to declare.

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