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Genetic characterisation of *Echinocephalus* spp. (Nematoda: Gnathostomatidae) from marine hosts in Australia

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ABSTRACT

We genetically characterised larval and adult specimens of species of *Echinocephalus* Molin, 1858 (Gnathostomatidae) collected from various hosts found within Australian waters. Adult specimens of *Echinocephalus* were collected from a dasyatid stingray [*Pastinachus ater* (Macleay); n = 2] from Moreton Bay, Queensland and larvae from a hydrophiine sea snake [*Hydrophis peronii* (Duméril); n = 3] from Cape York Peninsula, Queensland, from an octopus (*Octopus djinda* Amor & Hart; n = 3) from Fremantle, Western Australia and from a lucinid bivalve [*Codakia paytenorum* (Iredale); n = 5] from Heron Island, Queensland Australia. All nematode samples were identified morphologically and genetically characterised using the small subunit nuclear ribosomal DNA (SSU). Some morphological differences were identified between previous studies of *Echinocephalus* spp. and those observed herein but the significance of these differences remains unresolved. Molecular phylogenetic analyses revealed that larval *Echinocephalus* sp. from *H. peronii* and *C. paytenorum* in Australia were very similar (with strong nodal support) to larval *Echinocephalus* sp. infecting two fish species from Egypt, *Saurida undosquamis* (Richardson) (Synodontidae) and *Pagrus pagrus* (Linnaeus) (Sparidae). The SSU sequences of larval *Echinocephalus* sp. from *O. djinda* and adults from *P. ater* formed a well-supported clade with that of adult *E. overstreeti* Deardorff and Ko, 1983 from the Port Jackson shark, *Heterodontus portusjacksoni* (Meyer), as well as that of the larval *Echinocephalus* sp., from the common carp (*Cyprinus carpio* Linnaeus) from Egypt. This study extends the intermediate host range of *Echinocephalus* larvae by including a sea snake for the first time. Findings of this study highlight the importance of genetic characterisation of larval and adult specimens of *Echinocephalus* spp. to resolve the current difficulties in the taxonomy of this genus.

1. Introduction

The taxonomy of nematodes of the gnathostomatid genus *Echinocephalus* Molin, 1858 was recently reviewed. Currently, *Echinocephalus* contains 12 recognised valid species and 10 poorly described species, considered to be invalid by Moravec and Justine (2021). In the past, identification and characterisation of new species of *Echinocephalus* was based on inadequate morphological descriptions often from larval forms (Moravec and Justine, 2021). Elasmobranchs are currently the only recognised definitive hosts, primarily rays but also some sharks, and teleost fishes and a few marine invertebrates thought to only be paratenic or second intermediate hosts (Moravec and Justine, 2021). Importantly, Moravec and Justine (2021) emphasised that identification of larval stages to species level was not currently possible.

The identification and taxonomy of *Echinocephalus* in the past has been based on morphological features. This has led to some poorly described species that have confused taxonomy within the genus and may have potentially led to misidentification of new species of parasites (Moravec and Justine, 2021; van Megen et al., 2009). Modern technological methods such as molecular techniques, such as DNA sequence data, have now been developed to enable the definition and identification of genetic markers which can lead to the accurate identification of

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species (Morrison, 2006; van Megen et al., 2009).

This study aimed to genetically characterise larval and adult specimens of species of *Echinocephalus* collected from various hosts found within Australian waters, and provides taxonomic comments on the genus *Echinocephalus*.

2. Materials and methods

2.1. Collection of specimens

Adult specimens of *Echinocephalus* were collected from a dasyatid stingray [*Pastinachus ater* (Macleay); n = 2] from Moreton Bay, Queensland. Larval specimens were collected from a hydrophiine sea snake [*Hydrophis peronii* (Duméril); n = 3] from Cape York Peninsula, Queensland, from an octopus (*Octopus djinda* Amor & Hart; n = 3) from Fremantle, Western Australia and from a lucinid bivalve [*Codakia paytenorum* (Iredale); n = 5] from Heron Island, Queensland Australia. Specimens were collected under the state-issued permits, including Queensland (Queensland Marine Parks permit number: G19/42323.1) and Western Australia (Murdoch Animal Ethics – Cadaver and/or Tissue Notification, Permit No. 744).

2.2. Morphological identification of nematodes

Adult nematodes and samples from each group of larvae were cleared in lactophenol. Adults were identified following Moravec and Justine (2021). For representatives of larvae from octopuses and molluscs, the cephalic extremities were excised with a scalpel and viewed as apical preparations, with the distribution of papillae examined following Moravec and Justine (2006). This was not possible for the larvae from the sea snake as they had been fixed within the fibrous host capsule. The specimens have been deposited in the Australian Helminthological Collection (AHC) of the South Australian Museum, Adelaide (SAM) (hologenophores 49120, 49122, 49124; paragenophores 49121, 49123, 49125-6).

2.3. Molecular characterisation of nematodes

Genomic DNA (gDNA) was isolated from the mid-sections of nematode specimens using the DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufacturers' protocols. The concentration and purity of each DNA sample were determined spectrophotometrically (ND-1000 UV-VIS spectrophotometer v.3.2.1; NanoDrop Technologies, Inc., Wilmington, DE, USA).

The partial small subunit nuclear ribosomal DNA (SSU) region within the rDNA was amplified by Polymerase Chain Reaction (PCR) using the primers SSU F04 (GCTTGTCTCAAAGATTAAGCC) and SSU R26 (CATTC TTGGCAAATGCTTTCG) (Blaxter et al., 1998) in a T100 thermal cycler (BioRad, Hercules, CA, USA). PCR amplifications (initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 40 s, and a final extension at 72 °C for 5 min) were carried out in a final reaction volume of 50 μL, containing 3.12 mM of each deoxynucleotide triphosphate (dNTP), 12.5 pmol of each primer, and 10 mM Tris-HCl (pH 8.4), 7.5 mM $MgCl_2$ and 0.62 U of GoTaq Flexi DNA polymerase (Promega, Madison, USA). Known positive (genomic DNA of *Haemonchus contortus* and *Echinocephalus* spp.) and negative (Milli-Q H_2O) controls were included in each PCR run. Aliquots (5 μL) of individual amplicons were analysed on 1.5% (w/v) agarose gel in Tris-Borate-EDTA buffer stained with GelRed (Biotium) and visualised using a GelDoc system (BioRad, Hercules, CA, USA).

Amplicons were purified using shrimp alkaline phosphate and exonuclease I (ThermoFisher Scientific, Australia) before automated Sanger DNA sequencing using the PCR primers in separate reactions. The quality of the sequences was assessed using the Geneious Prime 2021.1.1 software (Biomatters Ltd., Auckland, New Zealand; www.geneious.com). The DNA sequences determined herein have been submitted to the GenBank database under the accession numbers OL415832-OL415835.

Published SSU sequences of *Echinocehalus* spp. were obtained from GenBank (Table 1) and aligned with new SSU data using MUSCLE in Mesquite v.3.61 (http://www.mesquiteproject.org) using default settings and were trimmed to uniform lengths of 783 bp. The evolutionary model (K2+I) of the DNA sequence dataset was determined using the Akaike and the Bayesian information criteria (AIC and BIC) tests in jModelTest v.2.1.5 (Darriba et al., 2012). Neighbour Joining (NJ) trees were constructed using MEGA 11 (Tamura et al., 2021), and Bayesian Inference (BI) trees were built using MrBayes software (Huelsenbeck and Ronquist, 2001). The NJ trees were constructed with 10,000 bootstrap replicates using the Kimura 2-parameter distance method. The BI analysis was run for 20,000,000 generations (ngen = 20,000,000) to calculate posterior probabilities (pp), with two runs, with every 200th tree saved (samplefreq = 200). The SSU sequence of *Gnathostoma lamothei* was used as an outgroup. Tree topology was checked for consensus between NJ and BI analyses.

3. Results and discussion

All of the larval stages examined conformed to earlier descriptions of this genus from Australia (e.g. Beveridge, 1987; Shamsi et al., 2021) with six rows of hooks on the cephalic inflation (Fig. 1A), two lips and clusters of tiny, spiniform papillae dorsally and ventrally (Fig. 1B and C) (Moravec and Justine, 2006). Moravec and Justine (2006) drew attention to differences in the spiniform papillae from the larvae of *E. overstreeti* Deardorff and Ko, 1983 they described from the type host, *Taeniurops meyeni* (Müller & Henle) as *Taeniura melanospila*, in the Pacific Ocean and the specimens described from scallops from South Australian Gulfs by Beveridge (1987). They noted that in *E. overstreeti* from the type host, the third row of papillae consisted of five papillae with two outlying areas of sclerotization lacking spines (Moravec and Justine, 2006, Fig. 7c) compared with the redescription of the species by Beveridge (1987, Fig. 25), in which the third and outer row consisted of three spiniform papillae. In all of the current larval specimens, only three spiniform papillae were present in the outer row, although in the specimens from *C. paytenorum,* they were joined by irregular areas of sclerotization, not seen in the specimens from *O. djinda*. The significance of these differences along with those noted by Moravec and Justine (2006) remains unresolved.

The pairwise comparison of each of the SSU DNA sequences between the new larval specimens and the reference sequences in GenBank ranged from 0 to 6.6% (Table 2; Supplementary Fig. S1). *Echinocephalus* sp. from *O. djinda* and *E. overstreeti*, from *Heterodontus portusjacksoni* (Meyer) from South Australia (GenBank no. OL415832- OL415835) were identical. The SSU sequence data generated from *Echinocephalus* larvae from *C. paytenorum* and *H. peronii* were most similar to those of *Echinocephalus* sp. larvae from the two teleost fish hosts from Egypt, *Saurida undosquamis* (Richardson) (Synodontidae) and *Pagrus pagrus* (Linnaeus) (Sparidae), with pairwise differences of 1.5% and 2.2%, respectively (Table 2; Supplementary Fig. 1).

Phylogenetic analyses derived from the SSU data from the *Echinocephalus* sequences generated similar tree topologies for the BI and NJ analyses; therefore, only the BI tree is presented herein (Fig. 2; alignment of the SSU sequences of *Echinocephalus* spp. is provided in the Supplementary material). Three principal clades were evident in the phylogenetic reconstruction. *Echinocephalus* cf. *pseudouncinatus* was sister to the remaining two clades. A second clade included the larval *Echinocephalus* sp. from *H. peronii* in Australia and the larval *Echinocephalus* sp. from *S. undosquamis* and *P. pagrus* from Egypt, with strong nodal support (BI: 1.0; NJ: 99%). Also associated with this clade, though with poor support (0.85, 51%) and differing at 2.6% of bases, were the larvae from *C. paytenorum* from Heron Island. The third clade included *E. overstreeti* from *H. portusjacksoni*, the larval *Echinocephalus* sp. from *O.*

Table 1
Details of small subunit nuclear ribosomal DNA sequences of *Echinocephalus* spp. included in the molecular analyses.

Parasite	Developmental stage	Host (scientific name)	Location	GenBank accession number	Reference
Echinocephalus sp.	Larvae	*Octopus djinda*	Western Australia	OL415832	This study
Echinocephalus sp.	Adults	*Pastinachus ater* (Macleay)	Morton Bay, Queensland, Australia	OL415833	This study
Echinocephalus sp.	Larvae	*Codakia paytenorum* (Iredale)	Heron Island, Queensland, Australia	OL415834	This study
Echinocephalus sp.	Larvae	*Hydrophis peronii* (Duméril)	Weipa, Queensland, Australia	OL415835	This study
Echinocephalus overstreeti	Adult	*Heterodontus portusjacksoni* (Meyer)	South Australia	JF934729	(Laetsch et al., 2012)
Echinocephalus sp. 2	Larvae	*Saurida undosquamis* (Richardson)	Egypt	KY972321	GenBank
Echinocephalus sp. 1	Larvae	*Pagrus pagrus* (Linnaeus)	Egypt	KY911549	BenBank
Echinocephalus sp.[a]	Larvae	*Cyprinus carpio* Linnaeus	Egypt	KC493258	Abdel-Ghaffar et al. (2013)
Echinocephalus pseudouncinatus	Larvae	*Atrina maura* (Sowerby I)	Mexico	MN514178	Gómez-Valdez et al. (2019)

[a] Identified as *Echinocephalus* sp. in GenBank but reported as *E. carpiae* in the publication; ˆ formerly *Octopus aff. O. tetricus*.

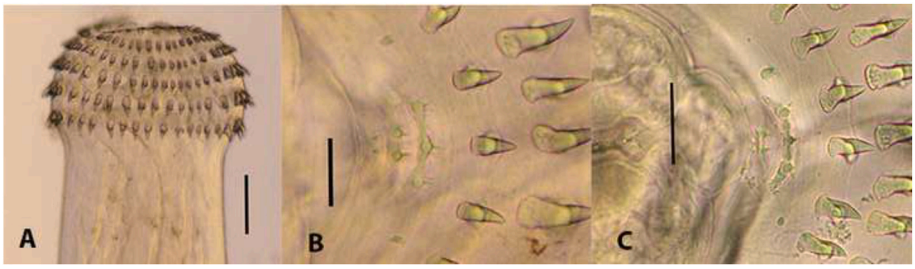

Fig. 1. A, Anterior end of *Echinocephalus* larva from *Octopus djinda* (formerly *Octopus O. aff. tetricus*), showing six rows of hooks on the cephalic inflation; **B**, Apical view of the spiniform papillae on the larva from *O. djinda*, showing a posterior row of three papillae; **C**, Apical view of the spiniform papillae on the larva from *Codakia paytenorum*, showing posterior row of three papillae joined by irregular areas of sclerotization. Scale bars: Fig. 1A and 40 μm; Fig. 1B and C, 10 μm.

Table 2
Pairwise comparison of percent differences of the small subunit nuclear ribosomal DNA sequences determined herein (**bold**) and the selected reference sequences of *Echinocephalus* spp.

Taxa	1	2	3	4	5	6	7	8	9
1. **OL415832 *Echinocephalus* sp.** (ex *Octopus djinda*, Western Australia)	ID								
2. JF934729 *Echinocephalus overstreeti* (ex *Heterodontus portusjacksoni*, South Australia)	0	ID							
3. **OL415833 *Echinocephalus* sp.** (ex *Pastinachus ater*, Moreton Bay, Queensland, Australia)	0.2	0.2	ID						
4. **OL415834 *Echinocephalus* sp.** (ex *Codakia paytenorum*, Heron Island, Queensland, Australia)	2.5	2.5	2.6	ID					
5. KY972321 *Echinocephalus* sp. (ex *Saurida undosquamis*, Egypt)	1.3	1.3	1.5	2.2	ID				
6. KY911549 *Echinocephalus* sp. (ex *Pagrus pagrus*, Egypt)	1.3	1.3	1.5	2.2	0	ID			
7. **OL415835 *Echinocephalus* sp.** (ex *Hydrophis peronii*, Weipa, Queensland, Australia)	1.2	1.2	1.3	2.1	0.2	0.2	ID		
8. KC493258 *Echinocephalus* sp. (ex *Cyprinus carpio*, Egypt)	3.1	3.1	3.2	5.5	4.4	4.4	4.3	ID	
9. MN514178 *Echinocephalus pseudouncinatus* (ex *Atrina maura*, Mexico)	3.9	3.9	4	5.2	4.5	4.5	4.4	6.6	ID

djinda and adults from *P. ater*, all from Australia, as well larval *Echinocephalus* sp. from the *C. carpio* Linnaeus from Egypt, with strong nodal support (0.99, 99%) (Fig. 2).

Adult specimens examined in this study from *P. ater* (Dasyatidae) were identified as *E. overstreeti* as the sequence data were only 0.2% different from those from *H. portusjacksoni* (Heterodontidae). The identification of the specimens from *P. ater* as *E. overstreeti* was also confirmed morphologically by measurement of the gubernaculum which was 0.8 mm in length, justifying its separation from *E. inserratus,* a species described recently, also from *P. ater*, from New Caledonia (Moravec and Justine, 2021). Moravec and Justine (2006, p.144) questioned the identity of *E. overstreeti* redescribed by Beveridge (1987) suggesting that it may represent another, probably undescribed, species as the type host of *E. overstreeti* was the blotched fantail ray, *Taeniurops meyeni* (as *Taeniura melanospilos*) (Dasyatidae). Beveridge (1987, 1991) reported adult *E. overstreeti* from a range of elasmobranch species from Australian waters, although gravid specimens were found only in *H. portusjacksoni*. In the current study, the female specimens from *P. ater* from Moreton Bay were gravid. The present evidence suggests that *E. overstreeti*, as described by Beveridge (1987), does in fact have a wide host range, occurring in both sharks and rays (Heterodontiformes, Orectolobiformes, Rajiformes, Myliobatiformes, Rhinopristiformes, Torpediniformes, Chimaeriformes). Furthermore, as the SSU sequence of *Echinocephalus* sp. larvae from *O. djinda* forms a clade with *E. overstreeti*, with strong nodal support (Fig. 2) and no nucleotide variation (Supplementary Fig. S1), we predict these larvae will represent *E. overstreeti*.

The two most phylogenetically distinct sequences (6.6% sequence difference) were those of larval *Echinocephalus* sp. from *O. djinda* and larval *E.* cf. *pseudouncinatus*, with the latter also sister to all remaining clades. The identification of the larvae of *E.* cf. *pseudouncinatus* was based on morphological features (Gómez-Valdez et al., 2019), although Moravec and Justine (2021) do not consider this type of identification to be possible. Milleman (1963) confirmed the identity of larvae and adults

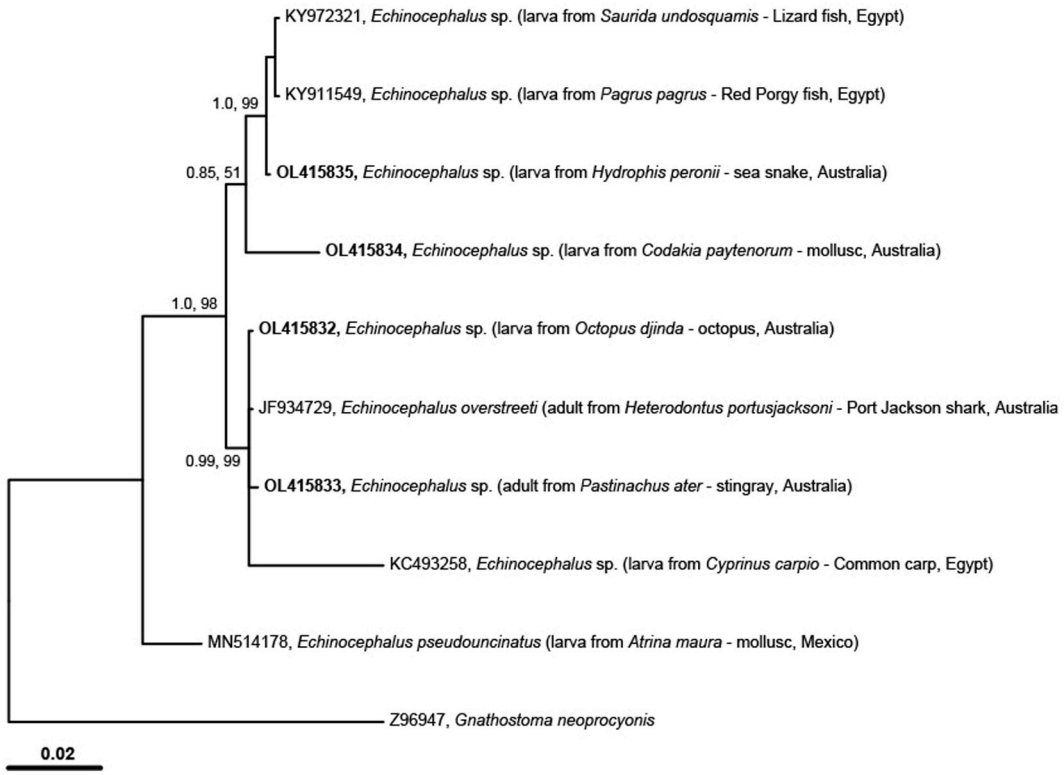

Fig. 2. Genetic relationship based on Bayesian Inference analysis of the small subunit nuclear ribosomal DNA (SSU) sequences of *Echinocephalus* spp. collected form sea snake, stingray and octopus in Australia determined in this study **(bold)**. Nodal support is given as a posterior probability for BI analysis followed by bootstrap values for NJ analysis on this tree. *Gnathostoma lamothei* (Bertoni-Ruiz et al., 2011) was used as the outgroup, however the GenBank entry for this parasite is with its old name, *Gnathostoma neoprocyonis* Z96947. The scale bar indicates the number of inferred substitutions per nucleotide site.

of *P. pseudouncinatus* by finding larval stage in the process of moulting to adults. However, this possibility did not exist in the study of Gómez-Valdez et al. (2019). For this reason, their sequence data have been indicated as belonging to *E.* cf. *pseudouncinatus*.

The larval *Echinocephalus* from *C. carpio* in Egypt, described as a new species, *E. carpiae* Abdel-Ghaffar et al. (2013) by Abdel-Ghaffar et al. (2013) belonged to the same clade as *E. overstreeti* and on a phylogenetic basis, *E. carpiae* is a junior synonym of *E. overstreeti*. However, the branch length and percentage difference in sequence similarity (97%) warrant further examination of this relationship. The specimens of *E. carpiae* were collected from a brackish lagoon bordering the Mediterranean coast of Egypt (Abdel-Ghaffar et al., 2013). The only species of *Echinocephalus* currently known from this region is *E. uncinatus*, found in the dasyatid rays *Bathytoshia lata* (Garman) [as *Dasyatis centroura* (Mitchill)] and *D. pastinaca* (Linnaeus) (see Beveridge, 1985), for which no molecular data are available.

Larval *E. overstreeti* have also been reported from *S. undosquamis* from the Red Sea off Egypt (Morsy et al., 2015). However, this identification was based exclusively on morphological features and therefore cannot be relied upon. It may be the same species as the specimens from the same host from Egypt listed as unpublished in GenBank and included in the current phylogenetic analyses, which clearly is not *E. overstreeti*.

Recently, larval *Echinocephalus* have been reported from the teleosts *Acanthopagrus australis* (Günther) and *Rhabdosargus sarda* (Forsskål) from Moreton Bay, Australia, but the generation of only ITS sequence data prevents comparison with the current data (Shamsi et al., 2021).

Moravec and Justine (2021) noted that resolution of the difficulties associated with the identification of the larval stages of *Echinocephalus* spp. would require molecular analyses. The current study has provided evidence for the validity of this approach in being able to associate a larval stage from an octopus with adult specimens of *E. overstreeti* from a shark in Australian waters, but the approach is severely limited by the lack of sequence data for adults of species of *Echinocephalus*, with *E. overstreeti*, as represented by the redescription of Beveridge (1987), being the only species to date with such data. In the Australian region, *E. sinensis* is also present although uncommon (Beveridge, 1991) and it is likely that *E. inserratus*, recently described from New Caledonia by Moravec and Justine (2021) will also be found in Australian waters as the same host species, *P. ater*, occurs in both Australian and New Caledonian waters. In European waters, molecular data for adult *E. uncinatus* are required to examine the purported presence of *E. overstreeti* suggested by the present data.

The current study extends the intermediate host range of *Echinocephalus* larvae in Australian waters. Larvae have been reported from bivalves and gastropods (Beveridge, 1987) but not previously from cephalopods. In the case of reptiles, *Echinocephalus* larvae have been reported from a turtle, Caretta, caretta (Linnaeus) (Lester et al., 1980) but not from sea snakes.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2021.12.012.

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