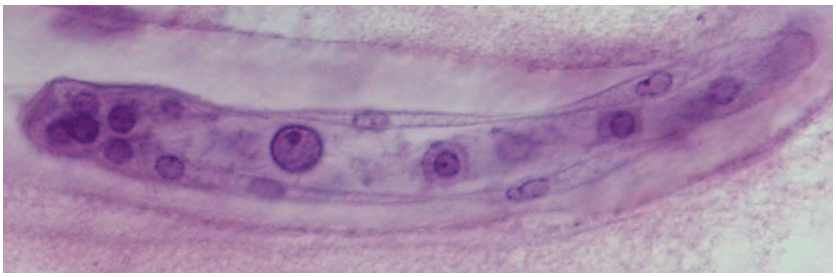
DICYEMID PARASITE FAUNA OF SOUTHERN AUSTRALIAN CEPHALOPOD SPECIES



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Declaration

I, Sarah Roseann Catalano, certify that the research presented in this thesis contains no material that has been accepted for the award of any other degree or diploma in any other university or tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institute without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Sarah Roseann Catalano

28/01/2014

Cover image: by Sarah Catalano

Top Left: *Sepioteuthis australis* (southern calamary)
Top Middle: *Octopus kaurna* (southern sand octopus)
Top Right: *Sepia apama* (giant Australian cuttlefish)
Bottom Centre: *Dicyemennea floscephalum* (Catalano 2013) ex *Octopus berrima*

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ABSTRACT

The dicyemid mesozoans (Dicyemida Van Beneden 1882) are a poorly-understood group of marine organisms that are found with high intensities in the renal appendages of benthic cephalopods. The majority of the research focusing on this group is from the northern hemisphere, with very few studies examining the dicyemid fauna of southern hemisphere cephalopod species. Confusion also exists in the literature on the validity of certain families, genera and species within this phylum, and the phylogenetic framework for the dicyemids is scarce. The few studies that have examined dicyemid molecular genetics focus only on single taxon or sole aspects of genome organisation. Furthermore, key parts of the life cycle of dicyemid parasites are unresolved and their position in the Tree of Life is uncertain.

My thesis highlights the taxonomic confusion in the literature that surrounds the Dicyemida, and presents a comprehensive list of all dicyemid species currently described to date (Chapter 2). Ten cephalopods species from Australian waters were collected and examined for dicyemids parasites, resulting in new dicyemid species descriptions (Chapters 3, 4 and 5). Host eggs and filtered seawater samples were collected from the cuttlefish mass breeding aggregation at Upper Spencer Gulf, South Australia, Australia, to assess the unknown host life cycle stage where new infection by the dispersive dicyemid embryo occurs. No dicyemid DNA was detected in any host egg or environmental samples, suggesting new infection occurs after the host embryo hatches rather than at the egg stage (Chapter 6).

Patterns of infections, prevalence, species richness, co-infection and co-occurrence of dicyemids among infected cephalopods species were explored (Chapter 7). Host size in general did not influence patterns of infection, however where dicyemid species co-occurred, restriction to discrete host sizes was observed, suggesting competition between species may be an important factor leading to niche separation. Calotte shape was found to vary between dicyemid species that co-occurred within a single host individual. Additionally, dicyemid fauna composition was found to vary with host geographical collection locality, alluding to the potential use of dicyemid parasites as biological tags (Chapter 7).

The complete cytochrome *c* oxidase subunit I (*COI*) minicircle molecule, including the *COI* gene plus a non-coding region, was sequenced from nine dicyemid species, and comparisons in sequence composition and size were made between and within species

(Chapter 8). The first phylogeny of dicyemids including multiple taxa from the two genera that combined contain over 90% of the nominal described species was estimated from Bayesian inference and maximum likelihood analyses. Monotypic species clades were observed, however the paraphyly to the genera suggests classification based on morphological traits may need revision (Chapter 8).

The hypothesis that parasite genetics of infected cephalopods will allow for a deeper insight into population structuring compared to that gained with complementary methods was tested, with dicyemid mesozoans infecting giant Australian cuttlefish (*Sepia apama*) as the chosen system (Chapter 9). The population structure of *S. apama* previously inferred from host morphology, behaviour and genetics was supported from dicyemid parasite mitochondrial haplotype phylogeography, with an analysis of molecular variance (AMOVA) providing an alternative insight into structuring of this cuttlefish species. This result suggests that in the future, a holistic approach that incorporates parasite and host data (morphology and genetics) should be used to assess cephalopod population boundaries.

An invited review article on the use of parasites as biological tags to assess the population structure of marine organisms is presented as the final data chapter (Chapter 10). Comments are made on the guidelines for selecting a parasite species as a reliable tag candidate, the need to incorporate parasite genetic information and the benefits of a multidisciplinary approach.

The direct outcomes of my study include the description of the first dicyemid species from Australian waters, insights into the unknowns in the dicyemid life cycle, presentation of the first dicyemid phylogeny allowing taxa classification to be assessed outside of the sole morphological approach and analysis of the use of dicyemid parasites as biological tags, supporting the integration of dicyemid parasite genetics alongside other complementary methods to assess cephalopod population structure. In summary, my study has significantly contributed to the field of dicyemid research, increasing both fundamental and applied knowledge on this enigmatic group of organisms.

ACKNOWLEDGEMENTS

What a journey! I never imagined that I would undertake a PhD, but it has been an amazing experience and one that I have truthfully enjoyed. There are numerous people to thank that have been so supportive of me along the way and that have helped me in the field with sample collections. To begin, thanks to Dave Barker, Cameron Dixon, Kathleen Hill, Graham Hooper, Adnan Moussalli, Bernadette Saunders, Brian Saunders, Richard Saunders, Alex Schnell, Errol Sporer and Lucas Woolford for either organising prawn boat surveys, research trawls, providing cephalopod samples or volunteering in the field. Thanks to all the prawn boat crew aboard Briana Rene Adele, Frank Cori, Kylie, Naturalist and Skandia, who were always accommodating and kept me entertained with their antics, stories and must-try, innovative tips for avoiding seasickness!

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I must thank my mentors Dr Rodney Ratcliff and Dr Kate Hutson. Rod, thanks for the opportunities, wisdom and friendship you have provided me along my research pathway, and Kate, I still remember your advice when I started Honours to 'just have fun with it', which has also served me well during my PhD (along with the parasite dances…!). Special mention to Terry Bertozzi for assistance with molecular genetic methods, sample collections and general chats – you are an indispensable resource to us students and your help has always been greatly appreciated.

Thanks to all the members of the marine, molecular and parasitology lab groups for fortnightly discussions, insights, ideas and informal corridor chats. I must thank Chris Izzo,

Juan Pablo Livore and Patrick Santos with whom I shared an office during my PhD, as well as the other Honours and postgraduate students that I have met along the way which made this journey that much easier and enjoyable. Special mention to Elizabeth Maciunas, the coffee and cheesecake breaks helped get me through!

To my supervisors, Prof Bronwyn Gillanders, Prof Steve Donnellan and A/Prof Ian Whittington, thank you for your guidance, support, knowledge and constructive comments on the numerous grant applications, funding reports, conference abstracts, manuscripts and thesis chapters that I sent your way during the course of my PhD. In particular, thank you Bronwyn for always making time for me, offering wise words of advice, but also providing me with the opportunity to drive the project in an independent manner. Steve, I have learnt so much from you with your wealth of knowledge on molecular ecology and evolution, and appreciate you always taking the time to explain new techniques as well as your enthusiasm for this project and group of parasites. To Ian, who has been there since Honours, I appreciate how meticulous and thorough you have been to all aspects of my research. The green pen, black listing at ASP, complaint letter, Small Researcher of Cephalopods, tall shelf jokes and Masterchef updates are just some of the many memories I have from this journey that will always bring a smile to my face. Tim Benson must be mentioned here too, thank you for all the help you have provided since our first encounter a few years back.

Last but by no means least, I would like to thank my family, who are my greatest support network and source of strength - I dedicate this thesis to you. In particular, my grandparents, Nonno Carmelo, Nonna Anna, Nonno Angelo and Nonna Rachele, for the sacrifices you made towards a better life for us – tanti baci. My brother Angelo and sister-in-law Stef, the commitment and perseverance you both show in your own career paths inspires me to strive to the best of my ability in all that I do. Thank you for always being there for me no matter what. My parents, Domenic and Margaret, words cannot express my gratitude. I look up to you both as role models and have been blessed to have such supportive, loving and selfless parents. Thank you. Finally, my fiancé and best friend, Kieran. Thank you for being patient and understanding when days were long. You have taught me that no problem is too large or difficult and that anything can be solved if you put your mind to it. Thank you for just being you. Grasshopper has done it!

CHAPTER 1: General Introduction

Media outreach: filming of my research at Point Lowly, South Australia, Australia, for two episodes which aired on Network 10 children's program *Scope* in September and November, 2012 (photo: Kieran Brazell).

General Introduction

Parasites and their relationship with the host

Parasitism can be simply defined as a relationship where one organism, the parasite, benefits at the expense of another organism, the host. This relationship represents a successful way of life, having evolved independently at least 60 times in animals (Poulin & Morand 2000). The most widely accepted definition of a parasite is an organism which lives in or on another organism (the host), which it feeds on, shows some degree of structural adaptation to and causes harm to (Poulin 2011). Parasites display a wide variety of life cycles, adaptations and diversification of forms which assist them in the colonisation of a host species (Poulin 2011). However they must also combat a number of challenges in order to successfully invade a host, including the ability to overcome environmental factors in the habitat, biological barriers provided by the host and constraints in their own life cycle (Roberts *et al.* 2002). Nonetheless, it is suggested that every free-living individual plays host to at least one parasite species at some stage during its life, highlighting that parasites are ubiquitous in both terrestrial and aquatic systems (Whittington & Chisholm 2003).

The relationship between a parasite and its host is an intimate one that is characterised by many complex interactions that are continuously changing (Bush *et al.* 2001; Roberts *et al.* 2002). The parasite depends on its host for food, habitat, shelter, transport, metabolic processes and completion of its life cycle. In turn, the parasite can influence and manipulate host appearance, behaviour, nutrient state, reproductive capacity and defence systems (Karvonen & Seehausen 2012). In aquatic systems, parasites may shape host population dynamics by imposing fitness costs, alter interspecific competition, influence energy flows, cause commercial losses in aquaculture systems and drive biodiversity (Brown *et al.* 2003; Hudson *et al.* 2006; Barber 2007). They have been applied as biological tags, providing insights into host population structure, habitat, range, migratory movements, diet and social behaviours (see Gibson 1972; Lester 1990; Moser 1991; Williams *et al.* 1992; Boje *et al.* 1997; MacKenzie 2002; Braicovich & Timi 2008; Mattiucci *et al.* 2008), and have also been used as bio-indicators of pollution (Poulin 1992; MacKenzie *et al.* 1995; MacKenzie 1999). Clearly due to this intimate relationship, parasites have significant ecological and evolutionary consequences for hosts and host populations (Marcogliese & Cone 1997; Karvonen & Seehausen 2012). However before these complex interactions, effects and

consequences can be evaluated and understood, it is important to identify the parasite species that are actually infecting a host species.

Identification of a parasite species as a distinct taxonomic entity is not without issues. Several parasite species may be mistakenly lumped into one, or multiple parasite species may be described from what is a single species which exhibits plasticity (Poulin & Morand 2000). Each of these scenarios respectively can lead to underestimates or overestimates of true parasite diversity and species richness in a system, and may also confound our understanding of parasite-host interactions. Therefore it is essential to use a robust approach which will lead to a high probability of correctly identifying what is actually a distinct species. In the past, the most common means to identify a species has been based on morphological characters, however this approach is increasingly being recognised as unreliable when used as the sole criterion (McManus & Bowles 1996). A more robust approach is to use a combination of morphological and molecular (i.e. identification on the basis of genetics) methods, which can assist with the identification of cryptic species and those that exhibit plasticity in morphological character traits (Poulin & Morand 2000; Littlewood *et al.* 2001).

This introduction into parasitism and parasites highlights the wealth of fundamental and applied knowledge that can be gained from studying parasite species and assemblages, not only for the parasite, but also for the host species. Parasites clearly play a role in the maintenance of biological and behavioural diversity of their hosts, so it is important to answer questions about what species occur where, what drives patterns of infection and prevalence, how parasites are maintained and proliferate in a system, the effect they can have on the host, and their biological identity compared to closely related taxa.

Dicyemid mesozoan parasites

The phylum Dicyemida Van Beneden, 1876 contains the poorly-understood and little-known group of marine organisms, dicyemid mesozoans. They are the most common and characteristic parasites in the renal appendages (synonyms with kidneys, renal sacs and renal organs) of benthic cephalopods (Furuya & Tsuneki 2003; Furuya *et al.* 2004). They occur in densities of thousands of animals per cm^3 and are highly host-species specific, although co-infection by more than one dicyemid species in a single cephalopod species has been documented (Furuya 1999). Dicyemids derive all their metabolic requirements from the dissolved nutrients in the host's urine (Hochberg 1982), though whether this relationship is purely parasitic remains unclear. Some studies state that dicyemids erode the renal surface

where they attach and also deprive the host of nutrients, therefore satisfying the definition of a parasite (Ridley 1968; Finn *et al.* 2005). However other studies suggest dicyemids do not harm the host and that this relationship is mutualistic, with the beating cilia covering the dicyemids body assisting with the excretion of urine from the renal sac (Lapan & Morowitz 1972; Lapan 1975b; Hochberg 1982; Furuya *et al.* 2004).

Morphological characters

The body organisation of a dicyemid is quite simple. They comprise only eight to 40 cells, with neither body cavities nor differentiated organs (Suzuki *et al.* 2010). They are characterised by having one long cylindrical axial cell which is surrounded by ciliated peripheral cells. At the anterior region, the top two tiers of cells (metapolar and propolar cells) are modified to form a calotte (attachment organ), allowing the dicyemid to maintain a foothold into the convoluted surfaces of the hosts renal appendages (Furuya *et al.* 2003a; Awata *et al.* 2006; Furuya *et al.* 2007).

The arrangement of the metapolar and propolar cells in the calotte traditionally determines the familial and generic placement of dicyemid species, with differences occurring both in the number and arrangement of these anterior cells (Hochberg 1982, 1983). Additionally, the size of the adult stages, the number of cells comprising the body, the shape of the calotte, the anterior extension of the axial cell, the presence or absence of verruciform cells and the structure of the infusoriform larvae are the most common morphological characters used to distinguish species (Hochberg 1982, 1983). Nonetheless, incomplete and information-poor descriptions, loss of type specimens, errors in taxonomy and conceptual differences have led to confusion over the validity of certain taxa within this phylum, with no molecular genetic studies being used in combination with classical morphological methods for species identifications.

Dicyemid life cycle

In contrast to the simple body organisation of dicyemids, their life cycle is complex with two stages of development (vermiform and infusoriform stages) and two modes of reproduction (asexual and sexual). The vermiform stages (vermiform embryo, nematogen adult and rhombogen adult) are formed asexually from an agamete in the axial cell of the nematogen adult and spend all of their life cycle attached to the host's renal appendages (McConnaughey 1951; Furuya *et al.* 2003b). The infusoriform stage, characterised by the morphologically-

distinct infusoriform embryo, escapes out into the ocean to find and infect a new host individual (Furuya & Tsuneki 2003). This embryo is produced via sexual reproduction from the infusorigen (hermaphroditic gonad) in the axial cell of the rhombogen adult (McConnaughey 1951; Furuya *et al.* 2003b).

This complex life cycle with two stages of development and two modes of reproduction allows the dicyemid parasite to switch between and fulfil population density strategies (ensuring persistence within a host individual) and dispersal strategies (ensuring persistence beyond the death of the host individual) (Furuya et al., 2003b). Certain transmission and developmental processes in the dicyemid life cycle, such as at which host life cycle stage new infection by the infusoriform embryo occurs, the mode of entry for new infection and how this embryo develops into the vermiform stages in the new host individual, presently remain unknown.

Position in the Tree of Life

The placement of Dicyemida in the Tree of Life is uncertain and controversial, due to dicyemids having both protozoan and metazoan features. They are bilaterally symmetrical with no tissues, body cavities or differentiated organs such as a gastrointestinal tract or nervous system, suggesting an affiliation with protozoans. Yet despite lacking these attributes that characterize metazoans, they are also multicellular with complex life cycles.

The Belgian biologist, Edouard Van Beneden (1876), created the name Mesozoa Van Beneden, 1876 to classify these organisms, as he believed this group occupied an evolutionarily intermediate position between the Protozoa and the Metazoa. Since then, dicyemids have been considered as evolved multicellular protozoans (Cavalier-Smith 1993), an extant link between protozoans and metazoans (Hyman 1959; Lapan & Morowitz 1974; Czaker 2000), chimeras of protozoans and metazoans (Noto & Endoh 2004), degenerate flatworms (Nouvel 1948; McConnaughey 1951; Stunkard 1972) and relatives of triploblasts (Katayama *et al.* 1995). Four recent molecular genetic studies on the presence and expression of regulatory genes, *Pax6*, *Zic* (Aruga *et al.* 2007), *Hox, otx* and *brachyury* (Kobayashi *et al.* 1999; Kobayashi *et al.* 2009) and innexin amino acid sequences (Suzuki *et al.* 2010) all suggest that dicyemids are simplified bilaterian metazoan animals that are most closely related to higher lophotrochozoans. However these molecular studies have not surveyed dicyemids from multiple taxa and there is no phylogeny examining the relationship between and within dicyemid species outside of the classical morphological approach.

Lack of research focus on southern hemisphere dicyemids

Dicyemids have been documented primarily from the northern hemisphere, including the western and north-eastern Pacific Ocean, northern Indian Ocean, Mediterranean Sea, north-western and eastern Atlantic Ocean, Gulf of Mexico and Arctic Ocean (Furuya 2010). There are relatively few published works on dicyemids from the southern hemisphere (i.e. Falkland Islands - Hochberg and Short (1970); Antarctic Peninsula - Short and Powell (1969), Short and Hochberg (1970) and Hochberg and Short (1983); and New Zealand - Short and Hochberg (1969) and Short (1971)), and only one study has documented dicyemid parasites from Australian cephalopods. Finn et al. (2005) collected 38 cephalopods species throughout southern, eastern and western Australia. They recorded the presence of dicyemids in 24 cephalopod species, but provided no formal descriptions of new dicyemid species.

Gaps in knowledge

There is a clear knowledge gap in dicyemid fauna and diversity from the southern hemisphere, particularly from Australian waters, for which no dicyemid species have been described formally. Subsequently, this knowledge gap led onto my research, investigating the dicyemid parasite species that infect Australian cephalopods, and comparing this fauna to that from northern hemisphere systems to gain a better understanding of the factors driving observed patterns of infection, prevalence and species richness for dicyemids. As molecular genetics studies are limited for the dicyemids, I wanted to use a combined morphological and molecular approach to describe new species and explore this relationship between and within species in greater depth. I made use of prawn trawl surveys in southern Australian waters to obtain fresh cephalopod material from the by-catch, allowing parasite smears to be prepared and dicyemid morphology to be characterised. Renal appendage material previously collected and stored in the South Australian Museum was also used in my study, adding to my dataset for molecular genetic analyses of dicyemid parasites. Furthermore, the outstanding localised abundance of giant Australian cuttlefish (*Sepia apama*) at a mass breeding aggregation in Upper Spencer Gulf, South Australia, Australia, allowed unknowns in the dicyemid life cycle to be explored, from the collection and molecular genetic analyses of host eggs and filtered seawater samples. I also explored further Hochberg's (1990) suggestion that dicyemids may be useful biological tags candidates in resolving cephalopod population structure and complex host taxonomic problems. In particular, the main aims of my research were to:

Main aims

1) Review the taxonomic confusion surrounding the Dicyemida in the current literature and present a comprehensive list of all species described to date (Chapter 2);

2) Document and formally describe the dicyemid parasite fauna of Australian cephalopod host species (Chapters 3, 4, and 5);

3) Explore the dicyemid life cycle and resolve at which host life cycle stage new infection by the dispersive infusoriform embryo occurs (Chapter 6);

4) Examine dicyemid patterns of infection and prevalence with respect to host size, host life history properties, host geographical collection locality, renal appendage (left *vs* right) and inter-parasite species competition for attachment sites, space and nutrient (Chapter 7);

5) Present the first phylogeny for dicyemid parasites to explore relationships between and within species based on molecular genetic data (Chapter 8);

6) Test the hypothesis that analysis of parasite population genetic structure will allow a deeper insight into cephalopod population structuring, compared to that gained using complementary methods (i.e. artificial tags, morphometrics, host genetics, life histories and behaviour) (Chapter 9); and

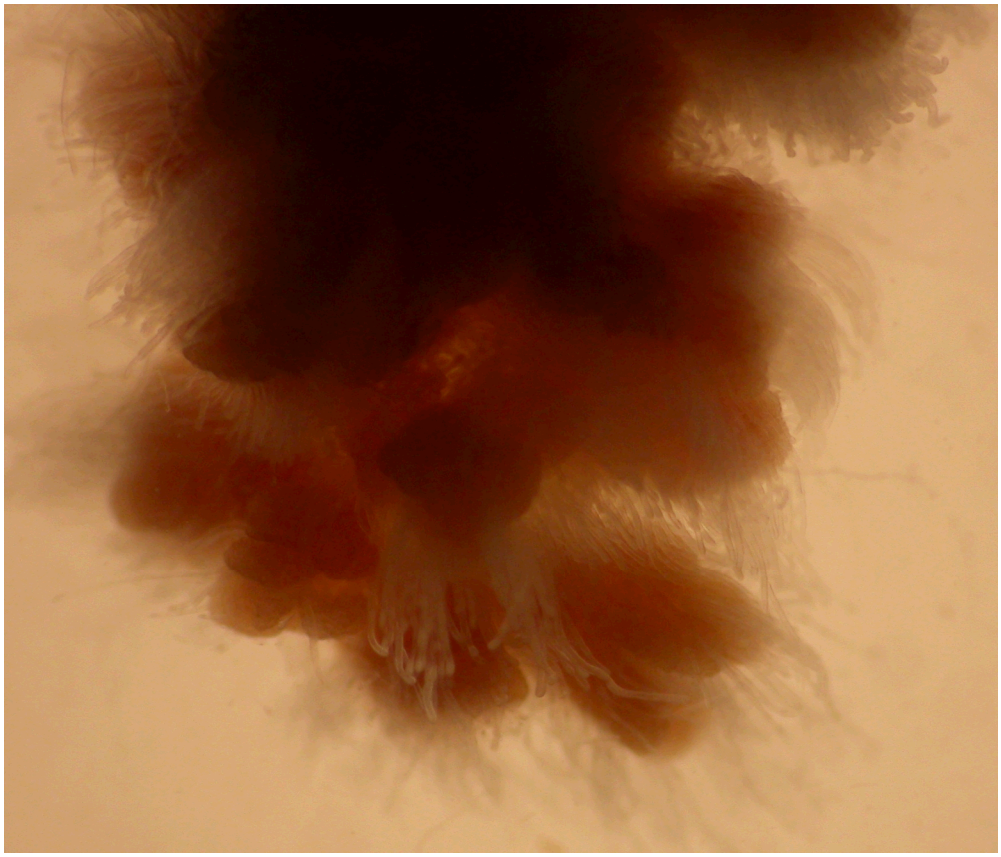
7) Review the use of parasites as biological tags providing comments on recent genetic advances and the benefits of a holistic approach (Chapter 10)

Chapters 2, 3, 4, 5 and 6 have been published in scientific journals, with Chapter 10 in press, Chapters 7 and 8 in review and Chapters 9 to be submitted shortly. The style and references for each chapter are therefore formatted according to the instructions for authors given by each scientific journal. Each chapter can be read as a separate paper, with separate introduction, methods, results and discussion, however collectively they form a logical progression of ideas that developed during my PhD study.

Each chapter is preceded by a preamble that provides information on the publication status at the time of submission along with the contributions of co-authors (where relevant). In the last section, Chapter 11, a general discussion is presented highlighting the main findings, implications and knowledge gaps filled from my study, concluding with likely avenues for future research. Copyright permission forms from publishers, granting the inclusion of published papers as chapters in my thesis, are provided in Appendix A.

This project was financially supported by funding from the Australian Biological Resources Study, Australian Federation of Graduate Women, Australian Society for Parasitology, Holsworth Wildlife Research Endowment, Lirabenda Endowment Fund, Nature Conservation Society of South Australia, Nature Foundation South Australia, Norman Wettenhall Foundation and Sir Mark Mitchell Research Foundation.

CHAPTER 2: A review of the families, genera and species of Dicyemida Van Beneden, 1876



Hundreds of dicyemid mesozoan parasites (white, fuzzy strands) attached to the renal appendage (in red) of *Sepia apama*.

Chapter 2 Preamble

This chapter is a review paper published in *Zootaxa*. It is included with permission from Magnolia Press (see Appendix A), and can be cited as: Catalano, S.R., 2012. A review of the families, genera and species of Dicyemida Van Beneden, 1876. *Zootaxa* 3479, 1–32. As five dicyemid species were omitted from Table 1 in the original publication, an erratum was published in *Zootaxa* 3646, 100, which is included here following the full length paper.

In this chapter, I searched through and collated information from the past literature in an attempt to unravel the confusion surrounding the Dicyemida. Prof Eric Hochberg (Santa Barbara Museum of Natural History, USA), Prof Hidetaka Furuya (Osaka University, Japan), the Barr Smith Library staff and Matt Taylor (PhD candidate, University of Adelaide) provided copies of older literature that was otherwise impossible to source, and Rebecca Kittel (PhD candidate, University of Adelaide), assisted with translation of German papers. I wrote the accepted manuscript and acted as the corresponding author. Drafts of this paper were reviewed by Bronwyn Gillanders, Steve Donnellan and Ian Whittington.

I certify that the statement of contribution is accurate

Signed:_____ Date:_____

(Sarah Roseann Catalano)

Catalano, S.R. (2012) A review of the families, genera and species of Dicyemida Van Beneden, 1876. *Zootaxa, v. 3479, pp. 1-32*

CHAPTER 3: Two new species of dicyemid (Dicyemida: Dicyemidae) from two Australian cephalopod species: *Sepioteuthis australis* (Mollusca: Cephalopoda: Loliginidae) and *Sepioloidea lineolata* (Mollusca: Cephalopoda: Sepiadariidae)

Striped pyjama squid, *Sepioloidea lineolata,* infected by *Dicyema pyjamaceum* Catalano & Furuya 2012.

Chapter 3 Preamble

This chapter is a co-authored manuscript that describes two new species of dicyemid parasites, the first from Australian cephalopod species, and is published in *Journal of Parasitology*. It is included with permission from Allen Press (see Appendix A), and can be cited as: Catalano, S.R. and Furuya, H., 2013. Two new species of dicyemid (Dicyemida: Dicyemidae) from two Australian cephalopod species: *Sepioteuthis australis* (Mollusca: Cephalopoda: Loliginidae) and *Sepioloidea lineolata* (Mollusca: Cephalopoda: Sepiadariidae). *Journal of Parasitology* 99, 203–211.

In this chapter, I collected the samples, performed dissections, prepared kidney smears and examined the smears for morphological analyses. I also supplied the funding and compiled the images (line drawings and photos). Lab space, microscope usage, data sheets and a tutorial on the cell types of dicyemid parasites was provided by my co-author, Prof Hidetaka Furuya (Osaka University, Japan). I wrote the manuscript, with drafts and proofs reviewed by Prof Hidetaka Furuya.

I certify that the statement of contribution is accurate

Signed:_____ Date:_____

(Sarah Roseann Catalano)

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis

Professor Hidetaka Furuya

J. Parasitol., 99(2), 2013, pp. 203–211

TWO NEW SPECIES OF DICYEMID (DICYEMIDA: DICYEMIDAE) FROM TWO AUSTRALIAN CEPHALOPOD SPECIES: *SEPIOTEUTHIS AUSTRALIS* (MOLLUSCA: CEPHALOPODA: LOLIGINIDAE) AND *SEPIOLOIDEA LINEOLATA* (MOLLUSCA: CEPHALOPODA: SEPIADARIIDAE)

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ABSTRACT: Two new species of dicyemid parasites from *Dicyema* are described from 2 species of Australian cephalopods, i.e., *Dicyema calamaroceum* n. sp. from *Sepioteuthis australis* Quoy and Gaimard, 1832 (southern calamary) collected from Spencer Gulf (SG) and Gulf St Vincent (GSV), South Australia (SA), Australia, and *Dicyema pyjamaceum* n. sp. from *Sepioloidea lineolata* Quoy and Gaimard, 1832 (striped pyjama squid), collected from SG, SA, Australia. *Dicyema calamaroceum* is a medium sized species that reaches approximately 2,400 μm in length. The vermiform stages are characterized by having 31–34 peripheral cells, a conical calotte, and an axial cell that extends to the propolar cells. An anterior abortive axial cell is absent in vermiform embryos, and verruciform cells were not observed in nematogens and rhombogens. Infusoriform embryos consist of 39 cells; 2 nuclei are present in each urn cell, and the refringent bodies are solid. *Dicyema pyjamaceum* is smaller than *D. calamaroceum*, with a body length that reaches approximately 1,950 μm. The vermiform stages are characterized by having 20–23 peripheral cells, a cap-shaped calotte that forms a cephalic swelling together with the parapolar cells, and an axial cell that extends to the propolar cells. An anterior abortive axial cell is absent in vermiform embryos. Verruciform cells and granules in propolar cells were observed in nematogens and rhombogens. Infusoriform embryos consist of 37 cells; 2 nuclei are present in each urn cell, and the refringent bodies are solid. This represents the first description of dicyemid parasites from Australia.

Dicyemid mesozoans (Dicyemida Van Beneden, 1876), small microscopic, worm-like organisms, are the most common and characteristic parasites in the renal appendages (synonymous with kidneys, renal sacs, and renal organs) of benthic cephalopods, occurring in densities of thousands of animals per cm^3 (Furuya et al., 1997; Furuya and Tsuneki, 2003; Furuya et al., 2004; Finn et al., 2005). Their body organization is simple, with neither body cavities nor differentiated organs, and they are comprised of only 8 to 40 cells (Furuya et al., 2007; Suzuki et al., 2010). They spend the majority of their life cycle attached to the host's renal appendages, deriving all their metabolic requirements from the dissolved nutrients in the host's urine (Hochberg, 1982). The majority of dicyemid species studied are host-specific, and, typically, 2, or more, species live in each host species or host individual (Furuya, 1999). To date, 112 species of dicyemids have been described, although confusion exists and surrounds the validity of certain families, genera, and species (see Catalano, 2012, for a review).

Dicyemids have primarily been documented from localities in the Northern Hemisphere, including the western and northeastern Pacific Ocean, northern Indian Ocean, Mediterranean Sea, northwestern and eastern Atlantic Ocean, Gulf of Mexico, and Arctic Ocean (Furuya, 2010). There are relatively few published works on dicyemids from the Southern Hemisphere, i.e., Falkland Islands (Hochberg and Short, 1970), Antarctic Peninsula (Short and Powell, 1969; Short and Hochberg 1970; Hochberg and Short, 1983), and New Zealand (Short and Hochberg, 1969; Short, 1971). Only 1 study has documented dicyemid parasites from Australian cephalopods. Finn et al. (2005) collected 38 cephalopods species from 8 families throughout southern, eastern, and western Australia, and recorded the presence or absence of dicyemid species in each host species. Twenty-four cephalopod species from 5 families were found to be infected, but no formal descriptions of new dicyemid species were given. The present study, therefore, represents the first description of new dicyemid parasites from Australian cephalopods. Here 2 new dicyemid species are formally described from 2 squid species representative of 2 families, i.e., *Sepioteuthis australis*, southern calamary (Loliginidae), and *Sepioloidea lineolata*, striped pyjama squid (Sepiadariidae). While Finn et al. (2005) recorded the presence of dicyemids in 12 of 15 *Sepioloidea lineolata* individuals, no dicyemids were recorded from 11 *Sepioteuthis australis* examined from southern Australian waters. Therefore, the documentation of a dicyemid species from *S. australis* in the present study also represents a new host record.

MATERIALS AND METHODS

Here 25 *Sepioteuthis australis* and 5 *Sepioloidea lineolata* were examined for dicyemids from November 2010 to October 2011. Six of 25 *Sepioteuthis australis* and all 5 *Sepioloidea lineolata* were infected, each with its own, new *Dicyema* species (Table I). Host specimens were obtained from the by-catch of prawn surveys (South Australian Research and Development Institute) in Spencer Gulf (SG) and Gulf St Vincent (GSV), South Australia (SA), Australia, and were examined immediately upon capture. Host mantle length (cm), weight (g), collection locality, and date examined were recorded for each cephalopod individual and are presented in Table I.

The body of each cephalopod was placed ventral side up in a tray and the mantle cavity was opened to expose the paired renal sacs. Small pieces of the left and right renal appendages were removed and smeared on to 76 × 25 mm glass microscope slides, thickness 1 mm (Livingstone). Four smears were made per renal appendage, with a total of 8 smears per host. Dissecting equipment was cleaned and sterilized in absolute ethanol for each renal appendage and for each host to avoid cross-contamination of preparations. The glass slide smears were fixed immediately to avoid parasite desiccation and stored in 70% ethanol in the field in Lock-Mailer™ jars (Ted Pella Inc.), then stained and mounted upon returning

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TABLE I. Dicyemid species from *Sepioteuthis australis* and *Sepioloidea lineolata* from South Australian waters. Abbreviations: GSV, Gulf St Vincent; ML, mantle length; SG, Spencer Gulf; W, weight.

Host no.	ML (cm)	W (g)	Locality	Date of examination	Dicyemids
Sepioteuthis australis (southern calamary)					
SRC11	10.0	54	SG	5 November 2010	None
SRC12	9.0	40	SG	5 November 2010	None
SRC13	9.0	44	SG	5 November 2010	None
SRC14	9.0	46	SG	5 November 2010	None
SRC15	7.5	28	SG	5 November 2010	None
SRC16	10.0	64	SG	5 November 2010	None
SRC17	9.1	48	SG	5 November 2010	None
SRC18*	9.0	42	SG	5 November 2010	*D. calamaroceum* n. sp.
SRC19	9.4	42	SG	5 November 2010	None
SRC20	10.5	60	SG	5 November 2010	*D. calamaroceum* n. sp.
SRC21	9.5	46	SG	5 November 2010	None
SRC22	9.7	45	SG	5 November 2010	None
SRC26	13.5	114	SG	5 November 2010	None
SRC37	15.0	145	SG	5 November 2010	*D. calamaroceum* n. sp.
SRC39	16.0	218	SG	6 November 2010	*D. calamaroceum* n. sp.
SRC49	15.5	150	GSV	1 December 2010	None
SRC50	15.0	150	GSV	1 December 2010	None
SRC52	17.0	190	GSV	1 December 2010	None
SRC53	17.5	215	GSV	1 December 2010	None
SRC54	15.0	210	GSV	1 December 2010	*D. calamaroceum* n. sp.
SRC55	18.0	240	GSV	1 December 2010	None
SRC58	20.5	350	GSV	1 December 2010	*D. calamaroceum* n. sp.
SRC60	16.0	190	GSV	2 December 2010	None
SRC61	14.0	130	GSV	2 December 2010	None
SRC66	16.0	200	GSV	2 December 2010	None
Sepioloidea lineolata (striped pyjama squid)					
SRC24	4.6	36	SG	5 November 2010	*D. pyjamaceum* n. sp.
SRC28	4.7	36	SG	5 November 2010	*D. pyjamaceum* n. sp.
SRC30	5.0	42	SG	5 November 2010	*D. pyjamaceum* n. sp.
SRC31	3.0	16	SG	5 November 2010	*D. pyjamaceum* n. sp.
SRC155*	4.5	35	SG	26 October 2011	*D. pyjamaceum* n. sp.

* Host tissue deposited in the Australian Biological Tissue Collection, SAMA, for the syntypes of *Dicyema calamaroceum* n. sp. (southern calamari, ABTC 122524) and *Dicyema pyjamaceum* n. sp. (striped pyjama squid, ABTC 122525).

to the laboratory in LockMailer™ jars containing each stain respectively. A mantle tissue sample was also taken for each host individual and preserved in 100% DNA grade ethanol.

Each smeared slide was rinsed in MiliQ water 3 times, stained in Ehrlich's acid hematoxylin diluted 20 parts MiliQ water to 1 part stain for 20 min, then rinsed again in MiliQ water. Slides were then dehydrated in an ethanol series and counterstained in eosin (70% ethanol for 10 min, 90% ethanol for 10 min, eosin 1% alcoholic solution diluted 20 parts MiliQ water to 1 part stain for 2 min and 100% ethanol for 15–20 min). Canada balsam was applied to 22 × 60 mm coverslips, which were placed on top of each stained glass slide. Mounted smears were dried on a hot plate at 50 C before examination with a compound light microscope (BX–51, Olympus, Tokyo, Japan) at magnifications up to ×1,500. Measurements and drawings were made with the aid of an ocular micrometer and a drawing tube (U–DA, Olympus), respectively, at Osaka University, Toyonaka Campus, Japan. All measurements are in micrometers (μm) as mean and 1 SD.

The terminology for cell names used in the description of infusoriform larvae is based on Nouvel (1948), Short and Damian (1966), and Furuya (1999, 2009, 2010). Syntypes of the dicyemids are deposited in the Marine Invertebrate Collection, South Australian Museum, Adelaide (SAMA), South Australia 5000, Australia (contact: Thierry Laperousaz, thierry.laperousaz@samuseum.sa.gov.au); and the National Museum of Nature and Science, Tokyo (NSMT), 4-1-4 Amakubo, Tsukuba City, 305-0005 Ibaraki, Japan (contact: Toshiaki Kuramochi, kuramoti@kahaku.go.jp). Tissue from the 2 cephalopods species harboring dicyemid parasites are deposited in the Australian Biological Tissue Collection (ABTC) of the SAMA, South Australia 5000, Australia (contact: Steve Donnellan, Steve.Donnellan@samuseum.sa.gov.au).

DESCRIPTION

Dicyema calamaroceum n. sp.

(Figs. 1, 2; Tables I, II)

Diagnosis: Medium-sized dicyemid; body length reaching 2,400. Calotte conical. Vermiform stages with 31 to 34 peripheral cells 4 propolars + 4 metapolars + 2 parapolars + 21 to 24 trunk cells; 33 peripheral cells most common; propolar cells opposite metapolar cells. Infusoriform embryos with 39 cells; refringent bodies solid; and 2 nuclei present in each urn cell.

Nematogens (Figs. 1A, C, 2A, C; n = 21 measured): Body length from 800 to 2,300, average length 1,459 ± 420, mode 1,370; width from 50 to 170, average width 110 ± 40, mode 70; trunk width mostly uniform. Peripheral cell number 31 to 34 (Table II): 4 propolars + 4 metapolars + 2 parapolars + 19 to 22 diapolars + 2 uropolars. Calotte conical, cilia on calotte approximately 5 long, oriented anteriorly. Propolar cells and their nuclei smaller than metapolar cells and their nuclei, respectively (Figs. 1A, 2A).

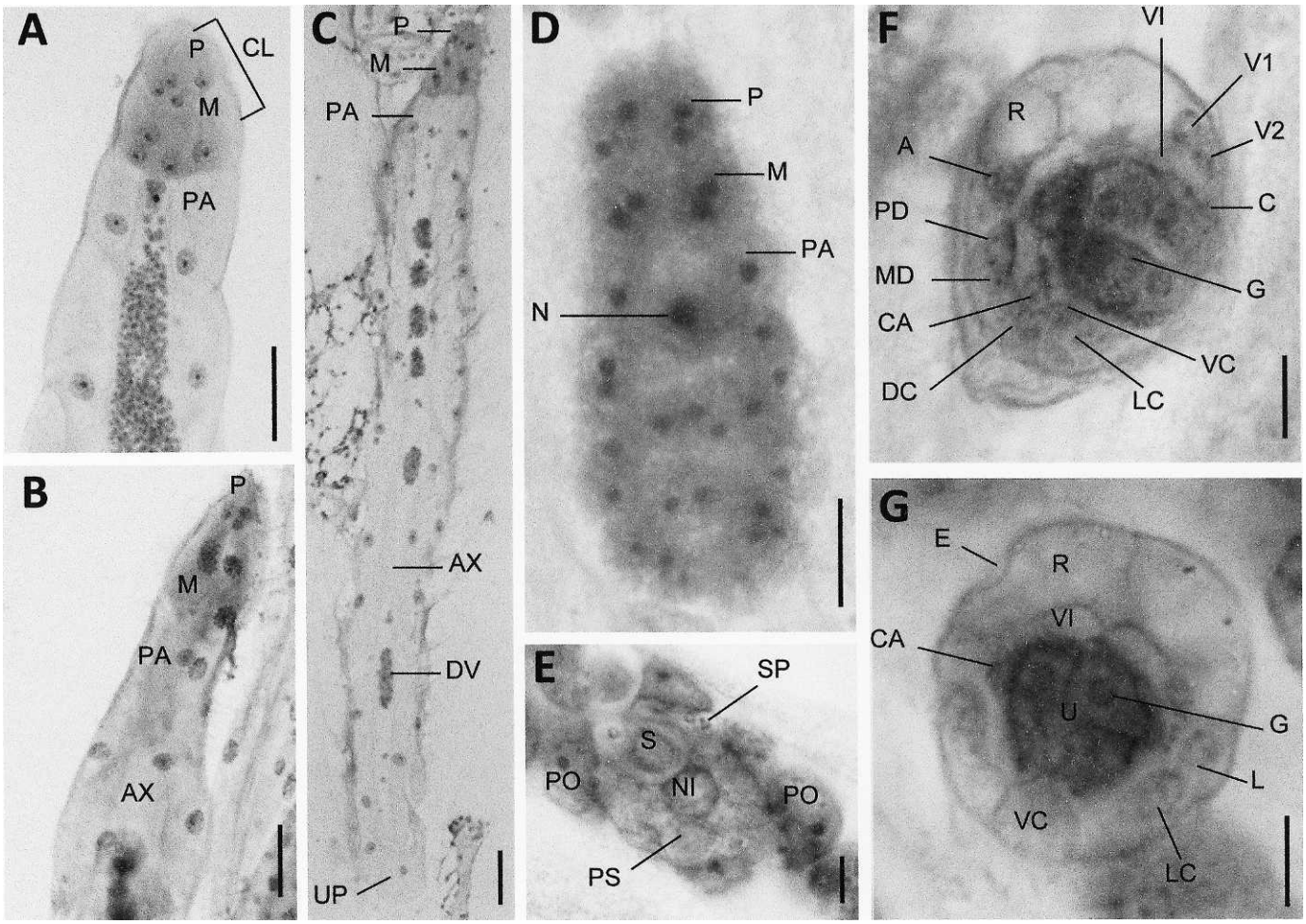

FIGURE 1. Light micrographs of *Dicyema calamaroceum* n. sp. infecting *Sepioteuthis australis*. (**A**) Anterior region of nematogen. (**B**) Anterior region of rhombogen. (**C**) Young nematogen, entire. (**D**) Vermiform embryo within axial cell. (**E**) Infusorigen. (**F**, **G**) Infusoriform embryos within axial cell: (F) optical sagittal section; (G) optical horizontal section. Scale bars represent 50 μm in (A–C), 10 μm in (D–G). Abbreviations: A, apical cell; AX, axial cell; C, couvercle cell; CA, capsule cell; CL, calotte; DC, dorsal caudal cell; DV, developing vermiform embryo; E. enveloping cell; G, germinal cell; L, lateral cell; LC, lateral caudal cell; M, metapolar cell; MD, median dorsal cell; N, nucleus; NI, nucleus of infusorigen; P, propolar cell; PA, parapolar cell; PD, paired dorsal cell; PO, primary oocytes; PS, primary spermatocytes; R, refringent body; S, spermatogonium; SP, sperm; U, urn cell; UP, uropolar cell; VC, ventral caudal cell; VI, ventral internal cell; V1, first ventral cell; V2, second ventral cell.

Cytoplasm of propolar and metapolar cells more darkly stained by hematoxylin and eosin than cytoplasm of other peripheral cells. Propolar cells opposite metapolar cells (Fig. 2C). Verruciform cells absent. Axial cell cylindrical, rounded anteriorly; extends through to middle of propolar cells. Average of 4 vermiform embryos present in axial cell of nematogens, with larger individuals having as many as 10 vermiform embryos in axial cell. Agametes present and numerous in axial cell; shape fusiform. Average diameter 11 ± 2, mode 8.

Vermiform embryo: (Figs. 1D, 2G, H; n = 21 measured): Full-grown vermiform embryos range from 220 to 580 long, average length 340 ± 80, mode 250; and range from 30 to 110 wide, average width 40 ± 20, mode 40. Peripheral cell number 31 to 34 (Fig. 2G, Table II): 4 propolars + 4 metapolars + 2 parapolars + 19 to 22 diapolars + 2 uropolars; 33 peripheral cells most common; propolar cells opposite metapolar cells. Anterior end of calotte rounded, conical. Axial cell rounded anteriorly; extends through to middle of propolar cells. Nucleus usually located in center of axial cell (Fig. 2H). Anterior abortive axial cell absent. Axial cell of full-grown embryos with as many as 2 agametes (Fig. 2H).

Rhombogens (Figs. 1B, 2B, D, E; n = 21 measured): Bodies similar in length and width to nematogens, slightly more slender than nematogens; length from 900 to 2,400, average length 1,590 ± 400, mode 1,770, width from 50 to 90, average width 70 ± 10, mode 70. Peripheral cell number 31 to 34 (Table II), 31 peripheral cells most common; propolar cells opposite metapolar cells (Fig. 2E). Calotte conical (Fig. 2D). Axial cell shape and anterior extent similar to nematogen. Verruciform cells absent. Average of 14 infusoriform embryos present in axial cell of rhombogens, with smaller individuals having as few as 3 and larger individuals having as many as 33. Usually 2, rarely 1, 3 or 4 infusorigens present in axial cell of each parent individual.

Infusorigens (Figs. 1E, 2F; n = 20 measured): Mature infusorigens medium-sized; usually irregular in shape; diameters of 22 to 45, average of 29 ± 7. Composed of 9–14 (mode 10)

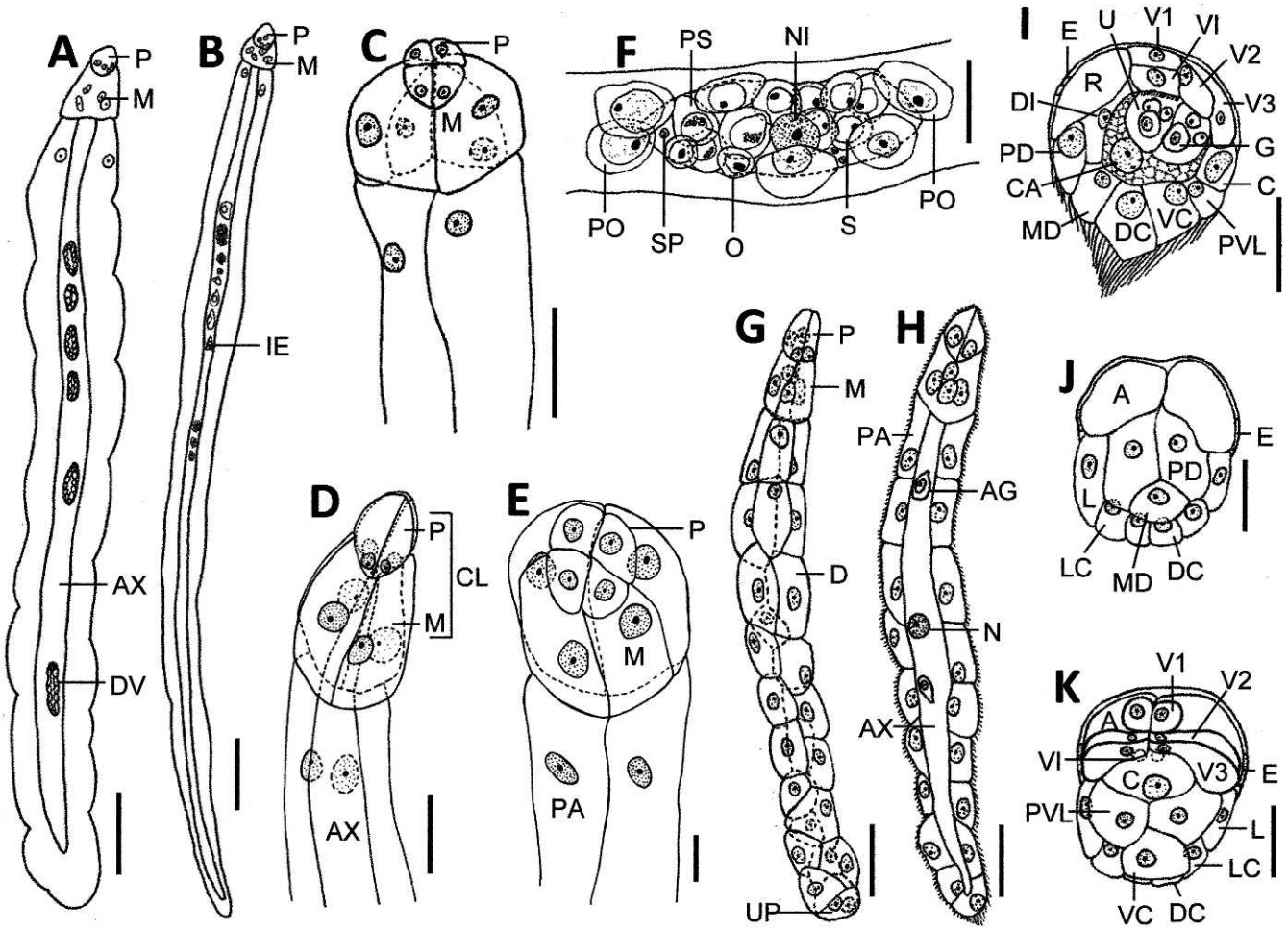

FIGURE 2. Line drawings of *D. calamaroceum* n. sp. infecting *S. australis*. (**A**, **B**) Vermiform stages, entire: (A) nematogen; (B) rhombogen. (**C**) Anterior region of nematogen. (**D**, **E**) Anterior region of rhombogen. (**F**) Infusorigen. (**G**, **H**) Vermiform embryo within axial cell: (G) cilia omitted; (H) optical section. (**I**–**K**) Infusoriform embryos within axial cell: (I) sagittal section; (J) dorsal view (cilia omitted); (K) ventral view (cilia omitted). Scale bars represent 100 μm in (A–B), 50 μm in (C), 20 μm in (D, E, G, H) and 10 μm in (F, I–K). Abbreviations: A, apical cell; AG, agamete; AX, axial cell; C, couvercle cell; CA, capsule cell; CL, calotte; D, diapolar cell; DC, dorsal caudal cell; DI, dorsal internal cell; DV, developing vermiform embryo; E, enveloping cell; G, germinal cell; IE, Infusoriform embryo; L, lateral cell; LC, lateral caudal cell; M, metapolar cell; MD, median dorsal cell; N, nucleus; NI, nucleus of infusorigen; O, oogonium; P, propolar cell; PA, parapolar cell; PD, paired dorsal cell; PO, primary oocytes; PS, primary spermatocytes; PVL, posteroventral lateral cells; R, refringent body; S, spermatogonium; SP, sperm; U, urn cell; UP, uropolar cell; VC, ventral caudal cell; VI, ventral internal cell; V1, first ventral cell; V2, second ventral cell; V3, third ventral cell.

external cells (egg line: oogonia and primary oocytes) + 7–16 (mode 10) internal cells (sperm line: spermatogonia, primary spermatocytes, and secondary spermatocytes) + 8–14 (mode 9) sperm. Mean diameter of fertilized eggs 12 ± 2, sperm 1 ± 0.

Infusoriform embryos (Figs. 1F, G, 2I–K; n = 100 measured): Full-grown embryos large, lengths average 34 ± 3 (excluding cilia); length: width: depth ratio 1.00: 0.73: 0.70. Shape ovoid, bluntly rounded to pointed posteriorly (Figs. 1F, 2I). Cilia at posterior end 10.5 long. Refringent bodies present, solid and large; occupy 57% of embryo length when viewed laterally (Fig. 2I). Cilia projecting from ventral internal cells to urn cavity (Fig. 2I). Capsule cells contain many large granules. Mature infusoriform embryos consisting of 39 cells: 35 somatic + 4 germinal. Somatic cells of several types present: external cells cover large part of anterior and lateral surfaces of embryo (2 enveloping cells); external cells with cilia on external surface (2 paired dorsal cells + 1 median dorsal cell + 2 dorsal caudal cells + 2 lateral caudal cells + 1 ventral caudal cell + 2 lateral cells + 2 posteroventral lateral cells); external cells with refringent bodies (2 apical cells); external cells without cilia (1 couvercle cell + 2 anterior lateral cells + 2 first ventral cells + 2 second ventral cells + 2 third ventral cells); internal cells with cilia (2 ventral internal cells); and internal cells without cilia (2 dorsal internal cells + 2

TABLE II. Number of peripheral cells in *D. calamaroceum* n. sp. infecting *S. australis*.

	No. individuals		
Cell no.	Vermiform embryos	Nematogens	Rhombogens
31	4	12	10
32	14	4	1
33	18	26	6
34	9	9	1

capsule cells + 4 urn cells). Each urn cell contains 1 germinal cell and 2 nuclei (Fig. 2I). All somatic nuclei appear pycnotic in mature infusoriform embryos.

Taxonomic summary

Type host: Southern calamary, *Sepioteuthis australis* Quoy and Gaimard, 1832 (Mollusca: Cephalopoda: Loliginidae), 9.0 cm mantle length, 42 g weight (ABTC 122524).

Type locality: Spencer Gulf, South Australia, Australia (33°18′37″S, 137°30′45″E).

Additional localities: Gulf St. Vincent, South Australia, Australia (35°16′12″S, 138°9′10″E).

Site of infection: Attached to the surface of the renal appendages.

Prevalence: Six of 25 hosts examined (24% prevalence).

Specimens deposited: Two syntype slides deposited in the Marine Invertebrates Collection, SAMA, Australia (E3731–2) and in the NSMT, Japan (NSMT-Me 19–20).

Etymology: The species name is derived from the common name of the host, southern calamary.

Remarks

Dicyema calamaroceum n. sp. with 31–34 peripheral cells is similar to 6 other species: *D. erythrum* Furuya, 1999 (28–36), *D. ganapatii* Kalavati et al., 1984 (28–32), *D. lycidoeceum* Furuya, 1999 (26–34), *D. macrocephalum* Van Beneden, 1876 (28–33), *D. oxycephalum* Furuya, 2009 (28–34), and *D. sullivani* McConnaughey, 1949 (28–33). However, *D. calamaroceum* differs from all 5 species except *D. erythrum* in regard to body shape, with either a greater length (cf. *D. ganapatii*, *D. oxycephalum*, and *D. sullivani*) or smaller length (cf. *D. lycidoeceum* and *D. macrocephalum*) of nematogens and rhombogens (Van Beneden, 1876; McConnaughey, 1949b, 1960; Kalavati et al., 1984; Furuya, 1999, 2009). Although *D. calamaroceum* and *D. erythrum* are similar in peripheral cell numbers and body size, they clearly differ in calotte size (large vs. small, respectively), number of cells of infusoriform embryos (39 vs. 37, respectively), and anterior length of refringent bodies (57% vs. 30%, respectively) (Furuya, 1999). Additionally, *D. erythrum* infects *Amphictopus fangsiao* collected from Japan, whereas *D. calamaroceum* is found in a squid species endemic to southern Australian waters.

Only 2 other *Dicyema* species have been described from a host in this genus, i.e., *D. orientale* and *D. koshidai* are both described from *Sepioteuthis lessoniana* collected from Japan (Nouvel and Nakao, 1938; Furuya and Tsuneki, 2005). However, *D. orientale* differs from *D. calamaroceum* in peripheral cell number (22 vs. 31–34, respectively), and *D. koshidai* differs from *D. calamaroceum* in the length of vermiform stages (larger vs. smaller, respectively) and number of cells of infusoriform embryos (37 vs. 39, respectively).

Dicyema pyjamaceum n. sp.

(Figs. 3, 4; Tables I, III)

Diagnosis: Small to medium-sized dicyemid; body length reaching 1,950. Calotte cap-shaped, forms cephalic swelling with parapolar cells. Vermiform stages with 20 to 23 peripheral cells 4 propolars + 4 metapolars + 2 parapolars + 10 to 13 trunk cells; 21 peripheral cells most common; propolar cells opposite metapolar cells. Infusoriform embryos with 37 cells; refringent bodies solid; and 2 nuclei present in each urn cell.

Nematogens (Figs. 3A, 4A, B, E; n = 21 measured): Body length from 330 to 850, average length 594 ± 171, mode 450; width from 30 to 70, average width 42 ± 11, mode 30; widest at parapolar cells. Peripheral cell number 20 to 23 (Table III): 4 propolars + 4 metapolars + 2 parapolars + 8 to 11 diapolars + 2 uropolars. Uropolar cells verruciform. Calotte cap-shaped, forming cephalic swelling together with parapolar cells (Figs. 3A, 4E). Cilia on calotte approximately 5.5 long, oriented forward. Propolar cells and their nuclei smaller than metapolar cells and their nuclei, respectively (Fig. 4E). Cytoplasm of propolar and metapolar cells more conspicuously stained by hematoxylin and eosin than cytoplasm of trunk cells; granules occasionally observed in cytoplasm of propolar cells. Propolar cells opposite metapolar cells. Axial cell cylindrical, rounded anteriorly; extends through to propolar cells; extends to end of uropolar cells posteriorly (Fig. 4A, B). Average of 2 vermiform embryos present in axial cell of nematogens. Agametes present and numerous in axial cell; average diameter 6 ± 1, mode 5.

Vermiform embryo: (Figs. 3E, 4H, I; n = 17 measured): Full-grown vermiform embryos range from 40 to 90 long, average length 60 ± 16, mode 40; and range from 15 to 31 wide, average width 20 ± 4, mode 20. Peripheral cell number 20 to 23 (Fig. 4H, Table III): 4 propolars + 4 metapolars + 2 parapolars + 8 to 11 diapolars + 2 uropolars; 22 peripheral cells most common; propolar cells opposite metapolar cells. Anterior end of calotte rounded (Fig. 3E). Axial cell rounded anteriorly; extends through to middle of propolar cells. Nucleus usually located in centre of axial cell (Fig. 4I). Anterior abortive axial cell absent. Axial cell of full-grown embryos with as many as 2 agametes.

Rhombogens (Figs. 3B–D, 4C, D, F, G; n = 21 measured): Body length greater than nematogens, similar in width; length from 490 to 1,950, average length 1,097 ± 411, width from 30 to 60, average width 40 ± 8, mode 35 (Fig. 4C, D). Peripheral cell number 20 to 23 (Table III), 21 peripheral cells most common; propolar cells opposite metapolar cells. Calotte cap-shaped, forms cephalic swelling with parapolar cell (Figs. 3B, 4F). Width greatest at parapolar cells, reaching 90. Granules occasionally observed in propolar cells. Axial cell shape and anterior extent similar to nematogen. Verruciform cells present. Average of 7 infusoriform embryos present in axial cell of rhombogens, with smaller individuals having as few as 3 and larger individuals having as many as 20. Usually 1 or 2 infusorigens present in axial cell of each parent individual.

Infusorigens (Figs. 3F, 4J; n = 20 measured): Mature infusorigens medium-sized; usually irregular in shape; diameters of 14 to 34, average of 23 ± 7. Composed of 5–13 (mode 6) external cells (egg line: oogonia and primary oocytes) + 4–8 (mode 8) internal cells (sperm line: spermatogonia, primary spermatocytes and secondary spermatocytes) + 6–16 (mode 10) sperm. Mean diameter of fertilized eggs 11 ± 1, sperm 1 ± 0.

Infusoriform embryos (Figs. 3G, H, 4K–L; n = 100 measured): Full-grown embryos medium-sized, lengths average 28 ± 4 (excluding cilia); length: width: depth ratio 1.00: 0.71: 0.95. Shape ovoid, bluntly rounded to pointed posteriorly (Figs. 3G, 4K). Cilia at posterior end 7 long. Refringent bodies present, solid; occupy 43% of embryo length when viewed laterally (Fig. 3H). Cilia projecting from ventral internal cells to urn cavity (Fig. 4K). Capsule cells contain many large granules. Mature infusoriform

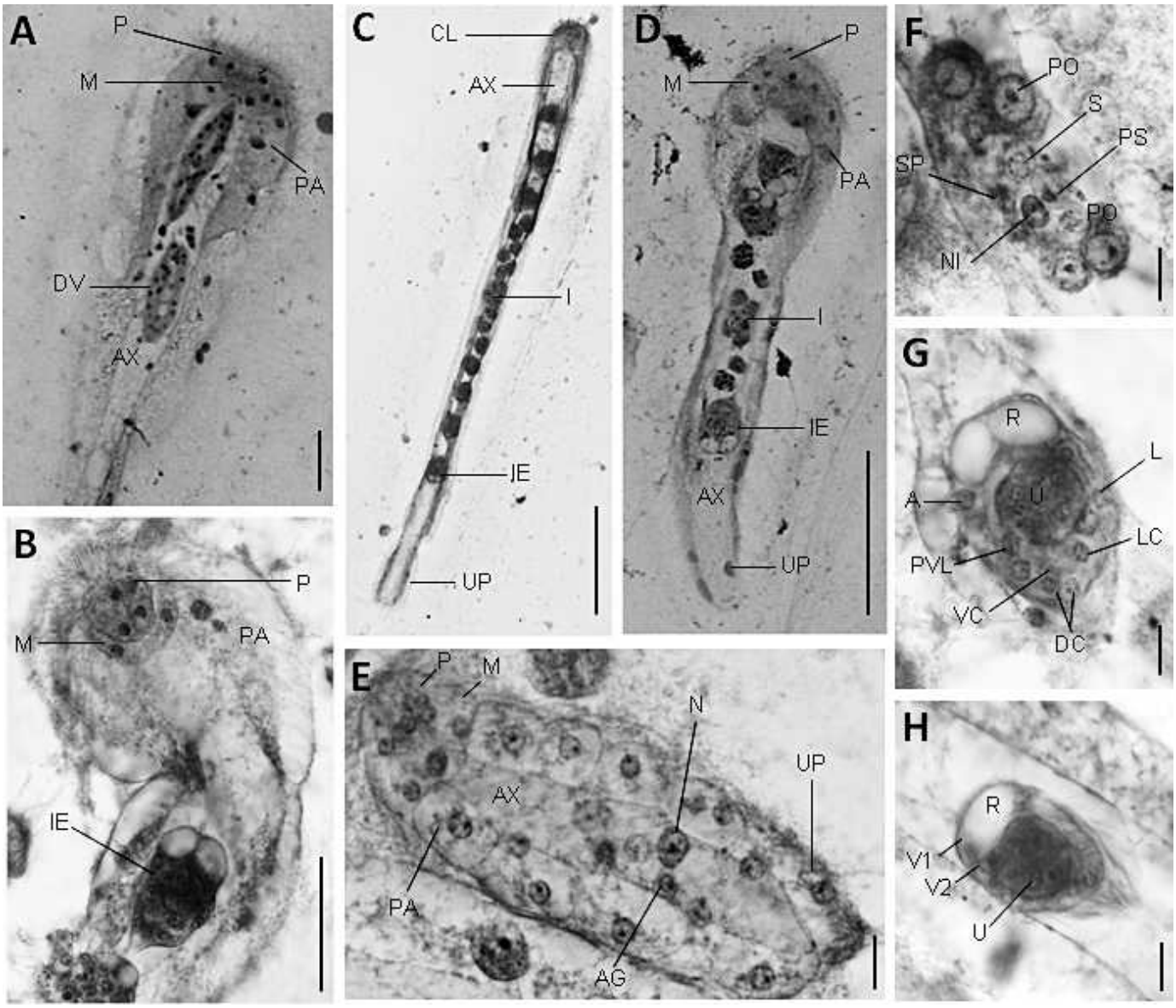

FIGURE 3. Light micrographs of *Dicyema pyjamaceum* n. sp. infecting *Sepioloidea lineolata*. (**A**) Anterior region of nematogen. (**B**) Anterior region of rhombogen. (**C**, **D**) Rhombogen, entire. (**E**) Vermiform embryo within axial cell. (**F**) Infusorigen. (**G**, **H**) Infusoriform embryos within axial cell: (G) optical horizontal section; (H) optical sagittal section. Scale bars represent 50 μm in (A, B), 100 μm in (C, D), 10 μm in (E–H). Abbreviations: A, apical cell; AG, agamete; AX, axial cell; CL, calotte; DC, dorsal caudal cell; DV, developing vermiform embryo; I, infusorigen; IE, Infusoriform embryo; L, lateral cell; LC, lateral caudal cell; M, metapolar cell; N, nucleus; NI, nucleus of infusorigen; P, propolar cell; PA, parapolar cell; PO, primary oocytes; PS, primary spermatocytes; PVL, posteroventral lateral cells; R, refringent body; S, spermatogonium; SP, sperm; U, urn cell; UP, uropolar cell; VC, ventral caudal cell; V1, first ventral cell; V2, second ventral cell.

embryos consisting of 37 cells: 33 somatic + 4 germinal. Somatic cells of several types present: external cells cover large part of anterior and lateral surfaces of embryo (2 enveloping cells); external cells with cilia on external surface (2 paired dorsal cells + 1 median dorsal cell + 2 dorsal caudal cells + 2 lateral caudal cells + 1 ventral caudal cell + 2 lateral cells + 2 posteroventral lateral cells); external cells with refringent bodies (2 apical cells); external cells without cilia (1 couvercle cell + 2 first ventral cells + 2 second ventral cells + 2 third ventral cells); internal cells with cilia (2 ventral internal cells); and internal cells without cilia (2 dorsal internal cells + 2 capsule cells + 4 urn cells). Each urn cell contains 1 germinal cell and 2 nuclei (Fig. 4K). All somatic nuclei appear pycnotic in mature infusoriform embryos.

Taxonomic summary

Type host: Striped pyjama squid, *Sepioloidea lineolata* Quoy and Gaimard, 1832 (Mollusca: Cephalopoda: Sepiadariidae), 4.5 cm mantle length, 35 g weight (ABTC 122525).

Type locality: Spencer Gulf, South Australia, Australia (33°12′39″S, 137°33′55″E).

Additional localities: None.

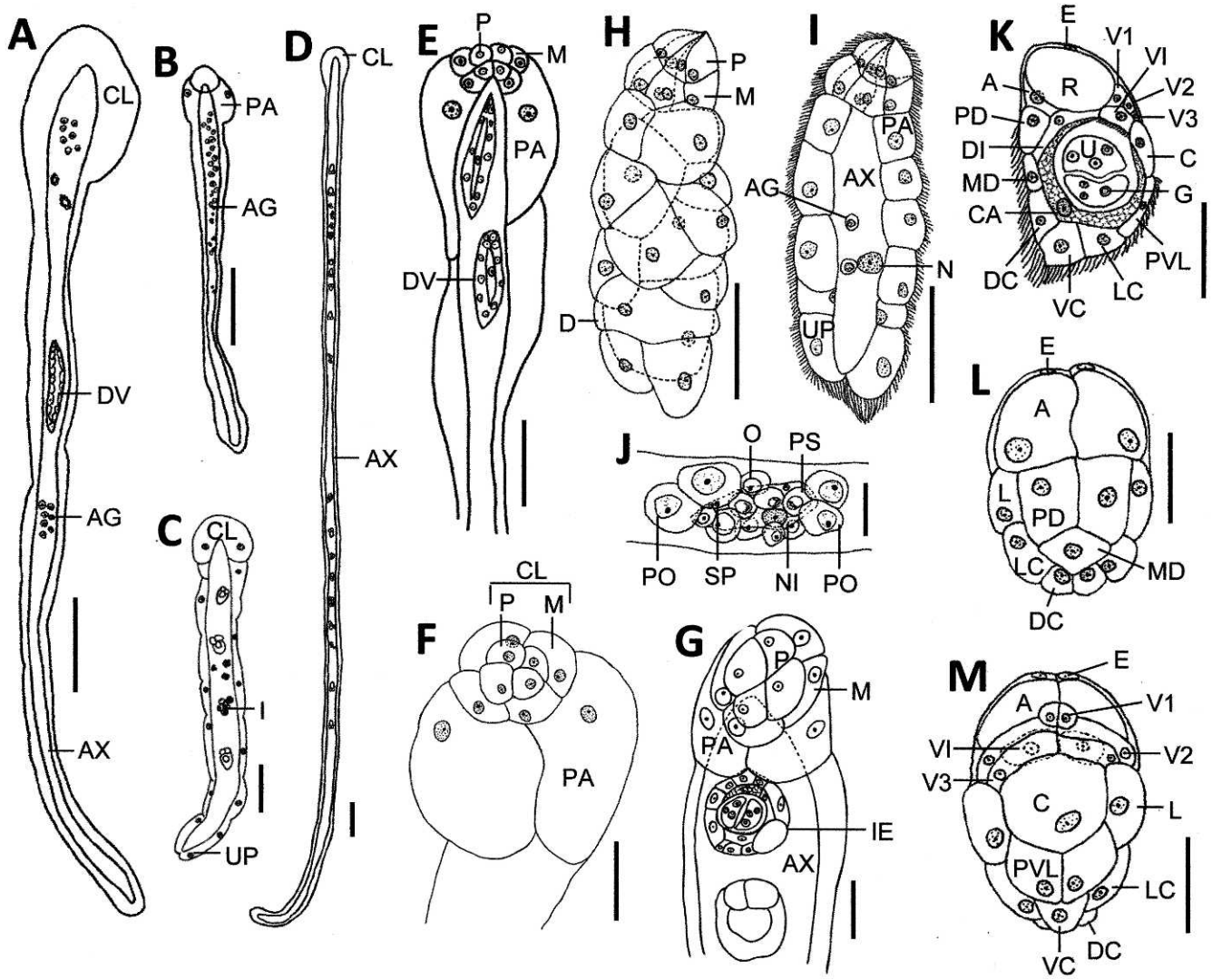

FIGURE 4. Line drawings of *D. pyjamaceum* n. sp. infecting *S. lineolata*. (**A**–**D**) Vermiform stages, entire: (A, B) nematogen; (C, D) rhombogen. (**E**) Anterior region of nematogen. (**F**, **G**) Anterior region of rhombogen. (**H**, **I**) Vermiform embryo within axial cell: (H) cilia omitted; (I) optical section. (**J**) Infusorigen. (**K, L**) Infusoriform embryos within axial cell: (K) sagittal section; (L) dorsal view (cilia omitted); (**M**) ventral view (cilia omitted). Scale bars represent 50 μm in (A–E), 20 μm in (F–I) and 10 μm in (J–M). Abbreviations: A, apical cell; AG, agamete; AX, axial cell; C, couvercle cell; CA, capsule cell; CL, calotte; D, diapolar cell; DC, dorsal caudal cell; DI, dorsal internal cell; DV, developing vermiform embryo; E. enveloping cell; G, germinal cell; I, infusorigen; IE, Infusoriform embryo; L, lateral cell; LC, lateral caudal cell; M, metapolar cell; MD, median dorsal cell; N, nucleus; NI, nucleus of infusorigen; O, oogonium; P, propolar cell; PA, parapolar cell; PD, paired dorsal cell; PO, primary oocytes; PS, primary spermatocytes; PVL, posteroventral lateral cells; R, refringent body; SP, sperm; U, urn cell; UP, uropolar cell; VC, ventral caudal cell; VI, ventral internal cell; V1, first ventral cell; V2, second ventral cell; V3, third ventral cell.

TABLE III. Number of peripheral cells in *D. pyjamaceum* n. sp. infecting *S. lineolata*.

	No. individuals		
Cell no.	Vermiform embryos	Nematogens	Rhombogens
20	2	4	1
21	2	16	11
22	3	6	3
23	2	9	9

Site of infection: Attached to the surface of the renal appendages.

Prevalence: Five of 5 hosts examined (100% prevalence).

Specimens deposited: Four syntype slides deposited in the Marine Invertebrate Collection, SAMA, Australia (E3733–6), and 2 syntype slides in the NSMT, Japan (NSMT Me 21–22).

Etymology: The species name is derived from the common name of the host, striped pyjama squid.

Remarks

Of the 61 presently described species within *Dicyema* (see Catalano, 2012), 32 partially overlap with the peripheral cell count range described for *D. pyjamaceum* n. sp. (20–23).

However, none matches with an identical peripheral cell count range of 20–23. Additionally, these 32 species can all be distinguished from *D. pyjamaceum* by differences in body size, calotte shape, cell number of infusoriform embryo, and number of nuclei in each urn cell of infusoriform larvae. *Dicyema helocephalum* Furuya, 2006, *D. japonicum* Furuya et al., 1992, and *D. robsonellae* Short, 1971, are the 3 most similar species to *D. pyjamaceum*, with cap-shaped calottes, infusoriform embryo cell number of 37, and similar body sizes. Nonetheless, *D. helocephalum* and *D. japonicum* can be distinguished from *D. pyjamaceum* by having 1 nucleus in urn cells compared to 2, and *D. japonicum* can further be distinguished by having anterior lateral cells instead of third ventral cells in infusoriform embryos (Furuya et al., 1992; Furuya, 2006). *Dicyema robsonellae* differs from *D. pyjamaceum* with the presence of an accessory nucleus in adult stages, postcapsular cells, and no cilia on ventral internal cells in infusoriform larvae (Short, 1971). This is the first *Dicyema* species to be described from a host in *Sepioloidea*.

DISCUSSION

Sepioteuthis australis is a common, inshore squid species endemic to southern Australia and northern New Zealand waters (Pecl, 2004). This large species, with a mantle length up to 50 cm, frequently moves over reefs and seagrass beds at night to forage and is a popular target for recreational anglers (Norman and Reid, 2000). A previous study by Finn et al. (2005), in which 11 *S. australis* individuals collected from southern Australian waters were examined for dicyemid parasites, revealed no infections. However, in the present study, 6 of 25 *S. australis* collected from temperate SA waters were infected by a single dicyemid species, *D. calamaroceum* n. sp.

Hochberg (1990) attempted to explain the trend in the distribution of dicyemids across latitude, based on findings of uninfected cephalopods from Hawaii and the Marshall Islands (McConnaughey, 1949a). He proposed that in tropical waters, cephalopods are uninfected, whereas in subtropical regions, prevalences ranged from 10 to 20%, and in temperate and polar waters, prevalences generally are 100%. The low dicyemid parasite occurrence of 24% observed in this study for a squid species in temperate waters clearly disagrees with his third statement. The study by Finn et al. (2005) also counters all of these statements, with 33.3% of tropical octopus, 31.6% of subtropical octopus, and 78.1% of temperate octopus infected by dicyemid parasites. Rather than set trends, it appears prevalence may be variable across latitudes. However, other factors, either singularly or in combination, may also influence prevalence, such as seasonality, host behavior, size, and life cycle, as well as variation in sampling methods and sampling sizes.

Therefore, the low prevalence observed here for *D. calamaroceum* infecting *S. australis* may be due to host size rather than latitude. Most individuals examined for dicyemid parasites were small, with mantle lengths (MLs) ranging from 7.5 to 20.5 cm (Table I). A common notion in the literature, for marine parasites in general, is that larger hosts are more likely to be infected than smaller ones, as large individuals provide more space, nutrients, and less competition (Kearn, 1967; Ho, 1991; Guegan et al., 1992; Poulin, 1997). For dicyemid parasites, similar relationships have been reported with a correlation between host size and dicyemid occurrence, whereby small or young cephalopods generally do not harbor dicyemids, while larger or older individuals are infected (Furuya et al., 1992). However, although the squid collected in this study were generally small, there was no trend observed with infection by dicyemid parasites restricted solely to the larger of these individuals (Table I). Specimens were also collected from SG and GSV in SA waters; however, no patterns were observed with infection being exclusive in individuals collected from 1 gulf compared to another (Table I). Clearly, more extensive sampling across localities that encompass the complete spatial distribution and all size ranges of this host is needed to evaluate and further elucidate dicyemid prevalence trends and the factors responsible, which would also add information on dicyemid distribution in temperate latitudes.

Sepioloidea d'Orbigny, 1845 presently contains 3 species, i.e., *S. lineolata*, *S. pacifica* Kirk, 1882, and *S. magna* Reid, 2009. The description provided here of a dicyemid parasite from *S. lineolata* represents the first from this cephalopod genus. *Sepioloidea lineolata* is endemic to southern Australian waters and is often found in sand and rubble around seagrass beds to depths of 20 m, spending most of its time buried in substratum (Norman and Reid, 2000; Talbot and Marshall, 2010). This squid species reaches 7 cm in length and can be identified by its distinctive color pattern of brown to black stripes over white (hence the common name "striped pyjama squid"), and finger-like papillae on the upper side of the mantle opening (Norman and Reid, 2000; Reid, 2009).

Here all 5 host individuals collected from the temperate waters in SA, irrespective of body size, were found to be heavily infected with *D. pyjamaceum* n. sp. (100% prevalence). As such, this agrees with Hochberg's hypothesis of 100% dicyemid prevalence of cephalopod species from temperate waters but, for *S. lineolata*, the high prevalence observed may be attributed to its behavior rather than latitude. The fact that *S. lineolata* is frequently associated and in contact with sandy substratum at the sea floor would allow for the dispersal larvae, infusoriform embryos, to readily infect this cephalopod species. Previous studies by Lapan and Morowitz (1972) and Lapan (1975) have shown that the infusoriform embryo, produced via sexual reproduction, contains refringent bodies in their apical cells, which are composed of highly hydrated magnesium salt of inositol hexaphosphate. The high specific gravity of this dense chemical, which accounts for more than one-third of the body weight of the infusoriform embryo, provides it with negative buoyancy. Therefore, the infusoriform embryo remains close to the sea bottom where it can encounter and infect a new host. Further support for this notion is provided by the fact that benthic cephalopods are commonly infected by dicyemid parasites, whereas dicyemid infection of pelagic species is rare. The study by Finn et al. (2005) also revealed high prevalence in *S. lineolata*, with 12 of 15 individuals collected from temperate southern Australian waters infected by dicyemids (80% prevalence).

In summary, the present study describes 2 new species of dicyemids, the first reported from Australia, from 2 cephalopod host species in southern Australian waters, with *D. calamaroceum* n. sp. from *Sepioteuthis australis* and *D. pyjamaceum* n. sp. from *Sepioloidea lineolata*. Considering Australia contains one of the most diverse cephalopod faunas in the world (Norman and Reid, 2000), the scope and potential for further study of dicyemid parasites in these waters is extensive and, subsequently, may add valuable new information to a parasite group that is currently best known from the Northern Hemisphere.

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LITERATURE CITED

Catalano, S. R. 2012. A review of the families, genera and species of Dicyemida Van Beneden, 1876. Zootaxa **3479:** 1–2.

Finn, J. K., F. G. Hochberg, and M. D. Norman. 2005. Phylum Dicyemida in Australian waters: First record and distribution across diverse cephalopod hosts. Phuket Marine Biology Research Center Bulletin **66:** 83–96.

Furuya, H. 1999. Fourteen new species of dicyemid Mesozoans from six Japanese cephalopods, with comments on host specificity. Species Diversity **4:** 257–319.

———. 2006. Two new species of *Dicyema* (Dicyemida: Dicyemidae) from *Amphictopus areolatus* (Mollusca: Cephalopoda: Octopodidae). Species Diversity **11:** 257–269.

———. 2009. Two new dicyemid from *Sepia longipes* (Mollusca: Cephalopoda: Decapoda). Journal of Parasitology **95:** 681–689.

———. 2010. A new dicyemid from *Benthoctopus sibiricus* (Mollusca: Cephalopoda: Octopoda). Journal of Parasitology **96:** 1123–1127.

———, F. G. Hochberg, and K. Tsuneki. 2007. Cell number and cellular composition in vermiform larvae of dicyemid mesozoans. Journal of Zoology **272:** 284–298.

———, M. Ota, R. Kimura, and K. Tsuneki. 2004. The renal organs of cephalopods: A habitat for dicyemids and chromidinids. Journal of Morphology **262:** 629–643.

———, and K. Tsuneki. 2003. Biology of dicyemid mesozoans. Zoological Science **20:** 519–532.

———, and ———. 2005. A new species of dicyemid mesozoan (Dicyemida: Dicyemidae) from *Sepioteuthis lessoniana* (Mollusca: Cephalopoda), with notes on *Dicyema orientale*. Species Diversity **10:** 45–62.

———, ———, and Y. Koshida. 1992. Two new species of the genus *Dicyema* (Mesozoa) from octopuses of Japan with notes on *D. misakiense* and *D. acuticephalum*. Zoological Science **9:** 423–437.

———, ———, and ———. 1997. Fine structure of a dicyemid mesozoan, *Dicyema acuticephalum*, with special reference to cell junctions. Journal of Morphology **231:** 297–305.

Guegan, J. F., A. Lambert, C. Leveque, C. Combes, and L. Euzet. 1992. Can host body size explain the parasite species richness in tropical freshwater fishes? Oecologia **90:** 197–204.

Ho, J. S. 1991. Two species of chondracanthid copepods parasitic on commercial fishes in the Pacific. Publications of the Seto Marine Biological Laboratory **31:** 1–10.

Hochberg, F. G. 1982. The "kidneys" of cephalopods: A unique habitat for parasites. Malacologia **23:** 121–134.

———. 1990. Diseases of Cephalopoda. Diseases caused by protists and mesozoans. *In* Diseases of marine animals, vol. III, O. Kinne (ed.). Biologische Anstalt Helgoland, Hamburg, Germany, p. 47–202.

———, and R. B. Short. 1970. *Dicyemennea littlei* sp. n. and *Dicyema benthoctopi* sp. n.: Dicyemid mesozoa from *Benthoctopus magellanicus*. Transcripts of the American Society for Microbiology **89:** 216–224.

———, and ———. 1983. *Dicyemennea discocephala* sp. n. (Mesozoa: Dicyemidae) in a finned octopod from the Antarctic. Journal of Parasitology **69:** 963–966.

Kalavati, C., C. C. Narasimhamurti, and T. Suseela. 1984. Four new species of mesozoan parasites (Mesozoa: Dicyemidae) from cephalopods of Bay of Bengal. Proceedings of the Indian Academy of Science (Animal Science) **93:** 639–654.

Kearn, G. C. 1967. Experiments on host–finding and host-specificity in the monogenean skin parasite *Entobdella soleae*. Parasitology **57:** 585–605.

Lapan, E. A. 1975. Magnesium inositol hexaphospate deposits in mesozoan dispersal larvae. Experimental Cell Research **94:** 277–282.

———, and H. J. Morowitz. 1972. The Mesozoa. Scientific American **227:** 94–101.

McConnaughey, B. H. 1949a. Mesozoa of the family Dicyemidae from California. University of California Publications in Zoology **55:** 1–33.

———. 1949b. *Dicyema sullivani*, a new mesozoan from lower California. Journal of Parasitology **35:** 122–124.

———. 1960. The rhombogen phase of *Dicyema sullivani* McConnaughey (Mesozoa: Dicyemidae). Journal of Parasitology **46:** 608–610.

Norman, M., and A. L. Reid. 2000. A guide to squid, cuttlefish and octopuses of Australasia. CSIRO Publishing, Collingswood, Victoria, Australia, 96 p.

Nouvel, H. 1948. Les Dicyémides II. Infusoriforme, tératologie, spécificité du parasitisme, affinités. Extrait des Archives de Biologie **59:** 147–224.

———, and Y. Nakao. 1938. Dicyémides du Japon. Bulletin de la Société Zoologique de France **63:** 72–80.

Pecl, G. T. 2004. The *in situ* relationship between season hatching, growth and condition in the southern calamary, *Sepioteuthis australis*. Marine and Freshwater Research **55:** 429–438.

Poulin, R. 1997. Species richness of parasite assemblages: Evolution and patterns. Annual Review of Ecology and Systematics **28:** 341–358.

Reid, A. 2009. *Sepioloidea magna* sp. nov.: A new bottletail squid (Cephalopoda: Sepiadariidae) from northern Australia. The Beagle, Records of the Museums and Art Galleries of the Northern Territory, 2009 **25:** 103–109.

Short, R. B. 1971. Three new species of Dicyema (Mesozoa: *Dicyemidae*) from New Zealand. *In* Biology of the Antarctic Seas IV, Antarctic Research Series Vol. XVII, G. A. Llano and I. E. Wallen (eds.). American Geophysical Union, Washington, D.C., p. 231–249.

———, and R. T. Damian. 1966. Morphology of the infusoriform larva of *Dicyema aegira* (Mesozoa: Dicyemidae). Journal of Parasitology **52:** 746–751.

———, and F. G. Hochberg. 1969. Two new species of *Dicyemennea* (Mesozoa: Dicyemidae) from Kaikoura, New Zealand. Journal of Parasitology **55:** 583–596.

———, and ———. 1970. A new species of *Dicyemennea* (Mesozoa: Dicyemidae) from near the Antarctic Peninsula. Journal of Parasitology **56:** 517–522.

———, and E. C. Powell. 1969. *Dicyemennea eltanini* sp. n. (Mesozoa: Dicyemidae) from Antarctic waters. Journal of Parasitology **55:** 794–799.

Suzuki, T. G., K. Ogino, K. Tsuneki, and H. Furuya. 2010. Phylogenetic analysis of dicyemid mesozoans (phylum Dicyemida) from innexin amino acid sequences: Dicyemids are not related to Platyhelminthes. Journal of Parasitology **96:** 614–625.

Talbot, C. M., and J. Marshall. 2010. Polarization sensitivity and retinal topography of the striped pyjama squid (*Sepioloidea lineolata*–Quoy/Gaimard 1832). Journal of Experimental Biology **213:** 3371–3377.

Van Beneden, E. 1876. Recherches sur les Dicyemides, survivants actuels d'un embranchement des Mésozoaires. Bulletins de l'Academis Royale Des Sciences et Belles Lettres de Bruxelles **41:** 1160–1205.

CHAPTER 4: First descriptions of dicyemid mesozoans (Dicyemida: Dicyemidae) from Australian octopus (Octopodidae) and cuttlefish (Sepiidae), including a new record of *Dicyemennea* in Australian waters

Aboard ‘Frank Cori’ - collecting cephalopod samples in Gulf St Vincent, South Australia, Australia (photo: Dan Gomon).

Chapter 4 Preamble

This chapter is a manuscript that describes three new species of dicyemid parasites, representing the first from Australian octopus and cuttlefish host species, as well as the first description of *Dicyemennea* from Australian waters. It is published in *Folia Parasitologica* and included with permission from the Editor-in-Chief, Tomáš Scholz (see Appendix A). It can be cited as: Catalano, S.R., 2013. First descriptions of dicyemid mesozoans (Dicyemida: Dicyemidae) from Australian octopus (Octopodidae) and cuttlefish (Sepiidae), including a new record of *Dicyemennea* in Australian waters. *Folia Parasitologica* 60, 306–320.

In this chapter, I collected the samples, performed all dissections, prepared kidney smears and examined the smears for morphological analyses. I also supplied the funding and compiled the images (line drawings and photos). I wrote the manuscript and acted as corresponding author. Drafts of this chapter prior to publication were reviewed by Bronwyn Gillanders, Steve Donnellan and Ian Whittington

I certify that the statement of contribution is accurate

Signed:_____ Date:_____

(Sarah Roseann Catalano)

Catalano, S. (2013) First descriptions of dicyemid mesozoans (Dicyemida: Dicyemidae) from Australian octopus (Octopodidae) and cuttlefish (Sepiidae), including a new record of *Dicyemennea* in Australian waters.
Folio Parasitologica, v. 60(4), pp. 306-320

CHAPTER 5: Five new species of dicyemid mesozoans (Dicyemida: Dicyemidae) from two Australian cuttlefish species, with comments on dicyemid fauna composition

Giant Australian cuttlefish (*Sepia apama*), host to four dicyemid species.

Chapter 5 Preamble

This chapter is a manuscript that describes five new species of dicyemid parasites from two cuttlefish species and includes a discussion on the factors responsible for trends in observed dicyemid parasite fauna composition. It is published in *Systematic Parasitology* and is included with permission from Springer (see Appendix A). This chapter can be cited as: Catalano, S.R., 2013. Five new species of dicyemid mesozoans (Dicyemida: Dicyemidae) from two Australian cuttlefish species, with comments on dicyemid fauna composition. *Systematic Parasitology* 86, 125–151.

In this chapter, I collected the cuttlefish samples from Spencer Gulf and Gulf St Vincent in South Australia. Samples from Coffin Bay, South Australia, were collected with help from Kieran Brazell, Kate Hutson, Brian Saunders and Richard Saunders, and samples from the western Tasman Sea, New South Wales (NSW), were collected by Alex Schnell (Macquarie University, NSW). All dissections, except for two cuttlefish samples from NSW, were performed by myself. I prepared kidney smears and examined the smears for morphological analyses. I also supplied the funding and compiled images (line drawings and photos). I wrote the manuscript, with drafts reviewed by Bronwyn Gillanders, Steve Donnellan and Ian Whittington.

I certify that the statement of contribution is accurate

Signed:_____ Date:_____

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Five new species of dicyemid mesozoans (Dicyemida: Dicyemidae) from two Australian cuttlefish species, with comments on dicyemid fauna composition

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Abstract Five new species of dicyemid mesozoans in two genera are described from two Australian cuttlefish species, *Sepia apama* Gray (giant Australian cuttlefish) and *S. novaehollandiae* Hoyle (nova cuttlefish): *Dicyema coffinense* n. sp. from *S. apama* collected from Coffin Bay, South Australia (SA), Australia; *D. koinonum* n. sp. from *S. apama* and *S. novaehollandiae* collected from Gulf St Vincent (GSV) and Spencer Gulf (SG), SA, Australia; *D. multimegalum* n. sp. from *S. apama* collected from Cronulla and North Bondi, New South Wales, Australia; *D. vincentense* n. sp. from *S. novaehollandiae* collected from GSV, SA, Australia; and *Dicyemennea spencerense* n. sp. from *S. novaehollandiae* and *S. apama* collected from SG, SA, Australia. Totals of 51 *S. apama* and 27 *S. novaehollandiae* individuals were examined, of which all except for four *S. apama* were infected by at least one dicyemid species. Dicyemid parasites were also observed in host individuals that were held in tanks for 2–3 months prior to examination, including nematogen-exclusive infections, leading to questions about persistence of dicyemids after host death and the mechanism responsible for the switch between a nematogen phase and a rhombogen phase. Variations in host size, calotte shape and collection locality are explored as predictors of differences in observed composition of the parasite fauna. In particular, dicyemid parasite fauna varied with host collection locality. As these parasites are highly host-species specific, their use as biological tags to assess cephalopod population structure using a combined morphological and molecular approach is discussed. This study increases the number of dicyemid species described from Australian cephalopods from five to ten, and from 117 to 122 species described worldwide.

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Introduction

The Dicyemida von Kölliker comprises a group of microscopic organisms, dicyemid mesozoans, which infect the nutrient-rich environment of the renal appendages (synonym of kidneys, renal sacs and renal organs) of benthic cephalopods at high intensities (Hochberg, 1982, 1983). Dicyemids are typically

highly host-species specific, although co-infection and co-occurrence have been recorded, where more than one dicyemid species infects a single cephalopod species, and more than one dicyemid species are found within a single cephalopod individual, respectively (Furuya, 1999; Furuya et al., 2003a). While dicyemid mesozoans are simple in morphology, comprising only eight to 40 cells with no body cavities or differentiated organs, they have a complex, partially unknown life-cycle characterised by two modes of reproduction and two stages of development (McConnaughey, 1951; Furuya et al., 2003b, 2007). This allows fulfilment of population density strategies (ensuring persistence within a host individual) and dispersal strategies (ensuring persistence beyond the death of the host individual) (Furuya et al., 2003b). The position of the Dicyemida in the Tree of Life is also uncertain and controversial (Noto & Endoh, 2004). Studies based on dicyemid morphology suggest a protozoan origin, whereas studies based on molecular analyses contradict each other (Lapan & Morowitz, 1974; Ohama et al., 1984; Katayama et al., 1995; Pawlowski et al., 1996; Kobayashi et al., 1999; Aruga et al., 2007; Kobayashi et al., 2009; Ogino et al., 2010; Suzuki et al., 2010).

Although 117 dicyemid species have been described to date (see Catalano, 2012, 2013; Catalano & Furuya, 2013), the majority of research on this group is northern hemisphere-centric, with published work on dicyemid infections in southern hemisphere cephalopods sparse in comparison. In Australia, only three studies have documented dicyemid parasites. The first study by Finn et al. (2005) recorded the presence of dicyemids in 24 cephalopod species, but provided no formal descriptions of new dicyemid species. The remaining two studies, by Catalano & Furuya (2013) and Catalano (2013), collectively described five dicyemid species in two genera from four cephalopod species collected throughout western and southern Australian waters.

Here, five dicyemid species are described from two cephalopod species in the Sepiidae, *Sepia apama* Gray (giant Australian cuttlefish) and *S. novaehollandiae* Hoyle (nova cuttlefish). *Sepia apama*, the largest species of cuttlefish in the world, is found in the shallow temperate waters of southern Australia distributed from Ningaloo in Western Australia (WA) to Moreton Bay in southern Queensland (Kassahn et al., 2003). This species is widely known for its unique breeding aggregation event, with thousands of animals congregating each year in the winter months on small, shallow, rocky reefs in upper Spencer Gulf, South Australia (SA), Australia, to spawn (Norman et al., 1999; Hall & Hanlon, 2002). *Sepia novaehollandiae* is also endemic to southern Australian waters and is often found with *S. apama* in the by-catch of prawn trawlers in SA waters (Reid et al., 2005). It can be distinguished from *S. apama* by a smaller body size and differences in cuttlebone characteristics, namely presence of a pointed spine, narrower width, V-shaped ventral striations and distinctive pink tinge which is visible to the naked eye when examining individuals dorsally.

In addition to the description of five new dicyemid species, the persistence of dicyemids within a host individual that has recently died, and the mechanism for the switch between the two stages of development in the dicyemid life-cycle, are examined in this study. The effect of host size, calotte shape and collection locality on observed dicyemid co-occurrence patterns in *S. apama* and *S. novaehollandiae* are explored. Finally, the application of using dicyemid parasites as biological tags to assess host population structure is discussed. This increases the dicyemid parasite fauna described from Australian cephalopods from five to ten, and the global total from 117 to 122 species described to date.

Materials and methods

Sample collection

In this study, 51 individuals of *Sepia apama* and 27 individuals of *S. novaehollandiae*, collected from southern Australian waters between July 2010 and August 2012, were examined for dicyemid parasites. *Sepia apama* individuals were collected from four localities: Coffin Bay (CB; n = 11 individuals), Spencer Gulf (SG; n = 26 individuals), Gulf St Vincent (GSV; n = 10 individuals) and Cronulla and North Bondi, New South Wales (NSW; n = 4 individuals). *Sepia novaehollandiae* individuals were collected from two localities: SG (n = 11 individuals) and GSV (n = 16 individuals) (see Fig. 1). Sampling was undertaken throughout SG and GSV, both of which encompass large areas (SG from Port Augusta to the tip of the Yorke Peninsula at Innes National Park *c*.300 km long, GSV from Port Wakefield to the tip of the Fleurieu Peninsula at Cape Jervis *c*.200 km long,

see Fig. 1). Therefore these two localities were divided into three regions each (upper, middle and lower), to investigate if dicyemid infection is region-specific. For SG, upper SG (USG) is designated to encompass the area from 32°33′55.2″S to 33°28′38.2″S (Port Augusta to Clements Gap Conservation Park), middle SG (MSG) is designated to encompass the area from 33°28′38.2″S to 34°21′9.0″S (Clements Gap Conservation Park to Maitland), and lower SG (LSG) is designated to encompass the area from 34°21′9.0″S to 35°13′39.6″S (Maitland to Innes National Park) (see Fig. 1). For GSV, upper GSV (UGSV) is designated to encompass the area from 34°12′9.8″S to 34°40′58.5″S (Port Wakefield to Port Gawler Conservation Park), middle GSV (MGSV) is designated to encompass the area from 34°40′58.5″S to 35°10′58.0″S (Port Gawler Conservation Park to Port Noarlunga South), and lower GSV (LGSV) is designated to encompass the area from 35°10′58.0″S to 35°39′10.2″S (Port Noarlunga South to Cape Jervis) (see Fig. 1).

Host specimens collected from SG and GSV were obtained from the by-catch of prawn surveys for the South Australian Research and Development Institute (SARDI) and *S. apama* individuals from CB and NSW were obtained *via* line fishing and SCUBA, respectively. All cephalopods were examined immediately upon capture, except those from NSW. The four individuals from this locality were collected by Alex Schnell (Macquarie University, NSW) in May and June 2012 and were housed at the Cronulla Fisheries Research Centre for 2–3 months. After breeding in captivity, these individuals deteriorated in health and subsequently died, when they were then examined for dicyemid parasites. The number of host individuals infected by each dicyemid species at each geographical locality is presented in Table 1, along with information on the host dorsal mantle length range (cm) and examination date.

Cephalopod dissections, smear preparations and morphological analyses

The method for dissecting each cephalopod individual follows Catalano & Furuya (2013). Note that 12–16 smears were made per renal appendage for *S. apama* individuals collected from NSW, as these

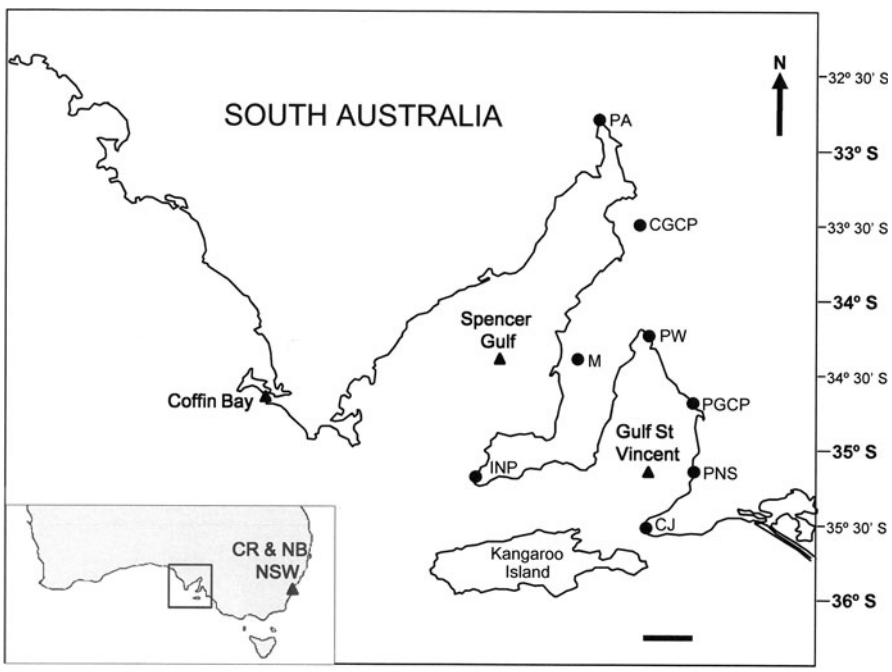

Fig. 1 Collection localities for *Sepia apama* and *S. novaehollandiae* in South Australian waters and off the coast of New South Wales (black triangles). Black circles indicate landmarks that make up the boundaries between upper, middle and lower Spencer Gulf and Gulf St Vincent, respectively (refer to text for specific latitude boundaries). Abbreviations: CGCP, Clements Gap Conservation Park; CJ, Cape Jervis; CR, Cronulla; INP, Innes National Park; M, Maitland; NB, North Bondi; NSW, New South Wales; PA, Port Augusta; PGCP, Port Gawler Conservation Park; PNS, Port Noarlunga South; PW, Port Wakefield. *Scale-bar*: 50 km

Table 1 Dicyemid species from *Sepia apama* and *S. novaehollandiae* from Australian waters

Locality	Dicyemid species	No. of infected host individuals	Host mantle length range (cm)	Date of examination
Sepia apama (giant Australian cuttlefish)				
CB (SA)	*Dicyema coffinense*	8	11.7–20.0	December 2011
USG (SA)	*Dicyema koinonum*	14	9.5–19.0	July 2010–October 2011
	Dicyemennea spencerense	6	10.5–16.5	July 2010–October 2011
MSG (SA)	*Dicyema koinonum*	7	10.0–18.0	March 2011
	Dicyemennea spencerense	4	10.0–18.0	March 2011
LSG (SA)	*Dicyema koinonum*	4	11.5–21.5	February 2012
	Dicyemennea spencerense	4	11.5–21.5	February 2012
MGSV (SA)	*Dicyema koinonum*	2	12.0–14.5	December 2010
LGSV (SA)	*Dicyema koinonum*	8	11.0–17.0	May 2012
CR, NB (NSW)	*Dicyema multimegalum*	3	38.0–49.5	August 2012
Sepia novaehollandiae (nova cuttlefish)				
USG (SA)	*Dicyema koinonum*	1	12.0	November 2010
	Dicyemennea spencerense	7	8.1–12.0	November 2010
MSG (SA)	*Dicyema koinonum*	3	5.5–10.5	March 2011
	Dicyemennea spencerense	3	9.0–10.5	March 2011
MGSV (SA)	*Dicyema koinonum*	3	8.0–10.5	December 2010–March 2011
	Dicyema vincentense	13	7.5–10.5	December 2010–March 2011

Abbreviations: CB, Coffin Bay; CR, Cronulla; LGSV, Lower Gulf St Vincent; LSG, Lower Spencer Gulf; MGSV, Middle Gulf St Vincent; MSG, Middle Spencer Gulf; NB, North Bondi; NSW, New South Wales; SA, South Australia; USG, Upper Spencer Gulf

individuals were much larger than those obtained in SA waters and therefore had larger renal appendages. A mantle tissue sample was also taken for each host individual examined and preserved in 100% DNA grade ethanol.

Smears were stained in Ehrlich's Acid Haematoxylin and counterstained in Eosin 1% alcoholic solution before being mounted in Canada balsam following the method outlined in Catalano & Furuya (2013). After being dried on a heat plate at 50°C, mounted smears were examined with a Nikon compound light microscope at the University of Adelaide, Australia at magnifications up to ×1,000. Measurements and drawings were made with the aid of an ocular micrometer and a drawing tube fitted to an Olympus compound microscope, respectively. Light micrograph images were taken with an Aptina 14MP ToupCam Camera (UCMOS14000KPA) and formatted with the supplied software (ToupView v3.7). All measurements are in micrometres and presented as the range followed by the mean ± standard deviation and the mode in parentheses.

The terminology for cell names used in the description of infusoriform larvae is based on Nouvel (1948), Short & Damian (1966) and Furuya (1999, 2009, 2010). Syntypes of the dicyemids are deposited in the Marine Invertebrate Collection (MIC), South Australian Museum, Adelaide (SAMA), South Australia 5000, Australia (contact: Thierry Laperousaz, thierry.laperousaz@samuseum.sa.gov.au); and the National Museum of Nature and Science, Tokyo (NSMT), 4–1–4 Amakubo, Tsukuba City, 305-0005 Ibaraki, Japan (contact: Toshiaki Kuramochi, kuramoti@kahaku.go.jp). Tissue samples from the cephalopod species harbouring dicyemid parasites is deposited in the Australian Biological Tissue Collection (ABTC) of the SAMA, South Australia 5000, Australia (contact: Steve Donnellan, Steve.Donnellan@samuseum.sa.gov.au).

Note that in the literature, there is no established or accepted common name for *S. novaehollandiae*. This species has been referred to as 'cuttlefish', 'southern cuttlefish' and 'New Holland cuttlefish' in different studies with no standard given. In this paper, I refer to

S. novaehollandiae in the common sense as 'nova cuttlefish', shortened from the species name.

Family Dicyemidae van Beneden, 1882
Genus *Dicyema* von Kölliker, 1849

***Dicyema coffinense* n. sp.**

Type-host: Giant Australian cuttlefish *Sepia apama* Gray (Mollusca: Cephalopoda: Sepiidae); mantle length 13.5 cm; weight 250 g (SRC 167, ABTC 126397).
Type-locality: Coffin Bay, South Australia (34°36′00″S, 135°25′55″E); collected *via* line fishing.
Site of infection: Attached to the surface of the left and right renal appendages.
Prevalence: In 8 out of 11 hosts (infection rate 73%).
Specimens deposited: Three syntype slides are deposited in the Marine Invertebrates Collection, SAMA, Australia (E3747–9) and two in the NSMT, Japan (NSMT-Me 31–32).
Etymology: The species name *coffinense* is derived from the type-locality, Coffin Bay.

Description (Figs. 2–3)

Diagnosis: Medium-sized dicyemid; body length reaching 2,920. Calotte conical; propolar cells and metapolar cells opposite; granules occasionally seen in parapolar cells of adults. Vermiform stages with 30–32 peripheral cells: 4 propolars + 4 metapolars + 2 parapolars + 20 to 22 trunk cells; 30 peripheral cells most common. Infusoriform embryos with 37 cells; refringent bodies solid; urn cell with 2 nuclei each.

Nematogens (Figs. 2A, D, 3A, D) [Based on 21 specimens.] Body 516–1,584 long (mean 944 ± 291, mode 1,000) and 16–30 wide (mean 22 ± 4, mode 22); trunk width mostly uniform (Figs. 2A, 3A). Peripheral cell number 30 to 32 (Table 2): 4 propolars + 4 metapolars + 2 parapolars + 18 to 20 diapolars cells + 2 uropolars. Calotte conical (Figs. 2A, D, 3D), granules occasionally seen in parapolar cells. Cilia on calotte approximately 4.8 long, orientated anteriorly. Propolar cells and their nuclei smaller than metapolar cells and their nuclei, respectively (Figs. 2D, 3D). Cytoplasm of propolar and metapolar cells more darkly stained by haematoxylin and eosin than cytoplasm of other peripheral cells. Verruciform cells absent. Axial cell cylindrical, rounded anteriorly, extends forward through metapolar cells to base of propolar cells; posterior extent of axial cell through uropolar cells (Figs. 2A, 3A). Average of 3 vermiform embryos present in axial cell of nematogens, with larger individuals having as many as 16 vermiform embryos in axial cell. Agametes present in axial cell, circular in shape, with average diameter 6.5 ± 0.5 (mode 6.2) (Figs. 2A, D, 3D).

Vermiform embryos (Figs. 2F, G, 3B, C) [Based on 21 specimens.] Full-grown vermiform embryos 114–272 long (mean 180 ± 46, mode 148) and 13–20 wide (mean 17 ± 3, mode 20). Peripheral cell number 30 to 32 (Table 2): 4 propolars + 4 metapolars + 2 parapolars + 18 to 20 diapolars cells + 2 uropolars; 30 peripheral cells most common. Anterior end of calotte rounded; metapolar cells and their nuclei larger than propolar cells and their nuclei, respectively (Figs. 2F, G, 3B, C). Axial cell rounded anteriorly, extends forward to base of propolar cells. Nucleus usually located in the centre of axial cell (Figs. 2F, G, 3B, C). Anterior abortive axial cell absent. Axial cell of full-grown embryo with as many as 4 agametes.

Rhombogens (Fig. 2B, C, E) [Based on 21 specimens.] Longer and slightly wider than nematogens, shape otherwise similar; length 1,384–2,920 (mean 1,842 ± 381, mode 1,448), width 24–64 (mean 32 ± 11, mode 24) (Fig. 2B, C). Peripheral cell number 30 to 31 (Table 2), 30 peripheral cells most common. Calotte conical, granules occasionally seen in parapolar cells, propolar cells and metapolar cells opposite (Fig. 2E). Axial cell shape and anterior extent similar to nematogen (Fig. 2B, C). Verruciform cells absent. Average of 8 infusoriform embryos present in axial cell of rhombogens, with smaller individuals having only 2 and larger individuals having as many as 21. Usually 1 or 2, rarely 4 or 5, infusorigens present in axial cell of each parent individual.

Infusorigens (Fig. 2K) [Based on 21 specimens.] Mature infusorigens small-sized; axial cell of infusorigens ovoid, 16–34 in diameter (mean 22.3 ± 4.2) (Fig. 2K). Composed of 6–11 (mode 7) external cells (egg line - oogonia and primary oocytes) + 4–13 (mode

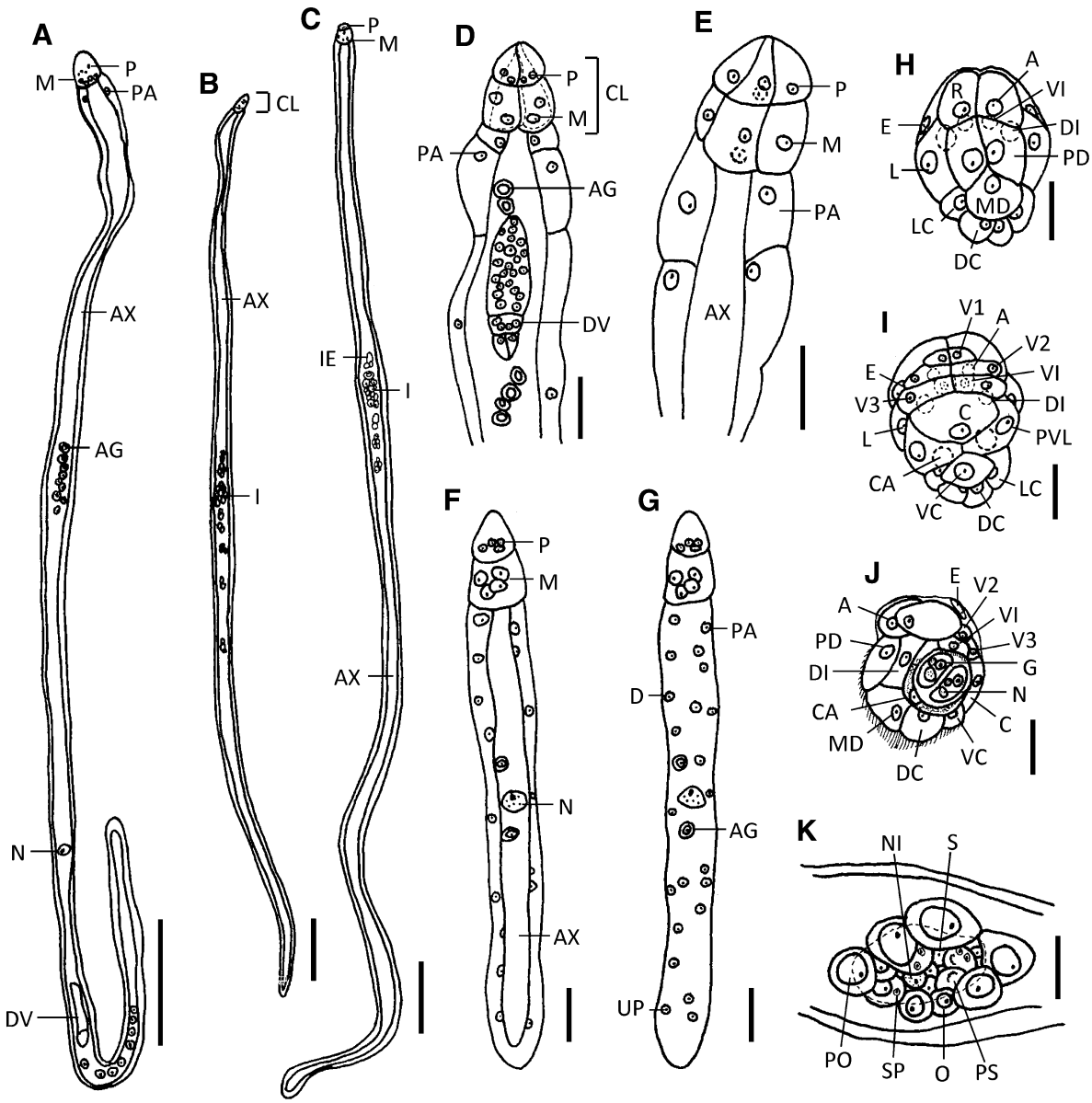

Fig. 2 Line drawings of *Dicyema coffinense* n. sp. from *Sepia apama*. A, nematogen, entire; B, C, rhombogen, entire; D, anterior region of nematogen; E, anterior region of rhombogen; F, G, vermiform embryo (F, optical section; G, cilia omitted); H–J, infusoriform embryos within axial cell (H, dorsal view, cilia omitted; I, ventral view, cilia omitted; J, sagittal section); K, infusorigen. *Abbreviations*: A, apical cell; AG, agamete; AX, axial cell; C, couvercle cell; CA, capsule cell; CL, calotte; D, diapolar cell; DC, dorsal caudal cell; DI, dorsal internal cell; DV, developing vermiform embryo; E, enveloping cell; G, germinal cell; I, infusorigen; IE, infusoriform embryo; L, lateral cell; LC, lateral caudal cell; M, metapolar cell; MD, median dorsal cell; N, nucleus; NI, nucleus of infusorigen; O, oogonia; P, propolar cell; PA, parapolar cell; PD, paired dorsal cell; PO, primary oocytes; PS, primary spermatocytes; PVL, posteroventral lateral cells; R, refringent body; S, spermatogonium; SP, sperm; UP, uropolar cell; VC, ventral caudal cell; VI, ventral internal cell; V1, first ventral cell; V2, second ventral cell; V3, third ventral cell. *Scale-bars*: A–C, 100 μm; D, E, K, 20 μm; F–J, 10 μm

7) internal cells (sperm line - spermatogonia, primary spermatocytes and secondary spermatocytes) + 3–9 (mode 5) sperm (Fig. 2K). Mean diameter of fertilised eggs 12.2 ± 0.3; mean sperm diameter 1.4 ± 0.2.

Infusoriform embryos (Figs. 2H–J, 3E) [Based on 100 specimens.] Full-grown embryos small-sized (mean length excluding cilia 28.4 ± 2.9); length : width : depth ratio 1.00 : 0.73 : 0.98. Shape ovoid, rounded

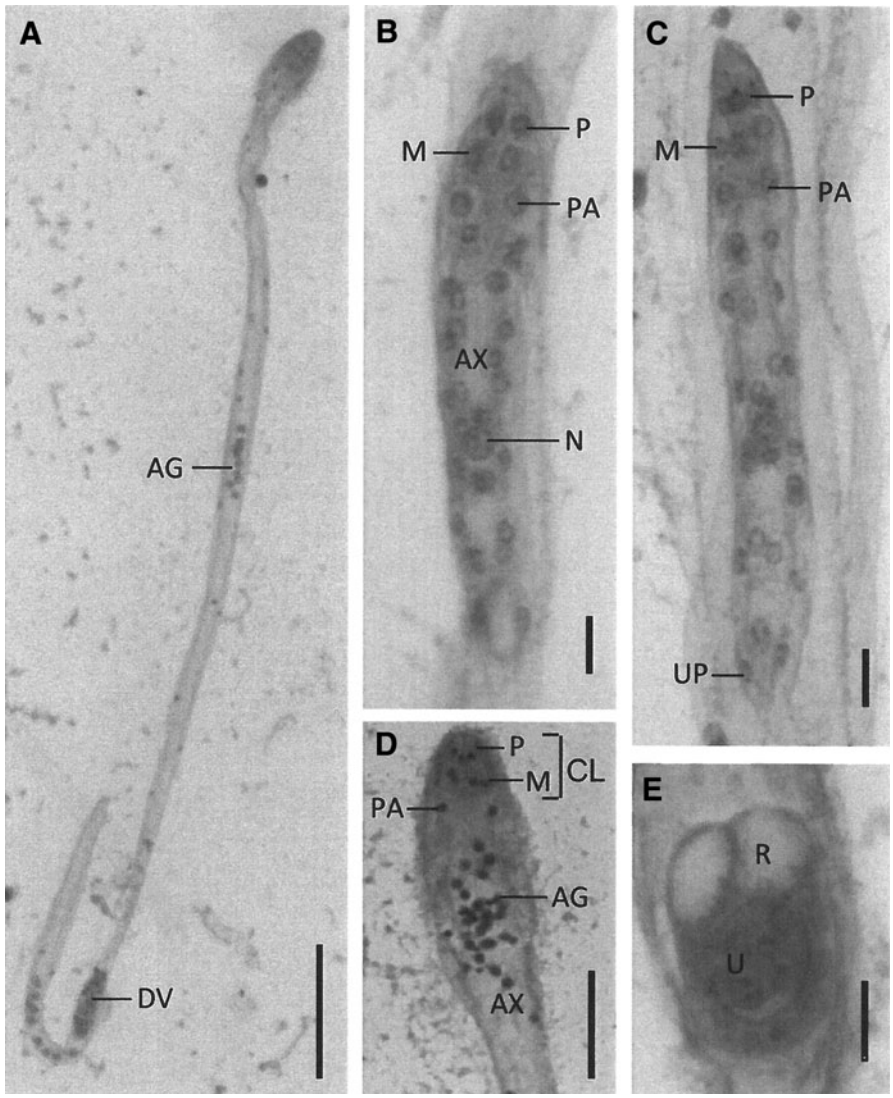

Fig. 3 Light micrographs of *D. coffinense* n. sp. from *S. apama*. A, nematogen, entire; B, C, vermiform embryo; D, anterior region of nematogen; E, infusoriform embryos within axial cell, horizontal section. *Abbreviations*: U, urn cell; see Fig. 2 for other abbreviations. *Scale-bars*: A, 100 μm; B, C, E, 10 μm; D, 50 μm

Table 2 Number of peripheral cells in *Dicyema coffinense* n. sp. infecting *Sepia apama*

Cell no.	No. of individuals		
	Vermiform embryos	Nematogens	Rhombogens
30	16	14	5
31	5	3	1
32	14	5	0

posteriorly (Figs. 2H–J, 3E). Cilia at posterior end 4.8 long. Refringent bodies present, solid and large, occupy 54% of embryo length when viewed laterally (Fig. 2J). Cilia projecting from ventral internal cells to urn cavity (Fig. 2J). Capsule cells contain many small granules, only on side adjacent to urn (Fig. 2J). Mature infusoriform embryos consisting of 37 cells: 33 somatic + 4 germinal cells. Somatic cells of several types present: external cells cover large part of anterior and lateral surfaces of embryo (2 enveloping cells); external cells with cilia on external surface (2 paired dorsal cells + 1 median dorsal cell + 2 dorsal caudal cells + 2 lateral caudal cells + 1 ventral caudal cell + 2 lateral cells + 2 posteroventral lateral cells); external cells with refringent bodies (2 apical cells);

external cells without cilia (1 couvercle cell + 2 first ventral cells + 2 second ventral cells + 2 third ventral cells); internal cells with cilia (2 ventral internal cells); and internal cells without cilia (2 dorsal internal cells + 2 capsule cells + 4 urn cells). Each urn cell contains 1 germinal cell and 2 nuclei (Fig. 2J). All somatic nuclei appear pycnotic in mature infusoriform embryos.

Remarks

Dicyema coffinense n. sp. is the first dicyemid species described from *S. apama*, and so far has only been found in hosts collected from Coffin Bay, South Australia. This species, with a peripheral cell count range of 30–32, partially overlaps with nine other species of *Dicyema*: *D. calamaroceum* Catalano & Furuya, 2013 (31–34), *D. erythrum* Furuya, 1999 (26, 28–36), *D. ganapatii* Kalavati, Narasimhamurti & Suseela, 1984 (28–32), *D. koshidai* Furuya & Tsuneki, 2005 (32–40), *D. macrocephalum* van Beneden, 1876 (28–33), *D. madrasensis* Kalavati, Narasimhamurti & Suseela, 1984 (24–27, 30–31), *D. oxycephalum* Furuya, 2009 (28–34), *D. papuceum* Catalano, 2013 (30–33) and *D. sullivani* McConnaughey, 1949 (28–33) (van Beneden, 1876; McConnaughey, 1949a; Kalavati et al., 1984; Furuya, 1999; Furuya & Tsuneki, 2005; Furuya, 2009; Catalano, 2013; Catalano & Furuya, 2013).

Dicyema coffinense can be distinguished from four of these species, *D. calamaroceum*, *D. ganapatii*, *D. madrasensis* and *D. oxycephalum*, based on differences in the number of cells in infusoriform embryos (37 *vs* 28–32, 39) and also from *D. sullivani*, based on differences in the length of infusoriform embryos (28.4 ± 2.9 *vs* 40–48) (McConnaughey, 1960; Kalavati et al., 1984; Furuya, 2009; Catalano & Furuya, 2013). Furthermore, *D. coffinense* can be distinguished from *D. koshidai* and *D. macrocephalum* based on smaller body length (2,920 *vs* 5,000 and 7,000, respectively) and from *D. papuceum* based on larger body length (2,920 *vs* 1,080) (van Beneden, 1876; Furuya & Tsuneki, 2005; Catalano, 2013). The remaining species, *D. erythrum*, shares with *D. coffinense* a similar body length, calotte shape, number of cells in infusoriform embryos and number of nuclei in each urn cell (Furuya, 1999). Nonetheless *D. erythrum* is distinguishable from *D. coffinense* by the presence of red granules in the cytoplasm of the peripheral cells, the wider body length range of adults, the presence of verruciform cells in nematogens and rhombogens, the smaller refringent bodies in infusoriform embryos and the larger diameters of fertilised eggs and sperm (Furuya, 1999).

Dicyema koinonum n. sp.

Type-host: Giant Australian cuttlefish *Sepia apama* Gray (Mollusca: Cephalopoda: Sepiidae), mantle length 12.0 cm; weight 220 g (SRC 70, ABTC 126396).
Other hosts: Nova cuttlefish *Sepia novaehollandiae* Hoyle (Mollusca: Cephalopoda: Sepiidae).
Type-locality: Gulf St Vincent (GSV), South Australia (SA), Australia (35°10′52″S, 138°23′25″E); collected from the by-catch of SARDI prawn trawl surveys.
Additional localities: Spencer Gulf (SG), SA (33°25′52″S, 137°31′02″E); collected from the by-catch of SARDI prawn trawl surveys.
Site of infection: Attached to the surface of the left and right renal appendages.
Prevalence: In 42 out of 63 hosts examined from both GSV and SG (overall infection rate 67%): in 10 out of 10 *S. apama* collected from GSV; in 25 out of 26 *S. apama* collected from SG; in 3 out of 16 *Sepia novaehollandiae* collected from GSV; and in 4 out of 11 *Sepia novaehollandiae* collected from SG.
Specimens deposited: Five syntype slides are deposited in the Marine Invertebrates Collection, SAMA, Australia (E3750–4) and five in the NSMT, Japan (NSMT-Me 33–37).
Etymology: The species name *koinonum* is derived from the Greek word "*koinonos*", meaning "share", because *D. koinonum* is shared between two cuttlefish species and from two different localities.

Description (Figs. 4–5)

Diagnosis: Small-sized dicyemid; body length reaching 1,104. Calotte relatively small, cap-shaped and conical; propolar cells and metapolar cells opposite. Vermiform stages with 28–29 peripheral cells: 4 propolars + 4 metapolars + 2 parapolars + 18 to 19 trunk cells; 28 peripheral cells most common. Verruciform cells present. Infusoriform embryos with 37 cells; refringent bodies solid; urn cell with 1 nucleus each.

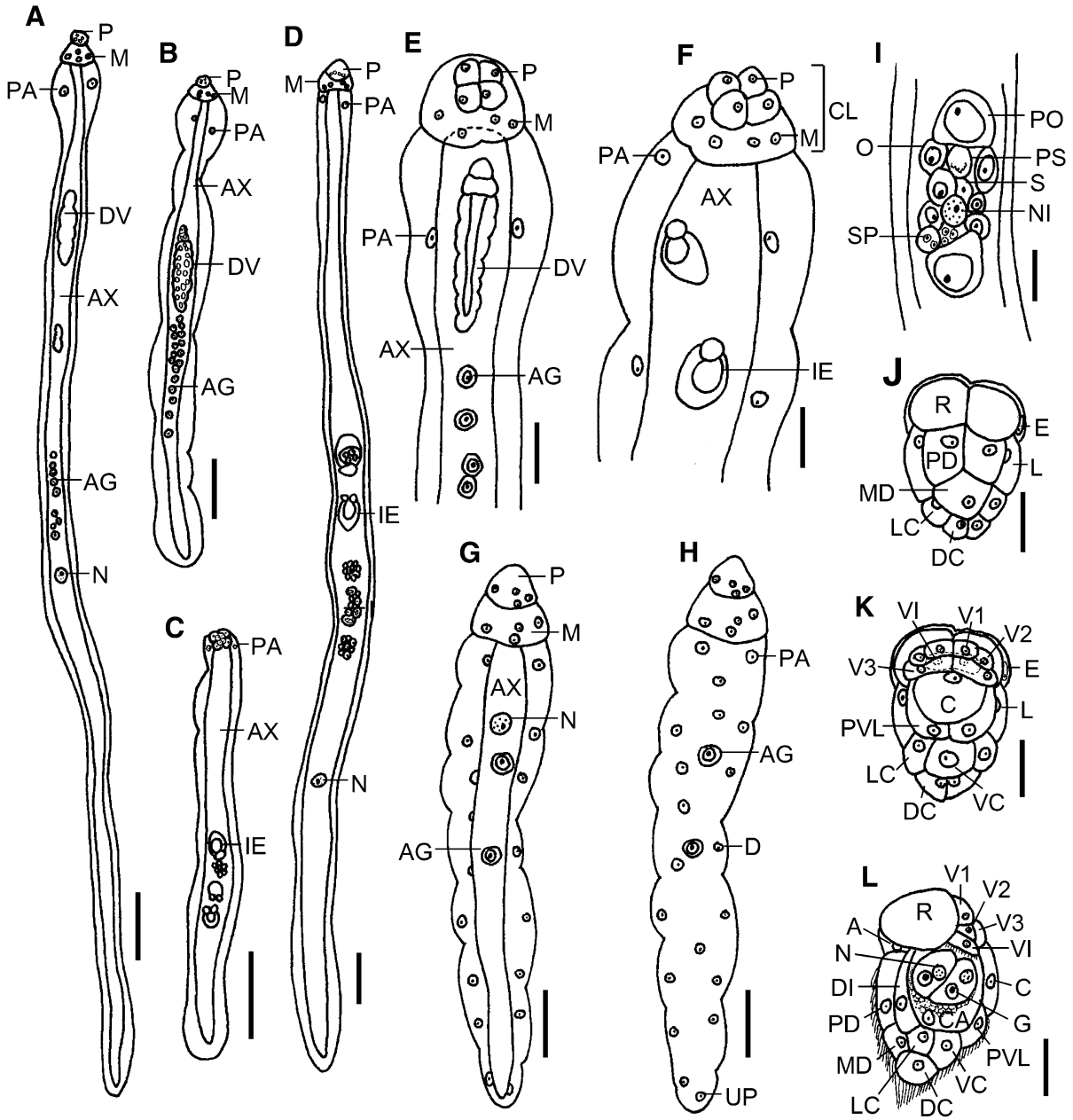

Fig. 4 Line drawings of *Dicyema koinonum* n. sp. from *Sepia apama* and *S. novaehollandiae*. A, B, nematogen, entire; C, D, rhombogen, entire; E, anterior region of nematogen; F, anterior region of rhombogen; G, H, vermiform embryo (G, optical section; H, cilia omitted); I, infusorigen; J–L, infusoriform embryos within axial cell (J, dorsal view, cilia omitted; K, ventral view, cilia omitted; L, sagittal section). See Fig. 2 for abbreviations. *Scale-bars*: A, B, D, 50 μm; C, 100 μm; E–H, 20 μm; I–L, 10 μm

Nematogens (Figs. 4A, B, E, 5B) [Based on 21 specimens.] Body 300–1,104 long (mean 618 ± 252, mode 1,016) and 24–48 wide (mean 34 ± 7, mode 32); trunk width mostly uniform, sometimes wider at parapolars (Fig. 4A, B). Peripheral cell number 28 to 29 (Table 3): 4 propolars + 4 metapolars + 2 parapolars + 16 to 17 diapolars cells + 2 uropolars. Calotte small, cap-shaped and conical, occasionally forming a cephalic swelling together with the parapolar cells (Figs. 4E, 5B). Propolar cells and metapolar cells

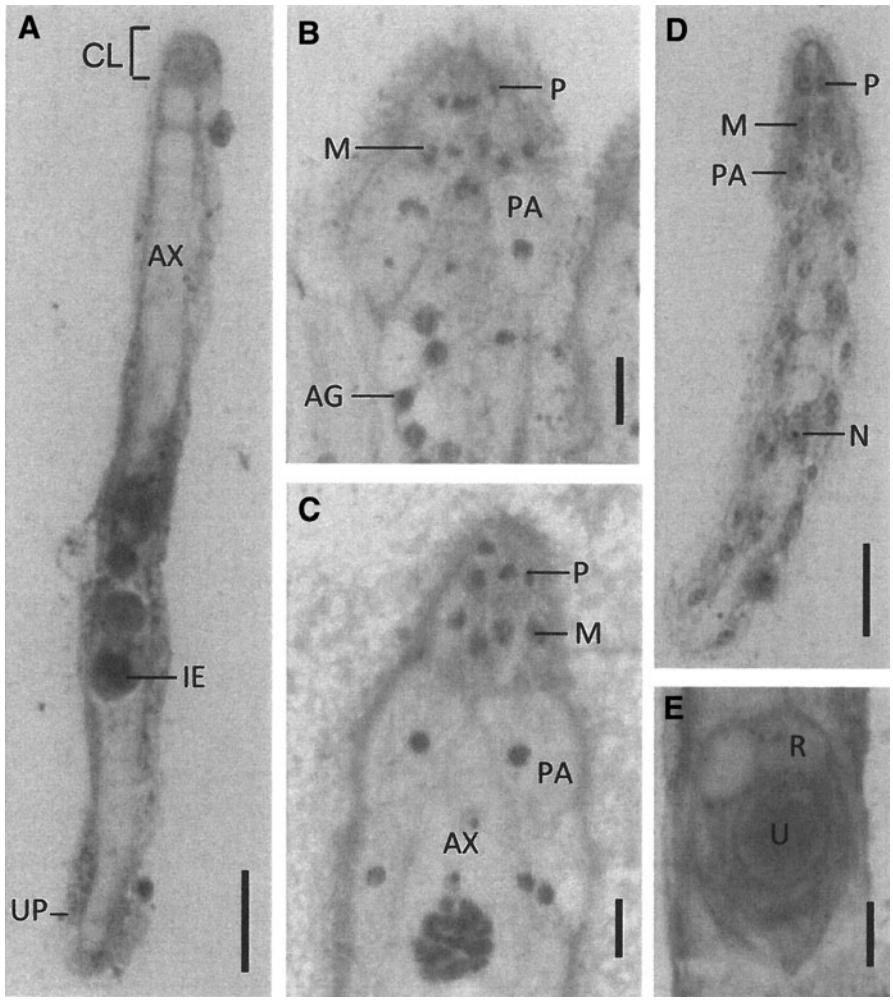

Fig. 5 Light micrographs of *D. koinonum* n. sp. from *S. apama* and *S. novaehollandiae*. A, rhombogen, entire; B, anterior region of nematogen; C, anterior region of rhombogen; D, vermiform embryo; E, infusoriform embryos within axial cell, horizontal section. See Figs. 2 and 3 for abbreviations. *Scale-bars*: A, 50 μm; B, C, E, 10 μm; D, 20 μm

opposite. Cilia on calotte approximately 6 long, orientated anteriorly. Propolar cells smaller than metapolar cells, nuclei similar in size (Figs. 4E, 5B). Cytoplasm of propolar and metapolar cells more darkly stained by haematoxylin and eosin than cytoplasm of other peripheral cells. Verruciform cells present in larger individuals, rare. Axial cell cylindrical, rounded anteriorly; extends halfway through metapolar cells; posterior extent of axial cell through uropolar cells (Fig. 4A, B, E). Average of 3 vermiform embryos present in axial cell of nematogens, with larger individuals having as many as 10 vermiform embryos in axial cell. Agametes circular, present in axial cell, with average diameter 7.0 ± 0.5 (mode 7.2).

Table 3 Number of peripheral cells in *Dicyema koinonum* n. sp. infecting *Sepia apama* and *S. novaehollandiae*

Cell no.	No. of individuals		
	Vermiform embryos	Nematogens	Rhombogens
28	25	17	18
29	3	1	0

Vermiform embryos (Figs. 4G, H, 5D) [Based on 29 specimens.] Full-grown vermiform embryos 68–308 long (mean 158 ± 59, mode 116) and 16–40 wide (mean 23 ± 5, mode 24). Peripheral cell number 28 to 29 (Table 3): 4 propolars + 4 metapolars + 2

parapolars + 16 to 17 diapolars cells + 2 uropolars; 28 peripheral cells most common. Anterior end of calotte rounded, conical (Figs. 4G, H, 5D). Metapolar cells larger than propolar cells, nuclei similar in size (Figs. 4G, H, 5D). Axial cell rounded anteriorly; extends forward to middle of metapolar cells. Anterior abortive axial cell absent. Axial cell of full-grown embryos with as many as 9 agametes, circular in shape.

Rhombogens (Figs. 4C, D, F, 5A, C) [Based on 21 specimens.] Length, width and shape similar to nematogens; length 360–1,000 (mean 555 ± 138, mode 472), width 30–40 (mean 35 ± 4, mode 32) (Figs. 4C, D, 5A). Peripheral cell number 28 (Table 3). Calotte small, cap-shaped, occasionally forms cephalic swelling with parapolar cells (Figs. 4F, 5C). Propolar cells and metapolar cells opposite. Axial cell shape and anterior extent similar to nematogen (Figs. 4B, 5C). Verruciform cells present, rare. Average of 4 infusoriform embryos present in axial cell of rhombogens, with smaller individuals having only 1 and larger individuals having as many as 10. Usually 1, rarely 2 or 3 infusorigens present in axial cell of each parent individual.

Infusorigens (Fig. 4I) [Based on 21 specimens.] Mature infusorigens small-sized; axial cell of infusorigens elongate, usually irregular in shape, 12–27 in diameter (mean 19.8 ± 3.6). Composed of 6–12 (mode 6) external cells (egg line - oogonia and primary oocytes) + 5–12 (mode 7) internal cells (sperm line - spermatogonia, primary spermatocytes and secondary spermatocytes) + 3–11 (mode 5) sperm (Fig. 4I). Mean diameter of fertilised eggs 11.7 ± 0.8; mean sperm diameter 1.0 ± 0.1.

Infusoriform embryos (Figs. 4J–L, 5E) [Based on 100 specimens.] Full-grown embryos small to medium-sized (mean length excluding cilia 28.7 ± 2.0); length : width : depth ratio 1.00 : 0.73 : 0.90. Shape ovoid, rounded posteriorly (Figs. 4J, K, 5E). Cilia at posterior end 8–12 long. Refringent bodies present, solid and large, occupy 63% of embryo length when viewed laterally (Fig. 4L). Cilia projecting from ventral internal cells to urn cavity (Fig. 4L). Capsule cells contain many small granules, only on side adjacent to urn (Fig. 4L). Mature infusoriform embryos consisting of 37 cells: 33 somatic + 4 germinal cells. Somatic cells of several types present: external cells cover large part of anterior and lateral surfaces of embryo (2 enveloping cells); external cells with cilia on external surface (2 paired dorsal cells + 1 median dorsal cell + 2 dorsal caudal cells + 2 lateral caudal cells + 1 ventral caudal cell + 2 lateral cells + 2 posteroventral lateral cells); external cells with refringent bodies (2 apical cells); external cells without cilia (1 couvercle cell + 2 first ventral cells + 2 second ventral cells + 2 third ventral cells); internal cells with cilia (2 ventral internal cells); and internal cells without cilia (2 dorsal internal cells + 2 capsule cells + 4 urn cells). Each urn cell contains 1 germinal cell and 1 nucleus (Fig. 4L). All somatic nuclei appear pycnotic in mature infusoriform embryos.

Remarks

Dicyema koinonum n. sp. infects two cuttlefish species, *S. apama* and *S. novaehollandiae*, and is recorded from two localities, Spencer Gulf and Gulf St Vincent, South Australia. Of the 65 species currently described within *Dicyema* (see Catalano, 2012, 2013; Catalano & Furuya, 2013), 14 partially overlap with the peripheral cell count range described for *D. koinonum* (28–29). However, *D. koinonum* can be distinguished from 12 of these species by differences in body length and infusoriform embryo cell counts, with only *D. acheroni* McConnaughey, 1949 and *D. sullivani* being similar (McConnaughey, 1949a, b, 1960). While *D. acheroni* is also characterised by an anterior extent of the axial cell to the metapolar cells, it can be distinguished from *D. koinonum* by the shape of the calotte (pointed with elongate metapolar cells *vs* small, cap-shaped and conical) and the absence of verruciform cells (McConnaughey, 1949b). *Dicyema sullivani* is distinguishable from *D. koinonum* by the longer infusoriform embryos (40–48 *vs* 28.7 ± 2.0), the differences in the number of nuclei in urn cells (2 *vs* 1) and the smaller infusorigens with less external and internal cells (McConnaughey, 1960).

Dicyema multimegalum n. sp.

Type-host: Giant Australian cuttlefish *Sepia apama* Gray (Mollusca: Cephalopoda: Sepiidae); mantle length 38.0 cm; weight 9,500 g (SRC 233, ABTC 126398).

Type-locality: Cronulla (CR) and North Bondi (NB), New South Wales, Australia (34°04′20″S, 151°09′35″E); collected by SCUBA at depths up to 10 m.
Site of infection: Attached to the surface of the left and right renal appendages.
Prevalence: In 3 out of 4 hosts examined from CR and NB (infection rate 75%).
Specimens deposited: Five syntype slides are deposited in the Marine Invertebrates Collection, SAMA, Australia (E3755–9) and four in the NSMT, Japan (NSMT-Me 38–41).
Etymology: The species name *multimegalum* is composed of two Greek words, "*multi*", meaning "many" and "*megalo*", meaning "large", in reference to the characteristic large granules observed with high density in the urn and capsule cells of infusoriform embryos.

Description (Figs. 6–7)

Diagnosis: Small-sized dicyemid; body length reaching 1,672. Calotte small, conical and compressed; propolar cells and metapolar cells opposite. Vermiform stages with 26–28 peripheral cells: 4 propolars + 4 metapolars + 2 parapolars + 16 to 18 trunk cells; 26 peripheral cells most common. Small granules in parapolar and trunk cells of adults, only on side adjacent to axial cell. Infusoriform embryos large, with 37 cells; refringent bodies small, solid; capsule and urn cells with numerous, large, darkly-stained granules; urn cell with 2 nuclei each.

Nematogens (Figs. 6A, B, F, 7A, C) [Based on 21 specimens.] Body 536–1,280 long (mean 913 ± 188, mode 824) and 26–60 wide (mean 39 ± 9, mode 48); trunk width mostly uniform (Figs. 6A, B, 7A). Peripheral cell number 26 to 28 (Table 4): 4 propolars + 4 metapolars + 2 parapolars + 14 to 16 diapolars cells + 2 uropolars. Calotte small, conical; propolar cells and metapolar cells opposite (Figs. 6F, 7A, C). Cilia on calotte 4–8 long, orientated anteriorly. Propolar cells and their nuclei smaller than metapolar cells and their nuclei, respectively (Figs. 6F, 7C). Cytoplasm of propolar and metapolar cells more darkly stained by haematoxylin and eosin than cytoplasm of other peripheral cells. Granules observed in parapolar and trunk cells numerous, located only on side of cell adjacent to axial cell. Axial cell cylindrical, rounded anteriorly, extends forward through metapolar cells to propolar cells; posterior extent of axial cell through uropolar cells (Figs. 6A, B, 7A). Average of 2 vermiform embryos present in axial cell of nematogens, with larger individuals having as many as 5 vermiform embryos in axial cell. Agametes circular, numerous with as many as 250 agametes in axial cell, with average diameter 6.0 ± 0.4 (mode 5.6) (Figs. 6A, B, 7A).

Vermiform embryos (Figs. 6E, 7B) [Based on 21 specimens.] Full-grown vermiform embryos 136–388 long (mean 258 ± 84, mode 188) and 24–48 wide (mean 33 ± 8, mode 28). Peripheral cell number 26 to 28 (Table 4): 4 propolars + 4 metapolars + 2 parapolars + 14 to 16 diapolars cells + 2 uropolars; 26 peripheral cells most common. Calotte small, compressed; anterior end rounded; metapolar cells and their nuclei larger than propolar cells and their nuclei, respectively (Figs. 6E, 7B). Axial cell rounded anteriorly, extends forward to middle of propolar cells. Nucleus usually located in the centre of axial cell (Fig. 6E). Anterior abortive axial cell absent. Axial cell of full-grown embryos with as many as 24 agametes.

Rhombogens (Fig. 6C, D, G) [Based on 21 specimens.] Length, width and shape similar to nematogens; length 340–1,672 (mean 628 ± 306, mode 396), width 32–52 (mean 41 ± 6, mode 40) (Fig. 6C, D). Peripheral cell number 26 to 28 (Table 4), 26 peripheral cells most common. Calotte small, conical and compressed; propolar cells and metapolar cells opposite (Fig. 6G). Axial cell shape and anterior extent similar to nematogen. Granules observed in parapolar and trunk cells, numerous, located only on side of cell adjacent to axial cell. Average of 2 infusoriform embryos present in axial cell of rhombogens, with smaller individuals having only 1 and larger individuals occasionally having as many as 6. Usually 1, rarely 2 or 3 infusorigens present in axial cell of each parent individual.

Infusorigens (Fig. 6L) [Based on 21 specimens.] Mature infusorigens small-sized; axial cell elongate, usually irregular in shape, 20–40 in diameter (mean 28.3 ± 5.2). Composed of 6–15 (mode 10) external cells (egg line - oogonia and primary oocytes) + 4–14 (mode 7) internal cells (sperm line - spermatogonia,

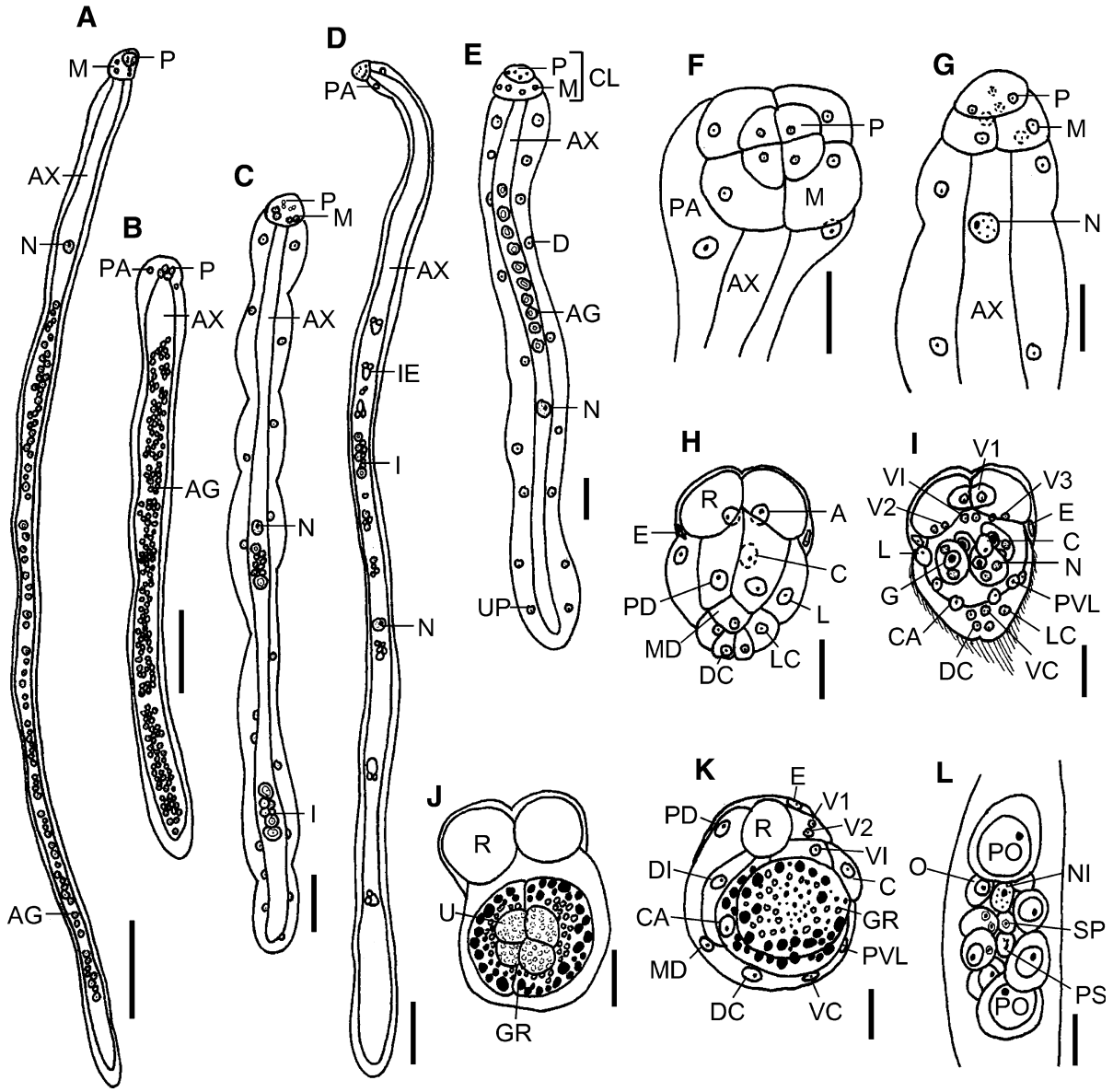

Fig. 6 Line drawings of *Dicyema multimegalum* n. sp. from *Sepia apama*. A, B, nematogen, entire; C, D, rhombogen, entire; E, vermiform embryo, optical section; F, anterior region of nematogen; G, anterior region of rhombogen; H–K, infusoriform embryos within axial cell (H, dorsal view, cilia omitted; I, ventral view, cilia omitted; J, ventral view, granules; K, sagittal section); L, infusorigen. *Abbreviations*: GR, granules; see Figs. 2 and 3 for other abbreviations. *Scale-bars*: A, B, D, 100 μm; C, 50 μm; E–G, 20 μm; H–L, 10 μm

primary spermatocytes and secondary spermatocytes) + 4–14 (mode 5) sperm (Fig. 6L). Mean diameter of fertilised eggs 10.3 ± 0.8; mean sperm diameter 1.4 ± 0.1.

Infusoriform embryos (Figs. 6H–K, 7D) [Based on 100 specimens.] Full-grown embryos large-sized, up to 40 long (mean length excluding cilia 33 ± 4.1); length : width : depth ratio 1.00 : 0.70 : 0.83. Shape ovoid to circular, rounded posteriorly (Figs. 6H–K, 7D). Cilia at posterior end 8.8 long. Refringent bodies present, solid and small, occupy 36% of embryo length when viewed laterally (Fig. 6K). Cilia projecting from ventral internal cells to urn cavity. Capsule and urn

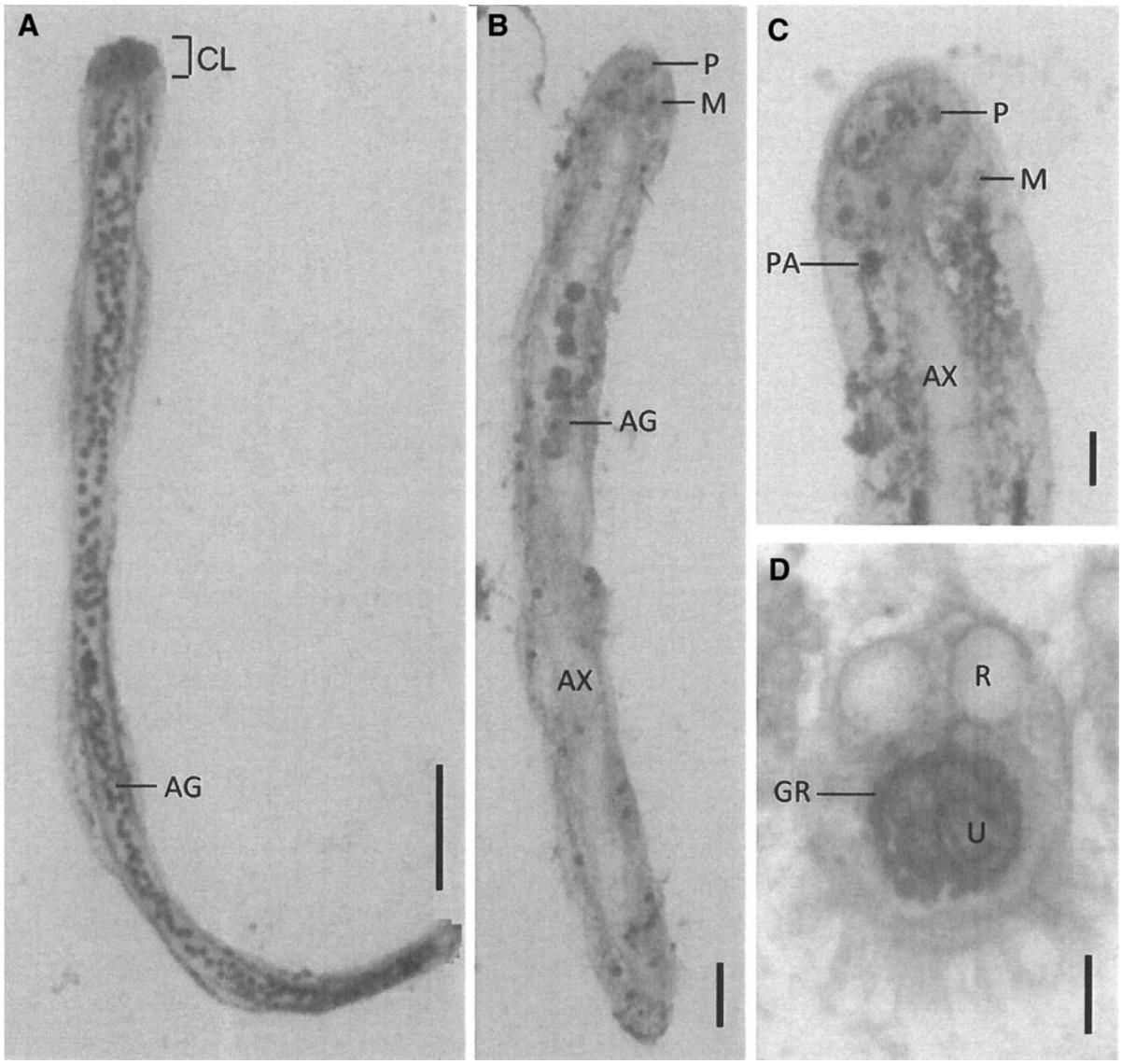

Fig. 7 Light micrographs of *D. multimegalum* n. sp. from *S. apama*. A, nematogen, entire; B, young vermiform embryo; C, anterior region of nematogen; D, infusoriform embryos within axial cell, horizontal section. See Figs. 2, 3, 6 for abbreviations. *Scale-bars*: A, 100 μm; B, 20 μm; C, D, 10 μm

Table 4 Number of peripheral cells in *Dicyema multimegalum* n. sp. infecting *Sepia apama*

Cell no.	No. of individuals		
	Vermiform embryos	Nematogens	Rhombogens
26	23	11	12
27	8	6	2
28	17	8	4

cells contain many darkly-stained granules, smaller and more numerous in urn cells (Figs. 6J, K, 7D). Mature infusoriform embryos consisting of 37 cells: 33 somatic + 4 germinal cells. Somatic cells of several types present: external cells cover large part of anterior and lateral surfaces of embryo (2 enveloping cells); external cells with cilia on external surface (2 paired dorsal cells + 1 median dorsal cell + 2

dorsal caudal cells + 2 lateral caudal cells + 1 ventral caudal cell + 2 lateral cells + 2 posteroventral lateral cells); external cells with refringent bodies (2 apical cells); external cells without cilia (1 couvercle cell + 2 first ventral cells + 2 second ventral cells + 2 third ventral cells); internal cells with cilia (2 ventral internal cells); and internal cells without cilia (2 dorsal internal cells + 2 capsule cells + 4 urn cells). Each urn cell contains 1 germinal cell and 2 nuclei (Fig. 6I). All somatic nuclei appear pycnotic in mature infusoriform embryos.

Remarks

Dicyema multimegalum n. sp. has only been found from *S. apama* collected from the east coast of Australia. It shares a peripheral cell count range with 16 other species within *Dicyema*: *D. acciacatum* McConnaughey, 1949 (22–28), *D. acheroni* (23–28), *D. erythrum* (26, 28–36), *D. ganapatii* (28–32), *D. lycidoeceum* Furuya, 1999 (26–34), *D. macrocephalum* (28–33), *D. madrasensis* (24–27, 30–31), *D. microcephalum* Whitman, 1883 (25–27), *D. moschatum* Whitman, 1883 (22–26), *D. nouveli* Kalavati, Narasimhamurti & Suseela, 1984 (26–28), *D. oxycephalum* (28–34), *D. paradoxum* von Kölliker, 1849 (25–28), *D. rhadinum* Furuya, 1999 (24–28), *D. sepiellae* Furuya, 2008 (24–29), *D. sullivani* (28–33) and *D. whitmani* Furuya & Hochberg, 1998 (26–28) (von Kölliker, 1849; van Beneden, 1876; Whitman, 1883; McConnaughey, 1949a, b; Kalavati et al., 1984; Furuya & Hochberg, 1998; Furuya, 1999, 2008, 2009). However, *D. multimegalum* differs from all of these species by the combination of a small, conical and compressed calotte, presence of small granules in the parapolar and trunk cells of adults that are only found on the side of the cell adjacent to the axial cell, and by the presence of many large, darkly-stained granules that fill the capsule and urn cells of infusoriform embryos.

***Dicyema vincentense* n. sp.**

Type-host: Nova cuttlefish *Sepia novaehollandiae* Hoyle (Mollusca: Cephalopoda: Sepiidae); mantle length 8.0 cm; weight 60 g (SRC 68, ABTC 126400).

Type-locality: Gulf St Vincent, South Australia (35°10′52″S, 138°23′25″E); collected from the by-catch of SARDI prawn trawl surveys.

Site of infection: Attached to the surface of the left and right renal appendages.

Prevalence: In 13 out of 16 hosts examined from the type-locality (infection rate 81%).

Specimens deposited: Three syntype slides are deposited in the Marine Invertebrates Collection, SAMA, Australia (E3760–2) and two in the NSMT, Japan (NSMT-Me 42–43).

Etymology: The species name *vincentense* is derived from the type-locality, Gulf St Vincent.

Description (Figs. 8–9)

Diagnosis: Large-sized dicyemid; body length reaching 5,700. Calotte elongate and pointed; propolar cells and metapolar cells opposite. Vermiform stages with 38–41 peripheral cells: 4 propolars + 4 metapolars + 2 parapolars + 28 to 31 trunk cells; 38 peripheral cells most common. As many as 500 agametes present in large nematogens. Infusoriform embryos with 37 cells; refringent bodies solid; urn cells with 1 nucleus each.

Nematogens (Figs. 8A, B, D, 9A, B) [Based on 21 specimens.] Body long and slender, length 904–5,700 (mean 2,684 ± 1,444), width 28–72 (mean 41 ± 12, mode 32); trunk width mostly uniform (Figs. 8A, B, 9A). Peripheral cell number 38 to 41 (Table 5): 4 propolars + 4 metapolars + 2 parapolars + 26 to 29 diapolars cells + 2 uropolars. Calotte elongate and pointed; propolar cells and metapolar cells opposite (Figs. 8D, 9B). Cilia on calotte approximately 7 long, orientated anteriorly. Propolar cells longer than metapolar cells, nuclei similar in size (Figs. 8D, 9B). Cytoplasm of propolar and metapolar cells more darkly stained by haematoxylin and eosin than cytoplasm of other peripheral cells. Verruciform cells absent. Axial cell cylindrical, rounded anteriorly, extends forward through metapolar cells to propolar cells; posterior extent of axial cell through uropolar cells (Figs. 8A, B, 9A). Average of 4 vermiform embryos present in axial cell of nematogens, with larger individuals having as many as 12 vermiform embryos in axial cell. Agametes circular, numerous, with as many as 500 agametes in axial cell, with average diameter 5.3 ± 0.5 (mode 5.6).

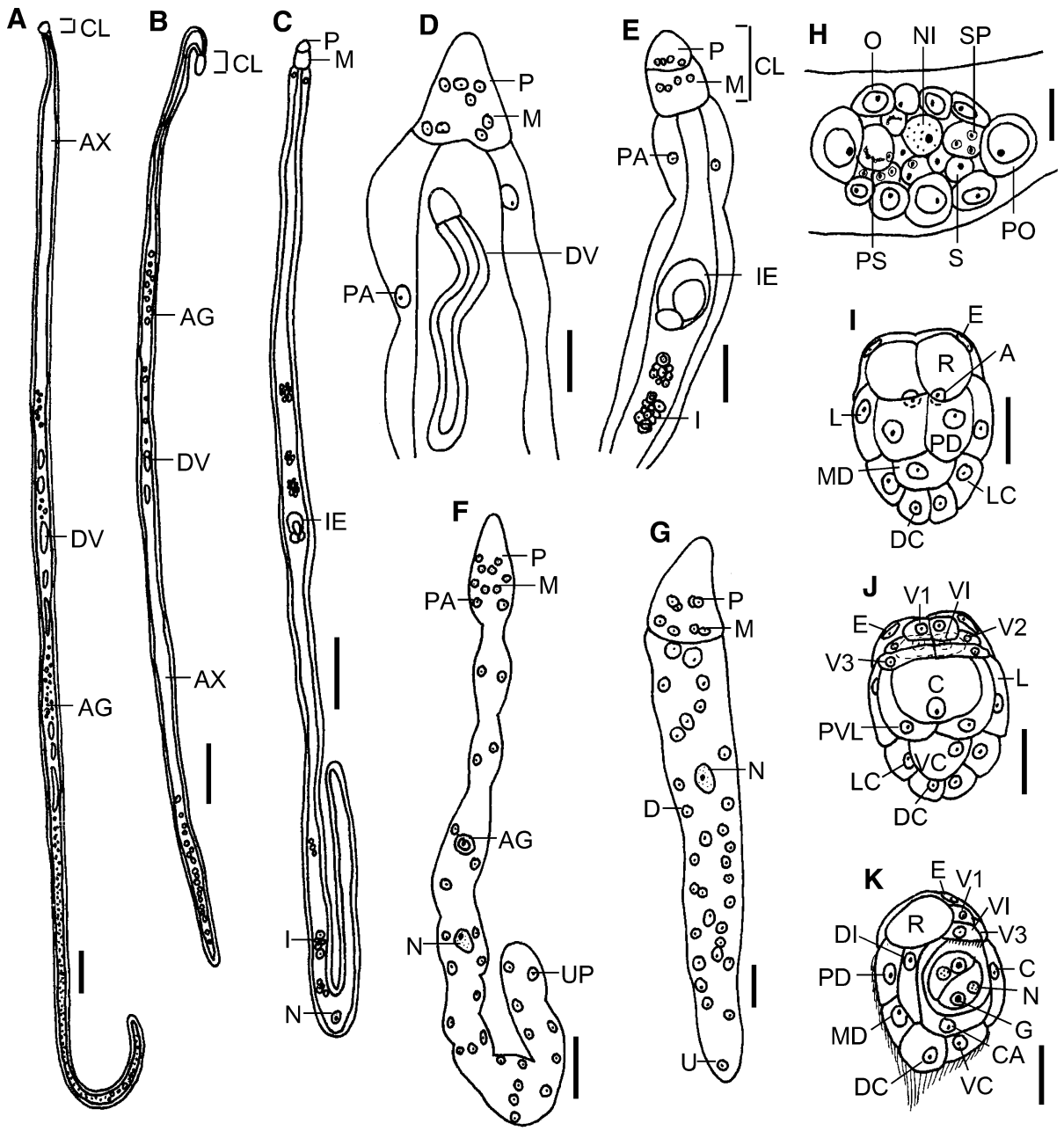

Fig. 8 Line drawings of *Dicyema vincentense* n. sp. from *Sepia novaehollandiae*. A, B, nematogen, entire; C, rhombogen, entire; D, anterior region of nematogen; E, anterior region of rhombogen; F, G, vermiform embryo, cilia omitted; H, infusorigen; I–K, infusoriform embryos within axial cell (I, dorsal view, cilia omitted; J, ventral view, cilia omitted; K, sagittal section). See Fig. 2 for abbreviations. *Scale-bars*: A, 200 μm; B, C, 100 μm; D–F, 20 μm; G–K, 10 μm

Vermiform embryos (Figs. 8F, G, 9C–E) [Based on 21 specimens.] Full-grown vermiform embryos long and slender, 112–416 long (mean 223 ± 99, mode 112) and 16–36 wide (mean 21 ± 6, mode 16). Peripheral cell number 38 to 41 (Table 5): 4 propolars + 4 metapolars + 2 parapolars + 26 to 29 diapolars cells + 2 uropolars; 38 peripheral cells most common. Anterior end of calotte distinctly pointed; propolar cells long; metapolar and propolar nuclei similar in size (Figs. 8F, G, 9C–E). Axial cell rounded anteriorly,

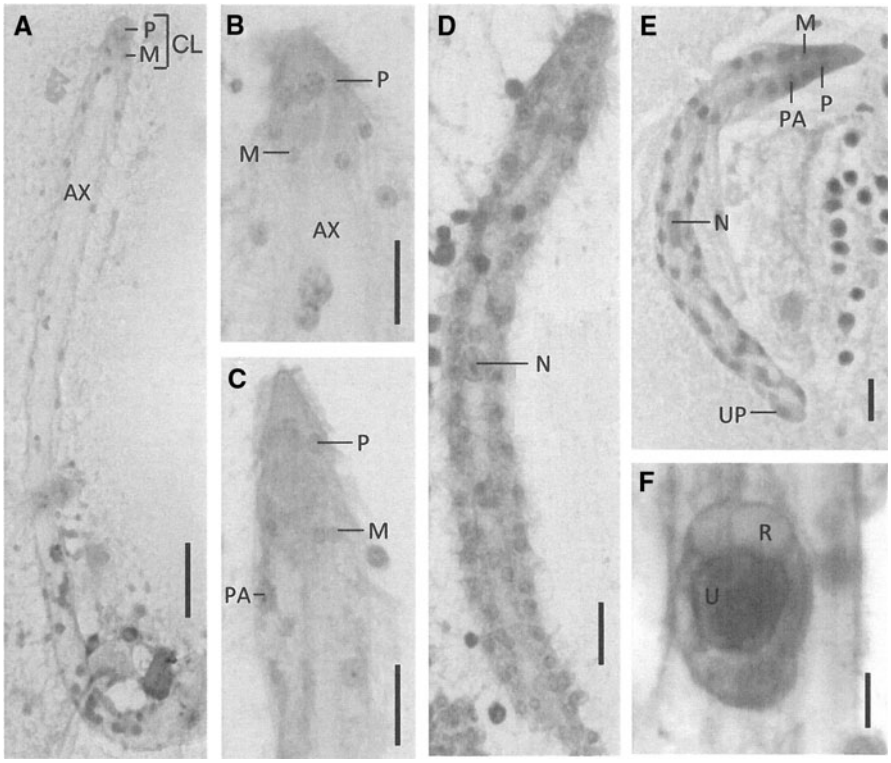

Fig. 9 Light micrographs of *D. vincentense* n. sp. from *S. novaehollandiae*. A, nematogen, entire; B, anterior region of nematogen; C, anterior region of young vermiform embryo; D, E, vermiform embryo; F, infusoriform embryos within axial cell, horizontal section. See Figs. 2 and 3 for abbreviations. *Scale-bars*: A, 50 µm; B–D, 20 µm; E, F, 10 µm

Table 5 Number of peripheral cells in *Dicyema vincentense* n. sp. infecting *Sepia novaehollandiae*

Cell no.	No. of individuals		
	Vermiform embryos	Nematogens	Rhombogens
38	20	7	5
39	4	3	1
40	2	2	4
41	5	1	1

extends forward to middle of propolar cells. Nucleus usually located in the centre of axial cell (Figs. 8F, G, 9D, E). Anterior abortive axial cell absent. Axial cell of full-grown embryos with as many as 9 agametes.

Rhombogens (Fig. 8C, E) [Based on 21 specimens.] Shorter than nematogens, width and shape otherwise similar, slender; length 1,032–3,560 (mean 1,868 ± 567, mode 1,520), width 29–56 (mean 36 ± 7, mode 40) (Fig. 8C). Peripheral cell number 38 to 41 (Table 5), 38 peripheral cells most common. Calotte elongate, pointed (Fig. 8E). Axial cell shape and anterior extent similar to nematogen. Verruciform cells absent. Average of 15 infusoriform embryos present in axial cell of rhombogens, with smaller individuals having only 1 and larger individuals having as many as 39. Usually 2, rarely 1, 3–7 infusorigens present in axial cell of each parent individual.

Infusorigens (Fig. 8H) [Based on 21 specimens.] Mature infusorigens small-sized; axial cell irregular in shape, 16–29 in diameter (mean 22.0 ± 3.8). Composed of 6–16 (mode 9) external cells (egg line - oogonia and primary oocytes) + 5–14 (mode 9) internal cells (sperm line - spermatogonia, primary spermatocytes and secondary spermatocytes) + 6–14 (mode 11) sperm (Fig. 8H). Mean diameter of fertilised eggs 8.8 ± 1.1; mean sperm diameter 1.3 ± 0.1.

Infusoriform embryos (Figs. 8I–K, 9F) [Based on 100 specimens.] Full-grown embryos large-sized, up to 40 in length (mean excluding cilia 35.8 ± 2.8); length :

width : depth ratio 1.00 : 0.70 : 0.89. Shape ovoid, rounded posteriorly (Figs. 8I–K, 9F). Cilia at posterior end 12 long. Refringent bodies present, solid and small, occupy 38% of embryo length when viewed laterally (Fig. 8K). Cilia projecting from ventral internal cells to urn cavity (Fig. 8K). Mature infusoriform embryos consisting of 37 cells: 33 somatic + 4 germinal cells. Somatic cells of several types present: external cells cover large part of anterior and lateral surfaces of embryo (2 enveloping cells); external cells with cilia on external surface (2 paired dorsal cells + 1 median dorsal cell + 2 dorsal caudal cells + 2 lateral caudal cells + 1 ventral caudal cell + 2 lateral cells + 2 posteroventral lateral cells); external cells with refringent bodies (2 apical cells); external cells without cilia (1 couvercle cell + 2 first ventral cells + 2 second ventral cells + 2 third ventral cells); internal cells with cilia (2 ventral internal cells); and internal cells without cilia (2 dorsal internal cells + 2 capsule cells + 4 urn cells). Each urn cell contains 1 germinal cell and 1 nucleus (Fig. 8K). All somatic nuclei appear pycnotic in mature infusoriform embryos.

Remarks

Dicyema vincentense n. sp. is found in *S. novaehollandiae* collected from Gulf St Vincent (GSV), South Australia (SA). Only two other species of *Dicyema*, *D. australis* Penchaszadeh, 1968 and *D. koshidai*, overlap partially with the peripheral cell count range observed for *D. vincentense* (38–41) (Penchaszadeh, 1968; Furuya & Tsuneki, 2005).

Dicyema australis is described from *Octopus tehuelchus* d'Orbigny collected in the Province of Buenos Aires, Argentina (Penchaszadeh, 1968). It is similar to *D. vincentense* in body length and anterior extent of the axial cell to the propolar cells. However *D. australis* differs in having a small, conical calotte, verruciform cells, small infusoriform larvae and two nuclei in each urn cell (Penchaszadeh, 1968).

Dicyema koshidai, described from the bigfin reef squid *Sepioteuthis lessoniana* Lesson, is very similar to *D. vincentense* in body length, body shape, length of cilia on calotte and number of cells in infusoriform embryos (Furuya & Tsuneki, 2005). However it differs from *D. vincentense* in having a conical calotte, anterior extent of the axial cell to the base of the metapolar cells, smaller lengths of infusoriform embryos, two nuclei in each urn cell and larger infusorigens with a higher number of external cells, internal cells and sperm (Furuya & Tsuneki, 2005).

Sepia novaehollandiae individuals collected from GSV, SA were also infected by *D. koinonum*, however co-occurrence of *D. koinonum* and *D. vincentense* in one host individual was not observed. *Dicyema vincentense* is easily distinguished from *D. koinonum* by body length (5,700 *vs* 1,104), calotte shape (elongate and pointed *vs* small, cap-shaped and conical), and peripheral cell count (38–41 *vs* 28–29).

Genus *Dicyemennea* Whitman, 1883

Dicyemennea spencerense n. sp.

Type-host: Nova cuttlefish *Sepia novaehollandiae* Hoyle (Mollusca: Cephalopoda: Sepiidae), mantle length 8.1 cm; weight 74 g (SRC 29, ABTC 126399).
Other hosts: Giant Australian cuttlefish *S. apama* Gray (Mollusca: Cephalopoda: Sepiidae).
Type-locality: Spencer Gulf, South Australia (33°09′08″S, 137°39′59″E); collected from the by-catch of SARDI prawn trawl surveys.
Site of infection: Attached to the surface of the left and right renal appendages.
Prevalence: In 10 out of 11 *Sepia novaehollandiae* (infection rate 91%) and in 14 out of 26 *S. apama* (infection rate 54%).
Specimens deposited: Four syntype slides are deposited in the Marine Invertebrates Collection, SAMA, Australia (E3763–6) and three in the NSMT, Japan (NSMT-Me 44–46).
Etymology: The specific name *spencerense* is derived from the type-locality, Spencer Gulf.

Description (Figs. 10–11)

Diagnosis: Medium-sized dicyemid; body length reaching 4,420. Calotte conical, metapolar cells elongate in adults. Vermiform stages with 24–26 peripheral cells: 4 propolars + 5 metapolars + 2 parapolars + 13 to 15 trunk cells; 24 peripheral cells most common. Infusoriform embryos with 37 cells; refringent bodies solid; 1 nucleus present in each urn cell.

Nematogens (Figs. 10A, B, G, H, 11A, B) [Based on 42 specimens.] Body slender, 924–2,900 long (mean

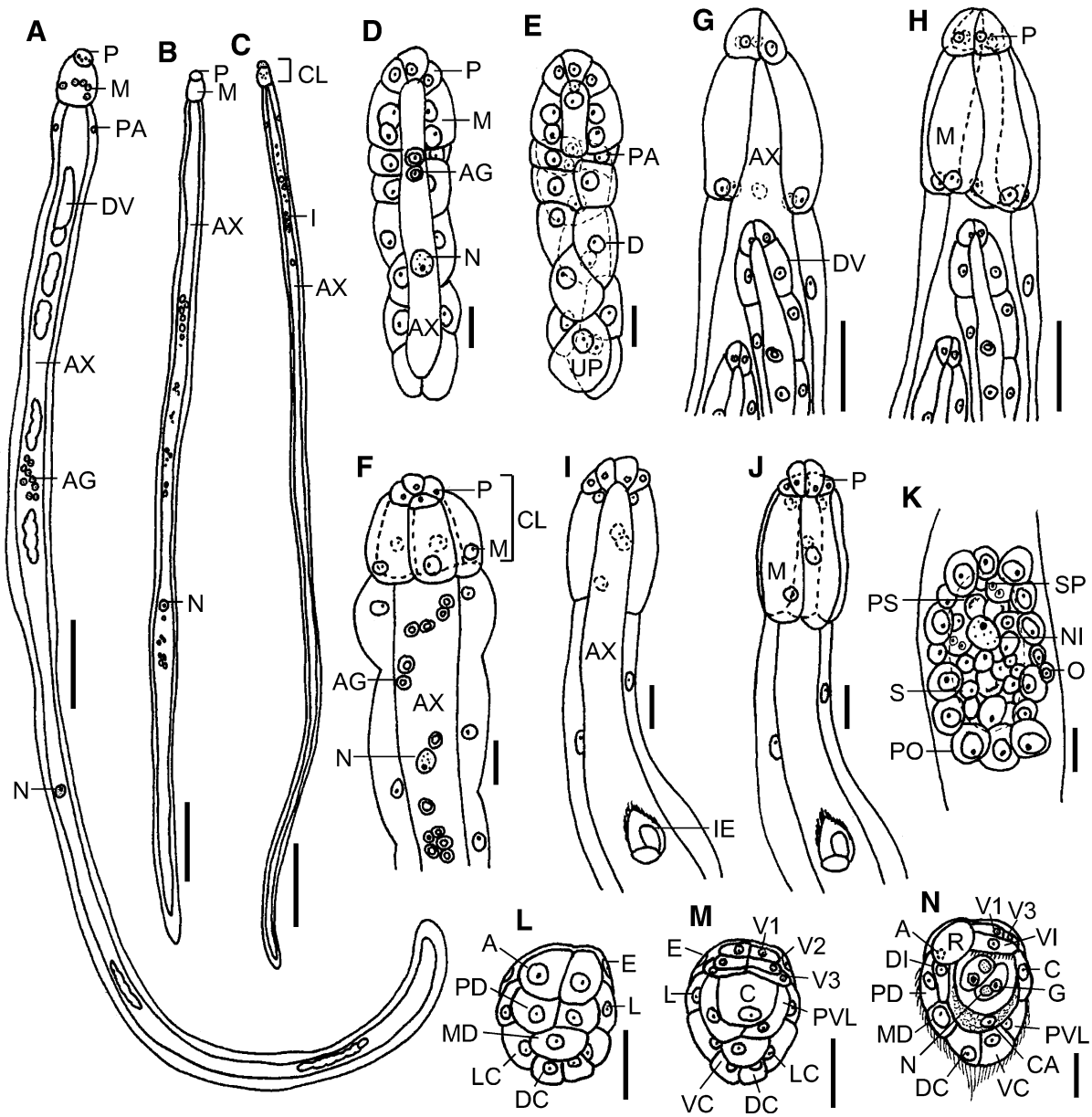

Fig. 10 Line drawings of *Dicyemennea spencerense* n. sp. from *Sepia novaehollandiae* and *S. apama*. A, B, nematogen, entire; C, rhombogen, entire; D, E, vermiform embryo (D, optical section; E, cilia omitted); F, anterior region of vermiform embryo; G, H, anterior region of nematogen; I, J, anterior region of rhombogen; K, infusorigen; L–N, infusoriform embryos within axial cell (L, dorsal view, cilia omitted; M, ventral view, cilia omitted; N, sagittal section). See Fig. 2 for abbreviations. *Scale-bars*: A, 100 μm; B, C, 200 μm; D, E, K–N, 10 μm; F, I, J, 20 μm; G, H, 50 μm

1,656 ± 472, mode 1,904) and 32–84 wide (mean 51 ± 12, mode 48); trunk width mostly uniform (Figs. 10A, B, 11A). Peripheral cell number 24 to 25 (Table 6): 4 propolars + 5 metapolars + 2 parapolars + 11 to 12 diapolars cells + 2 uropolars; 24 peripheral cells most common. Calotte conical, metapolar cells elongate (Figs. 10G, H, 11B). Cilia on calotte approximately 5.6 long, orientated anteriorly. Propolar cells and their nuclei much smaller than metapolar cells and their nuclei, respectively (Figs. 10A, G, H, 11B). Verruciform cells absent. Axial cell cylindrical, rounded anteriorly, extends

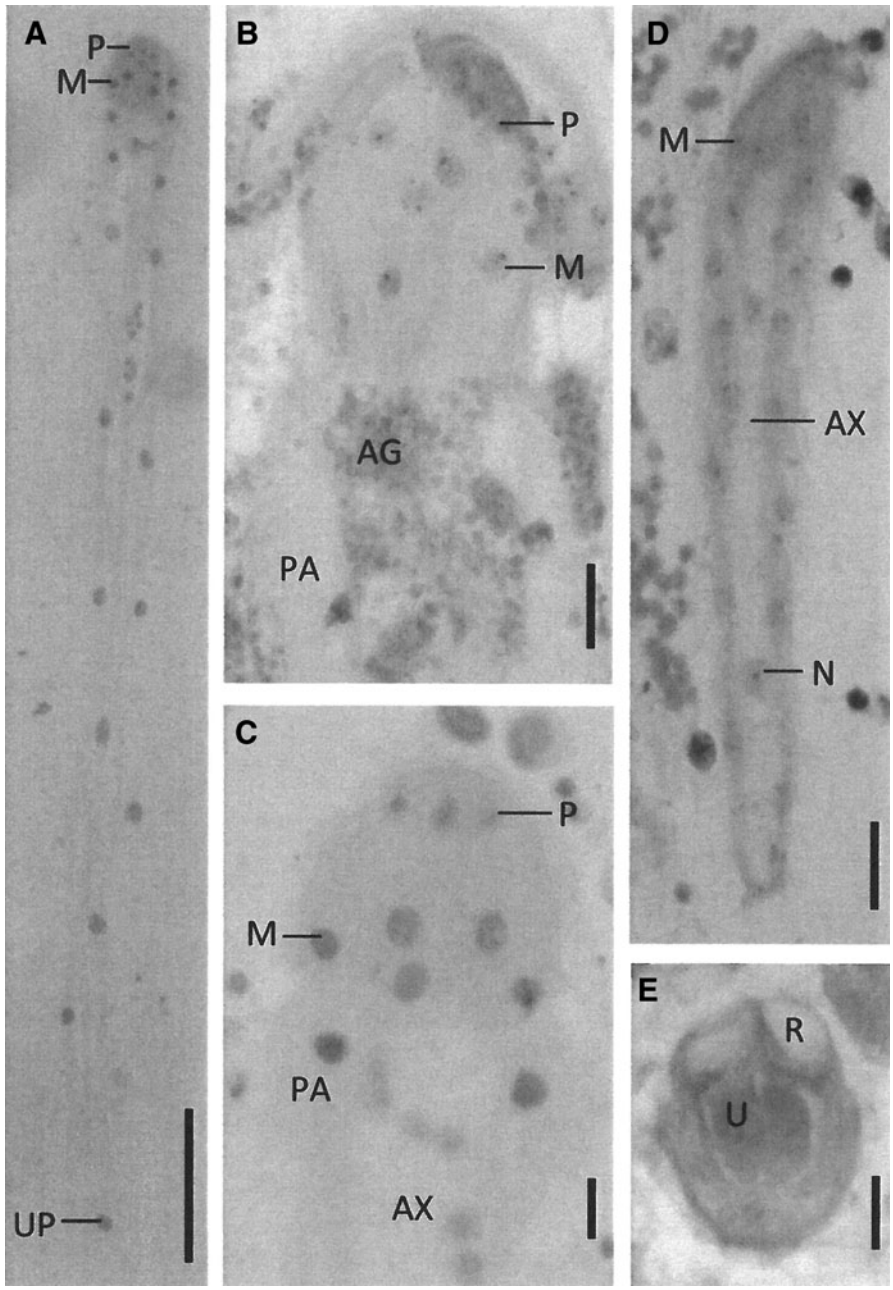

Fig. 11 Light micrographs of *D. spencerense* n. sp. from *S. novaehollandiae* and *S. apama*. A, nematogen, entire; B, anterior region of nematogen; C, anterior region of young vermiform embryo; D, vermiform embryo; E, infusoriform embryos within axial cell, horizontal section. See Figs. 2 and 3 for abbreviations. *Scale-bars*: A, 100 μm; B–E, 10 μm

forward to base of propolar cells; posterior extent of axial cell through uropolar cells (Fig. 10A, B, G). Average of 4 vermiform embryos present in axial cell of nematogens, with larger individuals having as many as 11 vermiform embryos in axial cell. Agametes present and numerous in axial cell, with average diameter 7.8 ± 1.1 (mode 8).

Vermiform embryos (Figs. 10D–F, 11C, D) [Based on 42 specimens.] Full-grown vermiform embryos

Table 6 Number of peripheral cells in *Dicyemennea spencerense* n. sp. infecting *Sepia novaehollandiae* and *S. apama*

Cell no.	No. of individuals		
	Vermiform embryos	Nematogens	Rhombogens
24	46	13	4
25	8	1	0
26	3	0	0

120–520 long (mean 301 ± 98, mode 352) and 24–50 wide (mean 37 ± 6, mode 40). Peripheral cell number 24 to 26 (Table 6): 4 propolars + 5 metapolars + 2 parapolars + 11 to 13 diapolars cells + 2 uropolars; 24 peripheral cells most common. Anterior end of calotte rounded; metapolar cells elongate, larger than propolar cells; metapolar nuclei larger than propolar nuclei (Figs. 10D–F, 11C, D). Axial cell rounded anteriorly; extends forward to base of propolar cells (Figs. 10D, 11C). Anterior abortive axial cell absent. Axial cell of full-grown embryos with as many as 10 agametes.

Rhombogens (Fig. 10C, I, J) [Based on 42 specimens.] Body slender, length longer than nematogens, otherwise similar in width and shape; length 1,496–4,420 (mean 2,640 ± 704, mode 2,000), width 39–104 (mean 62 ± 17, mode 56) (Fig. 10C). Peripheral cell number 24 (Table 6). Calotte conical, metapolar cells elongate, much larger than propolar cells and their nuclei (Fig. 10I, J). Axial cell shape and anterior extent similar to nematogen (Fig. 10I). Verruciform cells absent. Average of 12 infusoriform embryos present in axial cell of rhombogens, with smaller individuals having only 1 and larger individuals having as many as 28. Usually 1 or 2, rarely 3 or 4 infusorigens present in axial cell of each parent individual.

Infusorigens (Fig. 10K) [Based on 42 specimens.] Mature infusorigens medium-sized; axial cell of infusoriforms oval in shape, 16–87 in diameter (mean 41.5 ± 17). Composed of 10–31 (mode 14) external cells (egg line - oogonia and primary oocytes) + 10–31 (mode 19) internal cells (sperm line - spermatogonia, primary spermatocytes and secondary spermatocytes) + 3–9 (mode 6) sperm (Fig. 10K). Mean diameter of fertilised eggs 10.9 ± 1.2; mean sperm diameter 1.3 ± 0.2.

Infusoriform embryos (Figs. 10L–N, 11E) [Based on 146 specimens.] Full-grown embryos medium-sized (mean length excluding cilia 31.8 ± 4); length : width : depth ratio 1.00 : 0.81 : 0.93. Shape ovoid, some individuals pointed posteriorly, most rounded (Figs. 10L–N, 11E). Cilia at posterior end 7.2 long. Refringent bodies present, solid and small; occupy 38% of embryo length when viewed laterally (Fig. 10N). Cilia projecting from ventral internal cells to urn cavity (Fig. 10N). Capsule cells contain many small granules (Fig. 10N). Mature infusoriform embryos consisting of 37 cells: 33 somatic + 4 germinal cells. Somatic cells of several types present: external cells cover large part of anterior and lateral surfaces of embryo (2 enveloping cells); external cells with cilia on external surface (2 paired dorsal cells + 1 median dorsal cell + 2 dorsal caudal cells + 2 lateral caudal cells + 1 ventral caudal cell + 2 lateral cells + 2 posteroventral lateral cells); external cells with refringent bodies (2 apical cells); external cells without cilia (1 couvercle cell + 2 first ventral cells + 2 second ventral cells + 2 third ventral cells); internal cells with cilia (2 ventral internal cells); and internal cells without cilia (2 dorsal internal cells + 2 capsule cells + 4 urn cells). Each urn cell contains 1 germinal cell and 1 nucleus (Fig. 10N). All somatic nuclei appear pycnotic in mature infusoriform embryos.

Remarks

Dicyemennea presently contains 41 species (see Catalano, 2012, 2013), of which 18 show partial overlap with a peripheral cell count range similar to that described for *D. spencerense* n. sp. (24–26). However, out of these 18 species, only *D. brevicephaloides* Bogolepova-Dobrokhotova, 1962, *D. dorycephalum* Furuya & Hochberg, 2002, *D. floscephalum* Catalano, 2013, and *D. minabense* Furuya, 1999 share a similar body length of *c*.4,500 with *D. spencerense* (see Bogolepova-Dobrokhotova, 1962; Furuya, 1999; Furuya & Hochberg, 2002; Catalano, 2013).

Dicyemennea spencerense n. sp. can be distinguished from *D. brevicephaloides*, *D. dorycephalum* and *D. floscephalum* by calotte shape (conical and elongate in *D. spencerense vs* disc-shaped in *D. brevicephaloides* and *D. floscephalum*, and pointed in *D. dorycephalum*) (Bogolepova-Dobrokhotova, 1962; Furuya & Hochberg, 2002; Catalano, 2013). *Dicyemennea spencerense* differs further from *D. floscephalum* by smaller refringent bodies of the infusoriform

embryos (Catalano, 2013) and from *D. dorycephalum* by the absence of an anterior abortive cell in the vermiform embryos, the greater number of external cells and internal cells in the infusorigens and the spherical-shaped nucleus in apical cells, lateral cells and paired dorsal cells of the infusoriform embryos (Furuya & Hochberg, 2002).

Dicyemennea minabense is similar to *D. spencerense* with a conical calotte, ovoid-shaped axial cell in infusorigens and 37 cells in infusoriform embryos. However, it can be distinguished from *D. spencerense* by the shape and length of the metapolar cells in the calotte of the vermiform stages, the larger refringent bodies of the infusoriform embryos (occupying 60% of embryo length when viewed laterally *vs* 38%), and the number of nuclei in urn cells (2 *vs* 1).

Sepia novaehollandiae and *S. apama* individuals collected from Spencer Gulf, South Australia were also infected by *Dicyema koinonum*. Co-occurrence of both *D. koinonum* and *Dicyemennea spencerense* in one host individual was observed in three *S. novaehollandiae* and in 13 *S. apama*.

Comments on dicyemid fauna composition, infection rates and morphological variations

In this study, 47 out of 51 *S. apama* (overall infection rate 92%) were found to be infected by at least one dicyemid species (Table 1). A total of four dicyemid species were recorded from *S. apama*, however these were never observed together in the same host individual or from the same locality (Table 1). The individuals of *S. apama* collected from CB were exclusively infected by *Dicyema coffinense* n. sp. (infection rate 73%), those from SG were infected by *D. koinonum* n. sp. (infection rate 96%) and *Dicyemennea spencerense* n. sp. (infection rate 54%), the individuals from GSV were exclusively infected by *Dicyema koinonum* n. sp. (infection rate 100%) and those from NSW were exclusively infected by *D. multimegalum* n. sp. (infection rate 74%) (Table 1). Within SG and GSV, the fauna composition was not dependent on collection region (upper, middle and lower). Rather, *D. koinonum* and *Dicyemennea spencerense* were found throughout SG, and *Dicyema koinonum* was found throughout GSV (Table 1). High infection rates by each dicyemid species at each locality lend support to locality trends rather than sample bias trends. Although the specimens of *S. apama* collected from NSW were housed in tanks for 2–3 months prior to examination, infection by dicyemid parasites was still observed in three out of four host individuals.

All 27 *S. novaehollandiae* individuals were found to be infected (infection rate 100%), with a total of three dicyemid species recorded from this cuttlefish species (Table 1). Similarly to infections in *S. apama*, all three dicyemid species did not co-occur together in the same host individual or from the same locality. The specimens of *S. novaehollandiae* collected from SG were infected by *D. koinonum* (infection rate 36%) and *Dicyemennea spencerense* (infection rate 91%), whereas those from GSV were infected by *Dicyema koinonum* (infection rate 19%) and *D. vincentense* n. sp. (infection rate 81%) (Table 1). As for *S. apama*, dicyemid species were not restricted to certain regions within SG, rather *D. koinonum* and *Dicyemennea spencerense* were recorded in both regions sampled, USG and MSG. The high infection rates for *D. spencerense*, found exclusively in SG, and for *Dicyema vincentense*, found exclusively in GSV, support locality trends rather than sample bias trends (Table 1).

Calotte shape varied significantly among the five new dicyemid species, with differences in the length of the metapolar cells (elongate *vs* compact and compressed), the size of the nuclei (small *vs* large) and the shape of the anterior extremity (pointed *vs* rounded). Within each dicyemid species, calotte shape remained uniform.

Discussion

Dicyemid life-cycle

The dicyemid life-cycle is complex, with two stages of development and two modes of reproduction (see McConnaughey, 1951; Furuya et al., 2003b; Furuya et al., 2007 for a review). Asexual reproduction aids colonisation of the renal appendage leading to population density increase, whereas sexual reproduction ensures dissemination and continuation of the parasite life-cycle as the dispersive infusoriform embryo is released into the environment to find and infect a new host individual (Furuya et al., 2003b). The exact

mechanism for the switch between population density increase within the renal appendage (nematogen phase) to dispersal strategies (rhombogen phase) is unclear, although Lapan & Morowitz (1972) state that the production of the dispersive infusoriform embryo is the parasite's way of coping with a population explosion and at the same time ensuring that its species will survive beyond the eventual death of the host.

In this study, three out of four individuals of *S. apama* collected from NSW and examined 2–3 months after initial capture, were still found to be infected by a single dicyemid species, *Dicyema multimegalum*, which provides an indication of the generation time of dicyemids in their host, although this is likely species-specific and influenced by host characteristics such as size and behaviour. As a key requirement of the dicyemid life-cycle is to ensure survival beyond the death of its cephalopod host, it was surprising to observe all dicyemid life-cycle stages in the three captive infected cuttlefish, especially as all three individuals had recently mated and were rapidly deteriorating in health before they eventually died. Additionally, in the left renal appendage of one of these host individuals, only nematogens with large numbers of agametes in their axial cells were observed, with no rhombogens or infusorigens present in all 12 prepared smears. This indicates that dicyemids may persist and continue replicating even after host death, although it is unclear for how long they can continue to remain viable. Furthermore, although this host had died, an immediate priority was not to disperse; focus was still on increasing density within the renal appendage as indicated by the exclusive occurrence of nematogens. If chemical cues had been released by the dying host that signalled its deteriorating health, it can be assumed they were not detected or responded to by the dicyemid parasite. Other factors, such as nutrients, space and renal appendage size may therefore be responsible for the switch between nematogens and increasing density, to rhombogens and escape from the host, with this mechanism most likely being parasite-driven. As the three host individuals examined were large (38–49.5 cm mantle length; Table 1), maximum dicyemid density may not have been reached in the renal appendages over the elapsed time period of 2–3 months prior to examination. Hence if a smaller individual was examined after being maintained in tanks for the same time period, rhombogens may exclusively be found, or the host may be uninfected as the switch to the dispersal stage occurred earlier on and all infusoriform embryos had since escaped. Further investigation of these cues and mechanisms is required.

Host size and calotte morphology: dicyemid species co-occurrence patterns

A common notion in the literature for marine parasites in general is that larger host individuals are more likely to be infected by a greater number of parasite species compared to smaller host individuals, as they provide more space and nutrients with less competition (Kearn, 1967; Ho, 1991; Guegan et al., 1992; Poulin, 1997). For cephalopods, histological studies have shown that the complexity of the external surface of the renal appendage can be correlated with host size; small individuals tend to have simple external surfaces, whereas large individuals tend to have complex external surfaces (Furuya et al., 2004). This can allow for a greater availability of attachment sites and infection by a larger number of species in larger host individuals compared with smaller host individuals. Therefore, although three and four dicyemid species were recorded from *S. novaehollandiae* and *S. apama* respectively, small host size, which correlates with simple renal surface complexity and fewer attachment sites, may be the factor limiting the occurrence of dicyemid species in a single host individual.

The individuals of *S. apama* sampled in this study ranged in size from 9.5–49.5 cm (mantle length; Table 1), mantle length of 52 cm being the maximum recorded size for this species in the literature (Hall & Hanlon, 2002). In contrast to the common notion, the largest individuals of *S. apama* (i.e. mantle length 38–49.5 cm in this study) were only infected by a single dicyemid species, *D. multimegalum*, whereas smaller host individuals (i.e. mantle length 10.0–21.5 cm) were infected by two dicyemid species, *D. koinonum* and *Dicyemennea spencerense*. Therefore, although large individuals were examined, infection by a greater number of dicyemid species was not observed in *S. apama*. The individuals of *S. novaehollandiae* sampled in this study ranged in size from 5.5–12 cm (mantle length; Table 1), which extends the maximum size recorded for this species (see Reid et al., 2005; size previously recorded up to

7.7 cm mantle length). In general, larger individuals tended to be infected by more dicyemid species than small individuals. However this pattern may be due to sample size bias, as more large individuals were examined than small individuals.

Calotte shape is another factor that may explain co-occurrence patterns, provided that nutrients in the renal appendages are not limited. Furuya et al. (2003a) showed that co-occurrence is more likely for dicyemid species with distinctly different calottes compared to those that have similarly shaped calottes. This is because calotte shape dictates which surface of the renal appendage dicyemids can attach to, so if two or more species have differently shaped calottes, they can occupy different niches on the surface of the renal appendage and subsequently co-occur. The four dicyemid species that infect *S. apama*, i.e. *Dicyema coffinense*, *D. koinonum*, *D. multimegalum* and *Dicyemennea spencerense*, all have differently shaped calottes (see Figs. 2D, 4E, 6G, 10G), yet these species were not all found together in a single host individual. The same is true for the three dicyemid species that infect *S. novaehollandiae*, i.e. *Dicyema koinonum*, *D. vincentense* and *Dicyemennea spencerense* (see Figs. 4E, 8D, 10G) since co-occurrence of all three species in a single host individual was not recorded in this study. Therefore other factors, such as host behaviour and locality, rather than host size and calotte shape, may restrict co-occurrence of multiple dicyemid species in a single host individual.

Geographic patterns in dicyemid fauna composition: identifying host species complexes

Parasites are an important tool in studies of fish ecology and biology (Gonzalez et al., 2003). If parasites are treated as phenotypic characteristics of their hosts, they may be used as phenotype markers, providing a reliable guide to understanding the biology of their hosts (Pascual & Hochberg, 1996). As dicyemid parasites are highly host-species specific, differences in the composition of the parasite fauna within a host species may be an indication of the presence of a host species complex. The dicyemid fauna associated with *S. apama* and *S. novaehollandiae* varied with host collection locality, which suggests that there may be different subsets, populations or species within both cuttlefish in the southern Australian waters. Kassahn et al. (2003) used morphological and molecular techniques to examine the population structure of 173 individuals of *S. apama* collected from 19 localities across the species geographical range. Allozyme data identified three genetic groups which conformed to three spatially separated localities, WA, SA/Victoria and NSW, with further support for the separation of the SA/Victoria and NSW groups provided from microsatellite and morphometric data. These results, taken in combination with my findings of distinct parasite faunas at different host localities, provide strong support to the hypothesis that *S. apama* in southern Australian waters comprises a species complex. No studies have used morphological and molecular techniques to examine the population structure of *S. novaehollandiae* throughout its geographical range, although the results presented here suggest that two populations may occur in SA waters, one in SG and one in GSV.

Other cephalopod species have also been documented to be infected by a range of dicyemid species at various collection localities. For example, nine dicyemid species have been recorded from the stubby squid, *Rossia pacifica* Berry, collected from the Sea of Japan, Sea of Okhotsk and off the north-west coast of the USA (North Pacific Ocean) (see Catalano, 2012). Additionally, 11 dicyemid species have been recorded from the common octopus, *Octopus vulgaris* Cuvier, collected from the western North Pacific Ocean, western, north-eastern and north-southern North Atlantic Ocean and western Mediterranean Sea (see Furuya et al., 1992; Catalano, 2012). Such a high number of dicyemid species in a single host species is unusual and Hochberg (1990) suggested that *R. pacifica* and *O. vulgaris* represent a complex of host species. Within *Sepia* Linnaeus, the golden cuttlefish, *Sepia esculenta* Hoyle, is infected by the largest number of dicyemid species. Five species have been reported from three host collection localities in Japanese waters (Kii Strait off Minabe, Osaka Bay off Akashi and western Honshu), although all five dicyemid species were never found together in one individual or from the same locality (Furuya, 1999). This cuttlefish species is also found in the East and South China Seas to the Philippine Islands (Reid et al., 2005), although host species from these localities have not been examined for dicyemid parasites. Based on the species-complex observations for *R. pacifica* and *O. vulgaris* inferred from their dicyemid faunas, further research investigating the dicyemid species associated with *S. esculenta* throughout its geographical range would be beneficial.

Dicyemids as biological tags to assess cephalopod population structure: the way forward with molecular analyses

To resolve complex cephalopod taxonomy problems involving cryptic or sibling species, clarification may be achieved by a critical examination of their dicyemid parasite faunas (Hochberg, 1990). For example, the study by Pickford & McConnaughey (1949) showed that two cryptic octopus species off the Pacific coast of North America could be identified more readily by their associated dicyemid parasite fauna rather than host characters. Additionally, the results from the present study, showing a correlation of the composition of the dicyemid fauna with host collection locality, provides support for the existence of distinct populations of *S. apama* and *S. novaehollandiae* in southern Australian waters. However, I believe molecular analyses are needed in combination with classical morphological analyses of the dicyemid parasites to resolve the complex problems of cephalopod taxonomy. New molecular techniques and methods are readily available at decreasing costs with increasing throughput capacity. By examining genetic differences between dicyemid species within a cephalopod host species, a greater insight into the population structure of the host may be gained. For example, trematode parasite genetic studies have been used to infer steelhead trout host populations, whereby the odds of correct assignment to a trout population were four times greater using parasite genotypes than using host genotypes (Criscione et al., 2006). Additionally, previously undetected dicyemid species from morphological smear examinations may be found using molecular methods, therefore increasing dicyemid species diversity. Molecular genetics methods could also be used to test dicyemid species boundaries, shed light on the confusion surrounding the validity of certain genera and species within the Dicyemida, and address the unknowns in the dicyemid life-cycle and the position of the phylum in the Tree of Life.

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References

Aruga, J., Odaka, Y. S., Kamiya, A., & Furuya, H. (2007). *Dicyemid* Pax6 and Zic: tool-kit genes in a highly simplified bilaterian. *BMC Evolutionary Biology, 7*, 1–16.

Bogolepova-Dobrokhotova, I. I. (1962). Dicyemidae of far-eastern seas II. New species of the genus *Dicyemennea*. *Zoologicheskii Zhurnal, 41*, 503–518.

Catalano, S. R. (2012). A review of the families, genera and species of Dicyemida Van Beneden, 1876. *Zootaxa, 3479*, 1–32.

Catalano, S. R. (2013). First descriptions of dicyemid mesozoans (Dicyemida: Dicyemidae) from Australian octopus (Octopodidae) and cuttlefish (Sepiidae) species, including a new record of *Dicyemennea* in Australian waters. *Folia Parasitologica*, *60*, 306–320.

Catalano, S. R., & Furuya, H. (2013). Two new species of dicyemid (Dicyemida: Dicyemidae) from two Australian cephalopod species: *Sepioteuthis australis* (Mollusca: Cephalopoda: Loliginidae) and *Sepioloidea lineolata* (Mollusca: Cephalopoda: Sepiadariidae). *Journal of Parasitology, 99*, 203–211.

Criscione, C. D., Cooper, B., & Blouin, M. S. (2006). Parasite genotypes identify source populations of migratory fish more accurately than fish genotypes. *Ecology, 87*, 823–828.

Finn, J. K., Hochberg, F. G., & Norman, M. D. (2005). Phylum Dicyemida in Australian waters: First record and distribution across diverse cephalopod hosts. *Phuket Marine Biology Research Center Bulletin, 66*, 83–96.

Furuya, H. (1999). Fourteen new species of dicyemid Mesozoans from six Japanese cephalopods, with comments on host specificity. *Species Diversity, 4*, 257–319.

Furuya, H. (2008). A new dicyemid from *Sepiella japonica* (Mollusca: Cephalopoda: Decapoda). *Journal of Parasitology, 94*, 223–229.

Furuya, H. (2009). Two new dicyemid from *Sepia longipes* (Mollusca: Cephalopoda: Decapoda). *Journal of Parasitology, 95*, 681–689.

Furuya, H. (2010). A new dicyemid from *Benthoctopus sibiricus* (Mollusca: Cephalopoda: Octopoda). *Journal of Parasitology, 96*, 1,123–1,127.

Furuya, H., & Hochberg, F. G. (1998). Three new species of *Dicyema* (Phylum Dicyemida) from cephalopods in the western Mediterranean. *Vie et Milieu, 49*, 117–128.

Furuya, H., & Hochberg, F. G. (2002). New species of *Dicyemennea* (Phylum: Dicyemida) in deep-water *Graneledone* (Mollusca: Cephalopoda: Octopoda) from the Antarctic. *Journal of Parasitology, 88*, 326–330.

Furuya, H., & Tsuneki, K. (2005). A new species of dicyemid mesozoan (Dicyemida: Dicyemidae) from *Sepioteuthis lessoniana* (Mollusca: Cephalopoda), with notes on *Dicyema orientale*. *Species Diversity, 10*, 45–62.

Furuya, H., Tsuneki, K., & Koshida, Y. (1992). Two new species of the genus *Dicyema* (Mesozoa) from octopuses of Japan with notes on *D. misakiense* and *D. acuticephalum*. *Zoological Science, 9*, 423–437.

Furuya, H., Hochberg, F. G., & Tsuneki, K. (2003a). Calotte morphology in the phylum Dicyemida: Niche separation and convergence. *Journal of Zoology, 259*, 361–373.

Furuya, H., Hochberg, F. G., & Tsuneki, K. (2003b). Reproductive traits in dicyemids. *Marine Biology, 142*, 693–706.

Furuya, H., Hochberg, F. G., & Tsuneki, K. (2007). Cell number and cellular composition in vermiform larvae of dicyemid mesozoans. *Journal of Zoology, 272*, 284–298.

Furuya, H., Ota, M., Kimura, R., & Tsuneki, K. (2004). Renal organs of cephalopods: A habitat for dicyemids and chromidinids. *Journal of Morphology, 262*, 629–643.

Gonzalez, A. F., Pascual, S., Gestal, C., Abollo, E., & Guerra, A. (2003). What makes a cephalopod a suitable host for parasite? The case of Galician waters. *Fisheries Research, 60*, 177–183.

Guegan, J. F., Lambert, A., Leveque, C., Combes, C., & Euzet, L. (1992). Can host body size explain the parasite species richness in tropical freshwater fishes? *Oecologia, 90*, 197–204.

Hall, K. C., & Hanlon, R. T. (2002). Principal features of the mating system of a large spawning aggregation of the giant Australian cuttlefish *Sepia apama* (Mollusca: Cephalopoda). *Marine Biology, 140*, 533–545.

Ho, J. S. (1991). Two species of chondracanthid copepods parasitic on commercial fishes in the Pacific. *Publications of the Seto Marine Biological Laboratory, 31*, 1–10.

Hochberg, F. G. (1982). The "kidneys" of cephalopods: A unique habitat for parasites. *Malacologia, 23*, 121–134.

Hochberg, F. G. (1983). The parasites of cephalopods: A review. *Memoirs of the National Museum Victoria, 44*, 108–145.

Hochberg, F. G. (1990). Diseases caused by protists and mesozoans. In: Kinne, O. (Ed) *Diseases of Marine Animals, Volume III*. Hamburg, Germany: Biologische Anstalt Helgoland, pp. 47–202.

Kalavati, C., Narasimhamurti, C. C., & Suseela, T. (1984). Four new species of mesozoan parasites (Mesozoa: Dicyemidae) from cephalopods of Bay of Bengal. *Proceedings of the Indian Academy of Science (Animal Science), 93*, 639–654.

Kassahn, K. S., Donnellan, S. C., Fowler, A. J., Hall, K. C., Adams, M., & Shaw, P. W. (2003). Molecular and morphological analyses of the cuttlefish *Sepia apama* indicate a complex population structure. *Marine Biology, 143*, 947–962.

Katayama, T., Wada, H., Furuya, H., Satoh, N., & Yamamoto, M. (1995). Phylogenetic position of the dicyemid Mesozoa inferred from 18S rDNA sequences. *Biological Bulletin, 189*, 81–90.

Kearn, G. C. (1967). Experiments on host-finding and host-specificity in the monogenean skin parasite *Entobdella soleae*. *Parasitology, 57*, 585–605.

Kobayashi, M., Furuya, H., & Holland, P. W. H. (1999). Dicyemids are higher animals. *Nature, 401*, 762.

Kobayashi, M., Furuya, H., & Wada, H. (2009). Molecular markers comparing the extremely simple body plan of dicyemids to that of lophotrochozoans: insight from the expression patterns of *Hox*, *Otx*, and *brachyury*. *Evolution and Development, 11*, 582–589.

Lapan, E. A., & Morowitz, H. J. (1972). The Mesozoa. *Scientific American, 227*, 94–101.

Lapan, E. A., & Morowitz, H. J. (1974). Characterization of mesozoan DNA. *Experimental Cell Research, 83*, 143–151.

McConnaughey, B. H. (1949a). *Dicyema sullivani*, a new mesozoan from lower California. *Journal of Parasitology, 35*, 122–124.

McConnaughey, B. H. (1949b). Mesozoa of the family Dicyemidae from California. *University of California Publications in Zoology, 2*, 1–33.

McConnaughey, B. H. (1951). The life cycle of the dicyemid mesozoa. *University of California Publications in Zoology, 55*, 295–336.

McConnaughey, B. H. (1960). The rhombogen phase of *Dicyema sullivani* McConnaughey (Mesozoa: Dicyemidae). *Journal of Parasitology, 46*, 608–610.

Norman, M., Finn, J., & Tregenza, T. (1999). Female impersonation as an alternate reproductive strategy in giant cuttlefish. *Proceedings of the Royal Society of London, 266*, 1,347–1,349.

Noto, T., & Endoh, H. (2004). A "chimera" theory on the origin of dicyemid mesozoans: Evolution driven by frequent lateral gene transfer from host to parasite. *BioSystems, 73*, 73–83.

Nouvel, H. (1948). Les dicyémides II. Infusoriforme, tératologie, spécificité du parasitisme, affinités. *Archives de Biologie, 59*, 147–224.

Ogino, K., Tsuneki, K., & Furuya, H. (2010). Unique genome of dicyemid mesozoan: Highly shortened spliceosomal introns in conservative exon/intron structure. *Gene, 449*, 70–76.

Ohama, T., Kumazaki, T., Hori, H., & Osawa, S. (1984). Evolution of multicellular animals as deduced from 5S rRNA sequences: A possible early emergence of the Mesozoa. *Nucleic Acids Research, 12*, 5,101–5,108.

Pascual, S., & Hochberg, F. G. (1996). Marine parasites as biological tags of cephalopod hosts. *Parasitology Today, 12*, 324–327.

Pawlowski, J., Montoya-Burgos, J., Fahrni, J. F., Wuest, J., & Zaninetti, L. (1996). Origin of the Mesozoa inferred from 18S rRNA gene sequences. *Molecular Biology and Evolution, 13*, 1128–1132.

Penchaszadeh, P. E. (1968). Diciemidos (Mesozoa) en cefalopodos de Argentina *Dicyema australis* sp. nov. parasito del pulpo *Octopus tehuelchus* D'Orb. *Neotropica, 14*, 127–131.

Pickford, G. E., & McConnaughey, B. H. (1949). The *Octopus bimaculatus* problem: A study in sibling species. *Bulletin*

of Bingham Oceanographic College, Peabody Museum of Natural History, Yale University, 12, 1–66.

Poulin, R. (1997). Species richness of parasite assemblages: Evolution and patterns. *Annual Review of Ecology and Systematics, 28*, 341–358.

Reid, A., Jereb, P., & Roper, C. F. E. (2005). Family Sepiidae. In: Jereb, P. & Roper, C. F. E. (Eds.), *Cephalopods of the world. An annotated and illustrated catalogue of species known to date. Volume 1. Chambered nautiluses and sepioids (Nautilidae, Sepiidae, Sepiolidae, Sepiadariidae, Idiosepiidae and Spirulidae)*. Rome: Food and Agriculture Organisation of the United Nations, pp. 57–152.

Short, R. B., & Damian, R. T. (1966). Morphology of the infusoriform larva of *Dicyema aegira* (Mesozoa: Dicyemidae). *Journal of Parasitology, 52*, 746–751.

Suzuki, T. G., Ogino, K., Tsuneki, K., & Furuya, H. (2010). Phylogenetic analysis of dicyemid mesozoans (Phylum Dicyemida) from innexin amino acid sequences: Dicyemids are not related to Platyhelminthes. *Journal of Parasitology, 96*, 614–625.

van Beneden, E. (1876). Recherches sur les Dicyemides, survivants actuels d'un embranchement des Mésozoaires. *Bulletins de l'Academis Royale Des Sciences et Belles Lettres de Bruxelles, 41*, 1160–1205.

von Kölliker, A. (1849). Ueber *Dicyema paradoxum*, den Schmarotzer der Venenanhänge der *Cephalopoden* [in German]. *Berichte von der Königlichen Zootomischen Anstalt zu Wurzburg*, 59–66.

Whitman, C. O. (1883). A contribution to the embryology, life-history and classification of the dicyemids. *Mittheilungen Zoologischen Station Zu Neapel, 4*, 1–90.

CHAPTER 6: Using the giant Australian cuttlefish (*Sepia apama*) mass breeding aggregation to explore the life cycle of dicyemid parasites

Filtered seawater sampling to detect dicyemid mesozoan parasites in the environment at the mass *Sepia apama* breeding aggregation site - Stony Point, False Bay, South Australia, Australia (photo: Kieran Brazell).

Chapter 6 Preamble

This chapter is a co-authored manuscript that has been published in *Acta Parasitologica*, which describes the use of the mass breeding aggregation of *Sepia apama* (giant Australian cuttlefish) to explore the life cycle of dicyemid parasites. In particular, host eggs and filtered seawater samples were collected from the mass breeding aggregation area to examine the unknown host life cycle stage whereby new infection by the dispersive dicyemid embryo occurs. This chapter can be cited as: Catalano, S.R., Whittington, I.D., Donnellan, S.C. and Gillanders, B.M., 2013. Using the giant Australian cuttlefish (*Sepia apama*) mass breeding aggregation to explore the life cycle of dicyemid parasites. *Acta Parasitologica* 58, 599–602.

In this chapter, all co-authors and I developed the experimental collection approach for the filtered seawater sampling. Bronwyn Gillanders assisted with the exemption permit application for cuttlefish eggs collections. I collected the samples from the mass breeding aggregation area (with assistance from volunteers), performed the extractions and molecular tests. Steve Donnellan and I analysed the molecular results. I wrote the manuscript and acted as corresponding author. All co-authors provided feedback on manuscript drafts.

I certify that the statement of contribution is accurate

Signed:_____ Date:_____

(Sarah Roseann Catalano)

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis

Professor Bronwyn Gillanders Professor Steve Donnellan

A/Professor Ian Whittington

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VERSITA

RESEARCH NOTE

Using the giant Australian cuttlefish (*Sepia apama*) mass breeding aggregation to explore the life cycle of dicyemid parasites

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Abstract

Dicyemid mesozoan parasites, microscopic organisms found with high intensities in the renal appendages of benthic cephalopods, have a complex, partially unknown life cycle. It is uncertain at which host life cycle stage (i.e. eggs, juvenile, adult) new infection by the dispersive infusoriform embryo occurs. As adult cephalopods have a short lifespan and die shortly after reproducing only once, and juveniles are fast-moving, we hypothesize that the eggs are the life cycle stage where new infection occurs. Eggs are abundant and sessile, allowing a huge number of new individuals to be infected with low energy costs, and they also provide dicyemids with the maximum amount of time for survival compared with infection of juvenile and adult stages. In our study we collected giant Australian cuttlefish (*Sepia apama*) eggs at different stages of development and filtered seawater samples from the *S. apama* mass breeding aggregation area in South Australia, Australia, and tested these samples for the presence of dicyemid DNA. We did not recover dicyemid parasite cytochrome *c* oxidase subunit I (*COI*) nucleotide sequences from any of the samples, suggesting eggs are not the stage where new infection occurs. To resolve this unknown in the dicyemid life cycle, we believe experimental infection is needed.

Keywords

Dicyemida, infusoriform embryo, cephalopod host, life cycle, dicyemid *COI* gene, *Sepia apama*

Benthic cephalopods, including the giant Australian cuttlefish, *Sepia apama*, are hosts to a group of small, worm-like parasites, known as dicyemid mesozoans (Dicyemida Van Beneden, 1876), which infect the renal appendages at high intensities (Furuya *et al.* 2004). Although 122 species of dicyemids have been described to date (Catalano 2012, 2013a, 2013b, Catalano and Furuya 2013), their life cycle remains partially unknown. It is uncertain at which host life cycle stage (i.e. eggs, juvenile, adult) new infection by the dispersive stage (infusoriform embryo) occurs. Furthermore, the route of infection this embryo takes from the environment to the renal appendages of a new host individual is unknown. Note that past experimental studies have shown there is no intermediate host and that the infusoriform embryo is released from adult host individuals into the environment (see Lapan and Morowitz 1975). For a detailed review of the life cycle of dicyemid mesozoans, including information on the two stages of development and two modes of reproduction, see McConnaughey (1951) and Furuya *et al*. (2003).

As the infusoriform embryo is microscopic with an average length of 32–36 µm, average width of 26–28 µm and average depth of 24–25 µm (McConnaughey 1951), we suggest the best chance of such a tiny embryo finding and infecting a new host individual is when there is a high host density, for example, when cephalopods aggregate to breed. However, infection of an adult individual during breeding may be unfavourable because cephalopods only live for one to two years and die shortly after reproducing (Semmens *et al.* 2007).

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Therefore another host life cycle stage that is also abundant during breeding and may provide a better chance of survival beyond the eventual death of an adult is the host's newly deposited eggs. Eggs are sessile, meaning less energy expenditure by the infusoriform embryo to find, attach to and infect this stage. Eggs also provide the longest time period for survival compared to infection of host juvenile and adult stages.

Sepia apama, the largest species of cuttlefish in the world, is widely known for its unique mass breeding phenomenon (Kassahn *et al.* 2003). Each year between April and July, *S. apama* congregate in their thousands on small shallow rocky reefs in Upper Spencer Gulf (USG), South Australia (SA), Australia, to spawn (Norman *et al.* 1999, Kassahn *et al.* 2003). The USG breeding aggregation is the densest cuttlefish spawning aggregation reported in the world, with up to 105 cuttlefish per 100 m^2 (Hall and Hanlon 2002), and subsequently represents an ideal location and opportunity to explore the dicyemid life cycle. Note that in the years 2005, 2010 and 2011, we collected *S. apama* individuals from USG and examined them for dicyemid parasites. All 32 *S. apama* individuals, with total lengths ranging from 16.5 cm to 31 cm, were found to be infected by dicyemid parasites (Catalano 2013b, Catalano *et al.* unpublished result).

Therefore to examine the hypothesis that eggs are the host life cycle stage where new infection occurs, we collected *S. apama* eggs at different stages of development from the mass breeding aggregation in USG, SA, Australia, as well as filtered seawater (SW) samples before, during and after cuttlefish arrived in the area and tested for the presence of dicyemid DNA.

Specifically, between 15 and 30 eggs were collected each month from the underside of flattened rocks from July to October 2011 at three localities in False Bay, USG, SA, following Cronin and Seymour (2000) (see Table I). One hundred litres of filtered SW were also collected from Stony Point (see Table I) on five occasions in 2011 and six occasions in 2012 corresponding to periods before, during and after cuttlefish breed. Ten circular test sieves of 20 cm diameter were stacked upon each other vertically in descending order from coarse to fine mesh sizes (4 mm, 2 mm, 500 µm, 250 µm, 125 µm, 68 µm, 53 µm, 38 µm, 30 µm and 20 µm). The SW was slowly poured through the sieve stack and samples were collected from the 53 µm, 38 µm, 30 µm and 20 µm mesh size sieves. Filtered SW was stored in a 50 mL Falcon tube to which 45 mL of 100% undenatured ethanol was added.

DNA was extracted from eggs and filtered SW. For the eggs, a total of 20 egg membranes, 20 inside yolk sacs (from newly deposited eggs with undeveloped larvae; July and August 2011 collections) and 30 miniature cuttlefish at different stages of development (from eggs collected in September and October 2011) were extracted following the Gentra Kit (Gentra Systems) protocol, with a final elution volume of 50 µl in TLE. Total lengths were recorded for each of the fully formed cuttlefish from which DNA was extracted for September and October 2011 (see Table II). Dissection equipment was cleaned and sterilized in ethanol for each new egg to avoid cross contamination. For the filtered SW samples, each tube was spun at 1000 g for 10 min at room temperature, braking speed of 2, in a low speed bucket centrifuge (Centrifuge 5810R, Rotor A-4-81, Eppendorf). Excess ethanol was removed with a Pasteur pipette, 5 mL of 10 mM Tris was added and tubes were inverted 20 times to re-suspend the pellet. The spin and Tris wash steps were repeated twice, and then the pellet was re-suspended in 1 mL 10 mM Tris before transfer to a labeled 1.5 mL eppendorf tube. All remaining extraction steps followed the Gentra Kit protocol, with a final elution volume of 50 µl in TLE.

Each extract diluted 1:100 was tested with two cytochrome *c* oxidase subunit I (*COI*) primer pairs that are known to amplify partial dicyemid parasite DNA (from *S. apama* kidney samples): M1425 5'-GTTTTTTGGACATCCTGAGGT-3' and reverse M1426 5'-AGGACATAGTGGAAGTGTGCTACAAC-3' from Watanabe *et al.* (1999) (400 bp fragment), and newly designed primer pair M1435 5'- GCCTTATTTTAGTACAGTGTGC-3' and reverse M1436 5'-CGAGTATCAATATCTATACCAGATG-3' (1,000 bp fragment). Amplification reactions were conducted in a final volume of 25 µl containing 2.5 µl of GeneAmp 10x PCR Buffer II (Applied Biosystems, Inc. [ABI]), 4 µl of 25 mM $MgCl_2$ (ABI), 2 µl of 10 mM dNTP, 1 µl of each primer at 5 mM, 0.10 µl of AmpliTaq® Gold (ABI), 0.10 µl of BSA at 200 ng/µl and 2.5 µl gDNA extract. Cycling conditions were 95°C for 10 min with 35–40 cycles of amplification (94°C for 45 s, 50–52°C for 45 s and 72°C for 1–2 min). Negative controls were included in

Table I. Collection and locality data for monthly *Sepia apama* egg sampling. Abbreviations: SA, South Australia; USG, Upper Spencer Gulf

Collection	Date collected	Locality	Latitude Longitude
1	12th July 2011	1.1 km east of Black Point, False Bay, USG, SA	32°59′ 36.7″S 137°43′ 56.4″E
2	11th August 2011	Stony Point, False Bay, USG, SA*	32°59′ 45.1″S 137°45′ 6.08″E
3	14th September 2011	Black Point, False Bay, USG, SA	32°59′ 28.73″S 137°43′ 14.38″E
4	12th October 2011	Black Point, False Bay, USG, SA	32°59′ 28.73″S 137°43′ 14.38″E

*Locality of filtered seawater collections

Table II. Total lengths (mm) for extractions of miniature fully formed *Sepia apama* from September and October 2011 egg samples. Abbreviation: BC, Baby Cuttlefish

14th September 2011 egg samples		
Number	**BC number**	**Total length**
1	BC7	9
2	BC8	10
3	BC9	11
4	BC10	9
5	BC11	8
6	BC12	9
7	BC13	14
8	BC14	12
9	BC15	6
10	BC16	9
11	BC17	10
12	BC18	7
13	BC19	13
14	BC20	5
15	BC21	6
12th October 2011 egg samples		
Number	**BC number**	**Total length**
1	BC1	28
2	BC2	26
3	BC3	28
4	BC4	25
5	BC5	22
6	BC6	20
7	BC22	17
8	BC23	19
9	BC24	16
10	BC25	20
11	BC26	18
12	BC27	23
13	BC28	10
14	BC29	10
15	BC30	21

each reaction. PCR products were visualized with UV following agarose gel (1.5%) electrophoresis and staining for 30 min with GelRed (Biotium). Products were cleaned using a Multi Screen Vacuum Manifold and 384-well Multi Screen Filter Plate (Millipore), then sequenced with the forward primer only using dye terminator chemistry (BigDye Terminator v3.1 cycle-sequencing kit, ABI). Sequence alignments, error correction and similarity analysis (neighbour-joining trees) were performed using Geneious Pro 5.3.4 (Drummond *et al.* 2010 – Biomatters Ltd).

All egg membranes, yolk sacs, miniature cuttlefish and filtered SW samples tested with dicyemid specific primers M1435/M1436 did not produce PCR products. Using dicyemid specific primers M1425/M1426, 26 samples were positive corresponding to 1 egg membrane (0/10 July 2011, 1/10 August 2011), 8 yolk sacs (0/10 July 2011, 8/10 August 2011), 12 miniature cuttlefish (0/15 September 2011, 12/15 October 2011) and 5 filtered SW samples (20 µm, 38 µm April 2012; 38 µm, 53 µm August 2012; 38 µm September 2012; the remaining 39 filtered SW samples were negative). The 353 bp of *COI* sequences for these 26 samples were identical (representative sequence deposited in GenBank™, accession no. JX983108. However, when compared to sequences on GenBank™ and those obtained by the authors from dicyemid-infected *S. apama* kidney samples (Catalano *et al.* unpublished results), these sequences were clearly highly divergent from dicyemid *COI*. Rather this short fragment was 86% homologous to *COI* of the soil bacterium, *Mycobacterium rhodesiae.*

With no amplification of dicyemid DNA from any of the collected samples, our finding tends to refute the hypothesis that new infection by the infusoriform embryo is at the egg life cycle stage. However, in the years we sampled (2011 and 2012), cuttlefish numbers were reduced in USG compared to previous years, indicating dicyemid density in the environment may also have been low and may explain our result of not detecting dicyemid DNA in filtered SW samples. If these parasites were transmitted vertically though, we would expect to detect dicyemid DNA in the egg samples irrespective of the reduction in numbers at the mass breeding site. Nonetheless, bacterium DNA was amplified and the observed result may simply be explained by saturation of bacteria in the sample compared to parasites, meaning the detection of dicyemid DNA has been masked and subsequently missed even though they are present. Clearly a more reliable method is needed to address the challenging unknowns that still surround the dicyemid life cycle. A potential infection route survey could be undertaken, with different host tissues tested using the same dicyemid specific primers in this study. However, we do not believe such a test would be informative or enlightening. Larger sized adults may not be continuously infected, but instead infected when quite small and not re-infected since. Therefore such a route survey may return negative results, when instead the wrong sized host is being sampled. To shed further light on this conundrum, the first requirement is to establish when infection occurs and at what host life cycle stage. Then this stage could be targeted to establish the unknown route.

Lapan and Morowitz (1975) exposed uninfected *Sepia*, raised from eggs in isolated aquaria, to infusoriform embryos and found small vermiforms in the renal coelom. Successful infections, however, were in the order of 10% compared to 100% in nature, and they explicitly stated that their observations 'are not intended to represent firm experimental evidence'. Nonetheless, based upon this observation, we believe future research should follow on from Lapan and Morowitz (1975) to complete the life cycle of dicyemid parasites. Experimental infection in tanks is required. Such an experiment would provide a meaningful assessment of both the host life cycle stage whereby new infection occurs as well as the entry

route, allowing the complete life cycle of this bizarre group of poorly understood parasites to be resolved.

Ethical Note

Over one spawning season, each female cuttlefish can lay hundreds of eggs. From a past study by Naud *et al.* (2004), conservative estimates of fecundity and female population size yielded a total egg production of approximately 9,500,000 eggs per annum for an estimated 28,000 females. This equates to approximately 339 eggs per female. Hence, the total maximum number of 120 eggs collected in this study represents a very small proportion of the total egg production (over 200 eggs less than what one female may lay based upon the conservative estimates above) and should have little, if any, effect on population viability.

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References

Catalano S.R. 2012. A review of the families, genera and species of Dicyemida Van Beneden, 1876. *Zootaxa*, 3479, 1–32. DOI: z03479p032f.

Catalano S.R. 2013a. First descriptions of dicyemid mesozoans (Dicyemida: Dicyemidae) from Australian octopus (Octopodidae) and cuttlefish (Sepiidae) species, including a new record of *Dicyemennea* in Australian waters. *Folia Parasitologica*, 60, 306–320.

Catalano S.R. 2013b. Five new species of dicyemid mesozoans (Dicyemida: Dicyemidae) from two Australian cuttlefish species, with comments on dicyemid fauna composition relative to host size, calotte shape and host collection locality. *Systematic Parasitology*, 86, 125–151. DOI: 10.1007/s11230-013-9443-6.

Catalano S.R., Furuya H. 2013. Two new species of dicyemid (Dicyemida: Dicyemidae) from two Australian cephalopod species: *Sepioteuthis australis* (Mollusca: Cephalopoda: Loliginidae) and *Sepioloidea lineolata* (Mollusca: Cephalopoda: Sepiadariidae). *Journal of Parasitology*, 99, 203–211. DOI: 10.1645/GE-3252.1.

Cronin E.R., Seymour R.S. 2000. Respiration of the eggs of the giant cuttlefish *Sepia apama*. *Marine Biology*, 136, 863–870. DOI: 10.1007/s002270000274.

Drummond A.J., Ashton B., Buxton S., Cheung M., Cooper A., Heled J., Kearse M., Moir R., Stones-Havas S., Sturrock S., Thierer T., Wilson A. 2010. Geneious v5.3, Available from http://www.geneious.com.

Furuya H., Hochberg F.G., Tsuneki K. 2003. Reproductive traits in dicyemids. *Marine Biology*, 142, 693–706. DOI: 10.1007/s00227-002-0991-6.

Furuya H., Ota M., Kimura R., Tsuneki K. 2004. Renal organs of cephalopods: A habitat for dicyemids and chromidinids. *Journal of Morphology*, 262, 629–643. DOI: 10.1002/jmor.10265.

Hall K.C., Hanlon R.T. 2002. Principal features of the mating system of a large spawning aggregation of the giant Australian cuttlefish *Sepia apama* (Mollusca: Cephalopoda). *Marine Biology*, 140, 533–545. DOI: 10.1007/s00227-001-0718-0.

Kassahn K.S., Donnellan S.C., Fowler A.J., Hall K.C., Adams M., Shaw P.W. 2003. Molecular and morphological analyses of the cuttlefish *Sepia apama* indicate a complex population structure. *Marine Biology*, 143, 947–962. DOI: 10.1007/s00227-003-1141-5.

Lapan E.A., Morowitz H.J. 1975. The dicyemid Mesozoa as an integrated system for morphogenetic studies. 1. Description, isolation and maintenance. *Journal of Experimental Zoology*, 193, 147–160. DOI: 10.1002/jez.1401930204.

McConnaughey B.H. 1951. The life cycle of the dicyemid Mesozoa. *University of California Press*, *Berkeley and Los Angeles*, 55, 295–336.

Naud M., Hanlon R.T., Hall K.C., Shaw P.W., Havenhand J.N. 2004. Behavioural and genetic assessment of reproductive success in a spawning aggregation of the Australian giant cuttlefish, *Sepia apama*. *Animal Behaviour*, 67, 1043–1050. DOI: 10.1016/j.anbehav.2003.10.005.

Norman M.D., Finn J., Tregenza T. 1999. Female impersonation as an alternate reproductive strategy in giant cuttlefish. *Proceedings of the Royal Society of London*, 266, 1347–1349. DOI: 51624.

Semmens J.M., Pecl G.T., Gillanders B.M., Waluda C.M., Shea E.K., Jouffre D., Ichii T., Zumholz K., Katugin O.N., Leporati S.C., Shaw P.W. 2007. Approaches to resolving cephalopod movement and migration patterns. *Reviews in Fish Biology and Fisheries*, 17, 401–423. DOI: 10.1007/s11160-007-9048-8.

Watanabe K.I., Bessho Y., Kawasaki M., Hori, H. 1999. Mitochondrial genes are found on minicircle DNA molecules in the mesozoan animal *Dicyema*. *Journal of Molecular Biology*, 286, 645–650. DOI: 10.1006/jmbi.1998.2523.

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CHAPTER 7: Dicyemid fauna composition and infection patterns in relation to cephalopod host biology and ecology: exploring dicyemids as natural tags

Returning to land following a six night research trawl collecting *Sepia papuensis* samples in Shark Bay, Western Australia, Australia.

Chapter 7 Preamble

This chapter is a manuscript that is currently under peer-review in *Folia Parasitologica*, with Ian Whittington, Steve Donnellan and Bronwyn Gillanders as co-authors. It explores dicyemid parasite patterns of infection and fauna composition in relation to cephalopod host biology and ecology. This chapter can be cited as: Catalano, S.R., Whittington, I.D., Donnellan, S.C. and Gillanders, B.M., In Review. Dicyemid fauna composition and infection patterns in relation to cephalopod host biology and ecology: exploring dicyemid parasites as natural tags. *Folia Parasitologica*.

In this chapter, all co-authors and I assisted with the study design and planning for host and parasite collections. I collected the cephalopod samples, performed dissections and parasite preparations, wrote the manuscript and acted as corresponding author. Bronwyn Gillanders and I performed the statistical tests. All co-authors provided feedback on manuscript drafts.

I certify that the statement of contribution is accurate

Signed:_____ Date:_____

(Sarah Roseann Catalano)

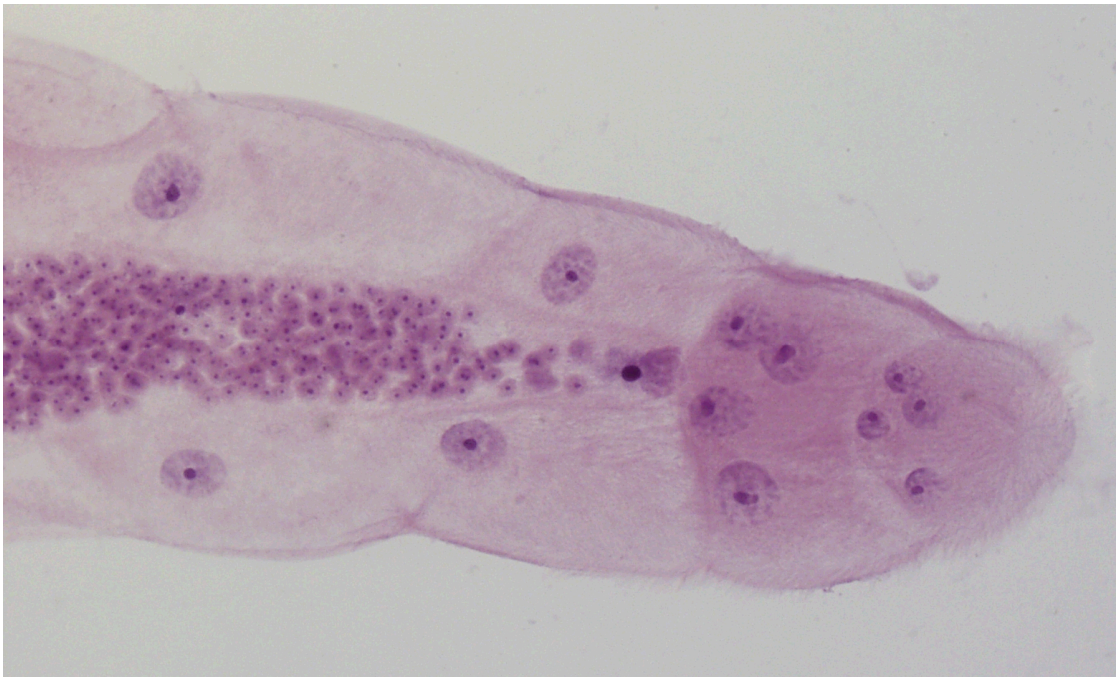
I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis

Professor Bronwyn Gillanders Professor Steve Donnellan

A/Professor Ian Whittington

Catalano, S.R., Whhittington, I.D., Donnellan, S.C. & Gillanders, B.M. (in review) Relationship marketing and university-industry linkages: a conceptual framework.
Folia Parasitologica.

CHAPTER 8: First insight into the phylogenetic relationship of dicyemid parasites: is classification based on morphological traits in need of revision?



Close-up of the anterior attachment region, termed ‘calotte’, of a dicyemid parasite. The calotte is inserted into the convoluted surface of the host’s renal appendage while the rest of the parasites body hangs free in the urine acquiring nutrients.

Chapter 8 Preamble

This chapter is a manuscript that is currently under peer-review in *Molecular Phylogenetics and Evolution*, with Terry Bertozzi, Ian Whittington, Steve Donnellan and Bronwyn Gillanders as co-authors. It presents the first phylogeny of dicyemid parasites, exploring the classification of these organisms outside of the classical morphological approach. This chapter can be cited as: Catalano, S.R., Whittington, I.D., Donnellan, S.C., Bertozzi, T. and Gillanders, B.M., In Review. First insight into the phylogenetic relationship of dicyemid parasites: is classification based on morphological traits in need of revision? *Molecular Phylogenetics and Evolution*.

In this chapter, all co-authors and I assisted with the molecular design and planning. Terry Bertozzi and I designed and tested the primers. I performed the molecular experiments and the results were analysed by Terry Bertozzi, Steve Donnellan and myself. I wrote the manuscript and acted as corresponding author. All co-authors provided feedback on manuscript drafts.

I certify that the statement of contribution is accurate

Signed:_____ Date:_____

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First insight into the phylogenetic relationship of dicyemid parasites: is classification based on morphological traits in need of revision?

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Molecular Phylogenetics and Evolution (In Review)

ABSTRACT

Dicyemid mesozoans are a little-known and poorly-understood group of marine organisms that parasitise the renal appendages of benthic cephalopods with high intensities. Phylogenetic studies are lacking for these organisms and their classification into distinct taxonomic groups is based purely on morphological traits. Here we characterised the complete cytochrome *c* oxidase subunit I (*COI*) minicircle molecule, containing the *COI* gene and a non-coding region, for nine dicyemid species, and used maximum likelihood and Bayesian inference analyses to estimate the relationship between and within dicyemid species. Divergence in *COI* nucleotide sequences between dicyemid species was high while within species diversity was generally lower. The non-coding region and putative 5′ section of the *COI* gene were highly divergent between dicyemid species. No tRNA molecules were found in the non-coding region, although palindrome sequences with the potential to form stem-loop structures were identified, which may play a role in replication of the minicircle molecule. In general, dicyemid species clades were monotypic, however the placement of certain species taxa in incorrect generic clades suggested classifications based on classical morphological traits may be in need of revision.

Keywords: Dicyemida; Mitochondrial DNA; Minicircle molecules; Cytochrome *c* oxidase; Molecular phylogeny; Taxonomic classification

1. Introduction

Within the eukaryotes lie the dicyemid mesozoans (Dicyemida Van Beneden, 1876), a poorly-understood group of marine parasites that are found with high intensity in the renal appendages of benthic cephalopods. Dicyemid mesozoans are simple in morphology, comprising 8–40 cells with one central axial cell surrounded by ciliated peripheral cells (Suzuki et al., 2010). In contrast, their life cycle is complex with two stages of development (vermiform and infusoriform) and two modes of reproduction (asexual and sexual) (McConnaughey, 1951; Furuya et al., 2003). To date, 122 dicyemid species have been described on the basis of variation in morphological characters, however there exists confusion over the validity of 20% of taxa, with errors in taxonomy highlighted as one of the factors responsible for this confusion (Catalano, 2012). Identification of a species based on morphological characters alone is increasingly being recognised as unreliable, especially for cryptic species or those that exhibit plasticity in morphological character traits (McManus and Bowles, 1996; Poulin and Morand, 2000; Littlewood et al., 2001). A more robust approach is to use a combination of morphological and molecular methods. For the dicyemids, molecular studies are scarce, and where they have been performed, they only include results for a single taxon. Additionally, there exists no preliminary phylogeny, which could be used to validate the current morphological taxonomic designations of dicyemids and assist in unravelling the confusion surrounding this group. Amplification of mitochondrial (mt) sequence data for dicyemid taxa may prove useful in resolving this confusion.

In general animal mt genomes exist in the form of a single, circular, double-stranded molecule (Boore, 1999), however in some cases, chromosomal fragmentation and the occurrence of minicircle molecules have been reported, either in conjunction with, or in place of, the typical mt genome. In animals, mt minicircle molecules have been reported in several nematode species (Armstrong et al., 1999; Gibson et al., 2011) as well as ten species of sucking and chewing lice (Shao et al., 2009; Cameron et al., 2011; Shao et al., 2012; Jiang et al., 2013). More widely among eukaryotes, minicircle molecules have been observed in the kinetoplast DNA of parasitic trypanosomatid protists (Ryan et al., 1988; Stuart and Feagin, 1992; Shapiro and Englund, 1995) and their sister group, diplonemid flagellates (Marande et al., 2005); the nuclear genomes of ciliates (Prescott, 1994); the chloroplast genomes of dinoflagellates (Zhang et al., 1999; Barbrook et al., 2001; Zhang et al., 2002); and the mt genome of the fungus *Spizellomyces punctatus* (Burger et al., 2003). This unusual genome

organisation has also been identified in the dicyemid mesozoans, with the mt cytochrome *c* oxidase subunit genes, *COI*, *COII* and *COIII*, found to exist on separate minicircular molecules in *Dicyema misakiense* (Watanabe et al., 1999). Aside from this single taxon, minicircle molecules have not been characterised from any other dicyemid species for a wider comparison.

In our study, we determined the complete *COI* minicircle sequence for seven species of *Dicyema* and two species of *Dicyemennea*, two of the nine genera within Dicyemida which combined contain 90% of the nominal dicyemid species. Our main aim was to present the first mt dicyemid phylogeny to explore the relationships between and within dicyemid species outside of the classical morphological approach. This will allow an evaluation on the accuracy of classifying dicyemids based on morphological traits alone, specifically addressing whether this approach is suitable for genus and species identification for the Dicyemida. We also explore properties of the mt *COI* minicircle between and within dicyemid species, examine the non-coding region for potential secondary structures which may be involved in gene transcription or minicircle replication, and discuss the evolutionary consequences of mt minicircular gene arrangement.

2. Materials and methods

2.1 Collection of hosts and parasites

Ten cephalopod species were collected between July 2010 and January 2013 at six localities in eastern, southern and western Australian waters as part of a broader study on dicyemid parasite infections in Southern Hemisphere cephalopods (see Catalano et al., in review, Fig. 1). Collections were made via line fishing, SCUBA, snorkelling and from the by-catch of research prawn trawl surveys (organized by the South Australian Research and Development Institute and Western Australian Fisheries). Immediately after capture, each cephalopod was measured, weighed and tentatively identified before the left and right renal appendages were removed. Multiple glass slide smears of each renal appendage were taken and fixed in 70% ethanol before each appendage was preserved separately in 100% ethanol (Catalano and Furuya, 2013). A mantle tissue sample was taken from each host individual examined and preserved in 100% ethanol to confirm host species identification. Frozen cephalopod renal appendages from the giant Australian cuttlefish, *Sepia apama*, collected in

South Australia (SA) (February-March 1998; June, September-December 2005; February, June 2006; October 2011) and New South Wales (NSW) (July 2011; March-May 2012), were also used. Since capture, the frozen material had been stored at -80 ºC at the South Australian Museum, Adelaide, SA and Cronulla Fisheries Research Centre, NSW.

2.2 Morphological identification of dicyemid species

Fixed renal appendage smears from fresh cephalopod samples were stained, mounted and then examined for dicyemid parasites with a compound microscope. Comparisons were made to previously described dicyemid species as presented in the synthesis by Catalano (2012) and from original descriptions. Measurements, drawings and formal descriptions were undertaken for all new dicyemid species discovered (see Catalano, 2013b, a; Catalano and Furuya, 2013). As *Dicyema* sp. 1 was collected from only one *Octopus kaurna* individual with infection exclusively by the rhombogen stage, it has not been described formally.

2.3 DNA isolation, PCR amplification and sequencing of COI *minicircles*

DNA was extracted from each renal appendage and mantle of each host individual separately using a Puregene DNA isolation kit (Gentra Systems) following the manufacturers protocol for DNA purification from solid tissue. The extracted DNA was then stored at 4 ºC until required. The primers COI-F and COI-R were used initially to amplify a 400 bp fragment of the *COI* gene (Table 1). To test for the presence of a *COI* minicircle molecule, new primers were designed to the ends of the 400 bp fragment, but in an inverted orientation (Table 1). Amplification of a product using these outward facing primers indicated a closed, circular conformation for the *COI* gene. For some dicyemid species, additional primer pairs were designed to span gaps and confirm nucleotide sequences (Table 1). A panel of the host cephalopod mantle DNA samples was screened with each primer pair to ensure they would not amplify host DNA.

Amplification reactions were conducted in a volume of 25 µl with a final concentration of 1x GeneAmp PCR Buffer II, 4 mM $MgCl_2$, 200 µM of each dNTP, 0.2 µM of each primer and 0.5 U of AmpliTaq® Gold DNA polymerase (Applied Biosystems). Amplifications consisted of an initial denaturation step of 95 ºC for 9 min, followed by 34

cycles of PCR with the following temperature profile: denaturation at 94 °C for 45 s, annealing at 50–60 °C (depending on the primers) for 45 s, and extension at 72 °C for 1 min, with an additional final extension at 72 °C for 6 min. PCR cycles were increased to 40–45 when PCR product yield was low. Amplicons were visualised on 1.5% agarose gels and purified using a Multi Screen Vacuum Manifold and 384-well Multi Screen Filter Plate (Millipore), then sequenced in both directions using the BigDye Terminator v3.1 cycle-sequencing kit (Applied Biosystems).

Table 1 Primers used for PCR amplification of each dicyemid species *COI* minicircle sequence and cephalopod *COI* partial fragment. All primers were designed in this study except those indicated otherwise. Orientation follows primer ID, either forward (F) or reverse (R).

Species	**Primer ID & orientation**	**Sequence (5′–3′)**
Dicyema coffinense	M1502R	ACTAAGAAGTTGCAAGACCT
	M1503F	TGTTGATTCCTTATCATTG
	M1530F	AGCTTGTGCTGGGTGAACTCT
	M1575R	GGGTAATAATCACAGGGAGAGAGC
	M1590F	CCGTTTGCAGTTAAGCTTTCCAGTGT
	M1591R	TGTTGCTGCAGTAAAATAAGACCGT
Dicyema furuyi	COI-F[1]	GTTTTTTGGACATCCTGAGGT
	COI-R[1]	AGGACATAGTGGAAGTGTGCTACAAC
	M1580F	AGCGAATATCTTCCCACACATTATCC
	M1581R	AGACCTTAATACCTGTAGGAACTGCAA
	M1586F	AGCAGGTGCTGGGTGAACTCT
	M1587R	AGCCACGCATACTCATAGCCGT
Dicyema koinonum	M1435F	GCCTTATTTTAGTACAGTGTGC
	M1436R	CGAGTATCAATATCTATACCAGATG
	M1452F	ATGCAACTTTATGGTGACTAT
	M1453R	TGCAGGTATGAGAGTGAGTA
Dicyema multimegalum	M1502R	ACTAAGAAGTTGCAAGACCT
	M1503F	TGTTGATTCCTTATCATTG
	M1530F	AGCTTGTGCTGGGTGAACTCT
	M1575R	GGGTAATAATCACAGGGAGAGAGC
	M1590F	CCGTTTGCAGTTAAGCTTTCCAGTGT
	M1591R	TGTTGCTGCAGTAAAATAAGACCGT
Dicyema papuceum	COI-F[1]	GTTTTTTGGACATCCTGAGGT
	COI-R[1]	AGGACATAGTGGAAGTGTGCTACAAC
	M1561F	AAGCGAATAACGTCTTATGGCTTGA
	M1562R	TCCCAGTAGGAACGGCAATAATCA
Dicyema sp.1	M1508F	TTGCTATTGGAGGWACATCGGG
	M1509R	CCTGCAGTATARATGTGATGTGCTCA

	M1522F	ACGTCGTAATACCTCAAGGGTACAGA
	M1523R	ACCAATAGCTACAGGCATTGCTGC
	M1524F	ACCGGACTTACTGTTTCCTCGGT
	M1525R	AGTGATCCACAGCTGCGTTCG
Dicyema vincentense	M1518F	TGGGCCCATCACATATATACTTCAGGA
	M1519R	ACTCACCTGGGATACGGTGTAGTG
	M1532F	TCGCCTTACATTTAGCAGGAGTGT
	M1533R	TGGGCCCATACAACTCTACCGA
	M1534F	TCTATTCAGCTATCTTCACTACACCGT
	M1535R	ATCGACGAGGTATCGCTGCAT
Dicyemennea floscephalum	M1469F	TCTGGTTCTTTGGCCACCCTGA
	M1470R	GGGCCATAACAATGTGGAAGTGACC
	M1473F	TGTCCTCTACTAACCATGTTGATGTGT
	M1474R	ACACAACAGATCCAACACCTGCG
	M1476F	TGTACTGTCGAATGCCTCTCTTGA
	M1478R	TCAGGGTGGCCAAAGAACCAGA
Dicyemennea spencerense	M1484F	CGTCCATAGTCAGCTCGTGCAA
	M1485R	ACCAAAGACACCTGCAAATACACCA
	M1489F	TGCGGCTTCTTTTAGGGTATTTGC
	M1490R	ACCTCAGGATGTCCAAAGAACCAG
Cephalopod universal primers	COIF[2]	TCWACNAAYCAYAARGAYATTGG
	COIR[2]	ACYTCNGGRTGNCCRAARARYCA

[1]Primers from Watanabe et al. (1999)
[2]Primers from Cooper et al. (2011)

2.4 Cephalopod host partial COI *amplification and sequencing*

A 400 bp fragment of *COI* was amplified using universal primers COIF and COIR (Table 1). Amplification, cycling and sequencing conditions were the same as for the dicyemid minicircles, except that a 48 ºC annealing temperature was used and sequencing was performed in a single direction using COIF. Sequences were compared to those on GenBank to confirm host cephalopod species identification.

2.5 Phylogenetic analyses of dicyemid parasite COI *gene*

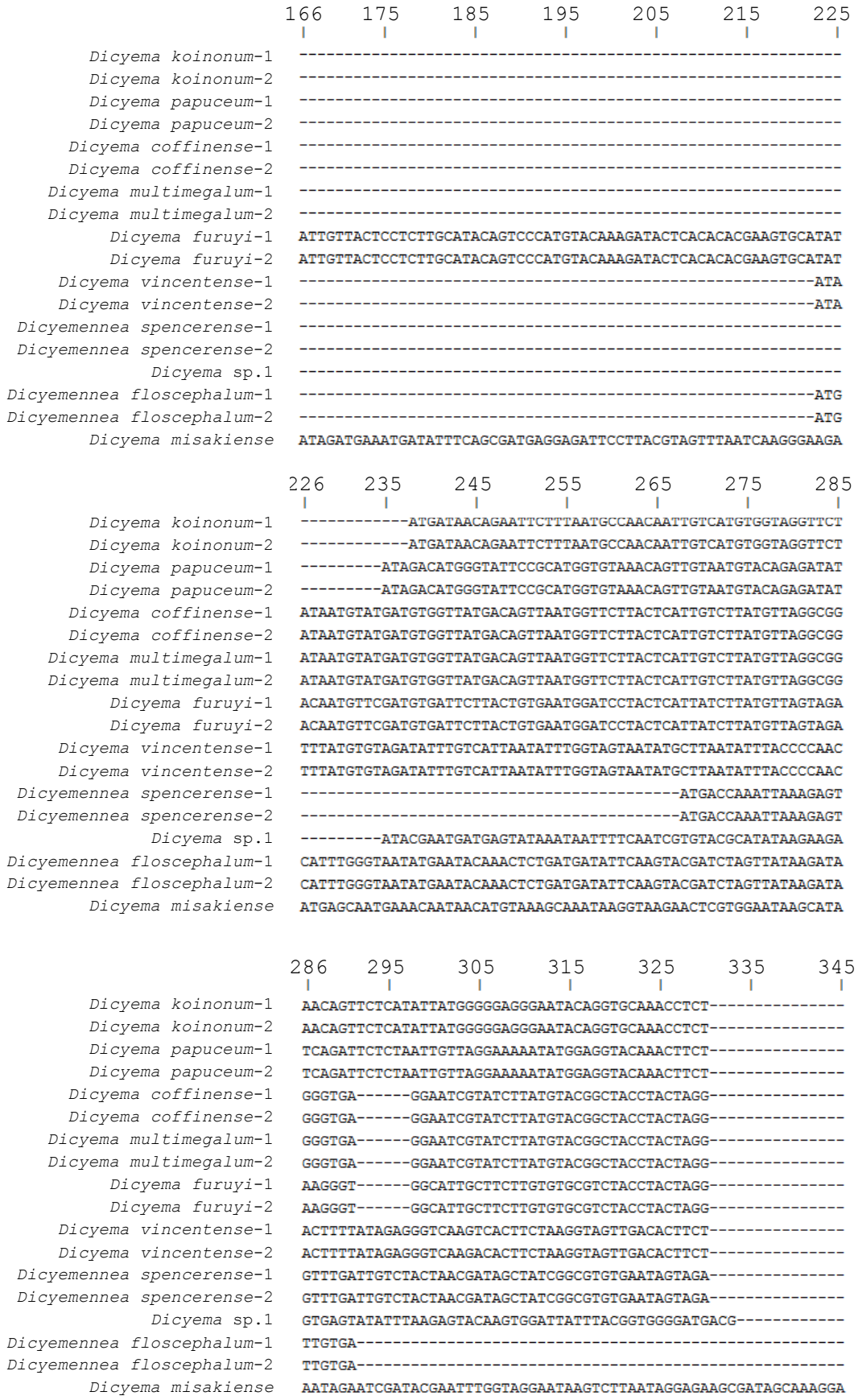
Sequences were edited and aligned using Geneious Pro 5.3.4 (Drummond et al., 2010). Due to co-infections by individuals with differing *COI* sequences, some base calls were ambiguous on both the forward and reverse strands. These base calls were inferred by comparison with reference haplotypes from the remainder of our data set. We designated the

start of the non-coding region directly after the stop codon of the *COI* gene, with a requirement that it contained no start codon in frame with the *COI* gene. However, because of the highly variable sequence composition and size of the non-coding region between dicyemid species, we were not able to align the non-coding region and so it was excluded from all phylogenetic analyses. Additionally, due to uncertainty in our alignment of the 5′end of the *COI* gene between dicyemid species the first 375 bp of sequence was also excluded from phylogenetic analyses (see Fig. 1a). Phylogenetic trees were reconstructed using maximum likelihood (ML) (PhyML 3.0; Guindon and Gascuel, 2003; Guindon et al., 2010) and Monte Carlo Markov Chain (MCMC) Bayesian inference (BI) (MrBayes 3.2; Ronquist et al., 2012).

For the ML analysis, the general time reversible (GTR) model (Rodŕiguez et al., 1990) with a proportion of invariant sites (I) and gamma distribution for rates across sites (G) was selected as the most likely nucleotide substitution model from the Akaike Information Criteria (AIC) scores in PartitionFinder 1.0.1 (Lanfear et al., 2012). The tree topology was optimised in order to maximise the likelihood and the best of NNI and SPR moves used to estimate tree topologies with five random starting trees. The starting tree was estimated using BioNJ and the degree of support for internal branches was assessed by 1,000 bootstrap pseudo-replications (BS).

MrBayes allows the input data to be partitioned with optimum nucleotide substitution models applied to each partition. The following nucleotide substitution models and partitioning scheme were identified as the most appropriate from the AIC scores in PartitionFinder: GTR + I + G for the first codon partition, GTR + G for the second codon partition and Hasegawa Kishino Yano (HKY) model (Hasegawa et al., 1985) + G for the third codon partition. Two MCMC runs of four chains each were performed simultaneously for 10 million generations, with a sample frequency every 200 generations, creating 50,000 trees. To confirm stationarity had been reached and ensure consistency between runs, the relationship between log likelihood scores and generation numbers were plotted and analysed in Tracer 1.5 (Rambaut and Drummond, 2007). We also used Tracer to determine whether our sample included sufficiently large effective sample sizes (ESS) for parameter estimates and to approximate the burn-in value. Subsequently, the first 12,500 trees (25%) were discarded from the sample as the burn-in period prior to summarising the result. A 50% majority rule consensus tree was constructed, with the robustness of nodes assessed by Bayesian posterior probability (PP) estimates.

a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Dicyema koinonum-2 ----------
Dicyema papuceum-1 ----------
Dicyema papuceum-2 ----------
Dicyema coffinense-1 ----------
Dicyema coffinense-2 ----------
Dicyema multimegalum-1 ----------
Dicyema multimegalum-2 ----------
Dicyema furuyi-1 ATTGTTACTCCTCTTGCATACAGTCCCATGTACAAAGATACTCACACACGAAGTGCATAT
Dicyema furuyi-2 ATTGTTACTCCTCTTGCATACAGTCCCATGTACAAAGATACTCACACACGAAGTGCATAT
Dicyema vincentense-1 ----------ATA
Dicyema vincentense-2 ----------ATA
Dicyemennea spencerense-1 ----------
Dicyemennea spencerense-2 ----------
Dicyema sp.1 ----------
Dicyemennea floscephalum-1 ----------ATG
Dicyemennea floscephalum-2 ----------ATG
Dicyema misakiense ATAGATGAAATGATATTTCAGCGATGAGGAGATTCCTTACGTAGTTTAATCAAGGGAAGA
226 235 245 255 265 275 285
Dicyema koinonum-1 ----------ATGATAACAGAATTCTTTAATGCCAACAATTGTCATGTGGTAGGTTCT
Dicyema koinonum-2 ----------ATGATAACAGAATTCTTTAATGCCAACAATTGTCATGTGGTAGGTTCT
Dicyema papuceum-1 ---------ATAGACATGGGTATTCCGCATGGTGTAAACAGTTGTAATGTACAGAGATAT
Dicyema papuceum-2 ---------ATAGACATGGGTATTCCGCATGGTGTAAACAGTTGTAATGTACAGAGATAT
Dicyema coffinense-1 ATAATGTATGATGTGGTTATGACAGTTAATGGTTCTTACTCATTGTCTTATGTTAGGCGG
Dicyema coffinense-2 ATAATGTATGATGTGGTTATGACAGTTAATGGTTCTTACTCATTGTCTTATGTTAGGCGG
Dicyema multimegalum-1 ATAATGTATGATGTGGTTATGACAGTTAATGGTTCTTACTCATTGTCTTATGTTAGGCGG
Dicyema multimegalum-2 ATAATGTATGATGTGGTTATGACAGTTAATGGTTCTTACTCATTGTCTTATGTTAGGCGG
Dicyema furuyi-1 ACAATGTTCGATGTGATTCTTACTGTGAATGGATCCTACTCATTATCTTATGTTAGTAGA
Dicyema furuyi-2 ACAATGTTCGATGTGATTCTTACTGTGAATGGATCCTACTCATTATCTTATGTTAGTAGA
Dicyema vincentense-1 TTTATGTGTAGATATTTGTCATTAATATTTGGTAGTAATATGCTTAATATTTACCCCAAC
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Dicyemennea spencerense-1 ----------ATGACCAAATTAAAGAGT
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Dicyema sp.1 ---------ATACGAATGATGAGTATAAATAATTTTCAATCGTGTACGCATATAAGAAGA
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Dicyema misakiense ATGAGCAATGAAACAATAACATGTAAAGCAAATAAGGTAAGAACTCGTGGAATAAGCATA
286 295 305 315 325 335 345
Dicyema koinonum-1 AACAGTTCTCATATTATGGGGGAGGGAATACAGGTGCAAACCTCT----------
Dicyema koinonum-2 AACAGTTCTCATATTATGGGGGAGGGAATACAGGTGCAAACCTCT----------
Dicyema papuceum-1 TCAGATTCTCTAATTGTTAGGAAAAATATGGAGGTACAAACTTCT----------
Dicyema papuceum-2 TCAGATTCTCTAATTGTTAGGAAAAATATGGAGGTACAAACTTCT----------
Dicyema coffinense-1 GGGTGA------GGAATCGTATCTTATGTACGGCTACCTACTAGG----------
Dicyema coffinense-2 GGGTGA------GGAATCGTATCTTATGTACGGCTACCTACTAGG----------
Dicyema multimegalum-1 GGGTGA------GGAATCGTATCTTATGTACGGCTACCTACTAGG----------
Dicyema multimegalum-2 GGGTGA------GGAATCGTATCTTATGTACGGCTACCTACTAGG----------
Dicyema furuyi-1 AAGGGT------GGCATTGCTTCTTGTGTGCGTCTACCTACTAGG----------
Dicyema furuyi-2 AAGGGT------GGCATTGCTTCTTGTGTGCGTCTACCTACTAGG----------
Dicyema vincentense-1 ACTTTTATAGAGGGTCAAGTCACTTCTAAGGTAGTTGACACTTCT----------
Dicyema vincentense-2 ACTTTTATAGAGGGTCAAGACACTTCTAAGGTAGTTGACACTTCT----------
Dicyemennea spencerense-1 GTTTGATTGTCTACTAACGATAGCTATCGGCGTGTGAATAGTAGA----------
Dicyemennea spencerense-2 GTTTGATTGTCTACTAACGATAGCTATCGGCGTGTGAATAGTAGA----------
Dicyema sp.1 GTGAGTATATTTAAGAGTACAAGTGGATTATTTACGGTGGGGATGACG----------
Dicyemennea floscephalum-1 TTGTGA----------
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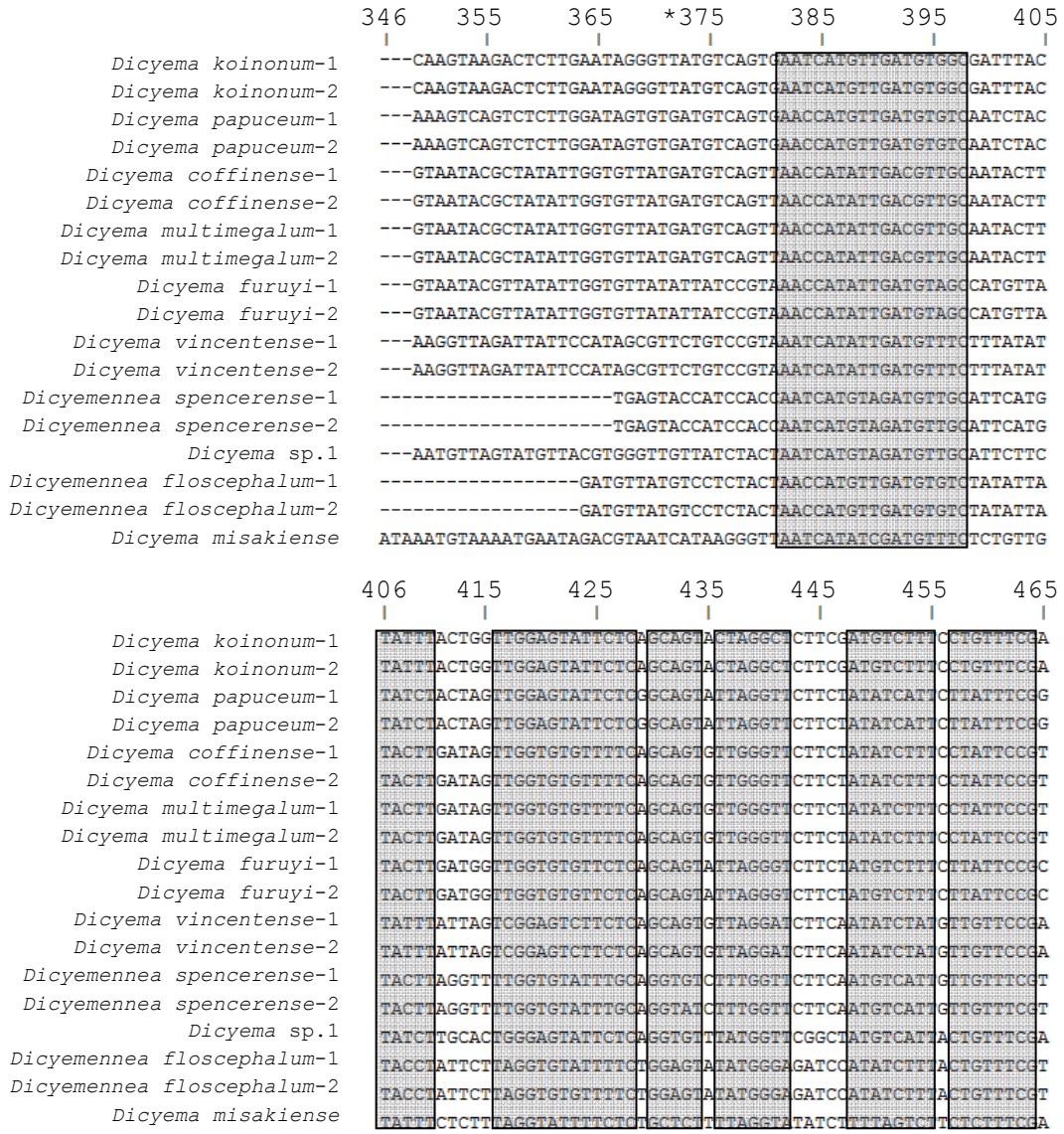
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Dicyema papuceum-1 ---AAAGTCAGTCTCTTGGATAGTGTGATGTCAGTGAACCATGTTGATGTGTCAATCTAC
Dicyema papuceum-2 ---AAAGTCAGTCTCTTGGATAGTGTGATGTCAGTGAACCATGTTGATGTGTCAATCTAC
Dicyema coffinense-1 ---GTAATACGCTATATTGGTGTTATGATGTCAGTTAACCATATTGACGTTGCAATACTT
Dicyema coffinense-2 ---GTAATACGCTATATTGGTGTTATGATGTCAGTTAACCATATTGACGTTGCAATACTT
Dicyema multimegalum-1 ---GTAATACGCTATATTGGTGTTATGATGTCAGTTAACCATATTGACGTTGCAATACTT
Dicyema multimegalum-2 ---GTAATACGCTATATTGGTGTTATGATGTCAGTTAACCATATTGACGTTGCAATACTT
Dicyema furuyi-1 ---GTAATACGTTATATTGGTGTTATATTATCCGTAAACCATATTGATGTAGCCATGTTA
Dicyema furuyi-2 ---GTAATACGTTATATTGGTGTTATATTATCCGTAAACCATATTGATGTAGCCATGTTA
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Dicyemennea spencerense-1 ----------TGAGTACCATCCACCAATCATGTAGATGTTGCATTCATG
Dicyemennea spencerense-2 ----------TGAGTACCATCCACCAATCATGTAGATGTTGCATTCATG
Dicyema sp.1 ---AATGTTAGTATGTTACGTGGGTTGTTATCTACTAATCATGTAGATGTTGCATTCTTC
Dicyemennea floscephalum-1 ----------GATGTTATGTCCTCTACTAACCATGTTGATGTGTCTATATTA
Dicyemennea floscephalum-2 ----------GATGTTATGTCCTCTACTAACCATGTTGATGTGTCTATATTA
Dicyema misakiense ATAAATGTAAAATGAATAGACGTAATCATAAGGGTTAATCATATCGATGTTTCTCTGTTG
406 415 425 435 445 455 465
Dicyema koinonum-1 TATTTACTGGTTGGAGTATTCTCAGCAGTACTAGGCTCTTCGATGTCTTTCCTGTTTCGA
Dicyema koinonum-2 TATTTACTGGTTGGAGTATTCTCAGCAGTACTAGGCTCTTCGATGTCTTTCCTGTTTCGA
Dicyema papuceum-1 TATCTACTAGTTGGAGTATTCTCGGCAGTATTAGGTTCTTCTATATCATTCTTATTTCGG
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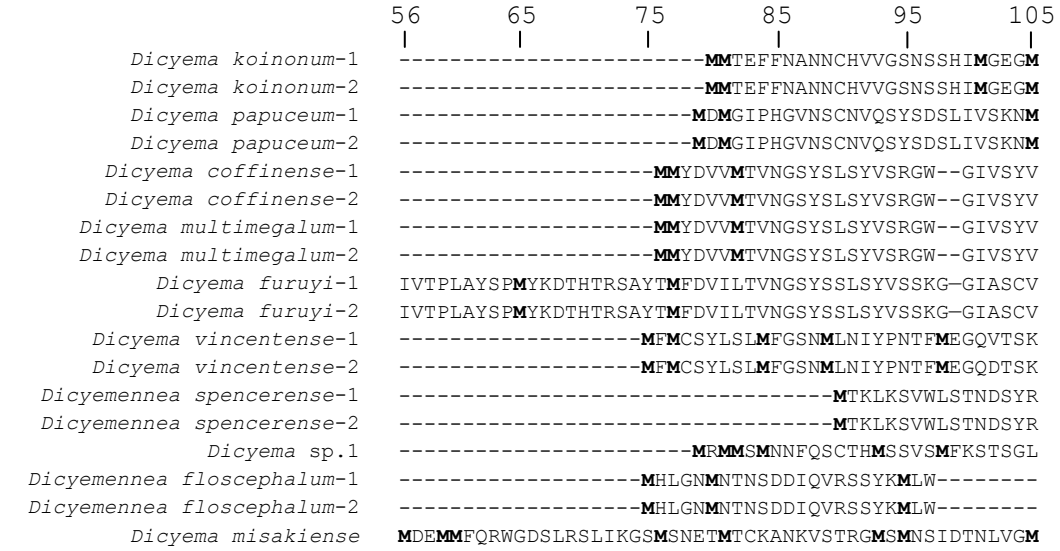
Dicyema sp.1 TATCTTGCACTGGGAGTATTCTCAGGTGTTTATGGTTCGGCTATGTCATTACTGTTTCGA
Dicyemennea floscephalum-1 TACCTATTCTTAGGTGTATTTTCTGGAGTATATGGGAGATCCATATCTTTACTGTTTCGT
Dicyemennea floscephalum-2 TACCTATTCTTAGGTGTGTTTTCTGGAGTATATGGGAGATCCATATCTTTACTGTTTCGT
Dicyema misakiense TATTTCTCTTTAGGTATTTTCTCTGCTCTTTAGGTATATCTTTTAGTCTTCTCTTTCGA

b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Dicyema papuceum-1 ----------MDMGIPHGVNSCNVQSYSDSLIVSKNM
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Dicyema coffinense-1 ----------MMYDVVMTVNGSYSLSYVSRGW--GIVSYV
Dicyema coffinense-2 ----------MMYDVVMTVNGSYSLSYVSRGW--GIVSYV
Dicyema multimegalum-1 ----------MMYDVVMTVNGSYSLSYVSRGW--GIVSYV
Dicyema multimegalum-2 ----------MMYDVVMTVNGSYSLSYVSRGW--GIVSYV
Dicyema furuyi-1 IVTPLAYSPMYKDTHTRSAYTMFDVILTVNGSYSSLSYVSSKG—GIASCV
Dicyema furuyi-2 IVTPLAYSPMYKDTHTRSAYTMFDVILTVNGSYSSLSYVSSKG—GIASCV
Dicyema vincentense-1 ----------MFMCSYLSLMFGSNMLNIYPNTFMEGQVTSK
Dicyema vincentense-2 ----------MFMCSYLSLMFGSNMLNIYPNTFMEGQDTSK
Dicyemennea spencerense-1 ----------MTKLKSVWLSTNDSYR
Dicyemennea spencerense-2 ----------MTKLKSVWLSTNDSYR
Dicyema sp.1 ----------MRMMSMNNFQSCTHMSSVSMFKSTSGL
Dicyemennea floscephalum-1 ----------MHLGNMNTNSDDIQVRSSYKMLW--------
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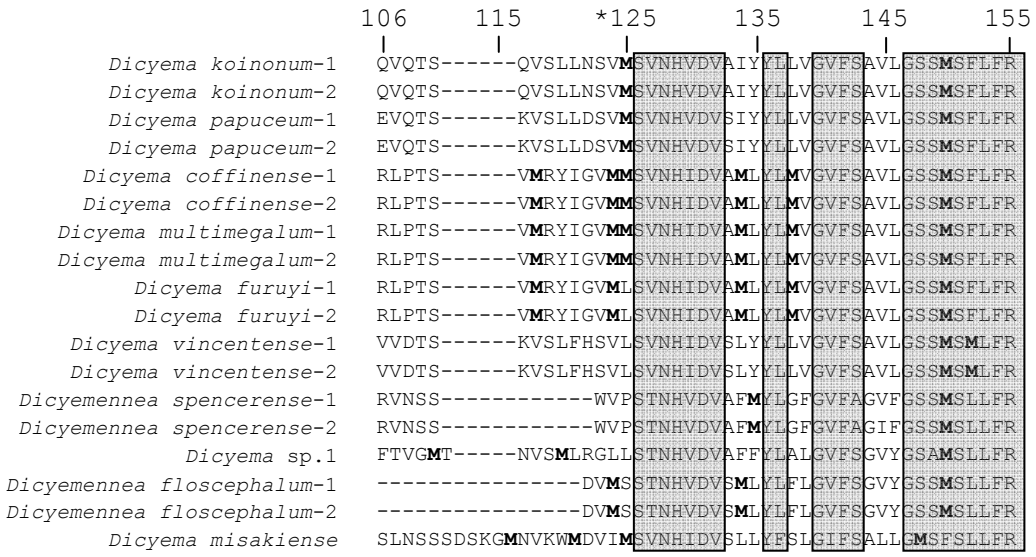


Fig. 1. a) nucleotide and b) amino acid sequence of the aligned 5′ end of *COI* for all dicyemid species showing high sequence heterogeneity. Two individuals of each species (where possible) are shown. Dashes indicate gaps for optimizing the alignment, and sequences identical or highly similar among all dicyemid species are boxed in grey. Numbers above the sequences indicate base and codon positions, numbered from the start of the *COI* gene for *Dicyema furuyi* (first 165 bp/55 codons, which are unique for this species only, are not shown). (*) signifies the start of the sequence used in our phylogenetic analyses. Methionines in the amino acid sequence are bolded to highlight the multiple possible gene initiation sites.

For both analyses, published *COI* sequences of the lophotrochozoans (Annelida) *Myzostoma seymourcollegiorum* and *Lumbricus terrestris*, which are suggested to be the closest ancestors of Dicyemida as inferred from molecular phylogenetic analyses (Kobayashi et al., 1999; Kobayashi et al., 2009; Suzuki et al., 2010), were included in the dataset as outgroups to facilitate rooting of the phylogenetic trees. The *COI* gene sequence of *D. misakiense* was also included in our analyses following modification. Watanabe et al. (1999) reported the *COI* gene sequence from this dicyemid species as 1,422 bp, with a 278 bp non-coding region (1,700 bp total minicircle size). After inspection, we observed start codons in frame with the *COI* gene in the non-coding region. Therefore, for consistency with our edited sequences, the last 195 bp in frame portion of the *D. misakiense* non-coding region was included as part of the *COI* gene, resulting in a gene size of 1,617 bp and non-coding region size of 83 bp (see Table 2). All trees presented here were visualised and edited in FigTree 1.3.1 (Rambaut, 2010), and representative sequences of all taxa and haplotypes have been deposited in GenBank (accession numbers KF208316–KF208361).

2.6 Secondary structure analyses of the non-coding region

We searched the non-coding region of all our dicyemid sequences for potential stem-loop structures using EMBOSS: palindrome (Faller, 1999) with a minimum palindrome length set at 6. The maximum palindrome length and maximum gap between repeated regions varied depending on the total size of the non-coding region of each dicyemid species, however both conditions were set to highest values so all potential palindromes would be identified. Three additional programs, tRNAscan-SE 1.2.1 (Lowe and Eddy, 1997), ARAGORN (Laslett and Canback, 2004) and ARWEN (Laslett and Canback, 2008), were used to search for potential transfer RNA (tRNA) genes in the non-coding region. In particular, tRNAscan-SE uses two previously described detection programs as first-pass prefilters followed by a highly selective covariance model to identify tRNA genes (Lowe and Eddy, 1997). ARAGORN and ARWEN both employ heuristic algorithms that search for hairpin structures, however whereas ARAGORN searches for tRNA and transfer-messenger (tmRNA) genes concurrently, ARWEN is specialised for finding mt tRNA genes (Laslett and Canback, 2004, 2008). These three programs were selected based on the findings of Morrison (2010) that no single computer program is necessarily capable of detecting all the tRNA genes in any given sequence.

3. Results

3.1 Dicyemid minicircles

A complete, closed minicircle was sequenced from 9 dicyemid species (Table 2). In some cases, although parasite infection was observed for a host individual from morphological examination of prepared smears, no *COI* dicyemid sequence could be amplified from the renal appendages of that individual. This was true for *Dicyema calamaroceum* infecting *Sepioteuthis australis* (southern calamary) and *D. pyjamaceum* infecting *Sepioloidea lineolata* (striped pyjama squid); all *COI* primer combinations were trialled at various cycling conditions but no *COI* dicyemid sequence was amplified. For the remaining 9 dicyemid species, the minicircle contained the *COI* gene plus a non-coding region. For all dicyemid species except *Dicyema* sp. 1, more than one haplotype was observed due to co-occurrence (infection by multiple dicyemid individuals of the same species within a renal appendage of one host individual).

Table 2 Length of the *COI* gene, non-coding region and minicircle molecule (bp) for each haplotype (Hap#) recorded from all dicyemid species analysed.

Host species	Dicyemid species	Number of haplotypes	*COI* gene	non-coding region	Minicircle molecule (total)	GenBank accession number
Sepia apama	*Dicyema coffinense*	5	1542 (Hap1–3)	245	1787	KF208316–8
			1542 (Hap4)	263	1805	KF208319
			1542 (Hap5)	269	1811	KF208320
Sepia papuensis	*Dicyema furuyi*	2	1767 (Hap1–2)	85	1852	KF208321–2
Sepia apama, *S. novaehollandiae*	*Dicyema koinonum*	20	1524 (Hap1–17, 19)	35	1559	KF208323–39, KF208341
			1524 (Hap18, 20–24)	36	1560	KF208340, KF208342–6
Octopus vulgaris	*Dicyema misakiense*[1]	1	1422	278	1700	AB011832
Octopus vulgaris	*Dicyema misakiense*[2]	1	1617	83	1700	
Sepia apama	*Dicyema multimegalum*	5	1542 (Hap1–4)	264	1806	KF208347–50
			1542 (Hap5)	265	1807	KF208351
Sepia papuensis	*Dicyema papuceum*	2	1518 (Hap1–2)	50	1568	KF208352–3
Octopus kaurna	*Dicyema* sp.1	1	1521 (Hap1)	7	1528	KF208354
Sepia novaehollandiae	*Dicyema vincentense*	2	1527 (Hap1–2)	20	1547	KF208355–6
Octopus berrima	*Dicyemennea floscephalum*	2	1506 (Hap1–2)	57	1563	KF208357–8
Sepia apama, *S. novaehollandiae*	*Dicyemennea spencerense*	3	1464 (Hap1–3)	57	1521	KF208359–61

[1]Original *D. misakiense* sequence on GenBank

[2] Modified *D. misakiense* sequence we used in our phylogenetic analyses

3.2 Properties of the dicyemid COI *gene: nucleotide sequence composition, amino acid translation and size*

Within each dicyemid species, both the nucleotide and amino acid *COI* sequences were highly conserved, with the length of the *COI* gene remaining constant (Table 2). Conversely, between dicyemid species, the nucleotide and amino acid *COI* sequences were highly variable, particularly at the 5′ end (Fig. 1a, b). The first ~300 bp of the *COI* gene for each dicyemid species contained multiple (3–9) methionine codons (Fig. 1b). The sequence AUA was identified as the start codon for five dicyemid species, with AUG the start codon for the remaining four dicyemid species. The length of the gene varied among dicyemid species, with the shortest *COI* recorded from *Dicyemennea spencerense* (1,464 bp), and the longest from *Dicyema papuensis* (1,767 bp) (Table 2). In general, the *COI* gene was shorter for *Dicyemennea* species (1,464–1,506 bp), compared to *Dicyema* species (1,518–1,767 bp) (Table 2).

3.3 COI *non-coding region*

The non-coding region varied in length from 7–269 bp among dicyemid species (Table 2) and was extremely variable in nucleotide composition and length between dicyemid species (Fig. 2). Within three dicyemid species, *Dicyema coffinense*, *D. koinonum* and *D. multimegalum*, the length of the non-coding region was also variable (Table 2, Fig. 2). Because we could not confidently align this region across species we excluded it from subsequent phylogenetic analyses.

No tRNA genes were identified in any of the non-coding sequences. However, palindrome runs were identified in *D. coffinense*, *D. multimegalum*, *D. papuceum*, *Dicyemennea floscephalum* and *D. spencerense* sequences with positions of potential stems and loops annotated in GenBank submissions (Table 3). All potential stem-loop structures associated with these palindromes were unique, except for palindromes 3 and 5, which were found in both *Dicyema coffinense* and *D. multimegalum*. The loops circumscribed by these two palindromes were of the same length and sequence composition in both *D. coffinense* and *D. multimegalum*, although they occurred at different places in the non-coding region for each dicyemid species (see annotated GenBank sequences KF208347–51 and KF208316–20).

Fig. 2. Unaligned nucleotide sequence of the *COI* minicircle molecule non-coding region showing variability in size and sequence content between and within dicyemid species. Corresponding haplotypes for each dicyemid species non-coding region sequence is given in parenthesis.

Table 3 Stem and loop features from palindrome sequences identified in the non-coding regions.

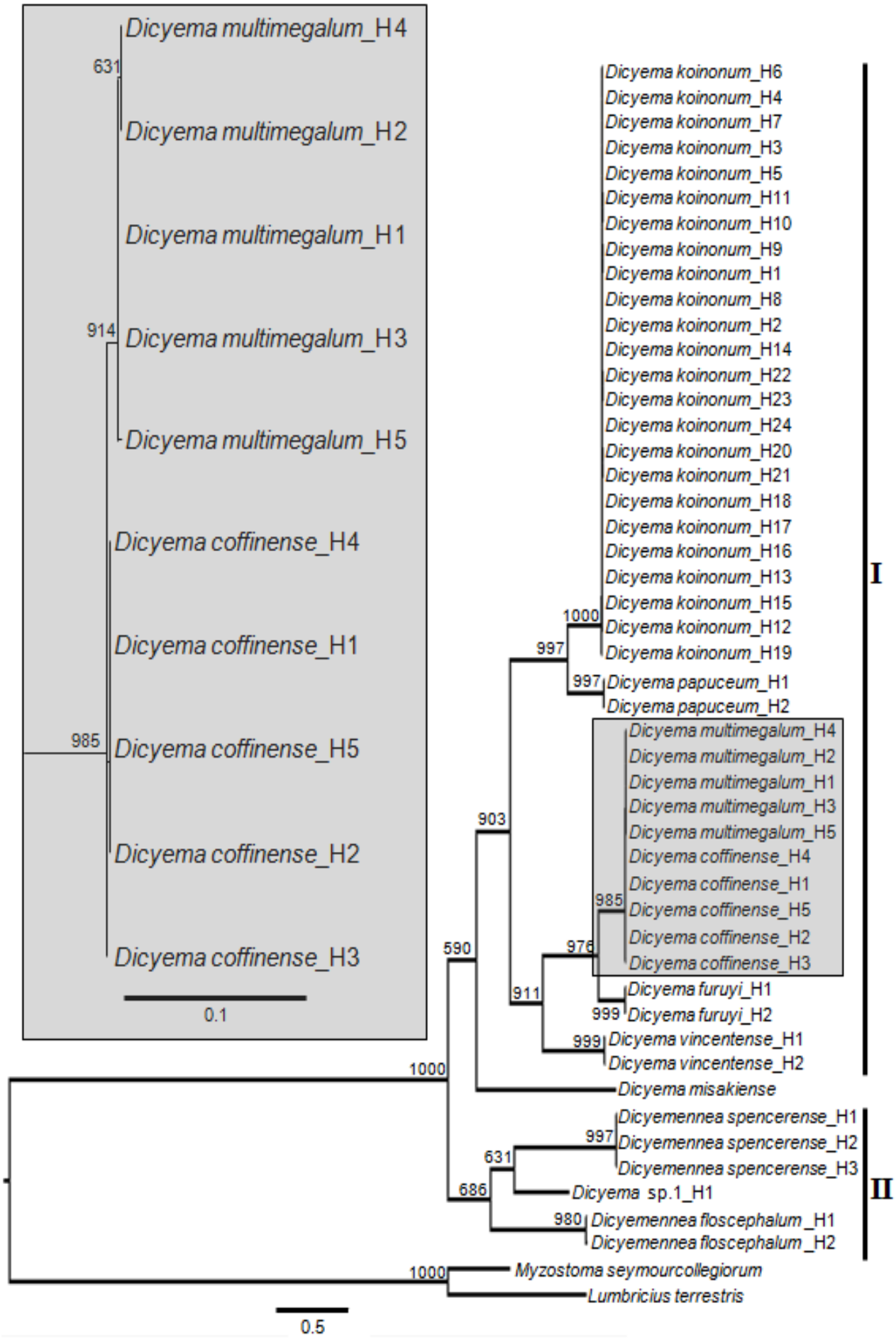
Dicyemid species	Palindrome number	Palindrome start sequence	Size of stem (bp)	Size of loop (bp)	GenBank accession numbers
Dicyema multimegalum	1	tgtttttataa	11	14	KF208347–51
Dicyema multimegalum	2	tactcctttc	10	53, 54	KF208347–51
Dicyema multimegalum, *Dicyema coffinense*	3	ttagagaat	9	11	KF208347–51, KF208316–20
Dicyema multimegalum	4	tcattata	8	7	KF208347–51
Dicyema multimegalum, *Dicyema coffinense*	5	aaggagt	7	18	KF208347–51, KF208316–20
Dicyema coffinense	6	ttaaaat	7	9, 16	KF208316–20
Dicyema papuceum	7	gggctta	7	30	KF208352–3
Dicyema papuceum	8	ttaaaag	7	15	KF208352–3
Dicyemennea floscephalum	9	atcaaat	7	13	KF208357–8
Dicyemennea spencerense	10	tatgag	6	26	KF208359–61

3.4 Phylogenetic analysis of dicyemid COI *gene*

We obtained an aligned dataset of 1,541 bp comprising 47 *COI* haplotypes from 10 dicyemid species including the previously sequenced *D. misakiense*. After 10 million generations in the Bayesian analysis, the standard deviation of split frequencies had reduced to 0.0026, with PSRF of 1.0 for all parameters, suggesting stationarity had been reached. Additionally, from the Tracer analysis, the log likelihood scores between runs were similar with ESS > 4,000 for all parameters for both runs.

The tree topology was similar between the ML and BI analyses (Fig. 3a, b). There was strong support for the majority of nodes, with BS and PP values of >90% and >0.98 respectively. Both analyses separated *Dicyemennea* spp. plus *Dicyema* sp. 1 (clade II) from all other *Dicyema* spp. (clade I) with strong support (BS, 1,000 replicates – Fig. 3a and PP – Fig. 3b). However, the placement of *Dicyema misakiense* varied, either as the sister to clade I but with low support (ML, Fig. 3a) or sister to both clades I and II with strong support (BI, Fig. 3b). Each dicyemid species formed distinct, monotypic subclades, except for *D. multimegalum* which was nested among the *D. coffinense* haplotypes in the ML analysis (Fig 3a inset) but not the BI analysis (Fig. 3b inset).

a


Dicyema multimegalum_H4
631
Dicyema multimegalum_H2
Dicyema multimegalum_H1
914
Dicyema multimegalum_H3
Dicyema multimegalum_H5
Dicyema coffinense_H4
Dicyema coffinense_H1
985
Dicyema coffinense_H5
Dicyema coffinense_H2
Dicyema coffinense_H3
0.1
Dicyema koinonum_H6
Dicyema koinonum_H4
Dicyema koinonum_H7
Dicyema koinonum_H3
Dicyema koinonum_H5
Dicyema koinonum_H11
Dicyema koinonum_H10
Dicyema koinonum_H9
Dicyema koinonum_H1
Dicyema koinonum_H8
Dicyema koinonum_H2
Dicyema koinonum_H14
Dicyema koinonum_H22
Dicyema koinonum_H23
Dicyema koinonum_H24
Dicyema koinonum_H20
Dicyema koinonum_H21
Dicyema koinonum_H18
Dicyema koinonum_H17
Dicyema koinonum_H16
Dicyema koinonum_H13
Dicyema koinonum_H15
1000
Dicyema koinonum_H12
997
Dicyema koinonum_H19
997
Dicyema papuceum_H1
Dicyema papuceum_H2
Dicyema multimegalum_H4
Dicyema multimegalum_H2
Dicyema multimegalum_H1
Dicyema multimegalum_H3
903
Dicyema multimegalum_H5
Dicyema coffinense_H4
Dicyema coffinense_H1
985
Dicyema coffinense_H5
Dicyema coffinense_H2
590
976
Dicyema coffinense_H3
911
Dicyema furuyi_H1
999
Dicyema furuyi_H2
999
Dicyema vincentense_H1
Dicyema vincentense_H2
1000
Dicyema misakiense
Dicyemennea spencerense_H1
997
Dicyemennea spencerense_H2
631
Dicyemennea spencerense_H3
686
Dicyema sp.1_H1
980
Dicyemennea floscephalum_H1
Dicyemennea floscephalum_H2
1000
Myzostoma seymourcollegiorum
Lumbricius terrestris
0.5
I
II

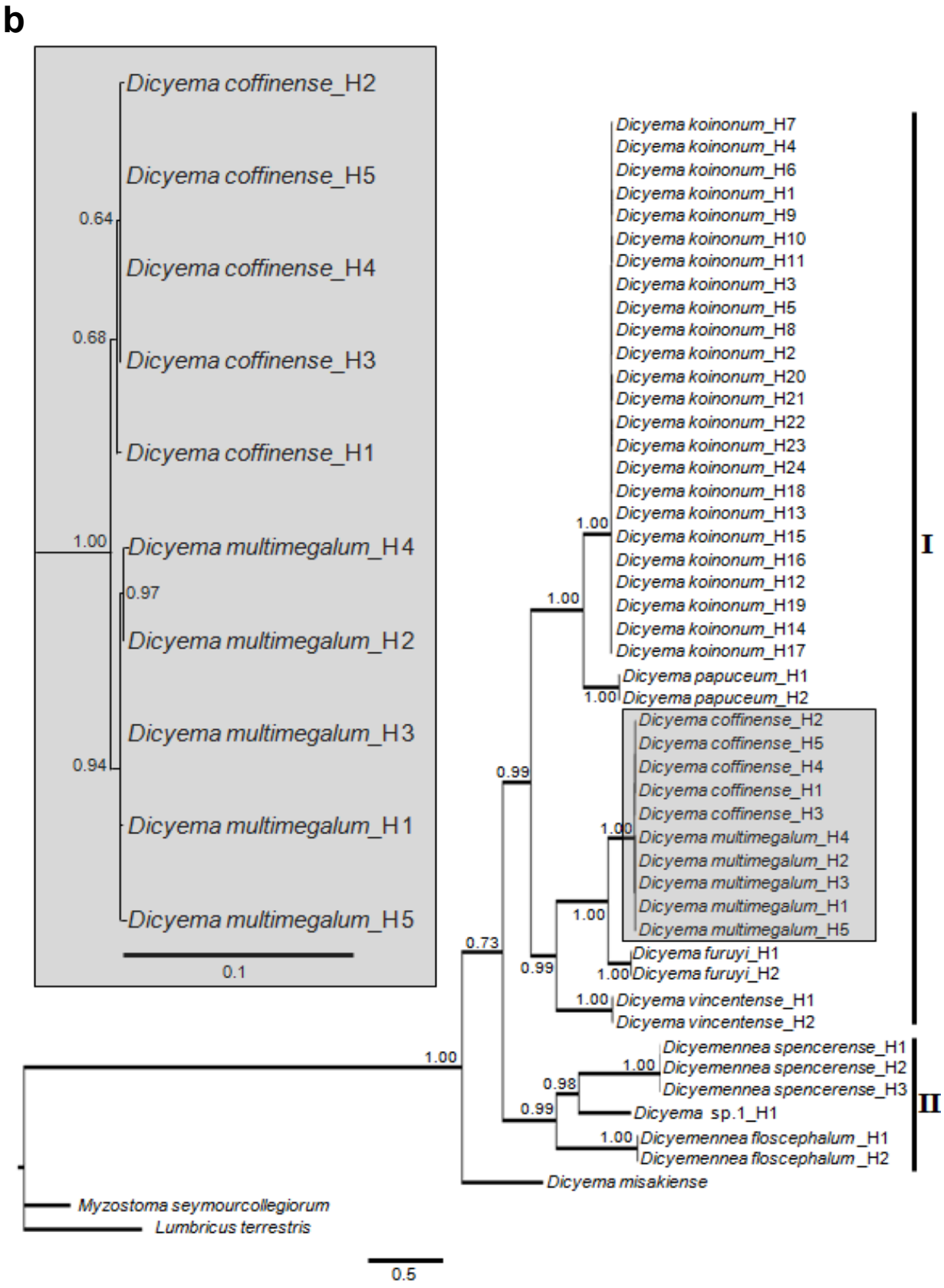


Fig. 3. Phylogenetic analyses of the complete mitochondrial *COI* gene for representative taxa in Dicyemida and two outgroup taxa in Lophotrochozoa determined from: a) maximum likelihood with bootstrap proportions (1,000 pseudo-replicates); and b) Bayesian inference with posterior probabilities. Resolution of the terminal nodes for the *Dicyema coffinense* and *D. multimegalum* clades is shown from the inset. Dicyemid host species are listed in Table 2 along with H# (haplotype number). All sequences were determined in the present study except for sequences from *D. misakiense* and the two outgroup taxa.

4. Discussion

Here we characterised the complete 1.5–1.8 kb *COI* minicircle molecule from nine dicyemid species, with this molecule including the full length *COI* gene and a non-coding region. We compare the properties of this molecule between and within dicyemid species in more detail below, and present the first phylogeny of dicyemids from ML and BI analyses to contrast with the current classifications of dicyemids based purely on morphological characters.

4.1 COI *gene: transcription initiation, size, sequence content and haplotype variation among and within dicyemid species*

For invertebrates, the typical *COI* initiation codon for transcription is methionine, encoded by either AUG or AUA. Subsequently for each dicyemid species that we sequenced, the first AUG or AUA in frame codon that resulted in the longest continuous sequence terminating at a stop codon was inferred as the gene initiation site. However, it is difficult to state with certainty if this represents the true initiation site(s), as we observed multiple in frame methionines at the 5′ end of each dicyemid minicircle (see Fig. 1b), suggesting the gene size may actually be shorter than recorded in Table 2. So is the presence of alternative initiation sites a mechanism used by the dicyemid parasite to rapidly adapt and evolve with their hosts, as multiple proteins may be obtained from the same minicircle sequence? This could be possible, although other authors have also expressed uncertainty in the identification of the initiation codon in mt genes for non-parasitic invertebrates (see Cantatore et al., 1989; Milbury and Gaffney, 2005). Due to the unresolved position of dicyemid parasites in the Tree of Life, it is difficult to make comparisons with closely related phyla to infer the true initiation site.

Like parasitic protozoans, where there is little similarity among minicircle sequences from different species (Ryan et al., 1988), the 5′ end of the *COI* gene was highly divergent in sequence between dicyemid species (Fig. 1a). The presence of asexual reproduction in the life cycle of dicyemid parasites may accelerate the accumulation of mutations, leading to a high amino acid sequence divergence rate (Aruga et al., 2007). However, both asexual and sexual stages of dicyemid parasites were collectively amplified and sequenced in our study, therefore amino acid sequence divergence cannot be solely attributed to asexual reproduction.

An alternative theory may centre on the parasites environment; the hosts renal appendages. This environment is ever-changing and dynamic through time, with the function, composition, shape and size of renal appendages varying between cephalopod species (Emanuel and Martin, 1956; Potts and Todd, 1965; Schipp et al., 1975). Additionally, the renal appendages are each enclosed within a separate renal sac, which also holds the urine (Harrison and Martin, 1965; Potts, 1967). The urine is constantly being produced and excreted, and the renal sacs can be empty, full or any measure in between at different times during the parasites presence in this environment. The composition of the urine, proportion of organic and inorganic ions and anaerobic state in the renal sacs can change across a temporal scale and between cephalopod species, and may differ depending on the health and nutrient state of the host individual (Schipp et al., 1975). Therefore, dicyemids must be able to regulate between these changes through time to ensure survival in their specific host species, with the mechanism/s responsible for adaption also needing to be dynamic, which could explain observed heterogeneity in amino acid sequence. A similar finding is presented for the acidithiobacilli, with correlations between genetic polymorphism of *Acidithiobacillus* strains and the microenvironment (acting as the selective pressure) from which the strains were isolated (Ni et al., 2008). As *COI*, together with *COII* and *COIII*, encodes the respiratory complex IV involved in oxidative phosphorylation (Gray, 2012), the anaerobic state within the renal sac of a host species may be the selective pressure responsible for the observed sequence heterogeneity in *COI* between dicyemid species.

4.2 Non-coding region: size and sequence content among and within dicyemid species, stem-loop structures and origin of replication

For dicyemid parasites, the non-coding region sequence and length varied greatly between species and even within species, so much so that this portion could not be aligned among dicyemid species and had to be excluded from our phylogenetic analyses. High sequence polymorphism and large size range differences in the non-coding region has also been reported for lice species, with a non-coding region of 48 bp in *Coloceras* compared to 2,050 bp in *Pediculus* (Shao et al., 2009; Cameron et al., 2011).

While the gene coding region defines genome functionality by specifying proteins, the non-coding region can define the architecture and regulation of the genome, often harboring the replication origin and the promoters for transcription (Le et al., 2002; Burger et al., 2012).

Although no tRNA molecules were found in any of the non-coding regions of the nine dicyemid species we characterised, palindrome sequences with the potential to form stem-loop structures were identified in five species. Palindromic sequences have been reported in the mtDNA of various other taxa before, with roles including mediating lateral gene transfer, mt recombination, chromosomal rearrangements and transcript stability (Burger et al., 2012). Palindrome regions may be involved in similar biological processes in dicyemid parasites, or the stem-loop stricture associated with them could function as the minicircle replication origin. Watanabe et al. (1999) reported one stem-loop structure in the non-coding region of the *D. misakiense COI* minicircle, although this becomes part of the 5′ region of the *COI* sequence with our reinterpretation of the structure of this minicircle. This highlights the need to be able to identify the gene initiation site of minicircle molecules with confidence, as incorrect assignments could lead to false assumptions in regards to the function of minicircle components.

4.3 Evolutionary considerations

Cytoplasmic organellar genome minicircle molecules represent a rare genome structure among the eukaryotes, therefore it is interesting to consider the factors that may have given rise to their evolution (Cameron et al., 2011). The typical mt genome, whereby all the genes are linked together on a chromosome, ensures the complete genetic information is passed on to the next generation, so why does mt fragmentation and minicircle molecules occur in some organisms? One explanation could be to increase protein synthesis output, as having single or only a few genes on small minicircles could allow for faster replication and transcription. Although Shao et al. (2009) suggests this alone does not appear to be sufficient enough to lead to chromosomal fragmentation. Another reason could be to increase sequence diversity through minicircle recombination events which could provide individuals with a selective advantage over others. Modification, acquisition, deletion and/or rearrangement of genetic information is thought to play a role in the evolution of organisms (Eichler and Sankoff, 2003). But this all relies on the minicircle being able to replicate so these advantageous changes can be passed on to the next generation. As the replication capability of dicyemid minicircles is unclear, there may be other reasons for their occurrence in these organisms. Awata et al. (2005) has also reported a canonical mt genome in the germ line of *D. japonicum* which they suggest represents the primary form of the genome whereas mt

minicircles represent a terminally differentiated form. By analysing the entire dicyemid mt genome, we may be able to elucidate why minicircle molecules are part of the genome organisation and what their overall function is.

4.4 First phylogenetic analyses of dicyemids

Currently the classification of dicyemid parasites and designation of new species is based purely on morphological characters. In general, the number and orientation of cells in each tier of the calotte, the presence or absence of abortive axial cells and the presence or absence of syncytial stages determines the genus, whereas the size of the adult stages, the number of cells comprising the body, the shape of the calotte, the anterior extension of the axial cell, the presence or absence of verruciform cells and the structure of the infusoriform embryo are characters used to distinguish species (Hochberg, 1982, 1983). Unfortunately, incomplete and information-poor descriptions, loss of type specimens, errors in taxonomy and conceptual differences has led to confusion over the validity of 20% of the taxa within this group (Catalano, 2012). In comparison, molecular genetic studies on dicyemid parasites are scarce. Our present study represents the first phylogenetic analysis of dicyemid parasites from the two genera that contain over 90% of the nominal species, and can be used to evaluate the accuracy of classifying dicyemid species based on the morphological traits listed above. In general, classifications based on morphological traits were supported by our molecular findings, as species clades were monotypic with strong support in both the ML and BI analyses (Fig. 3a, b). However, three unusual occurrences were observed: (1) the placement of *Dicyema misakiense* as the sister lineage to the *Dicyemennea* and *Dicyema* (clades I and II) in the BI analysis with strong support (Fig. 3b); (2) the placement of *Dicyema* sp. 1 in the *Dicyemennea* (clade II) in both analyses (Fig. 3a, b); and (3) nesting of the *D. multimegalum* haplotypes within the *D. coffinense* clade in the ML analysis (Fig. 3a inset).

Renal appendage smears of *Dicyema* sp. 1 were re-evaluated and the observation of four metapolar cells opposite four propolar cells in the calotte was confirmed supporting the placement of this species in *Dicyema* following the classical morphological approach (note that a defining character of species placed in *Dicyemennea* is the occurrence of five metapolar cells in their calotte). Therefore the placement of *Dicyema* sp. 1 in the *Dicyemennea* clade in both analyses, along with the basal placement of *D. misakiense* in the

BI analysis with strong support, suggests certain morphological traits, namely calotte cell counts, may not be an adequate character to classify dicyemids at the generic level. Phylogenetic analyses including taxa from all the suggested genera and inclusion of additional molecular markers are needed to further address this morphological discrepancy of what defines a distinct taxonomic species. As such, a complete revision of this phylum may be necessary.

The nesting of *D. multimegalum* within the *D. coffinense* clade for the ML analysis (Fig. 3a inset) suggests these two dicyemids are the same species, although the BI analysis does support monotypic clades (Fig. 3b inset). These two species were classed as distinct species based on differences in morphological characters, with variation in calotte shape (elongate metapolar cells vs compressed metapolar cells), maximum body length of adults (2,920 μm vs 1,672 μm), peripheral cell counts (30–32 vs 26–28) and maximum length of infusoriform embryos (31.3 μm vs 40 μm) for *D. coffinense* compared to *D. multimegalum*, respectively (Catalano, 2013a). Both these dicyemid species are recorded from the same host species, *S. apama*, although at different geographical localities, where sizes of the host are also markedly different; host mantle length range 11.7–20.7 cm at Coffin Bay, South Australia (infection by *D. coffinense*), whereas 38–49.5 cm at Cronulla and North Bondi, New South Wales (infection by *D. multimegalum*) (Catalano, 2013a). Therefore two potential outcomes can be inferred from our results; either *D. coffinense* and *D. multimegalum* are one taxon exhibiting morphological plasticity, or they are each distinct taxonomic entities with morphological variation reflecting adaptation to differing host environments. Examining the divergence in *COI* haplotype sequences between these two dicyemid species in a phylogeographic study may shed further light on whether these taxa represent separate species.

4.5 Conclusions

This study presents the first phylogeny of dicyemid parasites for an assessment of generic and species classification outside of the classical, morphological approach. The relationships between taxa in our analyses indicate that the morphological traits used to classify dicyemids may be in need of revision. Future studies should focus on including additional dicyemid taxa representative of all genera into this phylogeny, and also including further molecular markers. This may provide added support for a revision of the current

morphological characters that are used to distinguish between, and designate, dicyemid parasites into genera and species.

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References

Armstrong, M.R., Blok, V.C., Phillips, M.S., 1999. A multipartitie mitochondrial genome in the potato cyst nematode *Globodera pallida*. Genetics. 154, 181–192.

Aruga, J., Odaka, Y.S., Kamiya, A., Furuya, H., 2007. *Dicyemid* Pax6 and Zic: tool-kit genes in a highly simplified bilaterian. BMC Evol. Biol. 7, 1–16.

Awata, H., Noto, T., Endoh, H., 2005. Differentiation of somatic mitochondria and the structured changes in mtDNA during development of the dicyemid *Dicyema japonicum* (Mesozoa). Mol. Genet. Genomics. 273, 441–449.

Barbrook, A.C., Symington, H., Nisbet, R.E.R., Larkum, A., Howe, C.J., 2001. Organisation and expression of the plastid genome of the dinoflagellate *Amphidinium operculatum*. Mol. Genet. Genomics. 266, 632–638.

Boore, J.L., 1999. Animal mitochondrial genomes. Nucleic Acids Res. 27, 1767–1780.

Burger, G., Gray, M.W., Lang, B.F., 2003. Mitochondrial genomes: anything goes. Trends Genet. 19, 709–716.

Burger, G., Jackson, C.J., Waller, R.F., 2012. Unusual mitochondrial genomes and genes. In: Bullerwell, C.E. (Ed.), Organelle Genetics: Evolution of Organelle Genomes and Gene Expression. inger Berlin Heidelberg, pp. 41–77.

Cameron, S.L., Yoshizawa, K., Mizukoshi, A., Whiting, M.F., Johnson, K.P., 2011. Mitochondrial genome deletions and minicircles are common in lice (Insecta: Phthiraptera). BMC Genomics. 12, 1–15.

Cantatore, P., Roberti, M., Rainaldi, G., Gadaleta, M.N., Saccone, C., 1989. The complete nucleotide sequence, gene organization, and genetic code of the mitochondrial genome of *Paracentrotus ziuidus*. J. Biol. Chem. 264, 10965–10975.

Catalano, S.R., 2012. A review of the families, genera and species of Dicyemida Van Beneden, 1876. Zootaxa. 3479, 1–32.

Catalano, S.R., 2013a. Five new species of dicyemid mesozoans (Dicyemida: Dicyemidae) from two Australian cuttlefish species, with comments on dicyemid fauna composition. Syst. Parasitol. 86, 125–151.

Catalano, S.R., 2013b. First descriptions of dicyemid mesozoans (Dicyemida: Dicyemidae) from Australian octopus (Octopodidae) and cuttlefish (Sepiidae) species, including a new record of *Dicyemennea* in Australian waters. Folia Parasit. 60, 306–320.

Catalano, S.R., Furuya, H., 2013. Two new species of dicyemid (Dicyemida: Dicyemidae) from two Australian cephalopod species: *Sepioteuthis australis* (Mollusca: Cephalopoda: Loliginidae) and *Sepioloidea lineolata* (Mollusca: Cephalopoda: Sepiadariidae). J. Parasitol. 99, 203–211.

Catalano, S.R., Whittington, I.D., Donnellan, S.C., Gillanders, B.M., in review. Dicyemid fauna composition and infection patterns in relation to cephalopod host biology and ecology: exploring dicyemids as natural tags. Folia Parasit.

Drummond, A.J., Ashton, B., Buxton, S., Cheung, M., Cooper, A., Heled, J., Kearse, M., Moir, R., Stones-Havas, S., Sturrock, S., Thierer, T., Wilson, A., 2010. Geneious v5.3, Available from <http://www.geneious.com>.

Eichler, E.E., Sankoff, D., 2003. Structural dynamics of eukaryotic chromosome evolution. Science. 301, 793–797.

Emanuel, C.F., Martin, A.W., 1956. The composition of octopus renal fluid: I. Inorganic constituents. Zeitsehrift fiir vergleichende Physiologie. 39, 226–234.

Faller, M., 1999. EMBOSS: Palindrome. <http://emboss.bioinformatics.nl/cgi-bin/emboss/palindrome>.

Furuya, H., Hochberg, F.G., Tsuneki, K., 2003. Reproductive traits in dicyemids. Mar. Biol. 142, 693–706.

Gibson, T., Farrugia, D., Barrett, J., Chitwood, D.J., Rowe, J., Subbotin, S., Dowton, M., 2011. The mitochondrial genome of the soybean cyst nematode, *Heterodera glycines*. Genome. 54, 565–574.

Gray, M.W., 2012. Mitochondrial evolution. Cold Springs Harbour Perspectives in Biology. 4, 1–16.

Guindon, S., Gascuel, O., 2003. A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52, 696–704.

Guindon, S., Dufayard, J.F., Lefort, V., Anisomova, M., Hordijk, W., Gascuel, O., 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. 59, 307–321.

Harrison, F.M., Martin, A.W., 1965. Excretion in the cephalopod, *Octopus dofleini*. J. Exp. Biol. 42, 71–98.

Hasegawa, M., Kishino, H., Yano, T., 1985. Dating of the human ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Ecol. 22, 160–174.

Hochberg, F.G., 1982. The "kidneys" of cephalopods: a unique habitat for parasites. Malacologia. 23, 121–134.

Hochberg, F.G., 1983. The parasites of cephalopods: a review. Mem. Nat. Mus. Vic. 44, 108–145.

Jiang, H., Barker, S.C., Shao, R., 2013. Substantial variation in the extent of mitochondrial genome fragmentation among blood-sucking lice of mammals. Genome Biol. Evol.

Kobayashi, M., Furuya, H., Holland, P.W.H., 1999. Dicyemids are higher animals. Nature. 401, 762.

Kobayashi, M., Furuya, H., Wada, H., 2009. Molecular markers comparing the extremely simple body plan of dicyemids to that of lophotrochozoans: insight from the expression patterns of *Hox*, *Otx*, and *brachyury*. Evol. Dev. 11, 582–589.

Lanfear, R., Calcott, B., Ho, S.Y.W., Guindon, S., 2012. PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. Mol. Biol. Evol. 29, 1695–1701.

Laslett, D., Canback, B., 2004. ARAGORN, a program to detect tRNA genes and tmRNA genes in nucleotide sequences. Nucleic Acids Res. 32, 11–16.

Laslett, D., Canback, B., 2008. ARWEN, a program to detect tRNA genes in metazoan mitochondrial nucleotide sequences. Bioinformatics. 24, 172–175.

Le, T.H., Blair, D., McManus, D.P., 2002. Mitochondrial genomes of parasitic flatworms. Trends Parasitol. 18, 206–213.

Littlewood, D.T.J., Cribb, T.H., Olson, P.D., Bray, R.A., 2001. Platyhelminth phylogenetics-a key to understanding parasitism? Belg. J. Zool. 131, 35–46.

Lowe, T.M., Eddy, S.R., 1997. tRNAscan-SE: A program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res. 25, 955–964.

Marande, W., Lukes, J., Burger, G., 2005. Unique mitochondrial genome structure in diplonemids, the sister group of kinetoplastids. Eukaryot. Cell. 4, 1137–1146.

McConnaughey, B.H., 1951. The life cycle of the dicyemid Mesozoa. Univ. Calif. Publ. Zool. 55, 295–336.

McManus, D.P., Bowles, J., 1996. Molecular genetic approaches to parasite identification: their value in diagnostic parasitology and systematics. Int. J. Parasitol. 26, 687–704.

Milbury, C.A., Gaffney, P.M., 2005. Complete mitochondrial DNA sequence of the eastern oyster *Crassostrea virginica*. Mar. Biotechnol. 7, 697–712.

Morrison, D.A., 2010. How and where to look for tRNAs in Metazoan mitochondrial genomes, and what you might find when you get there. Online Archive arXiv.org, Cornell University Library, <arXiv:1001:3813v1>.

Ni, Y.Q., He, K.Y., Bao, J.T., Wan, D.S., Lui, H.Y., 2008. Genomic and phenotypic heterogeneity of *Acidithiobacillus* spp. strains isolated from diverse habitats in China. FEMS Microbiol. Ecol. 64, 248–259.

Potts, W.T.W., Todd, M., 1965. Kidney function in the octopus. Comp. Biochem. Physiol. 16, 479–489.

Potts, W.T.W., 1967. Excretion in the molluscs. Biol. Rev. 42, 1–41.

Poulin, R., Morand, S., 2000. The diversity of parasites. Q. Rev. Biol. 75, 277–293.

Prescott, D.M., 1994. The DNA of ciliated protozoa. Microbiol. Rev. 58, 233–267.

Rambaut, A., Drummond, A.J., 2007. Tracer v1.4. <http://beast.bio.ed.ac.uk/Tracer>.

Rambaut, A., 2010. FigTree, version 1.3.1 Institute of Evolutionary Biology, University of Edinburgh. <http://tree.bio.ed.ac.uk/software/figtree/>.

Rodŕiguez, F., Oliver, J.F., Mańin, A., Medina, J.R., 1990. The general stochastic model of nucleotide substitutions. J. Theor. Biol. 142, 485–501.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., H□hna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61, 539–542.

Ryan, K.P., Shapiro, T.A., Rauch, C.A., Englund, P.T., 1988. Replication of kinetoplast DNA in trypanosomes. Annu. Rev. Microbiol. 42, 339–358.

Schipp, R., von Boletzky, S., Doell, G., 1975. Ultrastructural and cytochemical investigations on the renal appendages and their concrements in dibranchiate cephalopods (Mollusca, Cephalopoda). Zeitschrift für Morphologie der Tiere. 81, 279–304.

Shao, R., Kirkness, E.F., Barker, S.C., 2009. The single mitochondrial chromosome typical of animals has evolved into 18 minichromosomes in the human body louse, *Pediculus humanus*. Genome Res. 19, 904–912.

Shao, R., Zhu, X.Q., Barker, S.C., Herd, K., 2012. Evolution of extensively fragmented mitochondrial genomes in the lice of humans. Genome Biol. Evol. 4, 1088–1101.

Shapiro, T.A., Englund, P.T., 1995. The structure and replication of kinetoplast DNA. Annu. Rev. Microbiol. 49, 117–143.

Stuart, K., Feagin, J.E., 1992. Mitochondrial DNA of kinetoplastids. Int. Rev. Cytol. 141, 65–87.

Suzuki, T.G., Ogino, K., Tsuneki, K., Furuya, H., 2010. Phylogenetic analysis of dicyemid mesozoans (Phylum Dicyemida) from innexin amino acid sequences: dicyemids are not related to Platyhelminthes. J. Parasitol. 96, 614–625.

Watanabe, K.I., Bessho, Y., Kawasaki, M., Hori, H., 1999. Mitochondrial genes are found on minicircle DNA molecules in the mesozoan animal *Dicyema*. J. Mol. Biol. 286, 645–650.

Zhang, Z., Green, B.R., Cavalier-Smith, T., 1999. Single gene circles in dinofagellate chloroplast genomes. Nature. 400, 155–159.

Zhang, Z., Cavalier-Smith, T., Green, B.R., 2002. Evolution of dinoflagellate unigenic minicircles and the partially concerted divergence of their putative replicon origins. Mol. Biol. Evol. 19, 489–500.

CHAPTER 9: Parasite mitochondrial phylogeography supports cephalopod population structure

Line fishing for *Sepia apama* samples to examine for dicyemid parasites at Coffin Bay, South Australia, Australia (photo: Kate Hutson).

Chapter 9 Preamble

This chapter is a manuscript that will be submitted to *Evolutionary Applications* with Terry Bertozzi, Ian Whittington, Steve Donnellan and Bronwyn Gillanders as co-authors. It is formatted according to the instructions to authors for this journal, and provides support for population structuring of *Sepia apama* (giant Australian cuttlefish) in southern Australian waters, as inferred from dicyemid parasite genetics.

In this chapter, all co-authors and I assisted with the molecular design and planning. I performed the molecular experiments and the results were analysed by Terry Bertozzi, Steve Donnellan and myself. I wrote the manuscript and acted as corresponding author. All co-authors provided feedback on manuscript drafts.

I certify that the statement of contribution is accurate

Signed:_____ Date:_____

(Sarah Roseann Catalano)

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis

Professor Bronwyn Gillanders Professor Steve Donnellan

A/Professor Ian Whittington Dr Terry Bertozzi

Catalano, S.R., Whittington, I.D., Donnellan, S.C., Bertozzi, T. & Gillanders, B.M. (Submitted) Parasite mitochondrial phylogeography supports cephalopod population structure. *Evolutionary Applications.*

CHAPTER 10: Parasites as biological tags to assess host population structure: guidelines, recent genetic advances and comments on a holistic approach

Examining dicyemid type material at the Santa Barbara Museum of Natural History, California, USA (photo: Daniel Geiger).

Chapter 10 Preamble

This chapter is an invited review manuscript that is in press and will appear in the next issue of *International Journal for Parasitology: Parasites and Wildlife.* It is co-authored with Ian Whittington, Steve Donnellan and Bronwyn Gillanders, and explores the use of parasites as biological tags to assess the population structure of marine organisms. It can be cited as: Catalano, S.R., Whittington, I.D., Donnellan, S.C. and Gillanders, B.M., 2013. Parasites as biological tags to assess host population structure: guidelines, recent genetic advances and comments on a holistic approach. *International Journal for Parasitology: Parasites and Wildlife.* The copyright and open access agreement for this manuscript is provided in Appendix A.

In this chapter, I searched through and collated information from the past literature. I wrote the manuscript and acted as corresponding author. All co-authors provided suggestions, comments and feedback on manuscript drafts.

I certify that the statement of contribution is accurate

Signed:_____ Date:_____

(Sarah Roseann Catalano)

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the paper in the thesis

Professor Bronwyn Gillanders Professor Steve Donnellan

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Invited Review

Parasites as biological tags to assess host population structure: Guidelines, recent genetic advances and comments on a holistic approach ☆

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ABSTRACT

We review the use of parasites as biological tags of marine fishes and cephalopods in host population structure studies. The majority of the work published has focused on marine fish and either single parasite species or more recently, whole parasite assemblages, as biological tags. There is representation of host organisms and parasites from a diverse range of taxonomic groups, although focus has primarily been on host species of commercial importance. In contrast, few studies have used parasites as tags to assess cephalopod population structure, even though records of parasites infecting cephalopods are well-documented. Squid species are the only cephalopod hosts for which parasites as biological tags have been applied, with anisakid nematode larvae and metacestodes being the parasite taxa most frequently used. Following a brief insight into the importance of accurate parasite identification, the population studies that have used parasites as biological tags for marine fishes and cephalopods are reviewed, including comments on the dicyemid mesozoans. The advancement of molecular genetic techniques is discussed in regards to the new ways parasite genetic data can be incorporated into population structure studies, alongside host population genetic analyses, followed by an update on the guidelines for selecting a parasite species as a reliable tag candidate. As multiple techniques and methods can be used to assess the population structure of marine organisms (e.g. artificial tags, phenotypic characters, biometrics, life history, genetics, otolith microchemistry and parasitological data), we conclude by commenting on a holistic approach to allow for a deeper insight into population structuring.

Contents

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1. Introduction

Determination of the biological identity of a population of marine organisms (for this review, limited to fishes and cephalopods), in relation to neighbouring populations of the same species, is a vital prerequisite in studying the biology, dynamics, interactions and ecological consequences of exploitation on that population (MacKenzie and Abaunza, 1998). This is particularly important given the rise in global fisheries as more species are being targeted and commercially exploited to keep up with increases in demand (Pierce and Guerra, 1994; Evans and Grainger, 2002). Marine species considered at risk as a result of overfishing, evident from declines in biomass and abundance, emphasise the importance of understanding the structure of populations across geographical distributions (Melendy et al., 2005; McClelland and Melendy, 2007). As alluded to already, before a stock can be efficiently managed and policies implemented for future sustainability, the stock needs to be correctly identified (Oliva and Sanchez, 2005).

Many techniques have been used to identify and discriminate stocks, including the application of artificial tags, such as acoustic tags, coded wire tags, passive integrated transponder tags and archival tags. Artificial tags are generally suitable for many species and sizes of organisms, with an added advantage of enabling discrete recognition of each tagged individual (Gillanders, 2009). However, they can be limited in signal detection range and retention over long term studies, with further uncertainties about the influence of the tag on the organism's behaviour and survivorship (Moser, 1991; Mosquera et al., 2003; Gillanders, 2009). Natural tags, including phenotypic characters (meristic, morphometric and life history traits), otolith chemistry, molecular genetic host markers and parasites, have also been used in population structure studies. In particular, parasites as biological tags have gained wide acceptance in recent decades (MacKenzie, 2002; Poulin and Kamiya, in press), as they can provide a reliable guide to understanding the biology of their host (Pascual and Hochberg, 1996). This is not to say parasites as tags are superior to other methods, but it is recognised that they have helped answer questions on host diet and feeding behaviour, movements and ranges, connectivity of stocks, recruitment patterns of juveniles and phylogenies (Sindermann, 1961; Moser, 1991; Williams et al., 1992; Criscione et al., 2006). Parasites have also been used as bio-indicators of pollution (Poulin, 1992; MacKenzie et al., 1995; MacKenzie, 1999a), and in population studies to discriminate stocks (MacKenzie, 1987, 2002; Lester, 1990; MacKenzie and Abaunza, 1998; Mosquera et al., 2003). Research on parasites as biological tags for marine organisms has increased at a steady rate, with nine papers on this subject published from the 1950s, more than 30 from the 1960s, more than 50 from the 1970s and more than 140 from the 1980s (Williams et al., 1992). Here, we focus on the use of parasites as biological tags for host population discrimination. We use the words 'stock' and 'population' interchangeably in this review, following the definition provided by Charters et al. (2010) of 'a spatially distinct group of marine organisms which exhibit no significant mixing with neighbouring individuals'. In agreement with Lester and MacKenzie (2009), we recognise the idea that this distinct group is essentially self-reproducing.

This review begins by briefly commenting on the importance of accurate parasite identification, followed by a summary of the use of parasites as biological tags in population structure studies of fishes and cephalopods. Due to the advent of molecular genetic technologies, the potential to incorporate genetic analyses of parasite population structure alongside genetic analyses of their host is discussed. An updated list of guidelines for selecting a parasite species as an adequate tag candidate is presented, and we conclude by highlighting the benefits of a multidisciplinary approach when investigating the population structure of marine organisms.

2. Parasite identification

Along with the need to correctly identify a stock before it can be appropriately managed, parasites also need to be correctly identified before they can be applied as biological tags. We add the caveat that in some cases the minimum necessary identification would be to discriminate each of the parasite species present without the further and potentially time consuming requirement of assigning scientific names. Classical methods commonly used for parasite taxonomic identification involve examining and measuring morphological character traits and using taxonomic keys to define a particular family, genus or species (Baldwin et al., 2012). Although widely used and relatively inexpensive, this form of identification can be difficult for larval stages and further hindered by poor specimen quality and taxonomic uncertainty in the literature. "Species" that exhibit a high level of morphological plasticity also pose a problem (Poulin and Morand, 2000). On one hand, several distinct species may be mistakenly identified as one, or a single morphologically plastic taxon may be interpreted as a species complex inferring significant host population structure.

Another approach to identify parasite species is to use molecular genetic methods (McManus and Bowles, 1996). Indeed, once a sound molecular genetic framework has been established for the species concerned, then higher throughput bar-coding can be applied to much larger sample sets. Another advantage of this approach would be that all stages of the parasite life cycle that could be sampled can be included, potentially increasing the matching parasite data for a larger number of host individuals collected over a longer period of the year. A combination of morphological and molecular genetic methods may therefore be more robust for identifying and discriminating parasite taxa, and should be considered in future studies using parasites as biological tags.

3. Parasites as biological tags in population studies of fishes

The two earliest records describing the application of parasites as biological tags in population studies of fishes are that of Dogiel and Bychovsky (1939), who distinguished between groups of sturgeon (*Acipenser* spp.) in the Caspian Sea using the monogenean parasites *Diclybothrium circularis* and *Nitzschia sturionis*, and Herrington et al. (1939), who examined redfish (*Sebastes marinus*) in the Gulf of Maine and suggested the existence of separate populations based on variations in infection levels of the parasitic copepod *Sphyrion lumpi*. Since these investigations over 70 years ago, the use of parasites as biological tags in population structure studies has flourished to include a wide range of fish species and geographical localities. Investigations have primarily focused on, although not limited to, fish species of economic importance, such as herring (e.g. Sindermann, 1961; Parsons and Hodder, 1971; Arthur and Arai, 1980; Moser and Hsieh, 1992), hake (e.g. MacKenzie and Longshaw, 1995; George-Nascimento, 1996; Mattiucci et al., 2004; Sardella and Timi, 2004), cod (e.g. Hemmingsen and MacKenzie, 2001; McClelland and Melendy, 2011), rockfish (e.g. Stanley et al., 1992; Moles et al., 1998; Oliva and Gonzalez, 2004) and hoki (e.g. MacKenzie et al., 2013). A diverse range of taxonomic groups of parasites have also been applied as biological tags (see Table 1 in Williams et al., 1992). In particular, parasites have been used for discovering multiple species in supposedly single species fisheries (e.g. Smith et al., 1981; George-Nascimento, 1996), for discriminating stocks within single species fisheries (e.g. Hemmingsen et al., 1991; Braicovich and Timi, 2008; Henriquez et al., 2011) and for

recognising single stocks from multiple breeding populations (e.g. Moser and Hsieh, 1992). Recently, Poulin and Kamiya (in press) performed a meta-analysis to examine the discriminatory power of using parasites to discriminate fish stocks, and found that overall, the probability of correct classification of fish to their group of origin based on parasite data was double that expected by chance alone, supporting the use of parasites as biological tags.

The benefits and limitations of using parasites as biological tags has been extensively reported by Sindermann (1961, 1983), Gibson (1972), MacKenzie (1987, 1999b, 2002), Lester (1990), Moser (1991), Williams et al. (1992), Pascual and Hochberg (1996) and Mosquera et al. (2003), and thus will not be repeated here. The use of parasites as biological tags in population structure studies has also been reviewed by many authors (Sindermann, 1983; MacKenzie, 1987; Lester, 1990; Williams et al., 1992), with a guide to the procedures and methods provided by MacKenzie and Abaunza (1998). The most recent reviews of parasites as biological tags in fish population studies are given by MacKenzie (1999b, 2002), Mosquera et al. (2003) and MacKenzie and Abaunza (2005). In the past 5 years, numerous studies have been published which used parasites as biological tags as the sole approach to discriminate fish stocks (for example Santos et al., 2009; Timi and Lanfranchi, 2009; Timi et al., 2009; Charters et al., 2010; Luque et al., 2010; Mele et al., 2010; Sequeira et al., 2010; Chou et al., 2011; Garcia et al., 2011; Henriquez et al., 2011; Hutson et al., 2011; Khan et al., 2011; McClelland and Melendy, 2011; Moore et al., 2011; Braicovich et al., 2012; Reed et al., 2012; Costa et al., 2013; MacKenzie et al., 2013; Oliva, 2013).

With this increase in the number of studies using parasites as biological tags to discriminate host stocks, Lester and MacKenzie (2009) provide a word of caution. They highlight that although differences in parasite fauna may be observed from fish collected at different geographical localities, it does not necessarily mean that there are multiple fish stocks, as many parasites are transient and may only be present sometimes. Leading on from this, it is suggested that a deeper insight or more robust conclusions may be gained from using a multidisciplinary approach to determine stock structure, a topic that will be discussed further in Section 7.

4. Parasites as biological tags in population studies of cephalopods, including comments on the dicyemid mesozoans

Over the last 20 years, the value of cephalopods in international commercial fisheries has increased rapidly (Pierce and Guerra, 1994; Pascual et al., 1996). However, cephalopods are highly susceptible to overfishing with little opportunity for recovery, owing to their short life spans, variable growth rates and semelparous breeding strategies (Pierce and Guerra, 1994; Boyle and Boletzky, 1996). Therefore, it is important to be able to recognise stock boundaries to ensure management policies governing commercial cephalopod fisheries are well-informed.

While numerous parasite species from a range of taxonomic groups have been described from cephalopods (Hochberg, 1983, 1990), their application as biological tags in population studies is rare (Pascual and Hochberg, 1996). The first study where parasites were used to examine cephalopod stock structure was performed by Smith et al. (1981). The authors used a multidisciplinary approach of allozyme electrophoresis, host morphology and prevalence of parasites in arrow squid *Nototodarus sloani* from New Zealand waters to assess stock structure, with the combined results supporting the occurrence of two species of arrow squid in these waters. It is doubtful whether the same result would have been concluded if parasites alone were used, as one parasite species did not support stock separation whereas the other did. A few years later, Dawe et al. (1984) addressed the issue of stock discrimination in the short-finned squid *Illex illecebrosus*, also employing a multidisciplinary approach by comparing data on host size, maturity, distribution of early life history stages and incidence of certain parasites. However the parasites examined were of little use as biological tags, as they had a broad geographic distribution, were generalist rather than specialist parasites, and could not be identified to species. Later, Bower and Margolis (1991) and Nagasawa et al. (1998) examined the helminth parasites of the flying squid, *Ommastrephes bartrami*, in the North Pacific Ocean. Bower and Margolis (1991) suggested that parasites may be useful tools in determining the stock structure of the flying squid, and Nagasawa et al. (1998) statistically tested parasite intensity of infection among collection localities to lend support to the occurrence of four flying squid stocks in these waters. The most recent study that has used parasites of cephalopods as biological tags is by González and Kroeck (2000). They studied the parasite fauna composition of shortfin squid *Illex argentinus* in San Matías Gulf, southwest Atlantic, with differences in composition, prevalence and mean intensity of enteric parasites between localities lending support to stock structuring.

An additional group of parasites, dicyemid mesozoans, have been suggested as potential tag candidates to help discriminate cephalopod stock structure (Hochberg, 1990; Catalano, 2013). These parasites are simple in morphology, highly host-species specific and found with high intensity in the renal appendages of almost all benthic cephalopod species examined to date (Furuya, 1999; Furuya et al., 2004). The use of dicyemid parasites as biological tags for cephalopod stock discrimination has been tested, with significant difference in dicyemid fauna composition between cephalopod species, and among cephalopod individuals of the same species collected from different geographical localities (Catalano et al., unpublished). However it must be highlighted that confusion exists in the literature on the validity of certain taxa within this phylum along with the morphological traits used to delineate species boundaries (Catalano, 2012). Nonetheless, by incorporating a molecular genetic framework, and comparing results between dicyemid parasite and host genetic analyses, this approach may still prove valuable in assessing cephalopod population structure beyond any single approach.

5. Recent genetic advances

Beverley-Burton (1978) was the first to use genetic analyses of parasite populations as a tool for host stock identification. The frequencies of different acid phosphatase allozymes in the larval nematode *Anisakis simplex* suggested that there may be two distinct groups of Atlantic salmon in the Atlantic Ocean. Other authors have used genetic methods (multilocus allozyme electrophoresis) to identify *Anisakis* larvae to species level, then by evaluating the relative proportions of these nematodes across sampling localities, recognised multiple discrete host stocks (Mattiucci et al., 2004, 2008).

In recent years there have been major technological advances in the field of molecular genetics, providing the ability to sequence multiple markers or whole genomes in a short time span with low costs, e.g. next generation sequencing (Schuster, 2007; Mardis, 2008; Quail et al., 2012). In fisheries science, multiple molecular markers such as allozymes, mitochondrial DNA, microsatellite and minisatellite loci, random amplified polymorphic DNA (RAPD) and single nucleotide polymorphisms (SNPs), have all been used to analyse stock structure of marine organisms directly (Carvalho and Hauser, 1994; Thorpe et al., 2000; Baldwin et al., 2012; Ovenden et al., 2013). This has proven useful for deep-sea species where tag-recapture techniques are difficult to apply (e.g. Roques et al., 2002; Friess and Sedberry, 2011; Varela et al., 2013). As candidate

molecular markers that are under selection can now be identified and genotyped readily, the capability also exists to assess genetic differences between recently diverged groups or between incompletely isolated groups, which was otherwise problematic with neutral molecular markers (Swain et al., 2005; Lamichhaney et al., 2012). These advances have subsequently helped to unveil previously unrecognised patterns of geographic genetic structure in marine organism (Sala-Bozano et al., 2009).

What is yet to be realised in any substantive way is the application of these new molecular genetic technologies, with high throughput and increased resolution, to parasitological studies of host population structure. Additional layers of information may be gained by contrasting the genetic population structure of parasite and host not just for determining host population structure but details of the hosts population biology (e.g. Pacific sardines – Baldwin et al. 2012). In the one example that we can find where the same class of high resolution population markers (microsatellites) were used in both parasite and host, Criscione et al. (2006) found that trematode parasite genetic structure identified source populations of host steelhead trout (*Oncorhynchus mykiss*) with four times more accuracy than the host's own genotype. This finding highlights how differences in host and parasite environmental tolerances, population size and connectivity influence their rates of population differentiation. We are not suggesting skipping over host genetics to assess population structure, but instead advocating the inclusion of parasite genetics into these studies for what may provide an additional line of confirmatory evidence or new insights into host structuring. As with any molecular genetic study, it is important to recognise that the results will depend on, and may differ, with the type of molecular genetic markers used, the number of loci examined, the geographical scope of the study, the number of fish sampled and the population biology of the parasite taxa examined (Baldwin et al., 2012). To boost the analytical power of these genetic analyses, it is recommended to increase the sample size, the number of molecular markers and loci used, and the number of parasite taxa included (Ovenden et al., 2013).

6. Guidelines for selecting an ideal parasite species as a tag candidate

According to Thorrold et al. (2002), the properties of a general tag, including artificial and environmental tags, genetic markers and parasites, should have the following characteristics:

1) Retention of the tag over an appropriate length of time.
2) No effect of the tag on the marked organism (invisible to predators, non-toxic, no effect on growth or survival).
3) Ability to mark a large number of individuals in a cost-effective manner.
4) Be relatively quick and inexpensive to detect.

In addition to these characteristics for tags in general, there are also a number of specific guidelines presented in the literature which highlight the desirable requirements a parasite species should have in being considered as a biological tag candidate. For example, the following are taken from Sindermann (1961, 1983), MacKenzie (1987, 1999b), Lester (1990), Moser (1991), Williams et al. (1992) and MacKenzie and Abaunza (1998):

1) The parasite species should have different levels of infection in the host at different geographical locations.
2) The life cycle of the parasite species should preferably involve only a single host as more information is needed on the biotic and abiotic factors influencing transmission between hosts for those parasite species with multi-host life cycles.
3) The life span of the parasite species in the host needs to cover the duration of the investigation as a minimum.
4) The prevalence of the parasite species should remain relatively stable between seasons and years.
5) The parasite species should be easily detected, preferably by gross examination.
6) The parasite species should have no effects on the behaviour or survival of the host.

It is wise to acknowledge that these guidelines are just that, recommendations rather than set rules. A single parasite species would rarely have all of these attributes, so compromises usually have to be made (Sindermann, 1983; MacKenzie and Abaunza, 1998). For instance, in some cases, anisakids and trypanorhynchs, parasites that require at least three host species to complete their life cycle (contravening guideline 2 above), have been found to be the best tag candidates (Boje et al., 1997; MacKenzie and Abaunza, 1998; Timi, 2007; Chou et al., 2011). Additionally, the use of several different parasites and even whole parasite assemblages as tags may be more reliable than using a single species, as a greater number of the guidelines may be met to yield a more complete assessment of host population structure (Timi, 2003, 2007; Sardella and Timi, 2004; MacKenzie and Abaunza, 2005). Note that if this approach is selected, only parasite species highlighted as permanent (recognisable for most of the life of the host) should be considered (Lester and MacKenzie, 2009).

7. Holistic approach to discriminate population structure of marine organisms

Rather than focusing on only a single approach to discriminate fish stocks, it may be of greater benefit to consider incorporating data across disciplines, as different stock identification approaches have different levels of sensitivities (Waldman, 2005). Meristics, parasite data and microsatellite markers can be used to detect differences that have arisen in the recent past, whereas other techniques which are more conservative, such as allozymes and coding DNA, require longer periods of isolation for differences to become recognisable (Cadrin, 2011). Therefore by combining approaches across disciplines, a more robust baseline is created and greater confidence in the observed result is gained (Cadrin, 2010). For example, Zischke et al. (2013) used morphometric measurements of 12 fixed anatomical characters and variation in parasite abundance of seven species to examine the stock structure of wahoo *Acanthocybium solandri* collected in three regions, with the results from both analyses complementing one another in stock boundary estimates. For future studies examining the global stock structure of wahoo, they suggest incorporating additional techniques such as otolith microchemistry and genetic microsatellites. In another study, life history data (age at first maturity, size structure and growth patterns), otolith microchemistry and parasite fauna composition were used to distinguish stocks of southern blue whiting *Micromesistius australis* between two main spawning grounds in the southwest Atlantic Ocean and southeast Pacific Ocean (Arkhipkin et al., 2009; Niklitschek et al., 2010). This contrasted with the results of earlier genetic studies based on mitochondrial DNA haplotype frequencies, which did not detect any significant differences between these areas (Shaw, 2003, 2005). More recently, Baldwin et al. (2012) reviewed the use of fish morphometrics, artificial tags, fish genetics, parasite genetics and parasites as biological tags to identify subpopulations of marine fishes and affirmed the merits of a holistic approach, integrating data from fish and parasite based techniques (both community and genetic), to resolve stock struc-

turing. Other authors also express support for using complementary methods from a broad spectrum, with different ecological and evolutionary characteristics, to provide a comprehensive picture of the population structure of marine organisms (Begg and Waldman, 1999; Cadrin et al., 2005; Sala-Bozano et al., 2009).

A multidisciplinary approach also provides additional techniques available for use in subsequent stock analysis studies (Cadrin, 2011). For example, Roques et al. (2002) used eight microsatellite loci initially to identify fish stocks of the western group of deepwater redfish *Sebastes marinum*, with these stocks subdivided into four smaller groups in a subsequent study that used parasite species prevalence data (Marcogliese et al., 2003). Early research on the winter flounder stock structure primarily focused on migration, life history rates and analysis of meristic characters, however over time, genetic analyses, parasite fauna composition, modelling analyses, otolith chemistry and telemetry tagging were incorporated, building on from this initial framework for a more robust and supported insight (Cadrin, 2011). Stock delineation of the Pacific hake *Merluccius productus* has also been assessed with a variety of techniques in different studies, including parasite analyses (Kabata and Whitaker, 1981), otolith morphology (McFarlane and Beamish, 1985), biological parameter estimates (Beamish and McFarlane, 1985; King and McFarlane, 2006) and mitochondrial sequence data (King et al., 2012). In summary, it seems viable that a multidisciplinary approach which integrates data across fields, such as molecular genetics, biometrics, life histories, modelling, otolith microchemistry analyses, artificial tagging studies and parasitological surveys, may provide a deeper and more robust insight into the population structuring of marine organisms in contrast to studies using a sole approach. There is also a need to utilise the recent advancements in these fields as tools to improve our understanding of stock boundaries.

8. Closing remarks

Our review highlights the usefulness of parasites as biological tags in population structure studies of marine organisms. However caution must be taken in selecting the most appropriate tag candidate species, as well as considerations on the number of taxa to include, the method of parasite identification and the way the data are analysed. With the recent advancement of molecular genetic techniques, we highlight the potential to include parasite genetic data alongside host intra-specific molecular genetic data, an area that is currently under-exploited. In particular the use of high resolution neutral markers or loci under selection in both the parasite and host to detect recent demographic driven host population structure is unexplored. As multiple approaches can be used to assess population structure of marine organisms, each with their own benefits and limitations, we ultimately advocate the integration of data from multiple disciplines for a deeper insight into population structuring. Due to the different levels of sensitivity of each method, additional layers of information may be gained or weak inferences may be better assessed using a holistic approach.

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References

Arkhipkin, A.I., Schuchert, P.C., Danyushevsky, L., 2009. Otolith chemistry reveals fine population structure and close affinity to the Pacific and Atlantic oceanic spawning grounds in the migratory southern blue whiting (*Micromesistius australis australis*). Fish. Res. 96, 188–194.

Arthur, J.R., Arai, H.P., 1980. Studies on the parasites of Pacific herring (*Clupea harengus pallasi* Valenciennnes): a preliminary evaluation of parasites as indicators of geographical origin for spawning herring. Can. J. Zool. 58, 521–527.

Baldwin, R.E., Banks, M.A., Jacobson, K.C., 2012. Integrating fish and parasite data as a holistic solution for identifying the elusive stock structure of Pacific sardines (*Sardinops sagax*). Rev. Fish Biol. Fisher. 22, 137–156.

Beamish, R.J., McFarlane, G.A., 1985. Pacific whiting, *Merluccius productus*, stocks off the west coast of Vancouver Island, Canada. Mar. Fish. Rev. 47, 75–81.

Begg, G.A., Waldman, J.R., 1999. An holistic approach to fish stock identification. Fish. Res. 43, 35–44.

Beverley-Burton, M., 1978. Population genetics of *Anisakis simplex* (Nematoda: Ascaridoidea) in Atlantic salmon (*Salmo salar*) and their use as biological indicators of host stocks. Environ. Biol. Fish. 3, 369–377.

Boje, J., Riget, F., Køie, M., 1997. Helminth parasites as biological tags in population studies of Greenland halibut (*Reinhardtius hippoglossoides* (Walbaum)), in the north-west Atlantic. J. Mar. Sci. 54, 886–895.

Bower, S.M., Margolis, L., 1991. Potential use of helminth parasites in stock identification of flying squid, *Ommastrephes bartrami*, in North Pacific waters. Can. J. Zool. 69, 1124–1126.

Boyle, P.R., Boletzky, S.V., 1996. Cephalopod populations: definition and dynamics. Philos. T. R. Soc. B 351, 985–1002.

Braicovich, P.E., Timi, J.T., 2008. Parasites as biological tags for stock discrimination of the Brazilian flathead *Percophis brasiliensis* in the south-west Atlantic. J. Fish. Biol. 73, 557–571.

Braicovich, P.E., Luque, J.L., Timi, J.T., 2012. Geographical patterns of parasite infracommunities in the rough scad, *Trachurus lathami* Nichols, in the Southwestern Atlantic ocean. J. Parasitol. 98, 768–777.

Cadrin, S.X., 2010. Interdisciplinary analysis of yellowtail flounder stock structure off New England. Rev. Fish. Sci. 18, 281–299.

Cadrin, S.X., 2011. An interdisciplinary assessment of winter flounder (*Pseudopleuronectes americanus*) stock structure. J. Northwest Atl. Fish. 43, 103–120.

Cadrin, S.X., Friedland, K.D., Waldman, J.R., 2005. Stock identification methods: an overview. In: Cadrin, S.X., Friedland, K.D., Waldman, J.R. (Eds.), Stock Identification Methods: Applications in Fishery Science. Elsevier, NY, pp. 3–6.

Carvalho, G.R., Hauser, L., 1994. Molecular genetics and the stock concept in fisheries. Rev. Fish Biol. Fisher. 4, 326–350.

Catalano, S.R., 2012. A review of the families, genera and species of Dicyemida Van Beneden, 1876. Zootaxa 3479, 1–32.

Catalano, S.R., 2013. Five new species of dicyemid mesozoans (Dicyemida: Dicyemidae) from two Australian cuttlefish species, with comments on dicyemid fauna composition. Sys. Parasitol. 86, 125–151.

Charters, R.A., Lester, R.J.G., Buckworth, R.C., Newman, S.J., Ovenden, J.R., Broderick, D., Kravchuk, O., Ballagh, A., Welch, D.J., 2010. The stock structure of grey mackerel *Scomberomorus semifasciatus* in Australia as inferred from its parasite fauna. Fish. Res. 101, 94–99.

Chou, Y.Y., Wang, C.S., Chen, H.G., Chen, H.Y., Chen, S.N., Shih, H.H., 2011. Parasitism between *Anisakis simplex* (Nematoda: Anisakidae) third-stage larvae and the spotted mackerel *Scomber australasicus* with regard to the application of stock identification. Vet. Parasitol. 177, 324–331.

Costa, G., Santamaria, M.T.G., Vasconcelos, J., Perera, C.B., Melo-Moreira, E., 2013. Endoparasites of *Trachurus picturatus* (Pisces: Carangidae) from the Madeira and Canary Islands: selecting parasites for use as tags. Sci. Mar. 77, 61–68.

Criscione, C.D., Cooper, B., Blouin, M.S., 2006. Parasite genotypes identify source populations of migratory fish more accurately than fish genotypes. Ecology 87, 823–828.

Dawe, E.G., Mercer, M.C., Threlfall, W., 1984. On the stock identity of short-finned squid (*Illex illecebrosus*) in the Northwest Atlantic. NAFO Sci. Coun. Stud. 7, 77–86.

Dogiel, V.A., Bychovsky, B.E., 1939. Parasites of fishes of the Caspian Sea. Trudy Kompleksnoi Izucheniyu Kaspiiskogo Morya 7, 1–150 (in Russian).

Evans, D., Grainger, R., 2002. Gathering data for resource monitoring and fisheries management. In: Hart, P.J.B., Reynolds, J.D. (Eds.), Handbook of Fish Biology and Fisheries, vol. 2. Blackwell Publishing, Oxford, pp. 84–102.

Friess, C., Sedberry, G.R., 2011. Genetic evidence for a single stock of the deep-sea teleost *Beryx decadactylus* in the North Atlantic Ocean as inferred from mtDNA control region analysis. J. Fish. Biol. 78, 466–478.

Furuya, H., 1999. Fourteen new species of dicyemid mesozoans from six Japanese cephalopods, with comments on host specificity. Spec. Div. 4, 257–319.

Furuya, H., Ota, M., Kimura, R., Tsuneki, K., 2004. Renal organs of cephalopods: a habitat for dicyemids and chromidinids. J. Morphol. 262, 629–643.

Garcia, A., Mattiucci, S., Damiano, S., Santos, M.N., Nascetti, G., 2011. Metazoan parasites of swordfish, *Xiphias gladius* (Pisces: Xiphiidae) from the Atlantic Ocean: implications for host stock identification. ICES J. Mar. Sci. 68, 175–182.

George-Nascimento, M., 1996. Populations and assemblages of parasites in hake, *Merluccius gayi*, from the southeastern Pacific Ocean: stock implications. J. Fish. Biol. 48, 557–568.

Gibson, D.I., 1972. Flounder parasites as biological tags. J. Fish. Biol. 4, 1–9.
Gillanders, B.M., 2009. Tools for studying biological marine ecosystem interactions - natural and artifical tags. In: Nagelkerken, I. (Ed.), Ecological Connectivity Among Tropical Coastal Ecosystems. Springer, Netherlands, pp. 457–492.
González, R.A., Kroeck, M.A., 2000. Enteric helminths of the shortfin squid *Illex argentinus* in San Matías Gulf (Argentina) as stock discriminants. Acta Parasitol. 45, 89–93.
Hemmingsen, W., MacKenzie, K., 2001. The parasite fauna of the Atlantic cod, *Gadus morhua* L. Adv. Mar. Biol. 40, 1–80.
Hemmingsen, W., Lombardo, I., MacKenzie, K., 1991. Parasites as biological tags for cod, *Gadus morhua* L., in northern Norway: a pilot study. Fish. Res. 12, 365–373.
Henriquez, V.P., Gonzalez, M.T., Licandeo, R., Carvajal, J., 2011. Metazoan parasite communities of rock cod *Eleginops maclovinus* along southern Chilean coast and their use as biological tags at a local spatial scale. J. Fish. Biol. 79, 1851–1865.
Herrington, W.C., Bearse, H.M., Firth, F.E., 1939. Observations on the life history, occurrence and distribution of the redfish parasite *Sphyrion lumpi*. In: United States Bureau of Fisheries Special Report, vol. 5, pp. 1–18.
Hochberg, F.G., 1983. The parasites of cephalopods: a review. Mem. Nat. Mus. Vic. 44, 108–145.
Hochberg, F.G., 1990. Diseases caused by protists and mesozoans. In: Kinne, O. (Ed.), Diseases of Marine Animals, Biologische Anstalt Helgoland, vol. 3. Hamburg, Germany, pp. 47–202.
Hutson, K.S., Brock, E.L., Steer, M.A., 2011. Spatial variation in parasite abundance: evidence of geographical population structuring in southern garfish *Hyporhamphus melanochir*. J. Fish. Biol. 78, 166–182.
Kabata, Z., Whitaker, D.J., 1981. Two species of *Kudoa* (Myxosporea: Multivalvulida) parasitic in the flesh of *Merluccius productus* (Ayres, 1855) (Pisces: Teleostei) in the Canadian Pacific. Can. J. Zool. 59, 2085–2091.
Khan, R.A., Chandra, C.V., Earle, P., 2011. Comparison of metazoan parasites of Atlantic cod, *Gadus morhua*, from three geographical areas of coastal Newfoundland. J. Parasitol. 97, 270–274.
King, J.R., McFarlane, G.A., 2006. Shift in size-at-age of the strait of Georgia population of Pacific hake (*Merluccius productus*). In: California Cooperative Oceanic Fisheries Investigations Report, vol. 47, pp. 111–118.
King, J.R., McFarlane, G.A., Jones, S.R.M., Gilmore, S.R., Abbott, C.L., 2012. Stock delineation of migratory and resident Pacific hake in Canadian waters. Fish. Res. 114, 19–30.
Lamichhaney, S., Barrio, A.M., Rafati, N., Sundström, G., Rubin, C., Gilbert, E.R., Berglund, J., Wetterbom, A., Laikre, L., Webster, M.T., Grabherr, M., Ryman, N., Andersson, L., 2012. Population-scale sequencing reveals genetic differentiation due to local adaptation in Atlantic herring. PNAS 109, 19345–19350.
Lester, R.J.G., 1990. Reappraisal of the use of parasites for fish stock identification. J. Mar. Freshwater Res. 41, 855–864.
Lester, R.J.G., MacKenzie, K., 2009. The use and abuse of parasites as stock markers for fish. Fish. Res. 97, 1–2.
Luque, J.L., Cordeiro, A.S., Oliva, M.E., 2010. Metazoan parasites as biological tags for stock discrimination of whitemouth croaker *Micropogonias furnieri*. J. Fish. Biol. 76, 591–600.
MacKenzie, K., 1987. Parasites as indicators of host populations. Int. J. Parasitol. 17, 345–352.
MacKenzie, K., 1999a. Parasites as pollution indicators in marine ecosystems: a proposed early warning system. Mar. Pollut. Bull. 38, 955–959.
MacKenzie, K., 1999b. Parasites as biological tags in population studies of marine organisms. Qatar Univ. Sci. J. 19, 117–127.
MacKenzie, K., 2002. Parasites as biological tags in population studies of marine organisms: an update. Parasitology 124, 153–163.
MacKenzie, K., Longshaw, M., 1995. Parasites of the hakes *Merluccius australis* and *M. hubbsi* in the waters around the Falkland Islands, southern Chile, and Argentina, with an assessment of their potential value as biological tags. Can. J. Fish. Aquat. Sci. 52 (S1), 213–224.
MacKenzie, K., Williams, H.H., Williams, B., McVicar, A.H., Siddall, R., 1995. Parasites as indicators of water-quality and the potential use of helminth transmission in marine pollution studies. Adv. Parasitol. 35, 85–144.
MacKenzie, K., Abaunza, P., 1998. Parasites as biological tags for stock discrimination of marine fish: a guide to procedures and methods. Fish. Res. 38, 45–56.
MacKenzie, K., Abaunza, P., 2005. Parasites as biological tags. In: Cadrin, S.X., Friedland, K.D., Waldman, J.R. (Eds.), Stock Identification Methods: Applications of Fisheries Science. Elsevier, NY, pp. 211–226.
MacKenzie, K., Brickle, P., Hemmingsen, W., George-Nascimento, M., 2013. Parasites of hoki, *Macruronus magellanicus*, in the southwest Atlantic and southeast Pacific oceans, with an assessment of their potential value as biological tags. Fish. Res. 145, 1–5.
Marcogliese, D.J., Albert, E., Gagnon, P., Sevigny, J.M., 2003. Use of parasites in stock identification of the deepwater redfish (*Sebastes mentella*) in the Northwest Atlantic. Fish. Bull. 101, 183–188.
Mardis, E.R., 2008. The impact of next-generation sequencing technology on genetics. Trends Gen. 24, 133–141.
Mattiucci, S., Abaunza, P., Ramadori, L., Nascetti, G., 2004. Genetic identification of *Anisakis* larvae in European hake from Atlantic and Mediterranean waters for stock recognition. J. Fish. Biol. 65, 495–510.
Mattiucci, S., Farina, V., Campbell, N., MacKenzie, K., Ramos, P., Pinto, A.L., Abaunza, P., Nascetti, G., 2008. *Anisakis* spp. larvae (Nematoda: Anisakidae) from Atlantic horse mackerel: their genetic identification and use as biological tags for host stock characterization. Fish. Res. 89, 146–151.
McClelland, G., Melendy, J., 2007. Use of endoparasitic helminths as tags in delineating stocks of American plaice (*Hippoglossoides platessoides*) from the southern Gulf of St. Lawrence and Cape Breton Shelf. Fish. Bull. 105, 180–188.
McClelland, G., Melendy, J., 2011. Use of parasites as tags in delineating stocks of Atlantic cod (*Gadus morhua*) from the southern Gulf of St. Lawrence and the Cape Breton Shelf. Fish. Res. 107, 233–238.
McFarlane, G.A., Beamish, R.J., 1985. Biology and fishery of Pacific whiting, *Merluccius productus*, in the Strait of Georgia. Mar. Fish. Rev. 47, 23–34.
McManus, D.P., Bowles, J., 1996. Molecular genetic approaches to parasite identification: their value in diagnostic parasitology and systematics. Int. J. Parasitol. 26, 687–704.
Mele, S., Merella, P., Macias, D., Gomez, M.J., Garippa, G., Alemany, F., 2010. Metazoan gill parasites of wild albacore *Thunnus alalunga* (Bonaterre, 1788) from the Balearic Sea (western Mediterranean) and their use as biological tags. Fish. Res. 102, 305–310.
Melendy, J., McClelland, G., Hurlbut, T., 2005. Use of parasite tags in delineating stocks of white hake (*Urophycis tenuis*) from the southern Gulf of St. Lawrence and Cape Breton Shelf. Fish. Res. 76, 392–400.
Moles, A., Heifetz, J., Love, D.C., 1998. Metazoan parasites as potential markers for selected Gulf of Alaska rockfishes. Fish. Bull. 96, 912–916.
Moore, B.R., Stapley, J., Allsop, Q., Newman, S.J., Ballagh, A., Welch, D.J., Lester, R.J.G., 2011. Stock structure of blue threadfin *Eleutheronema tetradactylum* across northern Australia, as indicated by parasites. J. Fish. Biol. 78, 928–936.
Moser, M., 1991. Parasites as biological tags. Acta Parasitol. 7, 182–185.
Moser, M., Hsieh, J., 1992. Biological tags for stock separation in pacific herring *Clupea harengus pallasi* in California. J. Parasitol. 78, 54–60.
Mosquera, J., de Castro, M., Gomez-Gesteira, M., 2003. Parasites as biological tags of fish populations: advantages and limitations. Comment. Theor. Biol. 8, 69–91.
Nagasawa, K., Mori, J., Okamura, H., 1998. Parasites as biological tags of stocks of neon flying squid (*Ommastrephes bartramii*) in the North Pacific Ocean. In: Proceedings of the International Symposium on Oceanic Large Squids. Japan Marine Resources Exploitation Center, Tokyo, pp. 49–64.
Niklitschek, E.J., Secor, D.H., Toledo, P., Lafon, A., George-Nascimento, M., 2010. Segregation of SE Pacific and SW Atlantic southern blue whiting stocks: integrating evidence from complementary otolith microchemistry and parasite assemblage approaches. Environ. Biol. Fish. 89, 399–413.
Oliva, M.E., 2013. Is *Anisakis simplex s.l.* a biological marker for stock identification of *Strangomera bentincki* from Chile? J. Fish. Biol. 83, 412–416.
Oliva, M.E., Gonzalez, M.T., 2004. Metazoan parasites of *Sebastes capensis* from two localities in northern Chile as tools for stock identification. J. Fish. Biol. 64, 170–175.
Oliva, M.E., Sanchez, M.F., 2005. Metazoan parasites and commensals of the northern Chilean scallop *Argopecten purpuratus* (Lamarck, 1819) as tools for stock identification. Fish. Res. 71, 71–77.
Ovenden, J.R., Berry, O., Welch, D.J., Buckworth, R.C., Dichmont, C.M., 2013. Ocean's eleven: a critical evaluation of the role of population, evolutionary and molecular genetics in the management of wild fisheries. Fish Fisher., 1–35.
Parsons, L.S., Hodder, V.M., 1971. Variation in the incidence of larval nematodes in herring from Canadian Atlantic waters. ICNAF Res. Bull. 8, 5–14.
Pascual, S., Hochberg, F.G., 1996. Marine parasites as biological tags of cephalopod hosts. Parasitol. Today 12, 324–327.
Pascual, S., Gestal, C., Estevez, J.M., Rodriguez, H., Soto, M., Abollo, E., Arias, C., 1996. Parasites in commercially-exploited cephalopods (Mollusca, Cephalopoda) in Spain: an updated perspective. Aquaculture 142, 1–10.
Pierce, G.J., Guerra, A., 1994. Stock assessment methods used for cephalopod fisheries. Fish. Res. 21, 255–285.
Poulin, R., 1992. Toxic pollution and parasitism in freshwater fish. Parasitol. Today 8, 58–61.
Poulin, R., Morand, S., 2000. The diversity of parasites. Quarter. Rev. Biol. 75, 277–293.
Poulin, R., Kamiya, T., in press. Parasites as biological tags of fish stocks: a meta-analysis of their discriminatory power. Parasitology 1–11. http://dx.doi.org/10.1017/S0031182013001534.
Quail, M.A., Smith, M.A., Coupland, P., Otto, T.D., Harris, S.R., Connor, T.R., Bertoni, A., Swerdlow, H.P., Gu, Y., 2012. A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific biosciences and Illumina MiSeq sequencers. BMC Genomics 13, 1–13.
Reed, C., MacKenzie, K., van der Lingen, C.D., 2012. Parasites of South African sardines, *Sardinops sagax*, and an assessment of their potential as biological tags. Bull. Eur. Assoc. Fish Pathol. 32, 41–48.
Roques, S., Sevigny, J.M., Bernatchez, L., 2002. Genetic structure of deep-water redfish, *Sebastes mentella*, populations across the North Atlantic. Mar. Biol. 140, 297–307.
Sala-Bozano, M., Ketmaier, V., Mariani, S., 2009. Contrasting signals from multiple markers illuminate population connectivity in a marine fish. Mol. Ecol. 18, 4811–4826.
Santos, M.J., Saraiva, A., Cruz, C., Eiras, J.C., Hermida, M., Ventura, C., Soares, J.P., 2009. Use of parasites as biological tags in stock identification of the black scabbardfish, *Aphanopus carbo* Lowe, 1839 (Osteichthyes: Trichiuridae) from Portuguese waters. Sci. Mar. 73, 55–62.
Sardella, N.H., Timi, J.T., 2004. Parasites of Argentine hake in the Argentine Sea: population and infracommunity structure as evidence for host stock discrimination. J. Fish. Biol. 65, 1472–1488.
Schuster, S.C., 2007. Next-generation sequencing transforms today's biology. Nature 5, 16–18.

Sequeira, V., Gordo, L.S., Neves, A., Paiva, R.B., Cabral, H.N., Marques, J.F., 2010. Macroparasites as biological tags for stock identification of the bluemouth, *Helicolenus dactylopterus* (Delaroche, 1809) in Portuguese waters. Fish. Res. 106, 321–328.

Shaw, P.W., 2003. Testing for genetic subdivision of the southern blue whiting (*Micromesistius australis*). In: Scientific Report, Fisheries Department, Falkland Islands Government, Stanley, Falkland Islands, pp. 22.

Shaw, P.W., 2005. Using mitochondrial DNA markers to test for differences between nuclear and mitochondrial genome genetic subdivision of the southern blue whiting (*Micromesistius australis*). In: Scientific Report, Fisheries Department, Falkland Islands Government, Stanley, Falkland Islands, pp. 20.

Sindermann, C.J., 1961. Parasite tags for marine fish. J. Wildlife Manage. 25, 41–47.

Sindermann, C.J., 1983. Parasites as natural tags for marine fish: a review. NAFO Sci. Coun. Stud. 6, 63–71.

Smith, P.J., Roberts, P.E., Hurst, R.J., 1981. Evidence for two species of arrow squid in the New Zealand fishery. New Zeal. J. Mar. Fresh. Res. 15, 247–253.

Stanley, R.D., Lee, D.L., Whitaker, D.J., 1992. Parasites of yellowtail rockfish, *Sebastes flavidus* (Ayres, 1862) (Pisces: Teleostei), from the Pacific coast of North America as potential biological tags for stock identification. Can. J. Zool. 70, 1086–1096.

Swain, D.P., Hutchings, J.A., Foote, C.J., 2005. Environmental and genetic influences on stock identification characters. In: Cadrin, S.X., Friedland, K.D., Waldman, J.R. (Eds.), Stock Identification Methods: Application in Fishery Science. Elsevier, NY, pp. 45–88.

Thorpe, J.P., Solé-Cava, A.M., Watts, P.C., 2000. Exploited marine invertebrates: genetics and fisheries. Hydrobiologia 420, 165–184.

Thorrold, S.R., Jones, G.P., Hellberg, M.E., Burton, R.S., Swearer, S.E., Neigel, J.E., Morgan, S.G., Warner, R.R., 2002. Quantifying larval retention and connectivity in marine populations with artificial and natural markers. Bull. Mar. Sci. 70, S291–S308.

Timi, J.T., 2003. Parasites of Argentine anchovy in the south-west Atlantic: latitudinal patterns and their use for discrimination of host populations. J. Fish. Biol. 63, 90–107.

Timi, J.T., 2007. Parasites as biological tags for stock discrimination in marine fish from South American Atlantic waters. J. Helminthol. 81, 107–111.

Timi, J.T., Lanfranchi, A.L., 2009. The metazoan parasite communities of the Argentinean sandperch *Pseudopercis semifasciata* (Pisces: Perciformes) and their use to elucidate the stock structure of the host. Parasitology 136, 1209–1219.

Timi, J.T., Lanfranchi, A.L., Etchegoin, J.A., 2009. Seasonal stability and spatial variability of parasites in Brazilian sandperch *Pinguipes brasilianus* from the Northern Argentine Sea: evidence for stock discrimination. J. Fish. Biol. 74, 1206–1225.

Varela, A.I., Ritchie, P.A., Smith, P.J., 2013. Global genetic population structure in the commercially exploited deep-sea teleost orange roughy (*Hoplostethus atlanticus*) based on microsatellite DNA analyses. Fish. Res. 140, 83–90.

Waldman, J.R., 2005. Definition of stocks: an evolving concept. In: Cadrin, S.X., Friedland, K.D., Waldman, J.R. (Eds.), Stock Identification Methods: Application in Fishery Science. Elsevier, NY, pp. 7–16.

Williams, H.H., MacKenzie, K., McCarthy, A.M., 1992. Parasites as biological indicators of the population biology, migrations, diet, and phylogenetics of fish. Rev. Fish Biol. Fisher. 2, 144–176.

Zischke, M.T., Griffiths, S.P., Tibbetts, I.R., Lester, R.J.G., 2013. Stock identification of wahoo (*Acanthocybium solandri*) in the Pacific and Indian Oceans using morphometrics and parasites. ICES J. Mar. Sci. 70, 164–172.

CHAPTER 11: General Discussion

Water sample collections at Stony Point in False Bay, South Australia, Australia (photo: Margaret Catalano).

General Discussion

The Dicyemida are a poorly-understood and little-known group of marine organisms that parasitise the renal appendages of benthic cephalopods. They are regarded as enigmatic with uncertain affinities in the Tree of Life and an unresolved life cycle. Taxonomic confusion also surrounds this group, with the validity of certain families, genera and species in doubt. Given the diversity of parasites and the way they can shape and influence ecosystems due to their intimate relationship with the host, it is important to recognise which species actually occur in a system, how they differ, their life cycle, the factors responsible for patterns of infection, their relationship with other species and if they can be applied in ecological and biological studies. My study aimed to address these important questions for the Dicyemida, outlined in the discussion below which is then followed by suggestions for future research direction.

Unravelling taxa confusion and establishing dicyemid species described to date

My study began with a review of the literature in an attempt to unravel the confusion surrounding the Dicyemida (Chapter 2 - Catalano 2012). After sourcing all past publications describing new dicyemid species, a comprehensive list of all the species described to date was developed, including information on morphological characters, host species, collection localities and reference to the original papers that described the new species. Uncertain taxa were highlighted and discussed in further detail, and from this synthesis, the number of dicyemid species described to date was established as 112. My review highlighted the gap in knowledge of dicyemid species from the southern hemisphere, with no new species described from Australian waters. Considering Australia has one of the most diverse cephalopod faunas in the world (Norman & Reid 2000), the scope and potential for systematic research on dicyemids from this area is extensive.

New dicyemid species descriptions

In my next three chapters, 10 new dicyemid species were described from six cephalopod species in four families, representing the first dicyemid descriptions from Australian cephalopods, and increasing the number described worldwide from 112 to 122 (Chapters 3, 4 and 5 - Catalano 2013a, b; Catalano & Furuya 2013). New host records of dicyemids were found from *Sepioteuthis australis* (southern calamary) and *Sepia papuensis* (Papuan

cuttlefish), and the occurrence of an uncertain stage in the dicyemid life cycle, the secondary nematogen, was confirmed. However, given that the observation of the secondary nematogen in prepared renal appendage smears is rare, I agree with McConnaughey (1951) that this stage is not a regular part of the dicyemid life cycle. Instead it may occur as a developmental error as the dicyemid transitions from an adult nematogen to rhombogen.

A total of three cephalopod species (*Sepia papuensis*, *S. apama* and *S. novaehollandiae*) were found to be infected by more than one dicyemid species, although co-occurrence of different dicyemid species within a single host individual was rare. This may be due to competition, limited resources, space and nutrients. When co-infection was observed, body size and calotte shape between the co-occurring dicyemid species were markedly different. As the calotte is used by the dicyemid parasite to attach to the renal appendages of the host, variations in shape morphology may help facilitate niche separation and hence give rise to the occurrence of one host individual infected by multiple dicyemid species (Furuya *et al.* 2003a). Sampling over time across the breadth of the achievable size limit for a host species may provide further insight into co-infection and co-occurrence patterns.

Insights into the dicyemid life cycle

For the first time, dicyemid parasites, including asexual stages, were recorded in host individuals that had recently died, providing further information on the dicyemid life cycle (Chapter 5 - Catalano 2013b). In particular, this finding contradicted what is currently presented in the literature about the need for the dicyemid to escape from the dying host individual to ensure future survival. Instead it appears as though dicyemids are able to persist and continue replicating even after host death, with other factors such as nutrients, space and renal appendage size possibly responsible for the switch between density increase strategies (asexual reproduction) and escaping dispersal strategies (sexual reproduction). Future research should investigate the cues and mechanism responsible for this switch between asexual and sexual reproduction, as well as how long after host death dicyemids can continue to replicate and remain viable.

I also aimed to resolve the unknown host life cycle stage where new infection by the infusoriform embryo occurs by sampling host eggs and filtered seawater from the giant Australian cuttlefish mass breeding aggregation in Upper Spencer Gulf, South Australia (Chapter 6 - Catalano *et al.* 2013). As cephalopods, including the giant Australian cuttlefish, are semelparous (bred once then die, with minimal overlap in generations), we predicted that

infusoriform embryo release would be high during this mass breeding event, ensuring that the parasites life cycle can continue beyond the death of the host.

Both host eggs and filtered seawater samples tested negative for the presence of dicyemid DNA when PCR amplified with dicyemid-species specific cytochrome *c* oxidase subunit I (*COI*) primers, suggesting initial infection by dicyemid parasites occurs after the host egg stage. However, the time period after the host embryo hatches and new infection occurs remains undetermined. Experimental studies performed by Lapan and Morowitz (1975) have shown infection is direct with no intermediate host, therefore tank experiments exposing newly hatched host individuals as well as juveniles to infusoriform embryos may establish the time after hatching whereby new infection occurs. Sampling cephalopod individuals across the breadth of their achievable size range could also be used to assess when new infection occurs. Finally, a route survey within the host may be beneficial, as the parasite may reside in a dormant stage in other host tissues before moving to the renal appendages. Only when the renal appendages develop in surface complexity may the parasite then be able to move to this microhabitat and maintain a foothold for subsequent colonisation.

Exploring factors responsible for observed dicyemid fauna composition and infection patterns

I surveyed a total of 149 freshly captured individual cephalopods comprising 10 species from six main localities in western, southern and eastern Australia for dicyemid parasites, allowing a range of factors that may be responsible for observed patterns of infection and fauna composition to be explored (Chapter 7 - Catalano *et al.* in review-a). Out of the 10 host species examined, three were found to be uninfected - *Nototodarus gouldi* (red arrow squid), *Euprymna tasmanica* (southern dumpling squid) and *Metasepia pfefferi* (Pfeffer's flamboyant cuttlefish). Hochberg (1990) and Furuya & Tsuneki (2005) have suggested that only benthic or epibenthic cephalopods species harbour dicyemid parasites, due to the negative buoyancy properties of the dispersal infusoriform embryo which infects a new host individual (Lapan 1975a). The lack of infection in *N. gouldi* supports this notion, as it is an oceanic species occurring offshore on the continental shelf (Norman & Reid 2000; Triantafillos *et al.* 2004). However both *E. tasmanica* and *M. pfefferi* frequently associate with the sea bottom (Norman & Reid 2000), providing the opportunity for encounter by the infusoriform embryo, yet individuals of both species were found to be uninfected. Therefore this result suggests that other factors, such as host size, geographical locality, host properties and host/parasite co-

history (i.e. the parasite may have become extinct in these host species), either singularly or in combination, may also influence the presence of dicyemids.

For cephalopod individuals harbouring dicyemid parasites, there was no clear pattern of infection in relation to host size, as small (35 mm mantle length) and large (496 mm mantle length) individuals were found to be infected with dicyemids (see Fig. 2, Chapter 7 - Catalano *et al.* in review-a). However, for the three cephalopod species infected by multiple dicyemid species, restriction to different size groups by each dicyemid species was generally observed. Therefore competition between dicyemid species, possible for attachment site, space and nutrients, may also be an important factor responsible for observed patterns of infection within a host individual.

Another predictor of infection patterns in parasites is host geographical collection locality, with past authors attributing occurrence patterns of different dicyemid species within a single host species to collection site (Furuya & Tsuneki 2005; Furuya 2008). Two cephalopod species in our study, *Sepia apama* and *S. novaehollandiae*, were collected from multiple localities in Australian waters, and in agreement with past studies, a distinct dicyemid fauna was recorded at each host collection site.

I lastly recorded the presence of asexual and sexual stages of dicyemid parasites between renal appendage sites (left *vs* right) of each host individual. Past authors generally treat the left and right renal appendages as a single entity, meaning cues responsible for dicyemid maturation cannot be evaluated. Therefore my observation of asexual stages exclusively in one renal appendage and sexual stage exclusively in the other renal appendage of a single host individual, which was observed more than once for two dicyemid taxa and two host species, has significantly added to what we know about dicyemid infections. In particular, this finding indicates i) infection of each renal appendage occurs independently and potentially at different times; ii) dicyemid individuals infecting one side of the renal appendage do not or are unable to move to the other side; iii) the developmental cues mediating the transition from the asexual to sexual stages is parasite-mediated rather than due to hormone fluxes associated with host maturation as suggested by Hochberg (1983); and iv) this pattern of asexual/sexual stage segregation between renal appendages of a single host individual is not dicyemid or host species-specific, as it was observed from more than one dicyemid and host species.

The first phylogeny hypothesis for dicyemid parasites

There have been few molecular genetic studies on dicyemid parasites, with those that are published in the literature focusing on single taxon and single individuals. The complete *COI* minicircle molecule for nine dicyemid species was sequenced, which included the *COI* gene and a non-coding region. From this, the first phylogeny, including representative taxa from the two genera which combined contain more than 90% of the nominal described species, was estimated (Chapter 8 - Catalano *et al.* in review-b). My finding of monotypic species clades, but paraphyly of genera estimated in Bayesian inference and maximum likelihood analyses, questions the notion that classification based on calotte cell counts is a viable character trait to distinguish genera. As such, the morphological taxonomy, and specifically, characters used for generic classifications for the Dicyemida, may be in need of revision.

One other study has characterised the *COI* minicircle molecule for a single dicyemid species, *Dicyema misakiense*. Watanabe et al. (1999) identified one tRNA-like stem-loop structure in the non-coding region of the minicircle molecule which may serve as the origin of replication. In my study, no tRNA structures were identified in the non-coding region of any of the nine dicyemid species minicircle molecules, however palindrome sequences with the potential to form stem-loop structures were found in five dicyemid species. Nonetheless, depending on where the *COI* gene initiation site was annotated, the resultant gene and non-coding region sizes and sequence compositions can change, leading to false assumptions on the ability of the molecule to replicate. For example, I re-interpreted the *COI* sequence annotated by Watanabe *et al.* (1999), as coding sequence was found in the non-coding region for this dicyemid species minicircle molecule. This coding sequence was included as the start of the *COI* gene in my analyses, meaning the size of the non-coding region reduced from 278 bp reported by Watanabe *et al.* (1999) to 83 bp. Subsequently, the tRNA-like stem-loop structure Watanabe *et al.* (1999) reported in the non-coding region became part of the *COI* gene sequence in my re-interpretation. This clearly highlights the need to be able to identify the gene initiation site of minicircle molecules with confidence, as it affects the ability to interpret the resultant function and properties of these molecules.

Dicyemids as biological tags

In the literature, dicyemid parasites are presented as being highly host-species specific (Furuya *et al.* 2003a; Furuya & Tsuneki 2003). My finding of significantly different dicyemid fauna composition between cephalopod species, and among cephalopod individuals of the same species collected from different geographical localities, supports this notion (see

Fig. 3, Chapter 7 - Catalano *et al.* in review-a). In addition, I found that correct classification of cephalopod individuals to their true biological identity based on the dicyemid parasite fauna composition was high (90%), along with classification of cuttlefish species to their true collection locality based on dicyemid fauna composition (74% for giant Australia cuttlefish, *Sepia apama* and 89% for nova cuttlefish, *S. novaehollandiae*). Collectively, these results suggest that dicyemid parasites can be used as biological tags to assess host population structure.

Few studies to date have incorporated parasite genetic analysis into population structure studies of marine hosts, with no studies using this approach for cephalopods. I therefore tested the hypothesis that parasite genetic structure will allow a deeper insight into cephalopod population structure compared to that previously inferred using complementary methods (Chapter 9). The phylogeographic structure of dicyemid mitochondrial *COI* haplotypes for four dicyemid species infecting *S. apama* supported the population structure of this cuttlefish species previously estimated from analyses of host morphology, behaviour and genetics. Furthermore, an analysis of molecular variance (AMOVA) of dicyemid sequences provided an alternative insight into structuring of this host species, suggesting different population boundaries of cuttlefish within the two gulfs in South Australian waters. This result provides support for the inclusion of parasite data (morphology and genetics) alongside complementary methods to examine cephalopod population structure.

The use of parasites as biological tags in population structure studies of marine organisms was reviewed in Chapter 10 - Catalano *et al.* in press, with comments provided on recent genetic advances and the benefits of a holistic approach. It is highlighted that missing in the literature is the application of new molecular genetic techniques with high throughput and increased resolution to parasitological studies of host population structure. As host gene flow does not necessarily equate to parasite gene flow (Criscione *et al.* 2006), an alternative or deeper insight into population structure may be recognised by examining parasite genetics, and subsequently warrants future attention. Ultimately my review advocated the use of a multidisciplinary approach in assessing the population structure of marine organism, integrating information across fields (i.e. artificial tags, morphology, life histories, otolith chemistry, genetics, parasitological data) for a deeper insight into population structuring where additional layers of information may be gained compared to using only a single approach.

Future research directions

My study contributed significantly to the literature on dicyemid parasite infections, however there still remains a wealth of knowledge to be gained from studying these organisms further. In particular, future research should focus on:

1) Resolving the confusion in the literature highlighted in Chapter 1 for the families, genera and species in Dicyemida of uncertain affinity. Additional samples from type localities and type hosts may need to be collected and analysed, with comparisons made to the original records.

2) Sampling cephalopod species from northern Australian where no dicyemid species have been documented or described previously. This will allow the baseline for infection in this region and for additional Australian cephalopod host species to be established, as well as provide the means to compare dicyemid infection patterns between temperate (southern Australia) and tropical (northern Australia) systems. Such a study can test the hypothesis presented by Hochberg (1990) that infections by dicyemid parasites, including species richness and prevalence, varies with latitude.

3) Performing tank experiments and route surveys to complete the dicyemid life cycle and establish i) at which host life cycle stage new infection occurs; ii) how long dicyemids can persist in a host individual that has died; iii) how the infusoriform stage develops into the vermiform stages in the new host individual; iv) cue and mechanisms responsible for the switch between vermiform and infusoriform stages; and v) generation time of dicyemids in a host individual, with observations covering a range of dicyemid species and host cephalopod species.

4) Establishing the effect (if any) that dicyemids have on their host. Some studies support the relationship between a dicyemid and its host as being parasitic, presenting the notion that the dicyemids are eroding the renal surface where they attach and also depriving the host of nutrients (Ridley 1968; Finn *et al.* 2005). However other studies suggest dicyemids do not harm the host and that this relationship is mutualistic, with the beating cilia covering the

dicyemids body assisting with the excretion of urine from the renal sac (Lapan & Morowitz 1972; Lapan 1975b; Hochberg 1982; Furuya *et al.* 2004).

5) Building on from the phylogeny and molecular genetic analyses presented in Chapter 8 to provide a deeper insight into the relationship between and within dicyemid species. This includes sequencing additional taxa from all described genera to add into the phylogenetic analyses, sequencing other molecular markers and characterising the complete genome of dicyemid parasites. Such phylogenetic studies may then allude to the unknown position of the dicyemids in the Tree of Life. Further molecular genetic studies on dicyemids from multiple taxa across all genera will also help to verify if descriptions based on current morphological characters are valid in distinguishing genera and species, and subsequently whether the taxonomy of Dicyemida needs to be revised.

6) Further applications of dicyemid parasites as biological tags to examine the population structure of cryptic cephalopods species or those that are commercially exploited where stock structure needs to be identified in order to inform management decisions. It would also be of benefit to obtain host and parasite samples from the fifth hypothesised population of *S. apama* off the east coast of Australia, as well as samples from additional sites across this cuttlefish species range to further explore the population structure of *S. apama*.

7) Examine high resolution neutral markers or loci under selection in both parasites and hosts to assess the robustness of this approach in recognising population structure of marine organisms. The results from such a study can then be compared with that obtained using other complementary methods (phenotypic characters, behaviour, life histories, otolith morphometrics and chemistry) for a complete evaluation of stock assessment methods.

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REFERENCES

Aruga, J., Odaka, Y.S., Kamiya, A. & Furuya, H. (2007) *Dicyemid* Pax6 and Zic: tool-kit genes in a highly simplified bilaterian. *BMC Evolutionary Biology*, 7, 1–16.

Awata, H., Noto, T. & Endoh, H. (2006) Peculiar behavior of distinct chromosomal DNA elements during and after development in the dicyemid mesozoan *Dicyema japonicum*. *Chromosome Research*, 14, 817–830.

Barber, I. (2007) Parasites, behaviour and welfare in fish. *Applied Animal Behaviour Science*, 104, 251–264.

Boje, J., Riget, F. & Køie, M. (1997) Helminth parasites as biological tags in population studies of Greenland halibut (*Reinhardtius hippoglossoides* (Walbaum)), in the north-west Atlantic. *Journal of Marine Science*, 54, 886–895.

Braicovich, P.E. & Timi, J.T. (2008) Parasites as biological tags for stock discrimination of the Brazilian flathead *Percophis brasiliensis* in the south-west Atlantic. *Journal of Fish Biology*, 73, 557–571.

Brown, M.J.F., Schmid-Hempel, R. & Schmid-Hempel, P. (2003) Strong context-dependent virulence in a host-parasite system: reconciling genetic evidence with theory. *Journal of Animal Ecology*, 72, 994–1002.

Bush, A.O., Fernandez, J.C., Esch, G.W. & Seed, J.R. (2001) *'Parasitism: The diversity and ecology of animal parasites*.' (Cambridge University Press: UK). 531pp.

Catalano, S.R. (2012) A review of the families, genera and species of Dicyemida Van Beneden, 1876. *Zootaxa*, 3479, 1–32.

Catalano, S.R. (2013a) First descriptions of dicyemid mesozoans (Dicyemida: Dicyemidae) from Australian octopus (Octopodidae) and cuttlefish (Sepiidae) species, including a new record of *Dicyemennea* in Australian waters. *Folia Parasitologica*, 60, 306–320.

Catalano, S.R. (2013b) Five new species of dicyemid mesozoans (Dicyemida: Dicyemidae) from two Australian cuttlefish species, with comments on dicyemid fauna composition. *Systematic Parasitology*, 86, 125–151.

Catalano, S.R. & Furuya, H. (2013) Two new species of dicyemid (Dicyemida: Dicyemidae) from two Australian cephalopod species: *Sepioteuthis australis* (Mollusca: Cephalopoda: Loliginidae) and *Sepioloidea lineolata* (Mollusca: Cephalopoda: Sepiadariidae). *Journal of Parasitology*, 99, 203–211.

Catalano, S.R., Whittington, I.D., Donnellan, S.C. & Gillanders, B.M. (2013) Using the giant Australian cuttlefish (*Sepia apama*) mass breeding aggregation to explore the life cycle of dicyemid parasites. *Acta Parasitologica*, 58, 599–602.

Catalano, S.R., Whittington, I.D., Donnellan, S.C. & Gillanders, B.M. (in review-a) Dicyemid fauna composition and infection patterns in relation to cephalopod host biology and ecology: exploring dicyemids as natural tags. *Folia Parasitologica*.

Catalano, S.R., Whittington, I.D., Donnellan, S.C., Bertozzi, T. & Gillanders, B.M. (in review-b) First insight into the phylogenetic relationship of dicyemid parasites: is classification based on morphological traits in need of revision? *Molecular Phylogenetics and Evolution*.

Catalano, S.R., Whittington, I.D., Donnellan, S.C. & Gillanders, B.M. (in press) Parasites as biological tags to assess host population structure: guidelines, recent genetic advances and comments on a holistic approach. *International Journal for Parasitology: Parasites and Wildlife*.

Cavalier-Smith, T. (1993) Kingdom Protozoa and its 18 phyla. *Microbiological Reviews*, 57, 953–994.

Criscione, C.D., Cooper, B. & Blouin, M.S. (2006) Parasite genotypes identify source populations of migratory fish more accurately than fish genotypes. *Ecology*, 87, 823–828.

Czaker, R. (2000) Extracellular matrix components in a very primitive multicellular animal, the dicyemid mesozoan *Kantharella antarctica*. *The Anatomical Record*, 259, 52–59.

Finn, J.K., Hochberg, F.G. & Norman, M.D. (2005) Phylum Dicyemida in Australian waters: first record and distribution across diverse cephalopod hosts. *Phuket Marine Biology Research Center Bulletin*, 66, 83–96.

Furuya, H. (1999) Fourteen new species of dicyemid mesozoans from six Japanese cephalopods, with comments on host specificity. *Species Diversity*, 4, 257–319.

Furuya, H. (2008) Three new dicyemids from *Octopus sasakii* (Mollusca: Cephalopoda: Octopoda). *Journal of Parasitology*, 94, 1071–1081.

Furuya, H. (2010) A new dicyemid from *Benthoctopus sibiricus* (Mollusca: Cephalopoda: Octopoda). *Journal of Parasitology*, 96, 1123–1127.

Furuya, H., Hochberg, F.G. & Tsuneki, K. (2003a) Calotte morphology in the phylum Dicyemida: niche separation and convergence. *Journal of Zoology*, 259, 361–373.

Furuya, H., Hochberg, F.G. & Tsuneki, K. (2003b) Reproductive traits in dicyemids. *Marine Biology*, 142, 693–706.

Furuya, H., Hochberg, F.G. & Tsuneki, K. (2007) Cell number and cellular composition in vermiform larvae of dicyemid mesozoans. *Journal of Zoology*, 272, 284–298.

Furuya, H., Ota, M., Kimura, R. & Tsuneki, K. (2004) Renal organs of cephalopods: A habitat for dicyemids and chromidinids. *Journal of Morphology*, 262, 629–643.

Furuya, H. & Tsuneki, K. (2003) Biology of dicyemid mesozoans. *Zoological Science*, 20, 519–532.

Furuya, H. & Tsuneki, K. (2005) A new species of dicyemid mesozoan (Dicyemida: Dicyemidae) from *Sepioteuthis lessoniana* (Mollusca: Cephalopoda), with notes on *Dicyema orientale*. *Species Diversity*, 10, 45–62.

Gibson, D.I. (1972) Flounder parasites as biological tags. *Journal of Fish Biology*, 4, 1–9.

Hochberg, F.G. (1982) The "kidneys" of cephalopods: a unique habitat for parasites. *Malacologia*, 23, 121–134.

Hochberg, F.G. (1983) The parasites of cephalopods: a review. *Memoirs of the National Museum Victoria*, 44, 108–145.

Hochberg, F.G. (1990) Diseases caused by protists and mesozoans. In 'Diseases of Marine Animals, Volume III.' (Ed. O. Kinne) pp. 47–202. (Biologische Anstalt Helgoland: Hamburg, Germany).

Hochberg, F.G. & Short, R.B. (1970) *Dicyemennea littlei* sp. n. and *Dicyema benthoctopi* sp. n.: Dicyemid mesozoa from *Benthoctopus magellanicus*. *Transcripts of the American Society for Microbiology*, 89, 216–224.

Hochberg, F.G. & Short, R.B. (1983) *Dicyemennea discocephala* sp. n. (Mesozoa: Dicyemidae) in a finned octopod from the Antarctic. *The Journal of Parasitology*, 69, 963–966.

Hudson, P.J., Dobson, A.P. & Lafferty, K.D. (2006) Is a healthy ecosystem one that is rich in parasites? *Trends in Ecology and Evolution*, 21, 381–385.

Hyman, L.H. (1959) *'The Invertebrates*.' pp. 323–365. (McGraw Hill: New York).

Karvonen, A. & Seehausen, O. (2012) The role of parasitism in adaptive radiations - when might parasites promote and when might they constrain ecological speciation? *International Journal of Ecology,* 1–20.

Katayama, T., Wada, H., Furuya, H., Satoh, N. & Yamamoto, M. (1995) Phylogenetic position of the dicyemid Mesozoa inferred from 18S rDNA sequences. *The Biological Bulletin*, 189, 81–90.

Kobayashi, M., Furuya, H. & Holland, P.W.H. (1999) Dicyemids are higher animals. *Nature*, 401, 762.

Kobayashi, M., Furuya, H. & Wada, H. (2009) Molecular markers comparing the extremely simple body plan of dicyemids to that of lophotrochozoans: insight from the expression patterns of *Hox*, *Otx*, and *brachyury*. *Evolution and Development*, 11, 582–589.

Lapan, E.A. (1975a) Magnesium inositol hexaphospate deposits in mesozoan dispersal larvae. *Experimental Cell Research*, 94, 277–282.

Lapan, E.A. (1975b) Studies on the chemistry of the octopus renal system and an observation on the symbiotic relationship of the dicyemid Mesozoa. *Comparative Biochemistry and Physiology*, 52A, 651–657.

Lapan, E.A. & Morowitz, H.J. (1972) The Mesozoa. *Scientific American*, 227, 94–101.

Lapan, E.A. & Morowitz, H.J. (1974) Characterization of mesozoan DNA. *Experimental Cell Research*, 83, 143–151.

Lapan, E.A. & Morowitz, H.J. (1975) The dicyemid Mesozoa as an integrated system for morphogenetic studies. *Journal of Experimental Zoology*, 193, 147–160.

Lester, R.J.G. (1990) Reappraisal of the use of parasites for fish stock identification. *Australian Journal of Marine and Freshwater Research*, 41, 855–864.

Littlewood, D.T.J., Cribb, T.H., Olson, P.D. & Bray, R.A. (2001) Platyhelminth phylogenetics-a key to understanding parasitism? *Belgium Journal of Zoology*, 131, 35–46.

MacKenzie, K. (1999) Parasites as pollution indicators in marine ecosystems: a proposed early warning system. *Marine Pollution Bulletin*, 38, 955–959.

MacKenzie, K. (2002) Parasites as biological tags in population studies of marine organisms: an update. *Parasitology*, 124, 153–163.

MacKenzie, K., Williams, H.H., Williams, B., McVicar, A.H. & Siddall, R. (1995) Parasites as indicators of water-quality and the potential use of helminth transmission in marine pollution studies. *Advances in Parasitology*, 35, 85–144.

Marcogliese, D.J. & Cone, D.K. (1997) Food webs: a plea for parasites. *Tree*, 12, 320–325.

Mattiucci, S., Farina, V., Campbell, N., MacKenzie, K., Ramos, P., Pinto, A.L., Abaunza, P. & Nascetti, G. (2008) *Anisakis* spp. larvae (Nematoda: Anisakidae) from Atlantic horse mackerel: Their genetic identification and use as biological tags for host stock characterization. *Fisheries Research*, 89, 146–151.

McConnaughey, B.H. (1951) The life cycle of the dicyemid Mesozoa. *University of California Publications in Zoology*, 55, 295–336.

McManus, D.P. & Bowles, J. (1996) Molecular genetic approaches to parasite identification: their value in diagnostic parasitology and systematics. *International Journal for Parasitology*, 26, 687–704.

Moser, M. (1991) Parasites as biological tags. *Acta Parasitologica*, 7, 182–185.

Norman, M. & Reid, A.L. (2000) *'A guide to squid, cuttlefish and octopuses of Australasia*.' (CSIRO Publishing, Collingswood: Victoria, Australia). 96pp.

Noto, T. & Endoh, H. (2004) A "chimera" theory on the origin of dicyemid mesozoans: evolution driven by frequent lateral gene transfer from host to parasite. *BioSystems*, 73, 73–83.

Nouvel, H. (1948) Les dicyémides II. Infusoriforme, tératologie, spécificité du parasitisme, affinités. *Archives de Biologie*, 59, 147–224.

Poulin, R. (1992) Toxic pollution and parasitism in freshwater fish. *Parasitology Today*, 8, 58–61.

Poulin, R. (2011) *'Evolutionary ecology of parasites*.' 2nd edn. (Princeton University Press: New Jersey, USA). 332pp.

Poulin, R. & Morand, S. (2000) The diversity of parasites. *The Quarterly Review of Biology*, 75, 277–293.

Ridley, R.K. (1968) Electron microscopic studies on dicyemid mesozoa. I. Vermiform stages. *The Journal of Parasitology*, 54, 975–998.

Roberts, M.G., Dobson, A.P., Arneberg, P., de Leo, G.A., Krecek, R.C., Manfredi, M.T., Lanfranchi, P. & Zaffaroni, E. (2002) Chapter 4: Parasite community ecology and biodiversity. In 'The ecology of wildlife diseases.' (Eds. P.J. Hudson, A. Rizzoli, B.T. Grenfell & H. Heesterbeek) pp. 63–82. (Oxford University Press).

Short, R.B. (1971) Three new species of Dicyema (Mesozoa: *Dicyemidae*) from New Zealand. In 'Biology of the Antarctic Seas IV, Antactic Research Series. Vol. 17.' (Eds. G.A. Llano & I.E. Wallen) pp. 231–249. (American Geophysical Union).

Short, R.B. & Hochberg, F.G. (1969) Two new species of *Dicyemennea* (Mesozoa: Dicyemidae) from Kaikoura, New Zealand. *The Journal of Parasitology*, 55, 583–596.

Short, R.B. & Hochberg, F.G. (1970) A new species of *Dicyemennea* (Mesozoa: Dicyemidae) from near the Antarctic Peninsula. *The Journal of Parasitology*, 56, 517–522.

Short, R.B. & Powell, E.C. (1969) *Dicyemennea eltanini* sp. n. (Mesozoa: Dicyemidae) from Antarctic waters. *The Journal of Parasitology*, 55, 794–799.

Stunkard, H.W. (1972) Clarification of taxonomy in the Mesozoa. *Systematic Zoology*, 21, 210–214.

Suzuki, T.G., Ogino, K., Tsuneki, K. & Furuya, H. (2010) Phylogenetic analysis of dicyemid mesozoans (Phylum Dicyemida) from innexin amino acid sequences: dicyemids are not related to Platyhelminthes. *Journal of Parasitology*, 96, 614–625.

Triantafillos, L., Jackson, G.D., Adams, M. & McGrath-Steer, B.L. (2004) An allozyme investigation of the stock structure of arrow squid *Nototodarus gouldi* (Cephalopoda: Ommastrephidae) from Australia. *Journal of Marine Science*, 61, 829–835.

Watanabe, K.I., Bessho, Y., Kawasaki, M. & Hori, H. (1999) Mitochondrial genes are found on minicircle DNA molecules in the mesozoan animal *Dicyema*. *Journal of Molecular Biology*, 286, 645–650.

Whittington, I.D. & Chisholm, L.A. (2003) Biodiversity of marine parasites in Australia: more than just a list of largely invisible creatures. *Records of the South Australian Museum Monograph Series*, 7, 51–60.

Williams, H.H., MacKenzie, K. & McCarthy, A.M. (1992) Parasites as biological indicators of the population biology, migrations, diet, and phylogenetics of fish. *Reviews in Fish Biology and Fisheries*, 2, 144–176.