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Source: Zoological Science, 32(5):465-473.
Published By: Zoological Society of Japan
DOI: http://dx.doi.org/10.2108/zs140245
URL: http://www.bioone.org/doi/full/10.2108/zs140245

ZOOLOGICAL SCIENCE **32**: 465–473 (2015)

A Flatworm from the Genus *Waminoa* (Acoela: Convolutidae) Associated with Bleached Corals in Western Australia

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A flatworm isolated from bleached colonies of the coral *Coscinaraea marshae* at Rottnest Island, Western Australia, is described using a combination of morphological and molecular systematics. This flatworm shares morphological features characteristic of the genus *Waminoa* (Acoelomorpha: Acoela), including the presence of two algal symbionts, but appears to have genital regions different from those of other described species of *Waminoa*. The design of new oligonucleotide primers enabled the amplification of partial 18S rDNA of the Rottnest Island acoel specimens, and phylogenetic analysis positioned them within *Waminoa*, confirming their placement in the genus. Furthermore, *Waminoa* specimens from Rottnest Island grouped into a sister clade to *Waminoa brickneri*, indicating that the morphological and genetic differences observed are most likely intraspecific and due to geographic variation. As such, we name these Rottnest Island specimens *W.* cf. *brickneri*, but highlight that key differences warrant further exploration before assignment to this species can be confirmed. This is the first acoel flatworm described from Western Australia and contributes to our understanding of the diversity and evolutionary relationship of the Acoela.

Key words: Acoela, Convolutidae, *Waminoa*, 18S rDNA, systematics

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INTRODUCTION

Acoel flatworms (Acoelomorpha: Acoela) are small, hermaphroditic metazoans that occur mainly in marine environments. Acoels are structurally very simple and lack a true epithelial gut, instead possessing a central syncytium (Achatz et al., 2013). Nearly 400 species of acoel flatworms have been described in 21 families (Tyler et al., 2006). The Convolutidae, Graff, 1905, is the most diverse family within the Acoela and contains 65 species in 12 genera (Tyler et al., 2006).

There is no fossil record for acoels, due to their soft bodies, and combined with their diverse morphological features this has made them difficult to classify (Tyler and Hooge, 1999). Understanding the evolutionary history of acoel flatworms plays an integral role in understanding the origin of bilaterian animals, and they are commonly used as representatives of a basal body plan (Hejnol and Martindale, 2008; Semmler et al., 2008; Jondelius et al., 2011). The phylogenetic position of acoels within the Bilateria is contentious; the debate centers around whether acoels are basal bilaterians (Ruiz-Trillo et al., 1999; Jondelius et al., 2011), deuterostomes (Dunn et al., 2008; Philippe et al., 2011), or within the Platyhelminthes (Egger et al., 2009).

While most acoel flatworms occur in marine sediment, they can occur living epizoically with other organisms, such as corals. All acoels found associated with corals house algal symbionts in their parenchyma (Winsor, 1990; Ogunlana et al., 2005), some of which are *Symbiodinium*, the same genus as the coral's algal symbiont (Barneah et al., 2007a). In such cases flatworms may be advantageous to the coral by partnering in a three-way symbiosis (Barneah et al., 2007a). In contrast, there have been concerns that flatworms may be detrimental to corals through shading (Haapkylä et al., 2009), consumption of algal symbionts (Hoeksema and Farenzena, 2012), or competition for food (Naumann et al., 2010; Wijgerde et al., 2012).

Acoel flatworms living epizoically with coral have been previously described in Queensland (Winsor, 1990), the Red Sea (Ogunlana et al., 2005), Indonesia (Ruiz-Trillo et al., 1999; Haapkylä et al., 2009) and Japan (Matsushima et al., 2010). In addition to those described in nature, at least three species of epizoic flatworms have been described on corals in aquaria (Shannon and Achatz, 2007). All of these flatworms have been associated with either genus *Convolutriloba*

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doi:10.2108/zs140245

Hendelberg & Akesson, 1988 or *Waminoa* Winsor, 1990 and all are classified within the family Convolutidae (Tyler et al., 2006). Only two species of *Waminoa* flatworms are currently described in full, *Waminoa litus* Winsor, 1990, found in North Queensland and *Waminoa brickneri* Ogunlana et al., 2005, found in the Red Sea.

Due to the vast diversity in morphology of acoels, a number of characteristics are used in their classification. The size and shape of flatworms, arrangement of their musculature, gland cells and cilia, as well as position of the mouth and parenchymal cells, are all used in morphological descriptions (Achatz et al., 2013). The most reliable form of identification is by features of their copulatory organs (Petrov et al., 2006; Achatz et al., 2009). However, it is becoming more common that distinctions between animal groups should not be decided by morphological means alone (Wiens, 2004).

Molecular studies on acoel flatworms have used 18S rDNA sequences to confirm identity to genus level (Ogunlana et al., 2005; Hooge and Tyler, 2005). However, the evolutionary rate of 18S rDNA is notably faster in Acoela relative to most other taxa, which has made it difficult to design PCR primers that successfully target Acoela alone. For example, Barneah et al. (2012) amplified coral and algal 18S rDNA sequences in addition to those of *Waminoa* when using primers designed by Norén and Jondelius (1999) for acoel flatworms, and noted the need for primers that specifically target *Waminoa*. Similarly, Hikosaka-Katayama et al. (2012) recovered 18S rDNA sequences from the dinoflagellate *Amphidinium* while using these same primers to amplify flatworm DNA.

There are no records of Acoela reported from Western Australia. This is surprising, as Rottnest Island is part of a biodiversity hotspot in southwest Australia (Myers and Mittermeier, 2000) and several census surveys have been undertaken in the Ningaloo Coast World Heritage area. Interestingly, in 2010/2011 the West Australia coastline experienced a warming event, during which ocean temperatures were up to 3°C higher than average over the summer months (Pearce et al., 2011). During this period *Coscinaraea marshae* corals inhabiting depths below 20 m bleached (i.e., expelled their algal symbionts). This was the only coral observed to bleach (Thomson et al., 2011), from the 25 species that inhabit the area (Wells et al., 1993). Following this bleaching event abundant acoel flatworms could be observed living on these *Coscinaraea marshae* corals (Thomson et al., 2011).

In this study we identify the first acoel flatworm from Western Australia, specifically Rottnest Island. We describe its morphology and compare partial 18S rDNA sequences of this specimen to that of other acoel flatworms. These data contribute to our knowledge of the geographic distribution and diversity of Acoela and provide specific primers for easier identification of *Waminoa* spp. in the future.

MATERIALS AND METHODS

Specimen collection

Flatworms were collected from 20 bleached *Coscinaraea marshae* coral colonies at 25–30 m depth at Rottnest Island (S 32 01.00, E 115 28.00), Western Australia in July 2011 and January 2012. Flatworms were located via SCUBA, collected along with the coral and placed in a container with seawater. Specimens were removed from coral fragments with a pipette as in Ogunlana et al. (2005) and placed in 20 ml glass scintillation vials containing either 100% ethanol (EtOH) or 10% formalin. Formalin-stored specimens were used for histological sectioning and microscopy analysis, while ethanol-stored specimens were used for genetic analysis.

Morphology

Whole fixed animals ($n \geq 10$) were imaged using conventional bright field, polarized light and phase contrast microscopy. Specimens ($n \geq 10$) preserved in formalin were processed following a standard processing schedule for histology using a Leica ASP 200 tissue processor. To ensure the specimens remained intact, individual flatworms were set in small blocks of 3% agar before being processed and embedded in wax. Paraffin sections 5 μm thick were cut either transversely or longitudinally through the entire animal and stained using two histological methods; toluidine blue or hematoxylin and eosin. A Leica Autostainer XL was used to stain the sections with hematoxylin and eosin following the manufacturer's protocols. Sections were stained with toluidine blue following a standard protocol (Bancroft, 1984) and following clearing, sections were mounted using Entellan New (Merck). One flatworm was embedded in epoxy resin and serially sectioned at 1 μm using a Leica EM UC6 microtome and a diamond knife. Each section was placed on a glass slide and stained using toluidine blue. All histology sections were viewed using a Zeiss Axioscope optical microscope.

Genetic analysis

DNA was extracted from entire flatworms using a QIAGEN DNeasy® blood and tissue kit (Qiagen Inc., Valencia, California) following the manufacturers' protocol. PCR primers were designed to target *Waminoa* 18S rDNA following morphological identification of the specimens belonging to this genus. For primer design 18S rDNA sequences from 69 acoel species representing all major acoel groups, and 84 *Waminoa* sequences (Barneah et al., 2012) were retrieved from the National Centre of Biotechnology Information (NCBI). Sequences were aligned and edited using Geneious v6.1 and compared to sequences from coral, algae, and fungi that were amplified in previous attempts to recover acoel 18S rDNA sequences using primers from Norén and Jondelius (1999) and Barneah et al. (2012; data not shown). The computer software program Oligo 6.8 was used to identify primers that target *Waminoa*. Two forward and two reverse primers were designed that could be used in the amplification of three regions within the 18S rDNA (Table 1). Amplification of 18S rDNA was performed using an Eppendorf Mastercycler® and the following conditions: 95°C at 5 minutes, followed by 35 cycles of 94°C at 45 seconds, 60°C at 45 seconds, and 72°C at 60 seconds with a final extension of 72°C for 5 minutes. PCR reactions were 30 μl in volume and contained 19.05 μl deionized water, 3 μl 10X PCR Buffer, 2.4 μl $MgCl_2$ (25 mM), 0.6 μl dNTP (2.5 mM each), 0.6 μl of each primer (10 μM), 1.2 μM BSA (Bovine Serum Albumin), 0.15 μl JumpStart™ Taq DNA polymerase (Sigma-Aldrich) and 2.4 μl of DNA. Target amplicons were purified and sequenced in both directions at the Australian Genome Research Facility, Perth node.

Table 1. Oligonucleotide primers designed to target *Waminoa* 18S rDNA. The primers name, and the sequence reading 5′ to 3′ are provided. Primers are listed in pairs where the forward primer is listed first.

18S rDNA Primer	Sequence (5′- 3′)
F1W *Waminoa*	GCGCGGAGAGGTAGTGACTAG
R1W *Waminoa*	GTAGGTCGTCTTGCTGGGATC
F2W *Waminoa*	CGTATGCTTTAGTTGCTGCGT
R2W *Waminoa*	CGATTTTGCAACCCATACTCC

Phylogeny

A multiple sequence alignment of partial 18S rDNA sequences recovered in this study and from GenBank was generated in Geneious v6.1 using MAFFT. The 18S rDNA sequence from a flatworm belonging to the family Mecynostomidae was used as an outgroup, based on the phylogenetic relationships established in Jondelius et al. (2011). Phylogenetic trees using maximum likelihood, maximum parsimony, neighbor-joining and Bayesian inference were generated in Geneious v6.1 using PAUP and Mr. Bayes. A GTR + G DNA substitution model selected by Modeltest was used in the construction of the maximum-likelihood tree, and bootstrap support for 1000 replicates was performed for maximum likelihood, maximum parsimony, and neighbor-joining tree searches.

RESULTS

Morphological description

The flatworm collected at Rottnest Island is placed in the family Convolutidae Graff, 1905, genus *Waminoa* Winsor, 1990 (Figs. 1–2). Specimens were found to be 1.97 (± 0.05) mm long and 1.53 (± 0.09) mm wide (Fig. 2B). The flatworms were 250–300 µm thick at their thickest point and were highly variable in shape; however, sexually mature specimens appear flat and disc-shaped, with a small notch at the posterior margin (Fig. 2B). Flatworms appear light brown in colour due to the presence of two endosymbionts

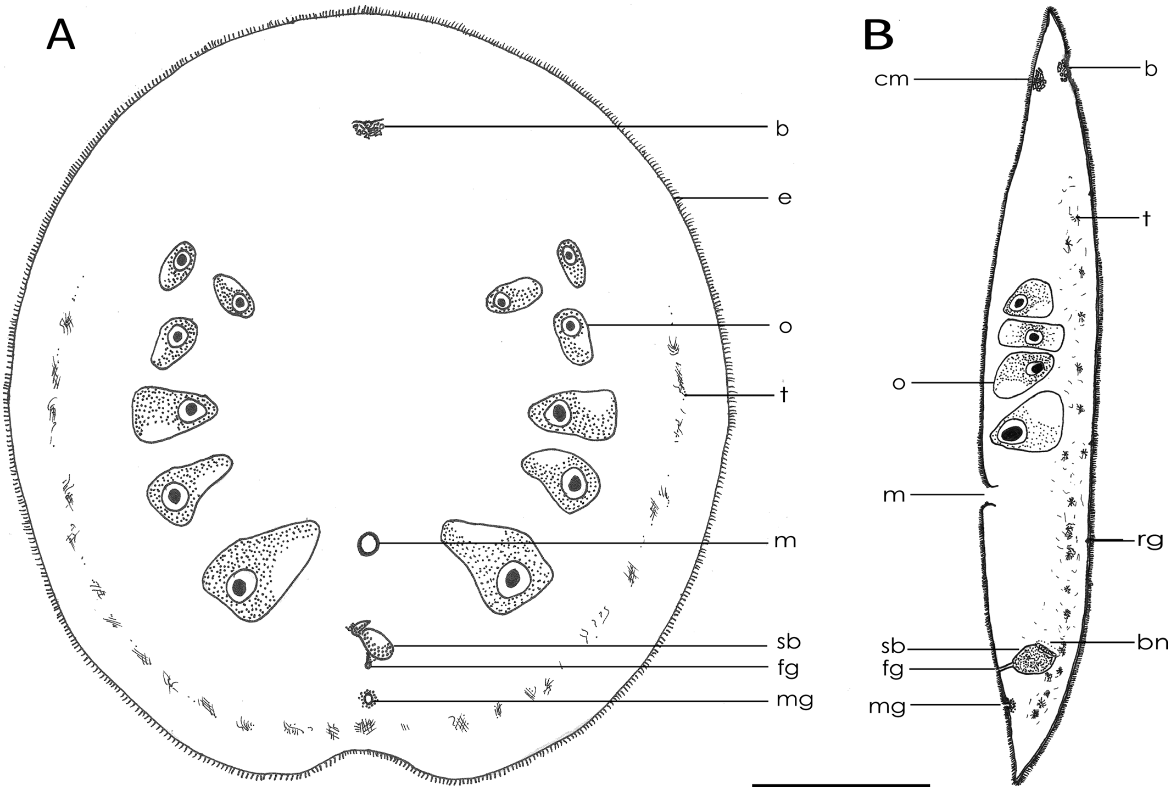

Fig. 1. Representative schematic of Rottnest Island *Waminoa* cf. *brickneri*. **(A)** Ventral reconstruction. **(B)** Longitudinal section. b, brain; cm, cell mass; e, epidermis; o, oocyte; t, testis; m, mouth; rg, rhabdoid gland; bn, bursal nozzle; sb, seminal bursa; fg, female gonopore; mg, male gonopore. Scale bar = 0.5 mm.

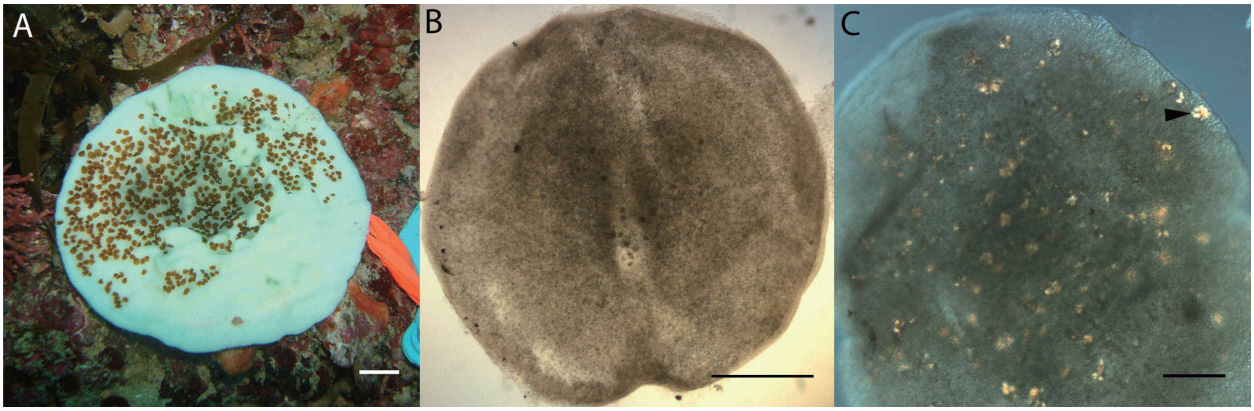

Fig. 2. The Rottnest Island *Waminoa* cf. *brickneri*. **(A)** Flatworms evident on bleached *Coscinaraea marshae* coral colonies. **(B)** Wholemount image fixed with 10% formalin. **(C)** Dorsal surface showing concrement cells (arrow). Scale bars: **(A)** = 1 cm, **(B)** = 500 µm, **(C)** = 200 µm.

that inhabit the parenchyma. Specimens were observed to have concrements described as inorganic masses (Winsor, 1990), on the dorsal surface (Fig. 2C). These were visible under differential interference contrast microscopy and appeared black under brightfield microscopy. These were not visible in histological sections, and thus appear to have undergone dissolution during histological processing.

The epidermis is fully ciliated, 5 μm thick on the dorsal side (Fig. 3A) and 2 μm on the ventral side (Fig. 3B). Epidermal cell nuclei are in-sunk, occurring below the body-wall musculature. The musculature consists of ventral cross-over muscle fibres and circular muscles surrounding the gonopores and mouth. Cilia are 5–7 μm long on the ventral surface and 7–9 μm on the dorsal surface and arranged in rows. Glands cells are present in the epidermis in small numbers on the dorsal and ventral surface. There are two distinct types of gland cells. Larger cyanophilic cells on the dorsal surface are 10 μm wide and 20 μm in length, appear slightly tooth shaped and show a complicated structure. They are most likely rhabdoid glands (Fig. 3A). Smaller gland cells that are not stained by toluidine blue are 2 μm in width and 5 μm in length. These are possibly mucous glands (Fig. 3B).

Mature specimens lack a statocyst, frontal gland and eyespots. It was expected that the statocyst would be crystalline and visible under polarized light microscopy. No statocyst was evident in sections, or whole mounts under polarized light. The brain appears to be in-sunk and present in the anterior end of the flatworm. This is usually referred to as a trunk nervous system and a commissural brain (Petrov et al., 2006), which is 90–100 μm in length. Posterior to the brain is a large cell mass slightly lighter when stained with toluidine blue, measuring 100 μm in length and 50 μm in width. Dorsal nerve cords could not be distinguished.

The mouth is located on the ventral surface, in the posterior third of the body and is 20–30 μm wide in fixed specimens (Fig. 3C). No pharynx was present. The parenchyma and central digestive syncytium contained no recognizable content. Paired ovaries and testes extend the length of the flatworm along the lateral sides of the animal, occurring freely throughout the digestive parenchyma (Fig. 3D). The testes were closer to the dorsal surface of the animal, grouped in paired clumps extending the length of the flatworm. The oocytes become larger as they migrate posteriorly towards the genital regions. They are recognizable by their enlarged nucleus with only a few of these becoming mature at a time.

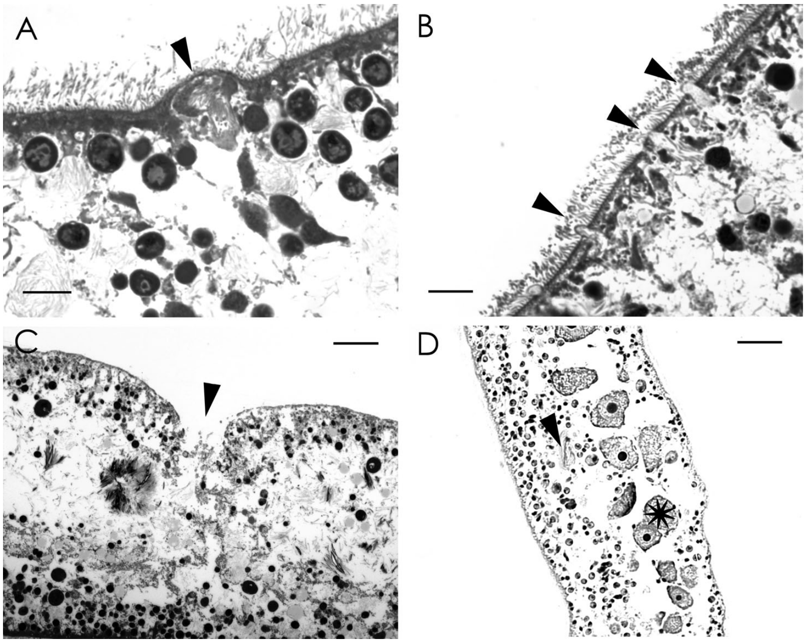

Fig. 3. Morphology of Rottnest Island *Waminoa* cf. *brickneri*. **(A)** Dorsal epidermis exhibiting rhabdoid gland cell. Transverse section 1 μm-thick, stained with toluidine blue. **(B)** Ventral epidermis showing mucous gland cells. Transverse section 1 μm-thick, stained with toluidine blue. **(C)** Mouth opening. Transverse section 1 μm-thick, stained with toluidine blue. **(D)** Oocytes developing close to the ventral surface (*). Arrowhead indicates testis developing closer to the dorsal surface. Longitudinal section 5 μm-thick, stained with haematoxylin and eosin. Scale bars **(A, B)** = 10 μm, **(C)** = 25 μm, **(D)** = 100 μm.

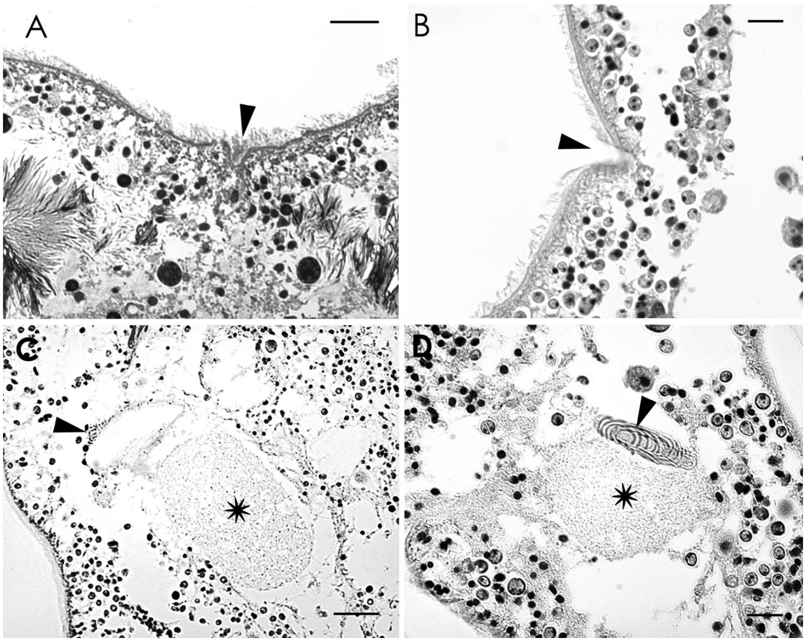

Fig. 4. Genital pores and female genital structures of Rottnest Island *Waminoa* cf. *brickneri*. **(A)**. The female genital pore. Transverse section 1 μm-thick, stained with toluidine blue. **(B)**. The male genital pore. Longitudinal section 5 μm-thick, stained with haematoxylin and eosin. **(C)**. Seminal bursa (*) and muscular structure (arrowhead). Transverse section 5 μm-thick, stained with haematoxylin and eosin. **(D)**. Seminal bursa (*) and muscular structure (arrowhead). Longitudinal section 5 μm-thick, stained with haematoxylin and eosin. Scale bars **(A, B, D)** = 25 μm, **(C)** = 50 μm.

The genital regions are found near the posterior tip of the body behind the mouth. The female gonopore is anterior to the male pore (Fig. 4A). The structure usually referred to as the vagina, which is the opening on the ventral side of the flatworm, leads from the female gonopore to the seminal bursa (Fig. 4D). The seminal bursa, which is slightly anterior to the gonopore is unpigmented and attached via connective tissue to a sac-like structure that is sometimes called the vestibulum (Achatz and Hooge, 2006). There is a tubular structure made of protein extending dorsolaterally from the bursa and extending the length of this vestibulum in transverse sections (Fig. 4C). This structure is quite large at 50 μm long (Fig. 4D). It appears to be made of protein due to eosinophilic staining with haematoxylin and eosin. However, the tubular nature of the structure suggests it could be a bursal nozzle. No other bursal nozzles were identified in any of the specimens examined and there should have been at least two. In the transverse sections from epoxy, the seminal bursa has two structures that are composed of matrix cells, with large nuclei, that extend antero-laterally into the parenchyma for approximately the length of the bursa; these are most likely bursal canals (Winsor, 1990). The male gonopore lies posterior to the female gonopore and is larger (Fig. 4B). No discernible copulatory organ is visible. However, the area anterior to the gonopore has cells arranged in a concentric manner that are denser than the surrounding material (Fig. 5A). The epidermis is thickened and in-sunk around the male gonopore. The gonopore is ciliated and seems to be an invagination of the body wall. A false seminal vesicle, dense with sperm, is present posterior to the testis, ending after the male gonopore in the majority of specimens examined, and these culminate in what is most likely the seminal vesicle (Fig. 5B).

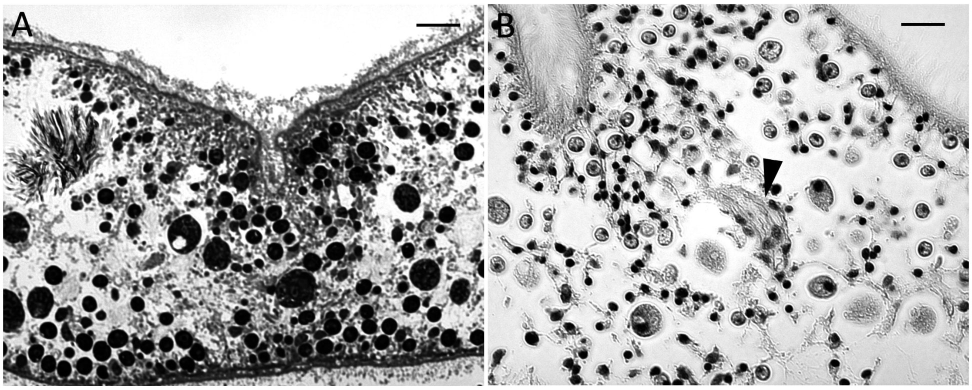

Fig. 5. Male genital structures of Rottnest Island *Waminoa* cf. *brickneri*. **(A)** Male gonopore, which is ciliated and surrounded by nucleated cells. Transverse section 1 μm-thick, stained with toluidine blue. **(B)** Seminal vesicle present posterior to the male gonopore dense with sperm (arrows). Longitudinal section 5 μm-thick, stained with haematoxylin and eosin. Scale bars = 20 μm.

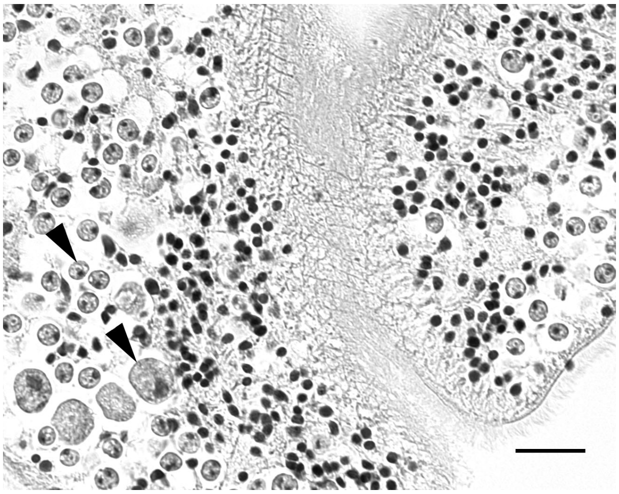

Fig. 6. Symbionts of the Rottnest Island *Waminoa* cf. *brickneri*. The smaller specimen is most likely *Symbiodinium* and the larger specimen most likely *Amphidinium* (arrowheads). Longitudinal section 5 μm-thick, stained with haematoxylin and eosin. Scale bar = 25 μm.

Two symbionts inhabit the parenchyma. One most likely belonging to the genus *Symbiodinium* sp. is 6–8 μm wide, and a larger, less-abundant symbiont most likely *Amphidinium* sp. is 12–16 μm wide (Fig. 6) (c.f. Winsor, 1990; Barneah et al., 2007b). *Symbiodinium* 18S rDNA sequences were recovered from the samples in initial attempts to amplify the flatworm using acoel primers from Norén and Jondelius (1999) and universal eukaryote primers as in Barneah et al. (2012) (data not shown). Characteristics of the larger symbiont, including that cell outlines possessed clefts and ridges and granulated chloroplasts were visible in the area outside the nucleus, are consistent with *Amphidinium*.

18S rDNA sequencing and phylogeny

The primer pair F1W/R1W amplified a 427 bp region of the 18S rDNA, the F2W/R2W primers amplified a 457–458 bp fragment, and the F2W/R1W primers amplified a 261 bp fragment from three flatworm specimens collected at Rottnest Island. Partial 18S rDNA fragments amplified using the three sets of primers were combined into single sequences that were 623–624 bp long, with a 99.7% sequence similarity (GenBank accession numbers KF719173–KF719175). A 589 bp multiple sequence alignment of partial 18S rDNA identified the flatworms collected at Rottnest Island as 97.5–98.3% similar to *Waminoa* spp. collected from the Red Sea, and 98.3% similar to *Waminoa* collected from Palau. Phylogenetic analysis supported the identification of the flatworms as belonging to the genus *Waminoa*, with the 18S rDNA sequences from Rottnest Island specimens forming a monophyletic group with other *Waminoa* sequences downloaded from GenBank (Fig. 7). The sequences recovered from *Waminoa* at Rottnest Island were further grouped into a separate clade compared to flatworm sequences from other regions of the world.

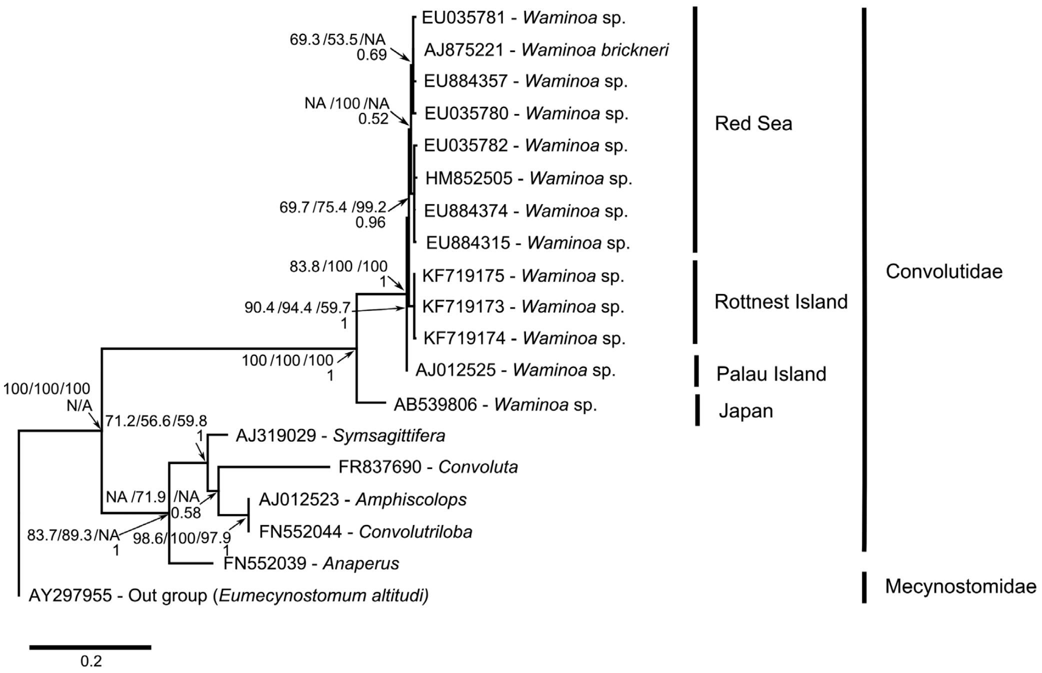

Fig. 7. Phylogeny of partial 18S rDNA showing the relationship of *Waminoa* collected at Rottnest Island and other flatworms in the family Convolutidae. Numbers adjacent to branch tips indicate Genbank accession numbers. This tree was generated using maximum likelihood with a GTR + G DNA substitution model. Bootstrap support for 1000 searches is shown for maximum likelihood, maximum parsimony and neighbour joining. Bayesian posterior probabilities are shown below these values. Mecynostomidae is used as an outgroup. Scale bar indicates substitution per site.

DISCUSSION

The *Waminoa* sp. found associated with bleached corals at Rottnest Island is the first acoel described from Western Australia, the first in this phylum of important worms. The morphology and 18S rDNA lineage of this flatworm confirm it is from the genus *Waminoa* and suggest that it is most likely *Waminoa brickneri*. However, as some genetic and morphological differences exist that require further exploration, we refer to the Rottnest Island species as *W.* cf*. brickneri*.

Morphology

Based purely on morphological features, this flatworm from Rottnest Island was thought to belong to the genus *Waminoa*. This is due to its round and flat shape with a posterior caudal notch and brown colour from two algal symbionts, as commonly seen in *Waminoa* spp. Additionally, the Rottnest Island flatworms were almost 2 mm in length, possessed a bilobed, in-sunk brain, the mouth was in the posterior third of the body, the two gonopores were close together, and no distinct stylet or penis papilla structure was present. All of these features are typical of currently described Waminoa (Tyler et al., 2006). By contrast *Convolutriloba* flatworms are brown, red, or green; shaped like a shield with a number of caudal notches; and possess sagittocysts, which were not present here (Shannon and Achatz, 2007).

It is likely that the specimen described here is *Waminoa brickneri* Ogunlana et al., 2005, due to similarities in their morphologies, including the absence of eyespots and a statocyst in mature specimens (their presence in juvenile Rottnest Island specimens could not be confirmed). However, these flatworms found at Rottnest Island differ from the Red Sea specimen in a number of ways. For example, *W. brickneri* was described as being 'speckled white' before fixation, which was not seen at Rottnest Island (Ogunlana et al., 2005). *Waminoa brickneri* is richly glandular and the dorsal surface contains abundant rhabdoid glands. These rhabdoids appear as large structures 20–50 µm in length, while the rhabdoids observed in the specimens described here were cyanophilic and did not exceed 10 µm. Achatz and Hooge (2006) describe a new Convolutidae specimen from the genus *Amphiscolops* and discuss the possibility of rhabdoids dissolving with the distal tips remaining. However, nothing resembling the distal tips of rhabdoids was observed.

While the genital regions of *W. brickneri* and the Rottnest Island flatworm are similar, there were a number of key differences observed. The seminal bursa, found in the same position as the one observed at Rottnest Island (above the female gonopore) could contain up to eight bursal nozzles in *W. brickneri* (Ogunlana et al., 2005). The only structure associated with the seminal bursa in the Rottnest Island specimen was almost 50 µm long, while the bursal nozzles of *W. brickneri* were 25 µm long. With this, we believe this single structure is a bursal nozzle, but further data is needed to confirm. The male gonopore in *W. brickneri* is smaller than the female gonopore, while in the Rottnest Island *Waminoa* the male gonopore was clearly larger than the female gonopore. *Waminoa brickneri* exhibited distinct penis glands in the male copulatory organ, but these were not observed in Rottnest Island specimens. However, they may not have been preserved in the specimens examined.

The only other *Waminoa* sp. to be designated a species name was *Waminoa litus* (Winsor, 1990) living on soft and stony corals in Queensland, Australia. *Waminoa litus* was not observed to exceed 2 mm the size of the Rottnest Island specimens. The dorsal and ventral epidermis of *W. litus* is similar to that of the specimens described here in that the cilia and thickness of the epidermis are different on dorsal and ventral sides. *Waminoa litus* had three types of gland cells - rhabdoids and two others that mainly occur in the dorsal epidermis. *Waminoa litus* was not observed to have concrement cells, while the specimen observed here does. Long sensory cilia were observed dispersed over the body of *W. litus* that were not seen in the Rottnest Island *Waminoa*. Where the species differ significantly is in the structure of the genital organs.

The *Waminoa* specimen from Rottnest Island clearly possessed two gonopores whilst *W. litus* was described as having only one. However, it may be possible that the female gonopore in *W. litus* was simply not found by Winsor (1990) as *W. litus* was sectioned at 5 µm thickness. Due to the small size, the female gonopore in the Rottnest Island *Waminoa* was not visible in sections cut at 5 µm thick, and could only be observed in 1 µm thick sections. The seminal bursa of *W. litus* was posterior to the female gonopore, which was quite large. The seminal bursa was clearly anterior to the female and male gonopores in the specimens investigated here and connects to the small female gonopore. The bursal canals described by Winsor (1990) matches those seen flanking the seminal bursa of the Rottnest Island *Waminoa*.

Two other species have been placed in the *Waminoa* genus, *Waminoa* sp. 1 and *Waminoa* sp. 2, which were found inhabiting the marine aquarium tanks at the Australian Institute of Marine Science (Winsor, 1990). Both of these specimens are too small to be the flatworm described here. When fixed, *Waminoa* sp. 1 was 1.2 mm in length and possessed iridescent white dendritic cells on its dorsal surface. *Waminoa* sp. 2 was only 1 mm in length. No further information on these two species was provided. From this it was suggested that sexuality in some acoel flatworms may be seasonal, as these worms were, despite lacking genitals, considered mature because they lacked a statocyst (Winsor, 1990). However, this may be more related to their stage of development and is an area that warrants further study.

Other flatworms currently referred to as *Waminoa* in the literature have not been formally described, and their identification is based off partial 18S rDNA sequence data. These include an undescribed acoel from Palau Island, which is distinctly different from the flatworm found at Rottnest Island due to the appearance of black stripes covering the flatworms' surface (Ruiz-Trillo et al., 1999), and a *Waminoa* sp. from Japan, which lacks a published description (Matsushima et al., 2010). It was 1 mm in length, distinctly narrower at the posterior end, and appears spotted, but other than this, no morphological data are available.

Further analysis of the genital regions in live specimens of the Rottnest Island *Waminoa* may reveal the species identity of these flatworms by confirming the number of bursal nozzles and the arrangement of the male copulatory organ. As there are only a few species described in *Waminoa*, it was unnecessary to characterize the muscles, nervous system, spermatozoa, or developmental biology in order to identify this specimen to genus. Nevertheless, further information on these systems would be useful in further distinguishing the *Waminoa* and delineating its position in the Convolutidae.

Genetic analysis

The partial 18S rDNA sequence confirmed that the specimens investigated in this study belong to the Convolutidae genus *Waminoa*. The *Waminoa* sp. from Rottnest Island was shown to group separately from all other *Waminoa* sequences currently available in Genbank, indicating it represents a unique lineage. However, the small sequence differences observed between the *Waminoa* spp. from the Red Sea (including *W. brickneri*) and the Rottnest Island specimens may be intraspecific and due to biogeographical differences. For other *Waminoa* spp. such as *W. litus*, *Waminoa* sp.1, and *Waminoa* sp.2, and the similar *H. piger*, molecular data are not available for comparison. This may be due to the difficulty in amplifying *Waminoa* DNA, as previously noted (Barneah et al., 2012).

Barneah et al. (2012) suggested that due to the similarity with coral and algae, more specific primers for amplifying *Waminoa* spp. should be designed. The primer pairs F1W/R1W and F2W/R2W developed here are shown to be highly suitable and an efficient method for amplifying partial 18S rDNA for diagnostic purposes. Furthermore, the fragment of 18S rDNA amplified by the primers has demonstrated good resolving power within *Waminoa*, resolving groups within the genus. Further DNA sequence data for *W. litus* and other suspected *Waminoa* specimens associated with coral using the primers developed here will enable researchers to better understand the evolutionary relationships of *Waminoa*.

While the 18S rDNA data available for *Waminoa* is limited, there appears to be a distinction in the geographic distribution of *Waminoa* that requires further investigation. The gene region amplified demonstrates the differences between the flatworms from the Indian Ocean, the Red Sea, and the Pacific. *Waminoa* from the Red Sea appears most closely related to the Rottnest Island *Waminoa*. The *Waminoa* sp. from Japan was found to be the most divergent *Waminoa* sp. from the Rottnest Island *Waminoa* (Matsushima et al., 2010).

As noted in other studies (Barneah et al., 2012; Hikosaka-Katayama et al., 2012), the non-specific binding of the nested 18S rDNA primers resulted in amplification of coral, fungal, and algal sequences (data not shown). As the 18S rDNA gene is highly conserved in animals, the differences between flatworm, coral, and symbiont 18S rDNA sequences is expected to be small. The 18S rDNA primers initially selected for this study were originally used in Norén and Jondelius (1999) and were used to amplify flatworm DNA from 87 acoel species in Jondelius et al. (2011). However, comparisons made between 69 representative species of acoel flatworm, fungi, and coral 18S rDNA sequences, show that the primers have a better match to fungi and coral DNA than to the Convolutidae. The variability exhibited in Acoel flatworm 18S rDNA is well documented (Ruiz-Trillo et al., 1999; Hooge et al., 2002; Jondelius et al., 2011) and while the primers previously utilised to amplify acoel DNA work for some taxa, they have limited capacity in the amplification of DNA from derived acoel lineages (such as Convolutidae), where sequence polymorphisms occur at the primer binding sites.

As the majority of the 400 species of acoel are found in Convolutidae (Tyler et al., 2006), the discovery of additional genetic markers to 18S rDNA would greatly improve the understanding of relationships within the Acoela and especially the Convolutidae. Issues in acoel and metazoan phylogeny arise from the lack of clarity in morphological characteristics and the vast differences found in the acoel gene regions used for these studies (Hooge et al., 2002; Jondelius et al., 2011). The cytochrome c oxidase subunit I (CO1) gene region is an important barcoding gene for most taxa, but appears problematic for Acoela. CO1 primers (Norén and Jondelius, 1999) were trialled with the Rottnest Island *Waminoa*, but only coral DNA in one sample was amplified (data not shown). This is most likely the reason that CO1 information does not currently exist for *Waminoa*. Future work should attempt to design new CO1 primers or investigate utilising other gene regions.

Symbiont and coral relationship

All acoels found associated with corals house algal symbionts in their parenchyma (Winsor, 1990; Ogunlana et al., 2005; Shannon and Achatz, 2007) and some of these (e.g., *Symbiodinium*) are also found in high numbers in coral. The relationship and ecological importance of these flatworms and their symbionts remain unclear. Our specimens contained two algal symbionts, one of which is most likely *Symbiodinium*, as indicated by morphology and recovered 18S rDNA (data not shown). The *Symbiodinium* 18S rDNA recovered here represents *Symbiodinium* belonging to the sub-generic group clade A (LaJeunesse, 2001). In *Coscinareae marshae* corals at Rottnest Island, colonies associate with *Symbiodinium* belonging to the sub-generic clade B and C lineages (Silverstein et al., 2011; Bessell-Browne et al., 2014). This suggests that the *Symbiodinium* sequence amplified in this study was from those within the flatworm rather than from those of the associated coral. This is consistent with previous findings of Barneah et al. (2007b) and Hikosaka-Katayama et al. (2012) who also determined that *Symbiodinium* from associated corals were genetically dissimilar to the *Symbiodinium* symbionts of the flatworms. The second algal symbiont is most likely *Amphidinium* based upon the description in Barneah et al. (2007b). *Amphidinium* has been confirmed in *W. litus* specimens in Japan via genetic analysis (Hikosaka-Katayama et al., 2012).

CONCLUSIONS

This description of *Waminoa* cf. *brickneri* from Rottnest Island contributes to the diversity and distribution of *Waminoa* flatworms, an understudied but important group of acoel flatworms. Although the flatworm specimen described here is most likely *Waminoa brickneri*, further morphological and molecular investigation is required to confirm this. Additional research into *Waminoa* flatworms and their symbionts in the future will contribute to understanding the unique relationship shared between the coral, algal symbionts, and flatworms. Controversial hypotheses based upon molecular investigations into Acoela flatworms have challenged the traditional views of metazoan phylogeny. Establishing the true nature of the evolutionary relationships between the metazoan animal groups is crucial to understanding evolution of the bilaterian body plan, and this must rely upon a correlative genetic and morphological approach.

ACKNOWLEDGMENTS

The authors thank Russ Babcock, Fiona Graham and Doug Bearham from the CSIRO. The authors acknowledge the facilities, and the scientific and technical assistance of the Australian Microscopy and Microanalysis Research Facility at the Centre for Microscopy, Characterisation & Analysis (CMCA), the University of Western Australia, a facility funded by the University, State and Commonwealth Governments. This research was supported in part by a UWA/AIMS/CSIRO Fellowship to MS.

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(Received October 30, 2014 / Accepted May 14, 2015)