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Re-description of *Thysanozoon nigropapillosum* (Polycladida: Pseudocerotidae) from the South China Sea, with observations on a novel pre-copulatory structure, sexual behaviour and diet

Wei-Ban Jie[1], Shih-Chieh Kuo[2] & Hin-Kiu Mok[1]

Abstract. *Thysanozoon nigropapillosum* (Hyman, 1959) is a marine polyclad flatworm found in the central Pacific. We believe that Hyman's original description of this species in having a yellow body margin and white papilla tips was in error, and we re-described its color patterns as having white body margin and yellow-tipped papillae based on fresh specimens collected in Taiwan. Dense rhabdites present on the tips of its surface papillae might serve to prevent dermal impregnation, whilst the forked penis is used to grip the margin of the body of its mate in a chopstick-like fashion during the process of dermal impregnation. Dense rhabdites were also found on the surface of the paired penis. The penis-gripping behaviour of *T. nigropapillosum* plays an important role during dermal impregnation. We suggest that this flatworm species experiences unilateral sex conflicts, not only in the structures of the dorsal papillae and the male genital organ, but also in the mating pattern.

Key words. polyclad flatworm, *Thysanozoon nigropapillosum*, dermal impregnation, pre-copulatory structure

INTRODUCTION

Polyclad flatworms are colourful and conspicuous inhabitants of coral reefs throughout tropical and subtropical oceans, but their diversity remains poorly documented, particularly in Taiwan. The abundance of marine flatworms and their biodiversity in Taiwan has been underestimated for a long time. The first report concerning polyclad flatworms in Taiwan was written by Kato (1943). Six species from Su-ao were examined and five of six species were described as new species. Since then, there has been little or no research carried out on marine flatworms in Taiwan.

Hyman (1959) described a new polyclad flatworm, *Acanthozoon nigropapillosum* from the Falarik Islet, Ifaluk Atoll in the central Pacific Ocean, on 18 October 1953 (Fig. 1). It was later transferred to the genus *Thysanozoon* by Faubel (Faubel, 1984).

Hyman's original description (1959: 581), which was based on a preserved specimen, stated, "It is black with a pale yellowish border… The dorsal surface is covered with low rounded black papillae tipped with white, hence to the naked eye the dorsal surface appears black dotted with white." It was also stated, "The black colour with black white-tipped papillae is distinctive." (Hyman, 1959).

Newman and the staff of the University of Guam published a checklist recording polyclad flatworm specimens and photographs from Micronesia for over 10 years (Newman et al., 2003). The sampling regions included Ifaluk Atoll—the type locality of *Thysanozoon nigropapillosum*. The photographic data in the publication is available at http://www.flmnh.ufl.edu/reefs. In the checklist, five undescribed species of *Thysanozoon* are included. *Thysanozoon nigropapillosum* (Hyman, 1959) is mentioned but there are no records about this species listed. Indeed, to date there is no record of a *Thysanozoon* species from the central Pacific that possesses white-tipped papillae and a yellow margin (Scott Johnson, pers. comm.). Although Newman did not give further descriptions of any of these five new species, one of them, i.e., *Thysanozoon* sp. 2 (photo no. GP365-20 from Guam and photo no. GP639-23 from Palau), has a white

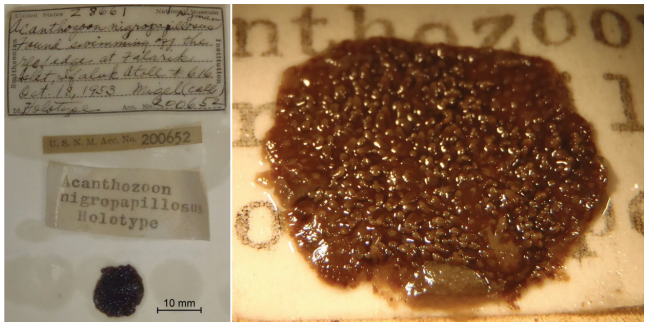

Fig. 1. Holotypes of *Acanthozoon nigropapillosum* Hyman, 1959 **(**Polycladida: Pseudocerotidae**)** at the Smithsonian Institution National Museum of Natural History, Washington D.C., USA. No. USNM 28661. Photograph by: Dr. Ning Chao.

[1]Institute of Marine Biology, National Sun Yat-sen University. No. 70, Lienhai Rd., Kaohsiung 80424, Taiwan; Email: wbjie@nehs.hc.edu.tw (JW) (*corresponding author); hinkiu@faculty.nsysu.edu.tw (MH)

[2]Department of Life Science, Chinese Culture University. No. 55, Hwa-Kang Road, Yang-Min-Shan, Taipei, 111, Taiwan; Email: guppy@staff.pccu.edu.tw

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body margin and yellow-tipped papillae, which is reverse of that described in Hyman's original paper. The pictures of the holotype in the Smithsonian Institution National Museum of Natural History show that, except for the colours of the papillae tips and border of the body, the shape of the dorsal papillae, the arrangement of the dorsal papillae, the proportion of the colours on the papillae and the width of the colored marginal band all closely agree with the original description of *A. nigropapillosum*. We postulate that Hyman mistakenly described the colour pattern of the holotype, which in fact has a yellow papillae tips and a white body margin.

This paper re-describes *T. nigropapillosum*, with additional observations on a novel pre-copulatory structure, geographical distribution, diet and sexual behaviour.

MATERIAL AND METHODS

All of the underwater photographs for this study were taken in Taiwan between 2005–2013 during snorkeling and scuba dives, generally at subtidal sites, tide pools, or under rubble. Specimens were photographed, whenever possible, either in situ or in the laboratory.

Observations on their behaviour were made and specimens collected over two years from 2012 to 2013. All specimens were fixed on frozen 10% seawater formalin (formaldehyde calcium acetate-propylene glycol, propylene phenoxytol) for at least two days. This is a fixation method that is modified from that of Newman & Cannon (1995). Specimens were then transferred into 70% ethanol for long term storage and histology. The identification of species is based on Newman & Cannon (2003). All of the specimens were deposited in the National Taiwan Museum. With the assistance of Ning Chao and Bill Moser, the pictures of the holotype specimen, USNM 28661, preserved in Smithsonian Institution National Museum of Natural History, were made available.

For histological study, longitudinal 5–8 μm serial sections of the reproductive organ, prepared by embedding the tissue in 56°C Paraplast wax, were cut and stained with haematoxylin and eosin. The resulting serial sections were deposited in the National Taiwan Museum.

TAXONOMY

Thysanozoon nigropapillosum (Hyman, 1959)
(Figs. 2, 3)

Acanthozoon nigropapillosus – Hyman, 1959: 581–583 (Type locality: Falarik Islet, Ifaluk Atoll, central Pacific Ocean).
Thysanozoon nigropapillosum – Faubel, 1984: 213 (New combination).

Material examined. 1 specimen (35 × 20 mm) (TMPL000001), subtidal zone at Hejie, Hengchun, Pingtung County. coll. W. B. Jie, 1 February 2012; 1 specimen (33 × 18 mm) (TMPL000002); subtidal zone at Hejie, Hengchun, Pingtung County. coll. W. B. Jie, 2 February 2012. 1 specimen (24 × 16 mm) (TMPL000003); subtidal zone at Longdong Bay, New Taipei City. coll. W. B. Jie, 6 July 2012. 1 specimen (10 × 9 mm) (TMPL000004), subtidal zone at Longdong Bay, New Taipei City. coll. W. B. Jie, 11 August 2012. 1 specimen (16 × 13 mm) (TMPL000005), subtidal zone at Badou Zi, New Taipei City. coll. W. B. Jie, 19 August 2012. 1 specimen (36 × 30 mm) (TMPL000006), subtidal zone at Hejie, Hengchun, Pingtung County. coll. W. B. Jie, 27 January 2013. 1 specimen (21 × 13 mm) (TMPL000007), subtidal zone at Hejie, Hengchun, Pingtung County. coll. W. B. Jie, 27 January 2013. 1 specimen (29 × 19 mm) (TMPL000008), subtidal zone at Shilang, Green Island, Taitung County. coll. W. B. Jie, 1 February 2013.

Redescription of *Thysanozoon nigropapillosum*. Broad and long body, with a deep black dorsal surface and a dark brown ventral surface; vivid opaque white and slightly wavy outer margin; numerous, evenly distributed, rounded yellow-tipped papillae of various sizes on the dorsal surface; one pair of ear-like, folded pseudotentacles in the middle of the anterior end; a cluster of cerebral eyespots, arranged in a horseshoe-shaped pattern, was present behind the tentacles (Fig. 2A); pharynx simply folded at the anterior; mouth opening in the middle of the pharynx; a pair of male pores behind the pharynx, followed by median female pore; sucker posterior to the female pore (Fig. 2B).

Supplementary description of pre-copulatory structure. Oblong seminal vesicle; un-branched vas deferens; coiled ejaculatory duct, ovate prostate, long, cone shaped stylet; shallow male and female antra (Fig. 3A, B).

Sagittal serial sections of the papillae showed that the dermal cells of each papilla contained a large number of dense

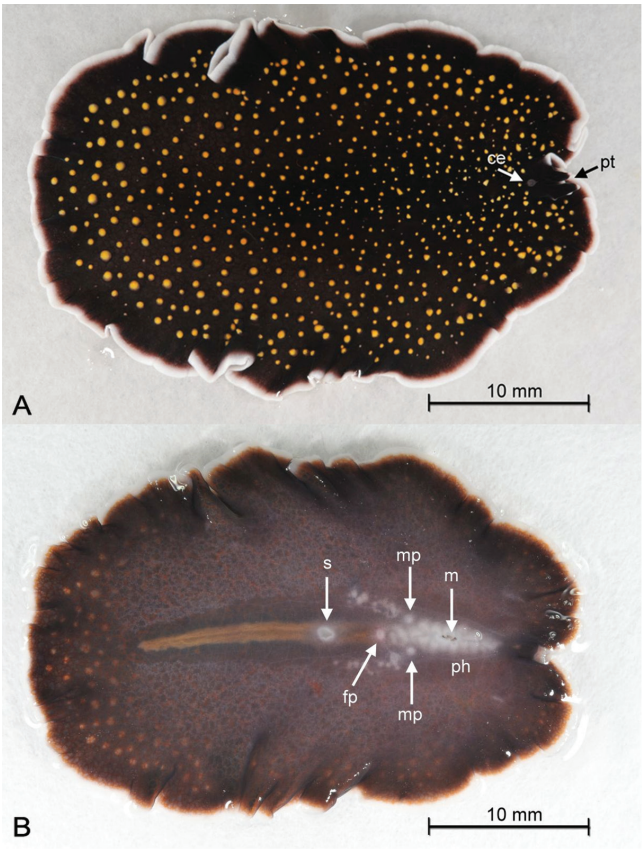

Fig. 2. *Thysanozoon nigropapillosum* (Hyman, 1959). Anterior is to the right. A, dorsal view; B, ventral view. ce, cerebral eyespots; fp, female pore; m, mouth; mp, male pore; ph, pharynx; pt, pseudotentacle; s, sucker.

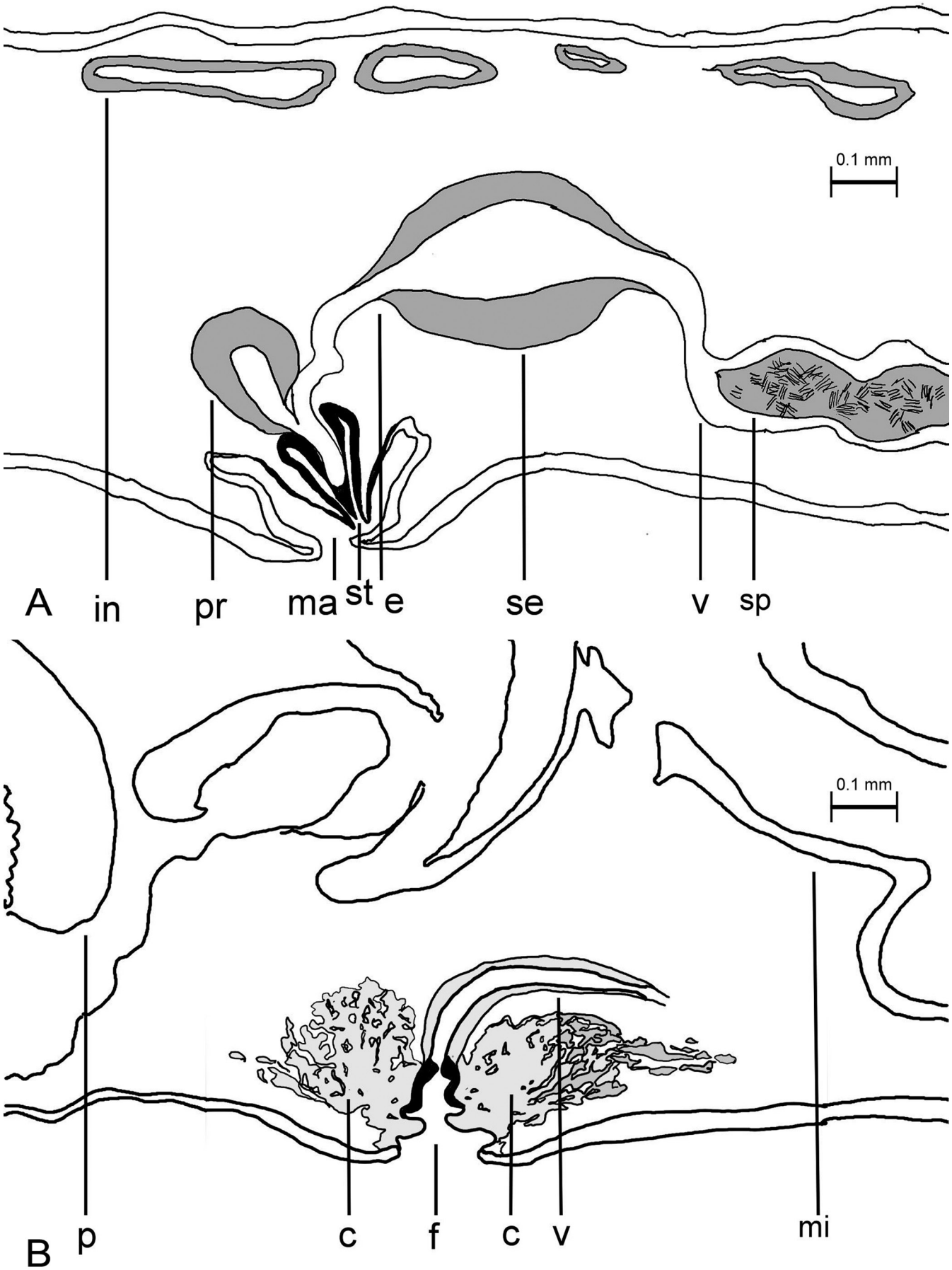

Fig. 3. Diagrammatic representation of the reproductive anatomy of *Thysanozoon nigropapillosum* (Hyman, 1959). A, male. e, ejaculatory duct; in, intestine; ma, male pore; pr, prostate gland; se, seminal vesicle; sp, sperm; st, stylet; v, vas deferens. B, female. c, cement gland; f, female pore; mi, main intestine; p, pharynx; v, vagina.

rhabdites, which were concentrated more at the tip of the papillae than on the surface between the papillae (Fig. 4A, B). These rhadites purportedly have either a defensive or a secretory role in all polyclad flatworms (Martin, 1978).

Rhabdites were also present on the surface of both the male and female reproductive organs. The inner surface of the male genital atrium (Fig. 4C) contained more rhabdites than the female pore (Fig. 4D).

Distribution and habitat. *Thysanozoon nigropapillosum* (Hyman, 1959) is not only abundant in Taiwan (Fig. 5A), but probably also in the Indian Ocean and tropical western Pacific (Fig. 5B). Gosliner et al. (1996) posted a picture of a flatworm having a white margin with yellow-tipped papillae that was labelled *T. nigropapillosum* (Hyman, 1959) and offered their collecting distribution including Maldives, Sri Lanka, Indonesia, New Guinea and Solomon Islands.

During the day, it is often seen in coastal reef waters at depths between 1 and 20 metres, but is rarely seen at night. When water currents are strong or subjected to disturbance by a diver, these flatworms can swim quickly by rhythmic contractions and undulation of their body margin.

Diet. *Thysanozoon nigropapillosum* (Hyman, 1959) has a simple folded pharynx and feeds on tunicates, using its mouth. Because it has a large pharynx, it can engulf colonial tunicates (e.g., *Didemnum* sp.) into the main intestine (Fig. 6A, B).

The species always occurs where its food is abundant (Fig. 7A). Regurgitated food pellets can occasionally be found in the field (Fig. 7B), but pellets are more easily observed in a tank, following collection of the flatworm. As observed under a microscope, each food pellet contains large amounts of condensed, calcareous, multi-rayed, star-like spicules of ca. 30 µm in diameter, derived from didemnids (Fig. 8).

Sexual behaviour. The mating behaviour of *Thysanozoon nigropapillosum* was fortuitously observed in the field on 25 July 2011, from 1530 hours to 1541 hours, 12 m depth, in Longdong bay (121°27′56″N, 25°0′43″E), northeast Taiwan. At the beginning of the encounter, two individuals circled each other. Subsequently one of the individuals extended its paired penis towards its partner and then gripped its partner's lateral outermost margin, in a chopstick-like fashion. In this unilateral insemination, after the spermatophore has been deposited on the epidermis, the sperm donor left, without receiving any sperm from its partner (Fig. 9A, B).

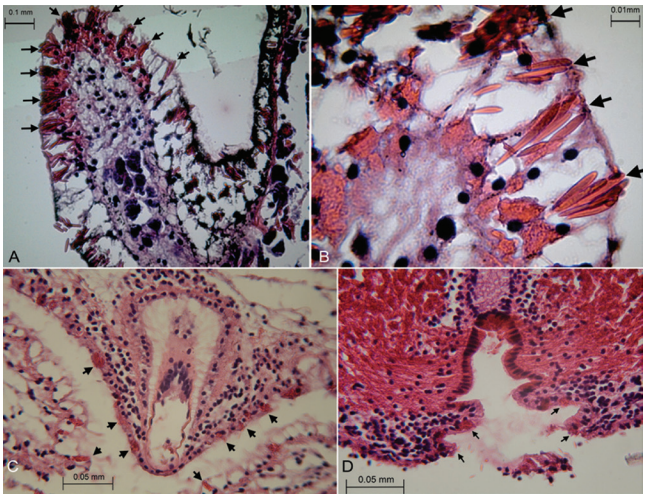

Fig. 4. *Thysanozoon nigropapillosum* (Hyman, 1959): rhabdites on epidermis (indicated by arrows). A, B, on the tips of papillae; C, distributed on the inner surface of the male atrium; D, distributed on the female pore.

DISCUSSION

We suggest that *Thysanozoon nigropapillosum* as described here is the most common shallow water marine flatworm

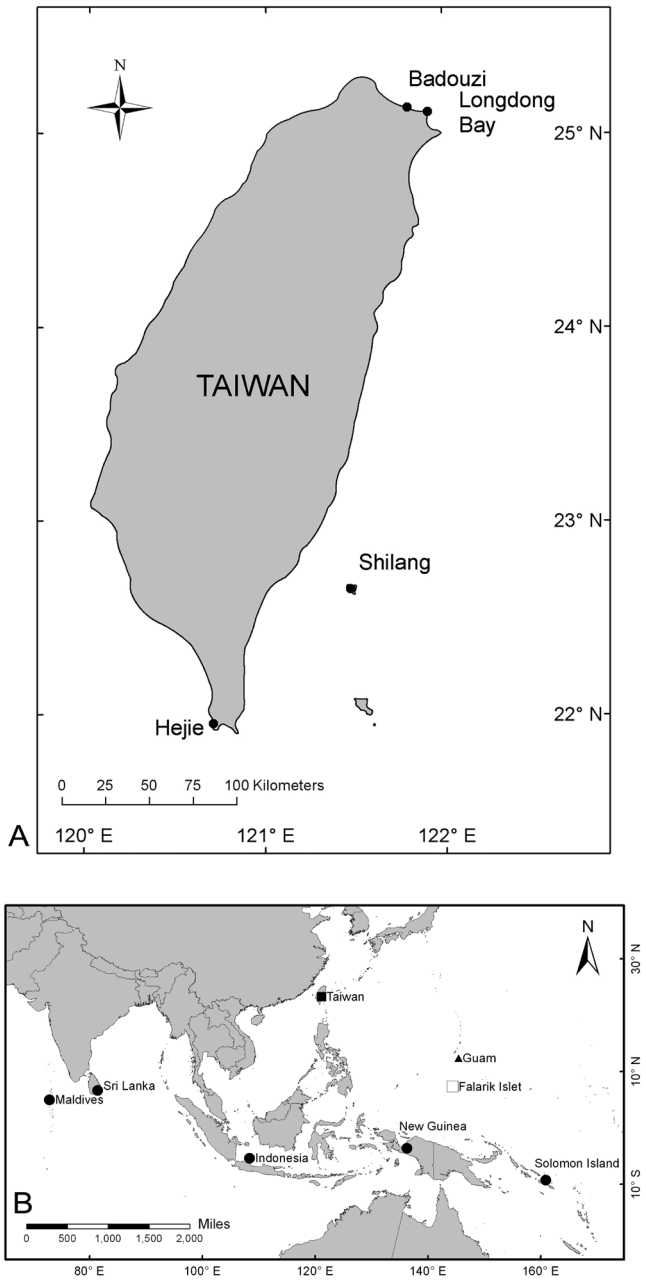

Fig. 5. Distribution of *Thysanozoon nigropapillosum* (Hyman, 1959) A, around Taiwan; B, type locality (hollow square), and the probably distribution of Newman et al. (2003) collection (filled triangles), the distribution of Gosliner et al. (1996) collection (filled circles) in Indo-west Pacific.

species in Taiwan, because, since 1959, no scientist or diver has recorded flatworms that fit the original description of the colour pattern in the holotype of this species. Despite the loss of the original colour in the preserved holotype, the shape of the papillae and the colour proportion of the papilla's tips remain visible in the holotype, and these are consistent with more recent observations made on living specimens. It is plausible that Hyman did not personally examine a fresh holotype, and the color pattern was reported according to the notes of the collector, Migel, who may have mistakenly described the color patterns. This paper therefore amends the description of this species.

The relationships between polyclad flatworms and nudibranchs have been studied (Newman & Cannon, 1994). Since both animals can bear toxins, the similarity of their colour patterns is generally believed to be due to a form of Mullerian mimicry. Both species benefit from being avoided by their predators. However, the extent to which this mimicry actually works in the sea still remains uncertain, as the potential common predators of polyclad flatworms and nudibranchs are unclear. Whilst nudibranchs are uncommon, the chances of encountering polyclad flatworms are even less. In eight years of observation (pers. unpubl. data), the most widespread and dominant nudibranch in Taiwan appears to be *Phyllidiella pustulosa*. It has a black dorsal coloration with pale-tipped compound tubercles. The colour of its dorsal surface has the same pattern as that of Taiwan's most common flatworm, *T. nigropapillosum*. Considering the encounter rate for these two common species, Mullerian mimicry between *Thysanozoon* and *Phyllidiella* is much more possible than for other flatworms or nudibranchs.

Polyclad flatworms are benthic predators. They are known to feed on a wide range of invertebrates, including bivalves, gastropods, sessile tunicates and barnacles (Newman & Cannon, 2003). They generally occur where their specific

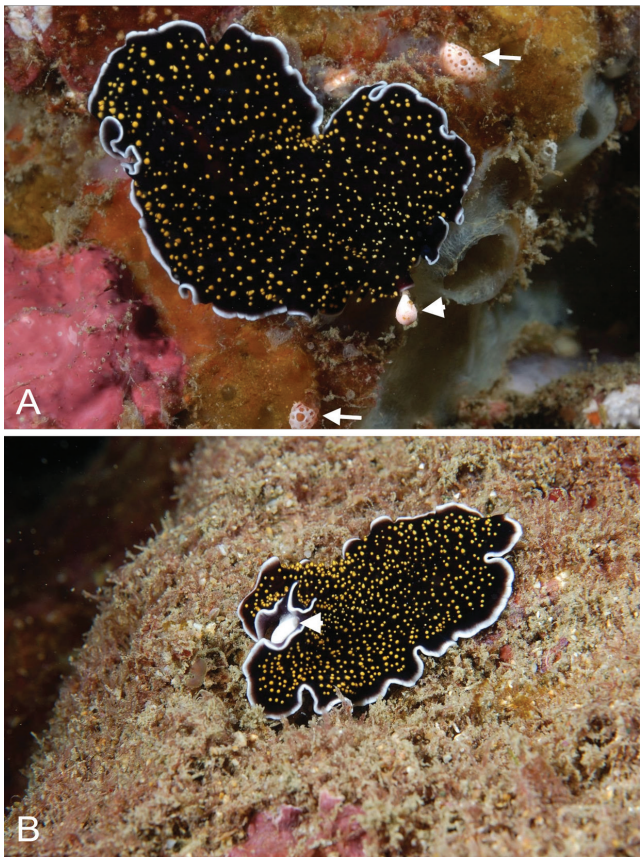

Fig. 7. *Thysanozoon nigropapillosum* (Hyman, 1959) A, with colonial tunicates (indicated by arrows), found at Longdong Bay, Northeast Coast of Taiwan, 28 August 2008; B, regurgitated food pellet (indicated by arrowhead). Chuanfanshr, Kenting, southern Taiwan, 9 February 2006.

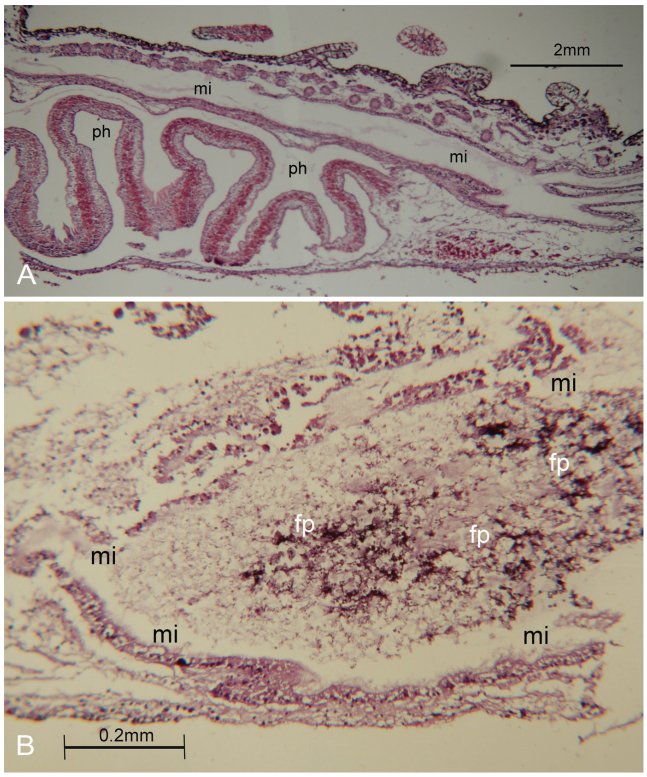

Fig. 6. Longitudinal section of *Thysanozoon nigropapillosum* (Hyman, 1959). A, pharynx and main intestine; B, food pellet remains inside the main intestine. fp, food pellet; mi, main intestine; ph, pharynx.

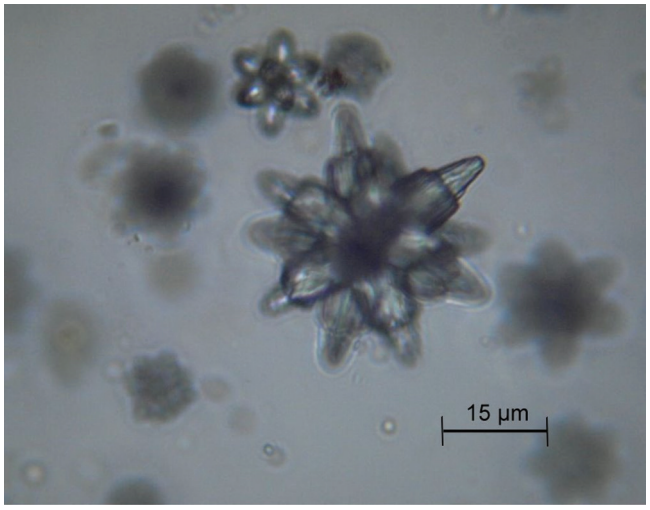

Fig. 8. Multi-rayed star-like spicules of colonial tunicates *Didemnum* sp. in food pellets of *Thysanozoon nigropapillosum* (Hyman, 1959).

food is abundant. In the case of *T. nigropapillosum*, they have been observed most often crawling over its favoured prey, colonial tunicates, and we report for the first time didemnid tunicate spicules in the food pellets and main intestine of *T. nigropapillosum*. These flatworms may well feed on other prey, but these have not been determined. When feeding, they stretch out the pharynx to engulf their prey. Their mouth opens just under their body so it is difficult to make direct observations. However, polyclads are mostly oligophagous animals, so if more pellets were available, the polyclad flatworms' diet would be more fully understood.

Marine flatworms are hermaphrodites; each individual has fully functional male and female reproductive systems. During copulation, at least three different types of inseminating behaviour have been recorded. The first type is reciprocal mating, or true copulation. When potential partners encounter each other, both individuals donate and receive sperm simultaneously. The second type of insemination is unilateral copulation, also called 'penis fencing'. When two potential partners encounter each other, only one stab the other with its penis and transfers its sperm via hypodermic insemination, as described in *Pseudoceros bifurecus* (Michiels & Newman, 1998). This behaviour results in a unilateral transfer of sperm, wherein the asymmetrical outcome favours the flatworm that injects its sperm first. In this case, logically, unilateral insemination probably favours those species that have a larger population or a greater likelihood of encounter. *Thysanozoon nigropapillosum* is a locally common species, so the relatively high rate of encounter may indicate that unilateral insemination is prevalent.

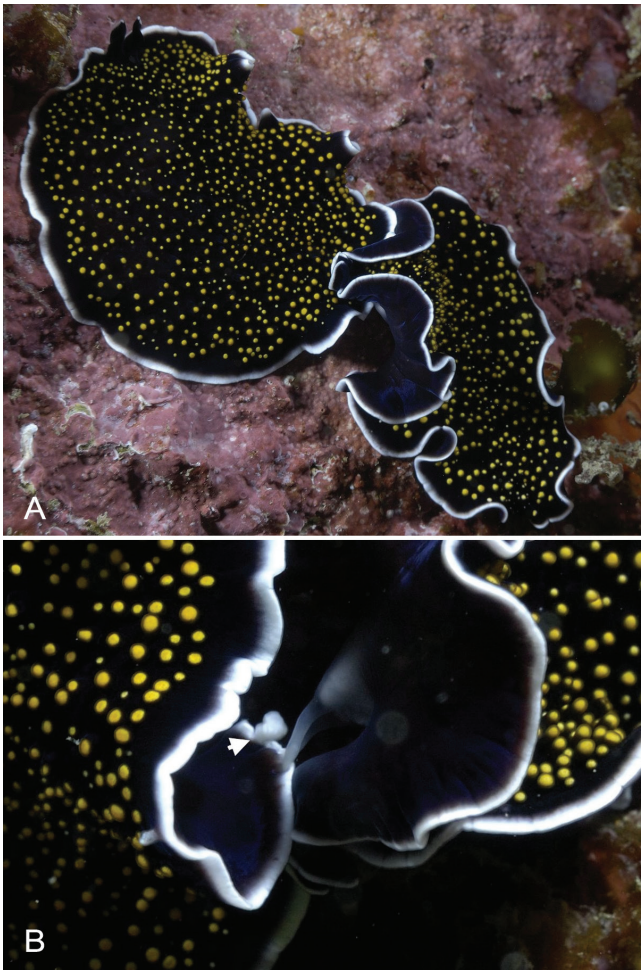

Fig. 9. *Thysanozoon nigropapillosum* (Hyman, 1959): copulation observed at Longdong bay, northeast coast of Taiwan, 25 July 2011. A, Dermal impregnation for unilateral insemination; B, One individual (right), with the paired penis stretched, gripping its partner (left) at the outermost margin. (spermatophore is indicated by an arrow)

The third type of inseminating behaviour is dermal impregnation. Gammoudi et al. (2012) provided histological evidence to show that ejaculated sperm are capable of entering the body directly from the skin. In *Thysanozoon brocchii*, heterogeneous spermatophores are found between the small dorsal papillae, in the marginal region of the body. It is suggested that these papillae probably have a function in avoiding becoming inseminated. The dermal impregnation behaviour of *T. nigropapillosum* is totally different from the penis fencing of *P. bifurcus*. The papillated *Thysanozoon* cannot leave their spermatophores on the receiver's dorsal papillae. Instead, *T. nigropapillosum* leaves its spermatophores in the marginal region of the receiver's body. This hypothetical pre-copulation defense has not been previously reported.

Under unilateral pressure of sexual selection, pre-copulatory (as opposed to post-copulatory) defensive structures and behaviour are worthy of study. Scharer et al. (2011) first reported post-copulation selection on sperm design. In the flatworm genus *Macrostomum*, the sperm from species employing dermal impregnation have a simple design that allows them to swim more easily through the receiver's tissue than complex sperm. In addition, species that undergo dermal impregnation tend to have a simple female genital atrium, which changed significantly when hypodermic insemination evolved. This implies that the female's defensive structures become unimportant during intercourse. Sagittal sections of the reproductive organ of *T. nigropapillosum* show that the penis is strengthened by rhabdites. This strengthened penis may allow the transfer of sperm more easily through dermal insemination. The female pore, which has much fewer rhabdites, is less important during intercourse. The presence of rhabdites on the tip of the papillae and inner male genital atrium that improves penis insemination may both be considered to be evolutionary advanced pre-copulation reproductive structures for polyclad flatworms.

Thysanozoon nigropapillosum (Hyman, 1959) often swims by undulating the margins of the body quickly during swimming. During dermal impregnation, the dorsal papillae of the body can prevent insemination. The swimming behaviour may also prevent insemination, when the body margin is moving quickly. Both the chopstick-like penis and the penis-gripping behaviour allow the flatworms to hold a partner securely that gives a better chance of successful insemination. This is another form of sexual behaviour that has not been previously reported. It is evident that the penis-gripping behaviour of *T. nigropapillosum* plays an important role during dermal impregnation. It also means that this

flatworm species experiences unilateral sex conflicts, not only in the structures of the dorsal papillae and the male genital organ, but also in the mating pattern. In pseudocerotid flatworms, there are two genera, namely, *Acanthozoon* and *Thysanozoon*, which have papillae on the dorsal surface. Members of the genus *Thysanozoon* have two penises, but those of *Acanthozoon* have only one penis. A comparison of the mating behaviour between these two genera would be of interest. Since copulation in the wild is rarely observed, more observations in captivity are required.

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