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Morphology and Phylogenetic Position of the Sargassum Nudibranch *Scyllaea fulva* Quoy & Gaimard, 1824 (Nudibranchia: Scyllaeidae): First Record in Hong Kong

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Scyllaeidae is a small group of nudibranchs comprising three genera (*Scyllaea*, *Notobryon*, and *Crosslandia*) with striking morphological similarities, making their identification challenging based on external features alone. Previous studies have highlighted the significance of central radular teeth in distinguishing *Notobryon* from *Scyllaea* and *Crosslandia*. The genus *Scyllaea*, commonly known as the sargassum nudibranch, currently consists of only two valid species, *Scyllaea pelagica* and *Scyllaea fulva*. These species inhabit seaweed *Sargassum* spp., feeding on hydroids. During a biodiversity survey conducted in April 2023, seven sargassum nudibranch individuals were collected from the seaweed *Sargassum* spp. at a depth of 2 meters in Tai She Wan through SCUBA diving. Initially, the specimens were misidentified based on their resemblance to *Notobryon wardi* and previous local records. However, thorough morphological and molecular examinations confirmed them to be *Scyllaea fulva*, representing the first record of this species in Hong Kong. Notably, our specimens lacked the blue spots observed in specimens from Thailand and the West Pacific Ocean, as reported in previous studies. Internally, a pineapple-like structure formed a honeycomb pattern on the surface of the masticatory edge of the jaw flap, with the presence of central radular teeth. A Maximum Likelihood tree analysis revealed *Crosslandia* to be the sister group of *Scyllaea*. Comparative analysis of intra-specific distances between individuals from the Philippines, French Polynesia, and Hong Kong indicated a close relationship between the Hong Kong specimens and those from the Philippines. Furthermore, we provide a detailed description of the external and internal morphology of *Scyllaea fulva* in this paper, integrating valuable morphological information for future species identification purposes.

Key words: Scyllaeidae, Sea slug, Mollusc, Subtropical reef, South China Sea

BACKGROUND

Scyllaeidae Alder & Hancock, 1855 is a small group of nudibranchs that consists of *Crosslandia* Eliot, 1902, *Notobryon* Odhner, 1936, and *Scyllaea* Linnaeus, 1758. They bear a striking resemblance to algae based on their body colour and wing-like lobes and primarily feed on epiphytic hydroids (Gosliner et al. 2008). Notably, they possess the ability to swim through the flexing of their rhinophoral sheaths and lateral appendages (Gosliner et al. 2008). Morphologically, Scyllaeidae can be distinguished by their dorsal margin,

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which extends into large lobes. These lobes, whether continuous or separated, often exhibit traces on the rhinophoral sheaths and are adorned with branchial tufts. The presence of an indistinct velum and the absence of frontal papillae are additional distinguishing features. Their rhinophore club is perfoliate, and the anus is positioned laterally or laterodorsally (Odhner 1936). However, due to the striking resemblance among Scyllaeidae members, the high similarity in their external morphology has led to significant confusion and misidentification (Pola et al. 2012).

Scyllaea, commonly known as sargassum nudibranch, can be distinguished from other scyllaeids by a radula with a central tooth and two pairs of well-separated dorsal lobes (Odhner 1936). *Scyllaea* comprises two recognised species including *Scyllaea pelagica* Linnaeus, 1758 and *Scyllaea fulva* Quoy & Gaimard, 1824 (MolluscaBase 2023) which both inhabit the seaweed *Sargassum* spp. *Scyllaea fulva*, a poorly studied species, was previously synonymized as *Nerea punctata* Lesson, 1830, *Scyllaea dracaena* Kelaart, 1858 and *Scyllaea quoyi* Gray, 1850. All of these species had been later synonymized as *Scyllaea pelagica* after Odhner (1936) considered *Scyllaea fulva* to be a spurious species. Pola et al. (2012) demonstrated that specimens of *Scyllaea pelagica* collected in the Indo-Pacific region were genetically distinct from those found in the Atlantic. The genetic difference between the Atlantic and Indo-Pacific specimens was determined to be 7% in the cytochrome *c* oxidase subunit I (*COI*) gene. This significant genetic distinction provided strong evidence for considering them separate species. Therefore, the name *Scyllaea fulva* was restored. Their study underscores the crucial role of molecular analysis in discerning between morphologically similar organisms.

In April 2023, we conducted a biodiversity survey in the *Sargassum* habitat. Seven individuals of *Sargassum*-like nudibranchs were found firmly adhering to *Sargassum* thalli. Upon initial examination, we identified the specimens as *Notobryon wardi* Odhner, 1936, based on a thorough review of external morphological features only (Rudman 2002b; Picton 2002; Chow et al. 2022). The external morphology (body colour, the shape of dorsal lobes, etc.) exhibited striking similarities between our specimens and those described in the literature. Furthermore, previous records had documented the presence of this species in Hong Kong waters (Rudman 2002b; Picton 2002; Chow et al. 2022). However, to ensure accurate identification, we employed molecular analysis to confirm the identities of our specimens. Surprisingly, the results revealed that our specimens were not *Notobryon wardi*, but instead belonged to the species *Scyllaea fulva*. It highlights the potential challenges of relying solely on external morphology for the identification of scyllaeids. Interestingly, the absence of previous records of the genus *Scyllaea* in Hong Kong literature (Jensen 1998; Chow et al. 2022; Astudillo et al. 2023) further emphasizes the significance of our findings. Additionally, we observed a significant dearth of well-documented morphological information (particularly internal morphology) pertaining to *Scyllaea fulva* in existing literature (Quoy and Gaimard 1824; Lesson 1830; Gray1850; Kelaart 1858; Odhner 1936). Hence, we initiated this study to present the first recorded occurrence of *Scyllaea fulva* in Hong Kong. This study aims to investigate both the external and internal morphology of this species. Furthermore, we seek to elucidate its phylogenetic position by analyzing three genetic markers: the cytochrome *c* oxidase subunit I (*COI*), the 16S ribosomal RNA (*16S rRNA*), and the Histone 3 (*H3*) genes. The data generated through this study will provide valuable insights into the morphology of scyllaeids, facilitating more accurate identification by researchers in the future.

MATERIALS AND METHODS

Sampling

Seven individuals of *Scyllaea fulva* were collected from *Sargassum* spp. at Tai She Wan (22°21'32.7"N, 114°20'15.1"E) by SCUBA diving on 28th March 2023 and 11th April 2023. The specimens were preserved in 95% ethanol for morphological and molecular analysis.

Morphological analysis

External morphological characteristics were examined under a Motic SMZ-171 stereomicroscope (Motic, China). Seven specimens were dissected to extract the buccal masses and reproductive systems. The buccal masses were dissolved in 20% diluted bleach for 30 min at room temperature to remove connective tissues and muscles. The jaws and the radula were isolated. The jaws and the radula were dried, coated with gold, mounted on a stub, examined and photographed under a LEO 1530 field emission scanning electron microscope (SEM) (Zeiss, Germany), and the reproductive system was observed and drawn under the stereomicroscope.

Molecular analysis

Genomic DNA of foot tissues from two specimens (NMMB-M011734 to 735) was extracted using Chelex

100 solution (Walsh et al. 1991). Concentration and purity of the DNA samples were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and DNA integrity was checked using electrophoresis on 1.0% agarose gel. Polymerase chain reactions (PCRs) were then conducted using the extracted DNA as templates to amplify the *COI*, *16S rRNA* and *H3* genes. Primer sequences for partial sequences of *COI* were sourced from Folmer et al. (1994) using pairs LCO1490 (5'-GGTCAACAAAT CATAAAGATATTGG-3') and HC02198 (5'-TAAACTT CAGGGTGACCAAAAAATCA-3'). Partial sequences of the *16S rRNA* region were amplified using the pairs 16Sar-L (5'-CGCCTGTTTATCAAAAACAT-3') (Palumbi et al. 1991) and 16s-xH (5'-CCGGTYT GAAMYYAGATCACGTAGG-3') (Mehrotra et al. 2020). Primers for the *H3* region were taken from Colgan et al. (2000) using the primers H3F (5'-ATGGCTCGTACCAAGCAGACVGC-3') and H3R (5'-ATATCCTTRGGCATRATRGTGAC-3'). The following PCR program was used for the *COI* gene: 1 min at 95°C (initial denaturation); 35 cycles of 30 s at 95°C (denaturation), 30 s at 52°C (annealing), and 45 s at 72°C (elongation); and 7 min at 72°C (final extension). The PCRs for the *16S rRNA* and *H3* genes were conducted using a similar programme, except that the annealing temperature was 50°C. PCR products were sent to BGI Hong Kong for sequencing on an ABI 310 Genetic Analyzer. All new sequences were deposited in GenBank (Table S1).

Alignments of the three genes were conducted separately and trimmed manually to 661 bp for *COI*, 466 bp for *16S rRNA* and 328 bp for *H3* using MEGA 7 (Kumar et al. 2016). The sequence of *CO1*, *16S rRNA* and *H3* genes from other scyllaeids and a distantly related outgroup (*Melibe viridis*) were downloaded from GenBank (Table S1). Sequences were concatenated using SequenceMatrix v.1.7.8 (Vaidya et al. 2011) and then imported to the website version of IQ-Tree (http://iqtree.cibiv.univie.ac.at/; Nguyen et al. 2015) for Maximum Likelihood tree reconstruction with 1,000 ultrafast bootstrap pseudoreplicates (Hoang et al. 2017). The best fit model (TPM3u+F+I+G4) was determined by IQ-Tree server. The phylogenetic trees were visualized and edited using FigTree v1.4.4. Pairwise genetic distances for the respective *COI*, *16S* and *H3* genes were estimated using MEGA 7 separately based on the *p*-distance method using the bootstrap method with 10,000 pseudoreplicates for variance estimation. Rates among sites were gamma distributed with invariant sites (G+I) and the gamma parameter was set to four.

RESULTS

TAXONOMIC ACCOUNT

Class Gastropoda Cuvier, 1795
Order Nudibranchia Cuvier, 1817
Family Scyllaeidae Alder & Hancock, 1855
Genus *Scyllaea* Linnaeus, 1758
Species *Scyllaea fulva* Quoy & Gaimard, 1824

Synonyms

Nerea punctata (Lesson 1830), *Scyllaea dracaena* (Kelaart 1858), *Scyllaea quoyi* (Gray 1850), *Scyllaea pelagica* (Pola et al. 2012).

Materials examined: NMMB-M011734: 0.9 cm in length; NMMB-M011735: 0.85 cm in length; NMMB-M011736: 1.4 cm in length; NMMB-M011737: 0.95 cm in length; NMMB-M011738: 0.5 cm in length; NMMB-M011739: 1.3 cm in length; NMMB-M011740: 2.9 cm in length.

Locality: Seven specimens were collected from *Sargassum* spp. at Tai She Wan (22°21'32.7"N, 114°20'15.1"E) at 2 m on the 28th March and the 11th April 2023.

Type locality: New Guinea. Geographic distribution (Fig. 1): Hong Kong (this study), Indo-Pacific region including Japan (Baba 1949), Papua New Guinea, Philippines (Pola et al. 2012), Réunion (Cadet 2012), Gump Station, Cook's Bay, Moorea, French Polynesia (Goodheart et al. 2017 2018); Mozambique (Tibiriçá et al. 2017) and Thailand (Mehrotra et al. 2021).

Habitat: Inhabit the seaweed *Sargassum* spp. (Fig. 2A).

External morphology (Fig. 2B–D and Fig. 3A–B): The body colour varies from transparent to semi-transparent green or light yellow. The body is slender, soft, flaccid, and elevated, but laterally compressed. The body surface is smooth. There are some black or brown spots on the body. The foot is narrow. The posterior crest is moderately large with an entire margin. The edge of posterior crest is brown. The front of the head is expanded in a semicircular veil. The rhinophores are perfoliate with 3–4 lamellae. There are two pairs of equal sized dorsolateral lobes. The lobes are wing-like and denticulated. Each dorsolateral lobe bears four to five large and transparent dendritic 'gills' on their upper surface and two on the tail as well as four on each side of the tail.

Internal morphology (Fig. 3C–F and Fig. 4): The buccal mass contained a pair of thin and triangular jaws. The masticatory edge of the jaws is expanded into a wing-like flap. Over the edge of this flap is a series of pineapple-like structures. These structures

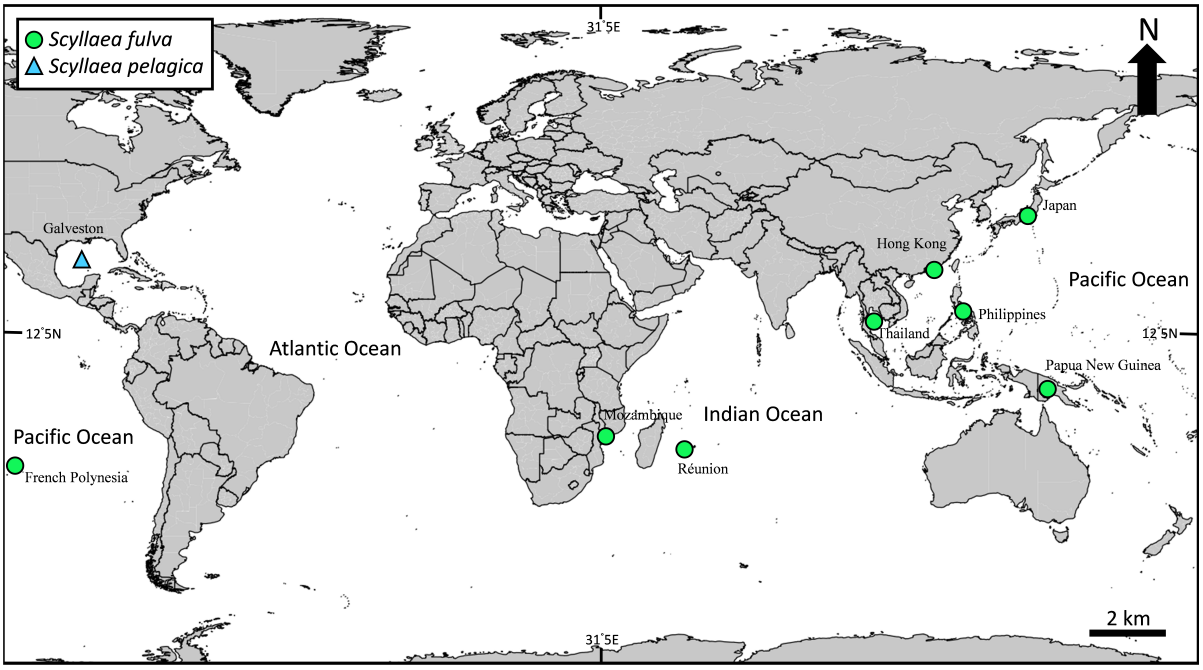

Fig. 1. Map showing the current known global distribution of *Scyllaea fulva* and *Scyllaea pelagica*.

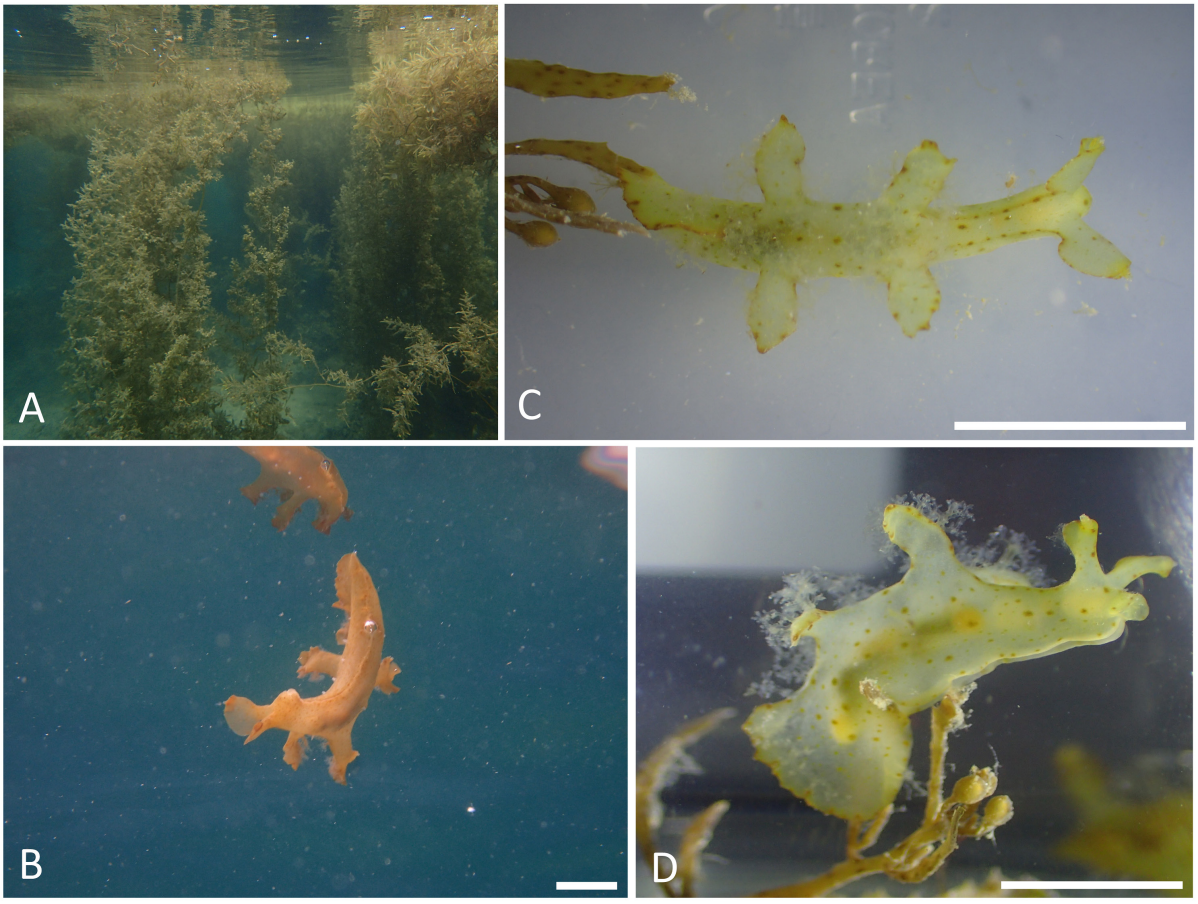

Fig. 2. Selected photographs showing the sargassum habitat and *Scyllaea fulva*. A, Sargassum bed at Tai She Wan; B, *In situ* swimming *Scyllaea fulva*; C, Dorsal view of *Scyllaea fulva* in a beaker; D, Lateral view of *Scyllaea fulva* in a beaker. Scale bar = 0.5 cm.

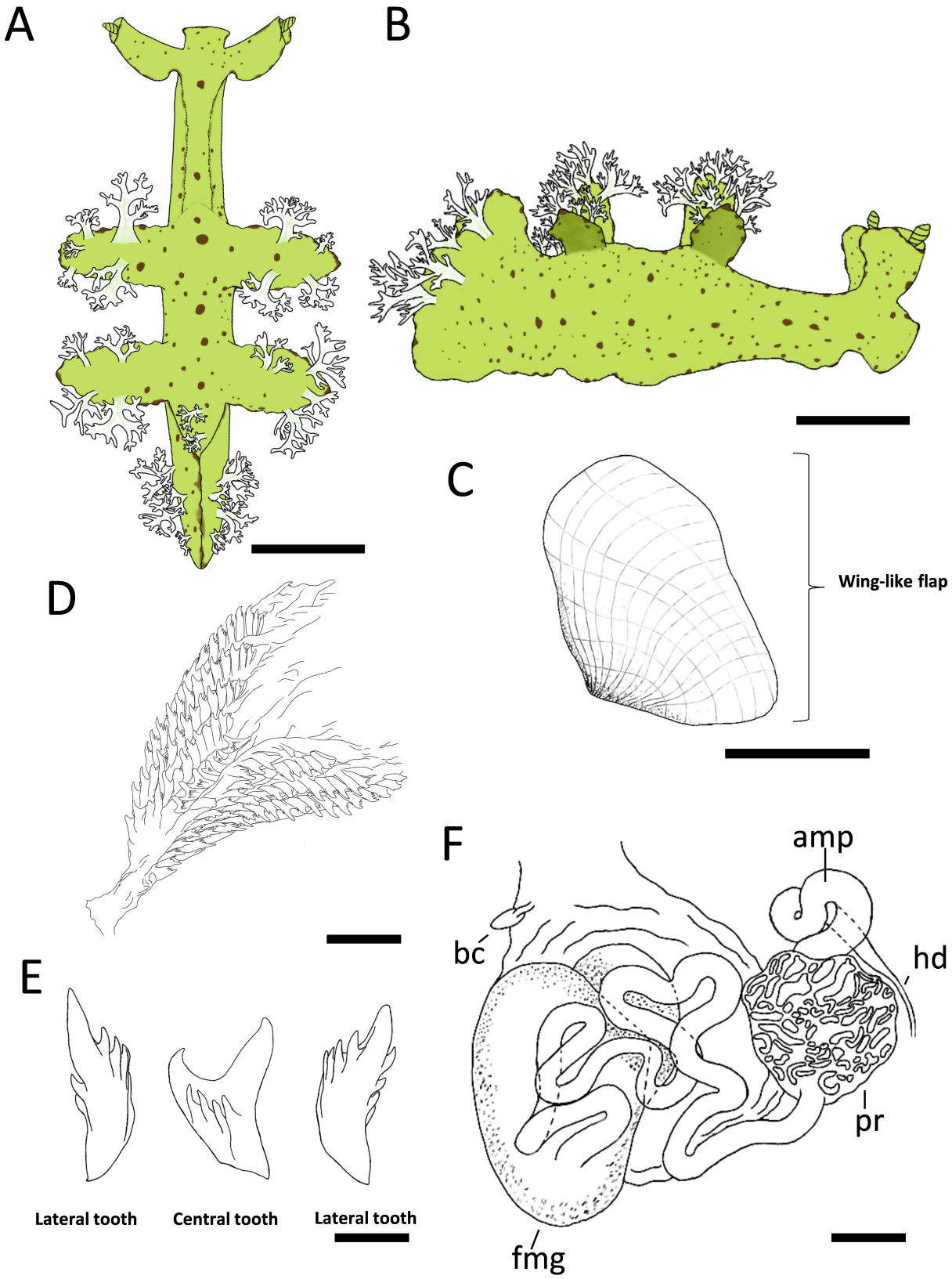

Fig. 3. Drawing showing the external and internal morphology of *Scyllaea fulva*. A, Dorsal view; B, Lateral view; C, Jaw; D, Radula; E, Teeth; F, Reproductive system. bc, bursa copulatrix; fmg, female gland mass; pr, prostate; amp, ampulla; hd, hermaphroditic duct. Scale bars: A and B = 0.5 cm; C = 0.5 mm; D = 250 µm; E = 20 µm; F = 0.2 mm.

form a honeycomb pattern over the entire surface of the masticatory edge, and the number of rodlets on the pineapple-like structure ranged from two to five. Central radular teeth were present. Each primary denticle bore two to five secondary denticles. The radula formula was 11 × 15.1.15. The reproductive system contained a small bursa copulatrix, a large female gland mass, and a duct connecting the female gland mass to the prostate and the ampulla.

Remarks: *Scyllaea fulva* can be distinguished from *Scyllaea pelagica* based on the radular formula. The radular formula of *Scyllaea pelagica* is 16–24 × 24–54.1.24–54 (Odhner 1936), which exhibits a higher number of denticles compared to *Scyllaea fulva*.

Molecular analysis

Three gene sequences were obtained from each of the two specimens (NMMB-M011734 to 735). Alignment and concatenation resulted in a dataset of 1455 bp (661 bp for *COI*, 466 bp for *16S rRNA* and 328 bp for *H3*). Our phylogenetic analyses showed that the two specimens we collected from the field were *Scyllaea fulva* (Fig. 5). Pair-wise sequence comparisons were conducted to determine the inter- and intra-specific *p*-distances (Table 1; Table S1). Interspecific *p*-distances amongst *Scyllaea fulva* and *Scyllaea pelagica* were 6.90%–7.20% for *COI*, 2.20%–2.50% for *16S* and 0.00% for *H3*. Intraspecific *p*-distances in *Scyllaea fulva* were generally very small, with 0.00%–0.60% for *COI*, 0.00%–0.20% for *16S*, and 0.00% for *H3*.

DISCUSSION

We have successfully confirmed the specimens to be *Scyllaea fulva*, commonly called sargassum nudibranch. After reviewing Astudillo et al. (2023), Jensen (1998), and Chow et al. (2022), we have documented the occurrence of *Scyllaea fulva* as the first record in Hong Kong waters, which is consistent with the known distribution of the species. Morphologically, our specimens did not bear the blue spots on the body, which is different from the individuals found by Gosliner et al. (2008), Goodheart et al. 2017 2018) and Mehrotra et al. (2021). This indicates that external

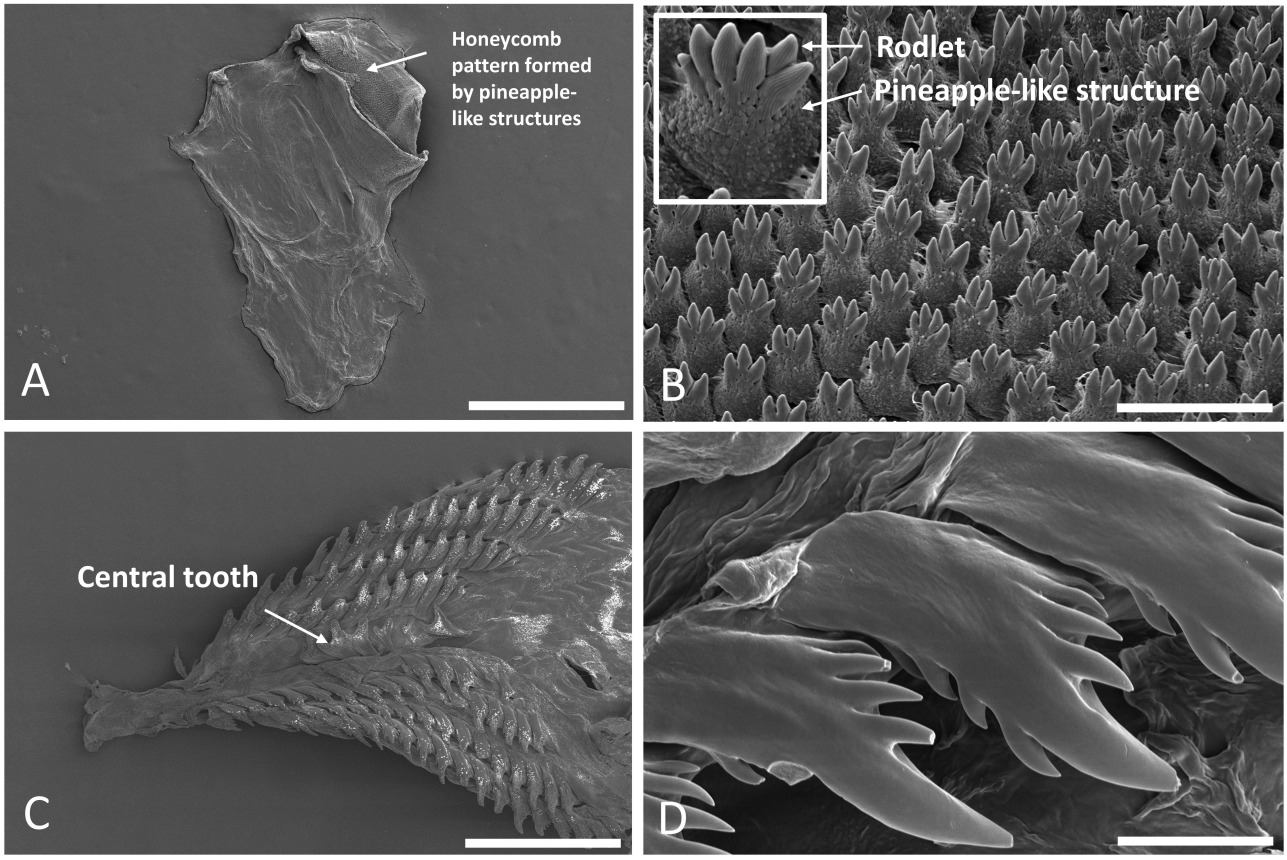

Fig. 4. SEM photos showing the jaws and radula. A, Overview of a jaw; B, Jaw element; C, Central teeth presented on the radula; D, Close-up image of the denticles. Scale bars: A = 500 μm; B = 20 μm; C = 500 μm; D = 20 μm.

morphological variation occurs within the species. Moreover, our specimens closely correspond to the original descriptions of *Scyllaea fulva* and its synonyms, exhibiting an elongated body, tawny color, grooved underside, and four slender wings, with the tentacles widened at the tip (Quoy and Gaimard 1824–1826; Lesson 1831; Gray 1850; Kelaart 1858). However, the literature did not provide detailed information on the morphology of *Scyllaea fulva*, particularly its internal morphology. Therefore, our findings on the internal morphology of *Scyllaea fulva* are important for integrating the morphological information of the species.

Before the work of Pola et al. (2012), *Scyllaea fulva* was considered a spurious species (Odhner 1936). Pola et al. (2012) demonstrated a 7% genetic distance for *COI* among specimens from the Atlantic and Indo-Pacific regions that confidently supported its recognition as a distinct species. Thus, *Scyllaea fulva* was re-established. Additionally, Odhner (1936) noted that scyllaeids can be differentiated at the genus level by the presence or absence of a central tooth on the radula. Rudman's (2002a b c) SEM photos of scyllaeids revealed that *Scyllaea pelagica* and *Crosslandia viridis* also had a central radular tooth, but *Notobryon wardi* did not. Several *Notobryon* species described by Pola et al. (2012) and Caballer and Ortea (2014) also lacked the central radular tooth, which is consistent with Odhner (1936) and Rudman's (2000a b c) conclusions.

To precisely identify scyllaeids, Pola et al. (2012) demonstrated that molecular analysis is a powerful tool for delineating species boundaries. In the present study, we also used molecular analysis to avoid misidentification and accurately identify our specimens. Our findings on inter- and intra-specific *p*-distances revealed that *H3* lacked a phylogenetic signal, but *COI*

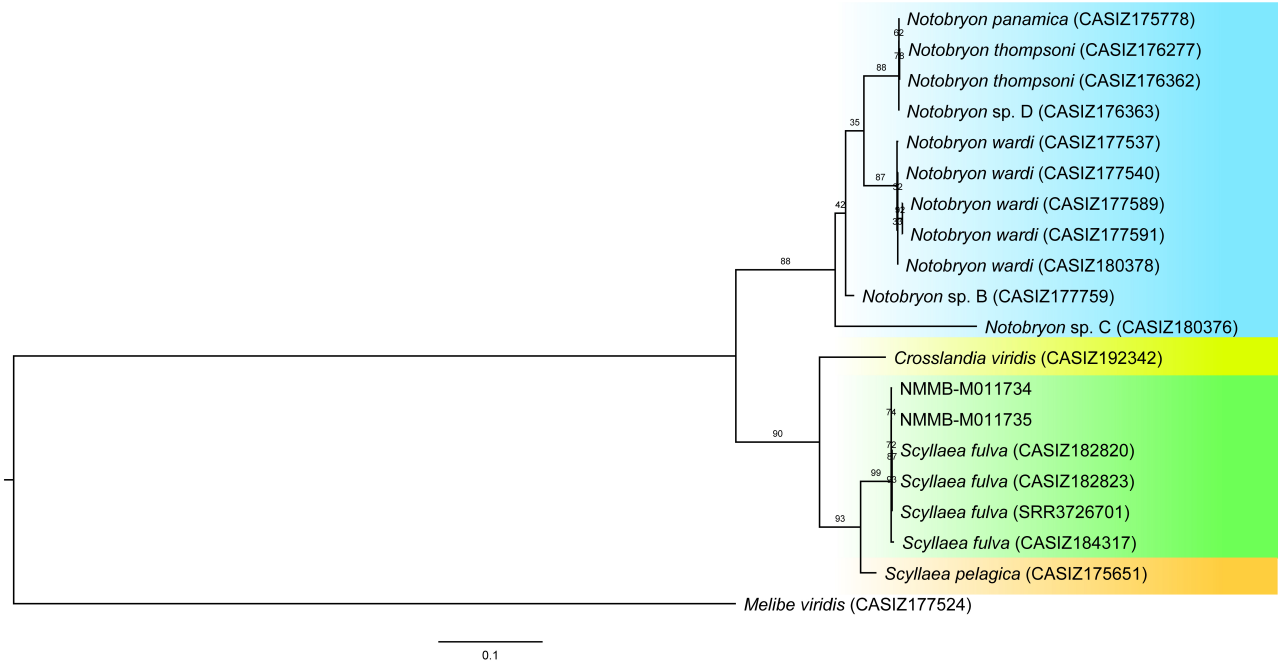

Fig. 5. Phylogenetic tree of the concatenated *COI/16S/H3* sequence dataset constructed using the maximum likelihood method. Bootstrap values > 50 are shown in the nodes.

Table 1. *Scyllaea* inter- and intra-specific uncorrected *p*-distances for *COI/16S/H3*. (P) indicates the specimens from the Philippine (Pola et al. 2012); (FP) indicates the specimen from French Polynesia (Goodheart et al. 2017 2018); (HK) indicates the specimens used in this study. For further details, refer to tables S2–4

Species	*COI*	*16S*	*H3*
Scyllaea pelagica vs *Scyllaea fulva*	6.90%–7.20%	2.20%–2.50%	0.00%
Scyllaea fulva (P) vs *Scyllaea fulva* (HK)	0.30%–0.60%	0.00%–0.20%	0.00%
Scyllaea fulva (FP) vs *Scyllaea fulva* (HK)	0.60%	0.00%	-
Scyllaea fulva (HK1) vs *Scyllaea fulva* (HK2)	0.30%	0.00%	0.00%

is a useful gene for delimiting the species. Compared with the genetic distance (0.30%–0.60%) for *COI* among *Scyllaea fulva* specimens from the Philippines, French Polynesia, and Hong Kong, the Hong Kong *Scyllaea fulva* is closely related to the species in the Philippines.

This occurrence of *Scyllaea fulva* expands the diversity of nudibranchs in Hong Kong, making it the second member of Scyllaeidae to occur in Hong Kong since Rudman (2002b) confirmed the Hong Kong specimen (AMC139151) stored in the Australian Museum to be *Notobryon wardi*. Additionally, the present study also reports the external and internal morphology to integrate the morphological data for *Scyllaea fulva*. These findings provide insights for the morphological identification of scyllaeids in the future.

CONCLUSIONS

Our study reports the first documented occurrence of *Scyllaea fulva* in Hong Kong waters. The confirmed presence of this species expands the diversity of nudibranchs in the region. The observed external morphological variation, coupled with detailed morphological data, provides valuable insights for future identification of scyllaeids and contributes to our understanding of *Scyllaea fulva*'s biology. This study highlights the importance of ongoing research to further explore the phenotypic and genetic diversity of marine organisms.

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Authors' contributions: SKFY and MY initiated the study, SKFY, TKTL and GYL conducted sampling, TKTL conducted the drawing, SKFY performed molecular and morphological analysis as well as drafted the manuscript.

Competing interests: SKFY, TKTL, GYL and MY declare they have no conflicts of interest.

Availability of data and materials: The accession numbers of specimens have been deposited in the GenBank. The examined specimens are deposited at the National Museum of Marine Biology and Aquarium in Taiwan.

Consent for publication: All of the authors agreed to publish the paper.

Ethics approval consent to participate: Not applicable.

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Supplementary materials

Table S1. Accession numbers of scyllaeids for three genes used in the phylogenetic analysis. (download)

Table S2. Uncorrected *COI p*-distances (%) calculated based on individual genes of the five available *Scyllaea* species in the NCBI and the sequences of specimens from this study. (download)

Table S3. Uncorrected *16S p*-distances (%) calculated based on individual genes of the five available *Scyllaea* species in the NCBI and the sequences of specimens from this study. (download)

Table S4. Uncorrected *H3 p*-distances (%) calculated based on individual genes of the four available *Scyllaea* species in the NCBI and the sequences of specimens from this study. (download)