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Original Article

Light-sheet microscopy for high-resolution imaging of *Caudoeuraphia caudata* (Pilsbry, 1916), a new record of acorn barnacle from Thailand's coast and its application in taxonomic identification and micro-morphological studies

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Abstract

The acorn barnacle (Cirripedia: Balanomorpha) is a sessile crustacean arthropod, distributing around the intertidal areas of tropical and temperate regions worldwide. Current practices for taxonomic identification are based on shell morphology and light microscopy, together with the use of scanning electron microscopy for arthropodal characters, which the latter technique requires complicated procedures. Through the recent technology of confocal light-sheet microscopy, here we demonstrate a clear description of *Caudoeuraphia caudata* (Pilsbry, 1916), a new record of its presence in eastern Thailand. This type of microscopy enables the high acquisition of fluorescent imaging of a whole barnacle's body and arthropodal structures, including cirri and mouthpart imaging in three dimensions, with simple procedures for sample preparation and through harboring autofluorescence of their own barnacle structures. Hence, this technology could potentially be an alternative way for identifying acorn barnacles at the species-level and visualizing the diversity of these marine arthropods.

Keywords: light-sheet microscopy, barnacle, Cirripedia, Chthamalidae, *Caudoeuraphia caudata*

1. Introduction

Acorn barnacles, a well-known biofouling organism, are well-characterized marine animals due to its invasions of oyster farms, aquaculture facilities, rehabilitated mangroves, offshore oil platforms and ships (Holm, 2012; Molnar, Gamboa, Revenga, & Spalding, 2008; Rawangkul, Angsupanich, & Panitchart, 1995; Sophia-Rani, Pmbhu, & Przyadharshini, 2010). This sessile barnacle inhabits tropical and temperate intertidal coastal zones where it adheres to hard substrates, particularly rock surfaces and produces calcareous and stable hard shells that serve to protect its soft body tissue. Some acorn barnacles flourish with other organisms such as Chelonibiidae with crabs (Hayashi, 2013), Bryozobiinae with sponges (Yu, Kolbasov, & Chan, 2016) Coronuloidea with turtles and whales (Hayashi, 2013), Pyrgomatidae with corals (Brickner & Høeg, 2010; Brickner, Loya, & Achituv, 2010; Chen, Lin, & Chan, 2012), Balanidae and Chthamalidae with mangrove roots, bivalve mollusks and other barnacles (Chen, Tsang, Chong, & Chan, 2014; Frith, Tantanasiriwong, & Bhatia, 1976; Lively & Raimondi, 1987). In Thailand, at least ten species of acorn barnacles have been identified along the Gulf of Thailand and the Andaman Sea coasts, including three families: Chthamalidae (3 species), Tetraclitidae (4 species) and Balanidae (3 species) (Pochai, Kingtong, Sukparangsi, &

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Khachonpisitsak, 2017). The species richness of acorn barnacles found in the stations along Andaman Sea ranged from 2-8 species while only 2-4 different species occur in the stations on the Gulf of Thailand (Pochai *et al.*, 2017).

Current practices for acorn barnacles' identification are based on morphological studies of: i) hard shell parts, ii) operculum (geometry of tergum and scutum), iii) arthropodal characters including 6 pairs of the biramous modified legs or cirri I-VI, iv) mouthparts (two each of maxillae, maxillules, mandibles, mandibular palps and a labrum), v) a penis, and vi) caudal appendages. The presence of caudal appendages is a unique feature found specifically in the genus *Caudoeuraphia* Poltarukha, 1997 and the only species in this genus is *Caudoeuraphia caudata* (Pilsbry, 1916). Identification of these fine arthropodal structures is generally conducted with light microscopy and high-resolution imaging with SEM (Chan *et al.*, 2008; Chan & Cheang, 2016; Shahdadi, Chan, & Sari, 2011). Information on these arthropodal characters is important for identifying acorn barnacles at the species-level while shell morphology is not always sufficient to clearly distinguish among acorn barnacles due to variations based on habitat, algae-furnished shell parts, and different diametric growth and/or ages (Chan, Chen, & Dando, 2016; Chan, Tsang, & Chu, 2007a, 2007b).

In recent years, fluorescence-based confocal light sheet microscopy (LSFM), using pure optics for sectioning helps to illustrate high resolution of three dimensional structures of large and live or fixed specimens through immunofluorescence and its own autofluorescence (de Medeiros *et al.*, 2015). Importantly, these can be done in a short period of imaging and require less complicated procedures for sample preparation, compared to laser scanning microscopy (LSM) and SEM. In this present study, we aimed to illustrate detailed morphology and three-dimensional imaging of arthropodal characters of a new record *C. caudata* by exploiting the benefit of light-sheet-based fluorescence microscopy.

2. Materials and Methods

2.1 Sampling sites and sample collection

Our survey covered coastlines of Eastern Thailand (13°20'31.8"N 100°56'34.6"E to 11°58'33.7"N 102°46'10.4 "E), including Chon Buri, Rayong, Chanthaburi, and Trat provinces. *Caudoeuraphia caudata* was found in two stations of all survey sites, including Mun Nork Island (MN), Klaeng district, Rayong province (12°34'03.5"N 101°42'05.5"E) and Kung Wiman Beach (KW), Na Yai Am district, Chanthaburi province (12°36'07.0"N 101°52'39.2"E) (Figure 1). A total of 64 individuals of *C. caudata* were collected from rocky shores during low tides, including 32 specimens from MN and 32 specimens from KW. Whole acorn barnacles were removed from the substratum using scalpels and immediately preserved in 95% alcohol for further examination. All work was done under certified animal research protocols of W.S. and S.K. (Certificate from Institute of Animal for Scientific Purposes Development-IAD, Royal Thai Government: U1-03103-2559 and U1-03104-2559, respectively).

2.2 Taxonomic identification

Samples were identified based on their shell morphology using a stereomicroscope. Taxonomic identification of all acorn barnacles was performed, using shell morphology and arthropodal characters, together with keys of Pilsbry (1916), Newman & Ross (1976), Chan, Prabowo, Lee, & Lee (2009), and Pochai *et al.*, (2017). All samples from each station were deposited in the Laboratory of Zoology, Department of Biology, Faculty of Science, Burapha University.

2.3 Sample preparation

Shells with intact body tissue were stored directly in 95% (v/v) ethanol at room temperature. Whole barnacle body tissue and arthropodal appendages were dissected from its shell under stereomicroscope and stored in 95% (v/v) ethanol until processing. To prepare samples for lightsheet imaging, body tissues were first rehydrated with sterile distilled water for 10 minutes and then immersed in warm 1% low melting point agarose (dissolved in Phosphate-Buffered Saline (PBS) pH 7.4) for 5 minutes. The sample immersion in agarose was done in 1.5 ml microcentrifuge tube and kept warm until embedding on heat block (50 ^{o}C). For embedding, the samples in warm agarose were pulled slowly into glass capillary tube (Blue cap, internal diameter 1.9 mm, Hilgenberg GmbH), and let the agarose gel harden, which should take about ten minutes. The gas capillary with sample was then assembled into a sample holder of lightsheet station and ready for imaging as manufacturer's instruction.

2.4 Microscopy and imaging

Light sheet fluorescence microscopy was performed under ZEISS Lightsheet Z1 Fluorescence illumination at the lightsheet laser's wavelengths of 405, 445, 488, 515, 561, and 638 nm. Sample positioning could be viewed by transmission LED. Emitted light was collected by an objective lens, W Plan-Apochromat 20x/1.0 (water immersion). Images were taken by AxioCam1, AxioObserver SPIM.

Recording time of a single section excited with both lasers was about 0.5-1 second. Number of optical sections depended on various parameter settings and sample size; however, generally only 500-1,400 autofluorescent sections were recorded, sufficient to create 3D images of a whole barnacle body. Average time of imaging was 5-8 minutes. Z-stacks/sections were fused computationally to obtain 3D images without further deconvolution via maximum intensity projection in ZEN 2014 SP1 (Black edition version 9.2.0.0, 64 bit, Carl Zeiss). 3D views, fluorescence intensity and brightness of arthropodal characters were edited and captured in ZEN 2.3 (Blue Edition, Carl Zeiss). The scale bars were obtained directly from the ZEN lite software.

Whole shell, shell plates and operculum images were captured with an Olympus SZ61 Stereo Microscope and digital camera Canon EOS 700D. Images were taken with EOS Utility and processed with Image Frame Work (Tarosoft).

3. Results and Discussion

3.1 Systematic taxonomy

Superorder Thoracica Darwin, 1854
Order Sessilia Lamarck, 1818
Suborder Balanomorpha Pilsbry, 1916
Superfamily Chthamaloidea Darwin, 1854
Family Chthamalidae Pilsbry, 1916
Subfamily Euraphiinae Newman & Ross, 1976
Genus *Caudoeuraphia* Poltarukha, 1997
Type species. *Chthamalus caudatus* Pilsbry, 1916
1 genus, 1 species recorded: *Caudoeuraphia caudata* (Pilsbry, 1916).

Caudoeuraphia caudata (Pilsbry, 1916)
Chthamalus caudatus Pilsbry, 1916: 314, fig. 92 A-C, pl. 73 figs 1, 1a, 1b.
Euraphia caudata – Newman & Ross, 1976: 41.
Caudoeuraphia caudata – Poltarukha, 1997: 464.

Non-type material examined. Gulf of Thailand: 32 specimens, Rayong province, Klaeng district, Mun Nork Island (MN), 27.I.2017, W. Sukparangsi (BUU17.CH.CC01-32) and 32 specimens, Chanthaburi province, Na Yai Am district, Kung Wiman Beach (KW), 01.IV.2017, S. Khachonpisitsak (BUU17.CH.CC33-64)

Diagnosis: Peduncle absent; shell conical and depressed, shell with 6 plates; membranous basis, non-tubiferous; caudal appendage present.

Distribution: *Caudoeuraphia caudata* is widely distributed in the Indo-Australian and Indo-Pacific regions. It has been recorded in Australia (Endean, Kenny, & Stephenson, 1956; Endean, Stephenson, & Kenny, 1956; Foster, 1974; Hosie, Sampey, Davie, & Jones, 2015; Jones, 2003, 2010; Jones, Anderson, & Anderson, 1990; Pope, 1965; Stephenson, Endean, & Bennett, 1958), China (Liu & Ren, 2007), Japan (Chan, 2006), Singapore (Jones & Hosie, 2016), Vietnam (Poltarukha & Zvyagintsov, 2008; Zevina, Zvyagintsev, & Negashev, 1992). In the present study, a new location record of *C. caudata* from the Eastern coast of Thailand, inhabiting Rayong and Chanthaburi provinces is documented (Figure 1A and 1).

Habitat: Inside the shaded areas of rocky crevices in the upper intertidal zone, periodic absence of seawater (during low tides), and no direct exposure to sunlight (Figure 1C and D). *C. caudata* were found mostly in big colonies in narrow rocky crevices, in which each individual was connected by shell parts (Figure 1D).

3.2 Taxonomic identification of *Caudoeuraphia caudata* based on shell morphology

Caudoeuraphia caudata lacks a peduncle, a distinctive feature identifying it as an acorn barnacle in the Suborder Balanomorpha. Body length ranges from 6-10 mm. Shell cone is flattened or depressed (Figure 2A left). *C. caudata* is easily distinguished from *Euraphia* and *Chthamalus*, in that the shell cone of *C. caudata* is wider and flatter. Densely packed colonies caused irregular shell margins and shapes. Shell parts adhere to rocky surfaces with a membranous basis, similar to that found in other Chthamalids. The edge of shell plates is solid or without a parietal tube. After removal from rock surfaces, the mantle around the

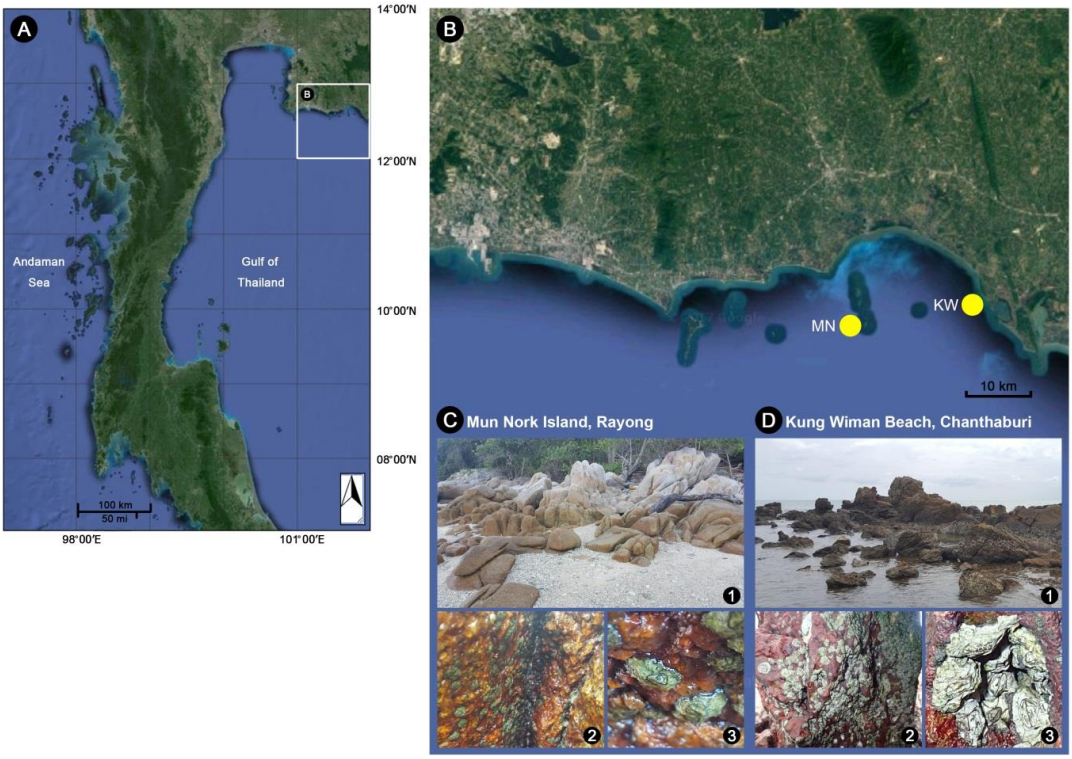

Figure 1. Distribution map and habitat characteristics of *Caudoeuraphia caudata* in the Eastern Thailand

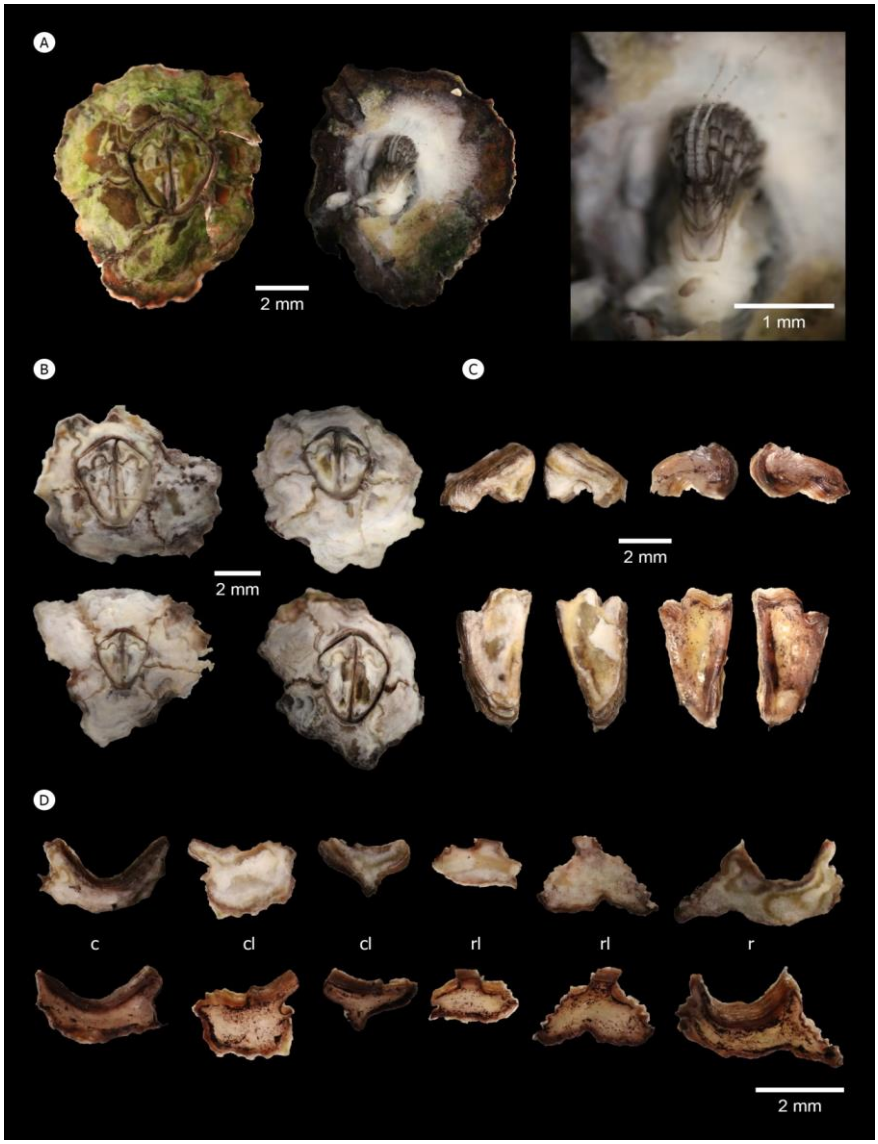

Figure 2. Shell morphology of *Caudoeuraphia caudata* (A) Dorsal (left panel), ventral (middle and right panel) view of external shell plate and intact body. (B) Variation of shell morphology. (C) External (left panel) and internal (right panel) view of tergum (upper panel) and scutum (lower panel). (D) External (upper panel) and internal (lower panel) view of each shell plate. Abbreviation: c, carina; cl, carinolateral; l, rostrolateral; r, rostrum

shell's edge is dark grayish pink, which became black in ethanol. Color of the inner mantle around the body is white (Figure 2A middle). On the ventral side, a long segmented caudal appendage close to cirri VI was apparent, extending from the posterior side of the body (Figure 2A right, Table 1). In all sampling sites, two patterns of *C. caudata* shell color were found. In shaded area of rocky crevices with low exposure to sunlight, the shell is green-brown perhaps due to the presence of algae (Figure 2A). On the edge of rocky shore with higher exposure to sunlight, the shell is white-light brownish (Figure 2B). The external surface of shell is usually smooth but some have an eroded surface exposing a deeper shell surface brownish-gray in color. The orifice containing an operculum is convex kite-shaped with rounded edges. The opercular plates inside the orifice are symmetrical. Color and surface of the exterior side of opercular plates resembles those of shell plates. The internal surface is yellow-light brown. The tergum is narrow while the scutum is long, covering most of the orifice area. The tergum is deeply interlocked or articulated with the scutum. The scutum is triangular with a slightly curved basal margin, and its external surface exhibits shallow and horizontal striations from the occludent margin to the tergal margin. The occludent margin of the scutum is without teeth and the tergal margin exhibits a clear sinus from exterior and interior view. Terga carry 4–5 lateral depressor crests (Figure 2C). In addition, the arrangement and number of shell plates around the orifice is the main characteristic for barnacles' identification. As in other Chthamalids, six shell plates are present, including a carina, two carinolaterals, two rostrolaterals and a rostrum; however, the size of each shell plate is greatly varied. The junction of each shell plate has small teeth or is irregular in shape (Figure 2D).

3.3 Morphological studies of acorn barnacles in high resolution and three dimensions

Without antibody staining and complication of immunofluorescence, barnacle body was directly excited with various wavelengths of laser light to search for the appropriate wavelength capable of stimulating the barnacle's own auto-fluorescence. Two excitation wavelengths, 405 and 488 appeared to be the best to visualize the structures of arthro-podal characters including soft structures. These excitation wavelengths lead to emission/detection wavelengths at 415 nm and 498 nm, respectively. Internal musculature is more excited at 488 nm laser, leading to a brighter red pseudocolor, while the cuticle or exoskeleton of cirri and mouthparts can be excited at both 405 and 488 nm (Figure 3C). However, the transparency of internal structures that can be seen through autofluorescence varies among samples depending on planes of dissection. This slight difference in excitation enhances cuticle separation from other internal soft muscles and provides more detail on muscular architecture. In combination two channels of laser excitation of pseudocolor green and red best demonstrates whole body morphology (Figure 3C) and micromorphology of specific structures of acorn barnacles and was used to describe fine structures.

3.4 Comparsion of cirral and penis morphology of *Caudoeuraphia caudata* to other Chthamalids

Here we describe morphology and setation present in cirri I-VI, penis, and caudal appendages of *Caudoeuraphia caudata*, following Chan *et al.* (2008) and based on lightsheet illumination.

3.4.1 Cirri I–II

The length and width of rami and setation of cirri I and cirri II are distinct from other cirri. Setal appendages protruding from cirral limbs can be observed. Numerous setae between these maxillipeds around oral cone resemble a mesh, serving a microfiltration function in capturing small food particles and preventing food escapement from the mouth (Figure 4A). Setae project in all directions from the shaft of limbs, in particular at the apex of both cirri I and II (Figure 4A1 and 4A2). In addition, a few short spines occur around segment junctions on exopod of cirri I (Figure 4A3). If compared to conical spines of *Chthamalus malayensis* (Figure 4B), these small spines on the exopod locate more anteriorly, in a similar manner to those in *Euraphia depressa* (Figure 4C).

In cirri I, oral setae on the anterior ramus are longer than those on the posterior ramus. The posterior side of protopod has long setae while few shorter setae are present on the anterior side (Figure 4D). Only serrulate type of setae are present around the limb of cirri I (Figure 4E and 4F). Similarly, long setae occur on the oral sites of the anterior ramus of cirri II and the protopods possess long setae on both anterior and posterior surfaces (Figure 4G). Unlike multicuspidate setae with a basal guard in *C. malayensis* (Tsang *et al.*, 2012), those on the oral site of all segments in the anterior ramus of cirri II in *C. caudata* are of the serrate type, composed of two rows of densely packed denticles (Figure 4H). The posterior and anterior surface of protopod in cirri II carry simple (Figure 4I) and serrate setae (Figure 4J), respectively. Setae at junctions around the aboral site bear only simple setae (Figure 4K).

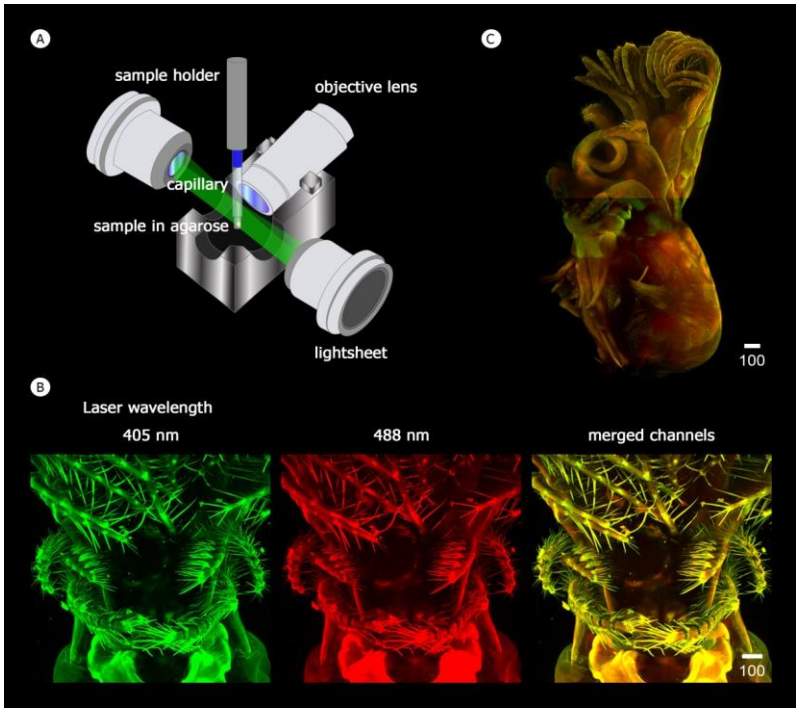

Figure 3. Schematic illustrations of whole procedures for light-sheet microscopy and barnacle sample preparation (A) High-speed imaging of whole barnacle body with light-sheet microscopy. (B) Wavelength of laser used for visualizing the barnacle body (*Caudoeuraphia caudata*) with autofluorescence. (C) The reconstruction of the whole Chthamalid barnacle in anterior side by imaging auto-fluorescence. Scale bar, µm

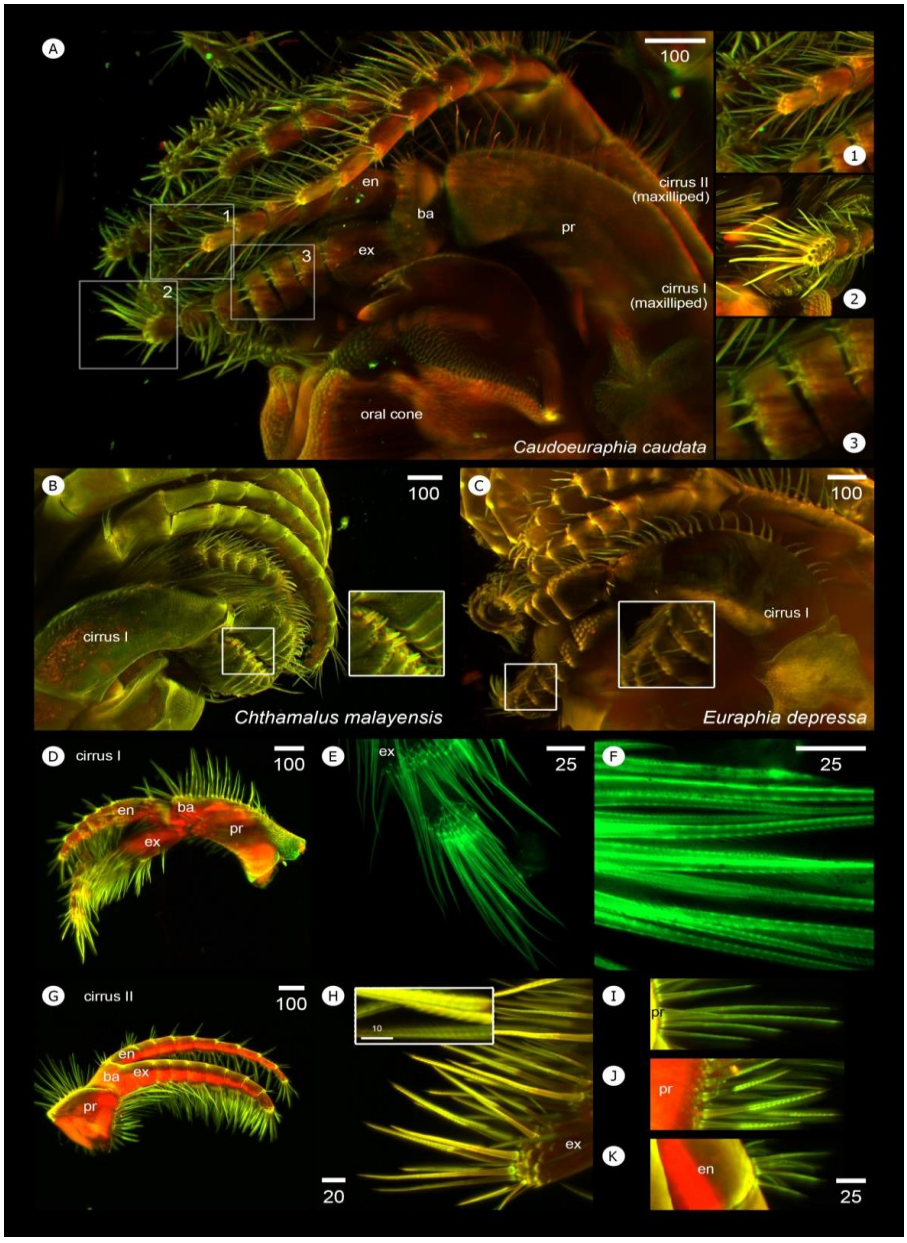

Figure 4. Light-sheet based visualization of 3D-autofluorescent cirri I and II of *Caudoeuraphia caudata* (A) Lateral view of *C. caudata*. (Inset 1) setation around anterior and (Inset 2) posterior rami of cirrus I. (Inset 3) spines protruding from anterior ramus of cirrus I. (B) Lateral view of *Chthamalus malayensis* showing conical spines (inset) on the cirrus I. (C) Lateral view of *Euraphia depressa* showing small spines (inset) on the anterior ramus of cirrus I. (D) overall morphology of cirrus I of *C. caudata*. (E) Apex of anterior ramus of cirrus I. (F) serrulate setae on cirrus I. (G) Overall morphology of cirrus II. (H) Close-up on apex of anterior ramus of cirrus II showing serrate setae (inset). (I) Simple setae found on the posterior side of protopod of cirrus I. (J) Serrate setae found on anterior surfaces of protopod of cirrus I. (K) Simple type of aboral setae at the segmental junction. Abbreviation: en, endopod; ex, exopod; basipod; pr, protopod. Scale bar, µm

Segment numbers of exopods and endopods vary in both cirri I-II and between left and right. Unequal number of segments is common between exopods and endopods. Asymmetry between right and left cirri I occurs also in some individuals (Table 1). Length of maxillipeds is clearly shorter than that of cirri III-VI. In cirri I, the anterior ramus is longer than the posterior, whereas cirri II exhibit shorter anterior ramus than the posterior. Exopod width of both cirri I-II is more than twice that of the endopod (Table 2).

3.4.2 Cirri III –VI

Cirri III-VI share similar features of limb morphology. Although cirri III are maxillipeds, their architecture resembles that of a cirral fan, except for setal type. Numerous setae between cirri III-VI are mesh-like but with larger mesh holes than those between cirri I-II, indicative of larger food particle gathering. The apex of anterior and posterior rami of cirri III has two large spines projected toward the oral cone

Table 1. Diverse segment numbers of arthropodal characters including cirri I – VI and caudal appendages found in *Caudoeuraphia caudata*.

Arthropodal characters	Number of segment	
	Right	
	Anterior ramus/ exopod	Posterior ramus/ endopod
cirrus 1	$5^{11\%}$, $6^{22\%}$, $7^{56\%}$, $8^{11\%}$	$5^{25\%}$, $6^{63\%}$, $7^{13\%}$
cirrus 2	$6^{22\%}$, $7^{56\%}$, $8^{11\%}$	$8^{44\%}$, $9^{44\%}$, $11^{11\%}$, $12^{11\%}$
cirrus 3	$11^{62.5\%}$, $13^{25\%}$, $14^{12.5\%}$	$11^{12.5\%}$, $12^{37.5\%}$, $13^{25\%}$, $14^{12.5\%}$, $15^{12.5\%}$
cirrus 4	$12^{37.5\%}$,$13^{12.5\%}$, $14^{37.5\%}$, $16^{12.5\%}$	$13^{25\%}$, $14^{12.5\%}$, $15^{50\%}$, $18^{12.5\%}$
cirrus 5	$13^{12.5\%}$, $14^{25\%}$, $15^{37.5\%}$, $16^{12.5\%}$, $19^{12.5\%}$	$13^{12.5\%}$, $14^{12.5\%}$, $15^{37.5\%}$, $16^{12.5\%}$, $17^{12.5\%}$, $18^{12.5\%}$
cirrus 6	$13^{14.3\%}$,$15^{14.3\%}$, $16^{57.1\%}$, $20^{14.3\%}$	$15^{28.5\%}$,$16^{42.9\%}$, $17^{14.3\%}$, $19^{14.3\%}$
caudal appendage	$19^{14.3\%}$, $20^{14.3\%}$, $21^{28.6\%}$, $23^{14.3\%}$, $24^{14.3\%}$, $25^{14.3\%}$	
Arthropodal characters	Left	
	Anterior ramus/exopod	Posterior ramus/endopod
cirrus 1	$6^{44\%}$, $7^{56\%}$	$5^{22\%}$, $6^{67\%}$, $7^{11\%}$
cirrus 2	$7^{33\%}$, $8^{22\%}$, $9^{44\%}$	$8^{22\%}$, $9^{67\%}$, $10^{11\%}$
cirrus 3	$10^{12.5\%}$, $11^{37.5\%}$, $12^{25\%}$, $13^{25\%}$	$11^{12.5\%}$, $13^{50\%}$, $14^{37.5\%}$
cirrus 4	$13^{37.5\%}$, $14^{25\%}$, $15^{25\%}$, $16^{12.5\%}$	$13^{12.5\%}$, $14^{50\%}$, $15^{12.5\%}$, $16^{12.5\%}$, $17^{12.5\%}$
cirrus 5	$13^{12.5\%}$, $15^{37.5\%}$, $16^{37.5\%}$, $19^{12.5\%}$	$13^{12.5\%}$, $15^{37.5\%}$, $16^{37.5\%}$, $20^{12.5\%}$
cirrus 6	$14^{16.67\%}$,$16^{66.66\%}$, $18^{16.67\%}$	$15^{33.33\%}$, $16^{16.67\%}$, $17^{33.33\%}$, $19^{16.67\%}$
caudal appendage	$20^{14.3\%}$, $21^{42.8\%}$, $22^{28.6\%}$, $27^{14.3\%}$	

Table 2. Average length and width of arthropodal characters including cirri I – VI and caudal appendages and penis found in *Caudoeuraphia caudata.*

Arthropodal characters	Endopod (endo) and exopod (exo) length	Fold difference	Endopod (endo) and exopod (exo) width	Fold difference
cirri 1	exo (444 μm) > endo (336 μm)	1.32	exo (171 μm) > endo (103 μm)	1.66
cirri 2	exo (653 μm) < endo (893 μm)	1.37	exo (167 μm) < endo (93 μm)	1.80
cirri 3	exo (1,309 μm) < endo (1,709 μm)	1.31	exo (154 μm) = endo (154 μm)	1.00
cirri 4	exo (2,311 μm) = endo (2,289 μm)	1.00	exo (144 μm) = endo (144 μm)	1.00
cirri 5	exo (2,273 μm) < endo (2,545 μm)	1.12	exo (160 μm) = endo (160 μm)	1.00
cirri 6	exo (2,080 μm) < endo (2,400 μm)	1.15	exo (140 μm) = endo (140 μm)	1.00
caudal appendage	right (2,130 μm) = left (2,130 μm)	1.00		
penis	2,080 μm	-		

and 2-3 thinner and shorter spines that protrude toward the posterior site (Figure 5A). Differently, the apex of cirri IV-VI has three large spines: two protruding toward the oral cone and one directed in a posterior direction (Figure 5B-5D). All of these cirri have long terminal oral setae and short setae at the junction of exopod and endopod segments. The protopods of cirri III contain setae on both anterior and posterior surfaces; whereas, cirri IV-VI have setae only on the anterior surface (Figure 5A-5D). All setae in Cirri III-VI are simple, except some serrate setae on the oral side of the anterior ramus-Cirri III (Figure 5A, inset). Cirri III and IV exhibit unequal numbers of segments between the anterior and posterior rami and between the left and right limbs. Cirri V and VI generally have equal segment numbers on the anterior and posterior rami; however, segment number can also vary in *C. caudata* with similar body length or age. Cirri VI exhibit the largest segment number in both exopod and endopod (Table 1). Length of cirri III is about twice longer than that of cirri I-II. In addition, cirri III have a shorter anterior ramus than the posterior; whereas, both rami in cirri IV-VI are approximately equal in length and width (Table 2).

3.4.3 Penis

C. caudata has a shorter penis, relative to that of *Euraphia* and *Chthamalus*. The whole penis including its apex in all dissected specimen were covered within the cirri; whereas, in other Chthamalids the penis is easily seen as a long appendage extending away from other cirri (Figure 5E and 5D). *C. caudata* penis length is similar to those of cirri IV-VI (Table 2). The penis apex is covered with 7-10 long simple setae, and shorter simple setae can also be found near

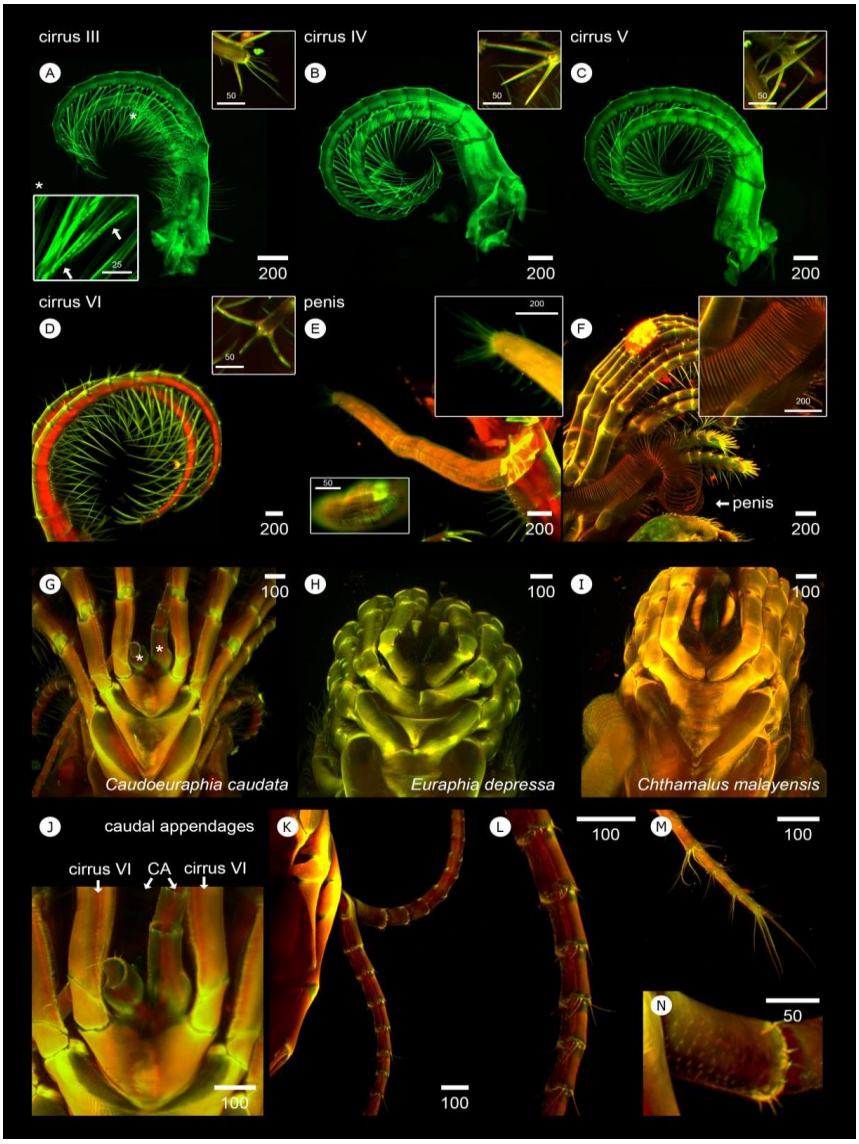

Figure 5. Light-sheet based visualization of 3D-autofluorescent cirri III-VI, penis and caudal appendages of *Caudoeuraphia caudata* (A)-(D) Morphology of cirrus III, cirrus IV, cirrus V and cirrus VI, respectively. (A) Top inset showing setation on apex of cirrus III. Bottom inset showing serrate type of setae found around oral site of anterior ramus of cirrus III. (B) Inset showing setation on apex of cirrus IV. (C) Inset showing setation on apex of cirrus V. (D) Inset showing setation on apex of cirrus V. (E) morphology of penis. Top inset illustrates pattern of setae around apex of the penis. Bottom inset shows surface of penis having pattern of irregular pattern of cuticle rings. (F) Penis of *Euraphia depressa*. Inset shows regular pattern of cuticle rings around its penis. (G)-(I) Posterior view of barnacles. (G) *C. caudata* carries caudal appendages (asterisks). (H)-(I) both *E. depressa* and *Chthamalus malayensis* have no caudal appendages. (J) Posterior view of *C. caudata* shows the position of caudal appendages (CA) joined to the base of sixth cirri. (K) Lateral view of caudal appendages. (L) Close-up on caudal appendage showing setae extending around segment junction. (M) Apex of caudal appendages. (N) Surface of caudal appendage covering with denticles. Scale bar, µm

the penis close to the apex (Figure 5E inset). In addition, *C. caudata* shows irregular shape of exoskeleton rings around the penis (Figure 5E inset) while that of *Euraphia depressa* exhibits obvious cuticular rings of cuticle (Figure 5F inset).

3.4.4 Caudal appendages

The most distinctive feature of *C. caudata* is the presence of caudal appendages at the posterior side (Figure 5G, asterisks), while these are absent in other chthamalids (Figure 5H and 5I). The base of caudal appendages is joined to the protopods of both sixth cirri (Figure 5J) and extends away from the cirral fan (Figure 5K). Simple setae (4-6) of unequal lengths occur at the junctions of segments (Figure 5L), whereas 4 longer simple setae are present at the apex (Figure 5M). Denticles with fine spines are present on the surface of caudal appendages (Figure 5N). Caudal appendages generally have 21 segments; however, in some specimens the number ranges from 19-27 segments (Table 1). Length is similar to that of cirri 6; however, width is much less than that of cirri I-VI (Table 2).

3.5 Three dimensional visualization of mouth appendages of *Caudoeuraphia caudata*

Mouthparts were illuminated and visualized through a light-sheet microscope (Figure 6A). Fan-like shape denticles with 5-6 small spikes occur around the labrum and on the base and body of each mouth appendage (Figure 6A1 and 6A2). Here we further describe each mouth appendage in detail (Figure 6 and Figure 7).

3.5.1 Maxillae

Maxilla has a bilobed shape (top and bottom lobes) and setae are grouped into three clusters: two clusters on the top lobe and one cluster on the bottom lobe (Figure 6B). The first cluster of setae faces upward away from the oral cone, carrying long simple and long serrulate setae (Figure 6B1 and 6B2). The second cluster on the top lobe contains two types of setae projecting toward the labrum: long-serrulate (Figure 6B3 inset) and short-simple (Figure 6B3 asterisk). The last cluster on the bottom lobe consists of a single row of simple setae, close to the base of maxilla (Figure 6B4). In addition, some small spines occur around the bottom lobe (Figure 6B4, asterisk).

3.5.2 Maxillules

Two cutting and notched surfaces occur on the maxillules (Figure 6C). Above the first notch, one large spine is located at the top followed by two smaller spines, then four

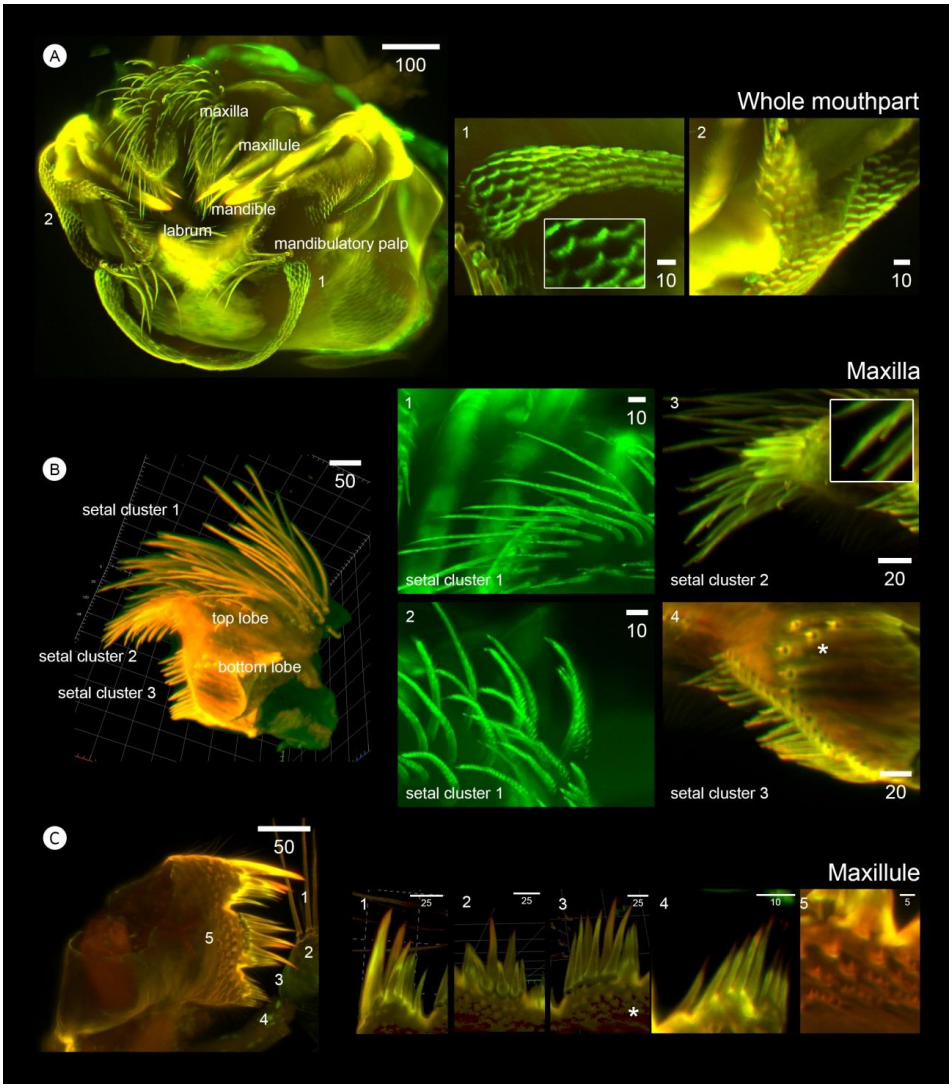

Figure 6. Light-sheet microscopy-based visualization of whole mouthparts and each individual mouthpart structures (maxilla and maxillule) of *Caudoeuraphia caudata* (A) Whole mouthpart or oral cone showing composition of mouth appendages. 1-2 is close-up of (A) and are shown on the middle (1) and right (2) panels. Panel 1 showing fan-like denticles (inset) around the surface of labrum. Panel 2 showing denticles around base of mandibular palp. (B) Overall morphology of maxilla. Panel 1-2 showing close-up view on setal cluster 1. Panel 3 showing close-up view on setal cluster 2. Panel 4 showing close-up view on setal cluster 3. (C) Overall morphology of maxillule. Number indicates the close-up pictures shown in panel 1-5. Panel 1-4 showing close-up of setae on maxillule. Panel 5 showing close-up on patterns of denticles on surface of maxillule. Scale bar, μm

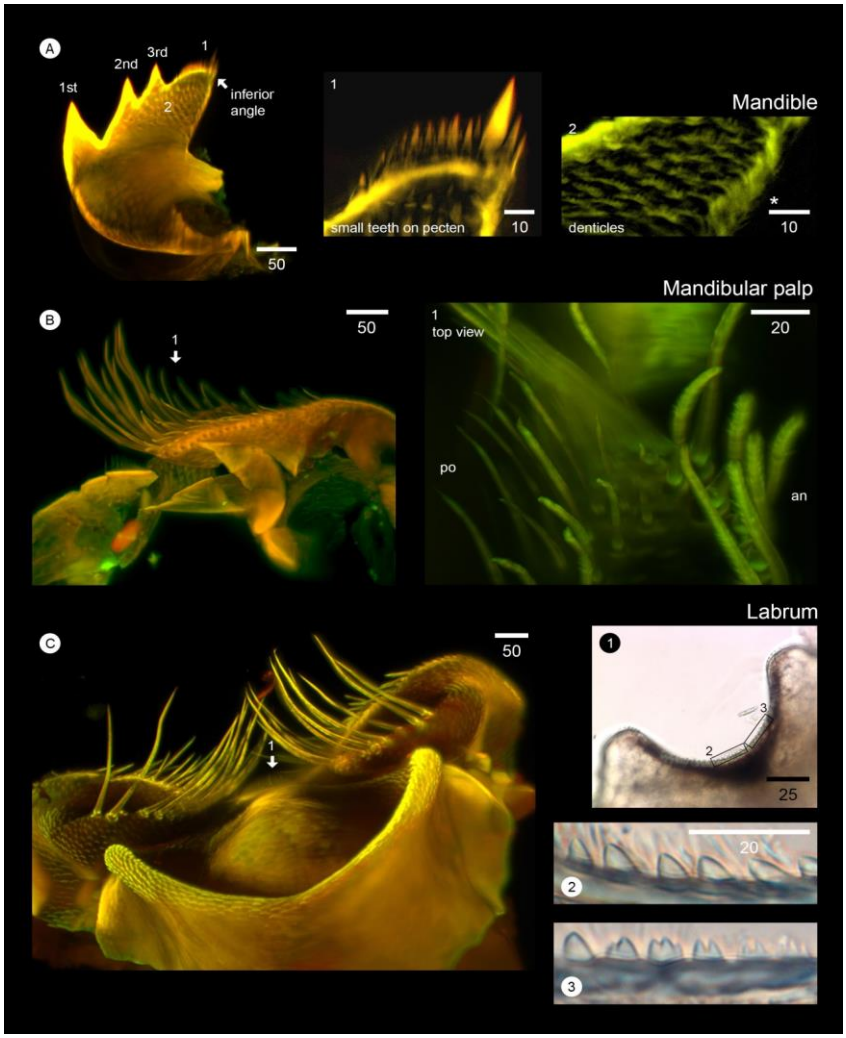

Figure 7. Light-sheet microscopy-based visualization of mouthpart structures (mandible, mandibular palp, and labrum) of *Caudoeuraphia caudata* (A) Overall morphology of mandible. 1st, 2nd and 3rd indicate teeth number. 1-2 indicate close-up view showing in the middle and right panel. Panel 1 showing close-up view on setae on pecten. Panel 2 showing close-up view patterns of denticles on surface of mandible. Asterisk indicates setae below the inferior angle. (B) Overall morphology of mandibular palp. Number 1 and arrow indicates picture from top view showing in right panel 1. Panel 1 illustrates setation on the surface of mandibular palp. (C) Overall morphology of labrum. Number 1 and arrow indicates picture a piece of labrum showing in right panel 1-3. Panel 1 illustrates whole labrum. Boxes are close-up of teeth showing panel 2 and 3. Abbreviation: in, inferior side; ex, exterior side. Scale bar, µm

smaller spines (Figure 6C1). At the middle of maxillules, two rows of spines are visible by 3D view: a first row with 4 small spines and a second row with two larger spines (Figure 6C2). Three additional spines are very close to each other, are below the six larger spines (Figure 6C3, asterisk). Below the second notch, eight small spines are located in a row. In this last lobe, two small notches further divide spines into a 1+4+3 pattern (Figure 6C4). The surface on the maxillule is decorated with denticles with small and sharp spines (Figure 6C5). This double notched maxillule is generally absent in *Chthamalus* and *Euraphia*.

3.5.3 Mandibles

Three large teeth occur on the mandibles (Figure 7A). The pecten of the mandible bears two rows of 12-18 small teeth. At an inferior angle of the mandible and located next to the pecten, are one large and two smaller teeth (Figure 7A1). In addition, numerous fine setae occur below the inferior angle (Figure 7A2 asterisk). Several rows of fan-shape denticles illuminated on the surface of mandible close to the teeth (Figure 7A2). The number of mandible teeth is a clear characteristic distinguishing *Chthamalus* from *Euraphia*. Previously, the presence of mandibles with three teeth in *C. caudata* led to its nomenclature as *Euraphia caudata* (Pilsbry, 1916).

3.5.4 Mandibular palps

Mandibular palps are rectangular in shape (Figure 7B). Two types of setae are present on the superior surface. Long and serrulate setae occur at the tip and along the exterior

side of the mandibular palps. Setae about one third shorter in length are present along the inferior surface of the palps (Figure 7B1).

3.5.5 Labrum

The shape of labrum is concave (Figure 7C1). Two types of teeth cover the length of the labrum: sharp teeth (similar to mammalian canine teeth) and teeth with two or three cusps (similar to mammalian premolar or molar teeth). The sharp teeth are located in the middle of concaved labrum (Figure 7C2) while the cuspidate teeth are present at both end of the labrum (Figure 7C3). This tooth pattern differs from that in *Euraphia depressa*, carrying only sharp teeth at the middle of the concaved labrum (Lively & Raimondi, 1987).

4. Conclusions

Lightsheet Fluorescence Microscopy (LSFM) provides ultrafast 3D imaging. With this technology, we provide a more detailed description of *Caudoeuraphia caudata* found on the rocky shores locating in the Gulf of Thailand, Eastern part of Thailand. The approach presented here exploits the barnacles' own fluorescence to visualize anatomical details of whole barnacle body structures, and this could be an alternative way to unveil nature of marine crustaceans.

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