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Author: Owada, Masato

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Radula and Shell Microstructure Variations are Congruent With a Molecular Estimate of Shallow-Water Japanese Chitons

Masato Owada*

Faculty of Science, Department of Biological Sciences, Kanagawa University, 3-27-1 Rokkakubashi, Kanagawa-ku, Yokohama, Kanagawa 221-8686, Japan

Variations of the radula and shell microstructures in 33 species of Japanese chiton were investigated along with molecular phylogenetic trees. The molecular phylogenetic trees indicated that Chitonida was composed of four clades, of which two clades formed Acanthochitonina and corresponded to Mopalioidea and Cryptoplacoidea, respectively, and the other clades formed Chitonina. In the radula, the shapes of the central and centro-lateral teeth and the petaloid process varied greatly among species or genera and were useful for the identification of particular species or genera. The presence of accessory and petaloid processes and the cusp shape were relatively conserved and useful for recognizing particular genera or even suborders. In the valves, four to six shell layers were found at the section, but the ventral mesostracum was not observed in Acanthochitonina. The shell microstructures in the ventral sublayer of the tegmentum varied at suborder, but those in the other layers were almost constant. The megalaesthete chamber type varied at superfamily and was helpful to identify particular families or superfamilies. The characteristics of the shell layers and shell microstructures appear to be a synapomorphy shared by the members of Acanthochitonina. The classification within Chitonina needs to be reexamined because the variations of the cusp shape and megalaesthete chamber type were relatively large and did not correspond to the current classification. *Callochiton* formed a sister group with Chitonida and would be equally closely related to Chitonina and Acanthochitonina because of possessing a mosaic of characteristics from both.

Key words: Mollusca, Polyplacophora, chiton, radula, shell microstructure, molecular phylogeny, cytochrome c oxidase subunit I, 16S ribosomal RNA, 18S ribosomal RNA, 28S ribosomal RNA

INTRODUCTION

Chitons, one of the minor groups in Mollusca, are elongate-oval and flattened in shape and have eight shell valves like armor. These valves are surrounded by a girdle, and the foot is on the ventral side. They have no head, and the oral region lacks eyes; instead, sensory organs called aesthetes are embedded in the valves. Chitons inhabit only seawater, clinging to the surface of something solid such as rocks and dead shells, and most eat algae or minute organisms with the radula. Their fossil records may date back to the Late Cambrian (Smith, 1960; Runnegar et al., 1979; Puchalski et al., 2008; Pojeta et al., 2010), and an obvious chiton fossil would be *Echinochiton* from Ordovician (Pojeta et al., 2003; Pojeta and Dufoe, 2008).

Approximately 1200 species of chitons have been discovered from shallow to deep seas in the world (Eernisse and Reynolds, 1994; Slieker, 2000; Schwabe, 2005; MolluscaBase editors, 2021). Chitons have been mainly classified based on the characteristics of shell, girdle, and radula (Bergenhayn, 1955; Kaas and Van Belle, 1980, 1990, 1994). Furthermore, Sirenko (2006) indicated a new classification by adding characteristics such as gills, glands, egg hull projections, and spermatozoids. The phylogenetic relationships of chitons have been examined using these, and other characters, such as DNA sequences (Okusu et al., 2003; Sigwart et al., 2010, 2013; Irisarri et al., 2014, 2020), aesthete canals and complexes (Fernandez et al., 2007; Vendrasco et al., 2008), and shell microstructures (Peebles et al., 2017). These phylogenetic relationships predominantly corresponded to each other in the higher taxa, but sometimes not in the lower taxa.

The characteristics of radulae are useful tools for the classification of gastropods (e.g., Sasaki, 2010; Ponder and Lindberg, 2020), and those of shell microstructures reflect the phylogenetic relationships of higher taxa, especially in bivalves (e.g., Taylor et al., 1969, 1973; Carter, 1990). As for chitons, Dall (1879) described the radula dentition of North Pacific chitons with comparing species. Thiele (1909–1910) provided monographs of worldwide chitons and emphasized the characteristics of the radula more than other chiton researchers. Sirenko has been actively publishing more than 100 publications covering most chiton taxa, and the majority of them include scanning electron microscope (SEM) images of the radula (e.g., Sirenko and Zhang, 2019; Sirenko, 2020). For Japanese species, Taki Is and Taki Iw published precise illustrations

* Corresponding author. E-mail: owada-bio@kanagawa-u.ac.jp
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of the radula without SEM (e.g., Taki Is and Taki Iw, 1929a; Taki Is, 1938). Saito (2004) analyzed the radula variations of many species in Cryptoplacoidea and reported that their characteristics were useful in identifying the species and genus and could be used to construct the phylogenetic relationships. Bergenhayn (1930) was the first to examine the shell microstructures of chitons, but the descriptions were not very detailed because SEM was not used. Recently, Peebles et al. (2017) described the mineralogy and shell microstructures of eight chiton species collected from New Zealand using SEM and discussed the phylogenetic relationships at family level. However, in both Saito (2004) and Peebles et al. (2017), the phylogenetic resolutions were not so clear. The characteristics of the radula alone were not enough to analyze the higher taxa, and the shell microstructures alone were not enough to analyze the lower taxa. Probably, in the phylogenetic analysis of chitons, combining the characteristics of the radula and shell microstructures is necessary.

More than 90 species of chitons have been discovered on or near the coasts of Japan. The classification of Japanese chitons was well arranged by Taki Is and Taki Iw (1929a, 1929b, 1929c, 1930, 1931a, 1931b), Taki Is (1938), and Saito (1994, 1995, 1998, 2000, 2001, 2006, 2011, 2017). Most Japanese chitons can be identified according to the taxonomic keys created by them.

In the present study, Japanese chitons living in the shallow sea were investigated. They inhabit various environments, and the species diversity is relatively high. First, phylogenetic relationships were constructed using the DNA sequences of mitochondrial cytochrome c oxidase subunit I (COI) and 16S ribosomal RNA (16S), and nuclear 18S ribosomal RNA (18S) and 28S ribosomal RNA (28S). Radula and shell microstructures were observed by SEM. Based on these results, the relationships among the variations of the radula and shell microstructures and the molecular phylogenetic tree of Japanese chitons living in the shallow sea are discussed.

MATERIALS AND METHODS

Sampling

All investigated specimens, identified as 33 chiton species,

Table 1. Species names, sampling localities, sampling dates, and accession numbers. Newly or renewedly determined DNA sequences are in bold.

order	suborder	superfamily	family	species name	sampling locality	sampling date	accession number			
							CO1	16S	18S	28S
Lepidopleurida	Lepidopleurina	Lepidopleuroidea	Leptochitonidae	*Leptochiton aequispinus*	Misaki	November, 2015	**LC718179**	**LC718131**	**LC718147**	**LC718163**
				Leptochiton hakodatensis	Asamushi	May, 2016	LC214408	LC214397	LC214369	**LC214386**
Callochitonida	Callochitonina	Callochitonoidea	Callochitonidae	*Callochiton foveolatus*	Shimoda	May, 2016	LC214414	LC214403	LC214380	**LC214392**
Chitonida	Chitonina	Chitonoidea	Ischnochitonidae	*Ischnochiton boninensis*	Manazuru	May, 2014	LC071647	LC071575	LC214370	**LC071611**
				Ischnochiton comptus	Manazuru	May, 2014	LC071627	LC071570	LC214371	**LC071606**
				Ischnochiton hakodadensis	Hakodate	August, 2015	LC214409	LC214398	LC214372	**LC214387**
				Ischnochiton hayamii	Zushi	May, 2015	LC214410	LC214399	LC214373	**LC214388**
				Ischnochiton manazuruensis	Manazuru	April, 2016	LC071619	LC071565	LC214374	**LC071601**
				Ischnochiton paululus	Asamushi	June, 2015	LC214411	LC214400	LC214375	**LC214389**
				Ischnochiton poppei	Isso	August, 2016	LC214412	LC214401	LC214376	**LC214390**
				Stenoplax alata	Isso	August, 2016	LC214413	LC214402	LC214377	**LC214391**
				Tripoplax albrechti	Hakodate	August, 2015	**LC718180**	**LC718132**	**LC718148**	**LC718164**
				Lepidozona coreanica	Manazuru	May, 2014	LC071669	LC071582	LC214378	**LC071618**
			Callistoplacidae	*Callistochiton jacobaeus*	Manazuru	May, 2014	LC071667	LC071580	LC214379	**LC071616**
			Chitonidae	*Rhyssoplax kurodai*	Manazuru	May, 2014	LC071668	LC071581	LC214381	**LC071617**
				Rhyssoplax komaiana	Isso	August, 2016	**LC718185**	**LC718137**	**LC718153**	**LC718169**
				Acanthopleura gemmata	Onna	June, 2017	**LC718175**	**LC718127**	**LC718143**	**LC718159**
				Acanthopleura loochooana	Onna	June, 2017	**LC718176**	**LC718128**	**LC718144**	**LC718160**
				Liolophura japonica	Manazuru	March, 2017	**LC718181**	**LC718133**	**LC718149**	**LC718165**
				Lucilina amanda	Zushi	September, 2019	**LC718187**	**LC718139**	**LC718155**	**LC718171**
				Lucilina lamellosa	Onna	June, 2017	**LC718188**	**LC718140**	**LC718156**	**LC718172**
				Onithochiton hirasei	Zushi	April, 2017	**LC718183**	**LC718135**	**LC718151**	**LC718167**
	Acanthochitonina	Mopalioidea	Tonicellidae	*Tonicella lineata*	Asamushi	May, 2016	LC214415	LC214404	LC214382	**LC214393**
				Tonicella zotini	Abashiri	August, 2018	**LC718189**	**LC718141**	**LC718157**	**LC718173**
			Schizoplacidae	*Schizoplax brandtii*	Shari	August, 2018	**LC718186**	**LC718138**	**LC718154**	**LC718170**
			Mopaliidae	*Mopalia retifera*	Shimoda	May, 2016	LC214416	LC214405	LC214383	**LC214394**
				Placiphorella stimpsoni	Shimoda	May, 2017	**LC718184**	**LC718136**	**LC718152**	**LC718168**
		Cryptoplacoidea	Acanthochitonidae	*Acanthochitona defilippii*	Manazuru	March, 2016	LC214417	LC214406	LC214384	**LC214395**
				Acanthochitona achates	Manazuru	March, 2016	**LC718178**	**LC718130**	**LC718146**	**LC718162**
				Acanthochitona dissimilis	Manazuru	July, 2017	**LC718174**	**LC718126**	**LC718142**	**LC718158**
				Notoplax conica	Shimoda	May, 2017	**LC718182**	**LC718134**	**LC718150**	**LC718166**
				Cryptochiton stelleri	Akkeshi	May, 2021	**LC718177**	**LC718129**	**LC718145**	**LC718161**
			Cryptoplacidae	*Cryptoplax japonica*	Manazuru	May, 2015	LC214418	LC214407	LC214385	**LC214396**

were collected from the adjacent water of Japan. They were collected from rocks or boulders on the shore during low tide, except *Leptchiton aequispinus* (Bergenhayn, 1993) collected at a water depth of 210 m by dredging, and *Lept. hakodatensis* (Thiele, 1909), *Ischnochiton paululus* Is. Taki, 1938, *Tonicella lineata* (Wood, 1815) and *Cryptochiton stelleri* (Middendorff, 1847) collected at a water depth of 3–8 m by SCUBA diving. The dates and localities of the samplings were shown in Table 1 and Fig. 1. All collected specimens were preserved in 100% EtOH directly or after freezing at –20°C. The species identification was performed as described by Saito (2017), and their classification followed the MolluscaBase editors (2021).

Phylogenetic analysis

Total DNA was extracted from the foot or rectal muscle of each specimen using a DNeasy Blood & Tissue Kit (QIAGEN). COI, 16S, 18S, and 28S gene regions were amplified by polymerase chain reaction (PCR) using Premix Taq (Takara) and a thermal cycler (T100, Bio-Rad). As for the 18S and 28S gene regions, two fragments that were separated into the upper and lower regions were amplified. The primers used are listed in Table 2. The conditions for PCR amplification were as follows: denaturation at 94°C for 30 s; annealing at 48°C (COI), 55°C (16S and 18S), or 58°C (28S) for 30 s; and extension at 72°C for 60 s. These steps were repeated 25–35 times. The PCR products were purified using ExoSAP-IT (Affymetrix), and cycle-sequencing reactions were performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (ABI). DNA sequences were analyzed using a genetic analyzer (3130 Genetic Analyzer, ABI) in both 5′ and 3′ directions. All sequences were registered in the DNA Data Bank of Japan (DDBJ) (Table 1).

The alignment of DNA sequences for each gene region was performed using MAFFT v7.475 (Katoh et al., 2005). Sites those contained gaps or were of questionable homology were trimmed using trimAl v1.4rev15 (Capella-Gutiérrez et al., 2009). A molecular phylogenetic tree was constructed from the concatenated sequence of the four gene regions by Maximum Likelihood (ML) and Bayesian methods. The ML method was performed using RAxML-NG v1.0.2 (Kozlov et al., 2019). A bootstrap test was performed 10,000 times. The model for the ML method was selected

Fig. 1. Sampling localities. Abashiri: 44°03′N, 144°16′E; Shari: 44°03′N, 144°57′E; Akkeshi: 43°01′N, 144°49′E; Hakodate: 41°45′N, 140°43′E; Asamushi: 40°54′N, 140°51′E; Zushi: 35°16′N, 139°34′E; Manazuru: 35°09′N, 139°09′E; Misaki: 35°10′N, 139°32′E; Shimoda: 34°40′N, 138°56′E; Isso: 30°27′N, 130°30′E; Onna: 26°29′N, 127°50′E.

Table 2. Primers used in the present study. Superscript numbers show the combination in PCR amplification.

region	direction	name	5'—3' sequence	reference
COI	forward[1]	CO1F	TCWACAAATCAYAAAGATATTGG	Owada et al. (2013)
	reverse[1]	CO1R	ACYTCMGGRTGMCCAAAAAATCA	Owada et al. (2013)
16S	forward[1]	primer A	CGCCTGTTTATCAAAAACAT	Xiong and Kocher (1991)
	reverse[1]	primer B	CTCCGGTTTGAACTCAGATC	Xiong and Kocher (1991)
18S	forward[1]	1F	TACCTGGTTGATCCTGCCAGTAG	Giribet et al. (1996)
	forward[2]	3F	GTTCGATTCCGGAGAGGGA	Giribet et al. (1996)
	reverse	4R	GAATTACCGCGGCTGCTGG	Giribet et al. (1996)
	reverse[1]	5R	CTTGGCAAATGCTTTCGC	Giribet et al. (1996)
	forward	18S a2.0	ATGGTTGCAAAGCTGAAAC	Whiting et al. (1997)
	reverse	18S bi	GAGTCTCGTTCGTTATCGGA	Whiting et al. (1997)
	reverse[2]	9R	GATCCTTCCGCAGGTTCACCTAC	Giribet et al. (1996)
28S	forward[1]	28S a	GACCCGTCTTGAAACACGGA	Whiting et al. (1997)
	reverse	28S b	TCGGAAGGAACCAGCTACTA	Whiting et al. (1997)
	forward	28S-NLF105-22	CCGAAGTTTCCCTCAGGATAGC	www.psb.ugent.be/rRNA
	forward[2]	28S Rd4.8a	ACCTATTCTCAAACTTTAAATGG	Whiting (2002)
	reverse[1]	28S rD5b	CCACAGCGCCAGTTCTGCTTAC	Whiting (2002)
	forward	28S-1600F	CCTGAAAATGGATGGCGCT	This study
	reverse	28S-1600R	AGCGCCATCCATTTTCAGG	Distel et al. (2011)
	reverse[2]	28S rD7b1	GACTTCCCTTACCTACAT	Whiting (2002)

for the sequence of each gene region using ModelTest-NG v0.2.0 (Darriba et al., 2020). The Bayesian method was performed using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). This program was run for 50,000,000 generations sampling every 1000th generation. The model for the Bayesian method was selected for the sequence of each gene region using Kakusan4 (Tanabe, 2011). Convergence in the Bayesian method was assessed using Tracer v1.7.1 (Rambaut et al., 2018).

Radula

The radula was carefully extracted from the soft parts of each specimen, which were fixed in 100% EtOH or frozen at –20°C. Each radula was cleaned with a dissecting needle under a binocular microscope (Olympus, SZ40), washed with an ultrasonic washer (Velvo-Clear, VS-100III), and preserved in 100% EtOH. Afterward, the radula was coated with 50-nm-thick platinum by ion sputter (JEOL, JEC-3000FC) and observed using SEM (JEOL, JCM-5000) at 10 kV accelerating voltage. The terminology for the radula followed Saito (2004) and Schwabe (2010).

Shell microstructures and aesthete canals and complexes

The investigated specimen was soaked in 10% chlorine bleach solution (Kao, Kitchen Haiter) for 1–4 days to remove the soft parts. The remaining eight valves were washed with pure water and desiccated at 60°C for 24 h. Valves III–VI were fractured by hand or with a micro-chisel and hammer. The sections along the antero-posterior axis were coated with 50-nm-thick platinum by ion sputter (JEOL, JEC-3000FC) and observed using SEM (JEOL, JCM-5000) at 10 kV accelerating voltage. The shell layers were identified as described by Connors et al. (2012); however, the anterior and posterior myostracums were not distinguished. The shell microstructures were identified according to Carter (1990). The aesthete canals and complexes were identified on the basis of Baxter and Jones (1981, 1984) and Currie (1992).

RESULTS

Molecular phylogenetic tree

The sequence lengths for the COI, 16S, 18S, and 28S gene regions were 557, 505–516, 1692–1709, and 1279–1313 base pairs (bp), respectively. After alignment and trimming, the analyzed lengths were 557, 499, 1693, and 1283 bp, respectively. The model for the ML method was GTR + G4 for the 16S, 18S, and 28S, TIM3 + I + G4 for the first base of the COI, TVM + I + G4 for the second base, and TIM2 + I + G4 for the third base. The likelihood index was *-ln* 26478.320061. The model for the Bayesian method was HKY85 + G for the 16S and the third base of the COI, SYM + G + I for the 18S, and GTR + G + I for the 28S and the first and second bases.

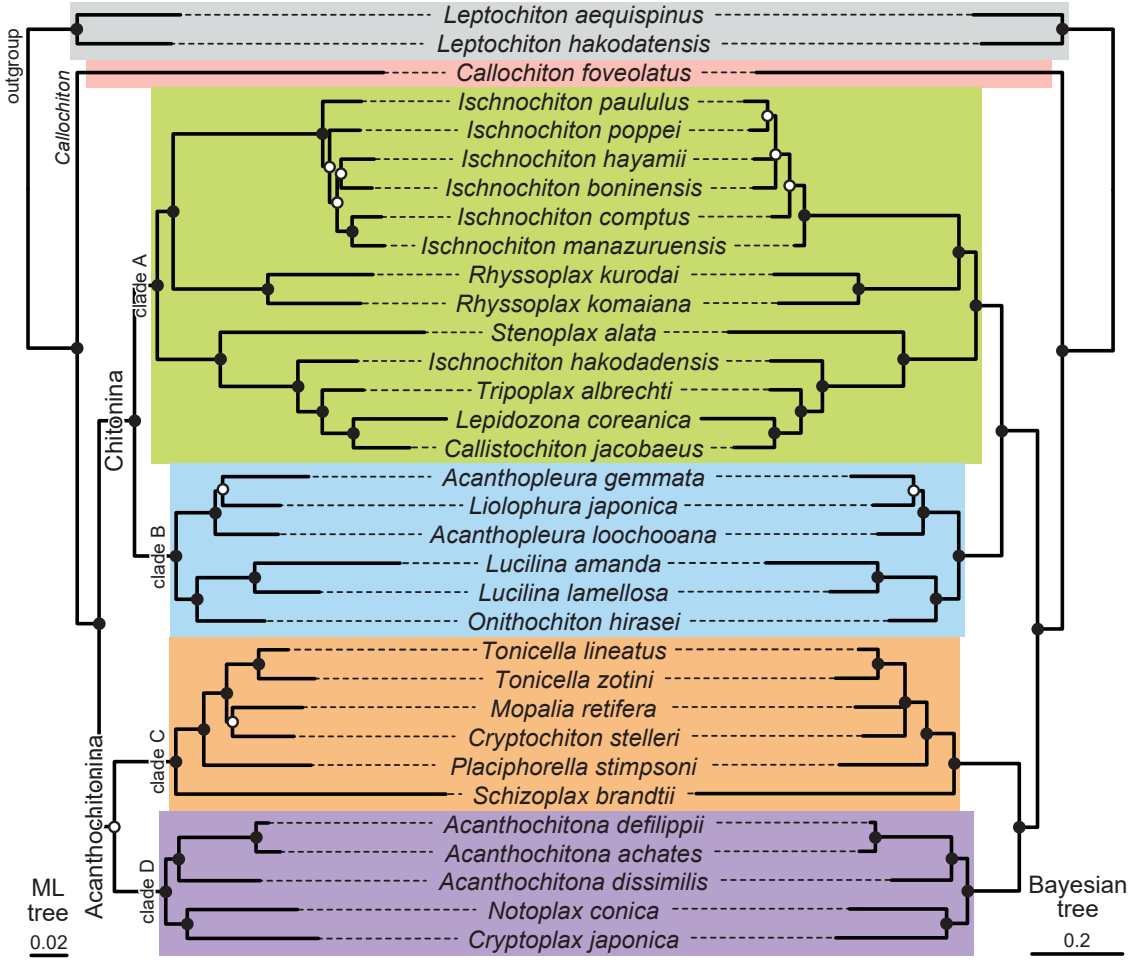

Fig. 2. Molecular phylogenetic trees. Clade A: some Chitonina, including the Chitoninae part of Chitonidae; Clade B: the Toniciinae plus Acanthopleurinae part of Chitonidae; Clade C: Mopalioidea; Clade D: Cryptoplacoidea. Filled circles on each node indicate bootstrap > 50% in the ML tree and posterior probability > 0.90 in the Bayesian tree, and open circles indicate not so.

The ML and Bayesian trees are shown in Fig. 2. The topologies of the two trees mostly corresponded to each other, and the bootstrap values and posterior probabilities on the nodes were significantly high. However, there were a few differences in the positions of *Ischnochiton* spp. and *Cryptoc*. *stelleri*. In both trees, Chitonida was monophyletic and contained four clades. Clade A was composed of some Chitonina, including the Chitoninae part of Chitonidae and actually including *Ischnochiton*, *Rhyssoplax*, *Stenoplax*, *Tripoplax*, *Lepidozona*, and *Callistochiton*. Clade B was composed of the Toniciinae plus Acanthopleurinae part of Chitonidae and including *Acanthopleura*, *Liolophura*, *Lucilina*, and *Onithochiton*. Clade C was composed of Mopalioidea and including *Tonicella*, *Mopalia*, *Cryptochiton*, *Placiphorella*, and *Schizoplax*. Clade D was composed of Cryptoplacoidea and including *Acanthochitona*, *Notoplax*, and *Cryptoplax*. Ischnochitonidae, Chitonidae, Mopaliidae, and Acanthochitonidae were polyphyletic, and additionally, *Ischnochiton* and *Acanthopleura* were not monophyletic. *Callochiton* formed a sister group with Chitonida.

Radula

SEM images of the representative radulae of the investigated specimens are shown in Fig. 3. The characteristics of the radula were more or less different among the species; however, these differences were relatively moderate within the genus, except for *Ischnochiton hakodadensis* Carpenter, 1893. The central teeth had various shapes, but they were grouped into six types: bulbous, cup-shaped, deltoid, hooked-needle, rectangular, and spatulate. The centro-lateral (first) teeth also varied, but they were grouped into six types: columnar, envelope-like, fingerlike, fungiform, sphenoid, and winglike. In addition, the accessory process, which is a minute process on the centro-lateral tooth (Saito, 2004), was observed in members of clades A and B, and *Lept*. *aequispinus*. The major (second) lateral teeth were grouped into four types: unicuspid, bicuspid, tricuspid, and patellar. Although the type of the major lateral tooth largely varied in clade A, it was conserved in the other clades. Furthermore, the petaloid process, which is a process below the head of the major lateral tooth (Thiele,

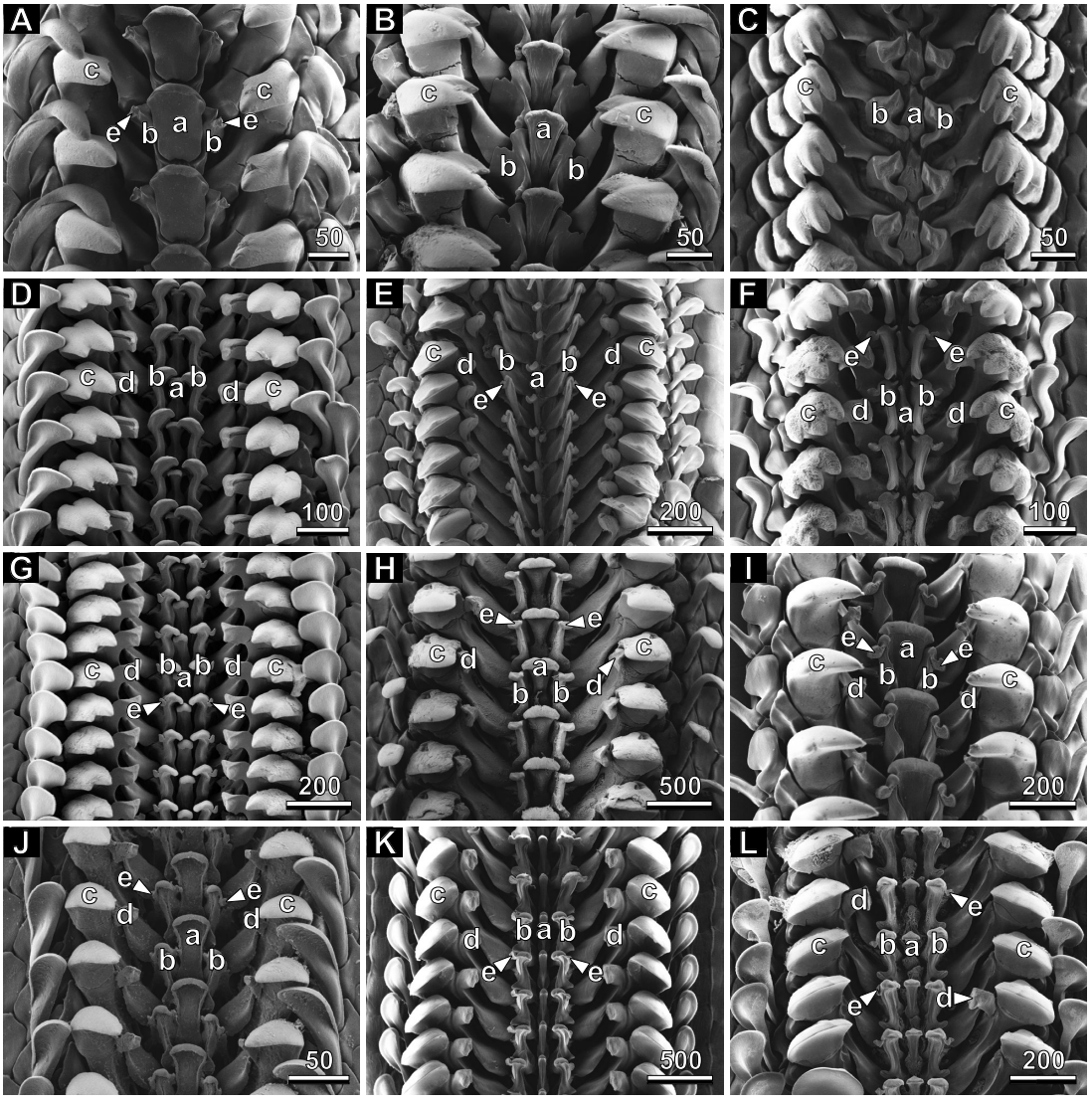

Fig. 3. Continued.

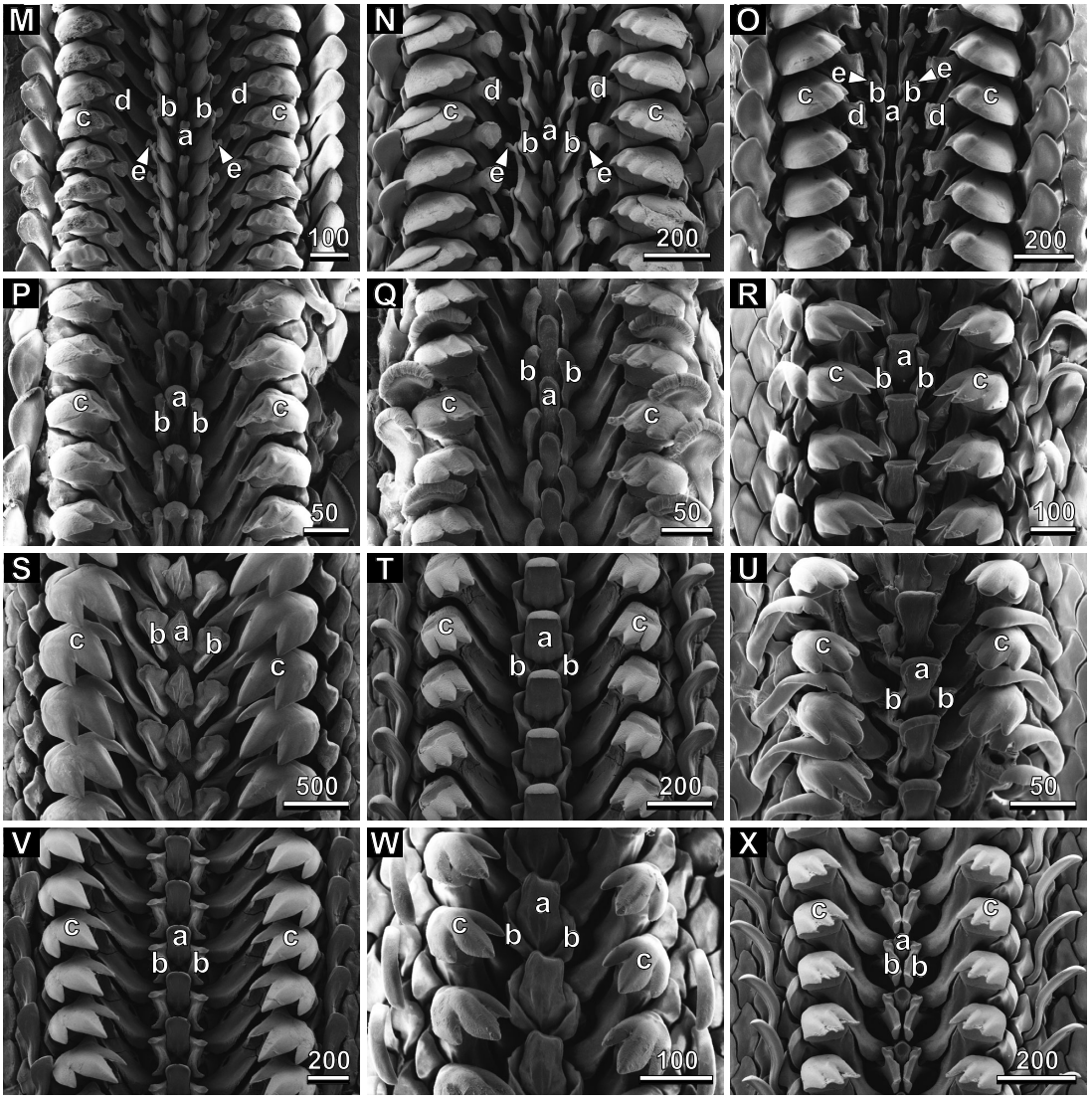

Fig. 3. SEM images of radula. A: *Leptochiton aequispinus*; B: *Lept. hakodatensis*; C: *Callochiton foveolatus*; D: *Ischnochiton comptus*; E: *Rhyssoplax komaiana*; F: *Stenoplax alata*; G: *I. hakodadensis*; H: *Tripopla albrechti*; I: *Lepidozona coreanica*; J: *Callistochiton jacobaeus*; K: *Acanthopleura gemmata*; L: *Acanthop. loochooana*; M: *Lucilina amanda*; N: *Lu. lamellosa*; O: *Onithochiton hirasei*; P: *Tonicella lineata*; Q: *To. zotini*; R: *Mopalia retifera*; S: *Cryptochiton stelleri*; T: *Placiphorella stimpsoni*; U: *Schizoplax brandtii*; V: *Acanthochitona defilippii*; W: *Notoplax conica*; X: *Cryptoplax japonica*. a: central tooth; b: centro-lateral (first) tooth; c: major (second) lateral tooth; d: petaloid process; e: accessory process. Scale bar unit indicates micrometer.

1893; Bullock, 1988; Saito, 2004), was found in clades A and B and was grouped into three types: cornered, nodule, and securiform. The characteristics of the radula of each species are shown in Table 3.

Shell microstructures

In the investigated specimens belonging to *Callochiton*, clades A and B, and the outgroups, six shell layers (i.e., tegmentum, dorsal mesostracum, articulamentum, ventral mesostracum, myostracum, and hypostracum) were observed in the valves. As for clades C and D, five shell layers, excluding the dorsal mesostracum from the six layers, were observed. Except in *Cryptoc. stelleri*, the number of shell layers was constant within the clade. In the valve of *Cryptoc. stelleri*, only four layers (i.e., articulamentum, ventral mesostracum, myostracum, and hypostracum) were found (Fig. 4).

The tegmentum of *Callochiton*, clades A and B, and *Placiphorella stimpsoni* (Gould, 1859) was composed of two layers. The dorsal sublayer was homogeneous (Ho) structure, and the ventral sublayer was mainly composite prismatic (CP) structure but partly Ho structure (Fig. 5A–H). In clades C and D, and the outgroups, the tegmentum was only one layer, the Ho structure (Fig. 5I–L). The dorsal and ventral mesostracums were constructed with crossed-lamellar (CL) structure (Fig. 5M–P). The articulamentum was composed of two layers in all investigated species. The dorsal sublayer was Ho structure, and the ventral sublayer was mainly CP structure but partly Ho structure (Fig. 5Q, R). In *Cryptoc. stelleri*, the dorsal sublayer often contained irregular simple prismatic (ISP) structure (Fig. 5S). The myostracum in all investigated species was observed between the

Table 3. Characteristics of radula.

clade	species name	central tooth	centro-lateral tooth		major lateral tooth	
		shape	shape	accessory process	cusp shape	petaloid process
outgroup	*Leptochiton aequispinus*	rectangular	envelope-like	present	bicuspidate	
	Leptochiton hakodatensis	cup-shaped	winglike		bicuspidate	
Callochiton	*Callochiton foveolatus*	bulbous	fungiform		tricuspidate	
clade A: some Chitonina, including the Chitoninae part of Chitonidae	*Ischnochiton paululus*	bulbous	fingerlike		bicuspidate	cornered
	Ischnochiton poppei	spatulate	fungiform		bicuspidate	cornered
	Ischnochiton hayamii	bulbous	fingerlike		bicuspidate	cornered
	Ischnochiton boninensis	bulbous	fingerlike		bicuspidate	cornered
	Ischnochiton comptus	bulbous	fingerlike		bicuspidate	cornered
	Ischnochiton manazuruensis	bulbous	fingerlike		bicuspidate	cornered
	Rhyssoplax kurodai	hooked-needle	sphenoid	present	patellar	nodulous
	Rhyssoplax komaiana	hooked-needle	sphenoid	present	patellar	nodulous
	Stenoplax alata	bulbous	fingerlike	present	tricuspidate	cornered
	Ischnochiton hakodadensis	bulbous	fingerlike	present	bicuspidate	cornered
	Tripoplax albrechti	cup-shaped	columnar	present	bicuspidate	securiform
	Lepidozona coreanica	cup-shaped	fingerlike	present	bicuspidate	securiform
	Callistochiton jacobaeus	cup-shaped	fingerlike	present	unicuspidate	cornered
clade B: the Toniciinae + Acanthopleurinae part of Chitonidae	*Acanthopleura gemmata*	bulbous	fingerlike	present	patellar	cornered
	Liolophura japonica	bulbous	fingerlike	present	patellar	cornered
	Acanthopleura loochooana	bulbous	fingerlike	present	patellar	cornered
	Lucilina amanda	bulbous	sphenoid	present	patellar	securiform
	Lucilina lamellosa	bulbous	sphenoid	present	patellar	securiform
	Onithochiton hirasei	bulbous	sphenoid	present	patellar	cornered
clade C: Mopalioidea	*Tonicella lineata*	cup-shaped	fingerlike		tricuspidate	
	Tonicella zotini	spatulate	fingerlike		tricuspidate	
	Mopalia retifera	rectangular	columnar		tricuspidate	
	Cryptochiton stelleri	deltoid	fungiform		tricuspidate	
	Placiphorella stimpsoni	rectangular	envelope-like		tricuspidate	
	Schizoplax brandtii	cup-shaped	winglike		tricuspidate	
clade D: Cryptoplacoidea	*Acanthochitona defilippii*	rectangular	columnar		tricuspidate	
	Acanthochitona achates	rectangular	columnar		tricuspidate	
	Acanthochitona dissimilis	rectangular	columnar		tricuspidate	
	Notoplax conica	rectangular	envelope-like		tricuspidate	
	Cryptoplax japonica	spatulate	columnar		tricuspidate	

ventral mesostracum and the hypostracum and/or beneath the hypostracum. This layer was mainly constructed with Ho structure (Fig. 5T, U) but sometimes contained ISP structure (Fig. 5V). The hypostracum was constructed with CL structure (Fig. 5W, X). The apophyses, the inner shell layers extending beyond the tegmentum, were composed of three layers: articulamentum, ventral mesostracum, and myostracum (Fig. 4).

Aesthete canals and complexes

Multiple-branch aesthete canals, which are a type of aesthete canals and complexes and widely distributed in all valves (Baxter and Jones, 1981, 1984; Currie, 1992), were observed in the tegmentum of all investigated species, except *Cryptoc. stelleri*. The shapes of the megalaesthete chambers in the aesthete complexes were observed and grouped into four types (Fig. 6). Type A had a cylindrical shape shortened more or less and was observed in members of clades A, B, and D. Type B had a sprawling shape into which micraesthete canals often merged before entering and was observed only in clade C. Type C had a wider shape in the middle and was observed in *Callochiton* and members of clades A and B. Type D had a bud-like shape with a stalk-like aesthete canal and was observed only in the outgroups. Table 4 lists the characteristics of the shell layers, shell microstructures, and megalaesthete chamber type.

The above results were listed and shown in relation to the phylogenetic tree in Fig. 7.

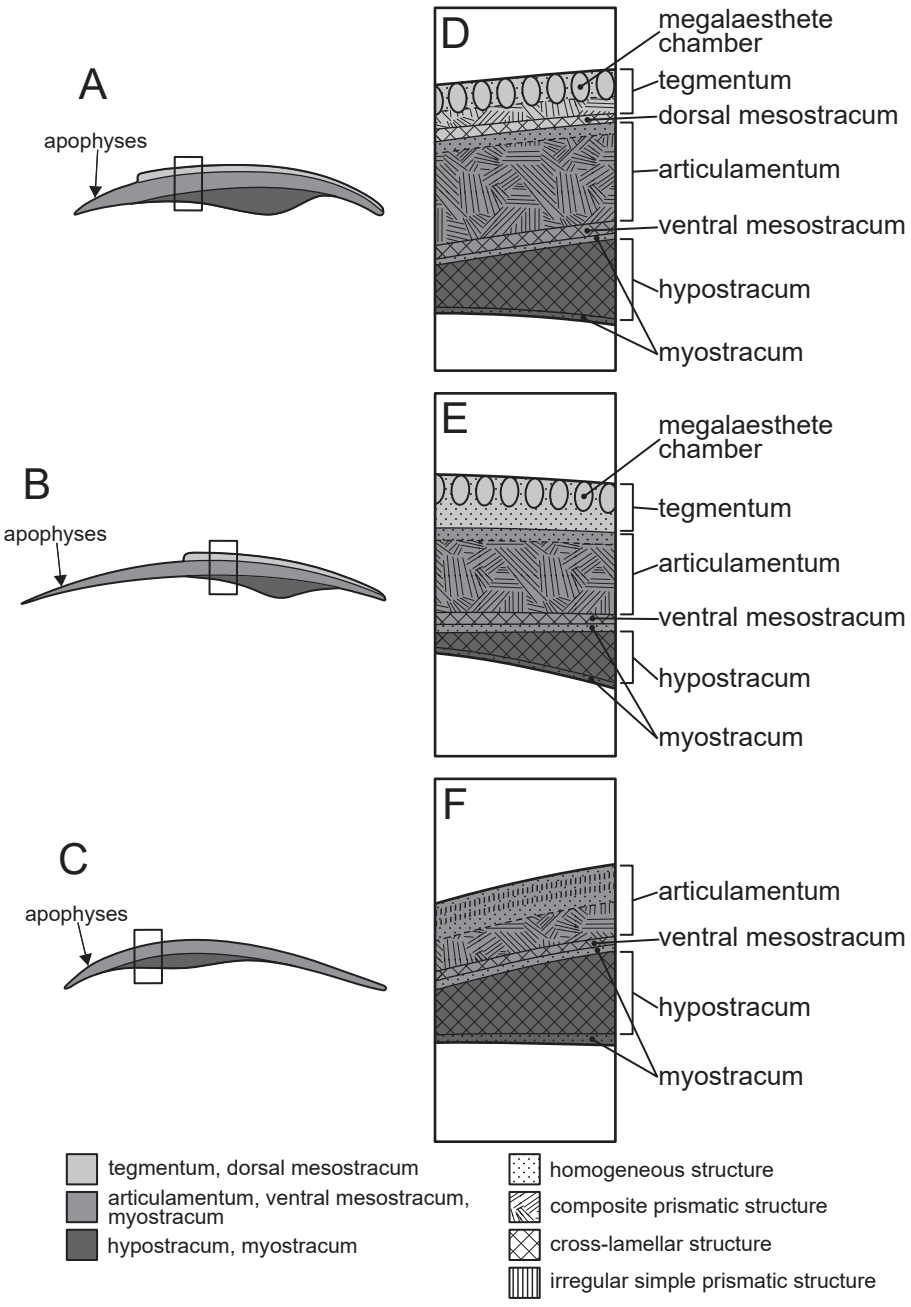

Fig. 4. Schematic of valve section. A: section along antero-posterior axis for species of *Callochiton* and clades A and B; B: clades C and D; C: *Cryptochiton stelleri*. D, E, F: close view of the box in the figure. In *Placiphorella stimpsoni*, the tegmentum was composed of two layers, homogeneous and composite prismatic structures.

DISCUSSION

Molecular phylogeny

The ML and Bayesian trees in the present study were highly robust, though the phylogenetic positions of *Ischnochiton* spp. and *Cryptoc*. *stelleri* were unclear. The topologies of these trees were predominantly similar to those in Irisarri et al. (2014, 2020), which reported that Chitonoidea (Chitonina) formed a sister group with a clade comprising Mopalioidea plus Cryptoplacoidea (Acanthochitonina), but not to those in Okusu et al. (2003). Additionally, the phylogenetic position of *Callochiton* in the present study corresponded to those in Sigwart et al. (2010), Irisarri et al. (2014, 2020), and Moles et al. (2021), but not to that in Sigwart et al. (2013), which described that *Callochiton* formed a sister group with a clade comprising Mopalioidea plus Cryptoplacoidea. The phylogenetic position of *Cryptoc*. *stelleri* in the present study made Acanthochitonidae and Mopaliidae polyphyletic groups. This position corresponded to those reported by Kelly and Eernisse (2008) and Irisarri et al. (2014), and the latter study considered *Cryptoc*. *stelleri* to be a member of Mopaliidae. Therefore, the present study also grouped *Cryptoc*. *stelleri* into Mopaliidae (Figs. 2, 7). *Ischnochiton hakodadensis*, the only species possessing an

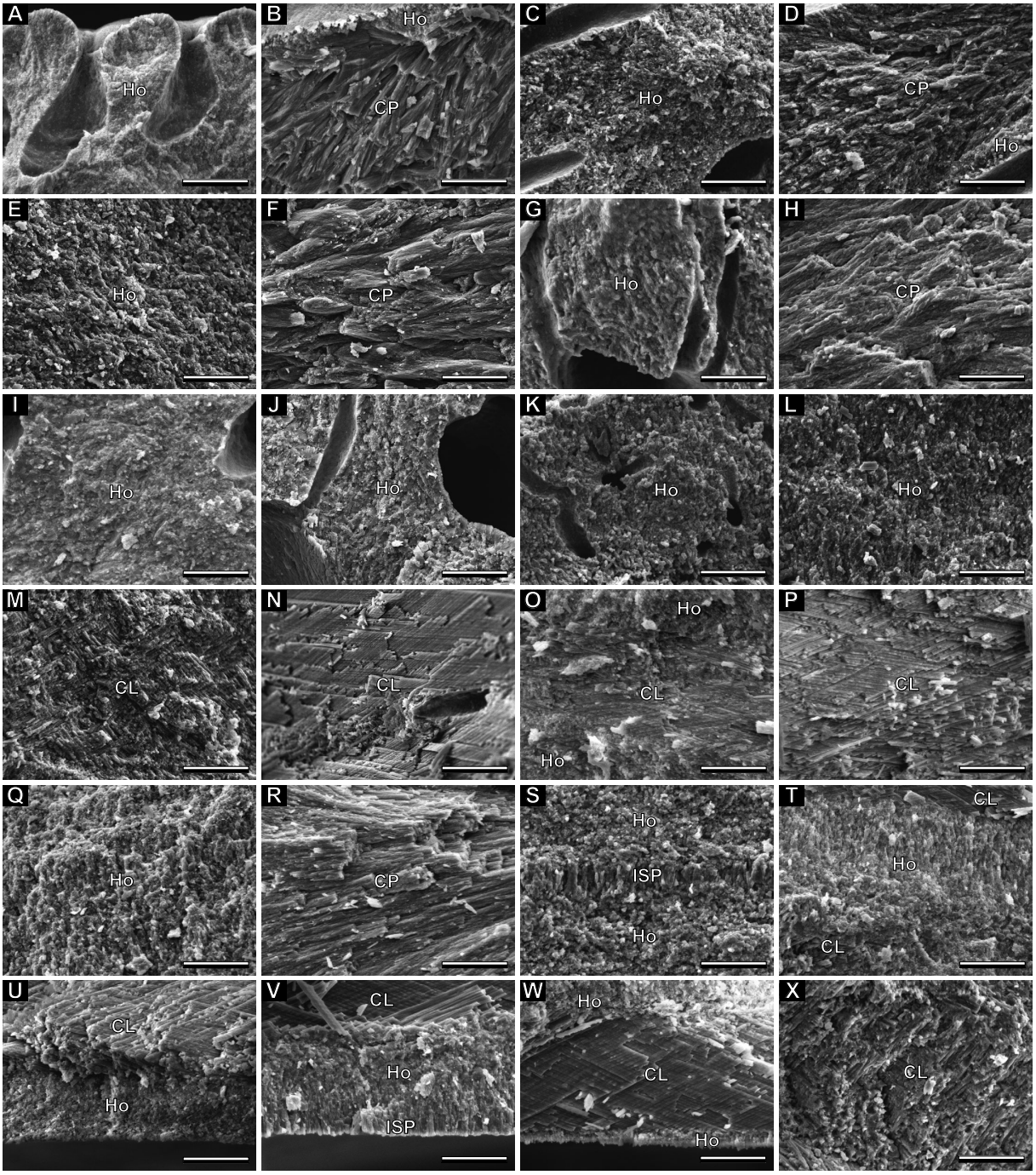

Fig. 5. SEM images of shell microstructures. Dorsal and ventral sublayers of tegumentum in **(A, B)** *Callochiton foveolatus*, **(C, D)** *Ischnochiton comptus*, **(E, F)** *Liolophura japonica*, **(G, H)** *Placiphorella stimpsoni*. Tegmentum in **(I)** *Leptochiton aequispinus*, **(J)** *Tonicella zotini*, **(K)** *Schizoplax brandtii*, **(L)** *Acanthochitona defilippii*. Dorsal mesostracum in **(M)** *Callo foveolatus*, **(N)** *Stenoplax alata*. Ventral mesostracum in **(O)** *Sc. Brandtii*, **(P)** *Cryptoplax japonica*. Dorsal and ventral sublayers of articulamentum in *Lucilina lamellose* **(Q, R)**. Dorsal sublayer of articulamentum in *Cryptochiton stelleri* **(S)**. Myostracum between the ventral mesostracum and hypostracum in *Lepidozona coreanica* **(T)**. Myostracum beneath the hypostracum in **(U)** *Tonicella lineata*, **(V)** *Rhyssoplax komaiana*. Hypostracum in **(W)** *I. boninensis*, **(X)** *Acanthoc. achates*. Ho: homogeneous structure; CP: composite prismatic structure; CL: crossed-lamellar structure; ISP: irregular simple prismatic structure. Scale bar indicates 10 μm. The upper direction of SEM images is dorsal side, and the lower direction is ventral side.

accessory process in the genus, made Ischnochitonidae a polyphyletic group, as reported by Owada (2016, 2018). Furthermore, the present study clarified that *Rhyssoplax*, which was classified as Chitonidae by Sirenko (2006), formed a sister group with *Ischnochiton*. However, the phylogenetic position of *Rhyssoplax* did not correspond to that reported by Irisarri et al. (2020). The DNA sequence of *Rhyssoplax* used by Irisarri et al. (2020) was determined by Riesgo et al. (2012). If their identification was correct, it would be suggested that *Rhyssoplax* is a polyphyletic group. The present

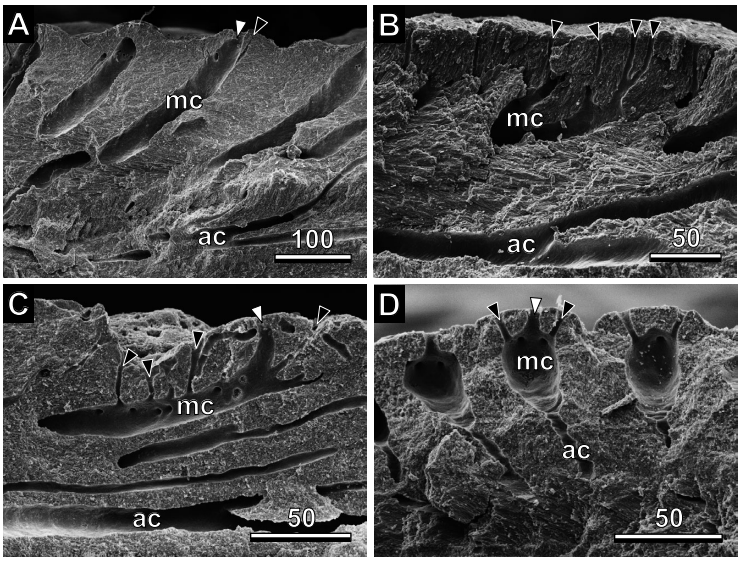

Fig. 6. Megalaesthete chamber type in multiple-branch aesthete canals. **(A)** type A in *Acanthopleura gemmate*, **(B)** type B in *Placiphorella stimpsoni*, **(C)** type C in *Ischnochiton comptus*, **(D)** type D in *Leptochiton hakodatensis*. ac: aesthete canal, mc: megalaesthete chamber. White arrowhead indicates the aperture of megalaesthete canal, and black arrowhead indicates that of micraesthete canal. Scale bar indicates 100 μm in **(A)**, 50 μm in **(B, C, D)**.

study also clarified that *Lepidozona coreanica* (Reeve, 1847), a common species along the coast of Japan, formed a sister group with *Callistochiton jacobaeus* (Gould, 1859) (Figs. 2, 7). These results indicate the need to reexamine the classification within Chitonina.

Radula

Saito (2004) suggested that the characteristics of the radula in chitons were useful not only for species identification but also for the assessment of the phylogenetic relationships at higher levels, emphasizing examples of Cryptoplacoidea in his comparisons. The present study indicated that the characteristics of the radula could identify the species (Table 3, Fig. 7), and support Saito (2004). The shapes of the central and centro-lateral teeth varied among species and genera and could be used for identification at least Japanese chiton species at those levels. Likewise, the shape of the petaloid process is informative at genus level, and considering all these tooth shapes helps to confirm identification. In contrast, the presence of accessory and petaloid processes and the major lateral cusp shape

Table 4. Characteristics of shell layers, shell microstructures, and megalaesthete chamber type.

clade	species name	tegmentum		dorsal	articulamentum		ventral	myostracum	hypostracum	megalaesthete
		dorsal	ventral	mesostracum	dorsal	ventral	mesostracum			chamber type
outgroup	*Leptochiton aequispinus*	Ho	Ho	CL	Ho	CP	CL	Ho	CL	D
	Leptochiton hakodatensis	Ho	Ho	CL	Ho	CP	CL	Ho	CL	D
Callochiton	*Callochiton foveolatus*	Ho	CP	CL	Ho	CP	CL	Ho	CL	C
clade A: some Chitonina, including the Chitoninae part of Chitonidae	*Ischnochiton paululus*	Ho	CP	CL	Ho	CP	CL	Ho	CL	C
	Ischnochiton poppei	Ho	CP	CL	Ho	CP	CL	Ho	CL	C
	Ischnochiton hayamii	Ho	CP	CL	Ho	CP	CL	Ho	CL	C
	Ischnochiton boninensis	Ho	CP	CL	Ho	CP	CL	Ho	CL	C
	Ischnochiton comptus	Ho	CP	CL	Ho	CP	CL	Ho	CL	C
	Ischnochiton manazuruensis	Ho	CP	CL	Ho	CP	CL	Ho	CL	C
	Rhyssoplax kurodai	Ho	CP	CL	Ho	CP	CL	Ho	CL	C
	Rhyssoplax komaiana	Ho	CP	CL	Ho	CP	CL	Ho	CL	C
	Stenoplax alata	Ho	CP	CL	Ho	CP	CL	Ho	CL	A
	Ischnochiton hakodadensis	Ho	CP	CL	Ho	CP	CL	Ho	CL	A
	Tripoplax albrechti	Ho	CP	CL	Ho	CP	CL	Ho	CL	A
	Lepidozona coreanica	Ho	CP	CL	Ho	CP	CL	Ho	CL	A
	Callistochiton jacobaeus	Ho	CP	CL	Ho	CP	CL	Ho	CL	A
clade B: the Toniciinae + Acanthopleurinae part of Chitonidae	*Acanthopleura gemmata*	Ho	CP	CL	Ho	CP	CL	Ho	CL	A
	Liolophura japonica	Ho	CP	CL	Ho	CP	CL	Ho	CL	A
	Acanthopleura loochooana	Ho	CP	CL	Ho	CP	CL	Ho	CL	A
	Lucilina amanda	Ho	CP	CL	Ho	CP	CL	Ho	CL	C
	Lucilina lamellosa	Ho	CP	CL	Ho	CP	CL	Ho	CL	C
	Onithochiton hirasei	Ho	CP	CL	Ho	CP	CL	Ho	CL	A
clade C: Mopalioidea	*Tonicella lineata*	Ho	Ho		Ho	CP	CL	Ho	CL	B
	Tonicella zotini	Ho	Ho		Ho	CP	CL	Ho	CL	B
	Mopalia retifera	Ho	Ho		Ho	CP	CL	Ho	CL	B
	Cryptochiton stelleri				Ho	CP	CL	Ho	CL	
	Placiphorella stimpsoni	Ho	CP		Ho	CP	CL	Ho	CL	B
	Schizoplax brandtii	Ho	Ho		Ho	CP	CL	Ho	CL	B
clade D: Cryptoplacoidea	*Acanthochitona defilippii*	Ho	Ho		Ho	CP	CL	Ho	CL	A
	Acanthochitona achates	Ho	Ho		Ho	CP	CL	Ho	CL	A
	Acanthochitona dissimilis	Ho	Ho		Ho	CP	CL	Ho	CL	A
	Notoplax conica	Ho	Ho		Ho	CP	CL	Ho	CL	A
	Cryptoplax japonica	Ho	Ho		Ho	CP	CL	Ho	CL	A

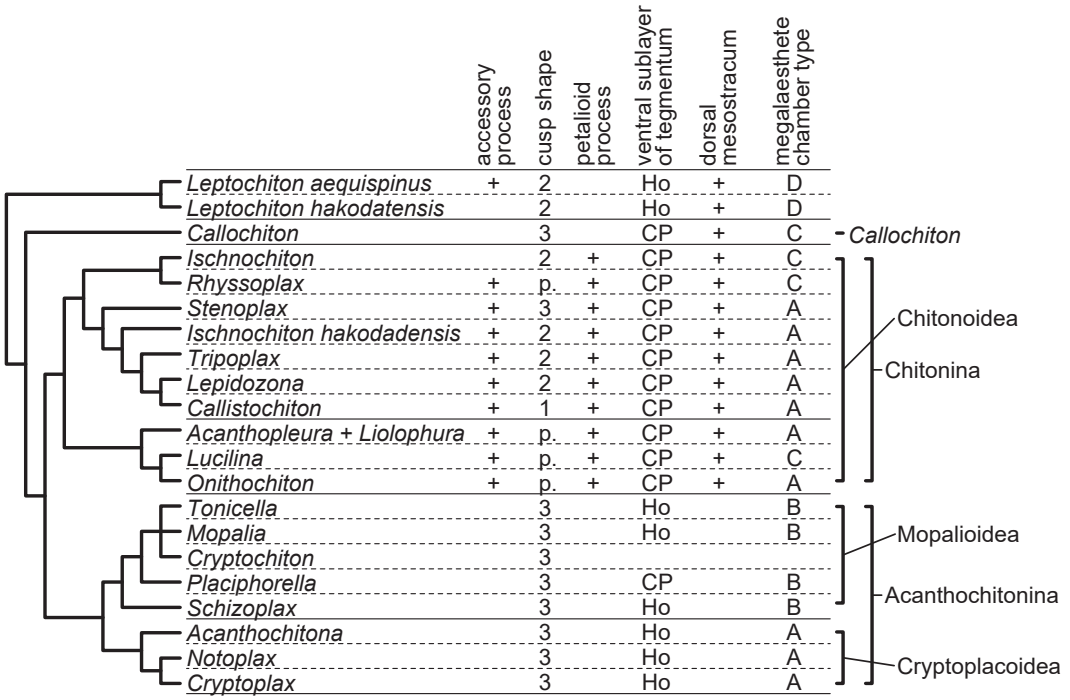

Fig. 7. Relationships among the molecular phylogenetic trees, the genera or species, the character state of radula, shell microstructures and megalaesthete chamber type, and the classification in the higher taxon. p.: patellar, Ho: homogeneous structure, CP: composite prismatic structure.

were found to be relatively conservative, but the cusp shape can be somewhat useful for distinguishing genera.

Thiele (1893) described the petaloid process as a diagnostic feature for groups corresponding to Chitonoidea in the present study, but until now it had not been tested with molecular phylogenetic analysis. Based on molecular phylogenetic analysis, the present study indicated that the petaloid process only occurs in Chitonina. The accessory process is likely convergent in Chitonina and *Lept. aequispinus* (Fig. 7) because it was not found in other Lepidopleurida, Callochitonida, or Acanthochitonina within Chitonida.

Shell microstructures

Bergenhayn (1930) reported that chiton valves consisted of four shell layers (i.e., tegmentum, articulamentum, hypostracum, and mesostracum), excluding the periostracum. However, Haas (1972, 1976, 1981) did not distinguish between articulamentum and hypostracum and described chiton valves as being constructed from three shell layers (i.e., tegmentum, hypostracum, and myostracum). Poulicek and Kreusch (1986) supported Bergenhayn (1930) but did not recognize mesostracum, and Carter (1990) supported Haas (1972, 1976, 1981). Connors et al. (2012) defined seven shell layers: tegmentum, dorsal mesostracum, articulamentum, ventral mesostracum, anterior and posterior myostracums, and hypostracum; *Tonicella marmorea* (O. Fabricius, 1780) was an exception in lacking the dorsal mesostracum. The present study adopted the definition of Connors et al. (2012) but did not distinguish between anterior and posterior myostracums because they did not appear to vary in the assembled information. Peebles et al. (2017) reported that the valves of chitons were composed of four to seven shell layers according to Connors et al. (2012) and suggested that the properties of the shell layers did not clearly reflect the phylogenetic relationships because of the large species variations. The present study indicated that the valves were constructed of four to six layers and that the tegmentum and articulamentum had sublayers. Additionally, the present study has extended the observation of Connors et al. (2012) that the dorsal mesostracum layer is absent in *To. marmorea*; it is also absent in the seven other Acanthochitonina genera, and other species of *Tonicella* examined in the present study (Fig. 7). The congruent distribution relative to the molecular result implies that a shared absence of the dorsal mesostracum layer is a synapomorphy of Acanthochitonina (Table 4, Fig. 7).

Differences in the shell microstructures were observed only in the ventral sublayer of the tegmentum. In *Callochiton*, Chitonina, and *P. stimpsoni*, the sublayer was mainly constructed with CP structure, and with Ho structure in the others. It is likely that a clade comprising *Callochiton* and Chitonida first obtained the Ho structure in the sublayer because the two species of *Leptochiton*, which are outgroups, had Ho structure. Presumably, the CP structure was independently acquired in *Callochiton*, Chitonina, and *P. stimpsoni*. In *Cryptoc. stelleri*, the structure of the valve differed greatly from the others because the tegmentum was not observed. This may be because the valves are always embedded in the soft part. Peebles et al. (2017) distinguished the CL structure in the mesostracum or hypostracum into at least two types, based on the size of the fibers that made the CL structure. However, the present study did not distinguish such types of CL structures because the difference between CP and Ho structures is much larger than that between the types of CL structures. Furthermore, individual variation in the size of the fibers was often observed.

Aesthete canals and complexes

Baxter and Jones (1981, 1984) and Currie (1992) observed aesthete canals and complexes in detail, and Fernandez et al. (2007) and Vendrasco et al. (2008) additionally constructed the phylogenetic relationships using their characteristics. Their phylogenetic conclusions are consistent with the present study. In the present study, the megalaesthete chamber type, which was applied as one of the characters for the phylogenetic analysis in Fernandez et al. (2007) and Vendrasco et al. (2008), was available for identification of particular families or superfamilies. However, in Chitonina, the variation in the type was relatively high compared with the others. Although the megalaesthete chamber type may be related to the life type, both *Acanthopleura* living on the surface of a rock in the intertidal zone and having a lens on the surface of the valve, and *Lepidozona* living on the back of a rock in the subtidal zone and lacking a lens, possessed the type A.

Conclusions

In the radula, the shapes of the central and centro-lateral teeth and the petaloid process were useful for the identification of particular species or genera. The presence of accessory and petaloid processes and the cusp shape were available for recognizing particular genera or even suborders. In the valves, the number of shell layers and the shell microstructure type in the ventral sublayer of tegmentum appear to be a synapomorphy shared by the members of suborder Acanthochitonina, and the megalaesthete chamber type was helpful for identifying particular families or superfamilies. These characteristics of the valve could also be useful for verifying the fossil records of chitons. The molecular phylogenetic tree implied that the above characteristics were significant and simultaneously indicated that the classification within Chitonina would need to be reexamined because the variations of the cusp shape and megalaesthete chamber type did not correspond to the current classification. *Callochiton*, which is contained in Callochitonida in the current classification, would be equally closely related to any member of Acanthochitonina and any member of Chitonina because of possessing a mosaic of characteristics from both. Buckland-Nics and Hodgson (2000) investigated the characteristics of the gametes in *Callochiton castaneus* (W. Wood, 1815) treated as *Callochiton dentatus* (Spengler, 1797) in MolluscaBase editors (2021), and reported that *Callo. castaneus* was basal to Chitonida and closely related to both Chitonina and Acanthochitonina. This result is consistent with the present study.

The radula and shell microstructures have been broadly conserved in chitons living in the shallow sea. Especially, shell microstructure characters are among the relatively few morphological features that can be observed in fossil chitons. They were found to be phylogenetically informative at both geologically ancient and recent levels.

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COMPETING INTERESTS

The author has no competing interests to declare.

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