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Source: Zoological Science, 35(3) : 281-291

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zs170106

ZOOLOGICAL SCIENCE **35**: 281–291 (2018)

Phylogenetic Relationships Among Japanese Species of the Genus *Ischnochiton* (Polyplacophora: Ischnochitonidae), Including a New Species

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Seven (including one new) species of the polyplacophoran genus *Ischnochiton* (Ischnochitonidae) from the Pacific coast of Japan, namely, *I. boninensis*, *I. comptus*, *I. manazuruensis*, *I. hakodadensis*, *I. hayamii* sp. nov., *I. paululus*, and *I. poppei*, were investigated on the basis of DNA sequence analyses of COI, 16S rRNA, 18S rRNA, and 28S rRNA gene regions. For the latter four species, SEM observations were simultaneously carried out. A molecular phylogenetic tree based on the four gene regions for 18 chiton species indicated that the seven Japanese *Ischnochiton* species are polyphyletic and originated from two different clades. A haplotype network based on the COI gene region for the six Japanese *Ischnochiton* species, except *I. hakodadensis*, showed that the genetic distances among them were large. The SEM observations revealed that the denticles of the major lateral teeth in the seven Japanese *Ischnochiton* species were bicuspid, and an accessory process was only observed in the minor lateral teeth of *I. hakodadensis*. *Ischnochiton hayamii* sp. nov. co-occurs with *I. boninensis*, *I. comptus*, and *I. manazuruensis* at the two investigated localities, and was difficult to distinguish from other, similar species by naked eyes. However, these can be discriminated based on a combination of adult body size, girdle scales, and valve sculpturing in the lateral and central areas.

Key words: Polyplacophora, *Ischnochiton*, phylogeny, radula, cytochrome *c* oxidase subunit I, 16S ribosomal DNA, 18S ribosomal DNA, 28S ribosomal DNA

INTRODUCTION

Over 900 species of chitons (Mollusca: Polyplacophora) have been described in environments ranging from the intertidal zone to deep-sea environments in the world's oceans. The body plan of these animals, which possesses eight valves, and their life-type attaching to hard substratum have changed little since the Late Cambrian (Smith, 1960; Runnegar et al., 1979; Puchalski et al., 2008). Approximately 100 species of chitons have been reported in the waters adjacent to Japan (Kaas and Van Belle, 1990, 1994; Slieker, 2000; Saito, 2017).

The current classification system of Polyplacophora was established by Kaas and Van Belle (1980). This is a revised version of Bergenhayn's (1955) classification scheme; which was based only on shell characters and can be applied to chiton fossils. In Kaas and Van Belle's (1980) classification, the family Ischnochitonidae is the largest taxon, comprising 18 genera and about 400 species, of which more than 40% includes known chiton species (Kaas and Van Belle, 1990, 1994; Slieker, 2000). Each species in this family was described based on its morphological characters, such as shell, girdle scale, and radula. However, the taxonomy of Ischnochitonidae has been considerably revised at the family and genus levels by subsequent researchers. Sirenko (1993, 2006) updated the taxonomy of the family using new taxonomical characters, e.g. aesthetes, gills, egg hull projections, and spermatozoides, in addition to the conventional shell characters. In Sirenko's (1993, 2006) classification scheme, the order Chitonida was reduced to only two suborders, Chitonina and Acanthochitonina. Several genera of Ischnochitonidae *sensu* Kaas and Van Belle (1980) were upgraded to new families, and the suborders of some of them were transferred to Acanthochitonina. Sirenko (2006) concluded that some characters of the valves, such as lateral extensions of the insertion plates and the presence or absence of slits, could be misleading when emphasized exclusively for classification at higher taxonomical levels. Today, Ischnochitonidae as restricted by Sirenko (2006) is still a large group comprising 10 genera and about 200 species (Schwabe and Gofas, 2009). The present study follows Sirenko's (2006) classification, as somewhat modified by subsequent authors (Schwabe and Gofas, 2009), because it corresponds more closely to the results of recent molecular phylogenetic analyses (Okusu et al., 2003; Irisarri et al., 2014).

The present study investigates the phylogenetic relationships among seven Japanese species of the genus *Ischnochiton*, namely, *I. boninensis* Bergenhayn, 1933, *I. comptus* (Gould, 1859), *I. manazuruensis* Owada, 2016, *I. hakodadensis* Carpenter, 1893, *I. hayamii* sp. nov., *I.*

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doi:10.2108/zs170106

paululus Is. Taki, 1938, and *I. poppei* Kaas and Van Belle, 1994, based on molecular analyses using DNA sequencing of mitochondrial cytochrome *c* oxidase subunit I (COI) and 16S ribosomal DNA (16S) gene regions, and nuclear 18S ribosomal DNA (18S) and 28S ribosomal DNA (28S) gene regions. For the latter four species, observations using a scanning electron microscopy (SEM) were simultaneously carried out. Because Japanese chitons in the genus *Ischnochiton* are morphologically quite similar to each other, DNA analysis and SEM observation are useful for better resolving their evolutionary history.

MATERIALS AND METHODS

Sampling

The investigated specimens were collected from the intertidal to subtidal zones at seven localities around the Pacific coast of Japan (Fig. 1): Hakodate, Hokkaido in August 2015 (n = 132, four species); Asamushi, Aomori Prefecture in June 2015 and May 2016 (n = 5, one species); Choshi, Chiba Prefecture in June 2015 (n = 192, two species); Zushi, Kanagawa Prefecture in June 2014, May 2015, and April 2016 (n = 304, four species); Manazuru, Kanagawa Prefecture in May 2014, March and May 2015, and March 2016 (n = 127, three species); Shimoda, Shizuoka Prefecture in May 2015 and 2016 (n = 359, four species); and Isso, Kagoshima Prefecture in August 2016 (n = 56, four species). The specimens from Asamushi were collected from the underside of pebbles or large bivalve shells at 5–8 m depth by SCUBA diving. All the remaining specimens were collected from the underside of boulders in the lower intertidal zone. All collected specimens were preserved in 100% ethanol directly, or after freezing at −20°C.

Morphological observations

All collected specimens were identified to species level under a stereomicroscopy system (SZ61, OLYMPUS). For 1–10 specimens of *I. hakodadensis*, *I. hayamii* sp. nov., *I. paululus*, and *I. poppei*, the body length and width and the height and width of the intermediate valve (IV) were measured using a digital caliper (CD-S15CT, Mitutoyo). Valves, radulae, and girdle scales were removed from several specimens of *I. hakodadensis*, *I. hayamii* sp. nov., *I. paululus*, and *I. poppei* for SEM observation. They were washed with pure water using an ultrasonic washer (VS-100III, Velvo-Clear), coated with 50 nm thick platinum using an ion spatter (JFC-166, JEOL), and observed using SEMs (JCM-5000 and JSM-840A, JEOL) at 5 kV accelerating voltage.

The type for the new species and the figured specimens have been deposited in the University Museum, University of Tokyo (UMUT) (UMUT RM 32611–32617). Terminology of morphological characters follows Schwabe (2010).

Genetic analyses

Total DNA was extracted using Dneasy Blood & Tissue Kit (QIAGEN) for foot tissue of specimens of 11 chiton species collected from each study locality: *I. boninensis*, *I. comptus*, *I. hakodadensis*, *I. hayamii* sp. nov., *I. manazuruensis*, *I. paululus*, *I. poppei*, *Stenoplax alata* (Sowerby II, 1841), *Lepidozona coreanica* (Reeve, 1847), *Callistochiton jacobaeus* (Gould, 1859), and *Rhyssoplax kurodai* Is. and Iw. Taki, 1929. The COI, 16S, 18S, and 28S gene regions of *I. hakodadensis*, *I. hayamii* sp. nov., *I. paululus*, *I. poppei*, and *S. alata*, the COI and 18S of *I. boninensis*, *I. comptus*, and *I. manazuruensis*, and the 18S of *L. coreanica*, *C. jacobaeus*, and *R. kurodai* were amplified using polymerase chain reaction (PCR) with Premix *Taq* (Takara) and thermal cycler (T100, Bio-Rad). The primers used are shown in Table 1. The conditions for the PCR amplification were as follows: denaturation at 94°C for 30 s; annealing at 48°C (COI), 55°C (16S and 18S) or 59°C (28S) for 30 s; and extension at 72°C for 60 s. These steps were repeated 25 to 35 times. The PCR products were purified by ExoSAP-IT (Affymetrix), and cycle-sequencing reactions were performed by BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The DNA sequences were analyzed using a genetic analyzer (3130, Applied Biosystems) in both 5′ and 3′ directions. All sequences were registered in the DNA Data Bank of Japan (DDBJ) (COI: LC214409–LC214413 and LC214419–LC214526; 16S: LC214398–LC214402; 18S: LC214370–LC214379 and LC214381; and 28S: LC214387–LC214391) (Tables 2, 3).

Seven selected species already registered in DDBJ were also included in the genetic analysis. All of the analyzed specimens are shown in Table 2. Alignment of the DNA sequences was performed in each gene region by MAFFT v7.212 (Katoh et al., 2005). Sites that contained gaps or those that were not homologous were trimmed using trimAl v1.2rev59 (Capella-Gutiérrez et al., 2009). A

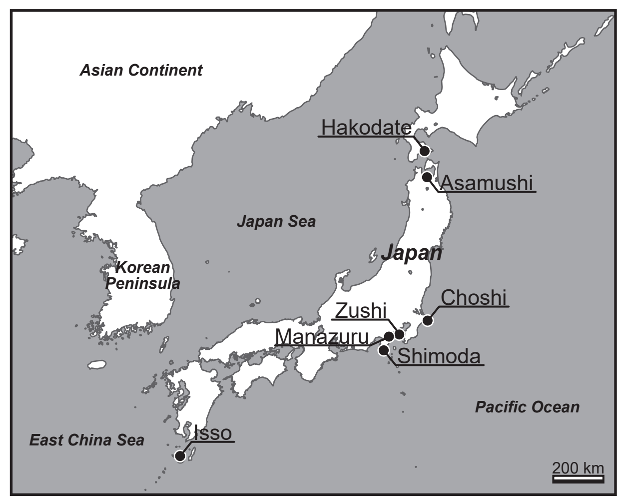

Fig. 1. Sampling localities of the investigated specimens. Hakodate: 41°45′N, 140°43′E; Asamushi: 40°54′N, 140°51′E; Choshi: 35°42′N, 140°52′E; Zushi: 35°16′N, 139°34′E; Manazuru: 35°09′N, 139°09′E; Shimoda: 34°40′N, 138°56′E; Isso: 30°27′N, 130°30′E.

Table 1. Primers used in the present study. Asterisks indicate the primers used only for DNA sequencing.

region	direction	5′–3′ sequence	reference
COI	forward	TCWACAAATCAYAAAGATATTGG	Owada et al. (2013)
	reverse	ACYTCMGGRTGMCCAAAAAATCA	Owada et al. (2013)
16S	forward	CGCCTGTTTATCAAAAACAT	Xiong and Kocher (1991)
	reverse	CTCCGGTTTGAACTCAGATCA	Xiong and Kocher (1991)
18S	forward	TACCTGGTTGATCCTGCCAGTAG	Giribet et al. (1996)
	forward*	GTTCGATTCCGGAGAGGGA	Giribet et al. (1996)
	reverse*	GAATTACCGCGGCTGCTGG	Giribet et al. (1996)
	reverse*	CTTGGCAAATGCTTTCGC	Giribet et al. (1996)
	forward*	ATGGTTGCAAAGCTGAAAC	Whiting et al. (1997)
	reverse*	GAGTCTCGTTCGTTATCGGA	Whiting et al. (1997)
	reverse	GATCCTTCCGCAGGTTCACCTAC	Giribet et al. (1996)
28S	reverse	GACCCGTCTTGAAACACGGA	Whiting et al. (1997)
	forward	TCGGAAGGAACCAGCTACTA	Whiting et al. (1997)

Table 2. Species names, localities, and accession numbers of the specimens used for molecular phylogenetic analyses. The newly determined DNA sequences are highlighted.

order	family	species	locality	accession number			
				CO1	16S	18S	28S
Chitonida	Ischnochitonidae	*Ischnochiton australis*	Australia	AY377707	AY377596	AY377641	AY377670
		Ischnochiton boninensis	**Manazuru**	LC071647	LC071575	LC214370	LC071611
		Ischnochiton comptus	**Manazuru**	LC071627	LC071570	LC214371	LC071606
		Ischnochiton elongatus	Australia	AY377708	AY377595	AY377642	AY377672
		Ischnochiton hakodadensis	**Hakodate**	LC214409	LC214398	LC214372	LC214387
		Ischnochiton hayamii sp. nov.	**Zushi**	LC214410	LC214399	LC214373	LC214388
		Ischnochiton manazuruensis	**Manazuru**	LC071619	LC071565	LC214374	LC071601
		Ischnochiton paululus	**Asamushi**	LC214411	LC214400	LC214375	LC214389
		Ischnochiton poppei	**Isso**	LC214412	LC214401	LC214376	LC214390
		Ischnochiton rissoi	Spain	AY377706	AY377594	AY377640	AY377671
		Stenoplax alata	**Isso**	LC214413	LC214402	LC214377	LC214391
		Lepidozona coreanica	**Manazuru**	LC071669	LC071582	LC214378	LC071618
		Lepidozona mertensii	USA	AY377710	AY377597	AY377643	AY377674
	Callistoplacidae	*Callistochiton antiquus*	Australia	AY377712	AY377599	AY377645	AY377676
		Callistochiton jacobaeus	**Manazuru**	LC071667	LC071580	LC214379	LC071616
	Chaetopleuridae	*Chaetopleura angulata*	Spain	AY377703	AY377591	AY377637	AY377668
		Chaetopleura apiculata	USA	AY377704	AY377590	AY377636	AY377667
	Chitonidae	*Rhyssoplax kurodai*	**Manazuru**	LC071668	LC071581	LC214381	LC071617

molecular phylogenetic tree was constructed from the concatenated sequence of the four gene regions using Maximum Likelihood (ML) and Bayesian methods, and a strict consensus tree was derived from these two trees. The ML method was carried out using RAxML v8.2.4 (Stamatakis, 2006), and the tree-search algorithm used shotgun search (n = 100). A bootstrap test was performed 1000 times. The model for the ML method was selected for the sequence of each gene region by Kakusan4 (Tanabe, 2011). The Bayesian method was carried out using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). This program was run for 5,000,000 generations with sampling at every 1000th generation. The model for the Bayesian method was selected for the concatenated sequence by Kakusan4 (Tanabe, 2011). As for the COI gene region, the haplotype network for *I. boninensis* (n = 60), *I. comptus* (n = 50), *I. hayamii* sp. nov. (n = 22), and *I. manazuruensis* (n = 24) was built by TCS v1.21 (Clement et al., 2000). The fix connection limit was set to 70 steps. All specimens of the haplotype network are shown in Table 3.

RESULTS

Morphological observations

The representative specimens of *I. hakodadensis*, *I. hayamii* sp. nov., *I. paululus*, and *I. poppei* were shown in Fig. 2. In *I. hakodadensis* (Figs. 2G, 3), the ratio of body length/width was 1.57 ± 0.019 (mean ± SE, n = 7), and that of height/length of intermediate valve was 0.382 ± 0.013 (n = 3). The tegmentum of the head valve and tail valve and the lateral area of the intermediate valve were sculptured with fine radial ribs. The central area of intermediate valve and the premucronal area of tail valve were approximately smooth. The girdle scale was 200–300 μm in width and smooth or very weakly sculptured. In *I. hayamii* sp. nov. (Figs. 2A–F, 4), the ratio of body length/width was 1.66 ± 0.026 (n = 10), and that of height/length of intermediate valve was 0.403 ± 0.007 (n = 4). The central area of the intermediate valve and the premucronal area of the tail valve were covered with low granules. The tegmentum of head valve, the postmucronal area of the tail valve, and the lateral area of the intermediate valve were smooth. The girdle scale was 150–200 μm in width and smooth or very weakly sculptured. In *I. paululus* (Figs. 2I, 5), the ratio of body length/width was 1.49 (Fig. 2I), and that of height/length of the intermediate valve was 0.424 (Fig. 5E). The tegmentum of valves was wholly covered with low granules. The girdle scale was 60–100 μm in width and sculptured with 5–8 coarse ribs. In *I. poppei* (Figs. 2H, 6), the ratio of body length/width was 1.46 ± 0.091 (n = 6), and that of height/length of the intermediate valve was 0.319 ± 0.009 (n = 5). The tegmentum of valves was covered with low granules. The tegmentum of the head valve, the postmucronal area of the tail valve, and the lateral area of the intermediate valve were sculptured with shallow radial ribs composed of granules. The girdle scale was 150–250 μm in width and very weakly sculptured.

The radulae of *I. hakodadensis*, *I. hayamii* sp. nov., *I. paululus*, and *I. poppei* are shown in Fig. 7. The denticle of the major lateral tooth in these four species was bicuspid. An accessory process was observed only in the minor lateral tooth of *I. hakodadensis* (Fig. 7B).

Genetic analyses

DNA sequences of COI, 16S, 18S, and 28S gene regions were determined in 11 chiton species for the construction of a molecular phylogenetic tree, and their lengths were calculated as 557, 504–516, 1694–1698, and 306–311 base pairs (bp), respectively (Table 2). For the construction of a haplotype network, the DNA sequences of the COI gene region were newly determined in 40 specimens of *I. boninensis*, 30 of *I. comptus*, 22 of *I. hayamii* sp. nov., and 16 of *I. manazuruensis* (Table 3).

Table 3. Species names, localities, isolate, accession numbers, and haplotypes of the specimens used for building a haplotype network. The newly determined DNA sequences are highlighted.

species name	locality	isolate	accession number	haplotype
Ischnochiton boninensis	Hakodate	B_Hkd1	LC214429	B-10
		B_Hkd2	LC214430	B-10
		B_Hkd3	LC214431	B-10
		B_Hkd4	LC214432	B-14
		B_Hkd5	LC214433	B-10
		B_Hkd6	LC214434	B-12
		B_Hkd7	LC214435	B-10
		B_Hkd8	LC214436	B-13
		B_Hkd9	LC214437	B-10
		B_Hkd10	LC214438	B-11
	Choshi	B_Chs1	LC071652	B-01
		B_Chs2	LC071653	B-01
		B_Chs3	LC071654	B-01
		B_Chs4	LC071655	B-01
		B_Chs5	LC071656	B-01
		B_Chs6	LC214424	B-01
		B_Chs7	LC214425	B-01
		B_Chs8	LC214426	B-01
		B_Chs9	LC214427	B-01
		B_Chs10	LC214428	B-01
	Zushi	B_Zsh1	LC071657	B-01
		B_Zsh2	LC071658	B-04
		B_Zsh3	LC071659	B-07
		B_Zsh4	LC071660	B-07
		B_Zsh5	LC071661	B-07
		B_Zsh6	LC214481	B-06
		B_Zsh7	LC214482	B-02
		B_Zsh8	LC214483	B-01
		B_Zsh9	LC214484	B-07
		B_Zsh10	LC214485	B-02
	Manazuru	B_Mnz1	LC071647	B-01
		B_Mnz2	LC071648	B-03
		B_Mnz3	LC071649	B-07
		B_Mnz4	LC071650	B-02
		B_Mnz5	LC071651	B-05
		B_Mnz6	LC214512	B-01
		B_Mnz7	LC214513	B-09
		B_Mnz8	LC214514	B-01
		B_Mnz9	LC214515	B-07
		B_Mnz10	LC214516	B-04
	Shimoda	B_Smd1	LC071662	B-07
		B_Smd2	LC071663	B-01
		B_Smd3	LC071664	B-07
		B_Smd4	LC071665	B-02
		B_Smd5	LC071666	B-01
		B_Smd6	LC214491	B-07
		B_Smd7	LC214492	B-02
		B_Smd8	LC214493	B-08
		B_Smd9	LC214494	B-01
		B_Smd10	LC214495	B-01
	Isso	B_Iso1	LC214451	B-17
		B_Iso2	LC214452	B-16
		B_Iso3	LC214453	B-18
		B_Iso4	LC214454	B-18
		B_Iso5	LC214455	B-18
		B_Iso6	LC214456	B-15
		B_Iso7	LC214457	B-18
		B_Iso8	LC214458	B-18
		B_Iso9	LC214459	B-18
		B_Iso10	LC214460	B-18
Ischnochiton comptus	Hakodate	Q_Hkd1	LC214439	Q-13
		Q_Hkd2	LC214440	Q-17
		Q_Hkd3	LC214441	Q-16
		Q_Hkd4	LC214442	Q-15
		Q_Hkd5	LC214443	Q-18
		Q_Hkd6	LC214444	Q-17
		Q_Hkd7	LC214445	Q-14
		Q_Hkd8	LC214446	Q-15
		Q_Hkd9	LC214447	Q-17
		Q_Hkd10	LC214448	Q-17
	Choshi	Q_Chs1	LC071642	Q-01
		Q_Chs2	LC071643	Q-20
		Q_Chs3	LC071644	Q-23
		Q_Chs4	LC071645	Q-01
		Q_Chs5	LC071646	Q-12
		Q_Chs6	LC214419	Q-01
		Q_Chs7	LC214420	Q-01
		Q_Chs8	LC214421	Q-01
		Q_Chs9	LC214422	Q-11
		Q_Chs10	LC214423	Q-01
	Zushi	Q_Zsh1	LC071632	Q-02
		Q_Zsh2	LC071633	Q-03
		Q_Zsh3	LC071634	Q-07
		Q_Zsh4	LC071635	Q-10
		Q_Zsh5	LC071636	Q-10
		Q_Zsh6	LC214506	Q-01
		Q_Zsh7	LC214507	Q-01
		Q_Zsh8	LC214508	Q-09
		Q_Zsh9	LC214509	Q-01
		Q_Zsh10	LC214510	Q-04
	Manazuru	Q_Mnz1	LC071627	Q-01
		Q_Mnz2	LC071628	Q-13
		Q_Mnz3	LC071629	Q-05
		Q_Mnz4	LC071630	Q-01
		Q_Mnz5	LC071631	Q-11
		Q_Mnz6	LC214476	Q-19
		Q_Mnz7	LC214477	Q-01
		Q_Mnz8	LC214478	Q-01
		Q_Mnz9	LC214479	Q-01
		Q_Mnz10	LC214480	Q-06
	Shimoda	Q_Smd1	LC071637	Q-08
		Q_Smd2	LC071638	Q-01
		Q_Smd3	LC071639	Q-22
		Q_Smd4	LC071640	Q-21
		Q_Smd5	LC071641	Q-01
		Q_Smd6	LC214486	Q-01
		Q_Smd7	LC214487	Q-01

		Q_Smd8	LC214488	Q-01
		Q_Smd9	LC214489	Q-10
		Q_Smd10	LC214490	Q-01
Ischnochiton hayamii sp. nov.	Hakodate	E_Hkd1	LC214449	E-05
		E_Hkd2	LC214450	E-05
	Zushi	E_Zsh1	LC214517	E-01
		E_Zsh2	LC214518	E-01
		E_Zsh3	LC214519	E-01
		E_Zsh4	LC214520	E-01
		E_Zsh5	LC214521	E-01
		E_Zsh6	LC214522	E-01
		E_Zsh7	LC214523	E-01
		E_Zsh8	LC214524	E-02
		E_Zsh9	LC214525	E-01
		E_Zsh10	LC214526	E-01
	Shimoda	E_Smd1	LC214496	E-04
		E_Smd2	LC214497	E-04
		E_Smd3	LC214498	E-01
		E_Smd4	LC214499	E-04
		E_Smd5	LC214500	E-04
		E_Smd6	LC214501	E-04
		E_Smd7	LC214502	E-01
		E_Smd8	LC214503	E-03
		E_Smd9	LC214504	E-04
		E_Smd10	LC214505	E-04
Ischnochiton manazuruensis	Zushi	S_Zsh1	LC214511	S-02
	Manazuru	S_Mnz1	LC071619	S-01
		S_Mnz2	LC071620	S-01
		S_Mnz3	LC071621	S-01
		S_Mnz4	LC071622	S-03
		S_Mnz5	LC071623	S-01
		S_Mnz6	LC214471	S-09
		S_Mnz7	LC214472	S-01
		S_Mnz8	LC214473	S-06
		S_Mnz9	LC214474	S-02
		S_Mnz10	LC214475	S-04
	Shimoda	S_Smd1	LC071624	S-07
		S_Smd2	LC071625	S-05
		S_Smd3	LC071626	S-08
	Isso	S_Iso1	LC214461	S-10
		S_Iso2	LC214462	S-15
		S_Iso3	LC214463	S-11
		S_Iso4	LC214464	S-16
		S_Iso5	LC214465	S-14
		S_Iso6	LC214466	S-10
		S_Iso7	LC214467	S-10
		S_Iso8	LC214468	S-12
		S_Iso9	LC214469	S-13
		S_Iso10	LC214470	S-10
Ischnochiton paululus	Asamushi	Him_Asm	LC214411	
Ischnochiton poppei	Isso	Kdb_Iso	LC214412	

The constructed molecular phylogenetic tree is shown in Fig. 8. The number of sites for each gene after alignment were 557, 505, 1696, and 307 bp in COI, 16S, 18S, and 28S, respectively. In the ML method, the GTR+G model was selected, and the likelihood index was −*ln* 13561.649. In the Bayesian method, the GTR+G model was selected. The obtained tree indicated that the Ischnochitonidae and *Ischnochiton* species were polyphyletic taxa. Four sympatric species—*I. boninensis*, *I. comptus*, *I. hayamii* sp. nov., and *I. manazuruensis*—formed a monophyletic group along with *I. paululus* and *I. poppei* (bootstrap value ≥ 50 and posterior probability ≥ 0.90). This clade was sister to *Ischnochiton elongatus* (Blainville, 1825) and, in turn, to *Ischnochiton australis* (Sowerby II, 1833), the latter two being distributed in Australian waters. *Ischnochiton comptus* constituted a clade with *I. manazuruensis*, but the phylogenetic relationships among *I. boninensis*, *I. hayamii* sp. nov., *I. paululus*, and *I. poppei* were not clear because of discordance in the topologies of the ML and Bayesian trees. *Ischnochiton hakodadensis* and *Ischnochiton rissoi* (Payraudeau, 1826) were phylogenetically distinct from the other eight *Ischnochiton* species which constituted a monophyletic group. *Ischnochiton hakodadensis* constituted a clade along with *Lepidozona mertensii* (Middendorff, 1847), *L. coreanica*, and *C. jacobaeus*. Similarly, *I. rissoi*, *Chaetopleura angulata* (Spengler, 1797), and *Chaetopleura apiculata* (Say in Conrad, 1834) formed a clade.

A total of 64 haplotypes were observed in the haplotype network (Fig. 9). They formed six groups corresponding to *I. boninensis*, *I. comptus*, *I. hayamii* sp. nov., *I. manazuruensis*, *I. paululus*, and *I. poppei*. The DNA substitutions between *I. boninensis* and *I. comptus* groups were 69 bp, those between *I. comptus* and *I. manazuruensis* groups were 63, and those between *I. boninensis* and *I. hayamii* sp. nov. groups were 56. The maximum number of DNA substitutions within each group was 10 bp for *I. boninensis* group, 26 for *I. comptus* group, 12 for *I. hayamii* sp. nov. group, and 16 for *I. manazuruensis* group. Sympatric populations of *I. boninensis*, *I. comptus*, *I. hayamii* sp. nov., and *I. manazuruensis* groups were genetically distinct.

Systematics

Family **Ischnochitonidae** Dall, 1889

Genus ***Ischnochiton*** Gray, 1847

Type species. *Ischnochiton textilis* (Gray, 1828)

Ischnochiton hakodadensis Carpenter, 1893

(Figs. 2G, 3, 7B)

Ischnochiton (*Ischnoradsia*) *hakodadensis* Carpenter in Pilsbry, 1893: 147, pl. 19, figs. 64–66. Is. Taki, 1962: 44 (in part). Iw. Taki, 1964b: 409 (in part).

Ischnoradsia hakodadensis.— Is. Taki, 1938: 373–375, pl. 15, fig. 8, pl. 26, figs. 1–5, pl. 27, figs. 1–5, pl. 28, figs. 19–20.

Ischnochiton (*Ischnochiton*) *hakodadensis*.— Kaas and Van Belle, 1990: 180–182, fig. 81.

Ischnochiton (*Ischnoradsia*) *hakodatensis* [sic].— Higo and Goto, 1993: 4 (in part).

Ischnochiton hakodadensis: Saito, 1994: 97 (in part). Saito, 1995: 103 (in part). Slieker, 2000: 98–99, pl. 37, fig. 12. Saito in Okutani, 2017: 49, 732, pl. 5, fig. 4.

Material examined. Thirty specimens from Hakodate, Hokkaido (41°45′N, 140°43′E), 12.7–32.9 mm in body length.

Description. Body large for genus, rarely exceeding 35

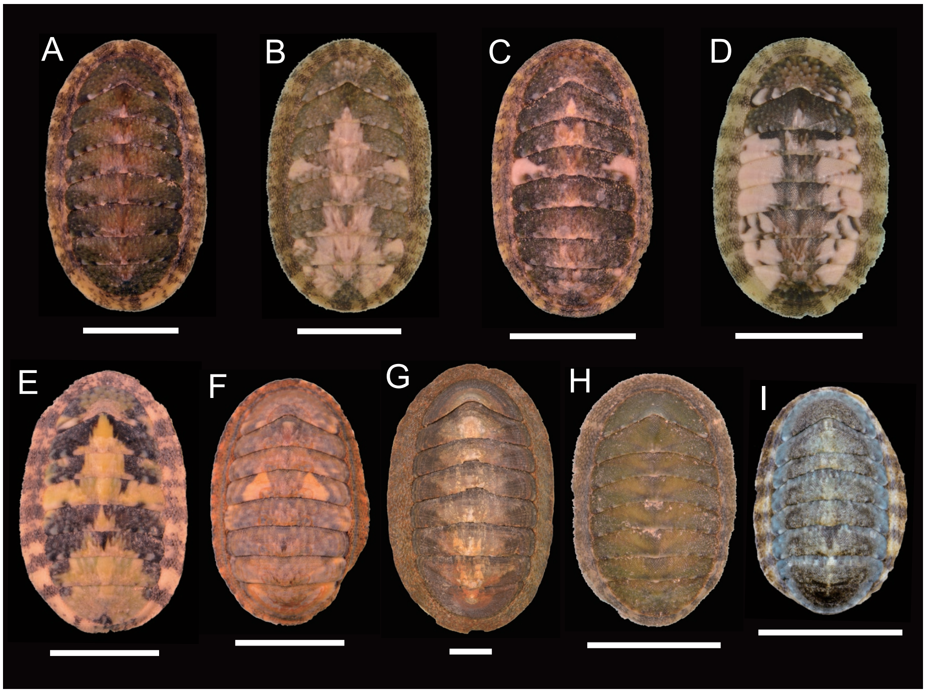

Fig. 2. Representative specimens of *Ischnochiton* spp. described in this study. **(A)** *I. hayamii* sp. nov. from Zushi (paratype, UMUT RM 32612); **(B)** *I. hayamii* sp. nov. from Zushi (holotype, UMUT RM 32611); **(C, D)** *I. hayamii* sp. nov. from Zushi (paratype, UMUT RM 32613, RM 32614); **(E)** *I. hayamii* sp. nov. from Shimoda (paratype, UMUT RM 32615); **(F)** *I. hayamii* sp. nov. from Hakodate (paratype, UMUT RM 32616); **(G)** *I. hakodadensis* from Hakodate; **(H)** *I. poppei* from Isso; **(I)** *I. paululus* from Asamushi (UMUT RM 32617). The scale bar indicates 5 mm.

mm in length, oval in outline, moderately elevated. Color of tegmentum mostly dark-olive or -brown with more or less black or brown stripes. Girdle rather narrow, colored like tegmentum with olive or brown transverse bands, obliquely imbricated with smooth or very weakly sculptured scales 200–300 µm in width. Head valve semicircular, sculptured with numerous fine radial ribs; posterior margin widely V-shaped; anterior slope steep, straight. Intermediate valves broadly rectangular, round-backed, not beaked; side slopes slightly rounded; central areas approximately smooth; lateral areas slightly arose, sculptured with 6–8 fine radial ribs. Tail valve semicircular, sculptured like head valve; posterior slope steep, straight; mucro subcentral; premucronal area approximately smooth. Slit formula 15–16/2–3/12–14. Central tooth oblongly blade-shaped; minor lateral teeth ornamented with accessory process; major lateral teeth bicuspid; denticles

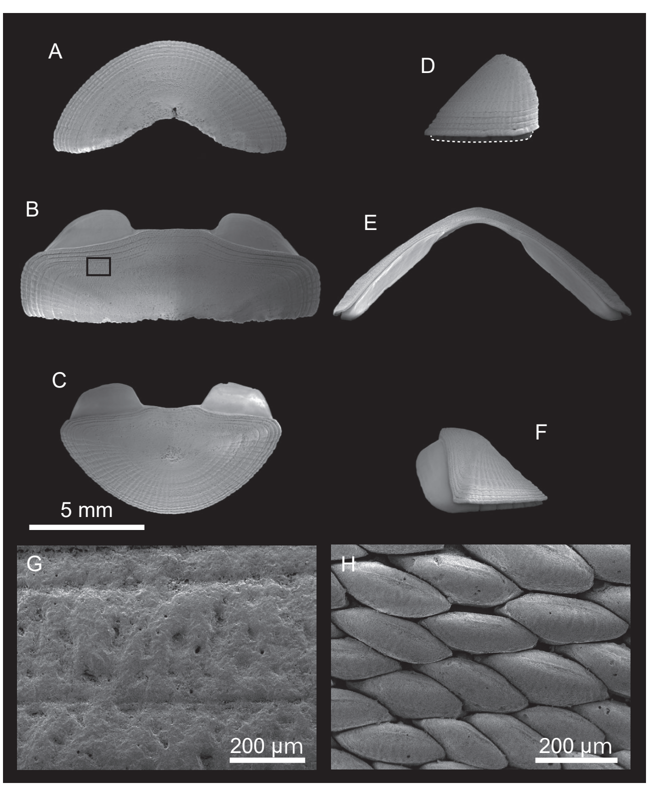

Fig. 3. SEM images of *Ischnochiton hakodadensis*. **(A)** head valve; **(B)** intermediate valve (valve VI); **(C)** tail valve; **(D)** lateral view of head valve; **(E)** horizontal view of intermediate valve; **(F)** lateral view of tail valve; **(G)** close up view of 3B; **(H)** girdle scales.

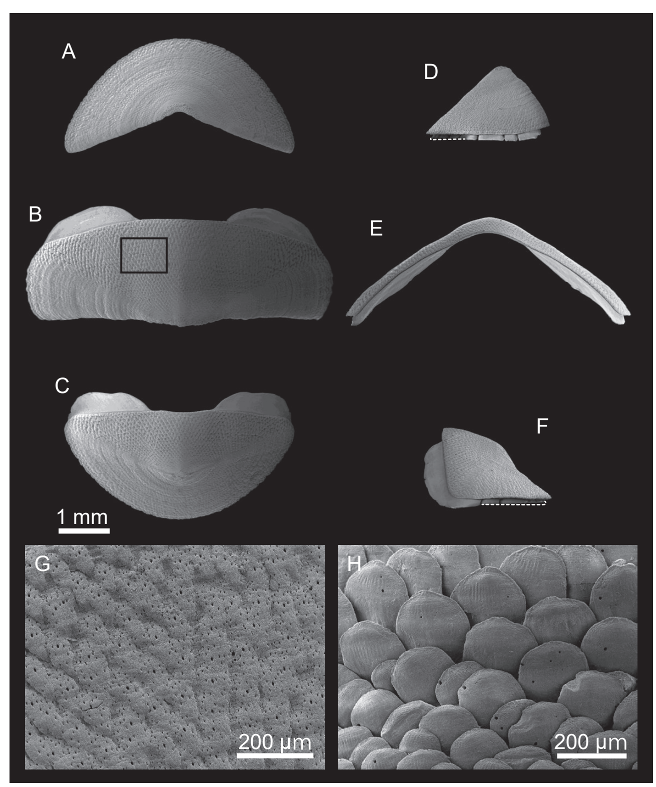

Fig. 4. SEM images of *Ischnochiton hayamii* sp. nov. **(A)** head valve; **(B)** intermediate valve (valve VI); **(C)** tail valve; **(D)** lateral view of head valve; **(E)** horizontal view of intermediate valve; **(F)** lateral view of tail valve; **(G)** close up view of 4B; **(H)** girdle scales.

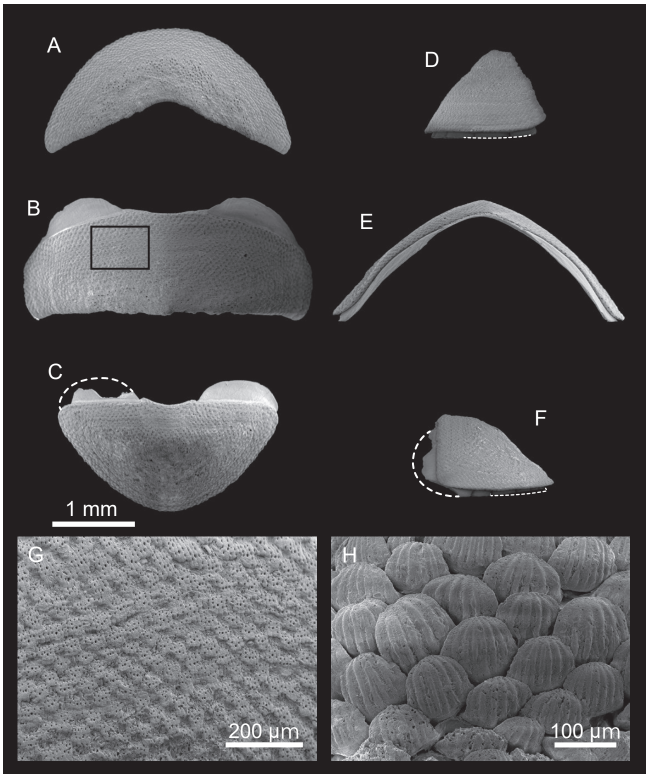

Fig. 5. SEM images of *Ischnochiton paululus*. **(A)** head valve; **(B)** intermediate valve (valve VI); **(C)** tail valve; **(D)** lateral view of head valve; **(E)** horizontal view of intermediate valve; **(F)** lateral view of tail valve; **(G)** close up view of Figure 5B; **(H)** girdle scales.

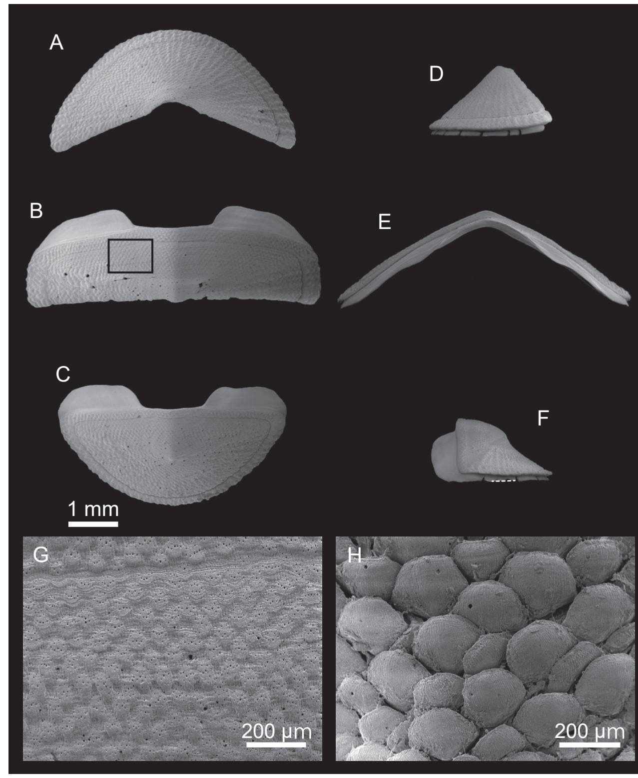

Fig. 6. SEM images of *Ischnochiton poppei*. **(A)** head valve; **(B)** intermediate valve (valve VI); **(C)** tail valve; **(D)** lateral view of head valve; **(E)** horizontal view of intermediate valve; **(F)** lateral view of tail valve; **(G)** close up view of 6B; **(H)** girdle scales.

rounded.

Remarks. *Ischnochiton hakodadensis* is distinguishable from the following three species on the basis of adult body size and valve sculpturing in head and tail valves and lateral area. *Ischnochiton comptus* resembles the present species; however, the intermediate valves of the present species possess 2–3 slits and its girdle scales obliquely arrange along valves. Is. Taki (1938) and Kaas and Van Belle (1990) described the radula of the present species in detail, but not the accessory process of minor lateral teeth.

Distribution. *Ischnochiton hakodadensis* is distributed in the intertidal zone from eastern Hokkaido to Tohoku and from Hakodate and Toyama Bay (Saito, 1994, 1995, 2017; Is. Taki, 1938).

Ischnochiton hayamii **sp. nov.**
(Figs. 2A–F, 4, 7A)

Type and material. Holotype: UMUT RM 32611 (Fig. 2B), Zushi, Kanagawa Prefecture (35°16′N, 139°34′E), 13.2 mm in body length. Paratypes: UMUT RM 32612–32614 (Figs. 2A, C, D), Zushi, Kanagawa Prefecture, 14.5, 13.3, 11.0 mm in body length; UMUT RM 32615 (Fig. 2E), Shimoda, Shizuoka Prefecture (34°40′N, 138°56′E), 12.0 mm; and UMUT RM 32616 (Fig. 2F), Hakodate, Hokkaido (41°45′N, 140°43′E), 11.3 mm. Non-type specimens: one specimens from Hakodate, Hokkaido, 13.3 mm in body length; 25 from Zushi, Kanagawa Prefecture, 8.7–14.8 mm; and 21 from Shimoda, Shizuoka Prefecture, 7.9–13.5 mm.

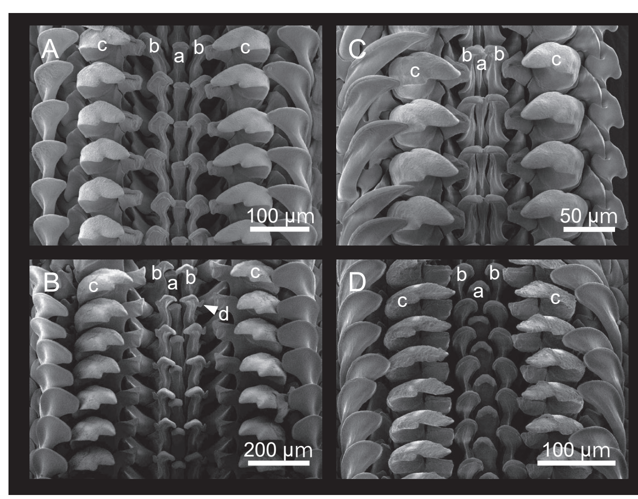

Fig. 7. SEM images of radulae. **(A)** *Ischnochiton hayamii* sp. nov.; **(B)** *I. hakodadensis*; **(C)** *I. paululus*; **(D)** *I. poppei*. a–central tooth; b–minor lateral tooth; c–major lateral tooth; d–accessory process.

Diagnosis. Medium-sized, moderately elevated species of *Ischnochiton*, characterized by oval outline, minute smooth girdle scales, semicircular smooth head valve, broadly rectangular carinated intermediate valves, semicircular smooth tail valve, central area covered with low granules, smooth lateral areas, rounded denticles of bicuspid major lateral tooth.

Description. Body medium for genus, rarely exceeding

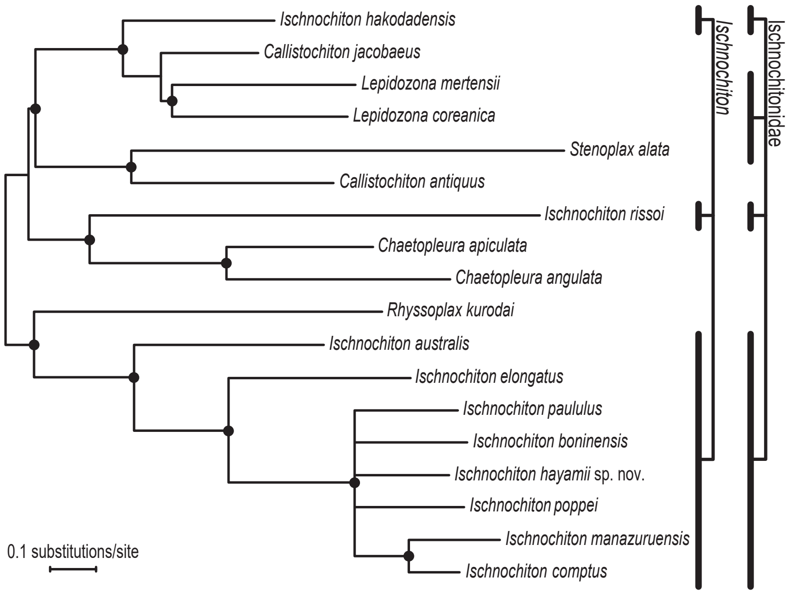

Fig. 8. Molecular phylogenetic tree based on the concatenated sequences of COI, 16S, 18S, and 28S gene regions. Solid circles on nodes indicate that bootstrap value ≥ 50 and posterior probability ≥ 0.90 fit in the same time. Branch length was calculated using the ML method.

15 mm in length, oval in outline, moderately elevated. Color of tegmentum variable, mostly brown with more or less yellow or white spots. Girdle rather narrow, brown with yellow or white transverse bands, imbricated with smooth or very weakly sculptured scales 150–200 μm in width. Head valve semicircular, smooth; posterior margin widely V-shaped; anterior slope steep, straight. Intermediate valves broadly rectangular, carinated, hardly beaked; side slopes almost straight; side margins slightly ridged; central areas covered with low granules forming quincuncial pattern; lateral areas hardly arose, smooth. Tail valve semicircular, smooth; posterior slope steep, slightly concave; mucro subcentral; premucronal area covered with low granules. Slit formula 10–12/1/8–10. Central tooth oblongly blade-shaped; major lateral teeth bicuspid; denticles rounded.

Remarks. Although *I. hayamii* resembles *I. comptus*, *I. manazuruensis*, and specially to *I. boninensis* in possessing moderately elevated and oval shell outline, semicircular head and tail valves, and broadly rectangular carinated intermediate valves, the four species differ with respect to their adult body size, girdle scales, and lateral and central areas (Gould, 1859; Bergenhayn, 1933; Owada, 2016). The adult body size of the present species rarely exceeds 15 mm, but those of the other three species commonly do. The girdle scale of *I. boninensis* is obviously sculptured with 8–18 ribs, whereas those of the other three species are smooth or very weakly sculptured. The widths of the girdle scale in *I. comptus* and *I. manazuruensis* are 300–400 μm, but those of the present species and *I. boninensis* are 150–250 μm. The lateral area of the present species is smooth, but those of the other three species are obviously sculptured with 5–8 ribs. The central areas in *I. hayamii*, *I. boninensis*, and *I. comptus* are covered with conspicuous quincuncial granulations, but that of *I. manazuruensis* is not. In addition, the color of the tegmentum in *I. boninensis*, *I. comptus*, and *I. manazuruensis* is more highly variable than that of the present species. However, in the juvenile specimens or specimens whose shell and girdle scale are secondarily abraded, it is difficult to distinguish the present species morphologically from the other three species.

Ischnochiton hayamii is clearly distinguishable from *Ischnochiton melinus* Dall, 1926 and *Ischnochiton mitsukurii* Pilsbry, 1898 based on head valve, lateral area, and girdle scale. In *I. melinus*, which Kaas and Van Belle (1990) treated as a synonym of *I. mitsukurii*, the head valve is sculptured with feebly radial striae (Dall, 1926). In *I. mitsukurii*, the lateral area is sculptured with three or four shallow inconspicuous radial sulci, and the girdle scale is coarsely striated (Pilsbry, 1898; Kaas and Van Belle, 1990; Saito, 2017). *Ischnochiton hayamii* differs from *I. paululus* possessing the head and tail valves and the lateral area covered with granules, the round-backed intermediate valve, and the sculptured girdle scale (Is. Taki, 1938). *Ischnochiton hayamii* is distinguishable from *I. poppei* possessing the head and tail valves and the lateral area sculptured with shallow radial ribs composed of granules (Kaas and Van Belle, 1994; Saito, 2017). In addition, the adult body size in *I. hayamii* is larger than those in *I. paululus* and *I. poppei*.

Distribution. *Ischnochiton hayamii* is distributed in the intertidal zone of Hakodate, Zushi, and Shimoda.

Etymology. The new species was named after my supervisor, the late Professor Itaru Hayami, who has greatly contributed to molluscan biology and paleobiology.

Ischnochiton paululus Is. Taki, 1938
(Figs. 2I, 5, 7C)

Ischnochiton paululus Is. Taki, 1938: 371–373, pl. 15, fig. 10, pl. 25, figs. 6–8, pl. 26, figs. 6–12, pl. 27, figs. 8, 9.
Ischnochiton (*Ischnochiton*) *paululus*: Kaas and Van Belle, 1990: 186–188, fig. 84. Higo and Goto, 1993: 4 (in part).

Material examined. Five specimens from Asamushi, Aomori Prefecture (40°54′N, 140°51′E), 5.7–8.3 mm in body length, including one figured and registered specimen (UMUT 32617, Fig. 2I).

Description. Body small for genus, not exceeding 10 mm in length, broad oval in outline, moderately elevated, wholly covered with granules forming quincuncial pattern. Color of tegmentum mostly dark-brown, pale-blue around shell margin. Girdle narrow, dark-brown with pale-yellow transverse bands, imbricated with scales 60–100 μm in width, those sculptured by 5–8 coarse ribs. Head valve semicircular, not sculptured; posterior margin widely V-shaped; anterior slope steep, slightly convex. Intermediate valves broadly rectangular, round-backed, not beaked; side slopes roundly convex; side margins slightly beaked; lateral areas hardly arose, not sculptured. Tail valve semicircular, not sculptured; posterior slope steep, slightly concave; mucro subcentral. Slit formula 10/1/12. Central tooth oblongly blade-shaped; major lateral teeth bicuspid; denticles rounded.

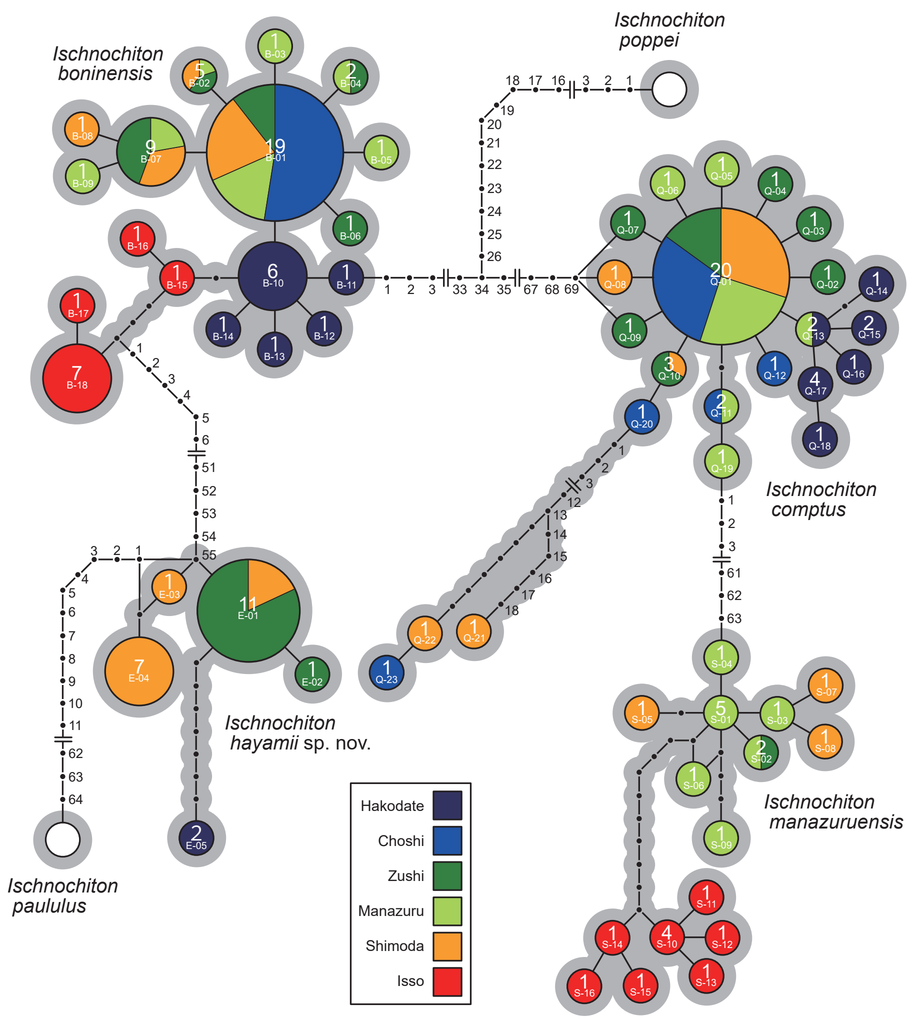

Fig. 9. Haplotype network based on the sequences of COI gene region. Numbers and characters in the center of closed circles indicate the number of specimens and haplotypes (Table 3), respectively. Numbers near filled circles indicate the number of DNA substitutions.

Remarks. *Ischnochiton paululus* is distinguishable from *I. melinus* and *I. mitsukurii* based on the head valves, side slopes, and lateral areas. The head valves of the present species and *I. mitsukurii* are covered with granules, whereas that of *I. melinus* is not. The side slopes of the present species are roundly convex, unlike the slightly convex side slopes in *I. mitsukurii*. The lateral areas of the present species and *I. melinus* are not sculptured, whereas that of *I. mitsukurii* is sculptured (Pilsbry, 1898; Dall, 1926). *Ischnochiton paululus* differs from *I. poppei* possessing the straight side slopes and the latera areas sculptured with shallow radial ribs composed of granules.

Distribution. *Ischnochiton paululus* has been reported only from Asamushi, Mutsu Bay, Aomori Prefecture (Is. Taki, 1938).

Ischnochiton poppei Kaas and Van Belle, 1994
(Figs. 2H, 6, 7D)

Ischnochiton (*Haploplax*) *poppei* Kaas and Van Belle, 1994: 72–74, fig. 29.
Ischnochiton poppei: Saito, 1998: 152, fig. 2E. Slieker, 2000: 98–99, fig. 14. Saito in Okutani, 2017: 49, 732, pl. 5, fig. 2.

Material examined. Thirty-three specimens from Isso, Kagoshima Prefecture (30°27′N, 130°30′E), 6.2–12.4 mm in body length.

Description. Body small for genus, not exceeding 15 mm in length, broad oval in outline, moderately elevated, wholly covered with low granules. Color of tegmentum variable, mostly red-brown. Girdle narrow, brown with light-brown spots, imbricated with very weakly sculptured scales 150–250 µm in width. Head valve semicircular, sculptured with shallow radial ribs composed of granules; posterior margin widely V-shaped; anterior slope steep, straight. Intermediate valves broadly rectangular, carinated, hardly beaked; side slopes straight; lateral areas hardly arose, sculptured like head valve. Tail valve semicircular, sculptured like head valve; posterior slope steep, concave; mucro subcentral. Slit formula 12–13/1/9–10. Central tooth spatula-shaped; major lateral teeth bicuspid; denticles pointed.

Remarks. *Ischnochiton poppei* is distinguishable from *I. boninensis*, *I. comptus*, and *I. manazuruensis* in adult body size and sculpturing in head and tail valves and lateral area. In addition, the present species differs from *I. melinus* possessing the head valve sculptured with feebly radial striae, and from *I. mitsukurii* possessing the sculptured girdle scale and lateral area. Kaas and Van Belle (1994) reported that the major lateral teeth of the present species were unicuspid. However, the SEM observation of the present study revealed that the major lateral teeth of the present species are bicuspid while the outer denticle is relatively small.

Distribution. *Ischnochiton poppei* is distributed in the intertidal zone of Goto Islands, Tanegashima Island, and Yakushima Island (Kaas and Van Belle, 1994; Saito, 1998, 2017).

DISCUSSION

The four sympatric species, namely, *I. boninensis*, *I. comptus*, *I. hayamii*, and *I. manazuruensis*, are morphologically similar, and it is difficult to distinguish from each other by naked eyes. Actually, Iw. Taki (1964a) described this *Ischnochiton* species complex as one species, and Is. Taki (1938) regarded it as two species. However, the four species can be morphologically distinguished by a combination of their adult body size, girdle scales, and valve sculpturing in both lateral and central areas, and the haplotype network based on the COI gene region indicated that the four species are clearly genetically different from each other.

The SEM observations demonstrated that the major lateral teeth of *I. poppei* are bicuspid, though Kaas and Van Belle (1994) reported that it was unicuspid. In addition, it revealed that there is an accessory process in the minor lateral teeth of *I. hakodadensis*, though Is. Taki (1938) and Kaas and Van Belle (1990) had not reported it. It is known that the characters of radula reflect phylogenetic relationships (e.g., Saito, 2004). Owada (2016) reported that the major lateral teeth of *I. boninensis*, *I. comptus*, and *I. manazuruensis* are similarly bicuspid. The molecular phylogenetic analysis indicates that *I. poppei* constituted a clade with *I. boninensis*, *I. comptus*, *I. hayamii*, *I. manazuruensis*, and *I. paululus* and that *I. hakodadensis* found to be in a separate lineage from them. These DNA-based results support the SEM observations.

The six Japanese *Ischnochiton* species except *I. hakodadensis* formed a monophyletic group in the molecular phylogenetic tree; however, the phylogenetic relationships among them were unclear. On the other hand, the morphological characters of *I. boninensis*, such as a sculptured girdle scale and lateral area, a quincuncially granular central area, and colored tegmentum patterns, greatly resemble those of *I. elongatus* which formed a sister group with the six Japanese *Ischnochiton* species. Presumably, the morphological character state of *I. boninensis* is relatively primitive in comparison with the other five species, because it is unlikely that all of such characters was acquired in parallel in both *I. boninensis* and *I. elongatus*. Furthermore, it is possible that *I. boninensis*, *I. comptus*, *I. hayamii*, and *I. manazuruensis* form a monophyletic group, given the similarity of the morphological characters.

Ischnochiton species are identified by very small differences, such as changes in the sculpture patterns of the girdle scale and valve central areas, and the shape of the minor lateral teeth, though these differences are difficult or impossible to detect in the field. In addition, it is highly likely that the characters of radula reflect the phylogenetic relationships among higher taxa rather than species level in Polyplacophora.

ACKNOWLEDGMENTS

I deeply thank Akiya Hino and Ken'ich Kanazawa (both Kanagawa University) for their support during the present study, and Masaya Saito, Tsuyoshi Kobayashi (both Kanagawa University) and Hirokazu Abe (Tohoku University) for their help in collecting specimens. I would like to express my appreciation to Kazushige Tanabe (University of Tokyo) and Toshiyuki Yamaguchi (Kanagawa University) for their critical review of the first draft, Yasuhiro Ito (UMUT) for repository of the type and figured specimens, and Douglas J. Eernisse (University of California) for his review and suggestions. I also thank to editors and reviewers for useful comments to improve the manuscript.

COMPETING INTERESTS

The author has no competing interests to declare.

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(Received July 1, 2017 / Accepted December 24, 2017)