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

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# 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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*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

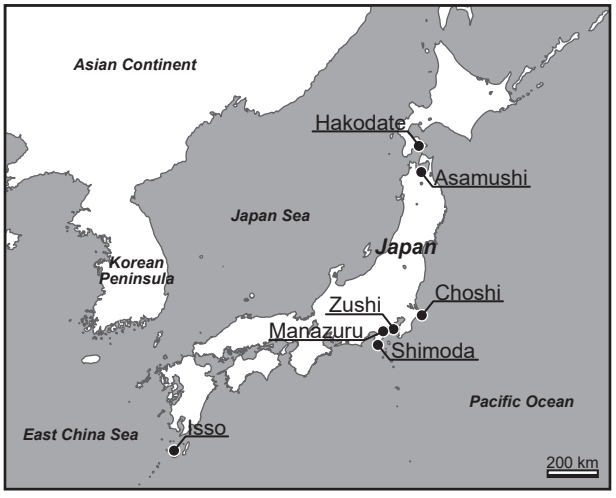


**Fig. 1.** Sampling localities of the investigated specimens. Hakodate: 41°45′N, 140°43′E; Asamushi: 40°54′N, 140°51′E; Choshi: 35°42′N, 140°52′E; Zushi: 35°16′N, 139°34′E; Manazuru: 35°09′N, 139°09′E; Shimoda: 34°40′N, 138°56′E; Isso: 30°27′N, 130°30′E.

**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

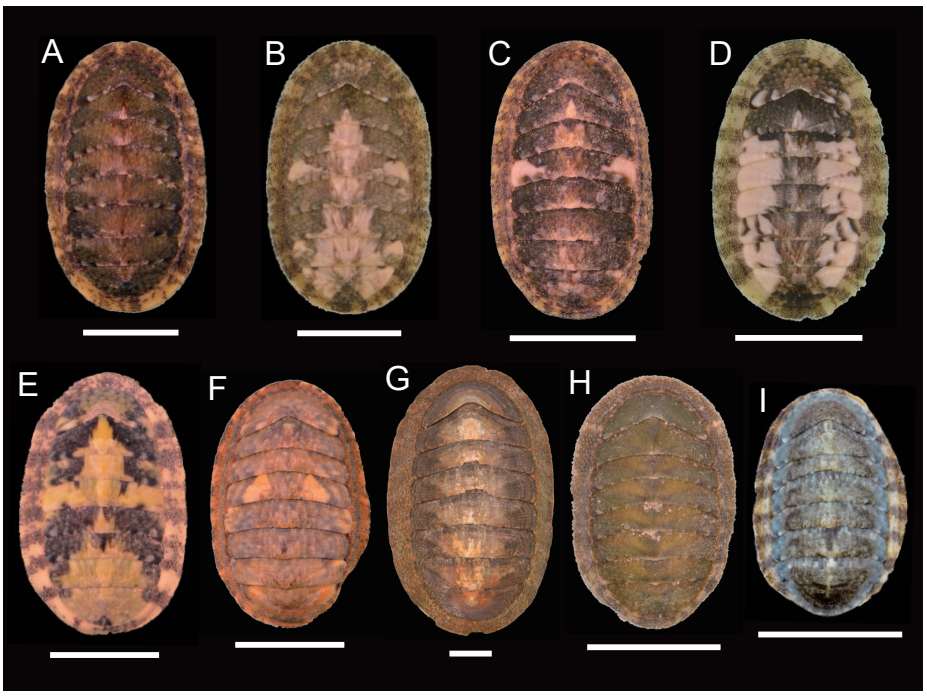


**Fig. 2.** Representative specimens of *Ischnochiton* spp. described in this study. **(A)** *I. hayamii* sp. nov. from Zushi (paratype, UMUT RM 32612); **(B)** *I. hayamii* sp. nov. from Zushi (holotype, UMUT RM 32611); **(C, D)***I. hayamii* sp. nov. from Zushi (paratype, UMUT RM 32613, RM 32614); **(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

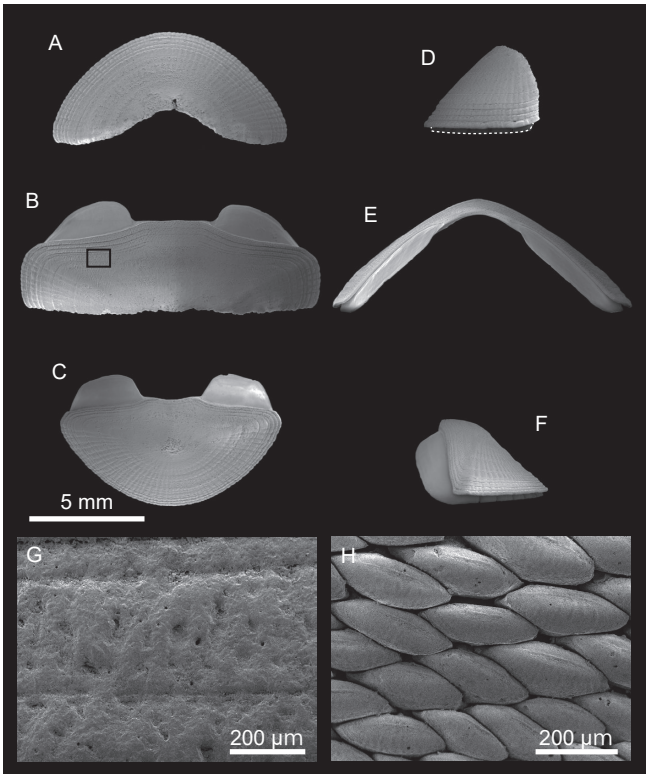


**Fig. 3.** SEM images of *Ischnochiton hakodadensis*. **(A)** head valve; **(B)** intermediate valve (valve VI); **(C)** tail valve; **(D)** lateral view of head valve; **(E)** horizontal view of intermediate valve; **(F)** lateral view of tail valve; **(G)** close up view of 3B; **(H)** girdle scales.

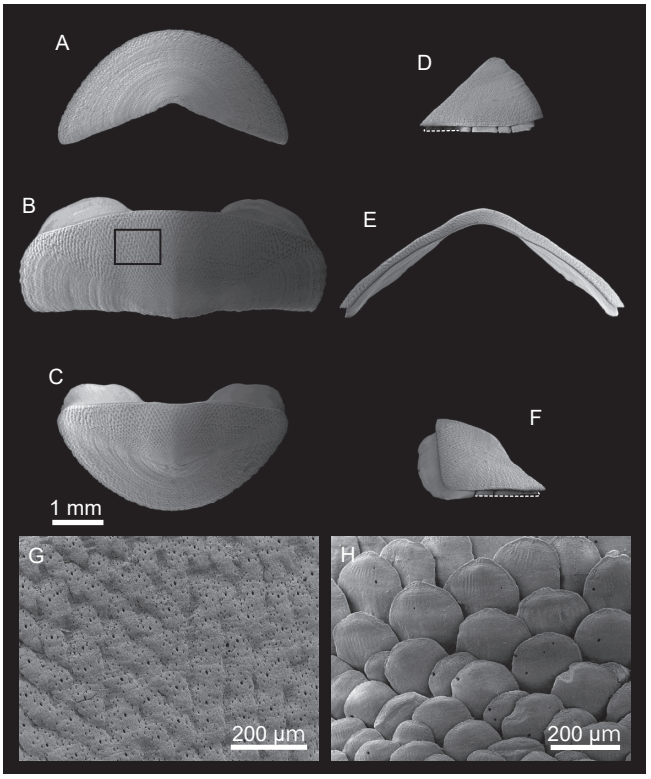


**Fig. 4.** SEM images of *Ischnochiton hayamii* sp. nov. **(A)** head valve; **(B)** intermediate valve (valve VI); **(C)** tail valve; **(D)** lateral view of head valve; **(E)** horizontal view of intermediate valve; **(F)** lateral view of tail valve; **(G)** close up view of 4B; **(H)** girdle scales.

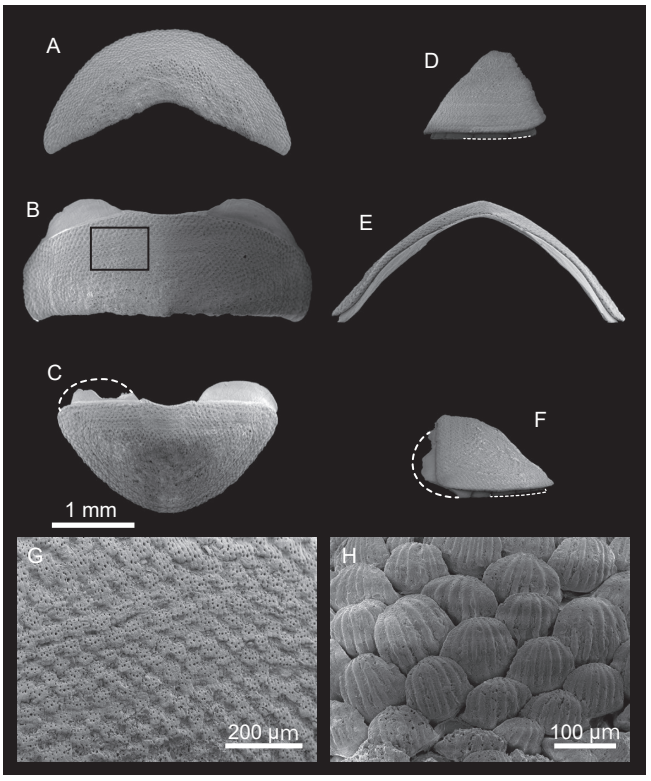


**Fig. 5.** SEM images of *Ischnochiton paululus*. **(A)** head valve; **(B)** intermediate valve (valve VI); **(C)** tail valve; **(D)** lateral view of head valve; **(E)** horizontal view of intermediate valve; **(F)** lateral view of tail valve; **(G)** close up view of Figure 5B; **(H)** girdle scales.

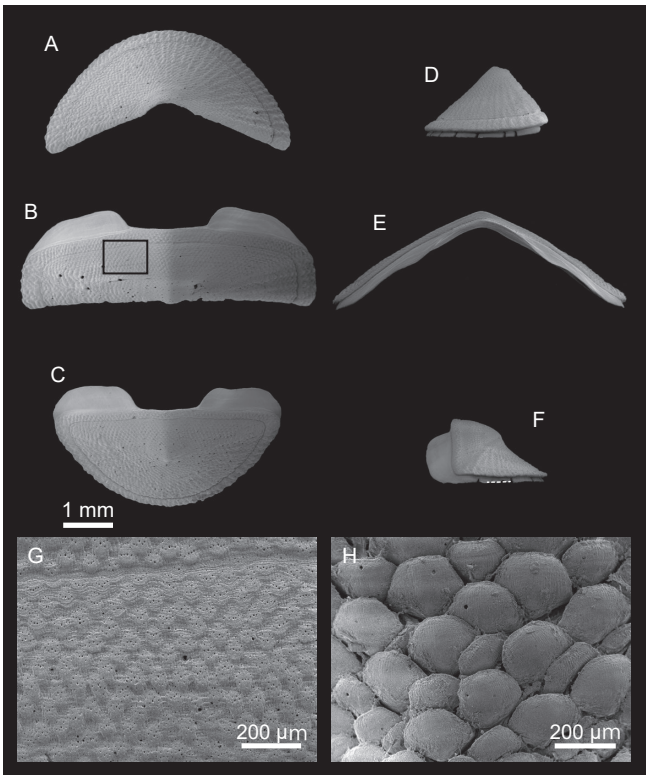


**Fig. 6.** SEM images of *Ischnochiton poppei*. **(A)** head valve; **(B)** intermediate valve (valve VI); **(C)** tail valve; **(D)** lateral view of head valve; **(E)** horizontal view of intermediate valve; **(F)** lateral view of tail valve; **(G)** close up view of 6B; **(H)** girdle scales.

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.

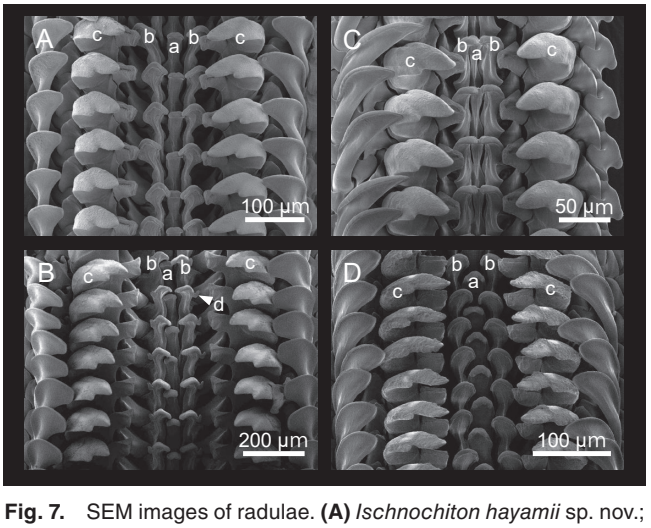


**Fig. 7.** SEM images of radulae. **(A)** *Ischnochiton hayamii* sp. nov.; **(B)** *I. hakodadensis*; **(C)** *I. paululus*; **(D)** *I. poppei*. a–central tooth; b–minor lateral tooth; c–major lateral tooth; d–accessory process.

**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

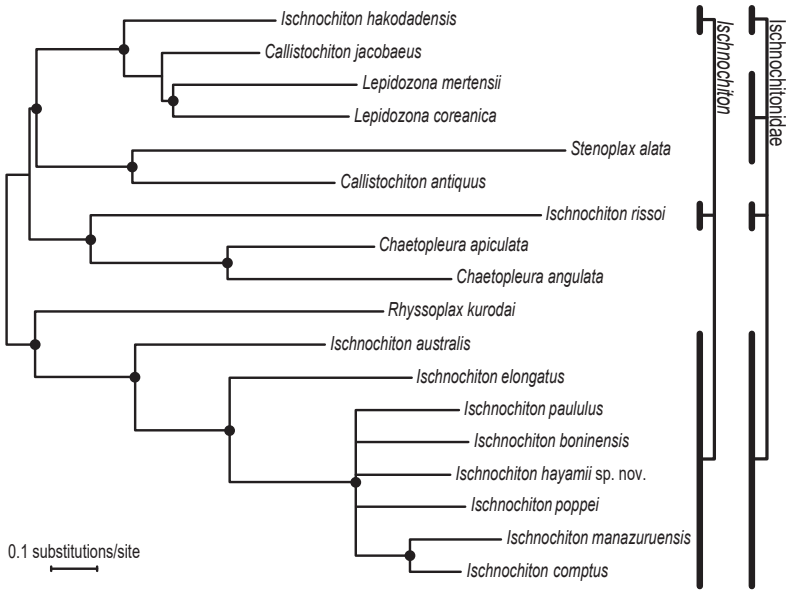


**Fig. 8.** Molecular phylogenetic tree based on the concatenated sequences of COI, 16S, 18S, and 28S gene regions. Solid circles on nodes indicate that bootstrap value ≥ 50 and posterior probability ≥ 0.90 fit in the same time. Branch length was calculated using the ML method.

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.

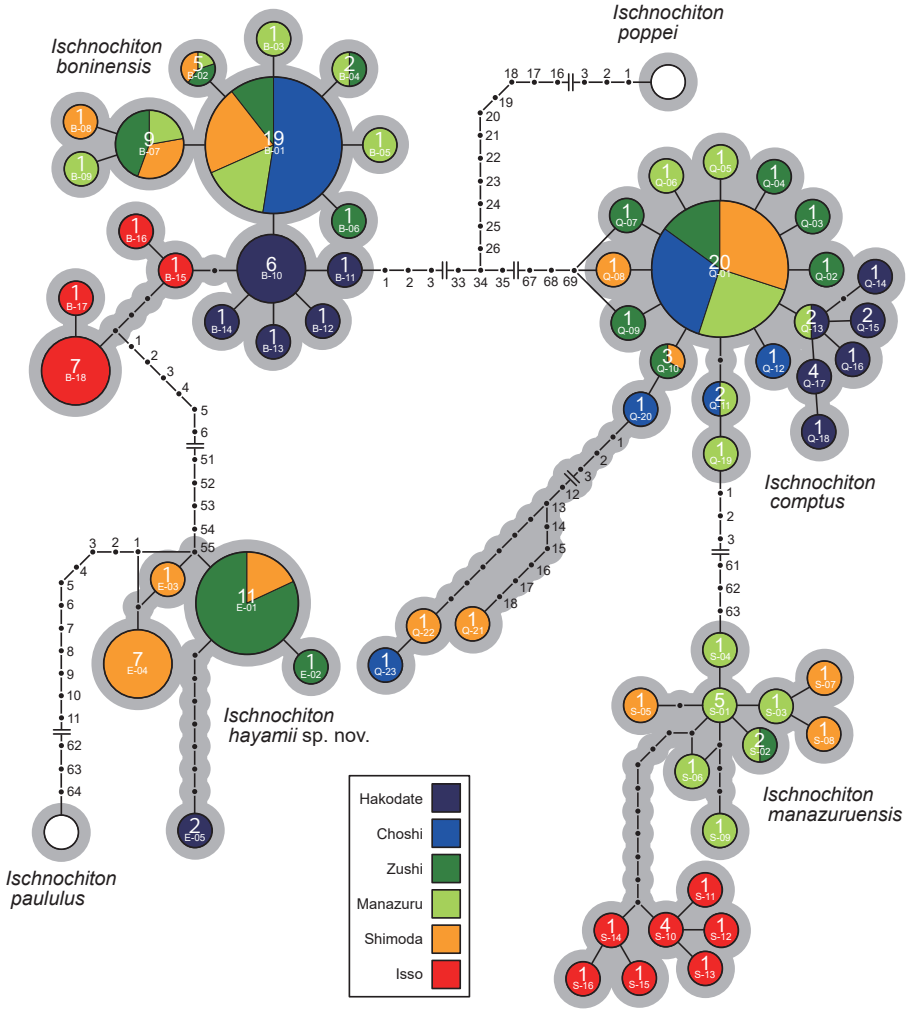


**Fig. 9.** Haplotype network based on the sequences of COI gene region. Numbers and characters in the center of closed circles indicate the number of specimens and haplotypes (Table 3), respectively. Numbers near filled circles indicate the number of DNA substitutions.

**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.

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## COMPETING INTERESTS

The author has no competing interests to declare.

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