Stirring the Deep, Disentangling the Complexity: Report on the Third Species of *Thermochiton* (Mollusca: Polyplacophora) From Haima Cold Seeps

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This study documents a new deep-sea chiton from the Haima cold seeps. Thermochiton xui. nov. is the third species of the genus Thermochiton and the first occurrence of this genus in the South China Sea. This species is identified by its morphological characteristics and the molecular sequence of a Thermochiton species is reported for the first time. The placement of the new species is determined in the phylogenetic tree of Ischnochitonidae by Maximum Likelihood (ML) and Bayesian inference (BI) methods, based on the sequences of the mitochondrial cytochrome c oxidase subunit I (COI), 16S ribosomal DNA (16S), and nuclear 28S ribosomal DNA (28S) gene regions. Bayesian evolutionary analysis with an uncorrelated relaxed clock approach indicated that this new species is estimated to have diverged from its most closely related shallow-water ischnochitonid taxa 5.10–10.07 million years ago in the Late Miocene. A regional ocean general circulation model was used to estimate the potential dispersal ability of the three species of Thermochiton. Because it is highly unlikely for one species to have spread between the northwest and southwest Pacific to the localities in which this genus has been found to date, we propose that 'stepping-stone' habitats and/or 'bridge species' were involved in the dispersal and evolution of these cold-seep endemic chitons.

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INTRODUCTION

The Haima cold-seep field, covering 618 km², was recently discovered on the northwestern slope of the South China Sea. Within this field, an area of 350 km² has had cold seep activity and developed into cold-seep carbonate rocks with crusts and nodules that harbor a distinct biological community (Liang et al., 2017; Zhao et al., 2020). To date, more than 80 species of macrobenthic organisms

have been sampled from the Haima cold seeps, including mostly mollusks, polychaetes, and crustaceans, a few species of echinoderms, and other animals. Since the discovery of the Haima cold seeps, many new molluskan species and the first recorded occurrences of species in the South China Sea have been reported while exploring the benthic communities associated with the cold seeps (Zhang et al., 2016; Zhang and Zhang, 2017; Chen et al., 2018; Sun et al., 2018; Jiang et al., 2019; Xu et al., 2019; Dong et al., 2021). Among them, primarily gastropods and bivalves have been found, with only one reported species of the class Polyplacophora (Dong et al., 2021).

Most polyplacophorans, also called chitons, live in shallow waters, usually in the intertidal zones on rocky substrates. Only a small fraction of chitons have been found in abyssal or bathyal habitats, especially in chemosynthetic environments such as sunken wood, cold seeps, or hydrothermal vents. Until 2010, only 18 chiton species had been found in chemosynthetic areas, of which 16 species were found in cold seeps (Schwabe and Sellanes, 2010). However, new deep-sea species have been discovered with the improvement of exploration technology (Sigwart et al., 2010; Sigwart and Sirenko, 2012; Sirenko and Sellanes, 2016; Sigwart and Chen, 2018; Sirenko, 2020). Saito and Okutani (1990) described a chiton of the order Chitonida from hydrothermal vents in the central Okinawa Trough, East China Sea. They established the genus *Thermochiton* for *Thermochiton undocostatus* Saito & Okutani, 1990 and placed the genus in the family Ischnochitondae. The second member of the genus, *Thermochiton papuaensis*, was reported in deep waters near Papua New Guinea, likely inhabiting communities with chemical activity (Sirenko, 2020). However, both species were described merely by their morphology without any genetic information. Their relationship within the family Ischnochitondae has not been determined by molecular analysis.

Ischnochitonidae is the largest family of Polyplacophora. Kaas and Van Belle (1980) classified the family into 18 genera, comprising approximately 400 species, which contained almost half of the known chitons at the time. Although the taxa were later rearranged (Sirenko, 2006), this large family currently includes 10 genera and approximately 228 species (World Register of Marine Species). Chitons of this family have distinct apophyses, well-developed slits on all valves, insertion plates that are not pectinated, and dorsal surfaces that lack ocelli. Their adanal gills do not extend to the anus. Additionally, all members of the family Ischnochitonidae have a scaly dorsal girdle and varying tegmental sculptures (Kaas and Van Belle, 1987). The inherent paraphyly and lack of well-defined diagnostic morphological features of this family have led to taxonomic controversy. Some molecular studies suggest that the family Ischnochitonidae is not monophyletic (Okusu et al., 2003; Owada, 2018; Irisarri et al., 2020). Even the monophyly of its type genus, *Ischnochiton*, remains doubtful (Owada, 2018; Irisarri et al., 2020). However, most ischnochitonids have only been described by their morphology, and molecular information is scarce. Indeed, the sequences of mitochondrial cytochrome c oxidase subunit I (COI), the most widely used effective DNA barcoding gene marker, have only been published for 24 species of Ischnochitonidae in the NCBI database.

The evolutionary history of geographically separated but closely related species is tangled but fascinating. Chemoautotrophic environments are ideal natural laboratories for studying the spread, connectivity, and evolution. Hydrothermal vents or cold seeps, are essentially isolated islands in the deep sea. As most vent-endemic species are benthic with limited mobility at their adult stage, the larval stage is vital for dispersal and colonization of species obligately living in chemoautotrophic environments (Tyler and Young, 1999; Tyler and Young, 2003). The wide geographic range of distributed species and long-distance dispersal or high connectivity of panmictic populations are achieved *via* long periods of planktonic larval duration (PLD) for swimming larvae (Adams et al., 2012; Fukumori and Kano, 2014; Yahagi et al., 2019). Some vent or seep mollusks, such as *Shinkailepas myojinensis* (Yahagi et al., 2017), *Shinkailepas briandi* (Yahagi et al., 2019), *Bathymodiolus childressi*, and *Thalassonerita naticoidea* (= *Bathynerita naticoidea*) (Arellano et al., 2014), develop long-lived planktotrophic larvae (Warén and Bouchet, 2001; Fukumori and Kano, 2014), which can vertically migrate hundreds of meters to photic and even euphotic zones to hunt for abundant food supplies (Bouchet and Warén, 1994; Arellano et al., 2014). These larvae were considered to float for at least 7–12 months in the pelagic water column before settling, based on the sizes of planktonic and settled larvae, as well as post-settlement juveniles (Arellano et al., 2014; Yahagi et al., 2017). The longer PLD of these species and stronger currents at the sea surface presumably fuel broad-ranged distribution and genetic homogeneity (Bouchet and Warén, 1994; Yahagi et al., 2017). Several crustaceans have developed the same strategy (Teixeira et al., 2011; Beedessee et al., 2013). However, the strategies employed by vent-associated chitons to colonize new territories and expand their distribution have not yet been reported, partly because of their rare occurrence.

During an exploratory cruise of the Haima cold seeps, two specimens of deep-sea chiton were collected and reported as *Leptochiton tenuidontus* Saito & Okutani, 1990 by Dong et al. (2021). This species was misidentified and should be classified as a new species of the genus *Thermochiton*. In this study, we clarify the identification of this new species. Our account is based on analyzing the valve, girdle, and radula morphological characteristics of the chiton, as well as DNA sequencing of the mitochondrial COI, 16S ribosomal DNA (16S), and nuclear 28S ribosomal DNA (28S) gene regions. DNA sequences were used to confirm the arrangement of this *Thermochiton* species within the family Ischnochitondae and to explore its evolutionary relationship with its shallow-water relatives. Further, a numerical model was used to simulate the dispersal range and estimate the potential dispersal ability, thus suggesting the evolutionary pathway of *Thermochiton* species.

MATERIAL AND METHODS

Sample Collection

The samples for this study were obtained during a cruise to the Haima cold seeps in the South China Sea on 19 May 2018 (**Figure 1**). The two individual specimens

were directly preserved in 75% ethanol. The specimens were initially identified at the species level based on their morphology according to identification guidelines (Saito and Okutani, 1990; Saito, 2004; Saito et al., 2008; Sirenko, 2021). The specimens were deposited in the Marine Biological Museum, Chinese Academy of Sciences (MBMCAS), Qingdao, China.

Morphological Observations

The two specimens were identified at the species level under a Zeiss SteREO Discovery.V12 stereo microscope (Zeiss, Wetzlar, Germany). The body length and width were measured, and the number of gills and gill types were observed. Specimens were placed in 7% NaOH and boiled for 10 min, followed by boiling twice in freshwater. Selected valves (head plate, 5th or 2nd plate of the intermediate plates, and tail plate), half of the radula, and a portion of the girdle were glued to the sample stage with conductive glue, gold plated in a vacuum sputter coater, and examined under a Hitachi S-3400N scanning electron microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 5 kV.

Genetic Study

Total DNA was extracted using a marine animal genomic DNA extraction kit (Tiangen Biotechnology, Beijing, China) according to the instructions of the manufacturer. To explore the genetic distance and relationship of the species to common coastal and deep-sea chitons of the family Ischnochitonidae, the mitochondrial COI, 16S rRNA (16S), and nuclear 28S rRNA (28S) gene regions were sequenced and analyzed.

Polymerase chain reaction (PCR) amplification of the mitochondrial COI gene was carried out using primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′). The mitochondrial 16S rRNA gene was amplified using primers 16Sa (5'-CGCCTGTTTATCAAAAACAT-3') and 16Sb (5'-CTCCGGTTTGAACTCAGATCA-3'). The nuclear 28S rRNA gene was amplified using primers 28Sa (5'-GACCCGTCTTGAAACACGGA-3') and 28Sb (5'-TCGGAAGGAACCAGCTAC-3'). PCR amplifications were carried out using a total reaction volume of 25 µl, with 12 µl 2×Es Taq MasterMix (CoWin Biosciences Co., Ltd., Beijing, China), 2 µl of template DNA (50 ng/µl), 1 µl of each primer (10 M), and 9 µl of dH$_2$O. The thermal cycling conditions for PCR amplification were as follows: initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C (30 s), annealing temperature of 50°C for 1 min, extension at 72°C for 1 min, and a final elongation at 72°C for 10 min. Double-stranded PCR products were purified and sequenced by BGI Tech Solutions Co., Ltd. PCR products were detected by an agarose gel.

The 24 species of the family Ischnochitonidae with available COI, 16S rRNA, and 28S rRNA gene sequences were also included in the analysis (**Table 1**). *Callistochiton jacobaeus* and *Callistochiton antiquus* of the family Callistoplacidae, *Rhyssoplax kurodai* of the family Chitonidae, and *Chaetopleura apiculata* and *Chaetopleura angulata* of the

FIGURE 1 | Map of samples sites of species of genus *Thermochiton*. Location of Haima cold seeps was indicated by a pentacle. Type localities of *Thermochiton undocostatus* and *Thermochiton papuaensis* were indicated by square and circle, respectively.
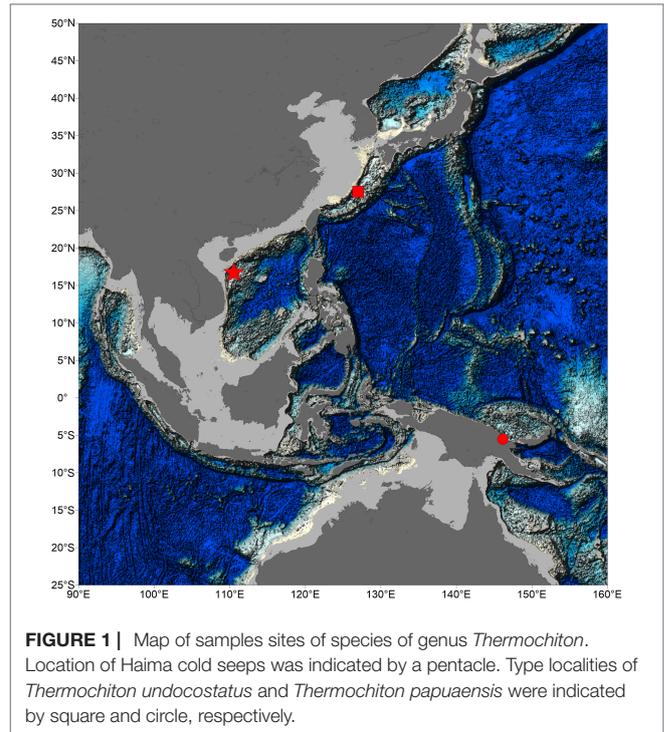
family Chaetopluridae were selected as outgroups based on their current taxonomic relationships.

All sequences were aligned using MAFFT v.7 software (Katoh and Standley, 2013) with the G-INS-I algorithm. Alignments were carried out by eye and BLAST analyses of the NCBI database. The online version of Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012) was used to detect barcode gaps and help support species delineation (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) using four randomly selected COI sequences of the 24 available species of Ischnochitonidae obtained from published studies, excluding outgroups (**Supplementary Table S1**). The analysis settings were as follows: Pmin = 0.001, Pmax = 0.1, Steps = 10, X (relative gap width) = 1.0. Then, the Kimura two-parameter model was used to measure a matrix of pairwise distances (Kimura, 1980). The COI, 16S, and 28S gene sequences from the same individual were concatenated using SequenceMatrix software (Vaidya et al., 2011) to conduct phylogenetic analysis of a combined-gene data set. Best-fitting sequence evolution models were calculated using jModelTest 2.1.10 software (Darriba et al., 2012), deriving the best-fit general time-reversible (GTR)+G model. The maximum likelihood (ML) method was executed using RaxML1.5b1 software (Stamatakis, 2014). The test was executed 1,000 times with the default settings. The support bootstrap scores of nodes were obtained from the best scoring tree after 1,000 bootstrap replications. A Bayesian phylogenetic analysis was carried out using MrBayes v.3.2.6 software (Ronquist et al., 2012). The program was run for 10,000,000 generations, sampling every 1,000 generations.

TABLE 1 | List of species and sequences used in this study for phylogenetic analysis.

Family	Species	COI	16S	28S	Reference
Ischnochitonidae	*Ischnochiton australis*	AY377707	AY377596	AY377670	Okusu et al. (2003)
	Ischnochiton boninensis	LC071647	LC071575	LC071611	Owada (2016)
	Ischnochiton boninensis	AY377709	AY377593	AY377673	Okusu et al. (2003)
	Ischnochiton comptus	LC071627	LC071570	LC071606	Owada (2016)
	Ischnochiton elongatus	AY377708	AY377595	AY377672	Okusu et al. (2003)
	Ischnochiton hakodadensis	LC214409	LC214398	LC214387	Owada (2018)
	Ischnochiton hayamii	LC214410	LC214399	LC214388	Owada (2018)
	Ischnochiton manazuruensis	LC071619	LC071565	LC071601	Owada (2016)
	Ischnochiton paululus	LC214411	LC214400	LC214389	Owada (2018)
	Ischnochiton poppei	LC214412	LC214401	LC214390	Owada (2018)
	Ischnochiton rissoi	AY377706	AY377594	AY377671	Okusu et al. (2003)
	Ischnochiton tridentatus	EF200831			Kelly and Eernisse (2007)
	Ischnochiton tridentatus	EF200834			Kelly and Eernisse (2007)
	Stenoplax alata	LC214413	LC214402	LC214391	Owada (2018)
	Stenoplax alata	AY377711	AY377598	AY377675	Okusu et al. (2003)
	Stenoplax conspicua	EF200968			Kelly and Eernisse (2007)
	Stenoplax mariposa	EF200987			Kelly and Eernisse (2007)
	Stenoplax limaciformis	KC669559			García-Ríos et al. (2014)
	Stenoplax purpurascens	KC669552			García-Ríos et al. (2014)
	Stenoplax floridana	KC669562			García-Ríos et al. (2014)
	Stenosemus albus	KF643687			Layton et al. (2014)
	Lepidozona cooperi	EF200850			Kelly and Eernisse (2007)
	Lepidozona pectinulata	EF200910			Kelly and Eernisse (2007)
	Lepidozona interstincta	EF200871			Kelly and Eernisse (2007)
	Lepidozona radians	EF200957			Kelly and Eernisse (2007)
	Lepidozona coreanica	LC071669	LC071582	LC071618	Owada (2018)
	Lepidozona mertensii	AY377710	AY377597	AY377674	Okusu et al. (2003)
	Thermochiton xui sp. nov.	OM896760	OM892876	OM892875	This study
	Thermochiton xui sp. nov.	OM896759	OM892877	OM892878	This study
Callistoplacidae	*Callistochiton jacobaeus*	LC071667	LC071580	LC071616	Owada (2016)
	Callistochiton antiquus	AY377712	AY377599	AY377676	Okusu et al. (2003)
Chitonidae	*Rhyssoplax kurodai*	LC071668	LC214381	LC071617	Owada (2016)
Chaetopluridae	*Chaetopleura apiculata*	AY377704	AY377590	AY377667	Okusu et al. (2003)
	Chaetopleura angulata	AY377703	AY377591	AY377668	Okusu et al. (2003)
Mopaliidae	*Mopalia cirrata*	EU409065	EU407007		Kelly and Eernisse (2007)
	Mopalia ferreirai	EF159654	EU407015		Kelly and Eernisse (2007)
	Mopalia acuta	EU409064	EU406984		Kelly and Eernisse (2007)

The uncorrelated relaxed clock method was used to estimate the divergence times of the different taxa using BEAST v. 1.10.4 software, based on the Bayesian interference (BI) tree of the concatenated sequences of three genes (Suchard et al., 2018). The earliest fossil record of *Mopalia*, 15–17.2 million years ago (MYA) (Kelly and Eernisse, 2008), was taken as the calibration point. An uncorrelated relaxed lognormal clock was derived for each partition using the GTR substitution model, assuming that the tree prior had a Yule species formation process. This analysis was run for 100 million generations, sampling every 1,000 generations with a 10% burn-in rate. The final result image was rendered using Figtree v1.4.4 software.

Tracer Simulation

The diffusion and advection processes of the biological tracer in the ocean were simulated using a regional ocean general circulation model (OGCM), which was constructed based on the Massachusetts Institute of Technology general circulation model (MITgcm) (Marshall et al., 1997). Our model domain ranged from 25°S to 45°N and from 110°E to 174°E, with a horizontal resolution of 1/10° × 1/10° and 82 z-levels in the vertical. Vertical mixing was represented using the K-Profile Paremeterization (KPP) scheme (Large et al., 1994), and background vertical viscosity and diffusivity were set as 10^{-4} and 10^{-5} m^2 s^{-1}, respectively. The potentially unresolved eddy processes were represented by the parameterizations described by Gent and McWilliams (1990) and Redi (1982), with both layer thickness and isopycnal diffusivity set at 500 m^2 s^{-1}. The initial temperature and salinity of the field were obtained from the climatological monthly mean data for January, which were derived from the Generalized Digital Environmental Model, version 3 (GDEMv3; http://www.

usgodae.org/pub/outgoing/static/ocn/gdem/) dataset. The climatological monthly mean wind stress was calculated from the cross-calibrated multiplatform (CCMP) datasets (Atlas et al., 2011), with a relaxation of the sea surface temperature and salinity to the monthly mean GDEMv3 climatology.

The model was started from an initial state of rest and ran for 100 years. The simulated total kinetic energy of the upper ocean layer reached a quasi-equilibrium state after a 100-year run. Subsequently, the simulated monthly mean temperature, salinity, and currents of the last month were used as the initial states of the following tracer experiments. The horizontal and vertical diffusivities were the same as those in the circulation model. The three localities of *Thermochiton* species were selected as the origins of the model: (1) the central Okinawa Trough, the type locality of *T. undocostatus* Saito et Okutani, 1990 (Saito and Okutani, 1990); (2) Papua New Guinea, the type locality of *T. papuaensis* Sirenko, 2020 (Sirenko, 2020); and (3) the Haima cold seeps in the South China Sea, where our specimens were collected. A tracer experiment was carried out for each origin. At the corresponding model grid of each origin, 100 million floats/m³ were released at the first model level of 2.5 m water depth. All three experiments ran for three years, with the monthly-mean tracer concentration fields used for analysis.

RESULTS
Systematics
Class Polyplacophora Gray, 1821
    Order Chitonida Thiele, 1909
      Family Ischnochitonidae Dall, 1889
        Genus *Thermochiton* Saito et Okutani, 1990
Thermochiton xui Wang, Zhang & Sirenko sp. nov.
 (**Figures 2-5**)
rn:lsid:zoobank.org:act:0C75D2E3-F30E-4970-9BC2-3363B397720C
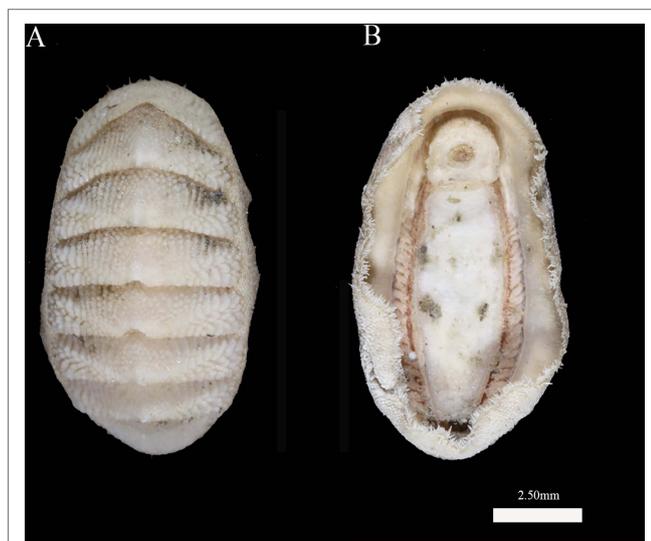
FIGURE 2 | *Thermochiton xui* sp. nov., holotype: MBM229030. **(A)** Dorsal view; **(B)** Ventral view.
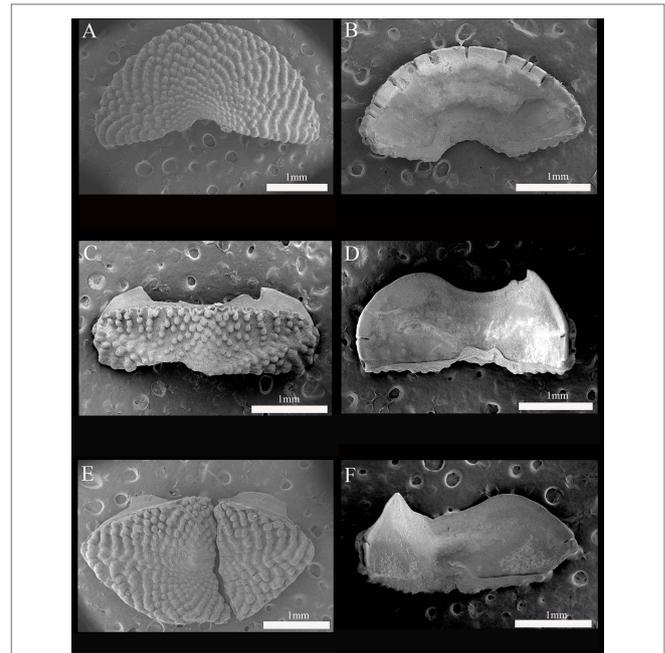
FIGURE 3 | *Thermochiton xui* sp. nov. holotype: MBM229030. **(A)** Head valve, dorsal view; **(B)** Head valve, ventral view; **(C)** Valve III, dorsal view; **(D)** Valve III, ventral view; **(E)** Tail valve, dorsal view; and **(F)** Valve IV, ventral view.

Type Material
Holotype: MBM229030, BL 9.0 mm. Paratype: MBM229031, BL 8.5 mm. All collected from type locality, May 19, 2018, and deposited in the Marine Biological Museum, Chinese Academy of Sciences (MBMCAS), Qingdao.

Type Locality
Haima cold seeps, South China Sea, off southern Hainan Island.

Distribution and Habitat
Only known from the type locality, Haima cold seeps, South China Sea, chemosynthetic habitat at a depth of 1,380–1,390 m.

Etymology
This species is named after Prof. Fengshan Xu for his contributions to Chinese malacology on Polyplacophora. He is the first Chinese author who has published the taxonomy of chitons and has described one genus, *Sinolorica* (= *Loricella* Pilsbry, 1893) and two species of chiton, *Sinolorica scissurata* (= *Loricella scissurata*) and *Deshayesiella sinica* (= *Hanleya sinica*) from the East China Sea (Xu, 1990).

Diagnosis
Animal small, oval to elongated oval in shape, dorsal elevation rather high, and subcarinated. The valves rather elevated, not beaked. Tegmentum white in color. Slit formula 11/1(2)/12. Girdle moderately wide and dorsally covered with dense, imbricating, obliquely set, squarish, curved, and wide scales with large round granules at the distal end and sculpted into approximately 18 longitudinal rows of smaller granules on the

FIGURE 4 | *Thermochiton xui* sp. nov. paratype: MBM229031. **(A, B)** Portion of radula; **(C, D)** Dorsal scales; **(E)** Dorsal scales and marginal spicules; and **(F)** ventral scales.

of intermediate valves covered with randomly arranged, large, and drop-shaped granules. Lateral area covered with elongated granules formed undulating costae. Numerous pores of aesthetes evenly covering both the costae and the interstices between them.

Articulamentum very thin, transparent, and glassy white; apophyses short, very wide, and rounded, coalescing across the small, shallow sinus; slit formula of insertion plates 11/1(2)/12; slit rays barely visible; and the solid teeth short, blunt, and the eaves are narrow. Four of the six intermediate valves with 2 slits on one side, whereas the other sides and other valves with only one slit per side. Ventral tegmental callus well developed.

Girdle rather wide, colored like the tegmentum, and dorsally covered with imbricating obliquely set, curved, wide

dorsal side of the scales. Two types of marginal spicules long, smooth, needle-like spicules and short, smooth, blunt-topped spicules. Central tooth of the radula elongated with a wide base. Centro-lateral tooth rough, wedge-shaped, attenuated posteriorly, taller than the central tooth, with a subtriangular blade at the antero-dorsal edge. The top of the lateral central tooth narrow with a sharp, oblique, inward tip. The head of the major lateral tooth of the radula is unicuspidal, bent, sickle-shaped, with an extended base ending with a long, sharp-pointed process directed inside.

Description

Animal elliptical in shape, with a body length of 9.0 mm. Valves rather elevated valves, not beaked, and the side slopes weakly convex. Color of the valves and girdle is white with friable, rusty brown deposits.

Head valve is semicircular, with a straight front slope, and the posterior margin is widely V-shaped and weakly notched in the middle. Intermediate valves rather short compared to their width, rectangular, with the front margin slightly convex, side margins rounded, the front margin slightly angular, the hind margin straight, with no beaks, and lateral areas strongly raised. Tail valve less than semicircular, front margin more or less angular, less wide than the head valve, the length is about half the width, the antemucronal and postmucronal slopes slightly convex, and the mucro central and not swollen.

All the valves in the jugal area delicately sculptured by randomly arranged, oval, flattened granules. Pleural area

FIGURE 5 | *Thermochiton xui* sp. nov. **(A)** Smooth marginal needle; **(B)** Short blunt-topped marginal spicules; **(C)** Ventral scales; **(D)** Head of major lateral tooth of radula. and **(E)** Central and first lateral teeth of radula.

scales, with large round granules at the distal end and about 18 longitudinal rows of smaller granules on the dorsal side of the scales. Scales near valves straight, but in other places angled in different directions. Some scales with 1–4 short longitudinal ribs near the side margin. Marginal spicules of two types: long, smooth spicules-needles and short, smooth blunt-topped spicules. Ventral side of the girdle is paved with smooth, elongate scales.

Radula 4.1 mm long with 110 transverse rows of mature teeth. Central tooth rectangular, oblong, parallel-sided, keeled medially, with a small blade and a large base. First lateral tooth is roughly wedge-shaped, attenuated posteriorly, taller than the central tooth, with a subtriangular blade at the antero-dorsal edge. Major lateral tooth is thin, long, with a shaft broadened near the base. Head of the major lateral tooth is unicuspid, bent, claw-shaped, with an extended base ending with a long, sharp-pointed process directed inside. Inner small laterals with two spatula-shaped processes and prow-shaped outer small laterals.

A total of 22 gills on each side, extending from valve II to valve VII. The gut containing rusty brown particles of detritus.

Remarks

The new species is the third species within the genus *Thermochiton* and has a very similar morphology to *T. undocostatus* and *T. papuaensis*. The new species differs from *T. undocostatus* by the tegmentum of intermediate valves being covered by granules (vs. undulating costae in *T. undocostatus*) and having two types of marginal spicules: long, smooth spicules-needles and short, smooth blunt-topped spicules (vs. only long, smooth spicules in *T. undocostatus*). *Thermochiton xui* sp. nov. differs from *T. papuaensis* by having granules in

the whole tegmentum of intermediate valves (vs. granules only in the jugal area and other areas with undulating costae in *T. papuaensis*); having two types of marginal spicules (vs. three types in *T. papuaensis*: long, smooth spicules and two kinds of ribbed spicules); and the first lateral tooth of the radula being approximately twice as long as the central tooth (vs. the first lateral tooth is approximately the same length as the central tooth in *T. papuaensis*).

Genetic Analysis

The ABGD analysis was performed on the COI gene sequences to determine whether the two specimens of *T. xui* sp. nov. were the same species and to conducted sequence-based species delimitation of the members of the family Ischnochitonidae (**Figure 6**). The ABGD analysis divided the 25 candidate species of chitons into reciprocal clades on the phylogenetic trees. The two specimens collected from the Haima cold seeps belonged to one species, *T. xui* sp. nov., which was consistent with the morphological identification. Additionally, both *Stenoplax alata* and *Stenoplax purpurascens* were respectively divided into two separate species. ML and BI analyses of the concatenated three-gene dataset facilitated the reconstruction of the phylogenetic tree, revealing similar topologies (**Figure 7**). The concatenated tree indicated that the new species was a sister group to most *Ischnochiton* taxa which is one of the four clades of the genus *Ischnochiton*. The species *Ischnochiton rissoi*, *Ischnochiton tridentatus*, and *Ischnochiton hakodadensis* were excluded from this main clade and formed three separate and distant clades. Likewise, the concatenated tree provided consistent support for the non-monophyly of the family Ischnochitonidae. The *Lepidozona* taxa formed a group but also contained *Callistochiton jacobaeus* and *Ischnochiton hakodadensis*. Furthermore, the outgroups were nested within the family Ischnochitonidae.

The evolutionary tree produced using BEAST (**Figure 8**) shows the estimated divergence time for Ischnochitonidae species. According to the analysis of the log files using Tracer 1.7 software, all searches reached convergence, and all effective sample size (ESS) values were greater than 200. The calibrated estimate showed that *T. xui* sp. nov. was separated from most members of the genus *Ischnochiton* in the Late Miocene, roughly 7.31 MYA, with a 95% highest posterior density (HPD) interval of 5.10–10.07 million years. Radiation of the family Ischnochitonidae occurred mainly from the Late Miocene to Pliocene.

Numerical Modeling

The numerical model in this study was designed to explore the evolutionary pathway of *Thermochiton* species and demonstrate how larvae in the three localities were connected by ocean currents. *Thermochiton* larvae were assumed to be passive floating particles in the OGCM to stimulate the spread process during PLD (**Figure 9** and **Supplementary Figures S1–S3**). Even ignoring their time swimming from the bottom to the sea surface and assuming that larvae use the optimal
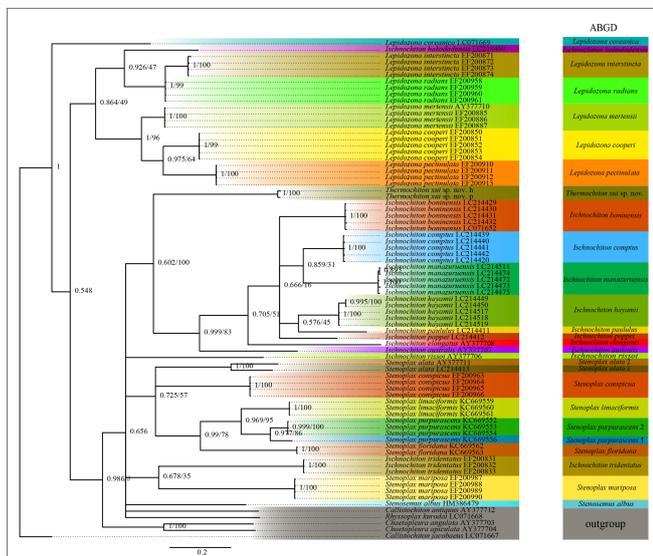
FIGURE 6 | Phylogeny tree of the chitons based on the COI dataset. Numbers above branches refer to BI posterior probability (PP) and ML bootstrap scores (BS). The ABGD based on the COI alignment with the model Kimura (K80) TS/TV (2.0).
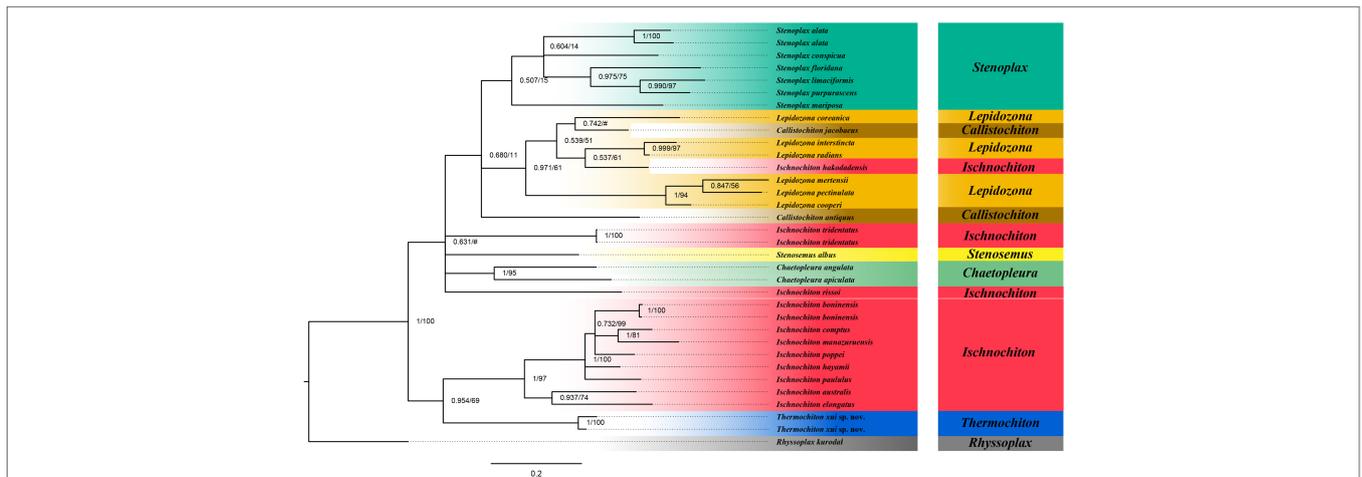
FIGURE 7 | Phylogeny tree based on three genes concatenated sequences. Numbers above branches refer to BI posterior probability (PP) and ML bootstrap scores (BS).

way to spread, i.e., being carried by surface currents, larvae from Papua New Guinea needed at least 15 and 26 months to spread to the Okinawa Trough and Haima cold seeps, respectively (**Supplementary Figure S1**). Meanwhile, larvae from the Haima cold seeps required eight months to spread to the Okinawa Trough (**Supplementary Figure S3**). Notably, the model started in January when northeast (winter) monsoons dominated the South China Sea. Thus, the simulated northeastward surface current would be relatively slower during the first few months than that simulated by a model starting when southwest (summer) monsoons dominated

the area. Nevertheless, the simulation ran for 36 months and did not reveal the possibility of the tracer diffusing from the Haima cold seeps or Okinawa Trough to Papua New Guinea (**Supplementary Figures S3**, **S2**).

Although development information about deep-sea chitons is lacking, larvae of shallow-water chitons are mostly lecithotrophic. As a result, deep-sea chitons are more likely spread to the seafloor or deep ocean layer than to float to the surface. Further, the current velocity at the bottom or in the deep ocean layer is much slower than that at the surface, suggesting that deep-sea chitons would need much longer to spread than those floating at the surface.
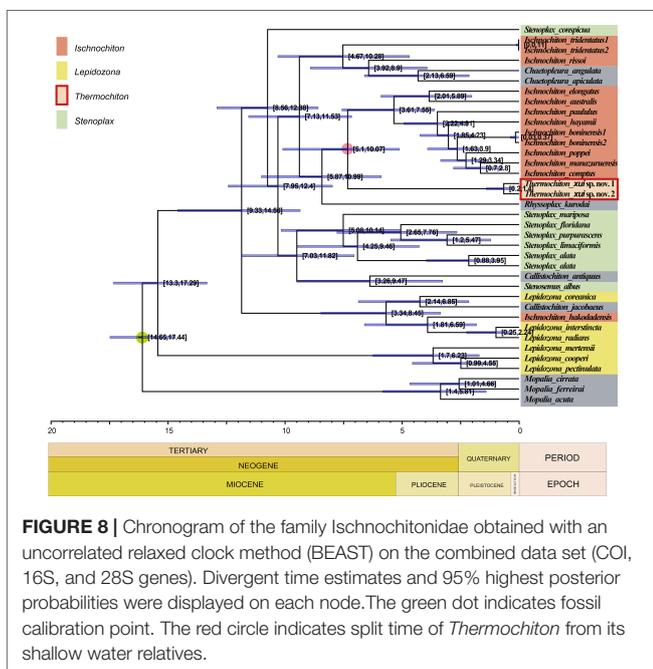
FIGURE 8 | Chronogram of the family Ischnochitonidae obtained with an uncorrelated relaxed clock method (BEAST) on the combined data set (COI, 16S, and 28S genes). Divergent time estimates and 95% highest posterior probabilities were displayed on each node. The green dot indicates fossil calibration point. The red circle indicates split time of *Thermochiton* from its shallow water relatives.

DISCUSSION

The discovery of *T. xui* sp. nov. increased the number of known members of the genus *Thermochiton*. Currently, the three species of the genus likely all live in areas of chemosynthetic activity. The genus *Thermochiton* was established for *T. undocostatus* Saito & Okutani, 1990, a chiton found in hydrothermal vents at the central Okinawa Trough, East China Sea (Saito and Okutani, 1990). The second member of the genus, *T. papuaensis*, was reported near Papua New Guinea and is likely to inhabit communities with chemical activity (Sirenko, 2020). Our new species was sampled from the Haima cold seeps in the South China Sea. The distribution areas of the genus are geographically far removed, located in the southwest and northwest Pacific. The type locality of *T. papuaensis* is approximately 4,637 km away from this sampling site and approximately 4,217 km away from the type locality of *T. undocostatus* (**Figure 1**).

The new species is the only species with molecular information in the genus. It is impossible to analyze the phylogenetic relationship between the three *Thermochiton*
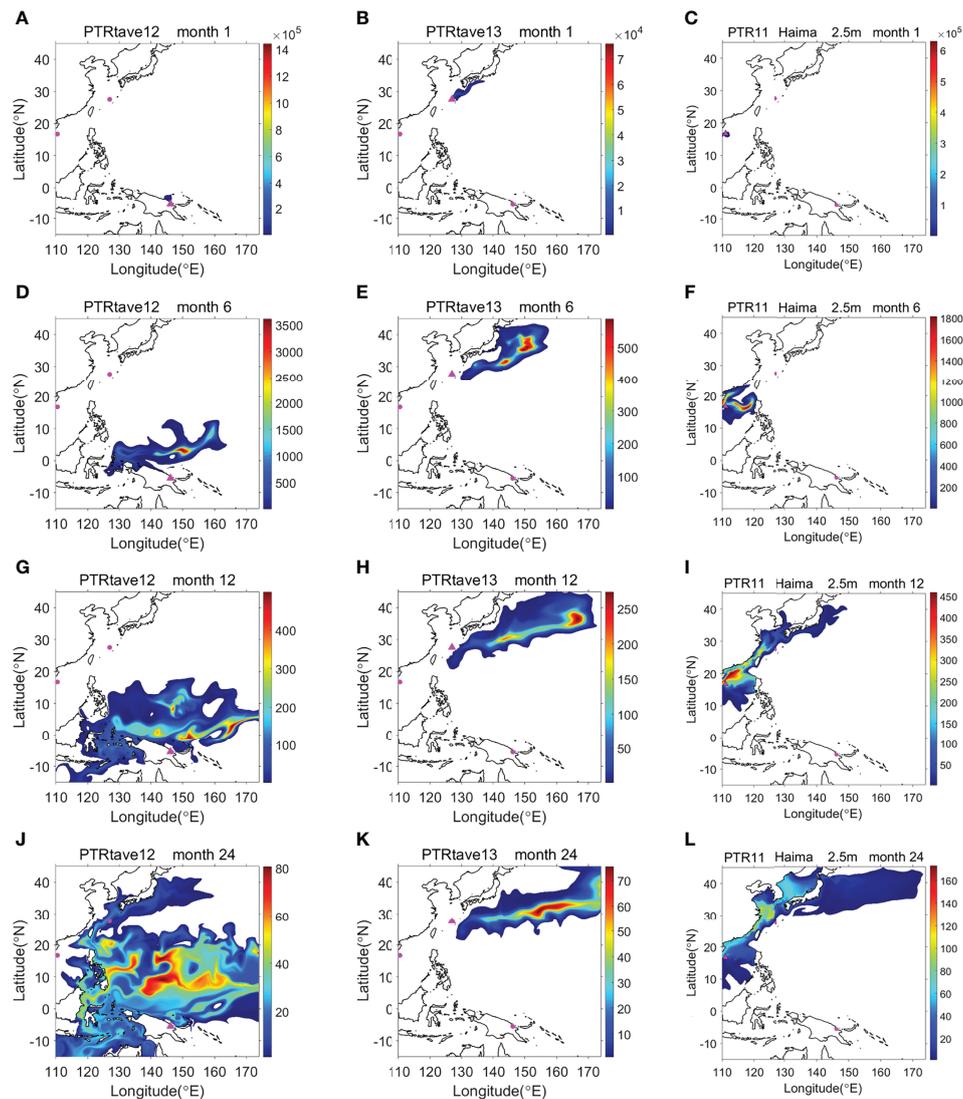
FIGURE 9 | Dispersal path and relative content of chiton larvae released from Papua New Guinea **(A, D, G, J)**, Okinawa Trough **(B, E, H, K)**, and Haima cold seeps **(C, F, I, L)** as modeled by the OGCM for 1 month **(A–C)**, 6 months **(D–F)**, 12 months **(G–I)** and 24 months **(J–L).**

species or infer how their ancestors spread based merely on morphological or molecular information. The modeling results in this study indicated that one *Thermochiton* species is unlikely to have crossed the complex Pacific western boundary currents to reach the three localities in which the genus has been reported. The western Pacific vents in distant basins are potentially connected by the Kuroshio Current (Mitarai et al., 2016). In addition, the South Equatorial Current can distribute vent species more than 4,000 km within the southwest Pacific. Nevertheless, the results of our numerical model suggested that at least 15 months were required for larvae to spread from Papua New Guinea to the Okinawa Trough *via* surface currents. Northward evolutionary migration is highly more likely than southward migration for Pacific deep-sea chitons. The upper prediction of the model reported by Yearsley and

Sigwart (2011) estimated no more than 240 days (i.e., 8 months) for the PLD of deep-sea chitons. The deep-sea mollusk with the longest PLD is the cold seep mussel *B. naticoidea*, whose larvae reportedly disperse for 7–12 months at the surface before settling (Arellano et al., 2014). The larvae of the seastar *Mediaster aequalis* can reportedly persist for approximately 13 months before settling (Birkeland et al., 1971). Nevertheless, it was estimated that even the longest PLD could not be expected to exceed 400 days (O'Connor et al., 2007). Thus, one *Thermochiton* species is unlikely to have a sufficiently long PLD and dispersal ability to cross the complex Pacific western boundary currents, even under ideal conditions and with extremely strong surface currents. It is more likely that currents carried the pelagic larvae of their ancestors to spread and radiate into different species along the evolutionary

pathway during colonization. We hypothesize that (1) 'stepping-stones' facilitated their colonization, i.e., suitable habitats for chitons between the southwest and northwest Pacific, such as seeps, vents, sunken wood, or carcasses, and/or (2) evolutionary 'bridge species' existed during their spread, i.e., chitons distributed at 'stepping-stone' sites that have not yet been found or became extinct with the disappearance of these ephemeral habitats. Therefore, additional expeditions are needed to further explore the deep-sea fauna and collect more data in order to reconstruct the evolutionary history of this genus.

Most deep-water chitons belong to the order Lepidopleurida, a basal clade of the class Polyplacophora. Sigwart (2017) suggested that this clade originated at a depth of approximately 500 m and radiated to either deep-sea or coastal areas. Most of the radiation to sunken wood occurred in the Jurassic. Many lepidopleuran chitons diversified during the Cenozoic and invaded the deep sea and different chemosynthetic

TABLE 2 | Summary of deep-sea chitons described from the East and South China Seas.

Taxon	Locality	Depth (m)	HV	CS	SW	NC	References
Leptochitonidae Dall, 1889							
Leptochiton tenuidontus Saito & Okutani, 1990	Iheya Ridge and off Kikaijima Island, Nansei Islands, East China Sea	1,395–1,442	√	√			Saito and Okutani (1990); Saito et al. (2008)
Leptochiton consimilis Sigwart & Sirenko, 2012	Solomon Islands, Taiwan	912–2,620			√		Sigwart and Sirenko (2012); Sirenko (2018)
Leptochiton habei Saito, 1997	Japan; Vanuatu, Solomon Islands, Taiwan	170–1,314			√		Saito (1997); Sirenko (2001); Sigwart and Sirenko (2012); Sirenko (2018)
Leptochiton longisetosus Sigwart & Sirenko, 2012	Vanuatu, Solomon Islands, Taiwan	492–1,317			√		Sigwart and Sirenko (2012); Sirenko (2018)
Leptochiton taiwanensis Sirenko, 2018	Taiwan	264				√	Sirenko (2018)
Leptochiton torishimensis (Wu & Okutani, 1984)	Izu Islands, Suruga Bay, Tosa Bay, Amami-Oshima Island, Taiwan	170–1,314			√		Wu and Okutani (1984); Saito (1997; 2001a; 2005); Sirenko (2018)
Leptochiton wui Sirenko, 2018	Taiwan, off Tashi	221			√		Sirenko (2018)
Leptochiton sp.	Taiwan, Bashi channel	1,314–1,317			√		Sirenko (2018)
Leptochiton vietnamensis Sirenko, 1998	South China Sea, Andaman Sea, New Caledonia, Philippines, Solomon Islands	651–1,313			√		Sirenko (1998); Sirenko (2001); Schwabe (2006); Sigwart and Sirenko (2012)
Hanleyella japonica Saito, 1997	Japan, Taiwan	85–440				√	Saito (1997; 2011); Sirenko (2018)
Hanleyella henrici Sigwart & Chen, 2018	Okinawa Trough	950–1,178	√				Sigwart and Chen (2018)
Parachiton laevisquamatus Sirenko, 2015	South China Sea	10–300				√	Sirenko (2015)
Nierstraszellidae Sirenko, 1992							
Nierstraszella lineata (Nierstrasz, 1905)	Japan, Taiwan, Philippines, Indonesia, Vanuatu, Solomon Islands	121–1,767			√		Sigwart and Sirenko (2012); Sirenko (2018)
Abyssochitonidae Dell'Angelo & Palazzi, 1989							
Ferreiraella soyomaruae (Wu & Okutani, 1984)	Near south of Japan, Taiwan	1,317–3,100				√	Wu and Okutani (1984); Kaas and Van Belle (1987); Sirenko (1988); Sirenko (2018)
Ferreiraella xylophaga karenae Sirenko, 2001	Near Vanuatu, Solomon Islands, off Taiwan	475–1,314			√		Sirenko (2001); Sigwart and Sirenko (2012); Sirenko (2018)
Protochitonidae Ashby, 1925							
Deshayesiella sinica (Xu, 1990)	Okinawa Trough, East China Sea; off Erimo-misaki, Hokkaido	1,680–2,043				√	Xu (1990); Saito (2012)
Loricidae Iredale & Hull, 1923							
Loricella scissurata (Xu, 1990)	East China Sea, Sagami Bay, Omurodashi Bank, Taiwan, South China Sea, Solomon Islands	187–563				√	Xu (1990); Saito (2005; 2011); Sirenko (2018; 2021)
Ischnochitonidae Dall, 1889							
Thermochiton undocostatus Saito & Okutani, 1990	Okinawa Trough, East China Sea	686–1,497	√	√			Saito and Okutani (1990); Saito et al. (2008)
Thermochiton xui sp. nov.	Haima cold seeps, South China Sea			√			This study
Lepidozona acostata Sirenko, 2016	South China Sea, near Vietnam; East China Sea, near Taiwan	170–760				√	Sirenko (2016a; 2018)
Tripoplax alba (Saito, 2013)	Okinawa Trough, East China Sea	605–611	√				Saito (2013)
Mopaliidae Dall, 1889							
Placiphorella isaotakii Saito, Fujikura & Tsuchida, 2008	Kuroshima Knoll off Yaeyama Islands area	691–692		√			Saito et al. (2008)
Acanthochitonidae Pilsbry, 1893							
Notoplax hilgendorfi Thiele, 1909	Japan, from Hakodate to Amami-Oshima Island, Taiwan	5–450				√	Saito (2001a; 2005); Sirenko (2018)

CS, cold seeps; HV, hot vents: SW, sunken wood; NC, non-chemosynthetic habitat.

habitats. Many other mollusks in hydrothermal vents, seeps, and organic falls have the pattern of Cenozoic radiation in the deep sea (Johnson et al., 2010; Lorion et al., 2013; Taylor et al., 2014). Indeed, our molecular clock analysis revealed that the genus *Thermochiton* originated from a shallow-sea ancestor around 5.10 to 10.07 MYA in the Late Miocene. The genus follows the "onshore to offshore" evolutionary pattern (Jablonski et al., 1983; Vrijenhoek, 2013), but occurred independently of the invasion of lepidopleuran chitons into chemosynthetic habitats.

A total of 23 species of deep-sea chitons have been identified in the bathyal and abyssal zones of the East and South China Seas to date (Kaas, 1979; Saito and Okutani, 1990; Xu, 1990; Saito, 2005; Saito, 2011; Saito, 2013; Sirenko, 2016b; Sigwart and Chen, 2018; Sirenko, 2018). Among them, 15 species live in hydrothermal vents, cold seeps, or sunken wood habitats (**Table 2**). Chemosynthetic areas are the main habitats of deep-sea chitons. Sirenko (2018) discovered 16 chiton species off Taiwan, 13 of which were new occurrences and two were new species, based on two exploration missions in 2000 and 2001. These data significantly increased the deep-sea chiton biodiversity in the East and South China Seas. To date, only four species of deep-sea chitons have been found in the South China Sea (*Leptochiton vietnamensis* Sirenko, 1998; *Parachiton laevisquamatus* Sirenko, 2015; *Loricella. scissurata* (Xu, 1990); *Lepidozona acostata* Sirenko, 2016; and *Thermochiton xui* sp. nov.). Sirenko and Zhang (2019) reported 30 chiton species from the vicinity of Hainan Island. However, the deep sea and numerous islands of the South China Sea have not been adequately sampled. The number of chitons currently recorded in the deep and shallow waters of the South China Sea is far from exhaustive compared with the nearly 100 species of chiton recorded in Japan (Slieker, 2000; Saito, 2001b; Saito, 2017) and the 68 species of chiton found in Vietnam (Sirenko, 2016b). The abundance of chiton species found in other locations suggests that the South China Sea has a high degree of biodiversity waiting to be discovered.

Our phylogenetic analysis based on the concatenated sequences from three gene regions supported the non-monophyly of the family Ischnochitonidae, as well as that of the genera *Ischnochiton*, *Lepidozona*, and *Callistochiton* (**Figure 7**). The outgroups were nested within the clade of the family Ischnochitonidae, similar to the findings reported by Owada (2018). The results suggest that the molecular data are not consistent with the assignment of species to genera and families based on plesiomorphic similarities. Both morphologic and genetic data indicated the complexity of the family Ischnochitonidae. Members of the genus *Connexochiton* Kaas, 1979 resemble those of the genus *Thermochiton*. Members of both genera have rounded granules at the distal end of the dorsal girdle scales, and the heads of the major lateral teeth of the radulae are unicuspidal, bent, and sickle-shaped. However, members of the genus *Thermochiton* have pores of aesthetes distributed throughout the tegmentum with concentric, undulating costae, whereas the pores of aesthetes of members of the genus *Connexochiton* are only distributed in granules. The genus *Subterenochiton* Iredale & Hull, 1924

is similar to members of the genus *Thermochiton* but differs by the presence of flattened, closely set, and same-sized tegmentum granules, as well as a single slit per side in the intermediate valves. The genus *Thermochiton* can be clearly distinguished from most *Ischnochiton* species by the following characteristics: small body size vs. medium to large body size; tegmentum granules raised vs. flat granules; intermediate valves with 2 slits per side vs. 1 slit per side; dorsal scales with granules vs. no granules; head of the major lateral tooth of the radula is unicuspidal vs. bicuspidal. Meanwhile, *Ischnochiton luteoroseus* Suter, 1907, *Ischnochiton granulifer* Thiele, 1909, and *Ischnochiton albinus* Thiele, 1911 also share similarities with members of the genus *Thermochiton*, including rounded granules on the dorsal girdle scales, unicuspidal head of the major lateral tooth of the radula, and a distinct protuberance on the major lateral tooth head. Interestingly, several species of the genus *Lepidozona*, *Ischnochiton*, *Subterenochiton*, and *Callistochiton* have dorsal scales with granules and/or longitudinal ribs, which is similar to members of the genus *Thermochiton*. Thus, these taxa need to be re-examined and more taxon sampling is needed. The current classification system of chitons was established based mainly on the morphology of valves, spicules, and girdle processes. Few taxonomic studies have combined morphology with molecular biology (Sigwart et al., 2010; Irisarri et al., 2020). However, as additional molecular data become available, classification uncertainty will undoubtedly arise. Emerging new data will provide insights into phylogenetic relationships and improve the stepwise systematics approach. For example, the previous morphological classification placed *Ischnochiton. hakodadensis* in the genus *Ischnochiton*, but molecular analysis revealed that the species was nested within the genus *Lepidozona* [(present study and findings reported by Owada (2018)]. The current information is insufficient to suggest any rearrangement of the family Ischnochitonidae; thus, this family remains poorly defined. Integrative taxonomy incorporating molecular data and morphological evidence will help solve this problem (Zhang et al., 2020). More evidence is needed to clarify phylogenetic relationships and improve stepwise systematics of chitons.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

JZ conceived and designed this project. HW, JZ, and BS performed morphological examination and description. HL and JZ conducted molecular analyses. XW and CL preformed the numerical modeling. DD and XL collected the samples. All

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