



## Duplicated paralog of *sulfide: quinone oxidoreductase* contributes to the adaptation to hydrogen sulfide-rich environment in the hydrothermal vent crab, *Xenograpsus testudinatus*

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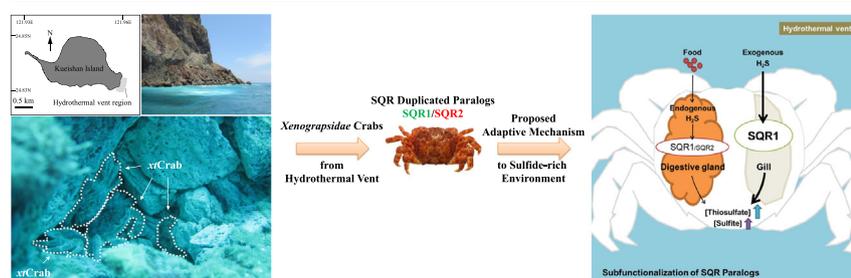
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### HIGHLIGHTS

- Hydrothermal vent *Xenograpsus testudinatus* (*xtcrab*) shows strong tolerance to high-hydrogen sulfide environment.
- *xtcrabs* are able to catalyze more toxic sulfide into less toxic thiosulfate.
- Duplicated paralogs of *Sulfide: quinone oxidoreductase* (*SQR*) were found in *xtcrab*.
- *SQR1* paralog expressed in the gills was specifically upregulated in sulfide-rich environment.

### GRAPHICAL ABSTRACT



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### ABSTRACT

The hydrothermal crab, *Xenograpsus testudinatus* (*xtcrab*) inhabits shallow-water, hydrogen sulfide ( $H_2S$ )-rich hydrothermal vent regions. Until now, the adaptative strategy of *xtcrab* to this toxic environment was unknown. Herein, we investigated the sulfide tolerance and detoxification mechanisms of *xtcrabs* collected in their high-sulfide hydrothermal vent habitat. Experimental immersion of *xtcrab* in various sulphide concentrations in the field or in aquaria assessed its high sulfide tolerance. HPLC measurement of hemolymph sulfur compounds highlighted *xtcrab* detoxification capacity *via* catabolism of sulfide into much less toxic thiosulfate. We focused on a key enzyme for  $H_2S$  detoxification, sulfide: quinone oxidoreductase (*SQR*). Cloning and phylogenetic analysis revealed two *SQR* paralogs in *xtcrab*, that we named *xtSQR1* and *xtSQR2*. As shown by qPCR, *xtSQR2* and *xtSQR1* were expressed in the digestive gland, suggesting the involvement of both paralogs in the detoxification of food-related  $H_2S$ . In contrast, *xtSQR1* transcript was highly expressed in the gill, while *xtSQR2* was not detectable, suggesting a specific role of *SQR1* in gill detoxification of  $H_2S$  of environmental origin. Comparison between *xtcrabs* in their hydrogen sulfide-rich hydrothermal habitat, and *xtcrabs* maintained for one month in sulfide-free seawater aquarium, showed higher transcript levels of gill *xtSQR1* in sulfide-rich habitat, further supporting the specific role of *SQR1* in environmental  $H_2S$  detoxification in the gill. Gill *SQR* protein level as measured by Western blot, and gill *SQR* enzyme activity were also higher in sulfide-rich habitat. Immunohistochemical staining showed that *SQR* expression was co-localized with  $Na^+ / K^+ -ATPase$ -positive epithelial and pillar cells of the gill filament. This is the first evidence of duplicate *SQR* genes in

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crustaceans. Overall, our study suggests that the subfunctionalization of duplicate *xtSQR* genes may play an important role in sulfide detoxification to maintain the sulfide homeostasis in *X. testudinatus*, providing an ecophysiological basis for its adaptation to the high-sulfide hydrothermal vent environment.

## 1. Introduction

Hydrogen sulfide ( $H_2S$ ) is a signaling molecule that plays various roles in the cardiovascular, gastrointestinal, and central nervous systems but is toxic at high concentrations (Kabil and Banerjee, 2010). In most animals, the toxicity of  $H_2S$  arises due to a blockage of the electron transport chain when high  $H_2S$  levels ( $\mu$ mole) inhibit mitochondria cytochrome *c* oxidase complex (COX) (Kabil and Banerjee, 2010). Thus, survival of the animal depends on its ability to maintain  $H_2S$  at low concentrations (Lagoutte et al., 2010). In mammals,  $H_2S$  is produced from the metabolism of L-cysteine by enzymes including cystathionine- $\beta$ -synthase (CBS), cystathionine- $\gamma$ -lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST) (Wang, 2002). Furthermore,  $H_2S$  that is synthesized by intestinal microflora can enter the circulation (Singh and Lin, 2015). In aquatic animals,  $H_2S$  is synthesized by endogenous catabolism and also taken up from the environment.  $H_2S$  is frequently produced in sediments by bacterial putrefaction, constituting a major source of exposure for aquatic animals that live in close association with the bottom. In fishes (Vetter and Bagarinao, 1989) and crustaceans (Kang and Matsuda, 1993; Johns et al., 1997), it was shown that tidal-marsh inhabitants have higher tolerances to sulfide than outer-bay and open-coast inhabitants. Furthermore, several crustacean and mollusk species are able to thrive in sulfide-rich deep-sea hydrothermal vent chemosynthetic ecosystems, such as blind crab (Bythograeidae), yeti crab (Kiwaidae), hydrothermal vent shrimp (Alvinocarididae), and hydrothermal vent mussel (*Bathymodiolus* spp.; *Gigantidas platifrons*) (Powell and Somero, 1986; Compère et al., 2002; Thatje et al., 2015; Leignel et al., 2018; Sun et al., 2022).

Most non-hydrothermal vent crabs suffer high rates of mortality when exposed to sulfide-rich environments for long periods (Caldwell, 1975). However, the hydrothermal vent crab *Bythograea thermydron* shows high tolerance and low sensitivity to sulfide compared to non-hydrothermal vent crab species (Vetter et al., 1987). Remarkably, another hydrothermal vent crab, *Xenograpsus testudinatus* (*xtcrab*), is known to reside in the shallow-water hydrothermal vent system off Kueishan Island, Taiwan, despite the sulfide-rich nature of the environment (Jeng et al., 2004). These animals likely have evolved novel mechanisms to adapt to the hydrogen sulfide-rich hydrothermal vent environment. Nevertheless, a comprehensive understanding of the molecular mechanisms underlying the sulfide tolerance of this hydrothermal vent crustacean is still lacking.

In eukaryotes, two main strategies are used to tolerate sulfide exposure, including heightened sulfide detoxification capacity and temporary implementation of anaerobic metabolism. Detoxification of sulfide is typically performed by a mitochondrial pathway that catalyzes sulfide to thiosulfate. This pathway is conserved in species ranging from Annelida to Chordata (Vetter and Bagarinao, 1989; Johns et al., 1997; Hildebrandt and Grieshaber, 2008). During sulfide detoxification, one thiosulfate can absorb two lethal sulfide molecules via the enzymatic action of sulfide:quinone oxidoreductase (SQR) (Grieshaber and Völkel, 1998). When this mechanism becomes saturated, the acclimation strategy may switch to anaerobiosis (Grieshaber and Völkel, 1998). In mammals, most intestinal bacteria-synthesized  $H_2S$  is oxidized in the colonic mucosa, while  $H_2S$  in circulation is mainly cleared by the liver (Furne et al., 2001; Norris et al., 2011). Unlike in mammals, the predominant sulfide-oxidizing tissue in fish and crustaceans varies according to species (Vetter and Bagarinao, 1989; Tobler et al., 2014). In various crustaceans, the digestive gland (also called the hepatopancreas) has been shown to exhibit high sulfide-oxidizing activity (Vetter et al., 1987). Like in mammals, SQR is a key enzyme and the first step of the pathway catalyzing sulfide oxidation (Ziosi et al., 2017). A single copy of SQR is conserved in eukaryotes from yeast to mammals, and no

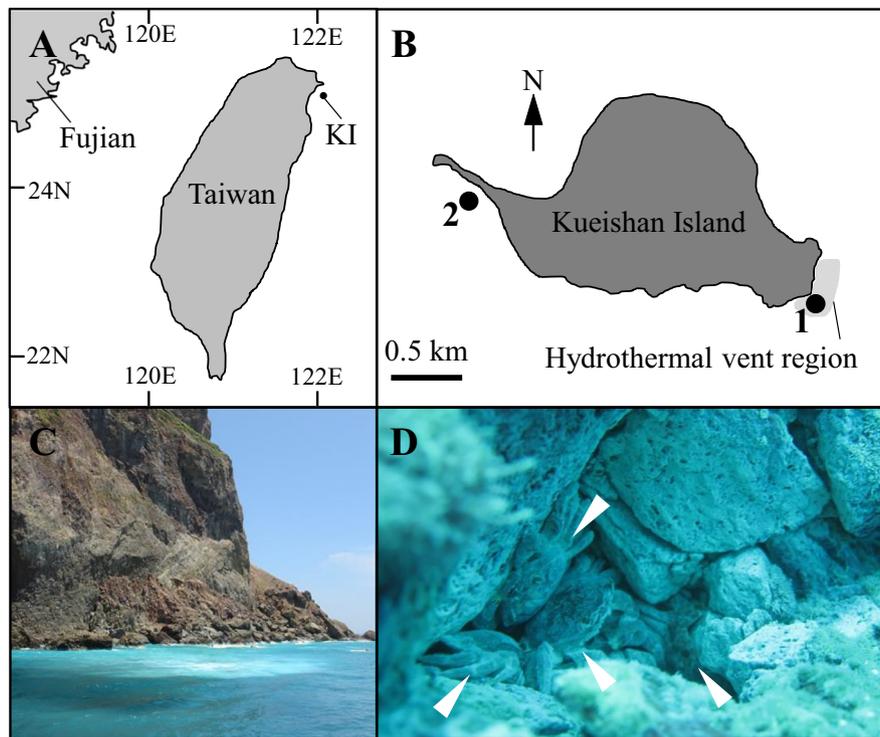
duplicated paralog has been found in teleosts, despite the whole-genome duplication that occurred in this lineage (Olson et al., 2012; Melideo et al., 2014). Upon exposure to sulfide-rich aquatic environments, SQR gene expression and sulfide-oxidizing enzymatic activity were both shown to be increased in several fishes (Vetter and Bagarinao, 1989) and crustaceans (Vetter et al., 1987; Zhu et al., 2021). Therefore, SQR may serve an essential regulator of sulfide homeostasis in aquatic animals.

*xtCrab* is a brachyuran that belongs to the *Xenograpsidae* family and resides in the hydrothermal vent region of Kueishan Island (Ng et al., 2000), Showa Iwo-Jima (Ng et al., 2014), and Shikine-Jima Islands in Japan (Miyake et al., 2019). Kueishan Island is located off the northeastern coast of Taiwan (Fig. 1A), with numerous shallow-water vents in its Eastern coastal region (Fig. 1B). In this area, the hydrothermal vent system generates turbid white sulfide-rich seawater (Fig. 1C), which is hospitable to very few organisms. Nevertheless, *xtcrabs* can be found in large numbers in and around this hydrothermal vent region (Fig. 1D). Thus, this *xtcrab* is an excellent model organism for studying the adaptive physiology of crustaceans in sulfide-rich environments. Compared to deep-sea hydrothermal vents, the shallow water environment is much more amenable to animal sampling and experiments. It also does not raise issues related to the potential physiological adaptations of resident animals to high pressures and high sulfide concentrations. A previous study revealed that *xtcrabs* have high capacity for  $H^+$  excretion (Hu et al., 2016). However, the mechanisms of sulfide detoxification in this species remain unclear. In light of the widespread key role for SQR in sulfide detoxification, we hypothesized the importance of SQR in the adaptation of this vent crab to the high sulfide environment. Therefore, we aimed to delineate the genetic basis and physiological significance of SQR in the *xtcrab*. In doing so, we were able to describe a key molecular mechanism underlying the remarkable sulfide tolerance of this species. In our experiments, we analyzed the physiological capacity of *xtcrab* to maintain sulfide homeostasis and survive in hydrogen sulfide-rich environments. We then identified two SQR paralogs (*xtSQR1* and *xtSQR2*) in *xtcrab* and used qPCR to determine the differential tissue distributions of the paralogs, including how the expression of both genes is regulated by exposure to different sulfide concentrations (sulfide-rich habitat versus sulfide-free aquarium). Next, we examined the cellular localization of SQR protein in the gill by immunohistochemistry and measured SQR enzymatic activity. Taken together, our findings suggest that a functional partitioning of duplicated SQRs plays an important role in the ability of *xtcrab* to thrive in the hydrogen sulfide-rich hydrothermal vent system.

## 2. Material and methods

### 2.1. Seawater survey

Seawater samples were collected with a 50 ml brown jar in the Kueishan Island hydrothermal vent region (24°50'31"N/121°57'6"E) in station 1 (*xtcrabs* habitat, <10 m from the vent, at depths of about 10 m) and station 2 (located on the other side of the island, far from the vent, and not inhabited by the *xtcrabs*, at depths of about 10 m) (Fig. 1B). Seawater parameters were surveyed using an Orion Star™ A329 Portable pH/ISE/Conductivity/DO meter (Thermo Fisher Scientific, Waltham, MA, USA). Measurements of pH, dissolved oxygen (DO), and sulfur compound (sulfide) concentrations were performed following the manufacturer's protocol. pH and DO were measured directly in the water samples. For sulfide analysis, 10 ml anti-oxidant buffer (SOAB, Orion 941,609 A with Orion 941,609 B, Thermo Fisher Scientific) was added to 10 ml of seawater sample to fix the sulfide compounds to obtain the fixed seawater sample for measurement by ISE meter.



**Fig. 1.** Location of the hydrothermal vent region in Kueishan Island and seawater parameters. (A) Kueishan Island (KI) is located 11 km from the Northeast coast of Taiwan's main Island. (B) Numerous shallow-water vents are located in the Eastern coastal region of the Kueishan Island (station 1). No vents are present on the other side of the island (station 2). (C) At station 1, turbid white (sulfidic) seawater is observed due to the hydrothermal vent system. (D) Hydrothermal vent crabs *Xenograpsus testudinatus* (xtcrabs) (white arrows) are distributed throughout this hydrothermal vent region, often inhabiting rock cracks. (E) Seawater parameters, including pH, dissolved oxygen (DO) and sulfide, were recorded for each sampling batch from station 1 and station 2.

## 2.2. Hydrothermal vent xtcrahs and control tdcrahs

xtCrabs with carapace lengths ranging from 15 to 25 mm and carapace widths ranging from 15 to 25 mm were collected from the Kueishan Island hydrothermal vent region (station 1) by SCUBA divers (Fig. 1B). Some xtcrahs were immediately anesthetized on the boat, using seawater containing 20 ppm (120  $\mu$ M) eugenol, for hemolymph and tissue sampling. Other collected xtcrahs were transferred to the aquaculture station of National Taiwan Ocean University (NTOU). For comparative analyses, crabs from a "control" intertidal species (*tcrab*; *Thranita danae*, Portunidae; intertidal swimming crabs) with carapace lengths ranging from 25 to 35 mm and carapace widths ranging from 50 to 60 mm were collected by net from the intertidal zone of a rocky seashore in Keelung, Taiwan (25°08'09.1"N/121°48'10.4"E) and transferred to NTOU.

All the transferred crabs were acclimated to a (300 l) tank system with normal seawater (sulfide-free, pH  $8.2 \pm 0.1$ , temperature  $28 \pm 1$  °C) and daylight conditions (12/12 h light/dark cycle). The crabs were fed once a day with shrimp commercial feed (Omega, Kaohsiung, Taiwan) during one month stay in aquarium. All procedures and investigations followed the standard guidelines and principles of the NTOU Institutional Animal Care and Use Committee.

## 2.3. Tissue sampling of xtcrahs from the sulfidic hydrothermal vent habitat and xtcrahs maintained in a normal seawater aquarium

xtCrabs were collected from the hydrothermal vent region (sulfidic environment). Some of the collected xtcrahs were immediately sampled on the boat ("habitat xtcrahs"), while other xtcrahs were kept and fed in the laboratory in normal seawater aquaria (non-sulfidic environment) for 1 month ("aquarium xtcrahs"). The sex of the xtcrahs was checked by external observation, according to the sexual dimorphism (males have narrow, triangular abdomens, whereas females have broader abdomens) (Hidir et al., 2021).

To analyze the tissue distributions of SQR transcripts by qPCR, 6 habitat xtcrahs were used. Various tissues (anterior gills, posterior gills, stomach, digestive gland, intestine, muscle, and heart) were obtained (Fig. S1A). According to their relative sizes, the gill tissues were pooled into small anterior gills (1st, 2nd, and 3rd gills) and large posterior gills (4th, 5th, 6th, and 7th gills) (Fig. S1B). Semi-quantitative RT-PCR was also performed on posterior gills and digestive glands of 3 "habitat xtcrahs". To determine the cellular localization of SQR by immunohistochemistry, posterior gills from 5 "habitat xtcrahs" were collected. For comparison of SQR transcript levels (RT-PCR and qPCR) between the sulfide-rich hydrothermal vent and sulfide-free conditions, posterior gills and digestive glands were collected from up to 35 "habitat xtcrahs" and 35 "aquarium xtcrahs" during 2016–2020, including the 6 habitat xtcrahs, which mentioned above, were used for tissue distribution analyses. Comparison of posterior gill SQR protein levels (Western blot) and SQR enzymatic activity between sulfidic-rich and sulfide-free conditions were also performed using 3 "habitat xtcrahs" and 3 "aquarium xtcrahs".

## 2.4. Survival of xtcrahs and control tdcrahs upon exposure to various sulfide levels

For this experiment, xtcrahs were collected from the hydrothermal vent region and maintained in the laboratory culture system under normal seawater (sulfide-free) conditions and feeding for 1 month. For sulfide exposure experiments, xtcrahs ( $n = 10$ /group) were then placed in 10 l seawater containers with different sulfide levels (0, 150, 500, and 1000  $\mu$ M; xtcrahs were not fed during the exposure). These sulfide levels were selected as each corresponds to a different habitat: intertidal sediments (150  $\mu$ M sulfide), hydrothermal vent regions (500  $\mu$ M sulfide), and crater of hydrothermal vent (1000  $\mu$ M sulfide). Air pumps continuously supplied air to maintain the oxygen levels.  $H_2S$  stock solutions (10 mM) were generated by dissolving sodium sulfide nonahydrate ( $Na_2S \cdot 9H_2O$ , Thermo Fisher Scientific) in deoxygenated seawater. The survival tests in

various sulfide concentrations were conducted for 48 h, and the tested seawater was renewed at 4 h intervals (because sulfide concentrations in water were decreased by 2- to 3-fold after 4 h). During the 48 h test, the mobilities and touch reactions of *xtcrabs* were checked every 4 h. For the control crab (*tdcrab*) experiment, the same procedures were used to expose *tdcrabs* to seawater with low (50  $\mu$ M) or no (0  $\mu$ M) sulfide. Immobilization and no reaction to touch, which are typical of moribund and dead crabs, were used as the criteria for mortality.

### 2.5. Immersion of *xtcrabs* and control *tdcrabs* in cages in hydrothermal vent and non-hydrothermal vent sites for the survival test in the field and measurements of hemolymph sulfur compounds in the *xtcrab*

Laboratory-maintained *xtcrabs* and control *tdcrabs* (cultured in normal seawater for 1 month) were placed in cages (two cages for each group,  $n = 6/\text{cage}$ ) and then immersed either in the hydrothermal vent region (sulfide-rich environment, station 1: N24° 50' 047", E121° 57' 707") or in a non-hydrothermal vent region (sulfide-free environment, station 2: N24° 50' 558", E121° 56' 212") off Kueishan Island (Fig. 1B) for 2 h. Hemolymph was collected from *xtcrabs* for sulfur compound analysis by HPLC.

### 2.6. HPLC analysis of sulfur compounds in *xtcrab* hemolymph

For the sulfur compound analysis, the analytical method was described previously (Lallier, 1998). Monobromobimane (mBBR, B4380, Darmstadt, Germany) was used to react selectively with thiols and reduced sulfur compounds to produce fluorescent derivatives, including sulfide, thiosulfate, and sulfite. These fluorescent derivatives were then measured by HPLC. Hemolymph was collected from *xtcrabs* on the boat and immediately frozen in liquid nitrogen. Upon return to the laboratory, the samples were transferred to  $-80^{\circ}\text{C}$ . Thawed hemolymph (100  $\mu$ l) was combined with 15  $\mu$ l mBBR and 200  $\mu$ l reaction buffer. Buffers at pH 8.0 (0.596 g HEPES and 0.093 g EDTA in 50 ml distilled water) and pH 4.27 (14 ml of 0.2 M acetic acid and 6 ml of 0.2 M anhydrous sodium in 50 ml distilled water) were respectively used for the thiosulfate/sulfite and sulfide reactions. The mixtures were left to react at room temperature in darkness for 20 h. Acetonitrile (200  $\mu$ l) was added to precipitate denatured proteins at  $60^{\circ}\text{C}$  for 10 min. Methanesulfonic acid (300  $\mu$ l) was added to stabilize the mBBR adducts (fluorescent derivatives), and the precipitated proteins were removed by centrifugation. The mBBR adducts were analyzed using a Waters 600E HPLC with a reverse-phase column (LiChrospher 60, RP select B,  $4 \times 125$  mm, 5  $\mu$ m). The excitation/emission wavelengths used for detection were 390/480 nm.

### 2.7. Total RNA extraction and cDNA synthesis

Tissues were homogenized in Trizol reagent (Invitrogen, Waltham, MA, USA). Total RNA was extracted following the manufacturer's protocol. The quality and quantity of total RNA were respectively determined using gel electrophoresis and a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific). First-strand cDNA was synthesized from 1  $\mu$ g total RNA with Superscript III reverse transcriptase (Invitrogen). cDNA synthesis was performed according to the manufacturer's protocol.

### 2.8. Cloning of *xtcrab* sulfide: quinone oxidoreductase (SQR) cDNAs

Total RNA was extracted from gills and digestive glands of *xtcrab* collected from hydrothermal vent regions, and reverse transcribed. To search for SQR sequence in *xtcrab*, we first retrieved SQR sequences from other crab species, using available Sequence Read Archive (SRA) raw reads of *Scylla olivacea* (GDRN00000000.1), *Cancer borealis* (GEFB00000000.1), and *Carcinus maenas* (GBXE00000000.1). These three sets of reads were separately used to conduct *de novo* assembly database with CLC Genome Workbench 8.0. Local BLAST with the human SQR sequence (NM\_001271213.2) from NCBI allowed us to annotate SQR sequences

from *Scylla olivacea*, *Cancer borealis*, and *Carcinus maenas* following CLC Genome Workbench manuals. SQR sequences from *Cancer borealis* and *Carcinus maenas* were aligned with DNAMAN sequence analysis software (Lynnon Corporation, Vaudreuil-Dorion, Canada; Li et al., 2019). This alignment of SQR sequences was used for designing "universal primers" for crab SQR sequences. With the universal primers (Fig. S2; Table S1), two SQR sequences (*xtSQR1* and *xtSQR2*) were found and cloned in full length with a rapid amplification of cDNA ends (RACE) kit (SMART RACE cDNA; BD Biosciences Clontech, Franklin Lakes, NJ) (Table S1). Full-length (containing ORF) cDNA sequences of the two *xtcrab* SQR genes, *SQR1* and *SQR2*, were respectively obtained from gills and digestive glands. To obtain partial sequences of SQR in various other crab species, gills and digestive glands were sampled in *Eriocheir sinensi*, *Candidiopotamon rathbunae*, *Eriphia ferox*, *Calappa lophos*, *Sesarmops impressum*, *Thranita danae*, *Cancer pagurus*, and *Grapsus albolineatus*. Total RNA were extracted and reverse transcribed. Using the "universal primers" for SQR, the partial sequences of SQR were obtained from the gills and digestive glands pooled cDNA in each species (Fig. S2; Table S1).

### 2.9. Sequence alignment and phylogenetic analysis

Sequence alignment was performed with DNAMAN. To infer the evolutionary relationships of *xtcrab* SQRs and the corresponding genes in arthropods and other metazoan species, phylogenetic analyses were performed using predicted amino-acid sequences. SQR sequences were listed in Table S2. The translated amino-acid sequences were automatically aligned with MUSCLE, as implemented in Mega7 (Kumar et al., 2016). The phylogenetic tree was constructed using the neighbor-joining method with the best model (LG + G, Gamma rate = 1.2644) in MEGA7 (Kumar et al., 2016). The number at each node represents the bootstrap value (% from 1000 replicates). The accession numbers of the sequences used in the analysis are listed in Table S2.

### 2.10. Semi-quantitative RT-PCR analysis of *xtcrab* SQR1 and SQR2

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis was used to assess the comparative expression of *xtcrab* SQR1 and SQR2 in gills and digestive gland. Tissues were collected from three "habitat *xtcrabs*" and subjected to the protocols for total RNA extraction and cDNA synthesis described above. *Elongation factor 1 alpha* (*EF1A*, MT459647) was used as a reference gene. All PCR products of *EF1A*, *xtSQR1*, and *xtSQR2* were designed to be 600 bp (Table S1). Densitometry analysis of RT-PCR products was performed using the ImageJ software (Schneider et al., 2012).

### 2.11. qPCR analysis of *xtcrab* SQR1 and SQR2

Specific qPCR primers were designed for each *xtcrab* paralog. The specificity of *xtSQR1* and *xtSQR2* qPCR primers for their respective *xtSQR* paralog were tested on full-length *xtSQR1* and *xtSQR2* plasmids. No cross-reaction was observed with *xtSQR1* primers on *xtSQR2* plasmid, nor with *xtSQR2* primers on *xtSQR1* plasmid (Fig. S3). Quantitative real-time PCR (qPCR) was used to analyze gene expression profiles. *EF1A* was used as an internal reference. Specific primers for *SQR1*, *SQR2*, and *EF1A* are listed in Table S1. qPCR was performed using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with SYBR green Master Mix (Bio-Rad Laboratories). The PCR thermal cycling process was as follows: one cycle of  $95^{\circ}\text{C}$  for 5 min, then 40 cycles of  $95^{\circ}\text{C}$  for 15 s, and  $60^{\circ}\text{C}$  for 1 min. The PCR specificity was confirmed by a single melting curve performed on unknown samples. No signals were detected in non-template controls by qPCR. The results were calculated according the  $2^{-(\Delta\Delta\text{Ct})}$  method (Livak and Schmittgen, 2001). The relative expression value for the target gene in each sample was normalized to *EF1A*, and the highest recorded value of target gene expression was defined as 100 %.

### 2.12. Recombinant *xtcrab* SQR production

The open reading frame (ORF) of SQR1 was inserted into pcDNA 3.1<sup>(+)</sup> Mammalian Expression Vector (Thermo Fisher Scientific). The PCR primers used to amplify the ORF of SQR1 are listed in Table S1. HEK293 (Human embryonic kidney) cells were used to host the recombinant construct. The *xtcrab* recombinant SQR1 (rSQR1) was produced in HEK293 cells as described in our previous study (Wu et al., 2012). rSQR1 was extracted in RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific) with cOmplete™ Mini Protease Inhibitor Cocktail (Roche, Penzberg, Germany). The HEK293-expressed rSQR1 was used to examine the specificity of the anti-SQR antibody by Western blot analysis.

### 2.13. Western blot analysis of *xtcrab* SQR

To analyze the intracellular localization of SQR, the posterior gills were homogenized in crab saline (400 mM NaCl, 11.3 mM KCl, 13.3 mM CaCl<sub>2</sub>, 26 mM MgCl<sub>2</sub>, 23 mM Na<sub>2</sub>SO<sub>4</sub>, 10 mM HEPES, pH 7.4). Then, the cytosol and organelle fractions were separated by centrifugation (11,000 ×g). Proteins in the cytosol (supernatant) and organelles (precipitate) were then extracted with T-PER™ Tissue Protein Extraction Reagent (Pierce Biotechnology, Waltham, MA, USA) and containing cOmplete™ Mini Protease Inhibitor Cocktail (Roche). To analyze the SQR protein expression pattern between sulfide-rich habitat and sulfide-free aquarium *xtcrab*, the posterior gills (4th to 7th gills. Fig. S1) were pooled and homogenized in Trizol reagent. 3 *xtcrabs* were used in each group. Proteins were extracted following the manufacturer's protocol. Protein concentrations were determined using the Bicinchoninic Acid Protein Assay Kit (Pierce Biotechnology). For Western blot analysis, we followed a previously described method (Chen et al., 2018). To detect the SQR and Actin proteins, rabbit anti-human SQR antibody (GenWay Biotech, GWB-MW6571, San Diego, CA, USA; diluted 1:8000 with 1.5 % nonfat milk powder) and mouse anti-human Actin antibody (MAB1501, Merck, Darmstadt, Germany; diluted 1:10,000 with 1.5 % nonfat milk powder) were used. For secondary antibody, alkaline phosphate-conjugated goat anti-rabbit IgG antibody (AS-28177, AnaSpec, San Jose, CA, USA; diluted 1:10,000 with 1.5 % nonfat milk powder) and alkaline phosphate-conjugated goat anti-mouse IgG antibody (#31320, Thermo Fisher Scientific; diluted 1:10,000 with 1.5 % nonfat milk powder) were used as appropriate. For detection, the NBT/BCIP Detection System (B1911, Sigma-Aldrich, St. Louis, MO, USA) was used. Quantification of signal intensity was performed with ImageJ software. Means ± SD (*n* = 3 *xtcrabs*/group).

### 2.14. SQR enzymatic activity analysis

To measure SQR enzymatic activity, an analytical method was modified from a previous publication (Weghe and Ow, 1999; Theissen and Martin, 2008). Briefly, tissues were homogenized in mitochondria isolation buffer (600 mM sucrose, 200 μM EGTA, 1 mM EDTA, 20 mM HEPES, and 0.5 % BSA). Cell debris were removed by centrifugation (1600 ×g) for 12 min. Mitochondria were then isolated by centrifugation (7100 ×g) for 15 min. Isolated mitochondria were then suspended in crab saline. The protein concentration was determined by Bicinchoninic Acid Protein Assay Kit (Pierce Biotechnology). SQR activity was measured at room temperature. The reaction mixture contained 10 mM Tris-HCl (pH 7.6), 100 μM decylubiquinone (dUQ, ubiquinone analog, D7911, Darmstadt, Germany), and isolated mitochondria. The assay was initiated by adding 200 μM sulfide (deoxygenated seawater; freshly prepared in N<sub>2</sub>-flushed seawater). After a 3-min incubation at room temperature, absorbance was measured at 284 nm (reduced dUQ) and 294 nm (oxidized dUQ) by spectrophotometry (Hitachi U-1900). SQR activity was determined by subtracting the absorbance at 294 nm from the absorbance at 284 nm. An extinction coefficient for dUQ of 15 mM<sup>-1</sup> cm<sup>-1</sup> was used to convert absorbance to molar concentrations (Morton, 1965).

### 2.15. Localization of SQR in the gills by immunofluorescence staining

The 4th (the largest) gills (Fig. S1B) from hydrothermal vent *xtcrabs* (*n* = 5) were collected immediately on the boat and fixed with RF solution (100 % formalin 349 ml, 95 % ethanol 407 ml, ammonium hydroxide 22 ml, and distilled water 222 ml) (Hasson et al., 1997) at room temperature for 16 h. Then, the tissues were placed in 70 % ethanol and stored at room temperature. Dehydrated gills were transferred from ethanol to xylene and then embedded in paraffin. For antigen retrieval, sections (4-μm thickness) were rehydrated with PBS (phosphate buffer with saline), and then treated with sodium citrate buffer (10 mM sodium citrate, 0.05 % Tween 20, pH 6.0) under 95 °C for 5 min. Cellular staining for SQR, Na<sup>+</sup>/K<sup>+</sup>-ATPase (NKA) and DNA (DAPI; 4',6-diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA, USA) was conducted in gill tissues (gill stem and filament). For co-staining of SQR and NKA, the rabbit anti-human SQR antibody (GWB-MW6571; diluted 1:1500 with 1.5 % nonfat milk powder) and the mouse anti-chicken NKA antibody (ATP1A1 a5, Developmental Studies Hybridoma Bank, Iowa city, IA, USA; diluted 1:200 with 1.5 % nonfat milk powder) (Hu et al., 2016) were applied to *xtcrab* tissue sections at 4 °C overnight. Secondary antibodies were Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody (A-11034, Thermo Fisher Scientific; diluted 1:200 with 1.5 % nonfat milk powder) and Alexa Fluor 546-conjugated goat anti-mouse IgG secondary antibody (A-11003, Thermo Fisher Scientific; diluted 1:200 with 1.5 % nonfat milk powder). These antibodies were used for the respective detection of anti-SQR and anti-NKA primary antibodies, and were applied at room temperature for 1 h. DAPI was used to label the nuclear DNA. The signals in gill stem and gill filament were observed and images were captured under fluorescence microscopy (Olympus BX53).

### 2.16. Statistical analysis

Data are shown as mean and standard deviation. Student's *t*-test was used to analyze the significance of differences between two groups. One-way ANOVA followed by a Tukey's *post hoc* analysis was performed with Statistical Package for the Social Science (SPSS) software to test the significance of differences among three or more groups. In all cases, *p* < 0.05 indicated statistically significant differences.

## 3. Results

### 3.1. Seawater parameters around Kueishan Island hydrothermal vents

Our survey of seawater from Kueishan Island (Fig. 1A) hydrothermal vent *xtcrabs* habitat (station 1) (Fig. 1B-D) showed that it differed from normal seawater (station 2) in terms of pH, dissolved oxygen (DO), and sulfide levels (Table 1). The measured ranges of pH (6.28–7.27) and DO (4.15–7.28 mg/L) in the hydrothermal vent region were much lower than those in the normal seawater (station 2; pH 8.01–8.17 and DO 7.83–8.29 mg/L) (Table 1). No detectable sulfide concentrations were found in the normal seawater (station 2; sulfide-free), while high levels of sulfide (17.80–1448.67 μM) were detected in the hydrothermal vent region (Table 1). The large variations in sulfide concentrations observed in the hydrothermal vent region may be due to the timing of measurements in relation to eruption of thermal vents, currents, and ocean waves.

### 3.2. Sulfide tolerance of *xtcrab*

*xtCrabs* collected in hydrothermal vent area and control species *tdcrabs* were maintained for 1 month in normal (sulfide-free) seawater aquaria and then subjected to a sulfidic environment in the aquarium, as prepared by the addition of sodium sulfide nonahydrate (Na<sub>2</sub>S·9H<sub>2</sub>O). The control *tdcrabs* survived and were able to move normally in the normal seawater during the 48 h experiment, but none of the control *tdcrabs* survived a 10 min exposure to 50 μM sodium sulfide nonahydrate in seawater. In contrast, all *xtcrabs* exposed to the different concentrations of sodium sulfide

**Table 1**  
Water Parameters in Kueishan Island.

Sampling batch	Station 1 (Hydrothermal vent region)			Station 2 (> 2 km from station 1)		
	pH	DO (mg/L)	Sulfide ( $\mu\text{M}$ )	pH	DO (mg/L)	Sulfide ( $\mu\text{M}$ )
1	6.28 $\pm$ 0.00 (6.28–6.29)	4.15 $\pm$ 0.15 (3.71–4.30)	566.27 $\pm$ 65.32 (464.50–647.26)	8.15 $\pm$ 0.01 (8.14–8.16)	7.89 $\pm$ 0.07 (7.79–7.93)	ND
2	6.50 $\pm$ 0.13 (6.33–6.66)	4.28 $\pm$ 0.01 (4.27–4.30)	237.33 $\pm$ 42.72 (182.00–286.00)	8.17 $\pm$ 0.02 (8.14–8.20)	7.85 $\pm$ 0.05 (7.81–7.92)	ND
3	7.27 $\pm$ 0.03 (7.24–7.31)	7.11 $\pm$ 0.13 (6.95–7.26)	17.80 $\pm$ 4.14 (12.00–21.40)	8.12 $\pm$ 0.02 (8.09–8.15)	8.29 $\pm$ 0.07 (8.23–8.39)	ND
4	6.52 $\pm$ 0.89 (5.62–7.42)	4.29 $\pm$ 0.53 (3.67–4.96)	1320.67 $\pm$ 1809.73 (36.60–3880.00)	8.05 $\pm$ 0.04 (8.01–8.11)	7.84 $\pm$ 0.05 (7.79–7.91)	ND
5	6.95 $\pm$ 0.01 (6.93–6.97)	7.28 $\pm$ 0.57 (6.83–8.49)	1448.67 $\pm$ 65.51 (1396.00–1572.00)	8.01 $\pm$ 0.01 (8.00–8.03)	7.83 $\pm$ 0.04 (7.78–7.88)	ND

ND: not detectable.

(150, 500 or 1000  $\mu\text{M}$ ) exhibited prolonged ability to move, but survival of *xtcrabs* decreased with increasing sodium sulfide nonahydrate concentrations (Fig. S4). The survival rates of *xtcrabs* in 0  $\mu\text{M}$  and 150  $\mu\text{M}$  sodium sulfide nonahydrate were 100 % over the 48 h experimental period. Meanwhile, all *xtcrabs* in the 1000  $\mu\text{M}$  sodium sulfide nonahydrate treatment group died between 24 and 48 h of exposure. Three of eight (37.5 %) *xtcrabs* in the 500  $\mu\text{M}$  sodium sulfide nonahydrate treatment group died between 36 and 48 h of exposure (Fig. S4). Taken together, the data showed that compared to intertidal *tdcrabs*, *xtcrabs* have much stronger sulfide tolerance.

### 3.3. Sulfide catabolism in *xtcrabs*

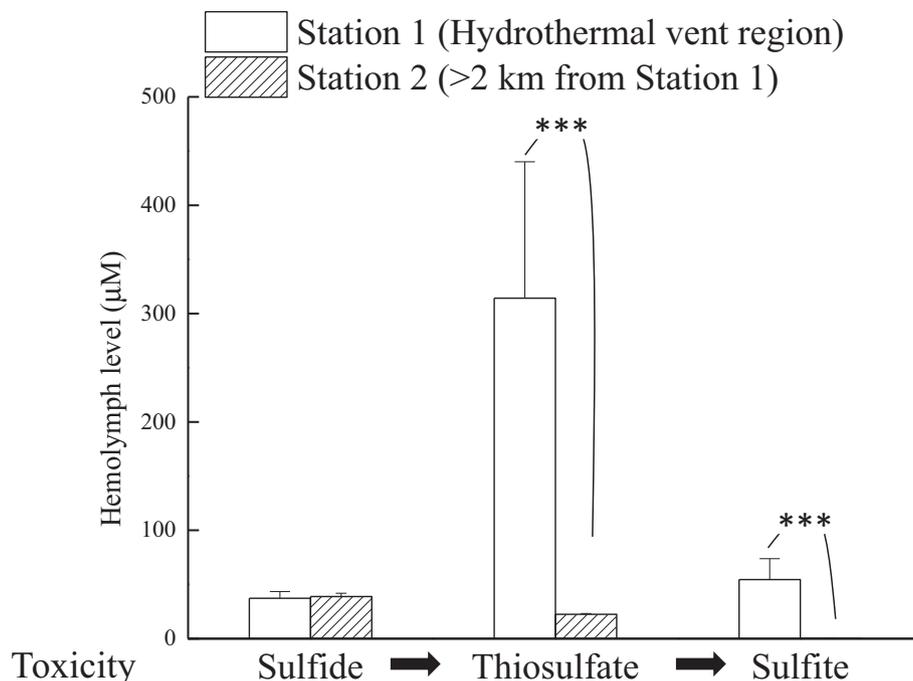
*xtCrabs* collected from the hydrothermal vent area and control *tdcrabs* were maintained for 1 month in normal (sulfide-free) seawater and then immersed for 2 h either in hydrothermal vent sulfide-rich seawater environment (station 1) or in normal seawater environment (station 2). All *xtcrabs* ( $n = 6$  in each station) survived immersion at either station, but *tdcrabs* ( $n = 6$ ) did not survive in station 1 compared to all survival in

station 2 ( $n = 6$ ), confirming the strong tolerance of *xtcrabs* to a high-sulfide natural environment.

HPLC analysis showed that the hemolymph levels of sulfide in *xtcrabs* were not different between individuals exposed to the two stations (Fig. 2). In contrast, the hemolymph levels of thiosulfate and sulfite (less toxic) in *xtcrabs* were significantly increased in the hydrothermal vent station 1 group compared to the normal seawater station 2 group (thiosulfate: 16-fold,  $p < 0.001$ ; sulfite 341-fold,  $p < 0.001$ ) (Fig. 2). In station 1 exposed hydrothermal vent *xtcrabs*, the order of sulfur compounds in terms of measured hemolymph concentrations was thiosulfate > sulfite > sulfide (Fig. 2).

### 3.4. Molecular identification of crab SQRs and phylogenetic analysis

*De novo* assembly of SRA small reads and Blast search using human SQR allowed us to identify two SQR sequences in Jonah crab, *Cancer borealis* transcriptome (assembly numbers: GEFB01005034.1; GEFB01000755.1), while only single SQR sequences were found in the transcriptomic analyses of green crab, *Carcinus maenas* (assembly number: GBXE01108184.1), and



**Fig. 2.** Sulfur compounds in hemolymph of *xtcrabs*. To study sulfide catabolism, *Xenograpsus testudinatus* (*xtcrabs*) were collected from the sulfide-rich hydrothermal vent region of Kueishan Island and maintained for one month in normal (sulfide-free) seawater aquaria. The crabs were then immersed for 2 h in cages in station 1 (hydrothermal vent region) or station 2 (sulfide-free region) (Fig. 1). Contents of sulfur compounds, including sulfide, thiosulfate and sulfite (low toxicity), were measured in hemolymph using HPLC. Means  $\pm$  SD ( $n = 6$  *xtcrabs*/group). Asterisks indicate significant differences between station 1 and station 2 *xtcrabs* ( $p < 0.001$ ).

mud crab, *Scylla olivacea* (assembly number: gi|936,310,034|gb|GDRN1110561.1). Using “universal primers” designed from all these crab SQR sequences, we identified two SQR-like sequences in *xtcrab*, named *xtSQR1* (accession number MT459642) and *xtSQR2* (MT459643) that we fully cloned from *xtcrab* gill and digestive gland, respectively.

To analyze the sequences of these two *xtcrab* SQR paralogs, amino-acid sequences of SQR from different species were aligned with DNAMAN software. Based on the findings of previous studies (Hell et al., 2008; Marcia et al., 2010), we were able to annotate three putative FAD-binding domains (I, II, and III), one sulfide oxidation domain, and one quinone reduction domain in the SQR sequences of various species (Fig. 3A). In *xtcrab*, the two predicted SQR-like proteins (named *xtSQR1* and *xtSQR2*) had open reading frames that each encoded 440 amino acid residues (Fig. 3B). Between these two SQR-like proteins, the amino-acid sequence identities between FAD-binding domains I, II, III, sulfide oxidation domain, and quinone reduction domain were respectively 100.00 %, 100.00 %, 78.57 %, 87.20 %, and 91.25 %. Overall, the sequence identity between the two SQR-like proteins was 86.36 % (Fig. 3B).

We also cloned two partial sequences of SQR-encoding genes from brown crab (*Cancer pagurus*) and one from Chinese mitten crab (*Eriocheir sinensis*). Their predicted amino acids sequences were also included in the phylogenetic analysis (Fig. 3C) together with sequences obtained from the NCBI database for SQR proteins of various other metazoan species (Table S2). The phylogenetic analysis showed that the two *xtcrab* SQR-like sequences (*xtSQR1* and *xtSQR2*) clustered with SQRs from arthropods (Fig. 3C). Among the crab sequences, the two *xtcrab* SQR1 and SQR2 sequences did not cluster with the *Cancer borealis*/*Cancer pagurus* SQR1 and SQR2 sequences, respectively (Fig. 3C). This suggests that *xtcrab* SQR paralogs and *Cancer sp* SQR paralogs would result from independent SQR gene duplication events.

To search for other SQR gene duplications in Brachyura, we further isolated SQR partial sequences by using the “universal primers” on gills and digestive glands pooled samples from other brachyuran crabs, included in *Candidiopotamon rathbunae*, *Eriphia ferox*, *Calappa lophos*, *Sesarmops impressum*, *Thranita danae*, and *Grapsus albolineatus*. A preliminary phylogenetic analysis was conducted with the identified short partial sequences (Fig. S5), which showed that several other independent SQR duplication events may have occurred across the Brachyura lineage included in *Sesarmops impressum*, *Grapsus albolineatus*, and *Thranita danae*. This suggests that multiple independent SQR duplication events are likely to have occurred in the brachyuran lineage.

### 3.5. Tissue distributions of *xtSQR1* and *xtSQR2* transcripts and regulation of expression by sulfidic environments

Habitat hydrothermal vent *xtcrabs* ( $n = 6$ ) were used for measurements of *xtSQR* paralog expression in various tissues. No significant difference in *xtSQR1* and *xtSQR2* transcripts levels were observed between males and females (data not shown) so that results for combined sexes are shown (Fig. 4A, B). qPCR results showed that *xtSQR1* was predominantly expressed in the gills and heart, but it was also detected in other tissues in habitat hydrothermal vent *xtcrabs* (Fig. 4A). The expression of *xtSQR1* in the posterior gills of habitat *xtcrabs* was 10.4-fold the level in the digestive gland ( $p < 0.001$ ). Meanwhile, *xtSQR2* was predominantly detected in the digestive gland and only slightly expressed in the stomach, intestine, muscle, and heart or no expressed in the gills of habitat *xtcrabs* (Fig. 4B). Taken together, these data showed a clear differential tissue distribution for the two *xtSQR* paralogs and a specific high expression of *xtSQR1* in the gills of hydrothermal vent habitat *xtcrabs*.

Semi-quantitative RT-PCR was performed on the posterior gill and digestive gland of 3 habitat hydrothermal vent *xtcrabs* to validate and complement the qPCR results (Fig. 4C). The results confirmed that in the gill *xtSQR1* was highly expressed while *xtSQR2* was not detectable ( $p < 0.001$ ). They also indicated that in the digestive gland, *xtSQR1* was expressed at a higher level than *xtSQR2* ( $p < 0.05$ ). Furthermore, the results declared that *xtSQR1* was expressed at a higher level in the gill compared to

the digestive gland ( $p < 0.001$ ) and *xtSQR2* was expressed at a higher level in the digestive gland compared to the gill ( $p < 0.05$ ) (Fig. 4C).

### 3.6. Regulation of *xtSQR1* and *xtSQR2* transcripts by sulfidic environments

To investigate the potential correlation between expression of the SQRs (*xtSQR1* and *xtSQR2*) and the sulfidic environment, gene expression in the gill and digestive gland was compared between *xtcrabs* immediately dissected after being collected from the hydrothermal vent region (habitat *xtcrabs*) and *xtcrabs* maintained for 1 month in normal (non-sulfidic) seawater (aquarium *xtcrabs*).

Comparison of *xtSQRs* expression in gill and digestive gland between sulfide-rich habitat and sulfide-free aquarium *xtcrabs* was performed on a large number of animals ( $n = 35$  per group). qPCR results showed that *xtSQR1* expression in *xtcrab* posterior gills was significantly increased in the sulfidic habitat group compared to the aquarium (sulfide-free environment) group (2.56-fold,  $p < 0.001$ ) (Fig. 5A). The coefficient of variation for gill *xtSQR1* transcript levels was higher in the habitat group (91 %) than the aquarium group (72 %) (Fig. 5A). This increased variation in habitat *xtcrabs* may be due to individual differences in response to the sulfide-rich environment or fluctuations in sulfide concentration at station 1 (Table 1). In contrast, *xtSQR1* expression levels in the digestive gland showed no significant difference between habitat and aquarium *xtcrabs* (Fig. 5B). *xtSQR2* expression was not detectable in the posterior gills (Fig. 5C), and its expression level in the digestive gland was significantly increased (1.78-fold) in the aquarium group (sulfide-free environment) compared to sulfidic habitat group ( $p < 0.001$ ) (Fig. 5D).

### 3.7. Specificity of anti-SQR antibody, and SQR protein levels in the gills of *xtcrabs*

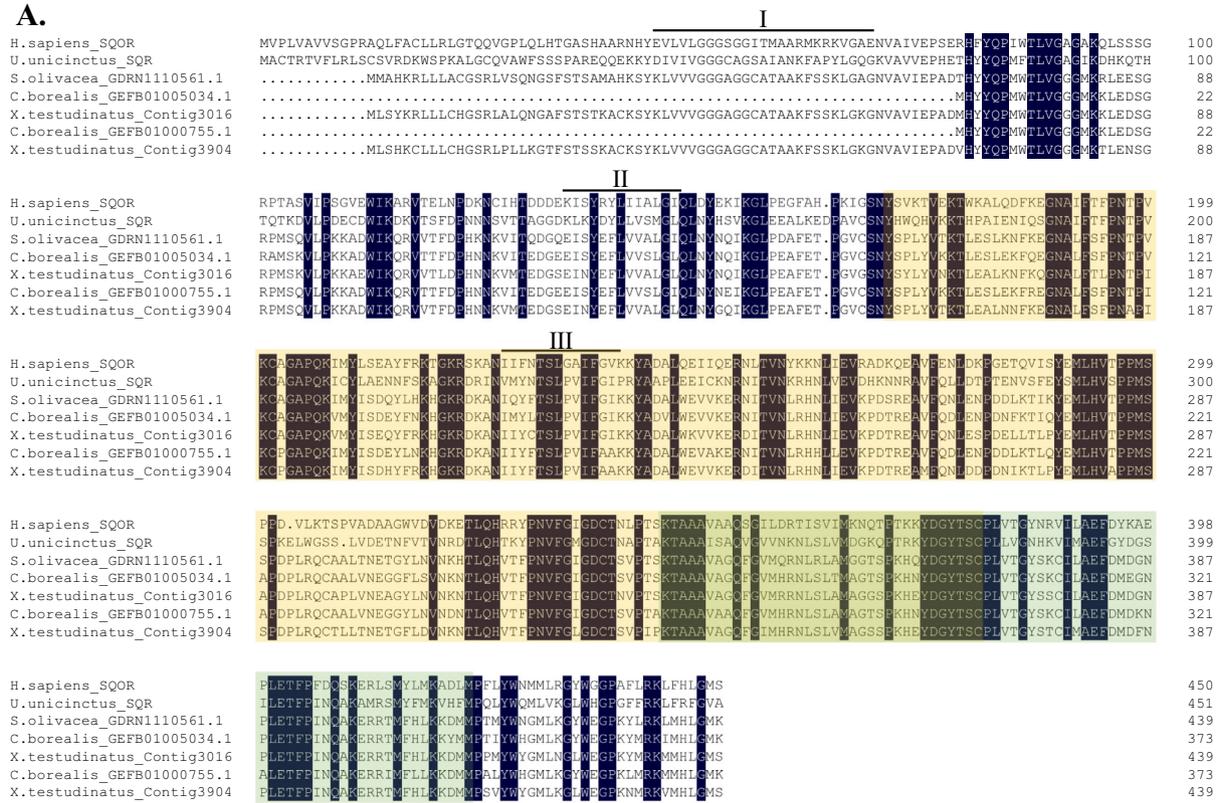
The specificity of  $\text{Na}^+/\text{K}^+$ -ATPase (NKA) antibody had been tested in *xtcrab* in the previous study (Hu et al., 2016). A commercially available anti-human SQR antibody was tested for its ability to detect *xtSQR*. Western blots were performed using *xtcrab* recombinant SQR1 (rSQR1) and gill extracts to test for the specific detection of *xtSQR*. Using the anti-SQR antibody, immunoblots of protein extracts from rSQR1-expressing HEK293 cells showed three bands, 48, 47, and 38 kDa (Fig. S6A); two of the bands (48 and 38 kDa) were also observed in control HEK293 protein extracts (no rSQR1 expression) (Fig. S6A). Thus, these results suggested that the antibody can detect a specific 47-kDa band corresponding to *xtcrab* rSQR1. A further immunoblotting analysis of gill extracts also revealed a single band at 47 kDa using the anti-SQR antibody (Fig. S6B). Actin was used as a reference in the Western blot analysis (Fig. S6C). Based on the ExPASy website ([http://web.expasy.org/compute\\_pi/](http://web.expasy.org/compute_pi/)), both SQR1 and SQR2 of *xtcrab* have a theoretical size of 49 kDa, which is roughly compatible with the observed signal at 47 kDa. Thus, we concluded that the anti-SQR antibody can most likely specifically recognize *xtSQR* proteins in *xtcrab* gill extracts. According to the known antigen sequence of the anti-SQR antibody, the detected *xtcrab* SQR in gill extracts could correspond to either or both SQR1 and SQR2. Importantly, the immunoblot signal was only observed in the organelle fraction (precipitate) (Fig. S6C), and no immunoblot signal was observed in the cytosol fraction (supernatant) (Fig. S6C). This result confirmed that *xtcrab* SQRs are contained in the organelle fraction, which includes mitochondria.

To compare the patterns of SQR protein expression between habitat and aquarium *xtcrabs*, we performed Western blot analysis with anti-SQR antibody on the gill extracts of habitat and aquarium *xtcrabs* ( $n = 3$  per group) (Fig. 5E). The immunoblot signal was significantly increased ( $p < 0.01$ ) in habitat *xtcrabs* compared to the aquarium group (Fig. 5E). After normalization of SQR signals to Actin levels, the habitat *xtcrabs* had 12.5-fold higher gill expression of SQR protein than the aquarium group (Fig. 5E).

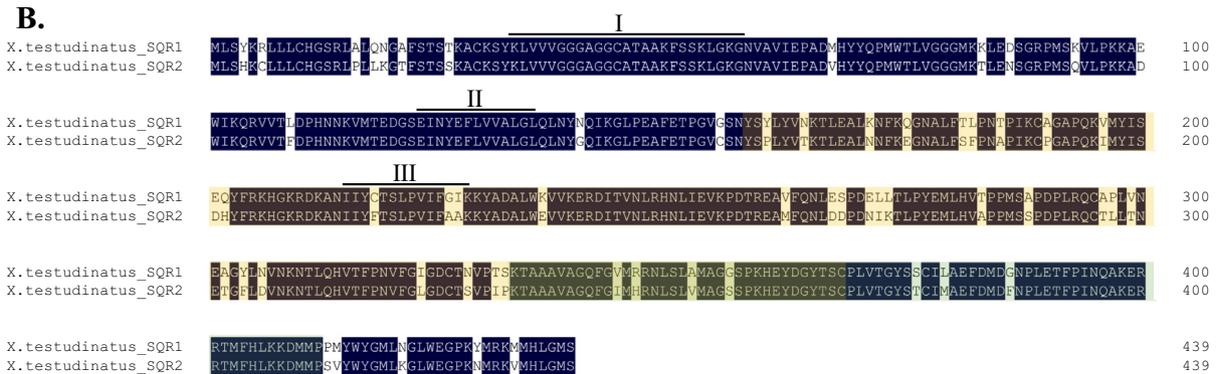
### 3.8. SQR enzymatic activity in *xtcrab* gills

The posterior gills of habitat *xtcrabs* ( $n = 6$ ) and aquarium *xtcrabs* ( $n = 6$ ) were also analyzed for SQR enzymatic activity. The sulfide-oxidation

**A.**



**B.**



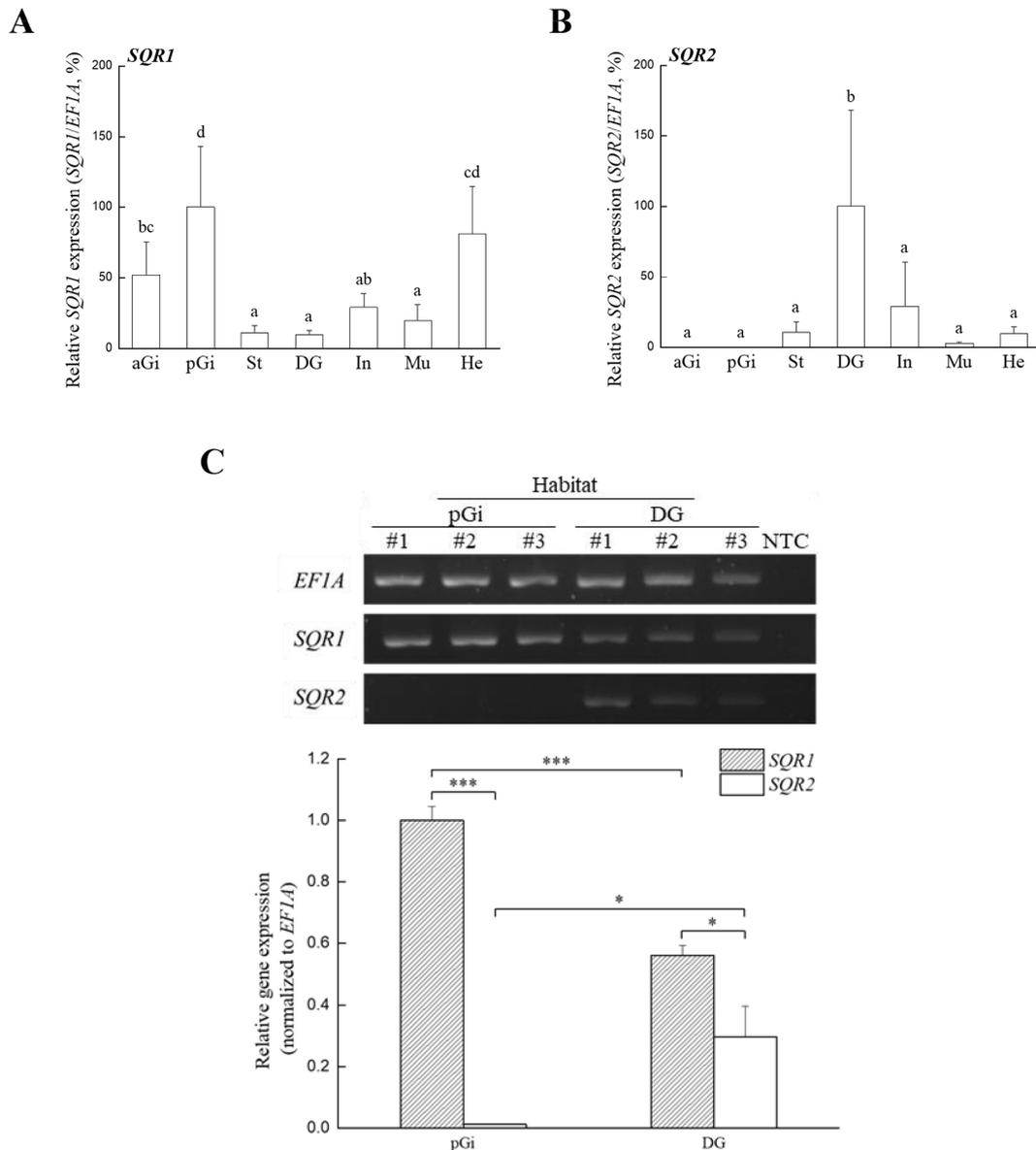
I – III : FAD-binding domain

Yellow box : Sulfide oxidation

Green box : Quinone reduction

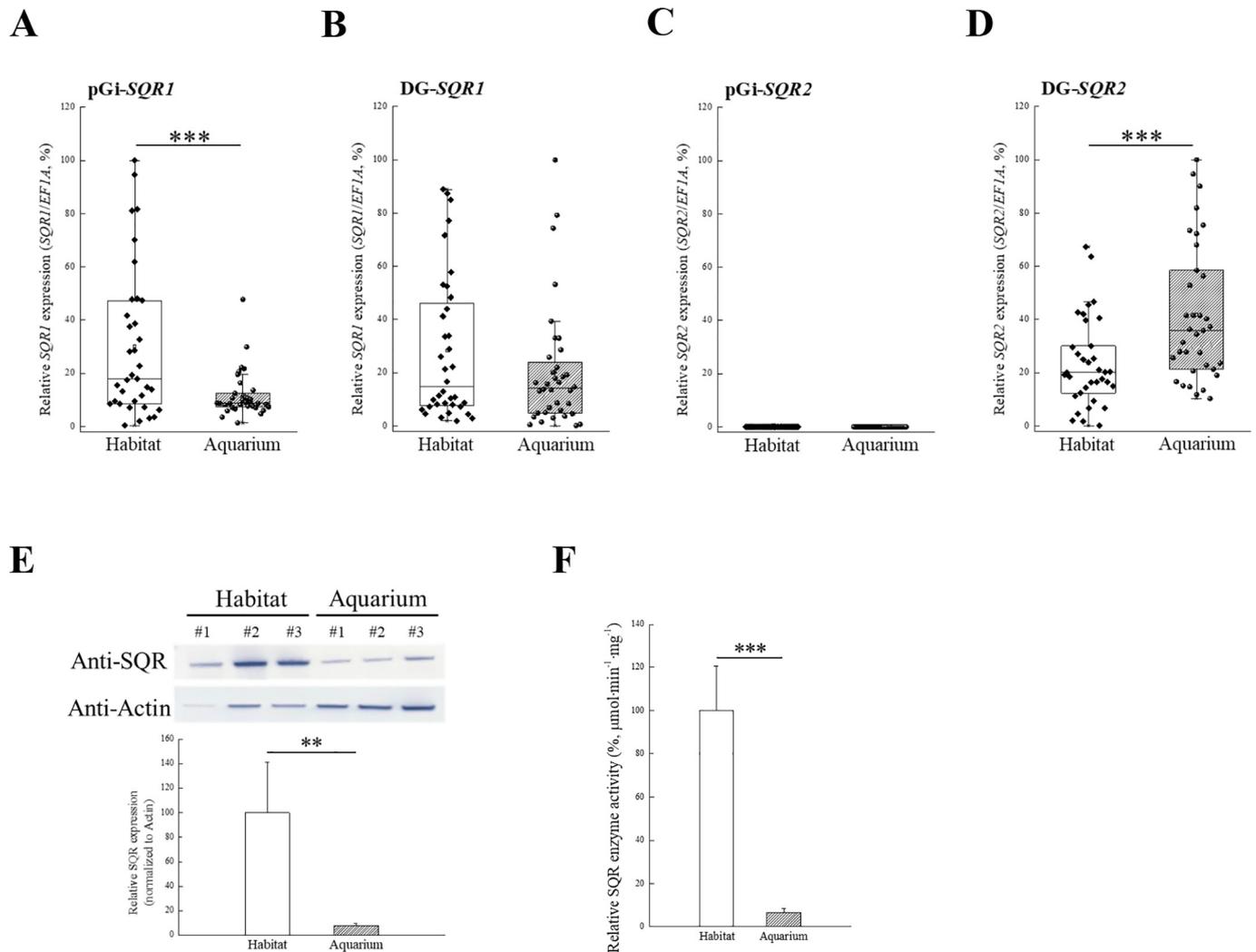
**C.**





**Fig. 4.** Tissue distributions of *SQR1* and *SQR2* transcripts in *xtrcrabs* residing in “habitat” and “aquarium”. Hydrothermal vent crabs *Xenograpsus testudinatus* (*xtrcrabs*) were collected from the Kueishan Island sulfide-rich hydrothermal vent region and immediately dissected (“habitat *xtrcrabs*”). Various tissues were sampled for qPCR analysis of SQR paralog transcript levels; (A) *xtSQR1* and (B) *xtSQR2*. Elongation factor 1A (*EF1A*) was used as a reference gene. Different letters indicate significant differences between tissues ( $p < 0.05$ ) (one-way ANOVA and Tukey multiple tests). Means  $\pm$  SD ( $n = 6$  *xtrcrabs*/group). aGi, anterior gill; pGi, posterior gill; St, stomach; DG, digestive gland; In, intestine; Mu, muscle; He, heart. (C) Transcript levels of *xtrcrab* SQR paralogs, *xtSQR1* and *xtSQR2*, as evaluated by semi-quantitative RT-PCR in the pGi and DG of “habitat” *xtrcrabs*. Signal quantitative analysis of RT-RCP products was calculated by ImageJ. Elongation factor 1A (*EF1A*) was used as a reference gene. NTC, no template control for RT-PCR reactions. Means  $\pm$  SD ( $n = 3$  *xtrcrabs*/group). Asterisks indicate significant difference between habitat and aquarium crabs (\*:  $p < 0.05$ ; \*\*\*:  $p < 0.001$ ).

**Fig. 3.** Sequences of *xtrcrab* *SQR1* and *SQR2* and comparison with other species. (A) Multiple sequence alignment of amino-acid sequences for SQRs from *Xenograpsus testudinatus* (*xtrcrabs*) and other species. In the hydrothermal vent *xtrcrab*, two SQR paralogs, *i.e.* *xtSQR1* (MT459642) and *xtSQR2* (MT459643), were identified and cloned in the present study. The alignment was performed with DNAMAN sequence analysis software. Consensus amino acids among the different SQR sequences are shown in white characters with black backgrounds. The SQR sequence of humans was obtained from NCBI (NP\_001258142.1). The SQR sequence of *Urechis unicinctus* was obtained from NCBI (ABR67864.1). Two sets of SRA raw reads were separately used to conduct the *de novo* assembly database with CLC Genome Workbench 8.0 to obtain the SQR sequences for *Scylla olivacea* (GDRN00000000.1) and *Cancer borealis* (GEFB00000000.1). (B) Sequences of *xtrcrab* SQR paralogs, *xtSQR1* and *xtSQR2*. Consensus sequences are shown in white characters with black backgrounds. I, II and III indicate the FAD-binding domains. The yellow box represents the sulfide oxidation domain. The green box represents the quinone reduction domain. (C) Phylogenetic analysis of SQR amino-acid sequences from hydrothermal vent crab *Xenograpsus testudinatus* (*xtrcrab*) and various other species, including other crustaceans, other invertebrates, and some vertebrates. Analysis was performed using neighbor joining method in MEGA7. The tree was rooted with bacteria (*Thiobacillus denitrificus*) SQR. Numbers indicate bootstrap values (% of 1000 replicates). *xtrcrab* SQR paralogs, *xtSQR1* and *xtSQR2*, were identified and cloned in the present study. The partial sequences of SQRs in *Eriocheir sinensis* and *Cancer pagurus* were cloned. The other SQR sequences were obtained from the NCBI database (Table S2). The red and green boxes indicate the two SQR paralogs identified and cloned in *xtrcrab* (*xtSQR1* and *xtSQR2*). Asterisks indicate partial sequences of SQR.



**Fig. 5.** SQR1 and SQR2 transcript levels, SQR protein expression and enzymatic activity in “habitat” and “aquarium” xtrabs. Hydrothermal vent crabs *Xenograpsus testudinatus* (xtrabs) were collected over multiple sampling periods from the Kueishan Island sulfide-rich hydrothermal vent region. xtrabs were immediately dissected (“habitat xtrabs”) or maintained for one month in normal (sulfide-free) seawater aquaria before dissection (“aquarium xtrabs”). Posterior gills (pGi) and digestive glands (DG) were sampled. (A–D) Transcript levels of xtrab SQR paralogs, *xtSQR1* and *xtSQR2*, as measured by qPCR in the pGi and DG of “habitat” and “aquarium” xtrabs. (A) Transcript levels of *xtSQR1* in pGi of “habitat” and “aquarium” xtrabs. (B) Transcript levels of *xtSQR1* in DG of “habitat” and “aquarium” xtrabs. (C) Transcript levels of *xtSQR2* in pGi of “habitat” and “aquarium” xtrabs. (D) Transcript levels of *xtSQR2* in DG of “habitat” and “aquarium” xtrabs. Elongation factor 1A (*EF1A*) was used as a reference gene. Means  $\pm$  SD ( $n = 35$  xtrabs/group). (E) SQR protein expression as measured by dot blot in the pGi of “habitat” and “aquarium” xtrabs. Signal quantitative analysis of dot blot was calculated by ImageJ. (F) SQR enzyme activity in the pGi of “habitat” and “aquarium” xtrabs. Mitochondria isolated from posterior gills were used for the SQR enzyme activity assay. Means  $\pm$  SD ( $n = 6$  xtrabs/group). Asterisks indicate significant difference between habitat and aquarium crabs (\*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ).

analysis showed that SQR enzyme activities in posterior gills of habitat xtrabs were significantly higher than that of aquarium xtrabs (15.5-fold increase,  $p < 0.001$ ) (Fig. 5F).

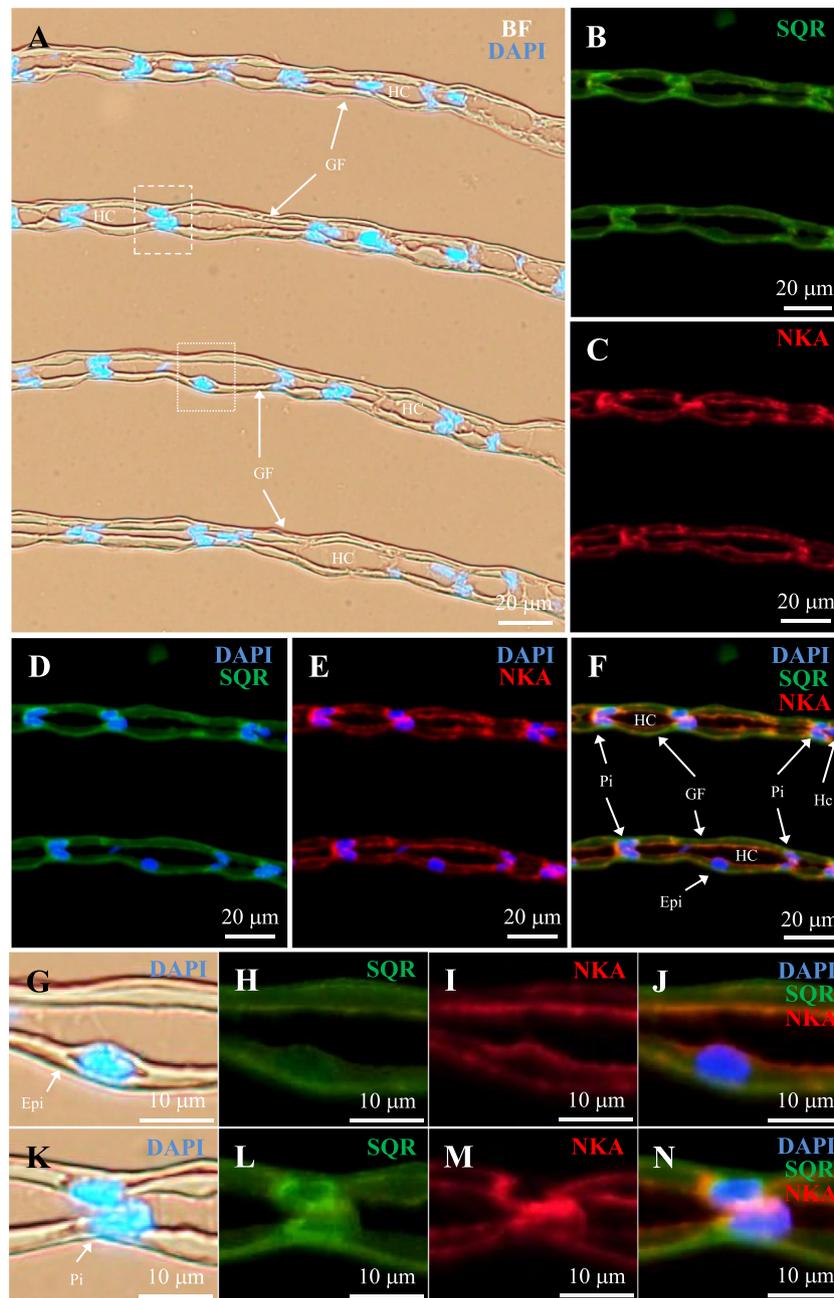
### 3.9. Localization of SQR-expressing cells in xtrab gills

Histological analysis with SQR-antibody immunofluorescence staining was then used to probe the localization of SQR expression in the gills of xtrabs. Cell nuclei were stained with DAPI and the structure of gill filaments was observed with the Bright Field vision under microscopy (Fig. 6A, G, K). Gill filaments consisted of two parallel layers of epithelial cells linked to each other by pillar cells, and surrounding the hemal channel (vessel) as described by Smolowitz (2021) (Fig. 6A, F). No positive signals for NKA or SQR were observed in the gill stem (data not shown). However, epithelial cells and pillar cells in the gill filament exhibited detectable levels of both NKA (Fig. 6C and E) and SQR (Fig. 6B and D). Notably, all SQR-positive cells also expressed NKA, according to sections that were co-

stained with DAPI, SQR, and NKA (Fig. 6F). The magnifications of epithelial cells (Fig. 6G–J) and pillar cells (Fig. 6K–N) in gill filament showed more details of the co-localization of SQR-positive cells and NKA-positive cells. Remarkably, we invariably found co-localization of SQR and NKA in each of >150 SQR-positive cells examined from five xtrabs.

## 4. Discussion

To cope with high sulfide ecological niches, animals have evolved diverse mechanisms for adaptation. For instance, avoidance behaviors that move to lower sulfide content environments in bivalves (Laudien et al., 2002), metabolic alternation that temporarily implements anaerobic respiration in crabs (Sanders and Childress, 1992; Thorpe et al., 1995), the symbiosis with bacteria that detoxifies sulfide in bivalves and crustacean hosts (Chiu et al., 2022; Sun et al., 2022), sulfide-resistant alternative oxidase (AOX) which substitutes the blocked COX in ascidian (Bremer et al., 2021), and the SQR enzymatic detoxification which detoxifies sulfide



**Fig. 6.** Localization of SQR-expressing cells in the gills. DAPI (4', 6-diamidino-2-phenylindole) staining was used to visualize cell nuclei (blue) in the gill filament (A, D, E, F, G, J, K, and N). Bright field under microscopy was used to observe the structure of gill filament (A, G, and K). Immunofluorescence staining was performed using the commercial anti-human SQR antibody (same as used for Western Blot, Fig. 6F). SQR (green) expression was observed in the gill filament (B). Immunofluorescence staining with  $\text{Na}^+/\text{K}^+$ -ATPase (NKA) antibody was performed to localize NKA (red) expression in the gill filament (C). The DAPI and SQR merged figure shows the localization of SQR in the gill filament (D). The DAPI and NKA merged figure shows the localization of NKA in the gill filament (E). The merged figure shows the co-localization of SQR and NKA signals in the gill filament (F). The magnified figures from the dot-square show the structure of the epithelial cell, the location of SQR, the location of NKA, and the co-localization of SQR and NKA signals in the epithelial cell (G-J). The magnified figures from the dash-square show the structure of the pillar cell, the location of SQR, the location of NKA, and the co-localization of SQR and NKA signals in the pillar cell (K-N). Note that the co-localization of SQR and NKA signals is observed in all the cells. HC: hemal channel; BF: Bright field; GF: gill filament; Hc: hemocyte; Pi: pillar cell; Epi: epithelial cell.  $n = 5$  xtrabs.

in various species (Vetter and Bagarinao, 1989; Johns et al., 1997; Hildebrandt and Grieshaber, 2008).

In this study, we performed a comprehensive analysis of the adaptive mechanisms of xtrabs to the shallow-water high-sulfide hydrothermal vent environment, with a special focus on SQR. To do so, we utilized methodological approaches ranging from field studies and ecophysiological experiments, to molecular and phylogenetic analyses. Overall, this study revealed that xtrabs have a remarkable tolerance to environmental  $\text{H}_2\text{S}$  via a strong detoxification capacity, and possess duplicate SQR genes, one

of which likely playing a key role in gill detoxification of aqueous environmental  $\text{H}_2\text{S}$ .

#### 4.1. High sulfide levels in seawater from Kueishan Island hydrothermal vent region

The fluids that erupt from its nearby shallow-water (<20 m depth) hydrothermal vents near Kueishan Island contain high concentrations of hydrogen sulfide, which is variably influenced by the surrounding waters depending on tidal flow (Chen et al., 2005). According to a previous

study on the site, the temperatures of bottom seawater where *xtcrabs* reside (Station 1) are  $30.0 \pm 1.5$  °C, which is significantly higher than the surface seawater ( $28 \pm 0.01$  °C) (Chan et al., 2016). Our seawater survey further showed broad ranges of pH (6.28–7.27), DO (4.15–7.28 mg/L), and sulfide (17.80–1448.67  $\mu$ M) in the *xtcrab* hydrothermal vent habitat. This variability in seawater parameters likely results from the intermittency of the vents, as well as from variations in wind and seawater currents. Despite the high variability of these parameters, the measured values were all significantly different from those in a non-hydrothermal vent region (Station 2; pH 8.01–8.17, DO 7.83–8.29 mg/L, no detectable sulfide). Similarly, the fluids from vent fissures in deep-sea (> 2500 m) hydrothermal vents are also quite different than the surrounding environments. A previous study by Arp and Childress (1981) revealed that seawater from a deep-sea hydrothermal vent had relatively high temperature (22 °C), anoxia, low pH (6.5), and high sulfide (350  $\mu$ M), and this water rapidly mixed with surrounding water that was characterized by low temperature (2 °C), hypoxia (110  $\mu$ M), higher pH (7.5), and no sulfide. As such, the deep-sea hydrothermal vent crab *Bythograea therymydon* is also exposed to highly variable conditions (Arp and Childress, 1981). Together, these data support the notion that Kueishan Island is an extreme high-sulfide environment similar to deep-sea hydrothermal vent regions, but in shallow-water.

#### 4.2. Remarkable survival and H<sub>2</sub>S detoxification by *xtcrabs* in high sulfide environments

We examined the sulfide tolerance of *xtcrabs* that had been previously maintained for 1 month in normal seawater by exposing the animals to high-sulfide seawater in aquaria. Even though the survival rates decreased with increased sulfide concentrations and exposure time, all *xtcrabs* exhibited prolonged ability to move after exposure to dissolved sodium sulfide nonahydrate up to 1000  $\mu$ M. In contrast, all *tdcrabs* in the control group died within 10 min exposure to 50  $\mu$ M sodium sulfide nonahydrate. Thus, *xtcrabs* have a much more robust sulfide tolerance than intertidal *tdcrabs*.

We also analyzed the responses of *xtcrabs* (previously maintained for 1 month in normal seawater aquarium) after immersion in either the high-sulfide hydrothermal vent region (station 1) or the normal (sulfide-free) seawater region (station 2). All *xtcrabs* survived immersion in either station. In contrast, all control species *tdcrabs*, died when immersed in station 1 (sulfide-rich), but the *tdcrabs* survived immersion in station 2 (normal seawater). This result further validated the strong sulfide tolerance of *xtcrabs* as compared to intertidal *tdcrabs*.

The toxicity of the sulfur compounds has been ordered from highest to lowest as follows: sulfide > thiosulfate > sulfite > sulfate (Ziosi et al., 2017). We found that the hemolymph concentrations of toxic sulfide in the *xtcrabs* immersed in the hydrothermal vent region (station 1) were similar to those in *xtcrabs* immersed in the sulfide-free seawater (station 2), likely reflecting a high capacity for sulfide catabolism. Conversely, the hemolymph concentrations of less toxic compounds, such as thiosulfate and sulfite, were significantly higher in the hydrothermal vent-exposed group compared to the normal seawater-exposed group. Thiosulfate concentrations were 6-fold higher than sulfite concentrations in the hemolymph of hydrothermal vent *xtcrabs*. Thus, our findings in *xtcrabs* are reminiscent of those in other crabs (Vetter et al., 1987), wherein thiosulfate is a predominant sulfur catabolic compound. These results suggest that *xtcrab* SQR enzymatic activity is highly effective at converting sulfide to thiosulfate and thereby keeping sulfide to low levels. The *xtcrabs* therefore exhibit a remarkable ability to maintain low levels of highly toxic sulfide in the body when facing a high-sulfide environment. Altogether these results suggest that even after *xtcrabs* are maintained in normal seawater for one month, the animals still retain a strong sulfide tolerance mechanism that likely involves efficient SQR-mediated detoxification.

#### 4.3. Duplicated SQR in *xtcrabs*

We cloned the cDNAs of two SQR gene paralogs from *xtcrabs* and named the genes *xtSQR1* and *xtSQR2*. The amino-acid sequences both

showed typical SQR functional domains, and our phylogenetic analysis revealed that *xtSQR1* and *xtSQR2* were both clustered with SQR genes from other crustaceans. This is the first demonstration of the presence of two SQR paralogs. In studies on various eukaryotes, single copies of the SQR gene were found in species ranging from yeast (Melideo et al., 2014) to vertebrates (Olson et al., 2012). Interestingly, we also found two paralogs of SQR in Jonah crab (*Cancer borealis*), edible crab (*Cancer pagurus*), mottled lightfoot crab (*Grapsus albolineatus*), terrestrial crab (*Sesarmops impressum*), and swimming crab (*Thranita danae*) while only single SQR genes were retrieved in green crab (*Carcinus maenas*), mud crab (*Scylla olivacea*), Chinese mitten crab (*Eriocheir sinensis*), red-eye reef crab (*Eriphia ferox*), freshwater crab (*Candidiopotamon rathbunae*), and common box crab (*Calappa lophos*).

Our phylogenetic analysis indicated that the duplication event that resulted in the two *xtcrab* paralogs was likely independent of the duplication event that occurred in the *Cancer* species, as there was no apparent orthology between *xtcrab* *SQR1/2* and *Cancer* *SQR1/2*. Additionally, our preliminary phylogenetic analysis on the identified short partial sequences from various crab species suggested that several other independent SQR duplication events may have occurred across the Brachyura lineage, such as in *Grapsus albolineatus* or in *Thranita danae*. Further investigation on whole sequences may allow to decipher the origin and evolution of duplicated SQR paralogs in Brachyura.

Thus, unlike the single SQR genes found in most eukaryotic genomes, duplicated SQR genes have been fixed and conserved through evolutionary selection in several crab lineages. In general, after a gene duplication event, one of the duplicated paralogs is typically lost due to degenerative mutations (non-functionalization). However, in some cases, both duplicated paralogs are conserved. In such cases, conservation of paralogs may be related to the amplification of their common function, or may allow for the development of functional differences between the paralogs, which could involve functional partitioning (subfunctionalization) or the emergence of a new function (neofunctionalization). Amplification, subfunctionalization, and neofunctionalization may all promote adaptation to environmental changes. For instance, in Antarctic notothenioid fish, the neofunctionalization of 118 duplicated genes is associated with an anti-freeze capability that is absent in non-Antarctic notothenioid fishes (Chen et al., 2008). In brachyuran crabs, the duplication of the key sulfide-detoxification enzyme SQR, observed in various species, is quite unique as a mechanism of animal adaptive evolution. As this group encompasses benthic species such as *Cancer* sp. that may inhabit H<sub>2</sub>S-rich underwater bottom sediments, the conservation of duplicated SQR paralogs may be related to enhancing H<sub>2</sub>S detoxification ability, which would deserve further investigation in these intertidal crab species. In the case of *xtcrab*, our findings suggest that the duplication and differential roles of SQR paralogs in *xtcrabs* may have been an important step in adaptation to the high-sulfide hydrothermal vent environment.

#### 4.4. Differential tissue distributions of duplicated SQR genes in *xtcrabs*

To the best of our knowledge, this study includes the first description of SQR tissue distribution in a crustacean. According to our qPCR analysis, *xtSQR1* was predominantly expressed in large posterior gills and heart, but it was also expressed in other tissues such as anterior gills, stomach, digestive gland, intestine and muscle. The wide *xtSQR1* expression pattern in various tissues might suggest the tissue protection function of *xtSQR1*. In contrast to the expression pattern of *xtSQR1*, *xtSQR2* was not detectable in the gill and was mainly expressed in the digestive gland. These clear differences between the two *xtSQRs* in terms of tissue distribution probably reflect distinct physiological roles for each gene and have likely contributed to the conservation of both paralogs. Expression of SQR in the gill has also been reported in deep sea hydrothermal vent shrimp (*Rimicaris* sp.); both RNA-seq and qRT-PCR showed higher transcript levels in sulfide-rich habitats compared to sulfide-free aquarium (Zhang et al., 2017). In different lineages of poeciliid fishes, RNA-seq on gill samples also showed upregulation of SQR transcript levels by exposure to a sulfide-rich environment (Greenway et al., 2020). Moreover, expression

of *SQR* in the digestive gland has been reported in other crustaceans, such as the oriental river prawn (*Macrobrachium nipponense*) (Zhu et al., 2021). In this species, *SQR* transcript levels were increased in the digestive gland under sulfide-rich water exposure (93.8  $\mu\text{M}$  sulfide for 48 h), as shown by both RNA-seq and qRT-PCR (Zhu et al., 2021). Expression of *SQR* in the digestive system was also reported in various metazoan animals, including the echiuran worm (*Urechis unicinctus*); in this worm, *usSQR* is most highly expressed in midgut, followed by anal sacs and coelomic fluid cells, with low expression levels in the hindgut and body wall (Ma et al., 2011).

#### 4.5. Potential detoxification of endogenously produced sulfur compounds by *xtSQR1* and *xtSQR2* paralogs in the digestive gland

In crabs, the digestive gland is responsible for the digestion of food, whereas the stomach is responsible for grinding food (Wang et al., 2014; Rady et al., 2018). Several studies reported that *xtcrab* are omnivores and likely feed on algae and zooplankton which are also the common diets for crab species (Jeng et al., 2004; Wang et al., 2022). We found that the digestive gland of *xtcrabs* expresses both *xtSQR1* and *xtSQR2* paralogs, with *xtSQR1* expressed at a higher level than *xtSQR2*. Notably, *xtSQR2* was predominantly expressed in the digestive gland. This expression pattern may suggest that in the crabs (similar to other terrestrial animals), hydrogen sulfide is endogenously produced from the metabolism of L-cysteine in foodstuffs (Wang, 2002).

Intriguingly, we found that *xtSQR2* transcripts were significantly upregulated in the digestive gland of crabs maintained in sulfide-free seawater aquarium as compared to those in the sulfide-rich habitat environment. In contrast, no change was observed in *xtSQR1* expression within the digestive gland. The increase in *xtSQR2* expression may be related to a higher rate of nutritional intake and the subsequent higher production of endogenous  $\text{H}_2\text{S}$  for *xtcrabs* maintained in normal seawater aquarium with abundant food as compared to their hydrothermal vent habitat. In any case, this pattern reveals a specific regulatory mechanism for *xtSQR2* expression in the digestive gland as compared to the stable expression levels of *xtSQR1*. Therefore, we suggest that in the digestive gland of the *xtcrab*, *xtSQR2* may play a key role (in concert with *xtSQR1*) in the detoxification of endogenous sulfide.

#### 4.6. Potential specific role of gill-expressed *xtSQR1* paralog in detoxification of environmental (exogenous) sulfur compounds

Gill is a major environment-facing organ in aquatic animals, which has essential functions in ion transport, osmoregulation and respiration. It may also be directly involved in stress acclimation, as evidenced by its expression of stress-related hormone receptors such as *corticosteroid receptors* and *corticotropin-releasing hormone receptors* in fish (Aruna et al., 2012, 2021). *SQR* is the enzyme that catalyzes the first step of sulfide detoxification in metazoans (Ziosi et al., 2017). In *xtcrab*, *xtSQR1* was widely expressed across various analyzed tissues, but it was predominantly expressed in gills and heart. In contrast, *xtSQR2* was barely detectable in these organs. Strikingly, *xtSQR1* expression in gills was significantly higher in *xtcrabs* residing in the hydrothermal vent region compared to *xtcrabs* maintained in normal seawater. Furthermore, we consistently found that *SQR1* transcripts as well as *SQR* proteins and enzymatic activity were significantly higher in the posterior gills of vent-residing habitat *xtcrabs* than aquarium *xtcrabs*. Thus, *xtcrabs* that have undergone long-term adaptation to the hydrothermal vent have developed high capacities for sulfide detoxification. Together, these results suggest a major and specific role for *xtSQR1* in the gills of *xtcrabs*. Furthermore, these findings highlight the implied role of *xtSQR1* duplicated paralog in the adaptation of gill function for detoxification of high concentrations of environmental sulfide, which may allow *xtcrab* adaptation to the hydrothermal vent environment.

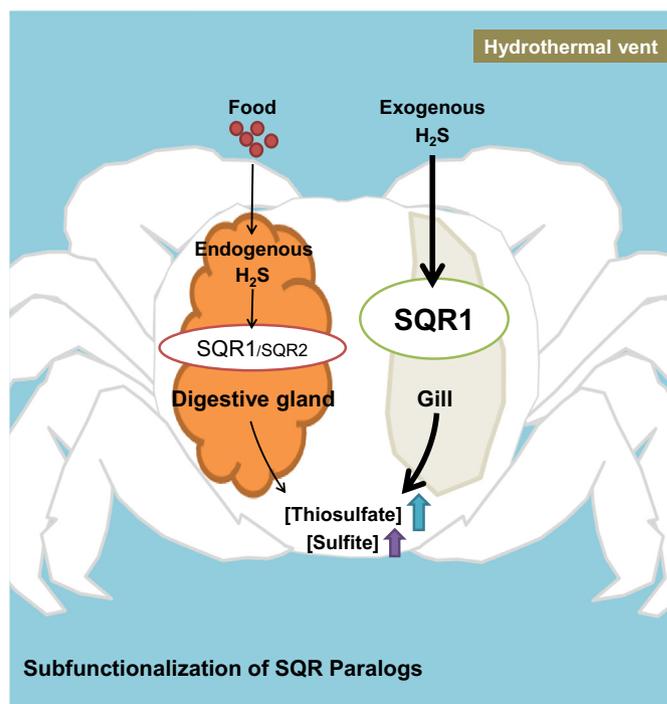
In different lineages of poeciliid fishes, RNA-seq on gill tissues revealed increased *SQR* transcript levels in sulfide-rich environments (Greenway et al., 2020). In addition, RNA-seq and qRT-PCR experiments on deep-sea hydrothermal vent shrimp showed increased *SQR* transcript levels in sulfide-

rich environments (Zhang et al., 2017). Together with our data, these findings suggest that upregulation of *SQR* expression by sulfidic environment may represent an ancient and conserved regulatory process in bilaterians. Since gill is a major organ exposed to the external environment, we suspect that the gill-expressed *xtSQR1* paralog might play a crucial role in *xtcrab* sulfide detoxification when environmental sulfide enters through the gills.

#### 4.7. Co-localization of *SQR1* and *NKA* in the pillar cells of gill filament

Using gill extracts, we showed that *SQR* protein detected with an anti-human *SQR* antibody were exclusively present in the organelle fraction, which includes mitochondria. In the echiuran worm, *SQR* signals are localized in mitochondria-rich regions of various tissues, including the body wall, hindgut, midgut and anal sacs (Ma et al., 2011). In mammals, *SQR* expression is also localized in mitochondria-rich regions of tissues, such as mouse colon, heart, lung, thyroid, penis and testis (Ackermann et al., 2014). Additionally, human *SQR* has been shown to co-localize with mitochondria (labeled with MitoTracker) in sperm, kidney and leukocytes (Ackermann et al., 2014).

In *xtcrab*, *SQR* expression was observed in some pillar cells of the gill filament. Remarkably, all observed *SQR*-positive cells were also *NKA*-positive cells. *NKA* cells are mitochondria-rich cells (chloride cells) known to be involved in the ion active transport, while *SQR* is part of the mitochondrial pathway of sulfide oxidation. Thus, *xtcrab* *SQR* is expressed



**Fig. 7.** Proposed sulfide detoxification mechanisms in the hydrothermal vent crab *Xenograpsus testudinatus*. Two paralogs of *SQR* were found in *X. testudinatus* (*xtcrabs*) and named *xtSQR1* and *xtSQR2*. Comparison of their tissue distributions revealed differential expression of the paralogs. *xtSQR1* is predominantly expressed in the posterior gill, and also expressed in various other tissues, including the heart and digestive gland. In contrast, *xtSQR2* is undetectable in the gill and mostly expressed in the digestive gland. Furthermore, *xtSQR1* expression is specifically upregulated in the gill in high-sulfide environments. We propose that *xtSQR1* paralog expressed in the gill should be largely responsible for the detoxification of exogenous (environmental)  $\text{H}_2\text{S}$ , while both *xtSQR1* and *xtSQR2* paralogs in the digestive gland would perform detoxification of endogenous (food-related)  $\text{H}_2\text{S}$ . *SQR* enzymatic detoxification of  $\text{H}_2\text{S}$  leads to increased thiosulfate levels in the hemolymph which maintains the sulfide homeostasis in *xtcrab*. The subfunctionalization of duplicated *SQR* genes may play an important role in sulfide detoxification in *X. testudinatus*, providing an ecophysiological basis for adaptation of this species to the high-sulfide hydrothermal vent environment.

in mitochondria-rich cells that consume energy (ATP) and are important for cellular respiration. Our seawater analysis showed that hydrothermal vent seawater not only has high sulfide concentrations, but it also has low oxygen levels. Therefore, we suggest that sulfide detoxification by mitochondria SQR may be critical for cellular respiration in *xtcrabs* in low oxygen conditions. We propose that *xtcrabs* have an evolutionarily selected SQR1 paralog that is mainly responsible for the detoxification of sulfide. By performing this function, the paralog would prevent the inhibition of cytochrome *c* oxidase by sulfide and maintain aerobic respiration in *xtcrabs* that reside in the sulfide-rich hydrothermal vent habitat.

## 5. Conclusion

In the present study we assessed the remarkable adaptation of *xtcrab* to their hydrothermal vent sulfide-rich environment, with high sulfide tolerance and sulfide detoxification capacity. We identified in *xtcrabs* two paralogs of SQR, providing the first evidence of the presence of duplicate SQR genes in crustaceans. *xtSQR1* is dominantly expressed in the gills and significantly increased in hydrothermal vent habitat compared to normal seawater environments. In contrast, *xtSQR2* is undetectable in the gill, and mostly expressed in the digestive gland. We suggest that the *xtSQR1* paralog in the gills might be majorly responsible for detoxification of exogenous sulfide from H<sub>2</sub>S-rich environment, while *xtSQR1* and *xtSQR2* in the digestive gland might cooperatively detoxify endogenous sulfide related to feeding (Fig. 7). The observed differential tissue expression and regulation of *xtSQR1* and *xtSQR2* reflects subfunctionalization of the duplicated genes, which may have led to the conservation of both paralogs through evolutionary selection. The duplication and subfunctionalization of SQR in *xtcrab* may thus play a key role in sulfide homeostasis and adaptation to the hydrogen sulfide-rich habitat of this hydrothermal vent crab.

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## Ethics

All procedures and investigations followed the standard guidelines and principles of the NTOU Institutional Animal Care and Use Ethics Committee.

## CRedit authorship contribution statement

**Chi Chen:** conducted the sample collection, developed the methodologies in *xtcrab*, performed the experiments, data curation and analyses of data, and wrote the original draft. **Guan-Chung Wu, Yung-Che Tseng and Ching-Fong Chang:** developed the concept of the study, guided the experiments, evaluated the data, edited the paper, and acquired funding. **Yao-Tse Chung, Hau-Wen Li, and Pei-Hsuan Chou:** contributed to sample collection, data curation, and analyses of data. **Sylvie Dufour:** gave important input into conceptual and mechanistic insights, reviewed and edited the paper. All authors approved the paper.

## Data availability

Data will be made available on request.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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