Molecular Mechanisms of the Convergent Adaptation of Bathypelagic and Abyssopelagic Fishes

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Abstract

Harsh environments provide opportunities to study how different species adapt, at the molecular level, to similar environmental stressors. High hydrostatic pressure, low temperature, and absence of sunlight in the deep-sea environment are challenging conditions for gene expression, cell morphology and vision. Adaptation of fish to this environment appears independently in at least 22 orders of fish, but it remains uncertain whether these adaptations represent convergent evolution. In this study, we performed comparative genomic analysis of 80 fish species to determine genetic evidences for adaptations to the deep-sea environment. The 80 fishes were divided into six groups according to their order. Positive selection and convergent evolutionary analysis were performed and functional enrichment analysis of candidate genes was performed. Positively selected genes (pik3ca, pik3cg, vcl and sphk2) were identified to be associated with the cytoskeletal response to mechanical forces and gene expression. Consistent signs of molecular convergence genes (grk1, ednr, and nox1) in dark vision, skin color, and bone rarefaction were revealed. Functional assays of Grk1 showed that the convergent sites improved dark vision in deep-sea fish. By identifying candidate genes and functional profiles potentially involved in cold, dark, and high-pressure responses, the results of this study further enrich the understanding of fish adaptations to deep-sea environments.

Key words: convergent evolution, positive selection, bathypelagic and abyssopelagic fishes, GRK1.

Introduction

The ocean occupies 70.55% of the Earth’s surface area, which constitutes the largest area of the natural environment (Costello et al. 2010). Shallow seas have been extensively studied because of their moderate temperatures and pressures as well as abundant food sources dependent and enriched by sunlight. These factors make shallow seas the most hospitable regions in the ocean. In contrast, fewer species inhabit deep-sea areas because of the extremely harsh living conditions, such as tremendous pressure,
Many deep-sea fish species possess physiological adaptations to high pressure, low temperatures, and darkness. In shallow-sea species, tens of atmospheres of pressure can disrupt enzyme ligand binding, kinetics and cytoskeletal protein assembly. In contrast, deep-sea fish are more resistant to high pressure (Lan et al. 2018). The ability of cells or tissues in an organism to respond to mechanical stimuli is important for normal functions, such as the formation and maintenance of bones, blood vessels, muscles, and other tissues, as well as blood pressure regulation, cell movement, and cell proliferation (Orr et al. 2006). The basis for many of these functions involves mechanosensitive adhesions mediated by membrane protein integrins. The force exerted by a cell’s own cytoskeleton or external sources enhances adhesions, resulting in various intracellular signals (Del Rio et al. 2009; Friedland et al. 2009). For instance, the expansion of two adhesion gene families (syndecan and protocadherin) occurs in deep-sea mussels (Sun et al. 2017). The helical structure of DNA and RNA is slightly affected by hydrostatic pressure, while other nucleic acid structures, such as hairpin loops and gene transcription, can be more sensitive to hydrostatic pressure (Macgregor Jr 1998; Munro et al. 2015; Patra et al. 2018). At low temperatures, DNA and RNA tend to tighten their structures, hindering the involvement of enzymes in DNA replication, transcription, and translation, as well as disrupting the transcription and translation processes (Lan et al. 2018). In a dimly light environment, rods are used for vision. Studies on rod opsins, photosensitive pigment, and the retina have been conducted on the vision of deep-sea fishes (Denton and Warren 1957; Wagner et al. 1998; Musilova et al. 2019). In low-light environments, the color of deep-sea fish is light compared to that of shallow-sea fish. Species living below 200 m are either red or black; the color of most benthic species is light yellow to red (Johnsen 2005). The bones of deep-sea fish have different levels of reduction (dimensional reductions, loss of skeletal elements, and reductions in bone density) that reach neutral buoyancy. For example, Gonostoma elongatum (Isospondyli) and Xenodermichthys copei (Isospondyli) have fragile, lightly ossified skeletons (Denton and Marshall 1958). Research on snailfishes indicates that, with an increase in depth, a decrease in light levels, and changes in temperature, the density and size of bones are affected (Gerringer et al. 2021). With increasing depth, a progressive decrease in skeletal ash content occurs (Childress and Ngageard 1973).

The deep sea is a less-explored part of the ocean; additionally, deep-sea fish adaptations independently appear in at least 22 orders (Randall and Farrell 1997). Due to the difficulty in obtaining deep-sea fish samples, most studies on deep-sea adaptations are based on the adaptation of single species, and the convergence of species has only been studied at the level of mitochondrial and opsin genes (Musilova et al. 2019; Shen et al. 2019; Lupse et al. 2021). As samples of deep-sea fish become more available and complete genomes are mapped, studies on the convergence of deep-sea adaptation will be conducted on a larger scale.

We analyzed 80 genomes of different bathypelagic and abyssopelagic fishes in six taxonomic groups. To identify candidate genes associated with deep-sea adaptations, single-copy genes were searched for positively selected genes associated with deep-sea stresses. To identify patterns of molecular convergence, convergent amino acid substitutions were identified.

**Results**

**Final Alignment Construction and Phylogeny**

We obtained genome-wide single-copy sequences from 80 fish species, including 36 bathypelagic and abyssopelagic fishes, 43 shallow-sea fishes and zebrafish (supplementary table 1, Supplementary Material online). The fish represent six orders. Gadiformes had 19 deep-sea fish, Beryciformes had seven deep-sea fish, Scorpaeniformes and Pleronectiformes each had three deep-sea fish, Perciformes had two deep-sea fish, and Ophidiiformes had one. The orthologous genes of the six orders were identified for construction of a phylogenetic tree (fig. 1).

**Positive Selection on Six Groups and Kyoto Encyclopedia of Genes and Genomes Function Enrichment**

To detect genes that evolving under positive selection in the branches leading to the deep-sea species, branch-site tests were run separately for the six groups. Positive selection was calculated for each deep-sea fish, with only one deep-sea fish in each group for each calculation. See Beryciformes (fig. 2A) as an example. Many positive selection genes were found in all orders (fig. 2B). Positive selection genes were enriched by kyoto encyclopedia of genes and genomes (KEGG) and plotted according to P-values showing that there were many pathways enriched in each order (fig. 2C). Several interesting enriched pathways were revealed. All six orders were enriched in “Focal
adhesion” pathway, and we found that Vinculin (vcl) was under positive selection in five species fish belonging to three orders (Beryciformes, Gadiformes, and Lophiiformes) (figs. 2B and 3A). Protein interaction networks linked two other positive selection genes, Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha (pik3ca) and phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit gamma (pik3cg), through ras, paxillin, and actin (fig. 3B). Pik3ca had one positively selected site in seven deep-sea fishes in the Gadiformes (fig. 3C) and pik3cg had one positively selected site in six deep-sea fishes belonging to Gadiformes and Beryciformes (fig. 3D). The positive-selection site of Pik3ca was located in the Ras-binding domain and that of Pik3cg was located in the C2 domain. Phosphoinositide 3-kinases (PI3Ks) are a family of lipid kinases that can phosphorylating the 3’OH of the inositol ring of phosphoinositides. Our results revealed that seven deep-sea fishes of pik3ca and six fishes of pik3cg did not overlap. The PI3K-related genes were positively selected in 13 deep-sea fish. “Sphingolipid metabolism,” “Calcium signaling pathway,” “Sphingolipid signaling pathway,” “Phospholipase D signaling pathway,” “VEGF signaling pathway,” “Apelin signaling pathway,” “Fc gamma R-mediated phagocytosis”; among the seven pathways, the Sphingosine kinase 2 (sphk2) gene was positively selected (fig. 2C). Three positively selected sites in Sphk2 involving 14 deep-sea fishes and three groups (Gadiformes, Beryciformes, and Lophiiformes) were identified (fig. 3E). No species in which Sphk2 showed all three positively selected sites, and five possessed two positively selected sites (fig. 3F).

Convergent Evolution in Six Groups and KEGG Function Enrichment

To estimate molecular convergence at the amino acid level, posterior expected numbers of convergent and divergent substitutions between pairs of deep-sea branches for the six groups were calculated separately. In each order, we first carried out the convergence between two branches, and then made statistics of all the convergent genes. Beryciformes is used as an example and shown in fig. 4A. Because Lophiiformes has only one deep-sea fish species, we were unable to do intra-order convergence calculations. In addition, we did not discover any convergent genes in Pleuronectiformes. All the convergent genes were analyzed statistically, and the vast majority of the convergent genes only converged in two branches (fig. 4B). KEGG enrichment was carried out for all the convergent genes, and the results showed that the convergent genes involved multiple pathways. There were not many overlapping genes among branches, but there were many overlapping pathways. KEGG pathway analysis showed that convergent genes were enriched in multiple adipose-related metabolic pathways.
Fig. 2.—Results of positive selection analysis were presented. (A) Using Beryciformes as an example, positively selected genes were calculated by labeling branches A, B, C, and D respectively. (B) Positively selected genes statistic of six groups. The left bar graph shows the number of positively selected genes in each group, and the upper bar graph shows the number of statistical units, which are shown by dots and lines. (C) In the KEGG functional enrichment pathway diagram of positively selected genes, the bubble size represents $-\log_{10}(P)$, and the color represents the enrichment fold, which is the ratio of the probability of actual enrichment to the probability of random enrichment. See supplementary table 4, Supplementary Material online for abbreviations of species names.
pathways, especially the “biosynthesis of unsaturated fatty acids” (fig. 4C). Previous studies demonstrated the positive role of unsaturated fatty acids in the deep-sea environment (Bell et al. 1986; Kamimura et al. 1993; Petursdottir et al. 2008; Saito and Hashimoto 2010). Gadiformes contained the largest number of deep-sea fish species in this study. We found several Gadiformes-specific convergent genes involving many fish. One site of NADPH oxidase 1 (nox1) was detected that showed a sign of convergent evolution in six species of Gadiformes (fig. 5A). The endothelin receptor-B gene (ednrb) was identified at one convergence site in six deep-sea species in the Gadiformes (fig. 5B).

Rhodopsin kinase (grk1) was identified at three convergence sites in 10 deep-sea species in the Gadiformes. Domain prediction revealed two convergence sites in the regulator of the G-protein signaling domain, and one convergence site in the serine/threonine protein kinases catalytic domain, which added a phosphorylation site (fig. 5C and supplementary table 2, Supplementary Material online). The effect of convergence site mutation on Grk1 was examined. A kinase assay showed that the enzyme activity of Grk1 was more active than that of wild zebrafish after the mutation of the converging sites, either separately or together. In particular, mutation of site 402...
Results of convergent evolutionary analysis were presented. (A) Using Beryciformes as an example, the A, B and C, D branches were labeled, and the gene convergence of the two branches was calculated. (B) Statistics of convergent genes per two branches. The left bar graph shows the number of the gene convergence of the two branches was calculated. (C) In the KEGG functional enrichment pathway diagram of convergence genes, the bubble size represents -log10 P-value, and the color represents enrichment fold, which is the ratio of the probability of actual enrichment to the probability of random enrichment. See supplementary table 4 for abbreviations of species names.
Molecular Mechanisms of the Convergent Adaptation

**Discussion**

**Visual Adaptation to the Dark Environment**

The vision of deep-sea fish vision is a topic of considerable interest. We found that convergent genes and positively selected genes were enriched in phototransduction signaling pathways. Grk1 was identified at three convergence sites in deep-sea species in the Gadiformes. Additionally the N402S site mutation due to the introduction of a phosphorylate at position 402 of Grk1. This process is usually called photoreceptor desensitization, and is physiologically related to adaptation to darkness (Kühn and Dreyer 1972; Ohguro et al. 1995; Hurley et al. 1998; Qingming et al. 1998) (fig. 6A). Deficiency or mutation of grk1 leads to impairment in scotopic vision (Yamamoto et al. 1997; Dryja 2000). Grk1 is also under positive selection in nocturnally active birds (Wu 2019). Some deep-sea demersal fishes migrate vertically or horizontally day and night (Gutsugu and Ohta 2005). Changes in grk1 may have enhanced dark vision in deep-sea fishes and improved their predation and predation-avoidance capabilities.

**Other Adaptations to the Deep-Sea Environment**

Hundreds of atmospheres of hydrostatic pressure can destroy the cytoskeleton organization and change cell morphology. The special connection between cells can maintain the tissue structure composition of the cells. Vcl, is an important component of focal adhesion, mediates the connection between the extracellular matrix and cytoskeleton, and participates in integrin-mediated cytomechanic-chemical signal transduction (Hu et al. 2009). Focal adhesions recognize mechanical forces generated either by either an external source or the cell itself and then translate them into a biological response. Vcl is a key protein regulating the

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**Fig. 5.**—(A) Convergence site of Nox1. (B) Convergence site of Ednrb. (C) Convergence sites of Grk1. Two convergence sites are located in RGS and S_TKc domain respectively. RGS: Regulator of G protein signaling domain; S_TKc: Serine/Threonine protein kinases, catalytic domain; S_TKc: Extension to Ser/Thr-type protein kinases. (D) Kinase activities of zebrafish Grk1, zebrafish G144N Grk1, zebrafish Q152R Grk1, and zebrafish N402S Grk1 and all three sites were mutated Grk1. The error bars are the 95% confidence interval. The color of the square in front of the name of a species represents the maximum depth that the species inhabits. Consensus (50%) represents a consensus sequence at 50% conservation. The consensus sequence greater than 50% of the threshold is uppercase, and the consensus sequence less than 50% is lowercase. The histogram represents the conservation of each site in all species of six orders.

N (Asn) to S (Ser) significantly increased kinase activity (fig. 5D).
transmission of contractile forces (Yang et al. 2010). The strength of the connection between the integrin adhesion receptors and the actomyosin cytoskeleton mediated by focal adhesion proteins determines the ability to generate and transmit forces. Contractile force generation is reduced when vinculin is absent and enhanced when vinculin is present (Mierke 2009). When pressure increases, vinculin is reduced and cell contraction is influenced (Yang et al. 2010). Focal adhesion complexes can connect the cytoskeleton with the adhesion site and trigger the downstream PI3K signaling pathway. Pik3ca and pik3cg regulate cell proliferation, differentiation, apoptosis, migration, and other physiological processes. This is accomplished by producing second messenger phosphatidylinositol 3,4,5 trisphosphate (PIP3) and acting on downstream molecules, such as mechanistic target of rapamycin complex 1 (mTORC1), Glycogen synthase kinase-3 (GSK3), and B-cell lymphoma 2 (Bcl-2) (König and Ostendorf 2015) (fig. 6B). Vcl is involved in sensing mechanical forces, and the cytoskeleton responds to changes in cell morphology. Pik3ca and pik3cg, as downstream genes, are involved in regulating various physiological processes and responding to them. We hypothesized that vcl, pik3ca, and pik3cg may contribute to a fish’s resistance to deep-sea pressure.

In addition, the KEGG pathway showed that nox1 is involved in osteoclast differentiation. Nox1 and mitochondria are involved in receptor activator of nuclear factor-κB ligand (RANKL)-mediated osteoclastogenesis. This is because Reactive oxygen species (ROS) produced by Nox1 acts as an intracellular signal mediator for osteoclast differentiation (Lee et al. 2005; Chen et al. 2016). Osteocytes are considered to be mechanosensory cells. The mechanical forces acting on tissues inform osteoblasts and osteoclasts to adapt to them by adjusting the amount and space of the tissue (Burger et al. 1995). The balance between osteoclasts and osteogenesis is the key to maintaining normal bone mass (Xu and Teitelbaum 2013). A deceleration of bone mineralization occurs due to an increase in the number of osteoclasts or a decrease in the number of osteoblasts (Roy et al. 2002). The bones of deep-sea fish have different levels of reduction and nox1 may provide a breakthrough.

Sphk2 catalyzes the phosphorylation of sphingosine to form sphingosine-1-phosphate (S1P), this could indicate an interaction with histone and inhibit its deacetylase 1/2 (HDAC1/2) activity, resulting in an increase in histone acetylation and transcription of downstream target genes (Hait et al. 2009). Core histone acetylation and deacetylation determine chromatin transcriptional activity and are closely related to gene regulation. Both low temperatures and high pressure inhibit the early stage of translation and reduce translation efficiency (Simonato et al. 2006). High pressure is stressful to life, because it forces a decrease in cell volume (Somero 1992). DNA is wrapped by histones on the chromosomes of eukaryotes. Histone acetylation is an epigenetic modification. Acetylation eliminates the positive charge of histone lysine and reduces its binding force to...
DNA (negatively charged), so that it can untwist the original tight chromosome structure and transform it into a looser form which is conducive to transcription and enhanced gene expression.

*Ednrb* is a G-protein-coupled seven-transmembrane receptor that interacts with a family of ligands, such as endothelins. Endothelin-1 is a major regulator of vascular function, and Ednrb is important for the control of vascular reactivity and blood pressure (Mazzuca and Khalil 2012). KEGG pathway analysis showed that ednrb is involved in melanogenesis. When the *ednrb* gene is deleted or suppressed, an almost complete loss of pigmentation occurs (Hosoda et al. 1994; Lee et al. 2003). The *ednrb* mutant shows a less dramatic phenotype with white spotting limited to about 20% of the coat (Hosoda et al. 1994). By studying mutations and polymorphisms affecting pigmentation and patterning, ednrb mutations are found to manifest in the white frame overo markings of horses, the white spots or fully white phenotype in mice, and the reduced melanophores in zebrafish (Mills and Patterson 2009).

The expression levels of Ednrb from four color skins tones in goldfish skin, ranging from red > cyan > black, and the rare white skin (Gan et al. 2021). Ednrb was convergent in six fishes of Gadiformes, but there was no obvious convergent body color in these six fishes, and its function needs further study.

### Conclusion

Determining the genetic basis of adaptive traits can be challenging. Through the positively selection and convergence analysis, to find a lot of interest genes, function enrichment shows lots different pathway. Adaptation to deep-sea environment may differ in the evolution of each species, but there are some common adaptive approaches, such as unsaturated fatty acid metabolism, reactions to mechanical force, histone acetylation, reduced bone content, and enhanced dark vision. The 80 fishes we studied included 28 fishes in the Gadiformes, a total of 36 deep-sea fishes including 19 fishes in the Gadiformes. The high proportion of Gadiformes also contributed to the majority of the convergent genes involved in Gadiformes to a certain extent. Gadiformes have also emerged as a unique lineage of all deep-sea fishes in the study of the evolution of the immune system in deep-sea fishes (Malmström et al. 2016). Andriashev divided the deep-sea fish fauna into ancient deep-sea forms and secondary deep-sea forms 1953. Gadiformes correspond to ancient deep-sea forms and are characterized by worldwide distribution, specialized morphology, and occupation of the deepest parts of the ocean (Priede and Froese 2013). Our results support the hypothesis that there are many specific sites in Gadiformes that may be functionally involved in deep-sea adaptation. Deep-sea invasive families, as secondary forms, such as Ophidiidae and Liparidae, make the greatest contribution to the fish fauna at depths >6000 m. With advances in ultra-abyss sampling technology, more abyss species will be discovered in the future, and these will allow Andriashev’s hypothesis to be tested at the molecular level.

### Materials and Methods

#### Source of Data

A total of 337 fish genomes were available at NCBI (https://www.ncbi.nlm.nih.gov/) as of July 2020. Information on the depth-ranges of these fish was collected from FishBase (https://www.fishbase.de/), OBIS (https://obis.org/), and Shen et al. (2019). Deep-sea fishes are usually considered those living at depths below 1,000 m (Angel 1997; Pradillon and Gaill 2007). The 337 species were classified by order. If no deep-sea fish species were found, the entire order was not studied further, leaving 180 species for consideration. The depth ranges of 34 species of the 172 fish species fell below 1,000 m (supplementary table 3, Supplementary Material online). For Perciformes, many shallow-water-dwelling sister taxa were available, and 10 species were selected according to the families of deep-sea fishes. In addition, four deep-sea fish genomes from the laboratory of this study were added. Considering that the zebrafish genome is well known, the zebrafish genome was used as the reference species. Finally, the remaining 36 deep-sea fishes and 44 shallow-living fishes were used in this study, and were placed into six groups according to the orders: 1) Beryciformes, 2) Gadiformes, 3) Lophiiformes, 4) Perciformes, 5) Pleuronectiformes, and 6) Scorpaeniformes.

#### Identification of Single-Copy Genes

The homologous genes in Fugu, Japanese medaka, Stickleback, Atlantic cod, Turbot, and Platyfish were researched in ENSEMBL (https://www.ensembl.org/) using BioMart tools with the Zebrafish genome as a reference based on one-to-one orthologs. Single-copy genes of these species were extracted based on one-to-one orthologs. The 10,058 single-copy homologous genes in at least six species were taken as the single-copy homologous gene set.

The genomes of 76 species from NCBI were downloaded. Our laboratory possesses the genomes of *Coryphaenoides rudis, Pseudoliparis swirei, Liparis tanakae,* and *Pachycara angeloii* sp.nov, and the genomes of these four species were included in this study (supplementary table 1, Supplementary Material online). Zebrafish protein sequences were extracted from protein data (Danio_rerio.GRCz11.pep.all.fa downloaded from NCBI) using a single-copy homologous gene set. TBLASTN (E-value cut-off 1e-3) (Altschul 2012) was used for the genomes of 80 species to determine the positional information.
of corresponding homologous genes. Scattered alignments were merged for each gene using Sorting Out Local Alignment Results v0.9.3 (Yu et al. 2006). The merged results with a cut-off <50 (cut-off being the aligned protein length/total protein length) were removed. After the cut-off selection, GENEWISE (Birney et al. 2004) was used to compare each zebrafish protein sequence with its optimal merged DNA segment sequences separately. GENEWISE scores >35 were used, and coding sequences were the resultant output. After the implementation of GENEWISE, homolog coding sequences of 80 species were obtained (supplementary table 4, Supplementary Material online).

Phylogenetic Inference

Phylogenetic trees were constructed of the six groups with zebrafish using single-copy orthologous genes. Each single-copy coding sequence of a group was translated into protein sequences and aligned against the zebrafish protein sequences using MAFFT v7.453 (L-INS-I) (Katoh and Standley 2013). Gblocks version 0.91b (Castresana 2000) extracted the conserved sites of multi-sequence alignment results of orthologous genes with option (−t = P, −b4 = 5, −b5 = h). A maximum likelihood (ML) tree was computed with IQ-TREE v2.0.3 (Lam-Tung et al. 2015) and RAxML v8.1.24 (raxmlHPC-HYBRID-SSE3 program) (Stamatakis 2014). ML trees of eight groups were constructed using the same method as previously described. ProtTest (Darriba et al. 2011) calculated the best amino acid substitution model required by RAxML (supplementary table 5, Supplementary Material online). These trees were visualized, rooted, and annotated using iTOL (Letunic and Bork 1988).

Positive Selection Analyses

The topologies of the six groups were used as the guide tree for positive selection analyses. Each group was separately tested for positive selection. The base-substitution mutation rate of non-synonymous mutation was dW, and the base-substitution mutation rate of synonymous mutation was dS. If dW/dS > 1, also called positive selection, this signified that most non-synonymous mutations are favorable and retained in long-term evolution, and positive selection is the main driving force of biological evolution. The branch-site model of CodeML implemented in the PAML package (Yang 2007) was used to test for positive selection on individual codons along the lineage leading to each of the deep-sea species. PRANK v.170427 (Löytynoja 2014) was used to conduct multiple nucleotide alignments for coding sequences of single-copy orthologous genes. In order to keep the positively selected sites conservative, Gblocks was used to filtered unreliable blocks (−t = c, −b5 = h). The dW/dS ratios of filtered reliable codons were calculated by setting each deep-sea fish as the foreground branch and other shallow-sea fishes of the same group as background branches. To detect whether the positive selection signs were significant, likelihood ratio test statistics were calculated and P-values were tested by the false discovery rate (FDR) method in R version 4.1.0 (Team 2019). The FDR used a P-value ≤ 0.05 as the significance level. Meanwhile, check the result file after “Positive sites for foreground lineages Prob(W > 1)” for positively selected sites, if there is more than one positively selected site is considered as positive selection gene. Unfiltered original sequences containing positively selected genes of six orders were extracted from all species and MEGA (Kumar et al. 2016) was used to check for conserved and consistency of positively selected sites in all deep-sea fishes.

Amino-Acid Convergence Analyses

PRANK v.170427 (Löytynoja 2014) was used to conduct multiple nucleotide alignments for coding sequences of single-copy orthologous genes and Gblocks to filtered unreliable blocks (−t = c, −b5 = h). In order to keep the convergence sites conservative, Gblocks was used to filtered the aligned bad blocks. Because the qualities of downloaded genomic data are different, if the number of species is too large, the filtered sequences will be too short, and many sites will not get convergence calculation. To ensure that convergence can be calculated for as many genes and sites as possible, each of the six orders was separately tested for convergent evolution. Grand Convergence (Castoe et al. 2009) was used to calculate the expected posterior numbers of convergent substitutions across all branch pairs. Since the Grand Convergence software was set up to mark only two branches at a time, we performed convergence calculations for every two branches of deep-sea species in each order. The program was run with the setting −free-b1 = 1 to automatically estimate branch length by using the LG amino acid substitution model. A P-value (convergence) > 0.80 was considered to be a convergent site. Genes containing at least one convergent site were considered convergent genes. The convergent genes of all species were extracted and aligned. Unfiltered original sequences containing convergence genes of six orders were extracted from all species and MEGA (Kumar et al. 2016) was used to check for conserved and consistency of convergence sites in all deep-sea fishes.

Gene Annotations and Functional Analysis

SMART (Letunic et al. 2011) and InterPro (Hunter et al. 2012) facilitated the identification and annotation of domains of positive selection genes and convergence evolution genes. The protein–protein interaction database STRING (https://version11.string-db.org/) was applied to explore the interactions among candidate genes. The KEGG (https://www.genome.jp/kegg/) was used to ascertain the
functions of candidate genes. The NetPhos 3.1 server predicted serine, threonine, or tyrosine phosphorylation sites in eukaryotic proteins using ensembles of neural networks (Blom et al. 2004).

Gene Synthesis, Plasmid Constructs, Cell Culture, and Transfection
To determine whether the convergence sites affected the Grk1 kinase activity, the grk1 gene and the mutant sites (G144N, Q152R, N402S and all three sites were mutated) from zebrafish were synthesized. The lentiviral vectors pCDH-CMV-MCS-EF1-copGFP-T2A-Puro were double-digested with NheI and BamHI endonuclease, and the T4 ligase was used to insert the synthesized genes into a plasmid. The recombinant plasmid was subsequently transfected to human embryonic kidney 293T (HEK293T) cells using Lipofectamine 2000 (Invitrogen). The 293T cells were homogenized for 20 s and immediately placed on ice, incubated for 5 min, centrifuged at 12,000 r/min for 10 min. We then removed the supernatant, added 200 µL chloroform, let stand at room temperature for 2 min, and centrifuged at 12,000 r/min at 4°C for 10 min. We then moved the supernatant into a new 1.5 mL centrifuge tube, added 600 µL isopropyl alcohol, let stand at room temperature for 15 min, centrifuged at 12,000 r/min at 4°C for 15 min, and discarded the supernatant. The precipitate was rinsed with 1 ml 75% ethanol, centrifuged at 12,000 r/min at 4°C for 5 min, and discarded the supernatant. We added 1 mL anhydrous ethanol, rinsed the precipitate, centrifuged the solution at 12,000 r/min at 4°C for 5 min, and then discarded supernatant. We allowed the precipitate to dry at room temperature for 10 min. We then add 40 µL DEPC water to dissolve the RNA and stored this solution at −80°C for later use. To remove of DNA from the total RNA, 1 µL DnaseI, 1 µL 10×DNase Buffer, and DEPC-H₂O were added to the total RNA to make a total volume of 10 µl. The mixture was subjected to 37°C for 30 min before 1 µl EDTA was added and the mixture was subjected to 65°C for 10 min. RNA was reverse transcribed into first stand cDNA using M-MLV reverse transcriptase and random primers. Grk1 and Grk1 N402S primers were designed using Primer 5 (Lalitha 2000). Primers for upstream and downstream 0.5 µL, SYBRGreen Mix 12.5 µL, and ddH₂O 9.5 µL were added to the cDNA template to achieve a volume of 25 µL. The internal reference gene used GAPDH. Reaction conditions were 95°C,10 min (95°C, 15 s; 60°C, 45 s)x40; 95°C,15 s; 60°C, 1 min; 95°C, 15 s; and 60°C, 15 s.

The expression intensity of cells was detected by qPCR (Real-time quantitative PCR). The primers used for loading control GAPDH were F: TCCGTCTTGAGAAACCTGCC, R: CAACCTGTTGCTCCGTGTAT, while those used for grk1 were F: TCCATGGCAGAGGAGATGA, R: CACATCCCCCTCCTTTGTCT. The qPCR results were tabulated and calculated with the software LightCycler® 96 SW 1.1.

Protein Extraction and Concentration Determination
We used the appropriate amount of cracking fluid and added phenylmethylsulfonyl fluoride (PMSF) within a few minutes before use so that the final PMSF concentration was 1 mM. We removed the culture medium and washed it again with phosphate buffered saline (PBS), normal saline, or serum-free culture medium. We added cracking fluid at the rate of 100–200 ul cracking fluid to each well of a 6-well plate. We blow several times to make full contact between lysate and cells. Cells were usually lysed 1 to 2 seconds after the lysate came into contact with them. Protein concentration was determined using a bichinchoninic acid kit for protein determination.

Kinase Activity Test
A kinase activity assay kit of Grk1 (GENMED Scientifics Inc., Wilmington, DE, USA) was used to measure the kinase activity according to the manufacturer’s instructions. The phosphorylation target of Grk1 was RRREEEEESAAA. In the presence or absence of Grk1 kinase sensitivity inhibitor gradient, the substrate RRREEEEESAAA was phosphorylated by Grk1 to generate the product phosphorylated polypeptide. Then, through the pyruvate kinase and lactate dehydrogenase reaction system, NADH (reduced nicotinamide adenine dinucleotide) was converted to NAD (nicotinamide adenine dinucleotide). Changes in peak absorbance (340 nm) were used to quantitatively analyze
the specific activity of Grk1. The experimental procedure was carried out using product number is GMS50160.7.3 v.A according to manufacturer instructions (GENMED SCIENTIFICS INC. U.S.A.). A Student’s t-test was used to analyze the data.

Supplementary Material

Supplementary materials are available at Genome Biology and Evolution online (http://www.gbe.oxfordjournals.org/).

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Authors Contributions

S.H. led the project. L.Y. conceived and designed the project. J.B. performed the computational analyses. H.X. provided data. W.L. and C.W. assisted in the experiments.

Data Availability

All data used in this study are provided in supplementary tables 1 and 3, Supplementary Material online.

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