New Insight Into Visual Adaptation in the Mudskipper Cornea: From Morphology to the Cornea-Related *COL8A2* Gene

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Much research has focused on visual system evolution in bony fishes. The capacity of visual systems to perceive and respond to external signals is integral to evolutionary success. However, integrated research on the mechanisms of adaptive evolution based on corneal structure and related genes remains limited. In this study, scanning electron microscopy (SEM) was used to assess the microstructure and adaptation of corneal epithelial cells. Then, the evolution of the cornea-related *COL8A2* gene was investigated. We found various projections (microridges, microplicae, microholes, and microvilli) on the corneal epithelial cells of amphibious mudskippers. Compared with those of fully aquatic fishes, these microstructures were considered adaptations to the variable environments experienced by amphibious mudskippers, as they can resist dryness in terrestrial environments and infection in aquatic environments. Moreover, strong purifying selection was detected for *COL8A2*. In addition, some specific amino acid substitution sites were also identified in the COL8A2 sequence in mudskippers. Interestingly, the evolutionary rate of the *COL8A2* gene was significantly and positively correlated with maximum diving depth in our dataset. Specifically, with increasing diving depth, the evolutionary rate of the *COL8A2* gene seemed to gradually accelerate. The results indicated that the cornea of bony fishes has evolved through adaptation to cope with the different diving depths encountered during the evolutionary process, with the corneal evolution of the amphibious mudskipper group showing a unique pattern.

Keywords: mudskippers, cornea, microstructure, *COL8A2*, purifying selection

INTRODUCTION

Independent adaptation to different aquatic niche across fishes has been accompanied by widespread changes in the visual system for coping with different light conditions (Reuter and Peichl, 2008). Due to adaptation to different water depths, the structure of fish eyes and their vision-related genes have undergone adaptive evolution accordingly. For example, the visual system of the Mariana lionfish (*Pseudoliparis swirei*) has been severely degraded due to its adaptation

to darkness (Jiang et al., 2019). In many deep-diving groups, the rhodopsin *RH1* gene, which mainly mediates scotopic vision (Lythgoe and Dartnall, 1970; Yokoyama et al., 2008), has not only evolved rapidly but also mutated in amino acid (AA) sequences with similar functional properties, resulting in adaptive evolution of protein functions (Xia et al., 2021). In addition, in the color vision system, cone cells and related genes (*SWS1*, *LWS*, etc.) also show adaptive evolutionary patterns (Levenson et al., 2006; Meredith et al., 2013; Emerling et al., 2015). Generally, the visual systems of fishes adapted to different living conditions have revealed new features of divergent structures and mechanisms of visual perception in various environments (Subramanian et al., 2008; Simmich et al., 2012; Hauser et al., 2017; Alexander et al., 2021).

The cornea is the outermost tissue of the eye and is an essential for animal vision, functioning in refraction and protection of the internal structure of the eye (Maurice, 1957; Bard et al., 1988). In contrast to the cone and retinal rod, the cornea may have different adaptation mechanisms due to its unique structure and physiological function in the eye (Maurice, 1957; Bard et al., 1988). Corneal morphology also shows complex diversity under different environmental conditions among different mammals and fishes (Mass and Supin, 2007; Subramanian et al., 2008), and the internal structure of the cornea has different cell types, densities, and microstructures, such as microvilli, microplicae, microridges and microholes (Collin and Collin, 2006; Simmich et al., 2012). A study on the corneas of 51 vertebrates revealed that corneal epithelial cells are mainly irregularly pentagonal or hexagonal in shape and show many microscopic structures that vary according to habitat (Collin and Collin, 2006). These structures are thought to be resistant to desiccation and abrasion in terrestrial environments and to infection in aquatic environments (Subramanian et al., 2008). In addition, Hu et al. (2016) found significant differences in corneal structure between juveniles and adults in Gobiinae due to their habitat differences (Hu et al., 2016). Generally, the cornea has undergone adaptive evolution in response to different water environments.

Belonging to a transitional group, most mudskipper species are amphibious fish (with both aquatic and semiterrestrial habitats) (Graham, 1997). They are members of family Oxudercidae and the subfamily Oxudercinae. They are divided into four main genera, including *Boleophthalmus*, *Periophthalmodon*, *Periophthalmus*, and *Scartelaos* (Ishimatsu and Gonzales, 2011). Their eyes placed on top of the head on short stalks and capable of being elevated or retracted, are well adapted for vision in air (Nelson et al., 2016).

Phylogenetic and comparative analyses revealed that the terrestrial transitions of mudskipper lineages are were independent of each other (Steppan et al., 2022). These species are mainly distributed in the intertidal mangrove or muddy beach areas of tropical, subtropical and temperate seas and exhibit a wide range of extreme adaptations to an amphibious lifestyle (Nursall, 1981; Burggren, 1997; Sayer, 2005; Ishimatsu and Gonzales, 2011). Because of the special habitat selection of this group, their visual evolution has always attracted much attention. Studies on corneal epithelial cells have shown that cell density in gobies usually decreases with decreasing corneal

osmotic stress, and corneal epithelial cells also adapt to different degrees (Hu et al., 2016). To cope with amphibious conditions, mudskippers have evolved a broader range of color sensitivities between LWS1 and LWS2 than other teleosts and a shift toward violet rather than ultraviolet light (Shi and Yokoyama, 2003; You et al., 2014). The cornea is an important structure for vision, and the *COL8A2* gene encodes the alpha 2 chain of type VIII collagen. This protein is a major component of the basement membrane of the corneal endothelium and forms homo- or heterotrimers with alpha 1 (VIII)-type collagens (GeneCard, 2021). In the normal cornea, there is a smooth monolayer of corneal endothelial cells (CECs), and the primary component is collagen type VIII (COL8), a non-fibrillar, short-chain collagen secreted by CECs (Meng et al., 2013). Investigations of the *COL8A2* gene in bony fish are rare. However, missense mutations in the gene encoding the α2 (VIII) subunit of collagen VIII are associated with an early onset form of Fuchs dystrophy in human (Biswas et al., 2001). Additionally, experiments on different mouse models with *COL82A* mutations recapitulated some of the findings for endothelial cells and Descent's membrane in humans (Jun et al., 2012). However, the variation in adaptive mechanisms among different diving depths, especially in amphibious species, remains unclear. In this study, some goby species were selected to dissect the morphology and structure of corneal epithelial cells, and their structures were compared with those of strictly aquatic fish to reveal the adaptation of fish corneas to different habitats.

MATERIALS AND METHODS

Specimen Selection
Specimens sampled from Xiapu, China, on December 2, 2014, were divided into three groups: *Scartelaos histophorus* (six individuals), *Boleophthalmus pectinirostris* (seven individuals), and *Periophthalmus magnuspinnatus* (six individuals). The examined specimens are listed in **Supplementary Table 1**. All individuals were mature and in good health without ocular disease or abnormality prior to euthanasia. Specimens were euthanized in accordance with ethical guidelines. After observing the location and shape of the eyes through a ZEISS STEISV II microscope, the right eye of each specimen was completely removed, and the cornea of each enucleated eye was pretreated. Higher-magnification images were recorded digitally (Hu et al., 2016).

Gene Collection and Primary Treatment
In this study, five species (i.e., *P. magnuspinnatus*, *Periophthalmodon schlosseri*, *S. histophorus*, *B. pectinirostris*, and *Mugilogobius chulae*) from Gobiiformes were used for phylogenetic and evolutionary analyses (**Supplementary Table 2**). To attain broad and balanced depth range coverage for analysis, 13 full-length mitochondrial DNA (mtDNA) and *COL8A2* sequences were obtained from Clupeiformes, Pleuronectiformes, Scombriformes, Acanthuriformes, Carangiformes, and Perciformes (35 species in total), which are able to dive to various depths (**Supplementary Table 2**).

Sequence Alignment and Identification of Specific Amino Acid Sites and Domains

Multiple sequence alignments (MSAs) of the 13 mtDNA sequences and COL8A2 sequences of 35 species were obtained by using webPRANK[1] based on codons. Afterward, the gaps and non-homologous regions were deleted by using GBLOCKS (Talavera and Castresana, 2007). The specific AA sites of COL8A2 MSA file were manually inspected by eye in MEGA 7 (Kumar et al., 2016). The domains of the protein secondary structure were identified by using UniProt.[2]

Three-Dimensional Structural Prediction and Specific Amino Acid Sites Distribution

We obtained the 3D structure of the COL8A2 protein from UniProt (see text footnote "2"). Here, the COL8A2 homolog of zebra fish (*Danio rerio*) was used as the model. To provide further insights into the functional significance of these specific AA sites, they were mapped onto the 3D structure of the COL8A2 gene using PYMOL.[3]

Phylogenetic Reconstruction

The 13 mtDNA sequences were assembled by SequenceMatrix (Vaidya et al., 2011). The PhyloSuite platform (Zhang et al., 2020) was used to construct a phylogenetic tree. Maximum likelihood (ML) and Bayesian inference (BI) phylogenies were inferred by using IQ-TREE (Lam-Tung et al., 2015) and MrBayes 3.2.6 (Ronquist et al., 2012), respectively. The best model for each analysis was GTR + R5 + F. The ML tree was constructed with 1000 bootstrap replications. For BI analysis, Markov chain Monte Carlo (MCMC) chains for 10 million generations, with sampling every 1,000 generations. The phylogeny generated in PhyloSuite was visualized in Figtree v1.4.4 software[4] and then used for subsequent evolutionary analyses.

Molecular Evolutionary Analysis

To determine whether adaptive evolution of the *COL8A2* gene might have occurred in bony fishes with different diving depths and the selection model of mudskipper, we used the PAML 4.9 package (Yang, 2007), which implements an ML method to calculate the ratio of non-synonymous (d_N) to synonymous (d_S) substitutions ($\omega = d_N/d_S$). $\omega > 1$, $= 1$ and <1 indicate positive selection, neutrality and negative selection, respectively.

To test for possible heterogeneity of ω ratios along independent branches, we used the *free-ratio branch model*, which allows each branch to have a separate ω value. The null model is the strict *one-ratio model* (M0, all branches have one estimated ω). To explore potential relationships between gene evolution and maximum diving depth values obtained from FISHBASE[5] (see **Supplementary Table 2**), we examined the association
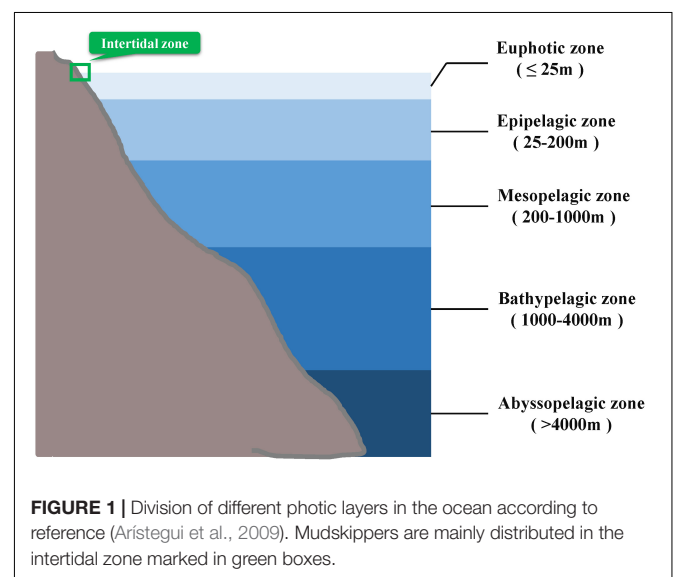
between these two factors according to the method described by Montgomery et al. (2011). The evolutionary rates of the *COL8A2* gene in the dataset were estimated by the root-to-tip ω values, which were computed in the CODEML program (Yang, 2007). Root-to-tip ω, which is regarded as a signal of an evolutionary trajectory and calculated through regression analysis against phenotypic data from extant species (Montgomery et al., 2011; Xu et al., 2017), is more inclusive of the evolutionary history of a locus than other metrics (Wolf et al., 2009). The mean ω value includes d_N/d_S ratios from an ancestral root to a terminal tip in a phylogenetic tree. Here, the value was calculated by the *free-ratio model* ($S*d_S = 0$ and $N*d_N = 0$ were not considered). Then, phylogenetic generalized least squares (PGLS) analysis was implemented in the R program (Orme et al., 2012). This method included the parameter lambda (λ), which was evaluated by the ML method. The parameter provided a quantitative estimate of the phylogenetic signal, which ranged from 0 (no phylogenetic signal) to 1 (significant phylogenetic signal).

To better understand the discrepancy in evolutionary rates between amphibious and fully aquatic fishes, a *two-ratio model* was implemented, in which the mudskipper clade was regarded as the foreground branch (**Figure 2A**). Then, the *six-ratio model* was further applied to uncover differences among fishes with different diving depths (**Figures 1**, **2B**). For all the analyses, the nested models were compared by using a likelihood ratio test (LRT) with various degrees of freedom. All analyses were run twice to ensure convergence. A general overview of this study is shown in **Figure 3**.

RESULTS

Type and Microstructure of Epithelial Cells

The epithelial cell microstructures were observed under a scanning electron microscope (**Figures 4–6**). In *B. pectinirostris*, the internal cells were differentiated into diverse structures, such
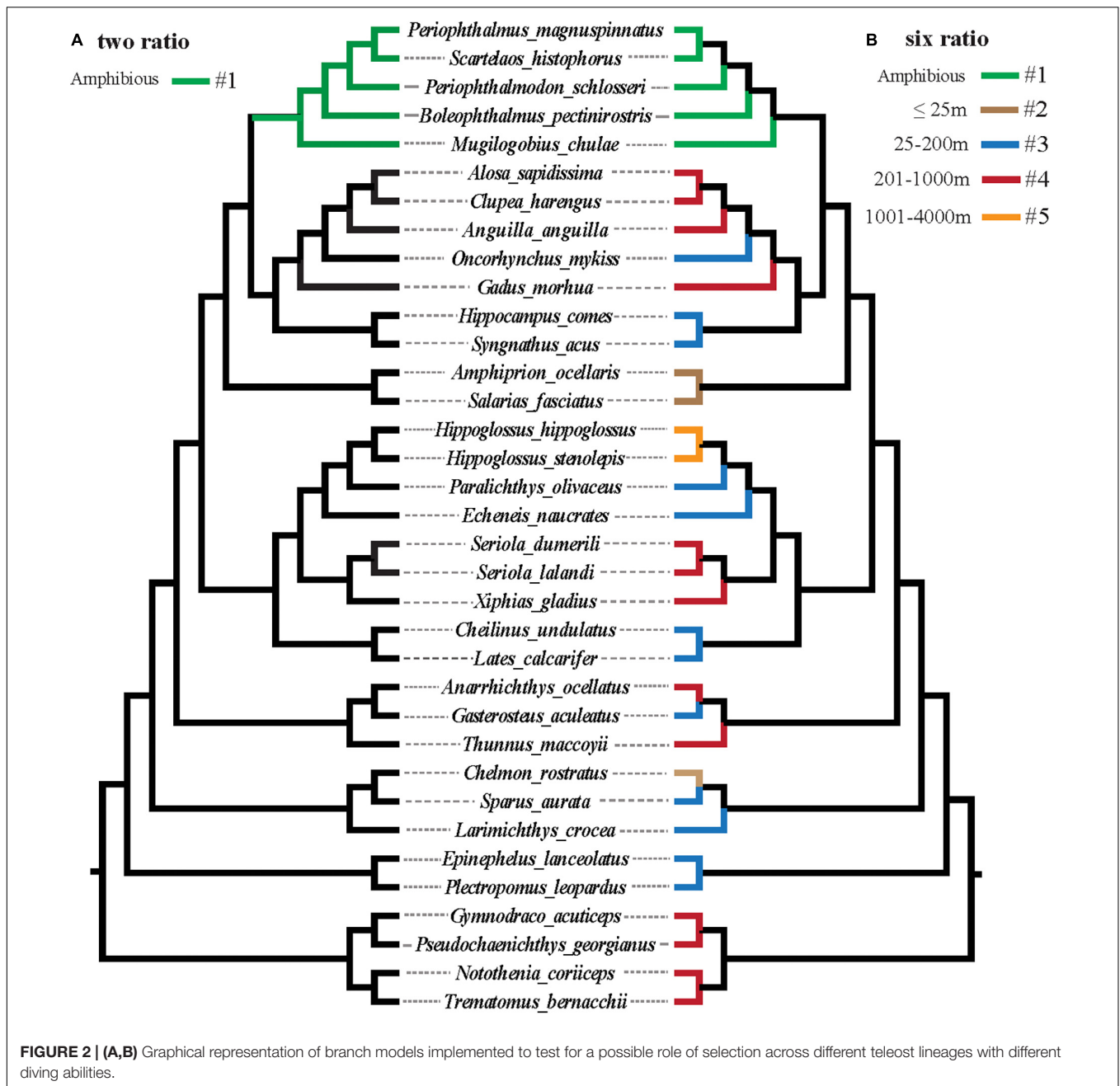

**FIGURE 1 |** Division of different photic layers in the ocean according to reference (Arístegui et al., 2009). Mudskippers are mainly distributed in the intertidal zone marked in green boxes.

FIGURE 2 | (A,B) Graphical representation of branch models implemented to test for a possible role of selection across different teleost lineages with different diving abilities.

as the ridge type (**Figures 4A,B**), reticular type (**Figure 4C**), and ridge-reticular type (**Figure 4D**). The border of corneal epithelial cells was clear, with many small projections (**Figure 4C**). The microplicae, microvilli, microholes (**Figure 4C**) and microridges (**Figure 4A**) were observed under high magnification.

In *P. magnuspinnatus*, the ridge cell type was observed (**Figure 5A**), and the border was clear with many small projections (**Figures 5B,D**). The corneal epithelial cells also consisted of four types of microstructures (**Figure 5**): microridges, microplicae, microvilli (**Figures 5B,D**) and microholes (**Figure 5C**). Compared those in *B. pectinirostris*, the microridges of corneal epithelial cells in *P. magnuspinnatus*

were rough, with more microplicae and microvilli. The corneal epithelial cell surface was characterized by fragmentation and a compact structure.

In *S. histophorus*, corneal epithelial cells were of the ridge type (**Figure 6**). The border of the cells was clear (**Figure 6C**). Microplicae, microvilli and microridges (but not microholes) (**Figure 6D**) were observed under high magnification.

Based on the study of Collin and Collin (2006), we further compared the corneal microstructures of mudskippers and fully aquatic fishes. Specifically, as shown in **Table 1**, corneal epithelial cell microstructure and type were compared. Four kinds of microstructures were present in *B. pectinirostris* and

FIGURE 3 | Overview of the study.
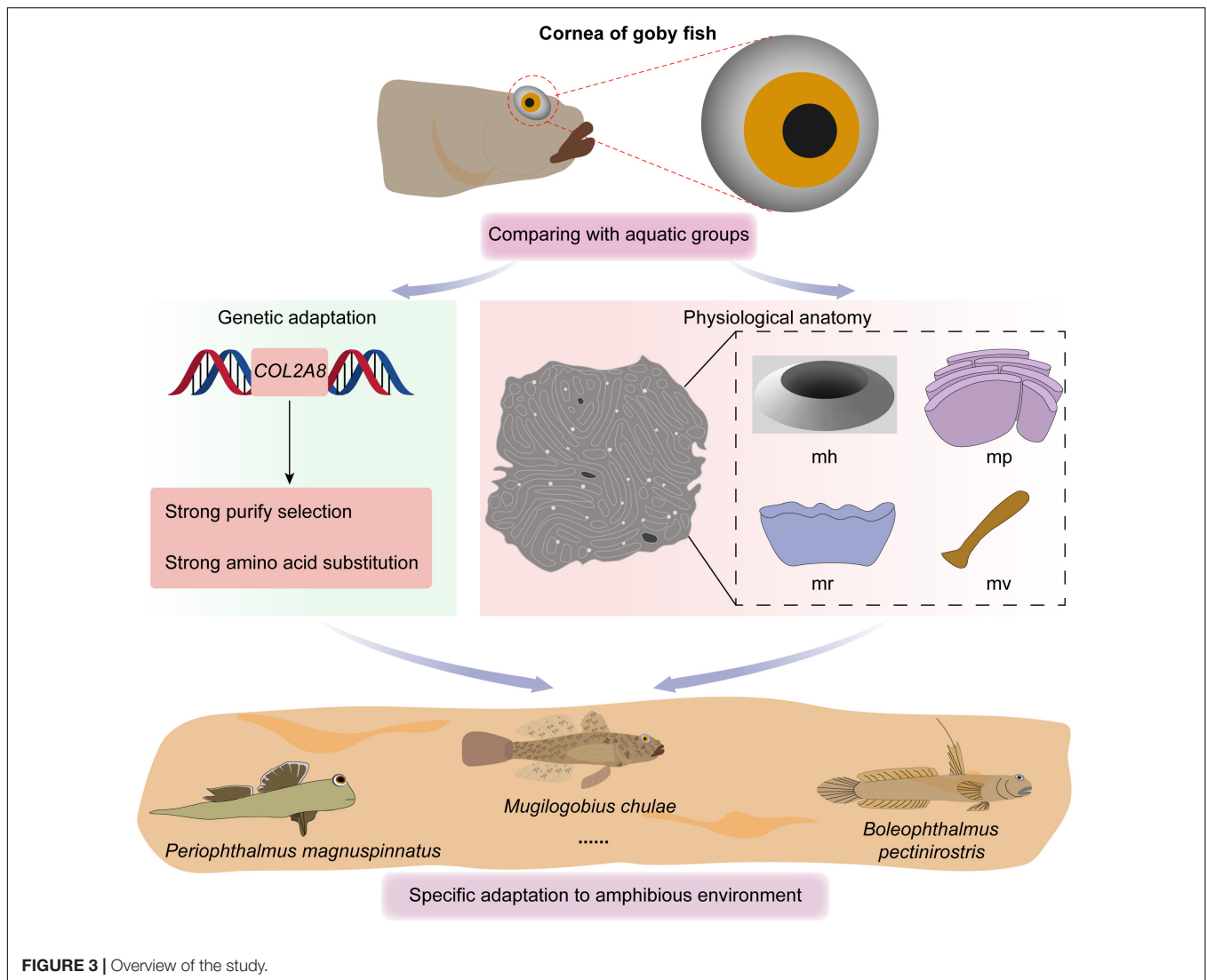
P. magnuspinnatus, while only microridges were recorded in *A. butcheri* and only microholes and microvilli were observed in *N. forsteri*. In *B. pectinirostris*, there were three corneal epithelial cell types, but only the ridge type was recorded in *A. butcheri*, *P. magnuspinnatus* and *S. histophorus*. In short, the mudskippers examined in our study may have richer cellular microstructures and cell types than fully aquatic fishes.

Phylogenetic Analyses

We constructed phylogenetic trees using IQ-TREE based on 13 mitochondrial coding genes. The relationships shown by the gene trees largely reflected known species relationships with high bootstrap support (**Supplementary Figure 1**).

Detection of Selection Pressure

The results showed that the *free-ratio model* (Model C) fit the data significantly better than the *one-ratio model* (Model A) for the *COL8A2* gene (**Table 2**), which suggested divergent selection pressures on different teleostean branches. Further *two-ratio*

model analysis revealed that the evolutionary rate of mudskipper fishes was significantly lower than that of other groups, and the whole mudskipper branch showed evidence of strong negative selection (**Table 2**). The results of the *six-ratio model* showed significant differences in evolutionary rates among fishes with various maximum diving depths (**Table 2**). Moreover, with increasing diving depth, the evolutionary rate of the *COL8A2* gene seemed to gradually accelerate, except in the bathypelagic group (**Figure 7**).

Association Between *COL8A2* Gene Evolution and Maximum Diving Depth

Corneal morphology is influenced by diving depth, and the rate of evolution of related genes is directly related to morphology. To explore the relationship between the evolutionary rate of the *COL8A2* gene and maximum diving depth, we implemented PGLS analysis to estimate the association between the root-to-tip ω of the *COL8A2* gene and maximum diving depth. The λ value was 0.699; however, $P > 0.05$, which indicated no phylogenetic
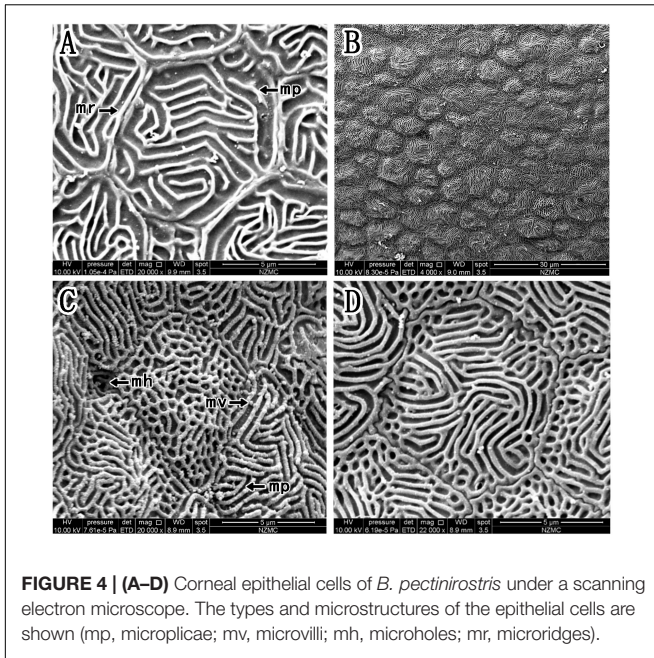
FIGURE 4 | (A–D) Corneal epithelial cells of *B. pectinirostris* under a scanning electron microscope. The types and microstructures of the epithelial cells are shown (mp, microplicae; mv, microvilli; mh, microholes; mr, microridges).
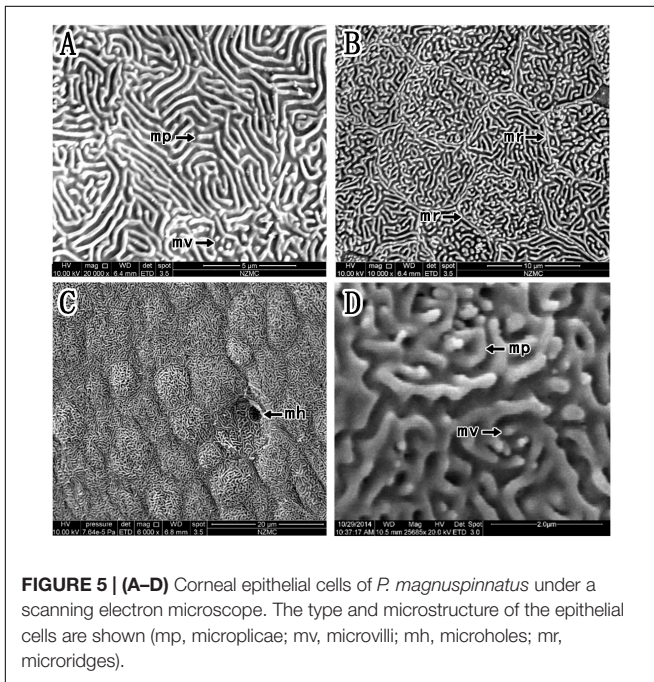

FIGURE 5 | (A–D) Corneal epithelial cells of *P. magnuspinnatus* under a scanning electron microscope. The type and microstructure of the epithelial cells are shown (mp, microplicae; mv, microvilli; mh, microholes; mr, microridges).
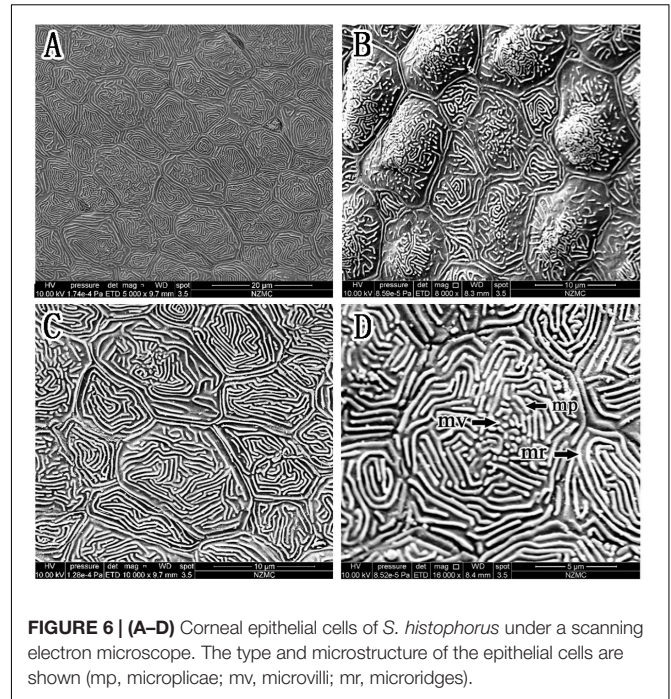

FIGURE 6 | (A–D) Corneal epithelial cells of *S. histophorus* under a scanning electron microscope. The type and microstructure of the epithelial cells are shown (mp, microplicae; mv, microvilli; mr, microridges).

signal in our analysis (**Supplementary Table 3**). Therefore, only the ordinary least squares (OLS) approach was implemented. The results showed a significant positive regression between the root-to-tip ω and maximum diving depth (**Figure 8**).

Specific Amino Acid Substitutions and Structural Links to Protein Function

Further sequence comparison revealed many AA site substitutions and deletions in the COL8A2 AA sequence of mudskipper fishes (**Figure 9**). Some sites were located in important functional sites or domains (**Figure 10**). In particular,

the absent region (sites 385–402) was located in the triple-helical region, and sites 5 and 13 belong to the signal peptide.

DISCUSSION

Corneal Adaptation of Mudskippers

Mudskippers are the largest group of amphibious teleost fish, most of which are uniquely adapted to live on mudflats (Graham, 1997). During their successful transition from aquatic to terrestrial living, they evolved morphological and physiological modifications in some systems, e.g., olfaction, vision, and aerial respiration (You et al., 2018). Regarding vision, for amphibious habitats lifestyle adaptation, some vision-related genes are differentially lost or mutated, such as *SWS1*, which exhibits a loss of function (You et al., 2014). In this study, adaptive characters of corneal physiological structure were found in mudskippers (**Figures 4**–**6** and **Table 1**). There were many types of corneal epithelial cells in mudskippers. For example, *B. pectinirostris* has three types of cells and mainly migrates to low tidal regions without vegetation, which suggests that it has various ecomorphologies to adapt to a diversity of habitats.

The main surface features of the corneal epithelium of terrestrial vertebrates are usually microplicae and microvilli, while the corneal epithelium of aquatic vertebrates usually has microridges (Beuerman and Pedroza, 1996). The results in **Table 1** show that amphibious mudskippers generally have microplicae, microvilli and microridges, while aquatic fish have only microridges. The presence of a variety of cellular microstructures seems to be characteristic of corneal epithelial cells of fishes with amphibious lifestyles.

Microvilli are usually vertical extensions of the surface of epithelial cells and are believed to be the most effective way to

TABLE 1 | Corneal epithelial cell microstructure and type comparison among different species and mudskippers.

Microstructural types and cell types	*A. butcheri*	*N. forsteri*	*S. histophorus*	*B. pectinirostris*	*P. magnuspinnatus*
Microridges	*	–	*	*	*
Microplicae	–	–	*	*	*
Microholes	–	*	–	*	*
Microvilli	–	*	*	*	*
Ridge type	*	–	*	*	*
Reticular type	–	–	–	*	–
Mixed type (microridge and reticular)	–	–	–	*	–

(*): present, (–): absent.
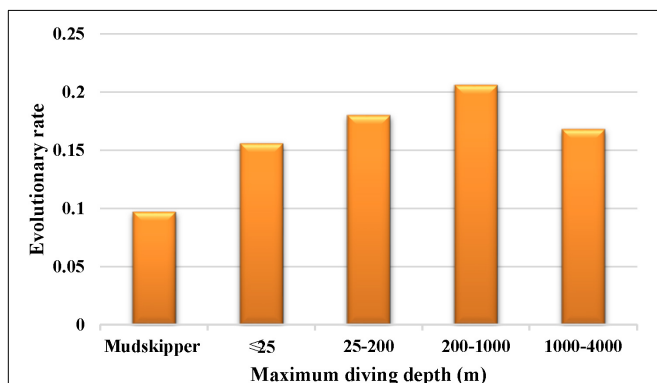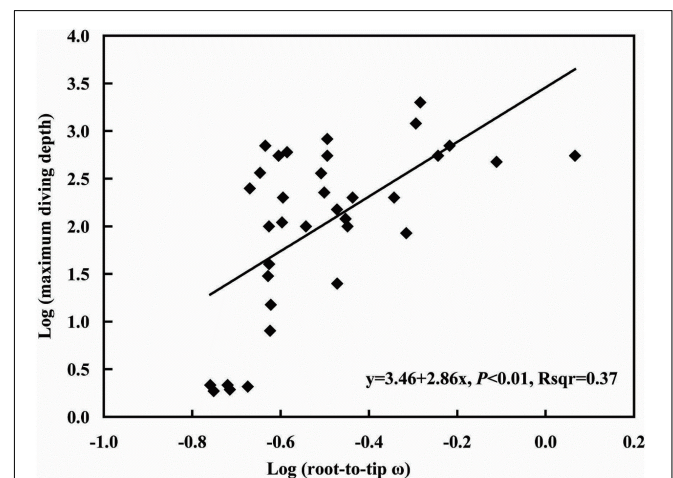The information related to corneal epithelial cell microstructure in fishes was obtained from *Collin and Collin (2006)*.

TABLE 2 | Likelihood and omega values estimated under branch models of selection pressures on the *COL8A2* gene.

Models	ω	$-\ln L$	np	Model comparison	LRT	*P*-value*
A. All branches have one ω	0.172	23670.186	70			
B. All branches have $\omega = 1$	1	25245.331	69	A vs. B	3150.290	<0.001
C. Each branch has its own ω	—	23559.605	137	A vs. C	221.162	<0.001
D. The Gobiiformes clade has ω_2, others have ω_1	$\omega_2 = 0.097$ $\omega_1 = 0.187$	23650.302	71	A vs. D	39.768	<0.001
E. The Gobiiformes clade has $\omega_2 = 1$, others have ω_1	$\omega_2 = 1$ $\omega_1 = 0.185$	23985.544	70	D vs. E	670.484	<0.001
F. The branches for a given diving depth have corresponding ω values, others have ω_1	$\omega_2 = 0.097$ $\omega_3 = 0.156$ $\omega_4 = 0.180$ $\omega_5 = 0.206$ $\omega_6 = 0.168$ $\omega_1 = 0.172$	23658.233	75	D vs. F	15.862	= 0.003

*All P-values reached significant levels.

increase the surface area of cells, which facilitates the uptake of oxygen and nutrients by the cell and the excretion of water and metabolites from the cell (Beuerman and Pedroza, 1996). These structures are generally found on the corneal surface of species that spend adequate periods out of an aquatic environment (Beuerman and Pedroza, 1996). Our study confirms the existence of microvilli in mudskippers. Phylogenetic work revealed molecular evidence to support the division of family Gobiidae into two major clades, family Oxudercidae (= Gobionellidae by some authors) and Gobiidea. Four formerly recognized gobiid subfamilies-Gobionellinae, Oxudercinae (including the mudskippers), Amblyopinae, and Sicydiinae, are included within family Oxudercidae (Thacker, 2003; Thacker, 2009; Thacker et al., 2011; Agorreta et al., 2013). Mudskippers (Teleostei: Gobiidae: Oxudercinae: Periophthalmini) are gobies that are

FIGURE 7 | The evolutionary rate of *COL8A2* among different maximum diving depths.

FIGURE 8 | Association between root-to-tip ω and maximum diving depth.

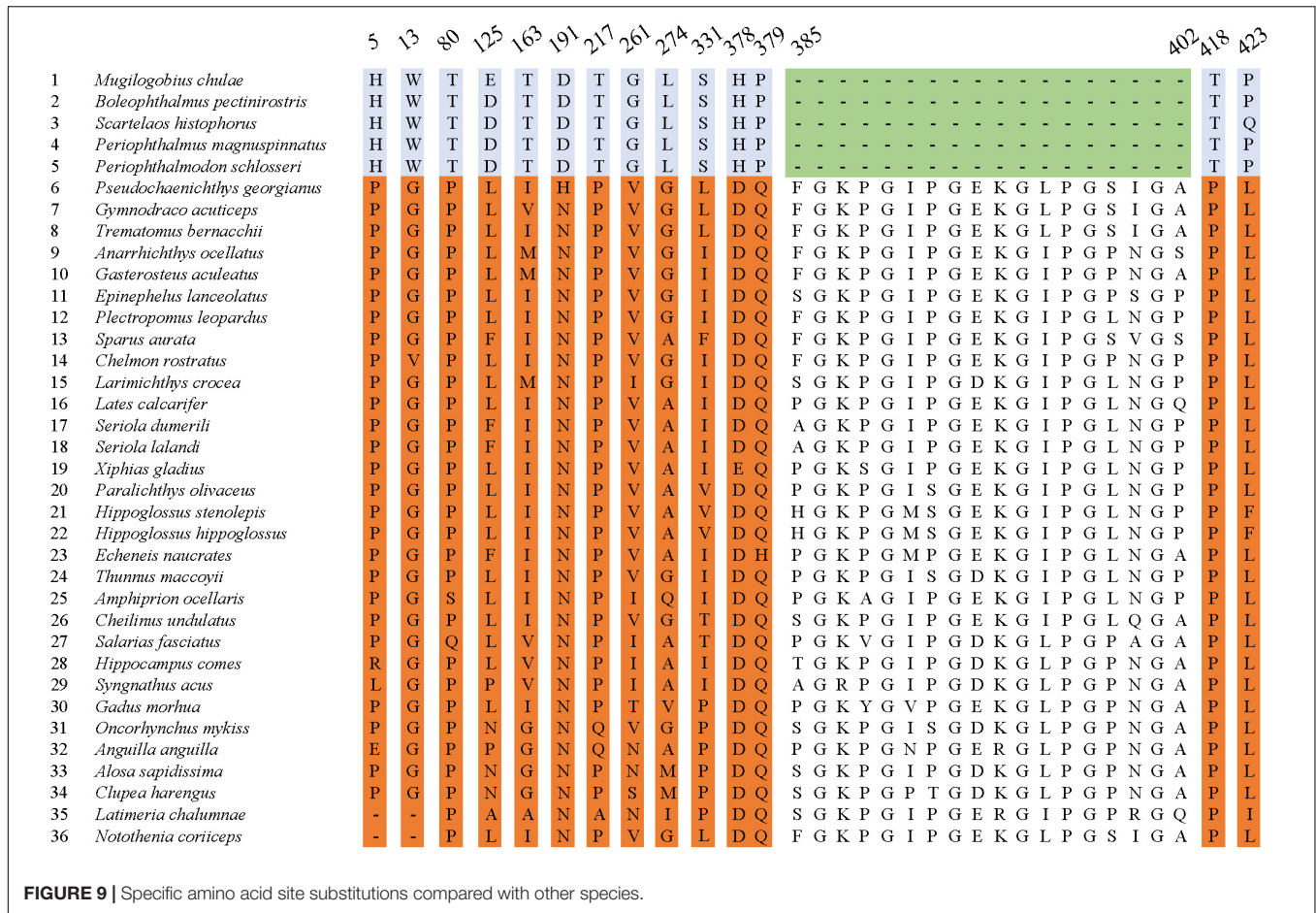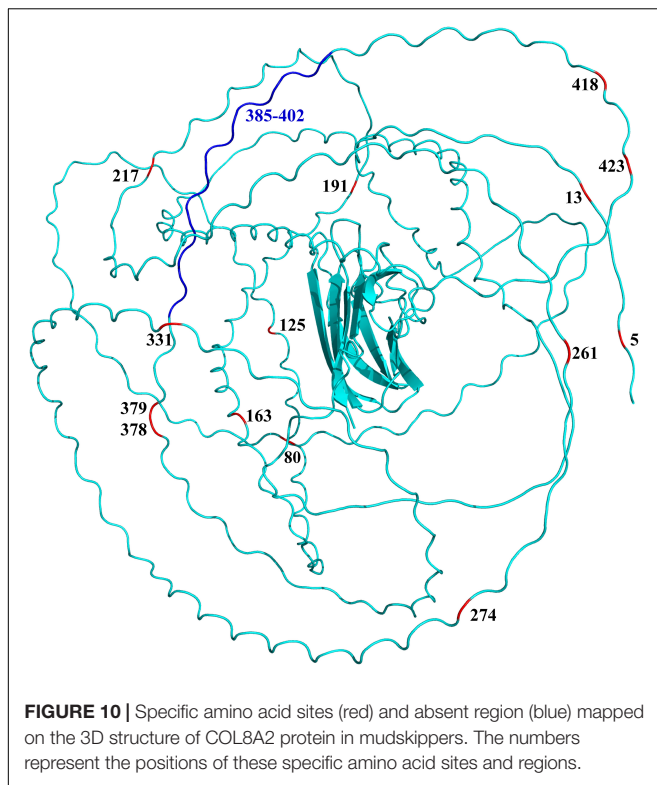| #	Species	5	13	80	125	163	191	217	261	274	331	378	379	385–402	418	423
1	*Mugilogobius chulae*	H	W	T	E	T	D	T	G	L	S	H	P	- - - - - - - - - - - - - - - - - -	T	P
2	*Boleophthalmus pectinirostris*	H	W	T	D	T	D	T	G	L	S	H	P	- - - - - - - - - - - - - - - - - -	T	P
3	*Scartelaos histophorus*	H	W	T	D	T	D	T	G	L	S	H	P	- - - - - - - - - - - - - - - - - -	T	Q
4	*Periophthalmus magnuspinnatus*	H	W	T	D	T	D	T	G	L	L	H	P	- - - - - - - - - - - - - - - - - -	P	P
5	*Periophthalmodon schlosseri*	H	W	T	D	T	D	T	G	L	S	H	P	- - - - - - - - - - - - - - - - - -	T	P
6	*Pseudochaenichthys georgianus*	P	G	P	L	I	H	P	V	G	L	D	Q	F G K P G I P G E K G L P G S I G A	P	L
7	*Gymnodraco acuticeps*	P	G	P	L	V	N	P	V	G	L	D	Q	F G K P G I P G E K G L P G S I G A	P	L
8	*Trematomus bernacchii*	P	G	P	L	I	N	P	V	G	L	D	Q	F G K P G I P G E K G L P G S I G A	P	L
9	*Anarrhichthys ocellatus*	P	G	P	L	M	N	P	V	G	I	D	Q	F G K P G I P G E K G I P G P N G S	P	L
10	*Gasterosteus aculeatus*	P	G	P	L	M	N	P	V	G	I	D	Q	F G K P G I P G E K G I P G P N G A	P	L
11	*Epinephelus lanceolatus*	P	G	P	L	I	N	P	V	G	I	D	Q	S G K P G I P G E K G I P G P S G P	P	L
12	*Plectropomus leopardus*	P	G	P	L	I	N	P	V	G	I	D	Q	F G K P G I P G E K G I P G L N G P	P	L
13	*Sparus aurata*	P	G	P	F	I	N	P	V	A	F	D	Q	F G K P G I P G E K G I P G S V G S	P	L
14	*Chelmon rostratus*	P	V	P	L	I	N	P	V	G	I	D	Q	F G K P G I P G E K G I P G P N G P	P	L
15	*Larimichthys crocea*	P	G	P	L	M	N	P	I	G	I	D	Q	S G K P G I P G D K G I P G L N G P	P	L
16	*Lates calcarifer*	P	G	P	L	I	N	P	V	A	I	D	Q	P G K P G I P G E K G I P G L N G Q	P	L
17	*Seriola dumerili*	P	G	P	F	I	N	P	V	A	I	D	Q	A G K P G I P G E K G I P G L N G P	P	L
18	*Seriola lalandi*	P	G	P	F	I	N	P	V	A	I	D	Q	A G K P G I P G E K G I P G L N G P	P	L
19	*Xiphias gladius*	P	G	P	L	I	N	P	V	A	I	E	Q	P G K S G I P G E K G I P G L N G P	P	L
20	*Paralichthys olivaceus*	P	G	P	L	I	N	P	V	A	V	D	Q	P G K P G I S G E K G I P G L N G P	P	L
21	*Hippoglossus stenolepis*	P	G	P	L	I	N	P	V	A	V	D	Q	H G K P G M S G E K G I P G L N G P	P	F
22	*Hippoglossus hippoglossus*	P	G	P	L	I	N	P	V	A	V	D	Q	H G K P G M S G E K G I P G L N G P	P	F
23	*Echeneis naucrates*	P	G	P	F	I	N	P	V	A	I	D	H	P G K P G M P G E K G I P G L N G A	P	L
24	*Thunnus maccoyii*	P	G	P	L	I	N	P	V	G	I	D	Q	P G K P G I S G D K G I P G L N G P	P	L
25	*Amphiprion ocellaris*	P	G	S	L	I	N	P	I	Q	I	D	Q	P G K A G I P G E K G I P G L N G P	P	L
26	*Cheilinus undulatus*	P	G	P	L	I	N	P	V	G	T	D	Q	S G K P G I P G E K G I P G L Q G A	P	L
27	*Salarias fasciatus*	P	G	Q	L	V	N	P	I	A	T	D	Q	P G K V G I P G D K G L P G P A G A	P	L
28	*Hippocampus comes*	R	G	P	L	V	N	P	I	A	I	D	Q	T G K P G I P G D K G L P G P N G A	P	L
29	*Syngnathus acus*	L	G	P	P	V	N	P	I	A	I	D	Q	A G R P G I P G D K G L P G P N G A	P	L
30	*Gadus morhua*	P	G	P	L	I	N	P	T	V	P	D	Q	P G K Y G V P G E K G L P G P N G A	P	L
31	*Oncorhynchus mykiss*	P	G	P	N	G	N	Q	V	G	P	D	Q	S G K P G I S G D K G I P G P N G A	P	L
32	*Anguilla anguilla*	E	G	P	P	G	N	Q	N	A	P	D	Q	P G K P G N P G E R G L P G P N G A	P	L
33	*Alosa sapidissima*	P	G	P	N	G	N	P	N	M	P	D	Q	S G K P G I P G D K G L P G P N G A	P	L
34	*Clupea harengus*	P	G	P	N	G	N	P	S	M	P	D	Q	S G K P G P T G D K G L P G P N G A	P	L
35	*Latimeria chalumnae*	-	-	P	A	A	N	A	N	I	P	D	Q	S G K P G I P G E R G I P G P R G Q	P	I
36	*Notothenia coriiceps*	-	-	P	L	I	N	P	V	G	L	D	Q	F G K P G I P G E K G L P G S I G A	P	L

FIGURE 9 | Specific amino acid site substitutions compared with other species.

"fully terrestrial for some portion of the daily cycle." They belong to four genera: *Boleophthalmus*, *Periophthalmodon*, *Periophthalmus*, and *Scartelaos* (Murdy, 1989). The diversity of both sensory structures and visually mediated behaviors of fish reflects the complexity of their environment. Visual stimuli vary in both physical and biological components (Evans, 2004). Aquatic and terrestrial environments typically present very different optical, osmotic and physico-mechanical problems to the cornea. In aquatic environments, the cornea contributes little to the refractive power of the eye due to the similarity of the refractive indices of water (Kröger, 1992). In terrestrial environments, the refractive index of air is 1.00 under standard temperature and pressure conditions. Based on our previous observation, terrestrial mudskippers tend to have eyes on the top of their heads, while aquatic mudskippers have eyes on both sides of their heads, similar to those of ordinary fish. These fishes inhabit the intertidal zone and mangrove respiratory root areas, and their eyes are exposed to air for a long time in tidal flats or shallow waters. Our results combined with those previously reported for *N. forsteri* (which lives in terrestrial habitats for a long time) (Collin and Collin, 2006) suggest that the corneal epithelium of amphibious fish has evolved adaptively in response to the relatively arid terrestrial environment.

Microplicae are mainly found in cartilaginous and jawless fishes and amphibians. They may represent a transitional form between microvilli and microridge that increases the osmotic exchange function of the cornea and the supporting strength of the cornea. All 30 mudskipper species are resident intertidal fishes, and are strictly found in temperate, subtropical and tropical mudflat, mangrove and lower fluvial ecosystems (Murdy, 1989; Jaafar and Larson, 2008). Mudskippers spend part of their lives out of water to feed, mate, and avoid capture by terrestrial predators. Therefore, they have developed behavioral and physiological specializations for adaptation to amphibious life. The most significant morphological and functional modifications are the trend to have closely set and moveable protuberant eyes located high on the head for escaping from predators on land and they are structurally adapted for accurate vision in both air and water (Sayer, 2005). Microplicae are observed in the corneal epithelial cells of amphibious fish, which may correspond to their amphibious lifestyle.

Among the microstructure types, the one that increases the cell membrane area the least is microridges. Previous studies suggested that microridges are found only on corneal surfaces exposed to hypertonic (mainly marine) environments, as commonly recorded in aquatic habitats (Hu et al., 2016). Microridges increase corneal strength and the surface area of

FIGURE 10 | Specific amino acid sites (red) and absent region (blue) mapped on the 3D structure of COL8A2 protein in mudskippers. The numbers represent the positions of these specific amino acid sites and regions.

the mucus layer, and the microridge structure helps stabilize the tear film of teleost fishes (Hu et al., 2016). Well-developed microridges occur only in bony fishes that live in hypertonic environments, such as marine or estuarine habitats (Hu et al., 2016). As shown in **Table 1**, the microstructures of the three mudskipper species were dominated by microridges, which is consistent with amphibious mudskippers having evolved adaptations to high-osmolarity seawater, similar to other marine fishes.

In aquatic species, unlike the pores found in most terrestrial species, microholes are found only in species that burrow into the substrate and may be exposed to air, e.g., *Geotria australis*, *Mordacia mordax*, *Ambystoma mexicanum* (Collin and Collin, 2000), and *Limnichthyes fasciatus* (Collin and Collin, 1997). Mudskippers usually nest in mudflats, building burrows where they can wait for an opportunity to surprise prey outside, as well as avoid predators and breed (Ishimatsu et al., 1998). In our study, *B. pectinirostris* and *P. magnuspinnatus* showed microholes. This reflects a great degree of adaptability to their habitats.

In addition, the evolutionary history of the cornea-related gene *COL8A2*, which plays key roles in the development of the corneal endothelium (GeneCard, 2021), also includes adaptive changes. We found that *COL8A2* has undergone strong purifying selection in some goby clades (**Table 2** Mudskipper Store Air in Their Burrows). Moreover, some specific AA substitution sites were found in the COL8A2 AA sequence, some of which were located in and/or near important functional sites and domains (**Figure 9**). For example, sites 385–402 are located in the triple-helical region; however, they are absent

in some goby clades. Mutation or deletion of these sites will lead to conformational changes in the three-dimensional structure of the COL8A2 protein, thereby affecting its overall function. Our results revealed that the cornea of gobies has evolved at the molecular level to enable adaptation to an amphibious lifestyle.

Visual Adaptation Among Bony Fishes at Different Water Depths

Due to the absorption and scattering of light in water, the photic environment of the aquatic system changes rapidly with depth, and the phenomenon of wavelength absorption leads to a blueshift with increasing water depth (Davies et al., 2012; Hauser et al., 2017; Hauser and Chang, 2017). In addition, due to the different levels of refraction between water and land, there are adaptive changes in the structure of fish eyes (Sayer, 2005). The cornea is the outermost structure of the eye and functions in refraction and protection of the internal structure of the eye. Its morphological changes largely reflect the adaptation of fish to an aquatic environment (Mass and Supin, 2007). In addition, genes might have undergone adaptive evolution. Our results revealed a significant correlation between *COL8A2* evolution and maximum diving depth (**Figure 8**), which indicated selection on genes imposed by diving depth. This conclusion is consistent with the finding of a previous study on *RH1* that water depth adaptation has a significant impact on the evolution of vision (Sugawara et al., 2005; Dungan et al., 2016; Xia et al., 2021). In addition, the analysis of differential selection further confirmed that *COL8A2* was subject to differential selection among different aquatic environments (**Table 2** and **Figure 7**). Through the comparison of anatomical data, obvious differences in the corneal structure of fishes in different habitats were also found. Meanwhile, the evolution of the *COL8A2* gene exhibits a special pattern. However, the relationship and mechanisms of these two types of evolutionary processes are unclear. Thus, further research is needed to confirm the mechanisms by which diving depth drives corneal adaptation.

CONCLUSION

This study provides new insight into the evolutionary mechanisms of visual adaptation in teleosts based on corneas. In this study, two parallel lines of evidence for visual adaptations in goby fish were uncovered. Phenotypically, the cornea of this clade might have evolved structures adapted to both aquatic (marine) and terrestrial habitats, i.e., microvilli and microridges. Furthermore, strong purifying selection was detected for the cornea-related *COL8A2* gene. In addition, some specific AA substitution sites were also identified in the *COL8A2* sequence in gobies, which might be associated with their amphibious lifestyle. Moreover, there was a significant and positive correlation between the evolution of the *COL8A2* gene and maximum diving depth. Further research is needed to determine the mechanisms explaining the relationship between morphological adaptation and functional genes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the experimental materials involved in this study are lower vertebrates, and there are few experimental materials. There are no inhumane procedures in the experiment.

AUTHOR CONTRIBUTIONS

WH participated in the determination of research and writing ideas, designed and completed the experiment of fish physiology and scanning electron microscopy, completed the data collection and manuscript writing of this part, and completed the manuscript submission. YM participated in the determination of research and writing ideas, and completed the data collection, and manuscript writing on the molecular part. JZ overall guidance to the research and writing ideas, and participated in the writing of the whole manuscript. FL participated in the collection of fish samples. XL participated in the writing and modification of part of the manuscript.

All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2022. 871370/full#supplementary-material

Supplementary Figure 1 | Phylogenetic tree of 13 mtDNA constructed by IQ-TREE.

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