Plastic loss of motile cilia in the gills of *Polypterus* in response to high CO$_2$ or terrestrial environments

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Funding information
Asahi Glass Foundation; Grant-in-Aid for JSPS Fellows, Grant/Award Number: 21J21544

Abstract
The evolutionary transition of vertebrates from water to land during the Devonian period was accompanied by major changes in animal respiratory systems in terms of physiology and morphology. Indeed, the fossil record of the early tetrapods has revealed the existence of internal gills, which are vestigial fish-like traits used underwater. However, the fossil record provides only limited data on the process of the evolutionary transition of gills from fish to early tetrapods. This study investigated the gills of *Polypterus senegalus*, a basal ray-finned/amphibious fish which shows many ancestral features of stem Osteichthyes. Based on scanning electron microscopy observations and transcriptome analysis, the existence of motile cilia in the gills was revealed which may create a flow on the gill surface leading to efficient ventilation or remove particles from the surface. Interestingly, these cilia were observed to disappear after rearing in terrestrial or high CO$_2$ environments, which mimics the environmental changes in the Devonian period. The cilia re-appeared after being returned to the original aquatic environment. The ability of plastic changes of gills in *Polypterus* revealed in this study may allow them to survive in fluctuating environments, such as shallow swamps. The ancestor of Osteichthyes is expected to have possessed such plasticity in the gills, which may be one of the driving forces behind the transition of vertebrates from water to land.

KEYWORDS
amphibious fish, cilia, gill, plasticity, *Polypterus*, terrestrial adaptation

TAXONOMY CLASSIFICATION
Zoology

1 | INTRODUCTION

Fish use their gills for respiration, but these gills progressively degenerated during the evolutionary transition of vertebrates from water to land, in parallel with a corresponding physiological and morphological remodeling of the respiratory system. For example, extant Amniota (mammals, reptiles, and birds) have completely lost their gills and depend on lungs for respiration. In some amphibians, the internal gills which are present during the larval stage degenerate during metamorphosis into adulthood. In tracing back to earlier stages of evolution, the fossil record of Acanthostega, and similar Paleozoic adult tetrapods, have shown the existence of internal gills (Coates & Clack, 1991; Schoch & Witzmann, 2011), which resembled those of lungfish (Clack, 2012). This suggests that the internal
gills were still functioning in the respiratory systems of these early tetrapods which inhabited the interface between water and land. Generally, gills are believed to function only in water because their lamellae collapse due to gravity and dry out in a terrestrial environment (Sayer, 2005). Therefore, the evolutionary degeneration of gills in response to the environmental changes associated with the water to land transition is an issue of interest. Indeed, a few extant species of teleost fish have adapted to terrestrial environments by modifying the structure of their gills (e.g., mudskippers (Low et al., 1988), and mangrove killifish (Kryptolebias marmoratus) (Ong et al., 2007)). In particular, plastic structural changes have been observed in the gill lamella of mangrove killifish during acclimation to terrestrial conditions (Ong et al., 2007; Turko et al., 2012).

To better understand the evolution of the respiratory system of stem Osteichthyes, the group Polypteridae (bichir, reedfish) provide appropriate model organisms. The Polypteridae are the group which is closest to the common ancestor of ray-finned fish and lobe-finned fish in extant ray-finned fishes, and retains several ancestral traits of the stem Osteichthyes. Importantly, some of these traits include the use of lungs or spiracles for air-breathing, and thus they show some of the tetrapod adaptations to survival in a terrestrial environment (Graham et al., 2014; Tatsumi et al., 2016). Some studies argued that the terrestrial adaptation of Polypterus occurred independently from that of the tetrapods (Damsgaard et al., 2020; Ord & Cooke, 2016).

In the meanwhile, recent genomic, transcriptomic, and histological analyses proposed that at least the lungs of Polypterus and tetrapods may have originated only once in their common ancestor (Bi et al., 2021; Cupello et al., 2022; Tatsumi et al., 2016). Indeed, Standen et al. have successfully kept Polypterus on land for up to eight months (Standen et al., 2014). Turko et al. (2019) found that in Polypterus reared on land, cells filled the area between the lamellae of the gills and reduced the size of the gill skeleton. Therefore, inspection of the gills of Polypterus before and after rearing in a terrestrial environment, thus mimicking the water to land transition, may help understand the remodeling process of the respiratory system which occurred during early tetrapod evolution.

In addition to the water to land transition, concentrations of oxygen and carbon dioxide in water also affect gill morphology (Phuong et al., 2017; Turko et al., 2012; Wright & Turko, 2016). Fish excrete carbon dioxide into water through their gills because of the high solubility of carbon dioxide in water (Rahn, 1966); a process which does not work in terrestrial (i.e., atmospheric) or high CO₂ environments. In fact, Polypterus was shown to depress gill ventilation in response to high CO₂ concentrations in an aquatic environment (Babiker, 1984). Additionally, Ultsch proposed that hypercarbic environments played a role in the water to land transition during vertebrate evolution (Ultsch, 1987, 1996). However, until now, morphological changes to gills upon exposure to such environments have not yet been documented.

In this study, we aimed to gain insight into the evolution of the respiratory system during the water to land transition of the stem Osteichthyes. Specifically, we examined whether changes in gills occur in Polypterus after the exposure to high CO₂ or terrestrial environments, using morphological comparison, immunostaining, and transcriptome as benchmarks.

### 2 | MATERIALS AND METHODS

#### 2.1 | Animals and rearing environment

The Polypterus senegalus Cuvier, 1829, used in this study were obtained from a commercial supplier (Nettaigyo-atsuhan forest, Japan) and kept in normal water conditions for at least 1 month before being transferred to either terrestrial conditions or high CO₂ conditions. The total length and the body weight of fish used in this series of experiments were shown in Table S1. The experiments were conducted in accordance with the Tokyo Institute of Technology Regulations for the Management of Animal Experiments.

The control fish were kept in a 600 L tank with a filtration system. The water temperature was approximately 28 ± 1°C, with a 12:12 light:dark cycle. Fish in a terrestrial environment were kept in a 25 cm × 35 cm mesh cage at a depth of about 1 mm with a supply of filtered water and foggers to prevent ventral and dorsal surface from drying, respectively (Figure 1; Standen et al., 2014; Turko et al., 2019). In the high CO₂ experiment, ambient temperature CO₂ gas was added continuously until the gill ventilation of Polypterus was depressed, in accordance with previous research (Babiker, 1984). The concentration of free carbon dioxide in the treatment tank was kept above 100 mg/L (pH 5.08–5.85). The concentration in the control tank was 8.8 mg/L (pH 6.89–7.11). The free carbon dioxide concentration was measured according to the method of the previous study Sharma, 1998). The 35 cm × 35 cm × 20 cm tank was continuously stirred using a water filter (Rio+ filter-set 2, Kamihata, Japan) to equalize and ensure a uniform carbon dioxide concentration. Fish were kept in the environments described above for at least 1 month. Then, some individuals were returned into aquatic environments and kept for 1 month.

**FIGURE 1** *Polypterus senegalus* in experimental terrestrial environments.
2.2 | Observation by scanning electron microscopy (SEM)

To examine changes in the microstructure of the surface of the gills, we conducted SEM observation. After keeping the animals in the respective environments, they were euthanized by decapitation and dissected. Specimens of gills for electron microscopy were washed in 0.7× PBS, then fixed in 2.5% glutaraldehyde, and treated with 8N HCl at 60°C for 30 min to remove surface mucus. The specimens were then dehydrated by ethanol series, and dried in a freeze dryer (ES-2030, Hitachi, Japan) using t-butyl alcohol. The specimens were osmium coated and observed by SEM (JSM-7001F, JEOL).

The length of the cilia in the SEM image was measured with Fiji (Schindelin et al., 2012). We selected five representative cilia for which the total length could be measured, and calculated the average and unbiased variance.

2.3 | Immunofluorescence staining

To examine the presence/absence of cilia and taste buds in the gills, we conducted immunostaining. The entire gills were fixed in 4% PFA and immunostained as follows: incubated with 0.5% Triton X-100 (v/v) in 0.7× PBS for 15 minutes at room temperature, washed with 0.7× PBS, and then blocked with normal goat serum 10% and bovine serum albumin 1% in 0.7× PBS for 1 h. Indirect immunofluorescence was performed using the following antibodies: anti-alpha tubulin (acetyl K40, rabbit monoclonal, abcam, ab179484, 1:2000), and anti-calretinin (mouse monoclonal, swant, 6B3, 1:2000) were used as primary antibodies for detecting cilia and taste buds, respectively. Three or more individuals were verified for each condition (Table S1). Anti-rabbit IgG (H+L) Alexa Fluor 594 (donkey polyclonal, Invitrogen, AB_141637, 1:2000), and anti-mouse IgG (gamma 1) Alexa Fluor 488 (goat polyclonal, Invitrogen, AB_2535764, 1:2000) were used as secondary antibodies. After washing with PBS, primary antibodies of anti-tubulin were added and incubated at room temperature for 1 hour. Secondary antibodies were reacted following the same procedure. The same procedure was also used to stain for calretinin. To prevent fading of the fluorescence, a mounting medium (VECTASHIELD with DAPI, Vector Laboratories) was added. The images were taken with a fluorescence microscope (Axioplan2, Carl ZEISS). After taking the photos, Photoshop was used to correct the levels and modify the colors.

2.4 | Histological observation with HE stain

We examined whether the inter-lamellar spaces between the gills of Polypterus become filled with cells or not in the terrestrial (Turko et al., 2019) and high CO2 environments. Gills specimens were fixed in Bouin’s fixative. The specimens were then dehydrated in ethanol, replaced by xylene, and embedded in paraffin. Thin sagittal sections with a thickness of 5μm were prepared. The sections were stained with hematoxylin and eosin.

To quantify changes in heights of inter-lamellar cell mass (ILCM) in terrestrial and high CO2 environments, we measured the heights of ILCM of individuals in control, terrestrial, and a high CO2 environment with ImageJ. Two gill lamellae per individual were selected and three ILCMs per gill lamellae were randomly selected. In this way, six ILCMs were measured from each of the three individuals in each environment. Finally, we calculated the average of the data for each individual for the test. To test for changes of heights of ILCM, we compared them using F-test and Student’s t-test with scypy version 1.9.3.

2.5 | RNA-seq analysis

To examine the differences in gene expression in gills between aquatic and terrestrial environments, we conducted RNA-seq analysis. RNA was extracted from whole gills of three individuals reared in the control water and in the terrestrial environment using TRI Reagent (Molecular Research Center, Inc.). The extracted total RNAs were sequenced at 100 bp paired-end on a NovaSeq 6000 from Macrogen Japan Corp., with the TruSeq stranded mRNA Library Prep Kit. The quality control of raw sequence data was performed with fastp (Chen et al., 2018) with the following options: -g, -q 20, -w 16. Next, the read data were mapped to the reference genome of Polypterus senegalus (Bchr_013 (Bi et al., 2021)) with STAR version 2.7.3a (Dobin et al., 2013). The mapped reads were then counted with featureCounts (Liao et al., 2014). Differentially expressed genes (DEGs) were estimated by TCC (Sun et al., 2013) with the iDEGES/edgeR-edgeR combination (p <.05). The Over-Representation Analysis (ORA) was conducted using WebGestalt under statistical tests of false discovery rate (http://www.webge stalt.org) (Liao et al., 2019).

2.6 | Recording gill ventilation and water flow

We recorded the head movement of the Polypterus to see the difference in gill ventilation behavior between terrestrial and aquatic environments. In the terrestrial environment, the Polypterus was placed on a damp non-woven cloth. A digital camera X100V (Fujifilm, Japan) was used for recording.

To detect water flow on the surface of the gills, Polypterus blood cells were dropped onto the surface of the excised gills of Polypterus. The blood cells were collected at the time of decapitation. The size of red blood cells of Polypterus palmas is 160μm2 (Hardie & Hebert, 2003). Before blood cell drops, we confirmed that there was no bleeding from the gills themselves. The movement of blood cells was recorded using microscopes (Axioplan2, Carl ZEISS, Germany and SZX7-TR2-APO-C, OLYMPUS, Japan). The movies were edited at 10x and 4x speed respectively.
3 | RESULTS

3.1 | The cilia on the surface of gills

SEM observations and immunostaining with an acetylated tubulin antibody revealed the existence of cilia as bundle-like structures distributed on the surface of primary gill lamella of *Polypterus senegalus* (gray bichir) in aquatic environments (Figures 2a–c and 3a). The average length of the cilia was 3.93 μm and unbiased variance was 0.16. The cilia were distributed in a dotted pattern. To investigate the effect of environmental conditions on the gill cilia, we reared *Polypterus* in either terrestrial environments or in high CO₂ water for more than 1 month. It is noteworthy that gill ventilation, which was observed in normal water environment, was suppressed in terrestrial environment (Video S1). The suppression of gill movement was also observed in the high CO₂ environment, as was also observed by Babiker (1984). Interestingly, SEM and immunostaining observation revealed that the cilia disappeared in the gills of *Polypterus* reared in both of these environmental conditions (Figures 2d–f and 3b). Subsequently, we returned the *Polypterus* individuals exposed to terrestrial or high CO₂ environments for one month back to the original water environment and reared them for an additional month. The immunostaining observations for the resultant *Polypterus* individuals revealed that the cilia in the gills had recovered (Figure 3b).

In addition to the gill cilia, the enlargement of the inter-lamellar cell mass (ILCM) between the lamellae of the gills was examined in the *Polypterus*, which were reared in both the terrestrial and high CO₂ environments. A previous study revealed that enlargement occurs in the ILCM of *Polypterus* reared on land (Turko et al., 2019), which was confirmed in the present study (Figure 4b,d). In addition, a statistically significant enlargement of the ILCM was also observed in the individuals reared in the high CO₂ environment (Figure 4c,d). As shown in the next section, RNA-seq analysis was performed on genes whose expression levels were increased in the terrestrial environment. We identified 1473 up-regulated genes. The over-representation analysis suggested genes involved in localization or tissue development were enriched (Figure 4e).

3.2 | Elucidation of cilia function by RNA-seq analysis

There are many types of cilia, which are classified into two main groups: motile and non-motile cilia. Although they are various in terms of their locations (e.g., airways, olfactory epithelium) and functions (Choksi et al., 2014), the function of the cilia present in the gills remains unknown.

The DEGs analyses were conducted on the whole exome sequencing data (RNA-seq data) for the gills of the *Polypterus* reared in both water and terrestrial environments. In particular, this study aimed to elucidate the function of cilia in the gills, which had not yet been documented. Considering that cilia were lost in the terrestrial environments, a focus was made on the DEGs of which the expressions were reduced in gills under terrestrial conditions. As a result, a total of 868 DEGs were obtained which was statistically significant. An ORA was then performed for the list of down-regulated DEGs (p < .05) using WebGestalt (Liao et al., 2019). The results of the ORA indicated that the down-regulated gene groups were related to 1. axonemal dynein complex assembly, 2. axoneme assembly, 3. cilium movement, and 4. cilium assembly (Figure 5a). Further inspection of

![Figure 2](image-url) Scanning electron micrograph of the surface of gill filaments of *P. senegalus*. The surface of *Polypterus* reared in water (a–c) and terrestrial environment (d–f). Scale bar in a, b, d, and e = 10 μm, in c and f = 1 μm.
these gene groups revealed that they were represented by foxj1a, which is included in one of the major genes involved in cilia movement (Figure 5b). foxj1a is a homolog of foxj1 (Forkhead box protein J1) (Aamar & Dawid, 2008; Hellman et al., 2010). The protein encoded by foxj1 is a master regulator of the motile ciliogenic program (Yu et al., 2008). The degree of expression of several genes, known to be regulated by foxj1 (Mukherjee et al., 2019) was examined, and found that they were also significantly down-regulated (Figure 5b). These results indicate that foxj1, and its downstream genes involved in cilia movement, were down-regulated in terrestrial environments.

To confirm whether the cilia were motile, we observed the water flows generated on the surface of the gills. The blood cells of Polypterus were used for visualizing the flow. The movement of blood cells was observed from the root to the tip of the gill lamella (Video S2). The water flow was also observed in the movie of the entire gill (Video S2), confirming that these cilia are motile.

Whether these cilia have specific functions beyond motility was also examined. It has been shown that taste buds of lampreys possess cilia, while those of bony fishes and mammals possess microvilli (Baatrup, 1983; Barreiro-Iglesias et al., 2008). However, until now, there have been no reports describing the taste buds of Polypterus in terms of the existence of cilia or microvilli. Therefore, an examination was conducted to determine whether the ciliated cells on the gills of Polypterus were taste buds or not. Immunostaining was performed using an anti-calretinin antibody which is used as a chemosensory marker in vertebrates, including for taste buds (Barreiro-Iglesias et al., 2008). As a result, clear signals of calretinin-positive cells were observed which show typical taste bud-like structures on the gill arches of Polypterus (Figure 6a). Importantly, no acetylated tubulin-positive signal was observed, indicating the absence of ciliated cells in the taste buds. Additionally, no calretinin-positive cells were observed where acetylated tubulin-positive cells were concentrated (Figure 6b). These results suggest that the ciliated cells on the gills of Polypterus are not taste buds.

Whether these cilia possessed functions of innate immunity was also examined, since the ciliated cells found in the airway epithelium of mammals have been shown to be involved in the immune system (Freund et al., 2018; Shah et al., 2009). Indeed, several taste 1 receptors (Tas1Rs) and taste 2 receptors (Tas2Rs) expressed in the upper airway respond to substances secreted by Gram-negative bacteria. These Tas1Rs and Tas2Rs are essential to trigger mucociliary clearance by controlling the beating of motile cilia (Lee et al., 2012). Therefore,
the expression of the Tas1Rs and Tas2Rs gene cascade was examined. The expression of Tas1Rs was not changed significantly (Figure 6c). The expression level of Tas2Rs was quite low and difficult to detect by RNA-seq. The next focus was on trpm5 and plcb2, which are located downstream of the Tas1Rs and Tas2Rs gene cascades (Ahmad & Dalziel, 2020; Tuzim & Korolczuk, 2021). These genes were expressed...
in gills, but there were no significant changes in expression levels between aquatic and terrestrial environments (Figure 6d). Next, the focus was on three genes of nitric oxide synthase (NOS). Nitric oxide controls the beating of the cilia in the airway epithelium of mammals (Li et al., 2000). It is noteworthy that one of the NOS genes was lost in teleost fish after the whole genome duplication Donald et al., 2015; however, all three NOS genes were identified in the genome of Polypterus. A comparison of the expression levels of the three types of NOS genes in the gills of Polypterus found no significant differences between the control and terrestrial environments (Figure 6e). These lines of data suggest that the cilia in the gills of Polypterus do not play a specific role in Tas1R/Tas2R-related innate immunity shown in the mammalian airway epithelium, but provide only motile functions, which may contribute toward producing water flow for efficient ventilation.

In addition to the cilia function, we examined the possibility of whether or not the loss of cilia in the terrestrial environment was the result of injury. We inspected the up-regulated DEGs which contain 1473 genes, showing that the genes associated with injury (e.g., inflammation) were not enriched (Figure 4e).

### DISCUSSION

#### 4.1 Origin of cilia in the gills

This study revealed the existence of cilia in the internal gills of Polypterus. This is the first report of ciliated cells of the internal gills in a ray-finned fish (Actinopterygii). Although several SEM images of gills have been presented in model organisms such as zebrafish (Danio rerio) and medaka (Oryzias latipes) (Leguen, 2018; Messerli et al., 2020), the cilia were not described in these studies. Cilia were not found in gills and/or specialized respiratory organs in walking catfish (Clariidae) nor in mangrove killifish (K. marmoratus), which are also adapted to the terrestrial environment in a manner similar to Polypterus (Maina, 2018; Ong et al., 2007). Additionally, sharks (cartilaginous fishes) do not possess cilia in their gills (Bullard et al., 2001). On the other hand, cilia have been reported in the internal gills of a lobe-finned fish, the Australian lungfish (Neoceratodus forsteri, Sarcopterygii) (Kemp, 1996), which diverged from the common ancestor of Osteichthyes 470 million years ago (Wang et al., 2021). It is plausible that cilia in the internal gills had...
been acquired in the common ancestor of Osteichthyans and retained in Polypterus and lobe-finned fish, but were lost in most other ray-finned fish, which diverged later than Polypterus. However, the size and arrangement of the cilia in the gills of Polypterus are distinct from those seen in lungfish (Kemp, 1996). Indeed, the cilia of Polypterus are noticeably short and are distributed in a circular dotted pattern (Figure 2), while those in lungfish are long and distributed in band-like patterns (Kemp, 1996). The morphological distinctness of the cilia observed in the gills of Polypterus and lungfish implies that specific roles of cilia have diversified between ray-finned and lobe-finned fish.

4.2 | Function of the cilia

In this study, immunostaining data showed that the cilia found in the internal gills of Polypterus were not taste buds. Additionally, transcriptome analyses suggested that these cilia did not possess functions in Tas1R/Tas2R related to innate immunity as seen in the airway epithelium of mammals. On the contrary, the cilia seen in the internal gills of Australian lungfish, which exist only during the larval stages, have been shown experimentally to produce water flow (Kemp, 1996). These cilia are responsible for increasing the efficiency of ventilation and eliminating small particles such as microorganisms from gill surfaces (Kemp, 1996). In this study, the cilia found in the gills of adult Polypterus were shown to be motile (Figure 5, Video S2). The roles of the cilia may be explained by the dual ventilation system used by Polypterus, which utilizes both lungs and gills. A previous study revealed that the frequency of gill ventilation in Polypterus decreases significantly before and after air-breathing (Magid, 1966). Polypterus may reduce its frequency of gill ventilation by complementing it with pulmonary ventilation. However, depression of gill ventilation leads to the reduction of other gill functions such as eliminating small particles, ion transport, excretion of carbon dioxide and ammonia (Ultsch, 1996). During such temporary depression of gill ventilation, the motile cilia may function to increase efficient water flow for excretion and transport. The constant direction of water flow produced by the cilia also reinforces the hypothesis (Video S2).

Considering that gill cilia and pulmonary ventilation are expected to be mutually reinforcing in function, it is plausible to speculate that they were acquired simultaneously in their evolution.

4.3 | Gill plasticity in response to environmental changes

This study found that the cilia in the internal gills of Polypterus were lost after the exposure to a terrestrial environment (Figures 2 and 3). It is noteworthy that gill ventilation was depressed in a terrestrial
environment (Video S1), implying that they do not function in such environment. Assuming that cilia play a certain role in efficient ventilation, excretion of carbon dioxide, and removal of small particles, it makes sense that they would disappear under conditions where water is unavailable. It is also likely that the loss of cilia may play a role of saving consumption of energy in a terrestrial environment, namely, reducing the cost for maintaining them. Previous study has revealed a reduction in the volume of mineralized bone in the gills on land, arguing that Polypterus may reduce investment in its gills when in a terrestrial environment (Turko et al., 2019). Importantly, the loss and regeneration of the cilia are plastic in response to the environmental change (Figure 3). Given that no experimental data showing the signal of inflammation was obtained (Figure 4e), the plastic loss and regeneration of cilia may be programmed biological responses rather than just an injury. This plasticity may have been acquired as a result of an adaptation to fluctuating environments, such as encountered in shallow rivers and swamps, which Polypterus, and possibly the ancestors of Osteichthyes inhabited.

Similar to terrestrialization, the high concentration of CO₂ in water resulted in the plastic loss and regeneration of cilia in the gills of Polypterus (Figure 3). Previous study has revealed that gill ventilation in Polypterus was depressed in water with high CO₂ concentrations (Babiker, 1984). In a high CO₂ environment, gill ventilation causes several negative impacts, represented by acidosis. Since CO₂ relies on passive transport, elevated CO₂ concentrations in environmental water may lead to an influx of CO₂ into the blood through the gills. Hence, suppressing gill ventilation is expected to be adaptive in a high CO₂ environment in Polypterus. The CO₂ concentration in the water tends to transiently increase in warm, relatively shallow and vegetated environments. The Devonian air-breathing vertebrates are considered to have inhabited environments with fluctuating CO₂ concentrations (Ultsch, 1996). Polypterus also inhabits similar environments (Magid, 1967). It is presumed that Polypterus survives in a high CO₂ environment by depressing gill ventilation and switching to ventilation through the lungs when needed. To prevent exposure of the gills to high CO₂ water, the frequency of gill ventilation as well as the motile cilia, would likely be reduced.

The enlargement of the ILCM was observed in response to high CO₂ and terrestrial environments (Figure 4). A previous study showed that the ILCM between the secondary gill lamellae was enlarged in the gills of Polypterus reared on land (Turko et al., 2019). The genes associated with localization and tissue development were up-regulated in a terrestrial environment (Figure 4e). This is consistent with the enlargement of the ILCM. The enlargement or reduction of ILCM are also observed in teleost fish such as smangrove killifish under terrestrial conditions (Ong et al., 2007). The enlargements of the ILCM may be adaptive for terrestrial environment in terms of reducing the surface of the gills in contact with air (Turko et al., 2019). The enlargement of the ILCM of Polypterus in high CO₂ conditions suggests a reduction of gas exchange surface area of the gills, leading to a presumed reduction of the passive import of CO₂ from the water. It is noteworthy that both the high CO₂ condition and terrestrial environment induced the similar morphological changes in the gills of Polypterus.

### 4.4 High CO₂ environments and the water to land transition

In previous studies, the possible link between hypercarbia and the water to land transition has been discussed. Because of its high solubility in water, CO₂ is normally excreted through the gills of most fish. In contrast, air-breathing fish take in O₂-rich air, resulting in a lower frequency of ventilation using gills. Therefore, the blood of air-breathing fish shows a higher P⁰ CO₂ relative to other fish (Bayley et al., 2019; Ultsch, 1996). Indeed, in some obligate air-breathing fish, the acid–base status of the blood is similar to that seen in amphibians (Bayley et al., 2019; Ultsch, 1996). Based on studies showing that hypoxic environments are also high in carbon dioxide, it has been proposed that air-breathing fish may have shifted to high P⁰ CO₂, [HCO₃⁻] blood prior to their terrestrial adaptation during vertebrate evolution (Ultsch, 1996).

It is noteworthy that both high CO₂ water and terrestrial environments were found to lead to similar results in Polypterus, namely, the depression of gill ventilation, ILCM enlargement, and the loss of cilia in the gills. Based on the present and previous findings (Bayley et al., 2019; Ultsch, 1996), we propose that adaptive traits to a high CO₂ environment shown above were acquired prior to terrestrial adaptation. Namely, the ancestors of stem tetrapods possibly become ready to adapt to land because some adaptive characters acquired in high CO₂ environments were also adaptive in terrestrial environments. For example, the depression of internal gill ventilation and enlargement of ILCM was adaptive in terrestrial environments in terms of preventing desiccation. The loss of cilia was also adaptive in saving consumption of energy in a terrestrial environment. Importantly, the above changes of internal gills were plastic in the ancestor of Osteichthyes, that may allow them to accomplish the drastic transition from water to land back and forward, and step by step.

In this study, SEM and immunostaining observations and transcriptome analyses revealed the existence of motile cilia in the internal gills of P. senegalus, which may create a flow of water on the gill surface leading to efficient respiration and material transport. These motile cilia were observed to have been plastically lost and regenerated in response to environmental changes resulting from rearing in terrestrial and high CO₂ environments. In some Devonian fish, some characters evolved under similar high CO₂ environments and may have been later co-opted during the adaptation to terrestrial environments by early tetrapods. The specific features characterized in the present study of Polypterus provide important insights into the evolutionary transition of vertebrates from water to land.

**AUTHOR CONTRIBUTIONS**

Yuki Kimura: Conceptualization (lead); formal analysis (lead); funding acquisition (equal); investigation (lead); visualization (lead); writing – original draft (lead). Nobuaki Nakamuta: Investigation (equal); writing – review and editing (equal). Masato Nikaido: Funding acquisition (equal); supervision (lead); writing – review and editing (lead).
ACKNOWLEDGMENTS
We thank Shizuka Oki, Yasuyo Shigetani, and Masataka Okabe of the Jikei University School of Medicine for teaching HE staining.

FUNDING INFORMATION
This work was supported by Grant-in-Aid for JSPS Fellows Grant Number 21J21544 and the Asahi Glass Foundation to M.N.

CONFLICT OF INTEREST STATEMENT
We declare we have no competing interests.

DATA AVAILABILITY STATEMENT
All sequence reads were deposited in the DDBJ Sequence Read Archive under BioProject accession no. PRJDB13172.

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