# Plastic loss of motile cilia in the internal gills of *Polypterus* in response to high $CO_2$ or terrestrial environments

Running title: Plasticity of gill cilia of *Polypterus*

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**Keywords**

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

## 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 to elucidate the process of the evolutionary transition of internal gills from fish to early tetrapods. This study investigated the internal 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 internal gills was revealed which may create a flow on the internal gill surface leading to efficient respiration. 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 loss of internal gills in *Polypterus* revealed in this study may allow them to survive in fluctuating environments, such as shallow swamps. The ancestor of Osteichthyes is also expected to have possessed such plasticity in the internal gills, which may be one of the driving forces behind the transition of vertebrates from water to land.

## Introduction

Fish use their internal gills for respiration, but these internal gills progressively degenerated during the evolutionary transition of vertebrates from water to land, and show a corresponding physiological and morphological remodeling of the respiratory system. For example, extant Amniota (mammals, reptiles, and birds) have completely lost their internal 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 Palaeozoic adult tetrapods, have shown the existence of internal gills (Coates and Clack, 1991; Schoch and 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, internal gills are believed to function only in water because internal gill lamellae collapse due to gravity and dry out in a terrestrial environment (Sayer, 2005). Therefore, how internal gills evolved in response to environmental changes during this 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 internal gills (e.g., mudskippers (Low et al., 1988), and mangrove killifish (*Kryptolebias marmoratus*) (Ong et al., 2007)). In particular, plastic structural changes were observed in the internal gill lamella of mangrove killifish during acclimation to terrestrial conditions (Ong et al., 2007; Turko et al., 2012). However, since teleost fish are once fully adapted to aquatic environments, the acclimation observed in these fish are unlikely to replicate the actual evolutionary transition that occurred in stem Osteichthyes during the Devonian period.

To better understand the evolution of the respiratory system of stem Osteichthyes, the group *Polypterus* (bichir, reedfish) is considered an appropriate model organism. The *Polypterus* is the most basal group of ray-finned fish (Fig. 1), and retains several ancestral traits of stem Osteichthyes. Importantly, some of these traits include the use of lungs or spiracles for air-breathing, and thus, are adapted to survival in a terrestrial environment (Graham et al., 2014; 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*. found that in *Polypterus* reared on land, cells filled the area between the lamellae of the internal gills and reduced the size of the internal gill skeleton (Turko et al., 2019). Therefore, inspection of the internal 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 internal gill morphology (Shartau and Brauner, 2014; Turko et al., 2012; Wright and Turko, 2016). Fish excrete carbon dioxide into water through their internal 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 $CO_2$ environments. In fact, *Polypterus* was shown to depress internal gill respiration in response to high $CO_2$ 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; Ultsch, 1996). However, until now, morphological changes to internal gills have not yet been documented after exposure to such environments over specific periods of time.

In this study, the alteration of the internal gills in terms of gene expression as well as morphology after the exposure to high $CO_2$ or terrestrial environments was investigated. In both experimental conditions, similar morphological changes were observed, in which the motile cilia of the internal gills of *Polypterus* disappeared, but then plastically re-appeared after the animal was returned to its original normal environmental conditions. The ability of such plastic changes in *Polypterus* provides crucial insights into their adaptation to a fluctuating environment, and possibly into the water to land transition of early tetrapods.

## Materials and methods

### Animals and rearing environment

The *Polypterus senegalus* Cuvier, 1829, used in this study were obtained from a commercial supplier (Nettaigyo-tsuhan forest, Japan) and kept in normal water conditions for at least one month before being transferred to either terrestrial conditions or high $CO_2$ conditions. Larva of *Polypterus* was obtained from the Kamihata Fish Industry Group in Japan. The larval stage was estimated to be Stage 36 or later according to a previous study (Bartsch et al., 1997). Axolotl (*Ambystoma mexicanum* Shaw and Nodder, 1798) ware obtained through a local pet shop (Suikei-Kobo, Japan). 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°C ± 1°C. The light/dark cycle changed every 12 hours. 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 as in previous studies (Standen et al., 2014; Turko et al., 2019). In the high $CO_2$ experiment, ambient temperature $CO_2$ gas was added continuously until the internal gill ventilation of *Polypterus* was depressed, in accordance with previous research (Babiker, 1984). 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 one month.

### Observation by scanning electron microscopy (SEM)

After keeping the animals in the respective environments, they were euthanized by decapitation and dissected. Specimens of internal and external gills for electron microscopy were washed in 0.7x PBS, then fixed in 2.5% glutaraldehyde, and treated with 8N HCl at 60°C for 30 minutes 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, Japan).

## Immunofluorescence staining

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. 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. The entire internal and external gills were immunostained as follows: incubated with 0.5 % Triton x-100 (v/v) in 0.7x PBS for 15 minutes at room temperature, washed with 0.7x PBS, and then blocked with normal goat serum 10% and bovine serum albumin 1% in 0.7x PBS for 1 hour. 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, US) was added. The images were taken with a fluorescence microscope (Axioplan2, Carl ZEISS, Germany). After taking the photos, Photoshop was used to correct the levels and modify the colors.

## Histological observation with HE stain

Internal 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 5 μm were prepared. The sections were stained with haematoxylin and eosin.

## RNA-seq analysis

RNA was extracted from three individuals reared in the control water environment and from three individuals reared 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 (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. The Over Representation Analysis (ORA) was conducted with WebGestalt (http://www.webgestalt.org) (Liao et al., 2019).

## Results

### 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 internal gill lamella of *Polypterus senegalus* (gray bichir) (Fig. 2A–C, Fig. S1A and Fig. 3A). These cilia were also observed on the external gills of axolotl by SEM observation (Fig. S1B). In *Polypterus*, the cilia were relatively short and distributed in a spotted pattern, whereas in axolotl, they were long and distributed in a band-like pattern. In addition to the internal gills, the existence of the cilia in the external gills of larval *Polypterus* was confirmed by immunostaining with an acetylated tubulin antibody (Fig. S1C).

Next, individual *Polypterus* were reared in either terrestrial environments or in high $CO_2$ water for more than one month. It is noteworthy that internal gill ventilation was observed when the water level was high enough to immerse the head, but was suppressed when the water level was too low to immerse the head (Fig. S2). The suppression of internal gill movement was also observed in the high $CO_2$ environment, as was also observed by Babiker (1984). The internal gills of *Polypterus* reared in terrestrial and in high $CO_2$ water environments were then investigated to identify the presence or absence of cilia. Interestingly, SEM and immunostaining observation revealed that the cilia disappeared in the internal gills of *Polypterus* reared in both of these environmental conditions (Fig. 2D–F, Fig. 3B).

In the next trial, we returned the *Polypterus* individuals exposed to terrestrial or high $CO_2$ environments for one month back to the original water environment and reared them for an additional month. The SEM and immunostaining observations for the resultant *Polypterus* individuals revealed that the cilia in the internal gills had recovered with no apparent differences relative to those reared only in control water (Fig. 3B). These results suggest that the *Polypterus* possesses the ability to undergo plastic morphological changes (formation and de-formation) of cilia in the internal gills in response to environmental changes they experience.

In addition to internal gill cilia, the enlargement of the interlamellar cell mass (ILCM) between the lamellae of the internal gills was examined in the *Polypterus*, which ware reared in both the terrestrial and high $CO_2$ 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 (Fig. 4), and a similar enlargement of the ILCM was also found to have occurred in the individuals reared in the high $CO_2$ environment (Fig. 4).

## Elucidation of cilia function by RNA-seq analysis

The DEGs analyses were conducted on the whole exome sequencing data (RNA-seq data) for the internal gills of the *Polypterus* reared in both water and terrestrial environments. In particular, this study aimed to elucidate the function of cilia in the internal 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 internal 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 (Fig. 5A). The further inspection of these gene groups revealed that they were represented by *foxj1a*, which is included in one of the major genes involved in cilium movement (Fig. 5B). *foxj1a* is a homolog of *foxj1* (Forkhead box protein J1)(Aamar and 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 regulated by *foxj1* was examined (Mukherjee et al., 2019), and found that they were also significantly down-regulated (Fig. 5B). These results indicate that *foxj1*, and its downstream genes involved in cilia movement, were down-regulated in terrestrial environments. These lines of data suggest that the cilia distributed on the internal gills of *Polypterus* 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 desccribing 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 internal 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 internal gill arches of *Polypterus* (Fig. 5C left). Importantly, no signal of acetylated tubulin positive cells 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 (Fig. 5C right). These results suggest that the ciliated cells on the internal 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 (*Tas1R*s) and taste 2 receptors (*Tas2R*s) 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 *Tas1R*s and *Tas2R*s gene cascade was examined. The expression level of *Tas2R*s was quite low and difficult to detect by RNA-seq. The next focus was on *trpm5* and *plcb2*, which are located downstream of the *Tas1R*s and *Tas2R*s gene cascades (Ahmad and Dalziel, 2020; Tuzim and Korolczuk, 2021). Both genes were expressed in internal gills, but there were no significant changes in expression levels observed between aquatic and terrestrial environments (Fig. S3A and B). Next, the focus was on three genes of nitric oxide synthase (NOS), which also contributes toward controlling the beating of motile cilia in the airway epithelium of mammals. 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 internal gills of *Polypterus* found no significant differences between the control and terrestrial environments (Fig. S3C). These lines of data suggest that the cilia in the internal 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 respiration.

## Discussion

### Origin of cilia in the internal 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 internal 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 internal 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 internal 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 Osteichthyes 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 internal gills of *Polypterus* are distinct from those seen in lungfish, which are morphologically more similar to those seen in the external gills of amphibians (Ichikawa and Toyoizumi, 2020; Kemp, 1996). Indeed, the cilia of *Polypterus* are noticeably obviously short and are distributed in a circular dotted pattern (Fig. S1A), while those in lungfish and amphibians are long and distributed in band-like patterns (Fig. S1B). The morphological distinctness of the cilia observed in the internal gills of *Polypterus* and lungfish implies that specific roles of cilia have diversified between ray-finned and lobe-finned fish.

### 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 in the external gills were believed

to produce water flow in amphibians and larval *Polypterus* (Bartsch et al., 1997; Ichikawa and Toyoizumi, 2020). Additionally, 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 respiration and eliminating small particles such as micro-organisms from internal and external gill surfaces (Bartsch et al., 1997; Ichikawa and Toyoizumi, 2020; Kemp, 1996). In this study, the cilia found in the internal gills of adult *Polypterus* were shown to be motile (Fig. 5). However, it appears that the internal gills of *Polypterus* have sufficient water exchange capacity even without cilia. This may be explained by the dual respiration system used by *Polypterus*, which utilizes both lungs and internal gills. A previous study revealed that the frequency of internal gill respiration in *Polypterus* decreases significantly before and after air-breathing (Magid, 1966). Additionally, it has been proposed that internal gill ventilation is inefficient in bony fish, consuming from 10% to up to 69% of the oxygen obtained through respiration (Milsom, 1989). *Polypterus* may reduce its frequency of internal gill respiration by complementing it with pulmonary respiration. However, depression of internal gill respiration leads to the reduction of other internal gill functions such as ion transport and the excretion of carbon dioxide and ammonia (Ultsch, 1996). During such temporary depression of internal gill respiration, the motile cilia may function to increase efficient water flow for excretion and transport. Considering that internal gill cilia and pulmonary respiration are mutually reinforcing in function, it is plausible that they were acquired at the same time in their evolution.

## The plasticity of cilia in internal gills in response to the environments

This study found that the cilia in the internal gills of *Polypterus* were lost after the exposure to a terrestrial environment (Fig. 2, 3), and it noteworthy that the cilia in the external gills of amphibians disappear first during metamorphosis (Hackford et al., 1977), which resembles the loss of cilia seen in *Polypterus* in a terrestrial environment. Considering that the cilia play essential roles in efficient respiration, excretion of the carbon dioxide, *etc*., it makes sense that cilia disappear under conditions where water is unavailable. Previous studies have revealed a reduction in the volume of mineralized bone in the internal gills after 8 months of rearing on land, suggesting that *Polypterus* may reduce investment in its internal

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 (Fig. 3), suggesting that the loss of cilia shown in this study does not result from an injury due to the forced terrestrialization. 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 $CO_2$ in water resulted in the plastic loss and regeneration of cilia in the internal gills of *Polypterus* (Fig. 3). Previous studies have revealed that internal gill ventilation in *Polypterus* was depressed in water with high $CO_2$ concentrations (Babiker, 1984), and this finding was confirmed in this study. In a high $CO_2$ environment, internal gill respiration causes several negative impacts, represented by acidosis. It is presumed that *Polypterus* survives in a high $CO_2$ environment by depressing internal gill ventilation and switching to respiration through the lungs when needed. Since respiration through internal gills are not adaptive in a high $CO_2$ environment, the frequency of internal gill ventilation as well as the motile cilia, would likely be reduced.

## ILCM size changes in response to environmental changes

In addition to these microstructural changes involving cilia, the enlargement of the ILCM was observed in response to high $CO_2$ concentrations or when the animal was in terrestrial environments (Fig. 4). A previous study showed that the ILCM between the secondary gill lamellae were enlarged in the internal gills of *Polypterus* reared on land (Turko et al., 2019). The enlargement or reduction of ILCM are also widely observed in teleost fish such as crucian carp (*Carassius carassius*) and mangrove killifish (Nilsson et al., 2012; Ong et al., 2007; Sollid et al., 2003). In crucian carp, the area of the ILCM reduces in response to low oxygen, leading to the increase in the surface area of the internal gills, and thus, the increase of oxygen absorption capacity (Nilsson et al., 2012). The enlargement of the ILCM of *Polypterus* in high $CO_2$ conditions suggests a reduction of surface area of the internal gills, further resulting in the reduction of the passive import of $CO_2$ from the water. It is noteworthy that both the high $CO_2$ condition and terrestrial environment induced the same

329 morphological changes in the internal gills of *Polypterus*, which has the ability to breathe air
330 (see latter discussion).

331

332 ## High $CO_2$ environments and the water to land transition

333 Previously, the possible link between hypercarbia and the water to land transition has
334 been discussed. Because of its high solubility in water, $CO_2$ is normally excreted through the
335 internal gills of most fish. In contrast, air-breathing fish take in $O_2$-rich air, resulting in a
336 lower frequency of ventilation using internal gills. Therefore, the blood of air-breathing fish
337 shows a higher $P_{CO2}$ relative to other fish (Bayley et al., 2019; Ultsch, 1996). Indeed, in some
338 obligate air-breathing fish, the acid-base status of the blood is similar to that seen in amniotes
339 (Bayley et al., 2019; Ultsch, 1996). Based on studies showing that hypoxic environments are
340 also high in carbon dioxide, it has been proposed that air-breathing fish may have shifted to
341 high $P_{CO2}$, [$HCO_3^-$] blood prior to their terrestrial adaptation during vertebrate evolution
342 (Ultsch, 1996).

343 In this study, both high $CO_2$ water and terrestrial environments were found to lead to
344 the similar results in *Polypterus*, namely, the depression of internal gill respiration, ILCM
345 enlargement, and the loss of cilia in the internal gills. Based on these finding, we propose the
346 following scenario in which the adaptive character(s) for high $CO_2$ environments in air-
347 breathing fish were later co-opted for terrestrial adaptation: (1) Air-breathing using lungs
348 evolved in the common ancestor of Osteichthyes, allowing them to survive in low $O_2$ and
349 high $CO_2$ environments. Additional to air-breathing, cilia were acquired to facilitate water
350 flow for efficient respiration and material exchange in the internal gills. (2) In response to
351 further elevation of $CO_2$ concentration in the water, air-breathing fish depressed internal gill
352 ventilation, reduced surface area, and lost cilia. (3) The early tetrapods were thus ready to
353 adapt to a terrestrial lifestyle because some adaptive characters acquired in high $CO_2$
354 environments were also adaptive in terrestrial environments. For example, the depression of
355 internal gill ventilation and the enlargement of the ILCM were adaptive to a terrestrial
356 environment as they both prevented desiccation. The loss of cilia was also adaptive in that
357 respiration and material transfer through internal gills are not functional on land. Importantly,
358 the above changes of the internal gills were plastic in the ancestor of Osteichthyes, and that

may have allowed them to progressively accomplish the incremental transition from water to land.

In this study, SEM 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 internal 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 $CO_2$ environments. In some Devonian fish, some characters evolved under similar high $CO_2$ environments 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*, the oldest lineage of amphibious ray-finned fish, provide important insights into the evolutionary transition of vertebrates from water to land.

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**Data availability**

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

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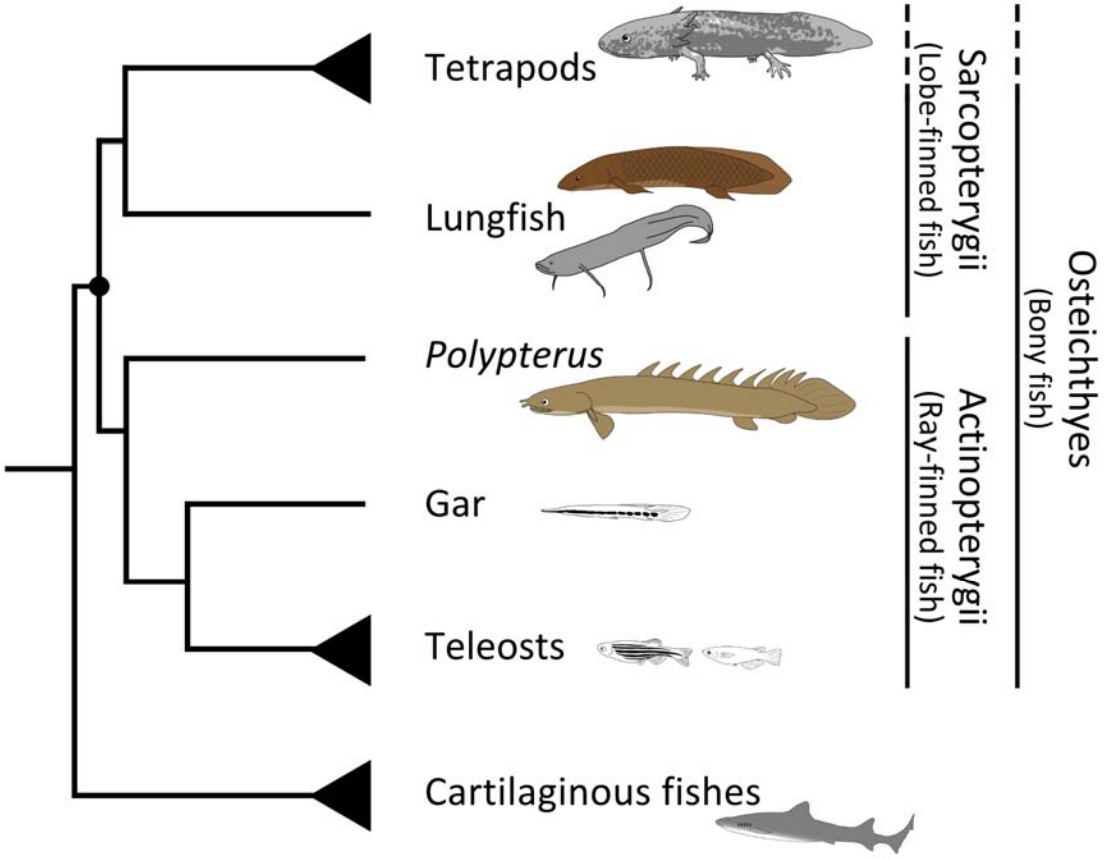
**Competing interests**

We declare we have no competing interests.

## Authors' contributions

Y.K.: Conceptualization, formal analysis, investigation, writing - original draft, visualization, funding acquisition; N.N.: Investigation, writing - review & editing; M.N.: Writing - review & editing, supervision, funding acquisition

389 **Figures**




390

391 **Figure 1. Phylogenetic relationships among cartilaginous fishes, tetrapods, lungfish and**

392 ***Polypterus*.** The black circle on the tree indicates the common ancestor of Osteichthyes.

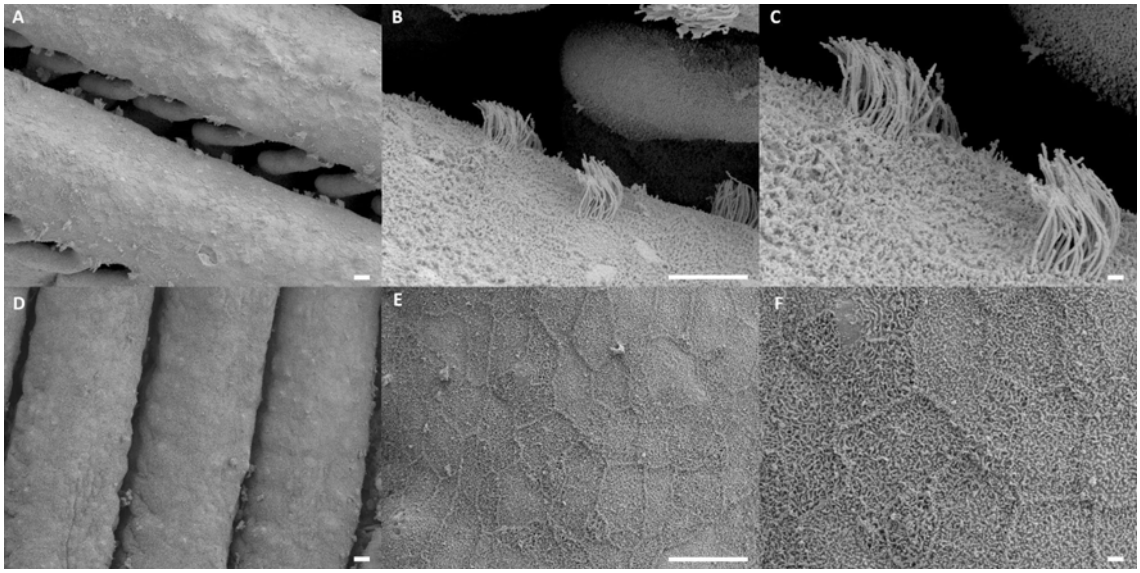



**Figure 2. Scanning electron micrograph of the surface of internal gill filaments of *Polypterus senegalus*.** The surface of *Polypterus* reared in water (A–C) and terrestrial environment (D–F). Scale bar in A, B, D and E = 10 μm, in C and F = 1 μm.

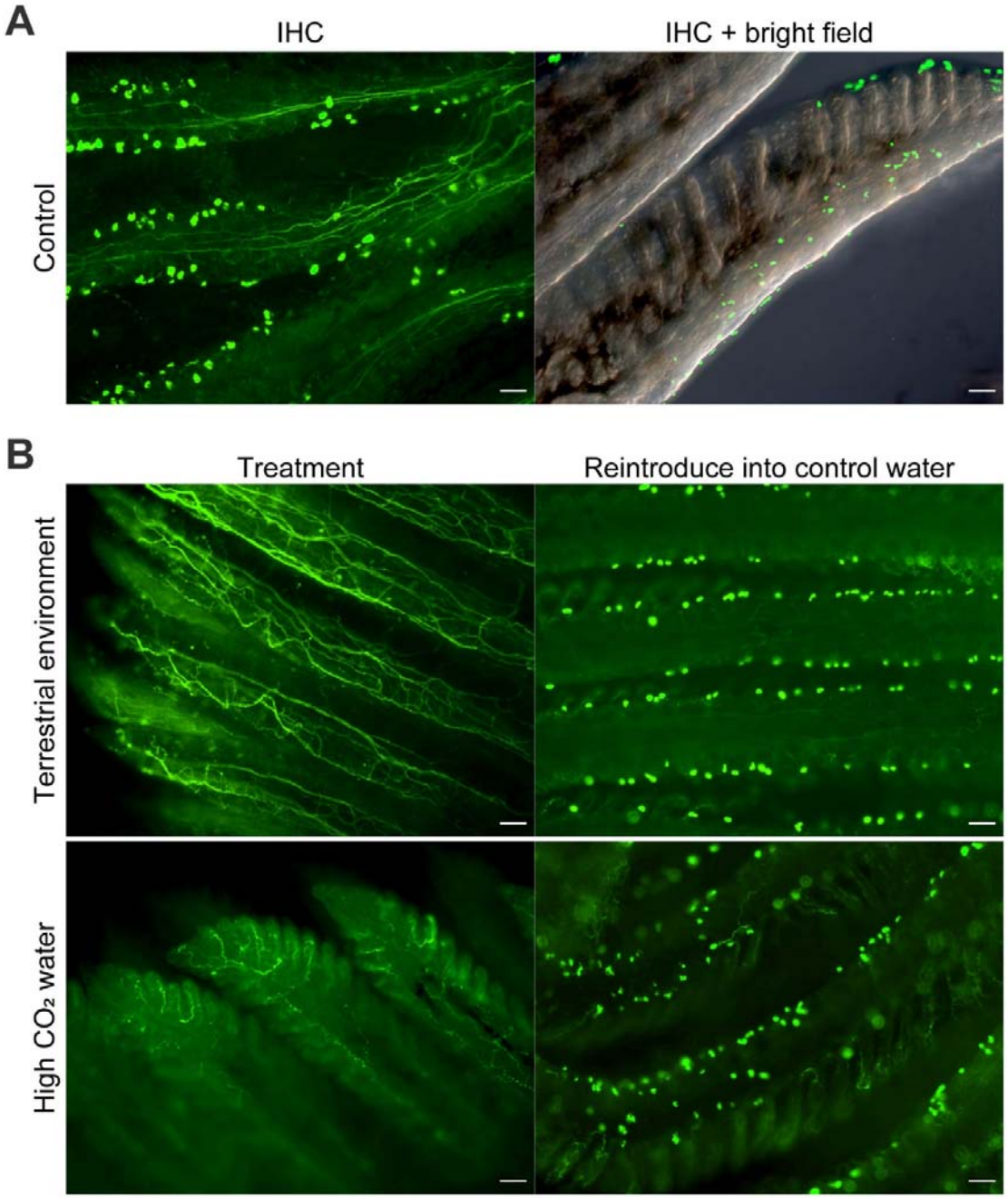



**Figure 3. Acetyl alpha Tubulin immunostaining of internal gills of *P. senegalus*.** The internal gills of *Polypterus* reared in control water (A), in a terrestrial environment (B: top part), and in high $CO_2$ water (B: bottom part). Punctate spots indicate the presence of multiple cilia. Scale bar = 50 μm.

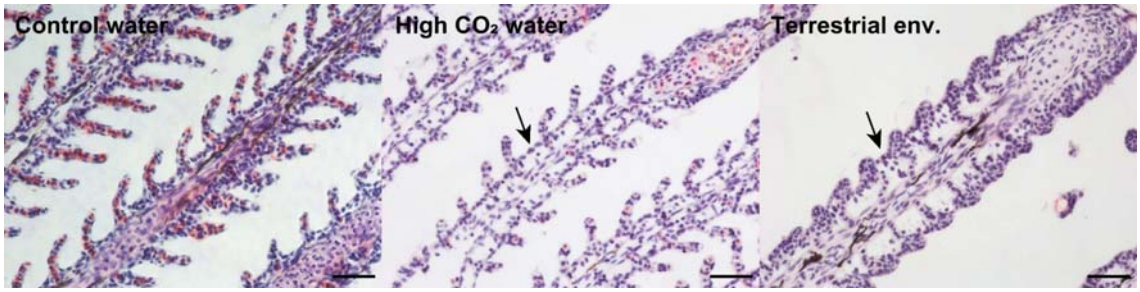



**Figure 4. Light micrographs of internal gills of *P. senegalus* stained with hematoxylin and eosin.** The inter-lamellar cell mass (ILCM) was enlarged in high $CO_2$ water condition and terrestrial environment compared to the control water (arrows). Scale bar = 50 μm.

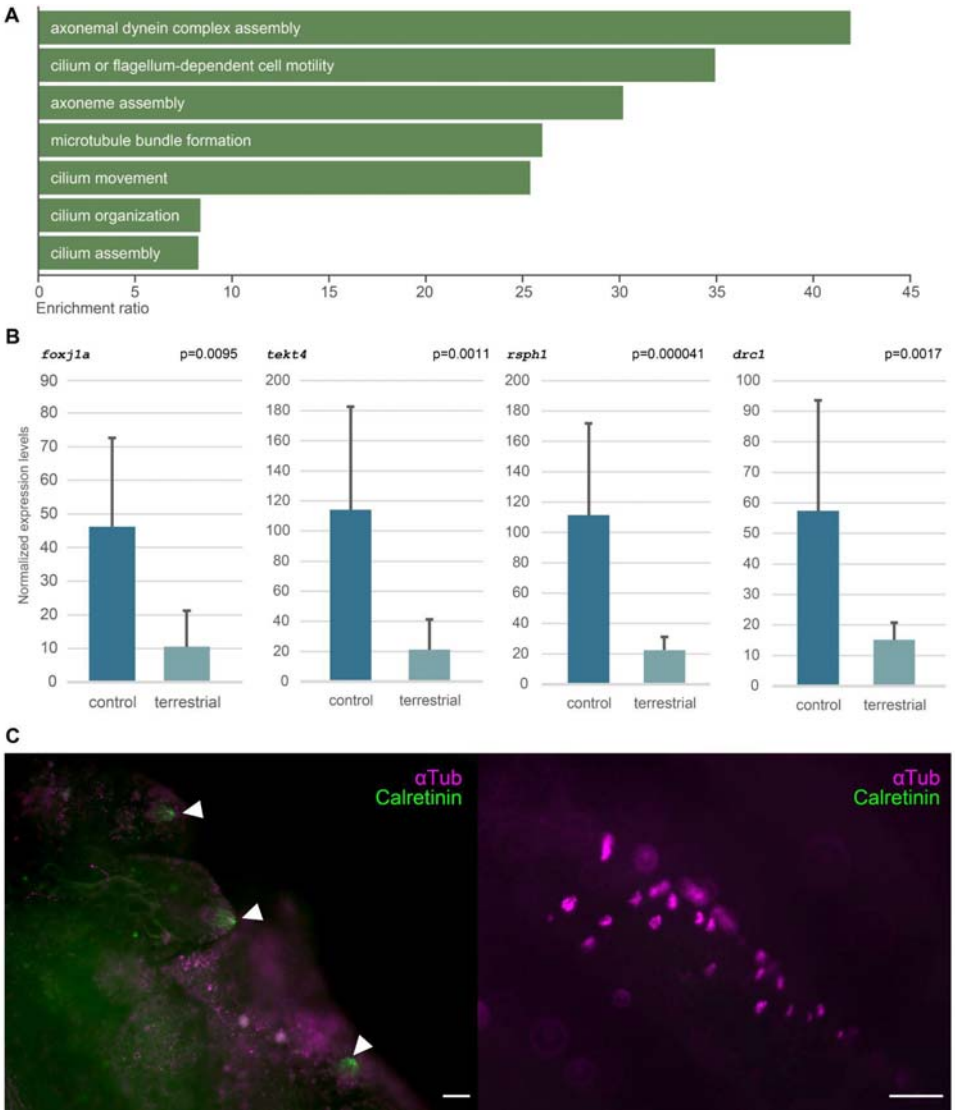



**Figure 5. Results of expression analysis and calretinin immunostaining in gills.** (A) The results of over representation analysis using genes whose expression levels were reduced in the internal gills of *Polypterus* reared in a terrestrial environment. (B) Normalized expression levels of four representatives of genes known to be involved in cilia movement. TCC was used for normalization (see methods section). (C) Micrograph of internal gills immunostained with anti-calretinin antibody (light green) and anti-acetylated alpha tubulin antibody (magenta). The left image shows the presence of taste buds (arrowhead) and absence of ciliated cells in the internal gill arches. The right image shows the presence of ciliated cells and absence of taste buds in the internal gill lamella. Scale bar = 50 μm.

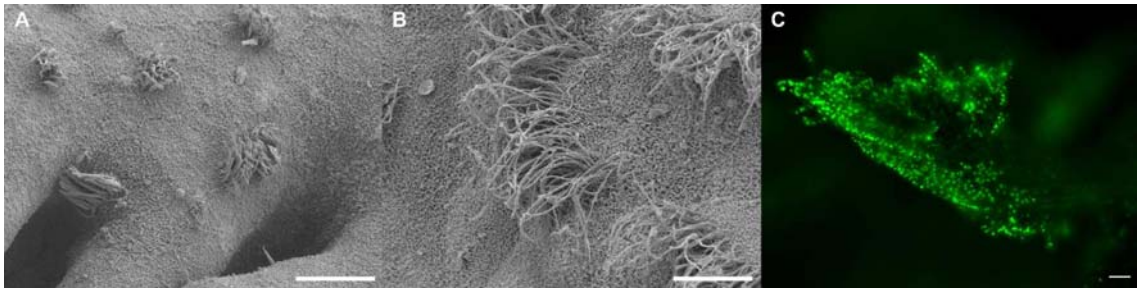



**Figure S1. SEM and immunostaining images of internal and external gills of *Polypterus* and axolotl.** (A) Scanning electron micrograph (SEM) of the surface of internal gill filaments of adult *P. senegalus* reared in water. (B) SEM of the surface of external gill filaments of *A. mexicanum*. (C) Micrograph of external gills of larva of *P. senegalus* immunostained with anti-acetylated alpha tubulin antibody. Scale bar in A and B = 10 μm, in C = 100 μm.

**Figure S2. Respiration of *Polypterus* in water and terrestrial environments.** The internal gill respiration of *Polypterus* in water (top part) and terrestrial environments (bottom part). The top and bottom still images are cropped at the same frame interval. The arrows indicate where the internal gills are moving during respiration in water.

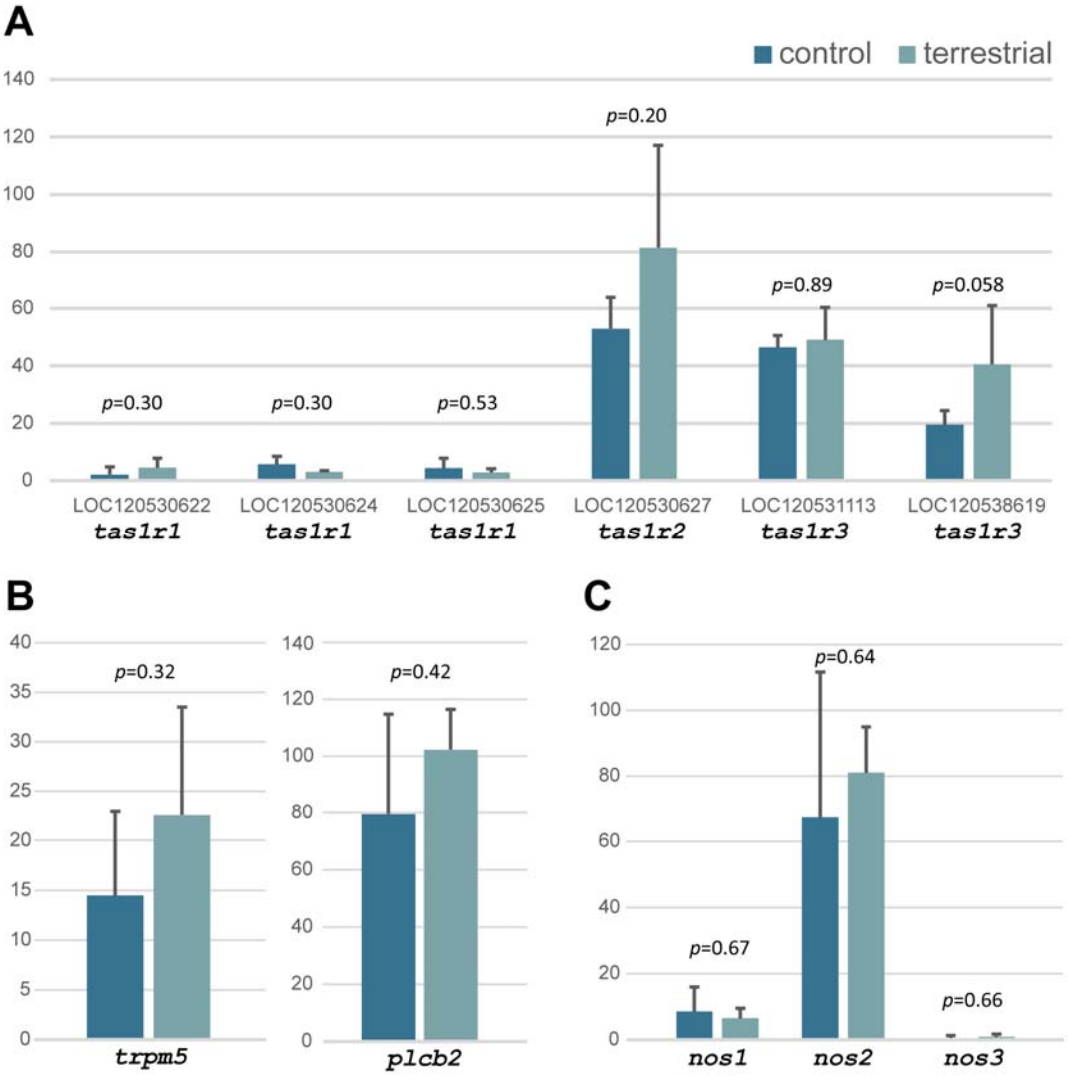



**Figure S3. Expression levels of some genes in gills in aquatic and terrestrial environments.** Normalized expression levels of (A) Tas1Rs and genes known to be involved in (B) Tas2R cascade (C) NOS (Nitric Oxide Synthase). TCC was used for normalization and testing (see methods section).

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