Morphological and Biochemical Variations in the Gills of 12 Aquatic Air-Breathing Anabantoid Fish

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ABSTRACT

All fish species in the Anabantoidei suborder are aquatic air-breathing fish. These species have an accessory air-breathing organ, called the labyrinth organ, in the branchial cavity and can engulf air at the surface of the water to assist in gas exchange. It is therefore necessary to examine the extent of gill modification among anabantoid fish species and the potential trade-offs in their function. The experimental hypothesis that we aimed to test is whether anabantoid fishes have both morphological and functional variations in the gills among different species. We examined the gills of 12 species from three families and nine genera of Anabantoidei. Though the sizes of the fourth gill arch in three species of *Trichogaster* were reduced significantly, not all anabantoid species had morphological and functional variations in the gills. In these three species, the specific enzyme activity and relative protein abundance of Na$^+$/K$^+$-ATPase were significantly higher in the anterior gills as compared with the posterior gills and the labyrinth organ. The relative abundance of cytosolic carbonic anhydrase, an indicator of gas exchange, was found to be highest in the labyrinth organ. The phylogenetic distribution of the fourth gill's morphological differentiation suggests that these variations are lineage specific, which may imply a phylogenetic influence on gill morphology in anabantoid species.

Introduction

The fish gill is a multifunctional organ that is in direct contact with the environment and is important for homeostatic activities, such as gas exchange and ionic regulation (Perry 1997,

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1998; Hirose et al. 2003; Evans et al. 2005). The four pairs of gills that are found in fish consist primarily of filaments and lamellae covered with epithelial cells. There are four major types of cells in the gill epithelia, including pavement cells, mitochondria-rich cells (MRCs), mucous cells, and undifferentiated cells (Perry 1997; Evans 1999). Pavement cells, which account for more than 90% of the gill surface (mostly in the lamellae), are the site of gas exchange (Perry 1998). MRCs are found mainly in the gill filament. In general, MRCs are the sites of ion extrusion in seawater and ion uptake in freshwater (Laurent and Perry 1990; Perry 1997; Evans 1999; Piermarini and Evans 2000). The membrane-spanning enzyme Na$^+$/K$^+$-ATPase (NKA) that is found in the MRCs is important for intracellular homeostasis and provides a driving force for many transport systems (Evans et al. 2005). Carbonic anhydrase (CA) is an important enzyme for rapid and reversible hydration/dehydration reactions of CO_2 (Henry and Swenson 2000).

Air-breathing fish are those that have the ability to exchange gases directly with the atmospheric environment (Graham 1997). These species are found not only in the well-oxygenated littoral zone but also in hypoxic wetlands and lakes (Randle and Chapman 2005). They are further classified into amphibious and aquatic air-breathing fishes. The accessory air-breathing organs are alternative gas exchange organs, including the labyrinth organ, skin, lungs, respiratory gas bladders, digestive tracts, and structures derived from buccal, pharyngeal, and branchial cavities (Graham 1997). The labyrinth organ is one of the accessory air-breathing organs that protrude from the first gill arch on both sides of the branchial cavities (Munshi et al. 1986). All anabantoid species have a labyrinth organ that is specialized for assisting in gas exchange and is involved in aquatic-surface respiratory behavior (Graham 1997). There are roughly 137 species and three families of air-breathing fish, including Anabantoidae (28 species), Helostomatidae (one species), and Osphronemidae (108 species). Previous studies on the Anabantoidei have focused mostly on their vascular organization, detailing the cardiac and/or vascular casts (Burggren 1979; Munshi et al. 1986; Olson et al. 1986, 1994, 1995; Olson 2002), and have shown that they possess branchial and systemic circuits similar to a double-circuit circulatory system. The anterior (first and second) gill arches receive blood from the heart and are the site for gas exchange, and the blood then flows to the labyrinth organ for further oxygen uptake before returning to the heart. Structural modifications and enlarged vessels in the posterior (third and fourth) gill arches assist in the transfer of oxygenated blood from the heart to the systemic circulation system (Munshi et al. 1986; Olson et al. 1986). We have previously shown that there are morphological and biochemical differences between the anterior and the posterior gill arches

in one species of Anabantoidei (*Trichogaster leeri*; Huang et al. 2008). In *T. leeri*, the NKA activity in the two anterior gills was upregulated significantly in deionized water and was found to be responsible under ionic stress. There were large-bore arterio-arterial shunts and shorter lamellae in the fourth gill arch, which is specialized for the transport of oxygenated blood and is less responsive to environmental stress (Huang et al. 2008). Recently, the molecular-based phylogeny of Anabantoidei was reconstructed using mitochondrial and nuclear DNA sequence data (Rüber et al. 2006), and this provides an opportunity to integrate the molecular data with the anatomical and biochemical characteristics of the gills.

The purpose of this study was to investigate whether morphological and potential functional variations in the gills among different species can be found widely in anabantoid species. Therefore, the specific objectives of this study were to (1) investigate the morphological variations in gill structure among different species of Anabantoidei, (2) examine the degree of the variations among the gills of different species, (3) describe the potentially functional differences within each species (evaluating the NKA activity at each gill site and the relative protein abundance of NKA in the anterior and posterior gill arches for ionic regulation and of CA for gas exchange) that could be correlated to the morphological modifications, and (4) discuss the possible evolutionary mechanisms underlying the morphological variations using a phylogenetic framework.

Material and Methods

Species Collection

Twelve anabantoid fish species were examined in this study (Table 1). These species are mainly distributed in Africa and Southeast Asia, including the Malay Peninsula, Thailand, and Indonesia (Sumatra and Borneo). Many of the streamlets or marshes that they inhabit accumulate humic substances and are often hypoxic. Most of the fish in this study are available year round from local commercial sources and are relatively easy to maintain in the laboratory. The fish were kept in holding aquaria (58 × 41 × 35.5-cm³ plastic tank) without sand on the bottom. Fish were held in recirculated and aerated tap water at 28° ± 1°C with a 12L : 12D photoperiod (osmolality: 0.5 ± 0.1 mOsm/kg; Na⁺ concentration: 1.24 mM) for more than 1 wk before the experiments. Fish were fed with commercial fish food (NOVO Bits, JBL) ad lib. daily until 1 d before sampling, and water was changed at least once per week. Oxygen levels were monitored (Orion model 810) and kept at 7.3 ± 1.13 mg/L and a pH of 7.12 ± 0.21 (pH vision 6071, Jenco, Hong Kong). The experiments and handling of the animals complied with the current laws of Taiwan.

Tissue Sampling

Fish were killed by severing the spinal cord, and all four gills were excised and fixed in Bouin's solution (Sigma) for 48 h at 4°C in the dark. These samples were then subjected to an ethanol-xylene series dehydration. After embedding in paraffin,

Table 1: List of the 12 anabantoid fish species examined

Family and Species	Abbreviation	Total Body Length (cm)
Anabantoidae:		
Ctenopoma acutirostre	*C. a.*	3.52 ± 1.27
Helostomatidae:		
Helostoma temminckii	*H. t.*	3.68 ± .70
Osphronemidae:		
Betta splendens	*B. s.*	3.78 ± .55
Colisa fasciata	*C. f.*	4.10 ± .12
Colisa lalia	*C. l.*	4.10 ± .23
Macropodus opercularis	*M. o.*	4.59 ± .73
Osphronemus goramy	*O. g.*	5.26 ± .16
Sphaerichthys osphromenoides	*S. o.*	2.60 ± .12
Trichogaster leeri	*T. l.*	6.96 ± .06
Trichogaster microlepis	*T. m.*	4.82 ± .41
Trichogaster trichopterus	*T. t.*	5.05 ± .53
Trichopsis vittatus	*T. v.*	2.60 ± .12

Note. Abbreviations used in the text and total body length (mean ± SEM) for each species are provided.

tissue sections were prepared at a thickness of 5 μm (RM2025RT, Leica) and placed on slides that were precoated with poly-L-lysine solution. Samples were dewaxed and rehydrated before staining with hematoxylin and eosin. Samples were then dehydrated once more and mounted (Permount, Sigma) before being photographed by a digital camera (D1, Nikon).

We collected the gill arches by alternating from both sides. For those gill filaments with intact cartilage rods, an indication of the axial position of each histological section, we sampled and measured the lengths of three filaments from the anterior, four from the middle, and three from the posterior portions of the gill arch. We measured 25 lamellae from the proximal to middle portion of these intact filaments. The length of each gill filament and lamella was determined by image processing (Image-Pro Plus 4.5, Media Cybernetics, Silver Spring, MD). Ten filaments and 25 lamellae in each gill from eight fish of each species were photographed and measured to quantify the lengths of these structures. All chemicals used in the experiment were purchased from Sigma and Merck.

Protein Extraction from Gills and the Labyrinth Organ

Each gill and labyrinth organ was homogenized (Ultrasonic Processor, Sonics) in 200 μL homogenizing medium containing a mixture of proteinase inhibitors (3.31 mM antipain, 2.16 mM leupeptin, and 1.92 M benzamidine in an Aprotinin saline solution [5–10 trypsin inhibitor units/mL, Sigma, no. A 6279] and buffer solution [100 mM imidazole {Imidazole-HCl buffer}, 5 mM Na₂EDTA, 200 mM sucrose, and 0.1% sodium deoxycholate]) at a ratio of 1 : 200 at a pH of 7.6. To separate the supernatants, the crude homogenate was centrifuged at 1,064 *g* for 10 min, followed by separation of supernatants from the first step that were then centrifuged at 20,160 *g* for 20 min

at 4°C; the supernatants from this step were then collected (EBR12R, Hettich) and immediately analyzed. A total of 2 μL of supernatant was diluted to 200 μL with deionized water. An aliquot of 100 μL of this mixture was further diluted to 800 μL with deionized water (i.e., 800-fold; Huang et al. 2008), and the diluted supernatant was thoroughly mixed with 200 μL of protein assay solution (Dye Regent Concentrate, Bio-Rad). Total protein was determined using a spectrophotometer (U-2001, Hitachi) at a wavelength of 595 nm. Bovine serum albumin diluted with deionized water at the same dilution factor was prepared as a control.

Enzyme Specific Activity

NKA activity was determined in triplicate, and eight fish of each species were examined to determine differences between the inorganic phosphate liberated in 350 μL of reaction medium in the presence and absence of 50 μL of 12.46 mM ouabain. We extracted proteins from the gill and used an aliquot of 10 μg of extracted proteins to determine enzyme activity. The inorganic phosphate concentration was measured according to the method of Peterson (1978). The reaction medium contained 142.85 mM imidazole (Imidazole-HCl buffer), 178.50 mM NaCl, 10.71 mM $MgCl_2$, and 107.14 mM KCl. The pH was maintained at 7.6, and the reaction was run at 37°C for 30 min and stopped by adding 200 μL of ice-cold 1.8 M trichloroacetic acid. Ouabain-sensitive ATPase activity was expressed as μmol Pi/mg protein/h.

Relative Proteins Abundance

Ten micrograms of gill tissue was homogenized in sample-loading buffer (20 mM Tris-HCl, pH 6.8, 8% sodium dodecyl sulfate [SDS], 10% β-mercaptoethanol, 40% glycerol, and 0.4% bromophenol blue) and was then denatured by heating at 37°C for 15 min for CA and NKA immunoblotting and finally separated on 12% and 10% SDS-polyacrylamide gels, respectively. After electrophoresis, the proteins were transferred from the gel to a polyvinyliden difluoride membrane (Amersham, NEN Life Science, Boston) and blocked in 5% nonfat milk in PBST (phosphate buffered saline Tween-20 containing 136.9 mM NaCl, 2.68 mM KCl, 6.39 mM $Na_2HPO_4.2H_2O$, 1.76 mM KH_2PO_4, and 20 mM 0.5% Tween-20, pH 7.4) for 1 h at room temperature (26°C). Membranes were then washed in PBST and incubated with either CA (1 : 10,000, CAII rabbit polyclonal primary antibody, NOVUS) or NKA (1 : 1,000, α-5 mouse monoclonal primary antibody against the α-subunit of chicken NKA, DSHB) for 1 h at room temperature. Membranes were washed again in PBST three times for 10 min and incubated in conjugated antirabbit secondary antibody (1 : 10,000, Jackson Immunoresearch) for CAII or antimouse secondary antibody (1 : 10,000, Jackson Immunoresearch) for NKA and β-actin (1 : 10,000, monoclonal antibody, Chemicon) for 1 h at room temperature. After washing in PBST three times for 10 min, we detected proteins using a chemiluminescence reagent plus system (NEN Life Science). The signals produced

were photographed using an Intelligent Dark Box II with a Fujifilm LAS-1000 digital camera. The relative protein abundance was analyzed by Image Gauge 4.0 (Fujifilm) and determined by dividing the intensity of CA or NKA with that of β-actin (internal control) in each set. The relative protein abundance in the three tissues was calculated as the percentage of the amount of protein in the first gill in each species. Since fish do not have the mammalian CA I, II, III, and XIII gene cluster (Tang and Lee 2007; Lin et al. 2008; Gilmour and Perry 2009; Gilmour 2010; Sattin et al. 2010), we expressed our finding as cytosolic CA (CAc).

Morphological and Biochemical Variability in the Gills

The gills of 12 species were analyzed for differences in morphology and potential function, including the lengths of filaments and lamellae as well as NKA enzyme activity and relative protein abundance in NKA and CAc. In this study, *Betta splendens*, *Colisa lalia*, *Helostoma temminckii*, and *Macropodus opercularis* represented the species that had no obvious morphological differences in their gills. In contrast, *Trichogaster microlepis*, *Trichogaster leeri*, and *Trichogaster trichopterus* had apparent variation in their gills. We then examined the potentially functional differences in the first and fourth gill arches and the labyrinth organ of these seven species by evaluating the NKA enzyme activity and protein abundance at each gill arch as an indicator of ionic regulation and CAc protein abundance as an indicator of gas exchange. From these data, we concluded that the functional differences within each species with respect to ionic regulation and gas exchange between the gills and the labyrinth organ were consistent with the morphological variations we observed in the gills.

Statistical Analyses

All data are presented as the means ± SEM. The differences among gills in terms of the length of filaments, lamellae, and NKA enzyme activity were determined using a one-way ANOVA followed by Tukey's pairwise comparison. The first and second gill arches were designated as the anterior gill arches and the third and fourth gill arches as the posterior gill arches. In addition, for NKA and CAc protein relative abundance, one-way ANOVA and Dunnett's tests were performed for further comparisons between the first and the fourth gill arches and the labyrinth organ. A value of $P < 0.05$ was deemed significant. All statistical analyses were conducted using SAS 8e for Windows (SAS Institute, Cary, NC).

Results

Intra- and Interspecies Variations in Gill Morphology

Morphological variations among the four gills were apparent in some but not all anabantoid species. Among the 12 species examined, in *Trichogaster leeri* (Fig. 1*Q*, 1*R*), *Trichogaster microlepis* (Fig. 1*S*, 1*T*), and *Trichogaster trichopterus* (Fig. 1*U*, 1*V*), we found an apparent modification in the fourth gill arch
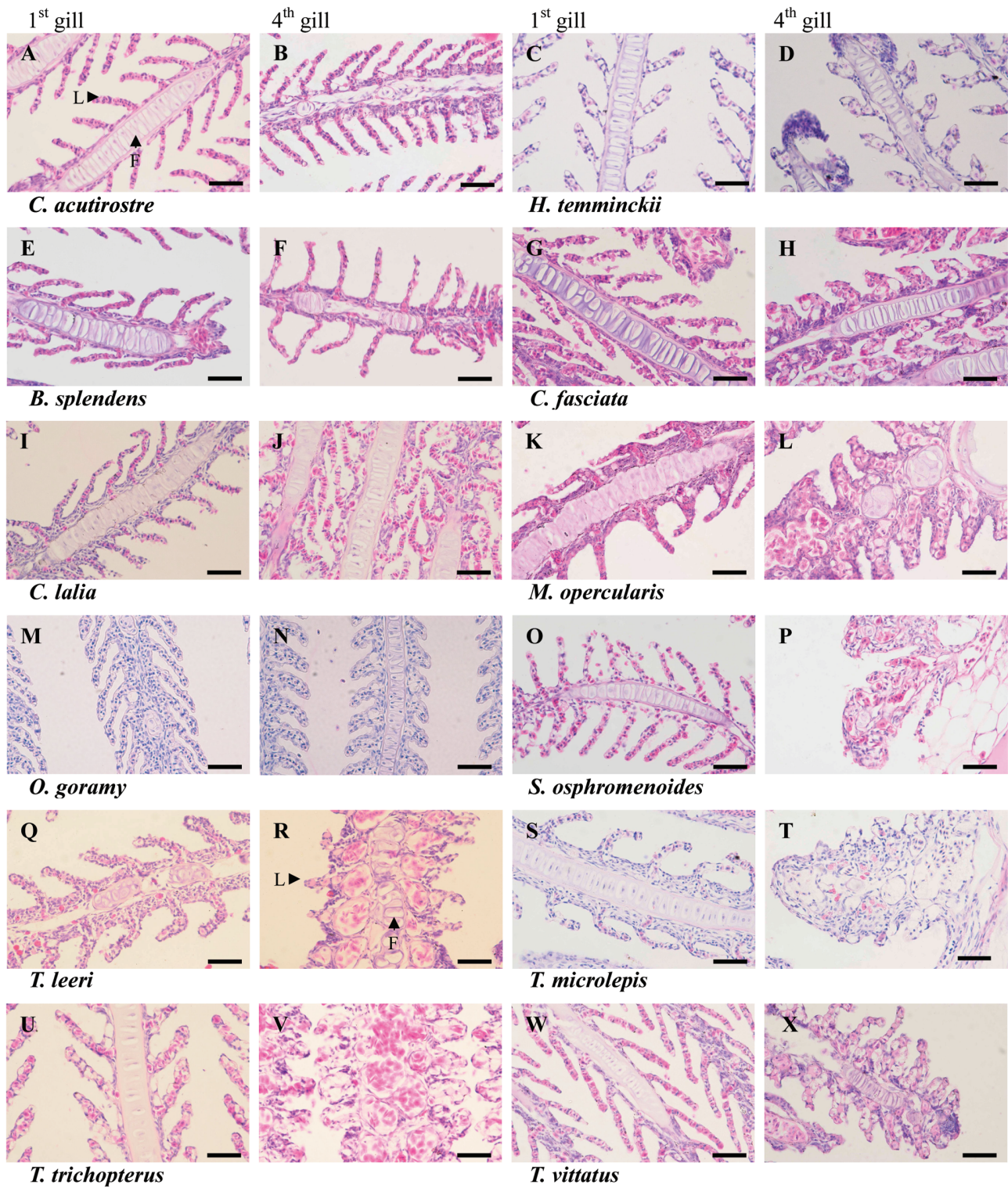
Figure 1. Gill morphology in the 12 species of anabantoid fishes. Among these, *Ctenopoma acutirostre* (*A*, *B*), *Helostoma temminckii* (*C*, *D*), *Betta splendens* (*E*, *F*), *Colisa fasciata* (*G*, *H*), *Colisa lalia* (*I*, *J*), *Macropodus opercularis* (*K*, *L*), *Osphronemus goramy* (*M*, *N*), *Sphaerichthys osphromenoides* (*O*, *P*), and *Trichopsis vittatus* (*W*, *X*) had no apparent morphological variations in the first and fourth gills. *Trichogaster leeri* (*Q*, *R*), *Trichogaster microlepis* (*S*, *T*), and *Trichogaster trichopterus* (*U*, *V*) had a significant modification in the fourth gill (*R*, *T*, *V*). The basal lamellae merged and became larger vessels. In addition, some of the filaments in the fourth gill did not possess visible lamellae. *F*, filament; *L*, lamellae. Scale bars = 30 μm.

(Fig. 1*R*, 1*T*, 1*V*). The merged basal lamellae had larger blood vessels in these species, and some of the filaments in the fourth gill arch did not possess any visible lamellae. The other nine had relatively less degrees of morphological variations in the fourth gill arches. These species are *Ctenopoma acutirostre* (Fig. 1*A*, 1*B*), *Helostoma temminckii* (Fig. 1*C*, 1*D*), *Betta splendens* (Fig. 1*E*, 1*F*), *Colisa fasciata* (Fig. 1*G*, 1*H*), *Colisa lalia* (Fig. 1*I*, 1*J*), *Macropodus opercularis* (Fig. 1*K*, 1*L*), *Osphronemus goramy* (Fig. 1*M*, 1*N*), *Sphaerichthys osphromenoides* (Fig. 1*O*, 1*P*), and *Trichopsis vittatus* (Fig. 1*W*, 1*X*). However, some species showed dramatic thickening of lamellae in the fourth gill arch compared with the first gill arch. These species were *H. temminckii* (Fig. 1*C*, 1*D*), *C. fasciata* (Fig. 1*G*, 1*H*), *M. opercularis* (Fig. 1*K*, 1*L*), *O. goramy* (Fig. 1*M*, 1*N*), and *T. vittatus* (Fig. 1*W*, 1*X*).

The lengths of the filaments among the gills in most species exhibited a tendency to gradually shorten from the first gill arch to the fourth gill arch (Fig. 2*A*). A significant difference between the anterior (first and second) and the posterior (third and fourth) gill arches, however, was found in five of the species (*O. goramy*: $F_{3,19} = 113.28$, $P < 0.0001$, $n = 8$; *S. osphromenoides*: $F_{3,31} = 22.15$, $P < 0.0001$, $n = 5$; *T. leeri*: $F_{3,39} = 65.25$, $P < 0.0001$, $n = 8$; *T. microlepis*: $F_{3,31} = 876.54$, $P < 0.0001$, $n = 8$; *T. trichopterus*, $F_{3,31} = 104.50$, $P < 0.0001$, $n = 8$). Significant differences in the lengths of the lamellae among gills were found in only three of the species we examined (Fig. 2*B*; *T. leeri*: Huang et al. 2008; *T. microlepis*: $F_{3,31} = 55.50$, $P < 0.0001$, $n = 8$; *T. trichopterus*: $F_{3,31} = 33.77$, $P < 0.0001$, $n = 8$).

Functional Differences in the Gill Arches and Labyrinth Organ

We chose seven species to further evaluate the potential functional variation in gills because the other five species were too small to effectively investigate potential functional differences. Comparisons were also conducted between the anterior (first and second) and the posterior (third and fourth) gill arches within these species. Only *T. leeri* (Huang et al. 2008), *T. microlepis* ($F_{3,31} = 10.62$, $P = 0.0002$, $n = 8$), and *T. trichopterus* ($F_{3,31} = 25.90$, $P < 0.0002$, $n = 8$; Fig. 3) differed significantly in the NKA activity between the anterior and the posterior gill arches, suggesting functional differences of gills among different species.

The immunoreactive band for NKA lies at approximately 95 kDa (Fig. 4*A*), and two CAc immunoreactive bands (bands I and II) are observed at approximately 29 kDa (Fig. 4*B*). We examined the first gill arch, the fourth gill arch, and the labyrinth organ in the seven species. Quantification of NKA immunoreactive bands among the seven species showed that both the fourth gill arch and the labyrinth organ had lower NKA protein abundance compared with the first gill arch in *C. lalia* ($F_{2,23} = 55.28$, $P < 0.001$, $n = 8$), *H. temminckii* ($F_{2,23} = 18.54$, $P < 0.001$, $n = 8$), *M. opercularis* ($F_{2,23} = 45.38$, $P < 0.001$, $n = 8$), *T. leeri* ($F_{2,23} = 130.28$, $P < 0.001$, $n = 8$), *T. microlepis* ($F_{2,26} = 45.15$, $P < 0.001$, $n = 8$), and *T. trichopterus* ($F_{2,23} = 5.62$, $P = 0.011$, $n = 8$). In *B. splendens*, only the labyrinth organ had lower NKA protein abundance than the first gill arch ($F_{2,23} = 3.75$, $P = 0.041$, $n = 8$; Fig. 4*C*).

When band I of the CAc immunoreactive bands was quantified, the fourth gill arch was found to have lower CAII protein abundance than the first gill arch only in *M. opercularis* ($F_{2,23} = 7.64$, $P = 0.003$, $n = 8$; Fig. 4*D*). There was no difference between the first and the fourth gill arches in the remaining six species. The CAc protein abundance of the labyrinth organ was lower than the first gill arch in *C. lalia* (one-way ANOVA, $F_{2,23} = 6.70$, $P = 0.006$, $N = 8$) but was higher in *T. leeri* ($F_{2,23} = 12.70$, $P < 0.001$, $n = 8$), *T. microlepis* ($F_{2,29} = 18.56$, $P < 0.001$, $n = 8$) and *T. trichopterus* ($F_{2,23} = 21.72$, $P < 0.001$, $n = 8$).

Discussion

Among the 12 anabantoid species that we examined, we found that not all species exhibit morphological variation among gills. For example, the gills of *Colisa lalia* did not vary morphologically with respect to the lengths of either filaments or lamellae. Conversely, some species, such as *Trichogaster microlepis*, had not only an apparent structural variation in the fourth gill but also a significant difference in the lengths of both filaments and lamellae between the anterior gill arches and the posterior gill arches. Additionally, the lengths of filaments among gills in *Sphaerichthys osphromenoides* differed but did not exhibit a structural modification in the fourth gill arch. Second, we found functional differences (indicated by NKA enzyme activity as well as NKA and CAc protein relative abundance) between the gills and the labyrinth organ in the three *Trichogaster* species.

Anabantoid fish exhibit a high diversity in branchial modifications. The three *Trichogaster* species that we examined displayed the most apparent structural variations in the gill arches. *Macropodus opercularis* and *Colisa fasciata* differed only in the lengths of the filaments and lamellae between the first and the fourth gill arches but not in the lengths between the anterior gill arches and the posterior gill arches (Fig. 2). Two other species, *Osphronemus goramy* and *S. osphromenoides*, exhibited variation in the length of filaments but not in the length of the lamellae between the anterior gill arches and the posterior gill arches. Finally, *Ctenopoma acutirostre* had no length variation in the gills. In addition, some species showed dramatic thickening of lamellae in the fourth gill arch compared with the first gill arch. These species were *Helostoma temminckii*, *C. fasciata*, *M. opercularis*, *O. goramy*, and *Trichopsis vittatus*. The structural variations in these species might decrease the blood-water diffusion pathway and result in more oxygenated blood from the labyrinth organ reaching the systemic circulation via the heart and the posterior gill arches (Munshi et al. 2001). Because it is difficult to standardize the thickness of lamellae, we did not measure the diffusion distance in these species.

Our integrative investigation of the morphological anatomy and biochemical variability of gills has therefore provided more new data to further our understanding of the anabantoid species. Fish were all acclimated in the same conditions before experiments were conduced, so their responses should all have
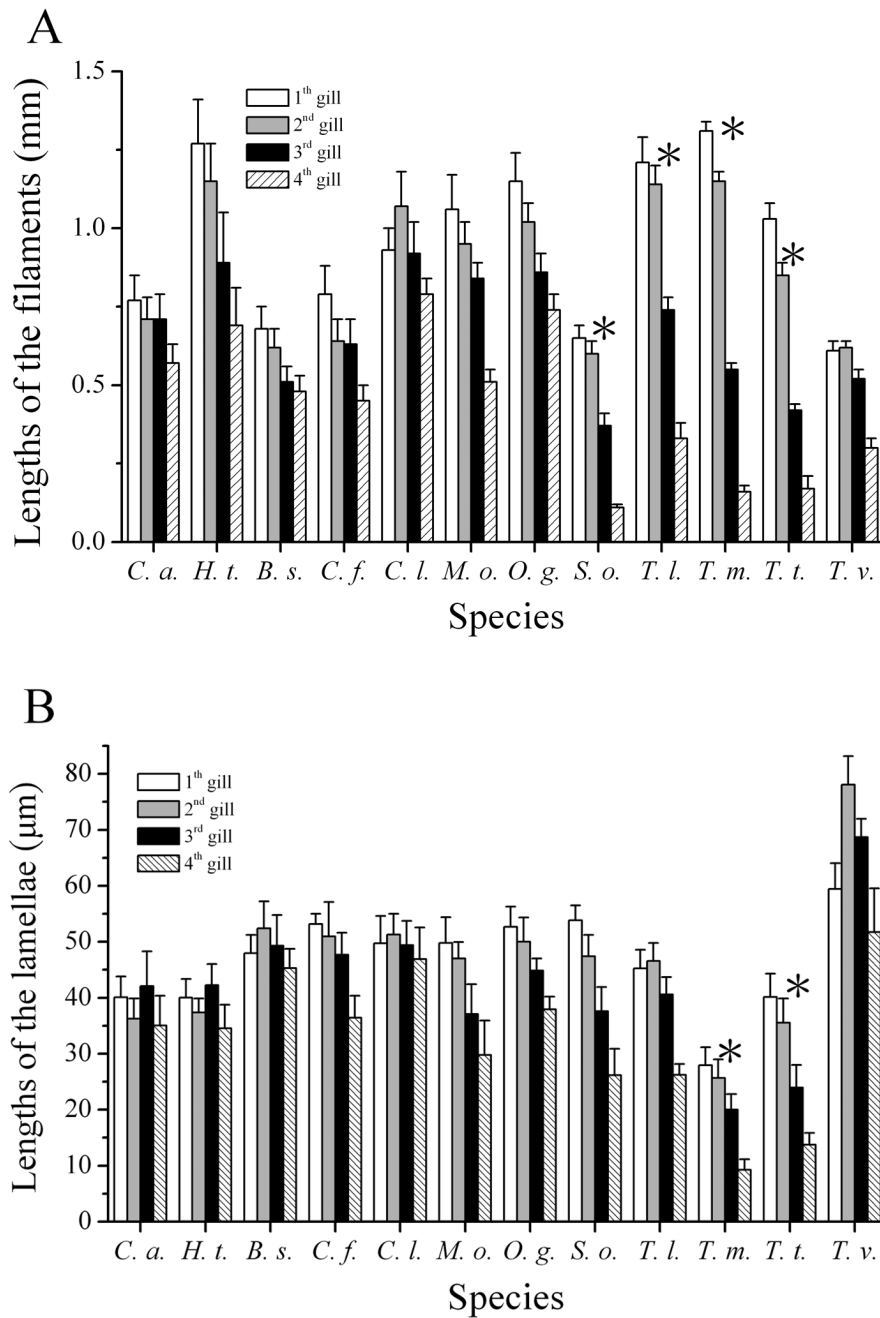
Figure 2. Lengths of the gill filaments and lamellae in the four gills in the 12 anabantoid species. *A*, Most species exhibited a gradual shortening pattern from the first to the fourth gill, but only five species showed a statistical difference between the anterior and the posterior gills. The lengths of the filaments in *Osphronemus goramy*, *Sphaerichthys osphromenoides*, *Trichogaster leeri*, *Trichogaster microlepis*, and *Trichogaster trichopterus* all had significant differences between the anterior (first and second gill arches) and the posterior (third and fourth gill arches) gills. *B*, Most species had a similar shortening pattern from the first to the fourth gill, and only *T. leeri*, *T. microlepis*, and *T. trichopterus* differed significantly between the anterior and the posterior gills. Values are means ± SEM. Significant differences between the anterior and the posterior gills (Tukey's test, $P < 0.05$, $n = 5$–10) are indicated with an asterisk. See Table 1 for species abbreviations.

been similar if the differences we observed were due to phenotypic plasticity. According to a previous study, morphological plasticity was observed in this group of fishes during different life stages or in more extreme environmental conditions than

natural habitat (Nilsson 2007). In *Arapaima gigas*, a South American air-breathing fish, gill structure changes at different life stages. This fish has no visible lamellae in the adult stage, in sharp contrast to what is observed in its juvenile stage
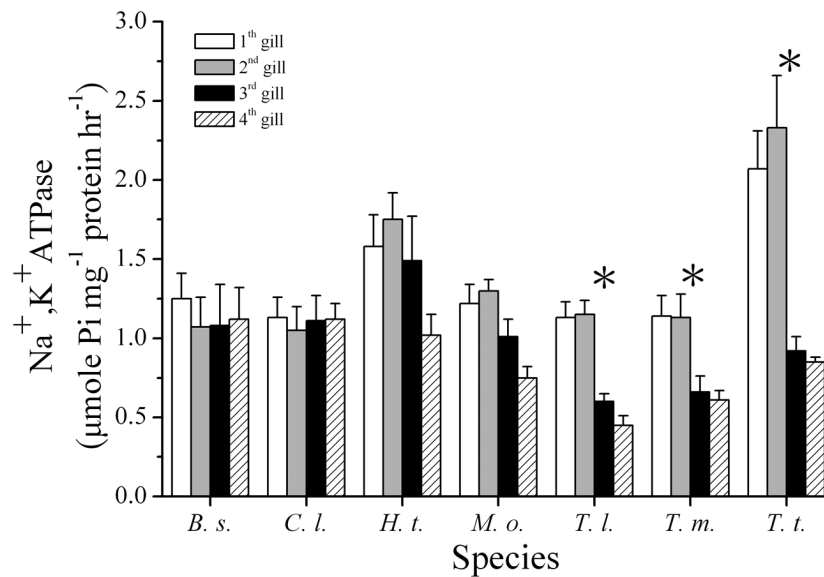
Figure 3. Na$^+$/K$^+$-ATPase enzyme activity in the four gills for seven anabantoid species. Only *Trichogaster leeri, Trichogaster microlepis*, and *Trichogaster trichopterus* differed significantly between the anterior and the posterior gills. Values are means ± SEM. Significant differences between the anterior and the posterior gills (Tukey's test, $P < 0.05$, $n = 8$) are indicated with an asterisk. See Table 1 for species abbreviations.

(Brauner et al. 2004). The water conditions we used were within the normal range for most fresh water and should therefore not induce phenotypic plasticity. Whether the morphological variations among gills within each species were a result of particular acclimation for specific environmental conditions deserves further study. For example, we found that gills underwent a substantial morphological change in *C. lalia*, when it was subjected to acidic conditions (pH 5.5) for 4 d, as did the gills of *T. microlepis*, when this species was prevented from engaging in aquatic surface respiratory behavior for 28 d (Huang and Lin 2010).

In the 1970s, most studies on the anabantoids focused on vascular organization by preparing cardiac and/or vascular casts for detailed structural information. For example, morphological modifications in the third and the fourth gill arches were found in *Anabas testudineus* and *Trichogaster trichopterus* (Hughes and Singh 1970; Kramer and Graham 1976; Burggren 1979), and the structure of the respiratory organs was also described in *A. testudineus* (Hughes and Munshi 1973; Hughes et al. 1973). The vascular anatomy of the gill and accessory air-breathing organ—or the branchial and systemic circuits, which are similar to a double-circuit circulatory system—were described in *A. testudineus* (Munshi et al. 1986; Olson et al. 1986). Auditory sensitivity and vocalizations were also investigated in these species (Ladich and Yan 1998), and structural examination of the heart was also carried out (Munshi et al. 2001). The vascular structure of gills was also examined in *Channa punctata, Channa gachua, Channa marulius*, and *Clarias batrachus* (Olson et al. 1994, 1995; Olson 2002), as was aquatic surface respiratory behavior (Randle and Chapman 2005; Alton et al. 2007; Chang et al. 2007). However, these studies mainly de-

scribed the fine structure of the gills and respiratory organ and did not provide any data on the potential functional modification of the gills that is associated with the morphological modifications that are observed. There was, in fact, no evidence showing that morphological variation correlates to functional differences until our recent report on the functional differentiation in the first and second gill arches within anabantoid species, which are responsible for regulating ionic stress in *Trichogaster leeri* and *T. microlepis* (Huang et al. 2008; Lee et al. 2008; Huang et al. 2010). From the results of the NKA activity and protein abundance assays that we conducted, we found that *T. microlepis* and *T. trichopterus* both exhibited functional differences between the first and the fourth gill arches. Nevertheless, *C. lalia* and *H. temminckii* exhibited different patterns with respect to enzyme activity and protein abundance of NKA in the first and fourth gill arches. This phenomenon could be a posttranslational modification, since different expression patterns between enzyme activity and protein abundance in NKA were also found in the milkfish *Chanos chanos* (Lin et al. 2003).

The relative abundance levels of NKA protein in the labyrinth organ were all lower than in the first gill arch among the seven species examined for this parameter. The labyrinth organ receives blood from the efferent artery of the first gill arch and is the potential site for additional gas exchange but appears not to be involved in ionic regulation (Burggren 1979; Munshi et al. 1986; Olson et al. 1986). The enzyme activity of CA in the labyrinth organ was only half of that in the gills in *T. trichopterus* (Burggren and Haswell 1979). The relative abundance of CAc protein might represent another functional difference between the gills and the labyrinth organ. Most species showed similar
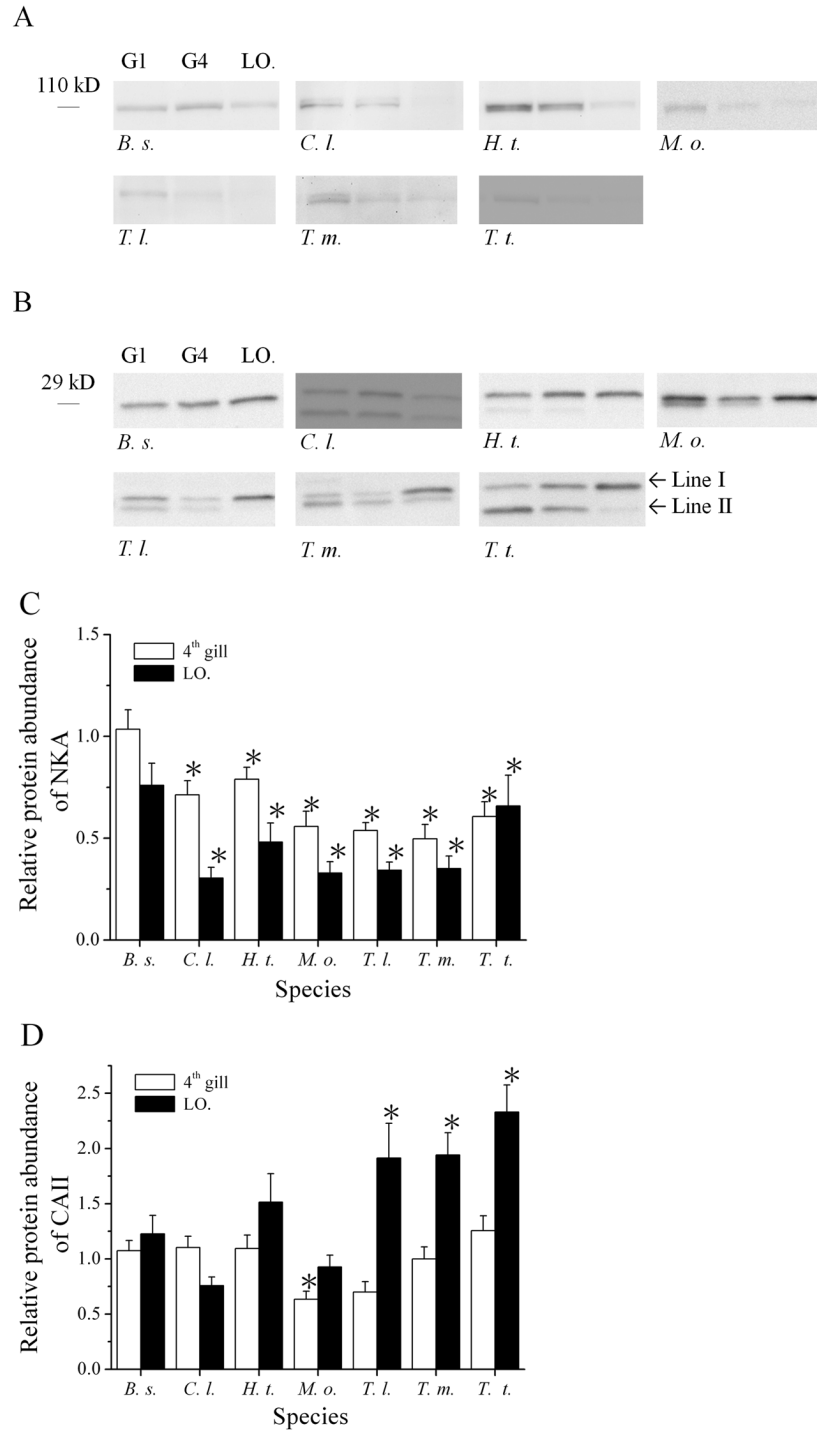
Figure 4. Western blot analyses showing the Na$^+$/K$^+$-ATPase (NKA) immunoreactive bands at approximately 95 kDa (*A*) and two cytosolic carbonic anhydrase (CAc) immunoreactive bands (bands I and II) at approximately 29 kDa in the first gill arch, fourth gill arch, and labyrinth organ of the seven species (*B*). Quantifications of NKA immunoreactive bands (*C*) and band I of the CAc immunoreactive bands (*D*) are reported relative to values measured in the first gill arch. Significant differences (Dunnett's test, $P < 0.05$, $n = 8$) are indicated with an asterisk. *G1*, first gill arch; *G4*, fourth gill arch; *LO*, labyrinth organ.

protein expression levels in the first and fourth gill arches, with the exception of *M. opercularis*. The three *Trichogaster* species had the highest CAc protein expression levels in the labyrinth organ compared with the first and fourth gill arches. In an earlier report, we found that the anterior gill arches were presumably used for ionic regulation (Huang et al. 2008). Assuming CAc protein abundance is correlated with the use of the labyrinth organ for air breathing, which needs to be investigated specifically, the results of the measurement of CAc protein abundance in this study may indicate that the degree of gas exchange that occurred in the labyrinth organ varied among anabantoid fish, which could compensate for the reduced respiratory surface area in the posterior gill. This compensates for the reduced respiratory surface area in the posterior gill. In addition to CAc, CAIV could play a role in gas exchange abilities in these fish (Gilmour et al. 2007), and further examination will be required to determine this. The second band that we observed here (band II) could represent nonspecific binding of the antibody we used against CAc (Fig. 4*B*). This speculation is supported by results obtained using a different antibody against CAc (Abcam, Cambridge) to repeat the immunoblotting experiment. With this antibody, we found a single band located at 29 kDa that corresponded to band I in this experiment (data not shown). This antibody (NOVUS antibody data sheet) does not recognize the type of the CAI that locates to erythrocytes. Immunohistochemistry staining of erythrocytes also showed no reaction for CAc (data not shown). Therefore, we can exclude the possibility of nonspecific influences of the erythrocytes in the gill vessels. In our experiment, the relative abundance of CAc protein was contributed from the gill tissue, not from the erythrocytes. While the basolateral NKA distribution is well known, the distribution of CAc deserves further study.

A molecular-based phylogenetic tree was previously reconstructed, and a total of 57 species representing all 19 anabantoid genera were included in the study by Rüber et al. (2006). On the basis of this phylogeny, we found that the morphological and functional variations of 12 anabantoid fish species are limited to one lineage (the genus *Trichogaster*). This lineage-specific distribution of morphological divergence in the fourth gill arch suggests that these variations may have evolved only once in the *Trichogaster* lineage, which may imply a phylogenetic influence on gill morphology in anabantoid species. An alternative explanation of this lineage-specific divergence is that the habitat-associated adaptation has occurred in *Trichogaster*, in which the common selective factors have driven convergent gill modification in these fishes. A broad sampling of gill variations among anabantoid is needed to distinguish these two alternative hypotheses.

Acknowledgments

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