JOURNAL OF MORPHOLOGY 276:681–694 (2015)

Evolution of the Branchiostegal Membrane and Restricted Gill Openings in Actinopterygian Fishes

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ABSTRACT A phylogenetic survey is a powerful approach for investigating the evolutionary history of a morphological characteristic that has evolved numerous times without obvious functional implications. Restricted gill openings, an extreme modification of the branchiostegal membrane, are an example of such a characteristic. We examine the evolution of branchiostegal membrane morphology and highlight convergent evolution of restricted gill openings. We surveyed specimens from 433 families of actinopterygians for branchiostegal membrane morphology and measured head and body dimensions. We inferred a relaxed molecular clock phylogeny with branch length estimates based on nine nuclear genes sampled from 285 species that include all major lineages of Actinopterygii. We calculated marginal state reconstructions of four branchiostegal membrane conditions and found that restricted gill openings have evolved independently in at least 11 major actinopterygian clades, and the total number of independent origins of the trait is likely much higher. A principal component analysis revealed that fishes with restricted gill openings occupy a larger morphospace, as defined by our linear measurements, than do fishes with nonrestricted openings. We used a decision tree analysis of ecological data to determine if restricted gill openings are linked to certain environments. We found that fishes with restricted gill openings repeatedly occur under a variety of ecological conditions, although they are rare in open-ocean pelagic environments. We also tested seven ratios for their utility in distinguishing between fishes with and without restricted gill openings, and we propose a simple metric for quantifying restricted gill openings (RGO), defined as a ratio of the distance from the ventral midline to the gill opening relative to half the circumference of the head. Functional explanations for this specialized morphology likely differ within each clade, but its repeated evolution indicates a need for a better understanding of diversity of ventilatory morphology among fishes. J. Morphol. 276:681–694, 2015. © 2015 Wiley Periodicals, Inc.

KEY WORDS: Actinopterygii; gill ventilation; convergent evolution

INTRODUCTION

Gill chamber morphology varies extensively among more than 31,000 living species of ray-finned fishes (Actinopterygii). This undoubtedly relates to performance of both gill ventilation and feeding, and understanding the evolutionary history of this variation can provide important context for functional studies (Lauder, 1990). Historically, broad surveys of morphological variation of actinopterygians categorized morphology without in-depth consideration of historical patterns. For example, with teleosts, McAllister's (1968) survey of branchiostegal rays, Monod's (1968) survey of the caudal skeleton, and Kusaka's (1974) survey of the urohyal, while anatomically detailed, could not be framed in a rigorous phylogenetic context because so little phylogenetic information for ray-finned fishes was available at that time. The more recent study of abdominal and caudal vertebral patterning by Ward and Brainerd (2007) exemplifies the continuing importance of broad morphological surveys as context for functional morphological research, but even in 2007, phylogenetic relationships among many subgroups of actinopterygians were poorly known (Stiassny et al., 2004). Recent large-scale efforts to improve resolution of the actinopterygian phylogeny using molecular data (Near et al., 2012; Betancur-R et al., 2013; Broughton et al., 2013; Near et al., 2013) now allow us to consider ancestral state reconstruction (e.g., Stewart et al., 2014; Davis et al., 2014) and frame questions about patterns of variation in structure across a broad distribution of actinopterygian species.

As actinopterygians ventilate their gills, water is pumped into the mouth, passed over the gill

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Correction added on May 6, 2015, after first online publication: LIETERATURE CITED changed to LITERATURE CITED.

Additional Supporting Information may be found in the online version of this article.

Contract grant sponsor: NSF Doctoral Dissertation Improvement Grant; Grant number: DEB-1310812.

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Received 29 May 2014; Revised 16 December 2014;
Accepted 20 January 2015.

DOI 10.1002/jmor.20371

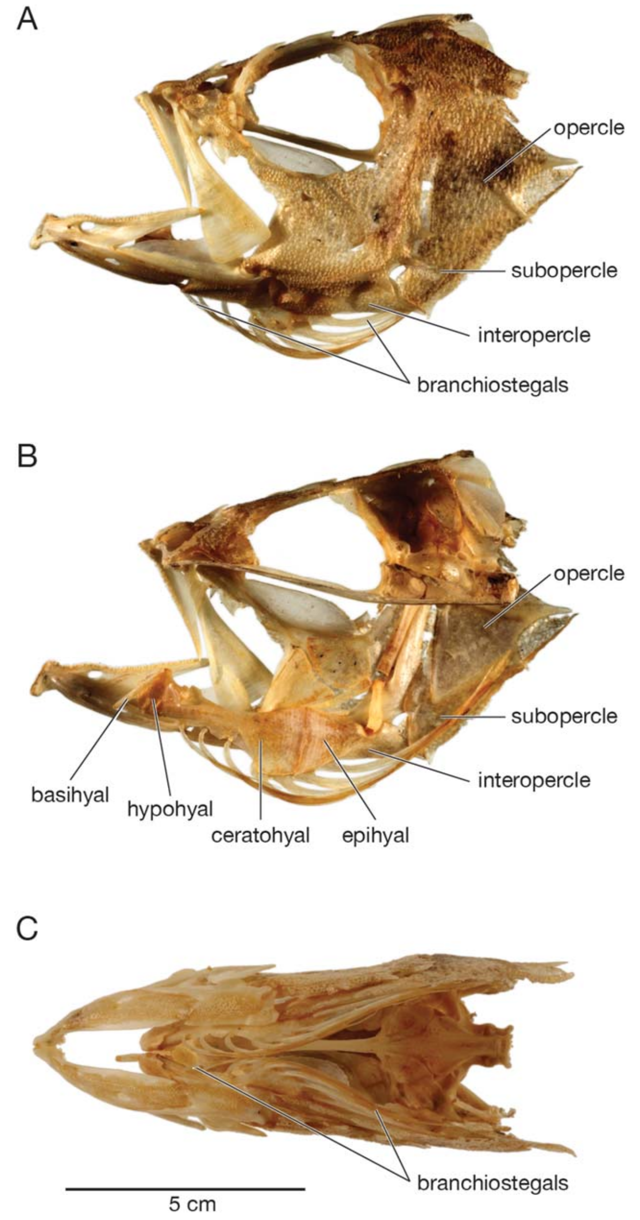

Fig. 1. Photograph illustrating the branchiostegal rays. Branchiostegal rays are dermal bones that articulate with ventral elements of the hyoid arch to form the ventrolateral surface of the gill chamber. The top photograph (**A**) is a lateral view of the cranial skeleton of *Sebastes norvegicus*. The middle photograph (**B**) is a lateral view of the interior cranial skeleton of *S. norvegicus* to demonstrate the ventral elements of the hyoid arch. The specimen has been bisected through the midsagittal plane, and the left half of the specimen is depicted. The photograph has been flipped to orient the mouth in the same direction as in the other photographs. The bottom photograph (**C**) is a ventral view of the cranial skeleton of *S. norvegicus* to depict a complete set of branchiostegal rays.

tissue, and pumped out through openings at the posterior edge of the gill chamber. The movements of the mouth and the gill covers (=opercula) have inspired experimental and morphological investigations that established a two pump model for ventilation (Hughes and Shelton, 1958; Hughes, 1960). Far less obvious is the often-substantial skeleton ventral to the gill covers, the branchiostegal rays, which contribute to the "suction" pump created by gill chamber expansion during ventilation. These rays are long struts of dermal bone that articulate with ventral elements of the hyoid arch to form the ventrolateral surface of the gill chamber (Fig. 1). In extant actinopterygians, the number of branchiostegal rays varies from 0 to 51 pairs, along with substantial variation in length, shape, and cross-sectional area (McAllister, 1968). The rays are actuated by several muscles, primarily the hyohyoideus abductor and adductor muscles, which are highly variable in their arrangement (Winterbottom, 1974). The relative size of the branchiostegal apparatus has been linked to differences in ventilatory function, with larger or more numerous branchiostegal rays often indicating a more prominent suction pump (Baglioni, 1907; Hughes, 1960; Liem, 1970).

The branchiostegal rays support the branchiostegal membrane, often referred to as the gill membrane. The branchiostegal membrane is ideally situated to have a potent effect on water transport during ventilation as a wall of the gill chamber and as a valve to control the flow of water out of the gill openings. In addition to its primary function in gill ventilation, this membrane can serve a variety of functions in fish behavior. For example, some males develop bright coloration of the branchiostegal membrane to attract females (Semler, 1971; Ragland and Fischer, 1987). Others flare their membranes in antagonistic displays against conspecific intruders or competitors (Baerends and Baerends-Van Roon, 1950). It is also a common substrate for photophores in light-producing fishes (Strum, 1969; Cavallaro et al., 2004). Our study focuses on an extreme modification of the branchiostegal membrane, the reduction of the gill opening to a small aperture, which presumably influences fluid flow in important though as yet poorly understood ways (Brainerd and Ferry-Graham, 2006; Graham, 2006).

Restricted gill openings are frequently mentioned as defining characteristics of taxonomic groups in classification references and field guides (e.g., Nelson, 2006; Lamb and Edgell, 2010). In species descriptions, these small gill openings are characterized relative to features such as eye size (e.g., Chernova, 2014) and position of the pectoral fin (e.g., Maldonado-Ocampo et al., 2014) to distinguish new species from close relatives. In some taxa, the gill opening is siphon-like or minuscule, presenting an obvious case of restricted gill openings that can be identified by qualitative assessment. For cases that are less obvious, a quantitative definition that can be applied across all ray-finned fishes may be useful for systematists. With only rare exceptions, "restricted gill

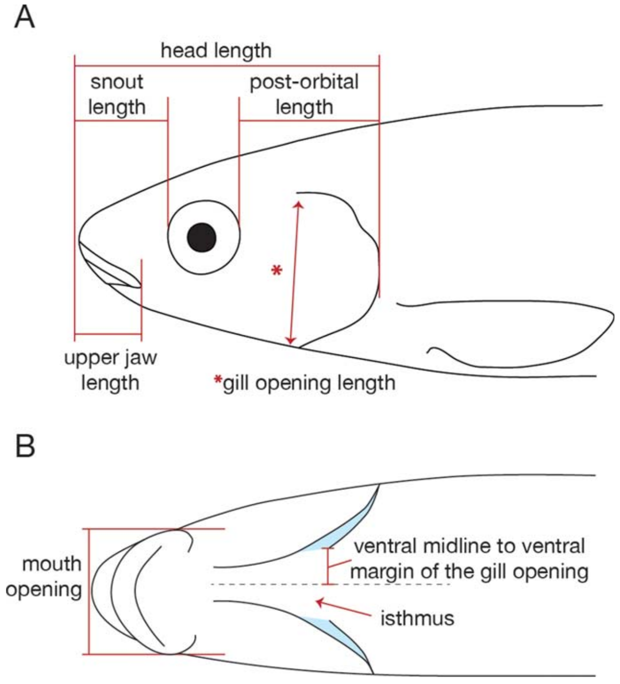

Fig. 2. Morphological measurements taken during survey. (**A**) Measurements taken from a lateral perspective included head length, snout length, postorbital length, upper jaw length, and gill opening length. (**B**) Measurements taken from a ventral perspective included mouth opening length and the length from the ventral midline to the ventral margin of the gill opening.

openings" and related terms are applied to fishes in which the branchiostegal membrane is broadly joined to the ventral surface of the body, restricting the aperture. Therefore, a useful quantitative definition of this trait could include the relative broadness of this attachment in addition to gill opening size.

To examine the evolutionary history and variation of gill openings, we surveyed branchiostegal membrane morphology and mapped it onto a comprehensive time-calibrated phylogeny of major actinopterygian lineages. Our goals were to: 1) reconstruct the evolutionary history of branchiostegal membrane morphology in Actinopterygii; 2) identify independent origins of restricted gill openings at the family level; 3) determine useful metrics for quantifying gill opening restriction; and 4) determine morphometric and ecological correlates of the occurrence of restricted gill openings. We highlight the extensive morphological, ecological, and phylogenetic diversity of actinopterygian fishes with restricted gill openings.

MATERIALS AND METHODS

We surveyed branchiostegal membrane morphology in specimens from the Cornell University Museum of Vertebrates (CUMV), the Academy of Natural Sciences of Drexel University (ANSP), the University of Washington Fish Collection (UW), and the Harvard Museum of Comparative Zoology (MCZ). Nelson (2006) recognized 453 families of actinopterygians, and we examined one specimen from 433 of these families (see Supporting Information Table S1). We selected individuals that were nonlarval and had a fully intact branchiostegal membrane, and whenever possible, we chose taxa of the same genus and species as those in the molecular data set used for ancestral state reconstruction of branchiostegal membrane morphology (see below). Because we only examined one specimen from each family, we did not capture the entire extent of variation that exists within families. We measured the following on each specimen: standard length, head length, upper jaw length, snout length, postorbital length (distance from posterior margin of orbit to posterior margin of the opercle), mouth opening (width of the maximum opening of the lower jaw near the jaw joint) and circumference of the head through the center of the opercle (or in the gill region in fishes without opercles, such as in saccopharyngiforms) (Fig. 2A). We also measured the length from the ventral midline of the body to the ventral margin of the gill opening (Fig. 2B). We characterized each specimen according to the following four branchiostegal membrane morphologies based on the terminology and descriptions of McAllister (1968).

Separate and Free from the Isthmus

Left and right branchiostegal membranes are separate from each other. Often, the branchiostegal membranes overlap ventroanteriorly, as in *Amia calva* (Fig. 3A, Amiidae). There is no attachment of the branchiostegal membrane to the isthmus of the body.

United and Free from the Isthmus

Left and right branchiostegal membranes are united with each other ventrally, and there is no attachment of the membranes to the isthmus. In some species, the unity of the membranes is broad and obvious, as in *Lepisosteus osseus* (Fig. 3B, Lepisosteidae). However, in other species, the membranes are united by only a small amount of tissue ventroanteriorly and can overlap slightly. This can make it difficult to distinguish between "separate" and "united" morphologies in certain groups. For such specimens, we made the distinction based on a qualitative assessment of whether or not the left and right membranes were continuous with one another. McAllister (1968) reported many clades as having examples of both "separate" and "united" configurations.

Joined to the Isthmus

The branchiostegal membranes are joined to the ventral surface of the body, separating the gill openings. The distance between the left and right gill openings can be small ("narrowly joined to the isthmus" according to McAllister, 1968) or large (broadly joined to the isthmus) as in *Eleotris vittata* (Fig. 3C, Eleotridae). The amount of space between gill openings exists along a continuum, and therefore we do not distinguish between "narrowly joined" and "broadly joined" in our survey, except that we consider extreme broadness to often indicate restricted gill openings (Fig. 3D). In some species, branchiostegal membranes are joined to the isthmus but appear externally to be united in a continuous membrane (e.g., many species of Cottidae). McAllister (1968) refers to these fishes as having a "gill membrane joined to isthmus and forming a free fold over it," because it can result in a folded appearance. In our survey, we consider this to be a case of "joined to the isthmus" morphology.

Joined to the Isthmus, Gill Opening Restricted

The branchiostegal membranes are joined very broadly to the ventral surface of the body, resulting in restricted gill openings, as in *Tetraodon lineatus* (Fig. 3D, Tetraodontidae). Fishes were

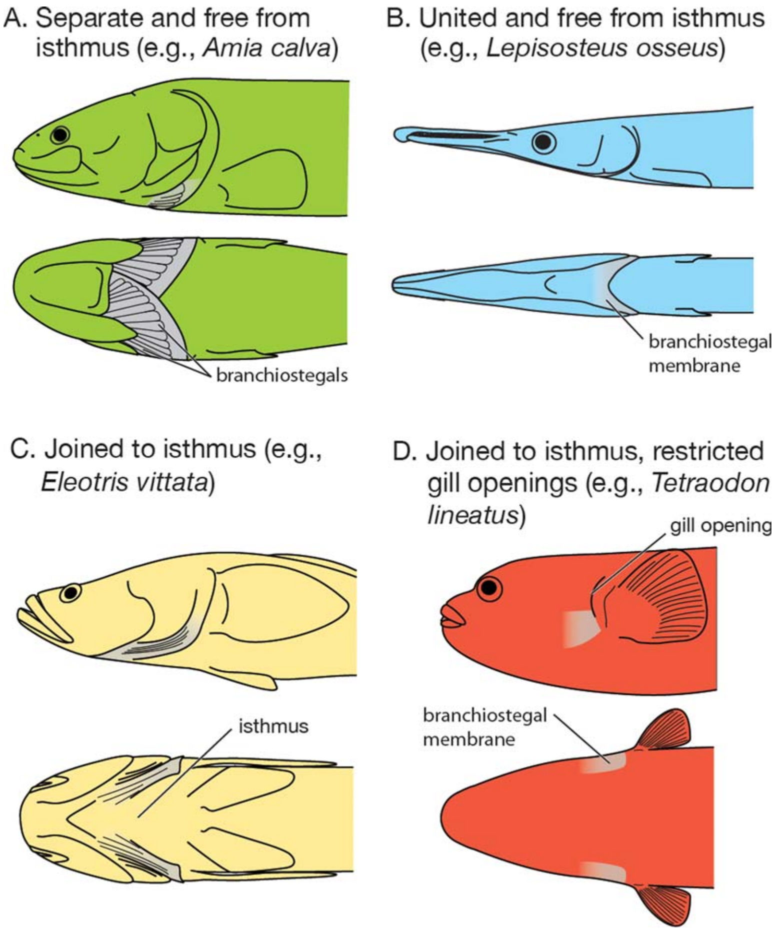

Fig. 3. Four conditions of the branchiostegal membrane. (**A**) Left and right membranes are separate from one another, often overlapping. (**B**) Left and right membranes are continuous with one another and free from the isthmus. (**C**) Membranes are joined to the isthmus of the body. (**D**) Membranes are joined to the isthmus of the body with restricted gill openings.

categorized as having this morphology if the branchiostegal membrane was attached to the isthmus and the gill openings were considerably smaller or more siphon-like in appearance relative to the more typical actinopterygian condition.

Ancestral state reconstruction. To reconstruct character state evolution of branchiostegal membrane morphology across Actinopterygii (Fig. 4), we first inferred a phylogeny using sequence data from two recent studies (Near et al., 2012; Near et al., 2013). This data set included 285 taxa representing 284 families recognized by Nelson (2006) and included sequence data for nine nuclear markers (*glyt*, *myh6*, *plagl2*, *ptr*, *rag1*, *SH3PX3*, *sreb2*, *tbr1*, and *zic1*). We used one species from each family, with the exception of Polypteridae, our outgroup to all other actinopterygians, for which we used two species (*Polypterus ornatipinnis* and *Erpetoichthys calabaricus*, Polypteridae). Using BEAST software (Drummond et al., 2012), we inferred a relaxed molecular clock phylogeny with relative divergence time estimates, rooting the tree with outgroup taxa *P. ornatipinnis* and *E. calabaricus*. Sequence data were partitioned using BEAUti software (Drummond et al., 2012) into nine unlinked GTR substitution models. An MCMC chain of 190 million generations was used to generate a maximum clade credibility tree in TreeAnnotator v1.8.0 software.

We assigned one of four branchiostegal membrane character states to each taxon, based on the condition for its family from our morphological survey (Supporting Information Table S1). Out of the 285 taxa used for phylogenetic reconstruction, 123 taxa (43%) are the same species that we examined in our survey. We calculated the marginal ancestral state reconstructions for each node using the rerootingMethod function with equal rates and symmetrical models from the phytools package in R (R Core Team, 2013; Revell, 2012), which is based on the rerooting method of Yang et al. (1995).

Testing metrics to define restricted gill openings. We investigated simple metrics that can be used to define restricted gill openings. We tested seven ratios of measurements for the ability to distinguish between restricted and nonrestricted gill openings. We only included data from specimens with an attachment to the isthmus (Fig. 3C and D; 138 families total). For each ratio, we determined the optimal cutoff value beyond or below which a fish could be considered to have restricted gill openings. To do this, we applied the optimize function from the stats package in R (R Core Team, 2013) to a function written to determine a cutoff value for each ratio based on presence or absence of restricted gill openings as determined by our qualitative assessment. The error (the number of individuals not properly categorized by each cutoff value) was used to calculate a precision estimate for the cutoff value for each ratio. A range of error values was calculated by performing this optimization procedure over 100 simulations, with each simulation using a randomly sampled 90% of the original data. We tested the following ratios in this manner: gill opening length relative to head length, distance from the ventral midline of the body to the ventral margin of the gill opening

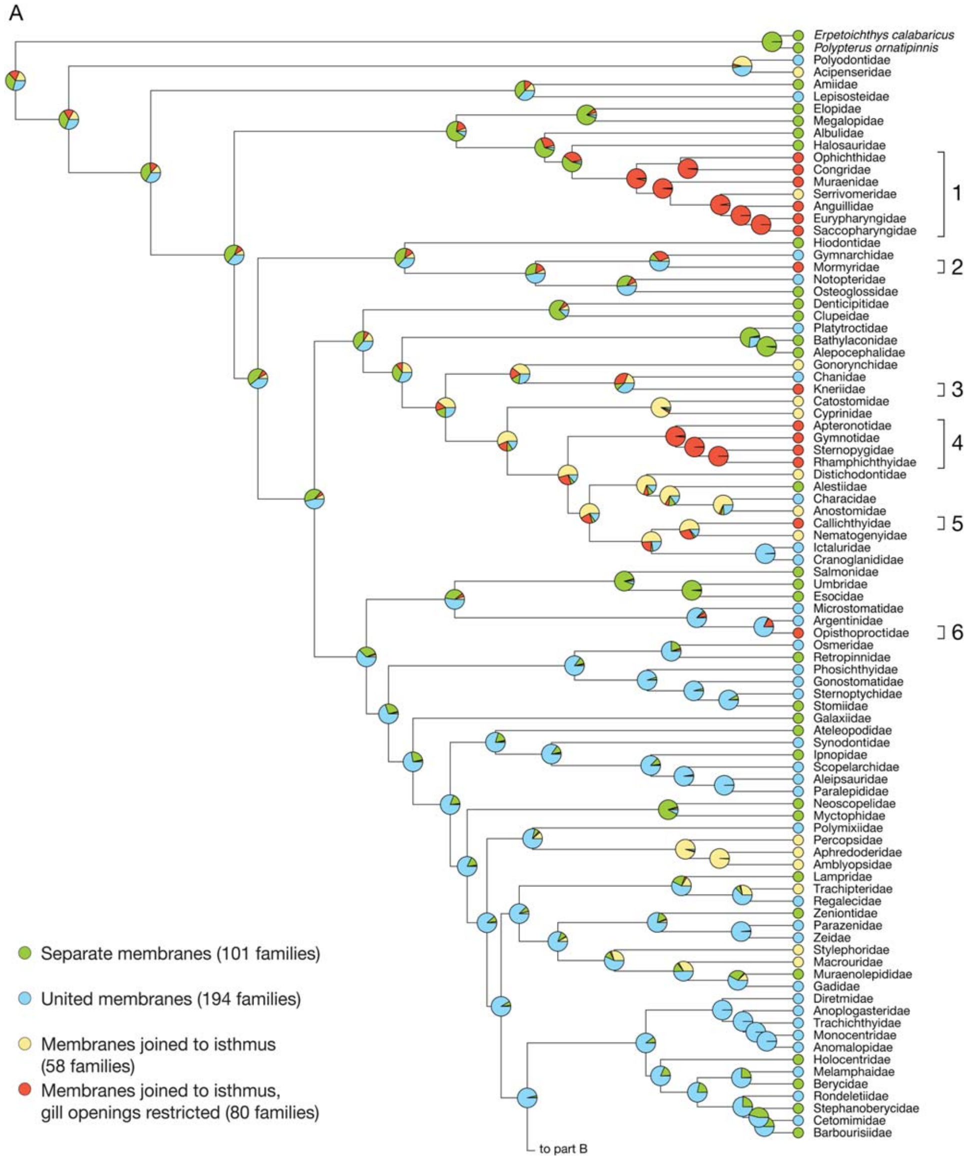

Fig. 4. Ancestral state reconstruction of the distribution of branchiostegal membrane morphology across Actinopterygii. A relaxed molecular clock phylogeny was constructed from 285 species representing 284 actinopterygian families. Pie charts at nodes show marginal ancestral state reconstructions for four branchiostegal membrane conditions. Brackets indicate clades in which restricted gill openings have independently evolved.

(ventral midline to ventral margin, VMVM) relative to head length, VMVM relative to half the circumference of the head in the gill region multiplied by 100 (herein referred to as the restricted gill opening ratio, or RGO ratio), VMVM relative to gill opening length, RGO ratio divided by gill opening length, RGO ratio multiplied by the inverse of the ratio of gill opening length to head length, and gill opening length relative to half the circumference of the head in the gill region.

Morphometric and ecological features co-occurring with restricted gill openings. To investigate the morphospace occupied by fishes with different branchiostegal membrane morphologies, we used JMP Pro 10.0.0 to conduct a principal component analysis on measurements taken from the 433 specimens examined. We did not adjust for phylogenetic relationships because phylogenetic information is not available for all 433 families examined. We used the following ratios: head length relative to standard length, upper jaw length relative to head length, snout length relative to head length, postorbital length relative to head length, mouth opening relative to head circumference, and half the head circumference relative to standard length; all ratios were arcsine transformed. We generated a plot of PC1 and PC2, a loading plot, and a table of factor loadings (Supporting Information Table S2). We performed a Bartlett's test of homogeneity in JMP Pro 10.0.0 to determine the number of components to retain and quantified the mean and standard deviation of each retained PC axis for each character state (Table 1).

To examine the relationship between ecology and branchiostegal membrane morphology, we used ecological data from FishBase (Froese and Pauly, 2011) to determine the basic ecology (pelagic, demersal, or reef-associated) and environment (tropical, subtropical, temperate, deep-sea, boreal, or polar) for each species in our survey. We performed a decision tree analysis in JMP Pro 10.0.0 as a data-mining technique to determine

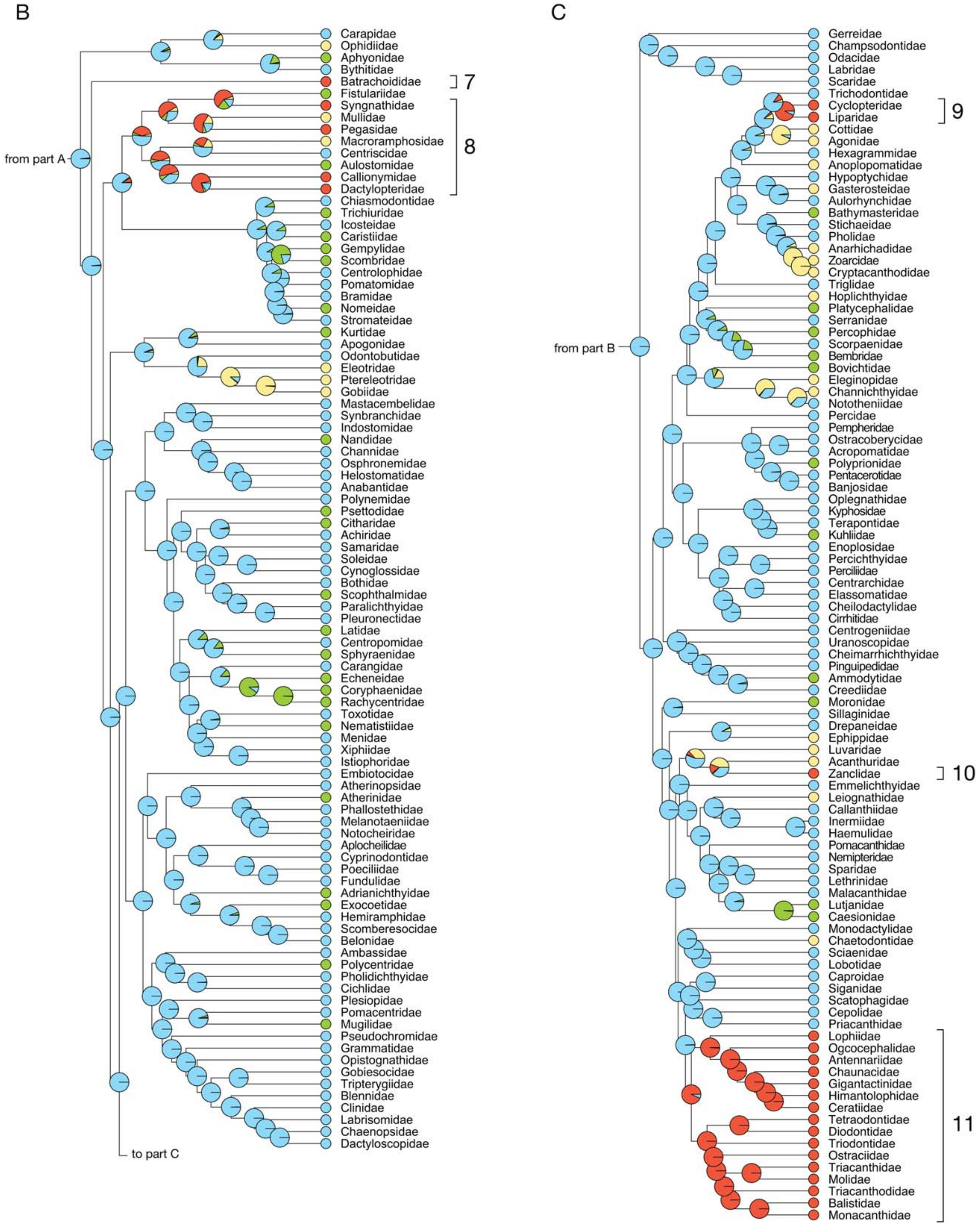

Fig. 4. Continued.

TABLE 1. Means and standard deviations for each significant PC axis of the principal component analysis for each branchiostegal membrane morphology condition

Membrane condition	PC1 mean	PC1 SD	PC2 mean	PC2 SD	PC3 mean	PC3 SD	PC4 mean	PC4 SD
Separate	−0.323	1.011	0.015	0.887	0.181	0.832	−0.250	0.784
United	0.093	1.283	0.114	1.008	−0.134	1.162	−0.125	0.779
Joined to isthmus	0.309	1.396	−0.348	1.007	−0.014	0.862	0.252	0.561
Restricted openings	−0.018	2.102	−0.058	1.839	0.133	1.514	0.441	0.978
All conditions	0.004	1.440	−0.003	1.186	0.005	1.139	0.000	0.834

which ecological variables are most recursively predictive of branchiostegal membrane morphology. No adjustment for phylogenetic relationships was made due to a lack of available molecular data for a large portion of the taxa surveyed.

RESULTS

Ancestral state reconstruction (Fig. 4) showed that restricted gill openings have evolved independently in each of the following 11 clades: 1) Anguilliformes and Saccopharyngiformes; 2) Mormyridae; 3) Kneriidae; 4) Gymnotiformes; 5) Callichthyidae; 6) Opisthoproctidae; 7) Batrachoidiformes; 8) Syngnathidae, Pegasidae, Dactylopteridae, and Callionymidae; 9) Liparidae and Cyclopteridae; 10) Zanclidae; 11) Tetraodontiformes and Lophiiformes. Fishes with restricted gill openings are spread throughout the phylogeny. Out of the 433 families surveyed, 101 families had separate membranes (23.3%), 194 families had united membranes (44.8%), 58 families had membranes joined to the isthmus (13.4%), and 80 families had restricted gill openings (18.5%).

We tested different ratios of measurements for the ability to distinguish between restricted and nonrestricted gill openings among fishes with an attachment to the isthmus. For each ratio, we determined a cutoff point between taxa with restricted and nonrestricted gill openings and quantified the range of error. The ratio of gill opening length relative to head length had a cutoff value of 0.385 (error = 3.2–5.3%), below which fishes were classified as having a restricted gill opening. The ratio of VMVM relative to head length had a cutoff value of 0.127 (error = 3.2–5.1%), above which fishes were classified as having a restricted gill opening. The RGO ratio had a cutoff value of 12.53 (error = 1.2–2.7%), above which fishes were classified as having a restricted gill opening. The ratio of VMVM relative to gill opening length had a cutoff of 11.89 (error = 1.2–12.5%), above which fishes were classified as having a restricted gill opening. The RGO ratio divided by gill opening length had a cutoff value of 51.22 (error = 10.2–12.7%), above which fishes were classified as having a restricted gill opening. The RGO ratio multiplied by the inverse of the ratio of gill opening length to head length had a cutoff value of 1,231 (error = 1.4–12.9%), above which fishes were classified as having a restricted gill opening. The ratio of gill opening length to half the circumference of the head had a cutoff value of 0.41 (error = 7.5–13.4%). The RGO ratio (Fig. 5) was the value that consistently categorized fishes with low error. Gill opening length relative to head length was the only value incorporating size of the gill opening that consistently categorized fishes with low error.

A plot of PC1 and PC2 from our principal component analysis (Fig. 6) shows the morphospace occupied by fishes with each branchiostegal membrane morphology as quantified by six ratios of body shape based on standard measurements. Fishes with restricted gill openings, when considered as a whole, occupy a morphospace larger than fishes with all other membrane morphologies, even when compared with the most common morphology of united branchiostegal membranes. Fishes occupying the upper right quadrant of the plot (e.g., Lophiiformes) have large heads relative to body length and are globular in shape. Fishes occupying the lower right quadrant of the plot (e.g., Tetraodontiformes) have eyes that are positioned far posteriorly from the tip of the snout. Fishes occupying the lower left quadrant are elongate with short snouts. The components PC5 and PC6 were found to have eigenvalues with

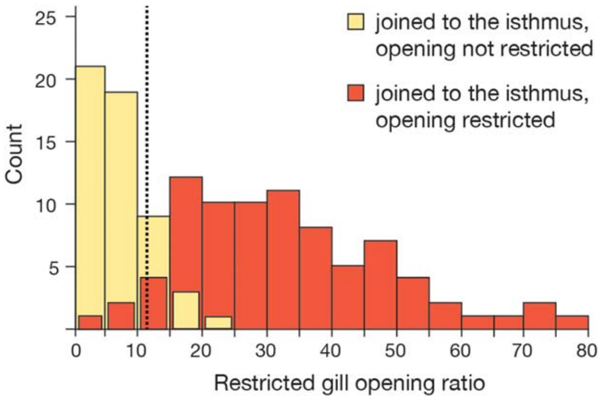

Fig. 5. Quantitatively defining restricted gill openings. These histograms show the distribution of the RGO ratio for fishes with membranes joined to the isthmus (yellow) and with membranes joined to the isthmus, gill openings restricted (red). The dashed line indicates the calculated cutoff value of 12.5, above which fishes can be considered to have a restricted gill opening (error = 1.2–2.8%).

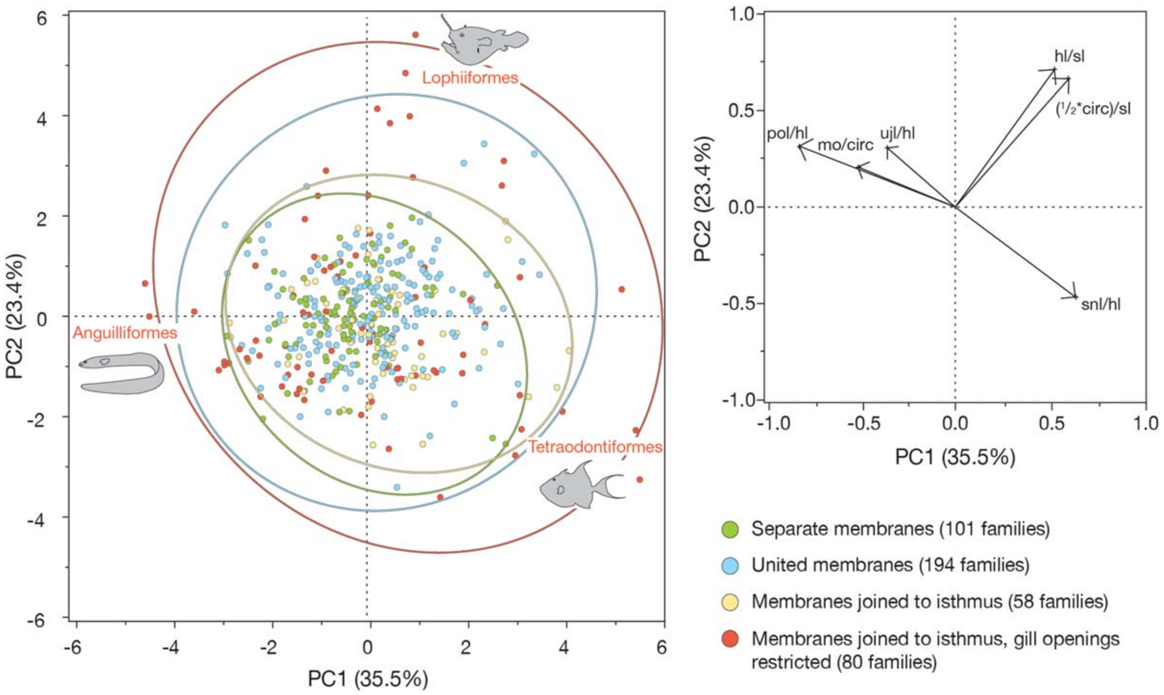

Fig. 6. Principal component analysis of morphometric data. The plot of PC1 and PC2 for measurements taken from specimens representing 433 actinopterygian families (left) shows that fishes with restricted gill openings occupy a more widespread morphospace, as defined by our morphometric data, than fishes with other branchiostegal membrane morphologies. Fishes with restricted gill openings occur at many of the extremes of our plot, and we have indicated examples of groups that represent these extremes: the highly elongate Anguilliformes (eels), the large-headed Lophiiformes (anglerfishes), and the often large-snouted Tetraodontiformes (e.g., triggerfishes). All measurements were arcsine transformed, and colors follow Figure 3. A corresponding loading plot for PC1 and PC2 is shown on the right.

significantly different variances from the first four principal components based on the Bartlett's test of homogeneity (for PC5, $X^2 = 2.49$ and $P = 0.43$; for PC6, $X^2 = 0$, and $P = 1$), so PC loadings (Supporting Information Table S2) and descriptive statistics (Table 1) are only provided for PC1–4. For each of these four principal component axes, the standard deviation among fishes with restricted gill openings was higher than any of the other branchiostegal membrane conditions (Table 1).

Decision tree analysis shows that the most important ecological factor recursively predicting branchiostegal membrane morphology is whether or not the fish is pelagic ($P = 0.008$) (Fig. 7). Among pelagic families ($P = 0.010$), most fishes with restricted gill openings are deep-sea; Molidae is the only pelagic family in our survey that has restricted gill openings but does not live in the deep-sea. Based on the taxa used for this study, most families with restricted gill openings are demersal ($n = 49$), and the rest are bathypelagic ($n = 16$), pelagic ($n = 1$), or reef-associated ($n = 14$). A high number of fish families with membranes joined to the isthmus are demersal ($n = 44$), with the rest occurring in bathypelagic ($n = 4$), pelagic ($n = 3$), and reef-associated ($n = 7$) habitats. The majority of reef fish families have membranes that are united and free from the isthmus ($n = 47$ out of 79), and most reef fishes ($P = 0.014$) with restricted gill openings inhabit subtropical waters.

DISCUSSION

Convergent Evolution of Restricted Gill Openings

Our phylogenetic analysis shows 11 independent origins of restricted gill openings within Actinopterygii, with the trait occurring in some of the earliest as well as the most recently diverged lineages (Fig. 4). While the ubiquity of this trait has likely been obvious to ichthyologists for decades, recent efforts to complete robust molecular analyses with a large number of taxa and markers (Near et al., 2012; Betancur-R et al., 2013; Broughton et al., 2013; Near et al., 2013) greatly enhance our ability to identify the number of times that this trait has evolved and its distribution across the phylogeny. The repeated evolution of restricted gill openings suggests that it is likely beneficial under a variety of conditions.

There are several more instances of convergent evolution of restricted gill openings not represented in Figure 4 due to the limited taxonomic coverage of the molecular phylogenetic data set. For example, within the species-rich clade of

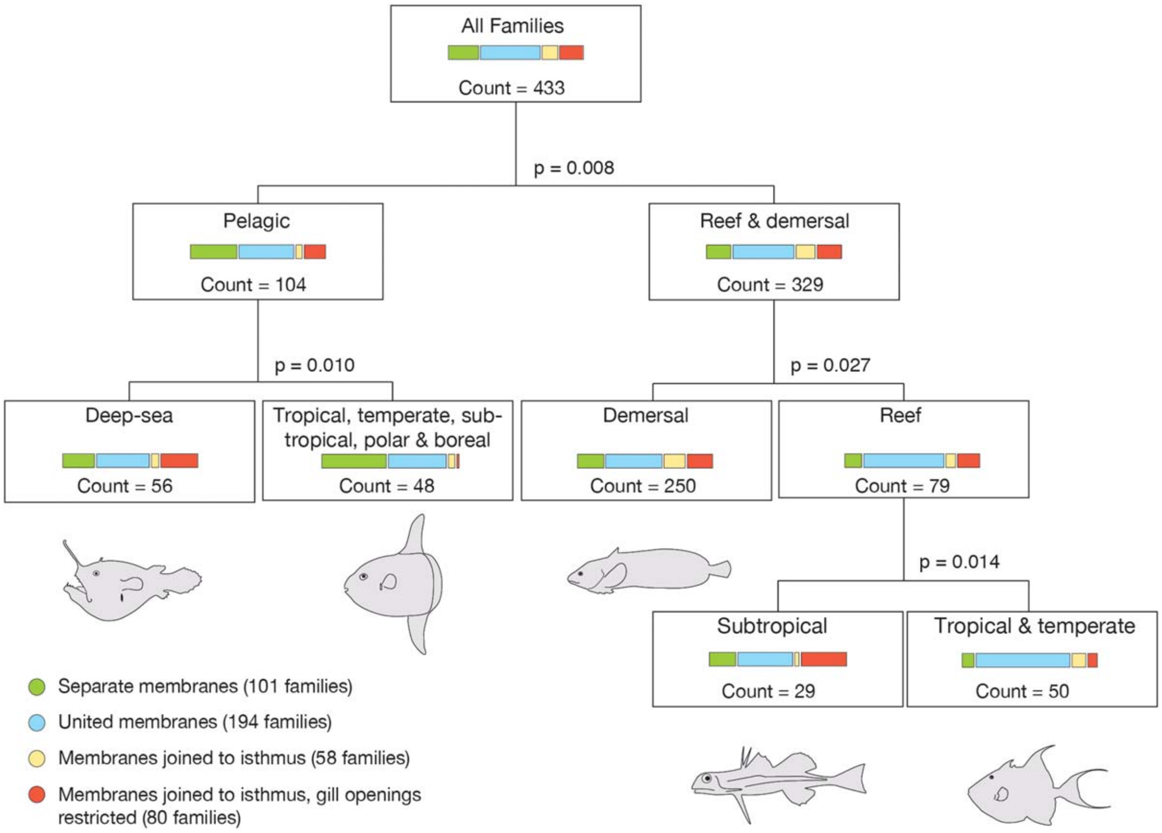

Fig. 7. Decision tree analysis of ecological variation. A decision tree analysis shows ecological variables that are recursively predictive of branchiostegal membrane morphology. All environments that we considered showed examples of all four membrane conditions. The primary ecological factor recursively predicting branchiostegal membrane morphology is whether a fish is pelagic ($P = 0.008$). Most families with restricted gill openings are demersal ($n = 49$).

Siluriformes, restricted gill openings occur in each of the following clades: Loricarioidei, Cetopsidae, Aspredinidae (Auchenipteridae + Doradidae), Ariidae, Erethistidae, Chacidae, and Mochokidae (Malapteruridae + Amphiliidae). Based on hypothesized phylogenetic relationships among these clades (Sullivan et al., 2006), restricted gill openings have evolved at least three times within Siluriformes, while the actual number is likely higher. Giganturidae is another example of an independent origin of this trait as the only family within Aulopiformes to have restricted gill openings. Microdesmidae, placed as the sister group to Gobiidae by Betancur-R et al. (2013) also have restricted gill openings. Congiopodidae is a clade of uncertain phylogenetic position within Scorpaeniformes (Lautredou et al., 2013) and Rhamphocottidae is nested within Cottoidei (*sensu* Nelson, 2006) according to Knope (2013) and Smith and Busby (2014); both of these clades likely represent additional instances of independent evolution of restricted gill openings.

Although membrane morphology is relatively conserved at a family level, it can vary within families and even within genera. Large, species-rich families such as Cyprinidae and Blenniidae contain species that have restricted gill openings, as seen in *Gila*, *Hypsoblennius*, and *Chasmodes* (Miller, 1945; Ditty et al., 2005). There can also be variation within species-depauperate families such as Aploactinidae, which contains species with membranes united and free from the isthmus as well as species with restricted gill openings (Poss and Johnson, 1991). As for intrageneric variation, *Ernogrammus hexagrammus* (Stichaeidae) has membranes joined to the isthmus whereas *Ernogrammus walkeri* has membranes united and free from the isthmus (Follett and Powell, 1988). There are also several families in which all or most members have an attachment of the branchiostegal membrane to the isthmus, with some members having restricted gill openings; these families include Cyprinidae, Auchenipteridae, Batrachoididae, Agonidae, and Gobiidae.

We identified a cryptic case of restricted gill openings in some barreleyes (Opisthoproctidae) that calls attention to the difficulty of determining branchiostegal membrane morphology from preserved specimens of deep-sea fishes. These specimens are typically delicate and easily damaged during collection and preservation, and tearing of the branchiostegal membrane can give the impression that membranes are free from the isthmus. This is exacerbated by the evolutionary trend of branchiostegal ray reduction in deep-sea fishes, because the rays cease to provide substantial skeletal support for the membrane (McAllister, 1968). Based on examination of two specimens in which the complete branchiostegal membrane is intact (*Opisthoproctus soleatus*, MCZ41536, and *Macropinna microstoma*, UW110014), we found that the gill opening is restricted to a small aperture covered by the opercular bones in these species. Other opisthoproctid genera such as *Dolichopteryx* and *Rhyncohyalus* have been noted to have united membranes that are free from the isthmus (Cohen, 1964), but the morphology of the gill opening was unambiguously restricted in the two aforementioned specimens of *O. soleatus* and *M. microstoma*. Among the Opisthoproctidae, these two taxa represent more extreme forms, with some skeletal modifications potentially linked to restricted gill openings. For example, *O. soleatus* possesses a large ventral keel that extends far forward on the ventral surface of the head, supported by anterior projections of the cleithrum (Cohen, 1964). *Macropinna microstoma* has large expansions of the interopercle and preopercle that extend ventrally to cover the gular region (Chapman, 1942). These structures may require the ventral space on the head provided by restricted gill openings. Also, both species have dorsally directed tubular eyes that require specialized configurations of the cranial elements, some of which may be facilitated by restriction of the gill openings.

Synbranchiform fishes (swamp eels) are often noted for their single, small gill opening on the ventral surface of the head. This is a specialized case of "united and free from the isthmus" (McAllister, 1968) in which the gill openings are covered with skin dorsally, giving the superficial appearance of one small ventral opening. Because the membranes remain free from the isthmus, we consider this to be a fundamentally different morphology from "restricted gill openings," as we have defined it in this study. This single opening likely has major functional consequences for the complex and unique synbranchiform aerial respiratory apparatus (Liem, 1980). Graham (1997) suggests that it may allow Synbranchiformes to better retain a volume of air in the gill chamber and keep out debris. Restricted gill openings may have similar functions in other air-breathing or burying fishes.

While restricted gill openings are distributed broadly throughout the ray-finned fishes, they are notably absent among much of the phylogenetic diversity of Percomorpha (Fig. 4B and C). The majority of families in these clades without restricted gill openings have previously been classified as belonging to "Percoidei," which has long been acknowledged as paraphyletic (Johnson, 1984). Many (but not all) "Percoidei" have a generalized, often perch-like form with few distinguishing specializations and occur among reefs and other near-shore environments (Johnson, 1984). These historical and ecological trends help to explain the high number of reef fishes in our survey with united membranes that are free from the isthmus (Fig. 7).

Quantifying Gill Opening Restriction

Although many species have clearly tiny gill openings, the difference between "membranes joined to the isthmus" and "restricted gill openings" morphology is frequently a matter of qualitative assessment by authors. Our survey provided an opportunity to use qualitative assessment of gill opening morphology to quantitatively define this feature. The most accurate and consistent metric tested in our study to quantify gill opening restriction was the restricted gill openings (RGO) ratio, which is a consideration of how broadly the branchiostegal membrane is attached to the isthmus relative to head circumference, as expressed in the following equation:

$$\text{RGO ratio} = \left(\frac{\text{VMVM}}{1/2 \text{ head circumference}}\right) \times 100$$

where VMVM is the distance from the ventral midline of the body to the ventral margin of the gill opening. This ratio quantifies the extent to which the gill openings are restricted ventrally by the branchiostegal membrane and the isthmus. Generally, a fish with an RGO ratio above 12.5 has restricted gill openings (Fig. 5), and this value can be used as a reference for species descriptions and other morphological assessments.

When diagnosing gill opening restriction, it is useful to consider gill opening length in addition to the RGO ratio. Gill opening length is a commonly reported metric that provides a direct indication of gill opening size. The ratio of gill opening length to head length had relatively low error in our subsampling simulations (error = 3.2–5.3%). Fishes with a gill opening length less than 38.5% of head length were most often characterized qualitatively as having restricted gill openings. Due to the importance of gill opening size in the definition of restricted gill openings, we attempted to refine our RGO ratio by testing metrics that included both VMVM and gill opening length. However, these more complex metrics showed higher errors and were unreliable for consistently distinguishing fishes that were characterized as having restricted gill openings. Therefore, the RGO ratio and the relative length of the gill opening should be considered separately in assessment of gill opening morphology.

Based on our simulations, the RGO ratio mischaracterized morphology in 1.2–2.7% of taxa. These mischaracterizations occur in cases of fishes with restricted gill openings that are positioned very close to the ventral midline (e.g., *Muraenesox bagio*, Muraenesocidae), which have a small RGO ratio and will therefore be erroneously characterized as "joined to the isthmus." Additionally, fishes with large gill openings but a broad attachment to the isthmus (e.g., *Rhyacichthys aspro*, Rhyacichthyidae) have a large RGO ratio and will be erroneously characterized as "gill openings restricted." The ratio of gill opening length to head length mischaracterized taxa in 3.2–5.3% of cases. In taxa with small head lengths, as in Gymnotidae (knifefishes), this ratio can be large despite a restricted gill opening. Taxa with large snouts, such as Acipenseridae (sturgeons), can have a small ratio of gill opening length to head length, despite a large gill opening. When diagnosing the presence of restricted gill openings in a species, it is useful to consider the following factors: the RGO ratio ("restricted" above 12.5), the gill opening length as a percentage of the head length ("restricted" below 38.5%), the position and appearance of the gill opening, and the relative condition of closely related taxa.

Relationship of Restricted Gill Openings with Morphometric and Ecological Factors

We defined a morphospace based on six ratios of measurements taken as a part of our survey. These measurements were selected because of their ability to capture the major axes of actinopterygian body shape variation, specifically relative body elongation, head size, snout size, horizontal position of the eye on the head, and mouth dimensions. Fishes with restricted gill openings occupy a large area of morphospace in our plot of PC1 and PC2 (Fig. 6), and for each of the first four principal components, the standard deviation is highest among fishes with restricted gill openings (Table 1). United branchiostegal membranes are the most common morphology in terms of number of families ($n = 194$ out of 433), and yet fishes with that condition occupy only a subset of the morphospace circumscribed by fishes with restricted gill openings ($n = 80$). Fishes with separate membranes and membranes joined to the isthmus occupy an even smaller portion of the overall morphospace. If fishes with restricted gill openings had occupied only a portion of our morphospace, then we could potentially infer body and head shapes that co-occur with restricted gill openings. However, fishes with a wide range of shapes possess restricted gill openings, indicating that small gill openings may be beneficial when co-occurring with a large variety of cranial morphologies. This trend is apparent even when superficially considering the diversity of fishes with restricted gill openings; moray eels, ocean sunfishes, and seahorses share very few similarities in body and head shape.

Furthermore, the presence of fishes with restricted gill openings at the extremes of this morphospace may indicate that a small, constrained gill opening is necessary for some extreme morphologies to be possible. Restricted gill openings co-occur with a number of highly specialized morphological systems. For example, some anguilliforms (eels) have evolved increased mobility of the pharyngeal jaws for prey capture and posteriorly displaced gill arches (Nelson, 1966; Mehta and Wainwright, 2008). Their ventilation relies mostly on a powerful buccal pump (Hughes, 1960), which may be due to the evolutionary restructuring of the pharyngeal chamber and a reduction of the branchiostegal apparatus. Lophiids (goosefishes) are cryptic ambush predators with large, up-turned mouths ideal for rapid ingestion of large prey. Lophiids have limited range of cranial motion during ventilation as a result of their extreme feeding morphology (Elshoud, 1986), and therefore ventilation is primarily driven by the action of a large branchiostegal apparatus closed off by a siphon-like restricted gill opening uniquely positioned behind the base of the pectoral fin. The inflation mechanism of burrfishes (Diodontidae) involves a kinematically complex expansion and compression of the buccal cavity, facilitated in part by a greatly enlarged first branchiostegal ray (Wainwright et al., 1995). Syngnathids use a powerful elastic recoil system, spanning from the epaxial muscles to the snout, to quickly rotate the snout upward towards a prey item during suction feeding (Van Wassenbergh et al., 2008), and their ventilation is primarily facilitated by a powerful gill-chamber pump (Hughes, 1960). While it is clear that restricted gill openings cannot be the sole explanation for the evolution of these complex biomechanical systems, it is possible that small gill openings played a critical role in their evolutionary history by freeing cranial morphology from the constraint of ancestrally large gill openings. Freeing constraints on morphological systems weakens evolutionary integration among structures and can result in rapid accumulation of disparities within a clade, producing extreme forms (Liem, 1973; Collar et al., 2014). The influence of the presence of restricted gill openings on patterns of diversification and morphological evolution could be tested in a group such as the catfishes (Siluriformes), in which the trait has evolved repeatedly in morphologically and ecologically disparate groups.

Our decision tree analysis (Fig. 7) demonstrates that fishes with all four branchiostegal membrane morphologies, including restricted gill openings, occur in a variety of habitats. However, fishes with restricted gill openings were notably sparse among pelagic (nondeep sea) fishes, with the Molidae as the only example in our survey (other pelagic species of Tetraodontiformes, such as the ocean triggerfish, *Canthidermis sufflamen*, Balistidae, have restricted gill openings). This indicates that restricted gill openings are not ideal for the high levels of activity required for a typical pelagic fish. For example, a small gill opening may not be suitable for fishes such as paddlefishes (Polyodontidae) that rely on ram ventilation (Burggren and Bemis, 1991). Benthic and structure-associated fishes are, to some extent, released from the selective pressures for and morphological constraints of extreme drag reduction and therefore may be more likely to possess modified ventilatory structures such as restricted gill openings.

Functional Implications of Restricted Gill Openings

Direct investigations of ventilatory pressures have revealed that fishes with restricted gill openings exhibit a variety of patterns of ventilatory function, ranging from dominant buccal pumpers to dominant branchiostegal pumpers (Hughes, 1960). This variation spans the continuum of known functional diversity in aquatically ventilating ray-finned fishes, and therefore it is difficult to link restricted gill openings to specific aspects of ventilatory function without further study. However, by considering the nature of the trait and the diversity of taxa in which it occurs, we can discuss potential functional implications. For example, there may be functional benefits for separating the left and right gill openings through an attachment to the isthmus. In fishes with "united and free from the isthmus" morphology, the unification of the left and right membranes results in the formation of a single gill opening from which water leaves the gill chambers. Separation of these two openings allows the gill openings to be positioned more variably on the head; in extreme cases, the gill openings can be positioned as small apertures on the dorsal surface of the head (e.g., Palefin Dragonet, *Synchiropus goodenbeani*, Callionymidae) or posterior to the pectoral girdle (e.g., American Angler, *Lophius americanus*, Lophiidae). This flexibility in gill opening positioning may be of great importance to fishes that rely on frequent and sustained contact of the ventral surface of the body with a substrate, because a gill opening that is pointed ventrally is likely to disturb sediment.

Broad attachment to the isthmus is also generally associated with a reduction in the size or number of branchiostegal rays, possibly because the branchiostegal membrane is more anchored by tissue and may not need robust and numerous rays to support it (McAllister, 1968). Reduction of the branchiostegals may be beneficial where general skeletal reduction is adaptive, as in deep-sea habitats. Also, reduction of the branchiostegal apparatus is associated with a reduced reliance on the gill chamber suction pump during ventilation (Hughes, 1960; Liem, 1970). This reduction is observed in many pelagic fishes and does not require an attachment to the isthmus. However, if selection favors a reduced branchiostegal apparatus and a stronger reliance on buccal pumping, a broad attachment to the isthmus may provide stability for the gill chamber and the opercular valve.

Some fishes have coopted their restricted gill openings for the more obvious function of opercular jetting. Many frogfishes (Lophiiformes: Antennariidae) force water from the gill openings to jet forward, either for a fast burst of movement or cryptic locomotion (Fish, 1987; Pietsch and Grobecker, 1987). Porcupine fishes (Tetraodontiformes: Diodontidae) expel a high-velocity jet of water from their gill openings, in conjunction with rapid movement of their fins and trunk, as an escape mechanism (Breder, 1924). Banjo catfishes (Siluriformes: Aspredinidae) use their ventrally positioned restricted gill openings to propel themselves along the benthos (Gradwell, 1971). However, this behavior has been observed in only a small subset of fishes with restricted gill openings, and it is likely a secondary advantage of this feature.

Lastly, the branchiostegal apparatus may be under fewer functional constraints relative to other components of the gill chamber, which are the opercular bones (opercle, subopercle, and interopercle) and pectoral girdle. The opercular bones are linked closely with the lower jaw and often function in feeding mechanics (Liem, 1970; Westneat, 2005); for example, these bones have been demonstrated to be strongly evolutionarily integrated with feeding structures in suction-feeding eels (Collar et al., 2014). The pectoral girdle functions in both feeding and locomotion and presumably experiences strong evolutionary pressures related to feeding and locomotor performance. However, the branchiostegal apparatus has a weaker association with feeding and locomotion, and therefore, it is likely the structure that is most directly influenced by selective pressures on ventilatory mechanics. Functional studies of variation in aquatic gill ventilatory systems in fishes will undoubtedly benefit from a closer examination of the branchiostegal apparatus.

While the direct effects of gill opening restriction on ventilatory biomechanics have yet to be determined (Brainerd and Ferry-Graham, 2006), the feature is associated with a large amount of morphological and ecological variation. It has evolved repeatedly throughout the evolutionary history of the ray-finned fishes, without a clear indication of its adaptive purpose. This relatively common modification of the gill chamber draws attention to the potentially critical influence of ventilatory morphology on the ecology, behavior, and evolutionary history of fishes.

ACKNOWLEDGMENTS

For access to specimens, we thank John P. Friel and Charles M. Dardia (CUMV), John Lundberg (ANSP), Karsten Hartel and Andrew Williston (MCZ), and Theodore W. Pietsch and Katherine Maslenikov (UW). For feedback on statistical analyses, we thank Collin B. Edwards, Nicholas A. Mason, Liam Revell, Peter C. Wainwright, and Francoise Vermeylen. For help with data collection and entry, we thank Matthew T. Gibbons. For comments on the manuscript, we thank John P. Friel, Harry Greene, Amy McCune, Adam Summers, and two anonymous reviewers. Author contributions: Stacy C. Farina designed and conducted the museum survey, collecting membrane morphology and morphometric data on each specimen. She performed all statistical analyses, prepared the figures, and wrote the text. Thomas J. Near contributed all molecular data used in this study. He assisted with the statistical analysis and interpretation of phylogenetic reconstructions and with development of the text. William E. Bemis contributed substantially to development and preparation of the survey, figures, and text.

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