

# Evolutionary innovation accelerates morphological diversification in pufferfishes and their relatives

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## Abstract

Evolutionary innovations have played an important role in shaping the diversity of life on Earth. However, how these innovations arise and their downstream effects on patterns of morphological diversification remain poorly understood. Here, we examine the impact of evolutionary innovation on trait diversification in tetraodontiform fishes (pufferfishes, boxfishes, ocean sunfishes, and allies). This order provides an ideal model system for studying morphological diversification owing to their range of habitats and divergent morphologies, including the fusion of the teeth into a beak in several families. Using three-dimensional geometric morphometric data for 176 extant and fossil species, we examine the effect of skull integration and novel habitat association on the evolution of innovation. Strong integration may be a requirement for rapid trait evolution and facilitating the evolution of innovative structures, like the tetraodontiform beak. Our results show that the beak arose in the presence of highly conserved patterns of integration across the skull, suggesting that integration did not limit the range of available phenotypes to tetraodontiforms. Furthermore, we find that beaks have allowed tetraodontiforms to diversify into novel ecological niches, irrespective of habitat. Our results suggest that general rules pertaining to evolutionary innovation may be more nuanced than previously thought.

**Keywords:** evolutionary innovation, trait integration, habitat association, Tetraodontiformes

## Introduction

Evolutionary innovations have shaped the process of evolution and can account for the evolutionary success of many clades (Heard & Hauser, 1995; Hunter, 1998; Miller, 1949). These novel traits are adaptations that directly enhance diversification by allowing species access to previously unattainable ecological niches and resources (Heard & Hauser, 1995; Miller, 1949), often via colonization of novel regions of morphological trait space (Evans et al., 2021). These adaptive breakthroughs can be found throughout the Tree of Life and include innovations such as flight in birds (Heard & Hauser, 1995; Mayr, 1963), adhesive toepads in tree-dwelling lizards (Miller & Stroud, 2022), orb-weaving in spiders (Blackledge et al., 2009) and pharyngeal jaws in cichlids and other fishes

(Liem, 1973). However, the ecological, developmental, and evolutionary mechanisms by which morphological novelties become innovations that enhance diversification remain poorly characterized (Erwin, 2015). Quantitative case studies integrating paleontological, phylogenetic, and ecological data are needed to better understand the processes underlying evolutionary innovation and the resulting downstream effects on the tempo and mode of morphological diversification across multiple time scales.

Studies of evolutionary modularity and integration can aid in the understanding of the evolution of innovation. Highly modular traits exhibit a strong degree of covariation within individual structural regions (modules) but much lower covariation between modules. In this way, modularity enables

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organisms to evolve semiindependent adaptations and can promote the evolution of complexity, diversity, and evolutionary innovations (Evans et al., 2017; Goswami et al., 2014; Jablonski, 2022; Larouche et al., 2018; Wagner & Altenberg, 1996; Yang, 2001). Because highly modular traits can explore a larger volume of trait space (Goswami et al., 2014), they can lead to the evolution of morphological innovation as more novel areas of the trait space become occupied and new morphologies evolve. Conversely, highly integrated traits covary strongly with each other and evolve together in a coordinated fashion (Olson & Miller, 1999). With such strong covariation, these traits have much less freedom to explore a large volume of trait space (compared to highly modular traits) (Evans et al., 2021; Goswami et al., 2014). As such, strong integration has been hypothesized to act as a constraint on diversification (Goswami & Polly, 2010; Goswami et al., 2014). This has been documented in cichlid fishes for over 50 years, where reduced integration in the functionally related oral and pharyngeal jaws has led to increases in diversity (Burruss et al., 2020; Liem, 1973). More recent analyses of this system suggest that increased integration decreases evolutionary rates of morphological change (Roberts-Hughes et al., 2023).

Despite integration being traditionally considered a constraint that hinders the evolution of complexity and diversity, some studies suggest that strong trait integration can also promote rapid morphological evolution in functionally linked traits (Evans et al., 2021; Felice et al., 2018). Recent simulation studies have shown that in this way, larger, more rapid morphological changes can evolve by directing evolution along a line of least phenotypic resistance (Goswami et al., 2014). Such a system would be more likely to evolve maximally disparate phenotypes by pushing species into the outer edges of trait space (Evans et al., 2021; Goswami et al., 2014; Navalón et al., 2020). This has been documented in the asymmetrical skulls of flatfishes (Evans et al., 2021), extreme variation in snake skull shapes (Ollonen et al., 2024), and the elongated beaks of Hawaiian honeycreeper birds (Navalón et al., 2020), suggesting that integration promotes the evolution of innovation by allowing clades to rapidly colonize into novel regions of trait space.

Innovations may also enable access to novel habitats. This can further shape evolution by providing ecological release from competition and predation, resulting in increased lineage and morphological diversification rates (Yoder et al., 2010). Certain habitats may promote more morphological diversification than others. Coral reefs exemplify such environments, containing 25% of all marine life despite occupying less than 0.2% of the ocean floor (Souter et al., 2021). Reefs host complex interactions between coral and fish species, facilitating fine-scale niche partitioning and associated morphological and functional specialization, which can lead to increased ecological opportunities (Alfaro et al., 2007; Brandl et al., 2018; Cowman & Bellwood, 2011; Evans et al., 2019b; Price et al., 2011). Additionally, coral reefs appear to spur the evolution of morphological innovations. Many reef-associated species bear novel innovations, such as the intramandibular jaw joint of butterflyfishes (Chaetodontidae), parrotfishes (Scarinae), and other fishes, which expands the gape, assisting in grazing algae and invertebrates from reef surfaces (Gibb et al., 2015).

Of the numerous evolutionary innovations that have evolved, the avian beak (particularly its shape) is an important feature that evolved prior to radiations into novel adaptive

zones (Bhullar et al., 2015; Guillerme et al., 2023; Jetz et al., 2012). Although birds represent the most speciose clade possessing beaks, they are found in other groups, including turtles and tortoises (Testudines) (Ingle et al., 2023), extinct pterosaurs (Martill et al., 2021), and certain groups of fishes. Despite their prevalence across the Tree of Life, beak evolution in these less speciose clades has received comparably less attention.

Among fishes, the evolution of highly modified teeth forming convergent beaked structures has evolved independently in at least two groups: parrotfishes (Labridae: Scarinae) (Evans et al., 2023a; Price et al., 2010) and tetraodontiforms (pufferfishes, ocean sunfishes, and allies) (Tyler, 1980). These dental features qualify as evolutionary innovations in both clades because they enable access to previously unattainable dietary and functional niches (Miller, 1949; Miller et al., 2023). Fish beaks facilitate durophagous feeding modes, either upon carbonate reef structures in the case of parrotfishes (Evans et al., 2023a; Lelley et al., 2019; Price et al., 2010) or hard-shelled invertebrates in the case of tetraodontiforms (Turingan, 1994; Tyler, 1980). Recently, an extensive morphological study investigating skull shape evolution in parrotfishes found that beaks are an evolutionary innovation that promoted rapid morphological diversification along certain evolutionary trajectories related to climatic and biogeographic variables (Evans et al., 2023a). Whether this pattern holds true for other clades with convergent beaked structures, such as tetraodontiforms, remains unknown.

Tetraodontiform fishes represent an excellent system for exploring patterns related to the evolution of innovation. These fishes possess a host of unique features and morphologies, including the cuboidal body of boxfishes, erectable body spines in porcupinefishes, and the fusion of the teeth into a beak in several families, including the most speciose family, the pufferfishes (Tetraodontidae) (Santini & Tyler, 2003; Tyler, 1980). This novel dentition develops from elongated tooth bands that continuously fuse together during ontogeny to form a mineralized beak (Fraser et al., 2012; Thiery et al., 2017).

The tetraodontiform beak represents an evolutionary innovation that has enabled the occupation of new dietary niches. Beaked species possess a pair of trituration tooth plates on the inside of the dentary and premaxilla that aid in crushing hard-bodied prey such as crabs and bivalves (Turingan, 1994; Tyler, 1980). The tetraodontiform beak is comprised of highly modified and fused jaw bones and represents a maximally disparate phenotype compared to nonbeaked tetraodontiforms. Given this extreme morphological disparity, we may expect that skulls of beaked species evolve rapidly and in a highly integrated fashion, similar to previous studies showing this in flatfishes, snakes, and certain birds (Evans et al., 2021; Navalón et al., 2020; Ollonen et al., 2024).

In addition to displaying novel phenotypes, tetraodontiforms permit tests for links between morphological diversity and habitat association. These fishes are circumglobally distributed and occupy a wide variety of ecosystems, from coral reefs to open oceans and even freshwater rivers (Tyler, 1980). This ecological diversity allows for the examination of how different habitats may influence patterns of morphological evolution and innovation. Furthermore, tetraodontiforms have a rich fossil record extending back to the Late Cretaceous, with many well-preserved specimens (Santini & Tyler, 2003). This enables evolutionary tests over deep time

scales, providing a more comprehensive understanding of the factors shaping their diversity.

Here, we investigate the role of an evolutionary innovation, the tetraodontiform beak, in shaping patterns of evolutionary integration, evolutionary rates, and morphological disparity. We quantify skull shape for 176 fossil and extant tetraodontiform species using a three-dimensional geometric morphometric approach. We use this dataset to test factors promoting the evolution of innovation under a comparative framework. Compared to its closest relatives, the tetraodontiform beak represents a maximally disparate phenotype. Thus, we hypothesize that skulls of beaked species have evolved this extreme morphology due to strong trait integration that evolved rapidly along a line of least phenotypic resistance. As such, we also expect that skulls of beaked species exhibit higher morphological disparity compared to skulls of nonbeaked species, as they would be able to occupy the more extreme edges of trait space. Lastly, we hypothesize that highly diverse habitats may have spurred the evolution of the tetraodontiform beak and resulted in increased rates of morphological disparity and evolution. By examining the impact of evolutionary innovation on multiple aspects of morphological diversification in a comparative framework, this study aims to provide new insights into the mechanisms driving patterns of phenotypic diversity across clades and habitats.

## Methods

### Taxonomic sampling and CT scan data acquisition

We analyzed the skull shape of 176 species of Tetraodontiformes, including 173 extant and three fossil species. This sampling encompasses all 10 living families, with fossil representatives from two families (Tetraodontidae and Triodontidae). A comprehensive list of the scanned species, scanning locations, and specimen voucher information is provided in [Supplementary Appendix 1 \(Supplementary Material\)](#). We included three of the only known cataloged three-dimensional fossil tetraodontiform skulls, †*Sphoeroides hyperostosus* (Tetraodontidae), †*Triodon antiquus* (Triodontidae), and †*Ctenoplectus williamsi* (stem Triodontidae). Each species was represented by a single adult specimen that underwent micro-CT scanning at the University of Washington Friday Harbor Laboratories (Bruker Skyscan 1173; 40 species), Rice University (Bruker Skyscan 1273; 92 species), the University of New England, Australia (General Electric phoenix vltomelx s; 13 species), Cornell University (General Electric 120 micro-CT; 1 species), or the University of Michigan (Nikon XT H 225 ST; 1 fossil species), totaling 147 new scans. Two previously scanned fossil specimens were also acquired—†*Ctenoplectus williamsi* from [Close et al. \(2016\)](#) and one unpublished scan of †*Triodon antiquus*, both scanned on a Nikon XT H 225 ST at the Natural History Museum, London. Finally, we sourced scans for 27 additional species from MorphoSource ([morphosource.org](#)) listed in [Supplementary Appendix 1 \(Supplementary Material\)](#).

### Segmentation, digitization, and fossil landmarks

Scans were segmented in 3D Slicer ([Kikinis et al., 2014](#)) to isolate the skull bones from the rest of the body. Within 3D Slicer, digitization of the specimens was conducted using a landmark scheme of 170 points (48 fixed landmarks and 122 sliding semilandmarks), as detailed in [Supplementary Figure S1](#) and [Supplementary Table S1](#). This scheme represents an

extended version of the general Eupercaria scheme from [Evans et al. \(2023b\)](#), ensuring a comprehensive representation of the diversity of tetraodontiform skull shapes. To ensure consistency of landmark placement, all landmarking was conducted by the same person. After landmarking all 176 specimens, each scan was inspected again for verification, with slight adjustments made when necessary.

All landmarks were placed on the left side of the skull. However, for one fossil specimen, †*Triodon antiquus*, the left side was unable to be landmarked due to taphonomic degradation. To address this, the CT scan was converted to a three-dimensional mesh and then inverted for landmarking using the *MeshLab* software ([Cignoni et al., 2008](#)). Additional taphonomic processes affecting our fossil specimens occasionally rendered some landmarks unplaceable. Instead of proceeding with a significantly reduced subset of landmarks shared across all extant and fossil specimens, we chose to estimate the missing landmarks for the fossil specimens using the ‘MissingGeoMorph’ function in the R package *LOST* ([Arbour & Brown, 2020](#)). We applied a Bayesian principal components analysis (BPCA) method to estimate missing landmark data, leveraging principal component regressions and Bayesian estimations to determine the position of absent landmarks ([Oba et al., 2003](#)). Empirical data set analyses by [Arbour & Brown \(2014\)](#) have shown this method to be highly reliable for landmark estimations. Moreover, these types of estimates produce a better fit to the original data than exclusion of specimens with incomplete landmarks ([Arbour & Brown, 2014](#)).

### Skull shape analyses

After digitization, we imported the landmark coordinates into R Statistical Environment version 4.2.3 ([R Core Team, 2023](#)) using a custom script from [Buser et al. \(2023\)](#) and processed them with the *geomorph* package version 4.0.5 ([Baken et al., 2021](#)). To remove the effect of nonshape variation, such as scale, size, and orientation across specimens, we performed a generalized Procrustes superimposition between specimens ([Rohlf & Slice, 1990](#)). Semilandmarks were slid along their tangent directions using the Procrustes distance criterion because sliding using bending energy may cause spurious correlations among landmarks that can bias modularity analyses ([Zelditch & Swiderski, 2023](#)). Given the biomechanical complexity of fish skulls, which contain multiple moving elements, analyzing shape can be challenging due to preservation artifacts affecting jaw positions ([Evans et al., 2019a](#)). We account for these biases in the rotation and translation of these mobile structures by performing a local superimposition, ensuring standardized positioning of the different skull elements ([Rhoda et al., 2021a, 2021b](#)). Following local superimposition, we then conducted a PCA to assess the main axes of skull shape variation. The first two principal components (PC1 and PC2) were visualized as a phylomorphospace using the pruned, time-calibrated phylogeny of [Troyer et al. \(2022\)](#). Additionally, we employed the “plotRefToTarget” function in *geomorph* to plot the primary and secondary axes of skull shape variation as ball and stick plots ([Supplementary Figure S2](#)).

### Phylogenetic estimation, trait coding, and ancestral trait reconstructions

To investigate skull evolution across Tetraodontiformes, we used the time-calibrated phylogeny proposed by [Troyer](#)

et al. (2022), which encompasses 239 extant and fossil species of Tetraodontiformes. Using the *ape* package (Paradis & Schliep, 2019), we pruned the tree to retain only the 176 taxa for which morphological data was available. Habitat associations for extant species were obtained from FishBase (Froese & Pauly, 2023) and Fishes of Australia (Bray & Gomon, 2023). Each species was categorized as being coral reef-associated or nonreef-associated, following previous studies (Alfaro et al., 2007; Santini et al., 2013a) (Supplementary Appendix 1). For fossil species, categorization was based on the paleobiotope where they were discovered, with reef association being determined by the presence of hermatypic corals (Friedman & Carnevale, 2018; Marramà et al., 2016). Dental morphology for each species was also characterized. Species were defined as beaked if they possessed highly modified and fused teeth, a characterization based on the criteria of Tyler (1980) (Supplementary Appendix 1). The beaked group consists of all species from the families Molidae, Diodontidae, Tetraodontidae, and Triodontidae. Nonbeaked species include those from the families Balistidae, Monacanthidae, Triacanthidae, Triacanthodidae, Ostraciidae, and Aracanidae. Nonbeaked species possess teeth that are discrete units and protrude out of the jaw sockets, while beaked species possess teeth that do not protrude and are incorporated into the matrix of the jaw bones. For the fossil species in our analysis, their classification as beaked or nonbeaked was based on characters 68–70 from the morphological matrix by Santini and Tyler (2003) defining beaked species as possessing teeth fused into a parrot-like beak and nonbeaked species having teeth as discrete units, either slender caniniform, stoutly conical, incisiform-molariform, or thick caniniform teeth.

To determine the timing of evolutionary transitions for each trait of interest in our analyses (habitat type, dentition type), we reconstructed ancestral states in *phytools* (Revell, 2012). We used a stochastic character mapping approach (Huelsenbeck et al., 2003) under a model with 1,000 simulations with the “make.simmap” function on the complete tree from Troyer et al. (2022), containing seven outgroup taxa, 52 fossil tetraodontiforms, and 187 extant tetraodontiforms. Model fitting was conducted using the “ace” function in *ape* for an equal rates model that assumes a single rate for all transitions, an all rates different model that allows transitions to have unique rates, and a symmetrical rates model where forwards and backwards transition rates are equal, but can be unique for each character state pair (Paradis & Schliep, 2019) (Table S2). Empirical Bayesian posterior probabilities for estimated ancestral states were plotted for each node of the phylogeny. After pruning outgroups from the tree, we estimated the number of character transitions between each state (i.e., reef to nonreef, beak to nonbeak, and vice versa) to determine the frequency of these transitions over time. Lastly, we recorded the proportion of time spent in each state, calculated in the “make.simmap” function that produces a matrix containing the total time spent in each state along each edge of the tree (Revell, 2012).

### Rates of skull shape evolution and morphological disparity

We quantified the rate of skull shape evolution between reef and nonreef-associated species, as well as between beaked and nonbeaked species using the “compare.evol.rates” function in *geomorph*. This method calculates the multivariate rate (the net rate of change over time along phylogenetic branches)

of evolution by comparing rates of groups (not necessarily clades) using a 3D array of Procrustes-aligned coordinates, a phylogenetic tree, and an array containing group state (i.e., habitat type) as input (Adams, 2014; Baken et al., 2021). Significance was assessed using the phylogenetic simulation approach run with 1,000 iterations (Adams & Collyer, 2018). Similarly, to compare skull morphological disparity, we employed the “morphol.disparity” function in *geomorph* for both reef vs. nonreef and beaked vs. nonbeaked states. This function calculates morphological disparity by estimating the Procrustes variance for each group using the residuals of a linear model fit. Additionally, we used the “compare.multi.evol.rates” function in *geomorph* to evaluate the net rates of shape evolution for each skull module and compare between beaked and nonbeaked species. This method calculates evolutionary rate parameters of multivariate traits ( $\sigma^2$ ) from predefined modules. Significance is assessed by comparing the observed rate to a null rate matrix derived from a random simulation using 1,000 permutations.

To quantify rates of tetraodontiform skull shape evolution across the phylogeny, we used BayesTraitsV4 (Pagel & Meade, 2022). To reduce dimensionality, we employed only the first 24 principal components, which account for 95% of the total shape variation. Principal component scores were multiplied by 1,000, following Evans et al. (2023b), as a way to correct for any computation issues arising from small numbers in BayesTraits. We account for evolutionary correlations in trait variation using the “TestCorrel” function in BayesTraits. We used a reversible-jump Markov chain Monte Carlo method with uniform priors and ran two independent chains for 200 million generations, sampling every 10,000 iterations, with the first 60 million discarded as burn-in. Convergence was visually assessed using Tracer v1.7.1 (Rambaut et al., 2018), with all ESS (Effective Sample Size) values exceeding 200. We tested two models of trait evolution: a single rate Brownian motion model that assumes one rate across the phylogeny, and a variable rates model that allows for changes in rates throughout the phylogeny and identifies where rates differ (Venditti et al., 2011). Model fitting was performed by calculating Bayes factors based on the marginal likelihoods from both models. A Bayes factor greater than 10 is regarded as strong support for that particular model (Pagel & Meade, 2022).

To assess changes in subclade morphological disparity for Tetraodontiformes, we implemented disparity through time (DTT) analyses under a Brownian motion model using the “geiger” package in R. We also compare DTT for reef vs. nonreef-associated species as well as beaked vs. nonbeaked species. We used the average squared Euclidean distance among all pairs of data points as our disparity index. The DTT method calculates changes in relative subclade disparity through time across nodes in the phylogeny. We compared the observed disparity to that under a simulated null Brownian motion model iterated over 1,000 generations. We used the observed and simulated disparities to calculate a morphological disparity index (MDI), which measures the deviation from expectations for relative within-clade disparities under a model of Brownian motion. A negative MDI indicates that disparity is distributed among subclades and is commonly interpreted as evidence for an early burst, characteristic of adaptive radiation (Harmon et al., 2010; Slater & Pennell, 2014). A positive MDI indicates that disparity is distributed within subclades. Because MDI estimations at multiple time

points suffer from a high false-positive rate, we use the two-tailed rank envelope method of Murrell (2018) to assess significance. This method provides an overall  $p$ -value as well as a  $p$ -interval because the ranks will almost always result in some ties. Because this is a two-tailed test,  $p$ -values below 0.025 are considered significant.

### Evolutionary modularity and integration

To test for patterns of evolutionary modularity between beaked and nonbeaked species, we defined eight a priori hypotheses of modularity that encompass a range of functional, embryological, and sensory hypotheses from previous literature (Evans et al., 2019a; Helfman et al., 2009; Kague et al., 2012; Westneat, 2005), as well as an 11-module individual bone hypothesis (Supplementary Figures S3). We evaluated modularity using the “phylo.modularity” function in the *geomorph* package. This function uses the covariance ratio (CR) method, which is a measure of the relative strength of covariation between modules compared to the strength within modules (Adams, 2016). A CR less than 1 indicates a more modular system, while a CR greater than 1 indicates less modularity (Adams, 2016). Then, under the best-supported hypothesis, we compared the effect sizes (strength of the modular signal) for beaked and nonbeaked species using the “compare.CR” function in *geomorph*. The best-supported model is indicated by the lowest effect size. Additionally, we evaluated our eight hypotheses of modularity with “phyloEMMLi” (Goswami & Finarelli, 2016), which applies maximum likelihood to compare different modularity hypotheses while also accounting for phylogenetic nonindependence. We visualized the results of the modularity analyses by creating network plots showing the magnitude of integration between each module.

Using our best-supported modular hypothesis, we tested evolutionary integration among modules using the “phylo.integration” function in *geomorph*. This method uses partial least squares (PLS) analysis to quantify the degree of covariation between our hypothesized modules (Rohlf & Corti, 2000). PLS values closer to 1 indicate higher integration. Because this method can be sensitive to sample size between groups (Adams & Collyer, 2016), we first randomly removed 42 nonbeaked species until both groups contained 67 species. Finally, we compared effect sizes between groups using “compare.PLS” in the *geomorph* package. All scripts, data produced, and Morphosource scan information used for this study can be found on the Dryad repository (<https://doi.org/10.5061/dryad.4f4qrfjxx>) in Supplemental Appendices 1 and 2.

## Results

### Skull shape evolution in Tetraodontiformes

Tetraodontiformes display a wide diversity of skull shapes (Figure 1A; Supplementary Figure S4A). The primary axis of shape variation (principal component 1, PC1) accounts for 37% of the overall variance and overwhelmingly corresponds to dentition type. Along PC1, beaked species occupy a separate area of the morphospace from their nonbeaked counterparts (Figure 1A). In addition to possessing an elongated distal margin of the first tooth, which corresponds to a beak, skulls of beaked species are characterized by an anteriorly positioned orbit, a long supraoccipital crest, and a wider proopercle (Supplementary Figure S2). Additionally, we find

that beaked species exhibit significantly greater ( $p = 0.002$ ) skull morphological disparity (variance = 0.0023) than nonbeaked species (variance = 0.0017). By contrast, reef and nonreef-associated species do not occupy different areas of the morphospace (Supplementary Figure S4C), and display no significant difference in skull disparity ( $p = 0.476$ ; reef variance = 0.0019, nonreef variance = 0.0020).

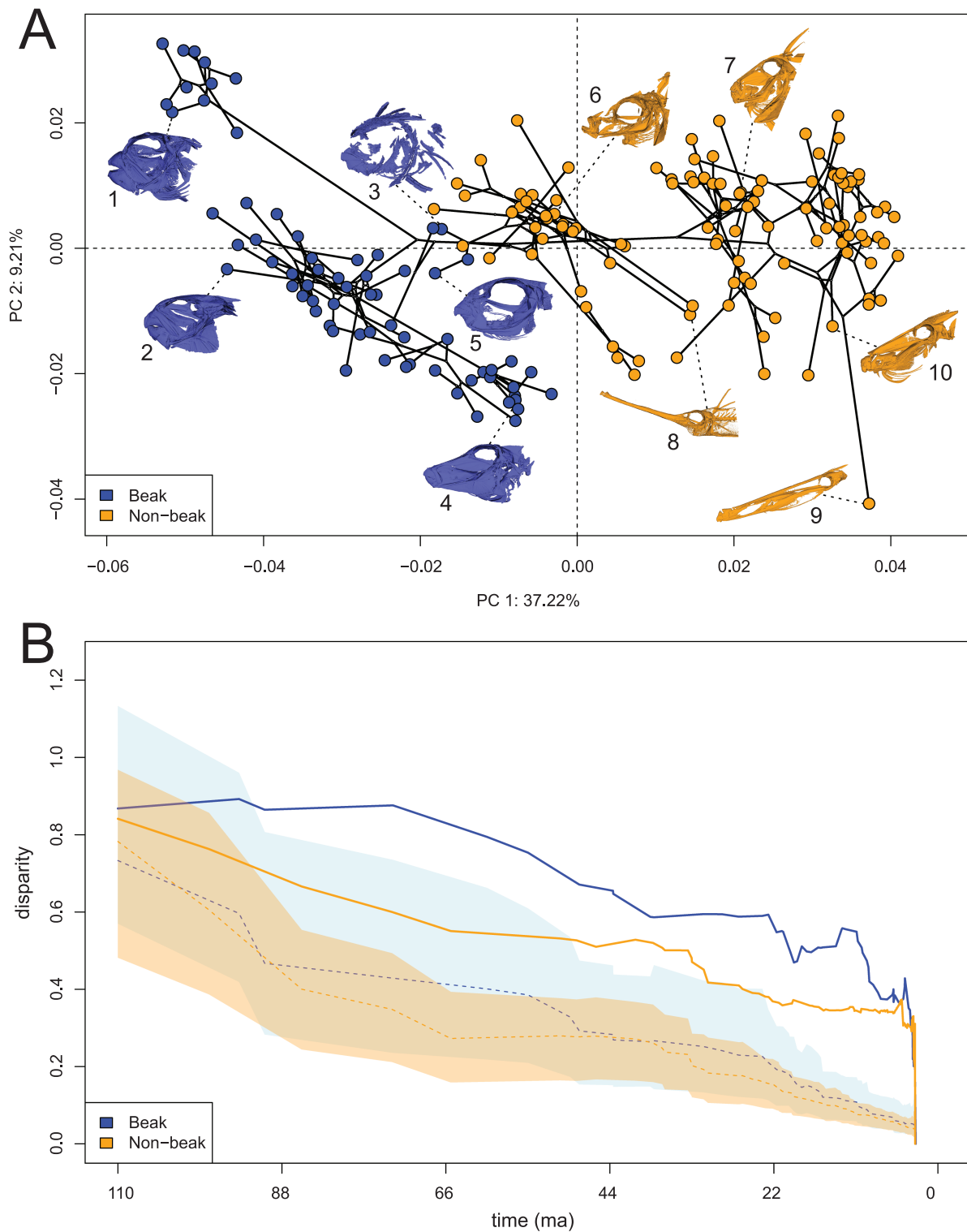
DTT analyses suggest that skull shape disparity for Tetraodontiformes is principally distributed within subclades based on a low yet positive morphological disparity index (MDI = 0.179, rank envelope test:  $p = 0.007$ ,  $p$ -interval = 0.0009–0.0139; Supplementary Figure S5). When comparing DTT between beaked and nonbeaked species, both sets of species deviate significantly from the null Brownian distribution beginning around 90 million years ago (Ma.) and continue to exceed Brownian expected disparity until the present day (Figure 1B). However, beaked species exhibit higher disparity over this time interval (MDI = 0.35; rank envelope test:  $p = 0.007$ ;  $p$ -interval = 0.0009–0.0139) compared with nonbeaked species (MDI = 0.22; rank envelope test:  $p = 0.005$ ;  $p$ -interval = 0.0009–0.0109). Additionally, beaked species display an upturn in disparity from 20 to 10 Ma. Reef-associated and nonreef-associated species also exhibit differences in DTT (Supplementary Figure S4D). Reef species display a lower MDI (MDI = 0.131; rank envelope test:  $p = 0.007$ ;  $p$ -interval = 0.0009–0.0149) compared with overall Tetraodontiformes, while nonreef species display a slightly higher MDI (MDI = 0.181; rank envelope test:  $p = 0.009$ ;  $p$ -interval = 0.0009–0.0189).

### Tempo and timing of transition rates and diversification

In examining overall rates of morphological evolution, we find significant differences in the tempo of beaked and nonbeaked tetraodontiforms. Beaked species display significantly higher rates of skull shape evolution ( $p = 0.001$ ; rate ratio = 1.42). When comparing by habitat, interestingly, nonreef-associated species show an evolutionary pace almost twice as fast as their reef-associated counterparts ( $p = 0.001$ ; rate ratio = 1.71).

On a branch-specific level, tetraodontiform fishes exhibit variable rates of skull shape evolution. Our BayesTraits analysis yielded strong support for a variable rates model of trait evolution over a single-rate Brownian motion model (Supplementary Table S3). The highest rate increases on the superfamily level occur on the two branches leading to the two beaked clades: Tetraodontoidea and Triodontidea (Figure 2). Within the pufferfishes (family Tetraodontidae), we find another high rate on the branch leading to the sharpnose pufferfishes (*Canthigaster* spp.), coinciding with a shift towards more elongated skulls compared to other pufferfish species (Figure 2). Beyond the beaked species, rate increases are found in the spikefishes (*Halimochirurgus* spp.) and the filefish *Anacanthus barbatus*, both of which exhibit remarkable snout elongations.

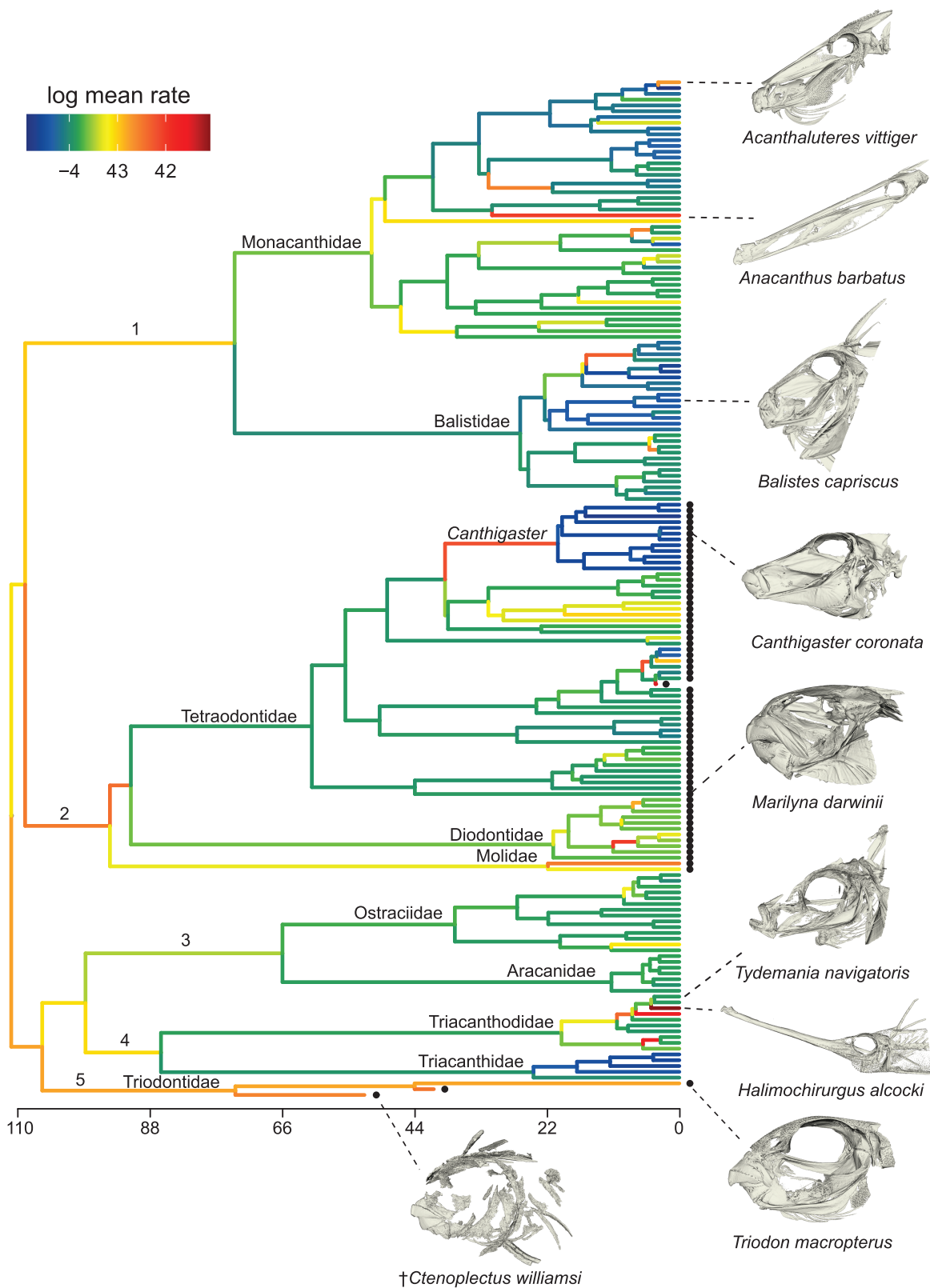
To determine the timing of evolutionary transitions to coral reef and nonreef habitats as well as the evolution of the beak, we performed ancestral character reconstructions for each trait using the time-calibrated phylogeny from Troyer et al. (2022). We recovered the ancestral state of all Tetraodontiformes as nonreef-associated, with multiple transitions to reef habitats occurring over their evolutionary history (Figure S6). Transitions from nonreef to reef habitats occur slightly more frequently across the phylogeny



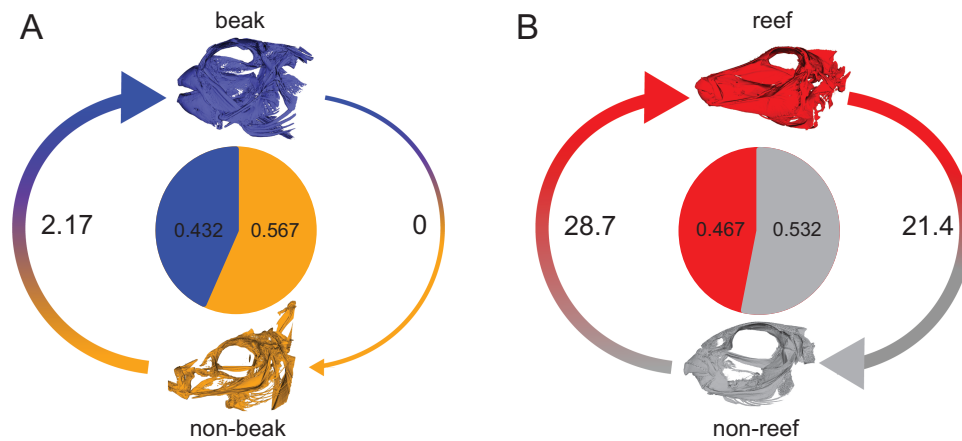
**Figure 1.** Skull shape disparity and evolution in tetraodontiform fishes. (A) Phylomorphospace of skull shape evolution colored by dentition type. Insets depict representative skull shapes for respective regions of the morphospace (1: *Chilomycterus reticulatus*, 2: *Marilyna darwinii*, 3: †*Ctenoplectrus williamsi*, 4: *Canthigaster coronata*, 5: *Triodon macropterus*, 6: *Tydemania navigatoris*, 7: *Balistes caprisicus*, 8: *Halimochirurgus alcocki*, 9: *Anacanthus barbatus*, 10: *Acanthaluteres vittiger*). (B) Disparity through time plots for beaked (blue) and nonbeaked species (orange). Dashed lines indicate the Brownian motion expectation, while shaded regions represent the 95% CI. Solid lines indicate actual measured disparity.

compared to transitions from reef to nonreef habitats. But on average, Tetraodontiformes have spent proportionally less time occupying reef habitats (0.467) compared to non-reef habitats (0.532; Figure 3). Additionally, we identify the ancestral dentition state as nonbeaked with two independent

transitions to a beak (Supplementary Figure S7). There are no transitions from a beaked dentition to a nonbeaked state (Figure 3). On average, the proportion of time spent in a beaked dentition state (0.43) is less than in a nonbeaked state (0.57; Figure 3). Notably, the oldest tetraodontiform fossils



**Figure 2.** Tempo of skull shape evolution in Tetraodontiformes. Estimated rate shifts of skull shape evolution mapped onto the time-calibrated Tetraodontiformes phylogeny from Troyer et al. (2022) under the best-supported model of trait evolution. Color gradient on branches indicates the rate of shape evolution (log mean rate: warmer colors have higher rates, while cooler colors have slower rates). Beaked species are indicated with black dots on branch tips. Representative CT scans of tetraodontiform skulls are shown for select branches. Numbers indicate branches leading to the five superfamilies (1: Balistoidea, 2: Tetraodontoidea, 3: Ostracioidea, 4: Triacanthoidea, 5: Triodontoidea). The ten extant families are labeled. Rates are estimated by using BayesTraitsV4 (Pagel & Meade, 2022).



**Figure 3.** SIMMAP character state transitions. A number of transitions from each state across the Tetraodontiformes phylogeny for (A) dentition state (beaks = blue, nonbeaks = orange) and (B) habitat state (reef = red, nonreef = grey). Pie charts represent the proportion of time spent in each state.

exhibiting beaked structures are estimated to be approximately 50 Ma. However, phylogenetic analyses reveal long stems for these groups extending back over 80 Ma, suggesting that the evolutionary origins of beak development in this group may precede the current fossil record (Supplementary Figure S7). Furthermore, the evolutionary origins of the beak precede Tetraodontiformes invasions into coral reef habitats by approximately 15 Ma (Supplementary Figures S6 and S7).

### Evolutionary modularity and integration

To assess if patterns of skull modularity and integration differ between beaked and nonbeaked tetraodontiform fishes, we compared multiple a priori hypotheses of modularity (Supplementary Figure S3) based on previous studies (Evans et al., 2019a; Helfman et al., 2009; Kague et al., 2012; Westneat, 2005) using the phylogenetically informed analyses phyloEMMLi (Goswami & Finarelli, 2016) and a CR analysis (Adams, 2016). Our analyses find strong model support for an eleven-module hypothesis of modularity where each individual skull bone is a separate module (Supplementary Table S4). Both beaked and nonbeaked species display similar levels of skull modularity (CR beaked = 0.813; CR nonbeaked = 0.865;  $p = 0.66$ ) and skull integration (PLS beaked = 0.768; PLS nonbeaked = 0.727;  $p = 0.87$ ).

Despite no significant difference in the degree of skull modularity and integration between beaked and nonbeaked species, we do observe a substantial difference in the overall net rates of morphological evolution in certain skull bones between groups. Notably, beaked species demonstrate higher overall rates of morphological evolution than their nonbeaked relatives (Figure 4). Among beaked species, the highest evolutionary rates are found in the premaxilla, maxilla, frontal, and dentary bones. Nonbeaked species, however, exhibit the highest evolutionary rates in the frontal and maxilla. For bones comprising the jaw (e.g., premaxilla, maxilla, dentary), beaked species exhibit evolutionary rates that are approximately two times faster than nonbeaked taxa (Figure 4).

### Discussion

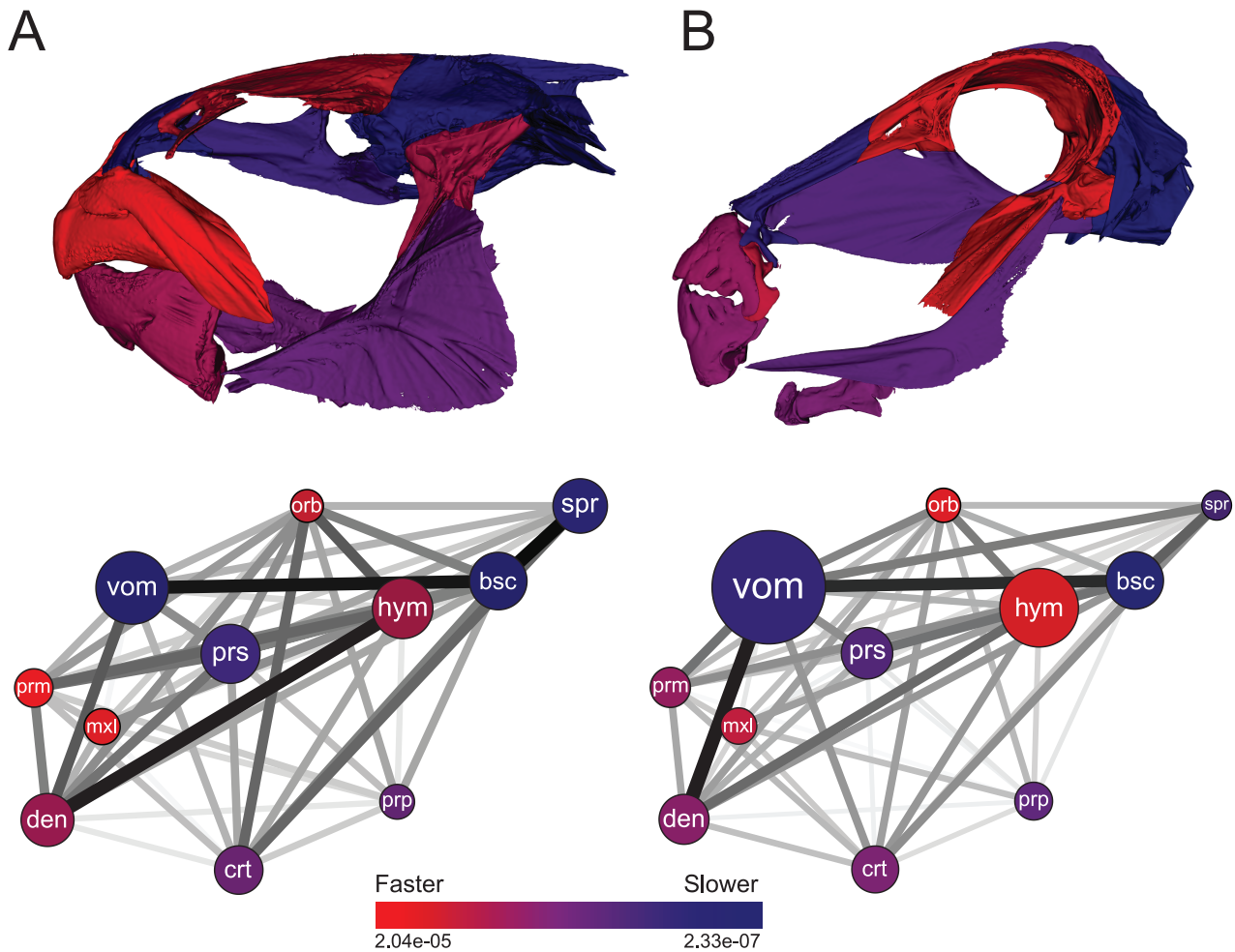
In this study, we used a three-dimensional geometric morphometric dataset of both extant and fossil species to investigate patterns of morphological disparity and rates of morphological evolution as they relate to innovation. Using tetraodontiform

fishes as a model system, we focus on the beak dentition present in several families. We observe similar patterns of modularity and integration among beaked and nonbeaked taxa. In fact, levels of modularity and integration are conserved, or unchanged, across the entire Tetraodontiformes clade. Despite this conservation, skulls of beaked tetraodontiforms evolve twice as fast and show higher levels of morphological disparity when compared to nonbeaked tetraodontiforms, especially in bones contributing to the jaws. Furthermore, contrary to findings from previous studies, we find that coral reef association does not promote skull evolutionary rates or morphological disparity. Instead, we suggest the evolutionary innovation of the tetraodontiform beak is a more important driver of their morphological diversification and increased evolutionary rates, making it a critical component of their evolutionary success.

The role of integration in morphological diversification and evolutionary innovation is currently debated. Initially, integration was thought to constrain phenotypic diversification, while modularity was viewed as a prerequisite to facilitate innovation, with the ability to increase morphological diversification (Goswami et al., 2014; Marroig et al., 2009; Yang, 2001). Recently, integration has been suggested to aid in the evolution of innovation by promoting evolution along specific trajectories and facilitating rapid diversification within a constrained region of trait space (Evans et al., 2021; Goswami et al., 2014; Navalón et al., 2020). In this way, integration can promote large responses to selective pressures by driving the evolution of maximally disparate phenotypes. However, our results show that despite beaked tetraodontiforms displaying rapid rates of morphological diversification, there are no significant differences in patterns of modularity and integration between beaked and nonbeaked species. This suggests covariation is unchanged and highly conserved throughout the entire clade.

It may seem counterintuitive that both integration and modularity can be conserved simultaneously; however, these concepts are hierarchically related. Modules are units that are tightly integrated internally yet relatively autonomous from other modules (Klingenberg, 2008; Zelditch & Goswami, 2021). Conserved patterns of skull integration and modularity are seen in other major vertebrate clades, including mammals, caecilians, and squamates (Marshall et al., 2019; Porto et al., 2009; Watanabe et al., 2019). Furthermore, strong trait integration may play a smaller role in the evolution of





**Figure 4.** Rates of bone module evolution in tetraodontiform skulls. Representative skulls from a (A) beaked (*Marilyna darwini*) and a (B) nonbeaked (*Balistes capriscus*) tetraodontiform depicting bone modules colored by rate of shape evolution under the best-fit modularity hypothesis. Network plots show the magnitude of integration between each bone module for both groups. Larger module circles indicate higher modularity, while darker lines between modules indicate higher integration. Bone modules are colored by rate of evolution. Bone abbreviations are: prm (premaxilla), den (dentary), mxl (maxilla), vom (vomer), prs (parasphenoid), crt (ceratohyal), prp (preopercle), hym (hyomandibula), orb (orbit/frontal), bsc (basioccipital), spr (supraoccipital).

innovation. For example, whereas Evans et al. (2021) suggested strong integration can play a major role in the evolution of innovation by coordinating responses to selective pressures across multiple integrated traits, our results suggest the opposite. We propose that rapid evolution of maximally disparate phenotypes, such as the tetraodontiform beak, can still arise while maintaining a conserved pattern and magnitude of trait integration across an entire clade.

Coral reefs have been previously linked to increases in rates of morphological evolution in fishes and the origin of various innovations (e.g., the teleost intramandibular jaw joint, pharyngeal jaws and beaks in parrotfishes, and long, flexible teeth associated with specialized detritivores) (Bellwood et al., 2014; Evans et al., 2023a; Gibb et al., 2015; Price et al., 2010, 2011, 2013). However, our results suggest a contrasting, more nuanced pattern. We observe that the tetraodontiform beak evolved ~15 Ma prior to their colonization of coral reefs. Additionally, nonreef-associated tetraodontiforms display elevated rates of skull shape evolution compared to reef-associated species.

Overall, our findings suggest reef association by itself may not be enough to promote large changes in morphological

diversification and evolution of divergent structures such as the beak. Recent studies lend support to these findings. Evans et al. (2019b) examined pharyngeal jaw morphology across reef and nonreef-associated wrasses but found no difference in rates of morphological evolution between groups. However, higher rates were found in specialized reef-associated clades, such as cleaner wrasses and parrotfishes (Evans et al., 2019b). We observe a similar result in Tetraodontiformes, where the branch leading to species in the genus *Canthigaster* displays a rapid increase in the rates of skull shape evolution (Figure 2). These are small, reef-associated pufferfishes that have evolved a pointed snout, perhaps allowing them to easily maneuver into tiny crevices and feed on small benthic organisms like sponges, corals, and invertebrates (Allen & Randall, 1977; Santini et al., 2013a). Overall, our findings corroborate those of Evans et al. (2019b) but in another clade of reef-dwelling fishes. The traditional notion that reefs are promoters of morphological diversity and innovation (Alfaro et al., 2007; Cowman & Bellwood, 2011; Kiessling et al., 2010; Price et al., 2011) may be more nuanced than previously thought. Instead, other factors, such as specialized trophic ecologies found on reefs may play a more important role in governing morphological adaptation.

Regardless of habitat, the beak in certain tetraodontiform families is likely an important factor in their high rates of morphological diversification. We observe that rates of skull shape evolution are almost twice as fast in beaked species compared to nonbeaked species, despite species having spent less proportional time in a beaked state compared to a nonbeaked state. This highlights the role that beaks may play in facilitating rapid evolution. Beak-like structures have evolved independently several times in ray-finned fishes. Notably, the beak-like teeth of parrotfishes (Labriformes) are estimated to have appeared around 32 Ma during the Oligocene (Evans et al., 2023a). In contrast, our ancestral trait reconstruction indicates that beaks evolved in Tetraodontiformes approximately 100 Ma (Supplementary Figure S7), making them one of the earliest and longest-established examples of this morphological innovation among fishes. The oldest unequivocal fossil evidence of the tetraodontiform beak comes from the holotype specimen of †*Balkaria histiopterygia* that dates to 55.8 Ma during the Eocene period. In contrast, some of the earliest parrotfish fossils date to the middle Miocene (15.9–11.6 Ma) (Bellwood & Schultz, 1988; Streebman et al., 2002).

Evolutionary innovations can be defined as traits that allow a lineage to exploit its environment in a novel way and enable access to previously unavailable resources (Miller, 1949; Miller et al., 2023). Some recent definitions have also included lineage diversification as a requirement for key innovations (Heard & Hauser, 1995; Hunter, 1998). However, it has been argued that these definitions are problematic in the sense that they conflate two separate phenomena (diversification and ecological shifts) (Miller et al., 2023). Furthermore, while certain innovations have resulted in increased rates of diversification, many do not (Wainwright & Price, 2016). For instance, several feeding innovations in wrasses (e.g., the split lower lip of cleaner wrasses) have led to reduced diversification but increased ecological specialization, potentially by reducing the size of available trophic niches (Wainwright & Price, 2016).

This pattern of specialization without diversification may explain why we observe a variable species richness among beaked tetraodontiforms. Despite sharing a similar functional innovation, the family Tetraodontidae contains over 200 species, while the deep-sea family Triodontidae represents a monotypic lineage. Perhaps there is an unknown element to the deep-sea trophic niche of Triodontidae that is responsible for this disparity in species richness. In addition to a beak, other tetraodontiform clades possess unique morphologies, such as body inflation (Bemis et al., 2023; Wainwright & Turingan, 1997), which may contribute to their diversification, and future studies should examine these aspects of their anatomy.

In this study, our ancestral character reconstruction infers beaks arose twice in the tetraodontiform phylogeny. These findings mirror those of another clade, where wrasses were observed to have three independent origins of a beak (Evans et al., 2023a). Evans et al. observed distinct differences in the beak shapes of these three clades and hypothesized this may be due to differing trophic ecologies (Evans et al., 2023a). While our study did not explicitly test this hypothesis, the two evolutionary origins and subsequent diversification of the tetraodontiform beak may have evolved similarly. For example, beaks of diodontids (e.g., *Chilomycterus reticulatus*) are comparatively thicker and wider than other beaked tetraodontiforms; beaks and snouts of *Canthigaster* spp. are

smaller and more narrow, and the skulls of triodontids (e.g., *Triodon macropterus*) are much more similar in shape to those of nonbeaked tetraodontiforms (Figure 1).

This partitioning of beaks into different areas of the morphospace may be paralleled in trophic niches as well. Although diet data is sparse, diodontids have been recorded using their powerful jaws to crush hard-shelled gastropods, mollusks, and crustaceans (Turingan, 1994; Vermeij & Zipser, 2015). By contrast, the *Canthigaster* diet tends to be more herbivorous with a smaller percentage of crabs and gastropods being ingested (Randall, 1967). Extremely little is known of the diet of the deep-sea *Triodon macropterus*; however, they have been observed eating crabs, fishes, squids, cuttlefishes, and shrimps in captivity (Bemis et al., 2023). Despite this study finding two transitions to a beak in tetraodontiforms, its evolutionary origins are still debated. Some studies have placed all beaked families into a clade (Santini & Tyler, 2003; Arcila & Tyler, 2017; Winterbottom, 1974), while others infer Triodontidae as separate (Arcila et al., 2015; Ghezelayagh et al., 2022; Santini et al., 2013b; Troyer et al., 2022). With difficulties of placing Triodontidae into the tetraodontiform phylogeny, this raises the question of whether the tetraodontiform beak has a single evolutionary origin or represents convergent or parallel morphologies arising multiple independent times. Future studies that fully resolve the placement of this clade will add clarity to the evolutionary origins of the beak.

Altogether, despite observing a conserved and unchanged pattern and magnitude of trait integration, we still see increased morphological rates in the jaws of beaked tetraodontiforms. These results suggest that the beak is an important innovation promoting their overall morphological diversification and rapid evolutionary rates. Additionally, rules that were previously thought to be broadly applicable, such as reef associations driving morphological diversity, are perhaps more nuanced. Instead, other factors, such as trophic specialization, may better explain this phenomenon.

## Supplementary material

Supplementary material is available online at *Evolution*.

## Data availability

Supporting figures and tables are found in the [Supplementary Information file](#). All scripts and data produced for this study can be found on the Dryad repository (<https://doi.org/10.5061/dryad.4f4qrfjix>).

## Author contributions

E.M.T and D.A. designed research; E.M.T. performed research and analyzed data; E.M.T., K.M.E., C.G., M.F., B.N., M.K., K.E.B. collected CT scan data; G.C. assisted with fossil habitat categorizations; and E.M.T. and D.A. wrote the paper with contributions from all authors.

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