Elevated Gene Copy Number Does Not Always Explain Elevated Amylase Activities in Fishes

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ABSTRACT

Amylase activity variation in the guts of several model organisms appears to be explained by amylase gene copy number variation. We tested the hypothesis that amylase gene copy number is always elevated in animals with high amylolytic activity. We therefore sequenced the amylase genes and examined amylase gene copy number in prickleback fishes (family Stichaeidae) with different diets including two species of convergently evolved herbivores with the elevated amylase activity phenotype. We found elevated amylase gene copy number (six haploid copies) with sequence variation among copies in one herbivore (*Cebidichthys violaceus*) and modest gene copy number (two to three haploid copies) with little sequence variation in the remaining taxa, which included herbivores, omnivores, and a carnivore. Few functional differences in amylase biochemistry were observed, and previous investigations showed similar digestibility among the convergently evolved herbivores with differing amylase genetics. Hence, the phenotype of elevated amylase activity can be achieved by different mechanisms (i.e., elevated expression of fewer genes, increased gene copy number, or expression of more efficient amylase proteins) with similar results. Phylogenetic and comparative genomic analyses of available fish amylase genes show mostly lineage-specific duplication events leading to gene copy number variation, although a whole-genome duplication event or chromosomal translocation may have produced multiple amylase copies in the Ostariophysi, again showing multiple routes to the same result.

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Introduction

Digestive enzymes provide the chemical mechanism by which animals digest different components of their food, and, thus, the activity levels of digestive enzymes are commonly used as a metric of an animal's digestive ability (Krogdahl et al. 2005; Karasov and Douglas 2013). Among the suite of digestive enzymes synthesized by vertebrates, the carbohydrase α-amylase frequently shows correlations between dietary substrate concentration and activity level, even when analyzed in a phylogenetic context in fishes (Horn et al. 2006; German et al. 2010), birds (Kohl et al. 2011), primates (Perry et al. 2007), and canids (Axelsson et al. 2013). α-amylase ($\alpha(1 \rightarrow 4)$-D-glucan glucanohydrolase, EC 3.2.1.1) catalyzes the endohydrolysis of $\alpha(1 \rightarrow 4)$-glucosidic linkages in starch and similar molecules, producing oligo- and disaccharides, which are subsequently hydrolyzed to monomeric glucose by intestinal disaccharidases (Ferey-Roux et al. 1998; Krogdahl et al. 2005). In fishes, herbivores and omnivores have the highest activity levels of amylase in their digestive tracts, and carnivores have the lowest, showing the importance of soluble polysaccharides in the nutrition of herbivores and omnivores (Zemke-White and Clements 1999; Horn et al. 2006; German et al. 2004, 2010; also see fig. 1). Moreover, herbivorous and omnivorous prickleback fishes (family Stichaeidae) have been shown to have elevated amylase gene expression and enzyme activities even when fed diets practically devoid of starch, thus suggesting that amylase genes are expressed constitutively in some species (German et al. 2004; Kim et al. 2014).

Elevated amylase activity may in fact represent a digestive specialization in herbivores and omnivores, allowing for efficient starch digestion (German et al. 2004, 2010, 2015; Skea et al. 2005, 2007; Karasov and Douglas 2013), yet we do not know the molecular underpinnings of these abilities. Elevated gene copy number appears to explain increased amylase activities in *Drosophila* (Shibata and Yamazaki 1995; Inomata et al. 1997; Inomata and Yamazaki 2000), salivary amylase in humans (Perry et al. 2007), and pancreatic amylase in mice (Sugino 2007) and dogs (Axelsson et al. 2013). But do these limited examples (an insect and three mammals) represent the only mechanism by which elevated amylase activities are achieved in animals and in fishes in particular? With the examples above in mind, we formulated three questions regarding amylase genetics and evolution and how these relate to digestion in fishes: (1) Are differences

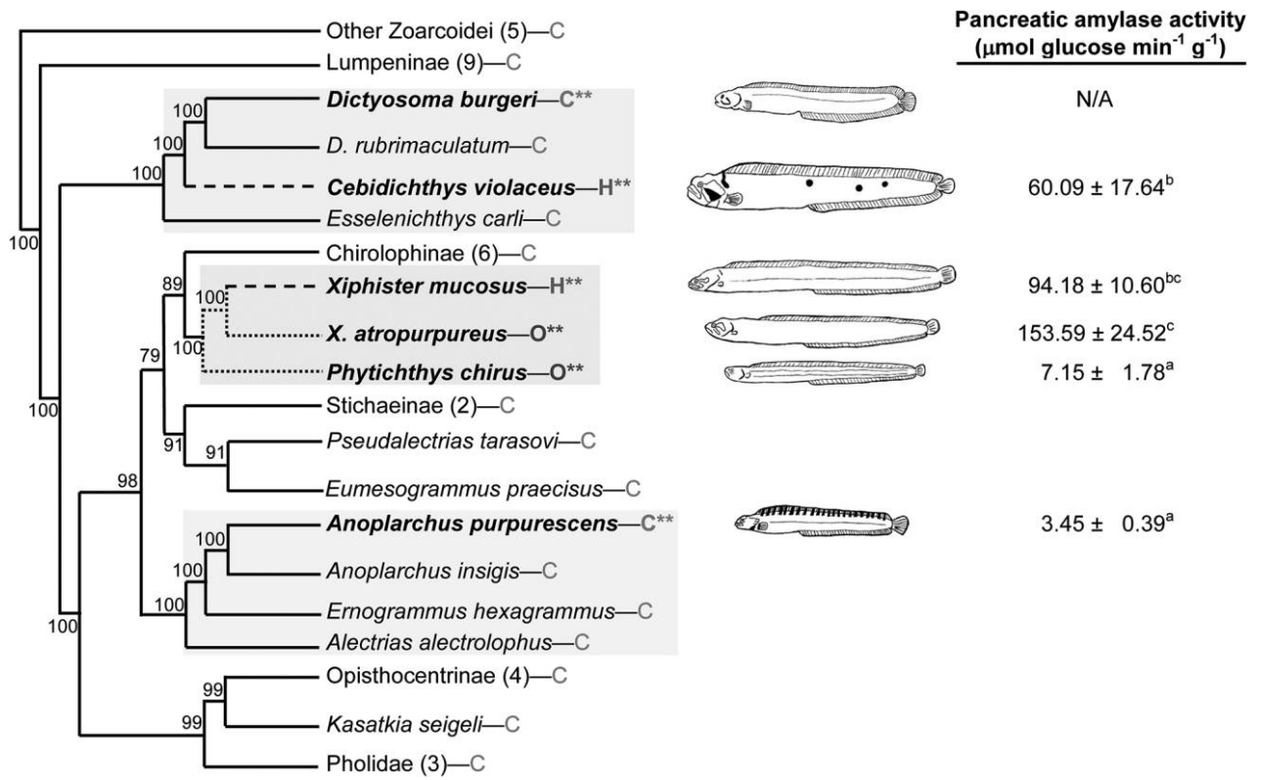

Figure 1. Phylogenetic relationships of the polyphyletic family Stichaeidae based on 2,100 bp of *cytb*, 16*s*, and *tomo4c4* genes (Kim et al. 2014). Bayesian posterior probabilities are indicated on nodes. Species used in this study are shown in bold and indicated with two asterisks. H = herbivory, O = omnivory, C = carnivory. Evolution of herbivory (dashed lines) and omnivory (dotted lines) is shown. Numbers in parentheses show number of taxa evaluated at that branch. Boxes highlight alleged families or subfamilies within the polyphyletic family Stichaeidae, with Cebidichthyidae (*top*), Xiphisterinae (*middle*), and Alectriinae (*bottom*) all highlighted (Chereshnev et al. 2013; Kim et al. 2014). Xiphisterinae is recognized as Xiphisteridae by Chereshnev et al. (2013). Pancreatic amylase activity values are mean ± SEM. Amylase activities were compared among taxa with ANOVA, followed by Tukey's HSD (ANOVA, $F_{4,32} = 23.50$, $P < 0.001$). Values not sharing a superscript letter are significantly different. A color version of this figure is available online.

in amylase activities among taxa always underpinned by differences in amylase gene copy number? (2) What are the functional consequences of increases in amylase gene copy number leading to increased expression of amylase genes and thus elevated amylase activity? (3) Across fishes, have changes in amylase gene copy number happened via lineage-specific duplication events, as appears to be common in other vertebrates (Perry et al. 2007; Axelsson et al. 2013), or by another mechanism, such as whole-genome duplication (WGD; Ohno 1970; Christoffels et al. 2004; Kasahara 2007; Glasauer and Neuhauss 2014) or chromosomal translocation (Fraser et al. 2005)?

Answering the above questions is crucial in the process of understanding whether this widespread phenomenon—a match between amylase activity and diet—is truly adaptive and always arises by the same mechanism (i.e., changes in gene copy number). We therefore examined amylase genetics and potential functional consequences in prickleback fishes with different diets and evolutionary histories (fig. 1). Prickleback fishes have been studied in the context of niche specialization spanning several layers of biological organization, including the isotopic (Saba 2004), molecular (Kim et al. 2014), biochemical (Chan et al. 2004; German et al. 2004, 2014, 2015), whole-animal (Horn et al. 1986), and ecological (Horn 1983; Horn et al. 1983) levels. Hence, with dietary diversity, ontogenetic dietary shifts, convergent evolution of herbivory, and sister taxa with different diets, the pricklebacks are a highly appropriate study system for understanding dietary specialization and the mechanisms underlying pancreatic amylase activity variation in particular. We focused on members of two parts of the stichaeid phylogeny: the Cebidichthyidae (Chereshnev et al. 2013; Kim et al. 2014), which features the evolution of herbivory in *Cebidichthys violaceus*, and the Xiphisterinae (Chereshnev et al. 2013; Kim et al. 2014), which features omnivory in two species (*Phytichthys chirus* and *Xiphister atropurpureus*), and the evolution of herbivory in *Xiphister mucosus*. For comparison, we included two carnivorous stichaeids, *Dictyosoma burgeri* and *Anoplarchus purpurescens*, as carnivory is the basal dietary condition of the family (fig. 1). Our study had five parts. First, we sequenced the amylase genes and examined sequence diversity of these genes in six stichaeid species (fig. 1). Second, we measured gene copy number in five of the species and expression of amylase genes in the two herbivores, *C. violaceus* and

X. mucosus. Because expression is impacted by regulatory elements, often in the 5′ flanking region of a gene (Ma et al. 2004; Wiebe et al. 2007), we sequenced the 5′ flanking regions (including their putative promoters) of *C. violaceus* and *X. mucosus* to examine these regions and hypothesize differences in how amylase expression is regulated among these two herbivorous taxa. Third, we examined the electrophoretic patterns of amylases in five of the species to confirm the active forms of different amylase gene variants (orthologs and paralogs) in the respective taxa. Fourth, we investigated potential functional outcomes of underlying amylase genetic variation by partially characterizing amylase activities in response to substrate, pH, and temperature in four of the species. To match genome with phenome, we then examined our results in the context of whole-animal digestibility trials from a previous investigation (Horn et al. 1986). Finally, we acquired all available fish amylase gene sequences and constructed an amylase phylogenetic tree in order to visualize the evolution of amylase gene copy number in various fish lineages. To aid in the determination of whether amylase gene copies arose via WGD events or lineage-specific duplication, we generated synteny maps for amylase genes from fishes in which the genome has been sequenced. We show that gene copy number does not always explain elevated amylase activities or starch digestibility and that most amylase gene copies likely arose through lineage-specific duplication events. Our results show the complexities of digestion and amylase gene evolution, thus illustrating that phenotypes can arise by more than one mechanism.

Material and Methods

Fish Capture, Tissue Extraction, Amylase Activity Assays, and Polyacrylamide Gel Electrophoresis

Twelve individuals each of *Cebidichthys violaceus*, *Xiphister mucosus*, *Xiphister atropurpureus*, and *Anoplarchuhs purpurescens* were collected by hand and dipnet variously between November 2011 and June 2013 at low tide from rocky intertidal habitats on the central California coast near Piedras Blancas (35.65°N, 121.24°W). Fifteen individuals of *Phytichthys chirus* were collected from rocky intertidal habitats at low tide on San Juan Island (Dead Man Bay; 48.50°N, 123.13°W) in July 2012. Three individuals of *Dictyosoma burgeri* were collected at low tide from Banda, Tateyama, Japan (34.98°N, 134.77°E), in August 2012. Each fish was euthanized with an overdose of MS-222 (1 g L^{-1} seawater), measured (standard length ± 0.5 mm), weighed (body mass ± 0.1 g), and dissected on a cutting board kept on ice (4°C) within 4 h of collection. Each digestive system was removed by cutting just anterior to the stomach and at the anus. The guts of five fish per species (three for *D. burgeri*) were gently uncoiled, and the pyloric cecal tissues (Gawlicka and Horn 2006; Kim et al. 2014) were excised with sterile instruments that had been treated with RNaseZap. The pyloric cecal tissues were immediately placed in 0.5-mL centrifuge vials containing chilled RNAlater and transported to University of California, Irvine, on ice. The guts of the remaining fishes were prepared for amylase activity measurement in the pyloric cecal tissue following German et al. (2015). Because gut fullness can affect digestive enzyme expression (Karasov and Douglas 2013), we confirmed that each individual had gut content masses on par with those observed for these species in previous investigations (German et al. 2015), and individuals with empty guts were excluded from analysis. Because of limited access to *D. burgeri*, we were not able to measure amylase activity levels in this species. One hundred milligrams of epaxial muscle was harvested with sterile instruments from each specimen for use in DNA extractions for genome walking (see "Total Genomic DNA Isolation and Sequencing of the *Amy2* 5′ Flanking Region"). The muscle was either snap frozen in liquid nitrogen or placed in 70% ethanol at the time of collection.

For *C. violaceus*, *X. mucosus*, *X. atropurpureus*, and *A. purpurescens*, amylase activity assays were performed at 15°C, pH 7.5, at saturating substrate concentrations (German et al. 2004), using potato starch, corn starch, amylopectin, and pure amylose, which represent various starches found in the red and green algae consumed by herbivorous and omnivorous prickleback fishes (Painter 1983; Zemke-White and Clements 1999). Amylase activity in the pricklebacks was also assayed at temperatures of 4°, 10°, 16°, 22°, 28°, 34°, 40°, 50°, 60°, and 70°C (all at pH 7.5) and pH values ranging from 5–10, in 0.5 pH unit intervals (all at 15°C), in order to assess optimal temperature and pH, respectively, of amylase activities in the target species (potato starch was used as the substrate for these trials). The Michaelis-Menten kinetics of prickleback amylases have been examined previously (German et al. 2004), so these analyses were not repeated here.

To visualize the amylases in the various species, we utilized native polyacrylamide gel electrophoresis (Fernandez et al. 2001; Skea et al. 2005). This technique does not use sodium dodecyl sulfate to denature proteins, and, thus, once the proteins have been run on a gel, amylases can be visualized using an enzyme activity stain. Twenty micrograms of protein from each species was loaded into 8% polyacrylamide continuous gels measuring 8 cm × 8 cm × 0.001 cm. Electrophoresis was performed at constant amperage (0.03 A) for 30–90 min, depending on sample, at 4°C, using a Mini-Protean Tetra Cell electrophoresis system (BioRad, Hercules, CA). After removal from the electrophoresis apparatus, gels were immersed in a 1% starch solution in 25 mM Tris-HCl, 1 mM $CaCl_2$, buffer, pH 7.5, for 40 min at room temperature (~22°C). Thereafter, gels were rinsed in deionized water and stained with Lugol's solution (I_2/KI) until appearance of bands. Activity is revealed as clear bands over a dark background. Gels were photographed for analysis. Isoelectric points (p*I*) of each amylase were estimated from the predicted amino acid sequences, which were inferred from the cDNA sequences of the full amylase genes (without the signal peptide sequence), using Protein Calculator v3.4 (http://protcalc.sourceforge.net/). From these p*I* estimations, conditions of the native PAGE were adjusted and appropriate running and loading buffers used to achieve the desired pH conditions (McClellan 1982). We attempted to run samples from all of the species under common conditions so that we could visualize them all on one gel (e.g., Fernandez et al. 2001) but were unsuccessful and thus optimized conditions for each species' amylases.

In the following subsections, we briefly describe the methods used for the molecular portion of this study. For more detailed methods, see the appendix.

RNA Isolation, Rapid Amplification of cDNA Ends, Reverse Transcription, Cloning, and Sequencing

In vertebrates, there are two main gene classes for amylases: *Amy1*, which is for salivary amylase in mammals, and *Amy2*, which is for pancreatic amylases (Ferey-Roux et al. 1998); this study is focused on the latter, as fishes appear to possess only *Amy2* (Janeček 1997). Total RNA isolation was performed using the RNeasy Plus mini kit (Qiagen, Valencia, CA), following the manufacturer's protocol, with the addition of an on-column DNase treatment with the RNase-Free DNase set (Qiagen) to remove genomic DNA.

We performed 5′ and 3′ rapid amplification of cDNA ends (RACE) with the SMARTer RACE cDNA amplification kit (Clontech Laboratories, Mountain View, CA). Ligation mixtures were used to transform JM109 chemically competent cells (Promega, Madison, WI) according to the manufacturer's instructions (technical bulletin TB095). Transformants were plated on Luria broth agar supplemented with ampicillin. For RACE and full-length analysis, plasmid DNA was purified from positive clones and sequenced in both directions (Eton Bioscience, San Diego, CA). See table S1 (tables S1–S4 available online) for all primers used in this study. All accession numbers for genes sequenced or used in this study are found in table S2.

Gene Copy Number Determination, Real-Time Quantitative Polymerase Chain Reaction, and Sequence Analysis

Real-time polymerase chain reaction (PCR) was performed in an iCycler iQ multicolor real-time detection system (BioRad) using the BioRad iTaq Universal SYBR Green supermix as per the manufacturer's protocol. To evaluate PCR efficiency, 10-fold dilution series were created with known concentrations of linearized TOPO TA pCR 2.1 vector carrying the *Amy2A* cDNA of each prickleback species or the ribosomal protein L8 cDNA fragment, generating a set of standards containing 3×10^5 to 3×10^1 copies of template per PCR reaction. Standard curves were generated by the iCycler iQ software by plotting the log of the DNA copy number against respective threshold cycles (C_T). All standard curves showed high correlation coefficients (between 0.998 and 0.999), with PCR efficiency between 94.2% and 102.5%, and were linear over the concentration range with standard and examined DNA.

All PCR runs were completed with a melting curve analysis to confirm the specificity of amplification and lack of primer dimers. For absolute quantification, the C_T values of the unknown samples were compared to a standard curve by interpolation in an automated process performed by the iCycler iQ software. The results of the analysis are presented as haploid copy number per cell. Relative quantification was performed according to the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). PAML 4.8a (Yang 1997) was used to examine amino acid sites under selection among the prickleback amylases.

Total Genomic DNA Isolation and Sequencing of the Amy2 *5′ Flanking Region*

Total genomic DNA was isolated from muscle by using the DNeasy kit (Qiagen) according to the manufacturer's instructions, with the exception that a DNase-free RNase A digestion step was added. To isolate the upstream sequence of the pancreatic amylase gene in *C. violaceus* and *X. mucosus*, we used the Universal GenomeWalker 2.0 kit (Clontech Laboratories).

Protein Modeling and Phylogenetic Analysis

Because of variation among α-amylase 2A and α-amylase 2B paralogs in *C. violaceus*, we used protein homology–based modeling to assess structural changes among the two paralogs (see fig. S1; figs. S1–S8 available online). To examine the similarity of the prickleback amylase sequences to other known fish sequences, the complete coding sequence of the *X. atropurpureus Amy* gene was used for a TBLASTX (NCBI blast, ver. 2.2.26) search of fish cDNA genomic libraries in Ensembl (Ensemble 80; Hubbard et al. 2005). Other databases were searched for *Ctenopharyngodon idella*, *Ictalurus punctatus*, and *Poecilia reticulata*, and a BLASTN was conducted on NCBI nr database to identify homologous sequences (see the appendix). Phylogenetic relationships were generated using a mixed-model, partitioned Bayesian method as implemented in the software package Mr. Bayes, version 3.1.2 (Huelsenbeck and Ronquist 2001). The genomic location of each *Amy* gene (along with the two to four closest flanking genes) was used to generate a synteny map for the *Amy* loci in various fish taxa (Castro et al. 2014). PAML 4.8a (Yang 1997) was used to examine amino acid sites under selection in specific lineages.

Statistics

All molecular data were analyzed as described above. Pancreatic amylase activities were compared among five stichaeid species with one-way ANOVA. Amylase activity data comparing enzymatic responses to substrate, temperature, or pH in *C. violaceus* and *X. mucosus* (or in *X. atropurpureus* and *A. purpurescens*) were analyzed with two-way ANOVA. Each of the ANOVAs was followed by Tukey's HSD multiple comparisons test. All ANOVA statistics were run in SPSS statistical software (ver. 20).

Results

Amylase Gene and Amino Acid Sequences

The amylase nucleotide sequences are similar in the six stichaeid species, each being 1,536 bp in length, with 95.7%–99.3% similarity among them (see fig. S2). We identified two separate sequence variants (paralogs) for *Cebidichthys violaceus* that we

called *Amy2A* and *Amy2B* (which share 99.3% nucleotide sequence identity and 11 nucleotide polymorphisms; see fig. S3), whereas all other species had only one *Amy2* gene sequence each; no variation was observed in numerous clones produced for each individual fish specimen for each species (totaling >60 clones per species). The other stichaeid taxa's *Amy2* gene sequences were most similar to *Amy2A* in *C. violaceus*, and, thus, based on our results, the other species essentially have only the *Amy2A* gene. The alignments of the amylase amino acid sequences showed variation among the different species and in the paralogs in *C. violaceus* (see fig. S4). All six species have amylase proteins that are 512 amino acids in length with conserved cysteine, Ca^{2+} binding, substrate binding, and active site residues. The amino acid sequences share 93.4%–98.0% sequence identity among the taxa. There are 10 nonsynonymous amino acid substitutions between α-amylase 2A and α-amylase 2B in *C. violaceus*, but according to screening for nonacceptable polymorphisms analysis (Bromberg and Rost 2007), only one change (E397G) is predicted to be nonneutral (see table S3). Although approximately 90% of all sequences for *C. violaceus* are clearly α-amylase 2A or α-amylase 2B, 10% of sequences show variation between these two paralogs, some of which suggest there has been crossing over among the genes following duplication (see fig. S3). The PAML analysis did not detect any amino acid sites under selection within the Stichaeidae (table S4).

Amylase Gene Copy Number, Expression, and the 5′ Flanking Region

Gene copy number analysis shows clear variation among five of the stichaeid species that were tested, with haploid copy number ranging from one to three in *Phytichthys chirus*, *Xiphister mucosus*, *Xiphister atropurpureus*, and *Anoplarchus purpurescens* to six in *C. violaceus* (table 1). For *C. violaceus*, primers targeting *Amy2A* or *Amy2B* individually, or both collectively, were used. The individual copy number estimates for *Amy2A* plus *Amy2B* sum to approximately five copies (3.36 for *Amy2A*, 2.15 for *Amy2B*), whereas the primer targeting both paralogs suggests that there are six copies in the haploid *C. violaceus* genome. In terms of expression, *C. violaceus* expresses both *Amy2A* and *Amy2B* at roughly equal ($P = 0.14$) levels, whereas *X. mucosus* expresses only a single *Amy2* (table 2). Because these herbivorous fish species were not raised under common-garden conditions, interspecific comparisons of expression were not made (Kim et al. 2014).

To better understand the evolution of *Amy2A* and *Amy2B*, we examined the 5′ flanking region of the *Amy2* genes in *C. violaceus* and *X. mucosus*. Consistent with the number of amylase paralogs observed in the two species, two flanking regions were recovered for *C. violaceus* but only one for *X. mucosus* (see fig. S5). Each of the 5′ flanking regions is associated with *Amy2A* and *Amy2B* in *C. violaceus* (see "Genomic DNA Isolation and 5′ Flanking Sequencing" in the appendix), suggesting that an apparent duplication and subsequent diversification of these 5′ flanking regions occurred before a duplication event generating *Amy2B* in *C. violaceus*. The two 5′ flanking regions in *C. violaceus* have only three nucleotide differences among them in the first 167 bp upstream from the start codon for the amylase gene (with two in the putative promoter), but there are nine nucleotide differences between the *C. violaceus* 5′ flanking region and that of *X. mucosus* in that same segment (fig. S5). After 167 bp upstream, *C. violaceus* and *X. mucosus* diverge, and there are few sequence similarities among them, including numerous indels.

Polyacrylamide Gel Electrophoresis

The amylase of *A. purpurescens* (α-amylase 2 p*I* 7.05) was run with porcine α-amylase at pH 7.0, and a single band was visible for *A. purpurescens*, while three bands were visible for porcine α-amylase 2 (fig. 2*A*). Three bands are expected for porcine α-amylase 2 (Strumeyer et al. 1988). *Cebidichthys violaceus* (α-amylase 2A p*I* 7.86), *P. chirus* (α-amylase 2 p*I* 7.59), *X. mucosus* (α-amylase 2 p*I* 7.73), and *X. atropurpureus* (α-amylase 2 p*I* 7.32) were run under common conditions, with a single band visible for each of them (fig. 2*B*). Because the molecular weights of the amylase proteins varied by <200 Da, the proteins migrated according to their predicted p*I*, suggesting that the estimated p*I* values are likely reflective of the actual p*I*. α-amylase 2B from *C. violaceus* was not visible under the conditions suitable for

Table 1: Amylase gene copy number determined with real-time quantitative polymerase chain reaction

Species	Haploid copy no.	*Amy2A* copy no.	*Amy2B* copy no.
Cebidichthys violaceus (H)	6.07 ± .95	3.36 ± .61	2.15 ± .14
Phytichthys chirus (O)	1.47 ± .14	NA	NA
Xiphister mucosus (H)	1.91 ± .27	NA	NA
Xiphister atropurpureus (O)	3.34	NA	NA
Anoplarchus purpurescens (C)	2.65	NA	NA

Note. $N = 2$ individual specimens for *C. violaceus*; $N = 1$ individual specimen for all other species. Values are mean ± SEM of three separate real-time quantitative polymerase chain reaction experiments on genomic DNA from the same samples (except *X. atropurpureus* and *A. purpurescens*, for which only two experiments were performed and hence no SEM is reported). For *C. violaceus*, a general amylase primer was used to obtain the overall haploid copy number, and *Amy2A*- or *Amy2B*-specific primers were used to obtain copy numbers for those specific variants. H = herbivore, O = omnivore, C = carnivore. NA = not applicable.

Table 2: Expression of amylase genes *Amy2A* and *Amy2B* relative to ribosomal protein L8 in the herbivorous fish species *Cebidichthys violaceus* and *Xiphister mucosus*

Species	*Amy2A*	*Amy2B*
C. violaceus	15.73 ± 3.23	27.79 ± 5.83
X. mucosus	3.14 ± .70	NA

Note. Interspecific comparisons in expression were not made because of too many confounding variables in sampling conditions. *Amy2B* is not expressed in *X. mucosus*. The expressions of *Amy2A* and *Amy2B* are not different in *C. violaceus* ($t = 1.81$, df = 4, $P = 0.14$). Values are mean ± SEM. $N = 3$ per species. NA = not applicable.

α-amylase 2A. Thus, to visualize α-amylase 2B in *C. violaceus* (p*I* 8.62), conditions were optimized for this paralog, whereas no other fish, or porcine α-amylase, were visible under the α-amylase 2B–specific conditions (fig. 2*C*). Thus, both α-amylase 2A and α-amylase 2B were detectable in *C. violaceus*, but, consistent with the molecular data, only a single α-amylase 2 was visible in the other species (fig. 2).

Amylase Activities in Response to Starch Type, Temperature, and pH

Amylase activity in *C. violaceus* and *X. mucosus* varied similarly ($P < 0.001$) in response to starch type, with both species showing greater activity against potato starch than other starches ($P < 0.001$), moderate activities against corn starch and amylopectin that did not vary among the two substrates ($P = 0.737$), and significantly lower activity against amylose ($P < 0.001$; fig. 3*A*). *Xiphister mucosus* had higher amylase activities than *C. violaceus* against potato starch ($P = 0.004$), corn starch ($P = 0.003$), and amylopectin ($P = 0.004$) but not for amylose ($P = 0.567$). *Xiphister mucosus* and *C. violaceus* amylase activities showed strong responses to temperature ($P < 0.001$), but the interaction of temperature × species was not significant ($P = 0.885$), showing that the amylases of these two species had similar temperature profiles (fig. 3*B*). The amylase activity of *X. mucosus* peaked at 50°C, whereas that of *C. violaceus* peaked at 40°C. Generally, *X. mucosus* showed higher amylase activities than *C. violaceus* at all temperatures, although the activities at any temperature were not statistically different (*P* values ranged from 0.031 to 0.645, with a significance of 0.005 cut off following a Bonerroni correction). *Xiphister mucosus* and *C. violaceus* amylase activities showed strong responses to pH ($P < 0.001$), but unlike temperature, the interaction of pH × species was significant ($P = 0.015$), showing that the amylases of these two species had different pH profiles (fig. 3*C*). *Xiphister mucosus* amylase activities peak at pH values of 8.0, whereas *C. violaceus* amylase activities peak at pH values of 7.5 (fig. 3*C*). *Xiphister atropurpureus* and *A. purpurescens* also show better abilities to hydrolyze starches other than amylose and amylase activities that peak at pH values of approximately 7.5–8.0, but temperature profiles were not evaluated in these species (see fig. S6).

Amylase Amino Acid Alignments, Phylogenetic Analysis, and Synteny Maps

The amylase amino acid alignments among all of the prickblebacks from this study and the 34 additional fish species show highly conserved cysteine, Ca^{2+} binding, substrate binding, and active site amino acid residues in all of the taxa (see fig. S7). The sequences shared ≥66.1% sequence identity, and these percent identities were greater in more closely related taxa. The phylogenetic hypothesis for amylases in fishes is well supported, with most nodes supported by ≥99% Bayesian posterior probabilities, with the lowest support at 54% (fig. 4). The tree recovers a phylogeny of utilized fish species that strongly resembles that of Near et al. (2013) in terms of larger taxonomic groupings. From the available genomes, it is clear that multiple amylase gene copies have evolved several times in different lineages, including *Danio rerio* (three copies), *Ctenopharyngodon idella* (two), *Xiphophorus maculatus* (two), *Poecilia formosa* (two), *Poecilia reticulata* (three), *Oreochromis niloticus* (three), *Takifugu rubripes* (two), *Tetraodon nigroviridis* (three),

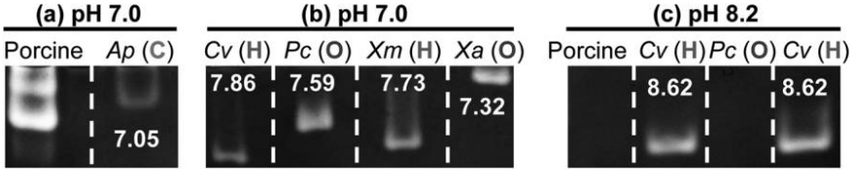

Figure 2. Native polyacrylamide gel electrophoresis of prickleback α-amylases at different pH values. Wells were loaded with pancreatic homogenates from the prickleback fishes. *a*, pH 7.0, α-amylase 2 from *Anoplarchus purpurescens* (*Ap*) and porcine amylase run with regular polarity (anode at bottom). *b*, pH 7.0, α-amylase 2A in *Cebidichthys violaceus* (*Cv*), *Phytichthys chirus* (*Pc*), *Xiphister mucosus* (*Xm*), and *Xiphister atropurpureus* (*Xa*) run with reversed polarity (cathode at the bottom). *Anoplarchus purpurescens* α-amylase 2 does not run into the gel at that pH and polarity. *c*, pH 8.2, α-amylase 2B in *C. violaceus* (*Cv*), *P. chirus* (*Pc*), and porcine amylase run with reversed polarity. No bands are visible for *P. chirus* or porcine amylase, but α-amylase 2B from *C. violaceus* is visible at that pH (replicate bands in *c* are different individual fish). Predicted isoelectric points of the amylases from each species, as inferred from their predicted amino acid sequences, are superimposed directly on their lanes on the gels in white. H = herbivore, O = omnivore, C = carnivore. A color version of this figure is available online.

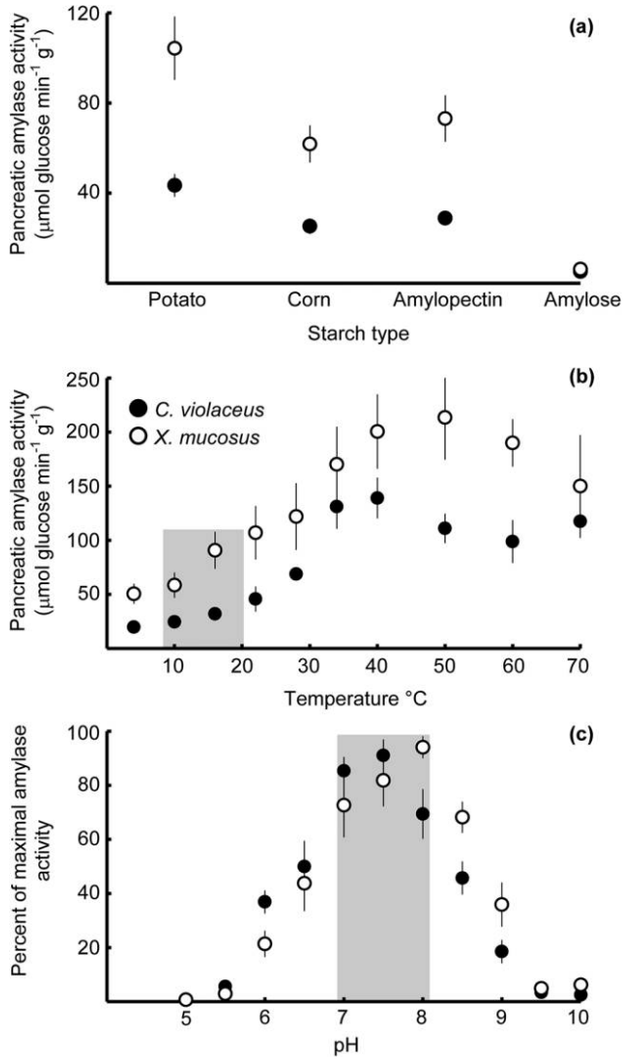

Figure 3. Pancreatic amylase activity in *Cebidichthys violaceus* and *Xiphister mucosus* as a function of starch type (*a*). For starch type, there were significant effects of starch type, species, and the interaction of starch type and species on amylase activity (two-way ANOVA, starch type: $F_{3,56} = 30.40$, $P < 0.001$; species: $F_{1,3} = 48.22$, $P < 0.001$; starch type × species: $F_{3,56} = 6.03$, $P = 0.001$). *b*, Amylase activity as a function of temperature. For temperature, there were significant effects of temperature and species but not interaction of temperature × species on amylase activity (two-way ANOVA, temperature: $F_{9,157} = 9.58$, $P < 0.001$; species: $F_{1,9} = 19.32$, $P < 0.001$; temperature × species: $F_{9,157} = 0.481$, $P = 0.885$). *c*, Percent maximal pancreatic amylase activity as a function of pH. For pH, there were significant effects of pH and the interaction of pH and species but not species alone on percent amylase activity (two-way ANOVA, pH: $F_{10,136} = 56.18$, $P < 0.001$; species: $F_{1,10} = 0.855$, $P = 0.357$; pH × species: $F_{10,136} = 2.34$, $P = 0.015$). Values are mean ± SEM. Shaded areas in *b* and *c* show biologically relevant temperatures and pH values, respectively.

Gasterosteus acculeatus (two), and *C. violaceus* (six, with two verified paralogs). The other pricklebacks have copies (gene copy number ranges from one to three), but there appears to be little variation among the copies.

The synteny maps show that there is one common syntenic region in most fish species and a second syntenic region in *D. rerio*, *C. idella*, and *A. mexicanus*, representing the Ostariophysi (Nelson 2006). Most fish amylases are found in syntenic region 1, flanked by *olfm3b* (noelin-3) and *col11A1b* (collagen type X1) on the 5′ end and *ntng1A* (netrin G1) and *vav3B* (guanine nucleotide exchange factor B) on the 3′ end (fig. 5). Some of the *ntng1A* genes have recently been renamed as *vav3B* in the Ensembl database, and, thus, *ntng1A* and *vav3B* are treated interchangeably in the synteny map. The arrangement of syntentic region 1 is highly conserved among all the taxa included in our analysis (Bouneau et al. 2003). Interestingly, *D. rerio* possesses two *Amy2* copies on a separate chromosome from syntenic region 1 (chromosome 17), separated by more than 14 kb, and these genes are flanked by a completely different set of genes (fig. 5). Thus, we called these different genomic arrangements syntenic region 2. *Ctenopharyngodon idella* and *Astyanax mexicanus* each have single *Amy2* genes that map to syntenic region 2, aligning with *Amy2B* from *D. rerio*; *A. mexicanus* also possesses two *Amy2*-like pseudogenes in syntenic region 1, suggesting that these genes were lost and that this species' only active amylases arise from the gene in syntenic region 2 (fig. 5). Based on the phylogenetic analysis (fig. 4), *Ictalurus punctatus* also possesses only an active *Amy2* gene that is most similar with these *Amy* genes from syntenic region 2, further supporting the point that this separate syntenic region is unique to the Ostariophysi (Nelson 2006). The PAML analysis showed that the amylases within the Cichlidae had numerous amino acid sites under positive selection, but this was not observed in any other lineage (table S4) and likely has to do with the narrow taxon sampling for a large family such as the Cichlidae.

Discussion

Our data support the hypothesis that convergent evolution of elevated amylase activity does not always happen by the same molecular mechanism in animals. The herbivorous *Cebidichthys violaceus* clearly has elevated amylase gene copy number (six haploid copies), whereas *Xiphister mucosus* (herbivore) and *Xiphister atropurpureus* (omnivore), which each have elevated amylase activities in their guts, have fewer amylase gene copies (two and three haploid amylase copies, respectively). Beyond the copy number variation, *C. violaceus* also has sequence variation among amylase paralogs that have arisen in this species, and these paralogs clearly have different isoelectric points and hence perform differently in PAGE. However, what these differences mean for amylase function (e.g., subfunctionalization) in *C. violaceus* requires further study. Interestingly, there are numerous differences in the 5′ flanking regions of *C. violaceus* and *X. mucosus* amylase genes, suggesting potential differences in how the amylase genes in these two species are regulated. Despite the molecular variation, few functional differences (other than activity level) are apparent in the amylase biochemistry in prickleback fishes, especially in the convergently evolved herbivores *C. violaceus* and *X. mucosus*. Therefore, *C. violaceus* and *X. mucosus* (and its sister taxon, *X. atropurpureus*) have taken different

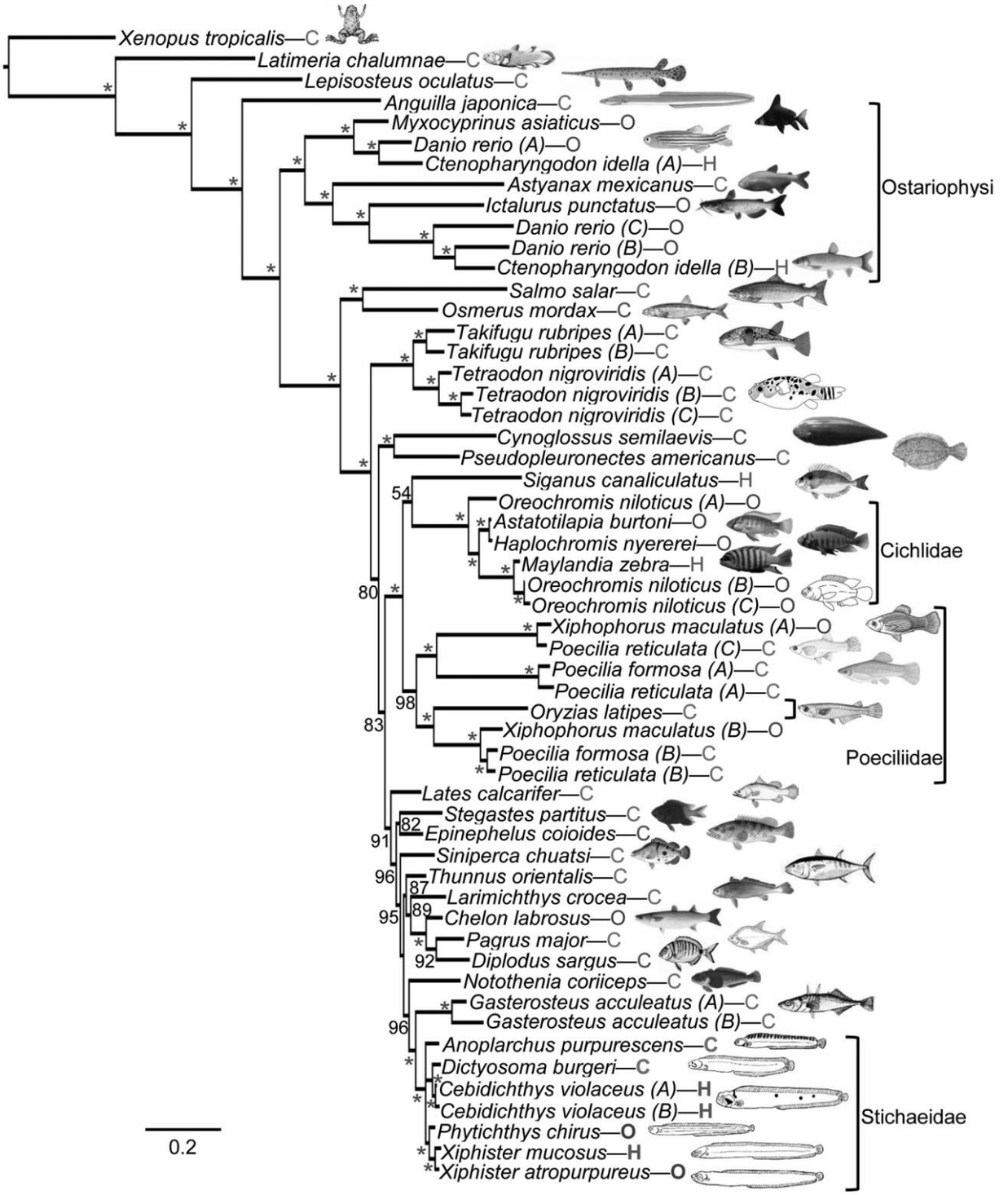

Figure 4. Bayesian phylogenetic hypothesis for amylase genes in fishes. The tree is rooted with the amylase gene of the western clawed frog, *Xenopus tropicalis*. Ten million generations of Markov chain Monte Carlo were performed with trees sampled every 200 generations and 10% of early runs counted as burn-in. The percentage of times that a particular node was recovered is listed as the Bayesian posterior probability: asterisk indicates ≥99%; otherwise, values are listed. Dietary affinities of species are as follows: C = carnivore, O = omnivore, H = herbivore. Lineages with discussed duplication events are bracketed. Note that *Oryzias latipes* is not a member of the family Poeciliidae. A color version of this figure is available online.

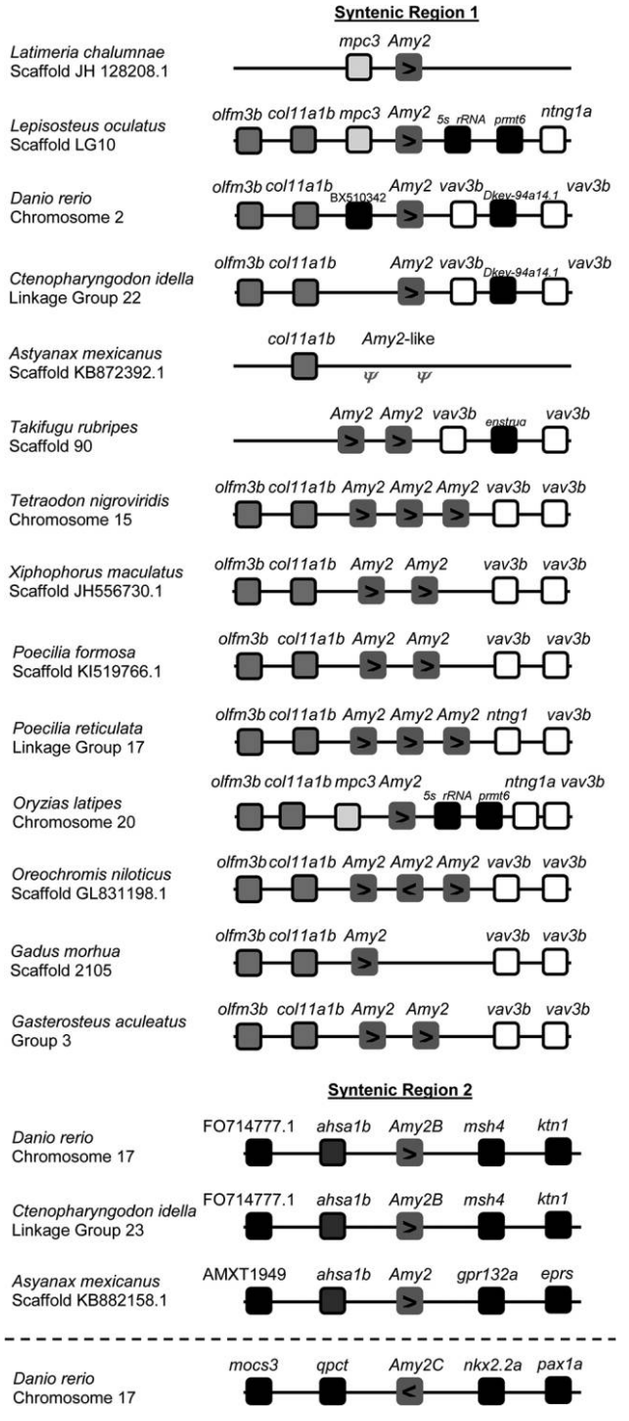

Figure 5. Synteny map for amylase genes in fishes. Common genes indicated by different shades of white and gray, and *Amy2* genes in dark gray, with arrows showing direction of orientation. Uncommon genes are shown in black. Two syntenic regions were identified, with syntenic region 1 representing the common *Amy2* locus. *Danio rerio, Ctenopharyngodon idella*, and *Astyanax mexicanus* have a syntenic *Amy2* locus on a different chromosome than syntenic region 1 (on what is chromosome 17 in *D. rerio*). Hence, syntentic region 2 is shown as a separate map. *Danio rerio* has a second *Amy2* copy (*Amy2C*) on that same chromosome (the bottom row of syntenic region 2) that is separated from the *Amy2B* gene by 14 kb, and thus, it does not actually align with syntenic region 2 (dashed line separates *Amy2C* map from the others). *Astyanax mexicanus* has two pseudogenes (ψ) that are *Amy2*-like in syntenic region 1, suggesting that these genes were lost in this species. A color version of this figure is available online.

routes of molecular evolution to achieve the same overall outcome and meet the need for elevated amylase activities to efficiently digest starch from red and green algae.

Gene Duplications and Significance to Prickleback Fishes

Duplication of amylase genes is common in animals, and these duplication events correlate with increased expression of the amylase protein (Inomata and Yamazaki 2000; Perry et al. 2007; Axelsson et al. 2013). Increased expression of amylases should have functional significance for fruit fly larvae, mice, dogs, humans, and herbivorous fishes that target starch as an important source of glucose for use in metabolism, so there is likely to be selective pressure on these genes. Indeed, an increase in amylase gene expression coinciding with increased gene copy number is an expectation, as increases in gene copy number tend to lead to increased expression in many genes not experiencing dosage compensation (Gout et al. 2010; Springer et al. 2010; Qian and Zhang 2014). Genes that are expressed at a high level tend to be reinforced via positive selection (Gout et al. 2010), and thus, when new copies arise in highly expressed genes, the increased dosage effect can be preserved. Moreover, protein-protein interactions between the copies can be beneficial enough (or even required) for the copies to coexist (Qian and Zhang 2014). Alternatively, expression of the existing gene copies can be lowered to maintain protein concentrations at an acceptable (selectable) level without the loss of the duplicated gene itself (Qian et al. 2010). The retention of the new copy then can allow for neo- or subfunctionalization of the new protein (Qian et al. 2010). Although we do not have the data, the structural (fig. S1) and p*I* (fig. 2) differences among α-amylase 2A and α-amylase 2B in *C. violaceus* hint at subfunctionalization for α-amylase 2B, but confirmation requires further investigation.

WGD events have occurred three times in the evolution of fishes: twice early in vertebrate evolution (Ohno 1970; Kasahara 2007) and a third time in the Actinopterygii in what is called the teleost-specific whole-genome duplication (TS-WGD), or 3R hypothesis (Christoffels et al. 2004; Glasauer and Neuhauss 2014). Based on these WGD events, most ray-finned fishes could theoretically possess eight copies of a pancreatic amylase gene, assuming a single copy existed before the original duplication event and all copies were subsequently retained (Gout et al. 2010; Qian et al. 2010). We estimated approximately two copies in most of the pricklebacks (with little sequence variation among the copies), although varying gene copy numbers are present in the genomes of other fishes (figs. 4, 5).

Cebidichthys violaceus has experienced gene duplication at the amylase locus, and this increased amylase gene copy number is likely leading to an increased amylase expression in this herbivorous fish species. However, changes in the regulatory

elements of amylase genes—even changes as short as 10–70 bases—can also lead to changes in expression (Cockell et al. 1989; Howard et al. 1989; Choi and Yamazaki 1994; Ma et al. 2004; Wiebe et al. 2007). The 5′ flanking regions are 95% similar among *C. violaceus* and *X. mucosus* for the first 167 nucleotides upstream from the start codon, and many important motifs (the putative promoter included) are within this section (Cockell et al. 1989; Howard et al. 1989; Wiebe et al. 2007). However, the 5′ flanking regions of these two species diverge moving further upstream, which suggests that there could be differences in how the expression of amylases is regulated among the two species. For instance, a conserved motif within the 5′ flanking region of amylase known as the TC box (Wiebe et al. 2007), amylase element I (Howard et al. 1989), or box A (Cockell et al. 1989), is divergent among *C. violaceus* and *X. mucosus*, although *X. mucosus* may have a putative TC box further upstream from that of *C. violaceus* (fig. S5). A putative hepatocyte nuclear factor-3 binding site, which is important in digestive enzyme expression (Rausa et al. 1997; Ma et al. 2004), is also divergent among *C. violaceus* and *X. mucosus*. The significance of these differences warrants further study, with the caveat that expression of amylase genes has been observed to be not different among *C. violaceus*, *X. mucosus*, and *X. atropurpureus* using in situ hybridization staining intensity as the metric of expression (Kim et al. 2014). Although it is apparent that the *Xiphister* taxa achieve elevated amylolytic activity via elevated expression of fewer genes, we cannot rule out that the amylases of the species of *Xiphister* are just more efficient than those of the other pricklebacks. More detailed biochemical tests of isolated amylase proteins are needed, but our PAML analysis (table S4) suggests that *Xiphister* amylases are not unique in the Stichaeidae, making more efficient proteins a dubious assumption, especially because of the similar Michaelis-Menten constant values among amylases from *C. violaceus* and the *Xiphister* species (German et al. 2004). Hence, the *Xiphister* species likely achieve elevated amylase activity by elevated expression of the genes they possess.

Although *C. violaceus* has elevated amylase activity in its gut in comparison to closely related carnivores or omnivores (fig. 1), *C. violaceus* consistently has lower amylase activities in its gut in comparison to the *Xiphister* taxa (Chan et al. 2004; German et al. 2004, 2014, 2015). One possible explanation is lineage-specific dosage compensation, where the expression of the *C. violaceus* paralogs is adjusted to prevent deleterious overexpression of a gene with high copy number (Qian et al. 2010). Indeed, expression of the *Amy2A* and *Amy2B* genes in *C. violaceus* is not statistically different (table 2). The lower amylolytic activity in *C. violaceus* holds true even when examining the ability of their amylases to hydrolyze different types of starch (fig. 3*A*). The red and green algae consumed by herbivorous and omnivorous prickleback fishes contain a mixture of branched-chain starches (similar to amylopectin) as well as straight-chained amylose (Love et al. 1963; Painter 1983), so our choices in starch types were driven by the starches in the diets of the fishes (German et al. 2004, 2015; German and Horn 2006). Nevertheless, in terms of digestive efficiency measured on the whole-animal level, *C. violaceus* appears to digest carbohydrates (including starch) from red and green algae as efficiently as or more efficiently than *X. mucosus* (Horn et al. 1986).

The fact that *C. violaceus* can absorb nutrients from algae as efficiently as *X. mucosus* underscores the complexity of the digestive process and how digestive efficiency extends beyond enzymatic activity levels (German 2011; Karasov and Douglas 2013; German et al. 2015). For instance, *C. violaceus* has longer retention of food in its gut and has greater levels of microbial fermentation in its hindgut than *X. mucosus* (German et al. 2015). Therefore, lower enzyme activity must also be viewed on a temporal scale: if *C. violaceus* can expose algae to the digestive process for more time than *X. mucosus*, the former species may not invest as many resources in amylase expression as does the latter, which passes food through the gut more quickly (German et al. 2015). Thus, functionally, both of these herbivores are successful in digesting algae but by slightly different digestive mechanisms, with amylase representing the centerpiece of these abilities.

The PAGE analysis and our protein model suggest that the amylase paralogs in *C. violaceus* may function differently in the digestive process. However, because we did not isolate these proteins (e.g., Ferey-Roux et al. 1998; Kushwaha et al. 2012), we cannot discern any differences among the active enzymes. By using crude tissue homogenates, we attempted to observe whether the amylase genetic diversity in *C. violaceus* produced an obvious advantage over having fewer gene copies, as in *X. mucosus*. In terms of temperature, *C. violaceus* and *X. mucosus* are sympatric in rocky intertidal habitats spanning from Southern California to southern Oregon, but *X. mucosus*'s range extends to the Bering Sea in Alaska (Eschmeyer et al. 1983). Thus, the latter species experiences a wider range of temperatures. Nevertheless, the temperature profiles for the amylases in these two species are similar (fig. 3*B*), and *X. mucosus* shows little amylase genetic diversity ranging from California to Washington (F. Chaabani and D. P. German, unpublished data). There are differences in the pH tolerances of the enzymes among the two herbivores, with amylase of *C. violaceus* having slightly lower pH (7.5) optima than that of *X. mucosus* (8.0; fig. 3*C*). Proteins have optima at pH values providing the greatest stability (as opposed to near their isoelectric points; Talley and Alexov 2010), and varying pH optima are known in other fish amylases (Fernandez et al. 2001; Kushwaha et al. 2012); how these varying pH optima affect the digestive process requires further study. For instance, the intestinal pH of *C. violaceus* (Edwards and Horn 1982) and *X. mucosus* (D. P. German, unpublished data) is approximately 7.5, but this could vary temporally in both taxa, perhaps requiring a higher pH optimum in *X. mucosus*. The Michaelis-Menten constants are similar for *C. violaceus* (0.36% ± 0.13% potato starch) and *X. mucosus* (0.33% ± 0.15% potato starch) amylases at pH 7.5 (German et al. 2004).

In our protein-modeling efforts, two amino acid substitutions between α-amylase 2A and α-amylase 2B in *C. violaceus* appeared to potentially affect the flexibility and/or stability of particular regions of the protein's 3D structure: D174N and E397G (fig. S1). The D174N substitution from aspartate to asparagine occurs as frequently as that of chance alone across all

possible amino acid substitutions (Yampolsky and Stoltzfus 2005), and only aspartate or asparagine is found at this position in amylases from each vertebrate class as well as select insect taxa (figs. S7, S8). In contrast, E397G makes the *C. violaceus* α-amylase 2A sequence unique in comparison to the other amylase orthologs across fishes (fig. S7) and even across vertebrates (fig. S8); in our analysis, only the Acari mite (*Blomia tropicalis*) also has a glutamate at this site in the protein. In addition, E397G constitutes a significant physiochemical change as it swaps an uncharged glycine for a negatively charged glutamate residue (table S3; fig. S1), again hinting at potential subfunctionalization of the two α-amylase proteins in *C. violaceus*.

Duplication Events across Fishes

Duplication events of amylase genes have occurred numerous times in the evolution of fishes (figs. 4, 5). Of the 41 species for which complete amylase gene sequences are available, 10 have identified paralogs. Most of these fishes are carnivorous or omnivorous, and *C. violaceus* and *X. mucosus* are among the first herbivorous fishes to have their complete amylase genes sequenced (with *Ctenopharyngodon idella*, *Signaus canaliculatus*, and *Maylandia zebra* representing the other herbivores). However, our PAML analyses suggest that within a given lineage, amylases of herbivorous fishes have not undergone any unique selection setting them apart from other fishes within those lineages (table S4).

The amylase gene phylogeny presented in figure 4 resembles a larger analysis of 10 nuclear genes that elucidated the relationships within the Acanthomorpha, the largest radiation of fishes that includes derived teleosts (Near et al. 2013). It appears that fishes have a pancreatic amylase (α-amylase 2) that is a member of glycoside hydrolase family 13 (Janeček et al. 2014) and that duplication events have mostly occurred within specific lineages and are in tandem on specific chromosomes (fig. 5).

Early duplication events followed by diversification can lead to separate gene families. In *Drosophila*, for example, duplication events produced two different amylase lineages, which each exist within different species (Inomata and Yamazaki 2000). Similarly, in mammals, salivary amylase (*Amy1*) and pancreatic amylase (*Amy2*) form distinctive gene lineages (Groot et al. 1989; Sugino 2007), but fishes appear to have only *Amy2*.

Within our phylogenetic analysis, there are three cases where ancient duplication events led to diversification of amylases within certain branches. The first is within the Ostariophysi, which include the minnows, carps, suckers, and tetras (Nelson 2006) in our study. *Danio rerio* and *C. idella* each have amylase loci on multiple chromosomes (fig. 5), and these two amylase types group by chromosome in our phylogenetic tree, with those *Amy* genes on what is chromosome 17 in *D. rerio* being more derived (fig. 4), again supporting WGD or chromosomal translocation as potential origins of these separate loci; *A. mexicanus* and *I. punctatus* have amylases only at this second locus (fig. 5). Neither *Latimeria chalumnae* nor *Lepisosteus oculatus*, the lineages of which evolved independently of teleost evolution in our analysis, have more than one amylase locus in their genomes, and this locus is syntentic with other fishes (fig. 5). Therefore, the duplication leading to *Amy* genes on separate chromosomes is unique in the Ostariophysi. Second, in the Poeciliidae, which includes *Xiphophorus maculatus* and the two *Poecilia* species in our analysis, there are two separate amylase branches represented by each of these species, suggesting that the duplication event leading to these amylases predates the evolution of these taxa and that each of these species has retained these *Amy2* genes (with *P. reticulata* generating an additional *Amy2* copy via lineage-specific duplication). Interestingly, all of these amylase genes are in tandem on a single chromosome (fig. 5), so these copies were themselves produced by an ancient, lineage-specific duplication event in the ancestor leading to the poeciliids. Finally, in the Cichlidae (which includes *Oreochromis niloticus* in our analysis), *Amy2A* from *O. niloticus* is a more ancient gene copy that likely gave rise to *Amy2B* and *Amy2C* in this species and likely other cichlids (fig. 4). All of these *Amy* genes are also on a single chromosome in syntenic region 1 (fig. 5), and the high level of selection in these amylases (table S4) warrants further investigation. We have recently sequenced the genome of *C. violaceus*, and our preliminary analysis suggests that the *Amy* locus (containing at least two *Amy* genes) in *C. violaceus* is part of syntenic region 1, and hence, the increased copy number in this species arose via lineage-specific duplication (data not shown). Thus, our phylogenetic and syntenic analyses uncovered putative WGD or chromosomal translocation and lineage-specific duplication events leading to different amylases in different fishes, showing that the mechanism of gene duplication is not always the same, as has also been seen for pepsinogen genes in tetrapods and fishes (Castro et al. 2012, 2014).

Conclusions

In conclusion, this study is unique in that it clearly shows that the evolution of the phenotype of elevated amylase activities can happen differently in different lineages. Studies in mammals (Perry et al. 2007; Axelsson et al. 2013) and *Drosophila* (Inomata and Yamazaki 2000) suggested that gene copy number was the primary determinant of increased amylolytic activity in animals. However, we show here that gene copy number alone does not explain elevated amylase activities in prickleback fishes, as carnivores with low amylase activities in their guts (e.g., *Anoplarchus purpurescens*) and herbivores with elevated amylase activities in their guts (e.g., *Xiphister mucosus*) alike have similar amylase gene copy numbers, yet some other herbivores (i.e., *C. violaceus*, with moderate amylase activity) have comparatively elevated amylase gene copy number. Thus, there is "more than one way to skin a cat" on the molecular level to achieve a common trait on the phenotypic level (Rodriguez et al. 2007; Castro et al. 2014). More detailed investigations (e.g., genomic and promoter analyses) are required to confirm amylase gene copy number and expression enhancement, and more detailed biochemical tests are needed to determine any effects on amylase biochemistry (e.g., subfunctionalization) and starch digestion. Nevertheless, our crude biochemical tests coupled to previous

whole-animal digestibility trials (Horn et al. 1986) suggest that elevated gene copy number versus elevated expression of fewer genes can have the same result.

APPENDIX

Supplemental Methods

RNA Isolation, Rapid Amplification of cDNA Ends, Reverse Transcription, Cloning, and Sequencing

Total RNA isolation was performed by using the RNeasy Plus mini kit (Qiagen, Valencia, CA). Up to 30 mg of freshly harvested or RNAlater-preserved pyloric cecal tissue was immersed in 600 μL of lysis buffer with β-mercaptoethanol and disrupted with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) for 90 s. The purification was continued as per the manufacturer's protocol, with the exception that an on-column DNase treatment with the RNase-Free DNase set (Qiagen) was added to maximize removal of genomic DNA. The RNA integrity was assessed by an Agilent bioanalyzer, and only samples with a RNA integrity numbers above 8.0 were subjected to further analysis.

The 5′ and 3′ rapid amplification of cDNA ends (RACE) was performed with the SMARTer RACE cDNA amplification kit (Clontech Laboratories, Mountain View, CA). On generation of RACE-ready cDNA samples according to the manufacturer's instructions, 5′ RACE and 3′ RACE touchdown polymerase chain reactions (PCRs) were performed with the Advantage 2 PCR kit (Clontech Laboratories) using the kit-provided Universal Primer Mix along with gene-specific primers AmyGSP1_5R (antisense, 5′ RACE) and AmyGSP2_3R (sense, 3′ RACE), which were designed to match all the previously published prickleback partial α-amylase mRNA sequences (Kim et al. 2014).

Primer PC_GSP1_5R was used for 5′ RACE PCR with *Phytichthys chirus*–specific RACE-ready cDNA. Based on the very high degree of homology among the prickleback *Amy2* cDNA sequences, the same primers were used to perform the 5′ and 3′ RACE with RNA from *P. chirus* and *Dictyosoma burgeri*, for which partial cDNA sequences were not available. The touchdown PCR program was as follows: five cycles at 94°C for 30 s and 72°C for 2 min; five cycles at 94°C for 30 s, 70°C for 30 s, and 72°C for 2 min; 25 cycles at 94°C for 30 s, 68°C for 30 s, and 72°C for 2 min. Finally, the PCR products were cloned and sequenced as described in "RNA Isolation, Rapid Amplification of cDNA Ends, Reverse Transcription, Cloning, and Sequencing" in the main text.

Two micrograms of RNA was reverse transcribed into cDNA by the Omniscript reverse transcription kit (Qiagen) according to the manufacturer's instructions. Oligo-dT was used as a primer at a final concentration of 1 μM. Each PCR was performed with a PfuUltra II Fusion HS DNA polymerase kit (Agilent Technologies, Santa Clara, CA) in a volume of 50 μL containing 2 μL of the reverse transcriptase reaction, 1 mM dNTPs (0.25 μM of each dNTP), 0.2 μM of each primer, and 1 μL of PfuUltra DNA polymerase. The reactions were carried out in a C1000 Touch thermal cycler (BioRad, Hercules, CA) using cycling parameters optimized for cDNA targets as per the manufacturer's instructions; briefly, 1 cycle: 95°C for 1 min; 40 cycles: 95°C for 20 s, 57°C for 20 s, 72°C for 45 s; 1 cycle: 72°C for 3 min. Based on the RACE results, forward and reverse primers were designed matching nucleotide sequences starting from the ATG and the stop codon, respectively, to amplify the full-length pancreatic amylase cDNA. Primer sets 5_amy_CV and 3_amy_CV (table S1) were used to amplify the *Amy2* cDNA obtained from *Cebidichthys violaceus* total RNA. Primer sets 5_amy_AP and 3_amy_AP were used for *Anoplarchus purpurescens* cDNA. Since the 5′ regions of *Xiphister mucosus* and *Xiphister atropurpureus* are identical, primer 5_amy_XM/XA was the forward primer in both *X. mucosus* and *X. atropurpureus* PCR, in the presence of species-specific reverse primers 3_amy_XM and 3_amy_XA (table S1). Primer sets 5_amy_PC and 3_amy_PC were used on *P. chirus* cDNA.

PCR products from five individual fish per species ($n = 3$ for *D. burgeri*) were visualized by ethidium bromide staining in 1.0%–1.5% agarose gels and purified with the NucleoSpin gel and PCR cleanup kit (Macherey-Nagel, Bethlehem, PA). The purified PCR products were treated with Taq DNA polymerase in presence of 10 mM dNTPs and Taq buffer for 10 min at 72°C in order to add 3′ A-overhangs and immediately ligated into pCR 2.1-TOPO TA vector using the TOPO TA cloning kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. One to three microliters of the ligation mixture was used to transform JM109 chemically competent cells (Promega, Madison, WI) according to the manufacturer's instruction (technical bulletin TB095). Transformants were plated on Luria broth agar supplemented with ampicillin (100 μg mL^{-1}) and ChromoMax IPTG/X-Gal solution (Fisher Scientific, Chino, CA) for blue/white colony screening. For RACE and full-length analysis, plasmid DNA was purified from 12–16 positive clones with NucleoSpin plasmid kits (Macherey-Nagel) and then sequenced in both directions (Eton Bioscience, San Diego, CA) with universal primers M13 RV and M13(-20) Fw and internal gene-specific primers SQ_574 RV, SQ_761 FW, and SQ_451FW (table S1). For the identification of the 5′ upstream region, 20 transformants for each primer set were grown in 96-well plates in 200 μL of lysogeny broth media containing 10% glycerol and shipped to Beckman Genomics (Danvers, MA) for further processing and sequencing. DNA sequence assembly and alignments were generated with CodonCode Aligner (Codon Code, Centerville, MA). Protein alignments were obtained with JalView. All accession numbers for genes sequenced or used in this study are found in table S2.

Gene Copy Number Determination, Real-Time Quantitative PCR, and Sequence Analysis

To evaluate the *Amy2* gene copy number, *Cebidichthys violaceus*–specific primers Ex2_CV_FW and Ex2_CV_RV were designed to amplify a 144-bp product within exon 2 of genomic DNA that contains the polymorphisms L66V, D67N, and R71K and thus would amplify the *Amy2A* and *Amy2B* variants in *C. violaceus*.

Primer Ex2_CV_RV matches all the prickleback species in this study, and hence it was used as the reverse primer in all the gene copy number–related PCRs. Because of sequence identity, primer Ex2_CV_FW was also used as the forward primer to amplify *Anoplarchus purpurescens* cDNA. Primer Ex2_XM_FW was designed to amplify the *Amy2* fragment from the cDNA templates of both *Xiphister* taxa, and primer Ex2_PC_FW was used to amplify from *Phytichthys chirus* cDNA template. PCR reactions were performed in triplicate with iTaq Universal SYBR Green supermix (BioRad) in a 20-μL volume containing 10 ng of genomic DNA template and primers at 300 nM final concentration. The following cycling program was used: 1 cycle: 95°C for 40 s; 40 cycles: 95°C for 15 s, 56°C for 15 s, and 72°C for 15 s; 1 cycle: 72°C for 7 min.

Primer set qUn_Amy_FW and qUn_Amy_RV amplifies a 176-bp region in all the prickleback species in our study and was used to quantify expression and copy number of *Amy2* genes in general. For *C. violaceus*, forward primer qUn_Amy_Fw and qUn_Amy_Rv targets a 176-bp region that contains three (LDR to VNK) of the 10 identified substitutions among *Amy2A* and *Amy2B* in this species. As such, the 176-bp PCR product generated with the qUn_Amy primer set is a mixture of the two isoforms and accounts for the total expression of pancreatic amylase in *C. violaceus*. To differentiate the expression of the two variants, variant-specific primers were designed that match 20–27 bp of the regions encompassing the nucleotides involved in the amino acid substitutions mentioned above. Specifically, primer ALD_SYB_FW binds to the region encompassing the triplet that encodes for A41, and LD_SYB_RV binds within the region containing the triplets for L66 and D67, and together they amplify 109 bp of *Amy2A* cDNA. Similarly, KVN_SYB_FW and VN_SYB_RV were designed to amplify a 110-bp product specific for *Amy2B*. Although matching in the same regions, the length of the oligos for each isoform had to be adjusted to keep the melting temperature at 66°–67°C. To amplify ribosomal protein L8 as a normalization gene, degenerate primer pairs previously described (Choe et al. 2005) were used to amplify a conserved region using *C. violaceus* cDNA template; the product was cloned and sequenced, and *C. violaceus*–specific primers L8-FW and L8-RV were designed to amplify a 204-bp fragment. Real-time PCR was performed in an iCycler iQ multicolor real-time detection system (BioRad) using the BioRad iTaq Universal SYBR Green supermix as per the manufacturer's protocol. DNA sequence assembly and alignments were generated with CodonCode Aligner (Codon Code). Protein alignments were obtained with Clustal Omega (Sievers et al. 2011) or JalView. Comparisons of amino acid substitutions among the α-amylase 2A and α-amylase 2B paralogs in *C. violaceus* were performed with screening for nonacceptable polymorphisms analysis (Bromberg and Rost 2007). Reliability index is a scale from 0 (no reliability) to 9 (high reliability). PAML 4.8a (Yang 1997) was used to examine amino acid sites under selection among the prickleback amylases. For this analysis, we estimated nonsynonymous/synonymous substitution rate ratio (dN/dS = ω), and we used CODEML to estimate a uniform ω with model 0 (one ratio) and tested for positive selection with M7 (β) and M8 (β and ω) models. Likelihood ratio tests (LRTs) were compared between the M7 and M8 models, and LRT values were compared with a χ^2 distribution with a significance cutoff of 0.05.

Genomic DNA Isolation and 5′ Flanking Sequencing

Total genomic DNA was isolated from muscle by using the DNeasy kit (Qiagen) according to the manufacturer's instructions, with the exception that a DNase-free RNase A digestion step was added. Twenty-five milligrams of fresh, frozen, or ethanol-preserved muscle was minced with a scalpel and immersed into 180 μL of lysis buffer ATL; 20 μL of proteinase K was added, and the sample was incubated at 56°C for 2–3 h or until lysis was completed. On lysis completion, 10 μL of a 10 mg mL^{-1} DNase-free RNase A stock (Thermo Fisher Scientific, Waltham, MA) was added to the lysate, and the sample was left at room temperature for 5 min. After RNA digestion, 200 μL of buffer AL was premixed with 200 μL of absolute ethanol, and 400 μL of this mixture was added into each lysate. The sample was applied onto a DNeasy column and the protocol continued as per the manufacturer's instructions. The DNA was eluted in a total volume of 200 μL and quantified in the BioTek Synergy H1 Hybrid spectrophotometer/fluorometer.

To isolate the upstream sequence of the pancreatic amylase gene in *Cebidichthys violaceus* and *Xiphister mucosus*, we used the Universal GenomeWalker 2.0 kit (Clontech Laboratories). Up to 2 μg of total genomic DNA from both species was digested with Eco RV restriction enzyme, and each batch of digested genomic DNA was then ligated separately to the GenomeWalker adaptor, generating uncloned Genome Walker libraries. A primary touchdown PCR was performed with the outer adaptor primer (AP1) provided in the kit and gene-specific primers CV_GW_GSP1 and XM_GW_GSP1 on *C. violaceus*– and *X. mucosus*–specific GW library templates. We observed two *Amy2* 5′ flanking sequences in *C. violaceus*, and thus, we sought to establish whether each one could be attributed to different *Amy2* isoforms in this species. For this purpose, we designed isoform-specific forward primers Ex2_LDR_FW and Ex2_VNK_FW, which, by matching within the region affected by the substitutions, amplify an isoform-specific gene fragment that is 120 and 121 bp long, respectively. Each 5′ untranslated region was associated with *Amy2A* and *Amy2B*, suggesting that these untranslated regions were not unique to each of the variants.

As per the manufacturer's instructions, the following program was used: 7 cycles: 94°C for 25 s, 72°C for 3 min; 32 cycles: 94°C for 25 s, 67°C for 3 min; 1 cycle: 67°C for an additional 7 min. An aliquot of the primary PCR was diluted 1 : 50 and used as template in a secondary touchdown PCR with nested outer adaptor primer (AP2) provided in the kit and gene-specific nested primer CV_GW_GSP2, which amplifies sequences from both species. *Cebidichthys violaceus*–specific GW_A_GSP2 and GW_L_GSP2 primers (table S1) were also separately used to target isoform-specific upstream regions.

The manufacturer's recommended touchdown PCR program for the secondary PCR was used, which included the following: 5

cycles: 94°C for 25 s and 72°C for 3 min; 20 cycles: 94°C for 25 s and 67°C for 3 min; 1 cycle at 67°C for an additional 7 min. The primary and secondary touchdown PCRs were performed with the Advantage 2 polymerase kit according to the manufacturer's instructions. The secondary PCR products were cloned and sequenced as descried earlier in "RNA Isolation, Rapid Amplification of cDNA Ends, Reverse Transcription, Cloning, and Sequencing" in the main text, and further reading was extended toward the 5′ end with primers Amy_GWsp3 and Amy_GWsp4 (table S1).

Protein Modeling and Phylogenetic Analysis

Because of the amino acid sequence variation among α-amylase 2A and α-amylase 2B paralogs in *Cebidichthys violaceus*, we used protein homology–based modeling to assess what structural changes might occur between the two paralogs (see fig. S1). To examine the similarity of the prickleback amylase sequences to other known fish sequences, the complete coding sequence of the *Xiphister atropurpureus Amy* gene was used for a TBLASTX (NCBI BLAST, ver. 2.2.26) search with the following cDNA genomic libraries taken from Ensembl (Ensembl 80; Hubbard et al. 2005): *Danio rerio, Poecilia formosa, Takifugu rubripes, Xiphophorus maculatus, Oreochromis niloticus, Oryzias latipes, Astyanax mexicanus, Lepisosteus oculatus, Gadus morhua, Tetraodon nigroviridis*, and *Latimeria chalumnae*. We also utilized a *T. nigroviridis* BAC library from Bouneau et al. (2003) for additional chromosomal information for this species. For *Ctenopharyngodon idella*, we searched the genome found on the National Center for Gene Research (Chinese Academy of Sciences) database (http://www.ncgr.ac.cn/grasscarp/); for *Ictalurus punctatus* we used c-BARBEL (http://catfishgenome.org/cbarbel/); and for *Poecilia reticulata* we used the genomics database on NCBI (http://www.ncbi.nlm.nih.gov/nuccore/658121090?report=graph&v=916735:923052). We used a threshold of 70% identity for the BLAST searches for amylase genes. In addition a BLASTN was conducted on NCBI nr database to identify homologous sequences. BLAST hits with a 90% identity were retained. Afterward, open reading frames were identified with OrfPredictor (Min et al. 2005), and only coding sequences that contained approximately 1,536 nucleotides (the general length of vertebrate amylase genes) were used for our phylogenetic analysis. This resulted in the amylase sequences (and their variants) from 34 fish species (in addition to the amylase sequences from the six stichaeid species), plus *Xenopus tropicalis*. Alignments were conducted with MAFFT (http://mafft.cbrc.jp/alignment/server/) for both nucleotide and amino acid sequences. Phylogenetic relationships were generated using a mixed-model partitioned Bayesian method as implemented in the software package Mr. Bayes, version 3.1.2 (Huelsenbeck and Ronquist 2001). Codon partitions were chosen with the model GTR + I + G used for each partition, as determined using the Akaike information criterion in the computer program Mr. Modeltest (Nylander 2004). Ten million generations of Markov chain Monte Carlo were performed using a random starting topology with trees sampled every 200 generations, with 10% of early runs counted as burn-in. The retained 45,000 trees were used to construct a 50% majority-rule consensus tree. The percentage of times that a particular node was recovered in the analysis is interpreted as the posterior probability of the occurrence of that node (Huelsenbeck and Ronquist 2001). The genomic location of each *Amy* gene was identified along with the two to four closest flanking genes on either side. These patterns were used to generate a synteny map for the *Amy* loci in various fish taxa (Castro et al. 2014). As with the pricklebacks, PAML 4.8a (Yang 1997) was used to examine amino acids under selection among amylases from fishes within the Ostariophysi, Poeciliidae, and Cichlidae (for more detail, see "Gene Copy Number Determination, Real-Time Quantitative PCR, and Sequence Analysis" in the appendix).

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