November 2016

A Genetic Analysis of Cichlid Scale Morphology

Kenta C. Kawasaki
University of Massachusetts Amherst

A GENETIC ANALYSIS OF CICHLID SCALE MORPHOLOGY

A Thesis Presented

by

KENTA C. KAWASAKI

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

September 2016

Molecular and Cellular Biology

A GENETIC ANALYSIS OF CICHLID SCALE MORPHOLOGY

A Thesis Presented

by

KENTA C. KAWASAKI

Approved as to style and content by:

_____
Craig Albertson, Chair

_____
Courtney Babbitt, Member

_____
Hélène Cousin, Member

_____
Dominique Alfandari, Program Leader /
GPD, Molecular and Cellular Biology
Program

ABSTRACT

A GENETIC ANALYSIS OF CICHLID SCALE MORPHOLOGY

SEPTEMBER 2016

KENTA C. KAWASAKI, B.S., UNIVERSITY OF MASSACHUSETTS AMHERST

M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Craig Albertson

Epidermal appendages are found on every vertebrate this world has to offer. In fish, these are commonly represented by scales. While we have a solid grasp of how scales develop, little is known about the underlying genetic mechanisms behind these phenotypic changes. Using two species of African cichlids (*Labeotropheus fuelleborni* and *Tropheops* “red cheek”) with varying scale phenotypes, we sought to examine their F2 hybrid offspring and statistically link the responsible genetic elements to their respective parental phenotypes through Quantitative Loci Trait (QTL) analysis.

Scales were removed from six different locations across the midline of each individual. Then, numerous traits on each scale were measured, and these values were used in the QTL analysis. 42 significant QTL were identified, with multiple QTL intervals possessing promising candidate genes. These genes include: *fgfr1b*, *efna5a*, *TGIF1*, *eIF6*, and *col1a1a*. Previous studies have implicated these particular genes and gene families to play important roles in scale and placode development. However, they represent the minority of QTL intervals discovered, providing direction for future research towards the other QTL intervals represented by this study.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

FROM GENOME TO SCALES

Introduction

The natural world never ceases to astonish, housing countless organisms of different shapes and sizes. However, how did all these morphological differences arise throughout history? As Mendelian genetics has taught us, almost everything about an organism is influenced by or dependent on its genetic code. This piece of information is essential to understanding how genotypes can influence phenotypic traits, but with genomes being extremely large and complex, how do we link specific phenotypes and their associated genotypes? This would be similar to looking for a needle in a nucleotide haystack. Thanks to continuing improving advancements in technology, particularly in the field of genomics, we now have the power to link specific phenotypic traits of interest to their respective genetic regions and elements.

Phenotype to Genotype

Identifying underlying genetic mechanisms that affect specific phenotypes is crucial to developmental biology, providing key insights into how organisms and their definitive structures develop at the molecular and cellular levels. This forward genetics approach can also identify the genetic etiology of developmental diseases that possess well-characterized phenotypes. Since many developmental structures and pathways are highly conserved across

species, using model organisms to conduct these analyses is beneficial and commonly used throughout the scientific community.

A Non-Traditional Model Organism

African cichlids, a non-traditional model organism, provide a valuable genetic tool to explore the causes of morphological variation within and between species, as well as provide insights to how environmental factors can affect different genotypes and phenotypes. These tropical freshwater fish consist of roughly 8% of all fish species, exhibiting remarkable diversity due to extraordinary adaptive radiations during their evolutionary history. For example, the Great Lakes of East Africa house nearly 2,000 recently evolved species of cichlids, of which an estimated 1,000 species can be found in Lake Malawi alone. While the cichlids of Lake Malawi have undergone an extensive adaptive radiation, it can be characterized by three general stages. First, cichlids diverged based on broad ecological niches, primarily splitting into rock-dwelling and sand-dwelling clades. This was followed by the diversification of the feeding apparatus. Finally, more recently evolved species diverged based on color patterning due to sexual selection (Danley and Kocher, 2001). This adaptive radiation has been extensively studied, particularly variation in cichlid jaws, teeth, brains, behavior, and color patterning (Powder and Albertson, 2015; Kocher, 2004; Kornfield and Smith, 2000; Streelman, Webb, Albertson, & Kocher, 2003; Sylvester et al., 2010). Importantly, the diversity among cichlids can now be studied at a molecular level in order to distinguish possible genetic influences and differences.

In this study, I propose to examine an understudied trait in this radiation, scales, which like teeth, are epidermal appendages.

Epidermal Appendages and the Evolution of Scales

Epidermal appendages, such as hair, teeth, feathers, and scales, are present throughout every vertebrate on this planet. Of these common epidermal appendages, scales appear to be the most ancestral form and share deep homology to other more derived types of appendages (Qu et al., 2015; Shubin, Tabin, & Carroll, 2009; Di-Poï and Milinkovitch, 2016). The Cambrian Explosion brought a transition from soft-bodied Ediacarian organisms to an ecological arms race of mineralized-skeletons and structures, providing a means for both predation and protection. Early scales were most likely large and heavy to provide basic protection from predators, but became smaller and lighter throughout evolutionary history in order to meet the demands of faster predators and thus faster escape mechanisms (Seilacher et al., 2005).

Today modern fish may possess one of four different types of scales- placoid, cosmoid, ganoid, or elasmoid. Cosmoid and ganoid scales represent more primitive forms and are bulky and heavily mineralized. These were replaced by thinner and more flexible elasmoid scales in modern teleosts, allowing for improved swimming and hydrodynamics. Elasmoid scales come in two forms, cycloid and ctenoid, with the main difference being the presence of small tooth-like structures (i.e., ctenii) on the posterior edge of ctenoid scales. They are thought to have evolved from ganoid scales due to the loss of ganoine and

thinning of the bony dermal plate (Zhu et al., 2012; Helfman, Collette, Facey, & Bowen, 2009; Lagler, 1947).

Diversity of Fish Scales

Fish exhibit tremendous diversity in scale number, shape, and size. In most cases, faster swimming fish possess small overlapping scales, which allows for a smoother flow of water over the body and thus less drag, as well as increased flexibility. This overlapping feature is true for all fish bearing cycloid and ctenoid scales, in contrast to ganoid and cosmoid scales. However, not all species of fish possess scales, such as lampreys and ocean eels. In some species of flatfish, both cycloid and ctenoid scales can be found, and scale type can vary based on sex and location on the body. Scale size also differs greatly between species. For example, most species of tuna possess body scales so small they are nearly invisible, while tarpon species possess rather large scales (Helfman et al., 2009; Patterson et al., 2002; Lagler, 1947; Zhu et al., 2011; Ehrlich, 2010).

Scales in Perciformes

Perciformes, including cichlids, possess ctenoid scales. Ctenoid scales are composed of a deep collagenous layer and a mineralized surface layer of hydroxyapatite and calcium carbonate (Ehrlich, 2010; Helfman et al., 2009; Zhu et al., 2012). Several structures on ctenoid scales have been used for fish taxonomy as well as inferring phylogenetic relationships (Jawad, 2005; Patterson

et al., 2002; Kuusipalo, 1998). These structures consist of the *circuli*, *focus*, *radii*, and *ctenii* (Patterson et al., 2002; Lagler, 1947). The circuli are elevated markings on the surface, typically following the outline of the scale and are concentrically arranged around the focus. The focus is the first part of the scale to appear in development. The radii are unmineralized grooves that radiate from the focus to the anterior margin of the scale. As mentioned before, tiny teeth called ctenii line the posterior edge of the scale (Lagler, 1947; Sire and Géraudie, 1983; Patterson et al., 2002). Remarkably, in spite of the morphological diversity of perciformes in general, and cichlids in particular, little is known about the evolution of scales in this group.

Development of Ctenoid Scales

Like most epidermal appendages, ctenoid scales arise from placodes and undergo a unique pattern of cellular differentiation and proliferation that distinguishes them from other epidermal appendages. They begin forming at the caudal peduncle at around 7-11 mm standard length, spreading anteriorly as the individual matures (Helfman et al., 2009; Sire and Géraudie, 1983). Signaled and organized by placodes, scale development starts with the formation of papillae caused by an accumulation of elongated fibroblasts beneath the basal layer, which lies between the dermis and epidermis. The fibroblasts eventually differentiate into scleroblasts, connected by short desmosomes to form a tri-stratified layer in the dermis. Between these scleroblast layers a central elongated space forms where the early scale will begin to differentiate. Two

different regions form around this space, a superficial region located directly beneath the epidermal-dermal boundary, and a deep region. Collagen fibrils, randomly organized in the *stratum laxum* of the upper dermal layer, start to align themselves into an organic matrix, which triggers the mineralization of the osseous layer. The developing scale then grows first in circumference, as the scleroblasts elongate, and then in thickness, caused by the mineralization of two overlapping layers of scleroblasts (Sire and Géraudie, 1983; Helfman et al., 2009).

Uncovering the Genetic Basis of Scale Development and Variation

While we know a fair amount about the genetic mechanisms that underlie scale development, we know virtually nothing about the genetics of scale shape. I seek to identify the underlying genetic mechanisms that affect scale development and variation in scale morphology. Two different species of cichlids, *Labeotropheus fuelleborni* (LF) and *Tropheops* "red cheek" (TRC), both inhabit Lake Malawi and have the potential to interbreed, but occupy different ecological niches and possess different scale phenotypes. Using a quantitative trait loci (QTL) mapping approach, I set out to statistically link specific scale traits to parental genotypes across the genome. From this study, multiple genetic signals across the genome for several different traits of F2 hybrid scales were discovered.

CHAPTER 2

METHODS

Characterization of Cichlid Scales

12 individuals of each parental species and 256 F2 hybrid individuals were phenotyped for this study. Scales were taken across the midline of the body at six different spots, spanning from just posterior of the opercle to the caudal peduncle (Figure 1). Each scale was then imaged using a digital camera mounted to a Leica stereomicroscope, taken at 15x magnification. From these images, various measurements were taken using the ImageJ software program (Schindelin et al., 2012). These measurements included the length of the scale anterior to posterior, the height of the scale dorsal to ventral, the length of the radii, the length of the anterior margin of the radii, the length of the posterior margin of the radii, the angle at which the radii extend to the focus, and the total number of radii present (Figure 2). To remove the effects of allometry on scale shape, all measurements were converted into residual data by normalizing to standard length.

Construction of Cichlid Genotypes

For all F2 hybrids and wild-caught parentals, SNPs were identified and genotyped using restriction-site-associated DNA sequencing (RAD-seq). After genomic DNA was extracted, digested, purified, and processed into RAD libraries from each individual, it was sequenced using Illumina HiSeq 2000 and was

aligned to the reference cichlid sequence (Metriaclima zebra v.0, http://cichlid.umd. edu/cichlidlabs/kocherlab/bouillabase.html). Once high-quality SNPs were identified, 3087 SNPs were selected for the final marker data set used for linkage map construction. These SNPs were narrowed further based on Mendelian inheritance to 948 loci, which assembled into 24 linkage groups. Levels of genetic divergence was estimated in the SNP dataset using F statistics (Fst), with 0.57 being an empirical threshold for a signature of divergence between cichlid genera (Mims et al., 2010). Genotyping and linkage map construction was done previously and details are provided in Albertson et al., (2014).

QTL Analysis

QTL analyses were conducted using R statistical language, following scripts in Broman & Sen (2009) and Arends et al., (2010). Significant QTL/markers were selected as potential cofactors and verified by backward elimination during multiple-QTL mapping (MQM) scans. This resulted in logarithm of the odds (LOD) scores for each trait, showing the number and genomic location of potential genes or genetic elements controlling each trait. Finally, markers of significant LOD scores were cross referenced to the cichlid reference genome and compared to a previously compiled Fst dataset from Albertson et al., (2014) to identify possible candidates (Albertson et al., 2014).

Figure 1: Position of Scales Taken from Each Individual. Scales were removed from the midline of the body at six different positions (1-6) from anterior to posterior, and imaged using a digital camera mounted to a Leica microscope.

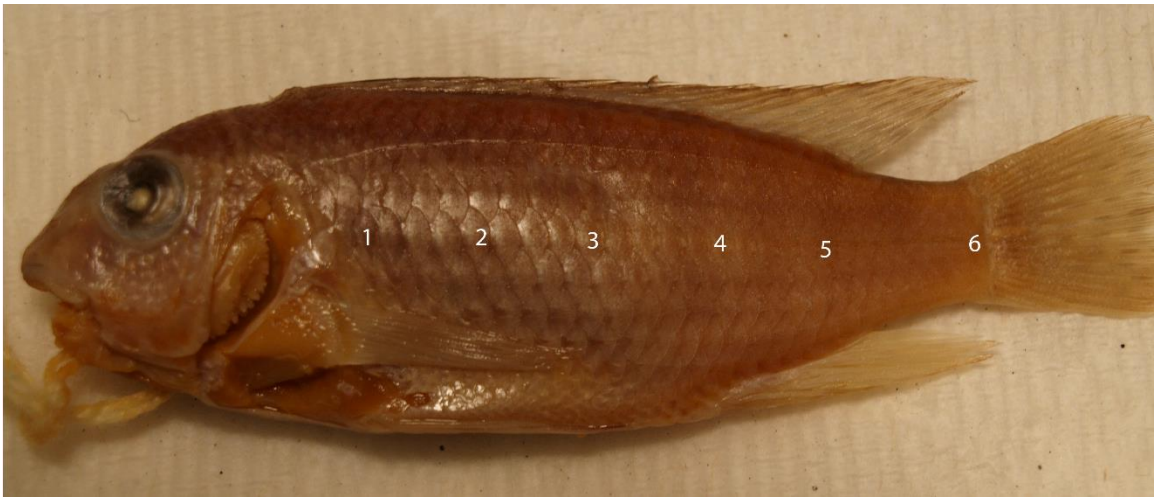
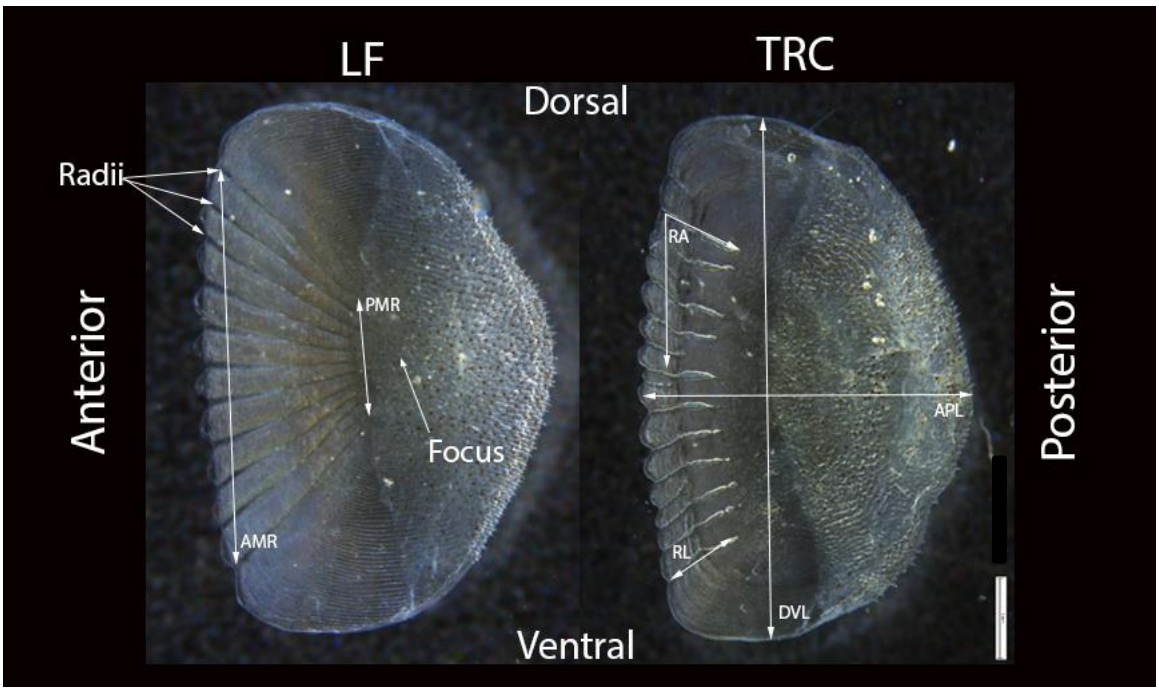


Figure 2: Scale Traits and Anatomy. Abbreviations for scale traits are as followed: DVL= Dorsal-Ventral Length, APL= Anterior-Posterior Length, RA= Radial Angle, RL= Radial Length, AMR= Length of the Anterior Margin of the Radii, PMR= Length of the Posterior Margin of the Radii



CHAPTER 3

RESULTS

Divergent Traits Between Parental Species

Phenotypic divergence was observed between scales of parental species. For all the traits except angle of the radii to the focus (Figure 3g), scale positions 3 and 5 consistently showed significant divergence between parental species (Figure 3a-f, 3h), with the most prominent divergence in the dorsal to ventral scale height, length of the anterior margin of the radii, and number of radii (Figures 3a, 3e, 3h). In other traits, significant divergences were evident to a lesser degree (Figures 3b, 3c, 3d, 3f).

F2 Hybrids as Intermediates

In F2 hybrids, there was a wide range of variation in scale morphology between individuals. When comparing measurements of F2 hybrids to parental species, they were largely intermediate LF and TRC. This is consistent with an incomplete dominant mode of inheritance.

Principal Component Analysis

In collaboration with a postdoc in the lab, we also mapped QTL for geometric measures (GM) of shape variation. This involved placing landmarks at homologous points on scales 3 and 5 in the F2 hybrids. The analysis itself is outside the scope of my thesis, but in brief the results yielded geometric

descriptors of shape variation, called partial warps. These were subjected to a data reduction analysis (i.e., PCA), which yielded a set of 3 principal component axes that collectively described the majority of the shape variation in the F2. The type of variation described by each axis can be depicted via deformation grids of x,y coordinate systems (Figures 4a-d; PC3 not shown). These shape variables (PCs) were also subjected to QTL mapping.

Results of the QTL Analysis

QTL analysis reveals numerous regions of the cichlid genome that underlie divergence of specific phenotypic traits. In total, 42 significant QTL were identified across all traits measured- 12 QTL for scale 3 traits, 21 QTL for scale 5 traits, and 9 QTL for morphometric measurements. Significant LOD scores were found on 19 of the 25 linkage groups (Table 1).

There are several linkage groups that possess overlapping significant QTL. Linkage groups 7 and 12 each have four overlapping QTL. QTL on linkage group 12 are all scale 5 traits as well as three of the four QTL on linkage group 7. Linkage group 17 possesses three overlapping QTL from both scale 3 and scale 5 traits, and nearly identical overlapping QTL are found on linkage groups 5, 6, and 20 (Figure 5). These overlapping QTL suggest that the associated traits may share a common genetic basis – e.g., pleiotropy.

Anterior Margin of the Scale Shows Strong Overlapping Signals

Linkage group 17 at 40 cM appears to have strong effects on the length of the anterior margin of the scale, as both scales 3 and 5 possess high LOD scores at this locus (Table 1). Both of these traits show a high allele effect from Lf/Lf genotypes and additive genetic variation. The marker closest to the peak LOD score falls right within the gene *snd1*, involved in viral carcinogenesis in humans and C-MYB transcription factor network pathways (Tsuchiya and Nakagama, 2010; Quintana et al., 2011). More interestingly, this locus is downstream from *col7a1,* a gene responsible for producing type VII collagen fibrils, or anchoring fibrils (Parente et al., 1991). These fibrils may be present in the deep collagenous layer, attaching the scale to the epithelium of the fish.

QTL for length of the anterior margin of the scale consistently shows high LOD scores across both scales 3 and 5. In addition to the QTL on linkage group 17, a high LOD score (5.54) was found at linkage group 18 at 0 cM for length of the anterior margin of scale 3 (Table 1). With the highest LOD score of all QTL (6.65), length of the anterior margin of scale 5 on linkage group 5 lies in a particular interesting interval (Table 1). The peak LOD score lies downstream of *eIF6*, a regulator of TGF-β1 and an important functional component of hemidesmosomes (Yang et al., 2015; Sanvito et al., 1999).

Candidate Genes for Linked Traits

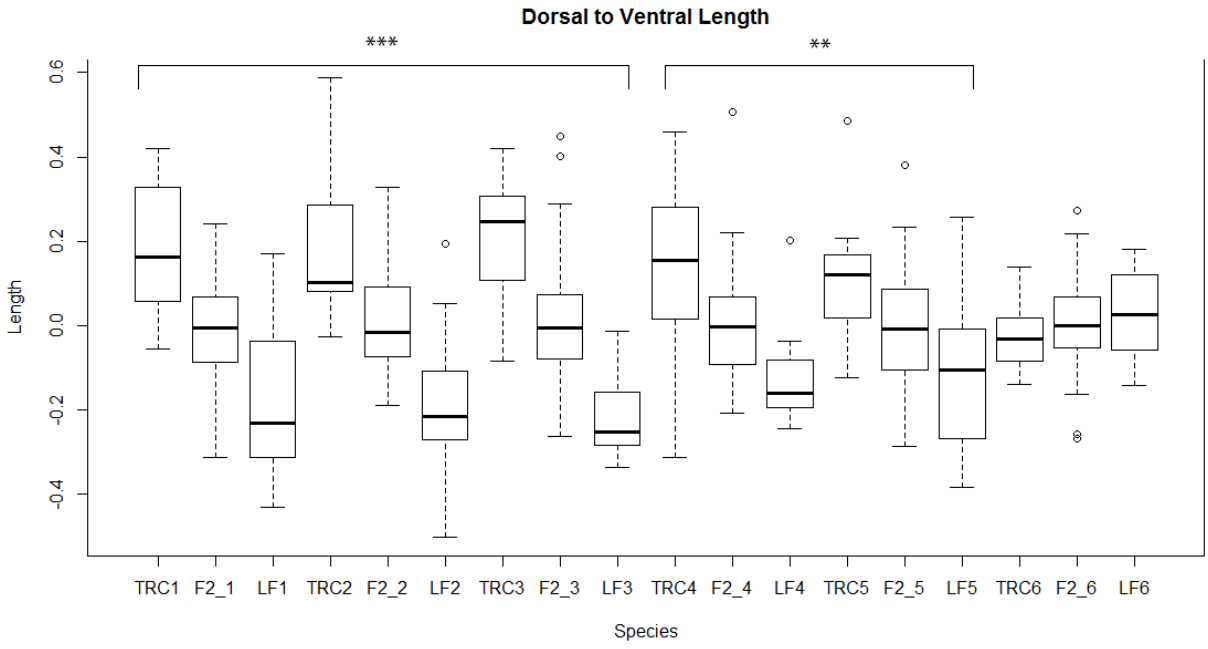
The locus that affected the most traits in this analysis was on LG 7. In particular, overlapping QTL for dorsal-ventral length for scale 5, PC2 scores for scales 3 and 5, and anterior-posterior length for scale 5 were all noted at this locus. Notably, two strong candidate genes are associated with these QTL. *Fgfr1b* and *Efna5a* are located on LG 7 at 50 cM and 45 cM, respectively, which is squarely associated with peak LOD scores (Figure 5, Table 1). Both are well known genes that play roles in scale and feather development. Previous studies have shown *fgfr1a* (the paralog of *fgfr1b*) to play a role in scale number and patterning (Rohner et al., 2009). *Efna5a* belongs to the ephrin ligand family, where numerous members of this family have been shown to play a role in placode development and polarity in pigeon feathers (Suksaweang et al., 2013; Vickrey et al., 2015).

Another strong candidate gene is *Col1a1a*, located on linkage group 4 at 57 cM (Figure 5). Linked to QTL for PC2 of scale 3 (Table 1), this gene is responsible for producing type I collagen, which are also found in the deep collagenous layer of scales (Le Guellec, Dubois, & Sire, 2004).

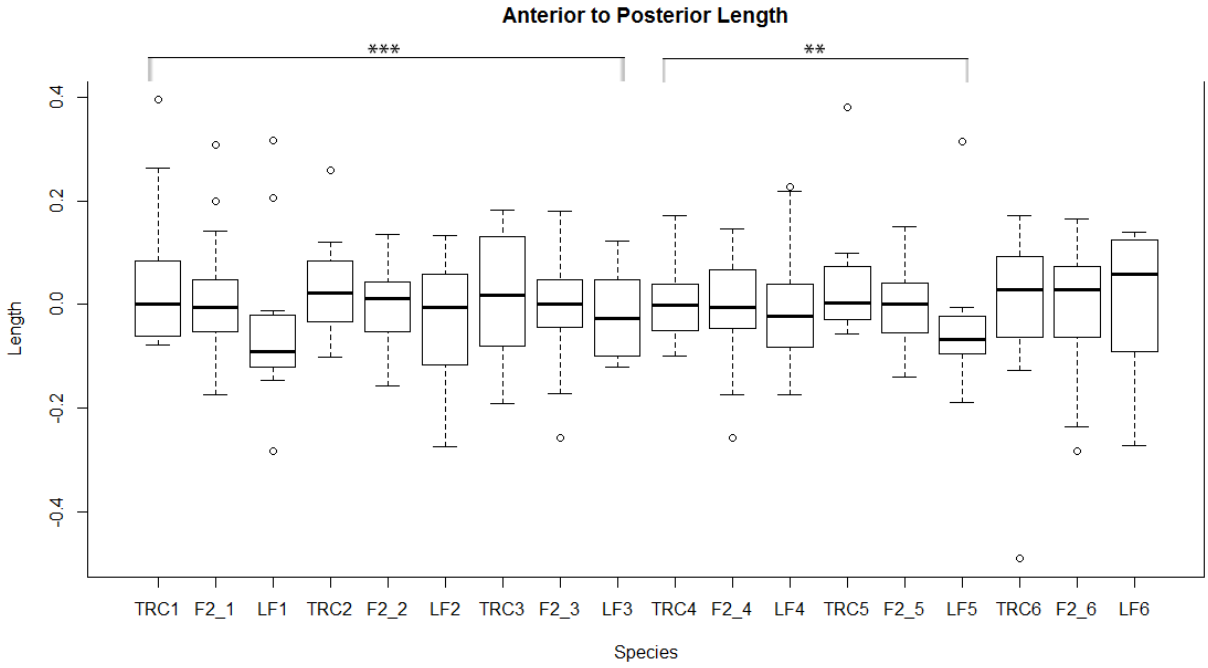
On linkage group 9 at 33 cM, *TGIF1* is linked to QTL for angle of the radii to the focus and PC1 of scale 3, and radii length of scale 5 (Figure 5, Table 1). This gene is known for its role in nodal signaling and brain development (Gondré-Lewis et al., 2015; Taniguchi et al., 2012).

Figure 3: Box plots for measurements across scales 1-6 in each species (TRC and LF) and the F2. Boxplots were created using R statistical language, including analysis of variance (ANOVA) to determine significant differences between parental traits. For all panels (a-h), significance is represented as follows: 0; '***' 0.001; '**' 0.01; '*' 0.05; '.' 0.1; ' ' >0.1. The X-axis of each panel (a-h) is labelled by species (TRC, LF, or F2) and scale position (1-6).

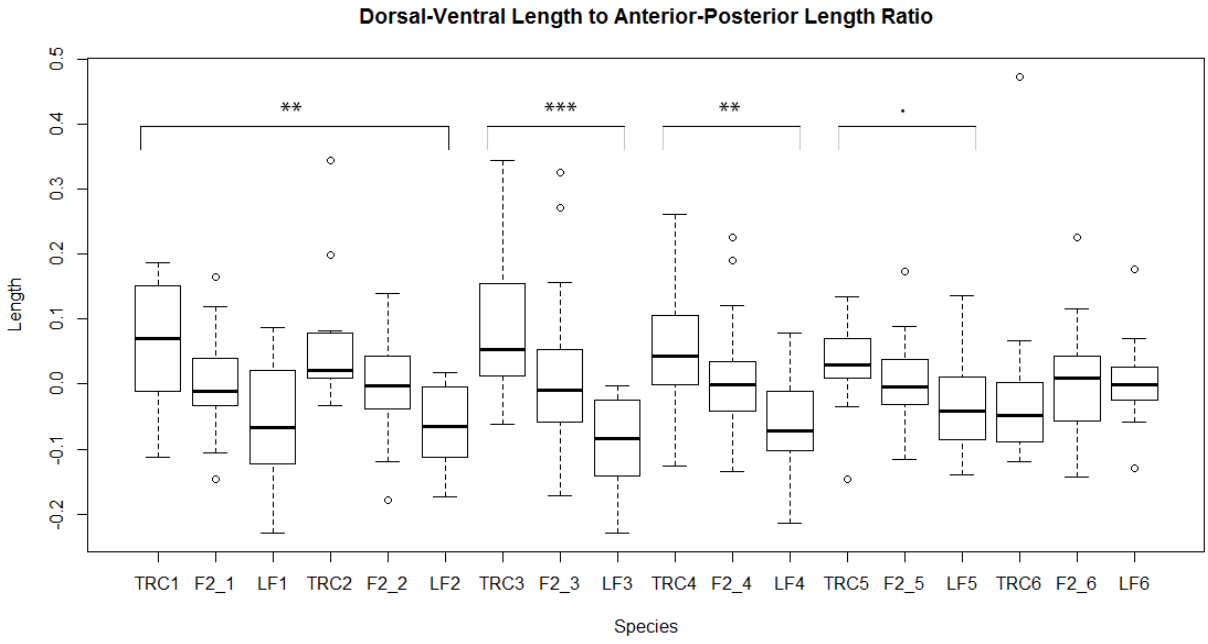
3a: Dorsal to Ventral Scale Length



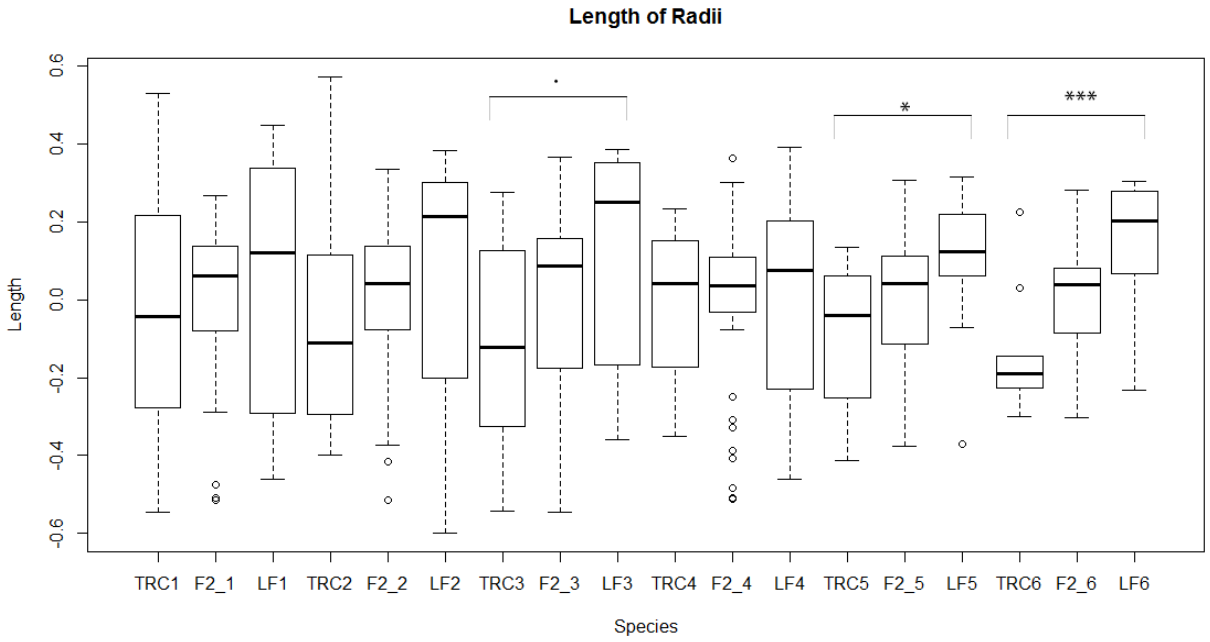
3b: Anterior to Posterior Scale Length



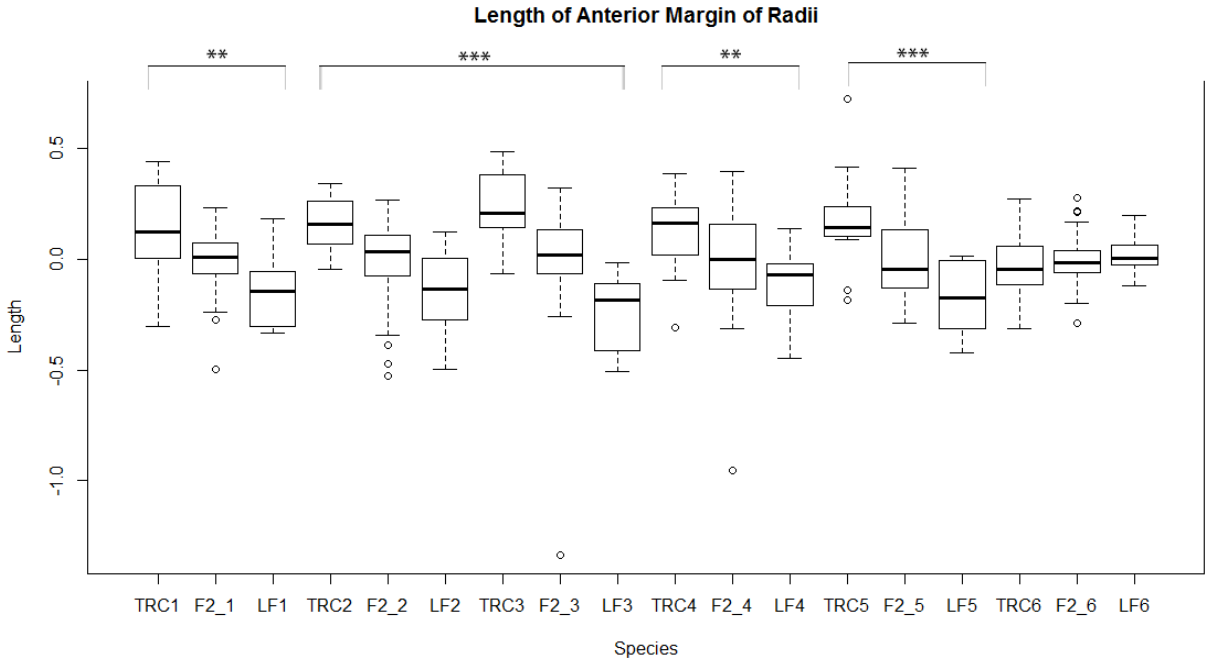
3c: Dorsal-Ventral Length to Anterior-Posterior Length Ratio



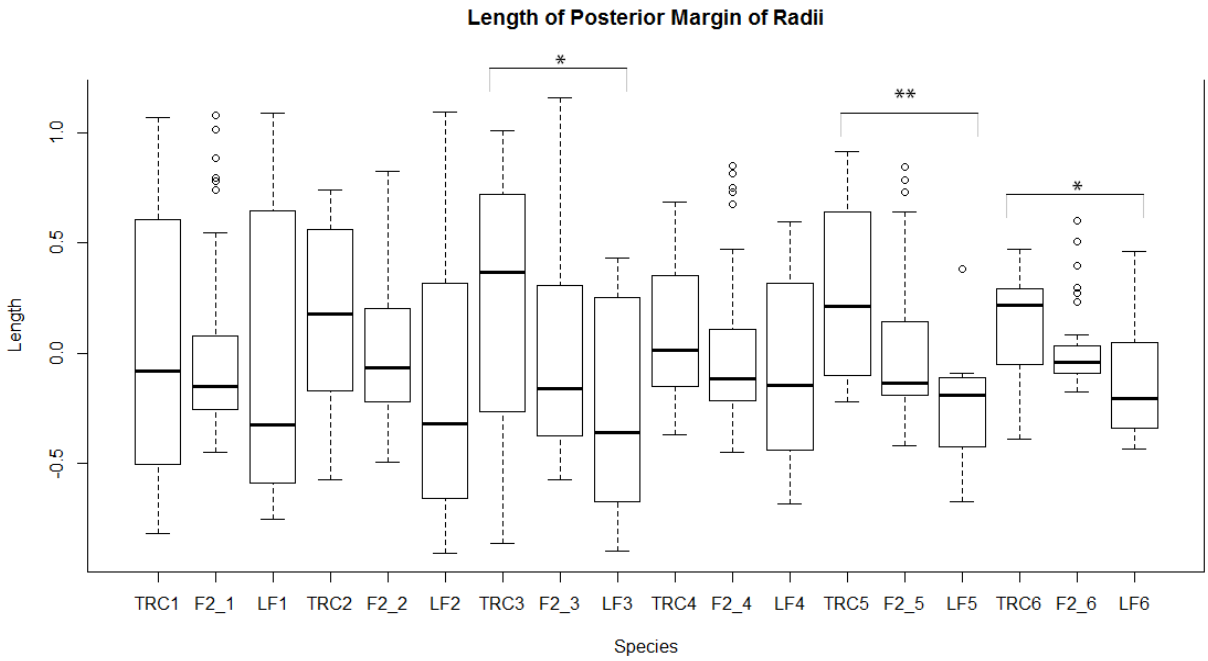
3d: Radii Length



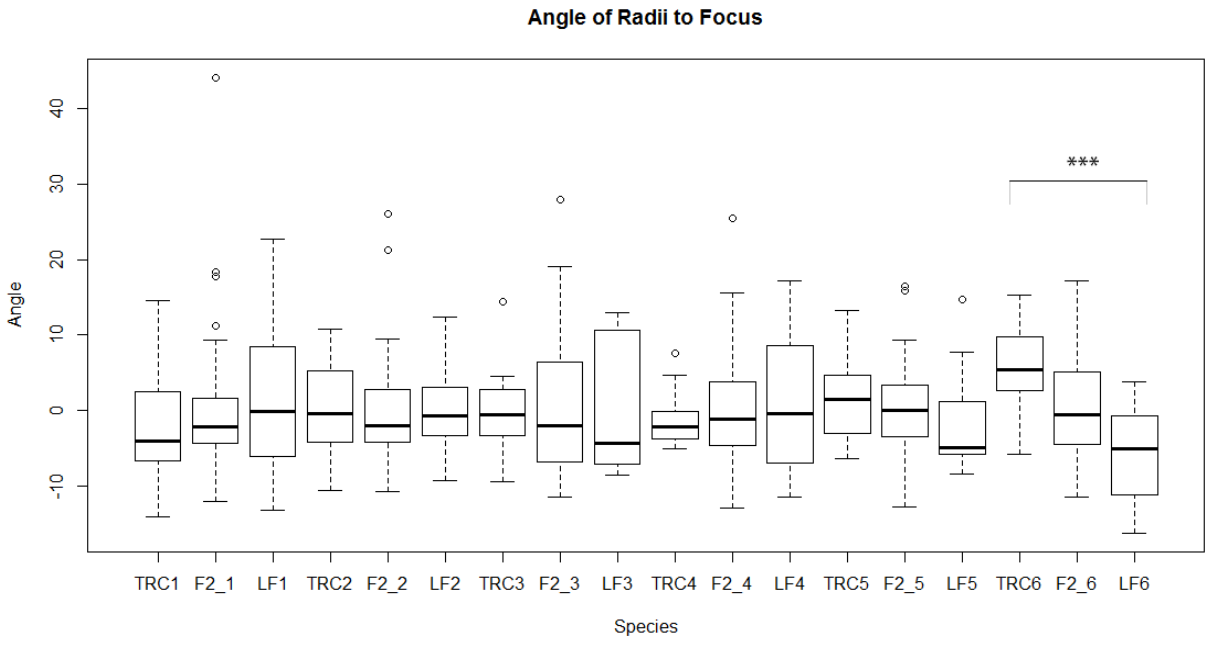
3e: Anterior Margin of Radii Length



3f: Posterior Margin of Radii Length



3g: Radii to Focus Angle



3h: Total Radii

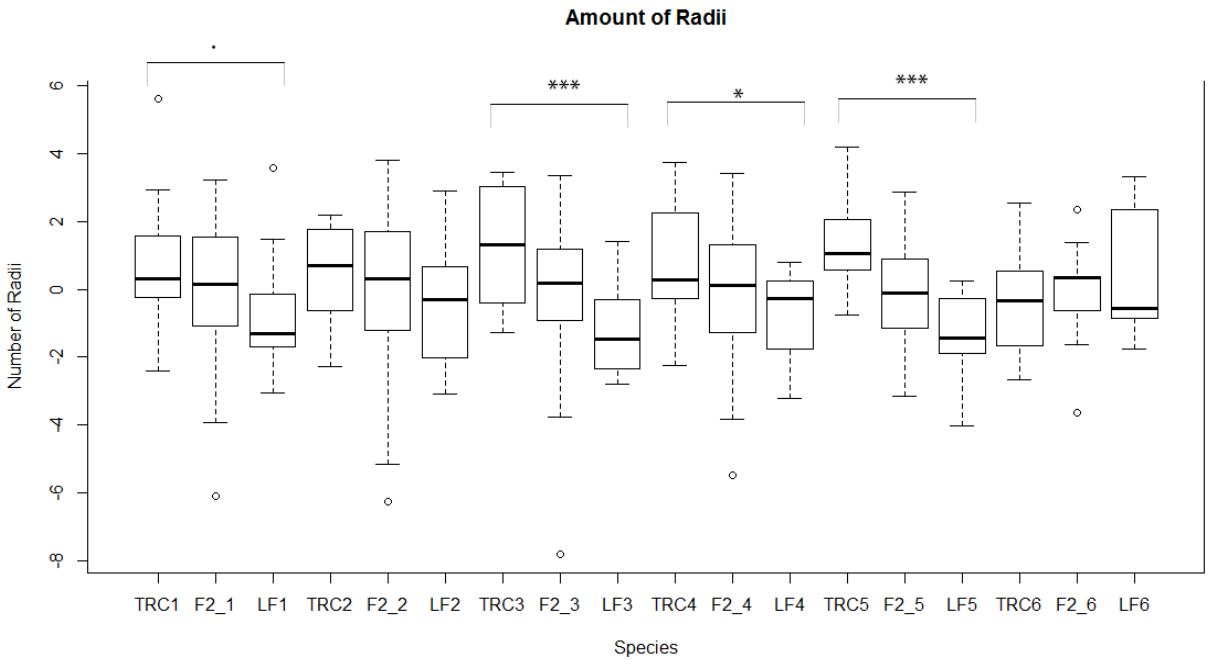
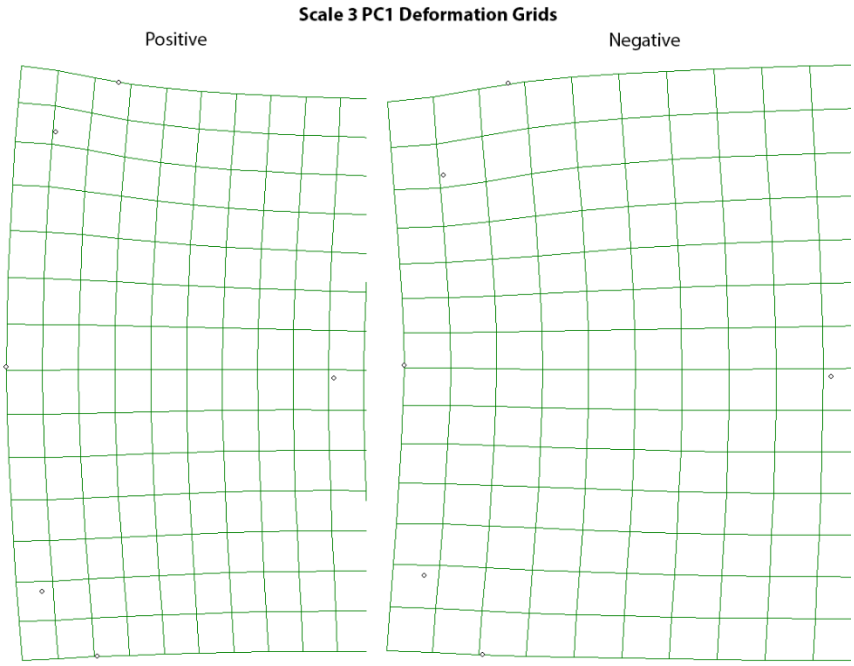
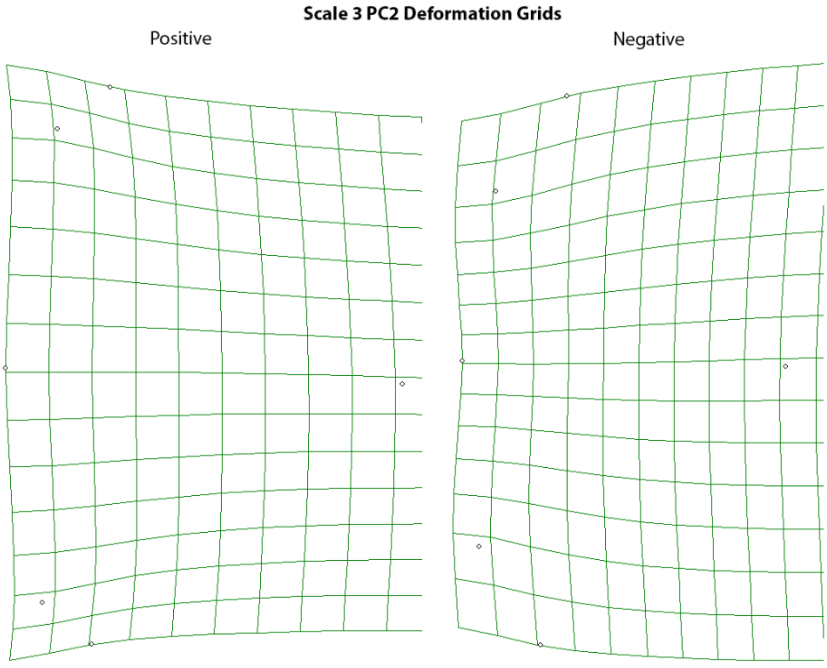


Figure 4: Deformation Grids from Principal Component Analysies of Scale Shape. All panels (a-d) show shape variation along an X-Y coordinate system, according to different principle components for scale positions 3 and 5. This data was gathered by a fellow post-doc (Kara Powder).

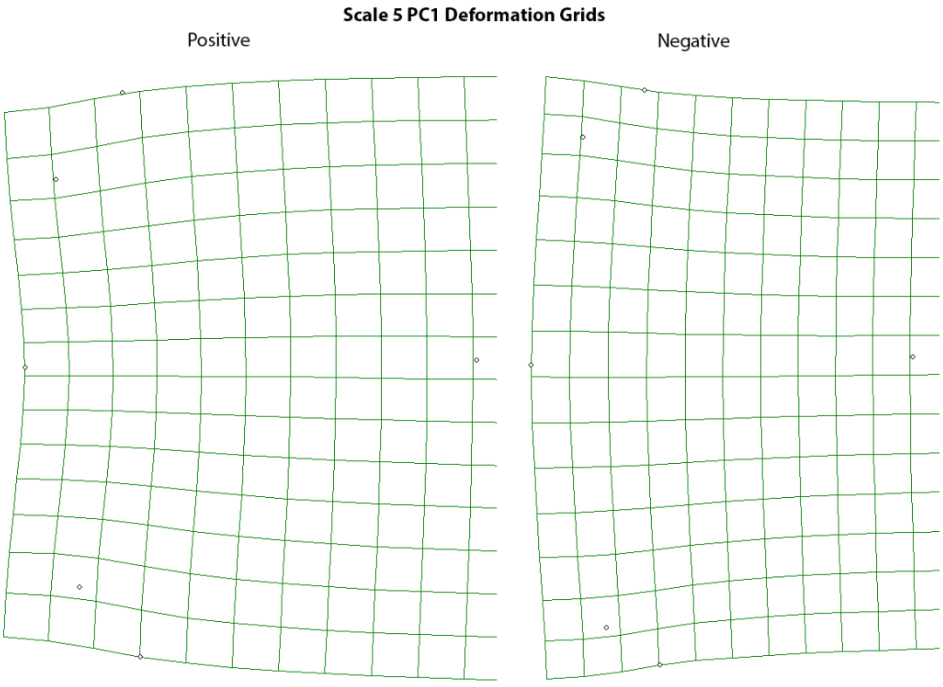
4a: Scale 3 PC1



4b: Scale 3 PC2



4c: Scale 5 PC1



4d: Scale 5 PC2

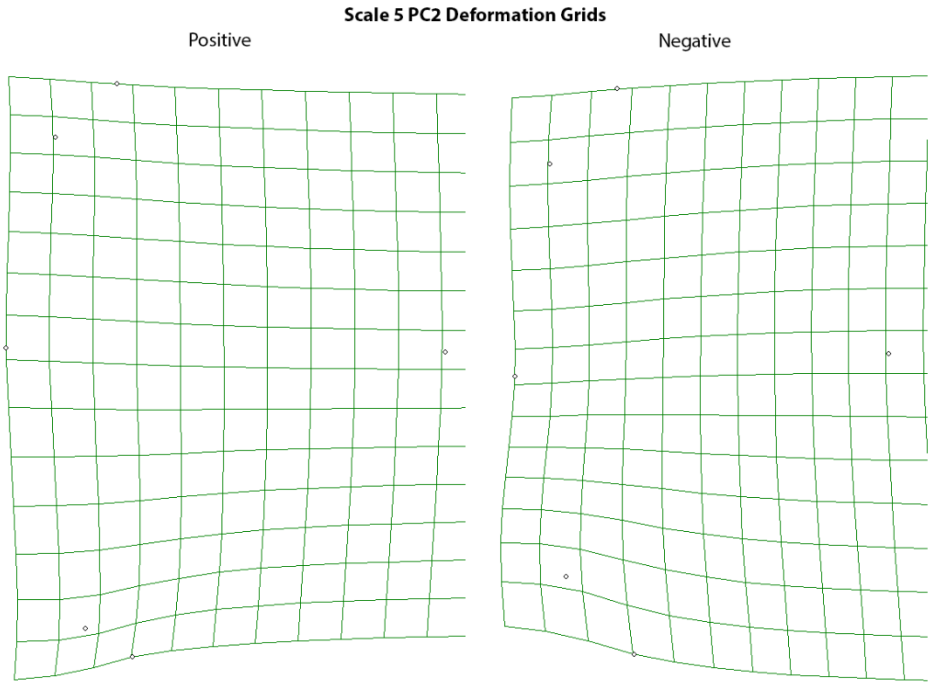


Figure 5: Genetic Map of Significant QTL. Only linkage groups with significant QTL are present (out of a total 24 LGs). Red lines represent scale 3 QTL, blue lines represent scale 5 QTL, and green lines represent QTL for scale shape disparity across the flank (not discussed). Putative candidate genes are also labelled according to their position in the cichlid genome (Cambridge Cichlid Browser)

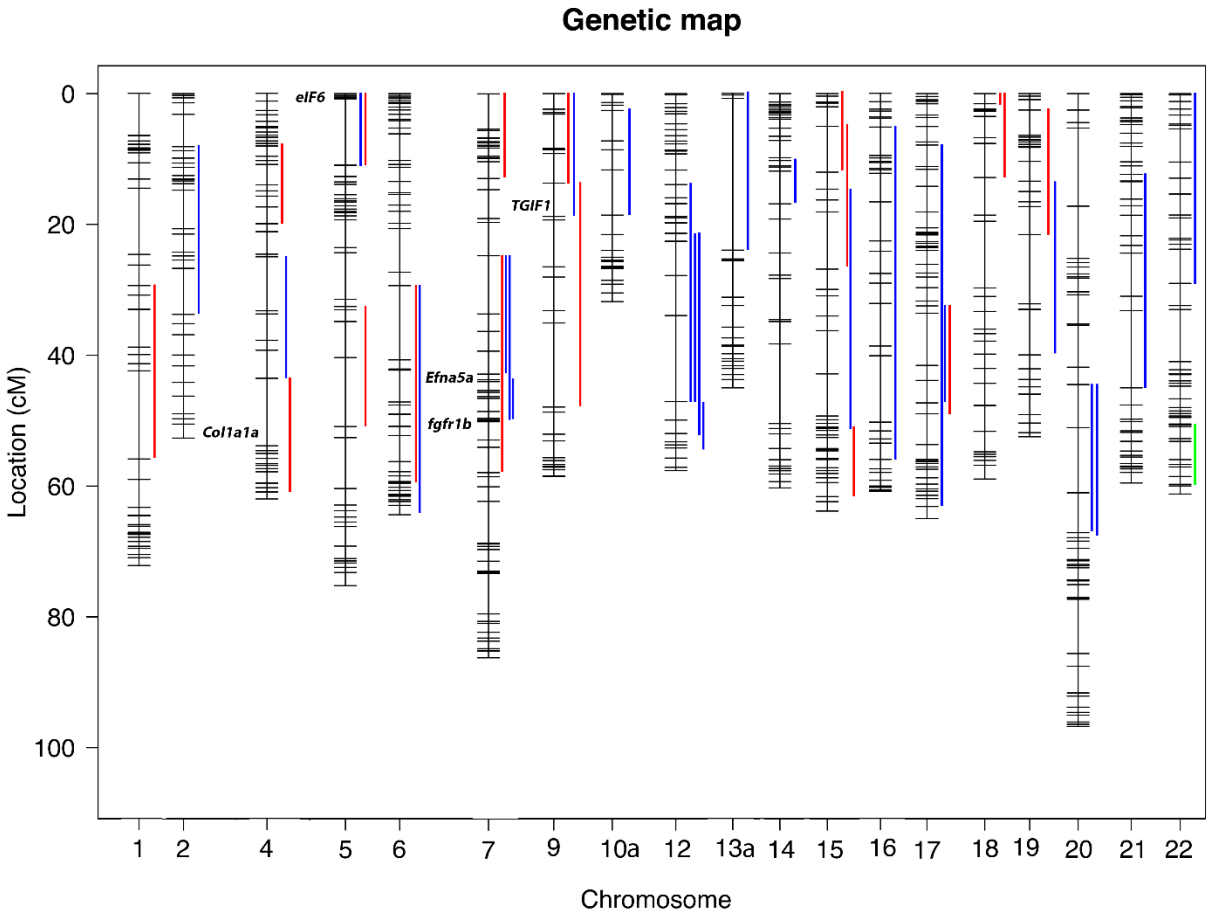


Table 1: List of quantitative trait loci (QTL) affecting scale shape variation in cichlids

							Allele effects				
Traits	**QTL**	**LG**	**cM**	**QTL interval**	**LOD**	**PVE (%)**	Lf/Lf	Lf/Trc	Trc/Trc	**Add**	**Dom**
Radii Angle 3	3RA1	15	0	0.0-12.0	3.75	7.34	0.5095	-1.3100	-2.7279	1.6187	-0.2008
	3RA2	4	20	9.6-21.2	4.41	8.60	-1.1471	0.4495	-3.9704	1.4116	3.0083
	3RA3	9	5	0.0-13.7	4.60	8.95	-0.4676	0.2223	-5.3008	2.4166	3.1065
Anterior Margin of Radii 3	3AMR1	17	40	32.5-53.7	3.74	7.35	0.0555	-0.0120	-0.0035	0.0295	-0.0380
	3AMR2	5	45	34.9-50.9	3.44	6.77	-0.0317	0.0159	0.0514	-0.0415	0.0061
	3AMR3	18	0	0.0-1.5	5.54	10.67	-0.0875	0.0408	0.0314	-0.0595	0.0688
	3AMR4	19	10	2.5-21.6	3.75	7.36	0.0052	-0.0113	0.0621	-0.0284	-0.0450
Posterior Margin of Radii 3	3PMR1	7	0	0.0-14.6	4.68	9.10	-0.0561	-0.0382	-0.1283	0.0361	0.0540
	3PMR2	6	45	29.4-59.3	3.27	6.45	-0.0175	-0.1431	0.0134	-0.0155	-0.1410
	3PMR3	15	15	5.0-26.8	4.47	8.71	0.0369	-0.1451	-0.0445	0.0407	-0.1413
	3PMR4	1	35	24.6-55.9	3.45	6.78	-0.0130	-0.1448	0.0331	-0.0231	-0.1548
Number of Radii 3	3NR1	18	5	0.0-12.8	5.00	9.69	-0.6920	0.4751	0.1887	-0.4404	0.7267
Dorsal-Ventral Length 5	5DVL1	7	45	24.7-50.1	5.04	9.76	-0.0398	-0.0106	0.0503	-0.0451	-0.0159
	5DVL2	12	25	13.9-47.1	3.70	7.27	-0.0284	0.0010	0.0505	-0.0392	-0.0100
Anterior-Posterior Length 5	5APL1	7	45	43.7-50.1	5.92	11.37	-0.0207	-0.0043	0.0367	-0.0287	-0.0123
	5APL2	21	35	13.5-45.0	4.74	9.21	0.0078	0.0104	-0.0094	0.0086	0.0112
Dorsal-Ventral to Anterior-Posterior Ratio 5	5DVAP1	6	60	29.4-64.4	5.66	10.89	-0.0054	-0.0105	0.0101	-0.0077	-0.0128
	5DVAP2	4	35	25.0-43.6	4.36	8.50	0.0007	0.0077	-0.0237	0.0122	0.0192
	5DVAP3	16	40	5.2-56.0	3.33	6.55	0.0075	-0.0143	0.0079	-0.0002	-0.0219
	5DVAP4	2	15	8.1-33.8	3.66	7.19	-0.0368	0.0020	0.0121	-0.0244	0.0143
	5DVAP5	12	25	22.6-47.1	6.30	12.04	-0.0100	-0.0143	0.0253	-0.0177	-0.0220
Radii Length 5	5RL1	22	5	0.0-29.0	3.85	7.55	-0.0140	0.0724	-0.0853	0.0357	0.1220
	5RL2	14	10	9.8-16.8	6.01	11.53	-0.0465	0.0190	0.0005	-0.0235	0.0420
	5RL3	20	50	44.5-67.9	5.45	10.51	0.0463	0.0177	-0.0881	0.0672	0.0386
	5RL4	9	15	0.0-19.3	3.60	7.08	0.0431	0.0069	-0.0450	0.0440	0.0079
Radii Angle 5	5RA1	17	45	7.9-63.1	3.20	6.32	0.0289	0.0252	1.0641	-0.5176	-0.5213
	5RA2	12	55	47.1-57.7	3.91	7.66	1.4209	1.0462	-1.9119	1.6664	1.2916
	5RA3	19	15	13.4-40.0	4.08	7.97	-0.7849	-0.0394	2.8132	-1.7991	-1.0536
Anterior Margin of Radii 5	5AMR1	17	40	32.5-47.3	3.76	7.38	0.0334	-0.0091	-0.0091	0.0213	-0.0213
	5AMR2	5	5	0.0-11.0	6.65	12.67	-0.0710	0.0330	0.0375	-0.0542	0.0497
	5AMR3	12	50	22.6-53.7	4.99	9.68	-0.0636	0.0045	0.0179	-0.0408	0.0273
Posterior Margin of Radii 5	5PMR1	13a	5	0.0-24.0	3.16	6.24	-0.0536	0.0480	-0.0543	0.0004	0.1019
Number of Radii 5	5NR1	15	35	18.1-51.5	4.65	9.05	0.3334	-0.2297	-0.6029	0.4682	-0.0950
Disparity	Dp1	22	60	52.8-61.2	4.81	9.33	0.0066	0.0084	0.0081	-0.0007	0.0011
Scale 3 PC1	3PC1a	9	35	13.7-47.9	3.74	7.33	-0.0150	0.0022	0.0015	-0.0082	0.0089
Scale 3 PC2	3PC2a	7	50	24.7-57.9	5.06	9.80	0.0138	-0.0019	-0.0100	0.0119	-0.0038
	3PC2b	15	50	49.3-61.6	4.18	8.16	0.0003	-0.0063	0.0202	-0.0099	-0.0166
	3PC2c	4	60	43.6-62.0	4.36	8.51	-0.0166	0.0027	0.0068	-0.0117	0.0076
Scale 5 PC1	5PC1a	5	5	0.0-11.0	4.93	9.55	-0.0193	0.0095	0.0009	-0.0101	0.0186
Scale 5 PC2	5PC2a	7	35	24.7-42.8	6.11	11.71	0.0012	0.0010	-0.0065	0.0039	0.0037
	5PC2b	10a	10	2.6-21.5	3.40	6.69	-0.0181	0.0068	0.0015	-0.0098	0.0152
Scale 5 PC3	5PC3a	20	55	44.5-67.1	3.83	7.51	-0.0025	0.0010	0.0011	-0.0018	0.0017

CHAPTER 4

DISCUSSION

Advantages of Scale Variation

Variation in scale morphology may provide unique advantages to each species. *Tropheops* “red cheek” cichlids tend to be more aggressive than the *Labeotropheus fuelleborni*, as well as possessing different feeding mechanisms. While both maintain a diet of primarily algae, TRC cichlids tend to feed by jerking and twisting their body and LF cichlids tend to scrape algae off rocks using a specialized feeding apparatus (Concannon and Albertson, 2015). Having shorter radii fashioned more parallel may provide a wider range of motion for TRC individuals, while also providing an easier escape mechanism from aggressive competitors. In general, shape variation in scales is essential for the adaptability of fish occupying different environments, as they provide protection from both predators and environmental pathogens while allowing for varying levels of hydrodynamics when swimming. Thus, the plasticity of fish scales is vital when introducing new environmental factors.

Fgfr1b is a Strong Candidate Gene

The results from the QTL analysis show promising candidate genes that underlie cichlid scale development, including *fgfr1b*, *efna5a*, *col1a1a*, *eIF6*, and *TGIF1*. Previous studies have shown many of these genes to play roles in scale development and morphology, particularly *fgfr1b*. This gene belongs to the family

of fibroblast growth factors, responsible for essential developmental events including cell proliferation, differentiation, migrations, and survival (Itoh and Ornitz 2004; Katoh and Katoh 2006). In a study by Rohner et al., *spiegeldanio* mutants (a mutation in fgfr1) show a near complete loss of scales along the flank, aberrant scale patterning, and dorsolateral elongation of the remaining scales, which appear to be larger than the wild-type. As one of the genes possessing the most overlapping QTL intervals, this study further strengthens the argument for *fgfr1b* involvement in scale development and suggests that studying mutants may inform us about the adaptation of scale shape and development (Rohner et al., 2009).

Candidate Genes Involved in Placode Formation

Almost adjacent to *fgfr1b* lies the gene *efna5a*. Belonging to the Ephrin family, these proteins play important roles in the regulation of cell migration and adhesion, as well as developmental patterning and morphogenesis. In general, Ephrin-A ligands preferentially bind to EphA receptors and are involved in adhesion while Ephrin-B ligands preferentially bind to EphB receptors and are involved in repulsion (Poliakov et al., 2004; Xu et al., 2000). However, Ephrin-A5 (produced by *efna5a*) is an exception, as it can interact with EphB2 receptors forming a heterodimer complex (Himanen et al., 2004). While no studies to date have shown the role of *efna5a* in scale development, other members in this family have been implicated in the development of pigeon feather placodes (Suksaweang et al., 2013). Interestingly, a study by Vickrey et al. demonstrated

two different missense mutations in the intracellular kinase domain of *EphB2* that were responsible for crests of reversed occipital feathers in two different species of pigeons (Vickrey et al., 2015). Thus, *efna5a* may have a role in placode development and shape in cichlid scales, but further research is required to confirm this.

TGF-β Pathway

The remaining candidate genes, *TGIF1*, *eIF6*, and *col1a1a*, each play a role in the transforming growth factor beta (TGF-β) pathway. This pathway is also responsible for cell growth, differentiation, and morphogenesis (Ignotz and Massagué, 1985; Massagué, 1990). In relation to scales, TGF-β increases the expression of extracellular matrix proteins, including type 1 collagen, which is produced by *col1a1a* (Ignotz and Massagué, 1985; Pan et al., 2013). *TGIF1* acts as a repressor of TGF-β-activated transcription while *eIF6* increases transcription of *TGF-β1* (Wotton et al., 1999; Sanvito et al., 1999). *TGF-β1* has been observed to induce expression of *col1a1* in cardiac fibroblasts (Pan et al., 2013). Also, previous studies have shown *TGF-β2* is expressed in developing chicken feathers, and can be ectopically expressed to induce dermal condensation, thus implicating its role in induction and differentiation of epidermal appendages (Ting-Berreth and Chuong, 1996; Jakowlew et al., 1994). Upon speculation, regulation of TGF-β-activated transcription and *TGF-β1* transcription through *TGIF1* and *eIF6*, respectively, could allow for specified proliferation and differentiation of scale papillae of as well as increased collagen production in scales.

Speculative Pathway of Scale Formation

Our data contributes to previous research that suggests the pathways responsible for scale development can be attributed to at least the genes aforementioned. However, the possible interactions between these genes is unknown. From what knowledge is available, we can surmise that *Efna5a* helps placode boundaries form, followed by *TGIF1* repressing TGF-β in cells outside the placode to initiate dermal condensation and formation of scale papillae. Then, expression of *eIF6* may eventually induce expression of *col1a1a* to help create the collagenous layer of the scale. Expression of *fgfr1b* is likely present throughout the developmental process, aiding in cellular differentiation and proliferation.

Targets of Future Research

While these genes appear to be strong candidates for scale development pathways based on strong QTL peaks, other significant QTL intervals that lie in gene deserts or do not appear to have obvious candidate genes may be targets of future research. It is possible that these QTL could lead to new insights into the molecular regulation of scale development.

Co-Localization of QTL and Pleiotropy

The co-localization of QTL intervals was noted for several traits. In particular, scales 3 and 5 QTL overlapped on linkage groups 5, 7, and 17. These traits include PC2 scores and length of the anterior margin of the radii for both scales. This observation strengthens the argument for these particular QTL intervals underlie the development of scales across the body. In addition, several linkage groups possess multiple QTL intervals for different traits. These traits may share a common genetic basis and possible pleiotropic effects, where one gene effects multiple aspects of scale development.

Pleiotropy is an important mechanism in evolution and development. Developmental pleiotropy occurs when a single gene acts in multiple tissues and can act in differing developmental stages. Thus, deleterious mutations in pleiotropic developmental genes can have devastating effects on the organism (Powder and Albertson, 2015). However, adaptive mutations can also have pleiotropic effects, with deleterious effects suppressed through the fixation of compensatory mutations. Studies have shown that pleiotropic effects may facilitate adaptation and increase the evolutionary rate of organisms (Camps et al., 2007; Razeto-Barry et al., 2011).

CHAPTER 5

CONCLUSION

Uncovering the Mysteries of Life One Scale at a Time

The results from this study enhance our knowledge of the genetic mechanisms of scale development and morphology, as well as provide an important example of how non-traditional model systems can be further utilized to expand our knowledge of developmental traits.

From the QTL analysis, the genes *fgfr1b*, *efna5a*, *TGIF1*, *eIF6*, and *col1a1a* may play important new roles in scale development and attribute to the difference in scale morphology between cichlid species. Further research should be performed on QTL intervals possessing unknown genetic regions as well as confirming the interactions between the selected candidate genes.

Studying the causes of scale variation provides insight on the adaptability of fish occupying different environmental niches, and represents how an organism's lifestyle can ultimately lead to evolved changes in their genome. This knowledge brings us one step closer to understanding the complex lifeforms that inhabit this planet.

BIBLIOGRAPHY

Albertson, R. C., Powder, K. E., Hu, Y., Coyle, K. P., Roberts, R. B., & Parsons, K. J. (2014). Genetic basis of continuous variation in the levels and modular inheritance of pigmentation in cichlid fishes. *Molecular Ecology*, *23*(21), 5135–5150. http://doi.org/10.1111/mec.12900

Arends D, Prins P, Jansen RC, Broman KW (2010) R/qtl: High- throughput multiple QTL mapping. Bioinformatics, 26, 2990– 2992.

Bloomquist, R. F., Parnell, N. F., Phillips, K. A., Fowler, T. E., Yu, T. Y., Sharpe, P. T., & Streelman, J. T. (2015). Coevolutionary patterning of teeth and taste buds. *Proceedings of the National Academy of Sciences*, 201514298. http://doi.org/10.1073/pnas.1514298112

Broman KW, Sen S (2009) A guide to QTL mapping with R/qtl. Springer, Dordrecht, Heidelberg, London, New York.

Camps, M., Herman, A., Loh, E., & Loeb, L. a. (2007). Genetic constraints on protein evolution. *Critical Reviews in Biochemistry and Molecular Biology*, *42*(768615718), 313–326. http://doi.org/10.1080/10409230701597642

Concannon, M. R., & Albertson, R. C. (2015). The genetic and developmental basis of an exaggerated craniofacial trait in East African cichlids. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution, 324(8), 662–670. http://doi.org/10.1002/jez.b.22641

Cooper, J. W., Wirgau, R. M., Sweet, E. M., & Albertson, R. C. (2011). Deficiency of zebrafish fgf20a results in aberrant skull remodeling that mimics both human cranial disease and evolutionarily important fish skull morphologies, *4*(164), 426–441. http://doi.org/10.1126/scisignal.2001449.Engineering

Daane, J. M., Rohner, N., Konstantinidis, P., Djuranovic, S., & Harris, M. P. (2015). Parallelism and Epistasis in Skeletal Evolution Identified through Use of Phylogenomic Mapping Strategies. *Molecular Biology and Evolution*, *33*(1), 1–12. http://doi.org/10.1093/molbev/msv208

Danley, P. D., & Kocher, T. D. (2001). Speciation in rapidly diverging systems: lessons from Lake Malawi. Molecular Ecology, 10(5), 1075–1086. http://doi.org/10.1046/j.1365-294X.2001.01283.x

Di-Poï, N., & Milinkovitch, M. C. (2016). The anatomical placode in reptile scale morphogenesis indicates shared ancestry among skin appendages in amniotes, (June), 1–9. http://doi.org/10.1126/sciadv.1600708

Ehrlich, H. (2010). Biological Materials of Marine Origin. *Biological Materials of Marine Origin Biologically-Inspired Systems V*, *1*, 335–340. http://doi.org/10.1007/978-90-481-9130-7

Francillon-Vieillot, H., de Buffrénil, V., Castanet, J., Géraudie, J., Meunier, F. J., Sire, J. Y., … de Ricqlès, A. (1990). *Skeletal Biomineralization: pattern, processes and evolutionary trends. Volume I*. (J. G. Carter, Ed.). New York: Van Nostrand Reinhold.

Ganguly, D. N., & Mookerjee, S. (1947). On the Structure and Development of Ctenoid Scales in Certain Indian Fishes. *Proceedings of the National Institute of Sciences of India*, *13*(6), 331–339. Retrieved from http://www.dli.gov.in/rawdataupload/upload/insa/INSA_1/20005b85_331.pdf

Gondré-Lewis, M. C., Gboluaje, T., Reid, S. N., Lin, S., Wang, P., Green, W., … Herman, M. M. (2015). The human brain and face: Mechanisms of cranial, neurological and facial development revealed through malformations of holoprosencephaly, cyclopia and aberrations in chromosome 18. *Journal of Anatomy*, *227*(3), 255–267. http://doi.org/10.1111/joa.12343

Haara, O., Harjunmaa, E., Lindfors, P. H., Huh, S.-H., Fliniaux, I., Aberg, T., … Thesleff, I. (2012). Ectodysplasin regulates activator-inhibitor balance in murine tooth development through Fgf20 signaling. *Development*, *139*(17), 3189–3199. http://doi.org/10.1242/dev.079558

Harris, M. P., Rohner, N., Schwarz, H., Perathoner, S., Konstantinidis, P., & Nüsslein-Volhard, C. (2008). Zebrafish eda and edar mutants reveal conserved and ancestral roles of ectodysplasin signaling in vertebrates. *PLoS Genetics*, *4*(10). http://doi.org/10.1371/journal.pgen.1000206

Helfman, G. S., Collette, B. B., Facey, D. E., & Bowen, B. W. (2009). *The diversity of fishes: biology, evolution, and ecology*. *Atlantic* (Vol. Second). http://doi.org/10.1007/978-1-4615-2664-3_1

Himanen, J.-P., Chumley, M. J., Lackmann, M., Li, C., Barton, W. a, Jeffrey, P. D., … Nikolov, D. B. (2004). Repelling class discrimination: ephrin-A5 binds to and activates EphB2 receptor signaling. *Nature Neuroscience*, *7*(5), 501–509. http://doi.org/10.1038/nn1237

Huysseune, a, & Sire, J. Y. (1998). *Evolution of patterns and processes in teeth and tooth-related tissues in non-mammalian vertebrates. European journal of oral sciences* (Vol. 106 Suppl).

Ignotz, R. a, & Massagué, J. (1986). Transforming growth factor-beta stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. *The Journal of Biological Chemistry*, *261*(9), 4337–45. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3456347

Itoh, N., & Ornitz, D. M. (2004). Evolution of the Fgf and Fgfr gene families. *Trends in Genetics*. http://doi.org/10.1016/j.tig.2004.08.007

Jakowlew, S. B., Ciment, G., Tuan, R. S., Sporn, M. B., & Roberts, A. B. (1994). Expression of transforming growth factor-beta 2 and beta 3 mRNAs and proteins in the developing chicken embryo. *Differentiation*, *55*(2), 105–118.

Jawad, L. A. (2005). Comparative scale morphology and squamation patterns in triplefins (Pisces: Teleostei: Perciformes: Tripterygiidae). *Tuhinga*, *16*, 137–167.

Katoh, M., & Katoh, M. (2006). FGF signaling network in the gastrointestinal tract (Review). *International Journal of Oncology*, *29*(1), 163–168.

Kocher, T. D. (2004). Adaptive evolution and explosive speciation: the cichlid fish model. *Nature Reviews. Genetics*, *5*(4), 288–298. http://doi.org/10.1038/nrg1316

Kornfield, I., & Smith, P. F. (2000). A FRICAN C ICHLID FISHES: Model Systems for Evolutionary Biology. *Annu. Rev. Ecol. Syst.*, (31), 163–96.

Kuusipalo, L. (1998). Scale morphology in Malawian cichlids. *Journal of Fish Biology*, *52*(4), 771–781. http://doi.org/10.1006/jfbi.1997.0623

Lagler, K. F. (1947). Lepidological Studies 1. Scale Characters of the Families of Great Lakes Fishes, Vol. 66, No. 2 (Apr., 1947), pp. Published by: Blackwell Publishing on beh. *Society*, *66*(2), 149–171.

Le Guellec, D., Morvan-Dubois, G., & Sire, J. Y. (2004). Skin development in bony fish with particular emphasis on collagen deposition in the dermis of the zebrafish (Danio rerio). *International Journal of Developmental Biology*, *48*(2-3), 217–231. http://doi.org/10.1387/ijdb.15272388

Lippitsch, E. (1990). Scale morphology and squamation patterns in cichlids (Teleostei, Perciformes): A comparative study. *Journal of Fish Biology*, *37*(June), 265–291. http://doi.org/10.1111/j.1095-8649.1990.tb05858.x

Massague, J. (1990). The Transforming Growth Factor-Bèta Family. *Annual Review of Cell Biology*, *6*, 597–641. http://doi.org/10.1146/annurev.cb.06.110190.003121

Meunier, F. J. (1984). Spatial organization and mineralization of the basal plate of elasmoid scales in osteichthyans. *American Zoologist*, *24*(4), 953–964. http://doi.org/10.1093/icb/24.4.953

Mims MC, Hulsey DC, Fitzpatrick BM, Streelman JT (2010) Geography disentangles introgression from ancestral poly- morphism in Lake Malawi cichlids. Molecular Ecology, 19, 940–951.

Pan, X., Chen, Z., Huang, R., Yao, Y., & Ma, G. (2013). Transforming Growth Factor β1 Induces the Expression of Collagen Type I by DNA Methylation in Cardiac Fibroblasts. *PLoS ONE*, *8*(4). http://doi.org/10.1371/journal.pone.0060335

Parente, M. G., Chung, L. C., Ryynänen, J., Woodley, D. T., Wynn, K. C., Bauer, E. a, … Uitto, J. (1991). Human type VII collagen: cDNA cloning and chromosomal mapping of the gene. Proceedings of the National Academy of Sciences of the United States of America, 88(16), 6931–6935.

Pasquale, E. B. (2005). Eph receptor signalling casts a wide net on cell behaviour. *Nature Reviews. Molecular Cell Biology*, *6*(6), 462–75. http://doi.org/10.1038/nrm1662

Patterson, R. T., Wright, C., Chang, A. S., Taylor, L. a, Lyons, P. D., Dallimore, A., & Kumar, A. (2002). Atlas of common squamatological (Fish scale) material in coastal British Columbia and an assessment of the utility of various scale types in paleofisheries reconstruction. *Paleontologica Electronica*, *4*(1), 1–88.

Poliakov, A., Cotrina, M., & Wilkinson, D. G. (2004). Diverse roles of eph receptors and ephrins in the regulation of cell migration and tissue assembly. *Developmental Cell*. http://doi.org/10.1016/j.devcel.2004.09.006

Powder, K. E., & Albertson, R. C. (2015). Cichlid fishes as a model to understand normal and clinical craniofacial variation. *Developmental Biology*, 1–9. http://doi.org/10.1016/j.ydbio.2015.12.018

Qu, Q., Haitina, T., Zhu, M., & Ahlberg, P. E. (2015). New genomic and fossil data illuminate the origin of enamel. *Nature*, *526*, 108–111. http://doi.org/10.1038/nature15259

Quintana, A. M., Liu, F., O'Rourke, J. P., & Ness, S. A. (2011). Identification and regulation of c-Myb target genes in MCF-7 cells. BMC Cancer, 11, 30. http://doi.org/10.1186/1471-2407-11-30

Razeto-Barry, P., Diaz, J., Cotoras, D., & Vasquez, R. A. (2011). Molecular evolution, mutation size and gene pleiotropy: A geometric reexamination. *Genetics*, *187*(3), 877–885. http://doi.org/10.1534/genetics.110.125195

Reinartz, A., Ehling, J., Franz, S., Simon, V., Bravo, I. G., Tessmer, C., … Gassler, N. (2010). Small intestinal mucosa expression of putative chaperone fls485. *BMC Gastroenterology*, *10*, 27. http://doi.org/10.1186/1471-230X-10-27

Rohner, N., Bercsényi, M., Orbán, L., Kolanczyk, M. E., Linke, D., Brand, M., … Harris, M. P. (2009). Duplication of fgfr1 Permits Fgf Signaling to Serve as a Target for Selection during Domestication. *Current Biology*, *19*(19), 1642–1647. http://doi.org/10.1016/j.cub.2009.07.065

Sanvito, F., Piatti, S., Villa, A., Bossi, M., Lucchini, G., Marchisio, P. C., & Biffo, S. (1999). Protein Involved in 60S Ribosomal Subunit Assembly. *Cell*, *144*(5), 823–838.

Seilacher, A., Buatois, L. A., & Mángano, M. G. (2005). Trace fossils in the Ediacaran-Cambrian transition: Behavioral diversification, ecological turnover and environmental shift. *Palaeogeography, Palaeoclimatology, Palaeoecology*, *227*(4), 323–356. http://doi.org/10.1016/j.palaeo.2005.06.003

Sharpe, P. T. (2001). Fish scale development: Hair today, teeth and scales yesterday? *Current Biology*, *11*(18), 751–752. http://doi.org/10.1016/S0960-9822(01)00438-9

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., … Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. Nature Methods, 9(7), 676–682. http://doi.org/10.1038/nmeth.2019

Shubin, N., Tabin, C., & Carroll, S. (2009). Deep homology and the origins of evolutionary novelty. *Nature*, *457*(7231), 818–823. http://doi.org/10.1038/nature07891

Sire, J., & Arnulf, I. (2000). Structure and development of the ctenial spines on the scales of a teleost fish, the cichlid Cichlasoma nigrofasciatum. *Acta Zoologica*, *81*(April), 139–158.

Sire, J., & Géraudie, J. (1983). Fine structure of the developing scale in the cichlid Hemichromis bimaculatus (Pisces, Teleostei, Perciformes). *Acta Zoologica*, *64*(1), 1–8. http://doi.org/10.1111/j.1463-6395.1983.tb00635.x

Streelman, J. T., Webb, J. F., Albertson, R. C., & Kocher, T. D. (2003). The cusp of evolution and development: A model of cichlid tooth shape diversity. *Evolution and Development*, *5*(6), 600–608. http://doi.org/10.1046/j.1525-142X.2003.03065.x

Suksaweang, S., Jiang, T.-X., Roybal, P., Chuong, C.-M., & Widelitz, R. (2013). Roles of EphB3/Ephrin-B1 Interactions in Feather Morphogenesis, *10*(1), 54–56. http://doi.org/10.1038/nmeth.2250.Digestion

Sylvester, J. B., Rich, C. a, Loh, Y.-H. E., van Staaden, M. J., Fraser, G. J., & Streelman, J. T. (2010). Brain diversity evolves via differences in patterning. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(21), 9718–9723. http://doi.org/10.1073/pnas.1000395107

Taniguchi, K., Anderson, A. E., Sutherland, A. E., & Wotton, D. (2012). Loss of tgif function causes holoprosencephaly by disrupting the Shh signaling pathway. *PLoS Genetics*, *8*(2). http://doi.org/10.1371/journal.pgen.1002524

Ting-Berreth, S. A., & Chuong, C. M. (1996). Local delivery of TGF beta2 can substitute for placode epithelium to induce mesenchymal condensation during skin appendage morphogenesis. *Developmental Biology*, *179*(2), 347–59. http://doi.org/10.1006/dbio.1996.0266

Tsuchiya, N., & Nakagama, H. (2010). MicroRNA, SND1, and alterations in translational regulation in colon carcinogenesis. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis. http://doi.org/10.1016/j.mrfmmm.2010.09.001

Vickrey, A. I., Domyan, E. T., Horvath, M. P., & Shapiro, M. D. (2015). Convergent evolution of head crests in two domesticated columbids is associated with different missense mutations in EphB2. *Molecular Biology and Evolution*, *32*(10), 2657–2664. http://doi.org/10.1093/molbev/msv140

Wells, K. L., Hadad, Y., Ben-Avraham, D., Hillel, J., Cahaner, A., & Headon, D. J. (2012). Genome-wide SNP scan of pooled DNA reveals nonsense mutation in FGF20 in the scaleless line of featherless chickens. *BMC Genomics*, *13*(1), 257. http://doi.org/10.1186/1471-2164-13-257

Wotton, D., Lo, R. S., Massagué, J., Swaby, L. C., & Massague, J. (1999). Multiple Modes of Repression by the Smad Transcriptional Corepressor TGIF Multiple Modes of Repression by the Smad Transcriptional Corepressor TGIF *, *274*(52), 37105–37110.

Xu, Q., Mellitzer, G., & Wilkinson, D. G. (2000). Roles of Eph receptors and ephrins in segmental patterning. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *355*(1399), 993–1002. http://doi.org/10.1098/rstb.2000.0635

Yang, S.-S., Tan, J.-L., Liu, D.-S., Loreni, F., Peng, X., Yang, Q.-Q., … Wu, J. (2015). eIF6 modulates myofibroblast differentiation at TGF-beta1 transcription level via H2A.Z occupancy and Sp1 recruitment. *Journal of Cell Science*, 3977–3989. http://doi.org/10.1242/jcs.174870

Zhu, D., Ortega, C. F., Motamedi, R., Szewciw, L., Vernerey, F., & Barthelat, F. (2012). Structure and mechanical performance of a “modern” fish scale. *Advanced Engineering Materials*, *14*(4), 1–10. http://doi.org/10.1002/adem.201180057