Genetic Basis of Vertebrate Diversity: the Cichlid Fish Model

Proposed by
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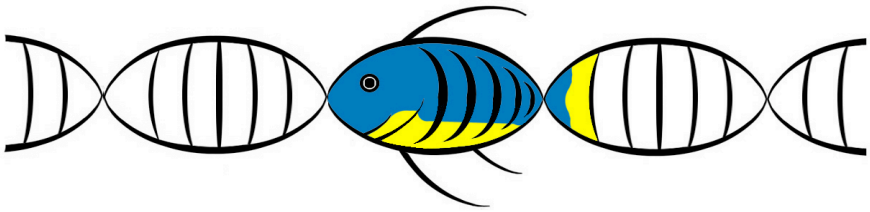

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Executive Summary

We advocate the development of genome sequences from cichlid fishes to support research into the genetic mechanisms which have contributed to the diversification of vertebrates. Cichlids have undergone a spectacular radiation in the lakes of East Africa which has generated a collection of 'natural mutants' that are amenable to genetic analysis in the laboratory. Cichlids exhibit tremendous diversity in features, such as jaws, teeth and pigmentation, that are derived from the neural crest. They also exhibit an extraordinary diversity and plasticity of behavior, which has contributed to their rapid speciation. Cichlids are perhaps the model system for understanding evolutionary mechanisms in vertebrates.

We request a total of 10Gb of sequence from this group. The Nile tilapia is the obvious candidate for full (5x) draft assembly in this group, because of the availability of homozygous clonal lines and extensive genetic and physical maps for this species. To complement this we request low-density draft coverage of three closely related haplochromine cichlids: *Astatotilapia burtoni* from Lake Tanganyika, *Paralabidichromis chilotes* from Lake Victoria and *Metriaclima zebra* from Lake Malawi, Comparisons among these species will help identify genetic changes responsible for the remarkable morphological and behavioral radiation of cichlids in the lakes of East Africa.

Table of Contents

THE QUESTIONS

Vertebrates have undergone a remarkably successful diversification over the past 500 MY.[1] The basic vertebrate body plan has been shaped and molded to adapt to life on the land, in the sea and in the air. We stand at the end of a long lineage of vertebrates whose evolutionary legacy has left its imprint on the structure and function of the human genome. Understanding the genetic and developmental mechanisms responsible for vertebrate diversity has broad implications for human health.

1. The neural crest

Much of the success of vertebrates can be traced to an evolutionary novelty – the neural crest – which either directs, or participates in, the development of most of the structures that are unique to vertebrates.[2] Migrating neural crest cells contribute to the development of the nervous and endocrine systems, pigment cells, and connective tissue. Neural crest migrations into the pharyngeal arches produce the skeletal elements of the head, including teeth and bones of the jaw and middle ear. While it is clear that this unique group of cells is responsible for the diversity of modern vertebrates, we still have a lot to learn about the molecular codes which modulate their activities and developmental fates. Because defects in neural-crest function are responsible for a wide range of human diseases, it is imperative that we understand the mechanisms by which this cell lineage is modulated to produce diverse adult structures.

2. Brain and behavior

Another key to the success of vertebrates is the evolution of a large and complex brain, and an extraordinary level of behavioral plasticity. The behavior of vertebrates, including humans, depends on their current social and physiological status, and on their prior experience. A fundamental goal of modern biology is to understand how the genome and environment interact to produce behavioral phenotypes. Plasticity in the nervous system consists of structural and functional changes in information processing after the initial formation of neural contacts. These changes are achieved initially by changes in neural activity or endocrine responses, but often lead to differential gene expression and subsequent alterations of structure and physiology. Functional genomics can contribute to an understanding of the complex interactions between genome and environment that result in highly plastic phenotypes.[3]

3. Rapid diversification

Almost 150 years after the publication of "On the Origin of Species",[4] understanding the mechanisms by which new species are formed remains a central problem in modern biology. We can point to only a few genes involved in the diversification of recent species. There is growing appreciation that genetic conflicts, and other unique selective forces, drive the divergence of some gene pathways. To understand the origins of many human diseases, and the differences between humans and our closest relatives, we must appreciate the selective forces driving the divergence of these most rapidly evolving portions of the genome.

THE MODEL SYSTEM

EAST AFRICAN CICHLID FISHES

One vertebrate system is particularly well suited to studying the genetic basis for vertebrate diversity. Fishes of the family Cichlidae are the most species-rich family of vertebrates. More than 3,000 species of cichlids are distributed from Central and South America, across Africa to Madagascar and southern India.[5] Cichlids are a diverse fauna in each of these areas, and have repeatedly demonstrated a capacity for rapid phenotypic radiation. However, it is the rapid radiation of cichlid fish species in the Great Lakes of East Africa which has attracted the most attention. By any measure, these species flocks are the most rapid radiations of any group of extant vertebrates.

Figure 1. The natural diversity of cichlid species represents a mutant screen ideal for studying post-embryonic morphogenesis.

The vast assemblage of cichlid species in East Africa can be viewed as a collection of mutants, screened by natural selection for differences in phenotype (Fig. 1). Natural variants offer several advantages. First, because of their unparalleled diversity, cichlid species can be used to assess an extremely broad range of traits. The natural mutant screen includes phenotypes that occur throughout the life cycle, including older stages not screened in traditional model systems. Second, mutations fixed by natural selection include changes in regulatory regions that are not typically identified in chemical mutagenesis screens of early embryonic development. Finally, cichlids offer an opportunity to study the evolution of developmental pathways among closely related species. Just as the dynamics of DNA sequence evolution are best revealed by comparisons among closely related sequences, so the evolution of developmental pathways is best studied by comparing closely related species.

The independent radiations of 500 species in Lake Victoria and 700 species in Lake Malawi have each occurred within the last 1 million years.[6,7] The radiation of more than 250 species in Lake Tanganyika has occurred over the last 6-8 MY.[8] So, most of the diversity in this cluster of more than 1500 species has arisen since the divergence of humans and chimpanzees. Similar radiations of cichlids have occurred in smaller lakes throughout the region, resulting in multiple instances of convergent evolution of morphology and behavior.[9] These replicate radiations are an ideal model system for studying the genetic mechanisms of adaptive evolution.

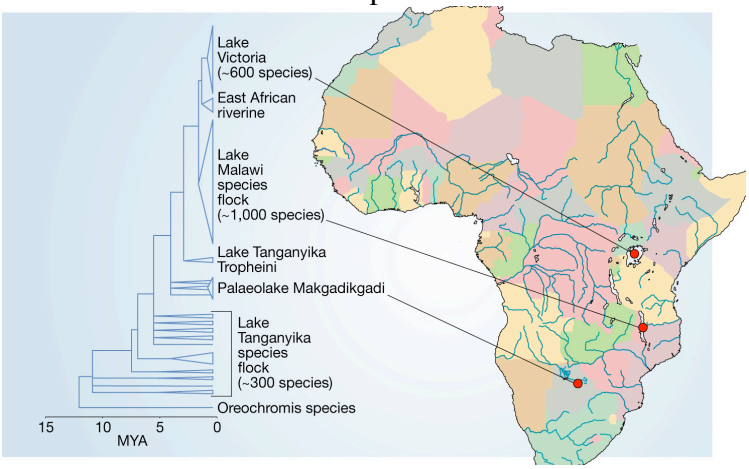


Figure 2. The radiation of cichlid fishes in East Africa.

The 'haplochromine' cichlids of these lakes shared a common ancestor with an important cultured cichlid, the Nile tilapia (*Oreochromis niloticus*), approximately 10-15 MY ago.[10,11] Cichlids are distantly related to other current fish models.

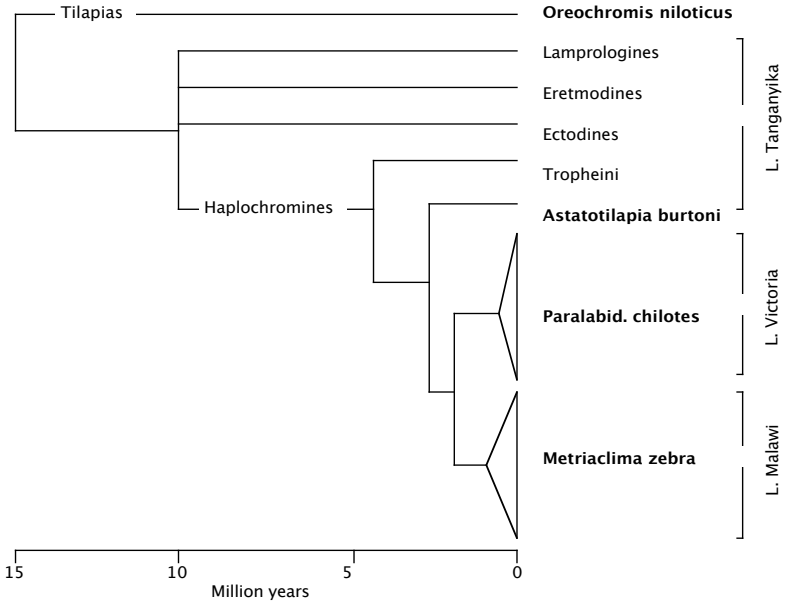
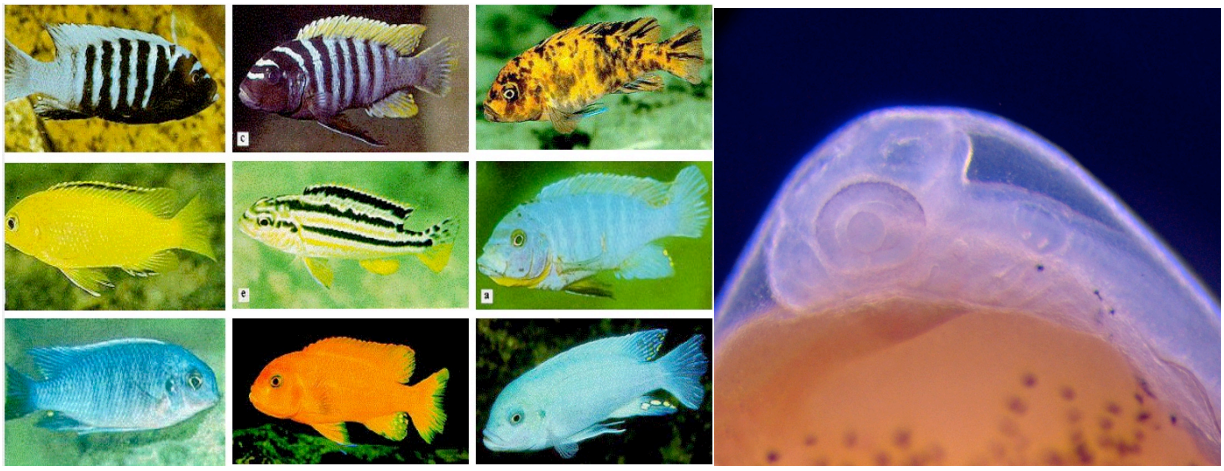


Figure 3. Phylogenetic relationships among East African cichlid fishes.

Cichlids as a laboratory organism

Cichlids readily adapt to captivity, and many species are available in local pet shops. Cichlids are known for their elaborate courtship and parental care. In many species females incubate their embryos in their mouths for 2-3 weeks after spawning. The larvae are robust and accept commercial flake foods as soon as their yolk is absorbed. Cichlids breed year round, and in the lab the generation time is as short as 6-9 months.

Differences among cichlid species are amenable to genetic analysis because fertile hybrids are easily obtained from most interspecific and many intergeneric crosses.[12] Methods for producing transgenic cichlids are well established,[13] and cell lines are in routine experimental use.[14,15] Moreover, we have developed comparative genetic maps that encompass the radiation of over 2,000 East African cichlid species. The very close relationship of these species makes it possible to use genetic analysis to identify the genes responsible for recent evolutionary novelties.



THE PARADIGMS

1. GENETIC ANALYSIS OF NEURAL-CREST DERIVATIVES

Constituents of the craniofacial skeleton, teeth, pigmentation, and nervous system are all derived, at least in part, from the neural crest, which is considered to be a critical innovation in the origin of vertebrates.[16,17,18,19] Comparisons among extant cichlid species will reveal how this cell type has been modulated to produce the diversity of vertebrate species.

Here we discuss just a few of the phenotypes currently being studied in cichlid fishes, including examples of traits not easily assessed in other model organisms.

Jaws

Cichlids are well known for the diversity of their feeding apparatus[20]. A second set of jaws in the throat (the pharyngeal jaws) has evolved to process food entering the gut, allowing the oral jaws to develop specializations for capturing different kinds of prey.

Albertson and colleagues[21] crossed two Lake Malawi cichlids (*Metriaclima zebra* and *Labeotropheus fuelleborni*) which show dramatic differences in oral jaw morphology. Mapping of quantitative trait loci affecting bone shape showed that a relatively small number of genes, with pleiotropic effects on several different structures, were responsible for differences between species. A major QTL for jaw shape was centered over the *bmp4* gene. Recent work[22] has shown that *bmp4* is differentially expressed in species with different jaw shapes, suggesting cis-regulatory elements are responsible for the changes in jaw shape (Fig. 4).

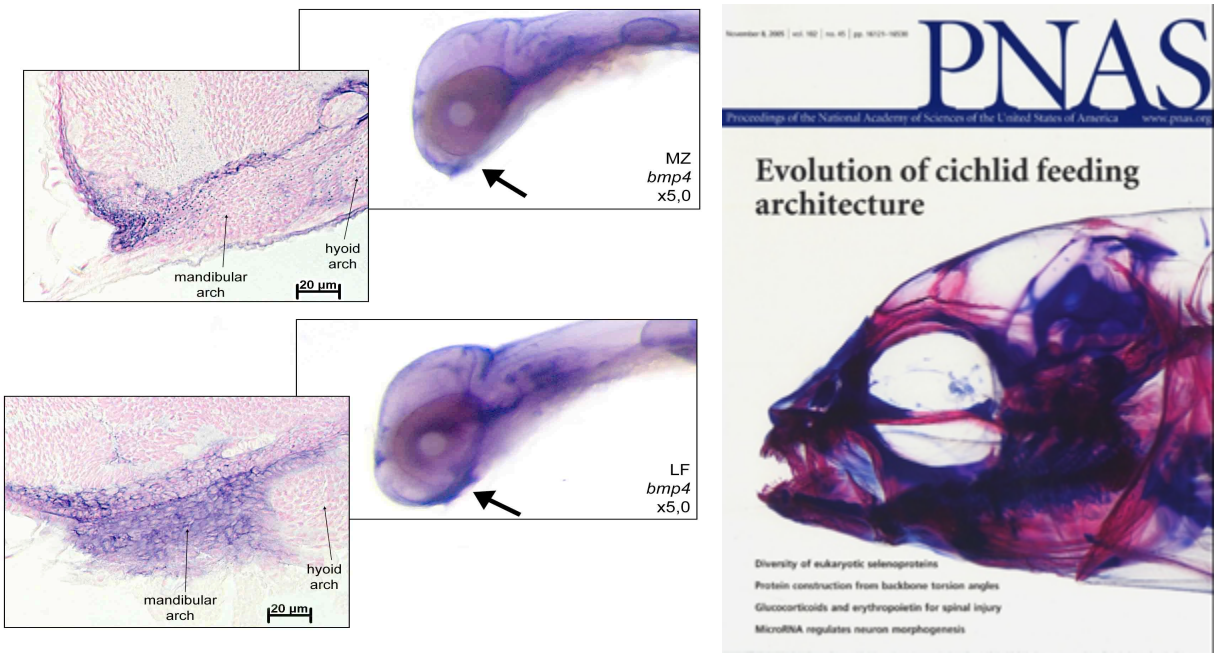


Figure 4. In situ hybridization reveals differences in expression of *bmp4* in the mandibular arch of Lake Malawi cichlid species (*Metriaclima zebra* and *Labeotropheus fuelleborni*) with different lower jaw shapes.

Teeth

Cichlid species have a variety of distinctive tooth shapes which enable their specialized modes of feeding. The genetic basis for diffences in tooth shape has been difficult to study in other model systems.[23] Other developmental models lack oral teeth, or have not produced mutants with dramatically different tooth shapes. Because cichlid adult teeth are replaced every 100 days,[24,25] odontogenesis can be studied at any life stage. Furthermore, whole heads can be cultured for up to 14 days, allowing studies on the effects of specific molecules on the development of jaws, teeth, and scales.[26]

The differences between the bicuspid teeth of *M. zebra* and the tricuspid teeth of *L. fuelleborni* (Fig. 5) are controlled by a single major gene on linkage group 5.[27] A genome sequence would facilitate positional cloning of the variation responsible for these differences in tooth shape.

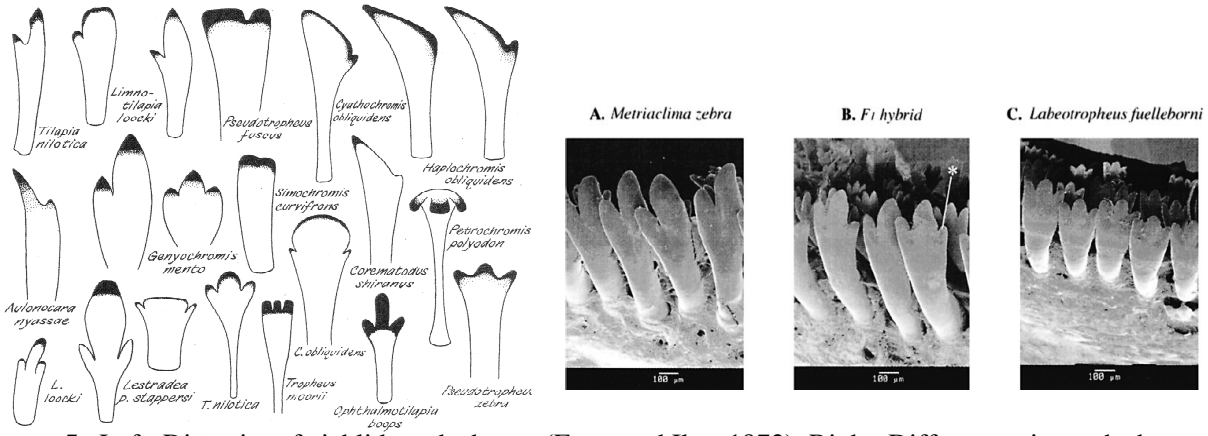


Figure 5. Left: Diversity of cichlid tooth shapes (Fryer and Iles, 1972). Right: Differences in tooth shape among closely related species of cichlid and their F_1 hybrid.

Pigmentation

The great diversity of color patterns in haplochromine cichlids provides rich material for understanding the evolutionary modification of neural crest derivatives. Studies of cichlid pigmentation will extend our understanding of the basic molecular pathways of specification, patterning and differentiation of pigment cells.[28,29,30,31]

Orange-blotch (OB) is a dramatic polymorphism found in many species of Lake Malawi and Lake Victoria cichlids (Fig. 6). The polymorphism is controlled by a single dominant gene on linkage group 5, which causes a metamorphic transformation of the wild-type blue-black (BB) pattern into the disorganized OB pattern in which pigment cells are highly clumped.[32]

A superficially similar phenotype occurs in cultured tilapia. Red tilapia have dramatically few melanophores (Fig. 6). The causative mutation maps to LG3, and fine mapping has localized it within a single BAC clone (Howe et al. in prep). Neither of the two genes in this clone have been implicated in pigment cell defects in other organisms. Thus cichlids offer a unique perspective on the development and regulation of pigment cells.

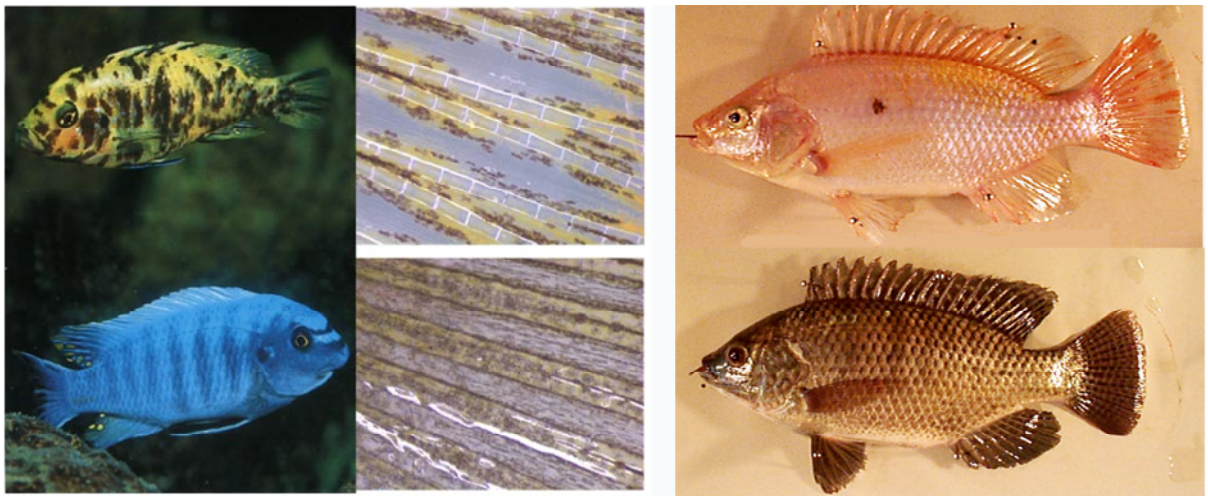


Figure 6. Left: Representative orange-blotch (OB, top) and blue with black bars (BB) cichlid color morphs. Enlargements show the distribution of pigment cells in the tail of each morph. Right: Normal and red mutant tilapia.

How will genome sequences help?

This sampling of neural crest phenotypes demonstrates the rich potential of the cichlid system for studying the modulation of neural crest cell fates. A high quality draft sequence of the tilapia genome would greatly accelerate the positional cloning of genes underlying these differences. It would also give rapid access to gene sequences for the design of microarrays and *in situ* probes for the analysis of developmental mechanisms. However, a collection of sequences from tilapia and several haplochromine cichlids will also enable an entirely different approach to identifying causative variation.

The first step in this strategy will be to identify conserved regulatory elements by comparison among highly diverged fishes. Comparison of the cichlid sequences to those of pufferfish, medaka and stickleback (all 80-100MY divergent from one another) and to zebrafish (300 MY divergent) would allow us to identify regulatory elements which are conserved among fish genomes. The second step will be to compare the derived haplochromine cichlids with each other, and with the tilapia outgroup, to identify recent unique changes in these regulatory elements. We aim to identify the changes in particular species which might be responsible for the phenotypes we have mapped genetically.

The extremely close relationships among East African cichlids make this strategy feasible and attractive. Most of the diversification within Lakes Malawi and Victoria has occurred within the last 200,000 years. Because the sequences of the diverse species within each lake are nearly identical, it will be relatively easy to correlate interesting changes in the phenotype with particular changes in genome sequence.

2. PHENOTYPIC PLASTICITY OF BEHAVIOR

Alterations in phenotype in response to changes in the social or physical environment are common in the animal world, either as short-term (i.e., modulatory) or as long-term modifications (e.g., via gene expression changes) in behavioral, physiological, or morphological properties. How does the environment influence an organism and what are the underlying physiological, molecular, and genetic mechanisms?

Understanding how social, physiological, and experiential factors influence the behavior of individual animals requires a behavioral model with rich social interactions and which allows access to key physiological processes. Cichlids have an astonishing diversity in behavior related to feeding, social dominance, pair bonding and parental care.[33] In the sections below we introduce some of the unique opportunities afforded by cichlids for the study of complex social behaviors and their genetic and (plastic) neural/molecular underpinnings.

Astatotilapia burtoni: a model system for behavioral plasticity

Astatotilapia (syn. Haplochromis) burtoni, a haplochromine cichlid from Lake Tanganyika, has been developed as a model system for studying behavioral plasticity under semi-natural conditions.[34] Males come in two different, though reversible, phenotypes: those with territories and those without.[35,36] Territorial (T) males, are brightly colored and have a dramatic black stripe through the eye. In contrast, nonterritorial (NT) males are cryptically colored, similar to females. A change in an animal's social status produces a change in the state of sexual maturation,[37] as well as a change in the rate of growth.[38] Testosterone levels are elevated in territorial fish,[39] and only territorial males have viable sperm.[40] A small population of forebrain neurons controls these changes: as a male ascends to territoriality, the size of neurons containing gonadotropin-releasing hormone (GnRH) in the preoptic area increases dramatically,[41] GnRH gene expression is upregulated,[42] and GnRH receptor levels in the pituitary are increased.[43] Recent studies have related these changes to expression of egr-1.[44] Animals grow faster while they are nonterritorial and more slowly while they are territorial.[45] This differential growth leads to changes in social dominance even in a physically stable environment. A small population of preoptic neurons, containing the growth-hormone-inhibiting neuropeptide somatostatin, mediates these changes in growth rate.[46] Circulating basal levels of the glucocorticoid stress hormone cortisol, as well as the cortisol response to an acute stressor, are reduced in territorial males as long as the social hierarchy is stable.[47,48] The *A. burtoni* system allows us to integrate molecular and genomic data with physiological processes and behavioral phenotypes to build a comprehensive conceptual framework for understanding phenotypically plastic traits as they result from interactions between genotype and environment.

Figure 7. Territorial male *Astatotilapia burtoni*.

Evolution of the brain

East African cichlid fish provide a singular opportunity to understand how social and habitat pressures sculpt the brain. For example, there is a close relationship between the relative sizes of various brain structures and variables related to the utilization of habitat such as prey size and agility, turbidity levels, depth, and substrate complexity.[49,50] Areas associated with primary sensory functions such as vision and taste relate significantly to differences in feeding habits. Taxa that utilize motile prey are characterized by a well developed optic tectum and a large cerebellum compared to species that feed on molluscs or plants. The genetic basis for these dramatic differences in brain structure can be addressed through microarray and QTL studies. Notably, cichlid brains do not show the obvious compensatory changes that have been taken as evidence of size and developmental constraints in brain organization across mammalian orders,[51,52,53] which suggests that the genetic basis for enhancement for particular sensory functions can be studied independently. Comparative studies of closely related species can be a particularly powerful approach,[54,55,56,57] which is strengthened by the well-resolved phylogenetic relationships among major lineages of haplochromine cichlids.[58]

Genetic basis of parental behavior

African cichlids are particularly diverse in their systems of mating and parental care.[59] Breeding behaviors include the formation of leks and the construction of sand-castle bowers with species-specific shapes.[60] *Lamprologus callipterus* has genetically determined large and dwarf male morphs which pursue alternative male mating strategies: large males collect piles of snail shells which attracts a resident harem of females, while dwarf males sneak copulations by hiding in the shells.[61] Parental care behaviors are equally diverse. Most species in lakes Malawi and Victoria are maternal mouthbrooders, and the males provide no care of the offspring. Species in Lake Tanganyika display a more diverse range of parental care behaviors, including the ancestral pattern of substrate guarding, and newly evolved systems of maternal, paternal and biparental mouthbrooding.[62] Juveniles of several species of *Neolamprologus* remain with the family and engage in a variety of helping behaviors long after they become sexually mature.[63] It is exciting to consider how linkage mapping and studies of gene expression might be applied to understand the genetic basis of these variations in behavior.

How will genome sequences help?

Most research on cichlid behavioral plasticity has focused on changes in the expression of a few neuropeptides.[64,65] While high-density cDNA microarrays have been constructed for this species,[66] genome sequences would facilitate the construction of a much more comprehensive expression profiling array. It would also allow a genome-wide analysis of regulatory sequences, thus providing another avenue for a molecular understanding of brain and behavior.

Applications of expression arrays could be expanded to encompass changes in gene expression among species with different levels of aggressive behavior and territoriality, and to examine changes in gene expression through the period of parental care in both maternal mouthbrooding species and pair-bonded substrate brooders. The unique advantage of the cichlid system is that these studies could be replicated over many different species to identify the key changes in gene expression which are responsible for changes in these behaviors.

3. RAPID EVOLUTION

It has become apparent that the selective forces championed by the Evolutionary Synthesis of the 1930's are inadequate to explain many patterns of evolution observed in genomes. A variety of new selective forces have been identified which can cause rapid divergence of gene sequences and molecular pathways. These include sexual selection, genetic conflicts between males and females, and genetic conflicts between different segments of the genome.[67,68,69] The effects of these selective forces on particular genes are often much stronger than the forces of classical natural selection.

Analysis of the human genome has identified genes which evolve both faster and slower than expected, indicating unusual patterns of both positive and negative selection.[70] Many of the most rapidly evolving genes are involved in tumor suppression, apoptosis and spermatogenesis, and may be the result of genetic conflicts.[71] These results have important implications for understanding the origins of human disease, and for the design and testing of drugs against particular molecular targets.

Rapidly diverging cichlids provide a powerful vertebrate model system for studying genetic conflicts and their effects on genome evolution. Cichlids are under strong natural selection to adapt to different feeding strategies. But they are also under strong sexual selection due to the unequal parental investment in broodcare, and there is evidence of strong genetic conflicts associated with the evolution of sex chromosomes.

Sexual selection

Dramatic sexual dimorphism and the great variety of male color patterns is *prima facie* evidence that sexual selection is a strong force in the evolution of cichlid species.[72] Recent models stimulated by the cichlid species flocks have suggested that disruptive sexual selection can lead to speciation even in full sympatry.[73,74,75] Sensory drive has been suggested as an important cause of divergent sexual selection.[76] While many different cues could be involved in mate choice, cichlids rely mainly on visual signals to communicate.[77,78,79] Visual cues appear to be most important for maintaining reproductive isolation among closely related species (Fig. 8).[80] Cichlids show surprising variation in visual sensitivity as measured by microspectrophotometry.[81] Some of this variability can be related to rapid evolution of opsin sequences,[82,83] while in other cases the differences are due to differential expression of the same set of cone opsin genes.[84] Changes in visual sensitivity likely affect many aspects of the ecology and behavior of these species.

Figure 8. Blue and red male *Pundamilia* spp. from Lake Victoria.

Sex determination

One of the most dramatic features of the human genome is the differentiation of the X and Y chromosomes, and the associated mechanisms of X-inactivation which equalize gene dosage. A detailed theory for the evolution of sex chromosomes has been developed, but the early stages in the development of human sex chromosomes have been obscured by 100 MY of additional evolution.

Cichlids offer a unique opportunity to understand early stages in the evolution of sex chromosomes. Cichlid sex chromosomes are not sufficiently divergent to be recognized by classical cytogenetics[85,86]. Molecular markers have identified different sex-determining systems among closely related species of tilapia. Nile tilapia (*O. niloticus*) have a XY sex-determining system on LG 1[87,88]. In its sister species, *O. aureus,* sex is determined by epistatic interactions between two loci, an XY system on LG 1 and a WZ system on LG3 (Fig. 9). The sex of tilapia is also influenced by additional genetic and environmental factors.[89] In the closely related haplochromine cichlids we have evidence for additional XY and WZ systems on linkage groups 5 and 7. By accessing the natural diversity of sex-determining systems among species, and by making appropriate hybrid crosses, it should be possible to enumerate the genes controlling sex in *O. niloticus*, and to develop a clearer picture for how this developmental pathway evolves.

UNH104 / UNH131	LG1 A / A	LG1 A /189
187/193	20 females 0 males	18 females 0 males
LG3 193/193	5 females 10 males	0 females 25 males

Figure 9. Epistatic interaction of sex-determining loci on linkage groups 1 and 3 in *Oreochromis aureus* (after Lee *et al*. 2004).

How will genome sequences help?

Classical models of allopatric speciation suggested speciation occurred by gradual divergence among geographically isolated populations. African cichlids provide the most stunning challenge to this theory. Cichlid speciation is rapid and occurs within closed lake basins. One lineage of Lake Victoria cichlids has speciated at least 700 times over the last 200,000 years.[90] Some of the most convincing examples of fully sympatric speciation are the radiations of cichlids within small crater lakes.[91,92] Because of their recent, rapid divergence, the thousands of cichlid species in the lakes of East Africa are the best model system for studying the genetic basis of vertebrate speciation.

There is now an abundance of theoretical models which have removed the conceptual barriers to sympatric speciation. However, these models remain untested because the genetic basis for speciation traits is largely unknown. Because cichlid species are easily hybridized in the laboratory, and because speciation traits have been phenotypically well-characterized in several cases, we have the opportunity to test key genetic assumptions of these models. Genes are the link between theoretical models and empirical observations of speciation, and genomic techniques promise to greatly accelerate the discovery of speciation genes.

The great potential of the cichlid system for studying the process of adaptive radiation has been recognized by the DOE-JGI Community Sequencing Program, which has funded a project to identify a large number of SNPs among Lake Malawi cichlids. Full genome sequences for tilapia and haplochromine sequences would greatly enhance the utility of these SNPs for positional cloning of QTL and studies of linkage disequilbrium in natural populations. Furthermore, comparison of the tilapia genome with the genomes of several haplochromine species would allow computational identification of rapidly evolving genes which are subject to unusual patterns of selection.

WHY SEQUENCE ANOTHER FISH?

Draft genome sequences of two pufferfish (Fugu and Tetraodon) have been published.[93,94] These species were chosen because of their small genome size, with an eye toward cost-effective annotation of the human genome. They have proven to be important reference sequences, and have provided important insight into the ancestral vertebrate karyotype. But they are relatively intractable as experimental organisms in the laboratory.

The shotgun sequencing of zebrafish did not provide a useful assembly, and clone-by-clone sequencing is in process. The zebrafish is an exquisite laboratory model which has helped reveal the molecular cascades which pattern the vertebrate embryo. Forward genetic approaches have been used to produce mutants with gross defects in early embryonic development. This focus on early embryonic phenotypes has provided little insight into the genetic basis for phenotypic differences among species. Zebrafish do not make fertile hybrids with other species, and with the exception of pigmentation phenotypes,[95] natural variation in adult phenotypes has remained relatively inaccessible. Zebrafish are distantly related from the other fish models (about as divergent as human and chicken; Fig. 10).

Shotgun sequencing of the medaka has been completed, and publication of the assembly is expected soon. The medaka provides a different perspective on early development of fishes.[96] Mutant screens in this species frequently identify new genes affecting processes that have been well-studied in other models. The genus *Oryzias* is not morphologically diverse, and provides little scope for studying variation in adult phenotypes.

The sequence of the stickleback genome is also imminent. The justification for sequencing the stickleback centered on identifying the genetic basis of differences between recently evolved forms. Most of the morphological differences among stickleback species are in mesodermally-derived structures (plates and spines). With respect to behavior, mechanisms of sex determination, and structures derived from the neural crest, the diversity of sticklebacks pales in comparison to African cichlids.

Why sequence cichlids?

Studies of cichlids nicely complement the research in other fish species. First, work on cichlids has focused on alterations of aspects of adult morphology (jaws, teeth, pigment) which are derived from the neural crest. The tremendous diversity of East African cichlids provide an unlimited source of natural mutants of these structures for genetic investigation. Second, cichlids present a rich and diverse behavioral repertoire which provides a fertile ground for studies of the evolution and plasticity of behavior.

Cichlids are a model for perciform fishes generally. The order Perciformes contains more than 9,300 species (25% of all living vertebrates), none of which have been targeted for genome sequencing. Tilapia is the obvious candidate for such a project, as it is the dominant experimental model within this group, the focus of hundreds of studies in physiology, endocrinology, immunology and toxicology.

Cichlids are 80-100MY divergent from the other sequenced fish models. Cichlid sequences will provide additional information to support the annotation of vertebrate genomes, and are at an appropriate level of divergence to identify regulatory elements. Cichlids also provide a unique resolution of the fish-specific genome duplication. We can therefore expect that studies of cichlids will reveal the function of different sets of genes than are being studied in other model organisms.

Cichlids are an ideal system for studying some of the 125 'Big Questions' identified by Science magazine,[97] including:

- How do limbs, fins, and faces develop and evolve?
- How did cooperative behavior evolve?
- What determines species diversity?

Our request to sequence tilapia to a depth of 5x, together with low coverage sequencing of *A. burtoni*, *P. chilotes* and *M. zebra* (1.5-2x each), amounts to a total of 10x coverage, or 10 Gb of sequence. This is equivalent in cost to sequencing another mammalian species to a depth of 3x. Sequencing these cichlid genomes would open rich new opportunities for research in the fields of development, behavior and evolution, and would provide important comparative sequence information to complement work on other fish models.

It is appropriate that this proposal focuses on sequencing the genome of Nile tilapia, a species which has been important to the human food supply for thousands of years. Depictions of tilapia farming grace the tombs of Egyptian pharaohs from 2500 BC, and it was probably a tilapia that St. Peter fed to the multitudes at the Sea of Galilee. Sometimes called the 'aquatic chicken', tilapia are sturdy and adaptable fish which have been exported from their native Africa, and are now cultured in more than 100 countries in Asia and the Americas[98]. Tilapia are a primary source of animal protein for millions people in the developing world. The sequence of the tilapia genome would be a gift to the world community, accelerating the selective breeding of tilapia strains to improve the human food supply.

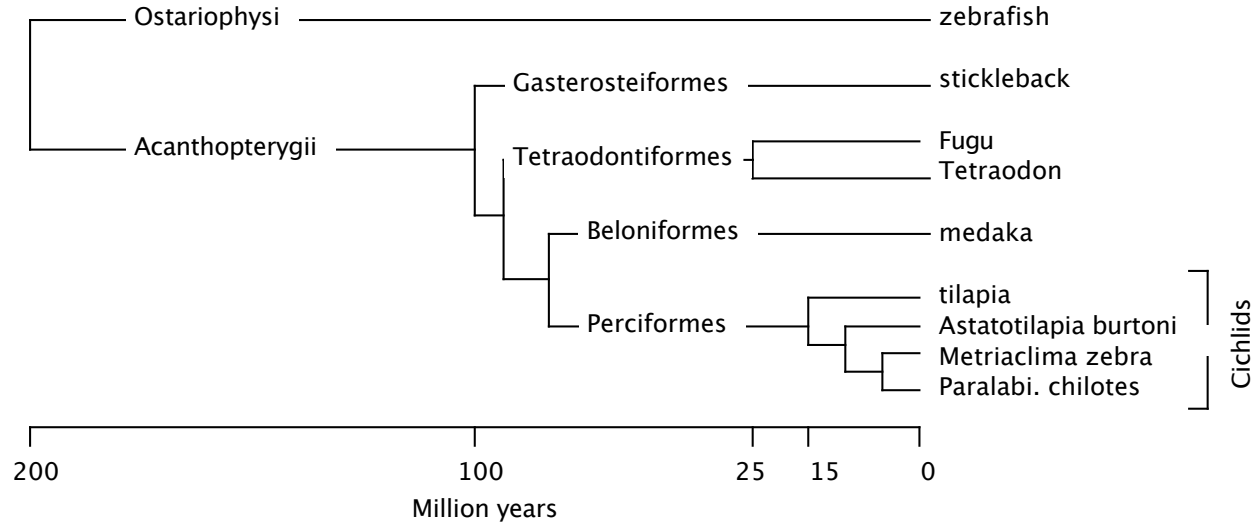


Figure 10. Relationship of cichlid fishes to other fish models.

Technical Information

Genome size - The size of the cichlid genome has been estimated from 30 measurements of 23 species.[99] The mean size is 1.1 pg. Five independent measurements of *Oreochromis niloticus* average 1.08pg. We therefore predict that a haploid Nile tilapia genome is 1.06 x 10^9 base pairs. This value is very close to the mean for non-polyploid teleosts (1.0pg).

Base composition - The base composition of the Nile tilapia genome is slightly AT-biased. An analysis of ~58kb of coding sequences from *Oreochromis niloticus* shows A:C:G:T in the proportions 0.27: 0.23: 0.23: 0.26. Sequence of 100kb cosmid containing a Hox cluster,[100] as well as three BACs containing opsin gene clusters (>300 kb, Carleton et al. in prep), shows the proportions 0.29: 0.21: 0.21: 0.29.

Polymorphism level - Fully inbred clonal lines of Nile tilapia have been developed by at least two groups using the techniques of gynogenesis.[101,102] Homozygous females are produced by blocking the first mitotic division of unfertilized eggs (mitotic gynogenesis – diploidization of a single chromatid). Clonal lines are then derived from these females by meiotic gynogenesis and the purity of the clones was verified by DNA fingerprinting. These clonal lines represent an essentially unlimited source of completely homozygous DNA for genome sequencing and analysis.

Repetitive DNAs - The major tandemly repeated DNAs of the Nile tilapia are well-studied and have been mapped by fluorescent in situ hybridization (Figure 11).[103] SATA is a ~230bp sequence present in ~10^5 copies and found in the centromeric regions of all chromosomes.[104] SATB is a ~1900bp sequence found primarily on the short arm of chromosome 4.[105] 18S rDNA sequences are found on chromosomes 8, 10 and 15. 5S rDNA repeats are found on chromosomes 3, 9 and 13. Cichlids have a standard telomeric repeat (TTAGGG) which is also found interstitially on chromosome 1, possibly indicating recent chromosome fusions.

The dispersed repetitive elements of the Nile tilapia are generally short and in low copy number, so they should not pose a major obstacle to shotgun assembly. ROn-1 is 345bp SINE present in 6000 copies.[106] ROn-2 is a 359bp SINE present in 10^4 copies.[107] Both are distributed in small clusters throughout the genome. A third type of SINE, AFC, is 320bp long and present in 10^4 copies per genome.[108,109] Insertions of this SINE have been used extensively for phylogenetic analysis of East African cichlids.[110,111] The tilapia genome also contains about 5,500 copies of an 1165bp LINE2 element with a sequence highly similar to other vertebrates.[112] These copies are arranged as small clusters, with higher concentrations being found at the termini of most chromosomes.

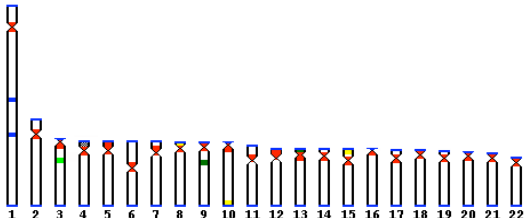


Figure 11. Karyotype of *O. niloticus*. Colors represent the distribution of different classes of repetitive DNA (Martins et al. 2004): red, SATA; speckled in black, SATB; yellow, 45S rDNA; blue, telomere; faint green, 5S rDNA type I; dark green, 5S rDNA type II.

Existing Genomic Resources:

Genetic map for tilapia

A second generation map of the tilapia genome has been constructed from the F_2 hybrid offspring of a cross between *O. niloticus* and *O. aureus*.[113] This map contains 550+ microsatellites and 30+ genes on 24 linkage groups, and has an average marker spacing of ~3cM. This work was funded by USDA-NRICGP #98-03476 and a collaboration with a Norwegian aquaculture company (Genomar asa).

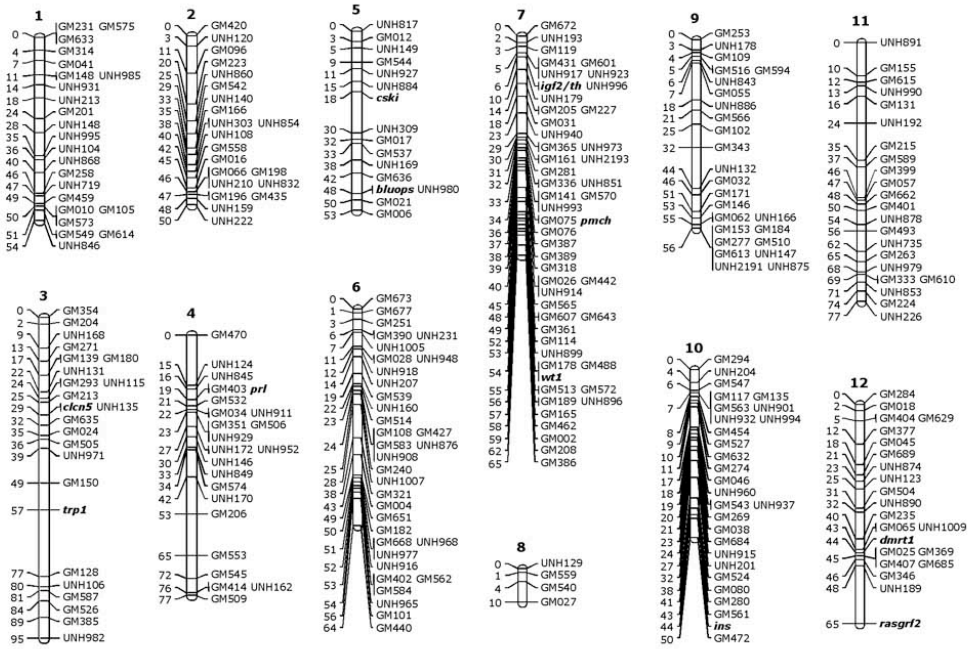


Figure 12. A portion of the current linkage map for tilapia (Lee et al. 2005).

Genetic map for haplochromines

The first genetic map for haplochromine cichlids was recently published as part of a QTL study of jaw morphology.[114] This map contains ~130 microsatellites in 24 linkage groups spanning 845 cM. A tilapiine/haplochromine comparative map is currently being constructed by typing additional Nile tilapia markers in the haplochromine family material (Streelman *et al.*, in prep). The data so far (~180 markers) indicate nearly complete co-linearity of the two maps. The current state of the comparative map can be viewed at http://hcgs.unh.edu/comp. Supported by NSF-IBN #9905127.

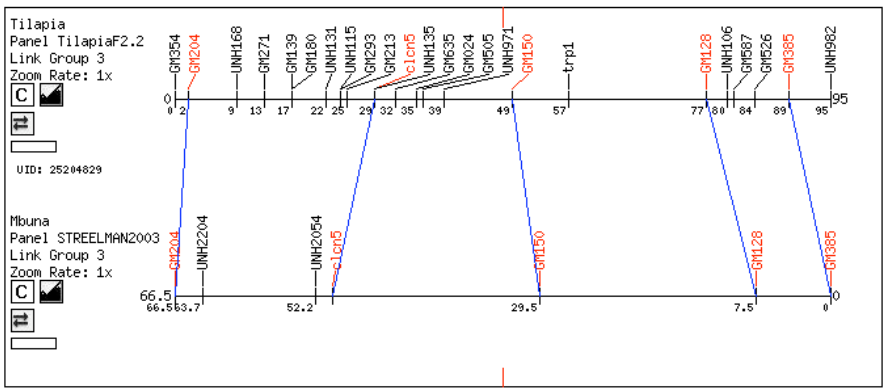


Figure 13. Comparative map of LG3 illustrating the co-linearity of Nile tilapia and Lake Malawi haplochromine linkage maps (hcgs.unh.edu/comp).

Tilapia BAC libraries

Four *O. niloticus* BAC libraries have been constructed from the same University of Stirling laboratory strain as the clonal line proposed for shotgun sequencing (Table 1)[115]. Nylon filters and copies of these libraries are available at cost from the Hubbard Center for Genome Studies.

Table 1. Tilapia BAC libraries (Katagiri et al. 2001).

Library	Insert size	Coverage
Tilapia 1	*65kb*	6x
Tilapia 2	*105kb*	65x
Tilapia 3	*145kb*	11x
Tilapia 4	*194kb*	6x

Tilapia physical map

With support from the USDA(NRICGP #00-03504), a restriction fingerprint database has been assembled for 35,000 clones (5x genome coverage) from the two largest Nile tilapia BAC libraries.[116] These fingerprints were obtained using simultaneous HindIII/HaeIII digestion. Fragment sizes were determined by capillary electrophoresis and the resulting high-resolution fingerprints clustered using FPC. Figure 3 shows that the number of contigs has stabilized at ~3500. The fingerprint database is freely available at http://hcgs.unh.edu/cichlid/#bacs. A proposal to sequence the ends of these 35.000 clones is pending at Genoscope.

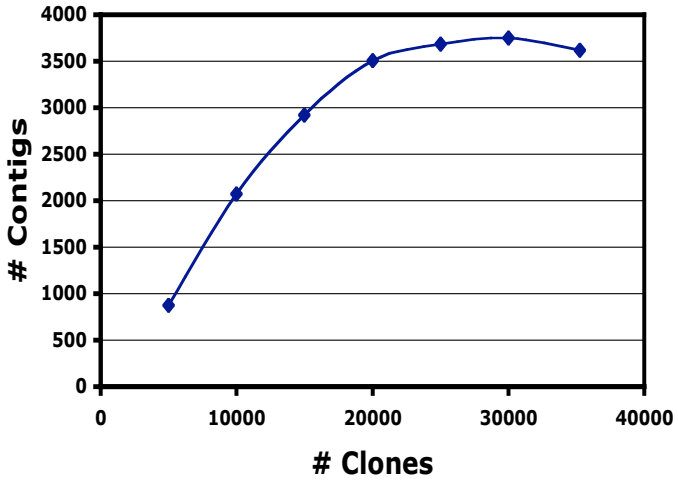


Figure 14. FPC contigs versus number of fingerprinted clones.

Haplochromine BAC libraries

BAC libraries have also been constructed from three species of haplochromine cichlids (Table 2).

Table 2. BAC libraries for haplochromine cichlids.

Species	Insert size	Coverage	Reference
Astatotilapia burtoni	*150kb*	14x	Salzburger *et al.* (submitted)
Paralabidochromis chilotes	*128kb*	10x	Watanabe *et al.* 2003
Metriaclima zebra	*120kb*	7x	DiPalma *et al.* (submitted)

cDNA libraries and ESTs

A large number of cDNA libraries have been constructed for East African cichlids (Table 3). Significant EST projects, currently totaling more than 75,000 sequences, have been completed and incorporated in TIGR's Gene Indices (www.tigr.org/tdb/tgi). A proposal to sequence another 100k ESTs from Nile tilapia is pending at USDA.

Table 3. Partial listing of cDNA and EST resources for East African cichlids.

Laboratory	Species	Tissue	# ESTs
Nagahama	*Oreochromis niloticus*	developing gonad	20,000
Genomar asa	*Oreochromis niloticus*	brain/gill	2,500
Baroiller/D'Cotta	*Oreochromis niloticus*	brain and gonad	in progress
Jaso-Friedman	*Oreochromis niloticus*	nonspec. cytotoxic cells	in progress
Martins	*Oreochromis niloticus*	brain/heart/muscle	in progress
Kocher	*Oreochromis niloticus*	various	in progress
Okada	*Paralabidochromis chilotes*	jaw	21,652
Okada	*Ptyochromis 'redtail sheller'*	jaw	14,073
Okada	*Lipochromis "matumbi hunter"*	jaw	in progress
Fernald/Hofmann	*Astatotilapia burtoni*	retina	in progress
Fernald/Hofmann/Meyer	*Astatotilapia burtoni*	brain	4,000
Hofmann/Meyer	*Astatotilapia burtoni*	mixed	12,000
Meyer	*Metriaclima zebra*	skin	in progress
		Total	> 75,000

Microarrays

The very close relationship among East African cichlids (<10MY divergence) means that ESTs from one species are useful for work in other species. Renn and colleagues[117] tested the utility of an *Astatotilapia burtoni* microarray for studying patterns of gene expression in several other East African cichlids. Essentially the same number of spots passes the signal threshold whether the cDNA sample is derived from *A. burtoni* or a Nile tilapia (*Oreochromis niloticu*s). This high degree of sequence similarity also means that ESTs from any of these species will be useful for annotation of genome sequences.[118] A second generation microarray with 18,000 features is under construction in the laboratory of Dr. Hans Hofmann.

Genome sequencing

Under the auspices of the Community Sequencing Program, the DOE-JGI is currently sequencing 0.1x genome coverage of each of five species of Lake Malawi cichlid (*Metriaclima zebra, Pseudotropheus tropheops, Melanochromis auratus, Copadichromis eucinostomus, Rhamphochromis esox*). The goal of this project is to identify SNPs for gene mapping, analysis of linkage disequilbrium and phylogenetics of this species flock. The first data from this project are expected in 2006. A network catalysis meeting is scheduled for Spring 2007 at the National Evolutionary Synthesis Center (NESCent) to coordinate the identification and subsequent applications of the SNP data.

Scientific Community

There is a large and diverse research community already working on the development, behavior, ecology, evolution, physiology and aquaculture of cichlid fishes around the world. The size of the cichlid research community can be estimated in many ways. A search for cichlid or tilapia produced more than 2000 citations in PubMed, over 4700 in Biological Abstracts and over 8000 in Aquatic Sciences and Fisheries Abstracts. More than 140 researchers subscribe to the cichlid genetics list server (hcgs.unh.edu/cichlid).

The Cichlid Genome Consortium was formally established at the Cichlid Evolution Workshop held in Karuizawa, Japan in September, 2003. This led to the organization of a Radcliffe Seminar on the Biology of Cichlid Fishes, held in Cambridge Massachusetts February 2-3, 2004, which brought together genome sequencing experts from the EMBL and MIT with cichlid researchers from the US and Europe. This whitepaper springs directly from that meeting.

Annotation and finishing

The Cichlid Genome Consortium is committed to completing the work of annotating and finishing the tilapia genome sequence. Basic annotation is easily obtained by applying standard gene-prediction algorithms to the shotgun sequence. However, this is only a first approximation, and there is always a need for continuing hand curation of the sequence. An essential tool in genome annotation is a relatively complete collection of EST sequences from full length clones.

Finishing a shotgun sequence is a challenging task, requiring experiments to link contigs and fill internal gaps in the assembly. We have already developed good resources for finishing, including a database of 35,000 Nile tilapia BAC fingerprints and extensive genetic maps for tilapia and the Lake Malawi haplochromines. BAC-end sequences will help link the shotgun assemblies to the BAC contigs and the genetic maps.

Collectively and individually, efforts are being made to complete the suite of genomic resources available, including sequencing of full-length cDNA libraries, construction of microarrays and linking the physical and genetic maps among species.

Comparative informatics

To facilitate comparative mapping among fish species, the Hubbard Center for Genome Studies has constructed a prototype comparative mapping database and viewer. These tools currently allow comparison of linkage maps and identify sequence similarities in comparisons to the Fugu and Tetraodon genome assemblies. The Cichlid Genome Consortium is committed to the further development of a cichlid model organism database and comparative genome viewers which accommodate the complexities of the fish-specific genome duplication.

Technical Challenges:

We do not anticipate any unusual technical challenges in this project. The use of a homozygous clonal line as a source of DNA for the tilapia genome will significantly enhance the whole-genome shotgun approach. Major classes of repetitive DNAs are either short and in low copy number, or are tandemly arrayed and well-characterized by fluorescent in-situ hybridization. Comparative assembly of the haplochromine cichlids on the tilapia genome should be straightforward, as these species diverged within the last 15MY and show high levels of sequence similarity.

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