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Comparative rates of lower jaw diversification in cichlid adaptive radiations

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Abstract

The lower jaw (LJ) provides an ideal trophic phenotype to compare rates and patterns of macroevolution among cichlid radiations. Using a novel phylogeny of four genes (*ND2*, *dlx2*, *mitfb*, and *s7*), we examined the evolutionary relationships among two of the most phylogenetically disparate cichlid radiations: (i) the Central America Heroines; and (ii) the East African Lake Malawi flock. To quantify jaw morphology, we measured two LJ lever systems in approximately 40 species from each lineage. Using geologic calibrations, we generated a chronogram for both groups and examined the rates of jaw evolution in the two radiations. The most rapidly evolving components of the LJ differed between the two radiations. However, the Lake Malawi flock exhibited a much faster rate of evolution in several components of the LJ. This rapid rate of divergence is consistent with natural selection, promoting unparalleled trophic diversification in Lake Malawi cichlids.

Introduction

The accumulation of phenotypic diversity varies across the tree of life. Phenotypic diversification is most rapid in adaptive radiations that are characterized by ecologically important phenotypes explosively diversifying (Schluter, 2000). The cichlid fishes in the East African Rift Lakes offer striking examples of adaptive radiation (Greenwood, 1964; Fryer & Iles, 1972). However, groups of cichlid fishes also appear to have radiated extensively in other parts of Africa and in lineages endemic to the Neotropics (Winemiller *et al.*, 1995; Genner *et al.*, 2007). Comparing rates of phenotypic evolution in East African lineages to rates in other cichlid groups should provide objective means to assess what patterns of diversification are specific to lineages in East Africa and what characteristics are shared with other cichlids.

In cichlids, jaws are critical to adaptive divergence. Extensive studies on the genetics, biomechanics and evolution of cichlid jaw elements (Albertson *et al.*, 2003; Albertson & Kocher, 2005; Albertson *et al.*, 2005; Hulsey *et al.*, 2006) provide an integrative framework to examine at what levels of biological design phenotypic diversification in these structures are both similar and different between radiations. To examine the patterns and rates of lower jaw (LJ) diversification in two phylogenetically disparate cichlid radiations, we compared the morphological and mechanical evolution of the LJ in the Neotropical Heroine cichlids and the cichlid flock of Lake Malawi.

Adaptive radiations of cichlids occur on both the African continent and in the Neotropics. The two monophyletic sister groups of cichlids on each continent likely diverged from one another with the splitting of Africa and South America at least 75 million years ago (Sparks & Smith, 2005). Two of the most evolutionarily disparate cichlid groups that have been suggested to be adaptive radiations are the Neotropical Heroine cichlids and the Lake Malawi haplochromine cichlid flock. Heroine cichlids are a trophically diverse radiation of Neotropical cichlids (Myers, 1966; Winemiller *et al.*, 1995), and the oral jaws of these fishes are thought to exhibit substantial anatomical diversity for exploiting disparate prey types (Eaton, 1943; Waltzek & Wainwright, 2003; Hulsey & Garcia de Leon, 2005; Hulsey *et al.*, 2008). However, in no group of cichlids has

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the rate and extent of trophic diversification likely to have been as extreme as in Lake Malawi in East Africa (Kocher, 2004). This monophyletic clade of approximately 500 recently evolved species exploits virtually every niche available in Lake Malawi (Greenwood, 1964; Fryer & Iles, 1972). If common patterns were found to govern diversification in these geographically and temporally divergent radiations, it could indicate similar mechanisms underlie all cichlid trophic diversification.

All cichlids share similar LJ structure and mechanical function. These elements (Fig. 1) have been extensively modelled as simple levers that confer mechanical properties that aid in exploiting different types of prey (Barel, 1983; Wainwright & Richard, 1995; Westneat, 1995; Albertson *et al.*, 2003). The consequences of morphological variation in these lever-like elements are highly predictable, mechanically informative and putatively adaptive (Albertson *et al.*, 2005). For instance, cichlid species exhibiting long dentary and articular LJ elements and short distances between the quadrate joint and the opening and closing ligaments generally exploit evasive prey such as fish and shrimp. Fish with this type of LJ morphology are generally more efficient at transmitting movement but have low mechanical advantage (Barel, 1983). Fish species with the morphologically opposite LJ morphology are likely modified for obtaining attached food such as algae or macroinvertebrates that require force to remove from the substrate (Wainwright & Richard, 1995). Critical morphological and mechanical variation in these LJ elements is also readily quantifiable and comparable across groups if information about evolutionary history is available.

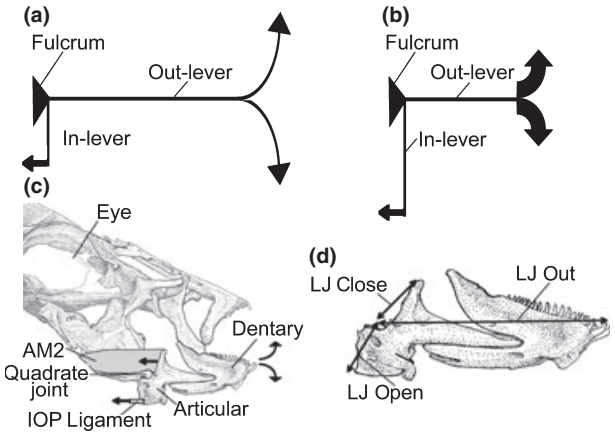

Fig. 1 Lower Jaw (LJ) Lever Systems. The LJ of cichlids can be modelled as a simple lever. The placement of the fulcrum and the relative length of in-lever and out-lever elements determine whether a lever can transmit lots of motion (a) or substantial force (b). Generally, a short in-lever and long out-lever result in the transmission of lots of motion as shown with the opening and closing arrows (a). Alternatively, a long in-lever and short out-lever result in the transmission of lots of force but relatively little motion as depicted with the arrows (b). In the LJ of cichlids (c), the interopercular (IOP) ligament attaches to the lower part of the articular, and this attachment serves as the input of motion and force to the LJ. The distance between this ligament and the quadrate joint serves as the in-lever, or LJ open, to the LJ system (d). Motion and force are then transmitted through the fulcrum at the quadrate-articular joint to the tip of the dentary. This distance serves as an out-lever for both the opening and closing of the LJ lever systems. The adductor mandibulae muscle 2 (AM2) that attaches on a process on the articular pulls the LJ closed. The closing motion and force from this muscle that acts through the fulcrum at the quadrate-articular joint to the tip can be measured as the LJ out of the LJ closing lever system. The ratio of LJ out to LJ open length can be used to calculate LJ open velocity ratio (VR). The ratio of LJ out to LJ close length can be used to calculate LJ close VR.

Phylogenies provide the macroevolutionary framework to examine how microevolutionary forces and constraints structure patterns and rates of phenotypic evolution in radiations (Hansen & Martins, 1996). Phylogenies are currently available for most species in these two radiations (Hulsey *et al.*, 2006, 2007). However, molecular phylogenies of both Lake Malawi and Heroine cichlids have largely been based on mitochondrial DNA. This could be problematic because of vagaries in the rates of mitochondrial evolution when the two groups are compared (Roca *et al.*, 2005; Mims *et al.*, 2010). Expanding the reconstructions of both groups to include genetic data from the nuclear genome would allow more robust analyses of the relationships among and between these cichlid lineages. Furthermore, to make inferences about the time frames of cichlid divergence, it would be ideal to have as many shared but genomically independent phylogenetic partitions as possible.

There are many ways to compare the phenotypic diversity within and among groups (Garland, 1992). Frequently, the phenotypic diversity among radiations is measured by the variance in these phenotypes or what is often termed disparity (Foote, 1993). To test whether one group is more phenotypically more diverse than another, the equivalence of variance in phenotypes can be straightforwardly tested. However, these measures of diversity in extant groups explicitly ignore the temporal accumulation of diversity (Collar *et al.*, 2005). Because one of the hallmarks of adaptive radiations is how quickly diversity accumulates (Schluter, 2000), the rate of phenotypic evolution in different cichlid radiations would be ideal to document. If groups exhibit similar amounts of putatively adaptive disparity and the accumulation of this diversity differs dramatically, we might infer differences in the relative strength of natural selection during the history of these radiations.

The relative importance of sexual selection vs. natural selection in the East African Rift Lakes is frequently debated (Streelman & Danley, 2003; Hulsey *et al.*, 2007; Salzburger, 2009). The diversity of colouration in these fishes and its apparent role in mate selection might have been the primary driver in the origin of the over 500 species of these fish present in Lake Malawi (Seehausen

& Schluter, 2004; Carleton *et al.*, 2005). However, natural selection may have also played a critical role in determining the forces governing their unparalleled speciation rates (Greenwood, 1964; Fryer & Iles, 1972; Albertson *et al.*, 2003). If the exceptionally rapid rate of speciation in Lake Malawi occurred without substantial divergence in ecologically important phenotypic characters like the LJ, it could indicate natural selection was not exceptionally critical in the diversification of these fishes. However, if there has been an increased rate of diversification in trophic characters like the LJ in Lake Malawi cichlids relative to other cichlid groups, this would be consistent with the idea that natural selection has played a fundamental role in the incredibly rapid rate of diversification of these fishes.

In this study, we generated time-calibrated phylogenetic hypotheses and estimated morphological and mechanical rates of evolution in the LJ of the Heroine and Malawi cichlid radiations. First, we reconstructed the phylogeny of these groups using one mitochondrial gene and three regions of the nuclear genome. Then, we measured components of the LJ that are thought to be important in the mechanics of these elements in a large number of species from both radiations. Finally, using the chronogram of both radiations, we tested for differences in the rate of LJ divergence in the two radiations.

Methods

Specimens

For the molecular analyses, all tissues utilized were reported in previous molecular studies of Heroine (Hulsey *et al.*, 2006) and Malawi cichlids (Hulsey *et al.*, 2007). For the morphological analyses, a combination of specimens collected from the wild and accessioned museum specimens were quantified. Collection localities for the phylogenetic and morphological analyses are available from the corresponding author. To analyse jaw morphology, all specimens were collected from their native range, preserved in formalin and then transferred to 70% ethanol.

Morphological measurements

Approximately three specimens of each species (Malawi: $n = 48$ species; Heroines: $n = 30$ species) were cleared using trypsin and double-stained using an Alcian-blue cartilage stain and alizarin red bone stain (Dingerkus & Uhler, 1977) for the examination of morphological characteristics of the jaws. In cleared and stained specimens, the bones are clearly discernable for morphological measurements, and the natural articulations of the skull are retained.

We measured three components of the LJ that can be used to model it as a set of simple levers (Fig. 1) including both the opening and closing lever systems (Barel, 1983; Wainwright & Richard, 1995; Westneat, 2003). First, the out-lever for both the opening and closing system (LJ out) was measured as the linear distance between the articular-quadrate joint and the anterior tip of the dentary bone on the LJ. Second, the in-lever for LJ opening (LJ open) was measured as the distance from the articular-quadrate joint to the midpoint of the interopercular–mandibular ligament on the articular bone that serves to pull open the jaw. Finally, the in-lever for closing (LJ close) was measured from the quadrate joint to the tip of the coronoid process on the articular. To remove the influence of size from the measurements made on individual specimens, we also measured standard length (SL) of each fish. The mean SL and mean of each of the three LJ morphometrics were first $\log_{10}$ transformed and then regressed against one another using reduced major axis regression. The residuals of each of the morphometrics were then used as the size-adjusted values in all subsequent comparisons of the morphometric variables.

We also transformed the three raw morphometric measurements into mechanical lever ratios. The ratio of out-lever to in-lever for opening and closing components of the LJ determine what fraction of motion input to the LJ is transmitted to the anterior tip of the jaw (Wainwright & Richard, 1995). For the analyses of the mechanical properties of the LJ, the inverse of the mechanical advantage, the velocity ratio (VR), was calculated (Westneat, 2003). The ratio of the length of LJ out divided by the length of LJ open was used to calculate LJ open VR. The ratio of the length of LJ out divided by the length of LJ close was used to calculate LJ close VR. Higher values of VR confer more rapid abilities to open and close the jaw and mechanically trade off with the ability to apply force in the LJ lever systems. Because these VR values are obtained from ratios of two linear measurements making them effectively nondimensional, we did not adjust these measurements for size in the comparisons of these mechanical variables.

DNA isolation and sequencing

Gene sequences from one mitochondrial and three nuclear gene regions were obtained for the Heroine and Malawi cichlids examined (Appendix S1). Sequences of the mitochondrial *ND2* gene previously published for the Malawi species (Hulsey *et al.*, 2007) were combined with new sequences from the Heroines using the same primers (Kocher *et al.*, 1995). The *s7* intron 1 was sequenced for both sets of species using published primers (Chow & Hazama, 1998). The *mitfb* gene was sequenced with primers used in Won *et al.* (2005) (*mitfb*For: 5′-CAGCCCTATGGCCTTATTGA; *mitfb*Rev: CCTTTTGATGTTTGGCAGGT). The *dlx2* gene was amplified from primers designed from cDNA sequence (*dlx2*F1: CGAACCAGATTACCTCAAGCA and *dlx2*R:

AGTTTGCCAAAAACGACGAA). Additional primers used to amplify *dlx2* in Malawi and Heroine cichlids (*dlx2*R3: GGCTGCTGCTCTGAGGTAAA; *dlx2*R4: CACGGGGGAG-ATTCACTG) were subsequently generated from these sequences. Because the temporal calibrations of phylogenetic divergence in the two radiations required an outgroup, sequences for all four genetic regions were obtained from the genome sequence of the Tetraodontiform *Takifugu rubripes*. Importantly, the sequences of this species were not utilized because of their potential to provide informative information about the cichlid relationships. These sequences were used because all the genes examined were well annotated in the *T. rubripes* genome, and the sequences were only included for the necessary removal of a clear outgroup in the time-calibration performed below.

For sequencing, total genomic DNA was isolated from caudal fin clips. A 1-l aliquot of extraction was used to provide a DNA template for polymerase chain reaction (PCR). Amplifications were carried out in a Perkin–Elmer DNA thermocycler using standard methods. Thermal cycling conditions for all regions sequenced consisted of an initial denaturation step of 94 °C (30 s), annealing step of 55 °C (30 s) and extension step of 72 °C (1.5 min). A final incubation of 72 °C for 5 min was added to ensure complete extension of amplified products. Subsequently, the PCR products were electrophoretically separated from unincorporated primers and dNTPs using electrophoresis in low-melting-point agarose gel with ethidium bromide (1 mg μL^{-1}) added and run in Tris-acetate buffer (pH 7.8). Positively amplified DNA was then purified using an enzymatic combination of 1 μL of exonuclease I (10.0 U μL^{-1}) and 1 μL shrimp alkaline phosphatase (2.0 U μL^{-1}) per 10 μL of PCR product. The PCR products were sequenced using Sanger sequencing at the High-Throughput DNA Sequencing Facility at the University of Washington. Gene sequences were assembled from individual sequencing reactions using the program Sequencher version 4.1 (Gene Codes, Ann Arbor, MI, USA). For analyses, sequences were aligned using Clustal X (Larkin *et al.*, 2007) and codon positions were defined using MacClade 4.0 (Maddison & Maddison, 2000). All sequences (Appendix S1) are available on Genbank.

Phylogeny reconstruction

For the Bayesian analyses, the *ND2* gene was partitioned into its three codon sites. The *s7* intron was treated as a single partition. The *mitfb* gene was partitioned into exonic and intronic regions using annotated partitions available for the Lake Malawi cichlid *Labeotropheus trewavasae* on Genbank (DQ239799). The *dlx2* gene was partitioned into exonic and intronic regions via comparisons to cDNA of this gene derived from the Lake Malawi cichlid *Metriaclima zebra* (Fraser *et al.*, 2009). The *mitfb* and *dlx2* intron regions were treated as unique data partitions, and the exons were partitioned into three codon sites for the phylogenetic analyses.

For the phylogenetic analyses, ModelTest 3.06 (Posada & Crandall, 1998) was used to identify the best model of molecular evolution for each partition. Once parameterized, the four genetic regions were concatenated to generate a single phylogenetic hypothesis. Then, Bayesian analyses were executed to find approximations of the maximum likelihood tree using MrBayes 3.0 (Ronquist & Huelsenbeck, 2003). The analyses treated the transition–transversion matrices, number of invariant sites and gamma shape parameters as unlinked or independent for each partition. Flat prior probability distribution for all parameters was assumed before analysis. We ran five separate Bayesian analyses for 5,000,000 generations with four Markov chains in each run. We sampled trees from the Markov Chain Monte Carlo search algorithm every 1000 generations. At the end of each analysis, the log-likelihood scores were plotted against generation time to identify the point at which log-likelihood values reached a stable equilibrium. In all five, the equilibrium appeared to be reached at approximately 100,000 generations, and therefore, sample points prior to generation 200,000 in each run were discarded as 'burn-in' samples. The remaining samples from all runs combined were used to produce a majority rule consensus tree in PAUP* 4.0b10 (Swofford, 2002). The percentages of trees that recovered a particular clade (the clade's posterior probability) were then depicted on the single best likelihood tree topology recovered.

Calibrating cichlid divergence

For the comparative analyses of LJ divergence, the phylogeny containing the two clades of cichlids was time-calibrated to produce an ultrametric chronogram. Branch lengths from our best phylogenetic hypothesis significantly rejected a single homogenous rate of molecular evolution (Fig. S1). Therefore, we used the penalized likelihood method (Sanderson, 2002) implemented in the program r8s 1.71 (Sanderson, 2003) to generate a time-calibrated chronogram for the two radiations. The outgroup branch for *T. rubripes* was first pruned from the analysis. Then, two external calibrations were used to generate the chronogram. The first calibration utilized the divergence between South America and Africa that occurred minimally 75.0 million years ago to calibrate the divergence between the two radiations of cichlids (Sparks & Smith, 2005). We randomly chose *Rhamphochromis esox* from the Malawi radiation and *Petenia splendida* from the Heroine radiation and fixed the most recent common ancestor between these Neotropical and African cichlids. The age calibration effectively calibrates the node and all its descendant species included in both radiations, and therefore, the two particular species chosen from each radiation should not influence the results. The second calibration utilized was the formation

of the Punta del Morro, a geologic boundary formed from the extension of the Mexican Neovolcanic Plateau to the Gulf of Mexico. By at least 5 million years ago, this boundary subdivided the genus *Herichthys* in Northeastern Mexico from all other Heroine cichlids to its south (Hulsey *et al.*, 2004). We used the fixage command in r8s to fix the divergence time between *Herichthys cyanoguttatus* to the north of the Punta del Morro and *Vieja fenestrata* south of the Punta del Morro at 5.0 million years. Similarly, the node that contains these two monophyletic groups was fixed. Both calibrations were used as absolute fixed age estimates. To estimate the time-calibrated ultrametric tree for both groups, cross validation was used to determine the optimal smoothing parameter for rate heterogeneity as outlined in Sanderson (2002) and implemented in r8s.

Comparative LJ diversity

The evolutionary divergence of LJ open, LJ out, LJ close, VR open and VR close estimated above was compared between the Lake Malawi and Heroine radiations. First, we analysed divergence in the LJ variables without taking evolutionary history into account. We examined the range of values in these variables to determine whether either radiation exhibited greater variation in LJ morphology or mechanics. Then, to determine whether either of the two radiations showed exceptional variation in any of the five LJ variables, an *F*-test was used to test whether the variance in each variable differed between the two cichlid radiations. Although the morphological and mechanical variables are clearly morphologically integrated, we believed treating the variables as independent would provide additional insight into the patterns of cichlid morphological and mechanical LJ evolution. These *F*-tests are also not phylogenetically independent tests of divergence but do provide information about the relative amounts of extant variation in the two clades. This type of comparison of nonphylogenetic disparity has often been considered of interest in studies of diversity (Hulsey & Wainwright, 2002), but they do not account for differences in phylogenetic structure or the time frame over which clades have been diversifying.

To test whether the evolutionary rate of phenotypic diversification in the LJ varied between the two radiations, the five LJ variables examined were imported into the program Brownie (O'meara *et al.*, 2006). Phenotypic rates (σ^2) of each of the five variables were obtained for the Lake Malawi and Heroine clades using maximum-likelihood estimates and the chronogram generated above. Under the Brownian motion model of character evolution implemented in Brownie, the maximum-likelihood estimator of the rate parameter and its likelihood score are estimated from the vector of species' character values, the ancestral value of the character, the number of taxa in the clade and the total branch length in the phylogeny. Thus, the input for the program was the matrix of LJ character values as they were reconstructed to have evolved on the chronogram (Fig. 2). The hypothesis that rates do not differ between clades was tested using a likelihood-ratio test. Support in Brownie for the null hypothesis would suggest that equal phenotypic rates in the two groups (i.e. one rate parameter for the phenotype in both radiations) could not be rejected. If the likelihood ratios were significant, it would suggest that rates are different in the two groups (i.e. separate rate parameters for the phenotype in each radiation). The program obtains *P*-values for the likelihood-ratio test statistic by comparison with a chi-square distribution with one degree of freedom. If there are substantial differences in the rates of evolution in the two groups, these analyses should explicitly account for that difference. However, if there is substantial rate variation within either the Heroine or Malawi cichlid clade, these analyses will not detect this type of within-clade rate divergence. Future analyses that include more species in each clade should be used to further explore these hypotheses.

Results

The total length of the concatenated alignment for all four partitions from both radiations was 2586 sites. In both radiations, the *ND2* gene was 1047 base pairs (bps) in length. For *s7*, the Heroine alignments ranged between 475 and 485 bps. In the Malawi cichlids, every sequence was 464 bps in length. The Malawi *mitfb* sequences generated ranged from 395 to 404 bps in length, whereas the Heroine sequences ranged from 378 to 420 bps. In the *dlx2* gene region examined, the intron in the Heroine cichlids contained a microsatellite that ranged from 2 to 66 TA repeats. This region was excluded from analyses. The remaining alignable Heroine sequences ranged from 873 to 884 bps and the Malawi sequences ranged only from 898 to 902 bps. Maximum pair-wise sequence divergence in Lake Malawi at the protein-coding and intron partitions examined ranged between 4.5 and 9 times less than the divergence recovered for Heroine cichlids (Table 1).

Most of the phylogenetic relationships recovered (Fig. 2) among the Heroine cichlids resemble those found in previous phylogenetic studies. The genera *Thorichthys* and *Herichthys* were recovered as monophyletic as in previous studies (Hulsey *et al.*, 2004). The relationships among species within the sister clade to *Herichthys* remain complicated, but the clade clearly contains the species *V. fenestrata* that was used to calibrate divergence across the Punta del Morro. The relationships of '*Cichlasoma*' *salvini*, *Rocio octofasciata* + *Astatheros macracanthus* and *Nandopsis tetracanthus* + *Nandopsis haitensis* remain ambiguous. Although the relationships among these species are generally not strongly supported, *P. splendida*, '*Cichlasoma*' *uropthalmus*, *'Cichlasoma' trimaculatum* and *Cryptoheros spilurus* fall into a monophyletic clade.

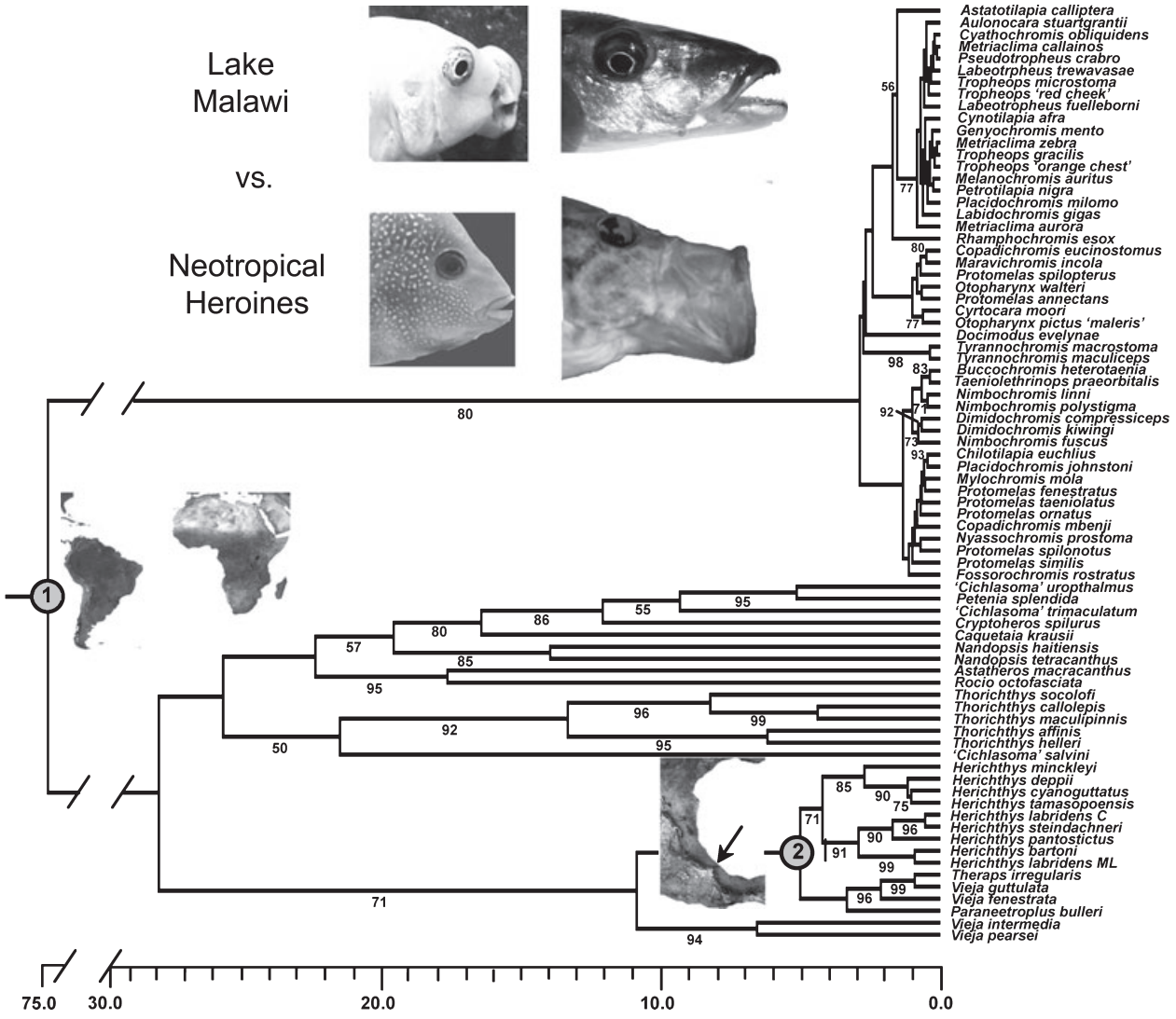

Fig. 2 Chronogram of Malawi and Heroine radiations. To create an ultrametric chronogram for both the Malawi (top) and Heroine (bottom) radiations, our best Mr Bayes phylogeny was time-calibrated using the program r8s (Sanderson, 2003). First, we utilized the divergence between South America and Africa (1) that occurred minimally 75 million years ago, to calibrate the divergence between the two radiations of cichlids. We also utilized the formation of the Punta del Morro (2) as a temporal calibration. This geologic boundary was formed as an extension of the Mexican Neovolcanic Plateau to the Gulf of Mexico. By at least 5 million years ago, this boundary subdivided the genus *Herichthys* in Northeastern Mexico from all other Heroine cichlids to its south. These calibrations were uses as fixed age estimates. To estimate the time-calibrated ultrametric tree for both groups, cross validations were examined to determine the optimal smoothing parameter for rate heterogeneity in the branch lengths determined from Mr Bayes. The maximum age of the Heroines appears to be about 30 million years, and the last common ancestor of the Malawi radiation began diversifying approximately 3 million years ago.

In contrast to the generally well-supported clades found in the Heroine cichlid, the relationships among the Lake Malawi cichlids are not well resolved as has generally been observed for this clade. There were a few interesting results, however. Although there was little support, *Rhamphochromis* was not recovered as the sister group to all other Malawi cichlids as has been found in previous studies (Hulsey *et al.*, 2007). There was some support for *Astatotilapia calliptera* grouping with the mbuna, or rock dwelling, species. As has been found in other analyses, species in the mbuna genera *Pseudotropheus*, *Labeotropheus*, *Genyochromis*, *Metriaclima*, *Petrotilapia*, *Melanochromis* and *Labidochromis* form a monophyletic clade. A large clade containing species in *Nimbochromis*, *Dimidochromis*, *Protomelas*, *Placidochromis* and *Mylochromis* was likewise recovered as a clade. In general, there is moderate to weak support for several clades, and the analysis of the four genetic partitions

Table 1 The per cent uncorrected maximum pair-wise sequence divergence for both the Heroine and Lake Malawi species flocks for six genetic partitions. Also shown is the age of the crown group of each species flock in millions of years estimated in the program r8s using the formation of the Punta Del Morro (5.0 million years ago) and the split between Africa and South America (75.0 million years ago) as minimum age calibration points.

	Heroines	Lake Malawi
ND2	17.5	3.0
S7	5.4	0.6
DLX2intron	4.8	0.7
DLX2exon	2.5	0.6
Mitfbintron	7.9	1.3
Mitfbexon	4.2	0.0
MRCA (mya)	28.0	2.9

provided little firm resolution for the phylogenetic relationships among Lake Malawi species. Using the best phylogeny and branch lengths estimated for these radiations, the ages estimated using r8s for the last common ancestor of all species in each clade indicate that the Heroine radiation is approximately 10 times older than the Lake Malawi radiation.

When the variance in the LJ variables (Table 2; Fig. 3) from the two radiations was compared with an *F*-test, the size-adjusted length of LJ open did not differ between the two radiations ($F_{47,29} = 1.32$; $P = 0.210$). Likewise, LJ out ($F_{47,29} = 0.76$; $P = 0.221$) and LJ close ($F_{47,29} = 0.67$; $P = 0.147$) did not differ significantly between the two radiations. The mechanics of LJ open VR ranged from 2.5 to 11.3 in the Malawi radiation showing greater variance than ($F_{47,29} = 3.36$; $P < 0.001$) and completely encompassing the Heroine radiation that ranged from 3.3 to 7.3. Malawi values for LJ close VR were generally lower, or more force modified, and ranged from 1.7 to 3.6, whereas the Heroines values ranged from 3.1 to 7.3. However, there was no difference in the variance between the two clades ($F_{47,29} = 1.49$; $P = 0.143$).

The relative rates of LJ divergence within each radiation differed substantially between the Lake Malawi and the Heroines (Table 3). In Lake Malawi, LJ out evolved at the fastest rate. LJ open evolved at about a third of the rate of LJ out in this East African clade. The slowest evolving element in Malawi was LJ close. With respect to mechanics, the rate of LJ open divergence was 6.6 times faster than LJ close in Lake Malawi. In the Heroine cichlids, LJ close evolved at the fastest rate. Both of the other LJ elements evolved at about half the LJ close rate, with LJ out evolving at a rate slightly higher than LJ open. Interestingly, in the Heroines, LJ close evolved at the fastest rate despite being the slowest evolving LJ element in Lake Malawi. Likewise, the rate of LJ close VR was approximately two times faster than the evolution of LJ open VR in Heroine cichlids. The fastest evolving morphological and mechanical elements of the LJ were completely different in the two radiations.

When the two radiations were compared (Table 4), LJ out was found to evolve at a 9.4 times faster rate in Lake Malawi than in Heroine cichlids ($P < 0.001$). LJ open also evolved at a 4.5 times faster rate in Lake Malawi than it did in the Heroine cichlid radiation ($P < 0.001$). Although the divergence in Lake Malawi of LJ close was also in the direction of evolving at a faster rate than in the Heroine cichlids (1.4 times), it was not found to be significantly faster ($P = 0.340$). Interestingly, the rate of LJ close VR was greater (1.7 times) but not significantly higher ($P = 0.111$) in Lake Malawi when compared to the Heroine cichlids. However, LJ opening VR evolved substantially faster ($P < 0.001$), approximately 19 times, in Lake Malawi when compared to the rate in Heroine cichlids.

Discussion

The amount of sequence divergence recovered for every genetic partition points to the Lake Malawi cichlid clade evolving over a much more rapid timescale than the Heroine cichlids. Generally, there was much less indel variation in the *s7*, *mitfb* and *dlx2* introns in the Malawi radiation. Although the protein-coding genes *mitfb* and *dlx2* could be involved in adaptive diversification of either cichlid clade, the relative rates of evolution in the exons of either gene were not relatively different from the rates of intron evolution when the two radiations were compared. The amount of sequence divergence in all protein-coding and intron regions examined supports the idea that the radiation in Malawi is substantially younger than the Heroine radiation. Additionally, the age estimates derived from mitochondrial DNA are not because of the potential phylogenetic vagaries (Roca *et al.*, 2005; Mims *et al.*, 2010) of this locus. The relative sequence divergence in the mitochondrial gene *ND2* (5.8 times) when maximum divergence within the two radiations was compared was not exceptional compared to the relative divergence observed for the nuclear markers.

Most of the phylogenetic relationships recovered among the Heroine cichlids resemble those found in previous phylogenetic studies that primarily rely on mitochondrial genes (Hulsey *et al.*, 2004). However, the placement of South American groups like *Caquetaia* and the Cuban and Haitian cichlids in the genus *Nandopsis* within the largely Central American clade of Heroine cichlids should be examined more extensively. Further analyses of Heroine cichlid phylogenetics will demand the incorporation of more nuclear markers, but the consistency of relationships and bifurcating histories recovered for species within this group suggests a consistent phylogeny could be determined relatively soon for these cichlids. Alternatively, despite the incorporation of three new nuclear markers, the relationships among the Lake Malawi cichlids are not well resolved and this has commonly been observed for this clade

Table 2 Species average morphometrics and mechanics used in the comparative analyses between the Malawi species flock and the Heroine species flock. The standard length (SL), lower jaw (LJ) opening in lever (LJ open), LJ out lever (LJ out), LJ closing in lever (LJ close), LJ opening velocity ratio (VR) (LJ open VR), LJ closing VR (LJ close VR) are presented.

Species	SL	LJ open	LJ out	LJ close	LJ open VR	LJ close VR
Malawi						
Astatotilapia calliptera	73.4	2.3	9.6	3.3	4.2	2.9
Aulonocara stuartgranti	70.7	2.4	9.5	3.4	4.0	2.8
Buccochromis heterotaenia	116.1	3.3	17.6	6.3	5.3	2.8
Chilotilapia euchilus	85.8	3.4	9.1	3.6	2.7	2.5
Copadichromis eucinostomus	90.7	1.9	9.1	3.6	4.8	2.5
Copadichromis mbenji	89.3	2.3	9.9	4.3	4.3	2.3
Cyathochromis obliquidens	78.6	2.1	8.2	3.3	3.9	2.5
Cynotilapia afra	71.2	2.0	8.1	3.4	4.1	2.4
Cyrtocara moori	131.3	3.7	17.1	5.3	4.6	3.2
Dimidochromis compressiceps	123.8	3.2	18.7	6.3	5.8	3.0
Dimidochromis kiwingi	202.9	4.6	25.1	8.3	5.5	3.0
Docimodus evelynae	59.1	1.9	6.5	3.4	3.4	1.9
Fossorochromis rostratus	80.8	1.9	11.2	3.6	5.9	3.1
Genyochromis mento	76.2	2.0	8.6	3.2	4.3	2.7
Labeotropheus trewavassae	89.6	1.9	5.8	3.3	3.1	1.8
Labeotrophopheus fuelleborni	99.1	2.6	6.6	4.0	2.5	1.7
Labidochromis gigas	75.1	1.9	6.4	3.1	3.4	2.1
Maravichromis incola	80.3	2.2	10.3	3.5	4.7	2.9
Maravichromis mola	113.0	3.3	13.4	4.6	4.1	2.9
Maylandia callainos	65.6	1.9	6.6	2.6	3.5	2.5
Melanochromis auritus	67.8	1.8	6.3	2.8	3.5	2.3
Metriaclima aureus	77.1	2.0	7.4	3.3	3.7	2.2
Metriaclima zebra	81.8	2.1	9.3	3.7	4.4	2.5
Nimbochromis fuscotaeniatus	76.9	2.3	12.7	4.0	5.5	3.2
Nimbochromis linni	112.1	3.4	12.8	4.7	3.8	2.7
Nimbochromis polystigma	99.4	3.4	16.1	5.5	4.7	2.9
Nyassochromisprostoma	94.9	2.2	10.2	4.1	4.6	2.5
Otopharynx pictus	80.3	1.9	9.9	3.7	5.2	2.7
Otopharynx walteri	91.2	2.5	12.7	4.3	5.1	3.0
Petrotilapai nigra	103.7	3.0	12.4	4.3	4.1	2.9
Placidochromis johnstonii	131.2	3.6	23.2	6.4	6.4	3.6
Placidochromis milomo	117.9	4.6	18.1	6.8	3.9	2.7
Protomelas annectans	112.3	3.9	14.4	5.0	3.7	2.9
Protomelas fenestratus	87.7	2.8	8.3	3.2	3.0	2.6
Protomelas ornatus	131.2	3.6	23.2	6.4	6.4	3.6
Protomelas similis	109.8	3.1	11.9	4.2	3.8	2.8
Protomelas spilonotus	154.2	4.4	21.3	8.9	4.8	2.4
Protomelas spilopterus	97.6	3.6	14.4	5.0	4.0	2.9
Protomelas taeniolatus	92.2	2.1	8.8	3.0	4.2	2.9
Pseudotropheus crabro	73.0	1.9	8.3	3.1	4.4	2.7
Rhamphochromis esox	93.8	1.2	13.5	4.1	11.3	3.3
Taeniolethrinops praeorbitalis	131.2	3.6	23.2	6.4	6.4	3.6
Tropheops gracilis	78.5	1.8	6.0	3.0	3.3	2.0
Tropheops microstoma	78.2	2.0	7.1	3.0	3.6	2.4
Tropheops orange chest	84.5	2.2	6.6	3.2	3.0	2.1
Tropheops red cheek	84.7	2.1	6.5	3.5	3.1	1.9
Tyrannochromis macrostoma	123.7	3.2	23.7	7.8	7.4	3.0
Tyrannochromis maculiceps	89.4	2.6	17.3	5.2	6.7	3.3
Heroine						
Astatheros macracanthus	83.9	3.0	10.9	1.7	3.6	6.4
Caquetaia kraussi	75.0	2.8	14.4	2.5	5.1	5.8
Cichlasoma octofasciatum	75.5	2.6	8.8	1.9	3.4	4.6
Cichlasoma' salvini	71.8	2.0	10.1	1.8	5.1	5.6
Cichlasoma spilurum	63.0	1.6	6.0	1.3	3.8	4.6
Herichthys tamasopoensis	89.8	2.9	10.8	1.6	3.7	6.8

Table 2 (Continued).

Species	SL	LJ open	LJ out	LJ close	LJ open VR	LJ close VR
Herichths deppi	77.7	2.2	11.0	1.7	5.0	6.5
Herichthys bartoni	81.9	2.6	11.7	1.6	4.5	7.3
Herichthys cyanoguttatus	88.1	2.7	11.5	1.6	4.3	7.2
Herichthys labridens C	91.4	2.7	10.1	2.1	3.7	4.8
Herichthys labridens ML	81.6	3.0	10.0	1.7	3.3	5.9
Herichthys pantosticus	76.8	2.4	10.0	1.6	4.2	6.3
Herichthys steindachneri	153.6	7.3	24.3	6.8	3.3	3.6
Herithchys minckleyi	89.5	3.1	12.4	2.7	4.0	4.6
Nandopsis haitiensis	70.1	2.2	9.6	1.8	4.4	5.3
Nandopsis tetracanthus	53.2	1.7	8.6	1.2	5.1	7.2
Nandopsis trimaculatus	74.7	2.3	10.1	1.9	4.4	5.3
Nandopsis uropthalmus	84.4	2.1	10.7	2.2	5.1	4.9
Paraneetroplus bulleri	98.2	2.0	7.3	1.4	3.7	5.2
Petenia splendida	99.7	3.0	22.0	3.7	7.3	5.9
Theraps irregulare	84.9	2.0	8.9	1.6	4.5	5.6
Thorichthsy helleri	82.2	2.5	9.5	1.6	3.8	5.9
Thorichthys affinis	62.3	2.0	9.3	1.6	4.7	5.8
Thorichthys callolepis	84.3	2.4	8.6	1.8	3.6	4.8
Thorichthys maculippinnis	95.6	2.9	11.0	2.2	3.8	5.0
Thorichthys socolofi	89.7	3.2	12.3	4.0	3.8	3.1
Vieja fenestratus	107.3	3.2	11.9	2.1	3.7	5.7
Vieja guttulatus	94.1	2.8	9.4	1.9	3.4	4.9
Vieja intermedium	59.9	1.8	7.8	1.5	4.3	5.2
Vieja pearsei	70.5	1.7	7.9	1.2	4.6	6.6

(Kocher *et al.*, 1995; Hulsey *et al.*, 2007). Additional analyses of Malawi cichlid evolutionary relationships will demand not only the analysis of more nuclear markers, but it will also require a greater appreciation for the potentially confounding effects of ancestral polymorphism and hybridization (Hulsey, 2009; Mims *et al.*, 2010) on the recovery of the evolutionary relationships among Lake Malawi cichlids.

The time frame of divergence in many cichlid radiations remains contentious (Genner *et al.*, 2007). Numerous fossil cichlids in both Africa and South America have been found suggesting cichlid diversification on both continents has been occurring for at least 50 million years (Murray, 2001; Malabarba *et al.*, 2006). However, because of the large number of cichlid lineages on these two continents, placing these fossils within a time-calibrated tree remains a considerable challenge. Nevertheless, cladistic analyses indicate that the pattern of branching among major lineages of cichlids is consistent with the break-up of Gondwana (Sparks & Smith, 2005). If this is true, it suggests a minimum time frame for cichlid divergence into the monophyletic sister groups in Africa and the Neotropics beginning around 75 million years ago. If this time frame is at least approximately correct, our time-calibrated chronogram suggests Heroine cichlids have been diversifying in Central America for approximately 30 million years, whereas Malawi cichlids have phenotypically exploded only in the last 3 million years.

The range of values of opening VR in the Heroine cichlids was completely bounded by the greater range of values in Malawi. The mechanics of closing VR in Malawi and in the Heroines was almost nonoverlapping and generally this value was much lower in the Malawi cichlids. This suggests that generally the Malawi cichlid radiation exhibits a much more force-modified range of closing jaw values than the Heroine cichlids. The trophic guilds modified to scrape algae and other prey off of rocky substrates in Malawi (Fryer & Iles, 1972) are largely absent in Heroine cichlids (Winemiller *et al.*, 1995; Hulsey & Garcia de Leon, 2005; Hulsey, 2006), and could be responsible for this striking difference in jaw mechanics between the two radiations.

The fastest evolving morphological and mechanical elements of the LJ were different in the two radiations. Whereas LJ out evolved most quickly in Lake Malawi relative to the other two LJ elements, LJ close evolved most rapidly compared to the other two LJ elements in the Heroines. These different rates of the LJ elements had consequences for the relative rates of mechanical evolution in the two radiations. In Lake Malawi, LJ open VR evolved most rapidly, but in the Heroines, LJ close VR evolved most rapidly. Because the relative rates of morphological evolution differed substantially between the two radiations, jaw mechanics likewise differed substantially. There do not appear to be general patterns for the relative rate of LJ divergence that is common to all cichlid radiations.

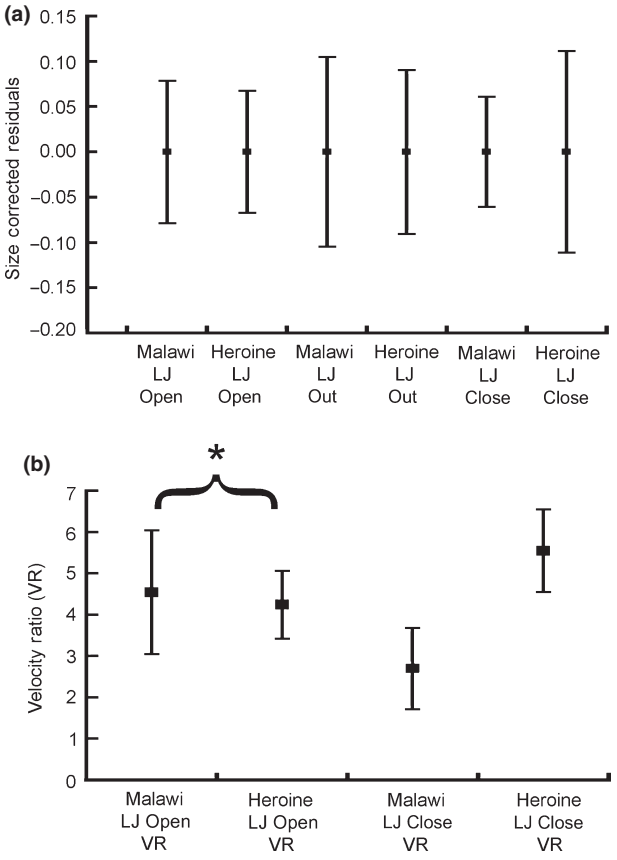

Fig. 3 Variation in the Malawi and Heroine lower jaw (LJ) variables. Prior to the phylogenetic rates analyses, we examined divergence in the LJ variables without taking evolutionary history into account. Shown above is the empirically estimated variance in the size-adjusted morphometric variables (a) LJ open, LJ out and LJ close as well as the mechanical variables LJ open velocity ratio (VR) and LJ close VR (b) in both radiations. To determine whether either of the two radiations showed exceptional variation in any of the five LJ variables, an *F*-test was used to test whether the variance in each variable differed between the two cichlid radiations. The *F*-tests provided information about the relative amounts of extant variation in the two clades. Size corrected LJ open ($P = 0.210$), LJ out ($P = 0.221$), and LJ close ($P = 0.147$) did not differ significantly between the two radiations. The mechanics of LJ open VR in the Malawi radiation exhibited greater variance than and completely encompassed the Heroine radiation ($P < 0.001$; noted with an *). In Malawi, values for LJ close VR were generally lower, or more force modified, and ranged from 1.7 to 3.6, whereas the Heroines values ranged from 3.1 to 7.3, but there was no significant difference in the variance between the two clades ($P = 0.143$).

Table 3 The relative rates within radiations when the three lower jaw (LJ) morphometrics and two velocity ratios (VRs) of these elements were compared to one another. Comparisons of rate magnitude are made relative to the slowest evolving phenotype in each radiation (*). For instance, LJ out evolved 3.7 times (X) faster than LJ close in the Malawi flock. Whereas rates of LJ open and LJ open VR were relatively faster within Malawi, LJ close and LJ close VR evolved at a relatively faster rate within Heroine cichlids.

	LJ open	LJ out	LJ close	LJ open VR	LJ close VR
Within Malawi	1.5 X	3.7 X	*	6.6 X	*
Within Heroines	*	1.2 X	1.9 X	*	1.9 X

Table 4 The rates of lower jaw (LJ) elements and LJ mechanics were compared between the Malawi and Heroine radiations. The Malawi radiation consistently showed a faster rate of evolution in morphometrics and mechanical characteristics of the LJ. However, LJ close and LJ close velocity ratio (VR) did not evolve at a significantly different rate in the two radiations. This is likely because of the fact that LJ close was the most rapidly evolving component of the LJ in Heroine cichlids and the slowest evolving component in Malawi cichlids.

	LJ open	LJ out	LJ close	LJ open VR	LJ close VR
Malawi vs. Heroines	4.4 X	9.4 X	1.5 X	14.3 X	1.1 X
Chi-square, *P*	0.000	0.000	0.220	0.000	0.777

The absolute rate of evolutionary change in LJ open (4.4 times) and LJ out (9.4 times) in Lake Malawi is substantially greater than the rate of change for these traits in the Heroine cichlids. Likewise, the rate of evolution of opening mechanics, LJ open VR, is also substantially higher in the Lake Malawi cichlids (14.3 times). However, despite the substantial differences of these rates in the two clades in the morphology and mechanics of LJ open VR, there was no significant difference between the rates of LJ close VR when the two clades were compared. The lack of a significant rate difference in LJ close between the two radiations is likely the reason for the lack of a rate difference in LJ close VR. The lack of independence of these morphological and mechanical phenotypes highlights the difficulty in pinpointing the exact traits that are diversifying in rapidly evolving groups like the Lake Malawi cichlids. However, untangling the levels of biological design (i.e. genetic, developmental, morphological or mechanical) that differ between radiations clearly provides novel insights into the factors promoting adaptive diversification at other levels of design (Protas *et al.*, 2006; Wainwright, 2007).

The rapid rate of morphological and mechanical evolution in the LJ of the Lake Malawi cichlids is consistent with enhanced natural selection playing a role in the unparalleled speciation rate of these fishes. Sexual selection is clearly an important driver of diversification in Lake Malawi cichlids (Seehausen & Schluter, 2004; Carleton *et al.*, 2005), but the rates of morphological and mechanical divergence in the LJ suggest adaptation to feeding on different prey types could play a critical role in ecological diversification of Malawi cichlids. Because we explicitly examined jaw variables that have been shown to be mechanically important in

feeding (Barel, 1983; Wainwright & Richard, 1995), the increased rate of LJ evolution observed in Lake Malawi is likely because of selection on feeding abilities imposed through competition or other negative ecological interactions. However, because female Malawi cichlids brood their young in their mouths, the importance of the jaws of Malawi cichlids in sexual selection cannot be completely discounted (Hulsey, 2009). It is also feasible that cichlids are able to discriminate conspecifics based on variation in jaw shape. Providing further explicit tests of the relative importance of natural vs. sexual selection in these two radiations is beyond the scope of this study. Nevertheless, assessing the relative importance of natural and sexual selection in the Lake Malawi cichlids will be critical for understanding what has primarily fuelled the rapid diversification in this unique lineage (Salzburger, 2009).

Understanding the mechanisms producing patterns and rates of phenotypic evolution in evolutionarily independent lineages will continue to provide novel insight into the importance of different levels of biological design in structuring adaptive diversification (Wainwright, 2007; Sidlauskas, 2008). The ever-increasing availability of phylogenetic markers coupled with a greater mechanistic understanding of adaptive evolution offer unprecedented opportunities for comparative biology (Losos *et al.*, 1998). By examining the rates of phenotypic evolution in a time-calibrated phylogenetic framework, the tempo of adaptive macroevolutionary diversification can now be explicitly assessed (Collar *et al.*, 2005). A greater appreciation of the rates of evolution in adaptive phenotypes among clades should greatly increase our understanding of whether general patterns exist in adaptive radiations.

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Supporting information

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

Appendix S1 Genbank numbers for the four gene regions sequenced.

Figure S1 The untransformed branch lengths of the phylogeny of Malawi and Heroine cichlids using four genetic partitions.

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