Ancestral splice variation is a substrate for rapid diversification in African cichlids

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Abstract

While the contribution of gene expression (GE) to adaptive evolution is widely accepted, the role of alternative splicing (AS) remains less understood. Here, we investigate AS and GE across three iconic adaptive radiations of African cichlid fishes that evolved within <16,000 to 3.8 million years. We show that AS evolves faster than GE, with both sources of variation being 'fine-tuned' over evolutionary time to become species-specific and clade-specific, respectively. Ecologically divergent species from younger radiations exhibit greater differences in splicing than those from older radiations. Most of these differentially spliced isoforms arose from standing variation, which was also present at low levels in non-radiating species, and increased in frequency during the adaptive radiation process. We identified several novel isoforms of craniofacial remodelling genes that emerged within each lake radiation and were differentially incorporated in the jaws of herbivorous vs. carnivorous species. Our findings indicate that a complex temporal interplay of GE and AS underlies adaptive radiation, with ancestral splice variation enabling rapid ecological diversification at early stages of speciation.

Key words: gene regulation, speciation, convergent evolution, trophic adaptation, morphology

Introduction

Since King & Wilson's[1] landmark paper, the role of gene expression (GE) is widely accepted in adaptive evolution but alternative splicing (AS) has been largely overlooked as a potential substrate for adaptation and

speciation[2]. Alternative splicing is a ubiquitous co-/post-transcriptional mechanism in multicellular organisms that can expand the transcriptomic and proteomic repertoire of a cell by generating multiple distinct messenger RNAs (mRNAs) from a single gene[3]. There exists a strong relationship between splicing complexity and organismal complexity across the tree of life[4]. Landmark cross-species comparisons across deeply divergent vertebrates have identified increased rates of AS evolution between species and linked it to some key evolutionary events[5–7]. However, the contribution of AS to the evolution of ecological and species richness, particularly at short evolutionary timescales, is not well understood as large-scale comparative studies are lacking[2].

Adaptive radiations of cichlid fishes are excellent models to study recent and rapid adaptive evolution in response to ecological opportunity[8]. They have evolved diverse jaw and teeth shapes and feeding habits to adapt to different food sources, forming species-rich communities spanning complex foodwebs[9,10]. Although the distinctive features of adaptive radiation are still debated[11,12], it has been proposed that both GE[13–16] and more recently, AS[17,18], may be important for eco-morphological divergence. However, it is not fully known how widespread the contribution of splicing is to adaptive radiation and which factors shape its evolution. Furthermore, the interplay of AS versus GE across varied phylogenetic timescales remains unclear. We propose that these two sources of heritable variation may play distinct roles in biological diversification, such as rapid functional diversfication during/near speciation through splicing versus much slower adaptive fine-tuning after speciation through gene expression. This hypothesis has not been tested. Differently aged radiations of cichlid fishes on the African continent provide a powerful framework to adress this knowledge gap (Figure 1A).

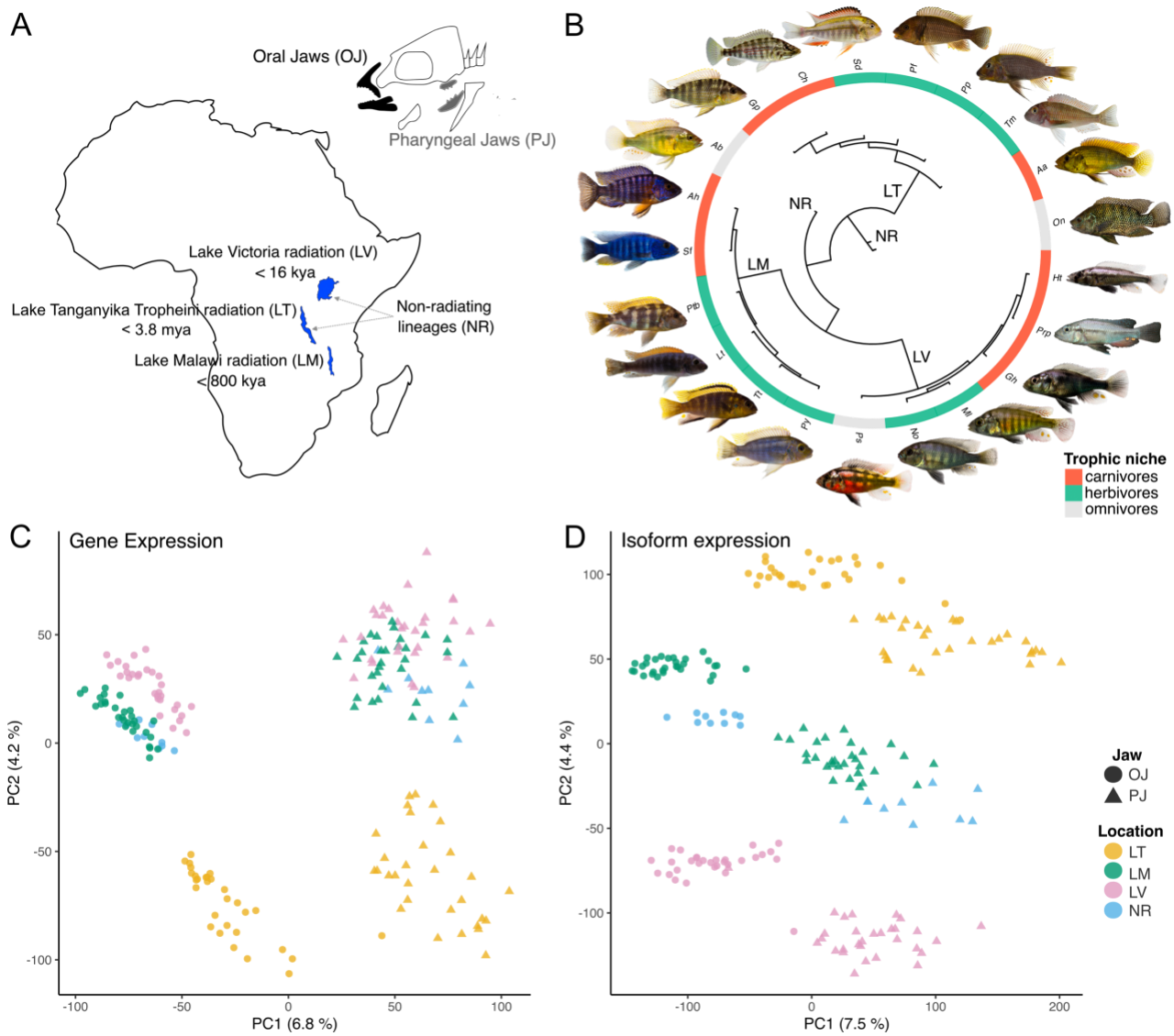


Figure 1 Study overview. (A, B) Biogeographical setting of species sampled in this study (20 species, 2 jaw tissues (oral and pharyngeal), 5 biological replicates per species per tissue, n=200). Phylogenetic relationships reconstructed using transcriptome SNPs. Data for *O. niloticus* (On) outgroup is from XX. Lake Victoria: *Paralabidochromis sauvagei* (Ps), *Neochromis omnicaeruleus* (No), *Mbipia lutea* (Ml), *Prognathochromis perrieri* (Pp); *Haplochromis thereuterion* (Ht); Lake Malawi: *Aulonocara hansbaenschi* (Ah), *Sciaenochromis fryeri* (Sf), *Petrotilapia* sp. *'Thick bars'* (Ptb), *Labeotropheus trewavasae* (Lt), *Tropheops tropheops* (Tt), *Petrotilapia* sp. 'yellow-chin' (Py); Lake Tanganyika: *Tropheus moorii* (Tm), *Petrochromis polyodon* (Pp), *Petrochromis famula* (Pf), *Simochromis diagramma* (Sd), *Ctenochromis horei* (Ch), *Gnathochromis pfefferi* (Gp). Non-radiating haplochromines, *Astatotilapia burtoni* (Ab) and *Astatoreochromis alluaudi* (Aa) are included as riverine lineages that entered lakes but did not radiate. **(C, D)** Principal component analysis of transcriptome-wide normalised gene and isoform expression from oral (OJ) and pharyngeal jaws (PJ) of all 20 species.

Here we investigate the evolution of GE and AS in cichlid adaptive radiations from the three African Great Lakes and test if they played a role in trophic diversification. Lake Victoria (LV) hosts the youngest radiation that arose over the past 16,000 years with ~500 species known, Lake Malawi (LM) hosts the second oldest radiation with ~700 species that arose over the past 800,000 years ago, and Tropheini cichlids from Lake Tanganyika (LT) that comprise the oldest radiation in our study with 31 species that arose < 3.8 Ma[19,20]. It is hypothesised that the radiations of Malawi and Victoria, as well as a subclade of the Tanganyika cichlid radiation (Tropheini) were seeded by a common riverine lineage, repeatedly giving rise to the similar eco-morphologies[21]. Thus, these three parallel radiations represent the spread and diversification of the 'modern haplochromines' that are famous for their species richness, morphological, and trophic diversity[21]. With a focus on (within radiations) divergent and (between radiations) convergent trophic adaptation, we sequenced the transcriptomes (mRNA) of oral jaws (OJ) and pharyngeal jaws (PJ) of juvenile larvae (developmental stage 26: yolk-sac absorbed into body cavity[22], an ecomorphologically informative developmental stage for cichlid jaws[17]) from 18 species representing corresponding trophic guilds of herbivory and carnivory from the three African Great Lakes (Figure 1B)[9,10]. Functional decoupling of cichlid oral and phryngeal jaws is thought to be a key innovation that facilitated their rapid diversification[21]. Two non-radiating species (NR) that live in lakes but did not radiate were also included in the analysis (Figure 1A, B).

Results

Global patterns of gene and isoform expression

To study the evolution of GE and AS during adaptive radiation, we generated transcriptomes of two tissues (OJ and PJ) from 100 cichlid fishes belonging to 20 species (Figure 1, Table S1) from three lake radiations and two species that did not make radiations (5 individuals per tissue per species, total N = 200). Reads were mapped to the phylogenetically equidistant *Oreochromis niloticus* reference genome[23] (median sequencing depth per tissue: median ~6.4 million 125 base-pair (bp) paired end reads per library; median ~80% uniquely mapped read; Table S1) using the genome-guided transcript assembly function. Gene annotations of species and tissues constructed using StringTie[24] were merged incrementally into one super-annotation[24] that was used for downstream analysis. This strategy helped to reduce false positive while increasing detection of novel and lowly expressed isoforms. Expression was quantified using established methods[24,25].

To explore global patterns of gene and isoform expression, we performed Principal component analysis (PCA) across all samples for all genes. Gene expression PC1 separated the two tissues, and on PC2, LT clustered separately from the younger radiations of LM, LV and the non-radiating species, the latter formed an overlapping cluster (Figure 1C). PCA clustering of isoform expression, which is a consequence of AS, was also tissue-specific on PC1, but clearly lake-specific on PC2 (Figure 1D) with non-radiating species clustering closer

104 to LM samples, particularly for the PJ. Both gene and isoform expression patterns within tissues reflect
105 phylogenetic relationships of radiations (Figure 1B), with stronger signals emerging from the isoform
106 expression PCA. This suggests that differences in splicing accumulate more rapidly than GE changes during
107 adaptive radiation, emerging as clades diverge, such that global splicing patterns across species reflects their
108 phylogenetic relationships.

109

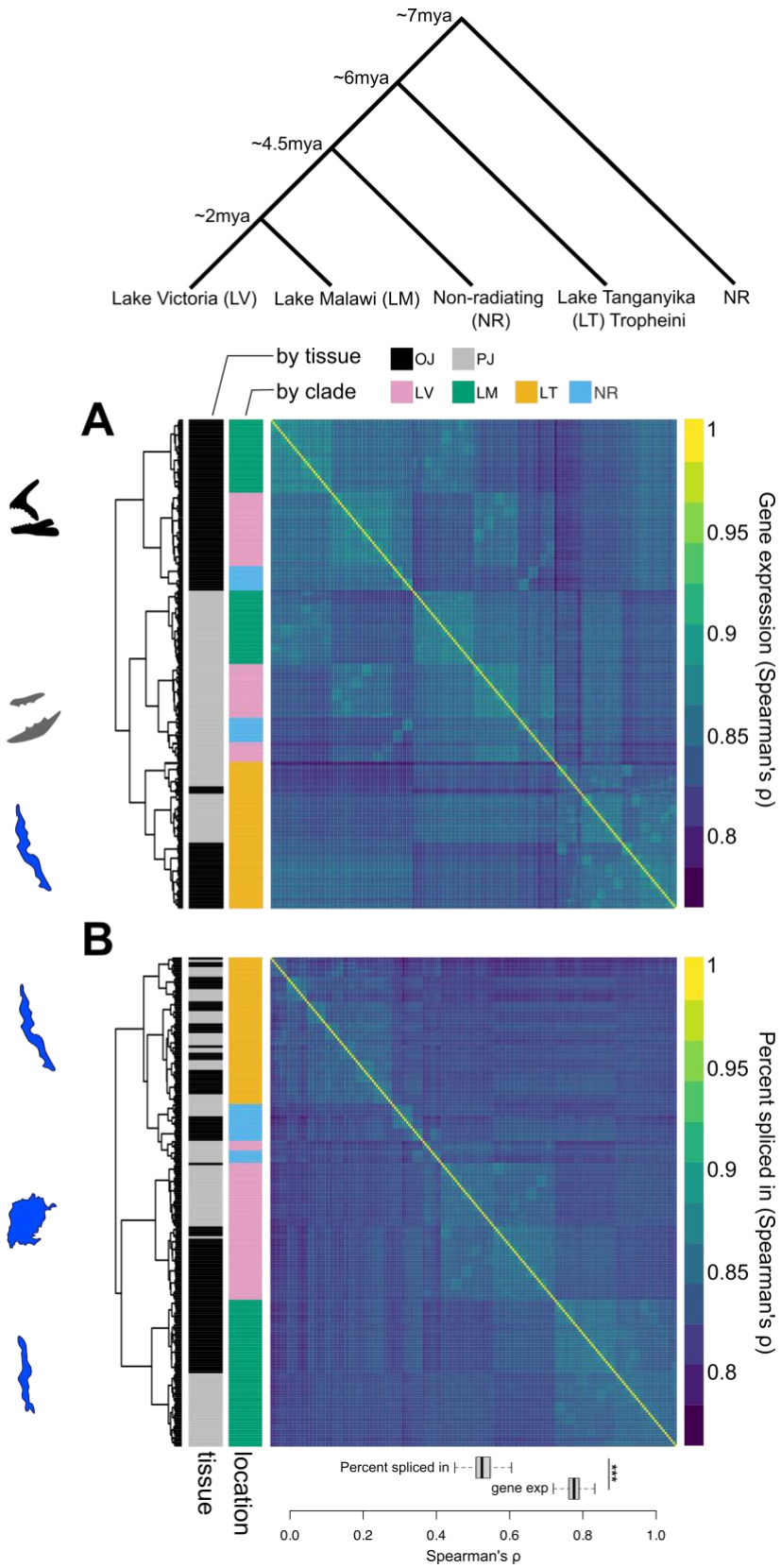
110 **Splicing and expression have contrasting evolutionary dynamics**

111 Alternative splicing results in the change of relative abundance of isoforms produced from the same gene[3],
112 which may have evolutionary consequences[2,26]. But little is known about how AS evolves at difference
113 timescales. To adress this, we calculated percent spliced in (PSI), a standard AS metric that represents the
114 relative abundance of an isoform as a proportion of the total GE[27,28] and computed Spearman's rank correlation
115 coefficients (Spearman's ρ) across all samples and tissues for GE (all genes) and PSI (only genes with more
116 than one isoform) to compare rate of change among these two processes. Our analysis revealed higher ρ values
117 for GE (Figure 2 boxplot, two-sided t-test $p < 0.001$). This suggets that GE was more conserved (less divergent)
118 across samples than PSI during cichlid evolution; while AS was evolving faster. We next tested if constitutively
119 expressed isoforms were more conserved during cichlid evolution than alternative isoforms. Indeed, we found
120 that Spearmans's ρ of PSI for alternative isoforms was significantly lower than that of constitutive isoforms
121 (Figure S1, two-sided t-test $p < 0.001$). This fits the expectation of alternative isoforms generally evolving under
122 weaker purifying selection[29,30].

123

124 Next, we analysed the hierarchical clustering of Spearman's ρ for GE and PSI. We found that GE grouped
125 samples by tissue for younger radiations (LV, LM) and nonradiating species (Ab, Aa). However, for the older
126 LT Tropheini radiation, samples clustered first by clade/radiation and then by tissue within the radiation (Figure
127 2A), defying well-established expectations of tissue-specific conservation of GE in vertebrates[6]. In contrast,
128 Spearman's ρ of PSI clustered samples first by clade/radiation, regardless of tissue. Samples from Lake
129 Tanganyika and nonradiating species further clustered by species, regardless of tissue (Figure 2B, S2).
130 Evolutionarily conserved (heritable) species-specific and clade-specific changes in splicing would result in
131 samples clustering first by species/clade and then tissue. This suggests that clade-specific divergence in splicing
132 exceeds tissue-specific conserved signatures of splicing at a phylogenetic distance of ~2mya, and species-
133 specific divergence in splicing exceeds tissue-specific divergence at ~6mya. Hierachical clustering relationships
134 generally reflected phylogenetic relationships of the radiations, however, NR species clustered with LV in the
135 gene expresssion analysis but with LT in the PSI analysis. Interestingly, PJ samples of *H. thereuterion* from LV
136 were placed outside of the LV clade, sister to the PJ's of the NR *A. burtoni*, which is distributed in Lake
137 Tanganyika basin and not found in Lake Victoria. Taken together with the PCA results (Figure 1A,B), our

results indicate that AS may have played an important role in the evolution of clade and species-specific jaw specialisations in comparison to GE. It may further suggest that heritable changes in splicing emerge rapidly during/near speciation in cichlid adaptive radiations, while GE changes may fine-tune morphology over longer evolutionary timescales.


~7mya
~6mya
~4.5mya
~2mya
Lake Victoria (LV)
Lake Malawi (LM)
Non-radiating (NR)
Lake Tanganyika (LT) Tropheini
NR
by tissue
by clade
OJ
PJ
LV
LM
LT
NR
A
B
1
0.95
0.9
0.85
0.8
Gene expression (Spearman's ρ)
Percent spliced in (Spearman's ρ)
tissue
location
Percent spliced in
gene exp
***
0.0
0.2
0.4
0.6
0.8
1.0
Spearman's ρ

143

144

Figure 2 Profiling of alternative splicing and gene expression in cichlid radiations. Hierarchical clustering of Spearman's rank correlation coefficient (ρ) of **(A)** normalised GE and **(B)** isoform percent spliced in (PSI) between pairs of samples (n=200). Sample clustering is represented as a tree on the left side of the heatmaps, with tissue and clade (radiations or nonradiating) of origin of species annotated in colours and the dominant cluster signature illustrated as radiation/non-radiation/jaw. Boxplots show significant difference in distribution of PSI and GE (two-sides t-test $p < 0.001$). Cladogram illustrates simplified cladogram of phylogenetic relationship of species shown in Figure S3A. Divergence times taken from Irisarri*, Singh* et al (2018)[19]. See Figure S2 for sample names.

Genes and isoforms evolve under stabilising selection

Next, we investigated the evolution of gene and isoform expression along the phylogeny (constructed using SNPs from the RNAseq data, Figure S3A) by fitting Brownian motion (BM) and Ornstein-Uhlenbeck (OU) processes. Ornstein-Uhlenbeck has been applied to model stochastic GE evolution towards a single optimum i.e. stabilising selection[31]. Brownian motion models neutral evolution i.e. genetic drift. The distribution of OU variance across the phylogeny, denoted 'evolutionary variance'[32], depicts how constrained expression evolution is. We found that expression of most genes and isoforms was evolving under stabilising selection (90–92%) and 8-10% of genes were evolving neutrally (chi-squared test q-value < 0.05, Figure S3B, File S1), as previously reported in model species[31]. However, isoform expression had significantly higher evolutionary variance (less constraint) than GE under the OU model for both jaws (Figure S3C), which would be the result of weaker stabilising selection acting on isoform expression than gene expression. This further explains the lower cross-species conservation of splicing we found above, even after taking the phylogeny into account.

Most splice variation is ancestral, but some may be novel

Ancestral variation is proposed to fuel rapid adaptive radiations[33]. One way that this could happen is through the adaptive sorting of ancestral gene regulation variation[34], such as splice variation. As a first step to test if ancestral splice variation contributed to trophic divergence, we quantified the number of genes and isoforms expressed uniquely in each radiation versus those shared between radiations and non-radiating species. Firstly, the total number of both genes and isoforms expressed in the OJ was higher than in the PJ; and regardless of tissue, the absolute number of genes and isoforms expressed were significantly higher in samples from the younger radiations (LM, LV) and the non-radiating lineages (NR) than the older radiation of LT (Mann-Whitney U test $p < 0.001$; Figure 3A,B; S4A,B). This pattern was also true when we analysed the number of isoforms per gene (Figure S5), but the variance in the number of isoforms among species from each radiation increased with age of radiation. However, the difference was only significant between the PJ's of LV and LM (Mann-Whitney U test $p < 0.05$). We tested the homogeneity of variance among groups using the Barlett's test and

found that significant differences for the number of isoforms per gene for PJ ($p = 0.017$) but not the OJ. This suggests that while the absolute number of isoforms expressed per gene is reduced over time, the transcriptional variance increases with time during adaptive radiation. Secondly, to understand how much of this expression variation was ancestral versus putatively *de novo*, we investigated if each gene or isoform was expressed in only one of the clades, or across several radiating and non-radiating clades (Figure 3C). The majority of genes (86.0%) and isoforms (73.0%) were expressed in at least one non-radiating species and one or more radiations – this variation is likely ancestral to all East African haplochromines. A small subset of isoforms (~12.0%) were unique to one radiation only – pointing towards the evolution of novel splice variation in the course of adaptive radiation – or less likely, repeated loss in all other lineages. It is of course possible that these putatively novel isoforms may be ancestral but the pattern is missed by our sample selection.Taken together, our data support the hypothesis that substantial transcriptomic variation existed in the common ancestor of all East African haplochromines that was likely shaped by selection and drift during adaptive radiations, with some isoforms being purged and others retained; with some evidence of putatively novel isoforms evolving in the course of adaptive radiation. But did this ancestral and novel isoform variation contribute to ecological adaptation in cichlid radiations?

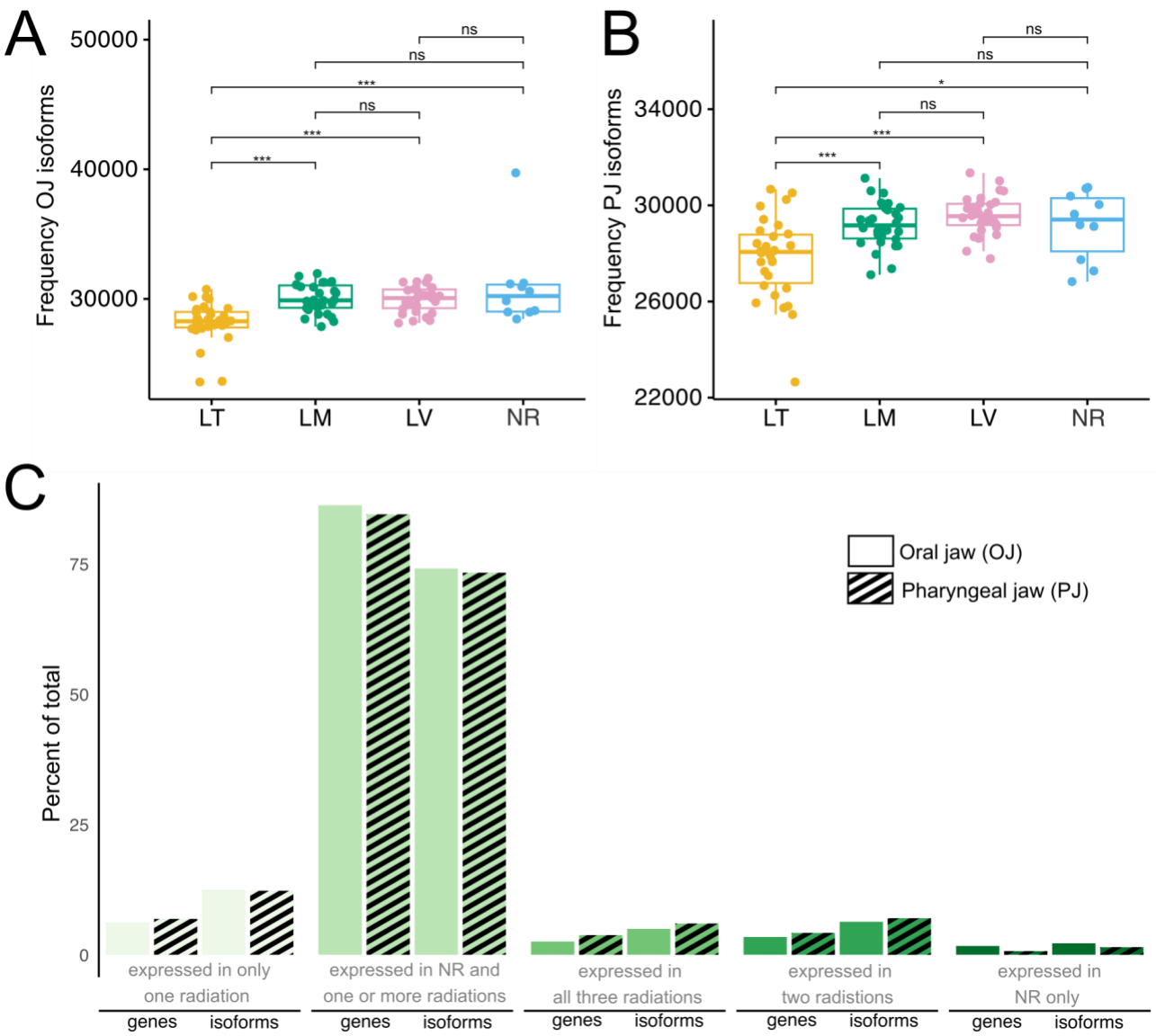
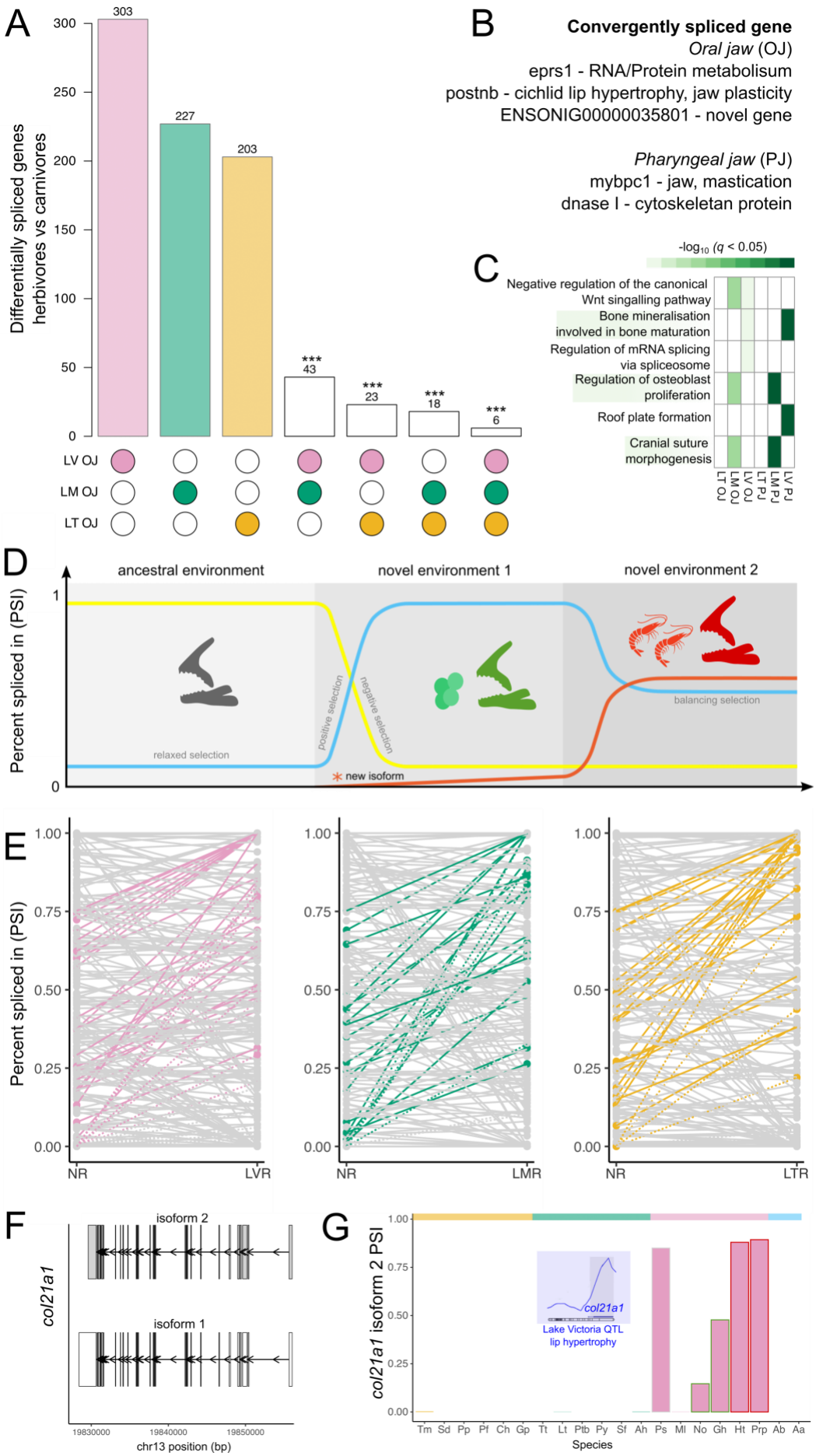


Figure 3 Transcriptomic variation in non-radiating and radiating cichlids. **(A, B)** The absolute number of expressed isoforms identified in the oral and pharyngeal jaw mRNA of all samples. The absolute number of genes expressed is shown in Figure S4. Asterisks denote Mann-Whitney U test significance: $p < 0.001$***, $p < 0.01$**, $p < 0.05$*. NS: no significance **(C)** Percent of total genes and isoforms expressed in non-radiating species and radiations or unique to one or more of the radiations for both jaws. LV: Lake Victoria, LM: Lake Malawi, LT: Lake Tanganyika, NR: non-radiating.

Trophic adaptation from old and new splice variants

Since the global patterns of AS revealed that it was evolving rapidly in the jaws of cichlids during adaptive radiation and that much of the splice variation was ancestral, we next asked if ancestral splice variants contributed to trophic divergence of cichlid jaws. We conducted differential PSI analysis between the jaws of species adapted to herbivorous and carnivorous diets (see Figure 1 for trophic specialisation of species) within

210 each lake to identify differentially spliced genes (DSGs) that may play a role in shaping their eco-morphological
211 differences. The largest number of genes were differentially spliced in LV (OJ 375, PJ 259) followed by LM
212 (OJ 294, PJ 266) and LT (OJ 250, PJ 150) (Figure 3A for OJ, Figure S6 for PJ, File S1). This indicates that
213 younger cichlid radiations leveraged splice variation relatively more than the older radiation in LT for trophic
214 adaptation. Differentially spliced genes were significantly enriched (Fisher's exact test $q < 0.05$) for GO terms
215 associated with craniofacial development such as 'regulation of osteoblasts', 'bone mineralisation involved in
216 bone maturation', 'roof plate formation', and 'skeletal muscle development' (subset shown in Figure 3C; full
217 data in Figure S7). The Wnt signalling pathway that has been associated with the evolution of new craniofacial
218 phenotypes at the larval stage in Malawi cichlids was enriched in divergent OJ and PJ from LV[35]. It is likely
219 that AS of Wnt pathway genes *gpc3* and *lzts2a* (File S1) that control skeletal development and dorsoventral
220 patterning contributed to adaptive jaw shape differences LV cichlids[36,37]. Two genes, *rnfox1* and *rbm5*, involved
221 in 'mRNA splicing via the spliceosome' were differentially spliced in LV oral jaws (File S1). This implicates
222 the spliceosome, the molecular machinery that controls AS, in cichlid adaptation. In LM OJ, differential splicing
223 of genes involved in bone maturation (*col1a1*) and cranial morphogenesis (*sp7*) were notable. Overall, twice as
224 many DSGs were shared between LV and LM, than either of these lakes shared with LT (Figure 4A, S6), which
225 is most consistent with these splice variants evolving and/or gaining function after the ancestor of these LT
226 haplochromines had split from the common ancestor of LV and LM, in line with the 'Out of Tanganyika' theory
227 of haplochromine cichlid evolution[21].


A
Differentially spliced genes herbivores vs carnivores
303
227
203
43
23
18
6
***
LV OJ
LM OJ
LT OJ
B
Convergently spliced gene
Oral jaw (OJ)
eprs1 - RNA/Protein metabolisum
postnb - cichlid lip hypertrophy, jaw plasticity
ENSONIG00000035801 - novel gene
Pharyngeal jaw (PJ)
mybpc1 - jaw, mastication
dnase I - cytoskeletan protein
C
-log10 (q < 0.05)
Negative regulation of the canonical Wnt singalling pathway
Bone mineralisation involved in bone maturation
Regulation of mRNA splicing via spliceosome
Regulation of osteoblast proliferation
Roof plate formation
Cranial suture morphogenesis
LT OJ
LM OJ
LV OJ
LT PJ
LM PJ
LV PJ
D
ancestral environment
novel environment 1
novel environment 2
Percent spliced in (PSI)
relaxed selection
positive selection
negative selection
balancing selection
new isoform
E
Percent spliced in (PSI)
NR
LVR
LMR
LTR
F
col21a1
isoform 2
isoform 1
chr13 position (bp)
G
col21a1 isoform 2 PSI
col21a1
Lake Victoria QTL
lip hypertrophy
Tm Sd Pp Pf Ch Gp Tt Lt Ptb Py Sf Ah Ps Ml No Gh Ht Prp Ab Aa
Species

228

229 **Figure 4** Evolutionary dynamics of differentially spliced genes between herbivores and carnivores species. **(A)**
230 Number of significantly differentially spliced genes between oral jaws (OJ) of carnivores and herbivores within
231 each lake radiation ($q < 0.05$) with overlaps shown (hypergeometric test $p < 0.001$). The number of differentially
232 spliced genes between the pharyngeal jaws (PJ) of carnivores and herbivores within each radiation is shown in
233 Figure S6A. **(B)** Genes that were convergently spliced in at least two radiations in the same direction (higher in
234 herbivores or higher in carnivores). No gene were convergently spliced in the same directly in all three
235 radiations. **(C)** Gene Ontology enrichment of differentially spliced genes between herbivores and carnivores.
236 Only a subset of terms are shown. Full dataset is presented in Figure S7. **(D)** Hypothetical expectations for
237 isoform frequency (measured as percent spliced in - PSI) during ecological adaptation. Figure adapted from
238 Singh and Ahi (2022). **(E)** Testing the hypothesis depicted in (D) in our transcriptome data from Lake Victoria
239 radiation (LV), Lake Malawi radiation (LM) and Lake Tanganyika radiation (LT) compared to non-radiating
240 species (NR). Mean PSI was calculated all individuals of all species in each group (NR, LV, LM, LT). All lines
241 represent all isoforms of genes that were signficantly differentially spliced between herbivores and carnivores
242 within each radiation. Solid grey lines represent all isoforms that were expressd at lower or similar levels in the
243 NR species and species from each radiation (PSI differences <= 0.2). Dotted grey lines represent all isoforms
244 that were not expressd in NR species but were expressed species from each radiation (PSI differences <= 0.2).
245 Coloured solid lines indicate isoforms that were expressed at low levels in NR species but were higher expressed
246 in radiating species (PSI differences > 0.2). Coloured dotted lines indicate isoforms that were not expressed in
247 NR species but were expressed in radiating species (PSI differences > 0.2). **(F)** The two isoform models for
248 *col21a1* in the gene annotation. **(G)** Species mean expression of *col21a1* isoform 2 across all species in our
249 dataset. Inset highlights a known QTL lip shape from LV containing *col21a1*.

250

251 Of the genes that were significantly differentially spliced between herbivores and carnivores in each lake (Figure
252 3A, S6A), a dynamic pattern of isoform evolution emerged: many of these were low expressed isoforms in NR
253 species that increased in expression in radiating species (Figure 3E for OJ, S6B for PJ). An increase in frequency
254 would be the expectation if these putatively ancestral isoforms gained adaptive function in response to newly
255 available food sources when the ancestor entered lakes (Figure 3D), as hypothesised by Singh & Ahi[2]. Many
256 highly expressed isoforms in non-radiating species also decreased in frequency or were lost in the lacustrine
257 radiations (presumably as the alternative isoform increased in frequency). We further identified 34 potentially
258 novel isoforms that were absent in non-radiating species and only expressed a subset of the three adaptive
259 radiations (Figure 3E dotted lines, File S1). Sixteen of these 34 novel isoforms were found in LV, followed by
260 12 in LM and six in LT. Novel isoforms of note were of genes *col21a1* and *lzts2a*. *Col21a1* had two isoforms
261 (Figure 3F) of which the main isoform is expressed in the OJ of LV herbivores as well as herbivores from LT
262 and LM (with one exception, File S1). The alternative isoform of *col21a1* is only expressed in LV species, with
263 higher expression in carnivores and omnivores (Figure 3G). *Col21a1* is associated with human cleft lip
264 syndrome and maps to a QTL for hypertrophic lips in LV cichlids[38]. It is conceivable that this isoform may have

265 arisen *de novo* in LV cichlids or arrived through hybridisation, which has been shown to contribute genetic
266 variation for rapid adaptive radiation in Lake Victoria[39]. In the future, integrating transcriptomic analysis with
267 population genetics and functional genomics (GWAS, spliceQTL) approaches would help to narrow down the
268 variants associated with splicing and the evolutionary processes that shaped them. Our results also highlight the
269 conservation of craniofacial development pathways across vertebrates, and the promise of cichlid fishes as a
270 natural model for studying human craniofacial disease[40].

271

272 **Differential expression of major craniofacial genes contributed to jaw divergence**

273 In addition to differences in splicing, we investigated GE differences between herbivores and carnivores in each
274 lake radiation to be able to compare the role of GE with AS in trophic adaptation. The largest number of
275 differentially expressed genes (DEGs) were in LT (OJ 6716, PJ 4871), followed by LM (OJ 2935, PJ 2133) and
276 LV (OJ 2619, PJ 1649) (Figure 5A for OJ, Figure S8 for PJ, File S1). Several interesting candidate genes for
277 craniofacial development stood out as the most extreme outliers, particularly in LT where GO terms such as
278 'neural crest cell development', 'skeletal system development, and 'ossification' were significantly enriched (q
279 < 0.05). One such gene for LT OJ was Frizzled 2 (*fzd2*), a Wnt pathway gene that is highly expressed in the
280 developing face of vertebrates, and mutations of this gene cause cleft palate and wider nose and upper jaw[41]. A
281 gene controlling prey crushing teeth morphology in cichlids, *odam*[42], and a gene linked to craniofacial disease,
282 *gja1,* were also significant for LT OJ[43]. Two interesting outliers in LV OJ were *kaznb*, a craniofacial
283 development gene that has been found to be upregulated in the jaws of herbivorous cichlids at larval and adult
284 life stages[44] and *egr1*, that forms part of the regulatory cascade modulating cranial bone morphogenesis through
285 the BMP pathway[45]. Interestingly, one of the most extreme outliers for LT OJ was *hnRNPk*, an RNA binding
286 protein that regulates pre-mRNA splicing[46].

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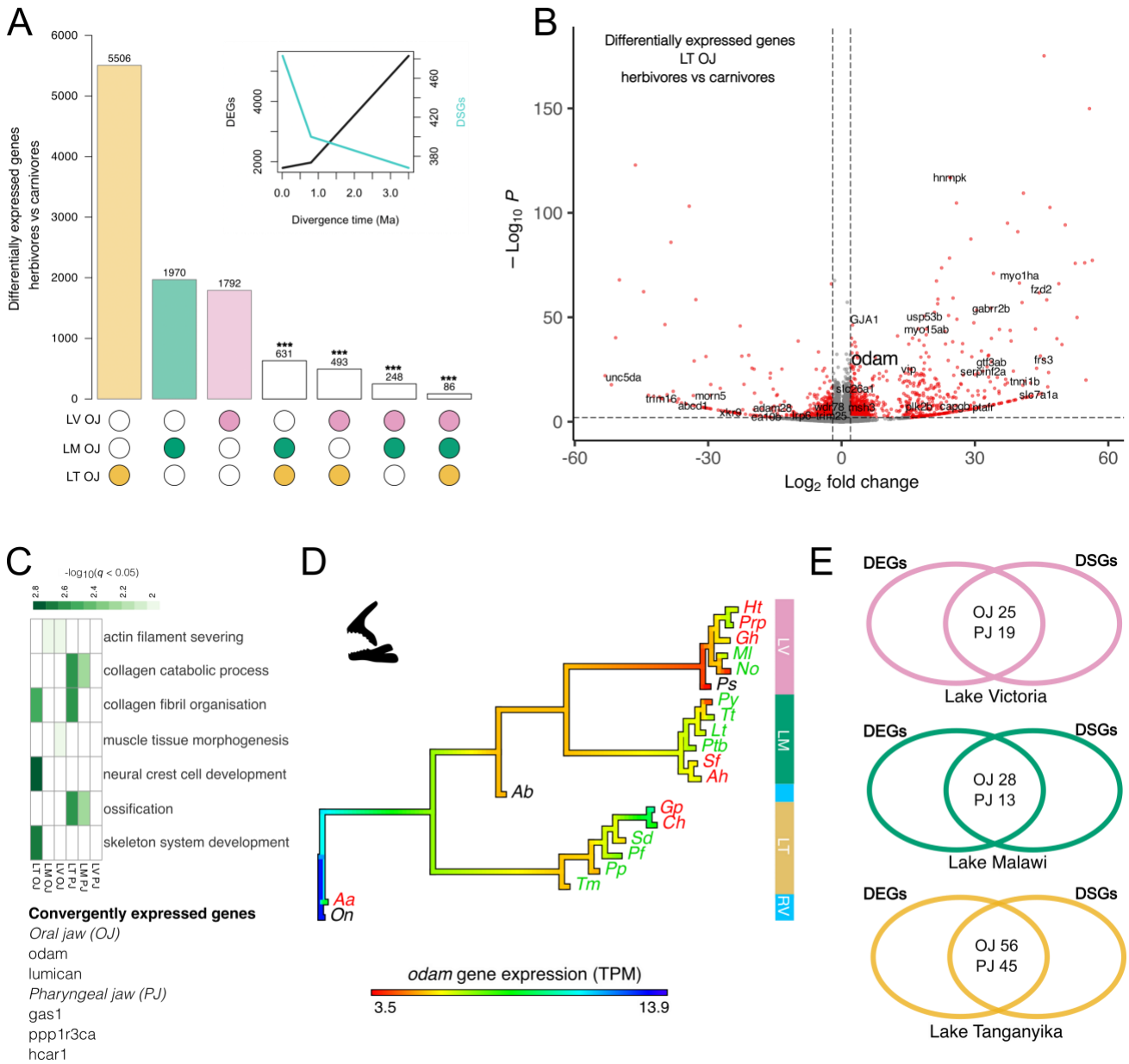

A
Differentially expressed genes
herbivores vs carnivores
5506
1970
1792
631
493
248
86
LV OJ
LM OJ
LT OJ
DEGs
DSGs
Divergence time (Ma)
B
Differentially expressed genes
LT OJ
herbivores vs carnivores
$-\text{Log}_{10}\,P$
Log_2 fold change
odam
C
$-\log_{10}(q < 0.05)$
actin filament severing
collagen catabolic process
collagen fibril organisation
muscle tissue morphogenesis
neural crest cell development
ossification
skeleton system development
Convergently expressed genes
Oral jaw (OJ)
odam
lumican
Pharyngeal jaw (PJ)
gas1
ppp1r3ca
hcar1
D
Ht
Prp
Gh
Ml
No
Ps
Py
Tt
Lt
Ptb
Sf
Ah
Ab
Gp
Ch
Sd
Pf
Pp
Tm
Aa
On
LV
LM
LT
RV
odam gene expression (TPM)
3.5
13.9
E
DEGs
DSGs
OJ 25
PJ 19
Lake Victoria
OJ 28
PJ 13
Lake Malawi
OJ 56
PJ 45
Lake Tanganyika

Figure 5 GE divergence in oral jaws of herbivore and carnivore species. (A) Number of differentially expressed genes (DEGs, $q < 0.05$) between oral jaws (OJ) of herbivores and carnivores in each lake, with overlaps annotated (hypergeometric test $p < 0.001$). Results for pharyngeal jaws can be found in Figure S8. Inset shows the relationships between number of DEGs and differentially spliced genes (DSGs) in each cichlid radiationnplotted as function of divergence time. **(B)** Volcano plot illustrating DEGs in Lake Tanganyika (LT) oral jaws. Significant DEGs are highlighted in red. Top gene outliers are annotated. **(C)** Gene Ontology enrichment of DEGs between herbivores and carnivores. Only a subset of terms are shown. Full list is presented in Figure S9. Some candidate genes for craniofacial development that were convergently expressed in all three radiations in the same direction (higher in herbivores or higher in carnivores). See File S1 for full list. **(D)** Ancestral state reconstruction of GE of the convergently expressed *odam* gene. **(E)** Overlap of DEGs and DSGs between herbivores and carnivores within each lake radiation.

Contrasting functions for alternative splicing and gene expression

We found dissimilar patterns in the extent of differential GE and AS underlying divergent trophic adaptation across cichlid radiations. Even though the overall number of DEGs was much higher than DSGs across all three radiations (Figures 4A, 5A, S6, S8), the relative number of DEGs across radiations was the highest in the oldest radiation and lowest in the youngest radiation, while the DSGs showed the opposite pattern (Figure 5A inset). Interestingly, more DEGs in each of the younger radiations overlapped with the older radiation, in contrast more DSGs overlapped among the younger radiations (Figures 4A, 5A, S6A, S8). This pattern suggests that splicing changes evolving rapidly to shape jaw divergence during speciation, while GE differences evolve much slower, mostly likely fine-tuning the adaptive morphology long after speciation.

It has been proposed that AS and GE perform contrasting biological functions[17,44,47–49]. However, these studies have lacked a phylogenetic perspective. In our dataset, the overlap of genes that were both differentially spliced and differentially expressed between herbivores and carnivores followed a phylogenetic and evolutionary pattern. The highest overlap was in LT (OJ 56, PJ 45), followed by LM (OJ 28, PJ 13) and LV (OJ 25, PJ 19) (Figure 5, S9). Our results reinforce the idea that putatively adaptive AS and GE changes act mostly at different genes – but we show that the extent of gene overlap depends on the evolutionary age of lineages, with fewer genes being regulated by both procesess in more recently diverged populations.

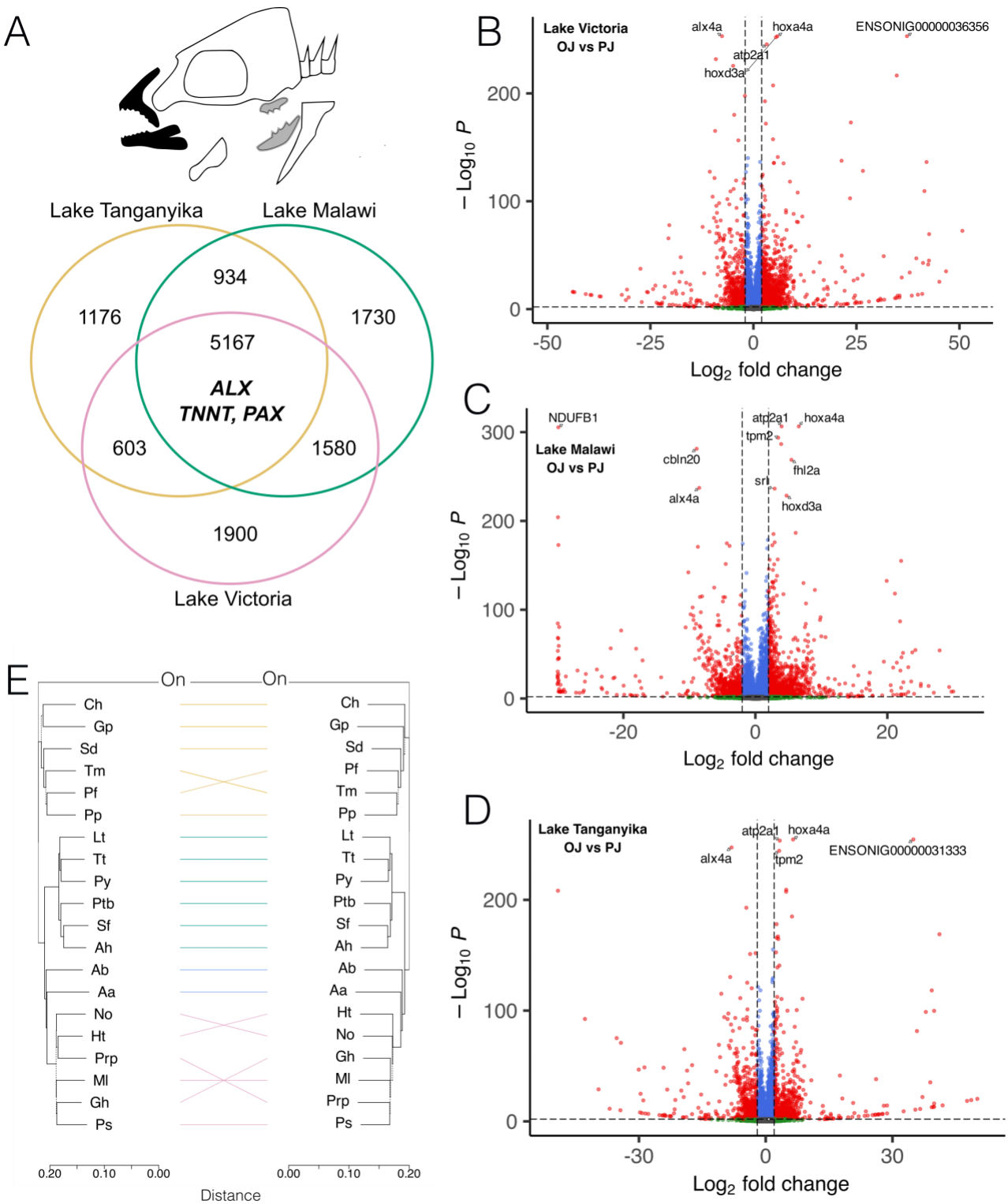
Convergent gene expression and splicing underlying trophic evolution

African cichlid radiations are an ideal sytem to study the repeatability of evolution as they have evolved convergent trophic adaptations, particularly in jaw and teeth shape, to exploit similar food sources in similar

327 ways in different lakes[50]. We asked if differentially expressed and spliced genes contributing to herbivory vs
328 carnivory showed parallel divergence across all three radiations. We find DEGs in both oral (23 upregulated,
329 12 downregulated in herbivores) and pharyngeal jaws (14 upregulated, 6 downregulated in herbivores) that fit
330 this pattern (Figures 5, S8). Several convergently used OJ genes have been associated with the development of
331 teeth (*odam*), bone (*lum*) and muscle *(myrip*, *myo15aa*) (Figure 5B,C, File S1). *Odam* expression has been
332 associated with larger vs smaller teeth in cichlid fishes and our ancestral state reconstruction suggests that this
333 gene increased in expression repeatedly in the oreal jaws of carnivores in each lake radiation (Figure 5D).
334 Convergent genes for PJ were *hcar1*, *ppp1r3c*, myl13, and *gas1*. *Gas1* interacts with *shh* is early face
335 development[51]. We validated the convergent expression of *odam, gas1, hcar1,* and *ppp1r3ca* using qPCR
336 (Figure S10). No DSGs were convergently spliced in the same direction in all three radiations. We only found
337 evidence of convergent splicing in the same direction in two of the three radiations in the following noteworthy
338 genes: OJ: *eprs1*, *postnb* and PJ: *mybpc1*, *Dnase1* (Figure 4A, B).
339

340 **ALX and PAX transcription factors are key drivers of cichlid jaw modularity**

341 Modularity of cichlid oral and pharygeal jaws is considered a key innovation that decoupled the task of prey
342 capture (OJ) and processing (PJ)[52]. Little is known about the gene regulatory basis of this functional
343 decoupling[16]. Differential GE analysis between the two jaw tissues within each lake revealed that the majority
344 of DEGs (5167) contribute to jaw divergence repeatedly in all three lakes, with 2555 being upregulated and
345 2591 downregulated in the PJ (Figure 6, File S1). These parallel expressed genes are associated with 'skeletal
346 muscle fibre/tissue development' and 'sarcomere organisation' GO terms ($q < 0.05$, File S1). Notable genes
347 with parallel expression were ALX skeletogenesis transcription factors (*alx1*, *alx4a*, *alx4b*) that were
348 upregulated in OJ versus PJ. PAX transcription factor family genes also had parallel expression patterns
349 (upregulated OJ: *pax3a*, *pax3b*, *pax6b*; upregulated PJ: *pax1a*, *pax5*) (Figure 6, File S1). PAX genes are master
350 regulators of early tissue development throught neural crest induction[53] and pax3a/b are expressed in a modular
351 fashion in some cichlid species[16]. Many HOX (*hoxa4a*, *hoxa5a*, *hoxb2a*, *hoxb3a*, *hoxb4a*, *hoxb5b*, *hoxb6b*,
352 *hoxb8*, *hoxc5a*, *hoxc6a*, *hoxc9a*, *hoxd10a*, *hoxd3a*, *hoxd9*) and Troponin (*tnni1b*, *tnni2a*, *tnnt1*, *tnnt2c*, *tnnt2d*,
353 *tnnt3a*) genes also showed signatures of parallel expression. Hox genes are implicated in cichlid dentition[54] and
354 bone development[55]. Furthermore, many OJ vs PJ DEGs were unique to each lake radiation (LV 1900, LM
355 1730, LT 1176).


A
Lake Tanganyika
Lake Malawi
934
1176
1730
5167
ALX
TNNT, PAX
603
1580
1900
Lake Victoria
B
Lake Victoria
OJ vs PJ
alx4a
hoxa4a
ENSONIG00000036356
atp2a1
hoxd3a
– Log10 P
Log2 fold change
C
NDUFB1
Lake Malawi
OJ vs PJ
cbln20
alx4a
atp2a1
hoxa4a
tpm2
fhl2a
sri
hoxd3a
D
Lake Tanganyika
OJ vs PJ
atp2a1
hoxa4a
alx4a
tpm2
ENSONIG00000031333
E
On
On
Ch
Gp
Sd
Tm
Pf
Pp
Lt
Tt
Py
Ptb
Sf
Ah
Ab
Aa
No
Ht
Prp
Ml
Gh
Ps
Distance

356

357 **Figure 6 Transcriptional modularity of cichlid oral (OJ) and pharyngeal jaws (PJ). (A)** Venn diagram
358 showing overlap of differentially expressed genes ($q < 0.05$) between oral and pharyngeal jaws of species within
359 each lake radiation. **(B, C, D)** Volcano plots illustrating DEGs in Lake Tanganyika (LT) and Lake Victoria (LV)
360 between oral and pharyngeal jaws. Significant DEGs are highlighted in red. Top gene outliers are annotated.
361 **(E)** Tanglegram of GE phylogenies of OJ (left) and PJ (right). Discordant tips are annotated with lines.
362
363 To further understand gene regulation evolutions of the jaws, we contrasted their transcriptome-wide GE
364 phylogenies of OJ and PJ for all species (Figure 6). Topological discordance in the phylogenies were interpreted
365 as evidence of decoupled GE evolution. Applying this interpretation, evidence of decoupled GE evolution
366 between OJ and PJ was found only in LV and LT cichlids, but not in LM. In LV, OJ of the two piscivores (Ht
367 and Prp) clustered together, but the PJ of Prp clustered with the omnivore (Ps). Similarly, in LT the OJ of the
368 two algae grazers (Pp, Pf) clustered together, but the PJ of Pp clustered with the algae browser Tm. These
369 patterns are consistent with prey capturing being functionally decoupled to prey prcoessing.
370

371 # Discussion

372 The role of AS in ecological adaptation has not been explored across broad phylogenetic timescales, in the
373 context of adaptive radiation. Through the analysis of 200 transcriptomes representing three independent but
374 phylogenetically nested adaptive radiations that evolved in the last <16,000 to 3.8 million years we show that
375 the cache of ancestral transcriptomic variation in East African haplochromine cichlids was shaped over time
376 presumably through selection[56] and stochastic processes[57] as lineages diversified. These evolutionary processes
377 most likely led to the faster evolution of AS than GE that we observe here. Signatures of splicing became
378 increasingly more species-specific, and GE became more lineage-specific over evolutionary time. However,
379 specific-specific signatures of splicing did not emerge until ~3.8 million years of evolution, a pattern not
380 observed in previous large-scale studies on older lineages[5,6]. This rapid turnover of AS is consistent with relaxed
381 selection pressures on splice-sites, as alternative isoforms can escape the effects of purifying selection as long
382 as the major isoform is expressed[29]. Theory suggests that relaxed selection can increase the rate of evolution of
383 complex adaptations in heterogenous environments, allowing populations to rapidly cross adaptive valleys[58].
384 Relaxed selection maintaining standing variation, followed by periods of positive selection on isoforms of
385 craniofacial genes may have contributed to the rapid evolution of trophic adaptations in cichlids as they entered
386 lakes and were exposed to new and ecologically more stable environments.

387

388 Our results on the evolutionary dynamics of GE are partially consistent with previous work on transcriptome
389 evolution at deeper timescales. In contrast to AS, GE is largely conserved at the tissue-level during vertebrate
390 evolution[5,6,13,31]. Remarkably, we found lineage-specific clustering of GE in Tropheini jaws. Tropheini are

named after their diverse trophic adaptations that allowed them to enter the littoral zones of Lake Tanganyika at a much later stage and successfully diversify alongside other established cichlid radiations[20,59,60]. They were able to do this by rapidly remodelling their jaw, dentition, and craniofacial ecomorphology to forage foods in new and different ways[59]. Our findings may reflect the particular importance of gene regulation for evolutionary transitions in Tropheini jaws during speciation. Furthermore, we find that the parallel modular expression of thousands of genes may underlie the differences between the functionally decoupled OJ and PJ across all three cichlid radiations. Of note were genes from the *alx* craniofacial skeletogenesis transcription factor family (*alx1*, *alx4*), of which *alx1* is associated with rapid diversification of beak shape in the adaptive radiation of Darwin's finches[61]. Bird beaks are a specialised modification of the upper and lower oral jaws found in all jawed vertebrates. It is conceivable that *alx* transcription factors are particularly responsive to environmental change and mediate large effect changes in jaw bone shape.

We found that both rapidly evolving AS and GE contributed to jaw adaptations in herbivorous and carnivorous cichlids, but their contribution was inversely predicted by evolutionary age. Relatively more differences in AS were found to underlie trophic differences in jaws of cichlids from the younger radiations, while GE differences dominated in the older radiation. This would suggest that rapidly evolving AS from standing variation is a substrate for rapid adaptation[17] as species diverge and may be one piece of the puzzle explaining how ecological and species richness can evolve when suffifient time is not available for new mutations to arise, fix, and be replenished. In contrast, ecologically important gene expression changes emerge at later stages of speciation. Consistent with previous studies, we found many genes implicated in jaw divergence suggesting that jaw remodelling is a complex phenotype governed by myriad genes[16,20,50,62]. The Wnt pathway emerged as a key player in the evolution of new jaw phenotypes at early stages of development, with genes regulated through splicing (*gpc3*, *lzts2a*) and differential expression (*fzd2*). Overall, more convergent differences in GE than AS were found across all three cichlid radiations. We propose that the lower cross-species and -radiation conservation of AS may underscore its functional role in shaping lineage and species-specific adaptations at short timescales.

It has been hypothesised that ancestral variation plays an important role in the rapid adaptive capacity of African cichlids[33,63]. But, little empirical evidence exists explaining how this ancestral variation mechanistically plays out. We show that ancestral splice variants (also lowly expressed in non-radiating species) that were differentially spliced between jaws of herbivores and carnivores increased in frequency in radiating cichlids, most likely enabling trophic divergence. First, this suggests that old genetic variation can fuel new adaptations[33]; and that the acestors of the East African cichlid adaptive radiations, which were most likely a widely dispersing riverine cichlids, acted as reservoirs of genetic variation for diversification on a continental scale[64]. And even if most ancestral alternative isoforms are non-functional[65], lowly expressed, and evolving stochastically[29], they

426 represent a cache of standing variation for adaptive evolution when ecological opportunity arises[2,66]. Standing
427 variation in AS has been shown to be important for the rapid domestication of sunflowers[66], which represents
428 only one ecological transition, while cichlid adaptive radiations involve multiple, often convergent, diverse
429 ecological transitions. In addition to ancestral variation, we found evidence of potentially novel isoforms
430 contributing to adaptive jaw differences. This suggests that AS itself can evolve during adaptive radiation,
431 generating new genetic material for evolutionary change at short timescales. Taken together, our findings may
432 support the flexible-stem model of adaptive radiation, whereby standing variation in the ancestral lineage
433 facilitates plastic responses in new environments, that become genetically assimilated and heritable over time[67].
434

435 # Materials and Methods

436

437 **Study design and sampling**

438 In this study we focused on six cichlid species from Lake Tanganyika, six cichlid species from Lake Malawi,
439 six cichlid species from Lake Victoria as well as two cichlid species from surrounding rivers, all of which are
440 part of the 'modern haplochromine' adaptive radiations (Figure 1). The 18 species from the three lakes represent
441 eco-morphological replicates of herbivore and carnivore trophic adaptations. We focused on the functionally
442 decoupled oral (OJ) and pharyngeal jaw (PJ) tissues, that are involved in food capture and processing.

443 Sample were collected by either through aquarium trade or fieldwork within the framework of collaborations
444 with Zambian and Tanzanian fisheries (TAFIRI). All fish were reared in the fish facility at University of Graz
445 under standardised conditions regarding tank environment, diet, and water parameters to minimise plastic
446 effects. Fertilised eggs were taken from the mouth of mouthbrooding females 24 hours after fertilisation, by
447 applying slight pressure onto their cheeks. The eggs were reared in a separate tank until the yolk sac was
448 completely absorbed, which is also known as the developmental stage 26[22]. Stage 26 larvae were euthanised
449 and stored in RNAlater in a fridge for seven days and then moved to a -80°C freezer. Stage 26 was selected as
450 it is an ecomorphologically informative developmental stage for cichlid with different trophic
451 specialisations[16,17,59].

452

453 **RNA extraction**

454 Each larva was dissected under a stereo microscope to dissect the oral jaws (OJ: bone, teeth) and the pharyngeal
455 jaws (PJ: bones and teeth) separately (Figure 1). The bones were cleaned as much as possible, but some
456 surrounding tissue (i.e. skin, cartilage, muscle) remained, especially for the OJ, as small size of the larvae
457 (<1cm) makes the dissection challenging. The dissected tissue was submerged in a lysis buffer and 1-
458 thioglycerol according to the protocol of the Promega RNA Reliaprep kit. The sample in the lysis buffer was

459 crushed with a ceramic bullet in a homogenizer (FastPrep-24, MP Biomedicals, Santa Ana, CA, USA) before
460 further processing. Two individuals were pooled per sample to increase the amount of RNA. For Lake Malawi
461 and Tanganyika, one individual yielded sufficient RNA. RNA quality was tested at a TapeStation 2200 with
462 RNA Screen Tapes. It was tried to reach a RINe number of >= 7 for each sample. We extracted RNA for 5
463 biological replicates per jaw per species, which resulted in 200 mRNA samples in total.

464

465 **Library preparation and sequencing**

466 Library preparation was done with the TruSeq Stranded mRNA Sample Prep Kit using 24 indexing adapters
467 and following the standard protocol. For the input RNA we tried to reach 1000ng for each sample. The quality
468 of the cDNA was tested on a TapeStation 2200 with D1000 Screen Tapes. Sequencing was executed at the
469 Vienna BioCenter Core Facilities on a HiSeq 2500 with paired end 125 cycles per read (2 x 125 cycles).
470 Demultiplexing was conducted by the same facility.

471

472 **Transcriptome assembly**

473 Each sample has approximately ten million paired-end reads. Raw sequence reads are available at NCBI
474 sequence read archive (Reviewer link:
475 https://dataview.ncbi.nlm.nih.gov/object/PRJNA640176?reviewer=p2dhiiern60seqodpuqbqb1o62). After a
476 quality check with Fastqc (v0.11.8)[68] and a trimming step with Trimmomatic (v0.3.9)[69], only reads with a phred
477 > 28 and a minimum length of 70 bp where retained. To enable the assembly of novel isoforms we developed
478 a bioinformatics pipeline with the following steps: Reads were assembled using STAR (v2.7.3.a)[70] in reference
479 guided mode using the *Oreochromis niloticus* reference genome[71]. This genome was selected because it is the
480 best assembled and annotated cichlid reference genome and is phylogenetically equidistant to the species
481 sampled in this study. Mapping statistics were generated with samtools idxstats (v1.9)[24,72]. StringTie (v2.0.6)
482 was run first both with a reference genome to assemble the RNA-Seq alignments into potential transcripts and
483 in genome-guided mode. This was done separately for the single files (per individual). The single gtf files for
484 each biological replicate were then incrementally merged according to species and tissue and finally into one
485 super annotation. This repeated merging steps were conducted to reduce the probability of false positives in
486 isoform assembly and accurately identify novel species-specific or lower expressed isoforms. To estimate the
487 accuracy of the produced annotation files we compared them with gffcompare (v0.11.2)[73] to our reference *O.*
488 *niloticus* annotation. We filtered for monoexonic transcripts not contained in the reference and a class code
489 assigned by gffcompare (v0.11.2), indicating 'possible polymerase run-on' fragments. The maximum intron
490 length was reduced to 200,000 bp, which is the maximum intron length found in the *O.niloticus* reference. Based
491 on the super annotation, the expression estimates were generated with StringTie allowing no multimapping.
492 From these expression estimates, count matrices were produced using a perl script from the griffith lab

493 (https://rnabio.org) to extract raw count data from StringTie results. The code used for our RNAseq pipeline in
494 on github[74]. SUPPA2 (v2.3)[28] was used to calculate percent spliced in (PSI) index for each isoform for each
495 gene.

496

497 **Differential gene expression analysis**

498 We used DESeq2[25]in R[75] to detect DEGs running comparisons (1) between herbivores and carnivores within
499 each lake radiation for each tissue separately and (2) between oral and pharyngeal jaws within each radiation.
500 DESeq2 uses raw read counts and estimates variance-mean dependence based on a model that utilises the
501 negative binomial distribution. The cutoff for DEGs was chosen at a false discovery rate of ($q < 0.05$).

502

503 **Alternative splicing analysis**

504 Alternative splicing analysis was conducted with SUPPA2 (v2.3)[28] to evaluate differential transcript usage
505 (DTU) between between herbivores and carnivores within each lake radiation for each tissue separately.
506 Visualisation of PSI analysis was conducted in R[75].

507

508 **Modeling gene and isoform expression**

509 We used the methods proposed by Chen et al (2019)[32] to model gene and isoform expression along the
510 phylogeny using Ornstein-Uhlenbeck (OU) and Brownian Motion (BM) processes. The OU process is a
511 modification of a random walk, describing the change in expression (dX_t) across time (dt) by $dX_t = \sigma dB_t + \alpha(\theta$
512 $- X_t)\, dt$, where dB_t denotes a Brownian motion process. The model elegantly quantifies the contribution of both
513 drift and selective pressure for any given gene: (1) Drift is modeled by Brownian motion with a rate σ while (2)
514 the strength of selective pressure driving expression back to an optimal expression level θ is parameterized by
515 α. The OU process accounts for phylogenetic relationships, thus allowing us to fit individual evolutionary
516 expression trajectories.

517

518 **Phylogenetic analysis and ancestral state reconstruction of gene expression**

519 We constructed expression trees using the neighbour-joining approach based on pairwise distance matrices
520 between samples using data from all genes and applying functions from the 'ape' package (v5.8.1)[76] in R[75].
521 Distances were computed as $1 - \rho$, where ρ is the Spearman's correlation coefficient, chosen due to its
522 robustness against outliers and any normalisation issues[31]. To visually evaluate the differences in expression
523 evolution, we constructed a tanglegram of the expression phylogenies of the two jaw tissues using the

524 'dendextend' package (v1.19.0)[77] in R. Ancestral state reconstruction was conducted using the 'phytools'
525 package (v2.4.4)[78] in R.
526
527 **Phylogenetic reconstruction (SNPs)**

528 SNP calling on mapped mRNAseq reads was conducted using bcftools[79] across all samples. Filtered high
529 quality SNPs were used to contruct a concatenated alingment with the most common allele at each position for
530 each individual. This alignment was used as input for RAxML (v8)[80] to contstruct a SNP phylogeny.

531

532 **GO term enrichment**

533 GO terms for *Oreochromis niloticus* were acquired in R via the biomaRt package (v2.46.1)[81] from ensembl[82].
534 Gene set enrichment analysis was conducted using topGO (v2.36.0)[83] using the method weight to account for
535 GO topology and Fisher's exact test to correct for multiple testing for the enrichment analysis. We used our
536 super annotation to build the gene universe.

537

538 **qPCR analysis**

539 From 4 replicates of RNA samples of each jaw, stage and species, we used 500ng of RNA to synthesize first
540 strand cDNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems). The cDNA from
541 each sample was diluted 1:10 times in nuclease-free water to be used for qPCR. We used *rpl18* as a reference
542 gene, which is validated as a stably expressed gene in both cichlid jaws across different trophic niches[14] to
543 normalize our expression data for qPCR. The primers were designed at conserved sequence regions of the
544 coding sequences from all species using CLC Genomic Workbench (v7.5 CLC Bio, Aarhus, Denmark) for
545 alignment and delineation of exon boundaries using *A. burtoni* genome[12]. Primer Express 3.0 (Applied
546 Biosystems, CA, USA) and OligoAnalyzer 3.1 (Integrated DNA Technology) were used to design the primers
547 as described in[14,84]. Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific, Germany)
548 was used to generate qPCR reactions. The amplification steps were conducted in 96 well-PCR plates on ABI
549 7500 real-time PCR System (Applied Biosystems). The primer efficiency calculation, qPCR program, plate set-
550 up and a dissociation step were performed as described in[14,84]. To normalize the expression levels, the Cq value
551 of *rpl18* was used as normalization factor (Cq reference), and ΔCq of each target gene was calculated
552 accordingly (ΔCq target = Cq target – Cq reference). Relative expression quantities (RQ) were calculated for
553 the normalized values using E−ΔΔCq and then fold difference values were calculated by transformation of RQ
554 values to logarithmic values in order to conduct further statistical analysis. The statistical expression differences
555 were identified using ANOVA statistical tests, followed by Tukey's HSD post hoc tests.

556

Data Availability

All data has been deposited to the NCBI SRA BioProject (PRJNA640176). Reviewer's link: https://dataview.ncbi.nlm.nih.gov/object/PRJNA640176?reviewer=p2dhiiern60seqodpuqbqb1o62

All bioinformatics code can be found on github: https://github.com/poojasingh09/2024_singh_transcriptomeanalysis_pipeline

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