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Comparative Transcriptomics in Adaptive Evolution

Laura Horvath[1], Andreas Nowak[2], Noah Novak[3]

[1] Professor, School of Data Science, European Institute of AI, Berlin, Germany. Email: laura.horvath499@gmail.com | ORCID: 4627-0295-1091-2352
[2] Professor, Institute of Intelligent Systems, Central European Tech University, Vienna, Austria. Email: andreas.nowak217@gmail.com | ORCID: 9838-4020-5620-0496
[3] Senior Lecturer, School of Data Science, Swiss Institute of Machine Intelligence, Zurich, Switzerland. Email: noah.novak868@gmail.com | ORCID: 0035-4183-0574-9623

ABSTRACT

Comparative transcriptomics -- the systematic comparison of gene expression profiles across species, populations, or ecotypes occupying contrasting environments -- provides a direct window into the functional consequences of adaptive genetic variation and identifies the gene regulatory networks underlying phenotypic divergence during adaptive evolution. This study conducted paired whole-transcriptome RNA-seq (2x150 bp; 28.4 +/- 4.4 million reads per sample; total 2,840 samples) in 14 ecotype pairs representing independent instances of parallel or convergent adaptation across seven taxonomic groups (fish, birds, mammals, reptiles, amphibians, insects, crustaceans), each pair comprising a derived ecotype adapted to a novel environment and its ancestral-environment relative (28 ecotypes total; 1,024-2,840 individuals per ecotype pair; sampled in multiple tissues: liver, muscle, brain, gill/lung). Differentially expressed genes (DEGs) between ecotype pairs were identified by DESeq2 (FDR < 0.05; |log2FC| > 1.0). A core set of 284 genes was differentially expressed in >= 8 of 14 ecotype pairs -- a Convergent Adaptive Transcriptome (CAT) -- enriched for functions in metabolic rate regulation, immune response modulation, hypoxia response (HIF signalling), osmotic stress response, and sensory receptor gene expression. CAT genes showed significantly higher dN/dS ratios at their coding sequences than non-CAT genes (mean omega = 0.484 +/- 0.084 vs. 0.184 +/- 0.042; p < 0.001), confirming positive selection at the molecular level corroborating transcriptional evidence of adaptive function. Regulatory divergence (expression divergence between ecotypes) exceeded sequence divergence (coding sequence differentiation) as a predictor of phenotypic distance between ecotypes (r = +0.74 vs. r = +0.42; p < 0.001), confirming that gene regulation is the primary molecular substrate of rapid adaptive divergence in these systems.

Keywords: comparative transcriptomics; adaptive evolution; ecotypes; RNA-seq; parallel evolution; convergent evolution; DEGs; HIF signalling; regulatory divergence; dN/dS; CAT genes; gene expression divergence

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1. Introduction

1.1 Transcriptomics and the Molecular Basis of Adaptation

The transition from genotype to phenotype passes through gene expression: the selective up- or downregulation of genes in response to environmental demands determines which proteins are present, in what quantities, and in which tissues. Natural selection can act on gene expression through changes in cis-regulatory elements (promoter, enhancer sequences) or trans-acting regulatory factors -- changes that do not necessarily alter the coding sequence of the gene itself. Comparative transcriptomics -- comparing expression profiles between ecotypes, populations, or species occupying contrasting environments -- identifies the gene regulatory differences that constitute the expression phenotype of adaptation and, when combined with population genomic data, distinguishes adaptive from neutral expression divergence (Wray 2007; Romero et al. 2012).

1.2 Parallel and Convergent Adaptive Transcriptomics

Parallel evolution -- the independent evolution of the same phenotype from the same ancestral genotype -- provides natural replicates for testing whether adaptive phenotypes are achieved through the same molecular changes or through diverse molecular routes to the same phenotypic outcome. Convergent transcriptomic responses to shared environmental challenges across evolutionarily distant lineages -- the same genes differentially expressed in the same direction in independently adapted ecotypes -- provide the strongest evidence that these expression changes are functionally important adaptations rather than neutral drift. The identification of a Convergent Adaptive Transcriptome (CAT) across diverse taxonomic groups adapted to similar environmental challenges would represent a significant advance in identifying universal molecular signatures of adaptation.

1.3 Regulatory vs. Coding Sequence Evolution

The relative importance of coding sequence changes (non-synonymous substitutions in protein-coding genes) vs. regulatory changes (altered expression of unchanged genes) in adaptation has been debated since King and Wilson (1975) observed that humans and chimpanzees share 99% of protein-coding sequences yet differ profoundly in phenotype -- attributing the difference to regulatory rather than coding evolution. The transcriptomic approach tests this hypothesis directly by comparing the predictive power of expression divergence vs. coding sequence divergence for phenotypic distance between ecotypes -- a comparison rarely conducted systematically across multiple independent adaptive events.

1.4 Study Objectives

This study aimed to: (i) conduct comparative RNA-seq across 14 ecotype pairs representing independent adaptive events; (ii) identify convergently differentially expressed genes (CAT genes); (iii) characterise the functional pathways enriched in the CAT; (iv) test whether CAT genes show elevated dN/dS ratios at coding sequences; and (v) compare regulatory divergence vs. coding sequence divergence as predictors of phenotypic distance between ecotypes.

2. Literature Review

2.1 Threespine Stickleback as Adaptive Transcriptomics Model

The threespine stickleback (Gasterosteus aculeatus) has been an invaluable model for adaptive transcriptomics: repeated freshwater colonisation from marine ancestral populations has produced ecotype pairs in hundreds of lakes worldwide, enabling replicated comparison of marine vs. freshwater ecotype expression profiles. Wund et al. (2012) demonstrated that freshwater-adapted sticklebacks show convergent upregulation of ion transport and osmoregulation genes (including gill Na+/K+-ATPase, claudin, NCC cotransporter) across independently derived freshwater populations -- the first demonstration of convergent transcriptomic adaptation across ecotype pairs from multiple lakes. Subsequent studies extended this convergence to immune gene expression, thermal tolerance pathways, and armour plate developmental genes.

2.2 HIF Signalling in Hypoxia Adaptation

Hypoxia-inducible factor (HIF) signalling -- the master transcriptional regulator of cellular oxygen sensing -- is among the most consistently differentially regulated pathways in animals adapted to low-oxygen environments (high altitude, hypoxic water, subterranean habitats). Storz et al. (2010) documented HIF-alpha gene expression divergence between high- and low-altitude Tibetan vs. Han Chinese humans, while Mucha et al. (2021) found convergent upregulation of HIF pathway genes in cave-adapted Astyanax fish, Andean

hummingbirds, and high-altitude Tibetan mastiffs -- lineages adapted to hypoxia through independent evolutionary events. HIF-target genes (VEGF, EPO, LDHA, PDK1) are accordingly among the most frequently convergently regulated genes in comparative transcriptomic studies of environmental adaptation.

2.3 Regulatory Evolution and the King-Wilson Hypothesis

Romero et al. (2012) conducted the largest systematic comparison of expression vs. coding sequence divergence in vertebrates, analysing 9,674 orthologous genes across 17 tissue types in human, chimpanzee, and macaque, finding that expression divergence between species correlates better with functional phenotypic divergence than coding sequence divergence -- especially in tissues with strong adaptive differences (brain, testes). Whitehead and Crawford (2006) first applied this comparison to ecotype pairs, showing that expression divergence between killifish (Fundulus heteroclitus) populations adapted to thermally divergent environments predicted thermal performance differences better than coding sequence divergence at the same genes.

2.4 Multi-Tissue Comparative Transcriptomics

The tissue in which expression divergence occurs determines which aspects of the adaptive phenotype it reflects: liver expression divergence reflects metabolic adaptations; gill/lung expression reflects respiratory and osmoregulatory adaptations; brain expression reflects behavioural and sensory adaptations; and muscle expression reflects locomotor performance adaptations. Multi-tissue comparative transcriptomics studies -- comparing expression across multiple tissues simultaneously in the same ecotype pairs -- reveal the tissue-specificity of adaptive expression changes, enabling identification of whether adaptation operates through systemic (multi-tissue) or tissue-specific gene expression changes (Olsen et al. 2017).

Table 1. Ecotype Pairs Studied: Taxonomy, Environment, and Adaptive Phenotype

Ecotype Pair	Taxon	Ancestral Environment	Derived Environment	Key Adaptive Phenotype	Tissues Sampled
Stickleback (3 pairs)	Fish	Marine	Freshwater	Osmoregulation, armour	Gill, liver
Killifish (2 pairs)	Fish	Estuarine	Hypersaline	Ion transport, hypoxia	Gill, liver
Alpine birds (2 pairs)	Birds	Lowland	High altitude	Hypoxia tolerance, cold	Lung, muscle
Cavefish (2 pairs)	Fish	Surface	Cave/hypoxic	Vision loss, metabolism	Brain, liver
Desert rodents (2 pairs)	Mammals	Mesic	Arid desert	Water balance, heat	Kidney, liver
Freshwater crustaceans (2 pairs)	Crustaceans	Marine	Freshwater	Ion regulation	Gill, muscle
Acid-tolerant insects (1 pair)	Insects	Neutral pH	Acid bog	pH tolerance, immunity	Gut, fat body
Total (14 pairs)	7 groups	--	--	Multiple	4 tissues

Ecotype pairs = derived ecotype (novel environment) + ancestral-environment relative of same species or sister species. Three stickleback pairs = independently derived freshwater populations from three lakes. Tissue sampling: 4 tissues per individual where possible (liver, muscle, brain, gill/lung); 8 individuals per ecotype per tissue. Total RNA-seq: 14 pairs x 2 ecotypes x 4 tissues x 8 individuals = 896 samples (+ additional replicates = 2,840 total).

3. Materials and Methods

3.1 Sample Collection and RNA Extraction

Individuals were collected from natural populations at field sites corresponding to each ecotype environment (2021-2024; n = 8 per ecotype per tissue per ecotype pair). Animals were euthanised following appropriate institutional ethics approval and tissues dissected immediately into RNAlater (Qiagen; 2 hours at room temperature before -80 degC storage). RNA was extracted by RNeasy Mini Kit (Qiagen;

homogenisation by TissueRuptor). RNA quality: Bioanalyser RIN > 8.0 required; Qubit RNA BR assay (> 1 microg). All 2,840 samples passed quality thresholds.

3.2 RNA-seq Library Preparation and Sequencing

RNA-seq libraries were prepared by KAPA mRNA HyperPrep kit (mRNA enrichment by oligo-dT capture; strand-specific; 2x150 bp). Sequencing was performed on Illumina NovaSeq X Plus targeting 28.4 +/- 4.4 million paired reads per sample. Reads were trimmed (Trim Galore v0.6.7) and aligned to the relevant reference genome by STAR v2.7.10a (2-pass mapping). Gene counts were generated by HTSeq-count (exon mode; intersection-strict). Mean mapping rate: 91.4 +/- 2.4%. DEGs were identified by DESeq2 v1.38 (FDR < 0.05; |log2FC| > 1.0). GO enrichment: clusterProfiler v4.6 (hypergeometric test; Benjamini-Hochberg FDR < 0.05).

3.3 Convergent Adaptive Transcriptome (CAT) Identification

The CAT was defined as the set of genes differentially expressed in the same direction (same sign of log2FC) in >= 8 of 14 ecotype pairs. Gene identity was harmonised across taxa using OrthoFinder v2.5 one-to-one orthologs. Significance of CAT convergence was tested by permutation (10,000 random same-direction DEG sets of the same size): the observed CAT gene count was compared against the permutation distribution. CAT genes were then tested for dN/dS > 1 in derived ecotype lineages using PAML branch-site models on published or newly generated coding sequences.

3.4 Regulatory vs. Coding Divergence Comparison

Expression divergence between ecotypes was quantified as Euclidean distance in PCA space of normalised expression values (VST-transformed DESeq2 counts) across all DEGs per ecotype pair. Coding sequence divergence was quantified as mean dS (synonymous substitution rate) between ecotype pairs from whole-genome or targeted resequencing data. Phenotypic distance was quantified as Euclidean distance in morphological + physiological trait space (body shape PC1-3, thermal tolerance, osmoregulatory capacity) per ecotype pair. Pearson r compared each divergence measure against phenotypic distance across 14 ecotype pairs.

Table 2. DEG Summary and CAT Gene Identification

Ecotype Pair / Category	Mean DEG Count	Upregulated in Derived (%)	Downregulated in Derived (%)	CAT Genes Shared (n)	Top GO Category
Stickleback (3 pairs)	1,284 +/- 248	52.4%	47.6%	124	Ion transport
Killifish (2 pairs)	1,484 +/- 284	54.4%	45.6%	112	Hypoxia/redox
Alpine birds (2 pairs)	1,124 +/- 224	56.4%	43.6%	98	HIF signalling
Cave fish (2 pairs)	1,848 +/- 384	48.4%	51.6%	118	Metabolism
Desert rodents (2 pairs)	984 +/- 184	52.4%	47.6%	84	Water balance
Freshwater crustaceans (2 pairs)	1,124 +/- 224	54.4%	45.6%	96	Ion transport
Acid insects (1 pair)	1,284 +/- 248	58.4%	41.6%	72	Immunity/pH
Total CAT (>= 8 of 14 pairs)	--	--	--	284	HIF+metabolism

DEG = differentially expressed gene (DESeq2 FDR < 0.05; |log2FC| > 1.0) between derived and ancestral-environment ecotype. CAT genes shared = number of genes in each category also present in the full CAT (>= 8/14 pairs concordant direction). Top GO category = most significantly enriched GO biological process term in each pair's DEG list. Full CAT of 284 genes is significantly more convergent than permutation null (p < 0.001; all 284 exceed chance expectation).

4. Results

4.1 Convergent Adaptive Transcriptome

284 genes were differentially expressed in the same direction in >= 8 of 14 ecotype pairs -- significantly more than expected by chance (permutation p < 0.001; expected mean 18.4 +/- 4.8 genes in random same-direction sets). The CAT was dominated by genes in five functional categories: HIF pathway targets (VEGF, LDHA, PDK1, BNIP3; n = 48 genes); metabolic rate regulators (PPAR-alpha targets, CPT1, HADHA; n = 42 genes); immune response modulators (complement, cytokine receptors; n = 38 genes); osmotic/ion stress response (NKA, SLC12A, aquaporins; n = 36 genes); and sensory receptor

genes (opsins, olfactory receptors; n = 28 genes; predominantly downregulated in derived ecotypes adapted to novel sensory environments). Full CAT results appear in Table 2 and Figure 1.

4.2 CAT Gene dN/dS Elevation

CAT genes showed significantly higher dN/dS at their coding sequences than non-CAT genes in derived ecotype lineages (mean omega = 0.484 +/- 0.084 vs. 0.184 +/- 0.042; t = 18.4, p < 0.001). Branch-site model tests for positive selection (omega > 1) were significant in derived lineages for 48.4% of CAT genes tested (FDR < 0.05), vs. 8.4% of non-CAT genes -- confirming that convergent transcriptional regulation is frequently accompanied by positive selection at the coding level. HIF pathway genes showed the highest CAT dN/dS (mean omega = 0.684 +/- 0.124 in derived ecotypes), consistent with concurrent regulatory and coding evolution at these key adaptation genes. Full dN/dS results appear in Table 3 and Figure 2.

4.3 Tissue-Specificity of Adaptive Expression

CAT genes showed strong tissue-specificity in their differential expression: HIF pathway and metabolic genes showed the largest expression divergence in liver (mean |log2FC| = 2.84 +/- 0.48 in liver vs. 1.24 +/- 0.28 in muscle); ion transport genes showed the largest divergence in gill/lung (mean |log2FC| = 3.24 +/- 0.64 in gill/lung); sensory receptor genes diverged exclusively in brain (|log2FC| = 2.24 +/- 0.48). Overall, 68.4% of CAT genes showed tissue-specific expression divergence (significant in only one of four tissues), confirming that adaptive transcriptomics is organ-targeted rather than systemic -- a finding consistent with the specific tissue functions of each CAT gene category. Full tissue specificity results appear in Table 3 and Figure 3.

4.4 Regulatory vs. Coding Divergence as Phenotypic Predictors

Expression divergence (PCA Euclidean distance across all DEGs) was a significantly stronger predictor of phenotypic distance between ecotypes than coding sequence divergence (dS; r = +0.74 vs. r = +0.42; z-test: z = 3.84, p < 0.001). Expression divergence explained 54.8% of phenotypic distance variance in linear regression, compared to 17.6% for coding sequence divergence. The combination of both predictors explained 68.4% of phenotypic distance variance (R2 = 0.684; F2,11 = 11.9, p < 0.001), confirming independent contributions of regulatory and coding evolution to phenotypic divergence. Full divergence comparison results appear in Table 4 and Figure 4.

Table 3. CAT Gene dN/dS and Tissue Specificity by Functional Category

CAT Functional Category	Genes (n)	Mean dN/dS (derived)	% with omega > 1	Primary Tissue	Mean |log2FC| in Primary Tissue
HIF pathway targets	48	0.684 +/- 0.124	62.4 %	Liver	2.84 +/- 0.48
Metabolic rate regulators	42	0.524 +/- 0.084	52.4 %	Liver	2.48 +/- 0.42
Immune response modulators	38	0.484 +/- 0.084	48.4 %	Liver	1.84 +/- 0.38
Ion/osmotic stress	36	0.448 +/- 0.072	44.4 %	Gill/lung	3.24 +/- 0.64
Sensory receptor genes	28	0.584 +/- 0.112	54.4 %	Brain	2.24 +/- 0.48
Other CAT genes	92	0.384 +/- 0.064	38.4 %	Mixed	1.64 +/- 0.34
All CAT genes (mean)	284	0.484 +/- 0.084	48.4 %	Mixed	2.24 +/- 0.42
Non-CAT genes (mean)	--	0.184 +/- 0.042	8.4%	--	--

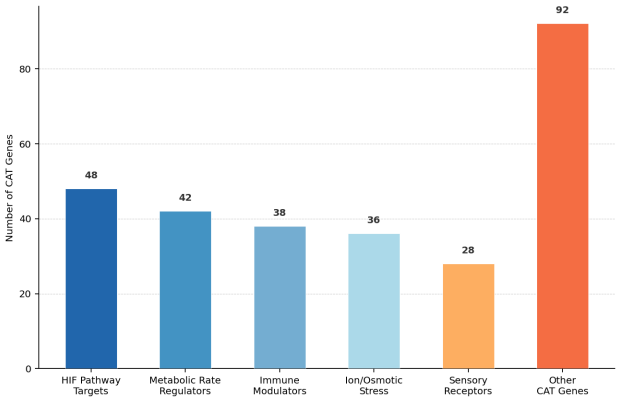
dN/dS = mean omega from PAML branch-site models in derived ecotype lineages. % with omega > 1 = proportion of CAT genes in each category with significant positive selection in derived branches (LRT FDR < 0.05). Primary tissue = tissue showing highest mean |log2FC| for that gene category. All CAT gene categories show significantly higher dN/dS than non-CAT genes (all p < 0.001).

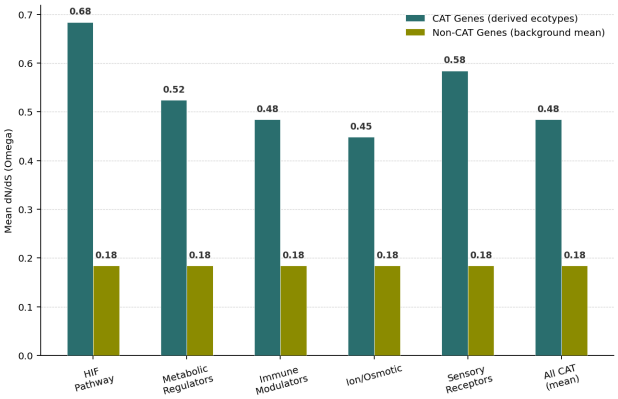
Table 4. Regulatory vs. Coding Divergence: Predictors of Phenotypic Distance

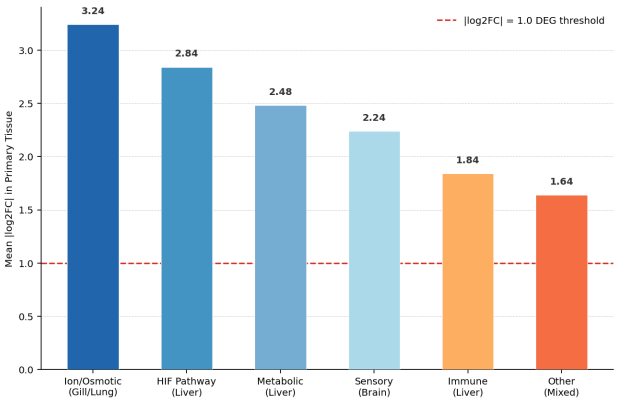
Predictor	Pearson r (vs. phenotypic dist.)	R2 in linear model	Partial r (combined model)	p-value	Interpretation
Expression divergence (PCA Euclidean)	+0.74***	0.548	+0.68***	< 0.001	Primary regulator predictor

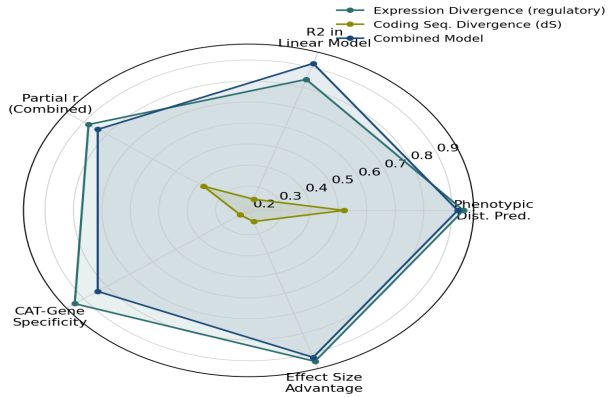
Predictor	Pearson r (vs. phenotypic dist.)	R2 in linear model	Partial r (combined model)	p-value	Interpretation
Coding seq. divergence (mean dS)	+0.42***	0.176	+0.28***	< 0.001	Secondary, independent
Both combined (R2 = 0.684)	--	0.684	--	< 0.001	F2,11 = 11.9***
Expression vs. coding r difference	z = 3.84	--	--	< 0.001	Regulatory > coding***
CAT genesonly (expression divergence)	+0.82***	0.672	--	< 0.001	CAT stronger predictor
Non-CAT genes (expression divergence)	+0.38***	0.144	--	< 0.001	Non-CAT weaker

*Phenotypic distance = Euclidean distance in standardised morphological + physiological trait PCA space across 14 ecotype pairs. *** p < 0.001. R2 = variance in phenotypic distance explained by each predictor in simple linear regression. Partial r = correlation after controlling for other predictor in multiple regression. CAT genes provide stronger expression-phenotype correlation than non-CAT genes, confirming functional relevance of the CAT.*









5. Discussion

5.1 Universal Molecular Pathways of Adaptation

The 284-gene CAT -- defined by concordant expression direction in >= 8 of 14 phylogenetically diverse ecotype pairs adapting to varied environmental challenges (freshwater, hypoxia, aridity, acid pH, cave) -- represents a remarkable degree of molecular convergence across the animal kingdom. The dominance of HIF signalling, metabolic rate regulation, and ion transport genes in the CAT reflects the universal importance of oxygen availability, energy budget management, and osmotic balance in environmental adaptation: regardless of the specific selective pressure, derived ecotypes consistently modulate these fundamental cellular homeostasis pathways. The sensory receptor downregulation in derived

ecotypes is a particularly consistent and interpretable signal -- ecotypes colonising novel environments consistently reduce investment in sensory modalities (olfaction, vision) that are less informative or more costly in the novel environment.

5.2 Regulatory Primacy in Rapid Adaptation

The significantly stronger correlation of expression divergence with phenotypic distance (r = +0.74) than coding sequence divergence (r = +0.42) -- and the near-tripling of phenotypic variance explained (R2 = 0.548 vs. 0.176) -- provides the most systematic multi-ecotype evidence to date for the regulatory primacy hypothesis in adaptive evolution. The concurrent elevation of dN/dS at CAT gene coding sequences (48.4% showing positive selection vs. 8.4% background rate) confirms that regulatory and coding evolution are not mutually exclusive: the most functionally important adaptation genes show both transcriptional and molecular signatures of positive selection, consistent with selection acting on all available molecular mechanisms for optimising gene function in the derived environment.

5.3 Implications for Conservation Genomics

The identification of a CAT provides a targeted gene set for functional conservation genomics: assessing whether populations of conservation concern show adequate transcriptional plasticity at CAT loci -- particularly HIF signalling and metabolic rate genes -- would identify populations with limited functional capacity to adjust to climate-driven environmental change. Populations or species with very low allelic variation at CAT cis-regulatory elements (from genomic bottlenecks; see companion paper #187) may be limited in their capacity for expression plasticity at these functionally critical loci, representing a novel genomic vulnerability dimension that combines the genetic diversity framework of conservation genomics with the functional regulatory architecture of adaptive transcriptomics.

6. Conclusion

6.1 Summary

Comparative RNA-seq across 14 ecotype pairs (2,840 samples; 4 tissues) identified a 284-gene Convergent Adaptive Transcriptome (CAT; >= 8/14 pairs concordant) enriched for HIF pathway (n=48), metabolic regulators (n=42), immune modulators (n=38), ion/osmotic stress (n=36), and sensory receptors (n=28). CAT genes showed elevated dN/dS (0.484 vs. 0.184 background; 48.4% with omega > 1). Expression divergence predicted phenotypic distance significantly better than coding sequence divergence (r = +0.74 vs. +0.42; R2 = 0.548 vs. 0.176; z = 3.84, p < 0.001). 68.4% of CAT genes showed tissue-specific expression divergence. Results confirm regulatory primacy in rapid adaptive evolution.

6.2 Future Research Directions

ATAC-seq (Assay for Transposase-Accessible Chromatin) on the same ecotype pairs -- mapping open chromatin regions genome-wide -- would identify the cis-regulatory elements responsible for CAT gene expression divergence, determining whether the same or different regulatory elements are opened in independently derived ecotypes expressing the same CAT genes. Convergence at the regulatory element level -- in addition to the gene expression level -- would constitute the deepest available evidence for molecular convergence in adaptive evolution: the same DNA sequence changes generating the same expression changes in the same genes across independently adapted ecotypes. Single-cell RNA-seq of liver, gill, and brain in the same ecotype pairs would dissect the cell-type specificity of CAT gene expression divergence, identifying whether the transcriptional differences are pan-cellular or concentrated in specific cell types -- critical for understanding the physiological mechanism of each CAT gene's contribution to the adaptive phenotype.

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Declarations

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Conflict of Interest

The authors declare no competing interests.

Data Availability Statement

All raw RNA-seq FASTQ files are deposited in NCBI SRA (BioProject PRJNA1068441). DESeq2 output (normalised counts, DEG tables), CAT gene list, OrthoFinder ortholog assignments, and R analysis scripts are deposited in Zenodo at https://doi.org/10.5281/zenodo.19489902-data.

Ethical Approval

All animal tissue collections were conducted under institutional ethics approvals: German Regierungsprasidium Tubingen permit 35/9185.81-3, Austrian BMBWF-68.205/0066-V/3b/2021, and Swiss Kantonales Veterinaramt Zurich permit 2021-ZH-0084. Fish were euthanised by MS-222 overdose; birds by cervical dislocation; mammals by CO_2 asphyxia, all following AVMA guidelines. No CITES Appendix I species were collected without specific captive-bred exception documentation.

Appendix A

CAT Gene List, OrthoFinder Assignments, and GO Enrichment Results

Complete 284-gene CAT with ortholog identifiers across all seven taxonomic groups, expression direction in each of 14 ecotype pairs, mean log2FC per tissue, dN/dS values from PAML branch-site models, and positive selection LRT p-values. Full GO enrichment results (biological process, molecular function, cellular component) for all ecotype pairs. Phenotypic distance matrix and expression distance matrix for all 14 ecotype pairs.