Parallel introgression, not recurrent emergence, explains apparent elevational ecotypes of polyploid Himalayan snowtrout

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(200/200) The recurrence of similar evolutionary patterns within different habitats often reflects

parallel selective pressures acting upon either standing or independently occurring genetic variation to produce a convergence of phenotypes. This interpretation (i.e. parallel divergences within adjacent streams) has been hypothesized for drainage-specific morphological ‘ecotypes’ observed in polyploid snowtrout (Cyprinidae: *Schizothorax*). However, parallel patterns of differential introgression during secondary contact is a viable alternative hypothesis. Here, we utilized ddRADseq ($N=35,319$ *de-novo* and $N=10,884$ transcriptome-aligned SNPs), as derived from Nepali/Bhutanese samples ($N=48$ each), to test these competing hypotheses. We first employed genome-wide allelic depths to derive appropriate ploidy models, then a Bayesian approach to yield genotypes statistically consistent under the inferred expectations. Elevational ‘ecotypes’ were consistent in geometric-morphometric space, but with phylogenetic relationships at the drainage-level, sustaining an hypothesis of independent emergence. However, partitioned analyses of phylogeny and admixture identified subsets of loci under selection that retained genealogical concordance with morphology, suggesting instead that apparent patterns of morphological/phylogenetic discordance are driven by widespread genomic homogenization. Here, admixture occurring in secondary contact effectively ‘masks’ previous isolation. Our results underscore two salient factors: 1) Morphological adaptations are retained despite hybridization; and 2) The degree of admixture varies across tributaries, presumably concomitant with underlying environmental or anthropogenic factors.

1. Introduction

Selection for local environmental conditions can drive rapid evolution, and occasionally does so within parallel systems [1]. This, in turn, can promote the emergence of unique ecotypes, or even novel species [2–4]. Adaptations to ecological gradients can also occur, especially in the absence of geographic isolation, and are commonly attributed to selection acting upon existing variation [either ancestral/standing [5,6], or that acquired through hybridization [7–11]]. These represent but two of several scenarios through which such patterns can be generated [12–14].

Divergent selection in response to ecological gradients can also facilitate speciation, even when gene flow is ongoing [15–17]. A hallmark of this process is the formation of genomic ‘islands of divergence,’ with selection and its cumulative effects serving to counterbalance homogenizing gene flow [18–22]. These so-called ‘islands’ may then expand via a hitchhiking mechanism, such that divergence is also initiated within linked genomic regions [23–26]. However, heterogeneous genomic divergence can also arise via entirely different processes, to include those wholly unrelated to speciation. This, in turn, introduces an analytical dilemma, in that the signatures of one can either obfuscate or instead emulate that of the other [27–29].

The resulting emergence of parallel ecotypes is often manifested phylogenetically as clusters within study sites or regions, rather than among eco-phenotypes [30]. However, an alternative is that ecological adaptations instead evolve via isolation followed by subsequent dispersal, such that genomic loci are now juxtaposed across both distributions and genomes [12,31,32]. ‘Genomic islands’ as well as phylogenetic patterns are then generated similar to those manifested by parallel divergence-with-gene-flow (per above). This can occur, for example, when introgression is constrained within localized genomic regions that underlie with adaptation to an ecological gradient [33]. Genome-wide homogenization is the result, with ancestral

branching patterns (e.g., those uniting ecotypes) now restricted to regions where permeability to
 46 gene flow is reduced by selection and/or low recombination [34,35]. However, the singular
 origin of an adaptive allele spread selectively via localized introgression can also yield relatively
 48 similar genomic patterns [36–38].

Together, this implicates four distinct scenarios that could generate genomic landscapes
 50 compatible with those expected under parallel ecotype emergence. Yet only two of these
 necessitate divergence-with-gene-flow, e.g., with selection acting in a repetitive fashion on either
 52 A) standing genetic variation or B) independent *de novo* mutations. In contrast, two alternative
 scenarios involve adaptive variability accumulated while in isolation, followed by secondary
 54 gene flow among dispersing ecotypes. This then results in: C) selective filtering against a
 background of genomic homogenization; or D) the selective introgression of adaptive alleles into
 56 novel populations (figure 1). Thus, while all are effectively operating in ‘parallel’ (e.g., in
 separate river drainages or habitat patches), their relationship to the diversification process
 58 differs substantially. This presents a challenge for the interpretation of such patterns, in that those
 superficially similar may in fact reflect markedly different processes that potentially act in
 60 concert [39–41]. A highly localized genomic architecture underlying ecotypes further
 complicates this situation [42].

(a) Can parallel emergence be discriminated from parallel introgression?

64 We posit these factors can indeed be discriminated by predicting the ancestries for replicated
 ‘pairs’ of ecotypes distributed among sites (although patterns may also depend upon migration
 66 rates among populations [43]). For hypotheses of ‘independent emergence,’ we would predict
 that genealogies with regions encapsulating targets of divergent selection [i.e., scenarios (A) or

(B); figure 1], would lack shared ancestry among ecotypes, reflecting their independent origins.

In a similar vein, a failure to correlate with unlinked targets of selection would also be expected

[12]. Thus, in the case of repeated selection upon standing genetic variation, adaptive alleles will

be identical-by-descent but with flanking regions excluded (given that populations will lack

identical, independently-fixed haplotypes) [13,14]. Furthermore, in a scenario of independent

emergence, mutations underlying adaptation may occur at different loci altogether.

By contrast, a 'divergence first' model with subsequent secondary contact [scenarios (C)

or (D); figure 1] would imply that ancestries of ecotypes are both correlated with and shared

among ecotypes at those loci targeted for selection. However, genomic patterns can be difficult

to disentangle from those expected under a scenario of adaptive introgression (scenario D). This

is because gene flow during secondary contact may effectively 'swamp' divergence developed in

isolation [12,44]. In the absence of genomic resources (a frequent scenario for non-model

organisms), additional clarification can often be inferred from biogeographic context.

(b) A case study involving *Schizothorax*

We here investigate the source of adaptive genetic variation among differentially adapted pairs

of rheophilic freshwater fishes occupying elevational gradients in Himalayan tributaries of the

Ganges and Brahmaputra rivers. Our study group (snowtrout; Cyprinidae: *Schizothorax* spp.) is

of interest in that it displays a legacy of whole-genome duplication (WGD), with variable

adaptations to high elevation habitat (both phenotypic and life-historic) [45–48]. Yet, in previous

studies patterns of convergence are also broadly apparent [49,50]. Additionally, the rapid

formation of multiple 'species flocks' has occurred within unique and isolated alpine lake

habitats [51–55], with recurrent divergences as an emerging consensus [51,56]. Here, two

Himalayan species are of particular interest: *Schizothorax progastus* and *S. richardsonii*. Both
 92 are distributed along an elevational gradient, with generalized morphologies within tributaries of
 the Ganges and Brahmaputra rivers converging upon ‘blunt-nosed’ (*S. richardsonii*) in upstream
 94 reaches, and ‘pointed-nosed’ (*S. progastus*) in downstream reaches [52,57–59].

The ‘pointed-nosed’ eco-phenotype displays a more terete, streamlined shape, and
 96 gradually replaces the ‘blunt-nosed’ form longitudinally along an elevational gradient, a pattern
 seemingly replicated in each of several collateral, southward-flowing tributaries in Nepal, which
 98 in turn suggests the presence of ecological non-exchangeability [60]. Recent phylogenetic
 analyses based on mtDNA failed to support the nominal species, with relationships occurring
 100 instead at the drainage level [56]. Despite this, morphologies are consistent across drainages
 [51], suggesting an independent emergence of ‘blunt-nosed’ phenotypes (i.e., those associated
 102 with *S. richardsonii*) within each highland drainage. Morphologically convergent ecotypes are
 also replicated within elevational gradients of Bhutan [61], again suggesting the formation of
 104 parallel ecotypes. This suggests the potential for rapid evolution, particularly when juxtaposed
 with the adaptive radiation by *Schizothorax* within isolated Rara Lake of Nepal [51,53].

Ploidy has been hypothesized as facilitating diversification within cyprinid fishes [62] ,
 106 with a rapid, positive shift in net diversification occurring within three predominantly polyploid
 108 subfamilies: Torinae, Schizopygopsinae, and Schizothoracinae, with the most profound effect in
 the latter. Of particular interest, all three subfamilies are endemic to the Himalayan and Qinghai-
 110 Tibetan Plateau (QTP) regions, and possess life-history specializations for high-elevation
 existence [63]. Thus, diversification of polyploid cyprinids on the QTP and adjacent regions is
 112 seemingly linked to extensive orogeny followed by periods of marked climate change [62,64–
 66].

However, population genomic methods are currently limited in their capacity to gauge how polyploidy has driven the adaptation by *Schizothorax* to elevational gradients. Studies involving non-model polyploid species (as herein) must employ methodological paradigms that assume diploidy, yet with fundamentally divergent theoretical expectations [67,68]. An additional complication involves the varying degrees of divergence and/ or conservation found among ohnologs (i.e., duplicated loci originating from WGD) that potentially occur in those species at intermediate stages of re-diploidization [69]. One positive is that models suitable for genotyping polyploid or mixed-ploidy data [70,71] now incorporate short-read sub-genomic methods (e.g., RADseq and related methods).

The identification of genomic adaptations to elevation in *Schizothorax* has two trenchant stumbling blocks: Drivers of morphological and phylogenetic discordance are not only numerous, but also constrained by polyploidy. To compensate, we combine novel statistical models and robust genotyping of non-model polyploids with expectations regarding how ancestries should be distributed across the genome. This allows us to address several questions regarding the evolution of diverse *Schizothorax* lineages seemingly replicated in the Himalayan drainages of Nepal and Bhutan:

1. Does morphological and phylogenetic discordance in parallel Himalayan elevational gradients stem from parallel ‘independent emergence (figure 1A, B), or secondary/ ongoing gene flow (figure 1C, D)?
2. If A or B, do ecotypic pairs in replicated drainages show evidence of co-divergence, thus indicating a shared underlying biogeographic process (e.g., periods of QTP uplift)?
3. If C or D, is there evidence for selection-mediated differential introgression?

4. Finally, is there evidence for either rapid co-divergence or hybridization as playing a
138 role in the formation of lacustrine ecotypes?

140 2. Methods

(a) Sampling and DNA preparation

142 Tissue samples ($N=96$) represent six *Schizothorax* species distributed throughout tributaries of
the Brahmaputra and Ganges rivers (Bhutan and Nepal, respectively) (figure 2a-c). Of these,
144 $N=48$ Nepali specimens were obtained from the University of Kansas Natural History Museum
(KUNHM) (figure 2b; table S1, S2) and represent two riverine species [‘high elevation’ *S.*
146 *richardsonii* ($N=16$) and ‘low elevation’ *S. progastus* ($N=8$)]. These were sampled from each of
the three major Nepali drainages (Kali Gandaki, Koshi, and Karnali), except for *S. progastus*
148 from Karnali. The remaining Nepali samples ($N=24$) represent a land-locked lacustrine radiation
endemic to Lake Rara [53]. These are: *S. macrophthalmus* ($N=8$), *S. raraensis* ($N=8$), and *S.*
150 *nepalensis* ($N=8$).

Bhutanese *Schizothorax* ($N=48$) were sampled from three major Bhutanese drainages
152 (Wang Chhu, Punatsang Chhu, and Mangde Chhu: figure 2c; table S1) and identified as *S.*
progastus and *S. richardsonii* based upon morphological diagnoses of vouchered specimens [52].
154 However, subsequent mtDNA sequence analysis has putatively identified Bhutanese *S.*
richardsonii as another convergent high-elevation specialist, *S. oconnori* [56], a species typically
156 found on the Qinghai-Tibetan Plateau (QTP [72,73]) within high elevation tributaries of the
upper Brahmaputra River [herein referred to as the Yarlung-Tsangpo River (YLTR)]. They are
158 subsequently referred to herein as *S. cf. oconnori* (figure 2c).

Tissues were processed using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Inc.),

following manufacturer's protocols. Extracts were evaluated for presence of high-molecular weight DNA using gel electrophoresis (2% agarose) and quantified at 2µl per sample in 200µl assays using Qubit broad-range DNA fluorometry assays (Thermo Fisher Scientific). DNA (500-1000ng of DNA per sample in 50µl reactions) was then fragmented using a restriction double-digest [74] with *Pst*I (5'-CTGCAG-3') and *Msp*I (5'-CCGG-3'). Digests were subsequently visualized on 2% agarose gels, purified using AMPure XP beads (Beckman Coulter, Inc.), and again quantified via Qubit fluorometer.

Samples were then standardized at 100ng of digested DNA and ligated in 30µl reactions using T4 DNA ligase (New England Biolabs, Inc.) following manufacturer's protocols. Barcoded oligonucleotide adapters were designed and employed following Peterson *et al.* [74]. After a second AMPure XP purification, samples were multiplexed in groups of $N=48$ and size-selected at 350-400bp (excluding adapters), using a Pippin Prep automated system (Sage Sciences). Adapters for Illumina sequencing were subsequently extended via a 10-cycle PCR with Phusion high-fidelity DNA polymerase (New England Biolabs, Inc.). Final reactions were purified via AMPure XP beads and standardized per submission requirements of the DNA Core Facility (University of Oregon Genomics & Cell Characterization Facility, Eugene, OR U.S.A.). Additional quality control at the core facility included fragment size analysis (to confirm successful amplification and the absence of artifacts) and qPCR (to assess the proportion of sequenceable library). Sequencing pooled two $N=48$ multiplexed libraries into a single lane of 1x100 sequencing on the Illumina HiSeq 4000.

(b) Data filtering and ploidy-aware assembly

Raw reads were demultiplexed and filtered for initial assembly using IPYRAD [75]. Reads having

>0 barcode mismatches or >5 low-quality bases were discarded, and adapter sequences trimmed

using the ‘strict’ option. Two assemblies were performed: one *de novo* (at an 85% identity threshold), and one using bwa to align against assembled transcriptome data (hereafter ‘transcriptome-guided’) [76]. Because the genotyping model in IPYRAD assumes diploidy [77], we used relaxed settings so as to retain assembled paralogs (e.g., electing for more stringent filtering following ploidy-aware genotyping; see below). These included a minimum depth threshold of only 6, allowing 20 heterozygous sites per consensus locus, with up to 4 alleles at a given locus within an individual.

To explore potential ploidy variation in our samples, we first employed BWA [78] to realign raw reads against the candidate locus catalog generated in our *de novo* assembly. For ploidy model selection, we then computed allelic read depths for bi-allelic sites using a denoising procedure (NQUIRE; [79] with expectations that ploidies represent allelic depths as follows: At ~50% in a diploid bi-allelic heterozygote (i.e., an approximate 50:50 representation of each allele in an AB heterozygote); at ~33% or ~66% for triploids (=ABB or AAB possible genotypes); and ~25%, 50%, or 75% for tetraploids (=ABBB, AABB, AAAB genotypes, respectively). Log-likelihoods of the observed allelic depth distributions were extracted for Gaussian expectations under each fixed model (e.g., 2n, 3n, or 4n), then normalized by that under a model of freely variable allele depths ($=\log L_{\text{Fixed}}/\log L_{\text{Free}}$), with chosen models representing the greatest normalized log-likelihood. Results were employed to generate prior expectations for subsequent ploidy-aware genotyping and downstream filtering.

Formatted SNPs (as .vcf) were genotyped for both *de novo* and transcriptome-guided assemblies using the Bayesian variant calling pipeline in POLYRAD [70]. We first computed per-locus $H_{\text{IND}}/H_{\text{E}}$ values, a statistic representing the probability of sampling reads from two

different alleles at a given locus in an individual [71]. We then simulated expected $H_{\text{IND}}/H_{\text{E}}$ values given the sample size and read depth distribution from the observed data, under expectations of either diploidy or tetraploidy, to define threshold values under which markers are statistically consistent with Mendelian behavior as a 95% upper-bound of the simulated distribution. Because expected values differ by ploidy, we used $H_{\text{IND}}/H_{\text{E}}$ values averaged across loci to segregate those statistically consistent with a diploid genotype model from those consistent with a tetraploid model. Using $H_{\text{IND}}/H_{\text{E}}$ thresholds and custom Python code (github.com/tkchafin/polyrad_scripts/filterPolyVCF.py), we partitioned genotypes for both assembled datasets into loci statistically consistent with tetraploid and diploid expectations. An additional requirement was that >50% of individuals be genotyped at an average mean per-individual depth of 20X.

(c) Geometric morphometrics of Nepali fishes

We first examined patterns of morphological convergence and divergence using geometric morphometric data [51] derived from 528 images captured from museum specimens representing all five Nepali species (for full sample metadata and KU voucher numbers, see Regmi et al. [51]; for those used for ddRAD, see table S2). Our analyses were restricted to the Nepali sub-tree in that voucher specimens for Bhutanese samples were not available. Briefly, 18 landmarks were targeted, focusing on head morphology (i.e., snout elongation, head depth, eye/nostril placement), and body shape (origin/insertion of fins, upper/lower bases of the caudal fin, and urostyle as most posterior point). Coordinates were then superimposed across samples using Generalized Procrustes Analysis implemented in GEOMORPH [80]. Full details on geometric morphometric data processing, including bias and sensitivity analysis, can be found in Regmi *et*

al. [51].

Procrustes-aligned coordinates were summarized using a principal component analysis (PCA) in GEOMORPH [80]. We then conducted a linear discriminant analysis (LDA) using the MASS R package [81], maximizing discriminant capacity with 80% of samples as a training set, and group classifications specified as species X drainage.

(d) Phylogenetic relationships

Given that most phylogenomic methods cannot take into account polyploid SNP genotypes [82], our initial phylogenies were instead built using an alignment-free method that computes distances from the intersection of k -mer distributions in unaligned reads [83,84]. Matrices of k -mer distances were then used to infer a phylogenetic tree with branch lengths using FASTME [85].

The resulting topology was additionally contrasted with that inferred under a polymorphism-aware (PoMO) model inferred in IQ-TREE [86,87], as evaluated across 1,000 ultrafast bootstraps, with a GTR substitution model, gamma-distributed rates ($N=4$ categories), and a virtual population size of 19.

(e) Population structure and molecular clustering

Most widely used clustering or ‘assignment test’ methods that examine population structure are either inappropriate for polyploid data or cannot handle mixed-ploidy genotypes [67]. However, STRUCTURE is robust in both situations [88,89], though more computationally intensive than alternatives. We thus coded our mixed-ploidy genotypes as input for STRUCTURE, following recommendations of Meirmans *et al.* [67] and Stift *et al.* [89], using 10 replicates each of K

values (= # sub-populations) ranging from $K=1$ to $K=20$. Analyses were additionally replicated across *de novo* and transcriptome-guided assemblies, using a total MCMC length of 100,000 iterations following a 50,000-iteration burn-in. Input files were generated using *polyVCFtoStructure.py* (github.com/tkchafin/polyrad_scripts), and results were aggregated/visualized using the CLUMPAK pipeline [90], with selection of optimal K following the Evanno method [91]. Final visualizations aligned the ancestry proportion bar plots aligned to our SKMER phylogeny using code from Martin *et al.* [92].

Results were contrasted with ordination via discriminant analysis of principal components (DAPC), performed in ADEGENET [93,94]. Samples were stratified according to species X basin assignment (e.g., *S. progastus* from Wang Chhu), and with analyses performed in three ways: 1) globally; 2) Nepal-only; and 3) Bhutan-only. The number of principal components (PCs) retained in each case was determined using the *xvalDapc* function as a cross-validation approach, with the optimal number of the root-mean-square-error (RMSE) of assignment derived from a 10% subset (with the remaining 90% serving as a training set). This was accomplished across 30 replicates per level of PC retention, up to a maximum of 300 retained PCs, resulting in 20 (globally-), 10 (Bhutanese-), and 5 (Nepali-) retained PCs.

(f) Modeling population mixture

We assessed hybridization within Nepali and Bhutanese sub-trees using TREEMIX [95], with a global search across numbers of migration events (m) ranging from 0–5. Because markers are assumed diploid, migration analyses were only performed on markers statistically-fitting diploid expectations, as designated by POLYRAD [70]. Optimal values of m for each subtree were determined from rates of log-likelihood change as computed in OPTM [96], which generates an

ad hoc statistic (ΔM) representing the second-order rate of change weighted by the standard deviation (e.g., the ‘Evanno’ method; Evanno et al. 2005). Independent replicates on the full dataset yielded identical likelihoods for some *m*-values (i.e., yielding an undefined ΔM) and, given this, we assessed variability using 100 bootstrap pseudoreplicates per migration model. To additionally discriminate among divergence scenarios among Nepali *S. richardsonii* and *S. progastus*, we calculated a 4-taxon Patterson’s *D* statistic and admixture fractions (f_4 -ratio) [97,98]. Both are formulated to test an enrichment of shared-derived site patterns between either component of a lineage pair (P1 or P2), and a third lineage (P3) relative to an outgroup. The assumed phylogenetic structure would be: (((P1, P2), P3), P4). Our interest was in shared patterns, both at the level of “conspecifics” among drainages and “heterospecifics” within drainage, and our tests were conducted such that P1 and P2 were defined by river [e.g., (*richX*, *progX*), (*richY*), Outgroup), where X and Y represent different drainages], and where P1 and P2 were defined per taxon-assignment [e.g., (*richX*, *richY*), *progX*), Outgroup)]. The outgroup in all cases was *S. cf. oconnori* from Bhutan.

(g) Testing models of co-divergence

To test if shared (e.g. geomorphic) events may have driven co-divergence, we used the program ECOEVOLITY [99], which employs a Bayesian method to compute probabilities on the number of independent divergences across a series of pairwise comparisons. Because ECOEVOLITY is most consistent when analyzing both constant and polymorphic sites [99,100], we sampled full-locus alignments (excluding sites with either >25% missing data globally or per-population) using the *phylip2ecoevolity.pl* script from Chafin et al. [101]. The event model followed a Dirichlet process, with the prior probability distribution for the number of divergence events skewed

towards a model of complete independence (i.e. no co-divergence). Posterior probabilities were computed over a total of 75,000 MCMC iterations, with a sampling frequency every 50th iteration. Burn-in was automatically computed using an automated iterative procedure which selected the number of burn-in iterations which maximized the effective sample size (ESS).

(h) Testing patterns of selection and locus-wise differentiation

Here we sought loci strongly associating with axes of population differentiation, and therefore employed the program PCADAPT [102] to identify loci putatively associated with differentiation of *S. richardsonii* and *S. progastus*. Detecting loci under selection is a persistent methodological gap in those studies examining polyploid SNP data [67] and, given this, we restricted our analysis to transcriptome-mapped loci that could appropriately be genotyped as diploid [70]. Analyses were performed by partitioning the data so as to target specific divergence events: *S. richardsonii* X *S. progastus* (Gandaki); *S. richardsonii* X *S. progastus* (Koshi); *S. richardsonii* X Lake Rara (*S. macrophthalmus*, *S. raraensis*, and *S. nepalensis*); *S. nepalensis* X *S. macrophthalmus*+*raraensis*; and *S. progastus* (Bhutan) X *S. cf. oconnori*. Loci were additionally restricted to those with a minor allele frequency (MAF) >0.05, with significance assessed using $\alpha=0.01$ adjusted via Bonferroni correction (where N tests = N SNPs). Transcripts encapsulating outlier SNPs were additionally annotated with gene ontology (GO) terms for biological functions using the BLAST2GO pipeline [103], searching against the SWISS-PROT and InterPro databases [104,105]. Redundancy was reduced in GO-term annotations by measuring semantic similarity using RRVGO [106] and the *Danio rerio* organismal database.

We additionally contrasted population pairs (delineated as above), using a ploidy-appropriate F_{ST} measure. We calculated heterozygosity (i.e. ‘gene’ diversity [92]) as: $H_s = 1 -$

Σp_i^2 , where p_i is the frequency of allele i [107]. Although in diploids this measure is often

referred to as ‘expected heterozygosity,’ it does not have the same relationship with

heterozygosity across ploidies [67]. Finally, we calculated genetic distance as Jost’s D [108], as

well as absolute distance (D_{XY}) for each SNP based on allele frequencies and summarized across loci as an arithmetic mean [109].

(i) Partitioned analysis across subsets of loci

As a final test of the hypothesis that divergence between pairs of *S. richardsonii*-*S. progastus* represents isolation prior to secondary hybridization, we performed a partitioned analysis across

subsets of our data. Our prediction was as follows: If differential adaptation reflects a retention of minor genomic regions from secondary introgression (i.e., as opposed to arising

independently), then disparate populations of *S. richardsonii* would be more similar at loci for which drainage pairs (*richardsonii-progastus*) are highly diverged. We diagnosed such loci in

two ways: First, we computed a ratio of D_{XY} between *richardsonii-progastus* pairs within Koshi and Gandaki rivers, and D_{XY} of *S. richardsonii* populations among drainages. Here, a value less

than 1.0 indicates a locus for which *richardsonii-progastus* pairs are more similar than

richardsonii-richardsonii comparisons. A value >1.0 indicates a locus for which *richardsonii-*

progastus are more highly diverged. If loci with a D_{XY} ratio >1.0 originated from hybridization,

genetic relationships among those loci should reflect a conspecific phylogeny. To assess the

latter, we replicated TREEMIX analyses (as above) for loci >1.0 and <1.0 . As a secondary test, we

also partitioned locus-wise *richardsonii-progastus* F_{ST} into four groups, each receiving the same

analytical treatment as the D_{XY} ratio partitions: 1) $F_{ST} = 0.0-0.249$; 2) $0.25-0.49$; 3) $0.50-0.749$;

and 4) $0.75-1.0$.

344

3. Results

346 (a) Sequence assembly and genotyping

Prior to downstream filtering, we assembled $N=48,350$ and $14,301$ SNPs for *de novo* and
 348 transcriptome-guided assemblies, respectively. Allele depths for biallelic sites were
 overwhelmingly trimodal, with NQUIRE likelihoods suggesting tetraploidy across all study taxa
 350 (figure 3). Raw outputs from *de novo* and transcriptome-aligned assemblies resulted in $48,350$
 and $14,301$ loci, respectively (table S3). Three samples failed to yield genotype data for $\geq 50\%$
 352 of loci and were removed. Simulated expectations in POLYRAD yielded H_{IND}/H_E thresholds of
 0.52 (diploid) and 0.65 (tetraploid). Post-filtering datasets consisted of $5,601$ ($2n$) and $5,284$ ($4n$)
 354 transcriptome-mapped, and $15,547$ ($2n$) and $19,772$ *de novo* SNPs (table S3). For each assembly
 method, these were divided into files containing all loci as well as subsets containing only those
 356 statistically consistent with a diploid model. Scripts for formatting datafiles and outputs can be
 found at: github.com/tkchafin/polyrad_scripts.

358

(b) Contrasting molecular and geometric morphometric results

360 Morphological DAPC showed a marked convergence of *S. richardsonii* and *S. progastus* body
 shapes, regardless of origin, with Lake Rara taxa (*S. macrophthalmus*, *S. nepalensis*, and *S.*
 362 *raraensis*) having weak or no differentiation (figure 4). Among the latter, *S. nepalensis* was most
 intermediate between a ‘Lake Rara’ cluster, and that for *S. richardsonii* (and to a lesser extent, *S.*
 364 *progastus*). This contrasted sharply with the clustering of Nepali specimens based solely on
 ddRAD data (figure 5a,c), where the predominant relationship was by drainage (e.g., with Koshi
 366 and Gandaki *S. progastus* and *S. richardsonii* grouping together).

The latter was mirrored in our phylogenetic analyses, with pairs of *S. progastus*-
 368 *richardsonii* from the Koshi and Gandaki rivers being monophyletic in the alignment-free
 phylogeny (figure 6a) and agreeing approximately with STRUCTURE-inferred assignment
 370 probabilities (figure 6b). Optimal Evanno-derived *K* in the latter identified peaks in *DeltaK* at
K=3 and *K*=7. The former yielded a homogenous Nepali sub-tree, with only the Koshi River
 372 samples somewhat distinct (figure 6a,b), whereas *K*=7 reveals weak differentiation, with mixed
 assignment spanning basins.

Inferred edges in TREEMIX suggested migration between *S. progastus* of the Koshi and
 374 Gandaki rivers, as well as between *S. progastus* of the Koshi with *S. raraensis* and *S.*
 376 *macrophthalmus* of Lake Rara (figure 6c). However, the latter may be mis-ascribed, particularly
 given the absence of sampling for *S. progastus* from the Karnali River. Furthermore, migration
 378 edges in TREEMIX may also explain the mixed inter-drainage assignment seen in STRUCTURE, as
 well as the non-monophyly of Koshi River *richardsonii*-*progastus* in our POMO analysis (figure
 380 7).

Bhutanese *Schizothorax* spp. differed in that no clear delineations were found between
 382 high- and low-elevation clades in *S. progastus*, and with clustering instead reflecting species-
 level assignments (figure 5, 6b). STRUCTURE results suggested mixed assignment of *S. progastus*
 384 and *S. cf. oconnori*, particularly in the Wang Chhu where both *de novo* and transcriptome-guided
 results agreed (figure 6b). TREEMIX results also revealed evidence in multiple sub-basins for
 386 exchange across species boundaries (figure 6c and S1). Of note, mixed assignment was also
 observed between *S. progastus* (Bhutan) with those from Nepal (figure 6c). We could not test
 388 whether this reflects retained ancestral variation (i.e., prior to divergence of Ganges and
 Brahmaputra rivers), or more contemporary mixture. Patterson's *D*-statistic likewise supported

this mixed ancestry, both at the level of conspecifics among drainages and heterospecifics within drainages (table S3).

(c) Co-divergence analysis

Co-divergence analysis rejected co-divergence for all riverine pairwise comparisons for both Nepal and Bhutan (figure 8). One notable exception was the inferred simultaneous divergence of the ‘Lake Rara’ lacustrine radiation (here represented as two comparisons: *S. macrophthalmus* x *S. raraensis*+*S. nepalensis*; and *S. raraensis* x *S. nepalensis*; figure 8a). Here, a model of 4 divergences was selected for Nepal (posterior probability > 0.9; figure 8c), with the relative timing for this radiation exceptionally recent compared to those inferred for within-drainage comparisons. We note, however, that inferred divergence times are likely skewed by introgression (e.g. figure 6c), but that the exact nature of this effect is unclear [101]. Parameter estimates for all analyses post burn-in were >600.

(d) Locus-wise differentiation and outlier analysis

The greatest number of significant SNPs following Bonferroni-correction occurred in outlier analysis involving Koshi and Gandaki *S. richardsonii-progastus* pairs, and from the comparison of *S. progastus* and *S. cf. oconnori* from Bhutan (i.e. $N=531$ and 54, respectively; figure S2a). Of these, the most substantial number of overlapping SNPs was found among Koshi and Gandaki comparisons. Outliers in both cases had higher F_{ST} than did the genome-wide distribution involving *de novo* or transcriptome-guided assemblies. This pattern was not repeated in the same loci for Bhutan (figure S2b), although outlier loci in Bhutan did share a negative relationship of gene diversity (H_E), as compared to *S. richardsonii-progastus* F_{ST} (figure S2c). Outlier F_{ST} for *S.*

richardsonii-progastus was correlated in the Gandaki *versus* Koshi rivers, but with no

discernible relationship with the same measure when compared to Karnali River *S. richardsonii* and Lake Rara species, Bhutanese *S. progastus-oconnori*, nor among Bhutanese populations of only *S. progastus* (figure S3). Relationships among different locus-differentiation statistics otherwise occurred per theoretical expectations (figure S4). Hence only comparisons (above) are presented for F_{ST} . Relating D_{XY} ratios to D_{XO} showed that high values were likely driven by near-zero distances among *S. richardsonii* pairs for a subset of loci (figure S5), in line with evidence from the D -statistics indicating shared inter-drainage ancestry among conspecifics (table S3).

Although ~75% of outlier transcripts could not be assigned candidate protein products, gene ontology (GO) term enrichment for shared outliers revealed a number of related biological processes, including anatomical structure formation (GO: 0048646), up-regulation of phosphoprotein phosphatase activity (GO: 0032516), and up-regulation of G protein coupled receptor signaling (GO: 0045745)(figure S2d). The few outlier transcripts that could be assigned candidate proteins in the overlapping Gandaki-Koshi set were: Aurora Kinase A (*AURKA*), Zinc Finger SWIM Domain Containig Protein 6 (*ZSWIM6*), Adhesion G protein-coupled receptor A3 (*ADGRA3*), and Caspase B (*CASPB*).

(e) Partitioned ancestry and mixture analyses

Loci for which *richardsonii-richardsonii* divergence was greater than *richardsonii-progastus* (i.e., D_{XY} ratio < 1.0; figure 9a) occurred in drainages irrespective of ecomorphological taxon-assignment and corresponded to ~68%. The remaining ~32% yielded groupings consistent with species-level taxonomy, but with migration among species suggested within the Gandaki and

Koshi rivers (figure 9a). Repeating the same analysis across loci binned by F_{ST} yielded a transition from drainage-level at low values (with migration among mid-highland *S. progastus*) towards species-level at high values (with migration within drainages) (figure 9b). This pattern was consistent when loci were binned with F_{ST} estimates derived either from *S. richardsonii*-*progastus* comparisons in the Gandaki or the Koshi, although the transition towards species-level grouping was slightly protracted in the latter (figure 9b).

4. Discussion

We apply genome-wide SNP data and ploidy-aware genotyping to demonstrate that genetic and morphologically distinct *Schizothorax* forms in Himalayan tributaries evolved prior to secondary gene flow, rather than as a parallel emergence of elevational forms. In addition, highland specialists in Nepal converged strongly onto a singular phenotype that was recapitulated in phylogenetic patterns at presumed targets of selection. However, neutral variation showed signs within each basin of homogenization among elevational pairs (*S. richardsonii* and *S. progastus*), suggesting a breakdown of reproductive isolation. In Bhutan, elevational pairs (*S. progastus* and *S. cf. oconnori*) exhibited low-level as mixture, suggesting that elevational pairs possess either greater reproductive fidelity or instead represent an earlier stage of homogenization via secondary gene flow.

(a) Parallel divergence *versus* parallel hybridization in Nepali snowtrout

Genome-wide relationships among Nepali and Bhutanese *Schizothorax* give the appearance of recurrently emerging ecotypes, with repeated colonization of highland habitats from a mid-highland progenitor occurring within each drainage (figure 5, 6). This seemingly fits the

narrative of WGDs as promoting the adaptive potential of schizothoracine fishes, as well as the
 460 broader pattern of convergence among high-elevation specialists [50,62]. Indeed, WGD-derived
 adaptive potential may have played a role in the parallel colonization of highland habitats by *S.*
 462 *richardsonii* in the Ganges tributaries of Nepal, and *S. cf. oconnori* in the Brahmaputra
 tributaries of Bhutan, although we note that the current study lacks geometric morphometric data
 464 for the latter.

In the case of elevational *Schizothorax* pairs in Nepal, we found that dominant ancestries
 466 shifted from grouping at the drainage-level (based on scarcely differentiated/ “neutral” loci)
 (irrespective of eco-morphology) towards the conspecific-level (via strongly
 468 differentiated/selected loci). However, this raises an additional question: How are adaptive
 phenotypes retained despite introgression being reflected within most of the genome? Here, one
 470 potential is that an allopolyploid origin of WGD in *Schizothorax* conflate the signal of
 hybridization. However, a lack of sub-genome divergence was found in *S. oconnori* [110], as
 472 would be expected under allopolyploidy.

It is also possible that polyploidy facilitates a greater degree of genomic exchange when
 474 compared with reproductively isolated diploid species [111]. For example, ploidy has been
 suggested to promoting introgression in diploid-tetraploid crosses of *Arabidopsis*, due to a
 476 circumvention of dosage-mediated postzygotic isolation [112–114]. Increased introgression has
 also been supported among tetraploid-tetraploid crosses (as herein), with an increase in local
 478 recombination rates as one potential mechanism. As ploidy increases, there is a relaxation of
 linkage as a component of purifying selection [115,116]. Similarly, increased dosage may ‘mask’
 480 deleterious loads, especially in young polyploid species [117,118]. Given the young age of WGD
 estimated for *S. oconnori* [110], these predictions implicate a genomic landscape vastly more

porous than might be expected in their diploid counterparts [114]. This may also be an underappreciated mechanism promoting the hypothesized increased adaptability of polyploids to stressful or novel environments [119,120].

(b) Varying rates of hybridization among drainages

In contrast to the substantially permeable genomic landscape seen among *S. richardsonii* and *S. progastus* pairs in Nepal, we saw a much smaller signature of gene flow in Bhutanese elevational pairs (*S. progastus*, *S. cf. oconnori*). In addition to gene flow obscuring species boundaries [92,121], contrasts between Himalayan streams and extreme freshwater habitats in the American Southwest [121–123] also implicate the potential for anthropogenically mediated hybridization. Here, ‘extinction vortices’ may be driven by a coupling of potentially maladaptive hybridization combined with declining population sizes. This provides a backdrop against which climatic expectations of shrinking habitats for vulnerable highland species such as *S. richardsonii* can be contrasted [124].

The potential role of hybridization in demographic trends or extinction risks [125,126] cannot be inferred from our data, nor can the environmental covariates potentially modulating species boundaries be inferred without further work. Given the potential for seasonal migratory behavior in *Schizothorax* [73,127], hydropower dams will disrupt movements, thus inadvertently promoting interspecific contact. Here, we again emphasize parallels with the heavily-modified and regulated rivers of western North America, where water policy and impoundments promote hybridization [122,128], define habitat suitability [129], and alter environmental cues [130]. Though both countries have a high percentage of protected areas, our results suggest that anthropogenically-mediated hybridization represents an additional dimension to consider when

balancing freshwater conservation planning [131,132].

506

(c) High-elevation adaptation

508 Although we found relatively little overlap in putative targets of selection (figure S2a), our
reduced-representation approach only surveyed a small percentage of transcripts, with poor
510 success in establishing functional annotations. Molecular convergence among high-elevation
populations has been observed in disparate human populations [133], and even among humans
512 and their domesticated species [134]. Other studies in *Schizothorax* have shown an array of
candidate genes underscoring adaptations to hypoxic and ultraviolet conditions associated with
514 elevation [135].

Schizothoracine fishes in general have been characterized as species similarly to trout
516 (Salmonidae) adapted to torrential flows, with positioning in rapid currents facilitated by body
shape [136]. A longitudinal survey of Nepali assemblages showed replacement of *S. richardsonii*
518 by *S. progastus* at decreasing elevations [60], suggesting a role for factors such as dissolved
oxygen and flow rates [137]. Turnover and narrow elevational ranges are found in other
520 Himalayan taxa as well [138,139]. Specialized sucker-like adaptations are present in *S.*
richardsonii but absent in *S. progastus* [60], and species richness in *Schizothorax* is greater at
522 mid-elevation [140], seemingly corroborating this relationship *Schizothorax*.

An interesting case of adaptation which we did not explore fully herein is that of the
524 putative species flock in Lake Rara (figure 8a). There, species overlap in geometric
morphometric space (figure 3), yet display marked differences in mouth morphology, gill raker
526 shape, spawning microhabitat choice, and diet (with *S. raraensis* insectivorous, *S. nepalensis*
herbivorous, and *S. macrophthalmus* planktivorous) [53]. We found them genetically

indistinguishable across several analyses, in agreement with previous studies [54,56], though this is unsurprising given their apparent recent and rapid diversification (figure 8) [101].

5. Conclusions

Our results indicate that population genomics can be accomplished in a statistically appropriate manner using non-model polyploid species with relatively minor adjustments to the traditional molecular ecology workflow. However, several limitations were encountered (such as the lack of ploidy-aware methods for outlier detection), signaling a need for further development. Despite these limitations, we were able to disentangle the parallel evolution of *Schizothorax* species in Himalayan tributaries of the Ganges and Brahmaputra rivers, and with recurrent processes implicated at differing timescales: convergent adaptation towards high-elevation environments among major drainages (e.g., Ganges *versus* Brahmaputra), and parallel selection against introgressive homogenization within drainages. With regards to the latter, we found heterogeneous levels of admixture among populations. Finally, we rejected the hypothesis of co-divergence between highland and mid-highland riverine forms, yet found the lacustrine radiation in Lake Rara to be both recent, and rapid.

Ethics. All methods were performed in accordance with relevant guidelines and regulations.

Bhutanese collecting permits were in conjunction with the National Research & Development Centre for Riverine and Lake Fisheries (NRDCR&LF), Ministry of Agriculture & Forests (MoAF), Royal Government of Bhutan. The export of fin clips was authorized through a Material Transfer Agreement (MTA) provided by the National Biodiversity Centre (NBC), with additional approvals provided by the Department of Forests & Park Services (DoFPs) and

Bhutan Agricultural and Food Regulatory Authority (BAFRA). Sampling protocols were approved by the University of Arkansas Institutional Animal Care and Use Committee (UA_IACUC_17064). The study was also carried out in compliance with ARRIVE guidelines (<https://arriveguidelines.org>).

Data and code accessibility. Raw sequence data will be made available via the NCBI Short Read Archive (SRA) pending acceptance, under BioProject PRJNA759907. Assembled datasets and input files are available in the Open Science Framework repository (doi: 10.17605/OSF.IO/TMJFK). Relevant code for this research work are stored in GitHub: github.com/tkchafin/polyrad_scripts and github.com/tkchafin/scripts, and have been archived within the Zenodo repositories doi: 10.5281/zenodo.5393418 and doi: 10.5281/zenodo.5181290.

Authors contributions. All contributed to conceptualization and study design. D.R.E. collected samples in Nepal. S. T., K. W., S. D., P. N., S. N., C. L., and G. P. K. coordinated and supervised field work in Bhutan. S.T., K.W., M.E.D., M.R.D, S. D., P. N., S. N., C. L., G. P. K., and T.K.C. collected samples in Bhutan. B.R. collected and curated geometric morphometric data. M.E.D. and M.R.D. generated molecular data. T.K.C. wrote necessary codes and performed bioinformatic work. B.R. and T.K.C. analyzed data. T.K.C. generated figures and drafted the manuscript. All authors contributed to revising the final product and subsequently approved its submission.

Competing interests. We declare we have no competing interests.

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982 Tables and Figures

984 **Figure 1:** Pattern and process in the formation of apparent ecotypes. Ecotypy is often inferred
 986 via a juxtaposition of discordant spatial, phylogenetic, and phenotypic patterns (left;
 988 differentially adapted forms represented in gold and purple). Evolutionary scenarios (right)
 990 which can generate these patterns include (A) Parallel selection upon standing variation; (B)
 Recurrent de novo adaptation via independent mutation; (C) Hybridization among co-occurring
 ecophenotypes following a common origin; or (D) The singular origin of an adaptive mutation,
 which is then spread via adaptive introgression.

994 **Figure 2:** Sampling locality information for $N=96$ *Schizothorax* spp. selected for ddRAD sequencing. Sites represent major
 995 Himalayan tributaries of the Brahmaputra and Ganges rivers from Nepal and Bhutan (A). Samples for five species (*S.*
 996 *macrophthalmus*, *S. nepalensis*, *S. progastus*, *S. raraensis*, and *S. richardsonii*) were collected from 11 sites in the major drainages of
 997 Nepal (Karnali, Gandaki, and Koshi; B). Major drainages of Bhutan (Wang Chhu, Punatsang Chhu, and Mangde Chhu) were
 998 represented by two species (*S. progastus* and *S. cf. oconnori*) collected from 11 localities (C). *Schizothorax progastus* from both Nepal
 1000 (*D*) and Bhutan (*E*) were collected from relatively lower elevations (~600-1200m), whereas *S. richardsonii* (Nepal; *D*) and *S. cf.*
oconnori (Bhutan; *E*) were generally found >1200m.

1002 **Figure 3:** Evaluation of ploidy from $N=48,350$ ddRAD loci for six *Schizothorax* species. (Left) Distributions of base frequencies
 1004 among raw reads representing bi-allelic SNPs for six *Schizothorax* spp. collected from Nepal [*S. macrophthalmus*, *S. nepalensis*, *S.*
progastus (solid line), *S. raraensis*, and *S. richardsonii*] and Bhutan [*S. cf. oconnori* and *S. progastus* (dashed line)]. (Right)
 1006 Likelihoods from Gaussian Mixture Models of fixed ploidy (e.g., diploid, triploid, tetraploid), divided by the likelihood under a model
 of freely variable base frequencies ($=\log L_{\text{Ploidy}}/\log L_{\text{Free}}$).

Figure 4: Discriminant analysis of principal components (DAPC) of morphological shape variation among five *Schizothorax* species. Results represent $N=528$ individuals of *S. macrophthalmus*, *S. nepalensis*, *S. progastus*, *S. raraensis*, and *S. richardsonii* collected from the Gandaki, Karnali (to include Lake Rara), and Koshi drainages of Nepal, and Procrustes-aligned two-dimensional Cartesian coordinates for 18 anatomical landmarks.

Figure 5: Visualization of genetic variation among six *Schizothorax* species, as assessed across $N=15,547$ statistically diploid SNPs. Visualizations represent the first two discriminant functions from a discriminant analysis of principal components (DAPC) computed across (A) all samples from Bhutan (*S. cf. oconnori* and *S. progastus*) and Nepal (*S. macrophthalmus*, *S. nepalensis*, *S. progastus*, *S. raraensis*, and *S. richardsonii*); (B) Only those samples from Bhutan, additionally partitioned by major drainage encompassing the samples locality (i.e., Wang Chhu, Mangde Chhu, or Punatsang Chhu); and (C) Only individuals samples in Nepal. Nepali samples are partitioned by major drainage (i.e., Gandaki, Koshi, and Karnali), with species from Lake Rara (*S. macrophthalmus*, *S. nepalensis*, and *S. raraensis*) additionally separated.

Figure 6: Phylogenetic and population structure of $N=93$ *Schizothorax* spp. collected in major drainages of Bhutan and Nepal. Results represent (A) an unrooted alignment/assembly free phylogeny inferred from k-mer distances, computed from raw ddRAD reads. Clades are grouped by drainage, and with colored bars denoting tips grouped by taxon (*S. macrophthalmus*, *S. nepalensis*, *S. cf. oconnori*, *S. progastus*, *S. raraensis*, and *S. richardsonii*). (B) Bar plots are provided for population assignments at $K=3$ and $K=7$ for (left) 10,884 transcriptome-aligned SNPs and (right) 35,319 *de novo* assembled SNPs. (C) TREEMIX analyses of population mixtures, with colored arrows representing inferred migration weights.

Figure 7: Population tree generated for $N=93$ *Schizothorax* spp. divided into 13 populations using a polymorphism-aware model applied to 15,543 ddRAD SNPs. Nodal values represent bootstrap support expressed as percentage of 1,000 ultra-fast bootstraps.

Figure 8: Tests of co-divergence among lineage pairs in Nepal and Bhutan. (A) and (C) show posterior probability distributions on divergence times for Nepal and Bhutan, respectively. Nepalese results (A) represent divergence of *Schizothorax richardsonii* (=rich.) and *S. progastus* (=prog.) in the Gandaki and Koshi rivers, *S. richardsonii* from the Karnali River with the ‘Lake Rara’ complex, *S. macrophthalmus* (=macrop.) with *S. raraensis* (=rara.) and *S. nepalensis* (=nepal.). Results for Bhutan depict *S. cf. oconnori* (=ocon.) and *S. progastus* from the Punatsang Chhu (=Puna.), Mangde Chhu, and Wang Chhu rivers. (B) and (D) depict posterior (dark bars) and prior (light bars) probabilities of the numbers of co-divergences.

1050 **Figure 9:** TREEMIX analyses of population mixtures among *S. progastus* and *S. richardsonii* pairs collected from the Gandaki and Koshi rivers of Nepal, based on partitioning different subsets of loci. (A) Loci were grouped by ratios of D_{XY} (computed between
1052 heterospecifics within each drainage) and D_{XO} (computed between conspecific *S. richardsonii*), with those loci having a D_{XY} ratio >
1.0 in both Gandaki and Koshi comparisons (i.e., greater distances among *S. richardsonii* and *S. progastus* within drainage than *S.*
1054 *richardsonii* among drainages) analyzed separately. (B) Loci were also partitioned into four bins defined by per-locus F_{ST} computed in
Gandaki (above; red) and Koshi (below; blue) pairs. Colored arrows indicate inferred migrations within each partitioned analysis, with
1056 the color indicating the migration weight.