Parallelism and Epistasis in Skeletal Evolution Identified through Use of Phylogenomic Mapping Strategies

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Abstract

The identification of genetic mechanisms underlying evolutionary change is critical to our understanding of natural diversity, but is presently limited by the lack of genetic and genomic resources for most species. Here, we present a new comparative genomic approach that can be applied to a broad taxonomic sampling of nonmodel species to investigate the genetic basis of evolutionary change. Using our analysis pipeline, we show that duplication and divergence of *fgfr1a* is correlated with the reduction of scales within fishes of the genus *Phoxinellus*. As a parallel genetic mechanism is observed in scale-reduction within independent lineages of cypriniforms, our finding exposes significant developmental constraint guiding morphological evolution. In addition, we identified fixed variation in *fgf20a* within *Phoxinellus* and demonstrated that combinatorial loss-of-function of *fgfr1a* and *fgf20a* within zebrafish phenocopies the evolved scalation pattern. Together, these findings reveal epistatic interactions between *fgfr1a* and *fgf20a* as a developmental mechanism regulating skeletal variation among fishes.

Key words: **: comparative genomics, nonmodel organisms, parallelism, zebrafish, fgf signaling, epistasis.**

Introduction

Conventional approaches toward identifying the genetic basis for evolutionary change center on genetic linkage analysis to uncover associations between genetic and phenotypic variations (Hoekstra et al. 2006; Shapiro et al. 2006; Rebeiz et al. 2009; Hopkins and Rausher 2011; Pardo-Diaz et al. 2012; Wray 2013). However, linkage-based approaches require distinct populations that exhibit morphological divergence but are still interfertile. Unfortunately, the vast majority of species cannot be intercrossed. These requirements limit the populations that can be used in these approaches, and thus constrain the types of morphological change that can be investigated. Another method used to identify genetic basis of phenotypic differences is by association mapping whereby specific polymorphisms within a population are found to correlate with a particular trait. As this method relies on variation for the phenotype of interest within a population, in cases in which traits are fixed among species this method is no longer applicable. Thus, although the vast majority of phenotypic variation is between species, there are a limited number of cases in which genetic causation can be formally addressed and as such, reliance on these approaches may bias our understanding of evolutionary change to cases of recent and rapid evolutionary radiations.

With the recent explosion of sequencing data and technologies, comparative genomic approaches have become a powerful means to address evolutionary change within natural populations and to perform genetic comparisons between often distantly related species (Wray 2013). These approaches include genome-wide scans for signatures of selection (Vitti et al. 2013), isolation of large-effect mutations (Enard et al. 2009; Andersson et al. 2012; Baldwin et al. 2014), analysis of copy number alterations (Perry et al. 2007), and identification of species-specific noncoding changes (Pennacchio et al. 2006; Prabhakar et al. 2008; Visel et al. 2008; McLean et al. 2011; Wray 2013; Boyd et al. 2015). These approaches are an effective way to identify genetic contributions to a phenotype in species not amenable to genetic linkage or association analysis (Hiller et al. 2012; Wray 2013). In conjunction with functional analysis within model organisms, particular candidate loci underlying morphological variation can be formally tested (Pennacchio et al. 2006; Prabhakar et al. 2008; Enard et al. 2009; McLean et al. 2011; Wray 2013; Baldwin et al. 2014; Boyd et al. 2015). However, comparative genomics is predominantly limited to species with well-annotated genomes and/or extensive transcriptome data (Gayral et al. 2013). Despite the rising numbers in species with available genomic resources, the number and scope of investigations into natural variation remains limited.

To facilitate genomic analysis of a wide range of species and phenotypic diversity, independent of existing genomic resources, we devised an experimental method, Phylomapping, which enables identification of signals of selection and variance associated with the evolution of particular groups. Key in this analysis is the derivation of a shared, "ancestral" exome between species to enable a systematic analysis of genetic variation and patterns of selection. To examine the utility of this

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approach, we analyzed scale-loss within a lineage of cypriniform fishes for which no previous genomic or genetic resources were available. Because we have data on variance across orthologous genes in the genome, the method allowed us to systematically test for changes in gene function and selection to generate hypotheses about how character change evolved. We were able to identify key genes and genetic mechanisms underlying the evolution of scale reduction occurring in this group.

Results

Phylomapping Methodology

We set out to develop an approach to facilitate comparative genomic analysis in species that lack prior genetic resources. As a first step, pooled genomic DNA from populations of individual species is enriched for conserved orthologous protein-coding sequence through cross-hybridization to oligonucleotide "baits" designed against the known exome of a related species or a combination of species (fig. 1*A*-I). The use of genomic DNA through targeted sequence capture enables study of rare samples and the use of vast natural history collections (Mason et al. 2011) while being independent of the tissue type and developmental stage of sampled individuals that occurs with transcriptome data. After massively parallel sequencing, reads from the captured DNA are then grouped into bins of orthologous sequences by BLASTN (Altschul et al. 1990) against the reference exome used to design the capture array (fig. 1*A*-II). These read sets are assembled into exon-sized contigs by CAP3 (Huang 1999) (fig. 1*A*-III). To establish a shared ancestral reference exome, orthologous sequences are paired between species for reconstruction by "prequel" from the PHAST package (Hubisz et al. 2011) (fig. 1*A*-III and -IV). The imputed ancestral sequence is annotated by a BLASTX (Altschul et al. 1990) comparison with the orthologous reference exome. To facilitate recovery of reads flanking each exon that were previously not identified by exon-centered BLASTN, the raw reads are then realigned to this ancestral scaffold by Stampy (Lunter and Goodson 2011). The realignment of the raw reads to this shared scaffold then permits a broad comparative analysis of variation between species (fig. 1*A*-IV).

To assess the limits of our approach, we asked how robust our pipeline was to variation between species and the reference genome used to guide exome reconstruction. Using the zebrafish genome, we artificially introduced variants into coding sequence at defined levels, created in silico reads, and then used the original zebrafish reference genome to scaffold the reconstruction of the variant exomes from the simulated reads. We were able to recover greater than 95% of reads containing even up to 15 single nucleotide polymorphisms (SNPs) per 100 bp of coding sequence (fig. 1*B*). Further, over 85% of reads with as many as 25 SNPs per 100 bp of coding sequence were able to be identified

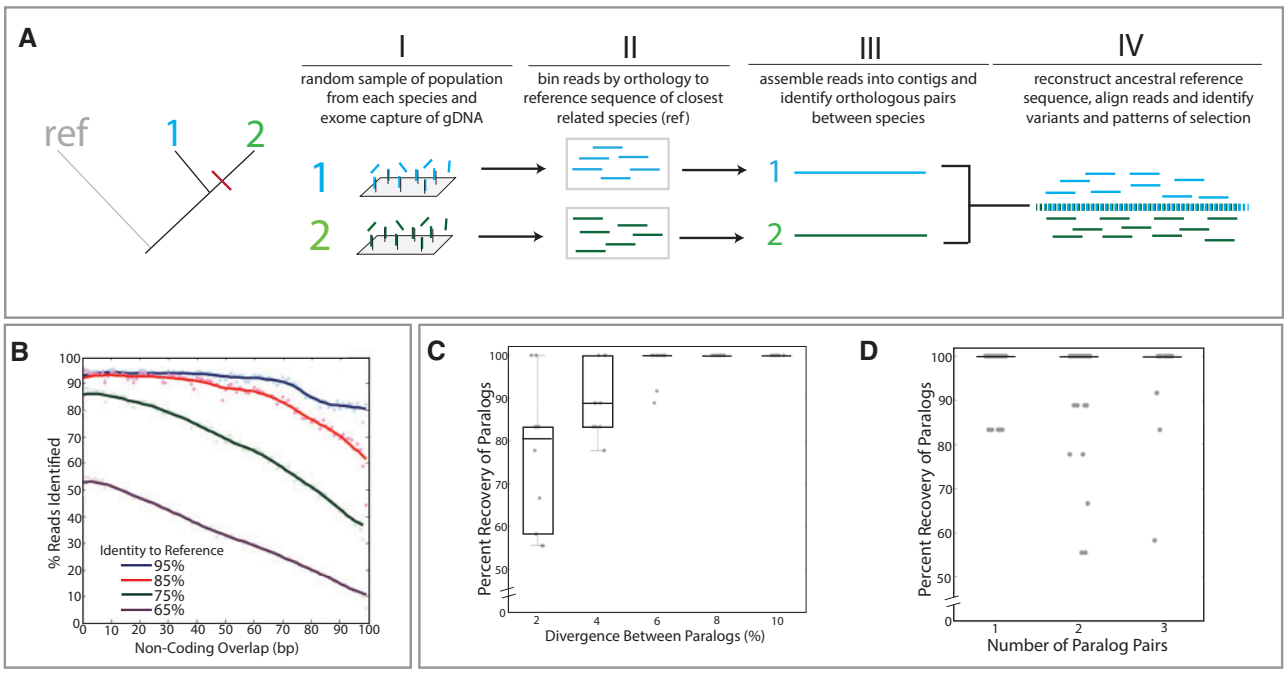

Fig. 1. Phylomapping approach for analysis of character change in species lacking prior genetic resources. (*A*) Overview of approach. In this example, there are two species, "1" and "2," with a common outgroup, "ref," that has existing genetic resources from which to design the targeted capture array (I) and guide identification of reads (II). Reads are binned by orthology and assembled into contigs (III). Pairwise analysis between orthologous contigs enables reconstruction of a shared ancestral sequence that is then used to align and compare sequence variance (IV). (*B*–*D*) Test limits of approach through simulated assembly and annotation of reads from divergent genomes. (*B*) In silico 100-bp reads were generated at varying levels of divergence from the zebrafish reference genome and put into the phylomapping pipeline, utilizing the zebrafish reference genome to scaffold the analysis. Alignment and identification of reads from varying levels of divergence relative to the reference genome showing read recovery with high levels of variation and extending into noncoding sequence flanking the exons. Lines represent kernel regression. Simulated reconstruction of paralogs from mixed read data (supplementary fig. S1, Supplementary Material online) as a function of the divergence between paralogous sequences (*C*) and the number of paralogs present within the mixed read pools (*D*). Each dot represents an individual simulation.

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(fig. 1B). Although the analysis pipeline ignores noncoding sequence for the original identification of the reads, we were able to recover large amounts of noncoding sequence flanking each exon after realigning reads back to the ancestral scaffold (fig. 1B). Thus, this approach has the potential to recover and identify variation from species that lack a close relative with annotated genome resources.

Given variation in gene copy number between genomes, we next asked whether this approach can distinguish paralogs from variant haplotypes. The de novo assembly within this pipeline reliably distinguished paralogs from haplotypes as long as there was greater than 6% variation contained within the coding and flanking noncoding sequence (supplementary fig. S1, Supplementary Material online, and fig. 1C). Further, we found that paralog phasing is robust to multiple copy number (fig. 1D). This enables prediction and parsing of exons with increase in copy number between species and further expands the ability to assess species-specific variation.

Analysis of Skeletal Reduction in the *Phoxinellus* Lineage

As a case study of natural variation, we examined skeletal variation in fishes. Diversity in scale patterning in fishes is a common and defining feature of many species and genera, with over 13 independent cases of scale loss within Cypriniformes alone (Harris 2012). To explore the genetic basis of this natural diversity we selected a clade of minnows, *Phoxinellus*, where loss of scales is a defining, derived trait for the group (Freyhof et al. 2006) (fig. 2A–C). The *Phoxinellus* clade is thought to have diverged from a scaled fish lineage about 2.5 Ma (Freyhof et al. 2006). Fish of the genus *Telestes* are a close outgroup to *Phoxinellus* that have retained the ancestral scaled character state. *Telestes* or *Phoxinellus* species presently lack experimental or genetic tools making study of these species difficult. To enable analysis of the genetic mechanisms underlying the evolution of scale pattern divergence, we applied our Phylomapping pipeline to look at patterns of selection within these groups.

During a field collection expedition in 2006, we collected *Phoxinellus alepidotus* from Sator Lake in Bosnia–Herzegovina and *Telestes ukliva* from locations in the Cetina river in Croatia (fig. 2D). Although *Phoxinellus*, *Telestes* and zebrafish are all cyprinids, they fall within different subfamilies and thus are quite distant relatives. Genomic DNA from 40 individuals of *P. alepidotus* and *T. ukliva* were pooled and hybridized to tiling arrays custom designed to cover the zebrafish exome (Zv9). Even given the evolutionary distance of approximately 50 My between zebrafish (Danioninae) and *Phoxinellus*/*Telestes* (Leuciscinae) lineages (Tang et al. 2010; Near et al. 2012), through use of the Phylomapping approach we were able to obtain genomic sequence from *P. alepidotus* and *T. ukliva* species pools orthologous to 77% of the zebrafish exome with an average depth of approximately 14–17× and 88% coverage of genes associated with development (fig. 2E and supplementary table S1, Supplementary Material online). The majority of zebrafish exons were represented equally by

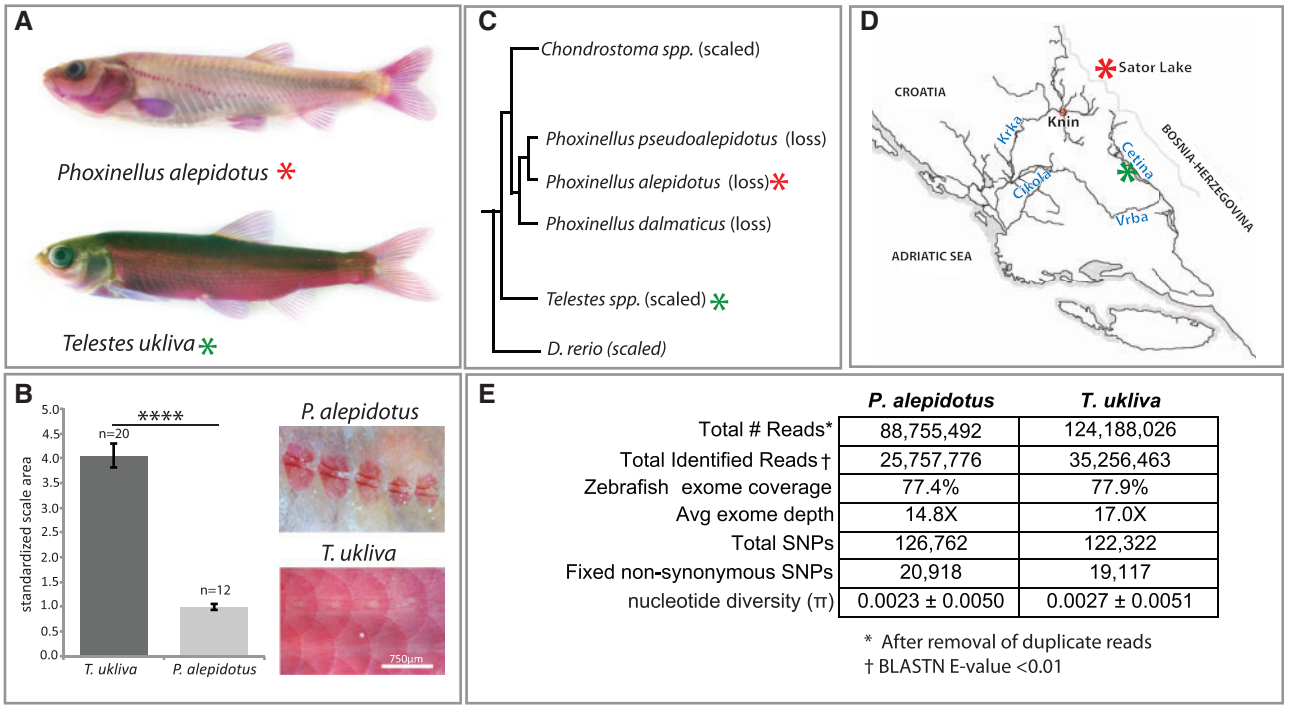

Fig. 2. Comparative genomic analysis of scale reduction in natural populations. (*A*) Alizarin red stain of *Phoxinellus alepidotus* and *Telestes ukliva* highlighting loss of scales within *Phoxinellus*. (*B*) Average scale area relative to fish standard length. Data from three individuals of each species, with a total of $n = 20$ scales in *T. ukliva* and $n = 12$ scales in *P. alepidotus*. ****Two-tailed *t*-test *P* value < 0.0001. Error bars represent ±SEM. (*C*) Phylogenetic relationship between *Phoxinellus* and *Telestes*, with *Danio rerio* as the referenced outgroup used for analysis. Modified from (Perea et al. 2010) with addition of *D. rerio*. (*D*) Collection sites of *P. alepidotus* (red asterisk) and *T. ukliva* (green asterisk) used in this study. (*E*) Exome sequencing statistics from cross-species exome capture using zebrafish-targeted capture array. Single-end 100-bp Illumina HiSeq2000 reads were assembled using the Phylomapping pipeline.

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both *P. alepidotus* and *T. ukliva* reads, with only 1.3% of zebrafish coding bases covered by just one species. Roughly 10% of zebrafish genes had no exons covered by *P. alepidotus* or *T. ukliva* reads (supplementary table S2, Supplementary Material online). These genes are significantly enriched for highly variable gene classes, including zinc finger transcription factors, cadherins, and nucleosome complexes (supplementary table S2, Supplementary Material online). Analysis of SNP variation within *T. ukliva* and *P. alepidotus* permitted analysis of nucleotide diversity within the populations (fig. 2*E*). The observed level of nucleotide diversity ($\pi = 0.0023$ in *P. alepidotus* and $\pi = 0.0027$ in *T. ukliva*) is on the low end of the range seen in other fishes such as zebrafish (Whiteley et al. 2011) and is in-line with that of other *Telestes* species and Balkan freshwater cypriniforms (Stefani et al. 2004; Marešová et al. 2011; Palandačić et al. 2012). This may reflect the small endemic range of *P. alepidotus* and *T. ukliva* and suggests recovery of population diversity through the Phylomapping approach. We identified around 20,000 fixed nonsynonymous changes between *P. alepidotus* and *T. ukliva* (fig. 2*E*). This diversity is consistent with large amounts of coding variation within other species of Cyprinidae, such as zebrafish, where there may be as many as 13,000 fixed nonsynonymous variants even between strains (LaFave et al. 2014).

Analysis of SNP diversity among the assembled exomes of *P. alepidotus* and *T. ukliva* identified a large set of genes with potentially deleterious variants fixed within the populations. To identify the changes associated with the reduction and altered morphology of scales in *P. alepidotus*, we restricted our analysis to genes associated with developmental processes based on gene ontology (fig. 3*A* and supplementary table S1, Supplementary Material online). As a measure of selection and function of particular variants, we looked for the accumulation of mutations beyond a neutral rate of drift likelihood ratio test (LRT) (LRT; Pollard et al. 2010) along with functional effect prediction of fixed nucleotide variants (SIFT; Kumar et al. 2009). Out of 1,022 development-associated genes identified in *P. alepidotus*, 248 contained unique, fixed SNPs predicted to be deleterious (SIFT < 0.05), 215 genes were predicted to be under selection or drift (LRT, q value < 0.05), and 70 genes had both signatures (fig. 3*A* and supplementary table S1, Supplementary Material online). Within these gene classes, several candidates of the Fgf and Eda signaling pathways were present (fig. 3*B*)—both signaling pathways are integrally associated with development of integumentary appendages such as scales (Kere et al. 1996; Headon and Overbeek 1999; Kondo et al. 2001; Colosimo et al. 2005; Harris et al. 2008; Rohner et al. 2009). Although Eda and Edar in particular are commonly associated with variation in scale number (Kondo et al. 2001; Harris et al. 2008), we found no evidence of selection on either of these loci in *P. alepidotus* (fig. 3*B* and supplementary table S1, Supplementary Material online).

Through our analysis, we detected a low level of duplication within both *T. ukliva* and *P. alepidotus* lineages (fig. 3*C* and supplementary table S3, Supplementary Material online). Of the 70 genes showing signatures of selection and predicted deleterious mutations, only four showed evidence of duplication (supplementary table S3, Supplementary Material online). Interestingly, one of these genes showing a shared duplication within the common ancestor of *Phoxinellus* and *Telestes* was *fgfr1a* (fig. 3*C* and supplementary table S3, Supplementary Material online). Reverse transcription polymerase chain reaction verified the presence of two independent paralogs of *fgfr1a* and indicated that both were expressed in skin (supplementary fig. S2, Supplementary Material online). Analysis of exon variation specifically within *P. alepidotus* indicated several fixed nonsynonymous changes in one *fgfr1a* paralog, *fgfr1ab*. The identified variants are predicted to be detrimental as they alter highly conserved residues within a hydrophobic binding interface between Fgf receptors and their ligands (Plotnikov et al. 2000) (fig. 3*D* and *E*). In this context, the P158S variant alters a specific hydrophobic contact (P169S; Plotnikov et al. 2000), suggesting a critical role of this change in regulating Fgfr1ab interaction with its ligands. These mutations are fixed and derived within the *Phoxinellus* genus, as they were not identified within *T. ukliva* or other closely related species of *Telestes*, *T. metohiensis* and *T. dabar* (Bogutskaya et al. 2012) (fig. 3*D*). Interestingly, identification of full-length *fgfr1ab* transcripts from *P. alepidotus* showed the presence of a truncated mature message with loss of the transmembrane and kinase domains (fig. 3*E* and supplementary fig. S2, Supplementary Material online). A splice acceptor mutation, consistent with this truncated mRNA, was observed in *P. alepidotus* but not *T. ukliva* sequencing reads. However, variance at this site within the *P. alepidotus* population suggests the *fgfr1ab* truncation is secondary to fixation of the binding interface mutations. A gene tree was constructed from these transcripts and the exome sequencing data (fig. 3*F*), revealing an extended branch length of *P. alepidotus fgfr1ab*, further supporting increased divergence of this paralog within this clade.

Identification of Key Modifiers of Skeletal Evolution

We extended our analysis to look for further clues to genetic regulators of scale evolution in this group. We identified a fixed, predicted function-altering, nonsynonymous mutation in the *fgfr1a* ligand *fgf20a* that is unique to *P. alepidotus* (fig. 3*B* and *D*). In mice, FGF20 is specifically upregulated downstream of EDA signaling (Lefebvre et al. 2012) and is sufficient to rescue phenotypes due to Eda deficiency during tooth development (Häärä et al. 2012). Further, Fgf20 is required for feather and scale development in chickens (Wells et al. 2012). The G80R SNP in *fgf20a* is fixed within the *Phoxinellus* genus and is not found within three different *Telestes* species (fig. 3*D*) suggesting that these mutations are unique to *Phoxinellus* and support a role of this ligand–receptor interaction in regulating scale patterning. However, no previous role was known for *fgf20a* in scalation as zebrafish mutants defective for *fgf20a* function do not have an apparent scale phenotype (Whitehead et al. 2005).

We asked whether the function of *fgfr1a* and *fgf20a* combine to affect the formation and phenotypic variation of scales. In zebrafish, *fgf20a* is expressed in the developing

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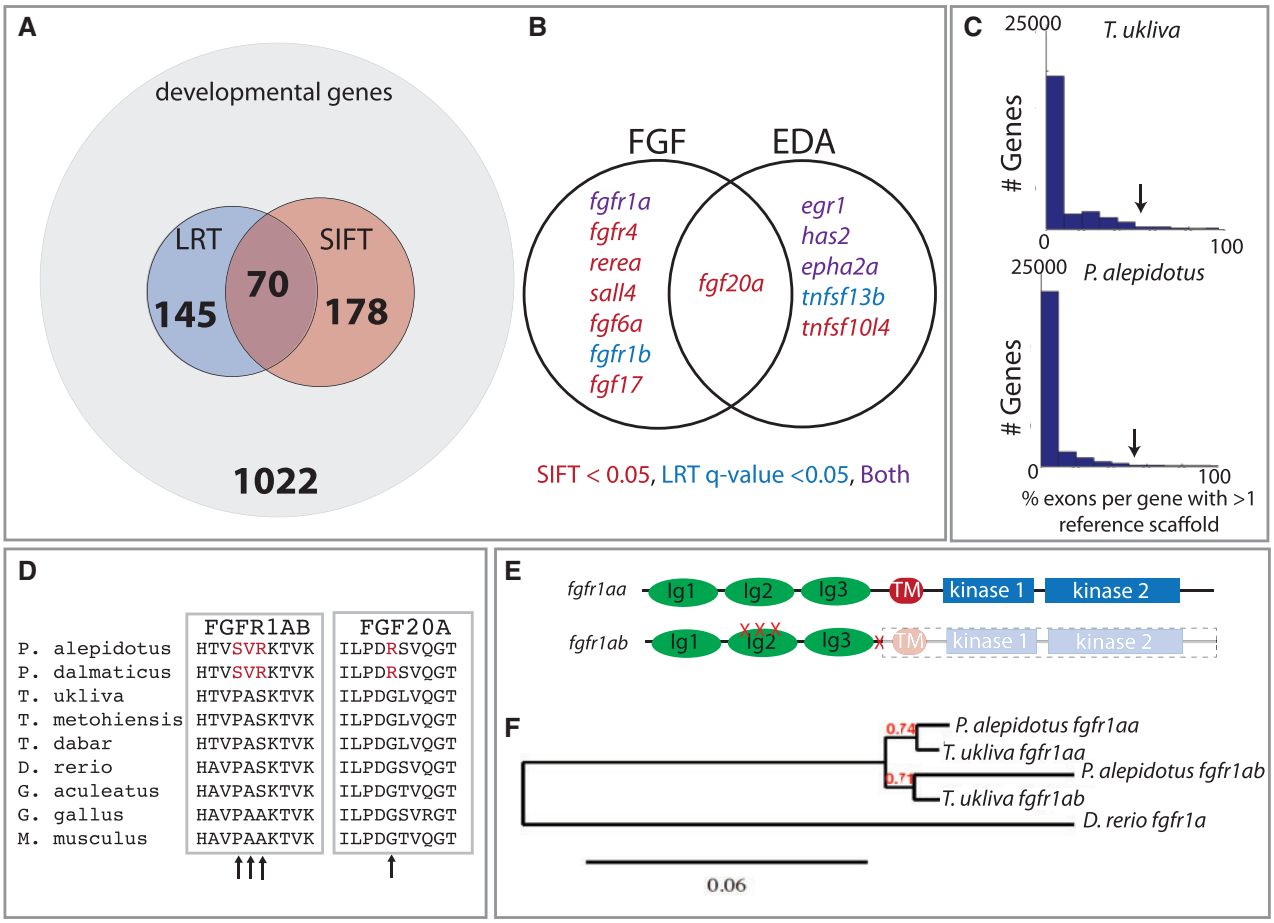

Fig. 3. Selection on an FGF ligand-receptor pair within *Phoxinellus* underlying scale reduction. (*A*) Analysis of a subset of developmental genes as defined by select gene ontology terms (supplementary table S1, Supplementary Material online) with exons showing signatures of nonsense or deleterious SNPs (SIFT) and accelerated sequence evolution relative to neutral drift (LRT) (supplementary table S1, Supplementary Material online). (*B*) Genes within the EDA and FGF signaling pathways showing either LRT q value < 0.05 or SIFT < 0.05 (supplementary table S1, Supplementary Material online). (*C*) Histogram of the percentage of exons per gene showing a signature of duplication (supplementary table S3, Supplementary Material online); arrow indicates location of *fgfr1a*. (*D*) Multiple protein alignment of Fgfr1ab and Fgf20a. Arrows indicate amino acid changes within the *Phoxinellus* genus. (*E*) Location of *Phoxinellus fgfr1ab* nonsynonymous mutations and premature truncation mapped onto Fgfr1a protein (supplementary fig. S2, Supplementary Material online). (*F*) Reconstructed gene tree from cDNA of *fgfr1aa* and *fgfr1ab* transcripts isolated from *Phoxinellus alepidotus* skin and *Telestes ukliva* exome sequencing data.

scale placodes and in the growing edges of scales in a domain overlapping with *fgfr1a* (fig. 4*A*). Zebrafish heterozygous for *fgf20a/dob*, or *fgfr1a/spd* loss-of-function mutations have normal scale numbers and morphology. However, a combined reduction in both ligand and receptor is sufficient to affect scale development, reducing the number of scales on the flank of each fish and limiting scale shape (fig. 4*B*–*D*). Although loss of *fgfr1a* alone leads to abnormally large, albeit fewer scales (fig. 4*C* and D), reduction of Fgf20a function in this context leads to scales that more closely resemble the small scale phenotype seen in *P. alepidotus* (fig. 2*A* and *B*). Thus, using a genomic approach centered on comparison between lineages with fixed morphological changes, we have identified a key role for Fgfr1ab–Fgf20a signaling in regulating the number and size of scales in zebrafish that reflects the phenotype observed in *Phoxinellus* (fig. 4*E*).

Identification of Global Patterns of Selection within *P. alepidotus*

In addition to *Phoxinellus*, unrelated fish have independently lost their scales within the Adriatic drainage basin (Delic et al. 2005; Bogutskaya et al. 2012), suggesting a selective benefit for scale reduction within this environment. Scales are thought to protect against predators and, as a calcified component of the fish skeletal system, function in regulating calcium and phosphate homeostasis. However, the selective advantage for scale reduction in these fishes was unclear: The Krka drainage basin derives from limestone beds, potentially limiting the need for additional calcium storage by scales. Further, in Sator lake, where an unrelated and scale-reduced fish is found, other species of fish that could serve as predators were not identified (Delic et al. 2005).

To explore the capability of our exome-wide data set to build hypotheses about selective causes for trait variance, we looked for accumulation of mutations beyond neutral drift by

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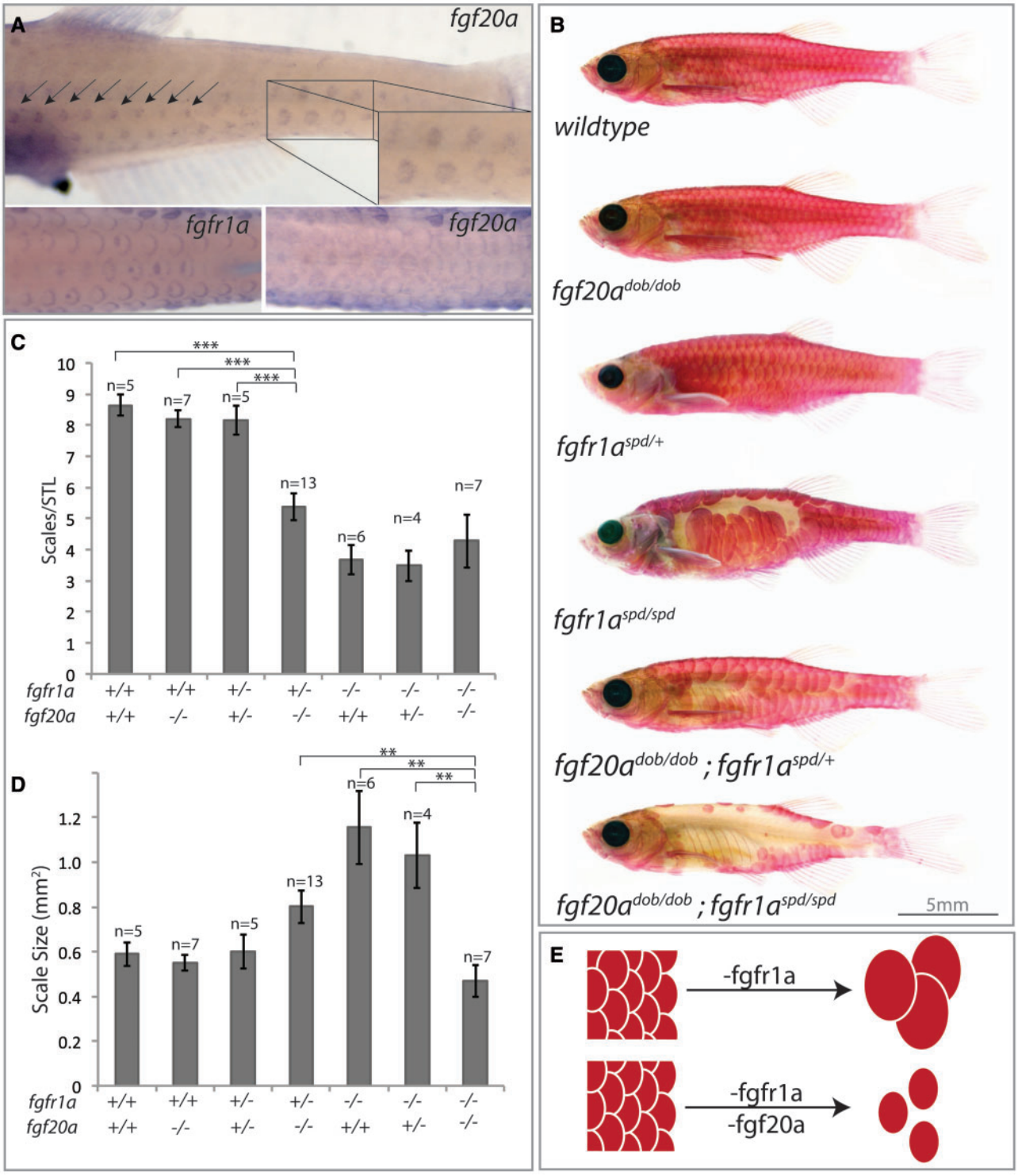

Fig. 4. Epistatic interaction between Fgf20a and Fgfr1a regulates scale development and is sufficient to phenocopy *Phoxinellus* scale reduced phenotype. (*A*) Expression of *fgfr1a* and *fgf20a* by whole-mount in situ hybridization in 30-day-old zebrafish. (*B*) Representative alizarin red-stained zebrafish with loss-of-function mutations in *fgf20a* (*dob*) and/or *fgfr1a* (*spd*). (*C*) Average number of scales on lateral flank normalized to the standard length of each fish. ***P value < 0.001. (*D*) Average area of scales (mm^2) in different genetic backgrounds. **P value < 0.01. Error bars represent ±SEM. (*E*) Model of epistatic interaction between *fgfr1a* and *fgf20a* regulating scale number and size.

LRT (q value < 0.05) within gene ontology groups between *P. alepidotus* and *T. ukliva* (fig. 5*A* and supplementary table S4, Supplementary Material online). In support of a role for calcium in fish scale variation, we identified a *P. alepidotus*-specific enrichment for selection on biological processes related to calcium homeostasis (fig. 5*B* and supplementary table S5, Supplementary Material online). Unexpectedly, we also observed a *P. alepidotus*-specific enrichment for selection on gene classes associated with neurobiology and behavior (fig. 5*A* and *B*; supplementary table S5, Supplementary

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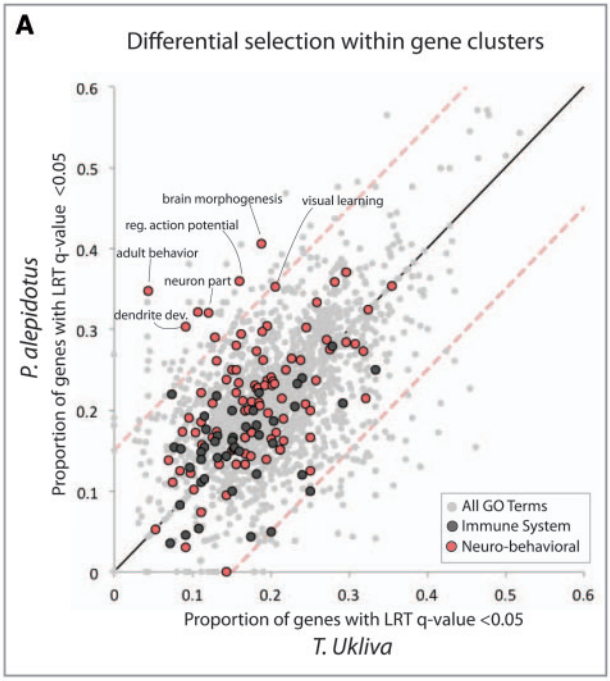

B

	total # genes	P. alepidotus # genes LRT (<0.05)	P. alepidotus p value	T. ukliva # genes LRT (<0.05)	T. ukliva p value
neuro/behavioral	2328	299	0.0137	241	0.6723
calcium homeostasis	384	59	0.0070	44	0.2722
UV response	185	25	0.1501	20	0.4157
immune system	1105	116	0.8056	128	0.1500
all genes	21967	3997		3842	

Fig. 5. Coordinated changes in *Phoxinellus alepidotus* point to multifactorial role of Fgfr1 signaling in trait evolution. (*A*) Proportion of genes within individual GO-terms with LRT q value < 0.05. Each dot represents a GO-term with a minimum of 20 genes. Mean represented by black line. Red-dashed lines represent ± 2 SD from the mean. (*B*) Enrichment for genes showing signature of accelerated sequence evolution relative to neutral drift (LRT q value < 0.05) within aggregate gene ontologies (supplementary table S4, Supplementary Material online).

Material online). Although it is unknown what behavioral traits are associated with *P. alepidotus*, these patterns provide evidence for broad changes in addition to scale reduction during evolution of *P. alepidotus*.

Discussion

Here, we outline an analytic and experimental approach to investigate the genetic causes of evolution in species that do not have existing genetic resources or lend themselves to classical linkage analysis. We find that this method is robust to sequence variation and can be efficiently used to generate a platform for comparative genomic analysis even between species that are evolutionarily distantly related to each other. As zebrafish and *Telestes*/*Phoxinellus* share a common ancestor at least 50 Ma, there is a large evolutionary window in which this method can be applied. With the current coverage of annotated reference genomes and transcriptomes growing at a rapid rate, this approach should enable comparative analysis within a large number of species, from fish and mammals to plants and invertebrate groups.

Because the Phylomapping approach centers on conservation of coding regions to identify and compare genomic sequence between distantly related species, it is not powered to directly identify *cis*-regulatory mutations. However, signatures of selection within exons may reflect a hitchhiking effect of changes in linked noncoding sequence. Further, coding sequence has been used to estimate other indicators of regulatory change, such as codon bias (Chamary et al. 2006; Cutter et al. 2006), translation efficiency (Hudson et al. 2011, 2014), and pathway-level selection (Kosiol et al. 2008) that could serve as a proxy to detect signatures of regulatory evolution. Thus, genome-wide detection of regulatory variation may be indirectly detected within exome data assuming variation is sufficient within the sample populations. Incorporation of known or conserved noncoding elements in the DNA capture step may enable direct identification of putative enhancer and promoter regions modified during evolution.

Using our Phylomapping approach, we detail the role of Fgf signaling in scale reduction through duplication and divergence of *fgfr1a* within *P. alepidotus*. We have previously shown that independent duplication and divergence of *fgfr1a* is the cause of scale reduction in a domesticated variant of the common carp, *Cyprinus carpio* (Rohner et al. 2009). This suggests that there is a common mechanism underlying both transitions in morphology. Unlike the domesticated carp, where remaining scales are larger in size (Rohner et al. 2009), the scales in *P. alepidotus* remain small. Our finding of the combined effect of *fgfr1a* and *fgf20a* in regulating scale size in zebrafish suggests that these two genes are associated with the scalation phenotype seen in *P. alepidotus*. Although we previously have showed linkage of *fgfr1a* to scale loss in carp (Rohner et al. 2009), strong selection from domestication is likely not representative of the types of changes that would occur during natural selection. Our finding of a parallel processes by which scales are reduced in both natural and artificial selections suggests that there is significant constraint on the genetic changes that can cause viable variation in scalation within cyprinids. Given the essential roles for *fgfr1a* during development, gene duplication and subfunctionalization of the paralogs would release constraint on variation. Many studies have pointed to the important role of *cis*-regulatory regulation in evolution of morphology (Carroll 2008; Wittkopp and Kalay 2012). Although it is clear change in the regulation of genes during development is important mediator of evolution, gene duplication and functional divergence of gene function is quite common in most eukaryotic species and represents an important mechanism for evolutionary change that can encompass both regulatory and coding variance (Ohno 1970; Force et al. 1999; Hoekstra and Coyne 2007).

Analysis of synapomorphy, or shared derived characters, is a key element in understanding the potential causes of evolutionary transitions. As morphology can be frequently shaped to the varied life-history strategies of a species, it is tempting to ascribe selection directly on these physical adaptations for their behavioral and physiological advantages. However, it is likely that many species are "making fruitful use of available parts" (Gould and Lewontin 1979) and

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particular morphological states or changes do not necessarily impart direct selective advantage but rather can become fixed by alternate means. Genetic correlation of traits, in which traits are physically linked in the genome or are linked through the use of shared genetic mechanisms, are cases that reveal bias in phenotypes that can arise though selection. Such correlative evolution can lead to aberrant adaptive hypotheses, as putative adaptations may be simply linked to selection on other diverse traits under strong selection and thus are not independent entities.

Adaptive hypotheses for loss of scales and armor in fish have been many, ranging from calcium conservation in calcium poor environments (Giles 1983; Spence et al. 2013; Smith et al. 2014) to changes in buoyancy (Myhre and Klepaker 2009) and increased motility without armor (Bergstrom 2002). Most arguments involve relaxation of selective pressures by predation (Bell et al. 1993; Marchinko 2009) with growth advantages through the reduced metabolic demands of developing and maintaining scales across the body (Marchinko and Schluter 2007; Barrett et al. 2008). But to date support for these hypotheses has been inconsistent. Loss of *fgfr1a* in the zebrafish leads to increased boldness and aggression behavior (Norton et al. 2011), suggesting functional evidence for pleiotropy between the genes involved in scale development and behavior. Further, mirror carp that have lost a duplicated paralog of *fgfr1a* during artificial selection for scale-reduction also display an increase in boldness and are more easily captured than their scaled variety (Klefoth et al. 2012, 2013). As boldness can lead to increased fitness through enhanced foraging and ability to find mates, the behavioral aspects of loss of Fgfr1a function could provide a strong selective pressure for correlative reduction of scales within a permissive environment. *Phoxinellus alepidotus* from the field were easily collected with small aquarium nets (Delic et al. 2005; M.P.H. personal observation) in contrast to *Telestes*, suggesting potential parallels in these fish. However, detailed behavioral studies are needed to identify behavioral changes associated with scale reduction in these species to directly address the possibility of pleiotropy driving correlated changes in behavior and morphology.

Here, we detail an analytic and experimental approach to investigate the genetic causes of evolution in species that do not lend themselves to classical linkage analysis. We detail the role of Fgf signaling in scale development through duplication and divergence of *fgfr1a* and epistasis with Fgf20a. In addition to scale reduction, this data set provides multiple entry points in understanding changes in morphology, physiology, and behavior as a consequence of adaptation and speciation. Thus, this comparative genomic approach should enable broad expansion of genetic information to new species and improve our ability to understand natural diversity.

Materials and Methods

Sample Collection and Identification

Samples of *Phoxinellus alepidotus* and *Telestes ukliva* were collected at described localities during a field expedition in 2006. Communication with local authorities was made prior to collection with permission. Fish were caught through use of minnow-traps baited with bread, or simply by use of a small dip net. The majority of fish captured were measured, fin clipped, and released. Ten *P. alepidotus* and four *T. ukliva* were anesthetized in the field and preserved for analysis of morphology. Genomic DNA and trizol fixed tissue from *P. Dalmaticus* was kindly provided by Dr Joerg Freyhof.

The identity of *P. alepidotus*, *P. dalmaticus*, and *T. ukliva* were determined by meristic characters and were verified through Sanger sequencing of a portion of cytochrome *B*, with BLASTN identification against the NCBI nucleotide database. Primers for cytochrome *b* sequencing from Freyhof et al. (2006) (F′: TGACTTGAARAACCAYCGTTG, R′: GGCAAATAG GAARTATCATTC).

Zebrafish Husbandry and Mutants

Zebrafish wild-type and mutant lines used were housed and maintained in accordance with institutional IUCAC protocols. Mutant lines used are *spiegeldanio* (*spd*; *fgfr1a*^{t3R705H}) (Rohner et al. 2009), *albino*, and *devoid of blastema* (*dob*) (Whitehead et al. 2005). *dob* were kindly provided by Dr Kenneth Poss.

Genotyping

Fish were anesthetized with tricaine and the tail or pectoral fins were clipped with standard surgical scissors. *fgf20a*^{dob} and *fgfr1a*^{spd} fish were genotyped by Sanger sequencing (*dob* primers-F′: TGCCTCTCTTAGGAAAAGCTG, R′:CATCTCTGGAC GTCCCATCT; *spd* primers-F′: TGATGACCTCAGCTGGTC TTT, R′:AGTCTTACAGCTCATGTGTGCAT).

RNA Isolation and cDNA Preparation

Tissue for RNA extraction was collected in the field and stored in Trizol until RNA was extracted. RNA was transcribed to cDNA using Cloned AMV First-Strand Synthesis Kit (Invitrogen) and SuperScript III Reverse Transcription Kit (Invitrogen) using oligo dT primers.

Reconstruction of *fgfr1aa* and *fgfr1ab* Transcripts

Full-length *fgfr1aa* transcripts were isolated from *P. alepidotus* skin with the following primers: 5′-AGTGCGGATGTCCTAGC AGT-3′ and 5′-AAAGCGTGTTCTCCGTGAGT-3′.

For reconstruction of *fgfr1ab*, 3′-Rapid Amplification of cDNA Ends (RACE) was performed with SMARTer 5′/3′ RACE kit (Clontech). A *fgfr1ab*-specific 3′-RACE touchdown primer was designed from the exon under selection in *P. alepidotus*: 5′-ATAAGATGGAGAAGAAGCTCCACACGGTTT CCGT-3′. Nested primers for *fgfr1ab*: UPM-short (Clontech), 5′-CGTAGTCGAGTGTCCACCACAT-3′.

5′-Sequence of *fgfr1ab* was reconstructed with a universal *fgfr1a* exon 1 reverse primer (5′-ACCATGCTGCTGCTGATCT-3′) and paralog-specific *fgfr1a* primer designed from the identified exon under selection in *P. alepidotus fgfr1ab* (*fgfr1aa* : 5′-CTGGTCTCTCTTAAACTCTTTGCCG-3′, *fgfr1ab*: 5′-CTGGT CTCTCTTAAACTCTTTGCCA-3′).

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Gene Tree Construction

Gene trees were constructed from using phylogeny.fr (Dereeper et al. 2008). Briefly, sequences were aligned by MUSCLE (Edgar 2004) and curated by Gblocks (Castresana 2000) before phylogenetic construction by PhyML (aLRT) (Guindon and Gascuel 2003). Tree rendering was performed with TreeDyn (Chevenet et al. 2006). Due to truncation of *P. alepidotus fgfr1ab*, only the first seven exons of *fgfr1a* were used to construct the gene tree.

Whole-Mount In Situ Hybridization

Whole-mount in situ hybridization was performed according to Harris et al. (2008) on 30-day-old *albino* zebrafish with a standard length of 10 mm. Primers for probe against *fgf20a*; f: GACCTGACGCATCTCAAAGG, r: GGGTGGTTTTGAGTTTGAGG. Primers for probe against *fgfr1a*; f: GCTTTGCTCAGGGACTCAAC, r: GCAGTCCATCAGGTCCGTAT.

Skeletal Stain and Morphometrics

Skeletal staining was performed using Alizarin Red in 1% KOH overnight and then cleared in 1% KOH (50 mg Alizarin Red per liter of 1% KOH). Scale area was measured using ImageJ. The scale area of five randomly selected scales was measured and normalized to the standard length of each fish, defined as the distance from the lower jaw to the end of the caudal peduncle.

Targeted Sequence Capture and Illumina Sequencing

Genomic DNA was extracted with the Qiagen DNAeasy Blood & Tissue DNA extraction kit from fin clips of 40 individuals from population samples of *P. alepidotus* and *T. ukliva*. Species-specific pools of 3–5 μg DNA were sheared according to manufacturer's protocol (Covaris) to an average size of 200 bp, as verified on a 4% agarose gel. DNA libraries were blunt-ended, 5′ phosphorylated, A-tailed, and adapter ligated as previously described (Bowen et al. 2011). Phusion High-Fidelity DNA Polymerase (NEB) was used to amplify pre- and postcapture libraries.

To enrich DNA libraries for coding sequence, a custom Agilent Technologies 1 M SureSelect DNA capture array containing 974,016 probes covering all 41,131,682 bp of the zebrafish known protein-coding exome (Zv9) was designed to minimize overlap of bait oligos with noncoding sequence. The 60 mer oligos were tiled on average every 40 bases, with 20 bp of overlap between probes. Cross-species hybridization was performed at 60 °C to allow for potential mismatches between the zebrafish bait and the cross-species libraries. Postcapture libraries were amplified as previously described (Bowen et al. 2011).

As longer reads have a higher probability of identification by BLASTN, we performed 100-bp single-end Illumina HiSeq 2000 sequencing, with one lane each for *P. alepidotus* and *T. ukliva*. Leveraging the sequence information from the first run, a second custom capture array was designed to reflect the common ancestor of *Phoxinellus* and *Telestes*. Libraries of the same 40 individuals were constructed as previously described, only this time with 6-bp barcoded adapters. Exome capture was performed as above, and the DNA from *P. alepidotus* and *T. ukliva* pooled into one lane of Illumina HiSeq 2500 for 100-bp single-end sequencing.

Adapter sequence for *P. alepidotus*:

5′-/5Phos/GCCTAAAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG-3′

5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTAGGC*T-3′.

Adapter sequence for *T. ukliva*:

5′-/5Phos/TGGTCAAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAG-3′

5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGACCA*T-3′.

Read Processing

Raw reads from sequencing were end quality trimmed (-Q 33 -t 33) and low-quality bases (-Q 33) were masked using the FASTX package (http://hannonlab.cshl.edu/fastx_toolkit, last accessed October 7, 2015). Identical reads were compressed using fastx_collapser (-Q 33).

Phylomapping Pipeline

Overview

Using the annotated reference sequence of a related species as bait, we perform targeted sequence capture to enrich DNA libraries for conserved, orthologous protein-coding genes in another species. After next-generation sequencing, reads are identified by BLASTN (*E*-value cutoff < 0.01, -outfmt 6; Altschul et al. 1990) and assembled into contigs by CAP3 (-o 25 -p 90, Huang 1999). Orthologous contigs are then identified between species through centroid clustering (k-means, SciPy) and are paired for the reconstruction of a shared ancestral sequence using prequel from the PHAST package (--subst-mod UNREST, --no-probs, --features; Hubisz et al. 2011). The ancestral sequence is annotated by BLASTX (*E* value < 0.001, -outfmt 6; Altschul et al. 1990) and used as a common reference scaffold for realignment of the original reads by Stampy (--substitutionrate = 0.09; Lunter and Goodson 2011). This shared genome serves as a scaffold for direct comparison of sequence variation within and between related species. Reads with greater than five mismatches to the ancestral sequence were removed as likely misalignments. See supplementary text, Supplementary Material online, for details.

Population Summary Statistics

Nucleotide diversity (π) was calculated and averaged from all coding sequence data using Popoolation (Kofler et al. 2011), with "--pool-size 80" and "--gtf" options.

Analysis of Variants

SAMtools (Li et al. 2009) was used to construct a shared mpileup file for *P. alepidotus* and *T. ukliva*. Variants were called from mpileup files using a custom python script. A minimum allelic depth of 3 was required for polymorphism identification. Heterozygous variants were called with an allele frequency greater than 25%, with homozygous variants

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having greater than 75% allele frequency. To increase confidence in identified variants for comparison between species, only variants with a read depth of at least three reads in each species were considered. Runs of greater than four consecutive SNPs between species were ignored as possible artifacts of the alignment and/or ancestral reference assembly. Variants were annotated using SnpEff (Cingolani et al. 2012). Fixed, nonsense and missense changes unique to *P. alepidotus* and *T. ukliva* were then isolated from the SnpEff file using custom python scripts.

PhyloP from the PHAST package (http://compgen.bscb.cornell.edu/phast/background.php) was used to identify exons under positive selection (Pollard et al. 2010). The consensus reference sequence for each species was reconstructed using the ancestral reference sequence as a template, and by amending bases in the imputed ancestral reference sequence to reflect fixed variants in a particular species as identified from the mpileup file. Neutral tree models were constructed using phyloFit (--tree "((Telestes,Phox), Zebrafish)") with "--features" to restrict analysis to coding sequence (Siepel and Haussler 2004). MAFFT (Katoh et al. 2005) was used to align sequences of *P. alepidotus*, *T. ukliva*, and *D. rerio*. Tree models were constructed for each of 1,000 randomly selected exons, and the values averaged to generate a neutral tree-model. PhyloP (Pollard et al. 2010) was then run with --subtree for both *Phoxinellus* and *Telestes*, with --method LRT, --mode CONACC, and --features options using the BLASTX-generated GTF file. For multiple hypothesis testing, the *P* values were converted to false discovery rate (FDR) *q* values according to Storey (2002).

Statistical Analysis of Gene Ontology Enrichment

To identify trends of sequence evolution within *P. alepidotus* and *T. ukliva* (fig. 5*B*), genes were pooled from a custom grouping of related gene ontologies (supplementary table S5, Supplementary Material online). Statistical significance for enrichment of genes under selection within these broad gene ontology groupings was analyzed by bootstrap resampling of 20,000 permutations against the entire gene set within each species.

To identify individual gene ontology terms for which there is an increase in the number of genes under selection specifically in *P. alepidotus* (supplementary table S5, Supplementary Material online), bootstrap resampling was performed with 15,000 permutations against a null distribution based on the number of genes with q value < 0.05 within the same gene ontology category in *T. ukliva*. For multiple hypothesis testing, the exact *P* values were converted to FDR *q* values according to Storey (2002) using custom python scripts.

The imputed reference exome for *T. ukliva* and *P. alepidotus* and python scripts with phylomapping protocol used for the analysis are provided at www.fishyskeleton.com.

Supplementary Material

Supplementary text, figures S1–S3, and tables S1–S6 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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