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Targeted mutagenesis of aryl hydrocarbon receptor 2a and 2b genes in Atlantic killifish (*Fundulus heteroclitus*)

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Abstract

Understanding molecular mechanisms of toxicity is facilitated by experimental manipulations, such as disruption of function by gene targeting, that are especially challenging in non-standard model species with limited genomic resources. While loss-of-function approaches have included gene knock-down using morpholino-modified oligonucleotides and random mutagenesis using mutagens or retroviruses, more recent approaches include targeted mutagenesis using zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 technology. These latter methods provide more accessible opportunities to explore gene function in non-traditional model species. To facilitate evaluations of toxic mechanisms for important categories of aryl hydrocarbon pollutants, whose actions are known to be receptor mediated, we used ZFN and CRISPR-Cas9 approaches to generate aryl hydrocarbon receptor 2a (AHR2a) and AHR2b gene mutations in Atlantic killifish (*Fundulus heteroclitus*) embryos. This killifish is a particularly valuable non-traditional model for this study, with multiple paralogs of AHR whose functions are not well characterized. In addition, some populations of this species have evolved resistance to toxicants such as halogenated aromatic hydrocarbons. AHR-null killifish will be valuable for characterizing the role of the individual AHR paralogs in evolved resistance, as well as in normal development. We first used five-finger ZFNs targeting exons 1 and 3 of AHR2a. Subsequently, CRISPR-Cas9 guide RNAs were designed to target regions in exon 2 and 3 of AHR2a and AHR2b. We successfully induced frameshift mutations in AHR2a exon 3 with ZFN and CRISPR-Cas9 guide RNAs, with mutation frequencies of 10% and 16%, respectively. In AHR2b, mutations were induced using CRISPR-Cas9 guide RNAs targeting sites in both exon 2 (17%) and exon 3 (63%). We screened AHR2b

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exon 2 CRISPR-Cas9-injected embryos for off-target effects in AHR paralogs. No mutations were observed in closely related AHR genes (AHR1a, AHR1b, AHR2a, AHRR) in the CRISPR-Cas9-injected embryos. Overall, our results demonstrate that targeted genome-editing methods are efficient in inducing mutations at specific loci in embryos of a non-traditional model species, without detectable off-target effects in paralogous genes.

Keywords

non-model organisms; gene knock-outs; mummichog; adaptation; zinc finger nucleases; CRISPR-Cas9

1. INTRODUCTION

The Atlantic killifish (*Fundulus heteroclitus*) is one of the most ecologically and environmentally important estuarine fish distributed along the East coast of the United States. Their ability to tolerate wide changes in environmental conditions, including temperature, salinity, oxygen and pH, have made them an ideal model species to investigate the biochemical, physiological and evolutionary basis of environmental adaptation (Burnett et al., 2007; Lister et al., 2011; Schulte, 2014; Schulte et al., 2011; Scott and Brix, 2013; Whitehead, 2010; Whitehead et al., 2012). Some populations of killifish are also valuable models for understanding the mechanisms of evolved resistance to toxicants (Hahn, 1998; Van Veld and Nacci, 2008; Wirgin and Waldman, 2004). Populations of killifish inhabiting contaminated coastal waters along the North Atlantic U.S. coast have evolved resistance to some contaminants representing major categories of aryl hydrocarbon pollutants, such as polynuclear aromatic hydrocarbons (PAHs), and halogenated aromatic hydrocarbons such as polychlorinated biphenyls (PCBs), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, 'dioxin') and other dioxin-like compounds (DLCs) (Bello et al., 2001; Elskus et al., 1999; Meyer et al., 2002; Nacci et al., 1999; Nacci et al., 2010; Powell et al., 2000). This evolved resistance involves alterations in signaling through the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor through which some PAHs, PCBs, and TCDD alter gene expression and cause toxicity. Killifish express four AHR paralogs (AHR1a, AHR1b, AHR2a and AHR2b), the products of distinct loci (Hahn et al., 1997; Karchner et al., 1999; Reitzel et al., 2014). Although the respective functions of these AHRs are not well understood, AHR2 proteins appear to play a major role in mediating the developmental effects of PAHs and PCBs in fish (Clark et al., 2010; Jonsson et al., 2007; Prasch et al., 2003).

While there is a great deal of understanding about the physiological and biochemical basis of environmental adaptation in killifish, very little is known about its genetic basis, mainly due to the lack of genetic tools to evaluate gene function. Recently, the antisense morpholino oligonucleotide (MO)-based gene knockdown method was adapted to killifish to determine the role of aryl hydrocarbon receptors and cytochrome P4501A (CYP1A) in developmental toxicity (Clark et al., 2010; Matson et al., 2008), and to study the role of aquaporins in adult killifish (Notch et al., 2011). However, MOs are effective only for a

limited period of time and do not completely eliminate gene expression. In order to conclusively demonstrate the function of any protein, gene knockouts are essential.

In the past decade, several targeted genome-editing methods have been successfully employed to manipulate genes in a variety of model and non-model organisms. They include zinc-finger nucleases (ZFNs), transactivator-like effector nucleases (TALENs) and the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system (Gaj et al., 2013; Kim and Kim, 2014; Peng et al., 2014; Sander and Joung, 2014) (Fig. 1). ZFN and TALENs utilize engineered nucleases consisting of a sequence-specific DNA-binding domain fused to a nonspecific DNA cleavage module. The DNA-binding domains can be customized to recognize any sequence of interest. Distinct from the site-specific nucleases, CRISPR-Cas9 is an RNA-guided DNA nuclease (RGN), where a small guide RNA (sgRNA) complementary to a 20 base target DNA sequence guides Cas9, a bacterially derived nuclease, to induce a double-strand break (DSB) at the target site (Hwang et al., 2013). Because the DSB repair process by non-homologous end-joining (NHEJ) is error prone, it leaves insertions or deletions (indels) at the target site. The relative ease of generating target-specific mutations using these techniques has opened up new avenues for conducting functional studies in any organism of interest. Several studies have demonstrated the efficacy of ZFNs, TALENs and RGNs in producing heritable mutations in non-model organisms such as rainbow trout, medaka, tilapia and yellow catfish (Dong et al., 2011; Li et al., 2013; Yano et al., 2014; Zhang et al., 2014) (Table 1). Mutagenesis rates of up to 95% have been observed with CRISPR-Cas9 (Jao et al., 2013; Li et al., 2014). So far, there are no studies describing the generation of gene knock-outs in killifish or of targeted AHR mutants in any non-mammalian vertebrate. In this study, we utilized ZFN and CRISPR-Cas9 approaches to induce mutations in paralogous killifish AHR2a and AHR2b genes, and we observed indels in the injected embryos. We discuss strategies for the application of this method to knock out closely related paralogs in a species with a long generation time.

2. MATERIALS AND METHODS

2.1. Killifish adults and embryos

Mature male and female Atlantic killifish were collected from Scorton Creek (Sandwich, MA) during full moon and new moon in Spring and Summer of each year (2011–2014) using minnow traps, as described previously (Karchner et al., 1999). Although killifish populations are known to vary dramatically in their sensitivity to DLCs, this source population is known to be relatively sensitive to DLCs, characteristic of other populations of this species resident to similarly uncontaminated sites (Nacci et al., 2010). Fish were maintained in tanks with continuous flow-through seawater (SW) at 18–20°C and 14h:10h light/dark photoperiod conditions. The animal husbandry practices were approved by the Animal Care and Use Committee of the Woods Hole Oceanographic Institution. Female fish were lightly anesthetized with Tricaine (MS222; buffered with sodium bicarbonate, Sigma-Aldrich, St. Louis, MO, USA) and oocytes were obtained for *in vitro* fertilization (IVF) by gently squeezing the abdomen. Oocytes were collected in glass petri dishes with filtered SW (25 parts per thousand; ppt). Milt was obtained by euthanizing mature males in MS222, dissecting out the gonads, and chopping them with a scalpel blade in seawater. A few drops

of milt were added to the oocytes for fertilization. Each IVF experiment included a pool of oocytes stripped from at least 2–3 females and milt from 1 or 2 males. Approximately 20 minutes after the addition of milt, embryos were rinsed with filtered SW to remove any excess sperm. Fertilized embryos were maintained at 23°C until further use.

2.2. Zinc Finger Nuclease design

AHR2a exon 2 and 3 (Fig. 2) were each targeted with a five-finger ZFN pair using the CompoZr® custom ZFN service (Sigma-Aldrich, St. Louis, MO, USA) (Table 2; Fig. 2). Left- and right-ZFN plasmids were *in vitro* transcribed and polyadenylated using the mMessage mMachine T7 kit (Life Technologies, Carlsbad, CA, USA). ZFN mRNA from each half-site was diluted to a concentration of 100–400 ng/μl and 2.5 nl of each was co-injected into 1 or 2-cell stage killifish embryos, as described below.

2.3. CRISPR-Cas guide RNA design and construction

Guide RNAs (gRNA) targeting AHR2a and AHR2b were designed using the ZiFiT Targeter website (http://zifit.partners.org/ZiFiT_Cas9). Exons 1, 2 and 3 of AHR2a and AHR2b were searched for target sites in order to obtain the shortest truncated mutant protein. A single target site was found in each exon. The AHR2a exon 1 target site did not begin with GG residues, which prevented it from being cloned into the gRNA expression vector. The AHR2b exon 1 target site was not optimal due to repetitive sequences. Therefore, the target sites for exons 2 and 3 of AHR2a and AHR2b were selected for gRNA synthesis (Table 2). PAGE-purified complementary oligonucleotides (Eurofins Genomics, Huntsville, AL, USA) were annealed and cloned into an expression vector following a published protocol (Hwang et al., 2013). The gRNA oligonucleotide sequences are provided in Table 2. Expression vectors for guide RNA (pDR274) and Cas9 endonuclease (MLM3613) were obtained from Addgene (<https://www.addgene.org/>). The gRNA was transcribed using the MAXIscript T7 kit, and the Cas9 mRNA was transcribed and poly(A)-tailed with the mMACHINE mMESSAGE mMACHINE T7 ULTRA kit (Life Technologies, Carlsbad, CA, USA). gRNA and Cas9 mRNA were mixed at a ratio of 1:12 for the microinjections.

2.4. Microinjection of ZFN mRNA and CRISPR-Cas9 gRNA

Microinjection of killifish embryos was performed following a previously established protocol (Matson et al., 2008) with some modifications. Briefly, prior to microinjection, 1-cell stage embryos were rolled on wet paper towels to prevent the filaments on the chorion from adhering to the microinjection needle. They were subsequently placed on custom-made agarose microinjection embryo trays and oriented so that the cell directly faces the microinjection needle. The microinjection setup consisted of a Zeiss Stemi 2000-C stereomicroscope, Narishige IM-300 microinjector and a micromanipulator (Narishige International, East Meadow, NY, USA). Aluminosilicate glass tubes (1.0 mm outer diameter, 0.64 mm inner diameter) were used to prepare microinjection needles. This type of needle was found to be superior to borosilicate needles for microinjection of killifish embryo, which have a thick chorion. A P-30 vertical micropipette puller (Sutter Instruments, Novato, CA, USA) at a heater output setting of 97 was used to pull aluminosilicate glass tubing into microinjection needles. The injection volume was calibrated by injecting the

solution into mineral oil and measuring the diameter of the droplet using a stage micrometer. The microinjection volume was approximately 5 nL per embryo. To compensate for variations in needle opening size, injection time and pressure were altered during microinjection. Injected embryos were maintained in filtered SW at 23°C. Some of the injected embryos were transferred to the fish-rearing facility at the National Health and Environmental Effects Research Laboratory, Atlantic Ecology Division, Environmental Protection Agency, Narragansett, RI, to be raised to adulthood. The numbers of injected fish that are being raised for identifying founders are provided in Supplementary Table 1.

2.5. ZFN protein expression

ZFN-injected and uninjected embryos were sampled at 3, 6, 9 and 24 hours post-injection to determine the temporal profile of ZFN protein expression. At each time point, one pool of 10 embryos was sampled. Embryos were homogenized in 1× sample treatment buffer with β-mercaptoethanol and boiled for 5 minutes to denature the protein. Samples were separated on an 8% polyacrylamide gel using the discontinuous buffer system (Laemmli, 1970). The proteins were transferred onto a nitrocellulose membrane with a semi-dry transfer unit (BioRad, Waltham, MA, USA), and the membrane was blocked with 5% milk in TBST (20 mM Tris pH 7.5, 300 mM NaCl and 0.1% (v/v) Tween 20) overnight. ZFN protein was detected using a mouse monoclonal anti-Flag antibody (Sigma, St. Louis, MO, USA) at 1:5000 dilution. The secondary antibody used was horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Santa Cruz Biotechnology, Dallas, TX, USA) at 1:5000 dilution. The membranes were incubated in the primary antibody for 60 min at room temperature, washed with TBST twice (10 min each), incubated with secondary antibody for 60 min, and finally washed with TBST (15 min). Detection was done using the ECL Prime Western blotting reagent (GE Healthcare, Pittsburgh, PA, USA).

2.6. Surveyor endonuclease assay

Somatic mutations were determined using the Surveyor[®] mutation detection kit (Transgenomic Inc., Omaha, NE, USA) following the manufacturer's instructions (Qiu et al., 2004). Embryos (pool of 5 embryos per replicate) were sampled at 48 hours post-injection and genomic DNA was isolated using the proteinase K digestion method (Goldenberger et al., 1995). The AHR2a and AHR2b target regions were amplified by polymerase chain reaction (PCR) using Advantage polymerase (Clontech, Mountain View, CA, USA). The primer sequences are given in Table 2. PCR cycling conditions were 94°C for 1 min, followed by 35 cycles of [94°C for 5 s, 68°C for 1 min], followed by 72°C for 7 min. The PCR products were melted and re-annealed to form hetero-duplex DNA and digested with Surveyor nuclease for 20 min at 42°C. The resulting products were electrophoresed on a 15% TBE gel and visualized by ethidium bromide staining.

2.7. DNA sequencing

PCR products from samples that were positive in the Surveyor endonuclease assay were cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) and sequenced in 96-well plates (Functional Biosciences, Madison, WI, USA). Insertions and deletions were identified by aligning the sequences with the wildtype AHR sequence (Sequencher 5.1, Gene Codes Corp., Ann Arbor, MI, USA). Off-target screening for AHR2b CRISPR-Cas9

exon2 was done by direct-sequencing of PCR products. The amplification reactions were purified using the GeneClean kit (MP Biomedicals, Santa Ana, CA, USA). Direct-sequencing of the amplicons for AHR1a, AHR2a, and AHRR was done with primers Fh2a-ex2rev, 2b-1aF, and RR-ex2fwd, respectively (Table 2). The chromatograms were visually inspected for regions of double peaks indicating insertions or deletions.

3. RESULTS

3.1. ZFN-induced AHR2a mutations

We tested different concentrations of ZFN mRNA on killifish embryos by injecting 500, 1000 and 2000 pg per embryo. No overt toxic effects or phenotypic abnormalities were observed at any of the concentrations as determined by microscopic observation of embryos during development. Hence, we selected 2000 pg to inject in subsequent experiments. ZFN protein expression was confirmed in the embryos injected with 2000 pg ZFN mRNA. Embryos were sampled at 3, 6, 9 and 24 hours post-microinjection. The maximum ZFN protein expression was observed at 6 hours post-microinjection (Fig. 3A). At 3 and 9 hours, ZFN protein expression was much lower, and at 24 hours, no expression was detected. These results suggest that the maximum efficiency of ZFNs in inducing DSBs in killifish is around 6 hours post-injection.

Approximately 400 embryos were injected with the ZFN pair targeting exon 2 of AHR2a, and all were screened for mutations using the Surveyor mutation detection assay. We did not detect any insertions or deletions in these embryos; therefore, we next tested the ZFN pair targeting exon 3. Using the Surveyor assay, we analyzed 14 pools of ZFN-injected embryos (5 embryos per pool), and detected the presence of mutations in 4 pools (Fig. 3B). Endonuclease digestion resulted in fragments of approximately 240 bp and 95 bp (Fig. 2B, 3B). The PCR product from one of the embryo pools that was positive in the Surveyor assay was cloned into the pGEM-T Easy vector. Sequencing of multiple clones revealed 6 mutant sequences out of 59 (10%), represented by four different types of deletions at the target site (2, 4, 5 and 28-base pair deletions) (Fig. 3C). We did not observe any cuts with the Surveyor endonuclease in the 2 pools of uninjected embryos. The remaining fish have been raised to adulthood, and their progeny are being screened to identify potential founders (Supplemental Table 1).

3.2. CRISPR-Cas9-induced AHR2a mutations

Microinjection of the CRISPR-Cas9 guide RNA targeting exon 2 of AHR2a was not successful in generating mutations (20 pools of 5 embryos/pool were analyzed). However, for the guide RNA targeting exon 3, Surveyor mutation detection assay results were positive in 2 out of 6 pools of injected embryos analyzed (30 embryos total; 5 embryos per pool) (Fig. 4A). The mutated target sites resulted in fragments of approximately 160 bp and 95 bp (Fig. 2B, 4A). The PCR products from the two positive samples (10 embryos total) were combined, cloned into the pGEM-T Easy vector, and sequenced. We observed 13 deletions in 81 sequences (16%). The 13 deletions varied in length from 1 to 58 bp, representing 10 distinct deletions (Fig. 4B).

3.3. CRISPR-Cas9-induced AHR2b mutations

CRISPR-Cas9 guide RNAs targeting exon 2 and 3 of the AHR2b locus were both efficient at generating mutations in the microinjected embryos. Surveyor mutation detection assay showed the presence of mutations in all 4 pools of AHR2b exon 2 CRISPR-Cas9-injected embryos analyzed (20 embryos total; 5 embryos per pool) (Fig. 5A). The PCR products from these 4 pools were combined and cloned into the pGEM-T Easy vector. Sequencing results revealed that 16 sequences out of 70 (23%) had indels at the target site, represented by 8 distinct mutations (5 deletions and 3 insertions) (Fig. 5B).

Similarly, injection of guide RNA targeting exon 3 of AHR2b induced mutations as observed with Surveyor mutation detection assay (Fig. 5C). All 4 pools of injected embryos showed the presence of mutations (5 embryos per pool). PCR products from two of the samples were combined, cloned into the pGEM-T easy vector and sequenced. We observed 55 sequences with indels out of 87 (63%). There were 20 different deletions and 7 different insertion mutants (Fig. 5D).

3.4. Screening for CRISPR-Cas9 off-target effects

In order to assess potential off-target effects of the CRISPR-Cas9 experiments, we chose one of the guide RNAs that gave positive results (exon 2 of AHR2b) to screen for possible mutations in other AHR paralogs and AHRR. The multiple AHR paralogs and the AHRR are closely related genes and share substantial sequence identity, especially in the N-terminus. Alignment of the AHR2b CRISPR-Cas9 target sequence with the other genes is shown in Fig. 6A. The numbers of mismatches between the 20 bp AHR2b target site and AHR1a, AHR2a, AHR1b, and AHRR are 4, 4, 6, and 3, respectively. These corresponding regions were amplified from the uninjected and AHR2b CRISPR-Cas9-injected embryo genomic DNA samples in which AHR2b mutations were observed. The PCR products were analyzed for mutations using the Surveyor endonuclease assay. The AHR1a, AHR2a, and AHRR samples contained small fragments indicating endonuclease digestion of the PCR products (Fig. 6B). These fragments were also present in the AHR2a and AHRR reactions from the uninjected embryos. These amplification reactions were purified and analyzed by direct-sequencing. The chromatograms of the AHR1a and AHR2a sequencing reactions contained double peaks at previously characterized SNP locations (#141 and #159 for AHR1a, and #156 for AHR2a) (Reitzel et al., 2014). Surveyor endonuclease is sensitive to even a single nucleotide mismatch between the two DNA strands, therefore it will cleave DNA at the SNP sites. The sizes of the observed Surveyor endonuclease fragments in these samples (Fig. 6B) can be explained by these SNPs, thereby suggesting that they do not represent off-target mutations. On the other hand, we did not detect a SNP in the AHRR sequences that would explain the fragments observed in the Surveyor assay. However the presence of these fragments in both uninjected and injected samples suggest that they are rare SNPs that may not be detected by direct sequencing.

4. DISCUSSION

In this study, we demonstrated the feasibility of targeted mutagenesis approaches in inducing mutations in an environmental model species with limited genomic and genetic

resources. The ease with which ZFN and CRISPR-Cas9 methods can be utilized to cleave specific genomic DNA sequences has led to the generation of gene knockouts in species not traditionally used for conducting genetic and functional studies (Table 1). Using these methods, we produced mutations in AHR2a and AHR2b loci in killifish embryos. These embryos are currently being raised to adulthood, and their progeny will be screened to identify potential founders. As killifish have a long generation time (2 years minimum) and lack established breeding techniques, the time it takes to generate homozygous mutants is relatively long. Here, we propose some strategies to expedite the process of generating homozygous mutants in killifish. AHR-null killifish will be valuable for characterizing the role of the individual AHR paralogs in evolved resistance, as well as in normal development.

We initially adopted the ZFN approach for generating mutations, as their design and activity are well characterized (Urnov et al., 2010). In addition, ZFNs were the first targeted mutagenesis approach that was successfully used in generating mutations in a number of model and non-model organisms (Bibikova et al., 2003; Doyon et al., 2008; Scott, 2005). ZFNs are assembled using zinc finger motifs, each of which recognizes a unique 3-bp DNA sequence (Kim et al., 2010; Urnov et al., 2010). Initial studies with ZFNs used 3-finger zinc-finger proteins (ZFPs), but recent studies have used up to six fingers per ZFP to increase specificity and target rare cleavage sites (Kim and Kim, 2014; Kim et al., 2010; Sander et al., 2011; Segal, 2011). We used five-finger zinc finger proteins that enabled ZFN dimers to specifically target a 30-bp region in exon 2 and exon 3 of AHR2a (Table 2).

In order to determine the translational efficiency of the injected ZFN mRNA, we measured ZFN protein expression in embryos using an anti-FLAG antibody. Our results demonstrated that maximal ZFN protein expression is observed at 6 hours post-injection, suggesting that ZFN protein binding to the target site and creating DSBs occurs around this time. In comparison, in zebrafish embryos injected with a ZFN targeting the aryl hydrocarbon receptor repressor (*ahrra*) gene, we observed maximal ZFN protein expression at 3 hours post-injection (Aluru et al., 2013). These species-specific differences in ZFN protein expression could be linked to the developmental rate in the two species. The developmental period for zebrafish from fertilization to hatching is 2 days, whereas killifish hatch at 15 days post-fertilization (Armstrong and Child, 1965). Another factor that could contribute to the differences in protein expression is the water temperature at which embryos are incubated. Zebrafish embryos are reared at a higher temperature (28°C) than killifish (23°C), which may affect the rate of transcription and translation.

We were successful in obtaining mutations in the AHR2a gene with a ZFN targeting exon 3. Sequencing results revealed the presence of 4 different deletions at the target site in 10% of the sequenced clones. These results are comparable to the frequencies observed in other species (McCammon et al., 2011; Ochiai et al., 2010; Young et al., 2011; Zhang et al., 2014). The CRISPR-Cas9 method has been reported to result in higher mutation frequencies in comparison to ZFNs or TALENs (Flowers et al., 2014). Therefore, we used the CRISPR-Cas9 approach to target AHR2a and observed indels in 16% of the sequences from embryos microinjected with a gRNA targeting exon 3. We detected mutations in the CRISPR-Cas9 injected embryos in the very first trial compared to several ZFN trials, even though the

proportion of indels showed only a modest increase with the CRISPR-Cas9 method. Similarly, microinjection of gRNAs targeting exon 2 and exon 3 of AHR2b produced indels in 17% and 63% of the sequences. We are currently raising the ZFN-AHR2a, CRISPR-Cas9-AHR2a and -AHR2b fish to reproductive maturity so that we can screen for founders (Supplemental Table 1). To our knowledge, this is the first study comparing the effectiveness of two different targeted genome-editing methods (protein and gRNA based) in a species with limited genomic resources. Additional comparisons with other target loci will be necessary to confirm our observations.

Genome-editing methods have become a powerful resource for generating mutants in non-model species, but there are some concerns with regard to their off-target effects (Gupta et al., 2011; Lin et al., 2014; Porteus, 2006; Porteus and Baltimore, 2003). ZFNs, as well as CRISPR-Cas9, have been associated with cytotoxicity, presumably due to binding and cleavage at non-target sites (Bibikova et al., 2002; Porteus, 2006; Porteus and Baltimore, 2003; Pruett-Miller et al., 2008), although toxic effects could also result from the target gene disruption. Screening the genome for off-target effects *a priori* by blast searches with the target sequence is not feasible for species that do not have sequenced genomes. However, paralogs and closely related genes that have been characterized can be screened for mutations, as these represent likely targets. We checked for off-target effects of CRISPR-Cas9 in all AHR paralogs, as well as AHRR, a gene closely related to AHR (Fig. 6). Based on the Surveyor assay and sequencing results, no off-target effects were detected, even though the number of sequence mismatches among these genes was as low as 3 residues. If genomic resources are available, several tools exist for *in silico* identification of potential off-target sites (Fine et al., 2014; Xie et al., 2014). Alternatively, potential off-target sites can be searched using the fuzznuc algorithm (<http://embossgui.sourceforge.net/demo/manual/fuzznuc.html>) in RNAseq data, if available.

In ZFN and CRISPR-Cas9 injected embryos we did not observe any developmental toxicity, including the highest concentration of ZFN (2000 pg of ZFN mRNA/embryo). These results are in contrast to studies in other fish species, in which overt developmental phenotypes were observed at these concentrations (McCammon and Amacher, 2010; McCammon et al., 2011; Zhang et al., 2014). One reason for the lack of toxicity could be the relative size of the killifish embryos in comparison to zebrafish embryos, in which most of these methods have been optimized. Freshly fertilized killifish embryos are 2 mm in diameter, and are larger than zebrafish (0.7 mm), where ZFNs above 100 pg per embryo have been shown to cause embryo toxicity (McCammon and Amacher, 2010). Similarly, in medaka with an egg diameter (1.2 mm), 160 pg of ZFN caused embryo toxicity (Zhang et al., 2014). In addition to the size of the embryo, rapid turnover of the ZFN protein might be responsible for the lack of embryo toxicity in killifish. Our results suggest that the ZFN protein is expressed transiently around 6 hours post-injection and is degraded by 9 hours (Fig. 3A), suggesting faster degradation of the injected mRNA in killifish. We cannot rule out the possibility of additional factors, yet to be determined, contributing to the low toxicity of injected mRNA in killifish.

Our results demonstrate the utility of the ZFN and CRISPR-Cas9 approaches in generating mutations in an ecologically relevant fish species that have successfully adapted to

environments contaminated with PCBs and PAHs. Generating AHR knockout mutants will provide a powerful tool to functionally characterize the role of these proteins in evolved resistance to PCBs and PAHs. We are currently raising the ZFN- and CRISPR-Cas9-injected fish to adults. The numbers of injected animals that are being raised and the breeding strategy that will be followed are provided in the supplemental information (Supp. Table 1 and Supp. Fig. 1). Killifish mature in 1 year; however, larger fish, producing sufficient offspring per pair for testing, require > 2 years of laboratory rearing. Most organisms in which these approaches are extensively used to generate mutants have short life history cycles (e.g. 2–3 months). However, in the past few years targeted mutagenesis approaches have been successfully used in species with longer life cycles such as yellow catfish (Dong et al., 2011), rainbow trout (Yano et al., 2014) and Atlantic salmon (Edvardsen et al., 2014). In order to expedite the process of obtaining homozygous mutants, several strategies can be followed. During certain steps of the homozygous mutant breeding, fin-clips from juvenile fish can be screened by direct-sequencing to identify mutants (Supp. Fig. 1). Direct-sequencing allows screening a single DNA sample per fish, as opposed to cloning the PCR product and screening multiple clones. Another possible approach to accelerate the identification of founders is to screen gametes stripped from juveniles. Once the founder animals are identified, it is possible to artificially induce precocious maturation of gametes by treating the founders with gonadotropins, a common practice in breeding aquaculture species (Chaube et al., 2014; Gwo et al., 1993; Watson et al., 2009).

Other strategies to consider while generating mutants in species with long life history cycles is to generate at least two mutant lines targeting different sites in each gene. As it is time consuming to raise embryos to reproductive maturity, targeting multiple regions of the same gene confirms the specificity of the observed phenotypes associated with the gene knockout. In addition, if a frameshift mutation is not produced in one of the targets, the other target will serve as an alternative. We followed these approaches and designed CRISPR-Cas9 guide RNAs against AHR2a, in addition to the ZFN method. Similarly, we designed two different gRNAs against AHR2b, and observed mutations at both target sites. Another important consideration in target site selection in species such as the Atlantic killifish is to avoid regions with known single nucleotide polymorphisms (SNPs) (Reitzel et al., 2014). Killifish have high genetic diversity (McMillan et al., 2006), and if the target site has multiple mismatches due to SNPs, the efficacy of the ZFN proteins or guide RNAs will be reduced. In addition, SNPs interfere with the mutation detection assays such as Surveyor endonuclease by creating cuts at polymorphic sites. An additional strategy to consider is optimizing injection conditions to maximize the chances for mutating both alleles at the target locus simultaneously. Such bi-allelic mutations in CRISPR-Cas9-injected embryos have been demonstrated previously (Blitz et al., 2013; Edvardsen et al., 2014; Flowers et al., 2014; Jao et al., 2013; Li et al., 2014). The presence of bi-allelic mutations in the germline will expedite the process of generating homozygous individuals.

Overall, targeted mutagenesis approaches have provided new opportunities for conducting functional studies in non-traditional model organisms. As a proof of concept, we and others (Table 1) have demonstrated that it is feasible to generate mutants in non-traditional model species. The relative ease of these methods, as well as the open-source sharing of reagents, have made these methods accessible to a wide range of laboratories.

5. CONCLUSIONS

In this study we demonstrated the utility of targeted gene-editing methods for generating *ahr* mutants in Atlantic killifish. We observed higher frequency of mutations with CRISPR-Cas9 compared to ZFN mRNA injected embryos. We are currently raising the injected embryos to identify potential germ line mutants. Some of the challenges in generating homozygous mutants in killifish include long generation time and lack of established breeding methods. We have proposed some strategies to overcome these challenges and expedite the process of identifying the founders and generating *ahr*-null mutants. The strategies proposed here are applicable to any fish species with limited aquaculture resources, providing an opportunity to conduct functional studies.

Supplementary Material

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Highlights

- AHR2 genes were mutated without detectable off-target effects in paralogs.
- CRISPR-Cas9 method was more efficient in inducing mutations compared to ZFN.
- We suggest strategies for generating knockouts in non-traditional fish models.
- AHR knockouts will be valuable for understanding evolved resistance to toxicants.

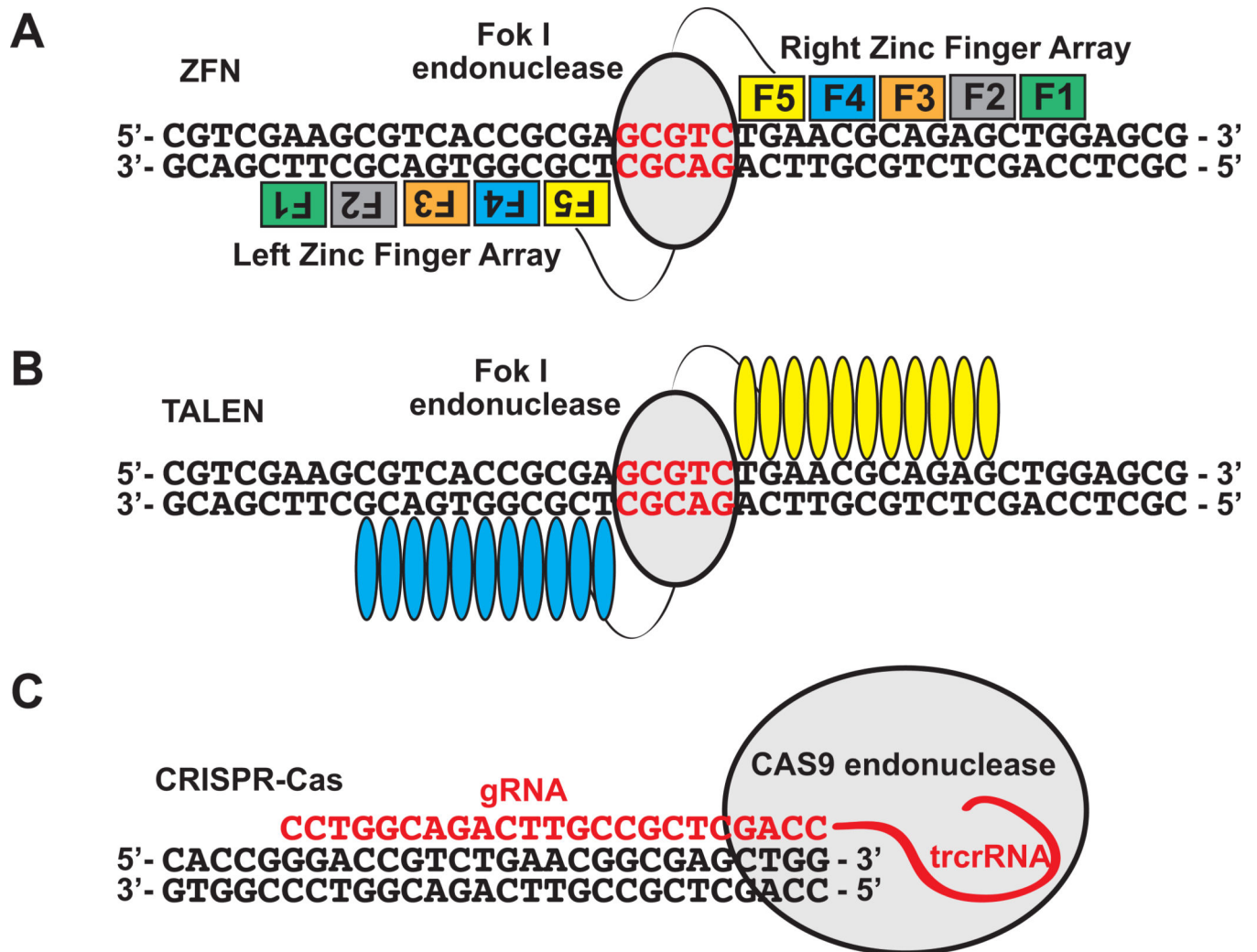


Figure 1. Outline of the targeted mutagenesis techniques currently used in generating mutants. **A.** Zinc finger nucleases **B.** TALENs and **C.** CRISPR-Cas system.

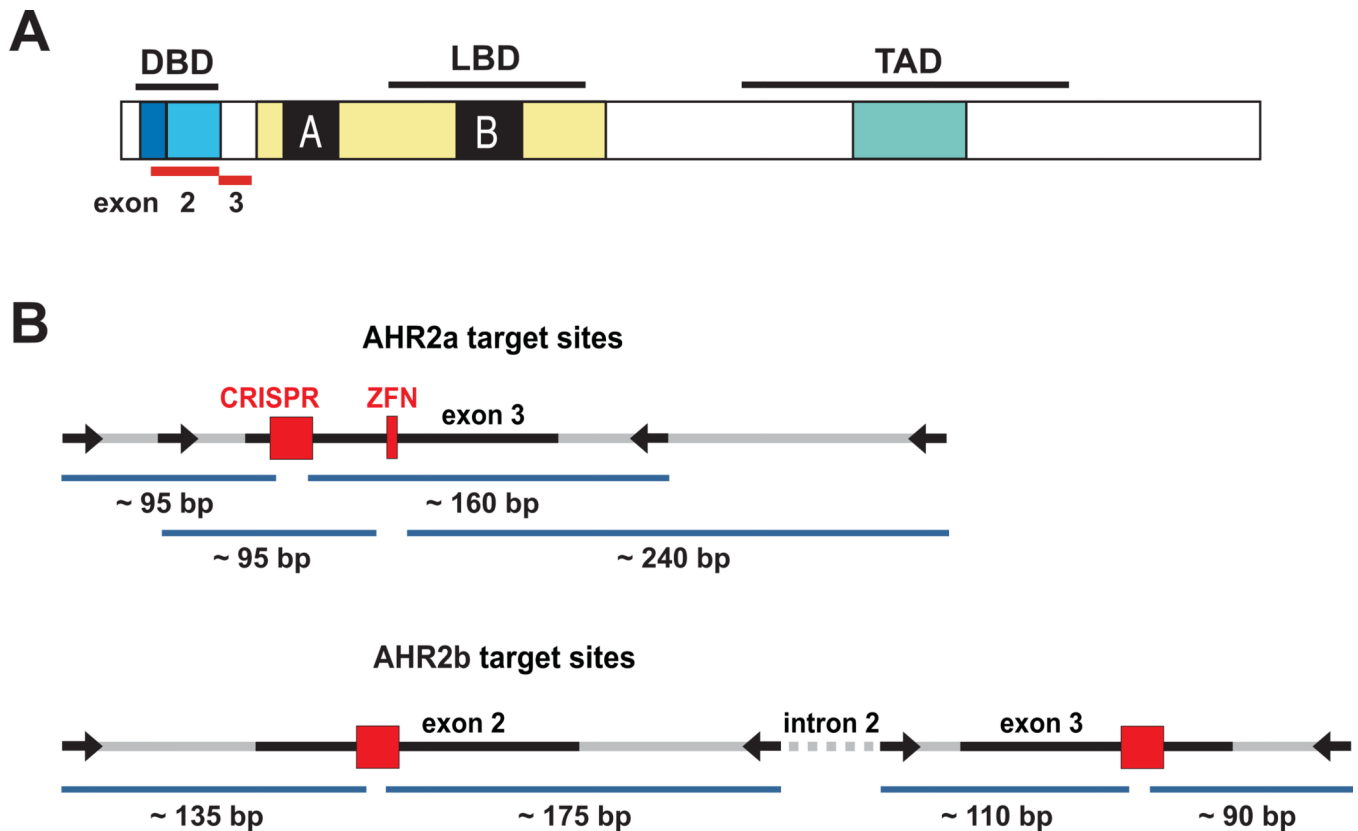


Figure 2.
 AHR target sites. **A.** Functional domain structure of the AHR protein. The location of the targeted exons 2 and 3 in relation to the functional domains is shown as red bars. DBD: DNA-binding domain, LBD: ligand-binding domain, TAD: transcriptional activation domain. **B.** Schematic representations of the ZFN and CRISPR-Cas target regions in AHR2a and AHR2b loci. Arrows represent the primers used in the PCR amplification of genomic DNA for screening. Surveyor nuclease fragments are shown below the target regions.

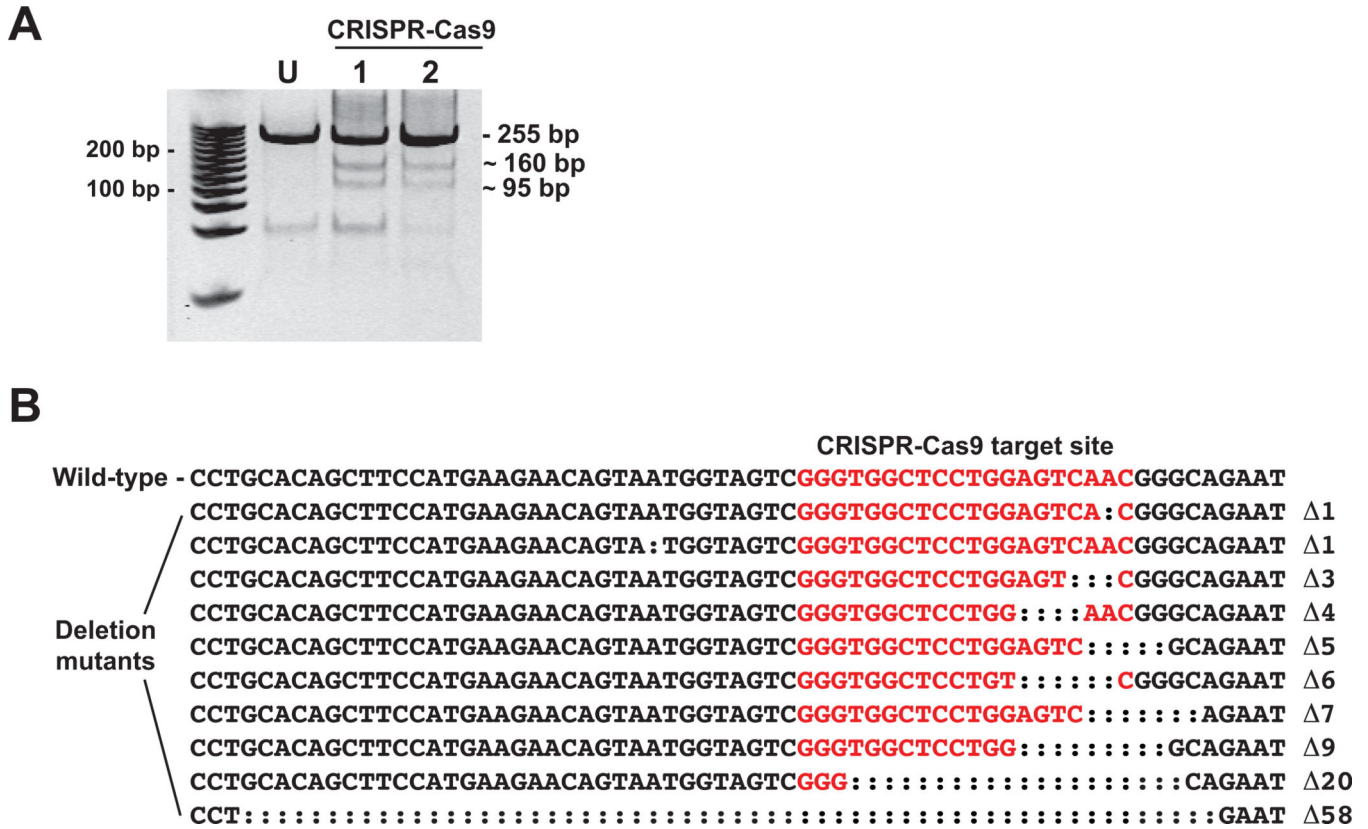


Figure 4. CRISPR-Cas9-mediated mutagenesis of killifish AHR2a targeting exon 3. **A.** Surveyor nuclease detection of mutations in the CRISPR-Cas9 target region of AHR2a exon 3. Each lane represents a pool of 5 embryos from which a 255 bp genomic DNA fragment was amplified. U: uninjected, lanes 1 and 2: CRISPR-Cas9-injected embryos. Approximate sizes of the digested fragments containing the deletions and insertions are shown (160 and 95 bp). The full-length PCR products from samples 1 and 2 were pooled, cloned, and sequenced. **B.** AHR2a exon 3 sequence surrounding the CRISPR-Cas9 target site (in red). Ten different deletion mutants were observed among the sequenced clones.

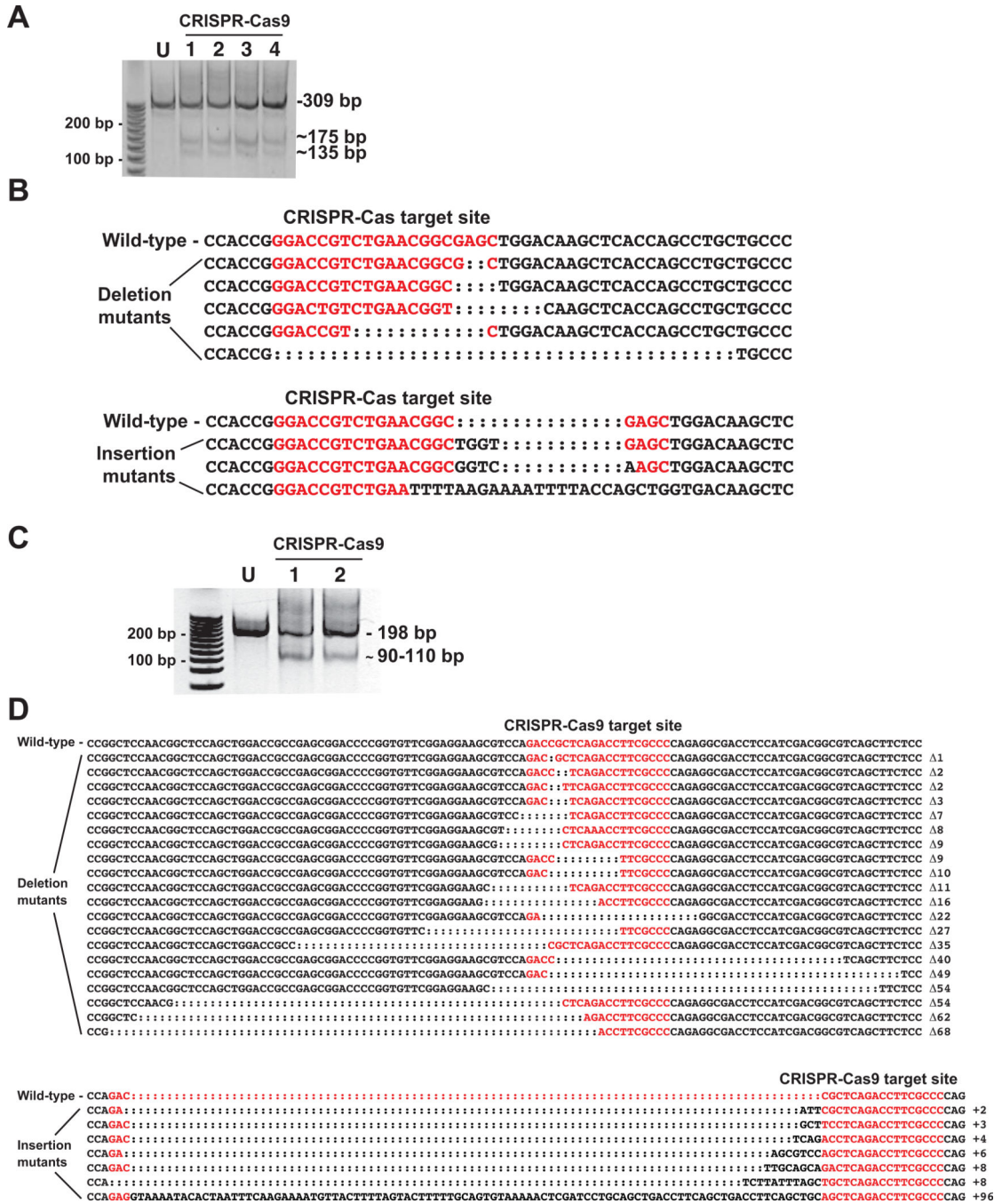
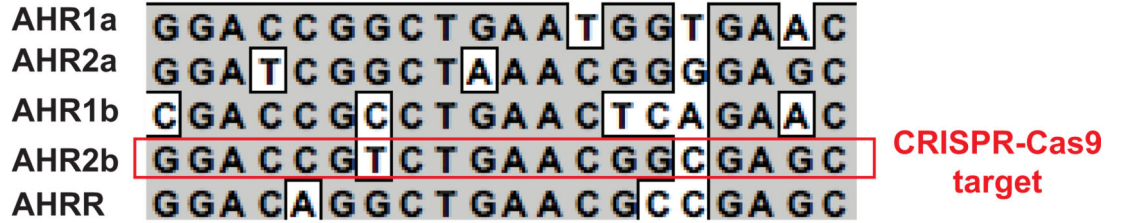


Figure 5. CRISPR-Cas9-mediated mutagenesis of killifish AHR2b targeting exon 2 and exon 3. **A.** Surveyor nuclease detection of mutations in the AHR2b CRISPR-Cas9 target region. Each lane represents a pool of 5 embryos from which a 309 bp genomic DNA fragment was amplified. U: uninjected control, lanes 1–4: CRISPR-Cas9-injected embryos. Approximate sizes of the digested fragments containing the deletions and insertions are shown (175 and 135 bp). The full-length PCR products from samples 1–4 were pooled, cloned, and sequenced. **B.** AHR2b exon 2 sequence surrounding the CRISPR-Cas9 target site (in red).

Five types of deletion mutants and 3 types of insertions were observed among the sequenced clones. **C.** Surveyor nuclease detection of mutations in the CRISPR-Cas9 target region of AHR2b exon 3. Each lane represents a pool of 5 embryos from which a 198 bp genomic DNA fragment was amplified. U: uninjected, lanes 1 and 2: CRISPR-Cas9-injected embryos. Approximate sizes of the digested fragments containing the deletions and insertions are shown (110 and 90 bp). The full-length PCR products from samples 1 and 2 were pooled, cloned, and sequenced. **D.** AHR2b exon 3 sequence surrounding the CRISPR-Cas9 target site (in red). Twenty different types of deletion mutants and seven types of insertion mutants were observed among the sequenced clones.

A



B

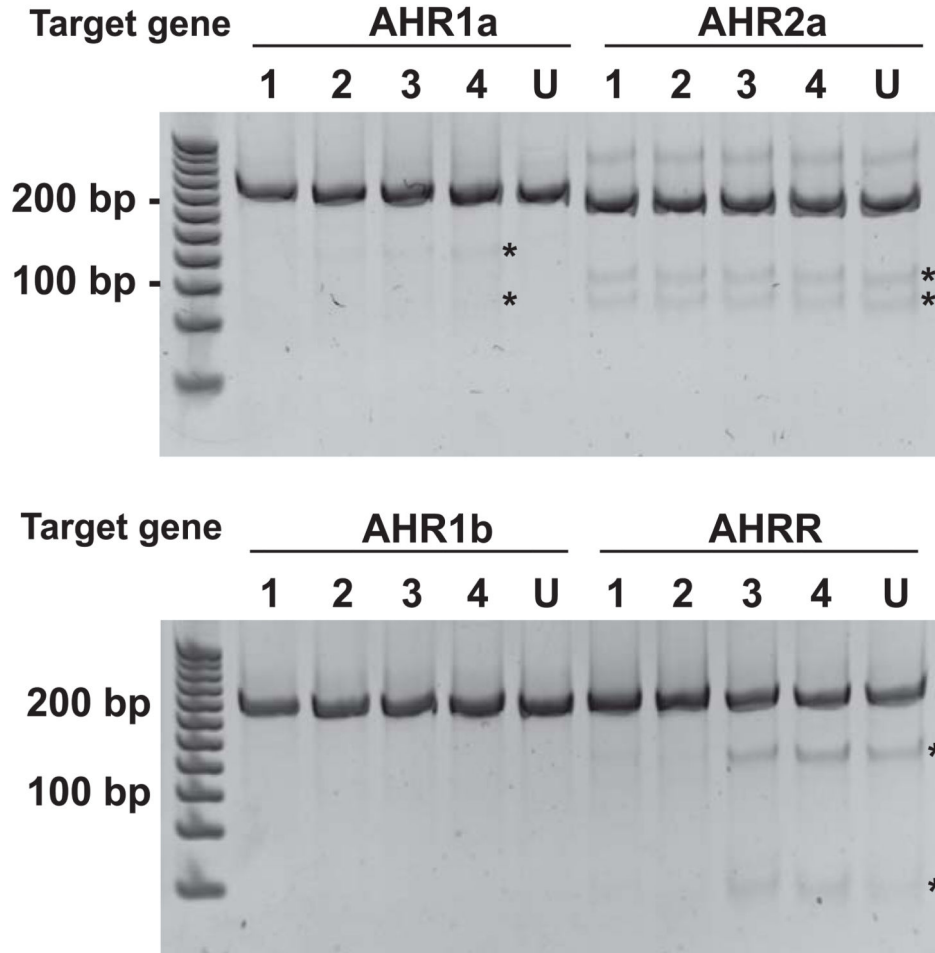


Figure 6. AHR2b CRISPR-Cas9 off-target analysis. **A.** Nucleotide sequence alignment of the AHR2b CRISPR-Cas9 target sequence with the corresponding regions of closely related genes. AHR1a, AHR2a, AHR1b, and AHRR have 4, 4, 6, and 3 mismatches to AHR2b in this region, respectively. **B.** Surveyor nuclease detection of mutations in AHR1a, AHR2a, AHR1b, and AHRR in the AHR2b CRISPR-Cas9 target region. Digested fragments are indicated by a *. Each lane represents a pool of 5 embryos from which genomic DNA fragments were amplified. U: uninjected control, 1–4: CRISPR-Cas9 injected embryos.

AHR1a, AHR2a, and AHRR PCR products from uninjected and injected embryos were sequenced.

Table 1

Published studies employing gene targeting in non-traditional model species.

Gene-editing approach	Species	Reference
ZFN	Monarch butterfly (<i>Danaus plexippus</i>) Two-spotted cricket (<i>Gryllus bimaculatus</i>) Sea urchin (<i>Hemicentrotus pulcherrimus</i>) Ascidian (<i>Ciona intestinalis</i>) Western clawed frog (<i>Xenopus tropicalis</i>) Yellow catfish (<i>Pelteobagrus fulvidraco</i>) Medaka (<i>Oryzias melastigma</i>) Rainbow trout (<i>Oncorhynchus mykiss</i>)	(Merlin et al., 2013) (Watanabe et al., 2012) (Ochiai et al., 2010) (Kawai et al., 2012) (Young et al., 2011) (Nakajima et al., 2012) (Dong et al., 2011) (Ansai et al., 2012) (Yano et al., 2014)
TALEN	Nematode (<i>Pristionchus pacificus</i>) Marine polychaete (<i>Platyneries dumerilii</i>) Culicine mosquito (<i>Aedes aegypti</i>) Silk worm (<i>Bombyx mori</i>) Sea urchin (<i>Hemicentrotus pulcherrimus</i>) Ascidian (<i>Ciona intestinalis</i>) Western clawed frog (<i>Xenopus tropicalis</i>) Iberian ribbed newts (<i>Pleurodeles waltl</i>) Tilapia (<i>Oreochromis niloticus</i>) Medaka (<i>Oryzias melastigma</i>) Yellow catfish (<i>Pelteobagrus fulvidraco</i>)	(Lo et al., 2013) (Bannister et al., 2014) (Aryan et al., 2013b) (Aryan et al., 2013a) (Ma et al., 2012) (Takasu et al., 2013) (Sajwan et al., 2013) (Wang et al., 2013) (Hosoi et al., 2014) (Treen et al., 2014) (Yoshida et al., 2014) (Ishibashi et al., 2012) (Lei et al., 2012) (Nakajima and Yaoita, 2013) (Liu et al., 2014) (Hayashi et al., 2014) (Li et al., 2013) (Wang and Hong, 2014) (Ansai et al., 2014) (Ansai et al., 2013) (Dong et al., 2011)
CRISPR-Cas9	Western clawed frog (<i>Xenopus tropicalis</i>) Axolotl (<i>Ambystoma mexicanum</i>) Atlantic Salmon (<i>Salmo salar</i>) Tilapia (<i>Oreochromis mossambicus</i>)	(Nakayama et al., 2013) (Guo et al., 2014) (Flowers et al., 2014) (Ji-Feng et al., 2014) (Edvardsen et al., 2014) (Li et al., 2014)

Table 2

AHR2a and AHR2b target regions and oligonucleotides used in ZFN and CRISPR-Cas9-based mutagenesis. The ZFN target site is shown in capital letters. The sequence flanking the target sites are recognized by the ZFN proteins.

Name	Description	Sequence
AHR2a exon 2	ZFN target site	ctgcgcctcagcgtgGGATAcctgagggtcaagag
AHR2a exon 3	ZFN target site	gactcattcagcttcTCTGAaggagagctgctgct
AHR2a exon 2	CRISPR-Cas9 target site	GGAGCGAATCTCATCAGGGA
AHR2a exon 3	CRISPR-Cas9 target site	GGGTGGCTCCTGGAGTCAAC
AHR2b exon2	CRISPR-Cas9 target site	GGACCGTCTGAACGGCGAGC
AHR2b exon3	CRISPR-Cas9 target site	GGGCGAAGGTCTGAGCGGTC
2-ex3Fwd	AHR2a ZFN pair3 screening	TGTTTCACCTCTGCACAGCTTCC
1XRR1	AHR2a ZFN pair3 screening	TACTAGCTAAACGTGACCTGTCTG
2a-ex3CrF	AHR2a CRISPR-Cas exon3 screening	CTGTCTATCAGGTGGAGAGTCC
2a-ex3CrR	AHR2a CRISPR-Cas exon3 screening	GTTGTGGATGAGTATGCAGGTGG
AHR2b-CrF	AHR2b CRISPR-Cas9 exon2 screening	AATCTTTGATCCGGTCTGATCTGG
AHR2b-CrR	AHR2b CRISPR-Cas9 exon2 screening	AGACACAACCAAGCAGCAACACG
2bex3-Fwd	AHR2b CRISPR-Cas9 exon3 screening	ATCGCCACGCCTTAGCTTCCAGTCCG
2bex3-Rev	AHR2b CRISPR-Cas9 exon3 screening	GCGTTTTTGCTTTGTCTGCTGTCTGG
Fh2A-ex3-CR1	AHR2a exon3 gRNA oligo1	TAGGGTGGCTCCTGGAGTCAAC
Fh2A-ex3-CR2	AHR2a exon3 gRNA oligo2	AAACGTTGACTCCAGGAGCCAC
FhAHR2b-CR1	AHR2b exon2 gRNA oligo1	TAGGACCGTCTGAACGGCGAGC
FhAHR2b-CR2	AHR2b exon2 gRNA oligo2	AAACGCTCGCCGTTCCAGACGGT
Fh2B-ex3-CR1	AHR2b exon3 gRNA oligo1	TAGGGCGAAGGTCTGAGCGGTC
Fh2B-ex3-CR2	AHR2b exon3 gRNA oligo2	AAACGACCGCTCAGACCTTCGC
2b-1aF	AHR1a off-target screening	GCTTAATCCTGCTTTCGTCTCTGC
Fh1a-ex2rev	AHR1a off-target screening	AAGAAGTGTGGCTCGTAGGTAGC
2b-2aF	AHR2a off-target screening	TTTTACCCCTCACGGAAGAAGATGG
Fh2a-ex2rev	AHR2a off-target screening	GTAGCTCTTGACCCTCAGGTATCC
2b-1bF	AHR1b off-target screening	TTTCCTCCTCTCCAGAGCCAAACC
Fh1b-ex2rev	AHR1b off-target screening	AAGAAGCTTTGGTGCGCAGGTAGC
RR-ex2fwd	AHRR off-target screening	CTCATCCCTCTGCGGCTCTCGTCC
RR-ex2rev	AHRR off-target screening	AAGAAGCTTTTGACGCGGAGGTAGG