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Research Article

# Annotation of the Nuclear Receptors in an Estuarine Fish species, *Fundulus heteroclitus*

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**Abstract.** The nuclear receptors (NRs) are ligand-dependent transcription factors that respond to various internal as well as external cues such as nutrients, pheromones, and steroid hormones that play crucial roles in regulation and maintenance of homeostasis and orchestrating the physiological and stress responses of an organism. We annotated the *Fundulus heteroclitus* (mummichog; Atlantic killifish) nuclear receptors. Mummichog are a non-migratory, estuarine fish with a limited home range often used in environmental research as a field model for studying ecological and evolutionary responses to variable environmental conditions such as salinity, oxygen, temperature, pH, and toxic compounds because of their hardiness. *F. heteroclitus* have at least 74 NRs spanning all seven gene subfamilies. *F. heteroclitus* is unique in that no RXR $\alpha$  member was found within the genome. Interestingly, some of the NRs are highly conserved between species, while others show a higher degree of divergence such as PXR, SF1, and AR $\alpha$ . *Fundulus* like other fish species show expansion of the RAR (NR1B), Rev-erb (NR1D), ROR (NR1F), COUPTF (NR2F), ERR (NR3B), RXR (NR2B), and to a lesser extent the NGF (NR4A), and NR3C steroid receptors (GR/AR). Of particular interest is the co-expansion of opposing NRs, Rev-erb-ROR, and RAR/RXR-COUPTF.

**Keywords:** RXR, phylogenetics, nuclear receptors, stress, endocrinology

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## 1. Introduction

*Fundulus heteroclitus* (mummichogs) are an atlantic killifish species found from Nova Scotia, Canada to northern Florida, USA. In some estuaries, they can account for as much as 25% of the total macrofauna [1]. Mummichog are noted for their hardiness, which includes the ability to survive a wide variety of temperatures, large salinity fluctuations, low dissolved oxygen, and heavily polluted ecosystems [1–7]. The ability to adapt and acclimate to these different conditions has made mummichogs a popular research subject in a number of fields, including ecology, evolution, physiology, and toxicology [1, 3, 8–12].

Coastal population growth, urban runoff from increased impervious cover, and industrial pollution results in significant anthropogenic stress in estuaries. The abundance of *F. heteroclitus* in estuaries, their association with sediments due to hiding from predators or searching for food, and their non-migratory nature makes them an excellent bioindicator species. *Fundulus* species have been used as bioindicators at contaminated sites [1, 2, 5, 13, 14] and to follow the evolution of pollution resistance in a vertebrate [3, 10, 11, 15–17]. Thus, mummichogs are an excellent vertebrate bioindicator species for understanding rapid adaptation to environmental change in



feral populations [5, 8], including adaptations in transcription factors such as estrogen receptor alpha (ER $\alpha$ ) splice variants [18], and evolutionary convergence in the aryl hydrocarbon receptor pathway (AHR1a, AHR2a, CYP1A) [5, 8].

NRs consist of five modules: A/B, C, D, E, and F. The A/B module contains activation function-1 (AF-1) sites crucial in binding coactivators. The C module contains the DNA-binding domain (DBD) that encompasses the zinc-fingers necessary for binding DNA at response elements and mediating the basic transcriptional functions of a NR. The DBD is also highly conserved between orthologs and in turn is used in phylogenetic analysis [19, 20]. The D module contains the hinge region and nuclear localization sites. The E module contains the ligand-binding domain (LBD) domain and AF-2 function crucial in binding coactivators. This module mediates receptor activity by sensing the cellular environment, binding ligand and responding by activating transcription [21, 22]. The LBD is larger than the DBD, moderately conserved among the orthologs of different species, and therefore used in phylogenetic analysis [20, 23]. The F module is of unknown function and is missing in some NRs [21, 24].

NRs regulate multiple physiological pathways such as cell differentiation, resource allocation, reproduction, development, and maintenance of homeostasis. They regulate these diverse physiological conditions by responding to both internal or external cues such as nutrients, steroids, heme, or xenobiotics [25–29]. Thus, NRs are considered conduits that help in the maintenance of homeostatic conditions by responding to various internal and external cues. NRs occupy crucial roles in the disciplines of environmental physiology, endocrinology, pharmacology, toxicology, nutrition, biochemistry, gene regulation, ecology, chemistry, and other fields of study [30].

In this study, we annotated the *F. heteroclitus* NRs through phylogenetic analysis. *F. heteroclitus* are keystone species in the estuaries of the eastern seaboard of North America that are hardy and able to thrive in various conditions of toxic insult, salinity, and temperature [1]. Therefore, we anticipate that analysis of the NRs will ultimately be useful in providing novel insight regarding their unique abilities to withstand diverse stressors and the subsequent transcriptional and physiological responses of these fish.

## 2. Methods

### 2.1. Identification of *Fundulus heteroclitus* nuclear receptors

*F. heteroclitus* NRs were identified using a Basic Local Alignment Search Tool (BLAST) [31] with NR DNA binding domains (DBDs) from human, *Takifugu rubripes*, or *D. magna* [20] against the assembled *F. heteroclitus* genome (<https://my.mdibl.org/display/FGP/Home>) as described previously [22]. Positive BLAST hits were confirmed as nuclear receptors with BLASTp on the NCBI database as were percent identity determinations between orthologous NRs [32].

## 2.2. Phylogenetics

Phylogenetic analysis was performed using methods described previously [20, 33]. The *F. heteroclitus* sequences are predicted protein sequences from the genome browser, and non-killifish sequences used for phylogenetic analysis were derived from the NCBI database, or the Takifugu genome browser (<http://www.fugu-sg.org/>). Phylogenetic comparisons included *F. heteroclitus*, *Takifugu rubripes*, *Danio rerio*, and *Homo sapiens*. A list of nuclear receptors used in comparisons to *F. heteroclitus* is provided in Additional File 1 available online at <http://www.agialpress.com/journals/nurr/2017/101285/>.

Phylogenetic analysis was performed using only the highly conserved DBD and moderately conserved LBD of each receptor. These domains were identified and isolated using the pfam00105 (Zf-C4) and pfam00104 (hormone receptor) designations on the conserved domain database CDD [34] as described previously [20, 23, 35]. The DBD and LBD from 246 NRs from three fish species and humans were aligned using ClustalX default parameters [36] (Additional File 2 available online at <http://www.agialpress.com/journals/nurr/2017/101285/>). Phylogenetic analysis was performed with Maximum Likelihood (ML) using MEGA 6.0 [37]. “Find Best Model” was used to determine the parameters for Maximum Likelihood. Further analysis was performed using the Bootstrap method with 500 replications, the JTT model with Gamma distributed rates among sites [3]. Tree inference options included SPR level 3, BIONJ with a very strong branch filter.

Maximum parsimony and distance parameters were used to provide additional support for the phylogenetic relationships observed. Distance parameters were measured using PAUP 4.0b10 with default characteristics (mean character difference and among site rate variation), and full heuristic searches. Branch support was measured by bootstrap analysis with 1000 replicates. Parsimony was constructed using PAUP version 4.0b10 with heuristic searches, tree-bisection-reconnection, topological constraints not enforced, and multiple tree option in effect with an initial maximum tree setting at 100,000. Branch support was measured by bootstrapping with 10,000 replicates [20, 38]. Maximum Parsimony trees were visualized with PAUP 4.0b10 and Neighbor-Joining trees were visualized with FigTree (<http://tree.bio.ed.ac.uk/software/>).

## 3. Results and Discussion

### 3.1. Nuclear receptor isolation from *Fundulus*

Analysis of the *F. heteroclitus* genome found 74 NRs, which include representatives of all seven subfamily members. Additional File 3 (available online at <http://www.agialpress.com/journals/nurr/2017/101285/>) provides links to the scaffold for each NR, its cDNA and its protein sequence. Seventy-four NRs are in range of what has been found in other fish species as 68 NRs were found in *Takifugu rubripes* [39] and 72 NRs in *Tetraodon nigriviridis* [40] although recent improvements in the Takifugu genome indicates 73 NRs [23]. The number of NRs in teleost genomes is significantly greater than those found in invertebrates and mammals because of whole genome duplication events [41]. The number of NRs in the common carp (*Cyprinus carpio*) is even greater (137) than other fish species because of an additional genome

duplication event [23]. Genome duplication is often followed by loss of some genes, gain of function of some genes (neofunctionalization) including differences in spatial or temporal gene expression between paralogs, or partitioning of ancient functions on duplicated genes (subfunctionalization) [42].

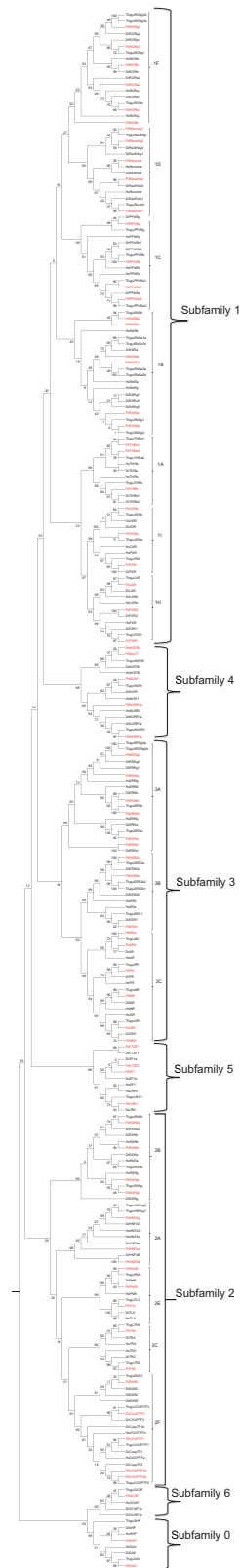
### 3.2. Phylogenetics

Phylogenetic analysis by Maximum Likelihood (ML) confirms the presence of all seven NR subfamilies in *F. heteroclitus*, and demonstrates that there are 2 NR0 members, 30 NR1 members, 17 NR2 members, 15 NR3 members, 5 NR4 members, 4 NR5 members, and 1 NR6 member (Figure 1). This file is also available as an expandable pdf (Additional File 4 available online at <http://www.agialpress.com/journals/nurr/2017/101285/>). The percentage of each subfamily of NRs is relatively similar between *F. heteroclitus*, the fish species examined and humans with minor exceptions. The three different phylogenetic models used (Maximum Likelihood, Maximum Parsimony, Neighbor-Joining) agreed at the group level and often at the subfamily level, but there are differences at the base of the phylogram; primarily the relationship of the 0-subfamily to the 5 and 6 subfamilies. However, the ancient bootstrap values are typically not significant using any of the analysis with Maximum Parsimony rarely being able to resolve distinct clades on the left hand side of the tree. The Neighbor-Joining and Maximum Parsimony phylogenetic trees are provided as additional files (Additional Files 5, 6 available online at <http://www.agialpress.com/journals/nurr/2017/101285/>).

### 3.3. NR0 Subfamily

The NR0 subfamily contains two groups, NR0A and NR0B. NR0A receptors lack a ligand-binding domain (LBD) and NR0B receptors lack a DNA-binding domain (DBD). Similar to other vertebrates, there are no NR0A members (knirps) in *F. heteroclitus* [20, 43]. Mummichogs contain two NR0B members, SHP and DAX (Figure 1; Table 1). We found that DAX/SHP fall within the 6 subfamily based on Maximum Likelihood but with low posterior probabilities (50%) (Figure 1). Maximum parsimony resolved the 2, 6, and 0 subfamilies as separate subfamilies but without resolving their evolutionary relationship to each other (Additional File 5 available online at <http://www.agialpress.com/journals/nurr/2017/101285/>). Previous work using parsimony and NJ suggested with 73% confidence that the NR0B group is evolutionarily related to 2 subfamily [44]. Bayesian Inference also indicated that these NRs evolved as part of the 2 subfamily [22]. However, most studies performed do not include them as part of a phylogenetic tree because of the reduced molecular information due to the lack of the LBD and subsequent uncertainty in the analysis [20, 45, 46].

Overall, the resolution of the 0 subfamily is poor. NR0B members clearly form their own group based on the lack of a C domain; however, their evolutionary relationship to other NRs is questionable. In turn, unlike other receptors, NR0 members are not named for their phylogenetic position but instead for their lack of a key domain [47]. The NR0B receptors SHP and DAX primarily work as co-regulators as they both contain LxxLL domains typically found in coregulators [48–50]. DAX is involved in reproductive development and steroidogenesis; SHP regulates cholesterol and glucose homeostasis [51].



**Figure 1:** Phylogenetic relationship of nuclear receptors as determined by ML. The phylogenetic tree is shown with bootstrap support values (frequency of occurrence) from ML at each node. Species included are *Takifugu rubripes* (*Tfugu*), *H. sapiens* (*Hs*), *Danio rerio* (*Dr*), and *Fundulus heteroclitus* (*Fh*). All *F. heteroclitus* sequences are in red.

**Table 1:** Nuclear receptors from *Fundulus* compared to those described in other species.

Group	<i>F. heteroclitus</i>	<i>H. sapiens</i>	<i>D. rerio</i>	<i>T. rubripes</i>	<i>T. nigroviridis</i>	<i>D. pulex</i>	<i>D. melanogaster</i>	
<b>0A</b>						KNR-R1	KNI	
						KNR-R2	KNRL	
							EGON	
<b>0B</b>	DAX	DAX	DAX	DAX				
	SHP	SHP	SHP	SHP				
<b>1A</b>	THR $\alpha$ 1	THR $\alpha$	THR $\alpha$	THR $\alpha$ 1	THR $\alpha$ 1	THRL11		
	THR $\alpha$ 2			THR $\alpha$ 2	THR $\alpha$ 2			
	THR $\beta$	THR $\beta$	THR $\beta$ 1	THR $\beta$	THR $\beta$			
			THR $\beta$ 2					
<b>1B</b>	RAR $\alpha$ 1	RAR $\alpha$	RAR $\alpha$	RAR $\alpha$ 1a	RAR $\alpha$ 1	RARL10		
	RAR $\alpha$ 2			RAR $\alpha$ 1b	RAR $\alpha$ 2	(unresolved may be member of 1M family)		
				RAR $\alpha$ 2a				
				RAR $\alpha$ 2b				
	RAR $\beta$ 1	RAR $\beta$		RAR $\beta$				
	RAR $\beta$ 2							
	RAR $\gamma$ 1	RAR $\gamma$	RAR $\gamma$ 1	RAR $\gamma$ 1	RAR $\gamma$			
	RAR $\gamma$ 2		RAR $\gamma$ 2	RAR $\gamma$ 2				
				RAR $\gamma$ 3				
	<b>1C</b>	PPAR $\alpha$ 1	PPAR $\alpha$		PPAR $\alpha$ 1	PPAR $\alpha$ 1		
		PPAR $\alpha$ 2		PPAR $\alpha$ 2	PPAR $\alpha$ 2	PPAR $\alpha$ 2		
PPAR $\beta$		PPAR $\beta$	PPAR $\beta$ 1	PPAR $\beta$	PPAR $\beta$			
			PPAR $\beta$ 2					
PPAR $\gamma$		PPAR $\gamma$	PPAR $\gamma$	PPAR $\gamma$	PPAR $\gamma$			
<b>1D</b>	Rev-erb- $\alpha$	Rev-erb- $\alpha$	Rev-erb- $\alpha$	Reb-erb- $\alpha$	Rev-Erb- $\alpha$ 1	E75	E75	
					Rev-Erb- $\alpha$ 2			
	Rev-erb- $\beta$ 1	Rev-erb- $\beta$	Rev-erb- $\beta$ 1	Rev-erb- $\beta$	Rev-erb- $\beta$ 1			
	Rev-erb- $\beta$ 2		Rev-erb- $\beta$ 2		Rev-erb- $\beta$ 2			
	Rev-erb- $\gamma$ 1		Rev-erb- $\gamma$ 1	Rev-erb- $\gamma$	Rev-erb- $\gamma$			
Rev-erb- $\gamma$ 2		Rev-erb- $\gamma$ 2						
<b>1E</b>					E78	E78		
<b>1F</b>	ROR $\alpha$ 1	ROR $\alpha$	ROR $\alpha$ 1	ROR $\alpha$	ROR $\alpha$ 1	HR3	DHR3	
	ROR $\alpha$ 2		ROR $\alpha$ 2		ROR $\alpha$ 2			
	ROR $\beta$	ROR $\beta$	ROR $\beta$	ROR $\beta$	ROR $\beta$			
					ROR $\beta$			
	ROR $\gamma$ 1	ROR $\gamma$	ROR $\gamma$ 1	ROR $\gamma$ 1	ROR $\beta$			
	ROR $\gamma$ 2		ROR $\gamma$ 1b	ROR $\gamma$ 2a				
RORc			ROR $\gamma$ 2b					

<b>Group</b>	<i>F. heteroclitus</i>	<i>H. sapiens</i>	<i>D. rerio</i>	<i>T. rubripes</i>	<i>T. nigroviridis</i>	<i>D. pulex</i>	<i>D. melanogaster</i>
<b>1H</b>	LXR $\alpha$	LXR $\alpha$	LXR $\alpha$	LXR $\alpha$	LXR $\alpha$	EcR $\alpha$	EcR
		LXR $\beta$				EcR $\beta$	
	FXR1	FXR	FXR1	FXR1	FXRa		
	FXR2		FXR2	FXR2	FXRa		
					FXRb		
<b>1I</b>	VDR-A	VDR	VDR-A	VDR-A	VDR-A1		
	VDR-B			VDR-B	VDR-A2		
					VDR-B		
	PXR	PXR	PXR	PXR	PXR		
		CAR					
<b>1J</b>						HR96	DHR96
<b>1L</b>						HR97a	
						HR97b	
						HR97g	
<b>2A</b>	HNF4 $\alpha$	HNF4 $\alpha$	HNF4 $\alpha$	HNF4 $\alpha$	HNF4 $\alpha$	HNF4	HNF4
	HNF4 $\beta$		HNF4 $\beta$				
	HNF4 $\gamma$	HNF4 $\gamma$	HNF4 $\gamma$	HNF4 $\gamma$ 1	HNF4 $\gamma$		
				HNF4 $\gamma$ 2			
<b>2B</b>		RXR $\alpha$	RXR $\alpha$	RXR $\alpha$	RXR $\alpha$	RXR	USP
	RXR $\beta$ -p				RXR $\alpha$		
	RXR $\beta$ 1	RXR $\beta$	RXR $\beta$ 1	RXR $\beta$	RXR $\beta$		
	RXR $\beta$ 2		RXR $\beta$ 2		RXR $\beta$		
	RXR $\gamma$ 1	RXR $\gamma$	RXR $\gamma$	RXR $\gamma$	RXR $\beta$		
	RXR $\gamma$ 2						
<b>2C</b>	TR2	TR2	TR2	TR2	TR2		
	TR4	TR4	TR4	TR4	TR4		
<b>2D</b>						HR78	DHR78
<b>2E</b>	TLX	TLX	TLX	TLX	TLX	TLL	TLL
	PNR 1	PNR	PNR	PNR	PNR	PNR	PNR
	PNR 2					DSF	DSF
						NR2E6	FAX-1
<b>2F</b>	COUP-TF1	COUP-TFa	COUP-TF1a	COUP-TF1	COUP-TFa	SVP	SVP
		COUP-TFb	COUP-TF1b	COUP-TF1	COUP-TFa		
	COUP-TF2a		COUP-TF2	COUP-TF2	COUP-TFb		
	COUP-TF2b				COUP-TFb		
	COUP-TF3		COUP-TF3	COUP-TF3	COUP-TFg		
	EAR2	EAR2	EAR2	EAR2	EAR2		
			EAR2	EAR2	EAR2		
<b>3A</b>	ESR1	ESR1	ESR1	ER1	ESR1		
	ESR2a	ESR2	ESR2a	ERb	ESR2a		
	ESR2b		ESR2b	ER2a	ESR2b		
				ER2b			



Group	<i>F. heteroclitus</i>	<i>H. sapiens</i>	<i>D. rerio</i>	<i>T. rubripes</i>	<i>T. nigroviridis</i>	<i>D. pulex</i>	<i>D. melanogaster</i>
<b>3B</b>	ERR $\alpha$	ERR $\alpha$	ERR $\alpha$	ERR $\alpha$	ERR $\alpha$	ERR	ERR
	ERR $\beta$ 1	ERR $\beta$	ERR $\beta$	ERR $\beta$	ERR $\beta$ 1		
	ERR $\beta$ 2				ERR $\beta$ 2		
	ERR $\delta$		ERR $\delta$				
	ERR $\gamma$ 1	ERR $\gamma$	ERR $\gamma$ 1	ERR $\gamma$ 2a	ERR $\gamma$		
	ERR $\gamma$ 2		ERR $\gamma$ 2	ERR $\gamma$ 2b	ERR $\gamma$		
				ERR $\gamma$	ERR $\gamma$		
					ERR $\gamma$		
<b>3C</b>	GR	GR	GR	GR	GR		
	GR2			GR2	GR2		
	MR	MR	MR	MR	MR		
	PR	PR	PR	PR	PR		
	AR $\alpha$	AR	AR	AR $\alpha$	AR $\alpha$		
	AR $\beta$			AR $\beta$	AR $\beta$		
<b>4A</b>	NGF1B	NGF1B	NGF1B	NGF1Ba	NGF1B	HR38	DHR38
	Nur77				NGF1B		
	NURR1a	NURR1	NURR1a	NURR1	NURR1		
	NURR1b		NURR1b				
	NOR1	NOR1	NOR1	NOR1	NOR1		
<b>5A</b>	SF1	SF1	SF1a	FTZ-F2	SF1	FTZ-F1	FTZ-F1
	FTZ-F2/SF1b		SF1b				
	LHR1	LRH1	LRH1	LRH1	LRH1		
	FTZ-F1		FTZF1	FTZF1	FTZ-F1		
<b>5B</b>					DpHR39	DHR39	
<b>6A</b>	GCNF	GCNF	GCNF1a	GCNF	GCNF	DpHR4	DHR4
			GCNF1b				

### 3.4. NR1 Subfamily

The NR1 subfamily is the largest subfamily in *F. heteroclitus* with 30 members. 40.5 Percent of mummichog NRs are in the 1-subfamily; 36-40% of NRs are in the 1-subfamily of other fish species and 40% of NRs are 1 subfamily members in humans. The 1-subfamily separates into two distinct clades; one clade includes the NR1B (RAR), NR1C (PPAR), NR1D (Reverb), and NR1F (ROR) groups, and the other one includes the NR1A (THR), NR1H (LXR/FXR), and NR1I (VDR) groups (Figure 1). There is disagreement in some of our different phylogenetic analysis at the extreme left of the trees where bootstrap values may drop as low as 23 indicating poor resolution.

The fish species, including *F. heteroclitus* show significant expansion of NR1B (RAR), NR1D (Rev-erb), and NR1F (ROR) groups relative to humans (Table 1). Interestingly, all of these NRs are found in the same NR1 clade. There are 6 members of the *F. heteroclitus* RAR group. Humans have 3 members and there are 3–7 RAR members in the other fish species investigated. There are 5 members of the Rev-erb clade and 6 members of the ROR groups in *F. heteroclitus*.

Five members in the Rev-erb group are typical in fish; however, the other fish species examined only have 5 ROR members. In comparison, humans have 2 Rev-erb and 3 ROR members (Table 1).

The RAR, Rev-erb, and ROR groups are important in lipid and glucose metabolism, gas-response, development, promoting T-cell differentiation, inflammation, and circadian rhythms [52–54]. Several genes are coordinately regulated by Rev-erb $\alpha$  and ROR $\alpha$  as they share the same response elements but exert opposing effects on transcription. Significant expansion of these two opposing sets of NRs in fish species including *F. heteroclitus* appears synchronous. It is thought that the crosstalk between the two receptor groups helps regulate their transcriptional and physiological networks, including circadian rhythms, lipid and glucose metabolism, and inflammation [55, 56].

In contrast, the NR1C (PPAR) group shows little expansion in *F. heteroclitus* or other fish species. There are three members in humans and four in each of the fish species investigated (Table 1). PPARs are crucial in the regulation of lipids [27, 57]. The pufferfish (*Takifugu* and *Tetraodon*) genomes both show two PPAR $\alpha$  NRs similar to *F. heteroclitus*, while the Atlantic salmon (*Salmo salar*) and zebrafish (*D. rerio*) genomes have two members of the PPAR $\beta/\delta$  group [41, 58, 59]. *Danio* are ancient Ostariophysi, *Salmo* are Salmoniformes, and *Fundulus* and *Takifugu* are both modern Percomorphs [60]. Because of whole genome duplication in teleosts [42], it is most likely that an ancient relative lost PPAR $\beta/\delta$  in the Percomorphs and PPAR $\alpha$  in the early teleosts. However, it cannot be completely ruled out that separate duplication events occurred in the Percomorphs and early teleosts as individual gene duplication events have occurred multiple times including cytochrome P450s, opsins, and NRs [20, 35, 61–63]. The organ distribution of PPARs differs from mammals. For example, pufferfish show wide tissue distribution of the PPARs; in mammals only PPAR $\beta/\delta$  is widely distributed [58]. Changes in organ distribution or domain structure and function is common in neofunctional retained duplicated receptors. Therefore, we performed pairwise comparisons between human NRs and their *F. heteroclitus* orthologs with duplications (Table 2). Mummichog PPAR $\alpha$ 2 shows greater differences in its LBD than PPAR $\alpha$ 1 compared to human PPAR $\alpha$  suggesting neofunctionalization (Table 2).

**Table 2:** Percent Identity comparisons of the DNA binding domain (DBD) and ligand binding domain (LBD) of orthologous human and *F. heteroclitus* nuclear receptors with Blastp.

Nuclear Receptor <sup>a</sup>	DBD	LBD	Ligands <sup>b</sup>
SHP		51	
DAX		51	
THR $\alpha$ 1-110	92	84	Thyroid hormones [114]
THR $\alpha$ 2-10098	96	91	
PPAR $\alpha$ 1	86	85	Leukotriene B4, 1-palmityl-2-oleoyl-sn-glycerol-3-phosphocholine,
PPAR $\alpha$ 2	86	73	Fatty acids [115]
Rev-Erb- $\beta$ 1-10090	94	83	Heme [114]
Rev-Erb- $\beta$ 2-10062	93	77	
ROR $\alpha$ -9958	99	93	
ROR $\alpha$ -369	91	75	
ROR $\gamma$ -9885	90	43	

Nuclear Receptor <sup>a</sup>	DBD	LBD	Ligands <sup>b</sup>
ROR $\gamma$ -10005	90	47	
FXR1-10028	96	72	5 $\beta$ -bile acids [83]
FXR2-9861	84	35	
VDR-A	93	79	1,25-OH vitamin D3, bile salts [116, 117]
VDR-B	94	80	
PXR	72	58	Xenobiotics, bile salts [73, 80]
RXR $\beta$ 1-483	94	88	9-cis retinoic acid, retinoids [114, 118]
RXR $\beta$ 2-9867	90	79	
RXR $\gamma$ 1-9885	94	95	
RXR $\gamma$ 2-9880	94	95	
PNR1-9949	93	74	
PNR2-195	91	59	
ESR1	96	66	17 $\beta$ -estradiol [119]
ESR2 $\alpha$ -10024	96	68	
ESR2 $\beta$ -10026	96	65	
ERR $\beta$ -9910	100	76	
ERR $\beta$ -3	100	84	
ERR $\gamma$ -10140	97	94	
ERR $\gamma$ 2-9925	91	81	
GR-0	96	74	Cortisol [120]
GR2-10031	96	74	
AR $\alpha$ -9941	65	59	Testosterone, 11-ketotestosterone, 17 $\alpha$ -methyltestosterone [121]
AR $\beta$ -9884	91	70	
NGF1B	99	69	
Nurr77	93	66	
NURR1a-9995	96	95	
NURR1b-114	97	58	
SF1a	72	58	Phosphatidylinositol, phosphatidylcholine [122]
FTZ-F2/SF1b-224	84	nd	
<sup>a</sup> Scaffold is often provided after the receptor to aid in determining the receptor in question.			
<sup>b</sup> Known or putative endogenous physiological ligands for fish (or human if fish unknown) are included for the receptors that show high divergence from humans or have a duplicated receptor with potential neofunctionalization.			

The presence of PPARs in fish may make them sensitive to peroxisome proliferation or perturbations in lipid allocation and homeostasis. For example, peroxisome proliferation or induction of biomarkers of peroxisome proliferation has been measured in fish exposed to PAHs, pharmaceuticals, phthalates, alkylphenols, and pesticides [64–69]. Peroxisome proliferation has been measured in mummichogs following 2,4-D exposure [70].

Furthermore, pharmaceuticals such as the PPAR $\alpha$  activator, gemfibrozil reduced n-3 fatty acids in rainbow trout (*Oncorhynchus mykiss*), which may reduce the nutritional quality of the fish, perturb their ability to acclimate to changes in water temperature, and repress their ability to reproduce following migration [64]. The PPAR $\gamma$  activator, TBT activated an obesogen

response by increasing body weight and whole-lipid content in Chinook salmon (*Oncorhynchus tshawytscha*) and condition factor, triglycerides, and hepatosomatic index in zebrafish [71]. Interestingly, toxicants that activate PPARs may enhance condition factor while also increasing other stress responses typically associated with poor physiological conditions [72].

The other NR1 clade includes the NR1A (THR), NR1H (LXR/FXR), and NR1I (VDR/PXR) groups. THR is split into two subgroups, THR $\alpha$  and THR $\beta$ . There are three THR members in each of the fish species examined compared to two in humans that only contain one THR $\alpha$  and THR $\beta$  member. Pufferfish and mummichogs contain two THR $\alpha$  members and one THR $\beta$  members; *D. rerio* contains one THR $\alpha$  member and two THR $\beta$  members (Table 1).

The NR1I group in fish that contains the vitamin D receptor (VDR), PXR, and CAR, has been relatively well studied because of the roles of PXR and CAR in acclimation to foreign chemicals [21, 73]. PXR was previously cloned in *F. heteroclitus* from a PCB polluted site and this PXR is nearly identical to the PXR sequenced during the genome project with the exception of a three amino acid region at amino acid 190 that is missing in the *F. heteroclitus* genome sequence [74].

PXR, but not CAR, has been identified in teleost species [39, 75]. Originally it was thought that CAR diverged from PXR at some later evolutionary point because CAR had only been found in mammals. However, recent phylogenetic data, examination of Saurapsid genomes such as reptiles and birds [76] and the lobe-finned fish *Coelocanth* indicates that CAR was lost in teleosts and PXR was lost in reptiles and birds [77]. Therefore, it is thought that the lobe-finned fish that arose 400 million years ago during the Devonian period and are the ancestors of all tetrapods, contained CAR and PXR [78, 79].

The DBD and LBD of *F. heteroclitus* PXR are highly diverged from humans with pairwise comparisons showing 72% identity to human DBD and 58% to human LBD (Table 2). This is typical of fish PXR as previous comparisons to human PXR show 61-73% sequence identity to the DBD and 52-57% identity to the LBD [78]. Only AR $\alpha$  (65%) and SF1a (72%) show as much divergence in the DBD as PXR; only ROR $\gamma$ , SF1, FXR2, SHP, and DAX show similar divergence within the LBD when compared to mammals [78]. However, with the exception of the NR0 members, SHP and DAX, all the other receptors showing high divergence have duplicated members and at least one of the duplicates appears to show neofunctionalization based on the large change in amino acid identity (Table 2).

The large differences in LBD sequences for PXR provides biochemical support for the significant differences in activation profiles between different fish species and between fish and humans, especially for fenvalerate and several organochlorine insecticides [73]. Ligand activation of zebrafish PXR matched only 30% of the representative human PXR ligands further indicating the divergence of fish and human PXR [75]. Ligands tested include primarily bile acids, steroids and pharmaceuticals. Of these, Phenobarbital, clotrimazole, dihydrotestosterone, androstanol and 5 $\beta$ -pregnane-3,20-dione all activated the zebrafish PXR. In general, human PXR can be activated by many bile salts, including both C<sub>24</sub> and C<sub>27</sub> bile salts. However, the zebrafish PXR was only activated by C<sub>27</sub> bile salts with a strong preference for sulfated bile alcohols [80]. The C<sub>27</sub> pathway is an ancient pathway found in early fish and amphibians and it has been hypothesized that PXR has evolved to deal with the increasingly complex bile salt synthetic pathways [76]. This is in contrast to other theories that suggest that the reason for the great divergence in the LBD of PXR between species, including zebrafish and *Fugu*,

is in response to the different sets of xenobiotic/environmental challenges encountered by the different species [81].

The FXR/LXR group (NR1H) in *F. heteroclitus* contains one LXR member, LXRA, and two FXR members. The FXR/LXR group is related to the ecdysone receptors in invertebrates (Table 1) [20, 82]. Humans have two LXR members and one FXR that is more closely aligned with FXR1 (Figure 1). The existence of two or more FXR members is common among fish species. The green pufferfish (*T. nigroviridis*) and Japanese medaka (*Oryzias latipes*) have at least 3 FXR members because they have two similar FXRA members (Table 1; Figure 1). In medaka, FXRA2 is activated by C24 bile acids and GW4064, but FXRA1 is not activated by these common ligands probably due to differences in the A/B domain [83]. Interestingly, in *Fundulus*, FXR2's LBD has the lowest percent identity when compared to humans (Table 2), indicating neofunctionalization and a different activation profile than humans.

In addition, there is significant diversity in FXR ligand responses in fish species [84]. Tetraodon FXR has a ligand selectivity profile very similar to human FXR, with activation by the synthetic ligand GW4064 and the bile acid, chenodeoxycholic acid. Furthermore, modeling and docking studies suggest that Tetraodon's ligand-binding pocket more similar to mammalian FXRs than to lamprey or zebrafish FXRs [85], which are activated by 5 $\alpha$ -bile alcohols but not by the evolutionarily more recent 5 $\beta$ -bile acids [86]. Based on phylogenetics, *Fundulus* FXR is more like Tetraodon and medaka than zebrafish (Figure 1) [78].

### 3.5. NR2 Subfamily

The NR2 subfamily is the second largest subfamily of NRs in *F. heteroclitus* with 18 NRs. NR2 is divided into 5 groups A, B, C/D, E and F. Of these, Group 2B (RXR) has expanded the most of the fish species investigated relative to humans, and group 2F (COUP) has expanded in all of the fish species investigated relative to human NRs (Table 1). In addition, *F. heteroclitus* has three Group 2E members, which are involved in eye development in zebrafish [87, 88], because it has two PNR receptors (Table 1). To our knowledge, other fish species only have one PNR with the recent exception of Nile tilapia (*Oreochromis niloticus*) and medaka [23, 78]. Considering the phylogenetic distance of the receptors it is unlikely that this is a recent event (Figure 1) and instead it is more likely that the second PNR was not lost following the genome wide duplication in fishes [41]. Interestingly, invertebrate species often contain more group 2E members than vertebrates (Table 1) [20].

RXRs bind to retinoids, are crucial for growth and development, and are key heterodimeric partners with several other NRs [89, 90]. COUP-TFs are necessary for growth and development, including venous and lymphatic development in zebrafish [91, 92]. Interestingly, COUP-TF members have also been shown to interact with a few other NRs including RXR $\alpha$ , RAR, THR, ERR $\gamma$ , ER $\alpha$ , and other COUP-TF members as hetero- and homodimers [90]; however they typically repress transcription including RAR/RXR responses [93]. Thus, the NRs that have shown expansion may have a repressive counterpart that also expanded.

There are three RXR members in humans and 4-5 members in *F. heteroclitus* of which none appear to be RXR $\alpha$  members. The exact number of RXR members is unknown because of the brevity of scaffold 2083 (Additional Figure 3 available online at <http://www.agialpress>).

com/journals/nurr/2017/101285/), which ends at 17,995 bp and in turn cuts off a potential RXR member at the AB domain (AF-1). Therefore, this RXR member appears to be a pseudogene but may not be. This partial gene contains a start site, two introns, and a stop codon. It is also structurally different than the other RXR $\beta$  genes that have two relatively close exons near the 5'-end. However given that the scaffold ends shortly after the stop codon it cannot be completely ruled out that there might be other splice sites and exons following. Interestingly, scaffold's 2083 gene fragment aligns well with RXR $\beta$  members from several fish species. If it is a RXR $\beta$  member than phylogenetic analysis would indicate that *F. heteroclitus* has 3 RXR $\beta$ , 2 RXR $\gamma$ , and 0 RXR $\alpha$  members (Table 1; Figure 1). Initial examination of the genome by BLAST suggested that the RXR at scaffold 9880 may be a RXR $\alpha$  member; however phylogenetic analysis indicates that this NR is an RXR $\gamma$  member with a weak posterior probability of 37. While the posterior probability is weak, Maximum Likelihood (Figure 1), Maximum Parsimony (Additional File 5 available online at <http://www.agialpress.com/journals/nurr/2017/101285/>), and Neighbor-Joining (Additional File 6 available online at <http://www.agialpress.com/journals/nurr/2017/101285/>) all agree that complete RXR members are found within RXR $\beta$  or RXR $\gamma$  subgroups and therefore at this time no RXR $\alpha$  member was found in *F. heteroclitus*. If the RXR at scaffold 2380 is a complete RXR $\alpha$  member than the *F. heteroclitus* genome will have at least 75 NRs. The lack of an RXR $\alpha$  member would be surprising if not unprecedented as we did not find other fish or mammalian species lacking RXR $\alpha$ .

Group 2A (HNF) is an ancient group [46] that expanded greatly in *C. elegans* [94]. HNF4 contains 3 members, which is typical of most fish species (Table 1). The HNF group of receptors has been highly studied in fish species, but what work has been done indicates that similar to mammals the HNF4 receptors are enrich in the liver and regulate liver enriched gene expression [95] sometimes in conjunction with other HNFs including HNF1 and COUP-TF1 [95, 96].

### 3.6. NR3 subfamily

There are 15 NR3 subfamily members in *F. heteroclitus*. In comparison, there are 15 in *T. rubripes*, 16 in *T. nigroviridis*, and 9 in humans. There are 6 estrogen related receptors (ERRs; NR3B) in *F. heteroclitus*, while most fish species contain 5-7 and humans contain 3 (Table 1). There are 3 estrogen receptors (ERs) (NR3A) in *F. heteroclitus*, while most fish species contain 3-4 receptors and humans contain 2 (Table 1). Overall, there is a significant expansion of the 3A and 3B group in *F. heteroclitus* and other fish species relative to humans. Interestingly, *Fundulus* chronically exposed to estrogenic pollutants developed heritable splicing variants that show different transcriptional responses than wild-type ER potentially as an adaptive mechanism to the estrogens found in the polluted environment [11, 18].

There are 6 NR3C (glucocorticoid, mineralcorticoid, androgen, and progesterone receptors) members in *F. heteroclitus*. There are also six members in *Tetraodon* and *Takifugu*, but only 4 NR3C members in zebrafish and humans (Table 1). *F. heteroclitus* has two glucocorticoid receptors (GR), two androgen receptors (AR), and one mineralocorticoid (MR) and one progesterone receptor (PR) within the NR3C group. This is consistent with several other fish species such as *Tetraodon* and *Takifugu* (Figure 1; Table 1) that have two GRs and two ARs [39, 40].

In addition, medaka, stickleback (*Gasterosteus aculeatus*), common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) all have two GRs [97, 98]; however, rainbow trout and common carp have undergone an additional round of genome duplication that may explain why these species have two GRs. Zebrafish (*Danio rerio*), only has one GR, GR2, that is missing a 9 amino acid sequence between the zinc fingers of the DBD [98, 99]. It is interesting to speculate that the increase in GRs are crucial in salt balance, especially in marine or estuarine species [12, 100, 101] as GR regulates salt balance in part by regulating the transcription of the sodium-chloride cotransporter and sodium-potassium ATPase [102, 103]. Carp, like zebrafish, are Ostariophysi and they contain two GRs. Thus, it appears that zebrafish unlike most other teleosts, lost a GR after the genome wide duplication in fish [104, 105] and carp (and rainbow trout) have two GR because of the second genome duplication.

The loss of the second AR occurred in several fish species unlike the loss of GR2, which occurred in only a few species. *F. heteroclitus*, *Takifugu* and *Tetraodon* all have two ARs. However, Cypriniformes such as zebrafish, Siluriformes such as catfish, and Salmoniformes, such as salmon and trout all have one AR. Interestingly, early teleosts such as Osteoglossiformes (arowana, knifefish) and Anguilliformes (eels) contain two very similar ARs, while Percomorphs such as pufferfish and *Fundulus* show divergence between their ARs [106] with different binding affinities for steroids and xenobiotics [107]. AR $\alpha$  which shows high divergence relative to humans and AR $\beta$  (Table 2), also has higher transactivation activity confirming neofunctionalization [108].

### 3.7. NR subfamilies 4-6

In *F. heteroclitus*, subfamilies 4-6 account for nine NRs; one more than *T. nigroviridis* because *F. heteroclitus* contains 5 NR4 members. Overall, there is a small expansion of the 4-subfamily as most fish species contain 4 members; however *F. heteroclitus* and Nile tilapia (*O. niloticus*) contain 5 members. Humans contain three NR4 members and most invertebrates contain one [20, 22]. Some NR4 members are crucial in brain differentiation, cell cycle, inflammation, and atherosclerosis [109–111]. The NR4 subfamily is related to the NR1 subfamily, and is an ancient family that is ligand independent [46, 112]. Several of the NR4 members show relatively large differences between mummichog and human LBDs. This divergence is unique to the duplicated NR4A2 member, Nurr1b, but occurs in both NR4A1 member (Table 2).

The NR5 subfamily contains FTZ members that regulate development. There are four members of the NR5 subfamily. SF-1 members are crucial in sex determination and down-regulated upon exposure to anthropogenic estrogenic chemicals during sex reversal [113]. SF1a and FTZ-F2 show very high divergence relatively to human SF1 (Table 2). GCNF, which is the only NR6 subfamily member, is involved in growth and maturation [112]. Zebrafish have two GCNF members but other fish species, *Daphnia*, and humans have one (Table 1) [23].

## 4. Conclusions

There are at least 74 full length NRs in the *F. heteroclitus* genome spanning all seven gene subfamilies of which 40% are in the NR1 subfamily often involved in circadian rhythms, development, energy metabolism and resource allocation. Fish species show expansion of the RAR

(NR1B), Reverb (NR1D), ROR (NR1F), COUPTF (NR2F), ERR (NR3B), RXR (NR2B), and to a lesser extent the NGF (NR4A), and NR3C steroid receptors (GR/AR). Of particular interest is the co-expansion of opposing NRs, Reverb-ROR, and RAR/RXR-COUPTF, and the potential lack of an RXR $\alpha$  member in the *F. heteroclitus* genome.

## Competing Interests

The authors declare that they have no competing interests.

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