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An Aryl Hydrocarbon Receptor Sequence from the Caecilian Gymnopis multiplicata Suggests Low Dioxin Affinity is Common to All Amphibian Orders

Sarah Kazzaz
Kenyon College

Wade Powell
Kenyon College

An Aryl Hydrocarbon Receptor Sequence from the Caecilian *Gymnopis multiplicata* Suggests Low Dioxin Affinity Is Common to All Amphibian Orders

Sarah Kazzaz, Wade Powell
Department of Biology, Kenyon College, Gambier, Ohio 43022 USA

Abstract

The aryl hydrocarbon receptor (AHR) mediates the toxic effects of dioxin-like compounds, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 6-formylindolo[3,2-b]carbazole (FICZ), in vertebrates. Ligand binding activates the receptor, causing alternate gene expression and toxicity. While most vertebrates possess at least one AHR that binds TCDD with high affinity (sub nM K_d), all characterized amphibian AHRs bind TCDD with at least 25-fold lower affinity. Our previous analyses of sequence, structure, and function of AHRs from *Xenopus laevis* (frog; order Anura) and *Ambystoma mexicanum* (salamander; order Urodela) identified three key amino acid residues in the ligand binding domain (LBD) that underlie low affinity binding. By studying the AHR of the caecilian *Gymnopis multiplicata*, we seek to determine whether all amphibian AHRs share the low affinity phenotype. This species represents order Apoda, a clade of legless amphibians that diverged early from the common lineage of the class, prior to the split between frogs and salamanders. RNA (derived from tissue collected in Nicaragua in 1984 and provided by the Museum of Vertebrate Zoology at the University of California, Berkeley) was used to clone cDNA. This 92.7 kDa encoded protein is monophyletic with vertebrate AHR1s and shares 59% identity with *Xenopus laevis* AHR1β and 63% identity with *A. mexicanum*. The LBD sequence includes all three residues that compromise the low TCDD affinity signature sequence, suggesting TCDD insensitivity. We tested this hypothesis with transactivation assays employing pGudLuc6.1, a luciferase reporter gene governed by the enhancer region from mouse CYP1A1. The EC_{50} for the reporter gene induction by TCDD and FICZ is similar to that of other amphibians. Together, sequence and structural analysis, as well as dioxin responsiveness suggests caecilian AHR binds dioxin-like compounds with low affinity. We predict that *Gymnpois multiplicata* is insensitive to the toxic effects of TCDD and other xenobiotic AHR agonists. Moreover, low affinity AHR and dioxin insensitivity emerged early in amphibian evolution.

TCDD Insensitivity in Amphibians

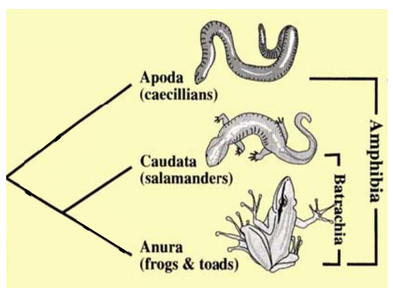
AHRs are found in all vertebrate species, illustrating its functional importance throughout evolution for physiology and development. Frogs and salamanders display low sensitivity to AHR agonists relative to all other vertebrates[2,3,4]. This low sensitivity can be explained by three conserved amino acid residues in AHR ligand-binding domain (LBD)—N325, A354, and A370 in frog AHR1β[3]. These residues confer low affinity for TCDD, and thereby lower AHR response and overall sensitivity of these species to TCDD toxicity.

These lower affinity residues have been observed in two of the three orders of amphibians, Anura and Urodela. The Anura order consists of toads and frogs and the AHRs of *X. laevis* and *X. tropicalis* have been thoroughly investigated. Likewise, the AHR of the Urodela order, which consists of salamanders, has been studied in the Mexican axolotl and mudpuppy. No AHRs from the order Apoda have yet been characterized.

6-formylindolo[3,2-*b*]carbazole (FICZ), a candidate endogenous agonist, binds these AHRs with higher affinity than TCDD[6,7].

Amphibian Evolution

Caecilians can help to determine when low affinity AHRs emerged during evolution.



Laboratory Manual for Animal Diversity: An Evolutionary Approach. San Diego University, 2002.

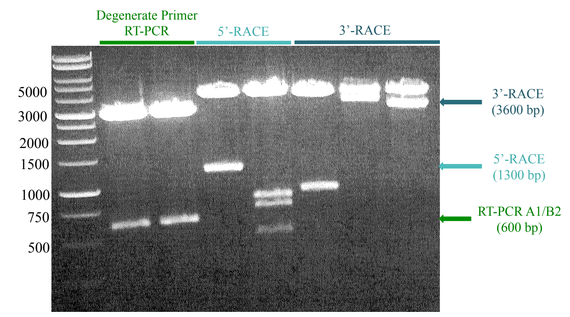
Objective

To determine whether low TCDD affinity is common to AHRs from all amphibian lineages or restricted to Anura and Urodela, we began characterization of AHR from the caecilian *G. multiplicata*. Analysis of AHR sequences and structure will clarify when low-affinity AHR arose—before or after caecilians diverged from the common amphibian lineage.

Results

Figure 1

a



b

RT-PCR			
Degenerate Primers	A1	Forward	5'-CGGGATCCARGCICTSAAYGGITT-3'
	B2	Reverse	5'-GCTCTAGAGCTCIRCYTCIGTRTAICC-3'
Cycling Conditions	94°C/ 5 min; (94°C/ 15 sec; 50°C/ 30 sec; 68°C/ 1 min)x43; 4°C/ hold		
RACE			
Gene Specific Primers	5'RACE	Reverse	5'-CCTGGGAAAAGCCATCATCATCTCCTTG-3'
	3'RACE	Forward	5'GTCACTTGGGGATTCCACCTCGC-3'
Cycling Conditions	(94°C/ 30 sec; 62°C/ 30 sec; 72°C/ 3 min)x25; 4°C/ hold		

Figure 1. Digestion of RT-PCR and RACE PCR clones of *G. multiplicata* AHR. (a) AHR cDNA of total RNA, extracted from *G. multiplicata* tissue, was amplified using degenerate primers and the GeneAmp® RNA PCR Core Kit (Applied Biosystems). The RT-PCR product was transformed into the pGEM-T vector (3000 bp) (Promega). The full AHR sequence of *G. multiplicata* was attained using the SMARTer® RACE Kit (Clontech). The RACE products were cloned using the Zero Blunt® TOPO® (3956 bp) PCR Cloning Kit (Invitrogen). Plasmids were digested with EcoR1 (Promega). The top band of each well represents the cloning vector. **(b)** The degenerate primers were designed from conserved regions within vertebrate AHR and have been previously shown to successfully amplify cDNA from many vertebrate species[5]. The 5'- and 3'-RACE primers were designed using the cDNA sequence and the primer design application within MacVector 14.5.3

Figure 2

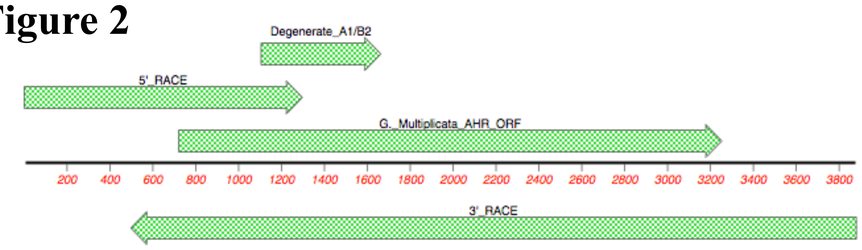


Figure 2. *G. multiplicata* AHR Open Reading Frame (ORF) Assembly. The full 3811 nucleotide (nt) long *G. multiplicata* AHR was determined using MacVector 14.5.3 Assembler of the RT-PCR cDNA sequence and the 5' and 3' RACE sequences. The 5'-untranslated region (UTR) is 721 nt long and the 3' UTR is 559 nt long. The 2533 nt ORF encodes 843 amino acids.

Figure 3

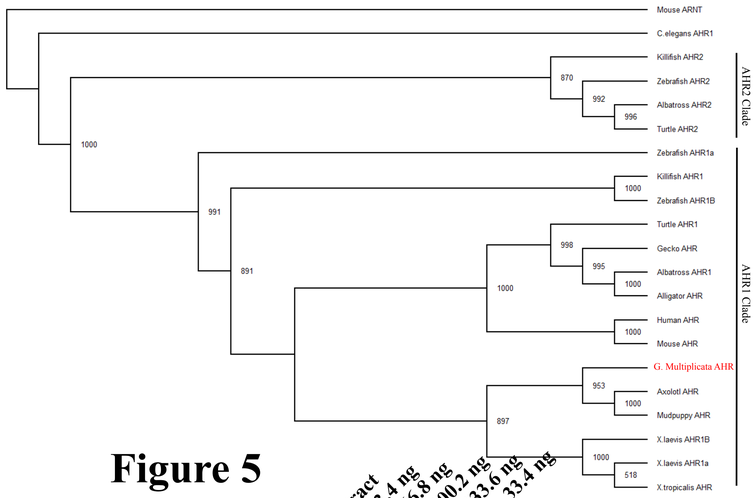
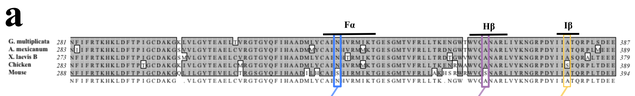


Figure 3. Phylogenetic Analysis of AHR Genes. The full 843 amino acid *G. Multiplicata* AHR sequence was aligned with other vertebrate AHRs using the Neighbor-Joining method in ClustalX. The tree was rooted with Mouse ARNT as the outgroup. Bootstrap values are indicated at each node.

Figure 4

a



b

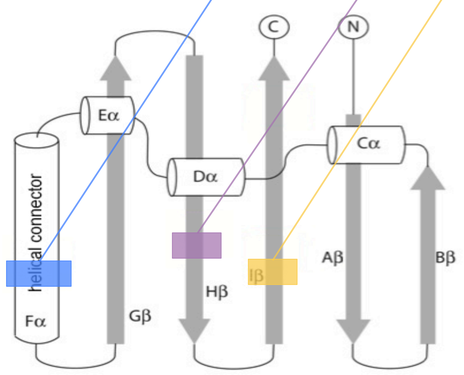
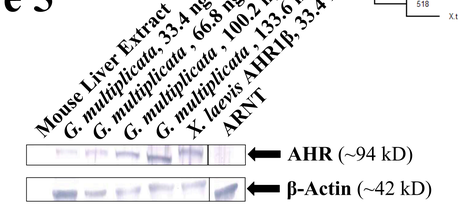


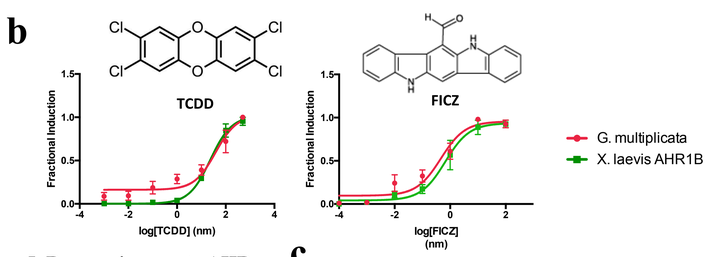
Figure 4. Sequence and Structural Model of *G. multiplicata* AHR LBD.
(a) Sequences were aligned with ClustalW. The variable residues consistent with low binding affinity are boxed.
(b) The low affinity binding residue locations of the LBD are highlighted on the schematic of the structural homology model of vertebrate AHRs[3,4].

Figure 5

a



b



c

EC_{50} Values	TCDD	FICZ
G. multiplicata	28.46 nM	0.79 nM
X. laevis AHR1B	26.84 nM	0.34 nM
Chimeric AHR[4]	0.08 nM	0.02 nM

Figure 5. Responsiveness to AHR agonists. COS-7 cells were transfected using Lipofectamine 2000 (Invitrogen). Cells were co-transfected with pGudLuc6.1 (reporter construct), *Renilla* (transfection control construct), *X. laevis* ARNT and AHR expression constructs.
a) AHR protein expression was detected using polyclonal antibody SA-210 (1:500, Enzo)
b) Five hours after transfection, cells were treated with TCDD, FICZ, or DMSO. Luminescence was measured 18 hours after dosing.
c) Nonlinear regression curves of the average fractional induction values were used to calculate EC_{50} values (n=3).

Conclusions

- The 3811 bp cDNA sequence of *G multiplicata* AHR was determined using RT-PCR and SMARTer® RACE
- *G. multiplicata* AHR is most similar to the AHR homolog of other amphibian orders and orthologous to AHR1s.
- The LBD of *G. multiplicata* AHR contains the three signature residues that predict low affinity for TCDD in frogs and salamanders
- Initial transactivation assays are consistent with low affinity binding
- The EC_{50} values obtained through the transactivation assay are relative indicators of the organism's sensitivity to AHR agonists. This characterization of *G. multiplicata* AHR predicts that like other amphibians, this caecilian is relatively insensitive to the toxic effects of TCDD and other xenobiotic AHR agonists.

Future Directions

- Determine the binding affinity of *G. multiplicata* AHR for TCDD

Acknowledgements

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References

1 Berta, A. (2002). Laboratory Manual for Animal Diversity: An Evolutionary History (4th edition). San Diego State University.
2 Hahn, M.E., Karchner, S.I., and Merson, R. R. (2017). Diversity as opportunity: Insights from 600 million years of AHR evolution. *Toxicology.* **2**:58-71.
3 Odio, C., Holzman, S. A., Denison, M. S., Fraccalvieri, D., Bonati, L., Franks, D. G., Hahn, M. E., and Powell, W. H. (2013). Specific Ligand Binding Domain Residues Confer Low Dioxin Responsiveness to AHR1beta of *Xenopus laevis*. *Biochemistry.* **52** (10): 10.1021/bi301722k.
4 Shoots, J., Fraccalvieri D., Franks D.G., Denison, M.S., Hahn M.E., Bonati, L., and Powell, W.H. (2015). An Aryl Hydrocarbon Receptor from the Salamander *Ambystoma mexicanum* Exhibits Low Sensitivity to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Environ. Sci. Technol.* **49,** 6993-7001.
5 Hahn, M.E., Karchner, S.I., Shapiro, M.A., Perera, S.A. (1997). Molecular Evolution of two vertebrate aryl hydrocarbon (dioxin) receptors A(HR1 and AHR2) and the PAS family. *Evolution*. **94**: 13743-13748.
6 Wincent, E., Amini, N., Luecke, S., Glatt, H., Bergman, J., Crescenzi, C., Rannug, A., Rannug, U. (2009). The Suggested Physiologic Aryl Hydrocarbon Receptor Activator and Cytochrome P4501 Substrate 6-Pormylindolo[3,2-*b*]carbazole Is Present in Humans. *The Journal of Biological Chemistry*. **284**: 2690-2696.
7 Laub, L.B., Jones, B.D., Powell, W.H. (2010). Responsiveness of a *Xenopus laevis* cell line to the aryl hydrocarbon receptor ligands 6-formylindolo[3,2-*b*]carbazole (FICZ) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). *Chem. Bio. Int.* **183**: 202-211.