RESEARCH ARTICLE

The Nuclear Receptors of *Biomphalaria glabrata* and *Lottia gigantea*: Implications for Developing New Model Organisms

Satwant Kaur[1], Susan Jobling[1], Catherine S. Jones[2], Leslie R. Noble[2], Edwin J. Routledge[1], Anne E. Lockyer[1]*

1 Institute of Environment, Health and Societies, Brunel University London, Uxbridge, United Kingdom,
2 School of Biological Sciences, University of Aberdeen, Aberdeen, United Kingdom

* anne.lockyer@brunel.ac.uk

Abstract

Nuclear receptors (NRs) are transcription regulators involved in an array of diverse physiological functions including key roles in endocrine and metabolic function. The aim of this study was to identify nuclear receptors in the fully sequenced genome of the gastropod snail, *Biomphalaria glabrata*, intermediate host for *Schistosoma mansoni* and compare these to known vertebrate NRs, with a view to assessing the snail's potential as a invertebrate model organism for endocrine function, both as a prospective new test organism and to elucidate the fundamental genetic and mechanistic causes of disease. For comparative purposes, the genome of a second gastropod, the owl limpet, *Lottia gigantea* was also investigated for nuclear receptors. Thirty-nine and thirty-three putative NRs were identified from the *B. glabrata* and *L. gigantea* genomes respectively, based on the presence of a conserved DNA-binding domain and/or ligand-binding domain. Nuclear receptor transcript expression was confirmed and sequences were subjected to a comparative phylogenetic analysis, which demonstrated that these molluscs have representatives of all the major NR subfamilies (1-6). Many of the identified NRs are conserved between vertebrates and invertebrates, however differences exist, most notably, the absence of receptors of Group 3C, which includes some of the vertebrate endocrine hormone targets. The mollusc genomes also contain NR homologues that are present in insects and nematodes but not in vertebrates, such as Group 1J (HR48/DAF12/HR96). The identification of many shared receptors between humans and molluscs indicates the potential for molluscs as model organisms; however the absence of several steroid hormone receptors indicates snail endocrine systems are fundamentally different.

Introduction

The tropical freshwater snail *Biomphalaria glabrata* is an intermediate host for several digenean trematode parasitic worms, including *Schistosoma mansoni*, the causative agent of human

intestinal schistosomiasis. Human schistosomiasis is the most widespread trematode infection affecting around 200 million people, leading to a chronic debilitating disease and up to 200,000 deaths per year, across 75 developing countries [1]. Due to its medical significance as an intermediate host, *B. glabrata* has been the focus for much research, including several gene discovery projects [2–4]. Tools have been developed for investigating genomic and transcriptomic attributes of this species, including a BAC library for genome sequencing [5]; a 5K cDNA microarray [6]; a 1.2K oligo microarray [7] and the means to selectively silence gene expression in the snail (RNAi: [8,9]). This background of research has culminated in the sequencing the *B. glabrata* genome [10] (http://129.24.144.93/blast_bg/2index.html). The progress made in developing these resources has also provided the potential for the snail to become a new model organism for other purposes, including testing and identification of endocrine disrupting chemicals (EDCs) and for understanding fundamental biology, including endocrinology.

Endocrine and metabolic disease are among the most common contemporary human afflictions, the prevalence of which has been well defined in large population-based studies (for example, [11,12]). Some of the causes of these are not immediately obvious, but may be related to increasing exposure to EDCs [13]. As a consequence, not only is more testing of potential EDCs needed, but a better understanding of endocrine function and disruption is also required. A solution that is appropriate and in keeping with the three R's (replacement, refinement and reduction) ethos of animal research [14], is to exploit the use of invertebrate organisms which may offer a simplified model for research, as well as providing a faster, cheaper and more ethically acceptable alternative to mammalian testing, at least during initial chemical screening [15]. As NRs play key roles in endocrine and metabolic functions [16], cross-species comparative studies of the conservation of these genes within invertebrate genomes may identify new model systems for the testing of chemicals with endocrine disrupting potential without using vertebrates. In addition to this, *B. glabrata* has recently been proposed specifically for developmental toxicity, acute toxicity and mutagenicity testing in order to establish standardised protocols to assess environmental risks [17]. Therefore a better knowledge of normal endocrine function in molluscs will enable us to understand the full impact of EDCs in the environment, where they have been shown to affect both vertebrate and invertebrate species [18].

One class of transcription factor involved in regulating endocrine function in vertebrates are the nuclear receptors (NRs). NRs regulate and coordinate multiple processes by integrating internal and external signals, thereby maintaining homeostasis (reviewed in [16,19]) These proteins exhibit strong similarities in their mode of action due to their conserved molecular structure, which includes a DNA-binding domain (DBD), consisting of two Cys4 zinc fingers, and the ligand-binding domain (LBD), which not only controls signalling by binding small lipophilic molecules, called ligands, but also binds co-activators and co-repressors [20]. Both the LBD and D box domain of the DBD mediate receptor hetero- or homo-dimerization (reviewed in [21]). Biochemical studies and crystal structure of the LBD reveal that ligand binding triggers a conformational change, causing bound co-repressors to be displaced by co-activators, leading to gene expression [22]. NRs are classified into six distinct families by sequence homology using a phylogenetic approach [23]. Based on their conserved nature and their biologically essential roles throughout the Metazoa, NRs are believed to have emerged early in animal evolution, prior to the bilaterian ancestor [24–26].

Molluscs are affected by EDCs and one of the most cited examples is that of the marine pollutant tributyltin (TBT), found in antifouling paint, which is responsible for imposex in at least 195 species of gastropods worldwide (reviewed in [27,28]). TBT has been shown to act through binding to an NR, the retinoid X receptor (RXR) [29,30]. Other environmental pollutants could act through the estrogen receptor (ER) or androgen receptor (AR) and there is also some evidence of a role for androgen and estrogen-like molecules in the reproductive cycle of

molluscs [31,32]. ER orthologues have previously been reported in molluscs (eg [33–37]) although their function is currently unconfirmed [38,36,39,37,40], since, for example, the identified ER homologue from the oyster *Crassostrea gigas* is unresponsive to estrogen [37], The presence of an ER in molluscs and the potential to respond to estrogens is confounded by the absence of aromatase (CYP19) [41], the enzyme required for the conversion of testosterone to estradiol, but it is possible that another enzyme catalyses the aromatase reaction [38]. The presence of ARs in molluscs has been inferred [32,42], but this remains an area of controversy [43] since homologues have not been identified despite investigations specifically searching for the gene [44]. A recent survey of NRs in *C. gigas* also did not identify an AR [45].

Previously only the RXR has been characterised in *B. glabrata* [46]. With the recent availability of a draft genome for *B. glabrata* it is particularly timely to search for the NRs of *B. glabrata*. The genome of the owl limpet, *L. gigantea* [47], a marine gastropod, for which 26 NRs have already been identified [25,48,49], was compared with the snail NR repertoire. These species, from different families, represent distinct gastropod lineages. The sequences of identified putative genes were confirmed using transcript data from the *L. gigantea* genome portal and by amplification of products from *B. glabrata* mRNA. We assess the conservation, divergence, and uniqueness of gastropod NRs in comparison with the previously characterised receptors of a vertebrate (*Homo sapiens*), insect (*Drosophila melanogaster*), nematode worm (*Caenorhabditis elegans*) (reviewed in [50]) and the parasitic trematode (*S. mansoni*) (reviewed in [51]).

Methods

Identification of nuclear receptors from *B. glabrata* and *L. gigantea* genomes

Human AR (GenBank: EAX05380) and ERα (GenBank: AAI28575) gene sequences were used to search for expressed sequence tags (ESTs) in *B. glabrata* and *L. gigantea* at National Centre for Biotechnology Information (NCBI) using TBlastX with default parameters [52]. These ESTs were then used to search for homologues at the preliminary *B. glabrata* genome (version 4.01: http://129.24.144.93/blast_bg/2index.html) (TBlastX expect limit:1e-04) and assembled *L. gigantea* transcripts from the filtered gene models in the JGI Genome Portal (http://genome.jgi-psf.org/Lotgi1/Lotgi1.info.html) using BLASTN (expect limit: 1e-99). This was followed by a wider search using NR gene sequences spanning all the groups present in humans and *D. melanogaster*. A secondary search using the identified *L. gigantea* and *B. glabrata* NR genes against both the gastropod genomes using a low expect threshold value identified additional family members, some of which were distinct from human and fly NRs. Query sequences were filtered for low complexity regions. Identified contigs from *B. glabrata* were downloaded and processed through GENSCAN [53] (http://genes.mit.edu/GENSCAN.html) using default parameters, to identify predicted coding regions, intron-exon boundaries, and peptides. Predicted peptides were assessed using BLASTP (expect limit: 1e-10) against the non-redundant GenBank database to check for the presence of the NR domains (LBD/DBD). The identified nucleotide sequences from both molluscs containing NR domains were analysed for redundancy using clustering analysis with sequence overlap cut-off set at 0.5 and segment coverage cut-off at 0.25 in Seqtools (8.4ver) (http://www.seqtools.dk/).

The identified genes were named based on the phylogenetic analysis and sequence similarity of the full-length sequence to previously characterized human and *D. melanogaster* NRs according to the recognised nomenclature [23]. Small sequence fragments of putative NRs from *B. glabrata* too short to be classified were left out of the subsequent analysis. Where there was no vertebrate or invertebrate homologue, subfamily classification was made, with a number representing the subfamily, a capital letter for the group, and a number for the individual gene.

RNA isolation and cDNA synthesis

Total RNA was isolated from whole homogenized adult *B. glabrata* snails using TRI-reagent (Sigma-Aldrich, St Louis, USA) according to the manufacturer's protocol and treated with DNAse to eliminate contaminating genomic DNA. Total RNA was extracted from embryonic samples using the RNeasy Fibrous tissue mini kit (Qiagen, Limberg, Netherlands), which included a DNAse treatment as part of the protocol. Quantification and purity of each RNA sample were determined by spectrophotometry (Nanodrop, Thermo Fisher Scientific Ltd. Waltham, USA), and the RNA integrity was visually checked by agarose gel electrophoresis. 4µg of total RNA from adult snails and 1.5µg total RNA from embryonic samples was reverse transcribed in a 20µl reaction using the Superscript III cDNA synthesis kit (Invitrogen, Life Technologies, Carlsbad. USA) with 5 µM of a custom oligo (dTAP) primer (TGACTC-GAGTCGACATCGAT$_{20}$) following the manufacturer's instructions. Residual RNA was removed by adding 1µl of RNase H (2 U/µl) to the reaction and incubating it at 37°C for 20 min. RT-PCR with 18S primers (18S-F: CGCCCGTCGCTACTATCG and 18S-R: ACGCCAGACC-GAGACCAA) [54] verified successful cDNA synthesis.

Polymerase chain reaction

Specific PCR primers (S1 Table) were designed using PRIMER3 (version 0.4.0: http://bioinfo.ut.ee/primer3-0.4.0/), to amplify fragments from DBD and/or LBD for each *B. glabrata* NR to confirm transcription and sequence. 25µl PCRs contained 2µl cDNA (diluted 1 in 20), 1 X PCR Buffer, 2.5mM MgCl$_2$, 0.2mM dNTPs, 0.5µM forward and reverse primers and 1.25U *Taq* DNA polymerase (Bioline, London, UK). Reaction conditions were 2 min at 95°C followed by 35 cycles of 30 sec at 95°C, 30 sec at 55–64°C (optimised for each primer pair (S1 Table)) 1 min 30 sec at 72°C, with a final extension of 5 min at 72°C. Amplified products were analysed by gel electrophoresis and products of an appropriate size were gel extracted where necessary and sequenced (Sequencing facility, Wolfson Wellcome Biomedical Laboratory, Natural History Museum, UK). Sequences were deposited in GenBank Accession Nos: JZ390894-JZ390939

Sequence alignments and phylogenetic analysis

The sequenced NR transcripts from *B. glabrata* were translated using proteomics tools at EXPASY (http://www.expasy.org/). The predicted peptide sequences from both *B. glabrata* and *L. gigantea* were analysed using PFAM domain analysis [55] (PF00104 and PF00105) and PANTHER [56], a hidden Markov model-based method (PTHR24082) confirming the NR domains. The NCBI program Simple Modular Architecture Research Tool (SMART, [57]) was used for the identification of DNA-binding domain (DBD) and ligand binding domain (LBD) regions which were then aligned with the DBD and LBD regions of human (*H. sapiens*), fruit fly (*D. melanogaster*) nematode (*C. elegans*) and parasitic trematode (*S. mansoni*) NRs (Table 1). The NR domains were aligned using default parameters in ClustalX and converted to Nexus format using default parameters in Mesquite v2.75. The DBD and LBD from NRs of the other species were obtained using the conserved domain database (CDD) and reconfirmed using SMART. The NR2A subfamily expansion that contains a large number of the *C. elegans* NRs [58] was disregarded and only 15 NRs from *C. elegans* that are broadly conserved among animal phyla were included in this study for comparative analysis.

Phylogenetic reconstruction was performed using Maximum Likelihood analysis with Bayesian inference and MEGA 6.06. Bayesian inference was conducted with MrBayes version 3.1.2 [59] using the WAG model; the best fitting substitution model determined by both Akaike information criterion and Bayesian information criterion frameworks (ProtTest (v1.4) [60]). Two independent runs of Markov Chain Monte Carlo (MCMC) analysis were

Table 1. Sequence identification of all nuclear receptors for species in the study: NRs from fly, human, nematode and trematode (NCBI accession numbers), compared to identified *L. gigantea* NRs (protein identification numbers: JGI genome portal version 1.1) and *B. glabrata* NRs (Contig numbers: Preliminary Bg Genomic Data (version 4.01)).

Group	C. elegans	Accession	S. mansoni	Accession	D. melanogaster	Accession	B. glabrata	Contig	L. gigantea	Protein ID	H. sapiens	Accession
0A					KNR	CAA31709						
					KNRL	AAF51627						
					EGON	CAA34626						
0B							BgDAX	182	LgDAX	153776	DAX1	AAH11564
											SHP	AAH30207
1A			THRa	AAR32912			BgTHR‡	3.1	LgTHR	207623	THRα	AAH08851
			THRb	AAR29359							THRβ	AAI06931
1B	ODR7	AAC46497	RAR-like*	CCD76558			BgRAR	398	LgRAR	207867	RARα	AAH08727
											RARβ	AAH60794
											RARγ	AAA63254
1C							BgPPAR1	2052	LgPPAR1	174409	PPARα	AAB32649
							BgPPAR2	1275	LgPPAR2	238472	PPARβ	AAA36469
											PPARγ	AAH06811
1D	HR85	AAO39185			E75	AAN11687	BgE75	29	LgE75	136477	Rev-erb-a	AAH56148
							BgRev-erb	160	LgRev-erb	168854	Rev-erb-b	AAH45613
							BgNR1D1†*	1939				
							BgNR1D2†*	1958				
							BgNR1D3†*	201				
1E			E78		E78	AAR30507	BgE78	534	LgE78	163301		
1F	HR23	P41828			HR3	AAA28461	BgHR3	73	LgHR3	167096	RORα	AAH08831
							BgROR	74	LgROR	155536	RORβ	AAH93774
											RORγ	AAA64751
1G	HR14	AAA96982										
1H					EcR	NP724456	BgEcR	1481	LgEcR	170342	LXRα	AAV38218
											LXRβ	NP_009052
											FXR	AAI30574
1I											VDR	AAB95155
											PXR	AAD05436
											CAR	AAY56401
1J	DAF12	AAD34462	HR96α(CAR) *	AAV80235	HR96	AAC46928	BgNR1J1	48	LgNR1J1	163618		
	HR8	AAP31437	HR96	AAW88541			BgNR1J2	954	LgNR1J2	63892		
	HR48	CAD36502					BgNR1J3	2148	LgNR1J3	163956		
							BgNR1J4	3233				
1K	HR1	AAC48174										
2A	HR49	CAD57702	HNF4	CCD80248	HNF4	AAF52702	BgHNF4	2702	LgHNF4	73247	HNF4	NP_849180
											HNF4γ	NP_004124
2B			RXR1	CCD59553	USP	AAF45707	BgRXR	3695	LgRXR	206562	RXRα	AAI10999
			RXR2	AAD33428							RXRβ	AAC18599
											RXRγ	AAA80681
2C/D	HR41	NP_500073	TR	AAV80236	HR78	Q24142	BgTR	2718	LgTR*	231979	TR2	AAA36761
											TR4	NP_003289

(*Continued*)

Table 1. (*Continued*)

Group	C. elegans	Accession	S. mansoni	Accession	D. melanogaster	Accession	B. glabrata	Contig	L. gigantea	Protein ID	H. sapiens	Accession
2E	FAX	AAD55066	TLL*	AAW88549	TLL	AAB71371	BgTLX*	3	LgTLX	130367	TLX	AAL05871
	HR67	CAA97428	PNR*	CCD82728	PNR	AAF58145	BgDSF	42	LgDSF	64143	PNR	AAD28301
			DSF*	AAW88537	DSF	AAF52303	BgFAX1	148	LgFAX1	168960		
					FAX1	AAF54133	BgPNR‡	3827	LgNR2E	232946		
									LgPNR	171552		
2F	UNC55	CAP16277	COUP-TFI*	CCD77973	SVP	NP_524325	BgCOUP-TFa	5703	LgCOUP-TFa	BgCOUP-TFa	COUP-TFα	AAH04154
			COUP-TFII*	AAW88536							COUP-TFβ	AAH42897
											EAR2	AAH02669
3A							BgER	469	LgER	132166	ERα	AAI28575
											ERβ	AAV31779
3B					ERR	AAL37554	BgERR	751	LgERR	168715	ERRα	AAH63795
											ERRβ	AAC99409
											ERRγ	EAW93335
3C											GR	AAH15610
											MR	AAA59571
											PR	AAA60081
											AR	EAX05380
4A	HR6	CAA85271	NR4A5*	AAR28090	DHR38	AAF53914	BgHR38	27	LgHR38	94341	NGFIB	CAG32985
							BgNR4a	2842	LgNR4a	182258	NURR1	AAH09288
											NOR1	CAI95138
5A	HR25	CAA91028	FTZ-F1α (SF1)*	CCD77387	FTZ-F1	AAN11667	BgFTZ-F1	1318	LgFTZ-F1	196916	SF1	AAH32501
											LRH1	AAI18572
5B			HR39(FTZ-F1)	CCD77680	DHR39	AAN11107	BgHR39	3667	LgHR39	94029		
6A	HR91	CAB60329			DHR4	AAX73355	BgHR4	1866	LgHR4a	128948	GCNF	AAB96828
									LgHR4b	162354		
2DBDNR			2DBDα*	AAW88533			Bg2DBDNR1*	304	Lg2DBDNR1*	168696		
			2DBDβ*	AAW88534			Bg2DBDNR2*‡	1296	Lg2DBDNR2*			
			2DBDγ*	AAW88550								
Unclassified							BgNRU1	221				
							BgNRU2	293				
							BgNRU3	180				
							BgNRU4*‡	250				

*NRs not used in phylogenetic tree;

+ NRs are short fragments that could be multiple partial genes or parts of a full transcript;

‡ NRs not found expressed in *B. glabrata*

performed, with four chains run for 7 million generations with the 'temperature' parameter at 0.10, prior probabilities with default values and sampling every 2000 generations. The first 2 million generations were discarded as burn-in because the Log likelihood values were plotted and found to be asymptotic well before the burn-in fraction. Convergence between the independent MCMC runs was examined by the average deviation of the split frequencies and the potential scale-reduction factor (PSRF), which was 1.00. Clades with posterior probabilities >95% were considered well supported. All PSRF values for MrBayes analyses were 1.00. For Maximun Likelihood analysis, the Jones-Taylor-Thornton (JTT) substitution model [61] was used, with a gamma distribution of rates between sites (eight categories, parameter alpha, estimated by the program). Support was evaluated by 1000 bootstrap replicates.

Maximum parsimony was also used to provide additional phylogenetic support for classification, naming, and the phylogenetic relationships observed between *B. glabrata*, *L. gigantea* and nuclear receptors from selected species. Maximum parsimony with heuristic searches, branch swapping set to tree-bisection-reconnection, topological constraints not enforced, and 1000 bootstrap replicates were performed using MEGA6.06 [62]. An appropriate out-group to root the sequences was difficult for such a diverse and ancient nuclear receptor family and so the phylogenies were mid-point rooted using Dendroscope (ver3.2.10) [63].

Results and Discussion

Nuclear receptor genes

Thirty nine NR sequences were identified from the genome of *B. glabrata* and a total of 33 from *L. gigantea* consisting of 7 newly identified NRs and confirming 26 sequences which had been previously identified from *L. gigantea* in previous studies [25,48,49] (Table 1). An exhaustive search with mammalian and *D. melanogaster* NRs and with the identified mollusc NRs found no further NR homologs. Representative genes for each of the major nuclear receptor groups were detected, suggesting the assembled genome for *B. glabrata* provides a good representation of total gene content with physical genome coverage of approximately 27.5X (Genbank: APKA00000000.1). The numbers found are comparable to the 48 NRs reported in the human genome [64], 49 in mice [65] and 47 in rats [66]. Insects have lower numbers; only 21 NR genes are found in *D. melanogaster* [67], 22 in *Apis mellifera* [68], and 21 in *Tribolium castaneum* [69]. Over 270 NRs have been found in *C. elegans* [70] but only 21 NRs in the trematode *S. mansoni* [71]. The unusually large number of NRs in *C. elegans* is due to extensive proliferation of one gene (HNF4) within the nematode phylum [58]. 43 NRs were recently identified from the Pacific oyster, *C. gigas*, a marine bivalve, [45], similar numbers to those we identified from the gastropods.

Expression of *B. glabrata* nuclear receptor mRNAs

Each predicted nuclear receptor was evaluated for expression in *B. glabrata* adult snails, by amplifying cDNA fragments with receptor-specific primers, to demonstrate that the predicted genes were expressed as transcripts and confirm their sequence. Primer pairs for which fragments were not obtained from adults were also tested on embryos. Expression was confirmed for 34 out of 39 identified receptor genes (Table 1), 31 from cDNA derived from whole adult snails, while 3 nuclear receptors, BgTLX, BgDSF and BgFAX1, were not expressed in adult snails but were identified in 96hrs/120hrs embryos. These receptors belong to NR2E sub family and may be involved in embryonic development as reported in *Daphnia pulex* [72]. All amplicons corresponded to the predicted sequence length and sequencing confirmed the predicted identity of the gene products (GenBank Accession Nos: JZ390894-JZ390939). For *L. gigantea*, *in silico* searches identified sequenced transcripts for 21 out of 33 NR genes (S2 Table). Three

B. glabrata putative NRs (BgNR1D1/2/3) for which only partial sequences were obtained from the genome were not included for further phylogenetic analysis, since it was not possible to ascertain if they are multiple partial genes or parts of the same transcript. In addition to this BgTLX, BgNRU4 and LgTR were not analysed further as these were also incomplete sequences. A separate analysis was made of 2DBD genes from *B. glabrata* and *L. gigantea* with other previously identified 2DBD sequences from *S. mansoni* and other species as the 2DBD region could not be aligned with the other NRs.

Phylogenetic analyses

The DBD and LBD regions for the full-length molluscan NRs (32 for *B. glabrata* and 30 for *L. gigantea*) were aligned with *S. mansoni*, *D. melanogaster*, *C. elegans* and *H. sapiens* NR sequences (S1 File). Phylogenetic trees were constructed using 3 different approaches: Bayesian Inference (BI), Maximum Parsimony (MP) and Maximum Likelihood (ML) and the resulting trees assessed for concordance. All trees agree at family and subfamily level (S1 Fig, S2 Fig, S3 Fig) but BI shows the greatest resolution at the base of the phylogram and is shown with nodal support generated from all the tree construction methods (Fig 1). The tree shows the main groupings of the NRs.

The position of the unclassified *B. glabrata* NRs (BgNRU) is the same in all trees, close to NR0A subfamily within the NR1 group; however the NR0 subfamily was derived to accommodate nuclear receptors that lack either the DBD or the LBD and these 3 *B. glabrata* NRs contain both domains (S4 Fig). Therefore these remain as unclassified. The position of members of NR2C/D is also not resolved within Group 2 (Fig 1). Both mollusc genomes each contain one member of this clade that we have designated LgTR/BgTR based on their sequence similarity with HsTR (Table 1). LgTR was not included in the phylogenetic analysis since it was missing the LBD, but BgTR clusters with the other NR2C/D group members Ce_HR41, SmTR and Dm_HR78. The Bayesian tree predicts BgTR as the most distal member of NR2C but the ML and MP trees show both the mollusc sequences to be closely related to DmHR78. However the MP tree placed Ce_HR41 in NR2D, although this NR is usually classified in NR2C. Clearly, the NR2C/D groups seem to be related and not well defined. Within NR1 NR1D was not clearly resolved. In the NR3 subfamily, only the position of the human GR showed non-concordance between the BI tree and the MP tree. All of the trees were in agreement for the NR4/NR5/NR6 subfamilies, with one difference in the position of BgNR4a/LgNR4a and NR5A in the MP tree.

The identified genes have been named in agreement with the unified nomenclature for NRs [23] based on their sequence homology and phylogenetic position. Overall, the identified mollusc NRs encompass almost all the subfamilies, with representatives divided between 21 groups (Table 1) and revealed both similarities and differences in the NR complement between the species used in the study. Seventeen of the 19 subgroups containing human NRs also had representatives in the invertebrate species, including molluscs although in general *H. sapiens* contained several NRs where snails had only one, most likely the result of gene or lineage-specific duplication events. The presence of these groups among both protostomes and deuterostomes suggests that these receptors originated in a common ancestor of the bilateria [24,25]. The 2 vertebrate groups not represented in molluscs were the Groups 3C and 1I. Three groups that were common to humans and molluscs, but not found in the other invertebrate organisms included here were 0B (DAX), 1C (PPAR) and 3A (ER). The presence of these receptors in molluscs suggests at least the possibility of commonality in shared signalling pathways, elucidating the evolutionary development of the endocrine system. Seven groups were found only in invertebrates; Groups 1G and 1K only in *C. elegans*; 0A only in *D. melanogaster*, while groups 1E (E78), 5B (DHR39) 1J and the 2DBD NRs were found in several of the invertebrate species.
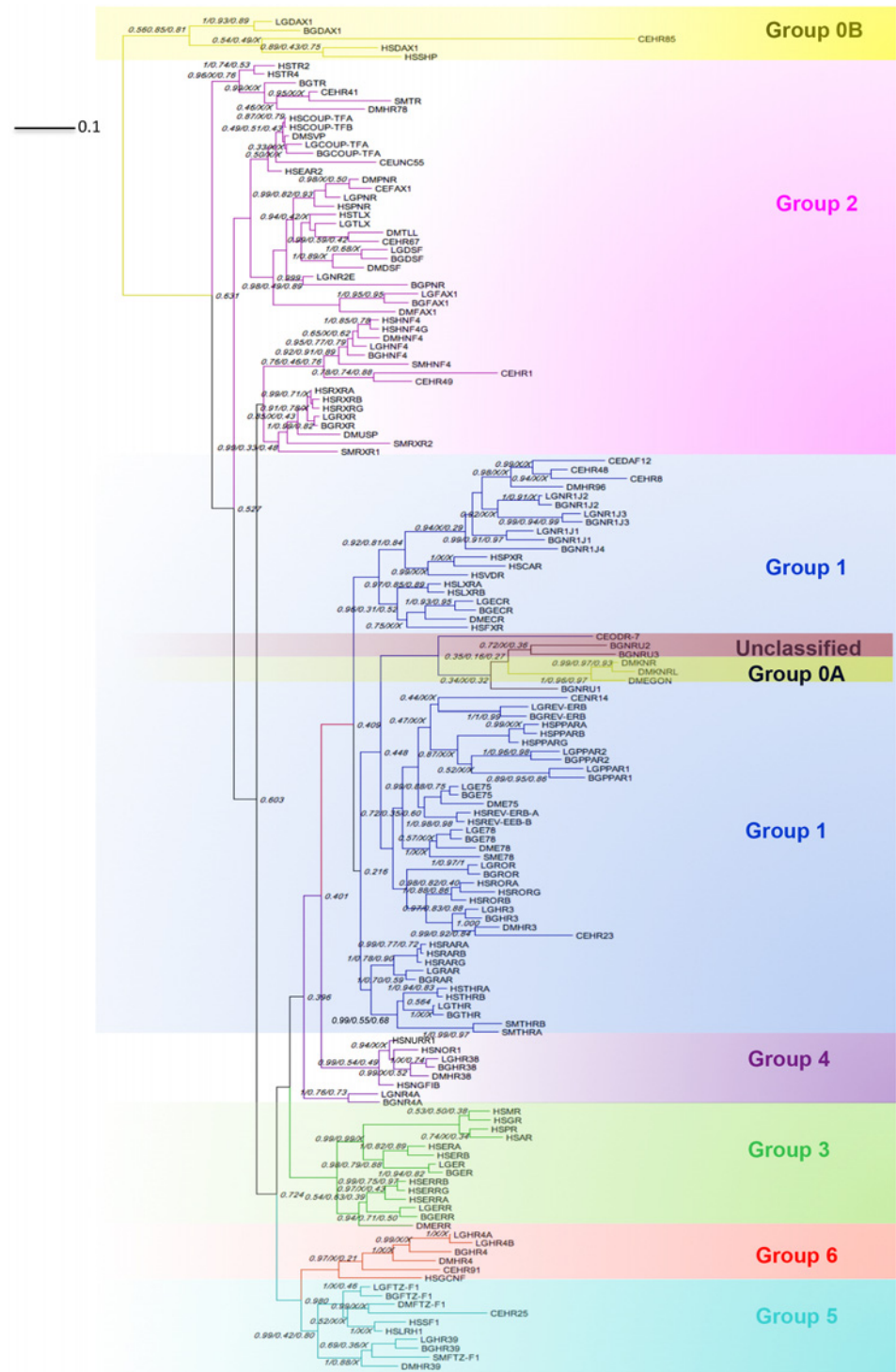
Fig 1. Phylogenetic relationships of NRs in molluscs, humans, fly, nematode and trematode. NRs from six species *B. glabrata (Bg)*, *L. gigantea (Lg)*, *H. sapiens (Hs)*, *D. melanogaster (Dm)*, *C. elegans (Ce)* and *S. mansoni (Sm)*, were subjected to phylogenetic comparisons using Bayesian inference, maximum parsimony and maximum likelihood methods. The Bayesian tree (midpoint rooted) is shown with posterior probability values from Bayesian inference and bootstrap values from maximum parsimony and maximum likelihood trees. The value of 1 on each node represents 100% posterior probability/bootstrap support; an X indicates an area of disagreement from the Bayesian tree (S1 Fig). Scale bar, 0.1 expected changes/site.

doi:10.1371/journal.pone.0121259.g001

Representation of these latter groups in *L. gigantea* and *B. glabrata* shows that these nuclear receptors pre-date the ecdysozoan/lophotrochozoan split.

Group 3 receptors

The nuclear receptors that bind to steroids, such as androgens, estrogens and progestogens, are responsible for the long-term effects of steroid hormones on reproduction, behaviour, immunity, stress responses and development. The Group 3 steroid receptor family includes the estrogen related receptor (ERR), as well as the estrogen receptor (ER) and group 3C subfamily with the androgen, progesterone, glucocorticoid and mineralocorticoid receptors. Steroid receptors were originally thought to be a vertebrate specific gene family but the identification of the genes with a clear homology to human ERR in trichoplax [73] and an ancestral steroid receptor in amphioxus [74] suggested that these receptors arose early in metazoan evolution and subsequently proliferated in vertebrates through series of gene duplication events [75]. We have identified ER homologues in *B. glabrata* and *L. gigantea* (Table 1, Group 3A), which have been previously reported in other molluscan species, including bivalves, *C. gigas* [37], *Mytilus edulis* [76] and *Chlamys farreri* [77] and gastropods, *Aplysia californica* [38], *Lymnaea ollula* [78], *Marisa cornarietis* [33], *Thais clavigera* [39], *Nucella lapillus* [79], *Bithynia tentaculata* [35] and the cephalopod, *Octopus vulgaris* [36]. The phylogenetic position of the mollusc ER homologue with the vertebrate ER is well supported with a BMCMC posterior probability of 98% and bootstrap values of 0.88 and 0.79 (Fig 1), supporting the suggestion that vertebrate and invertebrate ER diverged from a common ancestor, before the evolution of the deuterostomes [38,75,80]. Structural similarities between ER DBDs of molluscs, annelids, cephalochordates and vertebrates have been documented showing them to bind to and regulate transcription through estrogen response elements [36,40,74,81,82]. The ER LBD in invertebrates may have unique functions, since the mollusc ER homologue, at least *in vitro* (usually in mammalian reporter cell lines), appears to activate transcription constitutively in the absence of a ligand [36,37,39,40] although in annelids the ER has been shown to activate transcription in the presence of estrogens [82]. We also identified a single estrogen-related receptor (ERR) in both *B. glabrata* and *L. gigantea* (Table 1, Group 3B), which clusters with fly and human ERRs. ERRs have been reported previously in molluscs, including *M. cornarietis* [33], *C. gigas* (Genbank: EKC20050), *Mizuhopecten yessoensis* (GenBank: BAN84542.1) and *M. edulis* [76]. ERRs have no known endogenous ligand, although they are thought to bind to estrogen response elements and may play a role in estrogen signalling and energy metabolism [83].

We identified no convincing homologues in either of the two molluscs for the AR or for any other members of group 3C (Fig 1), which also contains the glucocorticoid receptor (GR), mineralocorticoid receptor (MR) and progesterone receptor (PR). The absence of an AR sequence homologue, both in our systematic exhaustive genomic searches of 2 gastropod species, as well as specific laboratory-based searches in other molluscs [44] using an approach which successfully identified other receptors and another systematic NR survey in the bivalve *C. gigas* [45] strongly suggests no vertebrate AR homologue exists in gastropod molluscs. The presence of an AR in molluscs has been hotly contested [84], its existence having been inferred from the effects of androgens and anti-androgens in several species of mollusc (eg. [42,85]), rather than by the identification of a homologous sequence. The absence of an AR means that the findings from previous papers requiring its presence to explain their results may require further analysis in the light of this information; although it should also be noted that the effects of steroids, including those reported for androgens in molluscs may not be mediated via NRs. Steroid hormones have also been shown to act via non-genomic mechanisms in vertebrates, using

membrane bound receptors from the G protein family [86–88], although homologues for these receptors have also not yet been identified in molluscs.

The absence of a molluscan AR and the constitutive expression of the ER *in vitro* suggest alternative pathways must exist for spermatogenesis/oogenesis in molluscs and other nuclear receptors have been proposed as initiating these pathways for reproductive processes, some of which exist in vertebrates and which may be particularly important in invertebrates. Although vertebrates have subsequently evolved further processes involving steroid hormones, the presence of orthologous NRs involved in other pathways in molluscs offers the potential opportunity to study these conserved networks in an invertebrate.

Group 1 and 2 nuclear receptors

Conservation between other NR-mediated pathways in molluscs and humans, even with the absence of Group 3C NRs, still provides the potential to develop a mollusc model for endocrine physiology. The retinoid X receptor (RXR, Group 2B) has been previously identified in molluscs including *B. glabrata*, [46], *L. gigantea* [25] and *L. stagnalis* [89]. We found that the molluscan RXR sequences grouped with good support (BMCMC posterior probability of 91%) close to the human RXR sequences, with the *D. melanogaster ultraspiracle* (USP) RXR homologue basal to the clade (Fig 1). BgRXR has been demonstrated to bind and be activated by retinoids, suggesting that retinoid signalling pathways are conserved in the Lophotrochozoa [46]. It was also shown to bind to the response element DR1 either as a homodimer or as a heterodimer with mammalian RARα, LXR, FXR or PPARα [46], which suggests the possibility of conservation of several important signalling pathways in molluscs. The ligand and the co-activator peptide have been previously reported to bind to snail RXR in essentially the same manner as observed in human RXR LBD structures [90], suggesting that the mechanisms of RXR-mediated transcription regulation are very similar in molluscs and humans. The significance of RXR signalling on reproductive physiology in molluscs is strongly supported by the finding that RXR expression is affected during TBT exposure and subsequent development of imposex in *N. lapillus* [91]; RXR is thought to be the receptor which mediates TBT induction of imposex in this species [29]. It has also been recently demonstrated that, in conjunction with RXR signalling, peroxisome proliferator-activated receptor (PPAR) pathways are activated by organotins [92] and may induce imposex in response to TBT in *N. lapillus* [93]. Convergence of 9-cis retinoic acid, a natural ligand for RXR, and PPAR signalling pathways through PPAR-RXRα hetero-dimerization is well established in mammals [94]. We identified two PPAR homologues in both *L. gigantea* and *B. glabrata* both containing identical sequence to the P-box of human PPAR (CEGCKGFFRRTI) which grouped with the human PPAR genes (Fig 1); however the 3 human genes seem to have diverged after the split with the molluscan paralogues. Examination of PPAR gene relationships in more detail and including some additional species in the phylogenetic analysis, confirms that the 2 molluscan paralogues diverged prior to the split with vertebrates. The genes we have designated PPAR1 lie basal to the clade, while the vertebrate α, β and γ PPAR genes are orthologues of the gene we have designated PPAR2 (Fig 2).

The RXR also binds with the thyroid hormone receptor (THR, Group 1A). Both gastropods contain a THR homologue (Table 1), which has been previously reported in *L. gigantea* and other invertebrates [49]. Wu et al (2007) confirmed that the *S. mansoni* THR was able to heterodimerise with the RXR. The molluscan THRs cluster with the human THRα/β (Fig 1). The presence of this NR makes *B. glabrata* a potential model for thyroid hormone processes. Thyroid hormones (THs) play key roles in growth development and metabolism in vertebrates. In invertebrates exogenous (food) sourced THs have been suggested to be involved in signalling [95].

Fig 2. Phylogenetic relationships of peroxisome proliferator-activated receptors (PPAR α, β and γ).
PPAR sequences from different species were subjected to phylogenetic analyses using Bayesian inference, maximum likelihood and maximum parsimony. Accession numbers of the PPARs of the different species in the phylogenetic tree: *Heterocephalus glaber* XP_004846774; *Physeter catodon* XP_007109986; *Leptonychotes weddellii* γ XP_006734113; *L. weddellii* α XP_006750071; *L. weddellii* δ XP_006737129; *Saccoglossus kowalevskii* XP_006819446; *Strongylocentrotus purpuratus* α XP_781750; *S. purpuratus* γ XP_784429; *C. gigas* EKC18691; *H. sapiens* α AB32649; *H. sapiens* γ AAH06811; *H. sapiens* β AAA36469; *Mus musculus* α NP_035274; *M. musculus* γ NP_035276; *M. musculus* β NP_035275; *Oryzias latipus* α XP_004069934; *O. latipus* β NP_001265836; *O. latipus* γ NP_001158348; *B. glabrata* 1 Contig2052; *B. glabrata* 2 Contig1275; *L. gigantea* 1 ProteinID174409; *L. gigantea* 2 ProteinID238472; *H. sapiens* ROR AAH0883. Scale bar, 0.1 expected changes/site.

doi:10.1371/journal.pone.0121259.g002

Ultraspiracle (USP), the fly orthologue of the RXR (reviewed in [96]), has been shown to heterodimerize with the ecdysone receptor (EcR) to control ecdysone signalling in insects driving metamorphosis and moulting [97]. EcR homologues (Group 1H) were identified in both molluscs (Table 1), the first time this has been reported for *B. glabrata*. The presence of the EcR ligand, ecdysone, has been previously reported in pulmonate snails, in *B. glabrata* and *Lymnaea stagnalis* [98], but its origin (endogenous or exogenous) is still contested [99]. Interestingly, ecdysone exposure was found to affect growth and egg production in *B. glabrata* and it has been suggested that ecdysone secreted from the *S. mansoni* parasite might provide a

mechanism for parasitic castration and gigantism seen in some intermediate hosts when infected [100]. The EcR of *L. gigantea* has been previously identified and its 3D structure investigated [101] revealing the ligand-binding pocket of the *L. gigantea* EcR homolog has the potential to bind to ecdysone-related steroids. The observation that EcR is expressed in testis tissue may also indicate a role in molluscan reproductive processes [101].

One retinoic acid receptor (RAR) was identified in each gastropod species. RARs have previously been identified in the gastropod *L. stagnalis* (GenBank: GU932671), the rock shell, *T. clavigera* [102], the bivalve, *C. gigas* [45] and recently in *N. lapillus* [103]. In vertebrates RAR binds retinoic acid, the biologically active form of vitamin A, which mediates cellular signalling in embryogenic antero-posterior patterning of the central nervous system [104]. However the RAR of *N. lapillus* did not bind to all-trans retinoic acid or any other retinoid tested nor was it able to activate the transcription of reporter genes in response to stimulation by retinoids [103]. More work is clearly necessary to elucidate the function of RAR in molluscs.

We also identified homologues of Group 2 C/D vertebrate testicular receptor 4 (TR4) in *B. glabrata* and *L. gigantea*, (designated as BgTR and LgTR) that in mice have been shown to control spermatogenesis [105] and folliculogenesis [106]. As an orphan nuclear receptor (with no known ligand) the physiological function of TR4 has been difficult to ascertain until the recent development of knock-out mice for this gene (reviewed in [107]). Thus determining the basic biology of vertebrate NRs is dependent on animal experimentation, some of which may be possible in a simplified model system such as a mollusc.

The NR2E nuclear receptors that have been functionally characterized have a common role in nervous system development, for example the *tailless* (*tll*) gene of *D. melanogaster* is involved in embryonic CNS and larval eye development [108] and the mouse *Tlx* gene has also been found to be a key component of retinal development and vision [109]. In *B. glabrata* we identified homologues of photoreceptor cell-specific nuclear receptor (PNR), *dissatisfaction* (DSF), TLX and FAX1, while in *L. gigantea*, we identified a further NR2E gene (Table 1).

Groups NR1J and NR1I cluster in the tree (Fig 1). No members of the NR1I group, including vitamin D receptor (VDR) or pregnane X receptors (PXR), were identified in *B. glabrata* and *L. gigantea*; however NR1J group in protostomes shares similarity with vertebrate NR1I group [110] and there is evidence that both NR1I/NR1J groups share a common ancestor [24]. NR1I receptors are considered as natural sensors and are involved in xenobiotic metabolism in vertebrates [111]; however, other studies have indicated that NR1J members might regulate xenobiotic responses in *D. melanogaster* and *C. elegans* [112,113]. Our results show that *L. gigantea* has three and *B. glabrata* four NR1J representatives (Table 1). The mollusc NR1J group members possess the well-conserved base contact residues (ESCKAFFR) within the DBD, characteristic of the NR1J sub family [114]. In molluscs the NR1J group receptors may be able to perform the same xenobiotic recognition functions as the closely related NR1I of vertebrates.

Group 5 and 6 nuclear receptors

Germ cell nuclear factor (GCNF) homologues (Group 6) have previously been reported in bilaterians (reviewed in [50]) and we identified them in both gastropod species. This is in contrast to the recently published survey of NRs in the pacific oyster *C. gigas* [45] which did not identify any NRs from Group 6. Both BgHR4 and LgHR4a/b cluster with good support (posterior probability 1) to the *D. melanogaster* GCNF homologue, Dm_HR4 (Fig 1). During embryonic stages in vertebrates, GCNF can interfere with retinoic acid signalling affecting the expression of *cyp*26A1, which is essential for normal hindbrain patterning and early developmental stages [104]. In Group 5 we identified BgFTZ-F1/LgFTZ-F1 and BgHR39/LgHR39, homologues of the *D. melanogaster* NR5 subfamily members, *fushi tarazu* factor 1 and hormone receptor 39

respectively, with a conserved stretch of 23 amino acids adjoining the C-terminal end of the zinc finger motif (AVR**S**DRMRGGRNKFG**P**MYKRDRA). This sequence is located immediately after the DBD and plays an important role in high affinity interactions of the receptor with DNA [115]. These Group 5 NRs are related to the vertebrate SF-1 which controls reproductive development and regulates the transcription of steroid-modifying cytochrome P450 genes. In *Drosophila* HR39 is essential for sexual development, required in females both to activate spermathecal secretion and repress male-specific courtship genes, and controls the expression of specific cytochrome P450 genes [116]. The conservation of function between invertebrates and vertebrates in these receptors in Groups NR5 and NR6, suggests the potential for molluscs to be used to model some aspects of mammalian reproductive biology and that further study into the reproductive biology of invertebrates is warranted.

2DBD nuclear receptors

We identified two 2DBD-NRs in *B. glabrata* as well as the two 2DBD-NRs previously reported from *L. gigantea* [48]. We compared these to the 3 previously identified from *S. mansoni* [71] as well as 2DBD NRs from other organisms (Fig 3). The gastropod 2DBD1 clusters with Sm2DBDγ while the position of the second gastropod 2DBD was not determined within the clade. Both the identified Bg 2DBD-NRs possess the two tandem DNA binding domains, and a well-conserved P-box sequence (CEACKK) in the first DBD and a P-box (CEGCK) in the second DBD. The P-box of the second DBD is similar to DBD of NR1 family but the P-box of the first DBD is unique to this NR and may determine novel targets [48].

NRs in parasite and intermediate host

The discovery of differences and similarities in the nuclear receptor repertoire of snail host and *S. mansoni* may open up avenues to further characterize host-parasite interactions and potentially to interfere with schistosome development within the host. Parasites are known to interfere with the snail reproductive system, since one effect of trematode infection in snails is parasitic castration (reviewed in [117]). The intimate host-parasite and intermediate host-parasite relationships, makes a comparison of nuclear receptors in all three organisms significant. It has been suggested, at least for the definitive host, that the ability of the parasite to exploit the hosts' hormonal microenvironment may be critical to allow it to establish, grow and reproduce [118]. This may also be significant in enabling the parasite to inhabit its intermediate host and may depend upon shared nuclear receptors. All 3 organisms have one or more THR, RAR, HNF4, RXR, TR, COUP-TF and similar NRs in Groups 2E, 4A and 5A. Only snails and schistosomes have the E78, HR39, HR96 and 2DBD NRs, while snails and humans only have DAX1, PPAR, Rev-erb, ROR, ER, ERR and group 6A NRs. NRs which are specific to the snails (in this three-way comparison) are the EcR and the group of unclassified NRs, designated as BgNRU1, BgNRU2, BgNRU3, and BgNRU4 respectively and placed in a separate group (Table 1). These unclassified receptors may have originated from a specific duplication event in a *B. glabrata* precursor, or alternatively, they could be remnants of ancient NR subfamilies, whose representatives have been secondarily lost in the other represented species. With regard to the EcR, given that snails have a EcR homologue and schistosomes do not, the suggestion that β-ecdysterone acts as an attractant in host location by miracidia and affects the rate of growth and maturation in snails [100] is potentially interesting, although the ability of snails to make ecdysone is not proven [99]. Both organisms have homologues of the *D. melanogaster* NRs E78 and HR39 which are active in ecdysone signalling [116,119].

In addition to discovering the intricacies of the molecular interactions between host and parasite with a view to disrupting the development of the schistosome, the other key strategy
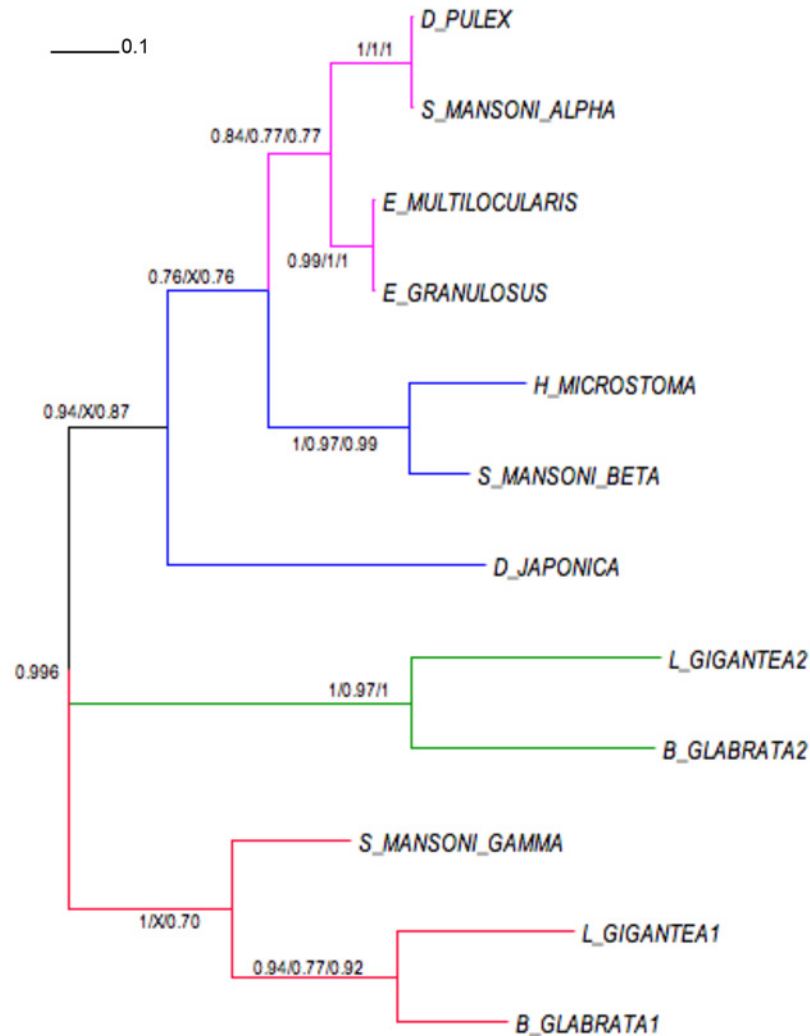
Fig 3. Phylogenetic relationships of 2DBD nuclear receptors (alpha, beta and gamma). 2DBD NR sequences from different species were subjected to phylogenetic analyses using Bayesian inference, maximum likelihood and maximum parsimony. Accession numbers of the 2DBD NRs of the different species in the phylogenetic tree: *D. pulex* FE382753; *Hymenolepis microstoma* CDS31978; *Echinococcus multilocularis* CDI99377; *Echinococcus granulosus* CDS24085; *S. mansoni* α AAW88533; *S. mansoni* β AAW88534; *S. mansoni* γ AAW88550; *Dugesia japonica* BP186725; *L. gigantea* 2DBD1 ProteinID168696; *L. gigantea* 2DBD2 ProteinID173632; *B. glabrata* 2DBD1 Contig304; *B. glabrata* 2DBD2 Contig1296. Scale bar, 0.1 expected changes/site.

for schistosomiasis control is to reduce the numbers and/or distribution of the intermediate host snails. Currently molluscicides, such as the commercially available niclosamide-based Bayluscide, are effectively employed in schistosomiasis control programs; however the problems of resistance and toxicity to other organisms means that the search for alternative, more selective, compounds is on-going. The capacity of nuclear receptors to bind small ligands, including exogenous substances such as natural products and synthetic chemicals, makes them potential targets for molluscicides [120].

Biomphalaria glabrata as a model organism

There is a long tradition in biology of examining biochemical processes in simplified models to elucidate mechanisms in more complex organisms. We have demonstrated that there are some NR groups with a single representative in the mollusc species' examined instead of the multiple genes from humans. For example in the NR1 subfamily, both molluscs have single homologues of several significant NRs, such as the retinoic acid receptor (RAR), THR and ROR. For these groups, *B. glabrata* could provide a simple model system, not only to study the development and diversification of endocrine systems, but also to investigate and test gene function and response to external stimuli. The identification of many vertebrate-like NRs in *B. glabrata* could make it a suitable model candidate to investigate functional relationships of individual receptors. The ultimate choice of model organisms lies not only with the biology of the organism, but also on its tractability for study and manipulation. Aside from the genome, the full range of genomic tools available for *B. glabrata*, such as RNAi [8,121], BAC library [5] and microarrays [6,122,123] as well as the potential to use classical approaches of enhancer and suppressor genetics and transgenics to explore regulatory networks, make *B. glabrata* a good candidate. However, the advent of next generation sequencing makes transcriptome study from non-model organisms a real possibility and therefore other mollusc species may ultimately make better model systems. In particular, this approach could be used to identify inputs from signal transduction pathways, potential hormone metabolic genes, co-activators, co-repressors and other unknown factors that may impinge upon receptor activity. Based on the absence of Group3C NRs, it can be concluded that *B. glabrata* is an inappropriate model for mammalian steroid hormone function mediated via NRs, as the genes for several major steroid hormone receptors are not present. However since steroid hormones have also been shown to act via non-genomic mechanisms in vertebrates, using membrane bound receptors (mPR and GPR30) [87] there is still potential to examine alternative pathways in these organisms.

Conclusions

We identified 39 nuclear receptor genes in *B. glabrata* and 33 in *L. gigantea* representing all seven principal vertebrate nuclear receptor groups. Molluscan endocrinology is poorly understood, so further study to determine the functionality of these identified NRs promises new insights, especially concerning the many unanswered questions regarding the effects of steroids and other EDCs on molluscs. The importance of the snail as an intermediate host for schistosomes, also justifies further investigation into the function of such genes. The absence of vertebrate NRs such as VDR, CAR and PXR, as well as steroid hormone NRs, AR/PR/GR/MR for which we found no orthologues in the two mollusc species examined in this study, indicates that several significant signalling pathways are absent in gastropods. Nevertheless, we have identified an array of NRs common to both vertebrates/molluscs and molluscs/flies. These results add weight to Thummel's speculation about the convergent regulation of NRs in vertebrates and invertebrates [124]. Elucidation of NR targets in molluscs may unlock their potential as new model organisms allowing the discovery of new pathways leading to similar phenotypes found in vertebrates or, indeed, similar pathways that produce a different phenotype; both of which could potentially form simple test assays, for determining gene function or drug/chemical testing. The range of phenotypes, and their underlying genetic mechanisms, available for study in different species may enable the identification of alternative pathways mediated by NRs that might also be exploited [125]. The potential of many invertebrate species for endocrine study is yet to be explored, but the underlying fundamental similarities and differences between molluscs and vertebrates may be the solution to determining not only the endocrine mechanisms of molluscs but also the full intricacies of our own.

Supporting Information

S1 Fig. Bayesian tree showing nuclear receptors in molluscs, human, fly, nematode and trematode. The NRs from six different species were subjected to phylogenetic comparisons using Bayesian inference. The Bayesian tree (midpoint rooted) with posterior probability values is shown. Notations Bg, Lg, Hs, Dm, Ce and Sm in association with receptor names denote sequences from *B. glabrata*, *L. gigantea*, *H. sapiens*, *D. melanogaster*, *C. elegans* and *S. mansoni* respectively.
(PDF)

S2 Fig. Maximum Likelihood tree showing nuclear receptors in molluscs, human, fly, nematode and trematode. The nuclear receptors from six different species were subjected to phylogenetic comparison using maximum likelihood method using Jones-Taylor-Thornton (JTT) substitution model. Node labels indicate bootstrap values. Notations Bg, Lg, Hs, Dm, Sm and Ce in association with the receptor name denote sequences from *B. glabrata*, *L. gigantea*, *H. sapiens*, *D. melanogaster*, *S, mansoni* and *C. elegans*, respectively.
(PDF)

S3 Fig. Maximum Parsimony tree showing nuclear receptors in molluscs, human, fly, nematode and trematode. The nuclear receptors from six different species were subjected to phylogenetic comparison using maximum parsimony. Node labels indicate bootstrap values. Notations Bg, Lg, Hs, Dm, Sm and Ce in association with the receptor name denote sequences from *B. glabrata*, *L. gigantea*, *H. sapiens*, *D. melanogaster*, *S. mansoni* and *C. elegans*, respectively.
(PDF)

S4 Fig. Sequence alignment file showing the unclassified NRs from *B. glabrata* as different genes with overlapping regions (DBD and LBD).
(PDF)

S1 File. A clustal alignment file of the conserved domain regions (DBD and LBD) of all the nuclear receptors included in the study. Notations Bg, Lg, Hs, Dm, Ce and Sm in association with receptor names denote sequences from *B. glabrata*, *L. gigantea*, *H. sapiens*, *D. melanogaster*, *C. elegans*, and *S. mansoni* respectively.
(TXT)

S1 Table. Oligonucleotide primers used in RT-PCR analyses of nuclear receptor genes. Forward and reverse primer sequences with their product size (as submitted to GenBank), their corresponding GenBank accession numbers and their optimised annealing temperatures.
(DOCX)

S2 Table. *L. gigantea* transcripts. Detailed summary of all the top BLAST expressed sequence tag (EST) hits for *L. gigantea* NRs, including the EST description, GenBank accession number and E-value.
(PDF)

Acknowledgments

Author Contributions

Conceived and designed the experiments: SK SJ CSJ LRN EJR AEL. Performed the experiments: SK AEL. Analyzed the data: SK AEL CSJ. Contributed reagents/materials/analysis tools: SJ CSJ. Wrote the paper: SK SJ CSJ LRN EJR AEL.

References

1. Chitsulo L, Loverde R, Engels D, Barakat R, Colley D, Cioli D, et al. Schistosomiasis. Nat Rev Microbiol. 2004; 2: 12–13. PMID: 15035004

2. Lockyer AE, Spinks J, Noble LR, Rollinson D, Jones CS. Identification of genes involved in interactions between *Biomphalaria glabrata* and *Schistosoma mansoni* by suppression subtractive hybridization. Mol Biochem Parasitol. 2007; 151: 18–27. doi: 10.1016/j.molbiopara.2006.09.009 PMID: 17081633

3. Lockyer AE, Spinks JN, Walker AJ, Kane RA, Noble LR, Rollinson D, et al. *Biomphalaria glabrata* transcriptome: Identification of cell-signalling, transcriptional control and immune-related genes from open reading frame expressed sequence tags (ORESTES). Dev Comp Immunol. 2007; 31: 763–782. doi: 10.1016/j.dci.2006.11.004 PMID: 17208299

4. Mitta G, Galinier R, Tisseyre P, Allienne JF, Girerd-Chambaz Y, Guillou F, et al. Gene discovery and expression analysis of immune-relevant genes from *Biomphalaria glabrata* hemocytes. Dev Comp Immunol. 2005; 29: 393–407. PMID: 15707661

5. Adema CM, Luo MZ, Hanelt B, Hertel LA, Marshall JJ, Zhang SM, et al. A bacterial artificial chromosome library for *Biomphalaria glabrata*, intermediate snail host of *Schistosoma mansoni*. Mem Inst Oswaldo Cruz. 2006; 101: 167–177. PMID: 17308766

6. Lockyer AE, Emery AM, Kane RA, Walker AJ, Mayer CD, Mitta G, et al. Early differential gene expression in haemocytes from resistant and susceptible *Biomphalaria glabrata* strains in response to *Schistosoma mansoni*. PLoS One. 2012; 7: e511020. doi: 10.1371/journal.pone.0051102

7. Adema CM, Hanington PC, Lun C-M, Rosenberg GH, Aragon AD, Stout BA, et al. Differential transcriptomic responses of *Biomphalaria glabrata* (Gastropoda, Mollusca) to bacteria and metazoan parasites, *Schistosoma mansoni* and *Echinostoma paraensei* (Digenea, Platyhelminthes). Mol Immunol. 2010; 47: 849–860. doi: 10.1016/j.molimm.2009.10.019 PMID: 19962194

8. Jiang YG, Loker ES, Zhang SM. In vivo and in vitro knockdown of FREP2 gene expression in the snail *Biomphalaria glabrata* using RNA interference. Dev Comp Immunol. 2006; 30: 855–866. PMID: 16442620

9. Hanington PC, Forys MA, Dragoo JW, Zhang S-M, Adema CM, Loker ES. Role for a somatically diversified lectin in resistance of an invertebrate to parasite infection. Proc Natl Acad Sci USA. 2010; 107: 21087–21092. doi: 10.1073/pnas.1011242107 PMID: 21084634

10. Raghavan N, Knight M. The snail (*Biomphalaria glabrata*) genome project. Trends Parasitol. 2006; 22: 148–151. PMID: 16497557

11. Ford ES, Li C, Zhao G, Pearson WS, Mokdad AH. Prevalence of the metabolic syndrome among US adolescents using the definition from the International Diabetes Federation. Diabetes Care. 2008; 31: 587–589. PMID: 18071007

12. Golden SH, Robinson KA, Saldanha I, Anton B, Ladenson PW. Prevalence and incidence of endocrine and metabolic disorders in the United States: a comprehensive review. J Clin Endocrinol Metab. 2009; 94: 1853–1878. doi: 10.1210/jc.2008-2291 PMID: 19494161

13. Schug TT, Janesick A, Blumberg B, Heindel JJ. Endocrine disrupting chemicals and disease susceptibility. J Steroid Biochem Mol Biol. 2011; 127: 204–215. doi: 10.1016/j.jsbmb.2011.08.007 PMID: 21899826

14. Schiffelers M-JWA, Blaauboer BJ, Bakker WE, Beken S, Hendriksen CFM, Koeter HBWM, et al. Regulatory acceptance and use of 3R models for pharmaceuticals and chemicals: Expert opinions on the state of affairs and the way forward. Regul Toxicol Pharmacol. 2014; 69: 41–48. doi: 10.1016/j.yrtph.2014.02.007 PMID: 24534000

15. Matthiessen P. An assessment of endocrine disruption in mollusks and the potential for developing internationally standardized mollusk life cycle test guidelines. Integr Environ Assess Manag. 2008; 4: 274–284. doi: 10.1897/IEAM_2008-003.1 PMID: 18393578

16. Sladek FM. What are nuclear receptor ligands? Mol Cell Endocrinol. 2011; 334: 3–13. doi: 10.1016/j.mce.2010.06.018 PMID: 20615454

17. Tallarico L de F, Borrely SI, Hamada N, Grazeffe VS, Ohlweiler FP, Okazaki K, et al. Developmental toxicity, acute toxicity and mutagenicity testing in freshwater snails *Biomphalaria glabrata* (Mollusca:

Gastropoda) exposed to chromium and water samples. Ecotoxicol Environ Saf. 2014; 110: 208–215. doi: 10.1016/j.ecoenv.2014.09.005 PMID: 25259848

18. Tyler C, Jobling S, Sumpter J. Endocrine disruption in wildlife: A critical review of the evidence. Crit Rev Toxicol. 1998; 28: 319–361. doi: 10.1080/10408449891344236 PMID: 9711432

19. Pawlak M, Lefebvre P, Staels B. General molecular biology and architecture of nuclear receptors. Curr Top Med Chem. 2012; 12: 486–504. PMID: 22242852

20. Glass CK, Rosenfeld MG. The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev. 2000; 14: 121–141. PMID: 10652267

21. Aranda A, Pascual A. Nuclear hormone receptors and gene expression. Physiol Rev. 2001; 81: 1269–1304. PMID: 11427696

22. Wurtz J-M, Bourguet W, Renaud J-P, Vivat V, Chambon P, Moras D, et al. A canonical structure for the ligand-binding domain of nuclear receptors. Nat Struct Mol Biol. 1996; 3: 87–94.

23. Auwerx J, Baulieu E, Beato M, Becker-Andre M, Burbach PH, Camerino G, et al. A unified nomenclature system for the nuclear receptor superfamily. Cell. 1999; 97: 161–163. PMID: 10219237

24. Bertrand S, Brunet FG, Escriva H, Parmentier G, Laudet V, Robinson-Rechavi M. Evolutionary genomics of nuclear receptors: from twenty-five ancestral genes to derived endocrine systems. Mol Biol Evol. 2004; 21: 1923–1937. PMID: 15229292

25. Bridgham JT, Eick GN, Larroux C, Deshpande K, Harms MJ, Gauthier ME, et al. Protein evolution by molecular tinkering: diversification of the nuclear receptor superfamily from a ligand-dependent ancestor. PLoS Biol. 2010; 8: e1000497. doi: 10.1371/journal.pbio.1000497 PMID: 20957188

26. Markov GV, Laudet V. Origin and evolution of the ligand-binding ability of nuclear receptors. Mol Cell Endocrinol. 2011; 334: 21–30. doi: 10.1016/j.mce.2010.10.017 PMID: 21055443

27. Matthiessen P, Gibbs P. Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in mollusks. Environ Toxicol Chem. 1998; 17: 37–43.

28. Oehlmann J, Schulte-Oehlmann U. Endocrine disruption in invertebrates. Pure Appl Chem. 2003; 75: 2207–2218.

29. Castro LFC, Lima D, Machado A, Melo C, Hiromori Y, Nishikawa J, et al. Imposex induction is mediated through the Retinoid X Receptor signalling pathway in the neogastropod *Nucella lapillus*. Aquat Toxicol. 2007; 85: 57–66. PMID: 17875330

30. Sternberg RM, Gooding MP, Hotchkiss AK, LeBlanc GA. Environmental-endocrine control of reproductive maturation in gastropods: implications for the mechanism of tributyltin-induced imposex in prosobranchs. Ecotoxicology. 2010; 19: 4–23. doi: 10.1007/s10646-009-0397-z PMID: 19653098

31. Lafont R, Mathieu M. Steroids in aquatic invertebrates. Ecotoxicology. 2007; 16: 109–130. doi: 10.1007/s10646-006-0113-1 PMID: 17238002

32. Koehler H-R, Kloas W, Schirling M, Lutz I, Reye AL, Langen J-S, et al. Sex steroid receptor evolution and signalling in aquatic invertebrates. Ecotoxicology. 2007; 16: 131–143. doi: 10.1007/s10646-006-0111-3 PMID: 17219085

33. Bannister R, Beresford N, May D, Routledge EJ, Jobling S, Rand-Weaver M. Novel estrogen receptor-related transcripts in *Marisa cornuarietis*; a freshwater snail with reported sensitivity to estrogenic chemicals. Environ Sci Technol. 2007;41. doi: 10.1021/es062565m

34. Stange D, Sieratowicz A, Horres R, Oehlmann J. Freshwater mudsnail (*Potamopyrgus antipodarum*) estrogen receptor: Identification and expression analysis under exposure to (xeno-) hormones. Ecotoxicol Environ Saf. 2012; 75: 94–101. doi: 10.1016/j.ecoenv.2011.09.003 PMID: 21944693

35. Hultin CL, Hallgren P, Persson A, Hansson MC. Identification of an estrogen receptor gene in the natural freshwater snail *Bithynia tentaculata*. Gene. 2014; 540: 26–31. doi: 10.1016/j.gene.2014.02.039 PMID: 24583164

36. Keay J, Bridgham JT, Thornton JW. The *Octopus vulgaris* estrogen receptor is a constitutive transcriptional activator: Evolutionary and functional implications. Endocrinology. 2006; 147: 3861–3869. doi: 10.1210/en.2006-0363 PMID: 16690796

37. Matsumoto T, Nakamura AM, Mori K, Akiyama I, Hirose H, Takahashi Y. Oyster estrogen receptor: cDNA cloning and immunolocalization. Gen Comp Endocrinol. 2007; 151: 195–201. doi: 10.1016/j.ygcen.2007.01.016 PMID: 17324427

38. Thornton JW, Need E, Crews D. Resurrecting the ancestral steroid receptor: Ancient origin of estrogen signaling. Science. 2003; 301: 1714–1717. doi: 10.1126/science.1086185 PMID: 14500980

39. Kajiwara M, Kuraku S, Kurokawa T, Kato K, Toda S, Hirose H, et al. Tissue preferential expression of estrogen receptor gene in the marine snail, *Thais clavigera*. Gen Comp Endocrinol. 2006; 148: 315–326. doi: 10.1016/j.ygcen.2006.03.016 PMID: 16782100

40. Bannister R, Beresford N, Granger DW, Pounds NA, Rand-Weaver M, White R, et al. No substantial changes in estrogen receptor and estrogen-related receptor orthologue gene transcription in *Marisa cornuarietis* exposed to estrogenic chemicals. Aquat Toxicol. 2013; 140: 19–26. doi: 10.1016/j.aquatox.2013.05.002 PMID: 23747549

41. Markov GV, Tavares R, Dauphin-Villemant C, Demeneix BA, Baker ME, Laudet V. Independent elaboration of steroid hormone signaling pathways in metazoans. Proc Natl Acad Sci USA. 2009; 106: 11913–11918. doi: 10.1073/pnas.0812138106 PMID: 19571007

42. Omran NE-SE-S. Testosterone, gonadotropins and androgen receptor during spermatogenesis of *Biomphalaria alexandrina* snails (Pulmonata: Basommatophora). Reprod Biol. 2012; 12: 301–308. doi: 10.1016/j.repbio.2012.10.002 PMID: 23153701

43. Scott AP. Do mollusks use vertebrate sex steroids as reproductive hormones? Part I: Critical appraisal of the evidence for the presence, biosynthesis and uptake of steroids. Steroids. 2012; 77: 1450–1468. doi: 10.1016/j.steroids.2012.08.009 PMID: 22960651

44. Sternberg RM, Hotchkiss AK, LeBlanc GA. The contribution of steroidal androgens and estrogens to reproductive maturation of the eastern mud snail *Ilyanassa obsoleta*. Gen Comp Endocrinol. 2008; 156: 15–26. doi: 10.1016/j.ygcen.2007.12.002 PMID: 18226815

45. Vogeler S, Galloway TS, Lyons BP, Bean TP. The nuclear receptor gene family in the Pacific oyster, *Crassostrea gigas*, contains a novel subfamily group. BMC Genomics. 2014; 15: 369. doi: 10.1186/1471-2164-15-369 PMID: 24885009

46. Bouton D, Escriva H, De Mendonca RL, Glineur C, Bertin B, Noel C, et al. A conserved retinoid X receptor (RXR) from the mollusk *Biomphalaria glabrata* transactivates transcription in the presence of retinoids. J Mol Endocrinol. 2005; 34. doi: 10.1677/jme.1.01766

47. Simakov O, Marletaz F, Cho S-J, Edsinger-Gonzales E, Havlak P, Hellsten U, et al. Insights into bilaterian evolution from three spiralian genomes. Nature. 2013; 493: 526–531. doi: 10.1038/nature11696 PMID: 23254933

48. Wu W, Niles EG, Hirai H, LoVerde PT. Evolution of a novel subfamily of nuclear receptors with members that each contain two DNA binding domains. BMC Evol Biol. 2007; 7: 27. doi: 10.1186/1471-2148-7-27 PMID: 17319953

49. Wu W, Niles EG, Loverde PT. Thyroid hormone receptor orthologues from invertebrate species with emphasis on *Schistosoma mansoni*. BMC Evol Biol. 2007; 7: 150. doi: 10.1186/1471-2148-7-150 PMID: 17727708

50. Enmark E, Gustafsson J-Å. Comparing nuclear receptors in worms, flies and humans. Trends Pharmacol Sci. 2001; 22: 611–615. PMID: 11730970

51. Wu W, LoVerde PT. Nuclear hormone receptors in parasitic helminths. Mol Cell Endocrinol. 2011; 334: 56–66. doi: 10.1016/j.mce.2010.06.011 PMID: 20600585

52. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic Local Alignment Search Tool. J Mol Biol. 1990; 215: 403–410. PMID: 2231712

53. Burge CB, Karlin S. Finding the genes in genomic DNA. Curr Opin Struct Biol. 1998; 8: 346–354. PMID: 9666331

54. Hertel LA, Adema CM, Loker ES. Differential expression of FREP genes in two strains of *Biomphalaria glabrata* following exposure to the digenetic trematodes *Schistosoma mansoni* and *Echinostoma paraensei*. Dev Comp Immunol. 2005; 29: 295–303. PMID: 15859234

55. Bateman A, Coin L, Durbin R, Finn RD, Hollich V, Griffiths-Jones S, et al. The Pfam protein families database. Nucleic Acids Res. 2004; 32: D138–D141. PMID: 14681378

56. Thomas PD, Campbell MJ, Kejariwal A, Mi H, Karlak B, Daverman R, et al. PANTHER: a library of protein families and subfamilies indexed by function. Genome Res. 2003; 13: 2129–2141. PMID: 12952881

57. Letunic I, Copley RR, Pils B, Pinkert S, Schultz J, Bork P. SMART 5: domains in the context of genomes and networks. Nucleic Acids Res. 2006; 34: 242–244. doi: 10.1093/nar/gkj079

58. Robinson-Rechavi M, Maina CV, Gissendanner CR, Laudet V, Sluder A. Explosive lineage-specific expansion of the orphan nuclear receptor HNF4 in nematodes. J Mol Evol. 2005; 60: 577–586. PMID: 15983867

59. Ronquist F, Huelsenbeck J. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 2003; 19: 1572–1574. PMID: 12912839

60. Abascal F, Zardoya R, Posada D. ProtTest: selection of best-fit models of protein evolution. Bioinformatics. 2005; 21: 2104–2105. doi: 10.1093/bioinformatics/bti263 PMID: 15647292

61. Jones D, Taylor W, Thornton J. The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci. 1992; 8: 275–282. PMID: 1633570

62. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol. 2013; 30: 2725–2729. doi: 10.1093/molbev/mst197 PMID: 24132122

63. Huson DH, Richter DC, Rausch C, Dezulian T, Franz M, Rupp R. Dendroscope: An interactive viewer for large phylogenetic trees. BMC Bioinformatics. 2007; 8: 460. doi: 10.1186/1471-2105-8-460 PMID: 18034891

64. Robinson-Rechavi M, Carpentier AS, Duffraisse M, Laudet V. How many nuclear hormone receptors are there in the human genome? Trends Genet. 2001; 17: 554–556. doi: 10.1016/S0168-9525(01)02417-9 PMID: 11585645

65. Robinson-Rechavi M, Garcia HE, Laudet V. The nuclear receptor superfamily. J Cell Sci. 2003; 116: 585–586. PMID: 12538758

66. Zhang ZD, Burch PE, Cooney AJ, Lanz RB, Pereira FA, Wu JQ, et al. Genomic analysis of the nuclear receptor family: New insights into structure, regulation, and evolution from the rat genome. Genome Res. 2004; 14. doi: 10.1101/gr.2160004

67. Adams MD, Celniker SE, Holt RA, Evans CA, Gocayne JD, Amanatides PG, et al. The genome sequence of *Drosophila melanogaster*. Science. 2000; 287: 2185–2195. PMID: 10731132

68. Velarde RA, Robinson GE, Fahrbach SE. Nuclear receptors of the honey bee: annotation and expression in the adult brain. Insect Mol Biol. 2006; 15: 583–595. PMID: 17069634

69. Bonneton F, Chaumot A, Laudet V. Annotation of *Tribolium* nuclear receptors reveals an increase in evolutionary rate of a network controlling the ecdysone cascade. Insect Biochem Mol Biol. 2008; 38: 416–429. doi: 10.1016/j.ibmb.2007.10.006 PMID: 18342247

70. Sluder AE, Maina CV. Nuclear receptors in nematodes: themes and variations. Trends Genet. 2001; 17: 206–213. PMID: 11275326

71. Wu W, Niles EG, El-Sayed N, Berriman M, LoVerde PT. *Schistosoma mansoni* (Platyhelminthes, Trematoda) nuclear receptors: Sixteen new members and a novel subfamily. Gene. 2006; 366: 303–315. doi: 10.1016/j.gene.2005.09.013 PMID: 16406405

72. Thomson SA, Baldwin WS, Wang YH, Kwon G, LeBlanc GA. Annotation, phylogenetics, and expression of the nuclear receptors in *Daphnia pulex*. BMC Genomics. 2009; 10: 500. doi: 10.1186/1471-2164-10-500 PMID: 19863811

73. Baker ME. Trichoplax, the simplest known animal, contains an estrogen-related receptor but no estrogen receptor: Implications for estrogen receptor evolution. Biochem Biophys Res Commun. 2008; 375: 623–627. doi: 10.1016/j.bbrc.2008.08.047 PMID: 18722350

74. Katsu Y, Kubokawa K, Urushitani H, Iguchi T. Estrogen-dependent transactivation of amphioxus steroid hormone receptor via both estrogen and androgen response elements. Endocrinology. 2010; 151: 639–648. doi: 10.1210/en.2009-0766 PMID: 19966182

75. Baker ME. Co-evolution of steroidogenic and steroid-inactivating enzymes and adrenal and sex steroid receptors. Mol Cell Endocrinol. 2004; 215: 55–62. PMID: 15026175

76. Puinean AM, Labadie P, Hill EM, Osada M, Kishida M, Nakao R, et al. Laboratory exposure to 17 beta-estradiol fails to induce vitellogenin and estrogen receptor gene expression in the marine invertebrate *Mytilus edulis*. Aquat Toxicol. 2006; 79: 376–383. doi: 10.1016/j.aquatox.2006.07.006 PMID: 16930737

77. Zhang H, Pan L, Zhang L. Molecular cloning and characterization of estrogen receptor gene in the scallop *Chlamys farreri*: Expression profiles in response to endocrine disrupting chemicals. Comp Biochem Physiol C-Toxicol Pharmacol. 2012; 156: 51–57. doi: 10.1016/j.cbpc.2012.03.007 PMID: 22507668

78. Kumkate S, Onmek N, Boonburapong B, Singhakaew S, Leardkamolkarn V. Estrogen-related fecundity reduction of *Lymnaea ollula* following *Fasciola gigantica* infection. J Parasitol. 2009; 95: 1391–1396. doi: 10.1645/GE-2080.1 PMID: 19642809

79. Castro LFC, Melo C, Guillot R, Mendes I, Queiros S, Lima D, et al. The estrogen receptor of the gastropod *Nucella lapillus*: Modulation following exposure to an estrogenic effluent? Aquat Toxicol. 2007; 84: 465–468. doi: 10.1016/j.aquatox.2007.07.008 PMID: 17727972

80. Eick GN, Thornton JW. Evolution of steroid receptors from an estrogen-sensitive ancestral receptor. Mol Cell Endocrinol. 2011; 334: 31–38. doi: 10.1016/j.mce.2010.09.003 PMID: 20837101

81. Bridgham JT, Brown JE, Rodríguez-Marí A, Catchen JM, Thornton JW. Evolution of a new function by degenerative mutation in cephalochordate steroid receptors. PLoS Genet. 2008; 4: e1000191. doi: 10.1371/journal.pgen.1000191 PMID: 18787702

82. Keay J, Thornton JW. Hormone-activated estrogen receptors in annelid invertebrates: Implications for evolution and endocrine disruption. Endocrinology. 2009; 150: 1731–1738. doi: 10.1210/en.2008-1338 PMID: 19036877

83. Bardet PL, Laudet V, Vanacker JM. Studying non-mammalian models? Not a fool's ERRand! Trends Endocrinol Metab. 2006; 17: 166–171. doi: 10.1016/j.tem.2006.03.005 PMID: 16580224

84. Scott AP. Do mollusks use vertebrate sex steroids as reproductive hormones? Part II. Critical review of the evidence that steroids have biological effects. Steroids. 2013; 78: 268–281. doi: 10.1016/j.steroids.2012.11.006 PMID: 23219696

85. Tillmann M, Schulte-Oehlmann U, Duft M, Markert B, Oehlmann J. Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part III: Cyproterone acetate and vinclozolin as antiandrogens. Ecotoxicology. 2001; 10: 373–388. doi: 10.1023/A:1012279231373 PMID: 11759570

86. Thomas P, Dong J. Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: a potential novel mechanism of endocrine disruption. J Steroid Biochem Mol Biol. 2006; 102: 175–179. PMID: 17088055

87. Levin ER. Rapid signaling by steroid receptors. Am J Physiol Regul Integr Comp Physiol. 2008; 295: R1425–R1430. doi: 10.1152/ajpregu.90605.2008 PMID: 18784332

88. Simoncini T, Genazzani AR. Non-genomic actions of sex steroid hormones. Eur J Endocrinol. 2003; 148: 281–292. PMID: 12611608

89. Carter CJ, Farrar N, Carlone RL, Spencer GE. Developmental expression of a molluscan RXR and evidence for its novel, nongenomic role in growth cone guidance. Dev Biol. 2010; 343: 124–137. doi: 10.1016/j.ydbio.2010.03.023 PMID: 20381485

90. De Groot A, De Rosny E, Juillan-Binard C, Ferrer JL, Laudet V, Pierce RJ, et al. Crystal structure of a novel tetrameric complex of agonist-bound ligand-binding domain of *Biomphalaria glabrata* retinoid X receptor. J Mol Biol. 2005; 354: 841–853. PMID: 16274693

91. Lima D, Reis-Henriques MA, Silva R, Santos AI, Castro LFC, Santos MM. Tributyltin-induced imposex in marine gastropods involves tissue-specific modulation of the retinoid X receptor. Aquat Toxicol. 2011; 101: 221–227. doi: 10.1016/j.aquatox.2010.09.022 PMID: 21036407

92. Le Maire A, Grimaldi M, Roecklin D, Dagnino S, Vivat-Hannah V, Balaguer P, et al. Activation of RXR—PPAR heterodimers by organotin environmental endocrine disruptors. EMBO Rep. 2009; 10: 367–373. doi: 10.1038/embor.2009.8 PMID: 19270714

93. Pascoal S, Carvalho G, Vasieva O, Hughes R, Cossins A, Fang Y, et al. Transcriptomics and in vivo tests reveal novel mechanisms underlying endocrine disruption in an ecological sentinel, *Nucella lapillus*. Mol Ecol. 2012; 22: 1589–1608. doi: 10.1111/mec.12137 PMID: 23205577

94. Kliewer S, Umesono K, Noonan D, Heyman R, Evans R. Convergence of 9-cis retinoic acid and peroxisome proliferator signaling pathways through heterodimer formation of their receptors. Nature. 1992; 358: 771–774. doi: 10.1038/358771a0 PMID: 1324435

95. Heyland A, Moroz LL. Cross-kingdom hormonal signaling: an insight from thyroid hormone functions in marine larvae. J Exp Biol. 2005; 208: 4355–4361. doi: 10.1242/jeb.01877 PMID: 16339856

96. King-Jones K, Thummel C. Nuclear receptors—A perspective from *Drosophila*. Nat Rev Genet. 2005; 6: 311–323. doi: 10.1038/nrg1581 PMID: 15803199

97. Li T, Bender M. A conditional rescue system reveals essential functions for the ecdysone receptor (EcR) gene during molting and metamorphosis in *Drosophila*. Development. 2000; 127: 2897–2905. PMID: 10851134

98. Whitehead DL, Sellheyer K. The identification of ecdysterone (20-hydroxyecdysone) in 3 species of mollusks (Gastropoda, Pulmonata). Experientia. 1982; 38. doi: 10.1007/BF01959769

99. Garcia M, Griffond B, Lafont R. What are the origins of ecdysteroids in gastropods? Gen Comp Endocrinol. 1995; 97: 76–85. PMID: 7713386

100. Shiff C, Dossaji S. Ecdysteroids as regulators of host and parasite interactions—a study. Trop Med Parasitol. 1991; 42: 11–16. PMID: 2052849

101. Laguerre M, Veenstra JA. Ecdysone receptor homologs from mollusks, leeches and a polychaete worm. FEBS Lett. 2010; 584: 4458–4462. doi: 10.1016/j.febslet.2010.10.004 PMID: 20934425

102. Urushitani H, Katsu Y, Ohta Y, Shiraishi H, Iguchi T, Horiguchi T. Cloning and characterization of the retinoic acid receptor-like protein in the rock shell, *Thais clavigera*. Aquat Toxicol. 2013; 142: 403–413. doi: 10.1016/j.aquatox.2013.09.008 PMID: 24096236

103. Gutierrez-Mazariegos J, Kumar Nadendla E, Lima D, Pierzchalski K, Jones JW, Kane M, et al. A mollusk Retinoic Acid Receptor (RAR) ortholog sheds light on the evolution of ligand binding. Endocrinology. 2014; en.2014–1181. doi: 10.1210/en.2014-1181

104. Chung AC, Cooney AJ. The varied roles of nuclear receptors during vertebrate embryonic development. Nucl Recept Signal. 2003; 1. doi: 10.1621/nrs.01007 PMID: 16604184

105. Mu X, Lee Y-F, Liu N-C, Chen Y-T, Kim E, Shyr C-R, et al. Targeted inactivation of testicular nuclear orphan receptor 4 delays and disrupts late meiotic prophase and subsequent meiotic divisions of spermatogenesis. Mol Cell Biol. 2004; 24: 5887–5899. PMID: 15199144

106. Chen L-M, Wang R-S, Lee Y-F, Liu N-C, Chang Y-J, Wu C-C, et al. Subfertility with defective folliculogenesis in female mice lacking testicular orphan nuclear receptor 4. Mol Endocrinol. 2008; 22: 858–867. doi: 10.1210/me.2007-0181 PMID: 18174360

107. Ding X, Yu S, Chen B, Lin S, Chang C, Li G. Recent advances in the study of testicular nuclear receptor 4. J Zhejiang Univ-SCI B. 2013; 14: 171–177. doi: 10.1631/jzus.B1200357 PMID: 23463759

108. Daniel A, Dumstrei K, Lengyel JA, Hartenstein V. The control of cell fate in the embryonic visual system by atonal, tailless and EGFR signaling. Development. 1999; 126: 2945–2954. PMID: 10357938

109. Yu RT, Chiang MY, Tanabe T, Kobayashi M, Yasuda K, Evans RM, et al. The orphan nuclear receptor *Tlx* regulates *Pax2* and is essential for vision. Proc Natl Acad Sci USA. 2000; 97: 2621–2625. doi: 10.1073/pnas.050566897 PMID: 10706625

110. Snow MI, Larsen PL. Structure and expression of daf-12: a nuclear hormone receptor with three isoforms that are involved in development and aging in *Caenorhabditis elegans*. BBA-Gene Struct Expr. 2000; 1494: 104–116. PMID: 11072073

111. Benoit G, Malewicz M, Perlmann T. Digging deep into the pockets of orphan nuclear receptors: insights from structural studies. Trends Cell Biol. 2004; 14: 369–376. doi: 10.1016/j.tcb.2004.05.007 PMID: 15246430

112. King-Jones K, Horner MA, Lam G, Thummel CS. The DHR96 nuclear receptor regulates xenobiotic responses in *Drosophila*. Cell Metab. 2006; 4: 37–48. PMID: 16814731

113. Lindblom TH, Dodd AK. Xenobiotic detoxification in the nematode *Caenorhabditis elegans*. J Exp Zool Part A Comp Exp Biol. 2006; 305: 720–730. PMID: 16902959

114. Antebi A, Yeh W, Tait D, Hedgecock E, Riddle D. *daf-12* encodes a nuclear receptor that regulates the dauer diapause and developmental age in *C. elegans*. Genes Dev. 2000; 14: 1512–1527. PMID: 10859169

115. Ueda H, Sun GC, Murata T, Hirose S. A novel DNA-binding motif abuts the zinc finger domain of insect nuclear hormone receptor *ftz-f1* and mouse embryonal long terminal repeat-binding protein. Mol Cell Biol. 1992; 12: 5667–5672. PMID: 1448096

116. Allen AK, Spradling AC. The SF1-related nuclear hormone receptor HR39 regulates *Drosophila* female reproductive tract development and function. Development. 2008; 135: 311–321. doi: 10.1242/dev.015156 PMID: 18077584

117. Lockyer AE, Jones CS, Noble LR, Rollinson D. Trematodes and snails: an intimate association. Can J Zool. 2004; 82: 251–269.

118. Escobedo G, Roberts CW, Carrero JC, Morales-Montor J. Parasite regulation by host hormones: an old mechanism of host exploitation? Trends Parasitol. 2005; 21: 588–593. doi: 10.1016/j.pt.2005.09.013 PMID: 16236553

119. Stone B, Thummel C. The *Drosophila* 78c early-late puff contains E78, an ecdysone-inducible gene that encodes a novel member of the nuclear hormone-receptor superfamily. Cell. 1993; 75: 307–320. doi: 10.1016/0092-8674(93)80072-M PMID: 8402914

120. Rapado LN, Freitas GC, Polpo A, Rojas-Cardozo M, Rincon JV, Scotti MT, et al. A benzoic acid derivative and flavokawains from *Piper* species as schistosomiasis vector controls. Molecules. 2014; 19: 5205–5218. doi: 10.3390/molecules19045205 PMID: 24762961

121. Knight M, Miller A, Liu Y, Scaria P, Woodle M, Ittiprasert W. Polyethyleneimine (PEI) mediated siRNA gene silencing in the *Schistosoma mansoni* snail host, *Biomphalaria glabrata*. PLoS Neglect Trop Dis. 2011; 5: e1212. doi: 10.1371/journal.pntd.0001212 PMID: 21765961

122. Lockyer AE, Spinks J, Kane RA, Hoffmann KF, Fitzpatrick JM, Rollinson D, et al. *Biomphalaria glabrata* transcriptome: cDNA microarray profiling identifies resistant- and susceptible-specific gene expression in haemocytes from snail strains exposed to *Schistosoma mansoni*. BMC Genomics. 2008; 9: 634. doi: 10.1186/1471-2164-9-634 PMID: 19114004

123. Hanington PC, Lun C-M, Adema CM, Loker ES. Time series analysis of the transcriptional responses of *Biomphalaria glabrata* throughout the course of intramolluscan development of *Schistosoma mansoni* and *Echinostoma paraensei*. Int J Parasitol. 2010; 40: 819–831. doi: 10.1016/j.ijpara.2009.12.005 PMID: 20083115

124. Thummel C. Flies on steroids—*Drosophila* metamorphosis and the mechanisms of steroid hormone action. Trends Genet. 1996; 12: 306–310. doi: 10.1016/0168-9525(96)10032-9 PMID: 8783940

125. Greenspan RJ. No critter left behind: An invertebrate renaissance. Curr Biol. 2005; 15: R671–R672. doi: 10.1016/j.cub.2005.08.018 PMID: 16139200