Evolutionary Fate of the Androgen Receptor—Signaling Pathway in Ray-Finned Fishes with a Special Focus on Cichlids

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ABSTRACT The emergence of the steroid system is coupled to the evolution of multicellular animals. In vertebrates in particular, the steroid receptor repertoire has been shaped by genome duplications characteristic to this lineage. Here, we investigate for the first time the composition of the androgen receptor—signaling pathway in ray-finned fish genomes by focusing in particular on duplicates that emerged from the teleost-specific whole-genome duplication. We trace lineage- and species-specific duplications and gene losses for the genomic and nongenomic pathway of androgen signaling and subsequently investigate the sequence evolution of these genes. In one particular fish lineage, the cichlids, we find evidence for differing selection pressures acting on teleost-specific whole-genome duplication paralogs at a derived evolutionary stage. We then look into the expression of these duplicated genes in four cichlid species from Lake Tanganyika indicating, once more, rapid changes in expression patterns in closely related fish species. We focus on a particular case, the cichlid specific duplication of the rac1 GTPase, which shows possible signs of a neofunctionalization event.

KEYWORDS androgen receptor ray-finned fish cichlids genome duplication sequence evolution

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Sex steroid hormones (androgens, estrogens, and progestogens) are involved in physiological processes as different as reproduction (Wierman 2007), the establishment of sex-specific traits [e.g., pigmentation (Lindsay et al. 2011)], communication (Arch and Narins 2009), parental care (Dey et al. 2010), the immune system (García-Gómez et al. 2013), and even vision (Shao et al. 2014). Androgens, and particularly the major circulating form testosterone, were long thought of as having a predominant male function, whereas estrogens often are thought of as female hormones; however, more and more evidence is accumulating that both types of sex hormones are present in the circulating blood of both sexes (e.g., Coumailleau et al. 2015), which is not least due to the fact that testosterone can be hydroxylated by the enzyme aromatase into estrogen.

Steroid-synthesizing enzymes and steroid hormone receptors have an ancient origin within the animal kingdom. The emergence of nuclear receptors, for example, is going along with the evolution of multicellular animals (Baker et al. 2015); estrogen signaling is already present in amphioxus (Callard et al. 2011). The true androgen receptor evolved only later in gnathostomes after their split from agnathans, which already show reactivity to androgens probably mediated by other receptors (Guerrero 2009 and references therein).

The sex steroid signaling system has been shaped throughout the evolutionary history of vertebrates by two whole-genome duplication events, characterizing this lineage (Dehal and Boore 2005). Although there is a decent understanding of the pathway’s general evolutionary origin, one particular vertebrate group (teleost fish) asks for a deeper investigation for at least two reasons. First of all, the teleost fish lineage represents, with ~30,000 described species, the largest and most diverse group of vertebrates (Nelson 2006). Second, this lineage experienced an additional whole-genome duplication event approximately 320–350 million years ago, also known as the teleost-specific whole-genome duplication (TSGD) (Meyer and Van De Peer 2005), which generated extra genomic material not present in other vertebrate groups.
One evolutionary fate of this extra genetic material is neofunctionalization of one of the paralogs, an event that has actually been proposed for the B-paralog of the androgen receptor in teleosts (Douard et al. 2008).

Here we set out to study the androgen receptor (AR) and its pathway in the teleost lineage with the aim to better understand its genomic composition shaped by gene and genome duplications. We investigate selection pressures acting on this pathway and examine gene expression profiles in a particularly diverse group of fishes, the East African cichlids (Santos and Salzburger 2012; Brawand et al. 2014).

The AR is a cytosolic protein that, upon fixation of a ligand, undergoes a conformational change and dissociates from chaperone proteins, dimersizes, and can translocate into the nucleus. The receptor subsequently binds to specific target sequences called androgen response elements and acts as a transcription factor together with coactivators or corepressors on specific target genes. This “classic” or “genomic signaling” is described as the general pathway of nuclear receptors (Figure 1, left panel).

Alternative signaling of steroids not immediately involving gene transcription can occur via a “nongenomic” or “extranuclear pathway,” implying a membrane and cytosolic protein cascade (reviewed in Foradori et al. 2008; Bennett et al. 2010) (Figure 1, right panel). This nongenomic response involves the rapid induction of second-messenger signal transduction cascades. For AR, they include the release of intracellular calcium, the activation of protein kinases such as extracellular signal-regulated protein kinases 1/2, protein kinase A, protein kinase C, and Akt, as well as actin cytoskeleton reorganization (Bennett et al. 2010 and references therein). These cascades usually trigger actions within seconds or minutes (Yamada 1979; Christian et al. 2000), whereas the activation of transcription usually peaks several hours after steroid exposure (Cato et al. 1988), although the latency for transcription has been reported to be as short as 7.5 minutes (Groner et al. 1983); however, the translational process then still may require hours.

To complete the picture of AR-signaling in teleosts we here investigate the described nongenomic and genomic cascades including orthologs to all human genes associated with the Gene Ontology term GO:0030521 “androgen receptor signaling pathway” and adding the nongenomic pathways as described in NetPath [(Kandasamy et al. 2010) and (Foradori et al. 2008; Bennett et al. 2010)]. The exhaustive gene list is given in Supporting Information, Table S1. We were particularly interested in duplicates derived from the TSGD and their evolution in cichlid fishes.

**MATERIALS AND METHODS**

**In silico screening of teleost genomes**

We initially searched spotted gar (Lepisosteus oculatus) orthologs to the human genes listed in Table S1 in the genome accessible over Ensembl (www.ensembl.org, Version 79). Subsequently, the spotted gar sequences were used as query in two searches. First, we exported all annotated Ensembl orthologs for each gene for the following teleost fish species: zebrafish (Danio rerio), cavefish (Astyanax mexicanus), platfish (Xiphophorus maculatus), Amazon molly (Poecilia formosa), fugu (Takifugu rubripes), green spotted puffer (Tetraodon nigroviridis), three-spined stickleback (Gasterosteus aculeatus), medaka (Oryzias latipes), and Nile tilapia (Oreochromis niloticus). If this search did not retrieve an ortholog for the given species, we used the spotted gar coding sequence of the corresponding gene in a second search with BLASTn (search sensitivity “Normal”) against the genome of the species of interest in Ensembl. If this again did not retrieve an ortholog for the species of interest, we extended our BLASTn search to Genbank (default settings).

Additionally, we used the sequences of the Nile tilapia as query in a BLASTn search against the available East African cichlid genomes/transcriptomes of Neolamprologus brichardi, Astatotilapia/Haplochromis burtoni, Pandamilia nyerei, and Maylandia zebra in Genbank at http://blast.ncbi.nlm.nih.gov/Blast.cgi.

If orthologous blast hits were found outside annotated genomic regions, coding sequences were annotated manually; in this case chromosome or scaffold information is given (Table S1).

In addition to identification of homologous genes using BLASTn, we confirmed gene localization and copy number using syntenic analysis over the Genomicsus genome browser [http://www.genomicus.biologie.ens.fr/genomicus-80.01/cgi-bin/search.pl (Louis et al. 2013, 2015)] and Ensembl gene trees. All gene IDs are listed in Table S1, and an illustration of all found genes is given in Figure 2. Tree topology represented on Figure 2 represents the consensus tree for the whole pathway and was built using DupTree (Wehe et al. 2008) using all single gene trees as input (see Phylogenetic reconstruction).

**Codon alignments**

For each gene, the longest available coding sequence was exported and loaded into SeaView 4.5.4 to be manually verified (Gouy et al. 2010). Subsequently, in-frame alignments from start to stop codon were performed using a codon empirical model with PRANK v.140110 (Löytynoja and Goldman 2005, 2008), which has been shown to outperform other alignment software for alignments of codon blocks and, in particular, generates less false-positive cases in selection analysis (Fletcher and Yang 2010).

**Phylogenetic reconstruction**

The best-fitting nucleotide substitution model for each codon-alignment was estimated using jModelTest 2.1.7 (Guindon and Gascuel 2003; Darriba et al. 2012) according to the corrected Akaike information criterion model. The generalized-time-reversible + gamma + I model was found to be the best-fitting model for most trees (40 of 61; see Table S2 for details). Hence, maximum-likelihood phylogenies for all genes were reconstructed using PhyML 3.1 (Guindon et al. 2010) under the generalized-time-reversible + gamma + I model, with 1000 bootstrap replicates (all single gene phylogenetic reconstructions are shown in Figure S1).

**Sequence evolution**

To determine selection patterns along the teleost phylogeny for genes involved in AR-signaling, we conducted the following analyses. In a first step, we performed site-wise Ka/Ks estimates by using Selecton (http://selecton.tau.ac.il/; Doron-Faigenboim et al. 2005; Stern et al. 2007) under the M8 model (M8, beta+w > 1) enabled for positive selection (Yang and Bielawski 2000). When positively selected sites were detected under this model, it was tested against the null model [no positive selection, M8a, beta+w = 1, (Swanson et al. 2003)]. In case of significance of the likelihood-ratio test, the p-value is indicated. These tests were run on multisequence alignments for each gene using the non-teleost spotted gar as reference (Figure S2).

Next, we ran the branch-site model aBS-REL implemented in HyPhy (HYpothesis testing using PHYlogenies, Version 2.2.4) (Pond et al. 2005; Smith et al. 2015), which allows for different Ka/Ks ratios among sites and among branches. Resulting trees with selection values were visualized using the http://veg.github.io/hyphy-vision/absrel/web interface, following the developer’s instructions (Figure S3). We finally ran gene-wide Ka/Ks estimates using Kaks_calculator_2.0 (Version 1.2) under the MA method (Zhang et al. 2006) on the following alignments: all cichlids [East African Lake (EAL) cichlids + Nile tilapia] compared with medaka and EAL cichlids compared with Nile tilapia. Ka/Ks comparisons were performed over the entire sequence length.
Gene expression in cichlid fishes

To examine gene expression profiles, we retrieved RNA-sequencing information for the gene members of the AR-signaling pathway for four species from Lake Tanganyika (A. burtoni, Eretmodus cyanosoticus, Julidochromis ornatus, and Ophthalmotilapia ventralis) from Böhne et al. (2014). The sequenced tissues of this study are brain and gonad from adult males and females. Note that genomic information is currently only available for A. burtoni (Brawand et al. 2014).

Cleaned reads for each individual sample for each species as described in Böhne et al. (2014) were mapped against our A. burtoni dataset of gene coding sequences. Mapping was done using Novoalign (Novocraft) with the following settings: -r All, -t 60, -l 30, and -s 5. Alignments were reported in SAM format and sorted, indexed, and transformed into count tables (number of mapped reads per transcript per sample) with the use of SAMtools (Li et al. 2009). In total, the data set comprised read counts for four male brain, four female brain, four ovary, and four testis samples per species except for A. burtoni, where three biological replicates per tissue type were available. Differential expression analyses on raw read counts were performed with the Bioconductor edgeR package (Version 3.1) (Robinson et al. 2010) by use of the “classical” model as described in Böhne et al. (2014). Count numbers were subsequently transformed into fragments per kilobase of transcript per million mapped reads (FPKM) to estimate relative transcript abundance within edgeR. FPKM values of paralogs were compared with a two-sided Welch t-test. Analyses using PRANK, PhyML, Novoalign, and SAMtools were run on sciCORE (center for scientific computing, University of Basel, http://scicore.unibas.ch/)

Data availability

Sequence data are available at GenBank and Ensembl, the corresponding accession numbers are list in Table S1. Gene expression data were taken from Böhne et al. 2014. Scripts are available upon request.

RESULTS

Tracing the AR pathway in fish genomes

We screened 14 ray-finned fish genomes (13 teleosts and the spotted gar) for 64 genes (Table S1) belonging to the AR-signaling pathway (Figure 1). The spotted gar is part of the family Lepisosteiformes, which diverged from the teleost lineage before the TSGD. Compared with teleost fishes, for which many genome rearrangements have occurred since the TSGD, the gar genome is closer in organization to the human genome (Amores et al. 2011) and was thus considered as the appropriate out-group for the here presented phylogenetic and sequence evolution analyses.

For two genes (nr1i3 and sgb2a1), we could not detect any ray-finned fish ortholog in the screened genomes, with nr1i3 being restricted to Sarcoterygii and sgb2a1 being a mammalian-specific gene. Out of the remaining 62 genes, 20 were retained as duplicates in most lineages after the TSGD (Figure 2). One gene, kdm3a is only present in the spotted gar, which indicates a loss of this gene at the basis of teleosts.

Some genes subsequently experienced lineage specific losses (such as cfl1 in pufferfishes, rnf14 in cichlids, rhoaab in Atherinomorphae, and rhoaaa in Eupercaria) and probably more recent losses on the species level (e.g., pik3r2b in the medaka, dnaj1 in fugu). We also found lineage- (e.g., rac1a in cichlids and medaka independently; rac1b in poeciliids) and species-specific duplication events (e.g., egfrb in tetraodon).

In summary, we detected 17 losses of one or both duplicates for genes otherwise retained in TSGD duplicates and further nine gene losses and four pseudogenes of otherwise single copy genes in teleosts. In total, we found additional lineage- or species-specific duplications for 19 genes.

Sequence evolution of the AR pathway in teleosts with a focus on cichlids

Branch-site models in Hy Phy overall suggested the action of purifying selection on most of the branches over the majority of all sites (Ka/Ks <1). We detected signs of significant positive selection

Genomic

Non-Genomic

- androgen steroid
- HSP
- AR
- androgen receptor
- ARE
- androgen response element
- Co
- cofactors of AR
- mAR
- membrane-bound AR
- NR
- nuclear receptor
- PI3K
- phosphatidylinositol 4,5-bisphosphate 3-kinase
- Ras/Raf/ERK/MAPK
- kinase signaling pathway
- Src
- proto-oncogene tyrosine-protein kinase
- TF
- transcription factor

AR or other receptors, which then dimerizes, interacts with cofactors (activators or repressors of transcription), and binds to androgen response elements, where it regulates expression of target genes. Nongenomic androgen actions are regulated over a membrane-bound AR or other receptors, which then transduces the signal into the cytoplasm, interacting directly with other membrane bound factors such as PI3K or activating other pathways, which lead to changes in second messengers, cytoplasmic calcium concentration, actin skeleton changes, and eventually also to the activation of other nuclear receptors and transcription factors. AKT, serine/threonine-protein kinase; AR, androgen receptor; ARE, androgen response element; Co, cofactors of AR, either activators or repressors of transcription; HSP, heat shock protein; mAR, membrane-bound AR; NR, nuclear receptor; PI3K, phosphatidylinositol 4,5-bisphosphate 3-kinase; Ras/Raf/ERK/MAPK, kinase signaling pathway; Src, proto-oncogene tyrosine-protein kinase; TF, transcription factor. Figure based on (Foradori et al. 2008; Guerriero 2009; Bennett et al. 2010; Kandasamy et al. 2010).
(Ka/Ks > 1) acting on four branches in four genes, namely on the branch at the basis of the teleosts after the split from spotted gar for ncoa3, pik3r2, and rock2 and on the branch of pik3r1b of A. burtoni (Figure S3). Site-wise models implemented in Selecton indicated strong purifying selection acting on all investigated genes, with only two genes (fkbp4 and rhob) having sites under significant positive selection (Figure S2). To test for selection and changes in selection regimes on a shorter evolutionary scale, we decided to focus on the only fish lineage, for which five genomes are currently available, the cichlids (Cichlidae). These include four species from the East African Great Lakes (A. burtoni and N. brichardi from Lake Tanganyika, M. zebra from Lake Malawi, and P. nyererei from Lake Victoria) and, with the Nile tilapia (O. niloticus), a member of a lineage basal to the lake cichlids. We tested selection acting on all cichlids compared with the next closest relative, the medaka (O. latipes) and also on East African Lake (EAL) cichlids compared with Nile tilapia by using gene-wide Ka/Ks estimates. Overall, Ka/Ks ratios were significantly greater on average after the split from the Nile tilapia (0.086 vs. 0.147, Figure 3). Interestingly, the greatest value in the comparison EAL cichlids vs. Nile tilapia is reached by ara, the key gene of the here investigated pathway.

Furthermore, after the split from Nile tilapia, the difference in selection regimes on (TSGD) duplicates increases significantly, especially so for ar, ptk2b, and rac1a. This pattern is particularly striking for the comparison between rac1aa and rac1ab. Ka/Ks for rac1aa is 4-fold and 9.2-fold greater than for rac1ab in the cichlids-medaka comparisons and EAL cichlids-Nile tilapia, respectively.

**Gene expression in cichlid fishes**

Gene duplicates seem to experience different selection regimes even at derived evolutionary stages as in the here investigated cichlids.
Previously, we showed that cichlids have a high turnover in gene expression patterns (Böhne et al. 2014). Hence, we next focused on expression differences of TSGD paralogs and the more recent lineage-specific duplication of rac1a in three tissues, brain, ovary, and testis. For this analysis, we used the RNA-sequencing data set of Böhne et al. (2014), which comprises four Lake Tanganyika cichlids (Figure 4), including again A. burtoni, for which the entire genome sequence is available (Brawand et al. 2014) and included in our sequence analyses.

Especially in the brain, we detected largely conserved expression patterns with always the same paralog being overexpressed in all four species (11 of 19 genes), which is indicative of ancestral subfunctionalization events of the two gene copies within this tissue. This pattern is less pronounced in testis (seven genes) and even less in ovary (three genes). Within a tissue, we rarely observed a change in paralog-overexpression (i.e., one duplicate is overexpressed in one species and the other in another species in the same tissue). This pattern was found for fhl2 as
the only gene in all three tissues, rhoaa in brain and testis, pten in ovary and testis, src in testis, and pik3r2 in the brain.

Focusing on between tissue comparisons, we found that in four cases (cdc42, ncoa3, pik3r1, and ar), the overexpression of the A or B duplicate changed depending on the tissue type, again indicative of different functionalities of the gene copies. This pattern is most pronounced for ncoa3, for which the A-copy is overexpressed in the brain whereas in general the B paralog is overexpressed in ovary and testis. In summary, this points to recent subfunctionalization events on the species and lineage level with an influence of the tissue inspected.

The strongest indication for a general subfunctionalization on a lineage level in gene expression comes from rac1a. This GTPase is the only gene for which one of the lineage-specific paralogs (the B-copy, rac1ab) is always overexpressed in all tissues and all species studied.

To assess whether this pattern was due to an increased expression of rac1ab or to a decreased expression of rac1aa, we compared expression levels (in FPKM) of both paralogs with the TSGD ohnolog rac1b and all other GTPase genes in the pathway: cdc42a, cdc42b, rhoaaa, rhoaab, rhoaab, rhoabb, and rhob (Figure 5). In all species, a 40-fold to 120-fold reduction in gene expression was observed for rac1aa compared with other GTPases, whereas rac1ab showed no difference with these ubiquitous proteins and strongly resembles rac1b in expression. This finding suggests that the difference in expression between the two more recent duplicates is due to a down-regulation of the general expression level of rac1aa compared with rac1ab in EAL cichlids.

**DISCUSSION**

Genome duplications, gene gains, and losses shape gene families and networks. Recently, the evolution of the androgen system in vertebrates has been described with special attention to the three rounds of whole-genome duplication that occurred in this lineage (Baker et al. 2013). With our study, we complete the picture focusing for the first time on
the genomic composition of the AR-signaling pathway in ray-finned fishes with special attention to East African cichlids. We identified fish orthologs for 62 of the 64 genes with one gene being present only in the spotted gar, indicating a loss at the basis of the teleost lineage. Of the remaining 62 genes, 20 were retained in duplicate after the TSGD, which is in the range of the overall loss rate estimates of 76–85% of duplicates after the TSGD in teleosts (Jaillon et al. 2004; Woods et al. 2005; Brunet et al. 2006). Interestingly, more duplicates were retained in the nongenomic signaling pathway (13 of 24) than the genomic one (7 of 37). We confirm a gene loss of arb (Douard et al. 2008) and rhob in zebrafish (Salas-Vidal et al., 2005) but show that the first one is, contrary to previous suggestions (Douard et al. 2008), not a loss in the entire Otophysi lineage, because we found arb to be present in the cavedish genome. Using only genomic information available in Ensembl, we identified in zebrafish only one copy for cdc42 and egfr and no rac1b gene, contrary to previous observations (Salas-Vidal et al. 2005; Laisney et al. 2010).

Evolutionary rates, as previously shown for at least two members of the AR pathway, ar (Douard et al. 2008) and fhl2 (Santos et al. 2014), were found to be different between A and B paralogs for most ohnologs. Such differences could be suggestive of possible neofunctionalization events in one gene copy with retention of the ancestral function in the more conserved, slower evolving copy.

Neofunctionalization of gene duplicates can be driven by positive selection (Beisswanger and Stephan 2008). Overall, we found no recurrent statistical support for positive selection on specific sites or branches acting on the AR pathway in teleosts, indicating conservation or constraint acting on the pathway. However, the investigated species cover a phylogenetic spectrum of ~350 million years. We thus decided to have a closer look into probable changes in selection regimes happening on a younger evolutionary scale. Within publicly available teleost genomes, only one particular group offers this possibility, the cichlids (Cichlidae), with five accessible genomes. In agreement with genome- and transcriptome-wide estimates (Baldo et al. 2011; Brawand et al. 2014), we could show that EAL cichlids have accelerated sequence evolution in the AR signaling pathway after their split from a common outgroup, the basal cichlid Nile tilapia around 25–50 million years ago (Brawand et al. 2014 and references therein), compared to values obtained in comparison all cichlids to the next closest relative with a sequenced genome, the medaka (split: ~140 million years ago; Azuma et al. 2008). This pattern could be indicative of either relaxed constraint or positive selection acting on the derived evolutionary stage of the cichlid lineage. Interestingly, this pattern is most pronounced for the A-copy of the AR itself. This analysis also showed that TSGD duplicates differ in their evolutionary rate at this derived evolutionary stage, opening up the possibility for late neofunctionalization events.

Changes in gene expression levels and locations also can be indicative of a neo- or subfunctionalization. It also has been shown previously in cichlids that paralogs can have species- and tissue-specific expression patterns. These data include TSGD and other whole-genome duplicates of neuroendocrine gene families such as the pro-opiomelanocortin (pomc) family (Harris et al. 2014), as well as the ARs (Harbott et al. 2007) and genes implicated in sexual development (Böhne et al. 2014). For duplicated genes of the AR-signaling pathway, we could show here that paralogs can have species- and tissue-specific overexpression patterns. General conservation of the same paralog overexpression is seen in the brain of cichlids, whereas the gonads and especially the ovary show more species-specific patterns.

A particularly strong indication for a late neofunctionalization event comes from the expression and selection pattern of the rac1a A and B copies, which are specific to the cichlid lineage (note that rac1a also has been duplicated in the medaka, but according to our phylogenetic reconstruction, this is an independent event). Ka/Ks in rac1ab is similar to rac1b, the corresponding TSGD paralog of rac1aa and rac1ab;
however, it is significantly greater in rac1aa, and it is rac1ab that is overexpressed in all tissues in all species compared with rac1aa, although at the same expression level than other GTPases and its ortholog rac1b, probably reflecting the ancient expression level. This finding suggests reduced gene expression for the faster-evolving rac1aa. Reduced gene expression can promote gene neofunctionalization, as shown for a sodium channel gene duplicate in electric fishes (Thompson et al. 2014). Indeed, a reduced gene expression of one paralog can enable faster gene evolution of this gene without altering the individual’s fitness provided that the other copy retains the “required” ancestral expression and function.

As other neuroendocrine pathways, the AR signaling pathway has been shaped by gene and genome duplications. We found that this pathway is generally under purifying selection in teleosts but shows accelerated evolutionary rates in cichlid fishes with indications for neofunctionalization on the lineage and species level.

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