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Phylogeny, taxonomy, and evolution of the endothelin receptor gene family

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ABSTRACT

A gene phylogeny provides the natural historical order to classify genes and to understand their functional, structural, and genomic diversity. The gene family of endothelin receptors (EDNR) is responsible for many key physiological and developmental processes of tetrapods and teleosts. This study provides a well-defined gene phylogeny for the EDNR family, which is used to classify its members and to assess their evolution. The EDNR phylogeny supports the recognition of the EDNRA, EDNRB, and EDNRC subfamilies, as well as more lineage-specific duplicates of teleosts and the African clawed frog. The duplications for these nominal genes are related to the various whole-genome amplifications of vertebrates, jawed vertebrates, fishes, and frog. The EDNR phylogeny also identifies several gene losses, including that of EDNRC from placental and marsupial (therian) mammals. When coupled with structural and biochemical information, site-specific analyses of evolutionary rate shifts reveal two distinct patterns of potential functional changes at the sequence level between therian versus non-therian EDNRA and EDNRB (i.e., between groups without and with EDNRC). An analysis of linkage maps and tetrapod synteny further suggests that the loss of therian EDNRC may be related to a chromosomal deletion in its common ancestor.

1. Introduction

Phylogenies track the interrelationships among descendants and ancestors and thereby the transmission of heritable traits across evolutionary lineages and time (Cracraft and Donoghue, 2004; Judd et al., 2008). Thus, as for a human pedigree or genealogy, phylogenies offer the natural historical framework for the definition, classification, and naming of groups and for the fuller understanding of the origins, context, and meaning of their heritable diversity. This central importance of phylogenies is as relevant to the study of genes and proteins as to a comparison of organismal groups (Thornton and DeSalle, 2000; Holland and Takahashi, 2005). Thus, a gene phylogeny or tree provides the proper setting for the taxonomic, functional, and genomic studies of a gene or protein family.

Endothelins (EDN) are small peptide paracrines and autocrines of tetrapods and teleosts, which are involved in many diverse physiological and developmental processes. These functions include the regulation of vascular tone (Yanagisawa et al., 1988a,b; La and Reid, 1995), alteration of ion transport (Zeidel et al., 1989; Garvin and Sanders, 1991; Prasanna et al., 2001; Evans et al., 2004), and migration of neural crest cells during craniofacial development (Kurihara et al., 1994; Clouthier and Schilling, 2004). The majority of current EDN research remains focused on biomedicine and mammalian model species, because numerous human pathologies have been linked to problems with EDN signaling (Kirby et al., 2008), including hypertension, atherosclerosis (Shreenivas and Oparil, 2007), congestive heart failure (Angerio, 2005), and glomerulonephritis (Richter, 2006).

Specifically, EDN operate through a signaling cascade that is initiated by a family of G-protein-coupled receptors for these secreted peptides (Hosoda et al., 1992; Arai et al., 1993). Traditionally, research on these membrane-bound, EDN receptors (EDNR) has focused on the EDNRA and EDNRB1 proteins that are encoded by two, unlinked, duplicate (paralogous) genes in humans and other known tetrapods and teleosts (Stenslokken et al., 2006; Scarparo et al., 2007). Since then, a number of additional *EDNR* genes has been reported and subsequently related to *EDNRA*, *EDNRB1*, and each other according to their overall sequence similarities and pharmacological properties (Karne et al., 1993; Kumar et al., 1994; Lecoin et al., 1998). However, this reliance on simple similarity measures and pharmacological features, rather than on a detailed gene phylogeny, has resulted in a confusing array of different gene designations and characterizations with limited explanatory and predictive power about the evolution, function, and structure of the EDNR family.

This study provides a well defined phylogeny for the EDNR family, which documents its history of gene duplications and losses. The gene duplications form the foundation for a revised taxonomy of its members, including the recognition of the EDNRA, EDNRB,

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and EDNRC subfamilies. The gene losses include the absence of EDNRC among placental and marsupial (therian) mammals, which remains of interest because of the known functional differences between therian versus non-therian EDNRA and EDNRB. In light of this interest, a series of site-specific analyses of evolutionary rate shifts is coupled with structural and biochemical information to test for potential functional changes at the sequence level between therian versus non-therian EDNRA and EDNRB (i.e., between groups without and with EDNRC). A comparison of linkage maps and tetrapod synteny is then combined with MEGABLAST searches to assess the potential role of chromosomal rearrangements in the loss of therian EDNRC.

2. Materials and methods

2.1. Nomenclature

Except where otherwise noted, the names and symbols of the EDNR family refer to those recommended by this study, as developed from its gene phylogeny. More generally, the names and symbols of this study also follow the guidelines of the HUGO Gene Nomenclature Committee (Wain et al., 2002). Thus, gene symbols are italicized, whereas gene names and protein symbols and names are not. Furthermore, "L," "P," and "U" are included as suffixes of the gene or protein symbols to designate distant ("like") relatives of an extended phylogenetic group, pseudogenes, and known but unidentified gene loci, respectively.

2.2. Datasets and multiple sequence alignment

EDNR were retrieved from the protein, genomic, and nucleotide databases of GenBank (release 164, February, 2008) and Ensembl (e! 44, April, 2007) by BLAST searches (Hubbard et al., 2007; Spudich et al., 2007; Wheeler et al., 2007). Seven EDNR from human, platypus, chicken, and killifish served as the query sequences in these BLAST retrievals (Table S1, available as Supporting Materials online). The killifish EDNR represent new sequences that were determined by this study (Appendix A). The initial EDNR from the BLAST searches were refined such that multiple alleles for a species were restricted to their single curatorial RefSeq sequence and alternative splice variants for a gene were limited to their longest transcript. Furthermore, EDNR for the more thoroughly sampled placental mammals were restricted to those of species with both complete EDNRA and EDNRB. These refinements resulted in a more taxonomically balanced EDNR sample that allowed for more thorough phylogenetic and evolutionary analyses.

A multiple sequence alignment was generated for the final EDNR set with Clustal X, version 2.0 (Larkin et al., 2007). This alignment was manually edited to move a few short gaps from inside to outside of the seven transmembrane domains (TMD) and other known structural regions of EDNR (Pollock and Highsmith, 1998). As the first 275 and last 58 positions of the final edited alignment included many gaps, we analyzed our aligned EDNR both with and without the inclusion of these gapped terminal regions (Swofford et al., 1996). However, as these two approaches supported the same conclusions, only those results for the full alignment are reported below.

2.3. Phylogenetic analysis

The EDNR alignment was analyzed with both maximum likelihood (ML) and Bayesian phylogenetics (BP) approaches. The evolutionary model for these analyses included the empirical rate matrix for amino acid replacements of Whelan and Goldman (2001). Their WAG matrix was adopted, because of its use of ML and a larger protein database to estimate the replacement rates. A series of likelihood ratio tests (LRT) was then conducted to verify that a gamma distribution for rate heterogeneity among sites (Γ) was also appropriate, whereas an invariable sites parameter was not (Huelsenbeck and Rannala, 1997). Thus, the final model for the ML and BP analyses consisted of WAG + Γ, with eight rate categories for the gamma distribution (Yang, 1996).

The ML (WAG + Γ) analysis of the EDNR alignment was performed with PHYML, version 2.4.4 (Guindon and Gascuel, 2003). This analysis was initiated from a BIONJ tree and group support for its final optimal solution was evaluated with nonparametric bootstrapping (1000 pseudoreplicates). The BP (WAG + Γ) analysis of EDNR was conducted with MrBayes, version 3.1 (Ronquist and Huelsenbeck, 2003). This analysis relied on Metropolis-coupled Markov chain Monte Carlo sampling with one cold chain and three heated chains that were run for 20 million generations. After a burn-in of 2 million steps, samples were taken every 500 generations, with the final set of sampled phylogenies (36,000) summarized as an extended majority-rule consensus tree. Group support for this solution was assessed with its bipartition posterior probabilities. This BP analysis was independently replicated three times to ensure the convergence of the final results.

A previous phylogenetic study suggested that the G-protein-coupled receptor 37 (GPCR37) and G-protein-coupled receptor 37-like 1 (GPCR37L1) proteins comprise the closest outgroup to the EDNR family (Fredriksson et al., 2003). Thus, a set of proteins for them was compiled, refined, aligned, and phylogenetically analyzed with ML as described for EDNR, except for the use of human GPCR37 and GPCR37L1 as the query sequences in the database retrievals. The final ML solution for the outgroup was then separately added to the different stem lineages of the three EDNR subfamilies according to the two-step Lundberg (1972) method. In this way, we first established the robustness of the major EDNR groups without interference from the highly diverged outgroup.

2.4. Site-specific evolutionary rate and statistical analyses

A series of LRT for evolutionary rate shift and conserved positions was performed to test for potential functional divergence at the sequence level between therian versus non-therian EDNRA and EDNRB. These tests were conducted with the Rate Shift Analysis server for type I, type II, and conserved positions (Knudsen and Miyamoto, 2001; Knudsen et al., 2003). A type I position is one whose site-specific evolutionary rate differs between two groups (Gu, 1999, 2001). The most obvious example of a type I site is a homologous position that is fixed for a specific amino acid in therians but is highly variable in non-therians (or vice versa). A type II position is one whose site-specific evolutionary rate is accelerated along the basal internode that interconnects the two most recent common ancestors of the two groups. The most obvious example of a type II position is one that is fixed for radically different amino acids between therians and non-therians. In contrast, a conserved position is one with a constant site-specific rate between the two groups, which is significantly slower than the average for the entire protein.

The rule of functional constraint in molecular evolution states that functionally important sites are under strong purifying selection and thereby evolve slowly (Kimura, 1983; Li, 1997). Thus, according to this rule, a type I position can be interpreted as of greater functional importance in the protein group with the slower site-specific rate (Knudsen and Miyamoto, 2001; Knudsen et al., 2003). In contrast, a type II position can be interpreted as one with equal functional significance in the two groups (Gu, 1999, 2001, 2006). A type II position then contributes to the functional differences between groups via the physicochemical properties that distinguish their specific amino acids. In these ways, type I and II

positions point to those sites that are most likely responsible for the functional differences between protein groups (Gaucher et al., 2002).

In the LRT of site-specific evolutionary rates, the aligned EDNRA and EDNRB for all species were separately removed from their full multiple sequence alignment and then individually analyzed with the WAG + Γ model of the Rate Shift Analysis server (Knudsen et al., 2003). The phylogenies for these LRT consisted of the separate EDNRA and EDNRB subtrees, as reproduced from the full ML phylogeny for all EDNR. These EDNRA and EDNRB subtrees were rerooted such that their therian and non-therian groups were now both monophyletic. Such rerooting is allowed, given the time reversibility of the WAG + Γ model (Felsenstein, 2004).

The potential functional importance of the identified rate shift and conserved sites was assessed against the known positions of EDNRA and EDNRB for EDN binding. The EDN family consists of the EDN1, EDN2, and EDN3 duplicates of tetrapods and teleosts (Inoue et al., 1989; Hyndman and Evans, 2007). EDNRA selectively binds to EDN1 (Arai et al., 1993), whereas EDNRB binds equally well to all three EDN (Hosoda et al., 1992). Correspondingly, EDNRA and EDNRB rely on different structures and residues to interact with their endogenous ligands (Adachi et al., 1993; Breu et al., 1995; Wada et al., 1995). EDNRA relies on 26 contiguous positions of its extracellular N-terminal head (its EDN1 binding domain) and on five additional ones of its TMD I–III and V for its EDN1 interactions. In contrast, EDNRB depends on 60 contiguous positions of its first extracellular loop and adjacent TMD II and III for its EDN binding. These 31 and 60 functionally important sites of EDNRA and EDNRB were determined with site directed mutagenesis, EDN binding assays, and other such laboratory experiments.

The potential functional importance of the type I and type I and II (I/II) positions was also assessed against whether therians or non-therians were the more slowly evolving group for these rate shift sites. A type I/II position is a site with different evolutionary rates in the two groups, as well as one with an accelerated rate along the basal branch that interconnects their two most recent common ancestors (Knudsen et al., 2003).

The LRT approach of Knudsen and Miyamoto (2001) and Knudsen et al. (2003) was chosen instead of other methods for this study (e.g., Gu and Vander Velden, 2002) on the basis of its successes in both evolutionary simulations and with real empirical datasets (e.g., Knudsen and Farid, 2004; Blouin et al., 2005; Godard et al., 2005; da Fonseca et al., 2006; Franchini and Elgoyhen, 2006; Goldstone et al., 2007). In particular, the simulations by Blouin et al. (2005) document that this LRT approach is excellent at recovering the true rate shift sites within a dataset. Furthermore, this power is achieved without an excessive inflation in the frequency of type I errors (i.e., the false positive rate remains less than 9% according to their simulations).

2.5. Chromosomal analyses

To assess the potential role of chromosomal rearrangements in the loss of therian EDNRC, linkage maps for the trimethyllysine hydroxylase, epsilon (*TMHLE*) and synaptobrevin-like 1 (*SYBL1*) loci of vertebrates were compiled from the April, 2008 builds of Entrez Gene (Maglott et al., 2005) and then compared for their patterns of synteny. These loci were targeted, since they belong to a single linkage group that includes *EDNRC* in non-therian tetrapods (see below). This single linkage group of non-therian tetrapods shares considerable synteny with those of therians, but not with those of teleosts. Thus, our analyses of the *TMHLE* and *SYBL1* regions were limited to tetrapods. As a part of these comparisons, a series of BLAST searches of the protein, genomic, and nucleotide databases in GenBank (version 164) was conducted with the inferred polypeptides of the unidentified "*LOC*" loci of the tetrapod linkage groups as the query sequences. These BLAST searches resulted in the identification of five previously unnamed loci.

As a follow-up to these linkage map comparisons, the nucleotide and genomic databases in GenBank (version 164) were searched for therian *EDNRC* genes and pseudogenes with discontiguous MEGABLAST. The seven exons of platypus *EDNRC* (Ensembl accession number ENSOANP00000013874) were used as the query sequences in these searches. Discontiguous MEGABLAST is designed to find more distantly related sequences within the databases (National Center for Biotechnology Information, 2002) and the platypus belongs to the monotreme sister group of therians (Warren et al., 2008). The power of these MEGABLAST searches was enhanced by allowing for mismatches to occur at the rapidly evolving, third codon positions of the platypus queries with the database sequences (Ma et al., 2002).

3. Results

3.1. Gene phylogeny

The ML and BP phylogenies for the EDNR alignment are identical in their branching patterns, except for three rearrangements of weakly supported species groups within two of the major duplicate EDNR clusters (Fig. 1 and Fig. S1 Supporting Materials online). Otherwise, these two solutions converge onto the same gene phylogeny that consists of three major groups of duplicate EDNR and a traditional higher-level arrangement for each of (((((placental mammals, marsupial), monotreme), birds), frogs) teleost fishes). The three major EDNR groups are well defined by ML bootstrap scores and BP posterior probabilities of ⩾99%. They are the products of two older gene duplications and are hereafter referred to as the EDNRA, EDNRB, and EDNRC subfamilies.

The ML and BP gene phylogeny reconfirms that therians are missing EDNRC (Fig. 1). Thus, this phylogeny reinforces the initial BLAST searches of the GenBank and Ensembl databases with platypus, chicken, and killifish EDNRC, which found no therian EDNRC. These searches were successful in their recovery of non-therian EDNRC and of therian EDNRA and EDNRB. However, even searches of the primate, rodent, and opossum genome databases failed to recover EDNRC from therians.

The gene phylogeny also supports two additional duplications of EDNRA and EDNRB among teleosts and EDNRA and EDNRC within African clawed frog (Fig. 1). The placement of the EDNRA and EDNRB duplications within the teleost common ancestor is well defined by bootstrap scores and posterior probabilities of 90–100%. The assignment of the EDNRA and EDNRC duplications to the African clawed frog is also strongly supported by values of 96–100%, except for the weak bootstrap score of only 60% for its EDNRA amplification.

Locating the root for a phylogeny is often the most difficult part of a phylogenetic investigation, because of the greater divergence between a study group and its outgroup (Swofford et al., 1996; Shavit et al., 2007). EDNRA, EDNRB, and EDNRC of the study group share amino acid identities of 48–76% with each other, but only 16–25% with their GPCR37 and GPCR37L1 outgroup. In recognition of this greater divergence, we separately added the ML subtree for the outgroup to the stem lineages of the three EDNR subfamilies and then recalculated the log likelihood of the EDNR phylogeny (Fig. 1). Unfortunately, little resolution about the location of the root is provided by this Lundberg (1972) rooting method, as indicated by the similar log likelihoods for these alternative placements. Thus, even though the three EDNR subfamilies are well defined, their exact order of gene duplications remains uncertain due to the extensive divergence with their closest known outgroup.

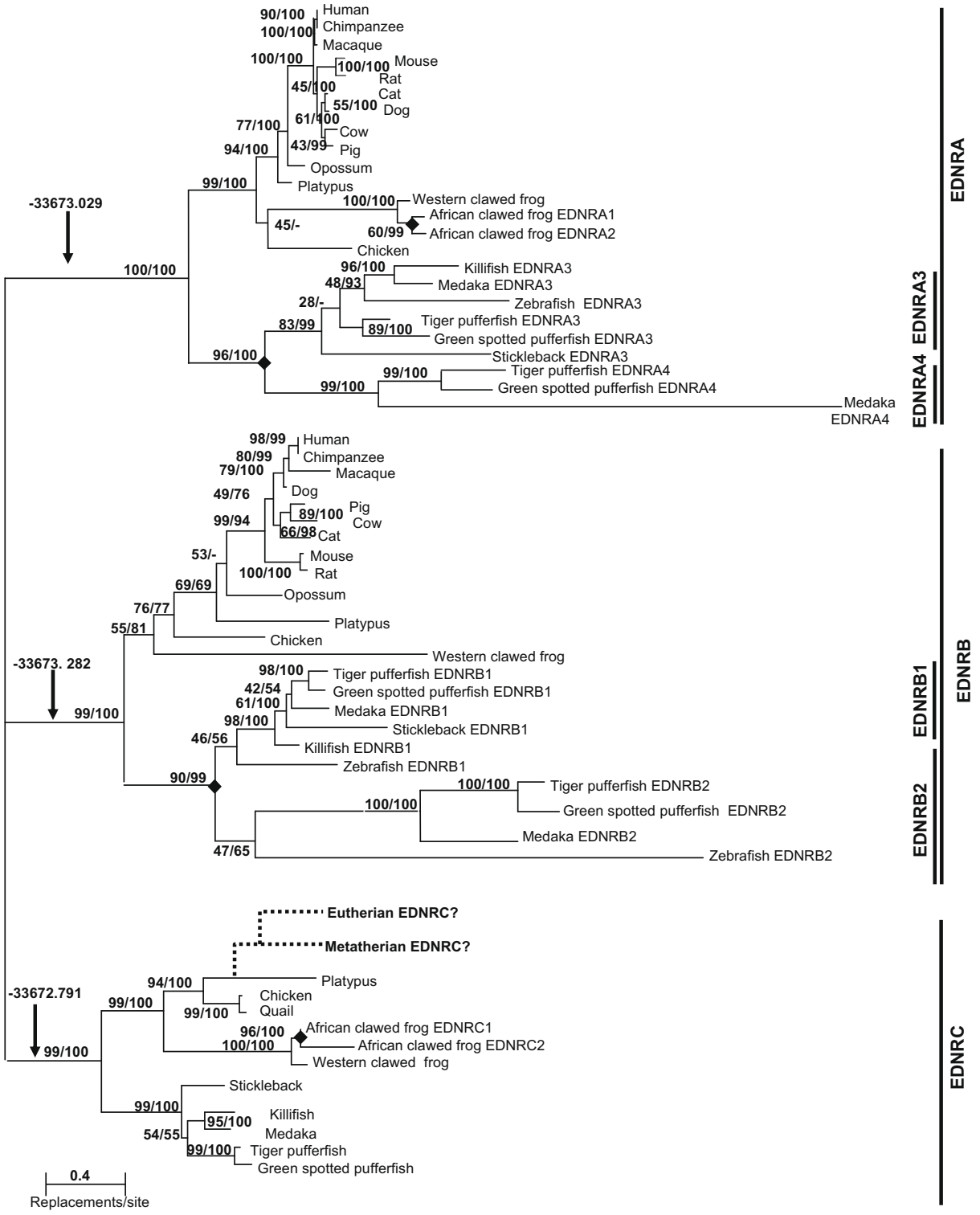

Fig. 1. EDNR gene phylogeny, as obtained from the ML (WAG + Γ) analysis. A directory of GenBank and Ensembl accession numbers is provided for these proteins in Table S1 (available as Supporting Materials online), along with the scientific names of their species. This ML phylogeny has the same branching pattern as that of the extended, majority-rule, consensus, BP tree for EDNR, except for three weakly supported groups that are demarcated with hyphens after the slashes along their stem branches. For all other groups (i.e., those recovered by both approaches), the numbers before and after these slashes correspond to their ML bootstrap scores and BP posterior probabilities. Diamonds refer to ancestral gene duplications within the EDNR subfamilies, whereas branch lengths are drawn proportional to their expected numbers of replacements per site. The dotted branches and question marks highlight the absence of therian EDNRC. Arrows refer to the log likelihood scores of this phylogeny after the separate addition of the GPCR37 and GPCR37L1 outgroup to the stem lineages of the three EDNR subfamilies. In light of the similar log likelihoods for these alternative rootings, the base of this EDNR gene phylogeny is left as an unresolved trichotomy among its three well-supported subfamilies.

3.2. Site-specific evolutionary rate and statistical results

A total of 21 type I, 14 type II, and 119 conserved positions are recovered in the LRT of therian versus non-therian EDNRA. Of the 21 type I positions, therians are more slowly evolving at 10 of these sites, whereas the same is true of non-therians at the other 11. In turn, a total of 40 type I, 26 type II, four type I/II, and 236 conserved positions are recovered in the LRT of therian versus non-therian EDNRB. Of the 40 type I sites, therians are more slowly evolving at 35 of these positions, whereas the same is true of non-therians at the other five. Of the four type I/II positions, therians are the more highly conserved group at three of these sites, whereas the same applies to non-therians at the remaining position.

The expected number of false positives for type I positions of EDNRA is calculated as 21.2; i.e., the length of this protein (425 sites) multiplied by the nominal significance level of $\alpha = 0.05$. This estimate of 21.2 is also the expected number of false positives for type II positions of EDNRA. In turn, the expected false positive rate for either the type I or type II positions of EDNRB is 31.0 (620 sites · 0.05). The expected number of false positives for type I/II sites cannot be directly calculated as the product of EDNRB length times 0.05 (Knudsen et al., 2003). However, this number is expected to be few according to the evolutionary simulations of these authors.

The observed numbers of type I and type II positions for EDNRA (21 and 14) and of type II sites for EDNRB (26) are less than their expected counts of 21.2 and 31.0, respectively. Nevertheless, the biological importance of these rate shift positions is validated by their nonrandom distributions among the known functionally important sites of EDNRA and EDNRB for EDN binding and between therians versus non-therians as the more highly conserved group (Table 1 and Fig. 2). Such nonrandom distributions are unlikely to arise, if the rate shift sites of EDNRA and EDNRB are primarily due to the type I errors.

The nonrandom distributions of the rate shift and conserved sites for EDNRA and EDNRB follow two different patterns. The type I and type II positions of EDNRA are overrepresented among its known functionally important sites for EDN1 binding (Table 1A and Fig. 2A). In contrast, the rate shift positions of EDNRB are underrepresented by only a single type I site among its known EDN binding residues (Table 1B and Fig. 2B). Instead, the EDN binding positions of EDNRB are enriched for conserved sites that comprise nearly 85% of these residues (Table 1C). Furthermore, therians are the more slowly evolving group at 38 of the 44 type I and type I/II positions for EDNRB (Table 1D). Collectively, these different distributions of rate shift and conserved sites point to two separate patterns of functional divergence between therian versus non-therian EDNRA and EDNRB.

Table 1
Results from the evolutionary rate shift and statistical analyses of therian versus non-therian EDNRA and EDNRB.

A EDNRA	Type I and/or II sites	Non-rate shift sites	Total sites
EDN1 binding sites	6 (2.6)	25 (28.4)	31
Other EDNRA sites	29 (32.4)	365 (361.6)	394
Total sites	35	390	425
B EDNRB	**Type I and/or II sites**	**Non-rate shift sites**	**Total sites**
EDN binding sites	1 (6.8)	59 (53.2)	60
Other EDNRB sites	69 (63.2)	491 (496.8)	560
Total sites	70	550	620
C EDNRB	**Conserved sites**	**Non-conserved sites**	**Total sites**
EDN binding sites	50 (22.8)	10 (37.2)	60
Other EDNRB sites	186 (213.2)	374 (346.8)	560
Total sites	236	384	620
D EDNRB			**Type I and I/II sites**
Therians slower			38 (22)
Non-therians slower			6 (22)
Total sites			44

EDN binding positions include those sites of EDNRA and EDNRB, which have been shown experimentally to be important in their EDN interactions (see text). (A) Overrepresentation of type I and type II sites among the EDN1 binding positions of EDNRA (Fisher's exact test of independence, $P = 0.032$). (B) Under-representation of type I and/or II positions among the EDN binding sites of EDNRB (G-test of independence, $P = 0.003$). (C) Enrichment of conserved sites among the EDN binding positions of EDNRB (G-test of independence, $P << 0.001$). (D) Overrepresentation of therians as the more conserved group among the type I and type I/II positions of EDNRB (G-test of goodness-of-fit, $P << 0.001$). Expected counts (in parentheses) are calculated for Table 1A, 1B, and 1C from their marginal totals, whereas those for Table 1D are based on an equal 1:1 ratio.

3.3. Chromosomal results

The *TMLHE* and *SYBL1* loci belong to a single linkage group in all included tetrapods, except in rat (Fig. 3). *EDNRC* lies within this syntenic block just upstream of RNA-polymerase 1, 16 kDA in all non-therian tetrapods. In contrast, *EDNRC* is missing from the *TMLHE* and *SYBL1* regions of all therians, including rat. In all therians except rat, the single *TMLHE*-to-*SYBL1* group is found on the X chromosome. In rat, these two regions are divided between its autosomes 12 and 20.

No therian *EDNRC* gene or pseudogene is retrieved in the discontiguous MEGABLAST searches of the nucleotide and genomic databases with the seven protein-coding exons of platypus *EDNRC* as the query sequences. In contrast, *EDNRA* and *EDNRB* are consistently recovered from therians in these searches, along with *EDNRA*, *EDNRB*, and *EDNRC* from non-therian tetrapods and teleosts. Thus, these discontiguous MEGABLAST searches are consistent with the linkage map comparisons in their support for a missing *EDNRC* gene as well as EDNRC protein in therians.

4. Discussion

4.1. Gene taxonomy, duplications, and losses

The ML and BP gene phylogeny supports the recognition of three ancient EDNR subfamilies that are well defined by bootstrap scores and posterior probabilities of $\geqslant 99\%$ (Fig. 1). The EDNRA subfamily directly corresponds in name and group membership to its previous references (Arai et al., 1990). Similarly, the EDNRB subfamily involves only a minor nominal change from its earlier EDNRB1 designation. In contrast, the EDNRC subfamily represents major changes in both name and group membership (Lecoin et al., 1998). This duplicate gene was initially referred to as "bird EDNR," because it was first isolated and characterized from the chicken and quail. This designation became more widely used after further studies failed to identify a related therian sequence (Pla et al., 2005; Scarparo et al., 2007). Today, it is known as "bird EDNRB2" in recognition of its greater sequence identity and pharmacological similarities with placental mammal EDNRB (Miwa et al., 2006, 2007).

However, biological similarity is often not a reliable indicator of historical relationships due to the prevalence of parallel and back changes and unequal evolutionary rates among lineages (Swofford et al., 1996; Felsenstein, 2004). By relying on full model-based approaches that account for these complicating factors, the ML and BP gene phylogeny offers the appropriate historical foundation to verify that "bird EDNRB2" is actually a shared duplicate of tetrapods and teleosts, which has been secondarily lost by therians. Thus, this gene is as phylogenetically ancient, widespread, and distinct as are EDNRA and EDNRB. Like them, it thereby warrants its own specific designation as EDNRC, as was first used by Karne et al. (1993).

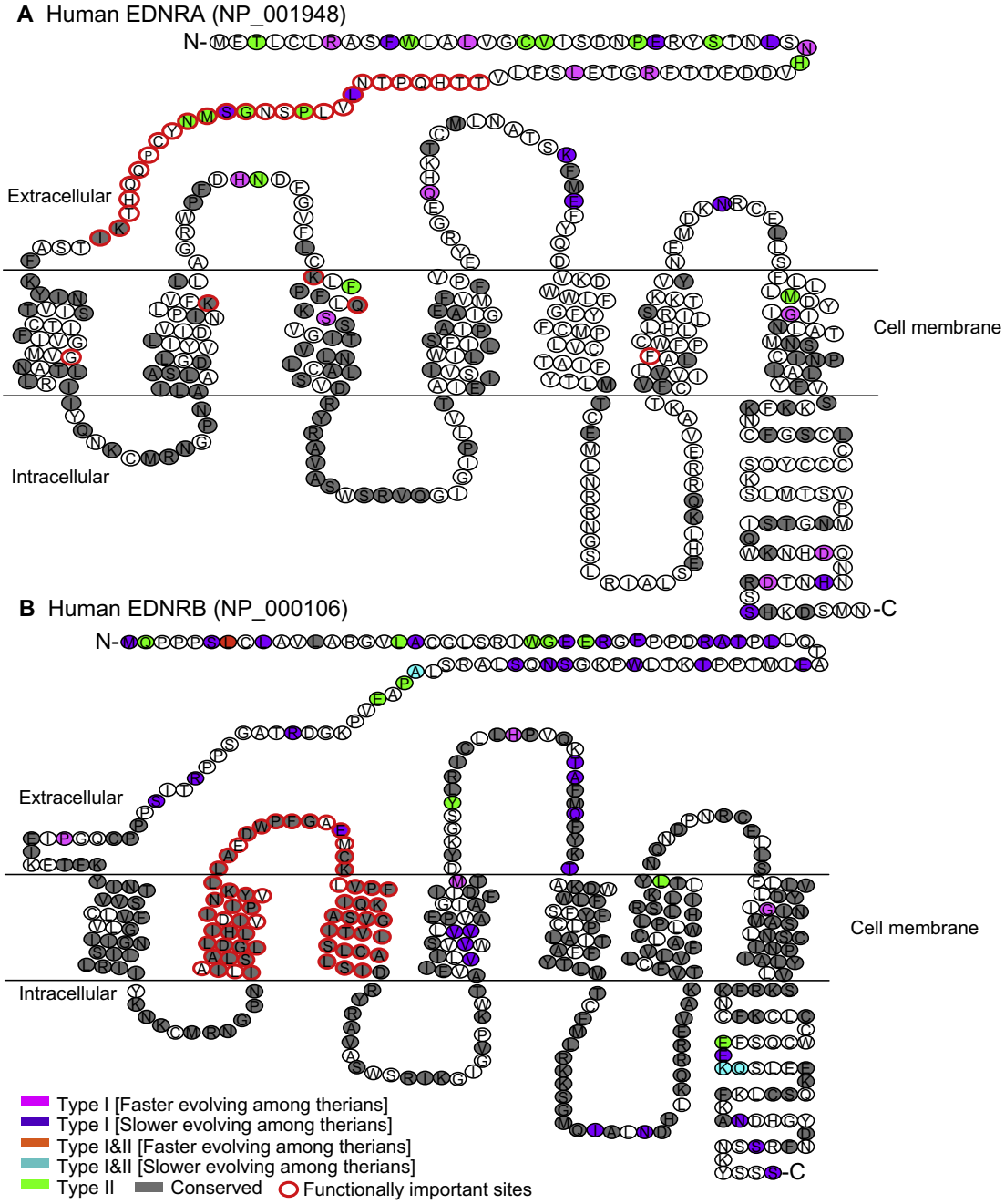

Fig. 2. Amino acid sequences of human EDNRA (A) and EDNRB (B), as drawn according to their extracellular and intracellular regions and seven TMD. The distribution of type I and/or II sites and conserved positions is presented relative to these structural features and their functionally important domains and residues for EDN binding. The GenBank accession numbers for these EDNR are given in parentheses.

As for its EDN family of ligands (Hyndman and Evans, 2007), the EDNR family of receptors has only been recovered so far from representatives of tetrapods and teleosts. Thus, the two gene duplications that underlie the three EDNR subfamilies may have occurred as recently as within the common ancestor of these two groups. In contrast, the genomes of lower vertebrates (i.e., sharks, lampreys, hagfishes, and their relatives) have not been nearly as well studied as those of tetrapods, teleosts, and non-vertebrate chordates (e.g., sea squirt and amphioxus) (Dehal et al., 2002; Volff, 2006; Putnam et al., 2008). Thus, EDNRA, EDNRB, and EDNRC may be more widely distributed phylogenetically than currently known and their gene duplications may therefore be considerably older than the tetrapod/teleost common ancestor. Indeed, the three EDNR subfamilies may be the products of the two rounds of whole genome duplications, which have been proposed for the common ancestors of vertebrates and jawed vertebrates (Dehal and Boore, 2005; Kasahara, 2007; Lynch, 2007; Putnam et al., 2008). If so, then these whole genome duplications were followed by the subsequent loss of one of the four original duplicates, thereby resulting in the three EDNR subfamilies that are currently known only for tetrapods and teleosts.

The ML and BP gene phylogeny also allows for the renaming of the additional duplicate genes of African clawed frog and teleosts according to their evolutionary histories (Fig. 1). The two EDNRA and two EDNRC of African clawed frog are now designated as EDNRA1/EDNRA2 and EDNRC1/EDNRC2, respectively. Similarly, the

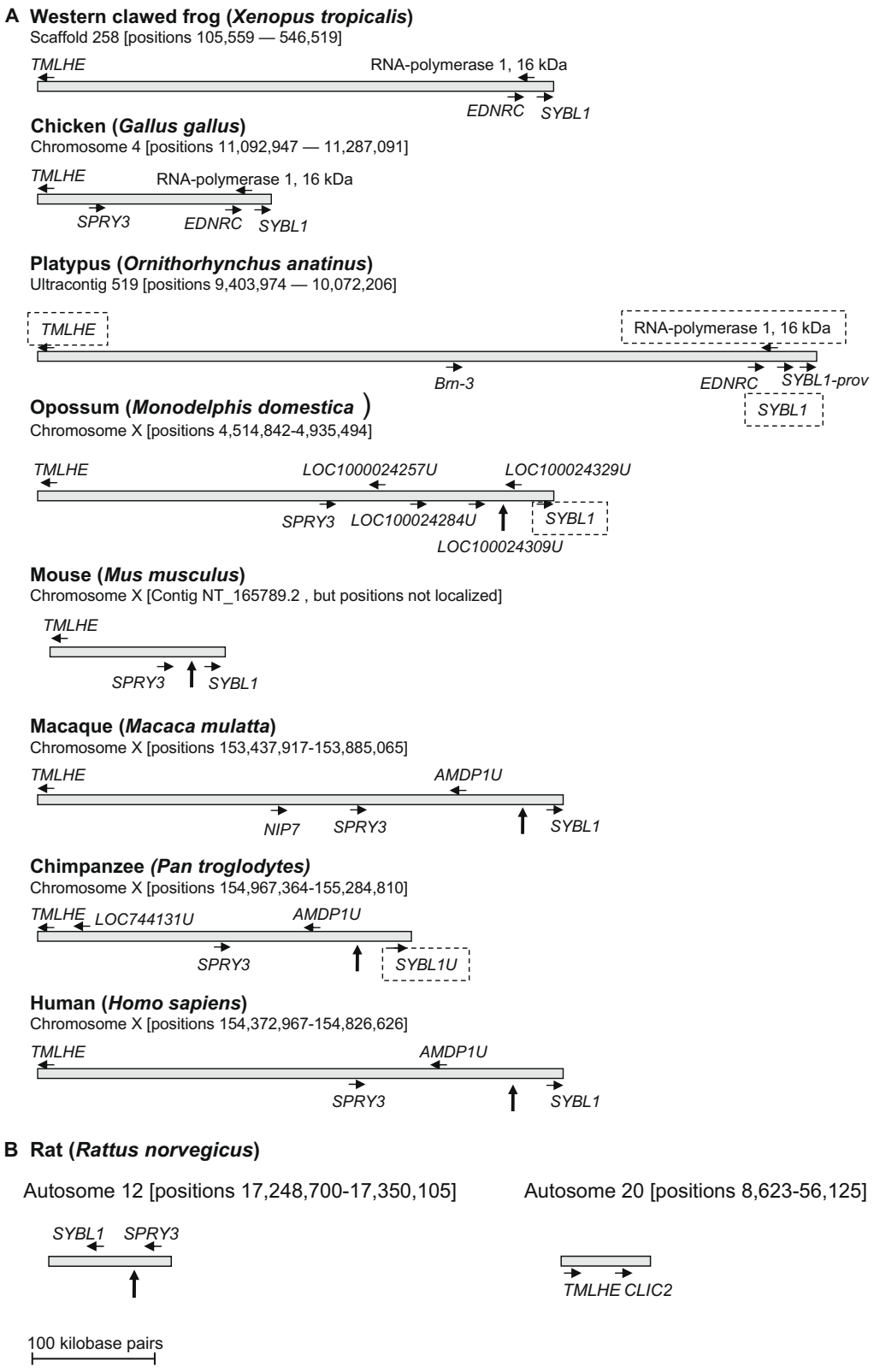

Fig. 3. Linkage maps for the *TMLHE* and *SYBL1* synteny among nine therian and non-therian tetrapods. (A) In all of these species except rat, the *TMLHE* and *SYBL1* loci belong to a single linkage group. (B) In contrast, these two loci occur on two separate autosomes in rat. The chromosome, scaffold, or ultacontig is noted for each linkage group, along with its sequence positions (when known) in brackets. Horizontal arrows designate the direction of transcription and thereby location of each mapped gene. Vertical arrows point to the inferred general position, where *EDNRC* was most likely non-functionalized in the therian common ancestor. The five boxed genes refer to those that were identified by the current BLAST searches of this study.

two EDNRA and two EDNRB of teleosts are now referred to as EDNRA3/EDNRA4 and EDNRB1/EDNRB2. The use of these different designations with their Arabic numeral suffixes is necessary to distinguish each pair of lineage-specific duplicates from the other

members of their subfamilies. These designations are particularly critical for distinguishing among the unrelated EDNRA duplicates of African clawed frog versus teleosts.

As suggested for the three EDNR subfamilies, these additional gene duplications may be part of the more recent whole-genome amplifications that have been supported for African (but not western) clawed frog (Evans, 2008) and the teleost common ancestor (Hoegg et al., 2004; Jaillon et al., 2004; Volff, 2005). If so, then the absence of EDNRB within the known genome of African clawed frog is ascribable to the loss of its ancestral EDNRB prior to its whole genome duplication. Similarly, the lack of a second EDNRC among the known genomes of zebrafish, medaka, and pufferfishes is attributable to the loss in the teleost common ancestor of one of its two duplicate EDNRC. This loss was followed by a second, more recent one in the zebrafish, which resulted in the complete absence of EDNRC in its known genome. A similar loss is also most likely responsible for the missing zebrafish EDNRA4.

4.2. Potential functional changes at the sequence level

The most common evolutionary fate for a duplicate gene is gene loss (Lynch and Conery, 2000; Walsh, 2003; Lynch, 2007). This typical fate is the result of the greater frequency of deleterious mutations and relaxed purifying selection due to the co-occurrence of alternative genes for the same or similar functions. Such losses have occurred several times within the EDNR family, as documented above for therians, African clawed frog, and teleosts (Fig. 1). Nevertheless, the loss of therian EDNRC remains of special interest, because of the known functional differences of EDNRA and EDNRB among model species of groups with and without EDNRC (e.g., birds and therians) (Pla and Larue, 2003). For example, EDNRA is first expressed in neural crest cells at an earlier developmental stage in mouse than in quail (Kempf et al., 1998; Nataf et al., 1998). In turn, EDNRB mediates the dorsolateral migration of neural crest cells in mouse (Pla and Larue, 2003), whereas EDNRC fulfills this role in chicken and quail (Lecoin et al., 1998). This latter difference between mice and birds exists, despite the overlapping co-expression of EDNRB and EDNRC in the neural crest cells of quail (Pla et al., 2005).

The LRT and statistical analyses of EDNRA provide evidence of functional divergence between therian and non-therian EDNRA due to changes in their EDN1 binding (Table 1 and Fig. 2). This implication of EDN1 binding as central to these biological differences is not too surprising, given that the functions of all EDNR are dependent on their interactions with their EDN (Arai et al., 1990; Sakurai et al., 1990; Schneider et al., 2007). In light of this fact, of greater significance is that the six recovered type I and type II positions from the larger set of EDN1 binding sites comprise a collection of specific targets for future functional experiments of therian versus non-therian EDNRA (Golding and Dean, 1998; Gaucher et al., 2002; Dean and Thornton, 2007). These future experiments will rely on site directed mutagenesis, EDN binding assays, yeast 2-hybrid systems, and other such laboratory approaches to more directly test the biological roles of these recovered rate shift sites.

In contrast, the LRT and statistical analyses of EDNRB provide evidence of functional divergence between therian and non-therian EDNRB, which is not related to EDN binding (Table 1 and Fig. 2). Unlike EDNRA, EDN binding is highly conserved between therian and non-therian EDNRB. Thus, the biological differences between therian and non-therian EDNRB are more likely due to changes in their interactions with their other cell-signaling partners (Clouthier and Schilling, 2004). Furthermore, therian EDNRB is under stronger functional constraints for these other interactions. As for EDNRA, the recovered type I and I/II positions for EDNRB provide a set of specific candidates for future functional experiments of the biological differences between therian versus non-therian EDNRB.

4.3. Potential loss of therian EDNRC by chromosomal deletion

In humans, the *TMLHE*-to-*SYBL1* linkage group lies just upstream of and within the pseudoautosomal region 2 (PAR2) at the end of the long arm of the X chromosome (Blaschke and Rappold, 2006). Similar to human PAR1 at the tip of the short arm of the X chromosome, this syntenic block has undergone numerous chromosomal rearrangements among both closely and distantly related mammalian groups, as well as other tetrapods (D'Esposito et al., 1997; Graves et al., 1998; Charchar et al., 2003; Waters et al., 2005). In particular, these rearrangements include the following, more recent, chromosomal mutations that have occurred over shorter periods of evolutionary time: (1) the loss of >70% of the intervening segment between *TMHLE* and *SYLB1* in the single linkage group of mouse, (2) the translocation of the *TMHLE* and *SYBL1* regions to two different autosomes in rat, and (3) the duplication of PAR2 onto the end of the long arm of the Y chromosome by XY recombination in human (Fig. 3). These chromosomal changes are most likely driven by the greater tendency of the unpaired ends of sex chromosomes and/or rearranged autosomes to interact non-homologously during meiosis (Charchar et al., 2003).

In light of these trends, we hypothesize that the loss of therian EDNRC is due to a chromosomal deletion of its gene within the therian common ancestor. Unfortunately, this hypothesis remains difficult to test, because of the uncertainties with searching for a putative, ancestral, *EDNRC* pseudogene within the therian genome. After >140 million years of rapid divergent evolution, an ancestral therian pseudogene is expected to show <45% nucleotide identity with the functional ortholog of platypus and other monotremes. This calculation assumes: (1) that the ancestral therian pseudogene was non-functionalized 140 million years ago (i.e., just prior to the split of eutherians and marsupials), (2) that therians and monotremes diverged 190 million years ago, (3) that the therian pseudogene is evolving under the Jukes/Cantor model at the average pseudogene rate of 3.87×10^{-9} substitutions per site per year, and (4) that the functional copy is changing 55% slower under the Jukes/Cantor model than the pseudogene (Li, 1997; Donoghue and Benton, 2007). Under these conditions, an ancestral therian pseudogene is expected to show limited sequence similarity to its other family members, thereby making its identification and recovery problematic.

5. Conclusions

This study combines a well supported phylogeny for the EDNR family with well established knowledge from molecular evolution (e.g., the rule of functional constraint and the frequent loss of duplicate genes). By relying on this integrative approach, our final biological statements are rooted in both the phylogenetic history and the known molecular evolutionary processes that underlie the diversity within a gene family. In particular, this integrative approach allows for the recognition of a natural phylogenetic classification for *EDNR*, for the design of additional experiments to test candidate protein positions for their functional significance, and for the generation of new hypotheses about the potential roles of chromosomal mutations in gene family evolution. In light of these successes, we recommend the use of this integrative approach for

the greater resolution of the taxonomy, evolution, function, and structure of gene families.

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Appendix A. Amplification, sequencing, and tissue expression analyses of the three new killifish *EDNR*

The killifish, *Fundulus heteroclitus*, is a non-traditional model species that is abundant in estuaries along the eastern coast of the United States and Canada (Bigelow and Schroeder, 2002). This fish is an ideal subject for physiological, toxicological, behavioral, ecological, and evolutionary studies, because of its abundance, small size (~8 cm), and easy laboratory maintenance (Marshall, 2003; Burnett et al., 2007).

Three new *EDNR* were amplified, cloned, and sequenced from the total cDNA of the killifish gill, as previously described (Hyndman and Evans, 2007). The primers for these amplifications and sequencing are given in Fig. S2 Supporting Materials online. These new *EDNR* were first identified on the basis of BLAST comparisons of their inferred polypeptides to the protein databases of GenBank (release 164) and Ensembl (e! 44). These initial designations were then confirmed by the ML and BP phylogenetic analyses (Fig. 1). Killifish *EDNRA3* (GenBank accession number EU391601), *EDNRB1* (EU391602), and *EDNRC* (EU391603) are 2543, 1629, and 1470 base pairs (bp) in length. Their inferred polypeptides align to positions 366-1643, 270-1520, and 162-1403 of human EDNRA, EDNRB, and quail EDNRC to which they share 63, 70, and 68% identity (Fig. S1 available as Supporting Materials online).

EDNR expression was examined in seven tissues of the adult killifish by semi-quantitative duplexing PCR, as previously described (Hyndman and Evans, 2007). The three new *EDNR* are expressed in all examined tissues, with the exception of no observed *EDNRB1* expression in the heart (Fig. S3, available as Supporting Materials online). All three are most strongly expressed in the kidney, with *EDNRA3*, *EDNRB1*, and *EDNRC* also showing strong expression in the heart, brain and gill, and brain and heart, respectively.

Appendix B. Supplementary data

Supplementary Table S1 and Figs. S1–S3 are available at *Molecular Phylogenetics and Evolution* online. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.04.015.

References

Adachi, M., Hashido, K., Trzeciak, A., Watanabe, T., Furuichi, Y., Miyamoto, C., 1993. Functional domains of human endothelin receptor. J. Cardiovasc. Pharmacol. 22 (Suppl. 8), S121–S124.

Angerio, A.D., 2005. The role of endothelin in heart failure. Crit. Care Nurs. Q. 28, 355–359.

Arai, H., Hori, S., Aramori, I., Ohkubo, H., Nakanishi, S., 1990. Cloning and expression of a cDNA encoding an endothelin receptor. Nature 348, 730–732.

Arai, H., Nakao, K., Takaya, K., Hosoda, K., Ogawa, Y., Nakanishi, S., Imura, H., 1993. The human endothelin-B receptor gene. Structural organization and chromosomal assignment. J. Biol. Chem. 268, 3463–3470.

Bigelow, H.B., Schroeder, W.C. (Eds.), 2002. Fishes of the Gulf of Maine. Blackburn Press, Caldwell, NJ.

Blaschke, R.J., Rappold, G., 2006. The pseudoautosomal regions, *SHOX* and disease. Curr. Opin. Genet. Dev. 16, 233–239.

Blouin, C., Butt, D., Roger, A.J., 2005. Impact of taxon sampling on the estimation of rates of evolution at sites. Mol. Biol. Evol. 22, 784–791.

Breu, V., Hashido, K., Broger, C., Miyamoto, C., Furuichi, Y., Hayes, A., Kalina, B., Loffler, B.M., Ramuz, H., Clozel, M., 1995. Separable binding sites for the natural agonist endothelin-1 and the non-peptide antagonist bosentan on human endothelin-A receptors. Eur. J. Biochem. 231, 266–270.

Burnett, K.G., Bain, L.J., Baldwin, W.S., Callard, G.V., Cohen, S., Di Giulio, R.T., Evans, D.H., Gomez-Chiarri, M., Hahn, M.E., Hoover, C.A., Karchner, S.I., Katoh, F., MacLatchy, D.L., Marshall, W.S., Meyer, J.N., Nacci, D.E., Oleksiak, M.F., Rees, B.B., Singer, T.D., Stegeman, J.J., Towle, D.W., Van Veld, P.A., Vogelbein, W.K., Whitehead, A., Winn, R.N., Crawford, D.L., 2007. *Fundulus* as the premier teleost model in environmental biology: opportunities for new insights using genomics. Comp. Biochem. Physiol. D Genom. Proteom. 2, 257–286.

Charchar, F.J., Svartman, M., El-Mogharbel, N., Venture, M., Kirby, P., Matarazzo, M.R., Ciccodicola, A., Rocchi, M., D'Esposito, M., Marshall Graves, J.A.M., 2003. Complex events in the evolution of the human pseudoautosomal region 2 (PAR2). Genome Res. 13, 281–286.

Clouthier, D.E., Schilling, T.F., 2004. Understanding endothelin-1 function during craniofacial development in the mouse and zebrafish. Birth Defects Res. C Embryo Today 72, 190–199.

Cracraft, J., Donoghue, M.J. (Eds.), 2004. Assembling the Tree of Life. Oxford Univ. Press, Oxford, UK.

da Fonseca, R.R., Antunes, A., Melo, A., Ramos, M.J., 2006. Structural divergence and adaptive evolution in mammalian cytochromes P450 2C. Gene 387, 58–66.

Dean, A.M., Thornton, J.W., 2007. Mechanistic approaches to the study of evolution: the functional synthesis. Nat. Rev. Genet. 8, 675–688.

Dehal, P., Boore, J.L., 2005. Two rounds of whole genome duplication in the ancestral vertebrate. PLoS Biol. 3, 1700–1708.

Dehal, P., Satou, Y., Campbell, R.K., Chapman, J., Degnan, B., De Tomaso, A., Davidson, B., Di Gregorio, A., Gelpke, M., Goodstein, D.M., Harafuji, N., Hastings, K.E.M., Ho, I., Hotta, K., Huang, W., Kawashima, T., Lemaire, P., Martinez, D., Meinertzhagen, I.A., Necula, S., Nonaka, M., Putnam, N., Rash, S., Saiga, H., Satake, M., Terry, A., Yamada, L., Wang, H.-G., Awazu, S., Azumi, K., Boore, J., Branno, M., Chin-bow, S., DeSantis, R., Doyle, S., Francino, P., Keys, D.N., Haga, S., Hayashi, H., Hino, K., Imai, K.S., Inaba, K., Kano, S., Kobayashi, K., Kobayashi, M., Lee, B.-I., Makabe, K.W., Manohar, C., Matassi, G., Medina, M., Mochizuki, Y., Mount, S., Morishita, T., Miura, S., Nakayama, A., Nishizaka, S., Nomoto, H., Ohta, F., Oishi, K., Rigoutsos, I., Sano, M., Sasaki, A., Sasakura, Y., Shoguchi, E., Shin-i, T., Spagnuolo, A., Stainier, D., Suzuki, M.M., Tassy, O., Takatori, N., Tokuoka, M., Yagi, K., Yoshizaki, F., Wada, S., Zhang, C., Hyatt, P.D., Larimer, F., Detter, C., Doggett, N., Glavina, T., Hawkins, T., Richardson, P., Lucas, S., Kohara, Y., Levine, M., Satoh, N., Rokhsar, D.S., 2002. The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. Science 298, 2157–2167.

D'Esposito, M., Matarazzo, M.R., Ciccodicola, A., Strazzullo, M., Mazzarella, R., Quaderi, N.A., Fujiwara, H., Ko, M.S., Rowe, L.B., Ricco, A., Archidiacono, N., Rocchi, M., Schlessinger, D., D'Urso, M., 1997. Differential expression pattern of XqPAR-linked genes SYBL1 and IL9R correlates with the structure and evolution of the region. Hum. Mol. Genet. 6, 1917–1923.

Donoghue, P.C.J., Benton, M.J., 2007. Rocks and clocks: calibrating the tree of life using fossils and molecules. Trends Ecol. Evol. 22, 424–431.

Evans, B.J., 2008. Genome evolution and speciation genetics of clawed frogs (*Xenopus* and *Silurana*). Front. Biosci. 1, 4687–4706.

Evans, D.H., Rose, R.E., Roeser, J.M., Stidham, J.D., 2004. NaCl transport across the opercular epithelium of *Fundulus heteroclitus* is inhibited by an endothelin to NO, superoxide, and prostanoid signaling axis. Am. J. Physiol. Regul. Integr. Comp. Physiol. 286, R560–R568.

Felsenstein, J., 2004. Inferring Phylogenies. Sinauer Assc., Sunderland, MA.

Franchini, L.F., Elgoyhen, A.B., 2006. Adaptive evolution in mammalian proteins involved in cochlear outer hair cell electromotility. Mol. Phylogenet. Evol. 41, 622–635.

Fredriksson, R.M., Lagerstrom, C., Lundin, L.G., Schioth, H.B., 2003. The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. Mol. Pharmacol. 63, 1256–1272.

Garvin, J., Sanders, K., 1991. Endothelin inhibits fluid and bicarbonate transport in part by reducing Na^+/K^+ ATPase activity in the rat proximal straight tubule. J. Am. Soc. Nephrol. 2, 976–982.

Gaucher, E.A., Xu, G., Miyamoto, M.M., Benner, S.A., 2002. Predicting functional divergence in protein evolution by site-specific rate shifts. Trends Biochem. Sci. 27, 315–321.

Godard, C.A.J., Goldstone, J.V., Said, M.R., Dickerson, R.L., Woodin, B.R., Stegeman, J.J., 2005. The new vertebrate CYP1C family: cloning of new subfamily members and phylogenetic analysis. Biochem. Biophys. Res. Comm. 331, 1016–1024.

Golding, G.B., Dean, A.M., 1998. The structural basis of molecular adaptation. Mol. Biol. Evol. 15, 355–369.

Goldstone, J.V., Goldstone, H.M.H., Morrison, A.M., Tarrant, A., Kern, S.E., Woodin, B.R., Stegeman, J.J., 2007. Cytochrome P450 1 genes in early deuterostomes (tunicates and sea urchins) and vertebrates (chicken and frog): origin and diversification of the CYP1 gene family. Mol. Biol. Evol. 24, 2619–2631.

Graves, J.A.M., Wakefield, M.J., Toder, R., 1998. The origin and evolution of the pseudoautosomal regions of human sex chromosomes. Hum. Mol. Genet. 7, 1991–1996.

Gu, X., 1999. Statistical methods for testing functional divergence after gene duplication. Mol. Biol. Evol. 16, 1664–1674.

Gu, X., 2001. Maximum-likelihood approach for gene family evolution under functional divergence. Mol. Biol. Evol. 18, 453–464.

Gu, X., 2006. A simple statistical method for estimating type-II (cluster-specific) functional divergence of protein sequences. Mol. Biol. Evol. 23, 1937–1945.
Gu, X., Vander Velden, K., 2002. DIVERGE: Phylogeny-based analysis for functional-structural divergence of a protein family. Bioinformatics 18, 500–501.
Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52, 679–704.
Hoegg, S., Brinkmann, H., Taylor, J.S., Meyer, A., 2004. Phylogenetic timing of the fish-specific genome duplication correlates with the diversification of teleost fish. J. Mol. Evol. 59, 190–203.
Holland, P.W.H., Takahashi, T., 2005. The evolution of homeobox genes: implications for the study of brain development. Brain Res. Bull. 66, 484–490.
Hosoda, K., Nakao, K., Tamura, N., Arai, H., Ogawa, Y., Suga, S., Nakanishi, S., Imura, H., 1992. Organization, structure, chromosomal assignment, and expression of the gene encoding the human endothelin-A receptor. J. Biol. Chem. 267, 18797–18804.
Hubbard, T.J.P., Aken, B.L., Beal, K., Ballester, B., Caccamo, M., Chen, Y., Clarke, L., Coates, G., Cunningham, F., Cutts, T., Down, T., Dyer, S.C., Fitzgerald, S., Fernandez-Banet, J., Graf, S., Haider, S., Hammond, M., Herrero, J., Holland, R., Howe, K., Howe, K., Johnson, N., Kahari, A., Keefe, D., Kokocinski, F., Kulesha, E., Lawson, D., Longden, I., Melsopp, C., Megy, K., Meidl, P., Ouverdin, B., Parker, A., Prlic, A., Rice, S., Rios, D., Schuster, M., Sealy, I., Severin, J., Slater, G., Smedley, D., Spudich, G., Trevanion, S., Vilella, A., Vogel, J., White, S., Wood, M., Cox, T., Curwen, V., Durbin, R., Fernandez-Suarez, X.M., Flicek, P., Kasprzyk, A., Proctor, G., Searle, S., Smith, J., Ureta-Vidal, A., Birney, E., 2007. Ensembl 2007. Nucleic Acids Res. 35 (Database issue), D610–D617.
Huelsenbeck, J.P., Rannala, B., 1997. Phylogenetic methods come of age: testing hypotheses in an evolutionary context. Science 276, 227–232.
Hyndman, K.A., Evans, D.H., 2007. Endothelin and endothelin converting enzyme-1 in the fish gill: evolutionary and physiological perspectives. J. Exp. Biol. 210, 4286–4297.
Inoue, A., Yanagisawa, M., Takuwa, Y., Mitsui, Y., Kobayashi, M., Masaki, T., 1989. The human preproendothelin-1 gene. Complete nucleotide sequence and regulation of expression. J. Biol. Chem. 264, 14954–14959.
Jaillon, O., Aury, J.M., Brunet, F., Petit, J.-L., Stange-Thomann, N., Mauceli, E., Bouneau, L., Fischer, C., Ozouf-Costaz, C., Bernot, A., Nicaud, S., Jaffe, D., Fisher, S., Lutfalla, G., Dossat, C., Segurens, B., Dasilva, C., Salanoubat, M., Levy, M., Boudet, N., Castellano, S., Anthouard, V., Jubin, C., Castelli, V., Katinka, M., Vacherie, B., Biémont, C., Skalli, Z., Cattolico, L., Poulain, J., de Berardinis, V., Cruaud, C., Duprat, S., Brottier, P., Coutanceau, J.-P., Gouzy, J., Parra, G., Lardier, G., Chapple, C., McKernan, K.J., McEwan, P., Bosak, S., Kellis, M., Volff, J.-N., Guigó, R., Zody, M.C., Mesirov, J., Lindblad-Toh, K., Birren, B., Nusbaum, C., Kahn, D., Robinson-Rechavi, M., Laudet, V., Schachter, V., Quétier, F., Saurin, W., Scarpelli, C., Wincker, P., Lander, E.S., Weissenbach, J., Crollius, R.C., 2004. Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype. Nature 431, 946–957.
Judd, W.S., Campbell, C.S., Kellogg, E.A., Stevens, P.F., Donoghue, M.J., 2008. Plant Systematics: A Phylogenetic Approach, third ed. Sinauer Assc., Sunderland, MA.
Karne, S., Jayawickreme, C.K., Lerner, M.R., 1993. Cloning and characterization of an endothelin-3 specific receptor (ETC receptor) from *Xenopus laevis* dermal melanophores. J. Biol. Chem. 268, 19126–19133.
Kasahara, M., 2007. The 2R hypothesis: an update. Curr. Opin. Immunol. 19, 547–552.
Kempf, H., Linare, C., Corvol, P., Gasc, J.M., 1998. Pharmacological inactivation of the endothelin type A receptor in the early chick embryo: a model of mispatterning of the branchial arch derivatives. Development 125, 4931–4941.
Kimura, M., 1983. The Neutral Theory of Molecular Evolution. Cambridge Univ. Press, Cambridge, UK.
Kirby, N.S., Hadoke, P.W., Bagnall, S.J., Webb, D.J., 2008. The endothelin system as a therapeutic target in cardiovascular disease: great expectations or bleak house? Brit. J. Pharmacol. 153, 1105–1119.
Knudsen, B., Farid, N.R., 2004. Evolutionary divergence of thyrotropin receptor structure. Mol. Genet. Metab. 81, 322–334.
Knudsen, B., Miyamoto, M.M., 2001. A likelihood ratio test for evolutionary rate shifts and functional divergence among proteins. Proc. Natl. Acad. Sci. USA 98, 14512–14517.
Knudsen, B., Miyamoto, M.M., Laipis, P.J., Silverman, D.N., 2003. Using evolutionary rates to investigate functional divergence and conservation: a case study of the carbonic anhydrases. Genetics 164, 1261–1269.
Kumar, C., Mwangi, V., Nuthulaganti, P., Wu, H.L., Pullen, M., Brun, K., Aiyar, H., Morris, R.A., Naughton, R., Nambi, P., 1994. Cloning and characterization of a novel endothelin receptor from *Xenopus* heart. J. Biol. Chem. 269, 13414–13420.
Kurihara, Y., Kurihara, H., Suzuki, H., Kodama, T., Maemura, K., Nagai, R., Oda, H., Kuwaki, T., Cao, W.-H., Kamada, N., Jishage, K., Ouchi, Y., Azuma, S., Toyoda, Y., Ishikawa, T., Kumada, M., Yazaki, Y., 1994. Elevated blood pressure and craniofacial abnormalities in mice deficient in endothelin-1. Nature 368, 703–710.
La, M., Reid, J.J., 1995. Endothelin-1 and the regulation of vascular tone. Clin. Exp. Pharmacol. Physiol. 22, 315–323.
Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947–2948.
Lecoin, L., Sakurai, T., Ngo, M.T., Abe, Y., Yanagisawa, M., Le Douarin, N.M., 1998. Cloning and characterization of a novel endothelin receptor subtype in the avian class. Proc. Natl. Acad. Sci. USA 95, 3024–3029.
Li, W.-H., 1997. Molecular Evolution. Sinauer Assc., Sunderland, MA.
Lundberg, J.G., 1972. Wagner networks and ancestors. Syst. Zool. 21, 398–413.
Lynch, M., 2007. The Origins of Genome Architecture. Sinauer Assc., Sunderland, MA.
Lynch, M., Conery, J.S., 2000. The evolutionary fate and consequences of duplicate genes. Science 290, 1151–1155.
Ma, B., Tromp, J., Li, M., 2002. PatternHunter: faster and more sensitive homology search. Bioinformatics 18, 440–445.
Maglott, D., Ostell, J., Pruitt, K.D., Tatusova, T., 2005. Entrez Gene: gene-centered information at NCBI. Nucleic Acids Res. 33 (Database issue), D54–D58.
Marshall, W.S., 2003. Rapid regulation of NaCl secretion by estuarine teleost fish: coping strategies for short-duration freshwater exposures. Biochim. Biophys. Acta 1618, 95–105.
Miwa, M., Inuoe-Murayama, M., Aoki, H., Kunisada, T., Hiragaki, T., Mizutani, M., Ito, S., 2007. Endothelin receptor B2 (EDNRB2) is associated with the panda plumage colour mutation in Japanese quail. Anim. Genet. 38, 103–108.
Miwa, M., Inoue-Murayama, M., Kobayashi, N., Kayang, B.B., Mizutani, M., Takahashi, H., Ito, S., 2006. Mapping of panda plumage color locus on the microsatellite linkage map of the Japanese quail. BMC Genet. 7, 2.
Nataf, V., Grapin-Botton, A., Champeval, D., Amemiya, A., Yanagisawa, M., Le Douarin, N.M., 1998. The expression patterns of endothelin-A receptor and endothelin 1 in the avian embryo. Mech. Dev. 75, 145–149.
National Center for Biotechnology Information. 2002. BLAST Lab: searching the trace archive with discontiguous MegaBlast. NCBI News Fall/Winter 7.
Pla, P., Alberti, C., Solov'eva, O., Pasdar, M., Kunisada, T., Larue, L., 2005. Ednrb2 orients cell migration towards the dorsolateral neural crest pathway and promotes melanocyte differentiation. Pigment Cell Res. 18, 181–187.
Pla, P., Larue, L., 2003. Involvement of endothelin receptors in normal and pathological development of neural crest cells. Int. J. Dev. Biol. 47, 315–325.
Pollock, D.M., Highsmith, R.F. (Eds.), 1998. Endothelin Receptors and Signaling Mechanisms. Springer, Berlin, Germany.
Prasanna, G., Dibas, A., Hulet, C., Yorio, T., 2001. Inhibition of Na^+/K^+-ATPase by endothelin-1 in human nonpigmented ciliary epithelial cells. J. Pharmacol. Exp. Ther. 296, 966–971.
Putnam, N.H., Butts, T., Ferrier, D.E.K., Furlong, R.F., Hellsten, U., Kawashima, T., Robinson-Rechavi, M., Shoguchi, E., Terry, A., Yu, J.-K., Benito-Gutiérrez, E., Dubchak, I., Garcia-Fernàndez, J., Gibson-Brown, J.J., Grigoriev, I.V., Horton, A.C., de Jong, P.J., Jurka, J., Kapitonov, V.V., Kohara, Y., Kuroki, Y., Lindquist, E., Lucas, S., Osoegawa, K., Pennacchio, L.A., Salamov, A.A., Satou, Y., Sauka-Spengler, T., Schmutz, J., Shin-I, T., Toyoda, A., Bronner-Fraser, M., Fujiyama, A., Holland, L.Z., Holland, P.W.H., Satoh, N., Rokhsar, D.S., 2008. The amphioxus genome and the evolution of the chordate karyotype. Nature 453, 1064–1072.
Richter, C.M., 2006. Role of endothelin in chronic renal failure—developments in renal involvement. Rheumatology 45 (Suppl. 3), iii36–iii38.
Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.
Sakurai, T., Yanagisawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K., Masaki, T., 1990. Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. Nature 348, 732–735.
Scarparo, A.C., Isoldi, M.C., de Lima, L.H., Visconti, M.A., Castucci, A.M., 2007. Expression of endothelin receptors in frog, chicken, mouse and human pigment cells. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 147, 640–646.
Schneider, M.P., Boesen, E.I., Pollock, D.M., 2007. Contrasting actions of endothelin ET(A) and ET(B) receptors in cardiovascular disease. Annu. Rev. Pharmacol. Toxicol. 47, 731–759.
Shavit, L., Penny, D., Hendy, M.D., Holland, B.R., 2007. The problem of rooting rapid radiations. Mol. Biol. Evol. 24, 2400–2411.
Shreenivas, S., Oparil, S., 2007. The role of endothelin-1 in human hypertension. Clin. Hemorheol. Microcirc. 37, 157–178.
Spudich, G., Fernandez-Suarez, X.M., Birney, E., 2007. Genome browsing with Ensembl: a practical overview. Brief. Funct. Genom. Proteom. 6, 202–219.
Stenslokken, K.O., Sundin, L., Nilsson, G.E., 2006. Endothelin receptors in teleost fishes: cardiovascular effects and branchial distribution. Am. J. Physiol. Regul. Integr. Comp. Physiol. 290, R852–R860.
Swofford, D.L., Olsen, G.J., Waddell, P.J., Hillis, D.M., 1996. Phylogenetic inference. In: Hillis, D.M., Moritz, C., Mable, B.K. (Eds.), Molecular Systematics, second ed. Sinauer Assc., Sunderland, MA, pp. 407–514.
Thornton, J.W., DeSalle, R., 2000. Gene family evolution and homology: genomics meets phylogenetics. Annu. Rev. Genomics Hum. Genet. 1, 41–73.
Volff, J.-N., 2005. Genome evolution and biodiversity in teleost fish. Heredity 94, 280–294.
Volff, J.-N. (Ed.), 2006. Vertebrate genomes. Genome Dynam. 2, 1–216.
Wada, K., Hashido, K., Terashima, H., Adachi, M., Fujii, Y., Hiraoka, O., Furuichi, Y., Miyamoto, C., 1995. Ligand binding domain of the human endothelin-B subtype receptor. Protein Expr. Purif. 6, 228–236.
Wain, H.M., Bruford, E.A., Lovering, R.C., Lush, M.J., Wright, M.W., Povey, S., 2002. Guidelines for human gene nomenclature. Genomics 79, 464–470.
Walsh, B., 2003. Population-genetic models of the fates of duplicate genes. Genetica 118, 279–294.
Warren, W.C., Hillier, L.W., Graves, J.A.M., Birney, E., Ponting, C.P., Grützner, F., Belov, K., Miller, W., Clarke, L., Chinwalla, A.T., Yang, S.P., Heger, A., Locke, D.P., Miethke, P., Waters, P.D., Veyrunes, F., Fulton, L., Fulton, B., Graves, T., Wallis, J., Puente, X.S., López-Otín, C., Ordóñez, G.R., Eichler, E.E., Chen, L., Cheng, Z., Deakin, J.E., Alsop, A., Thompson, K., Kirby, P., Papenfuss, A.T., Wakefield, M.J., Olender, T., Lancet, D., Huttley, G.A., Smit, A.F.A., Pask, A., Temple-Smith, P., Batzer, M.A., Walker, J.A., Konkel, M.K., Harris, R.S., Whittington, C.M., Wong, E.S.M., Gemmell, N.J., Buschiazzo, E., Vargas Jentzsch, I.M., Merkel, A., Schmitz, J.,

Zemann, A., Churakov, G., Kriegs, J.O., Brosius, J., Murchison, E.P., Sachidananda, R., Smith, C., Hannon, G.J., Tsend-Ayush, E., McMillan, D., Attenborough, R., Rens, W., Ferguson-Smith, M., Lefèvre, C.M., Sharp, J.A., Nicholas, K.R., Ray, D.A., Kube, M., Reinhardt, R., Pringle, T.H., Taylor, J., Jones, R.C., Nixon, B., Dacheux, J.-L., Niwa, H., Sekita, Y., Huang, X., Stark, A., Kheradpour, P., Kellis, M., Flicek, P., Chen, Y., Webber, C., Hardison, R., Nelson, J., Hallsworth-Pepin, K., Delehaunty, K., Markovic, C., Minx, P., Feng, Y., Kremitzki, C., Mitreva, M., Glasscock, J., Wylie, T., Wohldmann, P., Thiru, P., Nhan, M.N., Pohl, C.S., Smith, S.M., Hou, S., Renfree, M.B., Mardis, E.R., Wilson, R.K., . Genome analysis of the platypus reveals unique signatures of evolution. Nature 453, 175–184.

Waters, P.D., Delbridge, M.L., Deakin, J.E., El-Mogharbel, N., Kirby, P.J., Carvalho-Silva, D.R., Graves, J.A.M., 2005. Autosomal location of genes from the conserved mammalian X in the platypus (*Ornithorhynchus anatinus*): implications for mammalian sex chromosome evolution. Chrom. Res. 13, 401–410.

Wheeler, D.L., Barrett, T., Benson, D.A., Bryant, S.H., Canese, K., Chetvernin, V., Church, D.M., DiCuccio, M., Edgar, R., Federhen, S., Geer, L.Y., Kapustin, Y., Khovayko, O., Landsman, D., Lipman, D.J., Madden, T.L., Maglott, D.R., Ostell, J., Miller, V., Pruitt, K.D., Schuler, G.D., Sequeira, E., Sherry, S.T., Sirotkin, K., Souvorov, A., Starchenko, G., Tatusov, R.L., Tatusova, Wagner, L., Yaschenko, Y., 2007. Database resources of the National Center for Biotechnology Information. Nucleic Acids Res. 35 (Database issue), D5–D12.

Whelan, S., Goldman, N., 2001. A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. Mol. Biol. Evol. 18, 691–699.

Yanagisawa, M., Inoue, A., Ishikawa, T., Kasuya, Y., Kimura, S., Kumagaye, S., Nakajima, K., Watanabe, T.X., Sakakibara, S., Goto, K., 1988a. Primary structure, synthesis, and biological activity of rat endothelin, an endothelium-derived vasoconstrictor peptide. Proc. Natl. Acad. Sci. USA 85, 6964–6967.

Yanagisawa, M., Kurihara, H., Kimura, S., Goto, K., Masaki, T., 1988b. A novel peptide vasoconstrictor, endothelin, is produced by vascular endothelium and modulates smooth muscle Ca^{2+} channels. J. Hypertens. Suppl. 6, S188–S191.

Yang, Z., 1996. Among-site rate variation and its impact on phylogenetic analyses. Trends Ecol. Evol. 11, 367–372.

Zeidel, M.L., Brady, H.R., Kone, B.C., Gullans, S.R., Brenner, B.M., 1989. Endothelin, a peptide inhibitor of Na(+)–K(+)–ATPase in intact renaltubular epithelial cells. Am. J. Physiol. Cell Physiol. 257, C1101–C1107.