

Gene Duplications and Evolution of Vertebrate Voltage-Gated Sodium Channels

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Abstract. Voltage-gated sodium channels underlie action potential generation in excitable tissue. To establish the evolutionary mechanisms that shaped the vertebrate sodium channel α -subunit (*SCNA*) gene family and their encoded Na_v1 proteins, we identified all *SCNA* genes in several teleost species. Molecular cloning revealed that teleosts have eight *SCNA* genes, compared to ten in another vertebrate lineage, mammals. Prior phylogenetic analyses have indicated that the genomes of both teleosts and tetrapods contain four monophyletic groups of *SCNA* genes, and that tandem duplications expanded the number of genes in two of the four mammalian groups. However, the number of genes in each group varies between teleosts and tetrapods, suggesting different evolutionary histories in the two vertebrate lineages. Our findings from phylogenetic analysis and chromosomal mapping of *Danio rerio* genes indicate that tandem duplications are an unlikely mechanism for generation of the extant teleost *SCNA* genes. Instead, analyses of other closely mapped genes in *D. rerio* as well as of *SCNA* genes from several teleost species all support the hypothesis that a whole-genome duplication was involved in expansion of the *SCNA* gene family in teleosts. Interestingly, despite their different evolutionary histories, mRNA analyses demonstrated a conservation of expression patterns for *SCNA* orthologues in teleosts and tetrapods, suggesting functional conservation.

Key words: Voltage-gated sodium channel — Teleosts — Gene families — Genome duplication — Gene duplication

Introduction

Members of the voltage-gated sodium channel α -subunit family (*SCNA* genes) code for transmembrane Na_v1 proteins that allow the sodium influx required for action potential generation in excitable cells (for review, see Catterall et al. 2005). Non-vertebrate species typically have one to two *SCNA* genes (for review, see Goldin 2002). In contrast, molecular characterization of the mammalian *SCNA* gene family has revealed nine genes and an evolutionarily related one coding for a non-voltage-gated channel, Na_x . Each mammalian *SCNA* gene displays a unique expression pattern with respect to tissue specificity and developmental regulation (for review, see Goldin 2002). In addition, the encoded Na_v1 channels differ functionally on the basis of channel biophysical properties, toxin binding, and pharmacology (Catterall et al. 2005). Despite the extensive information existing about extant mammalian *SCNA* genes, important questions remain regarding their evolutionary relationships with nonmammalian vertebrate orthologues.

Phylogenetic analysis has been previously applied to *SCNA* genes to determine the evolutionary history

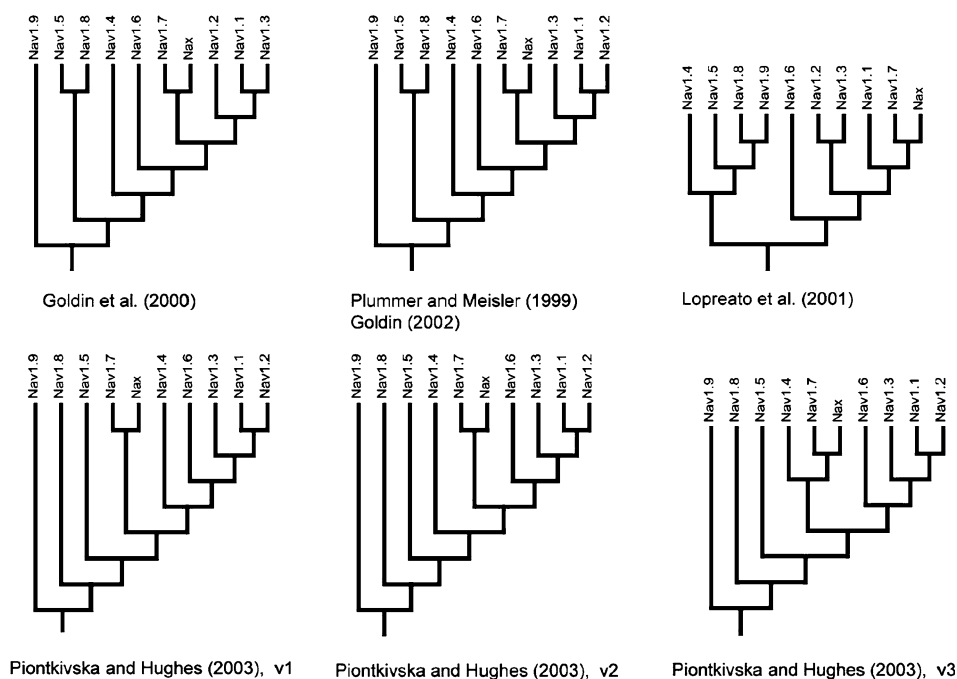


Fig. 1. Prior hypotheses of mammalian Na_v1 phylogeny. The basic topology of the mammalian Na_v1 family is a double-forked tree. The six trees presented are adapted from those of Goldin et al. (2000), Plummer and Meisler (1999), Goldin (2002), Lopreato et al. (2001), and Piontkivska and Hughes (2003).

of the vertebrate *SCNA* gene family (Goldin 2002; Goldin et al. 2000; Lopreato et al. 2001; Piontkivska and Hughes 2003; Plummer and Meisler 1999). The phylogenetic estimates of previous studies differ in significant ways (Fig. 1). For example, results of Plummer and Meisler (1999) and Lopreato et al. (2001) are distinct from other studies in that they both support the hypothesis that two early whole-genome duplications in vertebrates (known as the 2R hypothesis [e.g., Amores et al. 1998; Ohno 1970; Sidow 1996]) led to four *SCNA* genes in the most recent ancestor of teleosts and tetrapods (Teleostomi).

Prior studies have also indicated that, in mammals, three of the four *SCNA* monophyletic groups are linked to *HOX* gene clusters, and the fourth is part of a chromosome segment that has been separated from the fourth *HOX* gene cluster (Plummer and Meisler 1999). In zebrafish, seven *hox* genes exist, each on separate chromosomes and in close proximity to a cassette of associated genes (e.g., distal-less [*dll*], sonic hedgehog [*shh*], frizzled [*fzd*] [Amores et al. 1999]). Amores et al. (1998) proposed that the larger number of zebrafish *hox* and associated genes arose during a genome-wide duplication event unique to teleosts (see also Meyer and Schartl 1999; Wittbrodt et al. 1998; Vandepoele et al. 2004). Interestingly, Lopreato et al. (2001) suggested that, in teleosts, this genome-wide duplication event contributed to *SCNA* gene family evolution.

Several considerations now warrant reexamination of *SCNA* gene phylogeny. Since the completion of these studies, additional *SCNA* genes have been isolated in a number of teleost species. Furthermore, prior phylogenetic estimates were either unrooted or rooted using nonvertebrate gene sequences, the latter

increasing the risk of incorrectly reconstructed relationships as a result of long-branch attraction. Further, the existence of a completely sequenced genome and linkage maps for the zebrafish now permits the use of synteny to test hypotheses about evolutionary history. In addition, insights into evolutionary history of a gene family can also be obtained by examination of exon-intron organization (Plummer and Meisler 1999). In mammals, the nine different *SCNA* genes have common exon-intron boundaries in regions that code for transmembrane domains, but regions coding for intracellular loops show characteristic group-specific differences among the four different *SCNA* monophyletic groups (Plummer and Meisler 1999).

Here we identify *SCNA* genes in several teleost species and compare them to known mammalian genes. Then we use three different approaches to test whether an additional genome-wide duplication in teleosts shaped the evolution of the *SCNA* gene family. First, we use a phylogenetic analysis of a diverse taxon sample to compare the histories of gene duplication in teleosts and tetrapods. Next, we determine the chromosomal locations of zebrafish *SCNA* (*scna*, according to the zebrafish nomenclature convention; http://zfin.org/zf_info/nomen.html) genes and test for linkage with *hox* genes. Third, we compare the exon organization of specific domains of *D. rerio scna* genes to that of mammalian *SCNA* genes. In addition, we compare the potential functions of teleost and tetrapod sodium channels by determining expression patterns of newly identified *D. rerio scna* genes. By examining evolutionary relationships and gene expression, our comparative approach provides information about orthology and

Table 1. Degenerate primers used for RT-PCR

Nucleotide sequence (5'–3')	Targeted region
<i>D. rerio</i>	
GGNAARACNTAYATGATHTTYYTYGT	IS6/exon 9 (F)
ATGGCNTAYGARGARCARAAYCAG	IS6/exon 9 (F)
RTGYTCCATNGCCATRAANAG	IIS1/exon 11 (R)
TGGCAYATGAAYGAYTTYTYTYCAC	IIS1/exon 13 (F)
TAYTTYACHAAYGCHTGGTGYTGG	IIIS3/exon 17 (F)
TGGYTHATHTTYWSHATHATGGG	IIIS5/exon 19 (F)
GGNTGGATGGAYATHATGTANGC	IIIS2/exon 20 (F)
GGRTACATDATRTCCATCCANCC	IIIS2/exon 20 (R)
GGNGGNCARGAYATHTTYATGAC	DL III–IV/exon 22 (F)
GTCATRAADATRTCYTGNCNC	DL III–IV/exon 22 (R)
ATGGTNACHATGATGGTNGARACHG	IVS1/exon 23 (F)
TCDGTYTCNACCATCATDGTNACC	IVS1/exon 23 (R)
ATHGTNGTNAAYATGTAYATHGC	IVS6/exon 24 (F)
TGGGARAARTTYGAYCCNGAYGC	Carboxyl tail/exon 24 (F)
ACCATDGGCARRTCCATDGC	Carboxyl tail/exon 24 (R)
<i>S. macrurus</i> , <i>I. punctatus</i> , <i>G. petersii</i> , and <i>P. marinus</i>	
TGCTSGTGTGYYTGATYTTCTGG	IIIS5/exon 19 (F)
GTGAAGAAKGAACCCRAAGATGATG	IIIS6/exon 21 (R)

Note. Targeted regions are designated in terms of encoded protein domains (e.g., IIIS2). Exon positions are derived from rat *SCN6A* (Plummer et al. 1998). Forward and reverse primers are noted by F and R, respectively. The following abbreviations label degenerate residues: R = A/G, Y = C/T, K = G/T, S = C/G, H = A/C/T, and N = A/C/G/T.

function of vertebrate *SCNA* genes. Importantly, analysis of two distinct vertebrate lineages allows a rigorous test of the dominant paradigm for the rise of novel gene function (or subfunction) following gene duplication events.

Materials and Methods

Animals

Zebrafish (*D. rerio*) were obtained from a local pet store (PetSmart) and raised at 28.5°C according to established procedures (Westerfield 1995). Gold-lined black knifefish (*Sternopygus macrurus*) and elephant nose mormyrids (*Gnathonemus petersii*) were purchased from tropical fish importers and kept in laboratory tanks. Catfish (*Ictalurus punctatus*) were caught by hook and line near Austin, Texas. Lamprey (*Petromyzon marinus*) larvae and adults were provided by the Great Lakes Fishery Commission.

SCNA Gene Cloning

D. rerio. RNA was collected from zebrafish embryos (10–120 hpf) as well as from isolated adult tissues (brain, spinal cord, eye, cardiac and skeletal muscle) using the RNeasy Kit (Qiagen). An additional proteinase K digestion was performed with cardiac and skeletal muscle samples to remove proteins (e.g., contractile proteins) that interfered with the RNA isolation. Reverse transcription (RT) was performed using the Superscript II (Gibco) protocol. Degenerate primers (IDT) were designed to conserved regions, identified on the basis of mammalian *SCNA* sequence alignments (Table 1). These primers were used for both RT and polymerase chain reactions (PCR). Nested PCR reactions were performed and PCR products were resolved by gel electrophoresis. Products of interest were cloned into the PCR Script Amp Sk(+) vector (Stratagene) for further analysis, including sequencing.

Portions of the *D. rerio scna* gene sequences were also obtained from The Sanger Center zebrafish genomic database (http://www.ensembl.org/Danio_rerio/). Exon boundaries within genomic *scna* gene fragments were predicted using the NetGene2 Server (<http://www.cbs.dtu.dk/services/NetGene2>). PCR reactions were also performed to clone regions identified on the genomic database. Full-length sequences were assembled by combining overlapping cDNA sequences and analyzed using the ClustalW algorithm of the freeware BioEdit v7.05 program (<http://www.mbio.ncsu.edu/Bio-Edit/>; Tom Hall, Ibis Therapeutics). Sequences were deposited in GenBank (accession numbers DQ149503, DQ149504, DQ149505, DQ149506, DQ149507, DQ149508, DQ149509). Recently, *D. rerio scn4aa* and *scn4ab* (DQ22153, DQ221254) were independently cloned by Venkatesh et al. (2005).

S. macrurus, *I. punctatus*, *G. petersii*, and *P. marinus*. We sampled *SCNA* genes from a phylogenetically diverse sample of teleost taxa. *SCNA* gene sequences for *Takifugu rubripes* (Percomorpha) were extracted from the Takifugu genome database (<http://www.fugu-sg.org/>). Within the Ostariophysi, to which *D. rerio* belongs, we sequenced *SCNA* genes from a gymnotiform electric fish (*S. macrurus*) and the channel catfish (*I. punctatus*). We also sequenced *SCNA* genes from a mormyrid electric fish (*G. petersii*), which, as an osteoglossomorph, is a member of the most basal extant teleostean group, and from an agnathan vertebrate (*P. marinus*), which is sister to the gnathostomes, and whose sequences were designated for rooting our tree.

A region coding for Na_v1 transmembrane domains II and III of all *SCNA* genes of several teleost species was cloned. Muscle, brain, heart, or electric organ RNA was isolated by homogenization in guanidium thiocyanate (Ausubel et al. 1994) or RNA STAT-60 (Ambion). RT was performed with either random primers or the 3' reverse primer of the subsequent PCR reaction. Reaction parameters were optimized for each species. PCR with degenerate primers resulted in an initial PCR fragment of 370 base pairs (bp; Table 1). PCR products were cloned into the TOPO TA vector (Invitrogen) following the addition of terminal adenines (1 unit of Taq at 72°C for 10 min). Inserts were sequenced and additional rounds of PCR were performed with both specific and degenerate primers to extend sequences of interest. Resultant sequences were up to 2400

bases in length. Sequence alignments were utilized to ensure that each cDNA sequence represented a unique gene. Sequences were deposited in GenBank (accession numbers as follows: *S. macrurus* genes—AF378144, AF378139, DQ286578, AF378143, AF378142, AF378141, AF378140, AY183895; *I. punctatus* genes—AY204538, AY204535, AY204534, AY204533, AY204536, AY204537, AY204532, DQ385608; *G. petersii* genes—DQ275137, DQ275138, DQ275139, DQ275140, DQ275141, DQ275142, DQ275143; *P. marinus* genes—DQ275144, DQ275145).

Genomic DNA Isolation and Southern Blot Analysis

Genomic DNA was isolated from adult wild-type *D. rerio* using standard methods (Westerfield 1995). Following digestion with *Hind*III and *Eco*RI restriction enzymes, DNA was separated on a 1.5% agarose gel and then transferred to nitrocellulose (Sambrook et al. 1989). The ~200-bp probe, derived from a previously published zebrafish sequence (Tsai et al. 2001), was designed to hybridize to an extremely conserved region of all known mammalian *SCNA* genes (encoding the voltage sensor and part of the pore-forming domain—IVS4-S5). To optimize the detection of a single and not multiple bands per *SCNA* gene, the probe design met the following criteria: (1) lack of internal *Hind*III and *Eco*RI sites and (2) inclusion within a single exon. Probe template DNA was synthesized by PCR using DNA from *scn8aa* (zfNa_v1.6 [Tsai et al. 2001]) as input and the specific primers F-5' ACTTTGTGTCACC AACATTGTTTC and R-5' CTTACAGTAGGCAAAGTTTGAC. The probe was radioactively labeled with ³²P-dCTP using the Prime-it Kit (Stratagene). Hybridization was performed at 60°C using standard methods (Sambrook et al. 1989). Filters were washed (1×, 15 min, room temperature; 3×, 15 min, 37°C; 5× SSPE, 0.1% SDS) and exposed to X-ray film. Films were developed and digitally scanned. Digital files were imported into Adobe Photoshop.

Sequence Alignment and Phylogenetic Analyses

A matrix of *SCNA* nucleotide sequences coding for Na_v1 transmembrane domains II and III was constructed using coding region sequences from *D. rerio*, *S. macrurus*, *I. punctatus*, *G. petersii*, and *P. marinus*, as well as published sequences for *SCNA* genes from *Homo sapiens* and *Rattus norvegicus*, and matching sequences from the *Gallus gallus* and *T. rubripes* genome sequence databases. *SCNA* regions coding for transmembrane domains II and III represent the longest region of overlap over which we had confirmed sequence data in all species. *H. sapiens*, *R. norvegicus*, *G. gallus*, and *T. rubripes* sequences were acquired from GenBank (<http://www.ncbi.nlm.nih.gov/>).

Nucleotide sequences were initially aligned using default parameters in ClustalX (Thompson et al. 1994). The alignment was improved manually using MacClade 4.0 (Maddison and Maddison 1992), following predicted amino acid sequences for domains II and III of Na_v1 subunits from *H. sapiens* and *R. norvegicus*. Amino acid sequences and sequence lengths were highly conserved for transmembrane regions, making straightforward the alignment of nucleotides in these conserved regions using known amino acid sequences as a guide. However, the cytoplasmic loop between domain II and domain III varies extensively in length and sequence between Na_v1 isoforms and therefore we excluded this region from subsequent phylogenetic analyses.

PAUP* 4.0b10 (Swofford 2002) was used to calculate pairwise distances, and to test 24 models of nucleotide evolution using the MrModelblock file from Mr. Modeltest 2.0 (Nylander 2004). The latter program was also used to compare likelihood scores from the 24 models. For these data, we selected a general time-reversible model with invariable sites and a gamma distribution for variable rate sites (GTR + I + G). Phylogeny was estimated using MrBayes 3.0 (Huelsenbeck and Ronquist 2001), implementing the GTR + I + G model. Four Markov chains of 1 million generations

were run at the default temperature (0.2), and every 100th tree was saved to a file. The outgroup was set as *P. marinus*. The burn-in asymptote was determined by plotting tree number against log likelihood. Trees saved before the burn-in asymptote were discarded, and a majority rule consensus of the remaining trees was calculated in PAUP*4.0b10 to estimate posterior probabilities. Four replicates of these Bayesian runs were conducted to ensure convergence of the posteriors. The final estimate was rooted using the two Na_v1 sequences from *P. marinus*, which were recovered as sister genes with a posterior value of 100%.

Tree Comparisons

We used five different comparison tests to determine whether our estimate of mammalian *SCNA* gene phylogeny explained the molecular data better than previously published alternatives (Fig. 1) (Plummer and Meisler 1999; Goldin et al. 2000; Lopreato et al. 2001; Goldin 2002; Piontkivska and Hughes 2003). Because the topology of mammalian *SCNA* phylogeny of Goldin (2002) is identical to that of Plummer and Meisler (1999), these two trees were treated as a single hypothesis. In addition, because the phylogenetic tree of Piontkivska and Hughes (2003) contains a three-way polytomy, all three possible resolutions of this polytomy were tested as separate hypotheses.

Only human gene sequences were used in the tree comparison tests, because the minimum dataset used in previous studies only included mammalian Na_v1 proteins (Fig. 1). We created a NEXUS tree file for each competing topology and for the topology implied by our Bayesian estimate. We compared the seven trees presented in Figs. 1 and 5. Using the parsimony criterion, we used PAUP 4.0b10 to calculate tree lengths and to conduct Kishino-Hasegawa (K-H), Wilcoxon signed-ranks, and winning-sites tests. Using the likelihood criterion, we used PAUP 4.0b10 to calculate likelihood scores and a parsimony-based estimate of the rate matrix to conduct two-tailed K-H tests and one-tailed Shimodaira-Hasegawa tests, both using a RELI bootstrap with 1000 replicates. For all tree comparison tests, we excluded the same poorly conserved regions of data that were excluded from the parsimony and Bayesian phylogenetic analyses, as described above.

Gene Mapping

The chromosomal locations of *D. rerio* *scna* genes were determined using the LN54 radiation hybrid panel (Hukriede et al. 1999), which was generously provided by M. Ekker (Ottawa Health Research Institute, Canada). PCR was performed with gene-specific primers designed to amplify an ~200-bp region contained within a single exon. PCR was performed as described (Hukriede et al. 1999) using FastStart Taq DNA Polymerase (Roche Diagnostics) in 96-well plates on a GeneAmp 2700 PCR machine (Applied Biosystems). Products were analyzed by electrophoresis on 0.5% agarose gels. Results were interpreted at <http://mgchd1.nichd.nih.gov:8000/zfrh/beta.cgi>.

Genomic DNA Analysis

Regions of genomic DNA corresponding to *scna* genes of interest were identified using BLAST on the NCBI or Ensembl zebrafish database. RT-PCR sequences were used to identify the nucleotides corresponding to exons. The number of amino acids coded for by each exon were counted and compared between each zebrafish orthologue and rat *SCN5A* (M77235) and *SCN4A* (NM_000334).

Detection of *scna* mRNAs in Adult *D. rerio* Tissue

RT-PCR was performed to determine *scna* mRNA expression in excitable tissues of adult zebrafish. RT was performed with 1 µg of

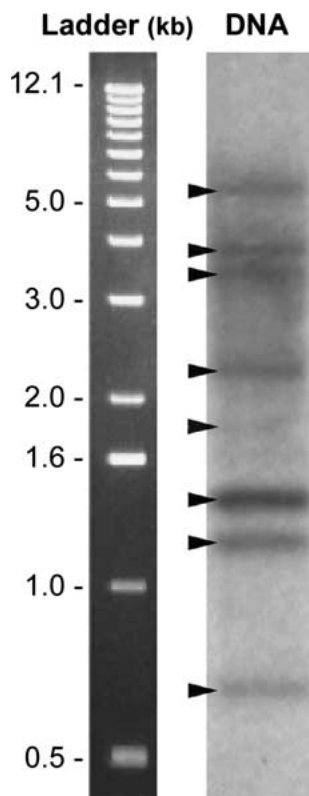


Fig. 2. Southern blot analysis suggests that the zebrafish *scna* gene family comprises eight genes. Southern blot analysis provided an initial estimate of the number of zebrafish *scna* genes. A “universal” probe, which recognized a conserved sequence in the regions coding for domains IVS4-S5 of all known vertebrate *SCNA* isoforms, was used. Arrowheads point to the eight hybridizing bands. Even though the teleost and tetrapod genomes have undergone divergent evolutionary pathways, the results indicated that the sizes of the zebrafish and mammalian *SCNA* gene families are similar (eight versus nine, respectively).

total RNA (brain, spinal cord, eye, cardiac and skeletal muscle) using gene-specific reverse primers designed for the radiation hybrid experiments. Two rounds of PCR (15 cycles each) were performed using 5 μ l of RT product in the first reaction at annealing temperatures empirically determined for each set. Positive (genomic DNA) and negative (no DNA) controls were routinely included.

Results

Southern Blot Analysis and Molecular Cloning Indicated That Teleosts Have Eight scna Genes

We used standard Southern blot analysis of genomic DNA to provide an initial estimate of the size of the zebrafish *scna* gene family. The probe targeted a region that is highly conserved in all known *SCNA* genes. Seven bands displayed strong hybridization signals (Fig. 2). The probe also recognized an additional band, albeit with a weaker hybridization signal. These data suggested the presence of eight *scna* genes in *D. rerio*.

Next, we isolated *D. rerio scna* genes using a combination of PCR and database mining. Partial

Table 2. Zebrafish *scna* gene and Na_v1 protein nomenclature

Gene name	Protein name	Chromosome (placement, cRay)	Previously cloned teleost gene ^a
<i>scn1Laa</i>	Na _v 1.1La	9 (532.65)	Na4
<i>scn1Lab</i>	Na _v 1.1Lb	6 (175.31) ^b	Na3
<i>scn4aa</i>	Na _v 1.4a	12 (184.67)	Na6
<i>scn4ab</i>	Na _v 1.4b	3 (174.07)	Na1
<i>scn5Laa</i>	Na _v 1.5La	2 (528.24)	Na2
<i>scn5Lab</i>	Na _v 1.5Lb	24 (473.14)	
<i>scn8aa</i>	Na _v 1.6a	23 (278.21)	
<i>scn8ab</i>	Na _v 1.6b	6 (421.08) ^b	Na5

^aFrom Lopreato et al. (2001).

^b*scn1Lab* and *scn8ab* mapped to the same chromosome. However, the two genes are located 245.8 cRay (~36.4 Mb) apart, suggesting that they did not evolve by tandem duplication of a common ancestral gene.

sequences for eight different genes were initially isolated using a PCR strategy. To obtain complete open reading frames, we performed additional PCR with specific primers designed on the basis of the partial sequence or information in the Ensembl genomic database (www.ensembl.org). Eight different open reading frames, each corresponding to one of the initial partial sequences, were obtained (Table 2; Supplemental Figure 1, Supplemental Table 1). Despite numerous efforts using PCR, library screening, and database mining, no additional *scna* genes were revealed. Thus, both the molecular cloning and the Southern analysis results revealed eight *scna* genes in zebrafish.

Independently, partial sequence information was obtained for several other teleost species. PCR analysis of genomic DNA or brain, heart, electric organ, and muscle cDNA identified eight *SCNA* genes each in the weakly electric fish *S. macrurus* and catfish (*I. punctatus*). Similar methods were used to identify seven mormyrid (*G. petersii*) genes. A search of the pufferfish (*T. rubripes*) genome database revealed eight *SCNA* genes. In sum, the molecular cloning data from several teleost species and Southern blot results of zebrafish genomic DNA revealed eight *SCNA* genes.

Phylogenetic Analyses

The total number of unique *SCNA* sequences in our alignment matrix was 70: 8 each from *T. rubripes*, *D. rerio*, *I. punctatus*, and *S. macrurus*; 7 from *G. petersii*; 10 each from *H. sapiens* and *R. norvegicus*; 9 from *G. gallus*; and 2 from *P. marinus*. The Bayesian estimate of phylogeny is given in Fig. 3, with branch lengths in Fig. 4. This tree is rooted with the two *P. marinus* sequences which group here as sister genes (100%). We believe that these two sequences represent a unique gene or genome duplication in lampreys, which are also thought to have

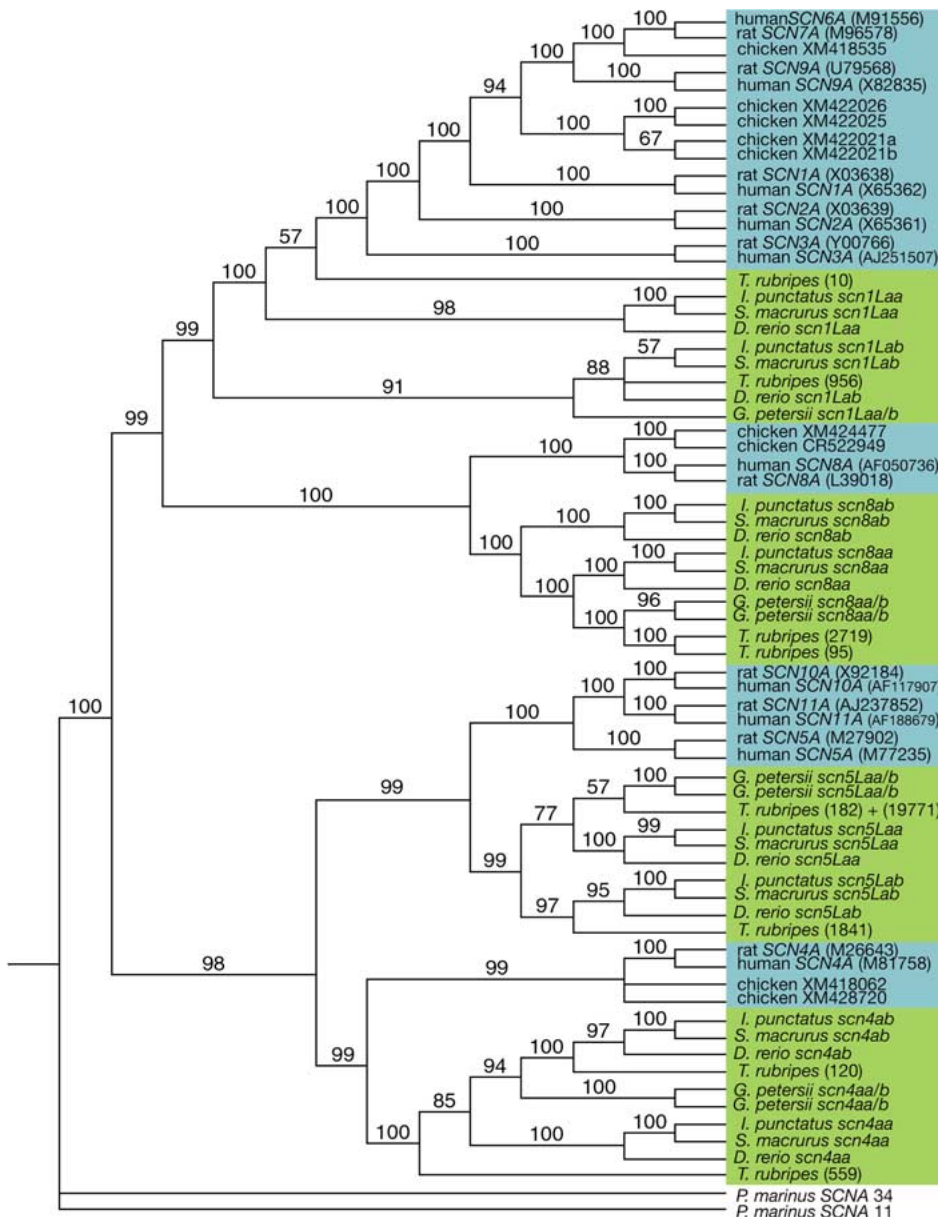


Fig. 3. Bayesian estimate of phylogeny for vertebrate voltage-gated sodium channel genes (MCMC \times 1,000,000 \times 4, GTR + I + G). Tetrapod and teleost sequences are highlighted in blue and green, respectively. The results are consistent with different gene duplication histories in tetrapods (tandem duplications) versus teleosts (whole-genome duplication). Numbers above branches indicate posterior probabilities. GenBank accession numbers are provided for tetrapod sequences. Only GenBank accession numbers appear for chicken sequences, as their nomenclature has not yet been established. Numbers in parentheses following *T. rubripes* entries indicate scaffold numbers from Assembly Release 3 (Mayfolds). For other teleost sequences, see Materials and Methods. The *scna* gene names given for *G. petersii* and *P. marinus* are temporary because their orthologous relationships are not yet fully established.

undergone independent *Hox* cluster duplications (see Fried et al. 2003; Stadler et al. 2004). However, re-rooting our tree with the lamprey genes as sister to one or the other of the two main groups does not change the relationships between genes in the teleost and tetrapod lineages.

We used phylogenetic analysis to test the evolutionary relationships between *SCNA* genes in teleosts and tetrapods. Our results indicated that, in teleosts, each of the four monophyletic gene groups contained two *SCNA* genes (Fig. 3). In contrast, in mammals, each of the four groups contained one to five genes. In three of the four groups, a monophyletic group of tetrapod genes was recovered as the sister to a monophyletic group of teleost genes, which is consistent with the hypothesis of independent duplica-

tion histories in the two vertebrate lineages. Although our Bayesian estimate did not strongly support a similar topology in the fourth group, composed of *scn1La* genes and their tetrapod orthologues, a Shimodaira-Hasegawa test using an estimated likelihood rate matrix and estimated gamma distribution could not reject an alternative tree rearranged so that monophyletic teleost *scn1La* genes were sister to their monophyletic tetrapod orthologues ($p = 0.21$).

Tree Comparisons

Our phylogenetic analysis resulted in a double-forked tree (Fig. 5). However, phylogenies from other laboratories have resulted in a “ladder” topology (Fig. 1). To test whether differences in the species used for

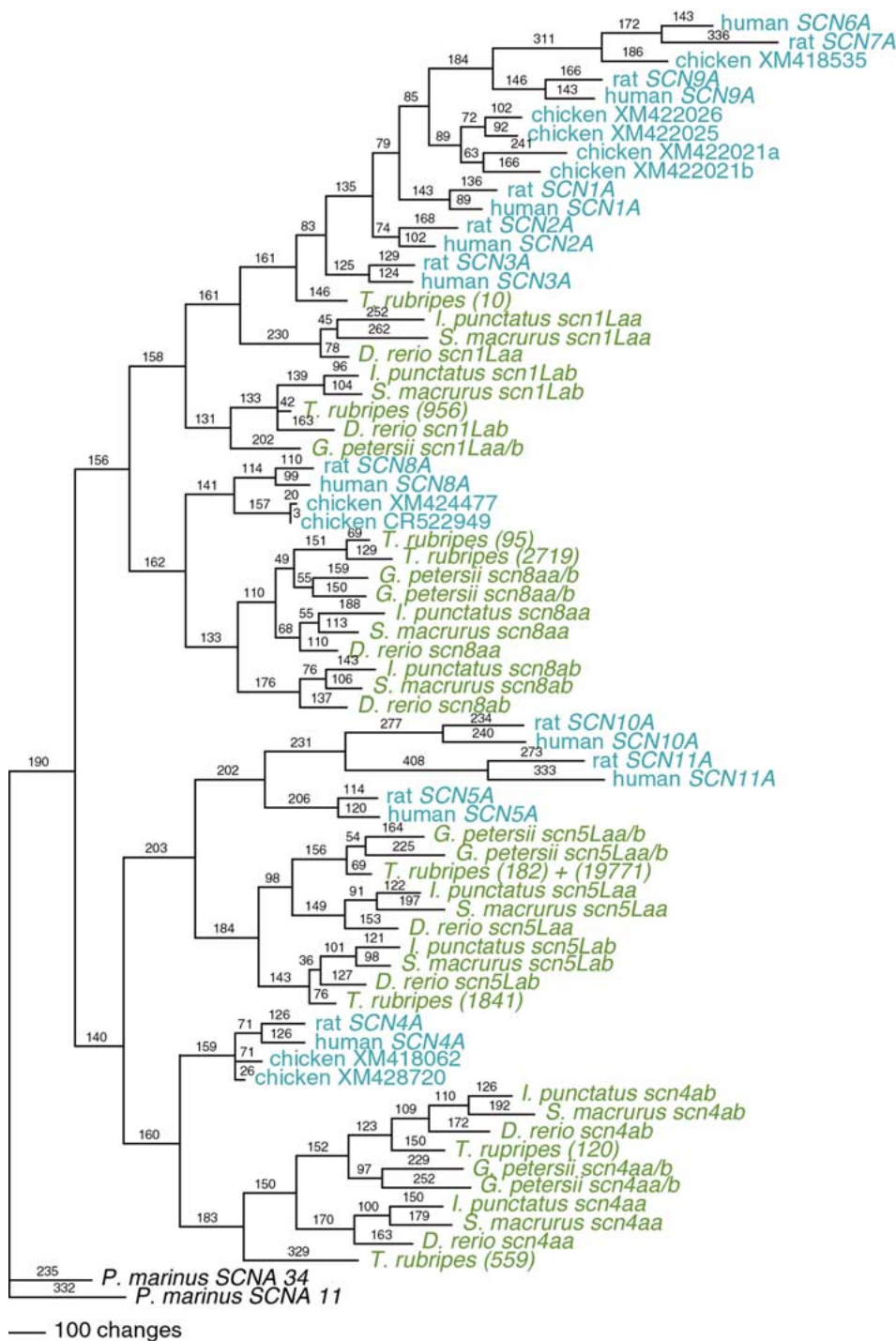


Fig. 4. Bayesian tree with branch lengths. Numbers above branches indicate length. Tetrapod and teleost sequences appear in blue and green text, respectively. GenBank accession numbers appear for chicken sequences, as their nomenclature has not yet been established. Numbers in parentheses following *T. rubripes* entries indicate scaffolds from Assembly Release 3 (Mayfolds). See Fig. 3 and Materials and Methods for other GenBank accession numbers.

rooting the tree could account for the differences, we tested the fit of our topology and the other published topologies using lamprey sequences for rooting.

Compared to all of the alternatives we tested, our topology had the shortest tree length and the highest consistency and retention indexes (Table 3). On the basis of several criteria and tests, our topology for mammalian *SCNA* gene phylogeny was significantly better at explaining the molecular data than the topology of Goldin et al. (2000) and all three possible

resolutions of Piontkivska and Hughes (2003) (Table 3). Our tree was not significantly better at explaining the molecular data than the topologies of Plummer and Meisler (1999), Goldin (2002), and Lopreato et al. (2001) (Table 3).

Under the likelihood criterion, our tree had the best likelihood score. Kishino-Hasegawa (K-H) and Shimodaira-Hasegawa tests using RELL bootstraps with 1000 replicates found our topology to be significantly better at explaining the data than all three

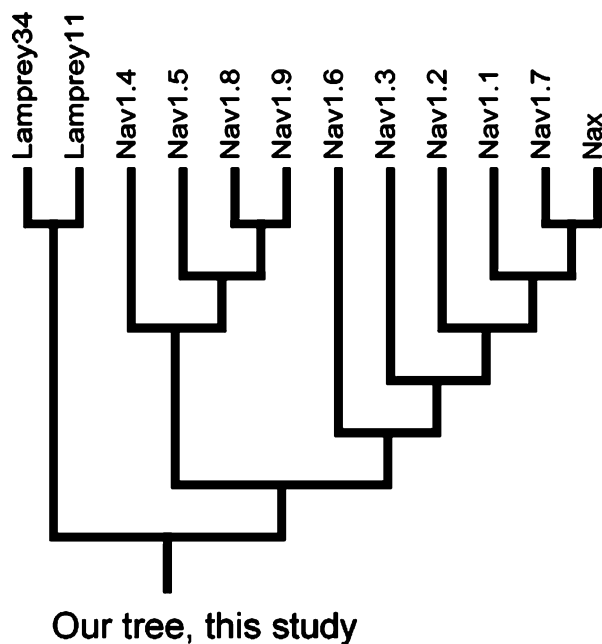


Fig. 5. Vertebrate Na_v1 phylogeny. Our phylogenetic tree for vertebrate Na_v1 proteins is most similar to that previously proposed by Lopreato et al. (2001) (Fig. 1).

possible resolutions of Piontkivska and Hughes (2003) (Table 3), and the K-H tests also found our topology to be significantly better than that of Goldin et al. (2000). However, the molecular data could not reject the topology of Plummer and Meisler (1999) and Goldin (2002) or the topology of Lopreato et al. (2001) (Table 3).

Nomenclature for Zebrafish scna Genes

We assigned names to the zebrafish scna genes on the basis of the existing mammalian nomenclature and guidelines established by the zebrafish community (Goldin et al. 2000; http://zfin.org/zf_info/nomen.html) (Table 2). When orthology between a pair of duplicated zebrafish scna genes and a single mammalian gene was obvious, assignment of names was straightforward (e.g., scn4aa and scn4ab are the co-orthologues of SCN4A ; Table 2). However, the four other scna genes were equally orthologous to more than one mammalian SCNA gene. For these cases, we adopted the zebrafish nomenclature convention and used the designation “like,” abbreviated by the letter “L,” to denote similarity but not orthology (http://zfin.org/zf_info/nomen.html). Further, the numerical identifier in zebrafish ($\text{scn}\#a$) assumed the lowest number in the mammalian group. For example, the zebrafish co-orthologues of the mammalian group containing SCN1A , SCN2A , SCN3A , and SCN9A are designated scn1Laa and scn1Lab . Previously published teleost genes have used an alternative nomenclature that we revise here (Table 2).

D. rerio scna Genes Map to Different Chromosomal Locations and Show Linkage with hox Clusters

It is thought that the four ancestral SCNA genes were each linked to a HOX cluster (Amores et al 1998; Plummer and Meisler 1999). If large-scale genome-wide, rather than select tandem, duplication expanded the teleost SCNA family, zebrafish scna genes would be expected (1) to map to locations not near each other and (2) to show linkage to a HOX cluster. We took advantage of the genomic databases and mapping strategies available for *D. rerio* to test these predictions.

The eight zebrafish scna genes mapped to seven different chromosomes, (scn1Laa , 9; scn1Lab , 6; scn4aa , 3; scn4ab , 12; scn5Laa , 2; scn5Lab , 24; scn8aa , 23; and scn8ab , 6; Fig. 6). Only two genes, scn1Lab and scn8ab , mapped to the same chromosome (6; Fig. 6). However, scn1Lab and scn8ab mapped to locations that are ~ 3636 Mb apart on chromosome 6. These results indicate that a mechanism involving tandem duplications is an unlikely basis for expansion of the SCNA gene family in teleosts.

The mapping results were also consistent with the second prediction. All of the eight zebrafish scna genes mapped to locations that were syntenically associated with a hox gene or genes that belong to a HOX cluster (Fig. 6). For example, although chromosome 6 did not contain a hox gene per se, some of the genes normally associated with a HOX cluster (e.g., engrailed [*en*], *dlx*) mapped to chromosome 6. Moreover, the members of a scna gene pair (e.g., scn1Laa and scn1Lab) each mapped near a member of the same duplicated hox gene pair (e.g., hoxaa and hoxab). These results support the hypothesis that a large genome-wide duplication of four ancestral genes was the basis for the expansion of the teleost SCNA gene family.

Exon Organization of scna Genes

We examined the exon organization of regions coding for interdomain loop regions of zebrafish scna genes to test predictions of genome-wide duplication as a mechanism for expansion of the teleost SCNA gene family. First, we focused on the interdomain loop region between transmembrane domains I and II (loop I–II). In mammals, SCN4A has two exons coding for loop I–II while genes in the three other SCNA groups have four (Dib-Hajj et al. 1999b; George et al. 1993; Plummer et al. 1998; Souslova et al. 1997; Wang et al. 1996). Similarly to mammals, one zebrafish gene, scn4aa , has two exons, E9 and E10 (Table 3). Interestingly, scn4ab has three exons in this region (E9, E10A, E10B). Thus, neither scn4aa nor scn4ab has four exons coding for the loop I–II

Table 3. Results of parsimony- and likelihood-based tree comparison tests

Tree comparisons: parsimony						
Topology ^a	Length (bp)	CI	RI	K-H	Wilcoxon	Winning-sites
1	3345	0.607	0.402	Best	Best	Best
2	3372	0.601	0.398	$p = 0.0521$	$p = 0.0523$	$p = 0.0575$
3	3389	0.599	0.382	$p = 0.0035^b$	$p = 0.0036^b$	$p = 0.0061^b$
4	3348	0.606	0.401	$p = 0.7099$	$p = 0.7098$	$p = 0.8041$
5	3493	0.581	0.335	$p < 0.0001^b$	$p < 0.0001^b$	$p < 0.0001^b$
6	3437	0.590	0.361	$p < 0.0001^b$	$p < 0.0001^b$	$p < 0.0001^b$
7	3491	0.581	0.336	$p < 0.0001^b$	$p < 0.0001^b$	$p < 0.0001^b$

Tree comparisons: likelihood			
Topology ^a	-lnL	2-tailed K-H with RELL	S-H with RELL
1	15828.40116	Best	Best
2	15862.58517	$p = 0.124$	0.146
3	15890.51693	$p = 0.001^b$	0.352
4	15836.75445	$p = 0.533$	0.465
5	16184.90340	$p < 0.001^b$	$p < 0.001^b$
6	16024.20430	$p < 0.001^b$	$p < 0.001^b$
7	16184.22587	$p < 0.001^b$	$p < 0.001^b$

Note. K-H, Kishino-Hasegawa; S-H, Shimodaira-Hasegawa.

^aTopology: 1, this study; 2, Plummer and Meisler (1999), Goldin (2002); 3, Goldin et al. (2000); 4, Lopreato et al. (2001); 5, Piontkivska and Hughes (2003), v1; 6, Piontkivska and Hughes (2003), v2; 7, Piontkivska and Hughes (2003), v3. Topology of Plummer and Meisler (1999), No. 2, is congruent with that of Goldin (2002), No. 2. Three versions of the tree of Piontkivska and Hughes (2003), Nos. 5–7, were tested, to account for all possible resolutions of a three-way polytomy.

^bSignificant at $p < 0.05$.

region (Table 3), similar to the gene-specific exon organization of mammalian *SCN4A*.

We also examined the regions coding for loop II–III, because it also has variable exon organization in mammals (Dib-Hajj et al. 1999b; George et al. 1993; Plummer et al. 1998; Souslova et al. 1997; Wang et al. 1996). For loop II–III, *SCN5A*, *SCN10A*, and *SCN11A* have three exons (E14, E15A, E15B), while other *SCNA* genes typically have only two (E14 and E15 only [Dib-Hajj et al. 1999b; George et al. 1993; Plummer et al. 1998; Souslova et al. 1997; Wang et al. 1996; Plummer and Meisler 1999]). Because the genomic databases were not sufficiently annotated in the relevant regions, we performed RT-PCR analyses. The sizes and sequences of the PCR bands that we cloned indicated that there were only two exons coding for loop II–III in zebrafish *scn5Laa* and *scn5Lab* (Table 3), unlike their mammalian co-orthologues (*SCN5A*, *SCN10A*, and *SCN11A*).

Zebrafish *scna* Genes Displayed Unique Expression Patterns

RT-PCR analyses revealed that zebrafish *scn1Laa* and *scn1Lab* were expressed in the central nervous system (brain, spinal cord, and eye) but not in skeletal or cardiac muscle. *Scn8aa* and *scn8ab* were also predominantly expressed in neural tissues (brain, spinal cord, and eye; Fig. 7). In contrast, *scn4aa*, *scn4ab*,

scn5Laa, and *scn5Lab* were expressed in a diverse range of excitable tissues (Fig. 7). For example, *scn4aa* and *scn4ab* mRNAs were detected in skeletal muscle, brain, spinal cord, and eye. In addition, *scn4ab* was expressed in cardiac muscle (Fig. 7). In sum, four zebrafish *scna* genes (*scn1Laa*, *scn1Lab*, *scn8aa*, and *scn8ab*) displayed predominantly neural expression patterns. In contrast, *scn4aa*, *scn4ab*, *scn5Laa*, and *scn5Lab* transcripts were present in both neural and muscle tissues.

Discussion

Evolution of the *SCNA* Gene Family in Vertebrates

Previous work indicated that tandem duplications of two of the four ancestral *SCNA* genes resulted in 10 *SCNA* genes in mammals, with 9 coding for Na_v1 channels (Plummer and Meisler 1999). However, how the *SCNA* gene family evolved in other vertebrates had not been resolved. In 2001, Lopreato et al. observed that the phylogenetic relationships emerging for the few previously identified teleost *SCNA* genes were not consistent with tandem duplication of selected ancestors and suggested that genome-wide duplication was perhaps involved.

Our data provide independent lines of evidence supporting the hypothesis that a genome-wide duplication event played a key role in evolution of the

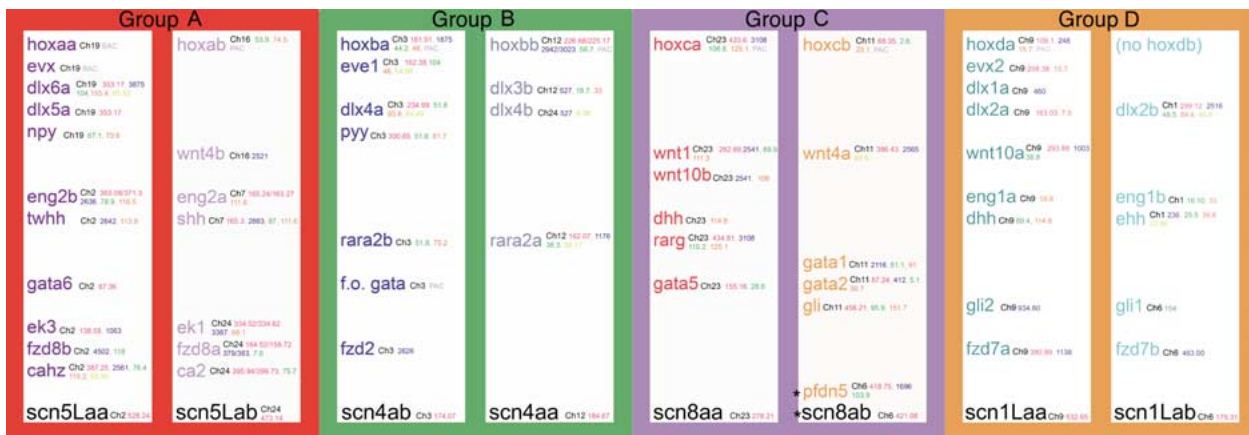


Fig. 6. Each zebrafish *scna* gene mapped to a chromosomal segment associated with a *hox* gene cluster. Radiation hybrid mapping using the LN54 panel revealed that zebrafish *scna* genes (in black) mapped to chromosomes containing HOX clusters. Depicted are paralogous chromosomal segments containing the four sets of duplicated HOX clusters, indicated by groups A–D. The chromosomal location for each gene is indicated, color-coded by mapping panel: LN54 (cR; red), T51 (cR; blue), HS (cM; green), MOP (icM;

orange), and GAT (cM; yellow) (www.zfin.org). In many cases, genes once thought to be found in continuous chromosomal regions have been broken up, and the genes are now found on multiple chromosomes, as has also occurred in humans (Plummer and Meisler 1999). Further, the chromosomal order of the genes shown here has been relaxed to show synteny, as intrachromosomal shuffling has altered the order of many genes. This figure was adapted from Amores et al. (1998).

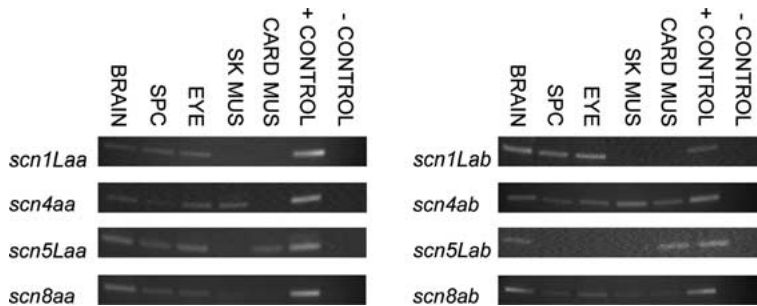


Fig. 7. RT-PCR revealed novel expression patterns for *scna* genes in electrically excitable tissues of the adult zebrafish. RT-PCR was performed with primers specific for each *scna* gene using RNA isolated from adult tissues. Genomic DNA was used in positive control PCR reactions to assess primer efficacy. Negative controls consisted of RT reactions that lacked the enzyme to detect possible amplification of genomic DNA rather than expressed mRNA.

teleost *SCNA* gene family. First, we identified *SCNA* genes in several teleost species and determined their phylogenetic relationships (Figs. 3 and 4). The results clearly indicate that teleost *SCNA* genes comprise four sets of duplicated genes. Second, the phylogenetic analyses also support genome-wide duplication in teleosts as the basis of expansion of the *SCNA* gene family. Third, we mapped *D. rerio* *scna* genes and examined their relationship to closely linked genes whose map positions were available on public databases (Fig. 6). The results revealed that the *SCNA* genes do not cluster near each other on a single chromosome, as expected if tandem duplications had occurred. Moreover, each member of a *scna* duplicated pair (e.g., *scna4aa* and *scna4ab*) is chromosomally linked to other duplicated genes (e.g., *hoxba*, *hoxbb*). Further, analysis of genomic structure of the domain I–II loop of the zebrafish genes supports identification of two *SCNA4A* co-orthologues (Table 4).

The hypothesis that a genome-wide duplication event, unique to teleosts, shaped the evolution of the *SCNA* gene family is consistent with the fact that

several developmental genes are duplicated in zebrafish (Amores et al. 1998; Ekker et al. 1995; Holland and Williams 1990; Stock et al. 1996; Vandepoele et al. 2004). Interestingly, for many developmental genes, genome-wide duplication would predict more genes than are present. For example, whole-genome duplication predicts eight *hox* genes, but only seven are present in zebrafish (Amores et al. 1998), suggesting loss of one after the genome-wide duplication event. For the seven duplicated *hox* genes that are present, studies of expression suggest that functional roles have been partitioned among duplicates (Jozefowicz et al. 2003; Prince 2002). In contrast to developmental genes, our data indicate that all *scna* duplicates have been retained in *D. rerio*, *T. rubripes*, *I. punctatus*, and *S. macrurus*.

The results for *G. petersii* sequences were unique in that this species appears to have orthologues for only one of the two teleost genes from each of the four monophyletic groups, with independent gene (or genome) duplications in *G. petersii* bringing the total number of genes up to seven or eight. It is known that osteoglossomorph fish did not diverge from other

Table 4. Exon organization of two cytoplasmic loops of zebrafish *scna* genes

Gene	Loop I-II		Loop II-III	
	Exons	mRNA (bp)	Exons	mRNA (bp)
<i>scn1Laa</i>	E9, E10A, E10B, E10C	959	E14, E15	614
<i>scn1Lab</i>	E9, E10A, E10B, E10C	921	E14, E15	683
<i>scn4aa</i>	E9, E10A	485	E14, E15	557
<i>scn4ab</i>	E9, E10A, E10B	419	E14, E15	527
<i>scn5Laa</i>	E9, E10A, E10B, E10C	852	E14, E15	581
<i>scn5Lab</i>	E9, E10A, E10B, E10C	848	E14, E15	638
<i>scn8aa</i>	E9, E10A, E10B, E10C	912	E14, E15	647
<i>scn8ab</i>	E9, E10A, E10B, E10C	921	E14, E15	653

teleosts before the third round of genome duplication (Crow et al. 2004; Hoegg et al. 2004; de Souza et al. 2005). The different results for *G. petersii* may be the result of a number of factors, either real (e.g., gene conversion) or artificial (e.g., long-branch attraction). However, they do not affect our overall conclusions about gene orthology and different duplication histories in tetrapods and teleosts.

Our parsimony- and likelihood-based topology tests using an extended dataset showed that our phylogenetic estimate is significantly better than that of previous analyses (Table 3). Our results differ most from those of Piontkivska and Hughes (2003) (Fig. 1). Interestingly, the tree constructed by Piontkivska and Hughes (2003) nests one *SCNA* gene (*SCN8A*) within a group of genes that reside in a cluster on another chromosome (*SCN1A*, *SCN2A*, *SCN3A*, *SCN9A*). Their tree also contains a phylogenetic polytomy with the [*SCN1A*, *SCN2A*, *SCN3A*, *SCN9A*] group and a gene (*SCN4A*) on yet another chromosome. In two of the three possible resolutions of the polytomy, *SCN4A* is also nested within the group of five genes residing on a different chromosome. All of the other phylogenies in Fig. 1 are fully resolved trees that are at least consistent with the chromosomal location of the genes.

The two early genome duplications in vertebrates (2R hypothesis [Lundin 1993; Meyer and Schartl 1999; Sidow 1996]) are believed to have occurred close to (one before, one after) the divergence of lampreys and hagfish. Importantly, rerooting the topology with the two lamprey genes as sister to one or the other of the two largest clusters does not change our conclusions of Teleostomei gene orthology or gene duplication mechanisms. Based on our findings, it appears that one lamprey *SCNA* gene may have been lost, followed by an independent duplication of the remaining gene, thus giving the sister relationship of the two genes we found. This possibility is consistent with the facts that (1) *SCNA* genes are linked to the HOX clusters (Plummer and Meisler 1999), and (2) lampreys are believed to have under-

gone independent HOX cluster duplications after their divergence from other vertebrates (Fried et al. 2003; Stadler et al. 2004). However, due to the fact that *SCNA* genes are closely affiliated with the HOX clusters, our results do not provide independent support for the 2R hypothesis.

SCNA Gene Expression in Teleosts and Mammals

Different evolutionary histories in gene families often result in orthologous genes that have divergent functions. We found that different genomic mechanisms increased the size of the *SCNA* gene family in mammals and teleosts (tandem duplication in mammals versus whole genome duplication in zebrafish). Our analysis of *scna* expression pattern (Fig. 7), however, raises the possibility that *SCNA* function may have been conserved despite different evolutionary mechanisms of the two vertebrate lineages.

For example, *scn8aa* and *scn8ab* are orthologous to the mammalian *SCN8A* gene. In the adult, both zebrafish genes are predominantly expressed in neural tissue (brain, spinal cord, and eye), similar to the expression pattern of *SCN8A* in mammals (Krzemien et al. 2000; Schaller et al. 1995; Tzoumaka et al. 2000). Interestingly our RT-PCR analyses also reveal expression in skeletal and cardiac muscle, as recently reported for mammalian *SCN8A* in cardiac tissue (Dhar Malhotra et al. 2001; Maier et al. 2002). Similarly, *scn4aa* and *scn4ab* are orthologous to mammalian *SCN4A*, a gene that is predominantly expressed in skeletal muscle (Trimmer et al. 1989). In zebrafish, we detected abundant expression of *scn4aa* and *scn4ab* in muscle (Fig. 7).

Four zebrafish *scna* duplicates were orthologous to more than one mammalian gene. For example, the mammalian genes *SCN1A*, *SCN2A*, *SCN3A*, and *SCN9A* are evolutionarily related to zebrafish *scn1Laa* and *scn1Lab*. Both the mammalian and the zebrafish genes in this group display expression patterns that are predominantly neuronal. Similarly, *scn5aa* and *scn5Lab* are orthologous to more than one mammalian gene (i.e., *SCN5A*, *SCN10A*, *SCN11A*). While *SCN5A*, *SCN10A*, and *SCN11A* group together phylogenetically, *SCN5A* shows low predicted amino acid identity to *SCN10A* (61.0%) and *SCN11A* (47.3%), suggesting divergent functions. Consistent with this notion, *SCN5A* is primarily expressed in cardiac tissue, while *SCN10A* and *SCN11A* transcripts are found in peripheral nociceptive sensory neurons (Akopian et al. 1999; Dib-Hajj et al. 1999a; Amaya et al. 2000). *SCN5A* expression also occurs in central nervous system regions associated with the limbic system (Donahue et al. 2000; Hartmann et al. 1999). In zebrafish, both *scn5Laa* and *scn5Lab* are expressed in adult cardiac tissue as well as in neurons, similar to *SCN5A*

(Fig. 7). However, neither *scn5Laa* nor *scn5Lab* is detected in sensory neurons during the first 5 days of development, the embryonic period and tissue type corresponding to the earliest expression of *SCN10A* and *SCN11A* (Novak et al. 2006). Thus, some of the functions noted for the *SCN5A*, *SCN10A*, and *SCN11A* group in mammals do not appear to be present in zebrafish (see also Sneddon et al. 2003).

Interestingly, we also detected expression of *scn4ab*, *scn5Laa*, and *scn5Lab* in tissues in which expression of their mammalian orthologues has not been observed (Fig. 7). Zebrafish *scn4ab* products were amplified from all tissues examined including brain, spinal cord, and eye, while *SCN4A* in mammals has been reported only in adult skeletal muscle and heart (Zimmer et al. 2002). Expression of *scn4aa* was also detected in all nervous tissues and skeletal muscle but not cardiac tissue (Fig. 7). Both *scn5Laa* and *scn5Lab* were also expressed in nervous and cardiac tissues. In mammals, *SCN5A* is expressed in cardiac muscle and limited regions of the brain (Hartmann et al. 1999; Rogart et al. 1989), while *SCN10A* and *SCN11A* are expressed in peripheral nociceptive sensory neurons (Akopian et al. 1996; Dib-Hajj et al. 1999a; Sangameswaran et al. 1996). However, mammalian expression of *SCN5A*, *SCN10A*, or *SCN11A* has not been reported in spinal cord or eye. Thus, genes that are primarily expressed in muscle types in mammals (*SCN4A* and *SCN5A*) may have different roles in the zebrafish.

Consideration of *SCNA* expression and voltage-gated sodium channels in invertebrate and chordate species provides an interesting perspective for our results (Plummer and Meisler 1999). Invertebrates such as insects and crustaceans have sodium-dependent action potentials in neurons but not muscle (Fatt and Ginsborg 1958; Suzuki and Kano 1977). In contrast, protochordates such as amphioxus have sodium-dependent actions potentials in both neurons and muscle (Hagiwara and Kidokoro 1971; Holland and Williams 1990). Despite these significant physiological differences regarding muscle electrical excitability, protochordates are believed to have had the same number of *SCNA* genes as do invertebrates. The protochordate may have evolved the ability to express a neural gene in muscle (Hagiwara and Kidokoro 1971; Holland and Williams 1990; Plummer and Meisler 1999). If so, the ancestral vertebrate *SCNA* genes were expressed in both neurons and muscle, and the zebrafish *scn4ab*, *scn5Laa*, and *scn5Lab* expression patterns may reflect this evolutionary history.

Concluding Remarks

Our results indicate that the final numbers of *SCNA* genes encoding Na_v1 proteins in mammals and tele-

osts are similar (eight versus nine, respectively). In contrast, invertebrate *SCNA* gene families consist of only one or two members (for review, see Goldin 2002). It is possible that the functional complexity of the diverse excitable tissues that have evolved in vertebrates requires multiple *SCNA* genes. Notably, the neural crest, a unique vertebrate specialization, gives rise to the peripheral nervous system where several *SCNA* genes display restricted expression. Further, in invertebrates, *SCNA* genes are expressed only in the nervous system, whereas they are additionally expressed in muscle and cardiac tissue in vertebrates. Therefore, the diversity of electrically excitable tissue types in vertebrates may have enhanced selection for multiple, yet a similar number of, *SCNA* genes despite different evolutionary histories.

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