Calcium-Activated Potassium (BK) Channels Are Encoded by Duplicate slo1 Genes in Teleost Fishes

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Calcium-activated, large conductance potassium (BK) channels in tetrapods are encoded by a single slo1 gene, which undergoes extensive alternative splicing. Alternative splicing generates a high level of functional diversity in BK channels that contributes to the wide range of frequencies electrically tuned by the inner ear hair cells of many tetrapods. To date, the role of BK channels in hearing among teleost fishes has not been investigated at the molecular level, although teleosts account for approximately half of all extant vertebrate species. We identified slo1 genes in teleost and nonteleost fishes using polymerase chain reaction and genetic sequence databases. In contrast to tetrapods, all teleosts examined were found to express duplicate slo1 genes in the central nervous system, whereas nonteleosts that diverged prior to the teleost whole-genome duplication event express a single slo1 gene. Phylogenetic analyses further revealed that whereas other slo1 duplicates were the result of a single duplication event, an independent duplication occurred in a basal teleost (Anguilla rostrata) following the slo1 duplication in teleosts. A third, independent slo1 duplication (autotetraploidization) occurred in salmonids. Comparison of teleost slo1 genomic sequences to their tetrapod orthologue revealed a reduced number of alternative splice sites in both slo1 co-orthologues. For the teleost Porichthys notatus, a focal study species that vocalizes with maximal spectral energy in the range electrically tuned by BK channels in the inner ear, peripheral tissues show the expression of either one (e.g., vocal muscle) or both (e.g., inner ear) slo1 paralogues with important implications for both auditory and vocal physiology. Additional loss of expression of one slo1 paralogue in nonneural tissues in P. notatus suggests that slo1 duplicates were retained via subfunctionalization. Together, the results predict that teleost fish achieve a diversity of BK channel subfunction via gene duplication, rather than increased alternative splicing as witnessed for the tetrapod and invertebrate orthologue.

Introduction

Calcium-activated, large conductance potassium (BK) channels are responsible for the currents that contribute to the electrical resonance defining the frequency tuning of hair cells in nonmammalian vertebrates including fish, amphibians, reptiles, and birds (Fettiplace and Fuchs 1999). The α-subunit of BK channels is encoded by a single Slo1 (KCNMA1) gene in tetrapods (Salkoff et al. 2006). Alternative splicing of Slo1, along with posttranslational modification and association with β-subunits, accounts for variation in BK channel properties that shape variable electrical resonance between hair cells in nonmammals (Jones et al. 1999; Ramanathan et al. 1999). Although frequency tuning of the peripheral auditory system in mammals is achieved through mechanical as opposed to electrical mechanisms, BK channels are necessary for normal function of the cochlea, the primary end organ of hearing in mammals. Knockouts of Slo1 in mice result in progressive hearing loss (Rutger et al. 2004) and impaired temporal precision of electrical signals in auditory afferents (Oliver et al. 2006).

BK channel currents have been identified as one of the major outward currents in the hair cells of teleost fish (Sugihara and Furukawa 1989; Steinacker and Romero 1991, 1992). Beisel et al. (2007) reported the genomic organization of a slo1 gene in the zebrafish Danio rerio from sequences deposited in electronic databases, whereas Lionetto et al. (2008) examined the expression of BK channel transcripts in the intestinal epithelium of the European eel Anguilla anguilla. The current study, however, is the first to examine the expression of genes encoding BK channels in the nervous system of this group of vertebrates which accounts for nearly half of all living vertebrate species (Nelson 2006). We identified two duplicate slo1 genes encoding BK channels in several teleosts and then conducted phylogenetic analyses to determine the timing of the duplications of slo1 genes in teleosts. Next, we examined the genomic sequences of teleost slo1 genes to determine whether each slo1 co-orthologue has the same complement of splice sites as the orthologous gene in tetrapods and calculated the relative rates of evolution between slo1 paralogues. Finally, we investigated the tissue-specific expression patterns of slo1a and slo1b in the plainfin midshipman fish (Porichthys notatus) that produce vocalizations with spectral content mainly below 1 kHz. Our initial goal was to identify the genes encoding BK channels in the peripheral auditory system of midshipman because the saccule, the main auditory division of the inner ear, and the eighth nerve that innervates the saccule (McKibben and Bass 1999; Sisneros and Bass 2003; Sisneros et al. 2004; Sisneros 2007) are most sensitive to 60–400 Hz, falling within the range of frequencies electrically tuned for by BK channels (Fettiplace and Fuchs 1999). However, as reported below, the discovery of two slo1 genes in midshipman led to the current study that included distantly related fishes to investigate how widespread this duplication event might be and when it might have occurred in the evolutionary history of fishes.

Materials and Methods

Taxon Sampling

There are two major lineages of living fishes, the actinopterygians and sarcopterygians (see Nelson 2006). Actinopterygians include the highly speciose teleosts and several smaller groups of nonteleosts, whereas sarcopterygians include lungfish, coelacanths, and tetrapods. We sampled teleosts commonly used in the study of developmental genetics (the zebrafish D. rerio [Nicolson 2005] and the
stickback *Gasterosteus aculeatus* [Peichsel 2005]), social behavior (the cichlid *Neolamprologus pulcher* [Fitzpatrick et al. 2008]), potassium channels (the rainbow trout *Oncorhynchus mykiss* [Henne and Jeserich 2004]), and auditory neurophysiology (*P. notatus* and the Gulf toadfish *Opsanus beta* [Bass and McKibben 2003]). The locally available American eel *Anguilla rostrata* was substituted for the European eel *A. anguilla* for which a partial *slo1* sequence has been reported (Lionetto et al. 2008). A chondrichthyan (the little skate *Leucoraja erinacea*) as well as several non-teleost actinopterygians (the bichir *Polypterus senegalus*, the longnose gar *Lepisosteus osseus*, and the bowfin *Amia calva*) were selected to determine whether duplication of the *slo1* gene occurred prior to the teleost whole-genome duplication event. The Ensembl genome browser and the National Center for Biotechnology Information databases were used to retrieve *slo1* sequences from tetrapods in which the gene has been studied extensively (the human *Homo sapiens* ENSG00000156113, the mouse *Mus musculus* ENSMUSG000000663142, the chicken *Gallus gallus* ENSGALG00000004980), and the turtle *Trachemys scripta* AF036625) as well as teleosts for which complete or nearly complete *slo1* entries were available (the stickleback *G. aculeatus*, the Japanese rice fish *Oryzias latipes*, the tiger puffer fish *Takifugu rubripes*, and the green spotted puffer fish *Tetraodon nigroviridis*; for Ensembl gene entry numbers, see table 2). A recently published partial sequence of a *slo1* transcript from the intestinal epithelium of *A. anguilla* was also included in analyses (Lionetto et al. 2008).

**slo1 Cloning and Sequencing**

Whole central nervous system (CNS, spinal cord, and/or brain) RNA was extracted from a single individual for each species except for *N. pulcher (n = 2)*, *G. aculeatus (n = 3)*, *P. notatus (n = 3)*, and *D. rerio (n = 11)* for which tissue was pooled from multiple animals and *T. nigroviridis* and *T. rubripes* for which only Ensembl sequence data were used. The Institutional Animal Care and Use Committee at Cornell University approved all procedures in this study. The samples from *P. notatus* and *O. beta* contained the anterior spinal cord because the vocal motor circuit extends from the caudal hindbrain into the anterior spinal cord in both species (Bass et al. 1994). Whole CNS RNA was isolated using the Trizol method (Invitrogen, Carlsbad, CA), treated with DNase I (Invitrogen) to remove genomic DNA contamination, and reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen) following the manufacturer’s protocols. Polymerase chain reaction (PCR) on cDNA from whole CNS was conducted using degenerate primers (forward primer: 5’-GGG AAG TTG GCC GGT CAG GAG CCN TGG GAR AAY TTY CAR AAY-3’, reverse primer: 5’-GGG AAG CTT NGC RTC RTG NCC YTC YTT CTA CTT CCA-3’) designed to the highly conserved pore region based on an alignment of published tetrapod *slo1* sequences corresponding to *H. sapiens* nucleotides 1177–1794. Tetrapod *slo1* sequences were used for primer design because at the time of primer design the teleost *slo1* sequences from Ensembl listed in the previous section were not yet available. PCR amplification was performed with an initial denaturation step of 94 °C for 4 min followed by 5 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 2 min; an additional 30 cycles of 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 2 min; and a final extension at 72 °C for 10 min resulting in a 690-bp product. Because the alternatively spliced exons (10 and 11) spanned by these primers were mutually exclusive in the transcript and were of the same length, this 690-bp product could have contained multiple alternatively spliced transcripts. Primers contained restriction enzyme sites at the 5’ end in order to facilitate ligation into Bluescript KS(+) plasmid. The resulting products were subcloned into ultracompotent DH5α cells (*Drosophila* Genomics Resource Center, Indiana University—18 November 2005), plated, grown in liquid culture, mini-prepped (Qiagen, Valencia, CA), and sequenced by the Cornell University Life Sciences Core Laboratory Center using universal primers. Sequences were analyzed and aligned using the ClustalW algorithm in Lasergene (DNASTAR, Inc., Madison, WI).

**Genomic Southern Blots**

Genomic Southern blots were conducted to confirm that the two *slo1* transcripts obtained from *P. notatus* were the products of two genes. The initial *slo1* sequences obtained as described above were expanded upon using an additional degenerate reverse primer (5’-GGG AAG CTT NNG RCA CCA RETG RAA CAT NCC NGT-3’) based on the same tetrapod alignment used to design the earlier primer pair. This reverse primer was paired with the forward primer used earlier using the same PCR conditions, and the resulting PCR product was subcloned and sequenced as described above to obtain *P. notatus slo1a* and *slo1b* sequences corresponding to *H. sapiens* nucleotides 1177–2475. To prepare specific probes that spanned more divergent sequences than the initial products sequenced that encode the highly conserved pore region of the BK channel, we used the expanded sequence data to design two *P. notatus* species-specific primer pairs corresponding to nucleotides 1886–2248 (exons 16–21) in the *H. sapiens* sequence (forward primer 1: 5’-GGG GAA TTC CAT GTT GGC CAA CCT GTT CTC CAT GAG G-3’, reverse primer 1: 5’-GGG GGA TCC GGT CAG TGA TGT CAT CAT GAC ACG CTT TAC-3’, forward primer 2: 5’-GGG GAA TTC TAT GCT GGC CAA GTT TTT CTC CAT GAC ACG CTT TAC-3’, and reverse primer 2: 5’-GGG GGA TCC GTA CAG TGA TGT CAT CAT GAC AAG CTT TAC-3’). Single exons of *slo1* were not sufficiently long to serve as effective templates for random primed probes (templates less than 300 bp work poorly) so we were forced to use templates that spanned multiple exons. PCR was performed on *P. notatus* whole CNS cDNA. PCR amplification was performed with an initial denaturation step of 94 °C for 4 min followed by 5 cycles of 94 °C for 45 s, 54.4 °C for 45 s, and 72 °C for 1 min; an additional 30 cycles of 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. The resulting ~350-bp products were subcloned and sequenced as above.

Genomic DNA was extracted from liver of *P. notatus* using the Puregene DNA Purification Kit (Qiagen). In all,
10 µg aliquots of DNA were digested with several different restriction enzymes (BamHI, EcoRI, and HindIII), run on a 0.8% agarose gel along with the probe templates as a control for probe specificity, and blotted onto a nylon membrane. We selected restriction enzymes that cut relatively infrequently within the target sequence in order to increase the odds that our probes would recognize a single band for each gene. Probes were synthesized from both plasmids using the High Prime DNA labeling Kit (Roche, Indianapolis, IN) and [32P]tagged dCTP. The blot was then probed and washed following the methods of Church and Gilbert (1984), and the blot exposed to Kodak BioMax MS film with an intensifying screen.

Phylogenetic Analyses

As mentioned above, all nucleotide sequences (618 bp long excluding primer sequences) were aligned using the ClustalW algorithm in Lasergene (DNA*; supplementary fig. 1, Supplementary Material online). Where available, the full-length nucleotide sequence from the Ensembl database was used in the alignment. In general, there was high sequence identity across the taxa sampled with slo1 paralogues sharing the high sequence identity with the single slo gene in chondrichthyan, nonteleost actinopterygians, and tetrapods. Phylogeny was estimated using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) using the facilities of the Computational Biology Service Unit of Cornell University. A general time reversible (GTR) model with invariable sites and a gamma distribution for variable rate sites (GTR + I + G) was applied. Four Markov chains of 10,000,000 generations were run sampling every 100th tree. A burn-in of 25% was applied to remove all trees generated in the first quarter of the analysis. From the remaining trees, a majority rule consensus tree was generated along with the posterior probabilities. The analysis was replicated to confirm both the consensus tree and the posterior probabilities. Modeltest 3.7 (Posada and Crandall 1998) was used to confirm that the same model could be applied to both slo1 and slo2 by analyzing slo1 + slo2 and slo1 + slo2 as two separate data sets.

The resulting consensus tree was edited in Mesquite 2.5 (Maddison WP and Maddison DR 2008) to reflect tree topologies of alternative hypotheses explaining the duplication of slo1. These hypothetical trees were compared with the consensus tree in PAUP*4.0 (Swofford 2002) using a Shimodaira–Hasegawa (SH) likelihood test with 1,000 bootstrap replicates and full optimization to test alternative hypotheses.

Relative Evolutionary Rate Analysis

The Tajima (1993) relative rate test was conducted using MEGA4 (Tamura et al. 2007) for slo1 paralogues with L. erinacea as an outgroup for the predicted 206 amino acid long protein sequences corresponding to the 618 bp sequence obtained above. Additionally, the complete amino acid sequences for the four teleost duplicate pairs available from Ensembl were compared with H. sapiens SLO1 as an outgroup.

Alternative Splicing Analysis

Genomic sequences were retrieved from Ensembl as of July 2008. Ensembl combines information on species-specific protein sequences, protein sequences from closely related species, and cDNA and expressed sequence tag sequences to produce annotated genomic sequences. Because of a lack of species-specific as well as closely related species protein sequences, the majority of supporting evidence used in Ensembl’s construction of genomic sequences for teleost slo1 genes appears to have been based on tetrapod genomic sequences. Exon boundaries as identified in Ensembl were manually compared with exon boundaries in

Qualitative Expression of slo1a and slo1b in P. notatus Tissues

Primers were designed to hybridize to short segments of both P. notatus slo1a and slo1b with 100% nucleotide identity within the priming site to amplify all slo1 transcripts in the given cDNA sample, including any splice variants within the region defined by the primers (forward primer: 5’-TTC ATG TGC TTC TTC ATC CT-3’, reverse primer: 5’-GAA CAA GGC TTC CAG CTC AAG). These primers amplified a region of P. notatus slo1a and slo1b corresponding to nucleotides 1279–1545 in the H. sapiens sequence. PCR amplification was performed with an initial denaturation step of 94°C for 4 min followed by 35 cycles of 94°C for 45 s, 46°C for 45 s, and 72°C for 1.5 min and a final extension at 72°C for 10 min resulting in 266-bp product.

The 266-bp PCR products were gel extracted, and half the volume of each sample (150–350 ng DNA) was digested with 40 units of XhoI which digests slo1b into two pieces (116 and 150 bp) while leaving slo1a intact. Digests were carried out at 37°C for 2 h. The digested PCR product was run alongside the undigested volume containing either slo1a or slo1b to produce known samples of pure slo1a and pure slo1b. To confirm complete digestion of all slo1b without digestion of slo1a, 400 ng of pure slo1a and pure slo1b were digested and run on a gel as described above. Note that more DNA was added to each control digest than for the digests of PCR products (see above) to be certain that the digest conditions were sufficient to cut all slo1b in any of the samples digested. The experiment was replicated on cDNA from either a second individual or a different pooled sample in order to confirm the results were not due to individual variability of slo1 expression. For those tissues that appeared to express exclusively one paralogue (vocal and trunk muscle and gill), the uncut purified PCR product (266-bp band) was sequenced by the Cornell University Life Sciences Core Laboratory Center using the PCR primers in order to confirm the identity of the gel band.
M. musculus and additional tetrapod sequences as needed (Beisel et al. 2007) to ensure that possible exons in teleost sequences were not excluded from the Ensembl sequences for lack of sequence similarity with tetrapod genes. Where alternatively spliced exons present in tetrapods where not labeled in Ensembl, unlabeled exons were identified by translation of the genomic sequence between previously identified exons.

Results

Two slo1 Genes in Midshipman

Two distinct slo1 transcripts were first identified in the cDNA from CNS and the sensory epithelium of the auditory sacculus using PCR with degenerate primers (supplementary fig. 1, Supplementary Material online; for GenBank accession numbers, see table 1). Additional degenerate primers were used to expand upon the initial sequence obtained in order to gather additional sequence information from which to produce probes for genomic Southern blots which differed enough in sequence to prevent cross-reactivity between the two sequences. Genomic Southern blots (supplementary fig. 2, Supplementary Material online) confirmed that the two slo1 transcripts identified in P. notatus were the product of two genes. It was unlikely that one of the two very similar slo1 transcripts identified was a product of another member of the slo gene family because of the high level of sequence divergence between slo1 and slo2 and the lack of expression of slo3 in the brain of mammals (Salkoff et al. 2006).

The expression of slo1a and slo1b was examined in both neural and nonneural tissues from adult male P. notatus using reverse transcriptase–polymerase chain reaction. PCR revealed that both slo1a and slo1b appeared to be expressed in the CNS, auditory (saccular) epithelium, heart, and pituitary (fig. 2A). Only slo1a was expressed in both trunk and vocal muscle, whereas in the gill and intestine, only slo1b was expressed (fig. 2A). Control digests of pure slo1a and pure slo1b confirmed that the digest conditions were sufficient to completely digest all slo1b while leaving slo1a intact (fig. 2B). Together, the results suggested that slo1 expression patterns differed between skeletal (trunk and vocal), smooth (gill and intestine), and cardiac (heart) muscle types which express slo1a, slo1b, and both slo1 genes, respectively. Sequencing the PCR products from muscle and gill confirmed the presence of only slo1a in trunk and vocal muscle and only slo1b in gill. Sequencing also revealed differences in splicing between the paralogues within these peripheral tissues where slo1a transcripts contained exon 10, as was the case with all slo1 transcripts sequenced from the CNS of all teleosts examined, whereas slo1b transcripts contained exon 11 (for splicing pattern, see fig. 4). Because we did not examine slo1 expression in non-CNS tissues in other species, we do not know if the differences in expression of exon 10 versus exon 11 in peripheral tissues were unique to P. notatus. Exons 10 and 11 are mutually exclusive in the final vertebrate slo1 transcript (Beisel et al. 2007), with exon 10 present in the majority of published transcript sequences from tetrapods.

In sum, two slo1 genes were identified in the CNS and peripheral tissues of P. notatus with some peripheral tissues showing expression of only one paralogue, with paralogues differing in alternative splicing patterns of potential importance to channel physiology. Exon 11 encodes a protein that lacks one of two glutamic acid residues present in exon 10 which provide electrostatic forces responsible for the large K\(^+\) current through BK channels (Brelidze et al. 2003; Nimigean et al. 2003).

Two slo1 Genes in Teleosts

The surprising discovery of two slo1 genes raised the question of whether this duplication was unique to P. notatus and, if not, when the duplication event occurred. We used the Ensembl genome browser to first obtain slo1 sequences of other teleosts including G. aculeatus, O. latipes, T. nigroviridis, and T. rubripes, all of which possessed two slo1 genes (table 2). Duplicate slo1 genes are each located on separate chromosomes in G. aculeatus (chromosomes 5 and 6), O. latipes (chromosomes 15 and 19), and T. nigroviridis (chromosomes 2 and 17); comparable data are unavailable in the current genome assembly for T. rubripes. For those species for which the chromosome location of slo1a and slo1b is known, both genes are flanked by some of the same genes including annexin A11, zinc finger CCHC domain-containing protein 24, and zinc finger protein 503 (ZNF503). The conserved synteny between slo1 duplicates in these species suggests that slo1 duplication was the result of a teleost whole-genome duplication event (Amores et al. 1998; Taylor et al. 2001). To further test this hypothesis, we examined basal teleosts and outgroups to the teleost whole-genome duplication event.

The same degenerate primers used to obtain both slo1 gene transcripts from P. notatus were used in PCR on whole CNS cDNA from other teleosts (A. rostrata, D. rerio, O. mykiss, O. beta, G. aculeatus, and N. pulcher), nonteleost basal actinoperygians (P. senegalus, L. osseus, and A. calva), and a chondrichthyan (L. erinacea). For all species examined, PCR produced a single band of the predicted size. When PCR products were subcloned and sequenced (table 1), it was revealed that whereas basal actinoperygians and chondrichthyans expressed only a single slo1 transcript, all teleosts examined expressed two slo1 transcripts similar to the two slo1 genes identified above in both nucleotide (supplementary fig. 1, Supplementary Material online) and predicted protein sequence alignments (fig. 1). The latter included D. rerio, despite the observation that the Ensembl database contained only one incomplete slo1 entry (table 2). Unlike the above species, O. mykiss expressed four slo1 transcripts. Slo1 paralogues showed high NT and AA sequence identities (84.1% D. rerio–92.7% A. rostrata, 97.6% P. notatus–99% multiple species, respectively). Both NT and AA sequence identities between teleost slo1 duplicates and H. sapiens SLO1 were similar between paralogues, differing by only 1–2% (81.7% P. notatus slo1a–84.8 D. rerio, 97.1% many species–98.5 O. latipes, T. rubripes, respectively). Similarly high protein sequence identity was observed between the full-length protein...
sequences of teleost slo1a and slo1b in Ensembl (86.0–89.8%). Together, our data demonstrate the duplication of the slo1 gene and retention of both copies in teleosts. The teleosts we examined had high sequence identity between slo1 duplicates over their length, and they both share the same highly conserved pore region of the BK channel found in tetrapods.

Multiple slo1 Gene Duplication Events

The nucleotide sequences of slo1 genes from 19 species including 11 teleosts were aligned, and a phylogenetic analysis was conducted to determine the relationship between tetrapod slo1 genes and duplicate teleost slo1 genes. The entire sequence was used as compared with the 618-bp region acquired through PCR for those species for which the entire coding sequence was available through sequence databases. This may help explain some of the long-branch lengths in those taxa for which there were more characters included in the analysis. A monophyletic group of tetrapod/sarcopterygian slo1 genes was recovered as a sister to the monophyletic actinopterygian slo1 genes (fig. 3A). Note that only a single slo1 sequence appears for A. anguilla reported by Lionetto et al. (2008). As reported above, we identified two slo1 transcripts in the brain of the cogener A. rostrata. Placement of A. anguilla slo1 as a sister to

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Table 1
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<td>slo1a</td>
<td>FJ269025</td>
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<td></td>
<td>slo1b</td>
<td>FJ269032</td>
</tr>
</tbody>
</table>

Table 2
Ensembl Database Entries of Teleosts for Which slo1 Exons Are Present in Tetrapods but Are Absent or Truncated in Teleosts

<table>
<thead>
<tr>
<th>Organism</th>
<th>Gene</th>
<th>Ensembl Gene</th>
<th>Absent Exons (alternative splice sites) Based on Mouse Nomenclature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Danio rerio</td>
<td>slo1a</td>
<td>No Ensemble entry</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>slo1b</td>
<td>ENSDARG000000060237</td>
<td>4 (1)^a</td>
</tr>
<tr>
<td>Oryzias latipes</td>
<td>slo1a</td>
<td>ENSORLG00000006622</td>
<td>4 (1)</td>
</tr>
<tr>
<td></td>
<td>slo1b</td>
<td>ENSORLG00000008798</td>
<td>19 (3)^b 19 (3)</td>
</tr>
<tr>
<td>Gasterosteus aculeatus</td>
<td>slo1a</td>
<td>ENSGACG00000008829</td>
<td>4 (1)</td>
</tr>
<tr>
<td></td>
<td>slo1b</td>
<td>ENSGACG000000002248</td>
<td>19 (3) 29 (6)^c</td>
</tr>
<tr>
<td>Takifugu rubripes</td>
<td>slo1a</td>
<td>ENSTRUG00000003720</td>
<td>4 (1)</td>
</tr>
<tr>
<td></td>
<td>slo1b</td>
<td>ENSTRUG000000014048</td>
<td>19 (3)</td>
</tr>
<tr>
<td>Tetraodon nigroviridis</td>
<td>slo1a</td>
<td>ENSTNIG00000008628</td>
<td>4 (1)</td>
</tr>
<tr>
<td></td>
<td>slo1b</td>
<td>ENSTNIG00000013160</td>
<td>19 (3)</td>
</tr>
</tbody>
</table>

^a Ensembl entry ends with exon 17.
^b Conserved 5' SRKR amino acid sequence with potential species-specific 3' end.
^c Potential partial/truncated exon present.
A. rostrata slo1a suggests that either slo1b was lost in A. anguilla or this paralogue is not expressed in the intestinal epithelium in this animal (see Discussion).

The Bayesian estimate of phylogeny (fig. 3A) supported three duplication events within the teleost lineage, the first occurring within the anguilliforms, the true eels. A second duplication event, possibly of the whole teleost genome, occurred prior to the ostariophysans that include the zebrafish Danio rerio. A third round of duplication, likely autotetraploidization, occurred in salmonids, represented here by O. mykiss (this event is referred to as autotetraploidization instead of ‘whole-genome duplication’ because diploidization of the salmonid genome is apparently incomplete [Allendorf and Thorgaard 1984; Phillips et al. 2006]).

Resolving the polytomy of D. rerio slo1a as a sister clade to the other ostariophysan slo1a genes (fig. 3B, bold) resulted in a tree that was not significantly different from the Bayesian consensus tree (SH test, \( P = 0.106 \)), with a more favorable likelihood score. A second SH test between the Bayesian consensus and a tree based on the hypothesis of a single slo1 duplication event at the origin of teleosts followed by a second duplication event in salmonids resulted in a significant difference between the trees (\( P = 0.041 \)) with likelihood favoring the consensus tree. As a result, we conclude that the duplication of slo1 in anguilliforms is a third duplication event independent from those in other teleosts and the salmonid lineage. Thus, additional SH tests were conducted to determine
the timing of the anguilliform *slo1* duplication relative to teleost whole-genome duplication.

We tested the hypothesis that *A. rostrata slo1* paralogues were the result of an independent gene loss and subsequent duplication event in anguilliforms following the divergence of *slo1a* and *slo1b* in other teleosts. Several previous studies have provided evidence for independent gene loss in elopomorphs, occasionally followed by a secondary duplication (Hoegg et al. 2004; de Souza et al. 2005; Crow et al. 2006; Hurley et al. 2007). SH tests could reject neither the hypothesis that anguilliform *slo1* genes are associated with teleost whole-genome duplication followed by a secondary duplication of the remaining *slo1* gene in *A. rostrata* (fig. 3B), a pattern observed in other anguilliform genes and consistent with the proposed timing of the teleost whole-genome duplication event (see Discussion).

In sum, the phylogenetic analyses identified a single *slo1* duplication event at the origin of teleosts followed by an independent loss and secondary *slo1* duplication event in anguilliforms and a third independent *slo1* duplication in salmonids.

*slo1* Paralogues Have Evolved at Similar Rates

Tajima (1993) relative rate tests were conducted to determine if either *slo1* paralogue evolved at an accelerated rate following duplication. There were no significant differences in relative rate of evolution between any teleost *slo1* paralogues within the 206AA long sequence, corresponding to the pore and S6 domains, obtained for all species with the chondrichthyan *L. erinacea slo1* serving as an outgroup. Examination of the entire protein-coding sequences from the teleosts *G. aculeatus*, *O. latipes*, *T. rubripes*, and *T. nigroviridis* with Homo SLO1 as an outgroup also resulted in no significant differences in relative rates of evolution between teleost *slo1* duplicate pairs with sequence identities between 78.1% and 81.8% at the nucleotide level.

Teleost *slo1* Genes Contain Fewer Sites of Alternative Splicing Than Tetrapods

Rates of alternative splicing are inversely correlated with gene family size (Kopelman et al. 2005). Thus, we examined the duplicate *slo1* genes of teleosts for evidence of reduced alternative splicing relative to the single *slo1* gene in tetrapods. The genomic sequences of *slo1* genes available from Ensembl for five teleosts ( *D. rerio*, *O. latipes*, *G. aculeatus*, *T. rubripes*, and *T. nigroviridis*) were compared with the genomic sequence and splice sites of mouse (*M. musculus*) *Slc1a* using the mouse numbering system (Beisel et al. 2007). Note that our analysis of *slo1* genomic sequences in *D. rerio* was limited by the presence of only a single Ensembl entry that terminates at exon 17 (out of the total 35). Table 2 and figure 4 provide an overview of the analysis as well as a diagrammatic representation of alternative splicing in *slo1* genes.

All exons involved in alternative splicing at sites 2, 4, 5, 6, and 7 were found to be present in both duplicate *slo1* genes in the teleosts examined. In all the teleosts examined, we found evidence for loss of the exons at splice sites 1 and 3 in either both *slo1* co-orthologues (site 1) or *slo1b* alone (site 3). Splicing in both sites is characterized by the insertion of none, part, or all of a nonconstitutively expressed exon (see fig. 4).

Exon 4 (splice site 1) in mouse was absent from both *slo1* duplicates in all five teleosts examined, including *D. rerio*. This is consistent with the findings of Beisel et al. (2007), who reported difficulty in identifying exon 4 in *D. rerio*. Excepting the *D. rerio* sequence that is truncated at exon 17, the teleost sequences corresponding to exon 19 in mouse (splice site 3) were highly divergent 3' of the conserved initial serine-arginine-lysine-arginine amino acid segment in *slo1a*, whereas the entire exon was absent in *slo1b*. Beisel et al. (2007) noted that the 3' end of exon 19, which is truncated in some tetrapod splice variants, appears to be species specific; this region has apparently diverged even further in teleost *slo1a*. The absence of even the highly conserved SRKR domain in *slo1b* suggests that exon 19 was differentially lost between *slo1a* and *slo1b* following duplication.

Lastly, we note that the only difference in either exon or splice site number between the *slo1* paralogues in teleosts was found in *G. aculeatus* in which exon 29 (splice site 6) was severely truncated at the 3' end. This truncated exon may, however, be the result of sequencing or alignment errors that may be resolved in new genome databases or through targeted genomic sequencing.

Discussion

As is the case with many genes and gene families in teleosts, we report a duplication of the *slo1* gene encoding the pore-forming α-subunit of BK channels in all the species examined. Of the species in which we sequenced transcripts from cDNA, all expressed the full complement of *slo1* genes in the CNS, while all *slo1* paralogues possessed similar nucleotide and amino acid sequence identities with orthologous genes in tetrapods.

The partial *A. anguilla slo1* sequence obtained by Lionetto et al. (2008) overlaps with the region sequenced from the CNS in the present study but came from cDNA reverse transcribed from RNA extracted from the intestine, a tissue which expresses only a single *slo1* duplicate in *P. notatus* (fig. 2). The presence of two *slo1* genes in the closely related *A. rostrata* along with evidence of tissue-specific loss of expression of a *slo1* duplicate in *P. notatus* suggests that *A. anguilla* possesses two *slo1* genes, only one of which is expressed in intestine. The expression of four different *slo1* transcripts in the brain of *O. mykiss* is consistent with it and other salmonids having at least 13 *hox* clusters (Moghadam et al. 2005; Mungpakdee et al. 2008) as compared with seven in *D. rerio* (Amores et al. 1998). An autotetraploidization event in salmonids about 25–100 Mya (Allendorf and Thorgaard 1984).
accounts for the apparent second round of \textit{slo1} duplication in \textit{O. mykiss} following the teleost whole-genome duplication event.

Whole-Genome Duplication Events across Teleosts

Bayesian estimate of phylogeny revealed relationships similar to what has been reported for actinopterygians (Nelson 2006). Interestingly, polypteriforms, lepisosteiforms (gar), and amiiforms (bowfin) formed a monophyletic group that is a sister group to teleosts, whereas both mitogenetic (Inoue et al. 2003) and nuclear genetic (Venkatesh et al. 2001) data have suggested polypteriforms are ancestral to the sister group of teleosts including gars and bowfin. This difference between our analysis and previous studies may be due to the relatively small sequences used in our analysis compared with previous studies using multiple genes and morphological traits to determine the relatedness of these groups.

Duplication of \textit{slo1} due to a teleost whole-genome duplication event at the origin of teleosts (Hoegg et al. 2004), followed by autotetraploidization in salmonids, would predict only two duplication events. Phylogenetic analyses support this hypothesis, identifying a duplication event at the origin of teleosts, an independent loss and secondary duplication event following the divergence of the anguilliforms from the teleost lineage, and a second round of duplication in salmonids. Crow et al. (2006) identified an independent duplication event of \textit{hoxd4} in elopomorphs (\textit{A. rostrata}, \textit{Megalops atlanticus}) with all four \textit{hoxd4} genes grouped with \textit{hoxd4a} of other teleosts, thus mirroring the data in our study. The same study reported the duplication of \textit{hoxa11} and \textit{hoxb5} as a result of a single duplication event in the ancestor common to all teleosts, including elopomorphs (Crow et al. 2006).

Other studies utilizing nuclear gene sequences to time the whole-genome duplication event in teleosts have reported only a single gene in elopomorphs, whereas other teleosts possess two, including proopiomelanocortin gene (de Souza et al. 2005), \textit{sox11}, and \textit{tyrosinase} (Hoegg et al. 2004; Hurley et al. 2007). In each case, the single elopomorph gene grouped onto a branch with one of the two teleost co-orthologues, suggesting that the teleost whole-genome duplication event occurred prior to the divergence of elopomorphs and that gene loss following duplication was higher in elopomorphs than in other teleosts for these particular genes.

Ion Channel Duplication Events

Studies of ion channel gene duplication in several species of teleosts support the hypothesis that a whole-genome duplication occurred prior to the divergence of basal teleosts (Amores et al. 1998; Taylor et al. 2001). This includes genes encoding the pore-forming \(\alpha\)-subunits of voltage-gated sodium channels (\textit{SCNA}; Novak et al. 2006; Zakon
et al. 2006), voltage-gated calcium channels (Wong et al. 2006), and shaker-related voltage-gated potassium channels (KCNA; Few and Zakon 2007; Hoegg and Meyer 2007). Analysis of the shaker-related voltage-gated potassium channel gene family (KCNA) showed that duplication of this family in the osteoglossomorph Gnathonemus petersii generally occurred as a result of the same event responsible for KCNA duplication in other teleosts (Hoegg and Meyer 2007).

**Fig. 3.**—(A) Bayesian estimate of phylogeny for vertebrate slo1 genes. Posterior probabilities are indicated above each branch. The Ensembl and GenBank accession numbers for each sequence can be found in the Materials and Methods and tables 1 and 2. (B) A portion of a tree modified (in bold) from the Bayesian consensus tree (A) in order to resolve the polytomy between Danio rerio slo1a and the slo1a genes from all other more derived teleosts. An SH test confirmed no significant difference ($P = 0.106$) between the two trees and that the tree in the lower panel (B) has a more favorable likelihood score. Slo1 genes of the genes in Anguilla rostrata have been relocated (in bold) as paralogues of slo1b of other teleosts, a move favored by likelihood over the Bayesian estimate ($P = 0.482$, SH test). The results indicate that a teleost whole-genome duplication event was followed by loss of slo1a in anguilliforms and that a second independent duplication event led to both slo1 paralogues present in A. rostrata.
By contrast, gene duplications in the voltage-gated sodium channel \( \alpha \)-subunit gene family (SCNA) giving rise to three pairs of \( \text{scn}a \) genes (\( \text{scn}4a \), \( \text{scn}5a \), and \( \text{scn}8a \)) in \( G. \ petersii \) were found to be independent from the duplication event in other teleosts (Novak et al. 2006). Novak et al. (2006) suggested that these results could be due to artificial factors such as long-branch attraction; nevertheless, the results may reflect different patterns of gene duplication between teleosts and tetrapods in a given ion channel gene family. As is the case with several of the studies described above, the exact timing of gene duplication in basal teleosts sometimes suggests the presence of independent events which ultimately result in basal teleosts possessing a similar complement of duplicate genes as more derived species.

Our data are consistent with the previous studies described above and suggest that the original \( \text{slo}1a \) paralogue in anguilliforms produced by a teleost whole-genome duplication was lost and the remaining paralogue duplicated in an independent event. Additional analyses are needed to determine whether the instances of gene loss and duplication observed in basal teleosts following teleost whole-genome duplication either occurred in a common ancestor of basal teleosts or were the result of a series of smaller, lineage-specific events. Because the sequence data from anguilliforms used in the current study are from the highly conserved pore domain, it may explain the inability to more definitely resolve the phylogenetic relationship between the \( \text{slo}1 \) genes of anguilliforms and other teleosts. Further sequencing of the \( \text{slo}1 \) genes of anguilliforms to include less highly conserved domains may help resolve this relationship more concretely.

Comparisons with Tetrapods and Invertebrates

Analysis of the genomic sequences of the teleosts \( O. \ latipes \), \( G. \ aculeatus \), \( T. \ rubripes \), and \( T. \ nigroviridis \) reveals a strict conservation of constitutive exons between both \( \text{slo}1 \) co-orthologues that extends to the single \( \text{slo}1 \) gene found in tetrapods and invertebrates (Fodor and Aldrich 2008). The presence of twice the complement of \( \text{slo}1 \) genes in teleosts has apparently not relaxed the constraints on constitutive exons. By contrast, alternatively spliced \( \text{slo}1 \) exons are not as highly conserved between tetrapods and teleosts where exon 1 is lost in both \( \text{slo}1 \) co-orthologues and exon 19 is lost in \( \text{slo}1b \). Thus, unlike constitutive exons, \( \text{slo}1 \) duplication in tetrapods and invertebrates may have relaxed constraints on alternatively spliced exons. The retention in \( \text{slo}a \), but loss in \( \text{slo}1b \), of exon 19 is evidence of a relaxed constraint that may have facilitated the retention of these duplicate genes via subfunctionalization (Hughes 1994; Force et al. 1999; Postlethwait et al. 2004).

The presence of at least two \( \text{slo}1 \) genes in teleosts may achieve the same transcript diversity as the single \( \text{slo}1 \) gene found in both tetrapods and invertebrates, which produce transcript diversity via more extensive alternative splicing. The question of whether tetrapod-specific splice sites are due to a gain in tetrapods versus a loss in teleosts requires investigating the genomic sequence of \( \text{slo}1 \) in a common vertebrate ancestor. Comparison with invertebrates such

![Figure 4](https://example.com/figure4.png)
Two slo1 Genes in Vocal Midshipman Fish

Differential expression patterns between slo1 paralogues in P. notatus, including divergent splicing patterns between paralogues in peripheral tissues (gill, trunk and vocal muscle), suggest differences between duplicates in cis-regulatory elements controlling tissue-specific expression and splicing of each gene. As is the case with the inclusion of exon 11 instead of exon 10 in P. notatus slo1b in gill (vascular smooth muscle), such differences between paralogues may code for ion channels with different functional properties. The exchange of exon 10 for exon 11 results in a change in several residues in this 31AA long sequence including a substitution of an alanine residue for the second glutamatic acid residue in the PEIIE domain labeled by an asterisk in figure 1. These glutamatic acid residues form a ring of eight negative charges (two from each of the four positively charged amino acids). These negative charges cause an electrostatic interaction resulting in the large K⁺ conductance for which BK channels are named. Reducing the amount of negative charge within this domain by mutating either or both glutamatic acid residues has been shown to decrease single-channel current amplitude (Brelidze et al. 2003; Nimigean et al. 2003). A change to the alternative exon at the same site in Drosophila has been shown to produce a BK channel with decreased single channel conductance, decreased open probability, decreased calcium sensitivity, and faster activation such as via interaction of the β1-subunit in the BK channel tetrameric structure) at the entrance to the channel’s intracellular vestibule, increasing K⁺ concentrations within the vestibule via electrostatic interactions resulting in the large K⁺ conductance for which BK channels are named. Reducing the amount of negative charge within this domain by mutating either or both glutamatic acid residues has been shown to decrease single-channel current amplitude (Brelidze et al. 2003; Nimigean et al. 2003). A change to the alternative exon at the same site in Drosophila has been shown to produce a BK channel with decreased single channel conductance, decreased open probability, decreased calcium sensitivity, and faster kinetics (Lagrutta et al. 1994). Together, the data suggest that P. notatus slo1a and slo1b have different mechanisms controlling their expression across tissue types and that alternative splicing of these paralogues differs in such a way as to produce channels which likely differ in their physiological properties. Further investigation of the promoter regions of these paralogues is necessary to determine the extent of these differences including whether either or both genes share the multiple promoters and estrogen-responsive elements which regulate transcription of M. musculus Slo1 (Kudn et al. 2007).

Subfunctionalization of slo1 genes between different muscle types may reflect the varying roles of BK channels in tissue types with radically different functional demands. The role of BK channels in vascular smooth muscle, such as that found in the gill, in maintaining blood pressure has been studied extensively (Salkoff et al. 2006). The demands of smooth muscle are much different from the vocal muscle of toadfish, including P. notatus and O. beta, which is among the fastest contracting vertebrate muscles (Rome 2006). Because toadfish vocal muscles have the fastest Ca²⁺ transients measured in any muscle (Rome 2006), BK channels in these tissues presumably need to be much more sensitive and/or rapid in their response to Ca²⁺ than BK channels in other tissues. Porichthys notatus slo1a, the only slo1 gene expressed in vocal muscle, may encode a channel with such differences compared with slo1b. In this scenario, following duplication of slo1 genes in teleosts, the physiological demands for rapid contraction in vocal muscle may have selected against the expression of slo1b that likely encodes a channel with reduced conductance/slower kinetics than slo1a (see above). Alternatively, slo1a may be posttranslationally modified in a way as to confer enhanced Ca²⁺ activation such as via interaction with the β1-subunit which enhances Ca²⁺ activation (McManus et al. 1995) and is expressed in skeletal muscle in mammals (Jiang et al. 1999).

To our knowledge, this study is the first to examine the expression of the genes encoding BK channels in the auditory division of the inner ear (saccule) of a teleost fish. Unlike all other vertebrates studied previously, BK channels in the midshipman saccule are encoded by two slo1 genes. Midshipman fish thus provide a unique opportunity to investigate how molecular plasticity, in this case the expression of BK channels, may contribute to the diversity in electrical tuning of the peripheral auditory system observed for tetraps with auditory ranges overlapping that of midshipman (Fettiplace and Fuchs 1999). Slo1 duplication may also contribute to the steroid-dependent shifts in frequency encoding by the midshipman saccule that enhance the detection of male advertisement calls (Sinseros et al. 2004). Studies of mammals have already shown that the expression and alternative splicing of slo1 are regulated by steroid hormones (Zhu et al. 2005; Kundu et al. 2007).

Concluding Comments

The frequency of alternative splicing is inversely correlated with gene family size (Kopelman et al. 2005). Consistent with this pattern, alternative splicing decreases in paralogues following gene duplication (Su et al. 2006). Although teleosts may have reduced alternative splicing within each slo1 co-orthologue compared with tetraps, they may achieve similar levels of protein diversity through the combination of two slo1 genes. In vitro expression studies are required to determine whether heteromeric channels can be assembled by combining transcripts of both slo1 co-orthologues. The mitf gene family, in which two teleost genes are homologous to two isoforms of a single mammalian gene (Lister et al. 2001; Altschmied et al. 2002), is exemplary of how these two mechanisms can be used interchangeably to achieve comparable levels of protein diversity as well as how subfunctionalization can preserve duplicate genes.

The conserved sentenly between duplicate genes, together with the reduction of alternative splicing compared with tetraps, support the hypothesis of a whole-genome duplication event followed by retention of duplicate genes via the subfunctionalization model (Hughes 1994; Force et al. 1999; Postlethwait et al. 2004). This model is further supported by the differential expression patterns of slo1
paralogues in midshipman fish. *In vitro* expression studies using patch clamp to characterize the physiological properties of the BK channels produced from slo1 paralogues will be necessary to determine the subdivision of functions between the duplicate genes in what accounts for approximately half of all living vertebrate species.

**Supplementary Material**

Supplementary table 1 and figures 1 and 2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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