DNA AND PROTEIN SEQUENCE ANALYSIS OF NEURONAL MARKERS NEURONAL NUCLEI (NEUN) AND DOUBLECORTIN (DCX) IN THE NORTHERN PACIFIC RATTLESNAKE (*CROTALUS OREGANUS*) AND WESTERN FENCE LIZARD (*SCELOPORUS OCCIDENTALIS*).

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Abstract

DNA and protein sequence analysis of neuronal markers Neuronal Nuclei (NeuN) and doublecortin (DCX) in the northern Pacific rattlesnake (*Crotalus oreganus*) and western fence lizard (*Sceloporus occidentalis*).

Brett Michael Vassar

Neuronal Nuclei (NeuN) and Doublecortin (DCX) are neuron specific proteins that are used in histological studies of brain structure in a variety of vertebrate taxa. Antibodies against NeuN (anti-NeuN) bind to the Fox-3 protein, an RNA binding protein common in mature neurons. Anti-DCX labels a microtubule-associated protein expressed in actively dividing neural progenitor cells and migrating neurons. The *DCX* gene encodes a protein that is well conserved across mammalian, avian, and a few reptilian species, therefore anti-DCX staining has been used successfully across a range of vertebrate taxa. Successful neuronal staining using anti-NeuN has been demonstrated in mammals, birds, and the Testudines order (turtles). However, herpetologists who study neurobiology in squamates have had limited success with anti-NeuN and anti-DCX binding to their respective antigens. All commercially available anti-NeuN and anti-DCX antiserums were designed to mammalian antigens, and significant differences in tertiary structure divergence at the epitope where these antibodies bind may explain the failure of anti-NeuN and anti-DCX immunohistochemistry in many squamate species. This study aims to characterize evolutionary differences in gene and protein structure between two species of reptiles (*Crotalus oreganus* and *Sceloporus occidentalis*) and mammals. We sequenced the *Fox-3* and *DCX* coding sequences using polymerase chain reaction (PCR) and Sanger sequencing, which allowed us to build phylogenetic trees comparing Fox-3 and DCX deduced protein structures. By identifying structural differences linked to evolutionary variation, new polyclonal antibodies specifically targeting Fox-3 and DCX in reptile brains can be developed to facilitate future investigations of neurogenesis and brain structure in squamate reptiles.

Keywords: NeuN, Fox-3, DCX, adult neurogenesis, reptiles, squamate, immunohistochemistry

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1. Introduction

Near the end of the 19th century, the neuron doctrine had been conceived through research conducted by notable neuroanatomist Santiago Ramon y Cajal and supported later by Heinrich Waldeyer (Purves et al., 2008). Neurons had just been discovered as the functional unit of the nervous system; neurons were discrete, and the structure of the brain remained fixed soon after birth. The idea that neurogenesis in the adult brain occurred was unimaginable. At the turn of the 19th century the leading hypothesis on neurogenesis, the production of new neurons, was that it only occurred during embryonic development and ceased before the onset of puberty (Gross, 2000). During the 1960s, the introduction of [^{3}H]-thymidine, a radiolabeled analog of thymidine, along with Joseph Altman's groundbreaking discoveries, followed by many others, began the effort to change the long standing view that neurogenesis was limited to life stages before sexual maturation (i.e., embryos, juveniles) (Altman and Das, 1965; Bonfanti, 2016).

Altman (1965) first demonstrated evidence of new neuron formation in the lateral ventricles and dentate gyrus of adult rats using autoradiography and histology. The scientific community, however, largely ignored Altman's and colleagues demonstrations of neurogenesis at that time because the available techniques were not adequate to differentiate neurons from glial cells (Gross, 2000).

Beginning in the early 1980's, multiple developments led to the acceptance of adult neurogenesis including experiments showing neurogenesis in adult birds, the introduction of immunohistochemical techniques ability to differentiate neurons from glia, and the up and down regulation of physiological variables on neurogenesis (Gross, 2000). Near the vocal control nucleus, HVC, of canaries, [^{3}H]-thymidine labeled cells

were identified in the ventricular zone, and found to migrate and differentiate into neurons and glial cells in the HVC (Goldman and Nottebohm, 1983). Newly generated neurons were also identified in male canaries using ^{3}[H]-thymidine along with autoradiography, light microscopy, and electron microscopy to show that synaptic terminals were contacting the newly formed neurons (Burd and Nottebohm, 1985). These studies conducted by Fernando Nottebohm were an integral part of the paradigm shift of neurogenesis and helped jumpstart the research conducted in mammals and other taxa.

By the 1990's, the previous stigma on adult neurogenesis had nearly vanished and many studies began to show evidence to support that adult neurogenesis occurred in two distinct regions of the mammalian brain: the subventricular zone and the dentate gyrus of the hippocampus (Richards et al., 1992; Reynolds and Weiss, 1992; Gage et al. 1995; Pérez-Cañellas and García-Verdugo, 1996; Palmer et al. 1997; Erikkson et al., 1998). With building evidence for adult neurogenesis, studies examining the effects of environmental conditions, stress, hormones, behavioral activity, and a myriad of other factors on adult neurogenesis began to arise (e.g., Aimone et al., 2014; Braun and Jessberger, 2014; Egeland et al., 2015; Mahmoud et al., 2016; Zhao et al., 2008).

Studies aiming to quantify changes in rates of neurogenesis, however, need to be able to identify which cells are newly proliferated, as well as which of these newly proliferated cells have differentiated into neurons. To accomplish this task, many studies have used immunohistochemical markers of cell proliferation such as bromo-deoxyuridine (BrdU), proliferating cell nuclear antigen (PCNA), or Ki67 to label mitotically-active cells in the brain (Brown and Gatter, 2002; Burns and Kuan, 2005; Hall et al., 1990; Encinas et al., 2008; Scholzen and Gerdes, 2000). The development of the

synthetic thymidine analog BrdU, for instance, allowed mitotically-active, proliferating cells in the brain to be labeled via immunohistochemistry for the estimation of the total number of new cells that were able to take up this marker (Gross, 2000).

Combining immunoreactivity localization of proliferating cells with molecular markers for other targets such as β-tubulin III, MAP-2, synaptophysin, doublecortin (DCX), or neuronal nuclei (NeuN) quickly became a commonly applied and effective method for localizing and distinguishing newly-proliferated cells in the brain (Gusel'nikova and Korzhevskiy, 2015). These markers can distinguish different types of cells in the brain because the proteins that they label are differentially expressed by cell type (i.e. neurons or glial cells) (Gusel'nikova and Korzhevskiy, 2015). For instance, co-labeling with NeuN - which is expressed in postmitotic neurons – and doublecortin (DCX) – a marker of migrating cells – allows discrimination of newly-proliferated, immature neurons (DCX^+, $NeuN^+$) from mature neurons (DCX^-, $NeuN^+$). These methods thus became critical for the identification of cell phenotypes because BrdU is injected before sacrifice to label dividing cells and cannot distinguish cell phenotype. Although many of these immunohistochemical markers were used, NeuN became one of the most widely used neural markers due to the fact its antigen is localized to the nucleus of mature neurons. This allowed for visualization of the co-localization of BrdU and NeuN in the nucleus, where cells could be labeled as both a proliferating cell as well as a mature neuron (Gusel'nikova and Korzhevskiy, 2015).

NeuN ('**Neu**ronal **N**uclei') was discovered through a screening of monoclonal antibodies generated against mice brain cell nuclei, which was identified as the neuron-specific antigen recognized by the antibody A60 (anti-NeuN) (Mullen et al., 1992). Since

then, NeuN has been one of the leading antigens used in the identification of many post-mitotic neural cell types. Antibodies against NeuN bind specifically to an antigen expressed exclusively in neural tissue, specifically in neurons (Mullen et al., 1992). More recently, Kim et al. (2009) identified Fox-3 as the NeuN antigen. Fox-3 – also referred to as RNA binding fox-1 homolog 3, hexaribonucleotide binding protein-3, Rbfox3, or feminizing locus on X-3 – is part of Fox-1 gene family of RNA binding proteins that regulate splicing (Kim et al., 2009). Because Fox-3 localizes to nuclei of maturing and differentiated neurons throughout the central and peripheral nervous systems, it became widely accepted as a robust marker for the identification of neurons in studies needing to quantify rates of neurogenesis (Wolf et al., 1996; Sarnat et. al., 1998, Mangavi et al., 2000; Weyer and Schilling, 2003).

Another widely used immunohistochemical neural marker is doublecortin (DCX), a microtubule-associated protein (MAP) expressed in actively dividing neural progenitor cells and migrating neurons in the brain (Cai et al., 2009, Francis et al., 1999). DCX is localized to the soma and leading processes of immature neurons where it plays a role in microtubule polymerization, organization, and stability (Francis et al., 1999; Gleeson et al., 1999). The DCX antigen has been used as a proxy for neurogenesis due to its spatial and temporal expression patterns during differentiation and migration in the central and peripheral nervous system (Francis et al., 1999; Gleeson et al., 1999; Brown et al., 2003). Similar to NeuN, DCX is a useful indicator for neurogenesis due to the fact that it can work independently from prelabeling methods, such as BrdU, because it can be detected in specimens postmortem and does not need to be injected into living organisms (Brown et al., 2003).

Successful neuronal staining using anti-NeuN and anti-DCX has been demonstrated in a wide variety of vertebrate taxa including mammals, birds, and amphibians (*Fox-3:* mammals, birds, & amphibians: Mullen et al., 1992; birds: Strand and Deviche, 2007; *dcx:* birds: Boseret et al., 2007; mammals: Cai et al., 2009; amphibians: Muñoz et al., 2015). However, mouse monoclonal antibody clone A60 MAB377 (Millipore Sigma, formerly Chemicon, Temecula, CA) and goat polyclonal antibody SC-8066 (Santa Cruz Biotechnology, Dallas, TX), two commercially available antibodies against NeuN and DCX respectively, have had limited utility in reptiles (personal observations). Anti-NeuN (MAB377) staining that has been successfully co-localized in D'Orbigny's slider turtle, *Trachemys dorbigni* (Fernández et al., 2002), but, to our knowledge, has not been shown to successfully bind in squamate reptiles (snakes and lizards; C. Strand, pers. Comm.; D. Lutterschmidt, pers. comm.). Although, a recent study by McDonald and Vickaryous (2018) demonstrated successful immunostaining of anti-NeuN (ab104225; Abcam, Cambridge, MA) in the leopard gecko, *Eublepharis* macularius. Anti-NeuN (ab104225) differs from MAB377 in that it is a rabbit polyclonal antibody against Fox-3. Similarly, anti-DCX (SC-8066) has been successfully used to label migrating neurons in the brain of the side-blotched lizard (*Uta stansburiana)* (LaDage et al., 2013), and Western fence lizard (*Sceloporus occidentalis*) (Wilson, 2015), but does not appear to bind specifically in snakes (C. Strand, personal communication). A separate anti-DCX antiserum (C18 antibody, Santa Cruz Biotechnology, Dallas, TX), however, was recently used to label DCX in Nile crocodile (*Crocodylus niloticus*) (Ngwenya et al., 2018), but, to our knowledge, hasn't been tested in squamates.

Current commercially available anti-NeuN and anti-DCX antiserums are generated against mammalian forms of the NeuN and DCX proteins, and significant divergence in the epitope or tertiary structure surrounding the epitope may explain the failure of anti-NeuN (MAB277) and anti-DCX (SC-8066) immunohistochemistry in squamate species. There is therefore a need to examine whether changes in protein structure explain the difficulty in using anti-NeuN and anti-DCX antisera in squamates, and develop neuron specific antibodies that can be used for squamate reptile research (Krohmer et al., 2011).

This study aims to characterize evolutionary variation in Fox-3 and DCX gene and protein sequence between squamate reptiles and their mammalian, avian and amphibian counterparts. Specifically, we amplified and sequenced the coding regions of *Fox-3* and *DCX* from the northern Pacific rattlesnake (*Crotalus oreganus*) and Western fence lizard (*Sceloporus occidentalis*), two squamates native to western North America. By examining the sequences of these proteins – in combination with protein sequences of other squamates – we found differences in the sequence of *Fox-3* in snakes and lizards compared to other vertebrates that may explain the difficulties. This information is critical for developing new polyclonal antibodies specifically targeting Fox-3 and DCX in reptile brains and enabling future investigation of neurogenesis in squamate reptiles.

2. Material and Methods

2.1 Animals

Whole brain tissue was obtained from a salvaged female northern Pacific rattlesnake (*C. oreganus*) collected from San Miguel, CA and a male western fence lizard (*S. occidentalis)* collected from Poly Canyon, San Luis Obispo, CA that had died during a separate experiment (IACUC #1406). The tissue was flash frozen in liquid N_2 and stored at −80˚C.

2.2 Isolation and sequencing of Fox-3 and DCX

2.2.1 Total RNA isolation

Total RNA was extracted from the whole brain using TRI Reagent® (Molecular Research Center, Inc., Cincinnati, OH, USA) with bromochloropropane as the phase separation reagent. The extracted RNA was then DNase I treated (TURBO DNA-free™ Kit, Ambion) and quantified by spectrophotometry (*C. oreganus* 260:280 = 2.112; *S. occidentalis* 260:280 = 2.189; NanoPhotometer P300 Implen, Inc., Westlake Village, CA, USA).

2.2.2 Isolation and sequencing of partial cDNAs encoding Fox-3 and DCX

First strand cDNA was synthesized in 20µl reverse transcription reactions containing 5µg of total RNA template, 0.5µg random hexadeoxynucleotide primers (Promega Corp., Madison, WI, USA), 4.0µl GoScript™ 5X Reaction Buffer, 3.75 mM $MgCl_2$, 0.5µM of dNTPs (Promega Corp.), 20U Recombinant RNasin® Ribonuclease Inhibitor (Promega Corp.), and 160U GoScript™ Reverse Transcriptase. The reverse transcription reaction was run on a T100™ Thermal Cycler (BioRad Laboratories, Inc., Hercules, CA, USA) under a thermal profile where the RNA template and primers were

heated at 70˚C for 5 min, chilled on ice for 5 min, centrifuged for 10 sec at 5000RPM, then added to remaining reagents and incubated at 25˚C for 5 min, 42˚C for 1 h, followed by 70˚C for 15 min to inactivate the reverse transcriptase.

Degenerate primers were designed from consensus regions of sequences for *Fox-3* and *DCX* cDNAs identified on GenBank (https://www.ncbi.nlm.nih.gov/) from the following reptilian species: *Fox-3*: green anole (XM_008104179) Chinese alligator (XM_006036922) and Burmese python (XM_007426269) [Figure 1 & 2]; and *DCX*: green anole (XM_008119210) and Burmese python (XM_007424922 and XM_007424923) [Figure 3]. Nucleotide sequences for each gene were aligned using Sequencher v5.1 software (Gene Codes Corp., Ann Arbor, MI, USA), and nested sets of degenerate primers were designed to consensus regions for both *Fox-3* and *DCX*. Nucleotide sequences for these degenerate primers are provided in Table 1.

Partial cDNAs encoding both *Fox3* and *DCX* were amplified by PCR in 50µl reactions containing 1X GoTaq® Colorless Master Mix (Promega Corp.), 0.2µM each forward and reverse degenerate primer in all combinations (Table 1), and 250ng of cDNA under a thermal profile of 95˚C for 2 min, 35 cycles of 95˚C 30 s, 50˚C 30 s and 72˚C for 90 s, followed by a final extension of 72˚C for 2 min. Subsequent PCR reactions using nested degenerate primers were conducted following the same thermal profile described above but with an annealing temperature of 52˚C. Nested PCR products were examined on 1.2% E-Gel® precast agarose gels (Invitrogen, Grand Island, NY) with 1µg/mL ethidium bromide, and PCR products of predicted size were subsequently cleaned (QIAquick PCR Purification Kit, Qiagen) and Sanger sequenced using all primers in Table 1 (Molecular Cloning Lab, Inc., South San Francisco, CA, USA).

2.2.3. Amplification of full-length Fox-3 coding region from C. oreganus

During the course of this research, a genome assembly for the southwestern speckled rattlesnake, *Crotalus mitchellii pyrrhus*, was completed (Genbank accession no. GCA_000737285.1), providing additional sequence information for isolating *Fox-3* cDNAs from the northern Pacific rattlesnake, *C. oreganus*. Reverse transcription was carried out using the GoScript™ Reverse Transcription System (Promega) with 0.5µg oligo-dT primers. A new set of gene-specific primers for *Fox-3* were designed to the 5' and 3' UTRs of *Fox-3* identified from the *C. mitchellii pyrrhus* genome, and the *C. oreganus Fox-3* primers found in Table 2 were used in different combinations (Figure 4A/4B) to amplify the full length *Fox-3* cDNA from *C. oreganus* under a thermal profile of 95˚C for 2 min, 35 cycles of 95˚C 30 s, 52˚C 30 s, 72˚C 100 s, followed by a final extension of 72˚C for 2.5 min. Resulting PCR products were examined on a 1.2% agarose gel (Fisher BioReagents) with 1µg/mL ethidium bromide, and then cleaned (QIAquick PCR Purification Kit, Qiagen) and Sanger sequenced using the *Fox-3* primers in Table 2 (Molecular Cloning Laboratories Lab, Inc., San Francisco, CA, USA).

2.3 Phylogenetic Analyses

Clustal X (v2.1) was used to align deduced protein sequences of *C. oreganus*, *S. occidentalis*, and other squamate reptiles and vertebrate species on the National Center for Biotechnology Information (NCBI) database (Larkin et al., 2007). Phylogenies were then constructed with Molecular Evolutionary Genetics Analysis software (MEGA v7; Tamura et. al., 2013) using Neighbor-Joining method with p-distance model and pairwise deletion of gaps. Each tree was bootstrapped using 1000 replicates. GenBank accession numbers for all sequences used in the phylogenetic analyses are provided in Table 3.

3. Results

3.1 Identification of Fox-3

Full-length cDNAs encoding *Fox-3* for *C. oreganus* were amplified via PCR using primers in the 5' and 3' UTR (Figure 5), which resulted in a 1125 nucleotide coding region. BLAST and phylogenetic analysis of deduced amino acid sequences of full-length cDNAs confirmed the identity of *Fox-3*, including 374 amino acids (Figure 6). Amplification of partial DNA sequence of *S. occidentalis* was successful using degenerate primers (Figure 7), although an estimated 266bp at the 5' end and 246bp at the 3' end of the *S. occidentalis* coding region were unsuccessful with amplification of cDNA ends, as estimated by comparison with the predicted coding region of *Anolis carolinensis* (XM_008119210). Kim et al. (2009) suggests that the epitope for anti-NeuN lies within the first 106 amino acids; therefore, *S. occidentalis* was omitted from the phylogenic analysis in Figure 6 and alignment in Figure 8 for Fox-3 because of this missing region at the 5' end.

3.2 Identification of DCX

Several partial cDNA amplicons were generated for *DCX* using combinations of UTR specific and degenerate primer sets (Table 1&2). The first 931bp of *C. oreganus* from the start codon was successfully amplified using the '*DCX*' primers in Table 2, while the middle 554bp of *S. occidentalis* of an approximate 1100bp coding region of *DCX* was amplified using the degenerate *S. occidentalis* (Figure 9)*; DCX* primers in Table 1, leaving 337bp at the 5' end and 285bp at the 3' end unknown. Anti-DCX (SC-8066) is a polyclonal antibody; therefore, a specific epitope could not be determined as to which section(s) of the coding region would be most important in the binding of anti-

DCX (SC-8066). Both *C. oreganus* and *S. occidentalis* were included in the DCX phylogeny, even though they were partially sequenced, to observe any phylogenetic differences based on deduced protein sequences for the partial regions of cDNAs obtained.

3.3 Comparison of Fox-3 Protein Sequence

After *C. oreganus Fox-3* was amplified by PCR, Sanger sequenced, and translated into deduced protein sequence using ExPASy Translate tool (https://web.expasy.org/translate/), one major difference in protein sequence between squamate reptiles and other taxon stands out. Kim et al. (2009) used a variety of molecular techniques to isolate the core part of the epitope for anti-NeuN binding, which is composed of a 4 amino acid sequence at the N-terminus, PPAQ (Kim et al., 2009). In many taxa including mammals, birds, amphibians, and even some reptiles (turtles), that PPAQ amino acid sequence is conserved in the Fox-3 protein. Our own *Fox-3* cDNA sequence obtained from *C. oreganus* as well as those for deduced Fox-3 proteins obtained from GenBank for other squamate species revealed that the putative epitope region in squamate Fox-3 evolved to an amino acid composition of PPAT, with a Threonine replacing the Glutamine at amino acid position 52 (Figure 8).

3.4 Phylogenetics of Fox-3

Phylogenetic analysis of deduced Fox-3 protein sequences in select vertebrate taxa indicated distinct clades for Fox-3 from reptiles, birds, mammals, and amphibians (Figure 6). The deduced protein for rattlesnake Fox-3 was part of the larger clade of Fox-3 sequences from vertebrates, and distinct from the select Fox-1 and Fox-2 sequences used to root the tree, confirming that the cDNA amplified from *C. oreganus* was indeed

encoding a Fox-3 protein. We also observed that Fox-3 from all species of Class Reptilia included in the phylogenetic analysis shared the PPAT epitope sequence as stated above, whereas the mammals and birds share the alternative sequence with a Glutamine in the 4th position of the putative epitope: PPAQ.

3.5 Comparison of DCX Protein Sequence

Due to unsuccessful PCR amplification of the full coding region of *DCX* in *C. oreganus* and *S. occidentalis*, a definitive comparison of the epitope(s) between taxa could not be made. Many of the commercial antibodies available are polyclonal with specificity to the C-terminal region of the protein, which could not be deduced from the sequence amplified in this project (SC-8066, Santa Cruz Biotechnology; ab18723, Abcam; AB2253, Millipore).

3.6 Phylogenetics of DCX

Similar to the phylogenetic analysis of Fox-3, deduced DCX protein sequences also show a distinct clade structure by vertebrate taxonomic class, with DCX sequences from reptiles, mammals, birds, and amphibians forming monophyletic groups (Figure 10). Although, contradictory to the literature, DCX has been shown to successfully label neurons in various lizard species, which doesn't coincide with the phylogenetic tree in Figure 10 (LaDage et al., 2013; Wilson, 2015; C. Strand, pers. comm.). Figure 10 supports the idea that anti-DCX antibodies binds to an epitope within the C-terminal region of the protein because the failure to amplify the 3' end of the coding region didn't allow for comparison between possible antibody binding sites. Therefore, with complete coding region amplification, squamate lizards may shift to a monophyletic group where anti-DCX antibodies are known to bind.

4. Discussion

Adult neurogenesis has been studied extensively in different taxa over the past few decades; therefore research consistently expands to more and more species. We have investigated sequence and evolutionary differences of two neuron specific immunohistochemical markers, NeuN and DCX, in two squamate reptile species. The complete coding region of a *Fox-3* cDNA from *C. oreganus* was amplified, along with a large portion of the *DCX* cDNA sequence from that species. Partial sequences of cDNAs encoding *Fox-3* and *DCX* also were amplified from *S. occidentalis*. This allowed for deduced protein sequence alignment and phylogenetic analysis of Fox-3 and DCX.

My finding that Fox-3 from *C. oreganus* and other squamate reptiles exhibits a single amino acid change in the region of the protein putatively targeted by the anti-NeuN (MAB377) antibody may provide an explanation for why this antibody has failed to work effectively for NeuN immunohistochemistry in these taxa. An important study showed that anti-NeuN (MAB377) cross-reacts with Fox-3 and synapsin I, sharing sequence specificity in a stretch of 14 amino acids position 6-19 in *Mus musculus*; (Figure 8), which overlaps with the putative binding epitope of Fox-3 (Kim et al., 2009). Within this region, the amino acids sequence PPAQ is a notable point of interest to our study due to an amino acid change from glutamine (Q) to threonine (T) (Kim et al., 2009). With the evidence provided by Kim et al. (2009), this new information suggests that the amino acids PPAQ are an important region of the NeuN epitope that is involved in anti-NeuN recognition.

Several non-mutually exclusive hypotheses may explain the conclusions that arise from an amino acid change at this position. First, there is a possibility that this single

amino acid change at position alters the tertiary structure change for this region of the NeuN protein. If this were the case, the amino acid change from a Q to T may cause altered protein folding, resulting in the epitope being hidden within the protein, thus masking the epitope in the internal structure of the protein. Although both Q & T are amino acids with uncharged polar side chains, a tertiary structural change due to a single amino acid change may not be the sole or correct explanation for the observed lack of anti-NeuN binding in squamates.

Second, various isoforms of Fox-3 exist, with the forms differing in polypeptide lengths and tertiary structure, thus potentially masking the epitope for anti-NeuN to bind the Fox-3 protein (Gusel'nikova and Korzhevskiy, 2015). Based on the NeuN sequence in *C. oreganus*, we determined that the protein contains an additional 46 amino acids ahead of the first 106 amino acids documented by Kim et al. (2009). With the added amino acids in *C. oreganus*, the first five amino acids would be MLCSM (Figure 8). Kim et al. (2009) identified 2 isoforms in their study, but an additional 47 amino acids were included beginning at position 252, which would not affect the N-terminus of Fox-3 isoforms that would begin with MAQPY. Therefore, the PPAQ region would begin at amino acid 6 in both isoforms and is consistent with anti-NeuN binding to both isoforms in their study. Compared to our findings, this would lead to a shift in the Fox-3 epitope being 46 amino acids downstream in the protein. The addition of 46 amino acids in these different isoforms may result in structural differences in squamate Fox-3, effectively burying the Fox-3 epitope inside its tertiary structure and not allowing for binding of anti-NeuN. With only "Predicted" sequences on Genbank and failure to have 3D protein structure and folding models available, this contradiction only leaves this as a plausible

explanation (see *G. gallus*; Figure 8) for the lack of anti-NeuN binding in squamate reptiles. Further investigations into the protein structure variation of Fox-3 among vertebrates is needed to resolve the possible role of these additional 46 amino acids in Fox-3 protein function in squamates.

Phosphorylation of Fox-3 may also play an important part in the changes of antibody binding to the Fox-3 protein. At position 54 (Figure 8), reptilian species besides *C. mydas* have an amino acid change from glutamine (Q) to threonine (T). Both Q and T are uncharged polar amino acids, but a major difference is that T is an amino acid that can be phosphorylated while Q is not. Other amino acid positions that may affect anti-NeuN binding include amino acids 85 and 113, which are changed from proline (P) to threonine and serine (S), respectively. Each of these previous amino acid changes go from non-phosphorylated amino acids to amino acids that can potentially be phosphorylated. Interestingly, Fox-3 is a multiply phosphorylated protein, which antibody binding is highly dependent on phosphorylation of the protein (Lind et al., 2005). A key finding is that high-salt nuclear extracts subjected to dephosphorylation with *E. coli* alkaline phosphatase typ IIIL showed no signs of immunoreactivity (Lind et al., 2005). Therefore, anti-NeuN does not recognize non-phosphorylated Fox-3 (Lind et al., 2005). Amino acid 77 contains an amino acids change from threonine to alanine (A), going from an amino acid that can be phosphorylated to one that is not. Similarly, amino acid 131 also has a change to a non-phosphorylated amino acid, serine to asparagine (N) in squamate reptiles. Although this amino acid change is seen in squamates, it is also seen in *M. musculus*, so the impact may not be as significant as other changes. In conjunction with the findings from Lind et al. (2005) and the key amino acid changes within the

epitope, there is evidence to support that these amino acid changes, which may lead to posttranslational modifications, could play an important role in the failure of anti-NeuN binding to its respective antigen in squamate reptiles.

Phylogenetic analysis of Fox-3 protein sequences in various taxa also coincides with research that supporting the evidence that anti-NeuN does not bind to Fox-3 in squamates (Figure 6). We are unaware of any studies showing successful anti-NeuN labeling (MAB377) of Fox-3 in neuronal tissue for any of the taxa that would be expected to be included in the reptile clade shown in Figure 6. Contrastingly, many of the species under the clades including birds and turtles, mammals, fish, and amphibians have evidence demonstrating the labeling of Fox-3 with anti-NeuN (MAB377) (Park et al., 2009; Bloom et al., 2014; Chang et al., 2018; Saito et al., 2017). In addition, the polyclonal anti-NeuN (ab104225) antibody has been used successfully to label Fox-3 in squamate reptile species, *E. macularius*, although further investigation into binding eptitopes is needed for anti-NeuN (ab104225) (McDonald and Vickaryous, 2018).

Similarly, phylogenetic analysis of DCX protein sequences has supporting evidence that anti-DCX does not label neurons in *Crotalus oreganus* and other snakes, although additional studies are needed to clarify why those antibodies do not bind snake DCX, including work to obtain the full coding region of DCX in several squamate species. Figure 10 shows reptiles in a different clade than mammals, where anti-DCX is known to bind to its antigen. Taxa including mammals, birds, and amphibians have evidence demonstrating the labeling of neurons with anti-DCX (Boseret et al. 2007, Dominguez et al., 2015; Yang et al., 2015; Pinkas et al., 2015). Although anti-DCX has not been shown to bind in snakes, anti-DCX (goat anti-DCX; SC-8066; Santa Cruz

Biotechnology) has been seen to bind in tissue from other reptiles, such as the squamate *Gallotia galloti* (Delgado-Gonzalez et al., 2011) *Uta stansburiana* (LaDage et al., 2013), and *Scelporus occidentalis* (personal observations). In our phylogenetic analysis, within the reptile clade, snakes fall into a separate monophyletic group (suborder Serpentes). Thus, it is possible that there are differences in the protein sequence of the snake group that may explain the lack of anti-DCX binding in this specific group.

Both NeuN and DCX have been important immunohistochemical markers that have been consistently used for neurogenesis research in a wide range of taxa. For squamate reptiles, the lack of neuron specific immunohistochemical markers has left many unanswered questions in this area of research. Through sequence and phylogenetic analysis of Fox-3 in *C. oreganus* and *S. occidentalis*, we provide evidence to support the hypothesis that the failure of commercial immunohistochemical antibody binding of anti-NeuN to Fox-3 in squamate reptiles is due to differences in the primary structure of the epitopes recognized by these antibodies. This information appears to help explain why prior studies using commercially available antibodies to mammalian Fox-3 and DCX proteins do not appear to bind the squamate forms of these proteins, and point to a need to develop new antisera to these proteins for use in reptilian taxa.

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Appendix

Table 1. Degenerate Primers used for amplification and sequencing of partial cDNAs from northern Pacific rattlesnake, *Crotalus oreganus* and western fence lizard, *Sceloporus occidentalis*.

Species	**Gene**	**Orientation**	**Nucleotide Sequence (5' to 3')**	**Abbr.**
C. oreganus	*Fox-3*	Python_FOX3_for1d	ATG GCT CAG CCC TAY CCC	P1
		Python_FOX3_for4d	ACG CCW TGA CSC TCT ACA C	P2
		Python_FOX3_for5d	AGC ACG CAG TCC ATA GCR G	P3
		Python_FOX3_rev3d	ACT CTG CCG TAR CTG TCR CT	P4
		Python_FOX3_rev4d	CCG TAG AAT CCG TCC TGA TAM AC	P5
		Python_FOX3_rev5d	GCT CCA TAR GTG GGG ATG G	P6
S. occidentalis	*Fox-3*	Anole_FOX3_for1d	CAC GCC WTG ACB CTC TAC AC	P7
		Anole_FOX3_for3d	AGC ACG CAG TCC ATA GCR G	P8
		Anole_FOX3_for4d	CCG CAG ACA GAT GAA GCV GC	P9
		Anole_FOX3_rev2d	CCC TCC TCG GTA TAG ACT AGC	P10
		Anole_FOX3_rev4d	AAT CCR TCC TGR TAM ACS ACY G	P11
		Anole_FOX3_rev5d	TCC ATA RGT GGG GAT GGG	P12
C. oreganus &	*DCX*	DCX_for1d	GGA ACT TGA TTT TGG ACA CTT TGA CG	P13
S. occidentalis		DCX_for2d	CGT CCA GAA ACA TGC GWG GTT	P14
		DCX_for4d	CCT TGC TGG CTG ACC TGA	P15
		DCX_rev3d	CTT GTG CTT ACG GAG GCT TC	P16
		DCX_rev4d	TGT GGA GAT KGG GGA YTG CTT	P17
		DCX_rev6d	CCA TCC AGG GTR TAS AGC TTY TT	P18

Table 2. Primers used for the amplification and sequencing of full-length cDNAs from northern Pacific rattlesnake, *Crotalus oreganus*.

Species	Gene	Orientation	Nucleotide Sequence (5' to 3')	Abbr.
C. oreganus	*Fox-3*	CoregF3For5'UTRupOut	AAG CAG AAG GAG CCT GCC AAC AT	FP1
		CoregF3For5'UTRupIn	AAC ATC GCC GCT TCT GGG TTT	FP2
		CoregF3For5'UTRdwnOut	GGC TTG AGA TTG TCA GCC ATG AG	FP3
		CoregF3For5'UTRdwnIn	AAT CAG GAC GCC ACG GCT	FP4
		CoregF3Rev3'UTRout	GGA GCA GTC AGT GTT ACT ACT GTG AGG A	RP1
		CoregF3Rev3'UTRin	TTA CTA CTG TGA GGA GGG TCT GGG T	RP2
C. oreganus	*DCX*	C.orgDCXForwOut	GCT TCC ATT CTT TAC CTC CTC CTT CTG	
		C.orgDCXForwIn	CCT CAG CTT CAC ATC TTT AGG GGT CA	
		C.orgDCXRevOut	AAA CAG AGC GTG TAC TAC GGA GAC	
		C.orgDCXRevIn	TGT ACT ACG GAG ACG GGG AAC T	

Table 3. **Genbank accession numbers of organisms used in Fox-3 protein phylogeny. *Crotalus oreganus* was sequenced in this study.**

Gene	Scientific name	Common name	Accession No.
Fox-3	*Crotalus oreganus*	northern Pacific rattlesnake	MK905740
	Anolis carolinensis	Green anole	XP_008102381
	Crotalus pyrrhus	Southwestern speckled rattlesnake	GCA_000737285.1
	Crotalus horridus	Timber rattlesnake	GCA_001625485.1
	Python bivittatus	Burmese python	XP_007426331
	Thamnophis sirtalis	Garter snake	XP_013915277
	Protobothrops mucrosquamatus	Brown spotted pit viper	XP_015676753
	Pogona vitticeps	Central bearded dragon	XP_020669446
	Phascolarctos cinereus	Koala	XP_020856497
	Sarcophilus_harrisii	Tasmanian devil	XP_012404076
	Monodelphis domestica	Gray short tailed opossum	XP_001371026
	Macaca mulatta	Rhesus monkey	XP_014975847
	Homo sapiens	Human	XP_011522668
	Chelonia mydas	Green sea turtle	XP_007069142.1
	Chrysemys picta bellii	Western painted turtle	XP_005283071.1
	Esox lucius	Northern pike	XP_010889997
	Poecilia mexicana	Shortfin molly	XP_014836955
	Lepisosteus oculatus	Gar	XP_006635193
	Danio rerio	Zebrafish	XP_021336052
	Aquila chrysaetos canadensis	Golden eagle	XP_011585014
	Gallus gallus	Chicken	XP_015151070
	Parus major	Great tit	XP_015501154
	Lepidothrix coronata	Blue crowned manakin	XP_017693214
	Nipponia nippon	Crested ibis	XP_009466044
	Xenopus laevis	African clawed frog	XP_018093838
	Xenopus tropicalis	Tropical clawed frog	XP_002932563
Fox-2	*Thamnophis sirtalis*	Garter snake	XP_013916012
	Homo sapiens	Human	NP_001026865
	Mus musculus	House mouse	NP_001104298.1
Fox-1	*Thamnophis sirtalis*	Garter snake	XP_013917625
	Homo sapiens	Human	NP_665898
	Mus musculus	House mouse	NP_899011.2

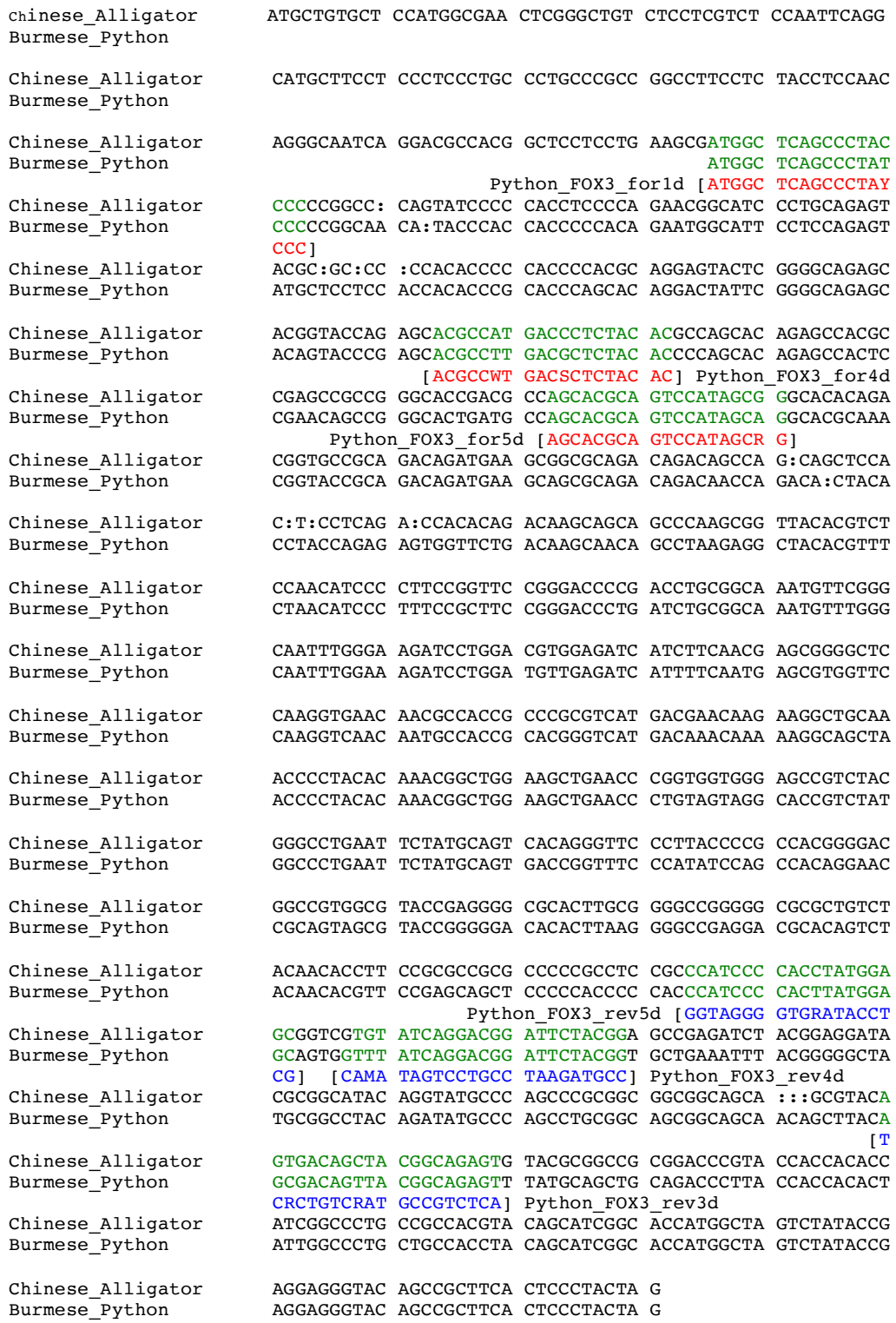

Figure 1. **Nucleotide consensus region alignment of Chinese alligator (XM_006036922) and Burmese python (XM_007426269) to determine degenerate primers for *C. oreganus* Fox-3**. Consensus regions between organisms are highlighted in green. Forward primers are written in red 5' to 3'. Reverse primers are written in blue and show the compliment strand 5' to 3'.

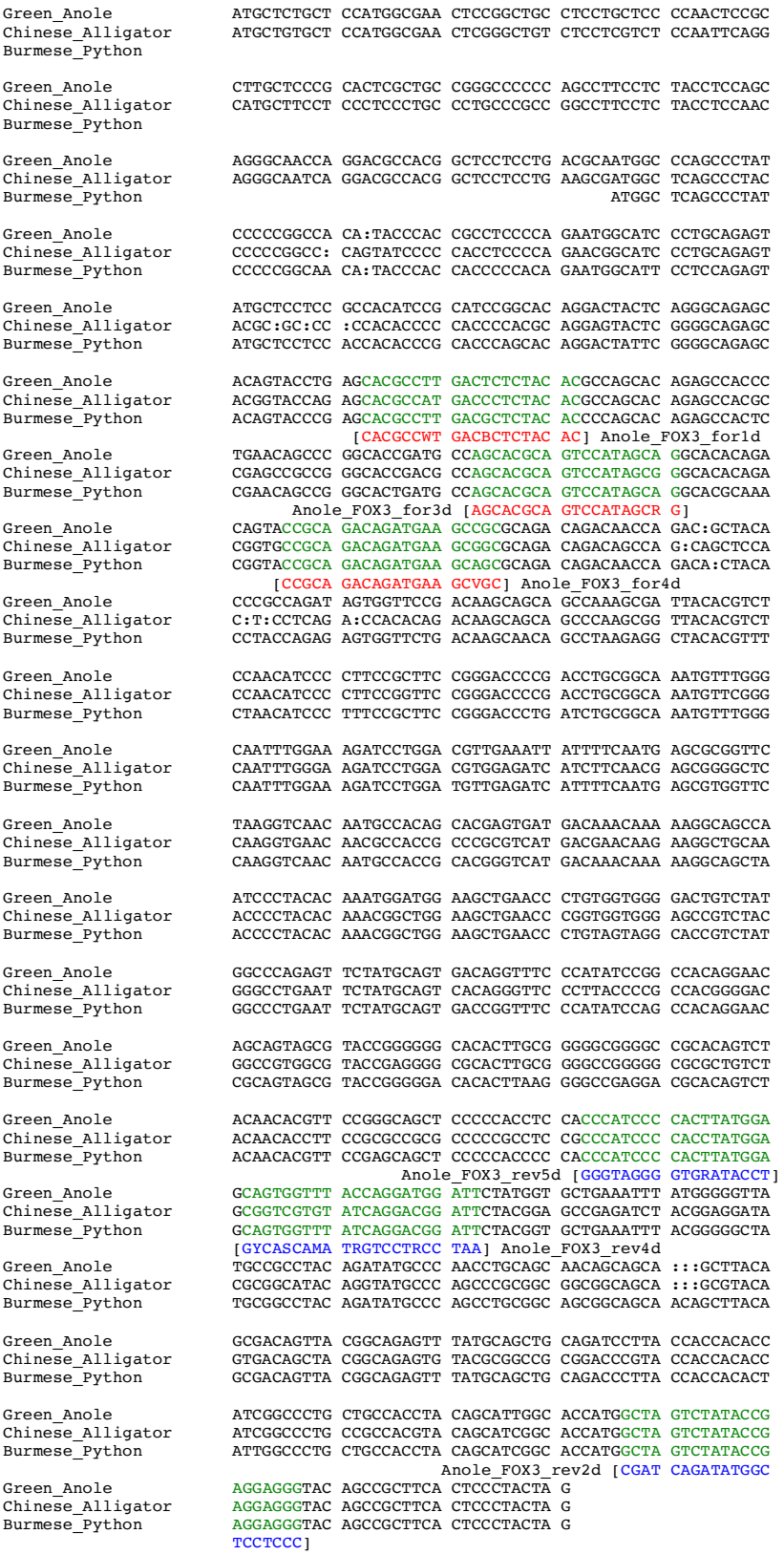

Figure 2. **Nucleotide consensus region alignment of Green Anole (XM_008104179), Chinese alligator (XM_006036922) and Burmese python (XM_007426269) to determine degenerate primers for *S. occidentalis* Fox-3**. Consensus regions between organisms are highlighted in green. Forward primers are written in red 5' to 3'. Reverse primers are written in blue and show the compliment strand 5' to 3'.

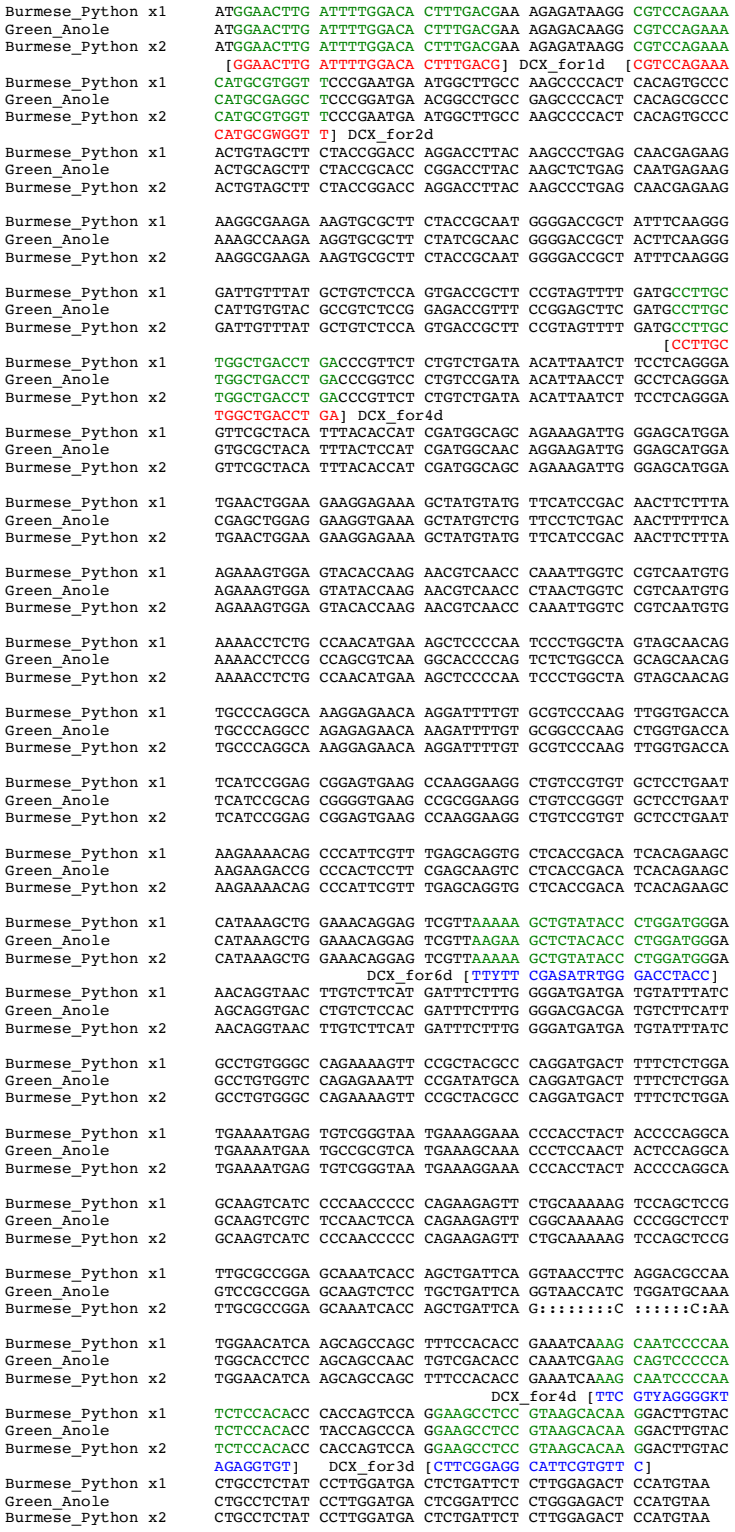

Figure 3. **Nucleotide consensus region alignment of Green Anole (XM_008119210) and Burmese python transcript variants x1 and x2 (XM_007424922 and XM_007424923) to determine degenerate primers for *C. oreganus* and *S. occidentalis* DCX**. Consensus regions between organisms are highlighted in green. Forward primers are written in red 5' to 3'. Reverse primers are written in blue and show the compliment strand 5' to 3'.

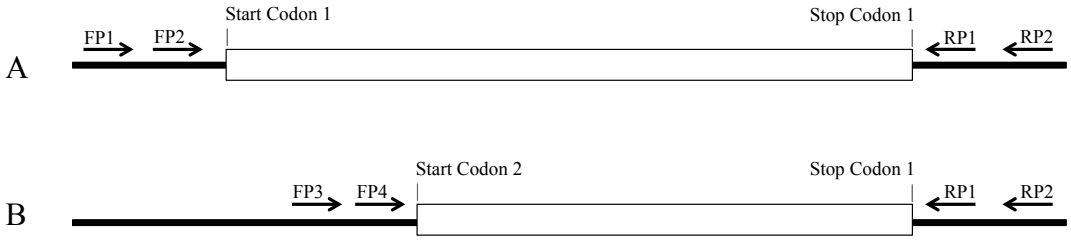

Figure 4. **Combinations of forward and reverse primers used to amplify full length coding regions of *Fox-3* cDNAs** (FP1-RP1, FP1-RP2, FP2-RP1, FP2-RP2, FP3-RP1, FP3-RP2, FP4-RP1, FP4-RP2). Each combination resulted in different amplicon lengths. A: PCR amplicon of *C. oreganus Fox-3* with a start codon (1) toward the 5' end; B: PCR amplicon of *C. oreganus Fox-3* with a start codon (2) downstream toward the 3' end. The amplicon in 'A' results in a protein with 45 additional amino acids at the N-terminus of the Fox-3 protein. For primer sequences, see Table 2.

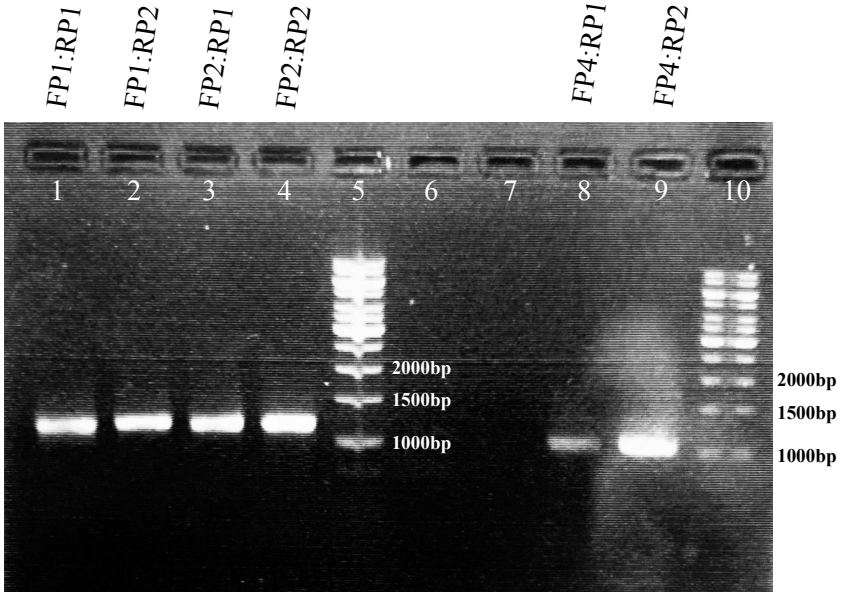

Figure 5. **1.2% agarose gel with *C. oreganus* full-length *Fox-3* cDNA PCR products.** Lane 5&10 contain a Promega 1kb DNA ladder (Promega Corp.). Lanes 1-4 and 8-9 contain forward and reverse primer combinations (Table 2).

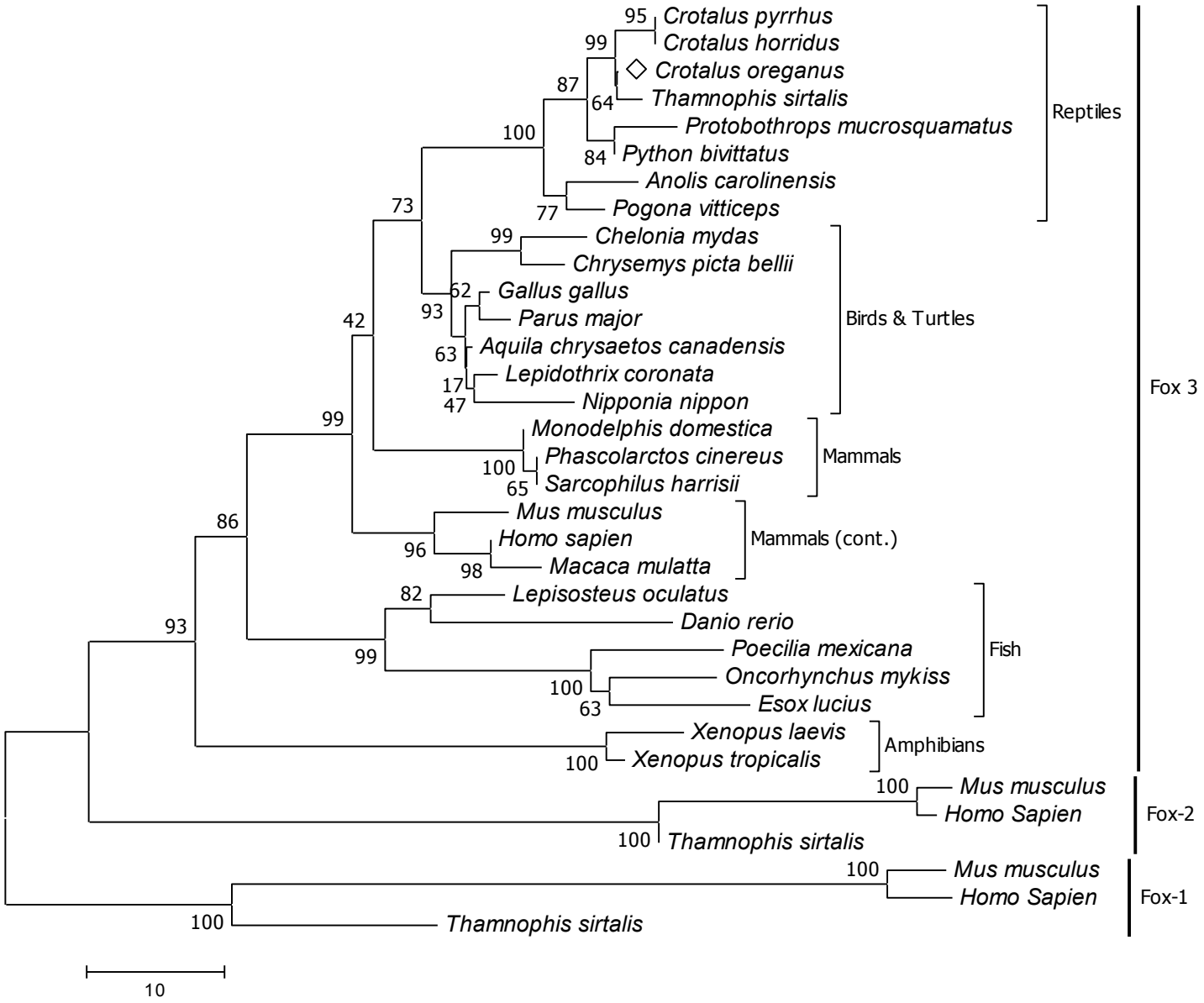

Figure 6. Phylogenetic tree of Fox-3, -2, and -1 based on deduced amino acid sequences from reptiles, birds, mammals, fish, and amphibians. Clades for various vertebrate taxa are marked by brackets, and clades for gene type, Fox-3, Fox-2, and Fox-1 are marked by bolded vertical lines. *C. oreganus* is marked with a diamond, representing the organism sequenced in this study. Tree was assembled with the Neighbor-Joining method with pairwise deletion of gaps and bootstrap values are shown at the nodes. Genbank accession nos. for all taxa are provided in Table 4.

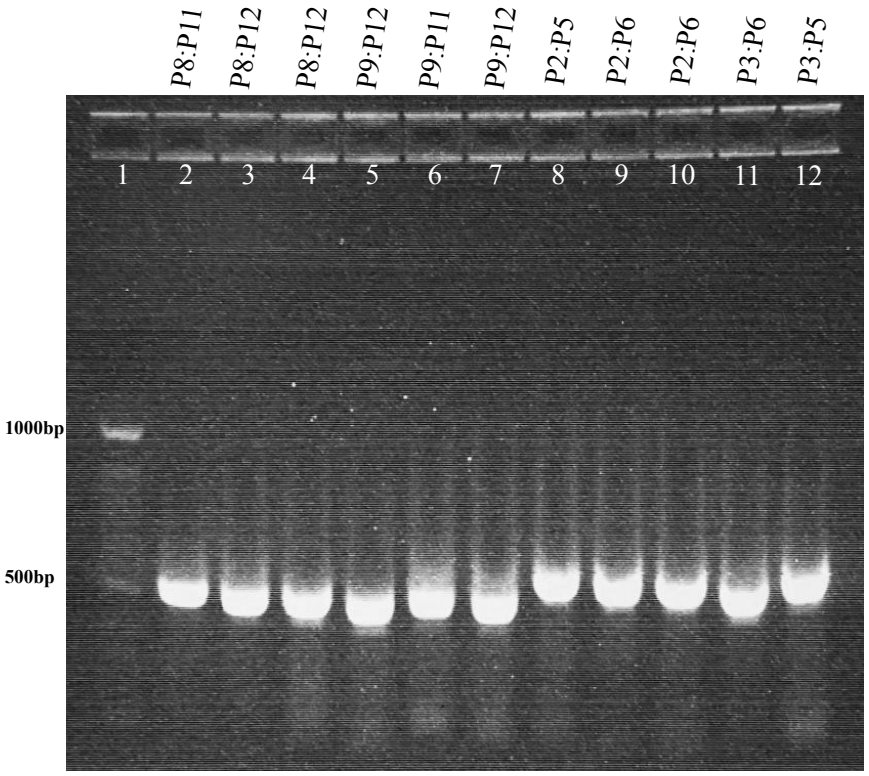

Figure 7 1.2% E-Gel® of nested degenerate primer products. Lane 1 contains Promega 100bp DNA ladder (Promega Corp., Madison, WI, USA). Lane 2-12 contains forward and reverse primer combinations (Table 1). Lanes 2-7 contains products amplified from *S. occidentalis Fox-3* cDNA, while lanes 8-12 contain products amplified from *C. oreganus Fox-3* cDNA.

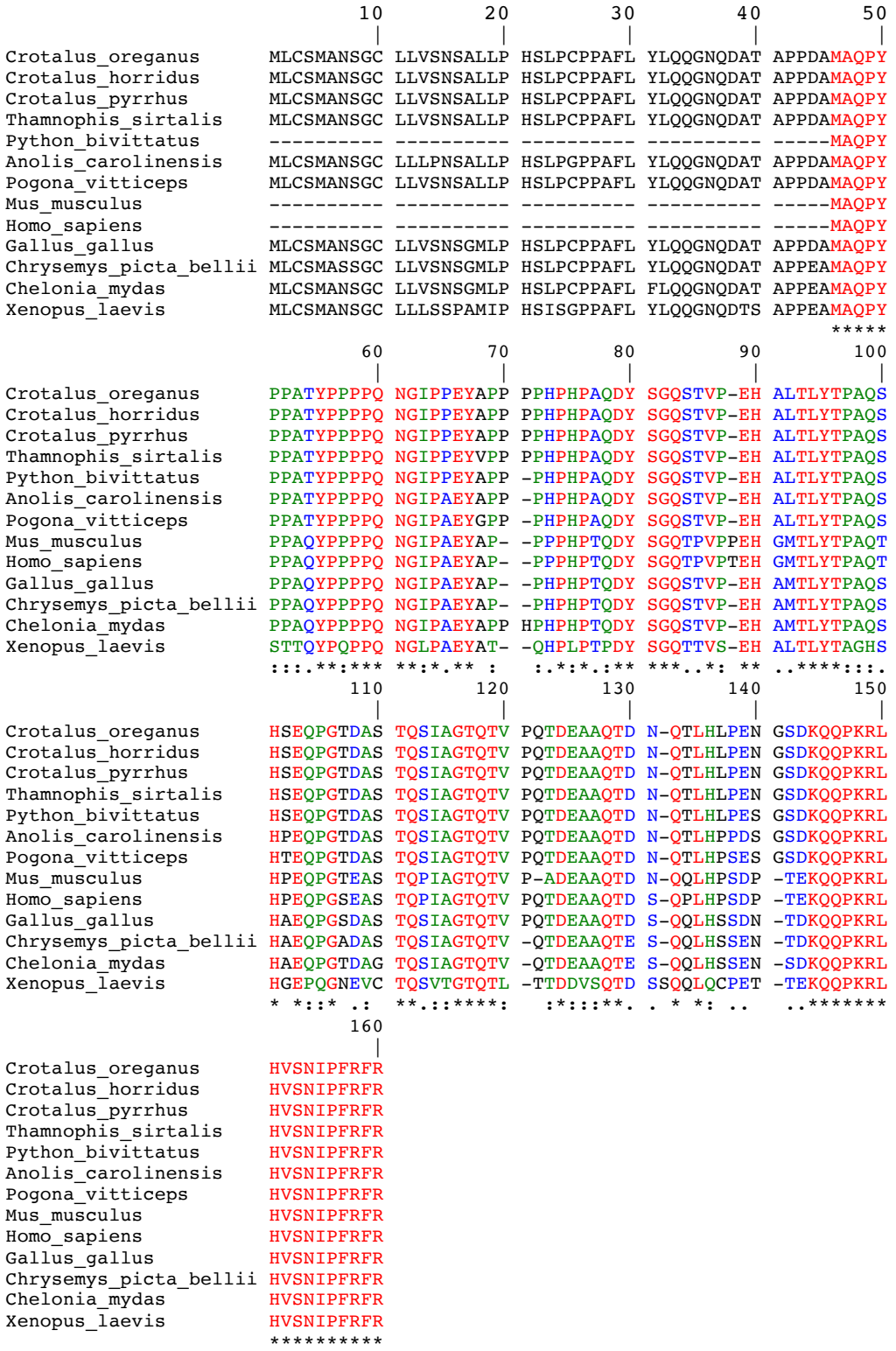

Figure 8. **Amino acid 1-160 alignment of Fox-3 from various taxonomic groups.** Identical amino acids (*; red), strongly similar amino acids (: ; green), weakly similar amino acids (. ; blue), and different amino acids (blank; black) are marked in the figure.

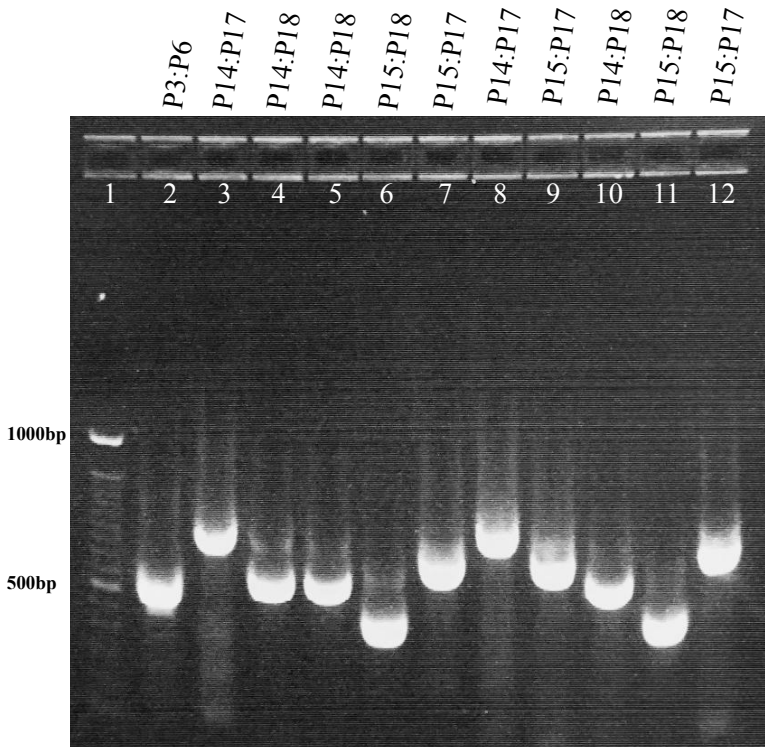

Figure 9. **1.2% E-Gel® of nested degenerate primer products for *S. occidentalis DCX* cDNA and *C. oreganus Fox-3* and *DCX* cDNAs**. Lane 1 contains Promega 100bp DNA ladder (Promega Corp.). Lane 2-12 contains forward and reverse primer combinations (Table 1). Lanes 3-7 contain products amplified from *S. occidentalis DCX* cDNA, while lane 2 (*Fox-3*) & lanes 8-12 (*DCX*) contain products amplified from *C. oreganus* cDNA.

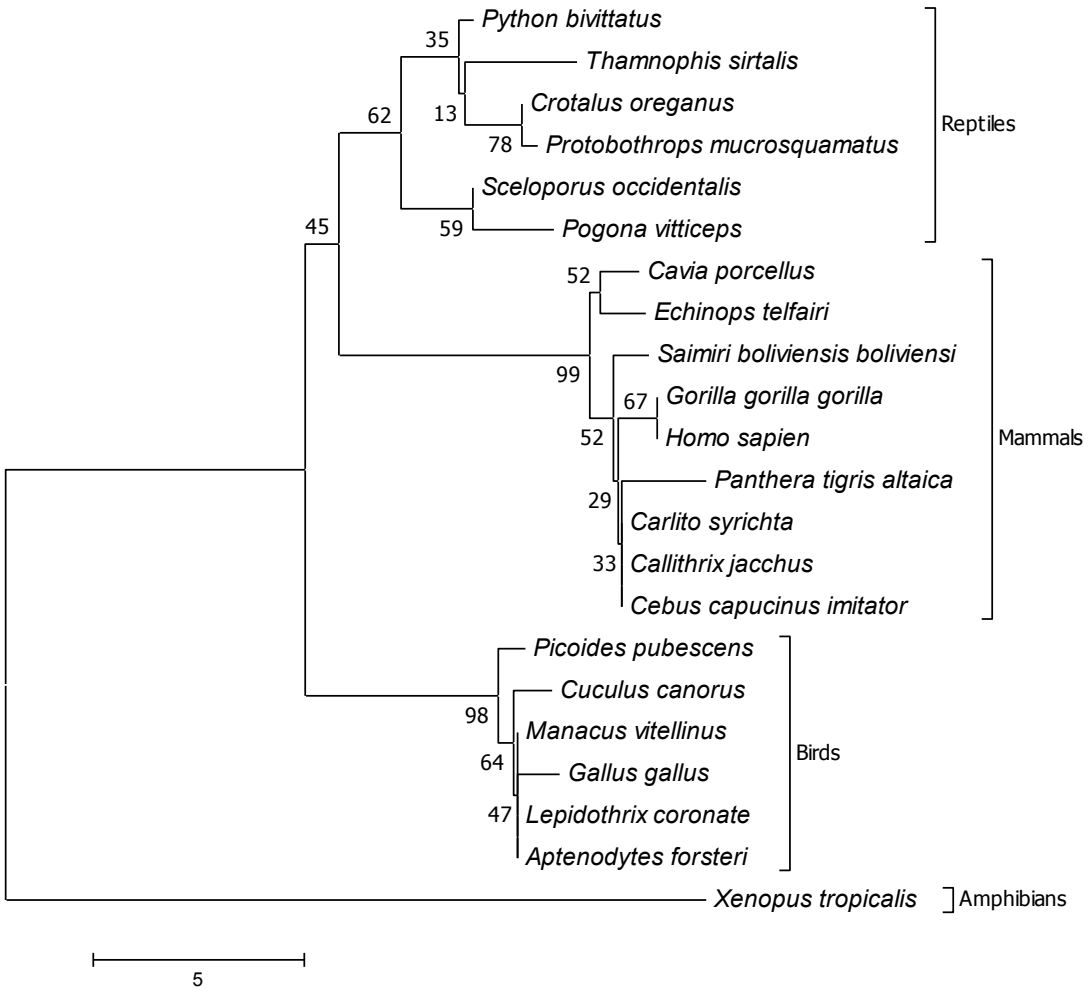

Figure 10. Phylogenetic tree of Doublecortin based on deduced amino acid sequences from reptiles, birds, mammals, and amphibians. Clades for various vertebrate taxa are marked by brackets. Tree was assembled with the Neighbor-Joining method with pairwise deletion of gaps and bootstrap values are shown at the nodes. Genbank accession nos. for all taxa are provided in Table 3.

Table 4. Genbank accession numbers used in DCX protein phylogeny. *Crotalus oregnaus* and *Sceloporus occidentalis* were partially sequenced in this study.

Gene	Scientific Name	Common Name	Accession No.
DCX	*Python Bivitattus*	Burmese python	XP_007424984
	Thamnophis sirtalis	Garter snake	XP_013913438
	Protobothrops mucrosquamatus	Brown spotted pit viper	XP_015677063
	Pogona vitticeps	Central bearded dragon	XP_020640047
	Homo sapiens	Human	NP_001182482
	Panthera tigris altaica	Siberian tiger	XP_015391350
	Callithrix jacchus	Common marmoset	XP_002763212
	Cebus capucinus imitator	Panamanian white-throated Capuchin	XP_017357640
	Echinops telfairi	Lesser hedgehog tenrec	XP_004710111
	Carlito syrichta	Philippine tarsier	XP_021573938
	Cavia porcellus	Guinea pig	XP_003467655
	Aimiri boliviensis boliviensis	Black-capped squirrel monkey	XP_003935896
	Gorilla gorilla gorilla	Western lowland gorilla	XP_004064764
	Manacus vitellinus	Golden-collared manakin	XP_017938755
	Lepidothrix coronata	Blue-crowned manakin	XP_017670887
	Aptenodytes forsteri	Emperor penguin	XP_009274787
	Cuculus canorus	Common cuckoo	XP_009558060
	Picoides pubescens	Downy woodpecker	XP_009898695
	Gallus gallus	Chicken	XP_015133663
	Xenopus tropicalis	Tropical clawed frog	XP_002938827