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Eggs-Only Diet: Its Implications for the Toxin Profile Changes and Ecology of the Marbled Sea Snake (*Aipysurus eydouxii*)

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Abstract. Studies so far have correlated the variation in the composition of snake venoms with the target prey population and snake's diet. Here we present the first example of an alternative evolutionary link between venom composition and dietary adaptation of snakes. We describe a dinucleotide deletion in the only three finger toxin gene expressed in the sea snake *Aipysurus eydouxii* (Marbled Sea Snake) venom and how it may have been the result of a significant change in dietary habits. The deletion leads to a frame shift and truncation with an accompanying loss of neurotoxicity. Due to the remarkable streamlining of sea snake venoms, a mutation of a single toxin can have dramatic effects on the whole venom, in this case likely explaining the 50- to 100-fold decrease in venom toxicity in comparison to that of other species in the same genus. This is a secondary result of the adaptation of *A. eydouxii* to a new dietary habit — feeding exclusively on fish eggs and, thus, the snake no longer using its venom for prey capture. This was parallel to greatly atrophied venom glands and loss of effective fangs. It is interesting to note that a potent venom was not maintained for use in defense, thus reinforcing that the primary use of snake venom is for prey capture.

Key words: Sea snake — Venom gland — Pseudogene — Three-finger toxin — Neurotoxin *Aipysurus eydouxii*

Introduction

The true sea snakes are the most derived lineage of snakes, having undergone many unique specializations to adapt to their marine environment (Heatwole 1999). These snakes are rooted deep within the Hydrophiinae subfamily (Australo–Papuan lineage) of the Elapidae family in the Colubroidea (advanced snakes) (Fig. 1) and remain genetically closely related to their terrestrial Australian ancestors. The sea krait and sea snake lineages independently colonized the oceanic environment (Voris 1977; Schwaner et al. 1985; McCarthy 1986; Minton and da Costa 1975).

Venom composition in snakes exhibits significant geographic variation (Chippaux et al. 1991; Jayanthi et al. 1988; Fry et al. 2002). Daltry et al. (1996) showed that these geographic variations were related to the prey composition of the region. The suscepti-

Nucleotide sequence data reported here have been deposited in the GenBank database under accession number AY559317.

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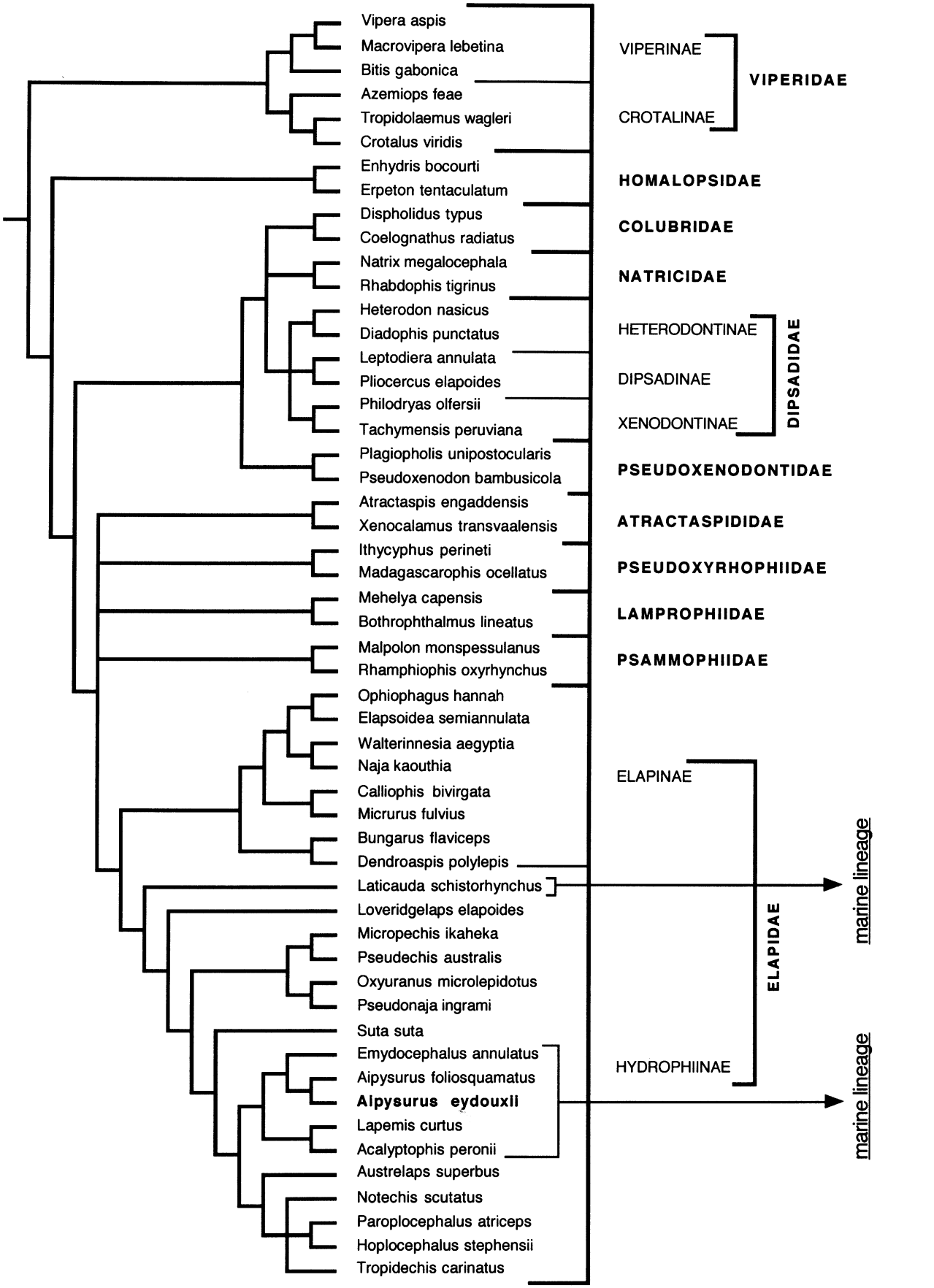


Fig. 1. Colubroidea snake taxonomy and the relative taxonomical positions of the marine lineages (Slowinski and Lawson 2002; Vidal and Hedges 2002; Scanlon and Lee 2004).

bility and availability of prey animals play important roles in the natural selection of the toxin composition. Thus the venom composition is thought to directly reflect the prey animals and hence the feeding habits of the snake (Daltry et al. 1996). Snakes that feed on a wide diversity of prey items need a multiplicity of toxin types in order to counter the variety of prey defense systems and physiological targets, the toxins evolving via the same "birth–death" mode of protein evolution utilized by immunological proteins (Fry et al. 2003a).

According to this process, gene families are created through the process of gene duplication (Nei et al. 1997; Rooney et al. 2002). Over time, some genes get deleted from the genome, through processes such as unequal crossing-over, while some become nonfunctional and degenerate into pseudogenes. As a result, paralogous groups of genes are generated across taxonomic lines if the gene duplication events giving rise to these groups took place before their divergence. These evolutionary patterns are similar to what has been observed in multigene families involved in the adaptive immune response (e.g., immunoglobulins and major histocompatibility complex genes [Nei et al. 1997]). It is believed that gene duplication and subsequent divergence contribute to an organism's ability to react to a wide range of foreign antigens. In an analogous manner, snake toxins must react with diverse compounds in their prey. Thus, a birth-and-death mode of evolution may generate a suite of toxins in order to allow snake predators to adapt to a variety of prey species (Fry et al. 2003a). This favors a diversity of toxin types and multiple isoforms within a particular toxin type. In contrast, fascinatingly both the sea snake and the sea krait venoms are remarkably streamlined in comparison to those of comparable land elapids, suggesting a strong functional association between the relatively simple venoms of these snakes and their teleost-based diet of a single vertebrate class (fish) (Fry et al. 2003b). These toxins have also undergone less functional diversification than in comparable terrestrial venoms (Fry et al. 2003b).

Two toxin types dominate sea snake venom: 3FTx (three-finger toxins) and type I PLA_2 (phospholipase A_2) (Fry et al. 2003b). Both of these toxin types are shared with all other Colubroidea venoms, excluding the Viperidae (Fry et al. 2003b, c; Fry and Wüster 2004). Several neurotoxic 3FTx have been isolated and characterized from sea snake venoms (Fryklund et al. 1972; Liu and Blackwell 1974; Maeda and Tamiya 1976; Tamiya et al. 1983). These toxins bind to the nicotinic site of acetylcholine receptors and block peripheral neuromuscular transmission (Chang 1979). These non enzymatic polypeptides contain 60–65 amino acid residues and belong to the three-finger toxin family; the best characterized are deeply rooted within the type I α-neurotoxin subfamily (Kini 2002; Fry et al. 2003a). In some sea snake venoms up to 70% of their venom proteins are postsynaptic neurotoxins (Guinea et al. 1983) and hence the toxicity of sea snake venoms is fairly high (LD_{50} values range from 0.04 to < 1 mg/kg) and very highly neurotoxic (Chetty et al. 2004). Thus, fast-acting, postsynaptic neurotoxins play a crucial role in immobilization and rapid killing of their prey. This is consistent with the unique problems associated with feeding on fast-moving prey in an aquatic environment, particularly these snakes not having the luxury afforded to their terrestrial cousins in being able to track their prey down at their leisure.

Although most sea snakes feed on fish and eels, two species, *Aipysurus eydouxii* (marbled sea snake) and *Emydocephalus annulatus* (turtle-head sea snake), have evolved to feed virtually exclusively on fish eggs (Voris and Voris 1983). During evolution, they have adapted to their new feeding habits through the evolution of strong throat musculature (to improve suction), consolidation of lip scales (to increase rigidity), reduction and loss of teeth, and greatly decreased body size (McCarthy 1987; Gopalakrishnakone and Kochva 1990). The venom glands and fangs show atrophy and are significantly reduced in size (Gopalakrishnakone and Kochva 1990) and the relative toxicity of the venom is greatly reduced (Tu 1974).

To understand how the unique dietary habit changes the selection pressure on the venom composition, we prepared a cDNA library from *A. eydouxii* venom glands and sequenced 200 clones. Here we report a dinucleotide deletion in the only expressed 3FTx gene, resulting in a truncated, inactive form. We correlate this with the decrease in toxicity and as a result of the change in its dietary habit from fish to fish eggs. Thus we describe how the change in ecology subsequently significantly affected the composition of the venom.

Materials and Methods

Construction of cDNA Library from A. eydouxii *Venom Gland*

A. eydouxii were collected from Albatross Bay in Weipa, Queensland, Australia. Total RNA was extracted from one pair of *A. eydouxii* venom gland using the RNeasy Mini kit (Qiagen, Hilden, Germany). The integrity of the total RNA was analyzed by denaturing formaldehyde agarose gel electrophoresis. The mRNA was purified using the NucleoTrap RNA kit (Macherey–Nagel, Düren, Germany). Enriched full-length double-stranded (ds) cDNA was prepared from mRNA using the Creator SMART cDNA library construction kit (Clontech, Palo Alto, CA). The ds cDNA molecules were digested by *Sfi*I and inserted directionally into a pDNR-LIB vector (Clontech) at the *Sfi*I position and subsequently electrotransformed into *E. coli* $DH_{5\alpha}$ using MicroPulser electro-

poration apparatus (Bio-Rad, Hercules, CA). The cDNA library was titered and amplified.

Screening of cDNA Library

Miniscale plasmid isolations from 200 randomly selected colonies were carried out using the QIAprep spin Miniprep kit (Qiagen). Purified plasmids were submitted to restriction enzyme analysis using *Sfi*I. Clones containing cDNA inserts were selected for sequencing.

DNA Sequencing and Computer Analysis

DNA sequencing was performed using M13 forward and reverse primers. The sequencing reaction was carried out using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA), with an Automated DNA sequencer (Model 3100A; Applied Biosystems). The sequences were compared to those in the GenBank databases using a BLAST search. Nucleic acid and amino acid sequence homologies were obtained using the Vector NTI program (Informax, Frederick, MD).

RT-PCR and TA Cloning

Fresh total RNA isolated from the same species of *A. eydouxii* venom gland was reverse transcribed to cDNA using PowerScript Reverse Transcriptase (Clontech). One pair of primers was synthesized (1st BASE Pte. Ltd., Singapore) based on the sequence encoding neurotoxin obtained from the cDNA library. RT-PCR was carried out on a thermal cycler (Eppendorf Mastercycler; Westbury, NY) using the upstream primer beginning from the start codon 5′-ATGAAAACTCTGCTGCTGACCTT-3′ and the downstream primer ending at the stop codon 5′-CTAATTGTTG CATTCGTTTGTTTG-3′. The samples were subjected to 2 min at 95°C followed by 30 cycles of 30 s at 95°C, 20 s at 56°C, and 30 s at 72°C. The run was terminated by a 5-min elongation step at 72°C. RT-PCR products were analyzed on 1% agarose gels and purified using the QIAquick gel extraction kit (Qiagen). The purified products were inserted into the pGEM-T Easy vector (Promega, Madison, WI) and transformed into *E. coli* $DH_{5\alpha}$. Plasmid isolation, DNA sequencing, and sequence analysis were performed as described above.

Southern Blot Analysis

Genomic DNA extracted from the liver of *A. eydouxii* was digested with *Eco*RI, *Bam*HI and *Cla*I respectively, electrophoresed on 0.8% agarose gels, and transferred onto Hybond-N^+ nylon membranes (Amersham, Piscataway, NJ). The membranes were hybridized with DIG-labeled probe derived from mutant neurotoxin (nucleotides 33–278) and analyzed by chemiluminescent detection (Roche Applied Sciences, Indianapolis, IN).

Peptide Synthesis

Peptide was synthesized by solid phase methods using *Fmoc* chemistry in an automated peptide synthesizer (Model Pioneer; Applied Biosystems). The peptide was cleaved and the protecting groups were removed from the amino acid side chains using a mixture of trifluoroacetic acid (TFA)/ethanedithiol/thioanisole/H_2O (45:2:2:1, v/v) in a final volume of 20 ml. The peptide was purified by HPLC on a Phenomenex Jupiter C18 column (5 μm; 250 × 10 mm) using a linear gradient of acetonitrile in 0.1% TFA in

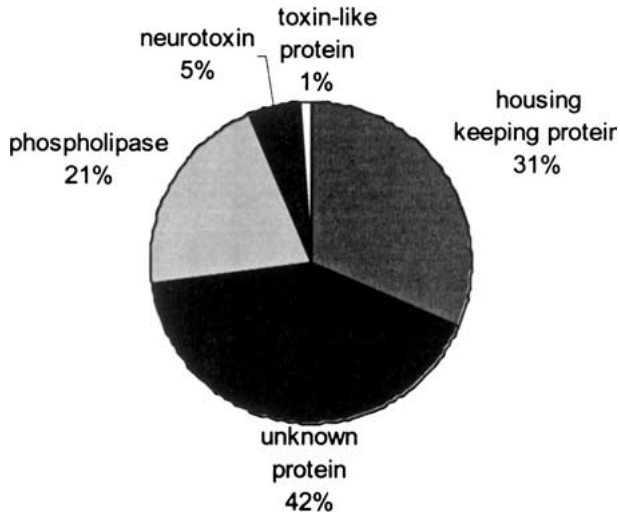


Fig. 2. Composition of the *A. eydouxii* venom gland cDNA library.

a BioCAD Sprint Perfusion Chromatography system (PerSeptive Biosystems, Framingham, MA). The linear peptide was dissolved in 5 ml of 0.1% TFA and immediately diluted into the folding buffer (100 m*M* Tris–HCl, 2 m*M* EDTA, pH 8.5) to a final concentration of 0.1 m*M* to allow oxidative folding (48 h, 22°C). The folded peptides were purified by RP-HPLC. The masses of the folded peptides were determined by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry.

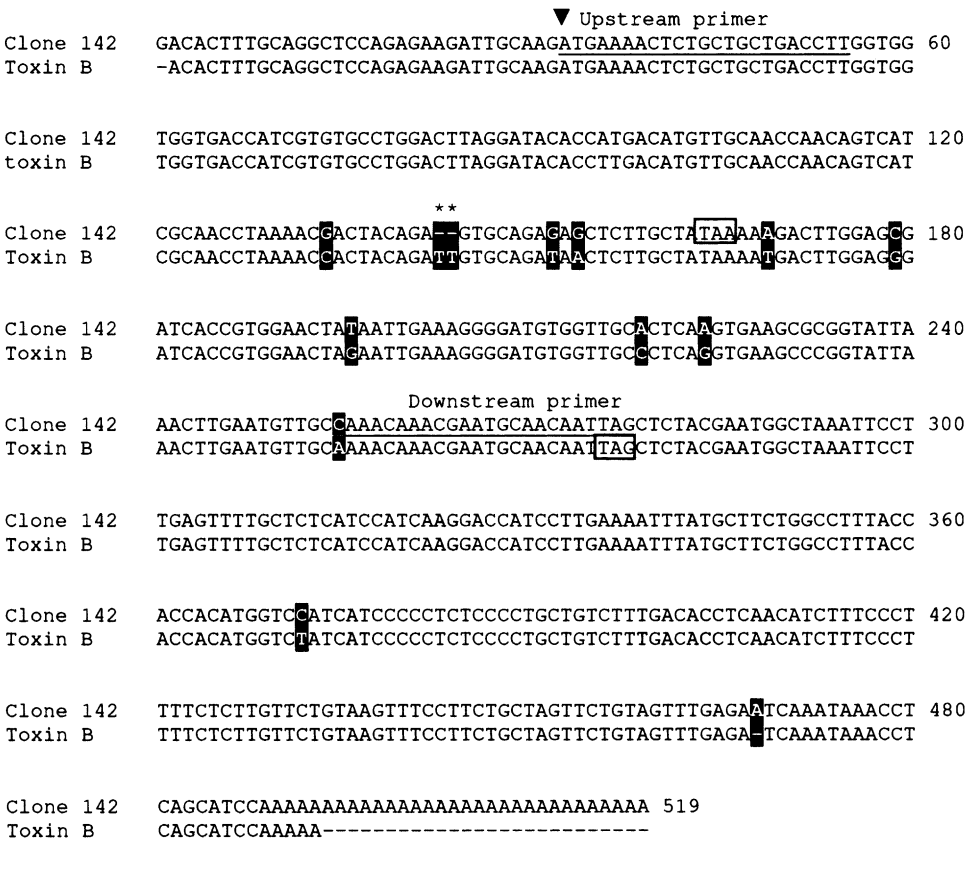
Bioactivity Assay

Swiss albino male mice (~20 g) and zebrafish (~1 g) were used to test the bioactivity of the three isoforms of folded synthetic peptides. The peptides were dissolved in 100 μl of 0.9% (w/v) NaCl and injected (intraperitoneal) into a group of three mice at a dose of 100 mg/kg. Adult zebrafish ($n = 3$) were injected (intramuscular) at three doses — 1, 10, and 100 mg/kg—in 2 μl of 0.9% NaCl. The fish were paralyzed by putting them into a mixture of 2-phenoxethanal:H_2O (1:1000, v/v). Then they were weighted and injected, α-Bungarotoxin and 0.9% NaCl (100 or 2 μl each in mouse and zebrafish, respectively) were used as positive and negative controls, respectively. The animals were monitored for any observable symptoms for 8 h and death in 24 h.

Phylogenetic Analysis

In order to minimize confusion, all proteins sequences are referred to by their Swiss-Prot accession numbers (http://www.expasy.org/cgi-bin/sprot-search-ful). Sequences were aligned using the program CLUSTAL-X (Thompson et al. 1997), followed by visual inspection for errors. The *Aipysurus eydouxii* and representatives of the full 3Ftx diversity (Fry et al. 2003a) were analyzed using Bayesian inference implemented on MrBayes, version 3.0b4 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). The method uses Markov chain Monte Carlo methods to generate posterior probabilities for each clade represented in the tree. The analysis was performed by running a minimum of $1*10^6$ generations in four chains and saving every 100th tree. The log-likelihood score of each saved tree was plotted against the number of generations to establish the point at which the log likelihood scores of the analysis reached their asymptote, and the posterior probabilities for clades were established by constructing a majority

A



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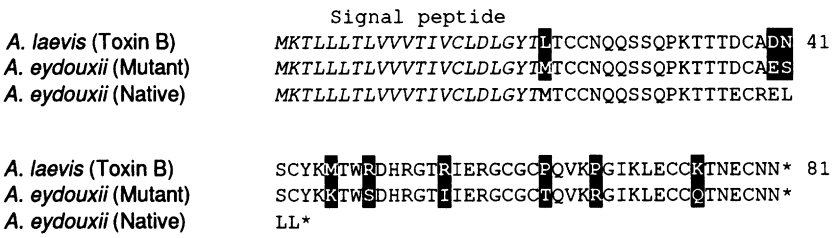

Fig. 3. **A** Comparison of cDNA sequences of neurotoxin isolated from *A. eydouxii* (clone 142; accession No. AY559317) with toxin B of *A. laevis* (accession No. X13373); http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = Nucleotide. The stop codons are boxed. The upstream and downstream primers used in RT-PCR are underlined. The nucleotide differences between clone 142 and toxin B are highlighted. **B** Comparison of amino acid sequences of neurotoxin toxin B isolated from venom of *A. laevis* with the *A. eydouxii* native and mutant (with insertion of the deleted TT dinucleotide) toxin. The signal peptide sequences are italicized. The amino acid differences between the "mutant" toxin and toxin B are shaded.

rule consensus tree for all trees generated after the completion of the burn-in phase. Alignments can be obtained by emailing Dr. Bryan Grieg Fry (bgf@unimelb.edu.au).

Results

We sequenced and analyzed 200 clones from the cDNA library of *A. eydouxii* venom gland (Fig. 2). Ten clones were identical and showed 97% identity to toxin B (Swiss-Prot accession number P19959), a type I α-neurotoxic 3FTx (Fry et al. 2003a) from *A. laevis* venom (Fig. 3A). These clones, however, show a dinucleotide (TT) deletion in the open reading frame resulting in a frame shift and premature termination (Fig. 3B). With the insertion of TT, the deduced amino acid sequence of the "mutant" neurotoxin shows 88% identity with toxin B, indicating that the neurotoxin gene is intact except for the dinucleotide

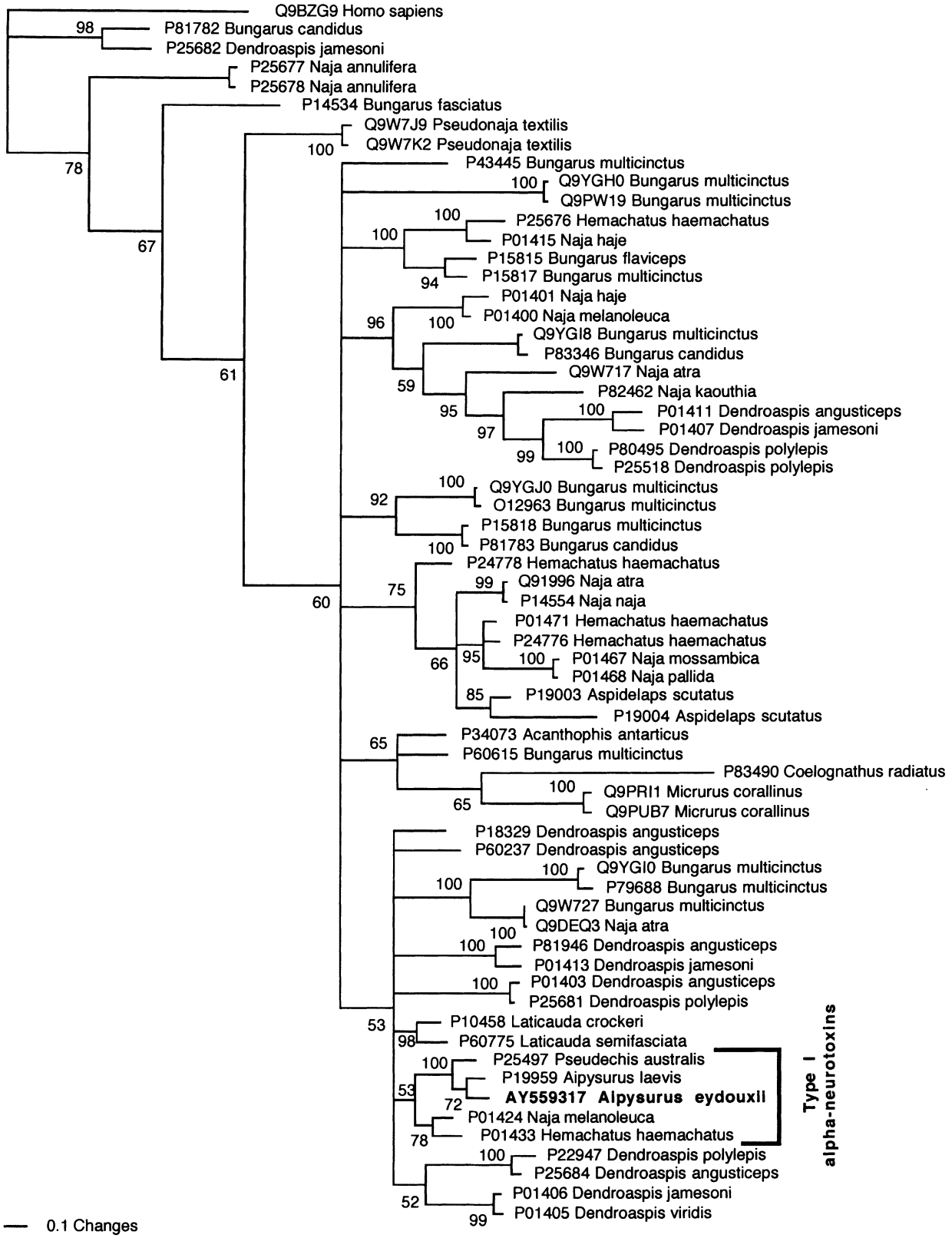

Fig. 4. Phylogenetic comparison of *A. eydouxii* sequence against the full breadth of three-finger toxin diversity (Fry et al. 2003a) utilizing Bayesian analysis (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). To minimize confusion, all sequences are referred to by their Swiss-Prot accession numbers (http://www.expasy.org/cgi-bin/sprot-search-full) except for the *A. eydouxii* sequence, which is referred to by its NCBI accession number (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = Nucleotide). The tree was rooted using Q9BZG9 from *Homo sapiens* as the outgroup.

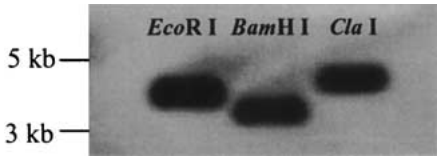

Fig. 5. Southern blot analysis of *A. eydouxii* genomic DNA. Genomic DNA extracted from the liver was digested with *Eco*RI, *Bam*HI, and *Cla*I, respectively, and electrophoressed on 0.8% agarose gels. After the transfer of DNA fragments to Hybond-N^+ nylon membranes, they were hybridized with the coding-region (246-bp) probe.

deletion (Fig. 3B) and also has the C^1C^{1-4A} double cysteine motif characteristic of Australo–Papuan elapids (Fry et al. 2003a). Phylogenetic analysis showed this toxin to be strongly associated with other type I α-neurotoxic 3FTx (Fig. 4).

None of the other toxin clones from the cDNA library showed homology with 3FTx, thus *A. eydouxii* venom gland appears to express only one 3FTx gene. To determine the number of 3FTx expressed, we cloned RT-PCR products from a fresh total RNA extract from *A. eydouxii* venom gland. Since the signal peptide and the carboxy-terminal regions are highly conserved among elapid venom 3FTx (Tamiya 1975), we used primers designed based on these regions as upstream and downstream primers (Fig. 3A) which cover the entire translated region. Fifty independent clones showed identical open reading frames with the same dinucleotide deletion. These results indicate the expression of a single 3FTx in the venom gland. Further, we determined the number of 3FTx genes in *A. eydouxii* by Southern hybridization experiments (Fig. 5). The genomic DNA from *A. eydouxii* was digested with three restriction enzymes and probed with a 246-bp gene-specific probe. All three restriction maps showed a single band. These results strongly support the presence of a single 3FTx gene expressed in *A. eydouxii* venom (Fig. 5).

The natural deletion results in a truncated protein with only 22 residues — almost one-third the length of normal 3FTx (Figs. 3B and 6). This truncated protein has only 3 of the 10 residues, which are identified as the functional site of erabutoxin, an α-neurotoxic 3FTx (Pillet et al. 1993; Trémeau et al. 1995; Antil et al. 1999). Thus one would expect that the trucated form does not show neurotoxicity. To test this hypothesis, we synthesized the 22-residue peptide using Fmoc chemistry. The synthesized peptide was folded and all three disulfide isoforms (Cys3–Cys4, Cys3–Cys17and Cys4–Cys17) were purified; their molecular mass was 2511.5, 2511.9, and 2511.7, respectively (data not shown), which is in agreement with the calculated mass. These three isoforms were injected into mice and zebrafish by intraperitoneal and intramuscular routes, respectively. The extremely

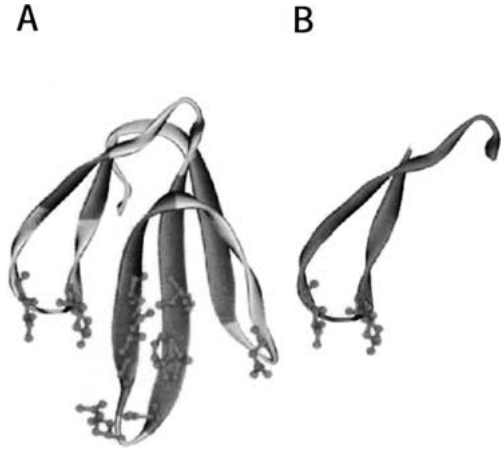

Fig. 6. Three-dimensional structural comparison of erabutoxin a and neurotoxin isolated from *A. eydouxii*. **A** Functional site of erabutoxin a. The residues involved in binding to muscle nicotinic acetylcholine receptors are shown as a ball and stick model. **B** A three-dimensional model of shortened neurotoxin from *A. eydouxii* venom gland. Only 3 of 10 functionally important residues are found.

well-characterized α-bungarotoxin (Chang 1999) was chosen as a positive control. The positive control α-bungarotoxin showed a LD_{50} of 1.41 and 0.31 mg/kg in mice and zebrafish, respectively. However, the animals injected with the synthetic peptides (100 mg/kg) did not exhibit any neurotoxic or other symptoms. Thus the deletion mutation in the *A. eydouxii* neurotoxin gene results in a biologically truncated, inactive polypeptide.

Discussion

In general, the venoms of sea snakes are less complex than those of comparable terrestrial snakes, containing fewer toxins overall and fewer isoforms of each toxin type (Fry 2003b). Postsynaptically active 3FTx account for 70% or more of the total venom and are responsible for the high toxicity of these venoms (Chetty et al. 2004) and consequently are integral to the ability of the snakes to capture prey. As shown here, the only 3FTx gene expressed in *A. eydouxii* venom shows a dinucleotide deletion. Thus it can produce only a truncated, inactive 22-mer peptide, in contrast to the 60- to 65-residue neurotoxic 3FTx active forms. The inactivation of the only major 3FTx gene may explain the relatively low toxicity of *A. eydouxii* venom compared to other species of sea snakes, with this venom being 50–100 times less toxic than other sea snake venoms (Tu 1974).

Due to the remarkable streamlining of sea snake venoms, a strong selection pressure exists against precisely this sort of mutation. Among sea snakes, *A. eydouxii* has an unusal feeding habit. This snake, similar to *Emydocephalus annulatus*, feeds almost

exclusively on fish eggs (Voris and Voris 1983). Had such a radical change in venom occurred prior to the profound dietary shift, it would have decimated the snake's ability to feed, and consequently the snake would most likely not have survived long after birth and almost certainly not for a long enough time to reproduce. In contrast, the change in ecology to a fish egg diet would have removed the selection pressure for the maintence of an effective prey-capturing venom. The fact that the venom was not maintained for defensive use reinforces the primary use of venom in snakes for prey capture rather than for defense (Fry and Wüster 2004). It would be interesting to examine the venom composition of *Emydocephalus annulatus* to see if a comparable change in the venom has occurred, as this sea snake also feeds exclusively on fish eggs (Voris and Voris 1983) and its venom is of an even lower toxicity (Minton 1983). Similarly, it would be worth examining the Australian terrestrial elapids in the *Brachyurophis* genus (shovel-nosed snakes), as these snakes have evolved to feed on lizard eggs and consequently have undergone a convergent loss of venom apparatus similar to that of *A. eydouxii* and *E. annulatus* (Scanlon and Shine 1988). These results reinforce the strong relationship between venom composition and feeding adaptations in snakes (Daltry et al. 1996).

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