Putting the Brakes on Snake Venom Evolution: The Unique Molecular Evolutionary Patterns of *Aipysurus eydouxii* (Marbled Sea Snake) Phospholipase A_2 Toxins

Min Li, Bryan G. Fry,†‡ and R. Manjunatha Kini*§*

*Department of Biological Sciences, Faculty of Science, National University of Singapore, Singapore; †Australian Venom Research Unit, Department of Pharmacology, School of Medicine, University of Melbourne, Parkville, Victoria, Australia; ‡Population and Evolutionary Genetics Unit, Museum Victoria, G.P.O. Box 666E, Melbourne, Australia; and §Department of Biochemistry and Molecular Biophysics, Virginia Commonwealth University, Medical College of Virginia

Accelerated evolution of toxins is a unique feature of venoms, with the toxins evolving via the birth-and-death mode of molecular evolution. The venoms of sea snakes, however, are remarkably simple in comparison to those of land snakes, which contain highly complex venoms. *Aipysurus eydouxii* (Marbled sea snake) is a particularly unique sea snake, feeding exclusively upon fish eggs. Secondary to this ecological change, the fangs have been lost and the venom glands greatly atrophied. We recently showed that the only neurotoxin (a three-finger toxin) gene found in the sea snake *A. eydouxii* has a dinucleotide deletion, resulting in the loss of neurotoxic activity. During these studies, we isolated and identified a number of cDNA clones encoding isozymes of phospholipase A_2 (PLA_2) toxins from its venom gland. Sixteen unique PLA_2 clones were sequenced from the cDNA library and TA cloning of reverse transcription-polymerase chain reaction products. Phylogenetic analysis of these clones revealed that less diversification of the PLA_2 toxins has occurred in the *A. eydouxii* venom gland in comparison to equivalent terrestrial and other marine snakes. As there is no longer a positive selection pressure acting upon the venom, mutations have accumulated in the toxin-coding regions that would have otherwise had a deleterious effect upon the ability to use the venom for prey capture. Such mutations include substitutions of highly conserved residues; in one clone, the active site His^{48} is replaced by Arg, and in two other clones, highly conserved cysteine residues are replaced. These mutations significantly affect the functional and structural properties of these PLA_2 enzymes, respectively. Thus, in *A. eydouxii*, the loss of the main neurotoxin is accompanied by a much slower rate of molecular evolution of the PLA_2 toxins as a consequence of the snake's shift in ecological niche. This is the first case of decelerated evolution of toxins in snake venom.

Introduction

Snake venom toxins form multigene families that are unique bioweapons in the predator-prey arms race (e.g., Daltry, Wuster, and Thorpe 1996). The ''birth-and-death'' model best describes the evolution of these large multigene families (Fry et al. 2003*a*). According to this process, gene families are created through the process of gene duplication (Nei, Gu, and Sitnikova 1997; Rooney, Piontkivska, and Nei 2002). Over time, some genes get deleted from the genome, through processes such as unequal crossing-over, while some others 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.

Previously, we found that the three-finger (3FTx) toxins duplicated and diverged early during the evolutionary history of elapids to form a broad superfamily (Fry et al. 2003*a*, 2003*b*, 2003*c*; Fry and Wuster 2004). However, this superfamily continues to diversify, as shown by our finding of taxon-specific gene clusters. 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, Gu, and Sitnikova 1997). It is believed that gene duplication and subsequent divergence contributes to an organism's ability to react to a wide range of foreign antigens. In an analogous manner, snake toxins must react with diverse molecular targets in their prey. Thus, a birth-and-death mode of evolution generates a suite of toxins in order to allow predatory snakes to adapt to a variety of different prey species.

The sea snakes and sea kraits are unique in the snake world in possessing remarkably streamlined venom (Fry et al. 2003*b*), despite these two lineages independently colonizing the ocean (fig. 1). The venoms of sea snakes are relatively simple because of their specialized diet, consisting of a single class of vertebrates (fish). Such a streamlining of venoms is reflected by the remarkable level of cross-reactivity of sea snake antivenom (Chetty et al. 2004). An extreme divergence in venom evolution can extend even to the secondary loss of toxins (or venom). This has happened independently two times in the sea snake lineages, with *Emydocephalus annulatus* (Turtle-Head Sea Snake) and *Aipysurus eydouxii* (Marbled Sea Snake) both evolving to become obligate fish egg eaters (Glodek and Voris 1982; H. K. Voris and H. H. Voris 1983) with an accompanying loss of fangs and greatly atrophied venom glands (McCarthy 1987; Gopalakrishnakone and Kochva 1990). The toxicity of *A. eydouxii* venom is 40–100 times lower than that of other sea snakes including the other six species in the same genus (Tu 1973, 1974). Similarly, the Australian terrestrial elapid *Brachyurophis* (shovel-nosed snakes) specializes in feeding upon reptile eggs (Scanlon and Shine 1988). The switch over to exclusive egg-feeding habit effectively removes the selection pressure to retain potentially toxic venom. Therefore, any change in toxin sequence (and hence bioactivity) would be a neutral mutation in these snakes as the toxins are not at all being used for prey capture.

Key words: gene duplication, sea snake, venom gland, phospholipase A_2, evolution of toxins.

E-mail: dbskinim@nus.edu.sg.

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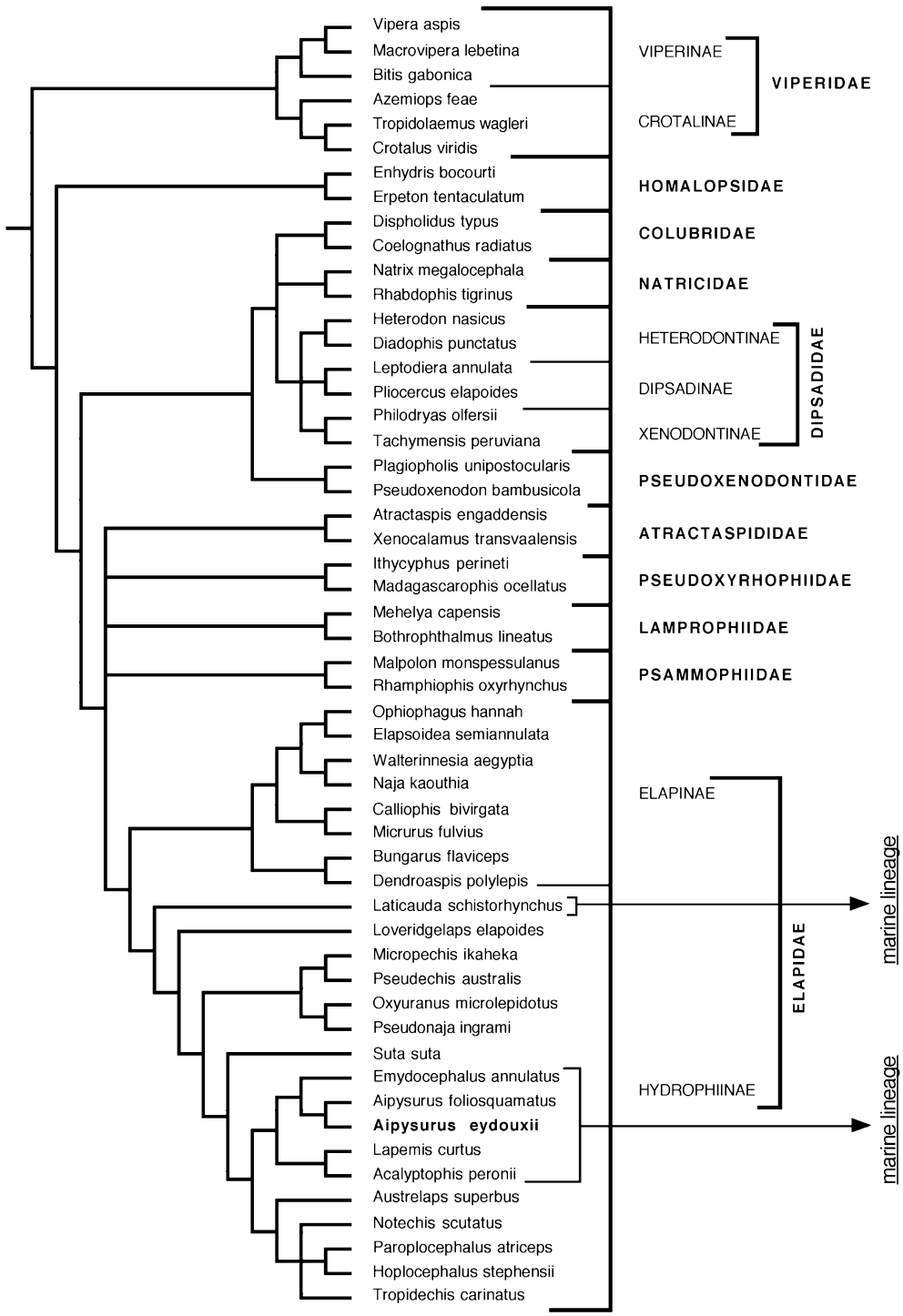

FIG. 1.—Colubroidea snake taxonomy and the relative taxonomical positions of the marine lineages (Slowinski and Lawson 2002; Vidal and Hedges 2002; Scanlon and Lee 2004).

Our earlier studies on the sole 3FTx present in *A. eydouxii* venom revealed that it has degenerated into a nonfunctional form (Li, Fry, and Kini 2005). The other major toxin family in elapid snake venom is the type IA phospholipase A_2 (PLA_2), and these toxins are present in greater diversity than the 3FTx in sea snake venom (Fry et al. 2003*a*). Similar to other toxins, PLA_2 toxins in elapid venoms show evidence of birth-and-death evolution (Slowinski, Knight, and Rooney 1997). These PLA_2 enzymes catalyze the hydrolysis of phospholipids at the *sn*-2 position to release fatty acids and the corresponding 1-acyl lysophospholipids. Unlike their mammalian counterparts, snake venom PLA_2 enzymes are toxic and induce a wide variety of pharmacological effects including neurotoxic, cardiotoxic, myotoxic, anticoagulant, antiplatelet, convulsive, hemolytic, and hemorrhagic effects. Such an

ability to induce diverse biological effects is due to their ability to interact with distinctly different target proteins located on the cell surface of the target tissues (Kini and Evans 1989; Kini 1997, 2003). Venoms from the land snakes, in particular, appear to contain a large number (more than 10) of PLA_2 isoforms due to frequent duplication events (Braganca and Sambray 1967; Shiloah, Klibansky, and de Vries 1973; Vishwanath, Kini, and Gowda 1987, 1988; Takasaki, Suzuki, and Tamiya 1990; Takasaki, Yutani, and Kajiyashiki 1990; Ogawa et al. 1992; Singh et al. 2000; Fry et al. 2003*a*). PLA_2 isozymes within the same snake venom can exhibit distinctly different pharmacological activities. Such a diversification in targeting and expression-induction of different pharmacological effects is due to accelerated evolution of snake toxin genes; the exons appear to mutate faster than the introns in these genes to effect high rate of substitution of amino acid residues (Ogawa et al. 1992; Nakashima et al. 1993, 1995; Nobuhisa et al. 1996; Chuman et al. 2000; Chijiwa et al. 2003). Interestingly, the mutation rates of the surface residues in PLA_2 enzymes are much higher than those of the buried residues, indicating that the accelerated evolutions appears to alter the molecular surface and hence the targeting of PLA_2 enzymes (Kini and Chan 1999).

The purpose of this study was to examine the molecular evolution of the type IA PLA_2 toxins present in *A. eydouxii* venom and to correlate any divergences relative to other snake venoms and to changes in the diet.

Materials and Methods

Construction of *Aipysurus eydouxii* Venom Gland cDNA Library

Aipysurus eydouxii were collected from Albatross Bay in Weipa, Queensland, Australia. Total RNA from one pair of *A. eydouxii* venom glands was extracted by using RNeasy Mini kit (Qiagen, Hilden, Germany). The integrity of total RNA was analyzed by denaturing formaldehyde agarose gel electrophoresis. One microgram of poly (A^+) mRNA was obtained by using NucleoTrap RNA kit (Macherey-Nagel, Düren, Germany). Enriched full-length double-stranded (ds) cDNAs were prepared from mRNA by using Creator™ SMART™ cDNA library construction kit (Clontech, Palo Alto, Calif.). To eliminate the possibility of polymerase chain reaction (PCR) mismatching, primer extension, instead of long-distance PCR, was employed in the generation of ds cDNAs. The ds cDNA molecules were digested by restriction enzyme *Sfi*I and inserted directionally into pDNR-LIB vector (Clontech) at the *Sfi*I position and subsequently electrotransformed into *Escherichia coli* $DH_{5\alpha}$ using MicroPulser™ electroporation apparatus (Bio-Rad, Hercules, Calif.). The successful *A. eydouxii* venom gland cDNA library contained $\sim 1.25 \times 10^6$ independent recombinants.

Screening of cDNA Library

Miniscale plasmid isolations from 200 randomly selected colonies were carried out using QIAprep® spin miniprep kit (Qiagen). Purified plasmids were submitted to restriction enzyme analysis using *Sfi*I. Clones containing cDNA inserts (196 clones; 98% efficiency) were subjected to DNA sequencing.

DNA Sequencing and Computer Analysis

Positive clones were subjected to sequencing using the M13 forward and reverse primers. The sequencing reaction was carried out using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, Calif.), with an automated DNA sequencer (Model 3100A, Applied Biosystems). All DNA sequencing reactions were repeated twice, and all the nucleotide sequences were checked using the Chromas (DNA sequence analysis software, Technelysium Pty Ltd., Tewantin Qld, Australia). The cDNA sequences were compared to those in the GenBank databases using BLAST network services at National Center for Biotechnology Information. Nucleic acid and amino acid sequence homologies were obtained using Vector NTI program (Informax, Frederick, Md.).

Reverse transcription-PCR and TA Cloning

To amplify all possible cDNAs encoding PLA_2, two rounds of reverse transcription (RT)-PCR was conducted. Fresh total RNA isolated from *A. eydouxii* venom gland was reverse-transcribed to cDNAs using PowerScript Reverse Transcriptase (Clontech). Gene-specific upstream primer P1 (5′-ATGTATCCTGCTCACCTTCTGGTC-3′) was designed according to the conserved signal peptide sequences, and the downstream primer P2 (5′-CCTTGCGCTGAAGCCTCTCAAATA-3′) was the sequences followed by the stop codon. PCR was carried out on a Thermal Cycler (Eppendorf Mastercycler, Westbury, N.Y.). The samples were subjected for 5 min to 95°C followed by 30 cycles of 1 min at 94°C, 20 s at 52°C, and 30 s at 72°C. The run was terminated by a 5 min elongation step at 72°C. High-fidelity polymerase of Advantage™ 2 (Clontech) was used during PCR amplification to eliminate the PCR mismatching. RT-PCR products were analyzed on 1% agarose gels and purified using QIAquick® gel extraction kit (Qiagen). Then, the purified products of ~470 base pairs were inserted into pGEM®-T Easy vector (Promega, Madison, Wis.) and transformed into *E. coli* $DH_{5\alpha}$. One hundred positive clones were sequenced, and the plasmid isolation, DNA sequencing, and computer analysis of these clones were performed as described above.

Phylogenetic Analysis

To minimize confusion, all protein sequences are referred to by their Swiss-Prot accession numbers (http://www.expasy.org/cgi-bin/sprot-search-ful) except for the toxins presented here, which are referred to by their GenBank accession numbers (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide&itool=toolbar). Sequences were aligned using the program CLUSTAL-X (Thompson et al. 1997), followed by visual inspection for errors. The *A. eydouxii* PLA_2 sequences and representatives of the full PLA_2 toxin diversity 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.

Table 1
Relative Abundance of PLA_2 Isoforms Isolated from *Aipysurus eydouxii* cDNA Library and TA Cloning

Clone	cDNA Library	TA Cloning
AY561154[a]	6	56
AY561155	1	1
AY561156	1	2
AY561157[a]	1	1
AY561158	1	1
AY561159	1	6
AY561160[b]	1	12
AY561161	1	2
AY561162[b]	1	1
AY561163		3
AY561164		2
AY561165[a]		1
AY561166		5
AY561167		2
AY561168		3
AY561169		2
Total	14	100

[a] Two separate single synonymous mutations occurred in AY561157 and AY561165 compared to AY561154.

[b] One single synonymous mutation occurred in AY561160 compared to AY561162.

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

Results

From *A. eydouxii* venom gland cDNA library, 14 clones showed homology to type IA PLA_2 enzymes, the complete cDNA sequences coded for nine unique isoforms (AY561154, AY561155, AY561156, AY561157, AY561158, AY561159, AY561160, AY561161, and AY561162). Among these clones, AY561154 was found to be the most abundant (six clones) and all others had only one clone each (table 1). These clones contained 34 nucleotides (nt) of 5′ untranslated regions (UTRs), 81 nt of signal peptide sequence, 354 nt of protein-coding region, and 183 nt of 3′ UTRs. Sequence analysis showed that 5′ and 3′ UTRs (99%) were more conserved than protein-coding regions (96%), similar to those of other snake PLA_2 genes. However, the sectional homologies for protein-coding regions in the *Laticauda semifasciata*, *Austrelaps superbus*, and crotalinae snake venom gland PLA_2 genes are ~90%, ~80%, and ~70%, respectively, indicating that *A. eydouxii* venom gland PLA_2 enzymes are less diversified than those from the terrestrial and other marine snakes (Kihara et al. 1992; Nakashima et al. 1993, 1995; John, Smith, and Kaiser 1994; Ogawa et al. 1992, 1995, 1996; Nobuhisa et al. 1996; Shimohigashi et al. 1996; Kordis, Bdolah, and Gubensek 1998; Ohno et al. 1998; Chuman et al. 2000; Singh et al. 2000). In addition, 12 other clones with shorter inserts contained only the 3′ UTRs of PLA_2 genes. To obtain more PLA_2 clones and to investigate in the nucleotide substitutions in coding region, two rounds of RT-PCR

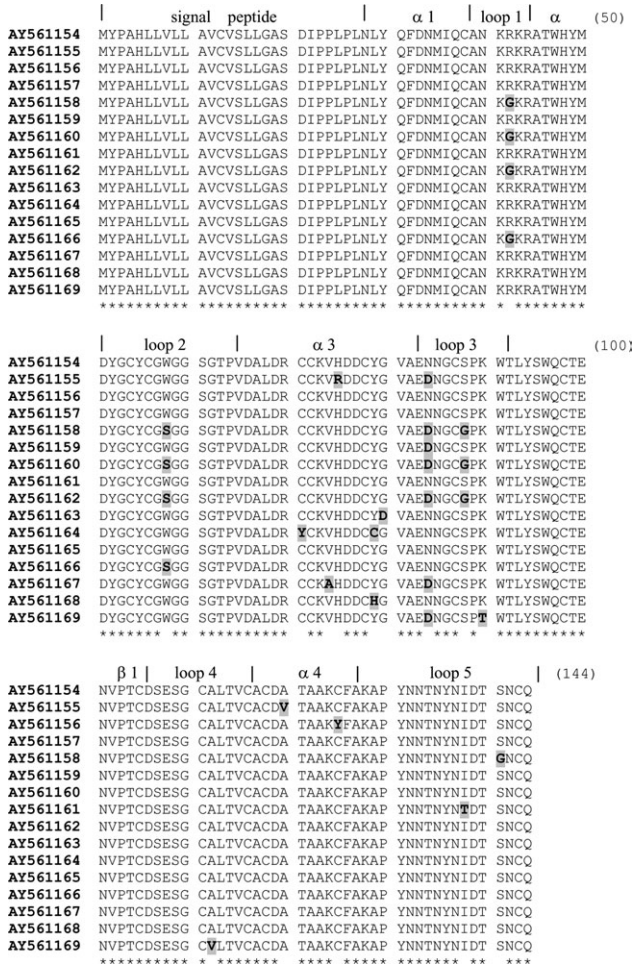

FIG. 2.—Amino acid sequences deduced from the nucleotide sequences of their cDNAs encoding *Aipysurus eydouxii* venom gland PLA_2. Asterisks show the consensus region. The bold residues indicate deviation from the consensus. The regions of α−helices (α 1–α 4), β-sheet (β 1), and loops 1–5 are indicated.

were performed and 100 positive clones were sequenced. While some of the clones were the same as sequenced from the cDNA library (table 1), seven additional sequences were obtained (AY561163, AY561164, AY561165, AY561166, AY561167, AY561168, and AY561169).

In total, 16 cDNAs encoding PLA_2 isozymes in the *A. eydouxii* venom gland were cloned employing both random screening of cDNA library and TA cloning of RT-PCR product. All of them have the same signal peptide sequences consisting of 27 amino acid residues, and overall, the sequences were highly similar (fig. 2). In the coding regions of 16 PLA_2 enzymes, a total of 33 bases mutated; 30 nt substitutions caused a change in the amino acid residue, while 3 nt substitutions caused no change in the amino acid residue. Thus, in most cases, the base substitution leads to substitution of amino acid residues. Two separate single synonymous mutations occurred in AY561157, and AY561165 compared to AY561154, and a single synonymous mutation distinguishes AY561160 from AY561162 without any change in their amino acid sequences. Thus, a total of 13 isozymes were identified with one through five

amino acid substitutions, which are classified as type IA PLA_2 enzymes based on the absence of pancreas loop.

Applying the methods described in *Materials and Methods*, we confirmed that all amino acid changes were caused by single nucleotide substitutions. Further, all these nucleotide sequences were observed in at least two independent clones, except AY561165, which shows a single synonymous substitution (table 1). Therefore, the observed changes in the nucleotide sequences most likely reflected the changes that occurred in the cDNAs encoding PLA_2 isozymes rather than due to PCR and sequencing errors. A detailed analysis showed that the nucleotide substitution rate on the first (13/33, 39.39%) and second positions (17/33, 51.51%) are at a higher frequency compared to the third position (3/33, 9.10%) of the codons.

Phylogenetic analysis revealed that the *A. eydouxii* PLA_2 toxins are all orthologs of each other relative to all other sequences (fig. 3). The relative distances among *A. eydouxii* PLA_2 enzymes were also much shorter than those shown in toxins from a single species in the terrestrial or other marine snakes.

Discussion

Multiple PLA_2 isozymes are found typically in individual snake venoms (Ogawa et al. 1992; Singh et al. 2000; Fry et al. 2003*b*). The phenomenon of accelerated evolution in the protein-coding region compared to UTRs was first observed in the cDNAs encoding PLA_2 isozymes from *Protobothrops flavoviridis* venom gland (Ogawa et al. 1992). Since then, accelerated evolution of PLA_2 enzymes is well documented in snake venom, such as in crotalid (e.g., *Crotalus scutulatus scutulatus*, *Ovophis okinavensis*, *Protobothrops flavoviridis*, and *Trimeresurus gramineus*), viperid (e.g., *Daboia palaestinae* and *Vipera ammodytes*), and elapid venom (e.g., *Austrelaps superbus* and *Naja kaouthia*) (Kihara et al. 1992; Nakashima et al. 1993, 1995; John, Smith, and Kaiser 1994; Ogawa et al. 1995, 1996; Nobuhisa et al. 1996; Shimohigashi et al. 1996; Kordis, Bdolah, and Gubensek 1998; Ohno et al. 1998; Chuman et al. 2000; Singh et al. 2000).

The results of this study show that *A. eydouxii* is unique among the snakes studied to date in that the venom is evolving at a much lower rate than comparative terrestrial snakes and even other marine snakes. While few species have been comprehensively studied and thus the sequences available do not fully represent the full diversity of toxins, a large number of terrestrial and marine snake PLA_2 toxin sequences are available in the databases (>100). Phylogenetic analysis of all the available marine snake toxins and representative terrestrial snake toxins is revealing. The toxins from the terrestrial snakes and all the other marine species (true sea snake as well as sea krait) were paraphyletic for a particular species that had multiple toxins sequenced, and species-level paralogs were widespread (fig. 3). In contrast, all the *A. eydouxii* sequences formed a monophyletic group and were species-level orthologs. In addition, the *A. eydouxii* sequences formed a very tight clade in comparison to toxins from other marine species or the terrestrial snake. Consistent with previous mass spectrometry results of sea snake venoms (Fry et al. 2003*b*), a single PLA_2 transcript was in much greater quantities than the others. Clone AY561154 was the most abundant PLA_2, being present in six times the amounts of the others (table 1). However, this transcript was neither the most basal nor the most recently derived sequence.

While several gene duplication events are evident in the *A. eydouxii* sequences, the highly conserved sequences and low distances separating the toxins reveal an extremely low rate of diversification. Similarly, while the mutations occur with the unique toxin gene bias toward positions 1 and 2 of the codons in *A. eydouxii* PLA_2 enzymes, there are very few mutations relative to PLA_2 enzymes from other marine or terrestrial snakes.

As there is no longer a positive selection pressure acting upon the *A. eydouxii* venom for prey capture, mutations have accumulated that would have otherwise had a deleterious effect. The few mutations present have occurred in key structural and functional residues, resulting in toxins likely to have greatly diminished usefulness in prey capture. In PLA_2 enzymes, several residues that are essential for the enzymatic activity are highly conserved. These residues are involved in the catalytic network (His^{48}, Asp^{49}, Tyr^{52}, Tyr^{73}, and Asp^{99}), the hydrophobic region around the enzymatic site contributing to the substrate binding (Leu^{2}, Phe^{5}, Tyr^{22}, Cys^{29}, Cys^{45}, Ala^{102}, Ala^{103}, and Phe^{106}), and in the catalytically important Ca^{2+} binding (Tyr^{28}, Gly^{30}, Gly^{32}, and Asp^{49}) (Scott 1997). Substitution of these residues often leads to loss of catalytic properties. For example, in a subgroup of group II PLA_2 enzymes, Asp^{49} is replaced by Lys^{49} (Maraganore and Heinrikson 1986) or Ser^{49} (Chijiwa et al. 2003). Asp^{49} is the most important residue involved in coordinating the Ca^{2+} ion that plays a crucial role in catalysis. Lys^{49}-PLA_2 enzymes lack the ability to bind Ca^{2+}, and hence, they exhibit very low or no catalytic activity (Maraganore and Heinrikson 1986; de Oliveira et al. 2001).

In the present study, a very interesting finding is that the site of active site His^{48} in AY561155 is replaced with Arg^{48} (fig. 2). The proposed catalytic mechanism of PLA_2 depends on the crucial role of His^{48}; it polarizes and attracts a proton from a positionally conserved water molecule, which then participates in the formation of a tetrahedral intermediate. Upon collapse of the intermediate, hydrolysis products are released and three water molecules move into the active site (Scott et al. 1990). The substitution of His^{48} in AY561155 by Arg^{48} most likely disrupts the catalytic network, leading to significant, if not complete, reduction in the catalytic activity. This is the first natural substitution of active site His^{48} in PLA_2 enzymes.

Snake venom PLA_2 enzymes possess a common scaffold with four α-helices and one β-sheet, which are connected by five loops (Ohno et al. 1998). This scaffold is held together by six to seven highly conserved disulfide bonds (Dufton and Hider 1983). Interestingly, in one of the sequences presented here, AY561164, Cys^{44} is replaced by a Tyr^{44} and Tyr^{53} is replaced by Cys^{53} (fig. 2). In AY561156 PLA_2, Cys^{125} is replaced by Tyr^{125}, resulting in odd number of Cys residues. It would be interesting to see how these substitutions affect the conserved disulfide bonds and folding as well as the catalytic properties of these proteins.

In general, most of the amino acid substitutions in the *A. eydouxii* sequences (22 out of 30) are found in the surface loops; 4, 4, 11, 1, and 2 amino acid changes occurred in

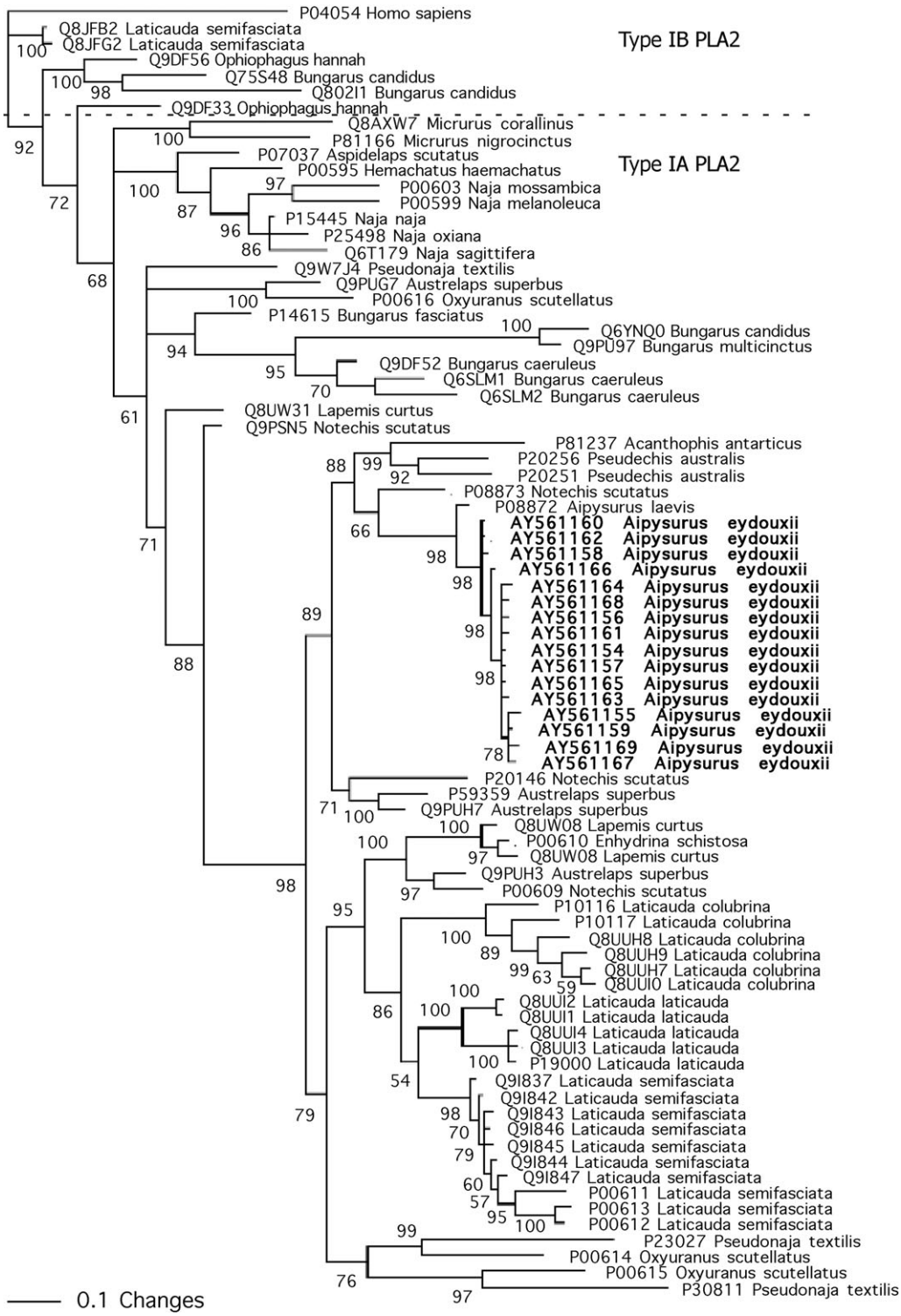

FIG. 3.—Phylogenetic comparison of *Aipysurus eydouxii* sequences against the full-breadth of type I PLA_2 toxin diversity (Fry et al. 2003*a*) utilizing Bayesian analysis (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). Included in the data set were all the available sea snake and sea krait sequences and representative terrestrial snake sequences. To minimize confusion, all sequences are referred to by their Swiss-Prot accession numbers (http://www.expasy.org/cgi-bin/sprot-search-ful) except the *A. eydouxii* sequences which are referred to by GenBank accession number (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide&itool=toolbar). The *Homo sapiens* sequence was used as the out-group.

loop 1 through 5, respectively (fig. 2). Six and two amino acid changes occurred in helices 3 and 4, respectively. This corroborates our earlier finding that the higher rate of accelerated mutation occurs in the surface residues than in the buried residues in snake venom PLA_2 enzymes (Kini and Chan 1999). Such natural substitution in the surface residues contributes to modifying the molecular surface to afford distinct and novel targeting to cells or tissues.

In snake venom PLA_2 enzymes, only one amino acid change has resulted in significant difference in their properties. For example, two Lys^{49}-PLA_2 called BP I and BP II from *P. flavoviridis* venom show only one amino acid replacement in loop 3 (Asp/Asn^{67}) but exhibit considerably different potencies in enzymatic activity and in contraction of the isolated muscle tissue (Shimohigashi et al. 1995, 1996; Ohno et al. 1998). Thus, while the mutational rate is greatly slowed down in *A. eydouxii* toxins, it is likely that some of the PLA_2 enzymes from *A. eydouxii* may have distinct physiological functions if they are indeed active.

Conclusion

Here, we report that PLA_2 enzymes from the *A. eydouxii* venom gland have the properties of accelerated evolution similar to other snake venom PLA_2 enzymes: (1) more substitutions occur in the protein-coding regions than in the UTRs; (2) nonsynonymous substitutions occur more frequently than synonymous ones; and (3) mutations occur more commonly on the molecular surface of the PLA_2 enzymes. Some of the point mutations in the protein-coding regions are likely to affect the functional and structural properties of *A. eydouxii* PLA_2 enzymes. However, phylogenetic analysis revealed an extremely low rate of diversification of PLA_2 enzymes in the *A. eydouxii* venom gland in comparison to terrestrial and other marine snakes. Therefore, in contrast to all the other snake venoms analyzed to date, gene duplication-diversification and accelerated evolution plays a minimal role in the evolution of *A. eydouxii* PLA_2 enzymes. While the proteins are evolving via the birth-and-death model, births are few, and the new toxins do not ''grow'' much and have premature deaths. We conclude that such a slower evolution of PLA_2 enzymes in *A. eydouxii* is secondary to the recent dietary switch of the snake to a food source (fish eggs) that does not require the use of venom for prey capture, and thus, the positive pressure of natural selection on the mutation of *A. eydouxii* PLA_2 enzymes is reduced significantly. Thus, in *A. eydouxii*, the loss of the main neurotoxin (Li, Fry, and Kini 2005) is accompanied by a much slower rate of molecular evolution of the PLA_2 toxins as a consequence of the snake's shift in ecological niche. This is a very unique situation. It is possible that the evolution of the toxins, particularly PLA_2 enzymes, was occurring at the same accelerated rate in *A. eydouxii* (or its ancestor) as in other snake venoms. However, when its diet changed to exclusively fish eggs, the evolutionary rate slowed down as the positive pressure of natural selection dropped significantly. This is the first time such a kind of evolution mode (initially accelerated evolution, actually decelerated evolution) has been reported for any snake venom toxin.

Supplementary Materials

Nucleotide sequence data reported here have been deposited in the GenBank database under the accession numbers AY561154–AY561169.

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