Regional evolution of venom-gland phospholipase A₂ isoenzymes of *Trimeresurus flavoviridis* snakes in the southwestern islands of Japan

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Conventional chromatographic analysis showed that phospholipase A₂ (PLA₂) isoenzymes of the venom of *Trimeresurus flavoviridis* (Habu snake) of Okinawa island are profoundly different in composition from those of *T. flavoviridis* of Amami-Oshima and Tokunoshima islands. The most striking feature was that myotoxic [Lys⁴⁹]PLA₂ isoenzymes, called BPI and BPII, which are expressed abundantly in the venoms of Amami-Oshima and Tokunoshima *T. flavoviridis*, are missing from the venom of Okinawa *T. flavoviridis*. Northern blot analysis of Okinawa *T. flavoviridis* venom-gland mRNA species showed the absence of BPI and BPII mRNA species. Analysis by single-stranded conformational polymorphism-PCR of venom-gland mRNA species of *T. flavoviridis* from three islands, with reference to five DNA species each encoding different PLA₂ isoenzymes from Tokunoshima *T. flavoviridis* venom gland, also suggested that BPI and BPII mRNA species are not expressed in Okinawa *T. flavoviridis* venom gland. In contrast, genomic Southern blot analysis with a variety of probes showed that only the bands corresponding to the upstream and downstream regions of the genes for BPI and/or BPII can be detected in Okinawa *T. flavoviridis*. These results suggested that the genes for BPI and BPII in Okinawa *T. flavoviridis* genome had been inactivated

to form pseudogenes. Differently from Amami-Oshima and Tokunoshima *T. flavoviridis* genomic DNAs, PCR amplification of the segments of BPI and BPII genes between the 5' moiety of second exon and the middle portion of second intron failed for Okinawa *T. flavoviridis* genomic DNAs. In sequence analysis of the two segments involving polymorphism between BPI and BPII genes, which are located in first exon and third exon, respectively, only one base was detected at the polymorphic positions for pseudogene in Okinawa *T. flavoviridis* genome. Based on these facts, it became evident for pseudogene that the upstream region of BPI gene down to the 5' moiety of second exon and the downstream region of BPII gene starting from the middle portion of second intron are in a linked form with a possible insertion. Such observations suggest that venom-gland genes for PLA₂ isoenzymes in *T. flavoviridis* snakes isolated for one to two million years have evolved independently. Their evolution is regional and seems, from several lines of consideration and observation, to be adaptive to the environment.

Key words: adaptation, chromatography, gene screening, pseudogene, species differentiation.

INTRODUCTION

Phospholipase A₂ (PLA₂; EC 3.1.1.4) isoenzymes, which constitute major toxic proteins in snake venoms, catalyse the hydrolysis of the 2-acyl ester bond of 3-*sn*-phosphoglycerides with the requirement of Ca²⁺ to produce 3-*sn*-lysophosphoglycerides and fatty acids. More than a hundred PLA₂ forms have been isolated from snake venoms and sequenced [1]. PLA₂ forms are classified into groups I and II on the basis of the mode of disulphide bond pairings [2]. Elapidae (Elapinae and Hydrophiinae) snake venoms contain group I PLA₂ forms, whereas Viperidae (Viperinae and Crotalinae) snake venoms contain group II PLA₂ forms. Group II PLA₂ forms are divided into two subgroups, [Asp⁴⁹]PLA₂ forms and [Lys⁴⁹]PLA₂ forms [3]. They are known to exhibit a variety of physiological functions [1,4], although they have the same scaffold [5,6].

Trimeresurus flavoviridis (Habu snake), which belongs to the Crotalinae subfamily, inhabit the southwestern islands of Japan: Amami-Oshima, Tokunoshima and Okinawa. These islands are thought to have been separated one to two million years ago [7].

Amami-Oshima island is the northernmost and Tokunoshima island is 30 km south of Amami-Oshima island; Okinawa island is located a further 120 km south of Tokunoshima island. Five PLA₂ isoenzymes have been isolated from Tokunoshima *T. flavoviridis* venom. They all consist of 122 amino acid residues and have been well studied so far: [Asp⁴⁹]PLA₂, called PLA2 (pI 7.9, highly lipolytic and myolytic) [8,9]; basic [Asp⁴⁹]PLA₂, called PLA-B (more basic than PLA2, oedema-inducing) (Y. Yamaguchi, Y. Shimohigashi, T. Chijiwa, S. Hattori and M. Ohno, unpublished work); the most basic [Asp⁴⁹]PLA₂, called PLA-N (neurotoxic) (S. Tsunasawa, N. Oda, D. Tsuru, S. Hattori and M. Ohno, unpublished work); and two [Lys⁴⁹]PLA₂ forms called BPI and BPII (both exhibiting extremely low lipolysis and strong myolysis) [10,11]. It should be noted that BPI and BPII are identical in sequence except at position 59: BPI contains an Asp residue and BPII contains Asn [10,11]. The cDNA species and genes encoding PLA2, BPI and BPII have been cloned [12,13]. Two cDNA species encoding PL-X', which is similar to PLA-B, and [Thr³⁸]PLA₂, which belongs to the [Asp⁴⁹]PLA₂ subgroup, have also been cloned [12]. All genes have a common

Abbreviations used: BPI, [Lys⁴⁹]PLA₂ (Asp⁵⁹); BPII, [Lys⁴⁹]PLA₂ (Asn⁵⁹); PLA₂, phospholipase A₂; PLA2, [Asp⁴⁹]PLA₂; PLA-B, basic, oedema-inducing [Asp⁴⁹]PLA₂; PLA-N, most basic, neurotoxic [Asp⁴⁹]PLA₂; PL-X', [Asp⁴⁹]PLA₂ similar to PLA-B; RT-PCR, reverse-transcriptase-mediated PCR; SSCP, single-stranded conformational polymorphism; UTR, untranslated region.

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Figure 1 Diagram of the structure common to *T. flavoviridis* venom-gland genes for PLA₂ isoenzymes together with sense (rightward arrows) and anti-sense primers (leftward arrows) for amplification of the fragments

The nucleotide positions indicated are those for the gene encoding BPI. Exons are shown as boxes and the protein-encoding regions are black. The hatched areas are the 5' and 3' UTRs. The primers are divided into common type, PLA2 type, BPI or BPII type and PL-X' type. Their positions and sequences are listed in Table 1.

structure consisting of four exons and three introns, as shown in Figure 1 [13]. Examination of the nucleotide sequences of these genes revealed that Darwinian-type accelerated evolution has occurred only in the mature protein-coding regions to acquire diverse physiological activities [13,14].

Our studies so far indicated that protein constituents of Amami-Oshima *T. flavoviridis* venom seem to be similar to those of Tokunoshima *T. flavoviridis* venom, although it recently became evident that metalloprotease isoenzymes are different in their sequences (M. Nakai, Y. Shimohigashi, T. Ogawa, S. Hattori and M. Ohno, unpublished work). The composition and sequence of PLA₂ isoenzymes in Amami-Oshima and Tokunoshima *T. flavoviridis* venoms are largely similar. However, in the study reported here, we found by chromatographic methods that the PLA₂ isoenzymes of Okinawa *T. flavoviridis* venom are quite different in composition from those of Amami-Oshima and Tokunoshima *T. flavoviridis* venoms and that, in particular, peaks corresponding to BPI and BPII are missing from Okinawa *T. flavoviridis* venom. For this reason an attempt was made to clarify, on the basis of genomic analysis, why BPI and BPII are not expressed in the Okinawa *T. flavoviridis* venom gland. The evolution of regional changes of venom-gland PLA₂ isoenzyme genes in isolated *T. flavoviridis* snakes is discussed.

MATERIALS AND METHODS

Materials

Restriction endonucleases and other enzymes were obtained from Takara Shuzo (Kyoto, Japan). *Taq* DNA polymerase was from Promega Biotech (Madison, WI, U.S.A.). The radioactive reagents [α -³²P]dCTP (3000 Ci/mmol), [γ -³²P]dATP (5000 Ci/mmol) and [α -³⁵S]dATP (1000 Ci/mmol) were obtained from Amersham (Little Chalfont, Bucks., U.K.) and Daiichi Pure Chemicals (Tokyo, Japan). Nitrocellulose and nylon membranes were products of Schleicher and Schuell (Dassel, Germany) and Amersham respectively. Specific oligonucleotide primers were custom-synthesized by Hokkaido System Service (Sapporo, Japan). Other reagents used were from Sigma and Wako Pure Chemicals (Osaka, Japan) and were of reagent grade.

Total RNA species were extracted from the venom glands of Amami-Oshima, Tokunoshima and Okinawa *T. flavoviridis* snakes and were purified by using a Dynabeads mRNA puri-

fication kit (Dynal, Lake Success, NY, U.S.A.). First-strand cDNA species were synthesized and amplified with an RNA PCR kit AMV (version 2.1) (Takara). Genomic DNA species were extracted from the liver of *T. flavoviridis* by the method of Blin and Stafford [15].

Column chromatography

The crude venoms (5.0 g) of Tokunoshima and Okinawa *T. flavoviridis* were fractionated on a Sephadex G-100 column (5.4 cm × 100 cm) [10,11]. The resulting second peak, containing PLA₂ activity, was chromatographed on a CM-cellulose (Whatman CM52) column (3.2 cm × 100 cm) under the conditions described in the legend to Figure 2.

Egg-yolk assay

The PLA₂ activity was routinely assayed by using an egg-yolk emulsion (pH 8.0), as described by Ishimaru et al. [16]. Fatty acids released enzymically were titrated with 0.01 M NaOH at pH 8.0 on a Radiometer RTS-5 titration assembly. The activity was assessed by calculating the rate of alkali uptake against fatty acids liberated.

Labelling of DNA species and oligonucleotides

DNA fragments were labelled with [α -³²P]dCTP by the random priming method [17]. Oligonucleotide primers and probes indicated in Figure 1 were labelled at the 5' end with [γ -³²P]dATP and T4 polynucleotide kinase [18]. Their positions and sequences are listed in Table 1.

Southern blot analysis

This was conducted as reported previously [18]. *T. flavoviridis* liver genomic DNA species (5 μ g) were digested with *Bam*HI, *Hind*III, *Sph*I or *Sma*I for 20 h at 37 °C. The resulting DNA fragments were subjected to electrophoresis on 0.7% agarose gels, transferred to nylon membranes and then hybridized with the probes, which were derived from cDNA species or genes encoding venom-gland PLA₂ isoenzymes of Tokunoshima *T. flavoviridis*. The probes were: U, *Eco*RI/*Acc*I fragment corresponding to the 5' untranslated region (UTR) of PLA2 cDNA [9]; A, *Acc*I/*Pst*I fragment corresponding to the protein-coding region of PLA2 cDNA [9]; B, *Kpn*I/*Xba*I fragment corresponding

Table 1 Primers used for PCR

The nucleotide positions refer to those in each of Tokunoshima *T. flavoviridis* venom-gland PLA₂ isoenzyme genes [13].

Type	Primer	Position	Sequence
Common	OGBS2	—114 to —92	TGGAGAGTTCCTGAAGATGGCCC
	CHO5	194–216	GATTCGGGAGGATGAGGACTCTC
	OIP-1	217–237	TGGATAATGGCCGTGTTGCTG
	CHO3	1879–1901	GCAAAGCTGGCACCTGTTTATTA
PLA2	CA3	549–570	TCCGTAAGCACTGTACGAAAGT
BPI or BPII	GL2EU	490–510	GATGGGAGCCTGGTCCAATTG
	GL2EUm	542–563	AGCTGCTAAAACTACGGCTTA
	GL2I1	1015–1035	GCCATCCAGGGTTTGTACAA
	GL3EUo	1315–1336	ATGTGCACAAATGCTGTACAA
	GL3EU	1349–1370	CTGCGACCCCAAAATGGACAGC
	OGBA1	494–516	CTTCCACAATGGACAGGCTCC
	GL2ED	543–563	TAAGCCGTAGTTTTAGCAGC
	GL2EDo	547–568	CCGTATAAGCCGTAGTTTTAG
	GL3ED	1374–1397	GGCCTTGTCTTCAGCTATAGGA
	CB3	1680–1700	CACACCTGCTTCAGGCATGGT
PL-X'	GL4ED	1777–1797	TGCCTTCTTGACGAAAGGTTT
	CX3	1776–1796	GCATTTCTCTGTAGGGTCCGT

to the protein-coding region of BPI cDNA [12]; 2B, PCR fragment amplified for genomic DNA species with sense primer GL2EU (positions 490–510) and anti-sense primer GL2ED (positions 543–563), corresponding to the middle portion of the second exon of the genes for BPI and BPII [13]; and 4B, PCR fragment amplified for genomic DNA species with sense primer GL3EU (positions 1349–1370) and anti-sense primer GL4ED (positions 1777–1797), corresponding to the region from the second half of the third exon to the first half of the fourth exon of the genes for BPI and BPII [13]. Hybridization was performed at 65 °C in 0.5 M Na₂HPO₄/NaH₂PO₄ (pH 7.2) containing 1 mM EDTA and 7% (w/v) SDS [19]. The filters were washed twice each in 2 × SSC (1 × SSC is 0.015 M sodium citrate/0.15 M NaCl), 0.5 × SSC and 0.05 × SSC at 65 °C for 30 min.

The nucleotide positions refer to those of the corresponding genes unless indicated otherwise.

Northern blot analysis

This was performed according to the method of Chirgwin et al. [20]. Total RNA species (10 µg) from the venom gland were denatured with glyoxal and DMSO, subjected to electrophoresis on 1.2% (w/v) agarose gels, transferred to nylon membranes and hybridized with the specific probes described above. The membranes were washed in a similar manner to that described above.

Reverse-transcriptase-mediated PCR (RT-PCR) for PLA₂ isoenzyme mRNA species

RT-PCR was conducted in accordance with the protocol of the RNA PCR kit AMV (version 2.1) for mRNA species from each of the venom glands of Amami-Oshima, Tokunoshima and Okinawa *T. flavoviridis*. Primers used for RT-PCR were prepared by reference to the structures of Tokunoshima *T. flavoviridis* PLA₂ isoenzyme cDNA species [12]. The sense primer was OIP-1, corresponding to positions 73–93 of PLA2 cDNA (positions 217–237 of its gene); it could anneal to all isoenzyme cDNA species and genes. The anti-sense primers were: CA3, positions 162–183 of PLA2 cDNA (positions 549–570 of its gene); CB3, positions 355–375 of BPI cDNA (positions 1680–1700 of its

gene); and CX3, positions 580–600 of PL-X' cDNA (corresponding to positions 1776–1796 of the gene for PLA2). The PCR conditions were as follows: 94 °C for 1 min, then 30 cycles of 94 °C for 30 s, 64 °C for 1 min and 72 °C for 1 min. DNA fragments were subjected to electrophoresis on 2% (w/v) agarose gels and stained with ethidium bromide.

Single-stranded conformational polymorphism (SSCP)-PCR for PLA₂ isoenzyme DNA species

SSCP-PCR is one of the simplest techniques for detecting mutations, in which the target sequence of interest can be amplified by PCR to separate mutated DNA fragments as single-stranded forms by non-denaturing PAGE [21]. Sequential variants usually show shifted mobilities; the shift is believed to be caused by a mutation-induced change in the tertiary structure of single-stranded DNA [21–23]. RT-PCR was conducted for venom-gland mRNA species from Tokunoshima, Amami-Oshima and Okinawa *T. flavoviridis* with CHO5 (positions 50–72 of PLA2 cDNA; positions 194–216 of its gene) and CHO3 (positions 557–579 of PLA2 cDNA; positions 1879–1901 of its gene) as sense and anti-sense primers respectively; their sequences are common to all PLA₂ isoenzyme cDNA species and genes. PCR was also made with the same primers for cDNA species encoding PLA2, BPI, BPII, PL-X' and [Thr³⁸]PLA₂ and the products, designated PLA2, BPI, BPII, PL-X' and [Thr³⁸]PLA₂ respectively, were employed as controls. The PCR products were mixed with formamide dye [95% (v/v) formamide/0.05% Xylene Cyanol/0.05% Bromophenol Blue/20 mM EDTA], denatured at 80 °C for 5 min, subjected to electrophoresis on non-denaturing 5% (w/v) polyacrylamide gels and stained with a silver stain kit (Wako Pure Chemicals, Osaka, Japan).

Copy numbers of PLA₂ isoenzyme genes

The numbers of genes encoding PLA₂ isoenzymes were estimated by dot-blot analysis. Liver genomic DNA (10 µg) of *T. flavoviridis* from three islands and the gene for BPI [13] diluted approx. to 1–18 copies of gene equivalence, where the genome size of *T. flavoviridis* is assumed to be equal to that of human (3 × 10⁹ bp), was applied to nylon membranes to be cross-linked by UV. The filters were hybridized with probes U and 2B and washed with 0.05 × SSC at 65 °C. Signal intensities were measured with a Fuji Bio Image analyser BAS 2000 (Fuji Film Co., Tokyo, Japan).

Screening of genomic DNA library with BPI cDNA

Extracted genomic DNA species were partly digested with *Sau*3AI. A fraction containing fragments (10–20 kb) obtained by sucrose-density-gradient centrifugation was incorporated into EMBL3 vector (Stratagene) (1.1 × 10⁶ primary recombinants). The DNA library was amplified once in *Escherichia coli* strain LE392 and screened by the plaque hybridization method [18] with BPI cDNA [12]. Hybridization was performed at 65 °C for 12 h in a mixture of 6 × SSC, 10 × Denhardt's solution (Denhardt's solution is 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% BSA), 1% (w/v) SDS and 100 µg/ml denatured and sonicated herring sperm DNA. The filters were washed twice each in 2 × SSC, 1 × SSC and 0.2 × SSC at 65 °C for 15 min.

PCR and direct sequencing of polymorphic segments

For amplification of the segments of the genes for BPI and BPII that contain polymorphisms at nucleotide positions 236 (first exon) and 1353 (third exon) between these genes [13], PCR was

performed for genomic DNA of Okinawa and Tokunoshima *T. flavoviridis*. The primers employed were CHO5 (positions 194–216)-OGBA1 (positions 494–516) for the first exon and GL3EUo (positions 1315–1336)-GL3ED (positions 1374–1397) for the third exon. The amplified DNA species were subjected to electrophoresis on 1.8% (w/v) agarose gels and the gel plugs containing the appropriate bands were cut out. The DNA species were recovered by extraction with phenol and sequenced by the direct sequencing method [24] with the use of Sequencing Pro (Toyobo, Osaka, Japan).

RESULTS

Fractionation of PLA₂ isoenzymes from the crude venoms

After gel filtration of the crude venoms of Tokunoshima and Okinawa *T. flavoviridis* snakes on a Sephadex G-100 column [10,11], the fraction containing PLA₂ activity (the second peak) was applied to a CM-cellulose column [10,11]. As shown in Figure 2, elution profiles are different between *T. flavoviridis* snakes from the two islands. In particular, the peaks corresponding to the BPI and BP II [Lys⁴⁹]PLA₂ forms of Tokunoshima *T. flavoviridis* venom are missing from Okinawa *T. flavoviridis* venom. Two peaks, a and b, from Okinawa *T. flavoviridis* venom exhibited PLA₂ activity. PLA₂ from peak a had the same amino acid sequence as PLA₂ of Tokunoshima *T. flavoviridis* venom. PLA₂ from peak b was similar to PLA-B from Tokunoshima *T. flavoviridis* venom, with three amino acid substitutions (T. Chijiwa, S. Hattori and M. Ohno, unpublished work).

Figure 2 Chromatography on a CM-cellulose column of Sephadex G-100-fractionated venom proteins from Tokunoshima and Okinawa *T. flavoviridis*

The crude venoms (5.0 g) were gel-filtered on a Sephadex G-100 column (5.4 cm × 100 cm) [10,11]. The proteins (2.15 g) from the second peak were charged on a CM52 column (3.2 cm × 100 cm) equilibrated with 0.02 M ammonium acetate, pH 6.8, and eluted with linear concentration gradient to 0.6 M ammonium acetate over 2.0 litres. The flow rate was 18 ml/h and 5 ml fractions were collected. PLA₂ from peak a of Okinawa *T. flavoviridis* venom is identical with PLA₂ from Tokunoshima *T. flavoviridis* venom. PLA₂ from peak b is similar to PLA-B from Tokunoshima *T. flavoviridis* venom, with three amino acid substitutions (T. Chijiwa, S. Hattori and M. Ohno, unpublished work).

Figure 3 Southern blot analysis of genomic DNA of *T. flavoviridis* from three islands

Liver genomic DNA (5 µg) was digested with *Bam*HI, *Hind*III and *Sph*I (each 25 units), subjected to electrophoresis on 0.7% agarose gels, blotted to nylon membranes and hybridized with probe U (the 5' UTR of PLA₂ cDNA). Lanes o, Okinawa; lanes t, Tokunoshima; lanes a, Amami-Oshima. Nucleotide size markers are shown to the right of the Figure.

Figure 4 Northern blot analysis of venom-gland mRNA of *T. flavoviridis* from three islands

Total RNA (10 µg) from venom gland was denatured with glyoxal and DMSO, subjected to electrophoresis on 1.2% (w/v) agarose gels, transferred to nylon membranes and hybridized with probes A (for PLA₂ mRNA) and B (for BPI and BP II mRNA). Lanes o, Okinawa; lanes t, Tokunoshima; lanes a, Amami-Oshima.

Southern blot analysis

The digests of liver genomic DNA species from Okinawa, Tokunoshima and Amami-Oshima *T. flavoviridis* with *Bam*HI, *Hind*III and *Sph*I were subjected to hybridization with probe U (the 5' UTR of PLA₂ cDNA), which is expected to hybridize with all PLA₂ isoenzyme genes [12,13]. The hybridization patterns of Okinawa *T. flavoviridis* genomic DNA were partly different from those of Tokunoshima and Amami-Oshima *T. flavoviridis* genomic DNA (Figure 3), reflecting the difference in chromato-

Figure 5 Electrophoretogram of DNA fragments obtained by RT-PCR for venom-gland mRNA of *T. flavoviridis* from three islands for examining the expression of the PLA₂ isoenzymes PLA2, BPI and/or BPII and PL-X'

Sense primer OIP-1 and a mixture of anti-sense primers of CA3 (for PLA2 mRNA), CB3 (for BPI and/or BPII mRNA) and CX3 (for PL-X' mRNA) were employed for amplification. DNA fragments were subjected to electrophoresis on 2% (w/v) agarose gels and stained with ethidium bromide. Lanes o1–o6, Okinawa; lanes t1–t3, Tokunoshima; lanes a1–a3, Amami-Oshima. Nucleotide size markers are shown to the right of the figure.

Figure 6 SSCP analysis of DNA fragments

DNA fragments *PL-X'*, *BPI*, *BPII*, [*Thr*³⁸]*PLA*₂ and *PLA*₂ (lanes 1–5 respectively) were prepared by PCR with primers CHO5 and CHO3 for cDNA species encoding *PL-X'*, *BPI*, *BPII*, [*Thr*³⁸]*PLA*₂ and *PLA*₂ respectively. DNA fragments (lanes 6–8 respectively) were amplified by RT-PCR for venom-gland mRNA of *T. flavoviridis* from Tokunoshima, Amami-Oshima and Okinawa as shown, with the same primers as above. DNA species were subjected to 5% PAGE and stained with a silver stain kit.

graphic profiles of the PLA₂ isoenzymes between Tokunoshima and Okinawa *T. flavoviridis* (Figure 2).

Northern blot analysis

This analysis of venom-gland mRNA species with probes A (the protein-coding region of PLA2 cDNA) and B (the protein-coding region of BPI cDNA) suggested that, in contrast with PLA2 mRNA, BPI and BPII mRNA species are not expressed only in Okinawa *T. flavoviridis* venom gland (Figure 4).

RT-PCR examination of the expression of BPI and BPII mRNA species

This was performed for venom-gland mRNA species of *T. flavoviridis* from three islands with sense primer OIP-1 (positions 73–93 of PLA2 cDNA, a common sequence for PLA₂ isoenzyme cDNA) and a mixture of anti-sense primers CA3 (positions 162–183 of PLA2 cDNA), CB3 (positions 355–375 of BPI and BPII cDNA) and CX3 (positions 580–600 of PL-X' cDNA). The numbers of specimens of *T. flavoviridis* were three for Tokunoshima, three for Amami-Oshima and six for Okinawa. An electrophoretogram of the PCR products showed that no expression of BPI and BPII mRNA species occurred for all specimens of Okinawa *T. flavoviridis* (Figure 5).

SSCP-PCR analysis of PLA₂ isoenzyme DNA species

Each of five DNA species, PLA2, [*Thr*³⁸]*PLA*₂, *BPI*, *BPII* and *PL-X'*, which were prepared by PCR for the corresponding cDNA species with CHO5 and CHO3 as primers, showed the two bands of sense and anti-sense strands after PAGE under non-denaturing conditions (Figure 6, lanes 1–5). SSCP analysis of the RT-PCR fragments amplified with CHO5 and CHO3 for Tokunoshima and Amami-Oshima *T. flavoviridis* venom-gland mRNA species (Figure 6, lanes 6 and 7) showed all the bands observed in Figure 6, lanes 1–5. In contrast, DNA species from Okinawa *T. flavoviridis* venom-gland mRNA species gave fewer bands in which the bands corresponding to those derived from BPI or BPII mRNA species could not be seen (Figure 6, lane 8). However, two sets of sense and anti-sense DNA species that were presumably derived from *PL-X'* mRNA (lane 1) and PLA2 mRNA (lane 5) could be seen. The existence of these two isoenzymes has been verified by the sequence determination of cDNA species isolated from the Okinawa *T. flavoviridis* venom gland cDNA library (results not shown).

Copy numbers of genes for PLA₂ isoenzymes

To estimate the numbers of genes encoding PLA₂ isoenzymes, genomic DNA from Amami-Oshima (two specimens),

Figure 7 Southern blot analysis of genomic DNA species of *T. flavoviridis* from three islands with probes 2B (A) and 4B (B)

Probes 2B and 4B corresponded to the internal portion of the second exon (positions 490–563) and the segment covering the third to fourth exons (positions 1349–1797) of the genes for BPI and BPII respectively, as shown in the top panel. Liver genomic DNA species were digested with *Sma*I/*Bam*HI, subjected to electrophoresis on 0.7% agarose gels, transferred to nylon membranes and hybridized with the probes. Lanes a, Amami-Oshima; lanes t, Tokunoshima; lanes o, Okinawa. Nucleotide size markers are shown to the right of the Figure.

Tokunoshima (three specimens) and Okinawa *T. flavoviridis* (four specimens) was subjected to hybridization with probes U (the 5' UTR of PLA₂ cDNA) and 2B (positions 490–563 of the genes for BPI and BPII, corresponding to the internal portion of the second exon). Because probe U can hybridize with the genes for all PLA₂ isoenzymes, the number of copies estimated by this probe would reflect the total number of genes for PLA₂ isoenzymes present. In contrast, probe 2B can hybridize only with the genes for BPI and BPII, so that the copy number estimated would reflect the number of genes for BPI and BPII. Approximately 20–30 genes for PLA₂ isoenzymes were estimated with probe U for genomes of all specimens of *T. flavoviridis* from three islands (results not shown), which is in accord with the value reported by Nakashima et al. [13]. The genes that hybridized with probe 2B were 1–4 for specimens of Okinawa *T. flavoviridis*, a substantially smaller number than the 4–13 estimated for specimens of Amami-Oshima and Tokunoshima *T. flavoviridis* (results not shown).

Screening of inactivated genes for BPI and BPII

An attempt to obtain inactivated genes encoding BPI and BPII from Okinawa *T. flavoviridis* was made for its genomic DNA library with BPI cDNA. However, we were unable to isolate it, in spite of repeated constructions of the library. Clones isolated were completely identical with pgPLA 1a–pgPLA 1b and pgPLA

Figure 8 Electrophoretogram of DNA fragments amplified by PCR with primers GL2EU-GL2EDo (A) and GL2EU-GL2ED (B) for liver genomic DNA species of *T. flavoviridis* from three islands

DNA fragments were subjected to electrophoresis on 2% (w/v) agarose gels and stained with ethidium bromide. Lanes a, Amami-Oshima (three specimens); lanes t, Tokunoshima (three specimens); lanes o, Okinawa (four specimens). Nucleotide size markers are shown to the right of the Figure.

2a–pgPLA 2b, which respectively encode PLA₂ and its analogue [13].

Structure of the pseudogene in Okinawa *T. flavoviridis* genome

Southern blot analysis and PCR to search for the missing regions in the genes for BPI and BPII

The genomic DNA species of *T. flavoviridis* from three islands were digested with a mixture of *Sma*I and *Bam*HI with reference to the restriction-enzyme map of Tokunoshima *T. flavoviridis* genes for BPI and BPII [13] and were subjected to hybridization with probes 2B (positions 490–563 of the genes for BPI and BPII, the intermediate region of the second exon) and 4B (positions 1349–1797, the region covering the second half of the third exon to the first half of the fourth exon of the genes for BPI and BPII) (Figures 7A and 7B). The approx. 900 bp band (positions –148 to 769) should come from the region involving the first and second exons of the genes for BPI and/or BPII. When hybridized with probe 2B, the band corresponding to approx. 900 bp was clearly detected for Amami-Oshima and Tokunoshima *T. flavoviridis* genomic DNA, as shown in Figure 7(A) (lanes a and t). However, the band corresponding to this fragment was not detected for Okinawa *T. flavoviridis* genomic DNA (Figure 7A, lane o). When hybridized with probe 4B, the approx. 1.5 kbp band involving the third and fourth exons of the genes for BPI and/or BPII was detected for the genomic DNA of *T. flavoviridis* from the three islands (Figure 7B).

Because no positive band was detected in a Southern blot analysis of Okinawa *T. flavoviridis* genomic DNA when probe 2B was employed (Figure 7A, lane o), PCR was conducted with sense primer GL2EU (positions 490–510) and anti-sense primer GL2EDo (positions 547–568) for the genomic DNA of *T. flavoviridis* from three islands in an attempt to amplify the internal portion of the second exon. Although a specific band of approx. 80 bp was detected for genomic DNA from all specimens of Amami-Oshima and Tokunoshima *T. flavoviridis*, no band was detected for genomic DNA from all specimens of Okinawa *T. flavoviridis* (Figure 8A). Similarly, no amplification occurred only for Okinawa *T. flavoviridis* genomic DNA when PCR was conducted with primers GL2EU-GL2ED (positions 543–563)

Figure 9 Electrophoretogram of DNA fragments amplified by PCR with primers OGBS2-OGBA1 (lanes 1), GL3EUo-CHO3 (lanes 2), GL2I1-CHO3 (lanes 3), GL2EU-GL3ED (lanes 4) and GL2EUm-GL3ED (lanes 5) for genomic DNA of *T. flavoviridis* from three islands

DNA fragments were subjected to electrophoresis on 2% (w/v) agarose gels and stained with ethidium bromide. Lanes o, Okinawa; lanes t, Tokunoshima; lanes a, Amami-Oshima. Nucleotide size markers are shown to the right of the Figure.

(Figure 8B). These results suggested that at least the intermediate region of the second exon of the genes for BPI and BPII is missing from the Okinawa *T. flavoviridis* genome.

In contrast, PCRs for Okinawa *T. flavoviridis* genomic DNA with OGBS2 (positions -114 to -92)-OGBA1 (positions 494–516) and GL3EUo (positions 1315–1336)-CHO3 (positions 1879–1901) as primers amplified the fragments [Figure 9(o), lanes 1 and 2] similarly to those for Amami-Oshima and Tokunoshima *T. flavoviridis* genomic DNA [Figure 9(a), lanes 1 and 2; Figure 9(t), lanes 1 and 2]. The sequencing confirmed that the two amplified fragments came from the upstream region including a part of the 5' moiety of the second exon and the downstream region, not including a part of the 5' moiety of the third exon, respectively, of the genes for BPI and/or BPII. PCR

with GL2I1 (positions 1015–1035)-CHO3 (positions 1879–1901) also amplified the fragment [Figure 9(a), lane 3; Figure 9(t), lane 3; Figure 9(o), lane 3]. However, PCRs with GL2EU (positions 490–510)-GL3ED (positions 1374–1397) and GL2EUm (positions 542–563)-GL3ED failed in amplification only for Okinawa *T. flavoviridis* genomic DNA (Figure 9(o), lanes 4 and 5). If all these results are taken together, it is likely that approx. 100 bp of the second exon at the 3' end and approx. 400 bp of the second intron at the 5' end of the genes for BPI and BPII are missing from the Okinawa *T. flavoviridis* genome.

PCR of two polymorphic segments and direct sequencing

BPI and BPII proteins are different at position 58; they contain Asp (GAC) for BPI and Asn (AAC) for BPII, which correspond to nucleotide positions 1353–1355 (numbering according to the gene for BPI) in the third exon [13]. Another silent mutation occurs at position 236 in the first exon, C in the gene for BPI and G in the gene for BPII [13]. Two fragments containing these positions were amplified from Tokunoshima and Okinawa *T. flavoviridis* (?) genomic DNA with primers CHO5 (positions 194–216)-OGBA1 (positions 494–516) and GL3EUo (positions 1315–1336)-GL3ED (positions 1374–1397) respectively. The fragments amplified were named 1E (containing position 236) and 3E (containing position 1353) respectively and were sequenced by the direct sequencing method (Figure 10). Fragment 1E from Tokunoshima *T. flavoviridis* gave G and C at position 236, whereas fragment 1E from Okinawa *T. flavoviridis* gave only C. Fragment 3E from Tokunoshima *T. flavoviridis* gave G and A at position 1353, whereas fragment 3E for Okinawa *T. flavoviridis* gave A alone. The detection of two bases for fragments 1E and 3E in Tokunoshima *T. flavoviridis* indicates that both are mixtures of two fragments that are derived from two isoforms of the genes for BPI and BPII. However, in Okinawa *T. flavoviridis*, the detection of only one base for fragments 1E and 3E at positions 236 and 1353 respectively shows that 1E came from the gene for BPI and 3E from the gene for BPII. Thus, in the pseudogene, the upstream region of the gene for BPI down to approx. nucleotide position 516 (the 5' moiety of the second exon) is followed by the downstream region of the gene for BPII starting from approx. position 1015 (the

Figure 10 Direct sequencing of the DNA fragments 1E (left panel) and 3E (right panel) amplified by PCR with primers CHO5-OGBA1 and GL3EUo-GL3ED respectively for genomic DNA of *T. flavoviridis* from Okinawa (o) and Tokunoshima (t)

Nucleotides detected at polymorphic positions 236 (1E) and 1353 (3E) between the genes for BPI and BPII are indicated by arrows.

Figure 11 Conceptual model showing the pseudogene derived from the genes encoding BPI and BPII in the Okinawa *T. flavoviridis* genome

middle portion of second intron), as depicted in Figure 11. It is likely that an insertion that might impede amplification exists between the fused fragments derived from the genes for BPI and BPII.

DISCUSSION

Trimeresurus flavoviridis snakes in southwestern islands of Japan, namely Amami-Oshima, Tokunoshima and Okinawa, belong to the same species. However, chromatographic analysis showed that BPI and BPII, which are strongly myotoxic [Lys⁴⁹]PLA₂ forms and that are expressed abundantly in venom glands from Amami-Oshima and Tokunoshima *T. flavoviridis*, are missing from Okinawa *T. flavoviridis* venom (Figure 2). In accordance with this, the absence of BPI and BPII mRNA species in Okinawa *T. flavoviridis* venom gland was suggested by Northern blot analysis (Figure 4), RT-PCR analysis (Figure 5) and SSCP-PCR analysis (Figure 6). The PCR experiment for Okinawa *T. flavoviridis* genomic DNA species with a variety of primers indicated that, although the upstream and downstream regions of the genes for BPI and/or BPII exist, most of the second exon at its 3' end and the first half of the second intron are lost. Analysis of the sequences containing polymorphisms (positions 236 and 1353) between the genes for BPI and BPII confirmed that the upstream region of the gene for BPI down to the 5' moiety of second exon is followed, with a possible insertion, by the downstream region of the gene for BPII starting from the middle portion of the second intron in the Okinawa *T. flavoviridis* genome (Figure 11). The fate of the missing downstream and upstream regions of the genes for BPI and BPII respectively is unknown.

It is considered from the above results that ancestral *T. flavoviridis* in the former Okinawa continent had genes for BPI and BPII that were arranged in tandem in this order on one chromosome. After separation into islands by eustasy (changes in sea level) in the orogenic stage one to two million years ago [7], the genes for BPI and BPII were disrupted to form a pseudogene only in *T. flavoviridis* of Okinawa island.

In multicopied genes, gene recombination and unequal crossover bring about gene inactivation or a change in the numbers of family genes [25,26]. These genetic changes can occur particularly when the homology between family genes is fairly high. The genes for BPI and BPII are highly homologous in sequence [13]. The pseudogene derived from the genes for BPI and BPII in the Okinawa *T. flavoviridis* genome seems to have been formed by unequal crossover between these genes. However, the inactivation of the genes for BPI and BPII only in Okinawa *T. flavoviridis* but

not in Amami-Oshima and Tokunoshima *T. flavoviridis* cannot be explained solely by the presence of highly homologous genes for PLA₂ isoenzymes. The accelerated evolution that we have discovered for venom-gland PLA₂ isoenzyme genes of Crotalinae snakes such as *T. flavoviridis*, *T. gramineus* and *T. okinavensis* [13,14,27] is not likely to be directly correlated with pseudogene formation in Okinawa *T. flavoviridis* because no such event has occurred in Tokunoshima and Amami-Oshima *T. flavoviridis*.

The loss of BPI and BPII greatly decreases the venom toxicity of Okinawa *T. flavoviridis* because their myotoxic activity is estimated to be several times stronger than that of PLA₂ [4] and the combined quantity of BPI and BPII is comparable with that of PLA₂ in the venom. From Amami-Oshima *T. flavoviridis* venom, haemorrhagic metalloproteases called HR_{1a}, HR_{1b}, HR_{2a} and HR_{2b} have been isolated together with non-haemorrhagic metalloprotease H₂ [28–30], of which HR_{1b}, HR_{2a} and H₂ have been sequenced [31–33]. HR_{2b} of Okinawa *T. flavoviridis* venom has also been sequenced [34]. HR_{1a} and HR_{1b}, which have a high molecular mass, show an approx. 10-fold stronger haemorrhagic activity than HR_{2a} and HR_{2b}, which are of lower molecular mass [29]. It has been reported that HR_{1b} is defective in Okinawa *T. flavoviridis* venom [35]. It is unknown what mechanism was operative for the inactivation of the HR_{1b}-encoding gene in Okinawa *T. flavoviridis*. The lack of strongly toxic BPI, BPII and HR_{1b} would seem to be of great disadvantage to Okinawa *T. flavoviridis*. However, they seem to survive well. It is unlikely that the simultaneous loss of three strongly toxic components in Okinawa *T. flavoviridis* venom was brought about by accidental mutations if its living surroundings were the same as those of Amami-Oshima and Tokunoshima *T. flavoviridis*. One possibility is that the prey species of Okinawa *T. flavoviridis* are partly or largely different from those of Amami-Oshima and Tokunoshima *T. flavoviridis*, such that strong venom toxicity might not be required for Okinawa *T. flavoviridis*. It is conceivable that the lack of necessity for a strong toxicity in the venom gave rise to the inactivation or disappearance of genes encoding such highly toxic proteins. According to a statistical investigation in Amami-Oshima, for example, *T. flavoviridis* snakes feed on rats (86%) and also birds, reptiles and amphibians (14% in total) in the farmlands and in the areas close to villages [36]. However, in Yambaru, a mountain area covered with forests in the northern part of Okinawa, *T. flavoviridis* snakes mostly (approx. 90%) prey on Holst's frogs (*Rana holsti*, a native animal designated by Okinawa Prefecture) inhabiting the streams in this area (K. Terada, Gyokusendo Snake Park of Okinawa, Japan, personal communication). This natural, wild area seems to reflect the environment in Okinawa in which *T. flavoviridis* snakes lived in

ancient times. It could be assumed that these feeding habits over a long period affect the venom components in Okinawa *T. flavoviridis*. This might be a sort of adaptation to the environment. In this connection, Daltry et al. [37] reported that the compositions of venom proteins have changed in the same snake species of the pit viper *Calloselasma rhodostoma* (Viperinae) inhabiting the remote areas of Southeast Asia to adapt to their prey species [37]. Independent genetic change observed in genes for PLA₂ isoenzymes of *T. flavoviridis* venom gland in isolated islands, and the accumulation of such changes in the genome with the passage of time, might lead to species differentiation in *T. flavoviridis*.

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