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Comparative Biochemistry of Disintegrins Isolated from Snake Venom: Consideration of the Taxonomy and Geographical Distribution of Snakes in the Genus *Echis*[1]

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Species in the genus *Echis* have been classified mainly based on their morphological appearance and the analytical patterns of their serum. However, re-classification of the genus *Echis* has recently been suggested by taxonomists, toxicologists, and clinicians, since there have been problems with the current classification, such as the efficacy of antivenoms used for treating bites and the broad geographical distribution of *Echis* snakes. In this study, we purified five novel disintegrins, the platelet aggregation inhibitors pyramidin A and B from the venom of *Echis pyramidum*, ocellatin from the venom of *Echis ocellatus*, and leucogastin A and B from the venom of *Echis leucogaster*, to compare their sequences and allow us to re-evaluate the classification of various species in the genus *Echis*. Comparison of the amino acid sequences of five new and four known isolated disintegrins from snake venoms of six *Echis* species and their distribution strongly support the recent re-classification of the genus *Echis*.

Key words: comparative biochemistry, disintegrin, *Echis carinatus*, snake venom, taxonology.

The taxonomy of venomous snakes is complicated because of the complex nature of the many groups of medically important species (*1*, *2*). Their classifications have been changed frequently based on new discoveries. Researchers have attempted to classify the many groups of snakes, but this has yet to be done completely due to the identification of novel species and variations in venom composition within subspecies.

In *Calloselasma rhodostoma*, Daltry *et al.* reported the association with differences in the diet of different populations, despite independent evolutionary descent, and thus intraspecific taxonomy (*3*). It is, therefore, difficult to classify the many groups of venomous snakes by either morphological or medical studies that include biochemical analysis of serum (*4–7*). *Echis* is one of the most complicated genera for taxonomic classification (*8*) due to the varied venom compositions in different species and the wide distribution from Asia and the eastern and southeastern coastal areas of the Arabian Peninsula to western Africa (*9*).

Disintegrin, a potent platelet aggregation inhibitor, has been shown to be present in almost all experimental snake venoms (*10*). Disintegrin molecules are small in size, with monomers of about 5 to 9 kDa (*11–14*) and dimers of about 14 kDa (*15*, *16*). Most monomeric disintegrins have an Arg-Gly-Asp (RGD) tripeptide sequence. Exceptions include barbourin from the venom of *Sistrurus miliarius barbouri*, which has a Lys-Gly-Asp (KGD) sequence that binds specifically to integrin αIIbβ3 (*17*). A number of high molecular weight ligands, such as fibrinogen, fibronectin, vitronectin, collagen and von Willebrand factor, also contain the RGD sequence. This sequence is recognized by several integrins including integrin αIIbβ3, αvβ3, and α5β1. Fibrinogen binds to integrin αIIbβ3 on platelet membranes, *via* the RGD sequence, resulting in the formation of platelet aggregates. Therefore, RGD-containing disintegrins act as competitive inhibitors of platelet aggregation against plasma fibrinogen (*12*). Furthermore, they also inhibit cell adhesion, which is related to integrin αvβ3 and vitronectin (*18*). Four disintegrins have been isolated from *Echis* snake venoms; echistatin α from *Echis carinatus sochureki* (*11*), which has been re-classified as *Echis sochureki*, echistatin β and γ from *Echis carinatus leakeyi* (*19*), which has been re-classified as *Echis pyramidum leakeyi*, and multisquamatin from *Echis multisquamatus* (*20*). With the exception of multisquamatin, the amino acid sequences of these molecules have been determined, and the tertiary structure of echistatin α has been reported (*21*).

In the present study, we purified six disintegrins from the venoms of four *Echis* species and determined their amino acid sequences. We will discuss the classification of the genus *Echis* from the viewpoint of the comparison of the amino acid sequences of new and known disintegrins, and the geographical distribution of *Echis*. The results of the present study support the recent re-classification of species of the genus *Echis*.

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Abbreviations: RGD, arginine-glycine-aspartate; KGD, lysine-glycine-aspartate; PRP, platelet-rich plasma; PPP, platelet-poor plasma.

MATERIALS AND METHODS

Materials—The lyophilized venoms of *Echis multisquamatus*, *Echis pyramidum*, *Echis ocellatus*, *Echis leucogaster*, and *Echis sochureki* were purchased from Latoxan (Rosans, France). The Superdex 200 pg FPLC column was from Pharmacia LKB Biotechnology Inc. The Vydac Protein & Peptide C18 HPLC column was from Jasco (Tokyo). Endoprotease Lys-C was purchased from Seikagaku Corporation (Tokyo). Endoprotease Asp-N was purchased from Boehringer Mannheim (Marburg, Germany). ADP was purchased from Sigma (St. Louis, MO). Chemicals used in this study were purchased from Sigma, Pharmacia Biotech, Wako Pure Chemical and Kanto Chemical.

Platelet Aggregation Assay—Human blood was obtained from healthy volunteers who gave their informed consent and denied taking any medications. The study protocol was approved by the ethics committee of our university. Blood was collected into 0.1 volume of 3.8% (w/v) sodium citrate by venipuncture and centrifuged at 150 ×*g* for 20 min at room temperature to prepare platelet-rich plasma (PRP). Platelet-poor plasma (PPP) was obtained from the PRP by further centrifugation at 300 ×*g* for 5 min. Disintegrin was added to PRP and incubated for 1 min at 37°C, after which ADP (20 μM final concentration) was added to initiate aggregation. Platelet aggregation was measured by determining the change in light transmission of PRP with a Niko Bioscience Hema Tracer 601 aggregometer.

Purification of Disintegrins—Lyophilized venom (*E. multisquamatus*, *E. pyramidum*, *E. ocellatus*, or *E. leucogaster*) was dissolved in 50 mM Tris-HCl, pH 8.0, and applied onto a column of Superdex 200 pg (1.6 × 60 cm) pre-equilibrated with the same buffer. The column was eluted at a flow rate of 60 ml/h with 1 ml fractions collected into glass tubes. Fractions were assayed for inhibitory activity of ADP-induced aggregation of human platelets. Active fractions were pooled and loaded onto a preparative C18 reverse-phase column equilibrated with 0.1% (v/v) trifluoroacetic acid in water. The column was developed at room temperature with a linear acetonitrile gradient (0 to 40% for 80 min) in 0.1% aqueous trifluoroacetic acid at a flow rate of 1 ml/min.

We also isolated echistatin α from the venom of *E. sochureki* by the same method; echistatin α was identified by amino acid sequence analyses of the *S*-pyridylethylated and endoprotease Asp-N digested samples (data not shown).

Amino Acid Sequence Analysis—Each disintegrin (about 0.5 mg protein), multisquamatin, pyramidin A, pyramidin B, ocellatin, leucogastin A, and leucogastin B, was reduced for 3 h at room temperature with 20 mM dithiothreitol in the presence of 0.5 M Tris-HCl, pH 8.5, 6 M guanidine hydrochloride, and 2 mM EDTA in a volume of 0.5 ml. Three microliters of 4-vinylpyridine was then added and alkylation was allowed to proceed for 3 h at room temperature. The *S*-pyridylethylated disintegrins were separated from reagents by C18 reverse-phase HPLC, and their complete amino acid sequences were determined by sequencing the peptides obtained by digestion with endoproteases Lys-C and Asp-N and by chemical cleavage with cyanogen bromide. All samples were analyzed on an Applied Biosystems protein sequencer (model 473A).

Sequence Analysis—The sequence alignment and evolutionary tree were constructed by MacVector (version 7.0; Genetics Computer Group, Madison, WI).

RESULTS AND DISCUSSION

Six disintegrins were purified from the venoms of *E. pyramidum*, *E. ocellatus*, *E. leucogaster*, and *E. multisquamatus* in two column chromatography steps on a Superdex 200 pg and a reversed-phase Vydac Protein & Peptide C18 column. We designated five new disintegrins as pyramidins A and B from the venom of *E. pyramidum*, ocellatin from the venom of *E. ocellatus*, and leucogastins A and B from the venom of *E. leucogaster*. Pyramidins A and B, ocellatin, leucogastins A and B, and multisquamatin were eluted at 17, 24, 21, 15, 17, and 15% acetonitrile from the C18 HPLC column, respectively (data not shown). The yield of each disintegrin from 100 mg of venom was as follows; pyramidin A, 0.46 mg; pyramidin B, 0.10 mg; ocellatin, 0.80 mg; leucogastin A, 0.30 mg; leucogastin B, 0.40 mg; and multisquamatin,

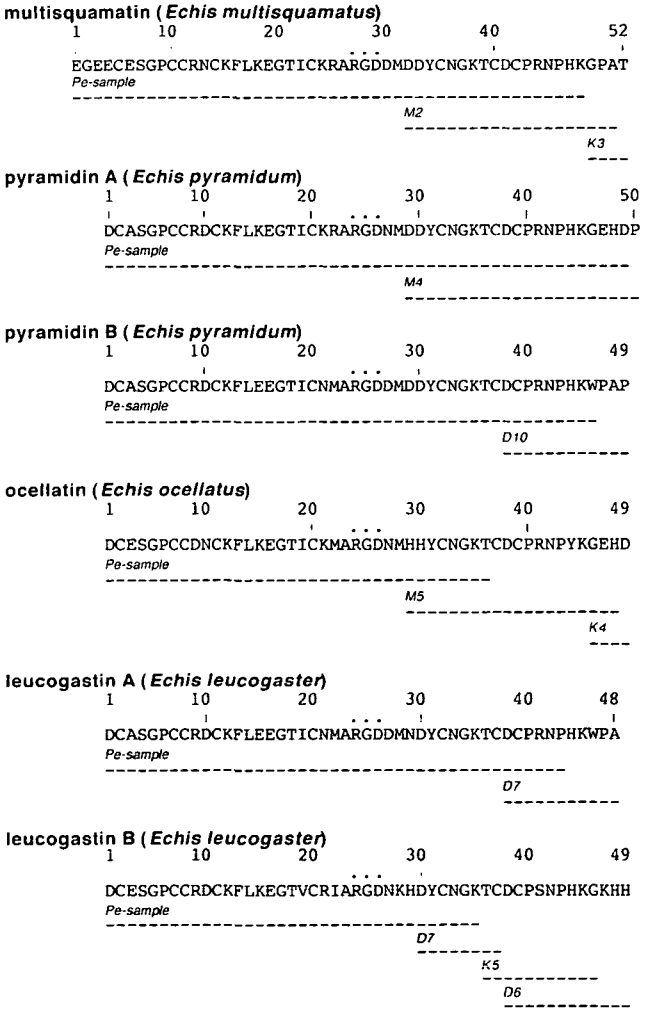

Fig. 1. **Amino acid sequences of purified disintegrins.** Amino acid residues are given in single-letter code. Residues determined by Edman degradation are indicated by broken lines. Dots show the position of the RGD sequence. Pe-sample, *S*-pyridylethylated disintegrin sample; M, CNBr peptides; K, endoprotease Lys-C peptides; D, endoprotease Asp-N peptides.

0.99 mg.

The IC_{50} values for the ADP-induced platelet aggregation of disintegrins were follows: pyramidin A, 160 ± 21 nM; pyramidin B, 233 ± 48 nM; ocellatin, 104 ± 33 nM; leucogastin A, 360 ± 67 nM; leucogastin B, 170 ± 41 nM; and multisquamatin, 93 ± 8 nM. These data are the averages of 2–3 independent experiments performed in duplicate (mean ± SE).

The amino acid sequences of the six disintegrins were determined by N-terminal Edman degradation of the *S*-pyridylethylated peptides, and the endoprotease Lys-C, endoprotease Asp-N, and CNBr-digested fragments. These peptide fragments were purified by reversed-phase HPLC. The complete amino acid sequences of the six disintegrins are shown in Fig. 1. Multisquamatin, pyramidins A and B, ocellatin, leucogastins A and B contain 52, 50, 49, 49, 48, and 49, respectively, amino acids with eight cysteine residues. Comparisons of the amino acid sequences show high degrees of identity among these six disintegrins derived from *Echis* snake venoms (Fig. 1).

Disintegrins show a high degree of homology to other disintegrins from the venoms of *Echis* genus snakes. The cysteine residues and RGD sequence alignment are completely conserved (Fig. 2). The venoms of *Echis* genus snakes contain small-type disintegrins. The small-type disintegrins, each of which contains about 50 amino acids, have a structure that inhibits platelet aggregation effectively, and this framework can not be changed without a loss of their biological activity. Therefore, we compared differences between their amino acid sequences to support the classification of venomous snakes from the standpoint of comparative biochemistry. With regard to the nomenclature of various species, the genus *Echis* remains one of the most taxonomically problematic groups of venomous snakes (*8*). In this study, we considered taxonomy and the geographical distribution of the snakes in the genus *Echis* by comparison of their disintegrin amino acid sequences.

Four disintegrins have been purified from the venoms of *Echis* snakes: echistatin α (*11*) from the venom of *E. carinatus sochureki* (renamed *E. sochureki*), echistatin β (*19*), and echistatin γ (*19*) from the venom of *E. carinatus leakeyi* (renamed *E. pyramidum leakeyi*), and multisquamatin (*20*) from the venom of *E. multisquamatus*. The amino acid sequences of the three former disintegrins have been reported, while that of multisquamatin remains to be determined. We purified five new disintegrins from the venoms of *E. pyramidum*, *E. ocellatus*, *E. leucogaster*, and multisquamatin from the venom of *E. multisquamatus* to determine their complete amino acid sequences.

These disintegrins consist of about 50 amino acid residues and show a high degree of identity to each other. The RGD sequence and eight cysteine residues are conserved. However, some amino acid differences were de-tected mainly around the RGD sequence and in the C-terminal region (Fig. 2). Saudek *et al.* have reported the three-dimensional structure of echistatin α (*21*). The RGD sequence is located in a flexible hairpin loop between two β strands of the protein core, and the C-terminal region exists in an adjacent RGD-loop (*21*). The residues flanking the RGD sequence and the C-terminal region in addition to the RGD sequence affect the binding specificity to integrins (*22–25*). The range of IC_{50} values for ADP-induced platelet aggregation of disintegrins from *Echis* snake venoms is 63 to 360 nM [echistatin α, 108 nM (*19*) and 136 nM (*23*); echistatin β, 63 nM (*19*); echistatin γ, 276 nM (*19*); multisquamatin, 97 nM (*20*), and our results described above]. No significant correlation was observed between the IC_{50} values for ADP-induced platelet aggregation and amino acid differences for the nine disintegrins shown in Fig. 2, because these nine disintegrins show a high degree of identity of the amino acid sequences in the RGD-loop and C-terminal region. Although the amino acid sequences of pyramidin A and echistatin β are identical, the IC_{50} for ADP-induced platelet aggregation of pyramidin A (160 nM) was found to be higher than that of echistatin β (63 nM) (*19*). Chen *et al.* have shown the effect of methionine oxidation of echistatin β by chloramine-T on ADP-induced platelet aggregation, and the IC_{50} value of methionine-oxidized echistatin β was found to be 130 nM (*19*), close to the IC_{50} of pyramidin A. This suggests that the methionine residue of pyramidin A may be partially oxidized, resulting in a high IC_{50} value. However, we have not examined the possibility of the methionine oxidation of pyramidin A.

Gasperetti reported the geographical distribution of the genus *Echis* (*9*) and Cherlin described the predicted migration of ancestral *Echis* snakes (*26*). The names of disintegrins from *Echis* snake venoms are overlaid with the species names in Fig. 3. We will discuss here the relationships between the amino acid sequences of disintegrins and the geographical distribution of *Echis* snakes in Africa and Asia.

E. multisquamatus is distributed in northwestern Baluchistan and *E. sochureki* is distributed in northern India, as shown in Fig. 3. Their habitats are very close to each other, and these species are considered to have diverged at a relatively late period during the evolution of the genus *Echis* (*26*). The amino acid sequences of multisquamatin and echistatin α are identical except for an extra three

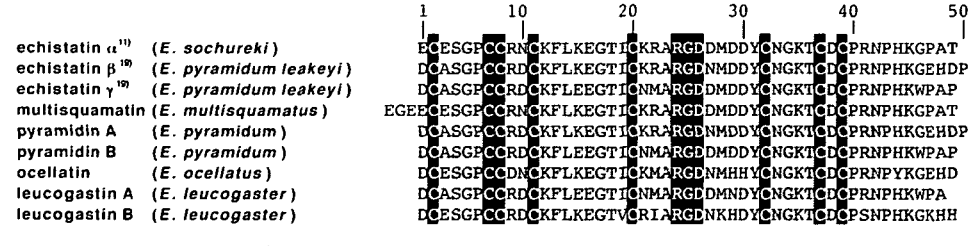

Fig. 2. **Alignment of disintegrins derived from *Echis* snake venoms.** The amino acid sequences of nine disintegrins from *Echis* snake venoms, echistatin α (*11*), echistatin β (*19*), echistatin γ (*19*), multisquamatin, pyramidin A, pyramidin B, ocellatin, leucogastin A, and leucogastin B. Identical amino acid residues are shaded and the conserved cysteine residues and RGD sequence are indicated by reversed characters. The amino acid sequences of pyramidin A and pyramidin B are identical to those of echistatin β and echistatin γ, respectively. At present, the species classification of some *Echis* snakes have been changed: *Echis carinatus sochureki* has been changed to *Echis sochureki*; *Echis carinatus leakeyi* to *Echis pyramidum leakeyi*; and *Echis carinatus leucogaster* to *Echis leucogaster*.

amino acids at the N-terminus of multisquamatin (Fig. 2). These results support the taxonomic classification of these species.

E. sochureki and *E. leucogaster* were previously classified as belonging to the same species as *E. carinatus*. However, the amino acid sequence identities between echistatin α and leucogastin A (83.3%) or between echistatin α and leucogastin B (75.5%) are relatively lower than the average between echistatin α and other *Echis* disintegrins (84.6 ± 8.4%, n = 8). Thus, our biochemical results support their taxonomic classification into different species: *E. carinatus sochureki* has been re-classified as *E. sochureki*, and *E. carinatus leucogaster* as *E. leucogaster*.

E. carinatus leakeyi, which is distributed in Kenya, Southern Ethiopia, and the Somali Republic, has been re-classified as *E. pyramidum leakeyi*. Gasperetti described the geographical point of identification of *E. carinatus leakeyi* as the hatched square in Fig. 3 (*9*). Chen *et al.* reported that echistatin β and echistatin γ were also isolated from the venoms of *E. carinatus leucogaster* and *E. carinatus pyramidum* (*19*). Nevertheless, we purified two other disintegrins, leucogastin A and leucogastin B, from the venom of *E. leucogaster*. Both amino acid sequences are different from those of echistatin β and echistatin γ. Next, we attempted to purify and examine other disintegrins from the venom of *E. pyramidum*, which has been classified as *E. carinatus pyramidum*. Two disintegrins were obtained, pyramidin A and pyramidin B, which are completely identical to echistatin β and echistatin γ, respectively. In contrast, the level of amino acid sequence identity between pyramidin A and echistatin α is 85.7%. This indicates that *E. carinatus leakeyi* is more closely related to *E. pyramidum* than to *E. carinatus*. These results support the re-classification of *E. carinatus leakeyi* as *E. pyramidum leakeyi*.

Although the geographical distributions of *E. leucogaster* and *E. ocellatus* partially overlap, a species of *Echis* snake ancestral to both species is thought to have migrated along

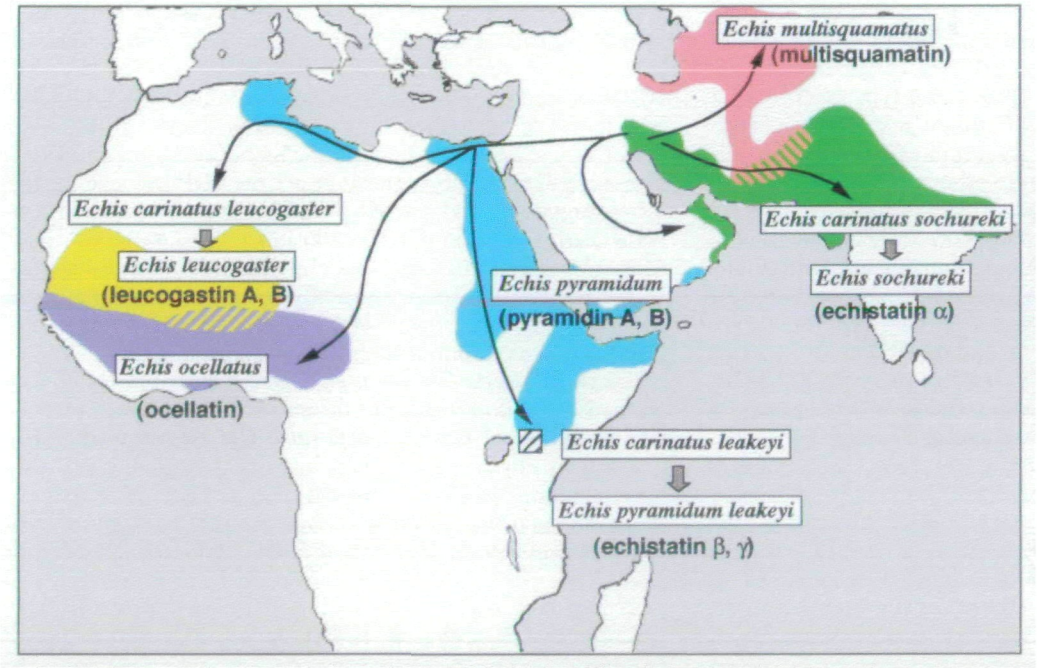

Fig. 3. **The relationship between isolated disintegrins and the present populations and migrations of genus *Echis* snakes in middle Asia and Africa.** Fig. 3 is a modification of Fig. 117 from Gasperetti (1988), and Figs. 4 and 5 from Cherlin (1990). The populations of *Echis* snakes (*9*) are shown in each area as follows; red, *Echis multisquamatus*; green, *Echis sochureki*; blue, *Echis pyramidum*; yellow, *Echis leucogaster*; purple, *Echis ocellatus*; hatched square, *Echis pyramidum leakeyi*. Arrows show the migration of *Echis* snakes from middle Asia to Africa (*26*). Bold arrows indicate changes in the names of some *Echis* snakes from the upper to lower name due to their re-classification. Disintegrins derived from *Echis* snake venoms are shown under the new species name.

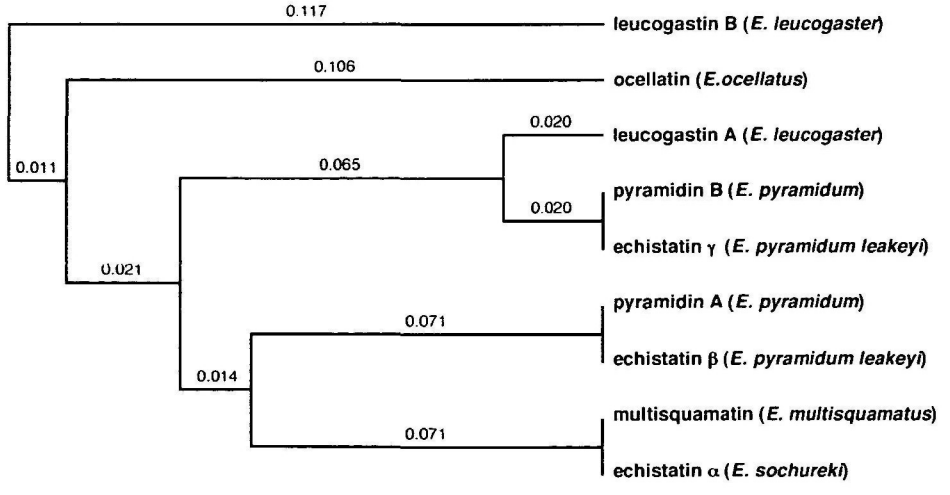

Fig. 4. **Evolutionary tree of disintegrins from *Echis* snake venoms.** An evolutionary tree of nine disintegrins from *Echis* snake venoms was constructed by the MacVector allowing UPGMA (unweighted pair group method with arithmetic mean) method. The numbers on the lines indicate evolutionary distances (calculated parameter on MacVector: absolute and gap sites ignored).

two routes *via* north and central Africa as shown in Fig. 3 (*26*). Our sequence analysis revealed the amino acid sequence identities between leucogastin A and ocellatin, and between leucogastin B and ocellatin to be 75.0 and 77.6%, respectively. These values are approximately the same (80%) as those obtained by comparing echistatin α with leucogastin A and leucogastin B, as described above.

An evolutionary tree for a total of nine disintegrins from *Echis* snake venoms is shown in Fig. 4. This evolutionary tree accords with the predicted migrations of ancestral *Echis* snakes as shown in Fig. 3. This evolutionary tree suggests that leucogastin A and pyramidin B (echistatin γ) may have diverged at a relatively late period from the same ancestral gene that yielded echistatin α, multisquamatin and pyramidin A (echistatin β). In contrast, a gene encoding leucogastin B and ocellatin may have diverged at the earliest period of disintegrin evolution. Although more studies involving the isolation, characterization and cDNA cloning of disintegrins from other *Echis* species will be necessary to describe the evolution of disintegrin, Fig. 4 suggests that most *Echis* snakes may have plural genes encoding RGD-containing disintegrins.

The taxonomic classifications of venomous snakes, such as those of the genus *Echis*, have been performed based on their morphology and biochemical analyses of serum. However, species have gradually been re-classified based on new observations. Species of the genus *Echis* were among the most problematic species to classify of in all venomous snakes (*8*). Biochemical analyses, and the structural and biochemical characterization of venom components are useful for supporting the classification of venomous snakes. Yamada *et al.* isolated and characterized structurally and functionally novel prothrombin activators derived from various *Echis* snake venoms (*27–29*). Therefore, biochemical studies of the amino acid sequences of novel prothrombin activators and disintegrins, as described here, will contribute to the phylogenetic and taxonomical classification of snakes of the genus *Echis*.

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