cDNA Cloning and Sequencing of Phospholipase A_2 from the Pyloric Ceca of the Starfish *Asterina pectinifera*

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Abbreviations: PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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Abstract

Three cDNA from the pyloric ceca of the starfish *Asterina pectinifera*, (namely, cDNA 1, 2, and 3), encoding phospholipase A_2 (PLA_2), were isolated and sequenced. These cDNAs were composed of 415 bp with an open reading frame of 414 bp at nucleotide positions 1 to 414, which encodes 138 amino acids including N-terminal Met derived from the PCR primer. The amino acid sequence deduced from the cDNA 1 was completely consistent with the sequence determined with the starfish PLA_2 protein, while those deduced from cDNA 2 and cDNA 3 differed at one and twelve amino acid residual positions, respectively, from the sequence of the PLA_2 protein, suggesting the presence of multiple forms in the starfish PLA_2. All of the sequences deduced from cDNA 1, 2, and 3 required two amino acid deletions in pancreatic loop region, and sixteen insertions and three deletions in β-wing region when aligned with the sequence of mammalian pancreatic PLA_2. In phylogenetic tree, the starfish PLA_2 should be classified into an independent group, but hardly to the established groups ⅠA and ⅠB. The characteristic structure in the pancreatic loop and β-wing regions may account for the specific properties of the starfish PLA_2, e.g., the higher activity and characteristic substrate specificity compared with mammalian pancreatic PLA_2.

Keywords: cDNA cloning; Group Ⅰ; Isoforms; Pancreatic loop; Phospholipase A_2; Phylogenetic tree; Starfish; β-wing

1. Introduction

Phospholipase A_2 (PLA_2; EC 3.1.1.4) is the enzyme that catalyzes the selective hydrolysis of the *sn*-2-acyl group in 1, 2-diacyl-*sn*-glycero-3-phospholipids and produces free fatty acids and lysophospholipids. PLA_2 consists of both extracellular- and intracellular-type enzymes (Dennis, 1997). Extracellular-type PLA_2 is abundant in mammalian pancreas and snake venom, and the enzymatic properties and amino acid sequences have been well characterized (Arni and Ward, 1996; Dennis, 1983). Thus far, the molecular mechanism of catalytic action of the PLA_2 has been investigated on the basis of three-dimensional structure (Arni and Ward, 1996; Dennis, 1983).

On the other hand, there appear to be a few studies on the digestive gland PLA_2 from marine invertebrates. In 1975, Okabe et al. partially purified PLA_2 from the pyloric ceca of the starfish *Asterina pectinifera* , and later, we prepared PLA_2-like enzyme from the starfish *Solaster paxillatus* (Kishimura and Hayashi, 1998). The basic properties of these enzymes such as Ca^{2+} requirement, optimum pH, and heat stability were similar to those of the mammalian pancreatic PLA_2. However, detailed properties and primary structures of both the purified enzymes remained to be investigated.

Recently, we isolated PLA_2 from the pyloric ceca of the starfish *A. pectinifera*, and studied its enzymatic properties comparing with those of mammalian pancreatic PLA_2 (Kishimura and Hayashi, 1999). The specific activity of the starfish PLA_2 for phosphatidylcholine was about 30 times higher than that of the commercially available PLA_2 from porcine pancreas (Sigma). In addition, the starfish PLA_2 hydrolyzes phosphatidylcholine

more efficiently than phosphatidylethanolamine like a snake venom PLA_2 but not a mammalian pancreatic PLA_2. These facts suggest that the starfish PLA_2 possesses some different features in primary and/or higher order structure from the mammalian pancreatic PLA_2. In fact, the amino acid sequence of the starfish PLA_2 showed some distinct features from mammalian PLA_2, e.g., two amino acid deletions in pancreatic loop region, and sixteen insertions and three deletions in β-wing region when aligned with the sequence of the mammalian pancreatic PLA_2 (Kishimura et al., 2000). Thus, we considered that the above sequential differences might cause for the specific properties of the starfish PLA_2. Accordingly, the studies utilizing recombinant DNA techniques have been expected to provide great advantages for further investigation of the structure-function relationships of the starfish PLA_2.

In the present paper, we describe the cloning and sequencing of the cDNAs encoding the *A. pectinifera* PLA_2.

2. Materials and methods

2.1 Materials

The starfish *A. pectinifera* was collected from the tideland at Usujiri near Hakodate in Hokkaido Prefecture, Japan, in January 1998. Cloning vector, pBluescript Ⅱ KS(+) and host strain, *Escherichia coli* XL1-Blue were purchased from Stratagene (La Jolla, CA). AMV reverse transcriptase XL,

TaKaRa Taq™ , T4 DNA polymerase, and restriction endonucleases were purchased from TaKaRa (Kyoto, Japan).

2.2 PCR and cDNA sequencing

Pyloric ceca (7.5g) were dissected from living starfishes and the total RNA was extracted by guanidium thiocyanate method described in the standard protocol (Maniatis et al., 1982). Poly $(A)^+$RNA was isolated from the total RNA with Oligotex-dT30 (TaKaRa, Kyoto, Japan). The first strand cDNA was synthesized with random hexanucleotide primers and reverse transcriptase, and the cDNA for the PLA_2 was amplified by PCR with mixed oligonucleotide primers designed on the basis of the N- and C-terminal amino acid sequences of the starfish PLA_2 protein (Fig. 1). The PCR products were subcloned to pBluescript Ⅱ KS(+) plasmid vector for sequencing. The nucleotide sequence of the cDNA was determined with a dye terminator cycle sequencing kit (Perkin Elmer-Applied Biosystems (Foster City, CA)) using a model 373A DNA sequencer (Perkin Elmer-Applied Biosystems (Foster City, CA)).

3. Results and discussion

3.1 cDNA clones for the starfish PLA_2

cDNAs of approximately 400 bp estimated by agarose gel electrophoresis

were obtained by PCR with a set of primers shown in Fig. 1. The amplified cDNAs were blunted by T4 DNA polymerase reaction and subcloned to an *Sma* Ⅰ site of pBluescript Ⅱ KS(+). By determination of nucleotide sequences of 8 independent clones obtained three species of cDNAs (cDNA 1, 2, and 3) were found to encode the PLA_2, i.e., one, three, and four clones contained the cDNA 1, cDNA 2, and cDNA 3, respectively. The nucleotide and deduced amino acid sequences of cDNA 1, 2, and 3 are shown in Fig. 2. The cDNA 1, 2, and 3 were all composed of 415 bp with an open reading frame of 414 bp at nucleotide positions 1 to 414, which encode 138 amino acids including N-terminal Met derived from the "ATG" in the PCR primer. Pancreatic PLA_2 protein generally contains signal- and pro-sequences before maturation, however, in the present study, with the aim to bacterially express the starfish PLA_2 in mature form, we added "ATG" as a translational initiation codon to 5'-terminus of the forward primer. At present, it is not clear whether or not the starfish PLA_2 has signal- and pro-peptides in premature form.

The amino acid sequence deduced from cDNA 1, namely, PLA_2 1, is completely consistent with the sequence determined previously with the PLA_2 protein (see Fig. 2) (Kishimura et al., 2000). While, the sequences deduced from cDNA 2 and cDNA 3 (termed PLA_2 2 and PLA_2 3, respectively) differed in one position (amino acid number 35) and twelve positions (numbers 32, 35, 55, 57, 65, 71, 80, 81, 87, 113, 116, and 120), respectively, from the sequence of the PLA_2 protein, suggesting the presence of multiple forms in the starfish PLA_2. Although PLA_2 1 and PLA_2 2 differ in one amino acid position, cDNA 1 and cDNA 2 differ in seven

nucleotide positions. Therefore, it seems that these differences are not due to the sequencing problem. On the other hand, separation and sequence determination of the isoforms were not achieved in the previous study (Kishimura and Hayashi, 1999). Since the PLA_2 2 and PLA_2 3 proteins can be expressed with the cDNAs in the appropriate host vector system, we will show their enzymatic activities elsewhere.

3.2 Comparison of the amino acid sequences of various PLA_2s

The amino acid sequences of the starfish PLA_2 1, 2, and 3, were aligned with those of porcine pancreatic PLA_2 (group Ⅰ B type) (Puijk et al., 1977), snake venom PLA_2s from elapinae (*Naja naja atra*, group Ⅰ A type) (Chang et al., 1997), crotalinae (*Crotalus atrox*, group Ⅱ A type) (Randolph and Heinrikson, 1982), viperinae (*Bitis gabonica*, group Ⅱ B type) (Botes and Viljoen, 1974), and rat brain PLA_2 (group Ⅱ C type) (Chen et al., 1994) (Fig. 3). The amino acid residues 26–53 of the starfish PLA_2 1, 2, and 3 showed fairly high sequence homology (75%) to the corresponding region of the group Ⅰ and Ⅱ type PLA_2s, and the residues involved in the catalytic network (His-49, Asp-111, Tyr-53, and Tyr-72) and the Ca^{2+}-binding site (Tyr-29, Gly-31, Gly-33, and Asp-50) of the group Ⅰ and Ⅱ PLA_2s were completely conserved in the starfish PLA_2 1, 2, and 3 (Fig. 3) (Arni and Ward, 1996; Renetseder et al., 1985). These data implies that the catalytic mechanism of the starfish PLA_2 is essentially the same as those of the group Ⅰ and Ⅱ type PLA_2s. Further, the starfish PLA_2 1, 2, and 3 conserved the 14 Cys residues at the appropriate positions which were

involved in the intramolecular disulfide bonds in the group Ⅰ type PLA_2 (Fig. 3) (Dennis, 1983). Accordingly, the starfish PLA_2 can be classified into the group Ⅰ type. On the other hand, the homology calculated with whole sequences between the starfish PLA_2 1, 2, and 3 and the other animal PLA_2s was relatively low (36-48%) since the high sequence divergency exists in the amino acid residues 54-107 including the pancreatic loop and β-wing regions (Fig. 3) (Arni and Ward, 1996; Renetseder et al., 1985). The starfish PLA_2 1, 2, and 3 possess the pancreatic loop-like sequence in the residues 63-66, however, two amino acid deletions were required in the residues 62(+1) and 66(+1) to align with the sequence of the porcine pancreatic PLA_2. In addition, two insertions of each of eight residues (residues 76-83 and 89-96) and deletion of three residues (residues 84(+1)-84(+3)) were required for the starfish PLA_2 1, 2, and 3 to align with the β-wing region of the porcine PLA_2. It has been reported that the PLA_2 of the groups Ⅰ and Ⅱ possesses N-terminal about ten residues forming the short amphiphilic helix (Arni and Ward, 1996). However, the secondary structure of the starfish PLA_2 predicted using SOPMA program (Geourjon and Deleage, 1995) showed to form extended strand structure in the N-terminal region (Fig. 4). Moreover, it was predicted that the starfish PLA_2 has an insertion of a long α-helix in the corresponding region to the β-wing of porcine pancreatic PLA_2 (Fig. 4). Therefore, we consider that the characteristic structures in these regions relate to the specific properties of the starfish PLA_2, such as the higher activity and characteristic substrate specificity comparing with those of the mammalian pancreatic PLA_2.

3.3 Phylogenetic relationship between the starfish PLA_2 and other PLA_2s

In order to clarify the molecular evolutional relationship between the starfish PLA_2 and the group Ⅰ and Ⅱ type PLA_2s, we made a phylogenetic tree using CLUSTAL W program (Thompson et al., 1994). As shown in Fig. 5, the starfish PLA_2 1, 2, and 3 are hardly placed in either group ⅠA or ⅠB. Therefore, the starfish PLA_2 should be classified into a new type of group Ⅰ PLA_2. The occurrence of a new PLA_2 group has also been suggested by McIntosh et al. with the PLA_2 from the venom of marine snail *Conus magus* (McIntosh et al., 1995). The enzyme has been classified into the group Ⅸ PLA_2 since it is comprised of the two polypeptide chains and shows little sequence homology to other PLA_2s (Dennis, 1997). On the other hand, Shiomi et al. purified two PLA_2s from the venom of crown-of-thorns starfish *Acanthaster planci* and determined the N-terminal 62 amino acid sequences (Shiomi et al., 1998). Although the sequences of the pancreatic loop and β-wing regions of these enzymes have not been determined, they identified the enzymes as the group Ⅰ type PLA_2 since these enzymes possess Cys-11 and elapid loop but without Cys-51. In a previous study, we reported that the molecular weight of *S. paxillatus* PLA_2 was slightly lower than that of *A. pectinifera* PLA_2 (approx. 13,000 on SDS-PAGE for *S. paxillatus* PLA_2 vs. 15,300 for *A. pectinifera* PLA_2) (Kishimura and Hayashi, 1998; Kishimura et al., 2000). In addition, the specific activity of *S. paxillatus* PLA_2 was not as high as that of *A. pectinifera* PLA_2 (26 units/mg for *S. paxillatus* PLA_2 vs. 119,000 units/mg for *A. pectinifera* PLA_2)

(Kishimura and Hayashi, 1998; Kishimura and Hayashi, 1999). Thus, it is unclear whether or not *S. paxillatus* PLA_2 should be classified into a new type of group Ⅰ PLA_2 like *A. pectinifera* PLA_2. Therefore, at present, *A. pectinifera* PLA_2 seems to be the only enzyme belonging to a new type of group Ⅰ PLA_2 among the starfish and other marine invertebrates.

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(Legends for figures)

Fig. 1. Oligonucleotide primers used for the amplification of DNAs by PCR. The primers were designed based on the amino acid sequence of the

starfish PLA_2 protein (Kishimura et al., 2000). Upper and lower rows show the nucleotide and amino acid sequences, respectively. The single-letter amino acid code is used. F, forward primer synthesized based on the amino acid sequence of residues 1-5 in the starfish PLA_2 protein along with 5'-terminal "ATG" as a translational initiation codon. R, reverse primer corresponding to the residues 132-137 in the starfish PLA_2 protein.

Fig. 2. The nucleotide and deduced amino acid sequences of the starfish PLA_2 cDNAs.

The deduced amino acid sequence and the residue numbers are shown below the codons. The single-letter amino acid code is used. Numbers in the right margin refer to the last nucleotide in each row. Annealing sites of PCR-primers, F and R (see in Fig. 1), are underlined. Amino acid residues different from those of the starfish PLA_2 protein (Kishimura et al., 2000)

are boxed. a, cDNA 1*[1]; b, cDNA 2*[2]; c, cDNA 3*[3].

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*[1]Accession No. AB022278 in DDBJ.

*[2]Accession No. AB032266 in DDBJ.

*[3]Accession No. AB032267 in DDBJ.

Fig. 3. Alignment of the deduced amino acid sequences of the starfish PLA_2s with the sequences of group Ⅰ and Ⅱ PLA_2s.

Residues identical in all the PLA_2s in this figure are boxed. Dashes indicate deletions introduced for maximizing the sequence similarity. The location of the active site, Ca^{2+}-binding loop, elapid and pancreatic loop, and β-wing region are shown with solid bars based on the crystallographic studies of bovine pancreatic and *Crotalus atrox* venom PLA_2s (Arni and Ward, 1996; Renetseder et at., 1985). The positions of deleted amino acids in pancreatic loop and β-wing regions of the starfish PLA_2s are

represented as 62(+1), 66(+1), 84(+1), 84(+2), and 84(+3). Starfish 1, 2, and 3, *A. pectinifera* PLA_2s from cDNAs 1, 2, and 3, respectively (present paper); Snake (ⅠA), *Naja naja atra* venom PLA_2 (Chang et al., 1997); Porcine (ⅠB), porcine pancreatic PLA_2 (Puijk et al., 1977); Snake (ⅡA), *C. atrox* venom PLA_2 (Randolph and Heinrikson, 1982); Snake (ⅡB), *Bitis gabonica* venom PLA_2 (Botes and Viljoen, 1974); Rat (ⅡC), Rat brain PLA_2 (Chen et al., 1994).

Fig. 4. Predicted secondary structure of the starfish PLA_2.

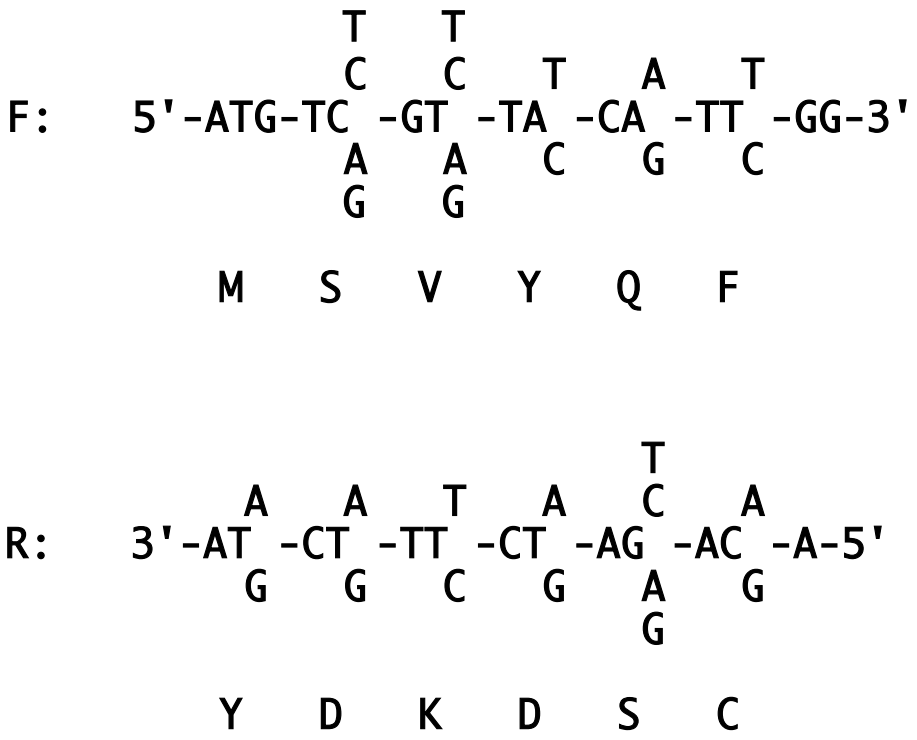
Secondary structures of the starfish PLA_2 and porcine pancreatic PLA_2 were predicted using SOPMA program (Geourjon and Deleage, 1995). Dashes indicate deletions introduced for maximizing the structure similarity. The location of the active site, Ca^{2+}-binding loop, elapid and pancreatic loop, and β-wing region are shown with solid bars based on the crystallographic studies of bovine pancreatic and *Crotalus atrox* venom PLA_2s (Arni and Ward, 1996; Renetseder et at., 1985). Starfish 1, predicted secondary structure of the starfish PLA_2 1 (present paper); Porcine (ⅠB), predicted secondary structure of porcine pancreatic PLA_2 (Puijk et al., 1977); h, α-helix; e, extended strand; t, β-turn; c, random coil.

Fig. 5. Radial rootless phylogenic tree of PLA_2s.

The phylogenetic tree was made using the programs of CLUSTAL W (Thompson et al., 1994) and TreeView (Page, 1996). The branch length represents the evolutionary distance between the proteins. PLA_2s belonging to the same group are boxed: group Ⅰ A (O.s.scu, *Oxyuranus s. scutellatus* venom (Lind and Eaker, 1982); L.sem, *Laticauda semifasciata* venom (Takasaki et al., 1988); E.sch, *Enhydrina schistosa* venom (Lind and Eaker, 1981); N.s.scu, *Notechis scutatus scutatus* venom (Lind and Eaker, 1980); P.aus, *Pseudechis australis* venom (Nishida et al., 1985); B.mul, *Bungarus multicinctus* venom (Kondo et al., 1981); N.n.atr, *Naja naja atra* venom (Chang et al., 1997); N.mel, *Naja melanoleuca* venom (Joubert, 1975a); N.m.mos, *Naja mossanbica mossanbica* venom (Joubert, 1977); N.nig, *Naja nigricollis* venom (Chwetzoff et al., 1989); H.hae, *Hemachatus*

haemachatus venom (Joubert, 1975b)), group ⅠB (Por, porcine pancreas (Puijk et al., 1977); Bov, bovine pancreas (Fleer et al., 1978); Hor, horse pancreas (Evenberg et al., 1977); Hum, human pancreas (Verheij et al., 1983); Dog, dog pancreas (Ohara et al., 1986); Rat, rat pancreas (Ohara et al., 1986); Red, red sea bream hepatopancreas*), group ⅡA (C.ada, *Crotalus adamanteus* venom (Heinrikson et al., 1977); C.atr, *Crotalus atrox* venom (Randolph and Heinrikson, 1982); T.oki, *Trimeresurus okinavensis* venom (Joubert and Haylett, 1981); T.fla, *Trimeresurus flavoviridis* venom (Oda et al., 1990); A.h.blo, *Agkistrodon halys blomhoffii* venom (Tomoo et al., 1989); Hum.s, human synovial fluid (Kramer et al., 1989); Rat.p, rat platelet (Hayakawa et al., 1988)), group ⅡB (B.gab, *Bitis gabonica* venom (Botes and Viljoen, 1974); B.cau, *Bitis caudalis* venom (Viljoen et al., 1982)), and group ⅡC (Rat.b, rat brain (Chen et al., 1994)). Star1, 2, and 3, *A. pectinifera* PLA_2 1, 2, and 3 from cDNA 1, 2, and 3, respectively (present paper).

*Accession No. AB009286 in DDBJ.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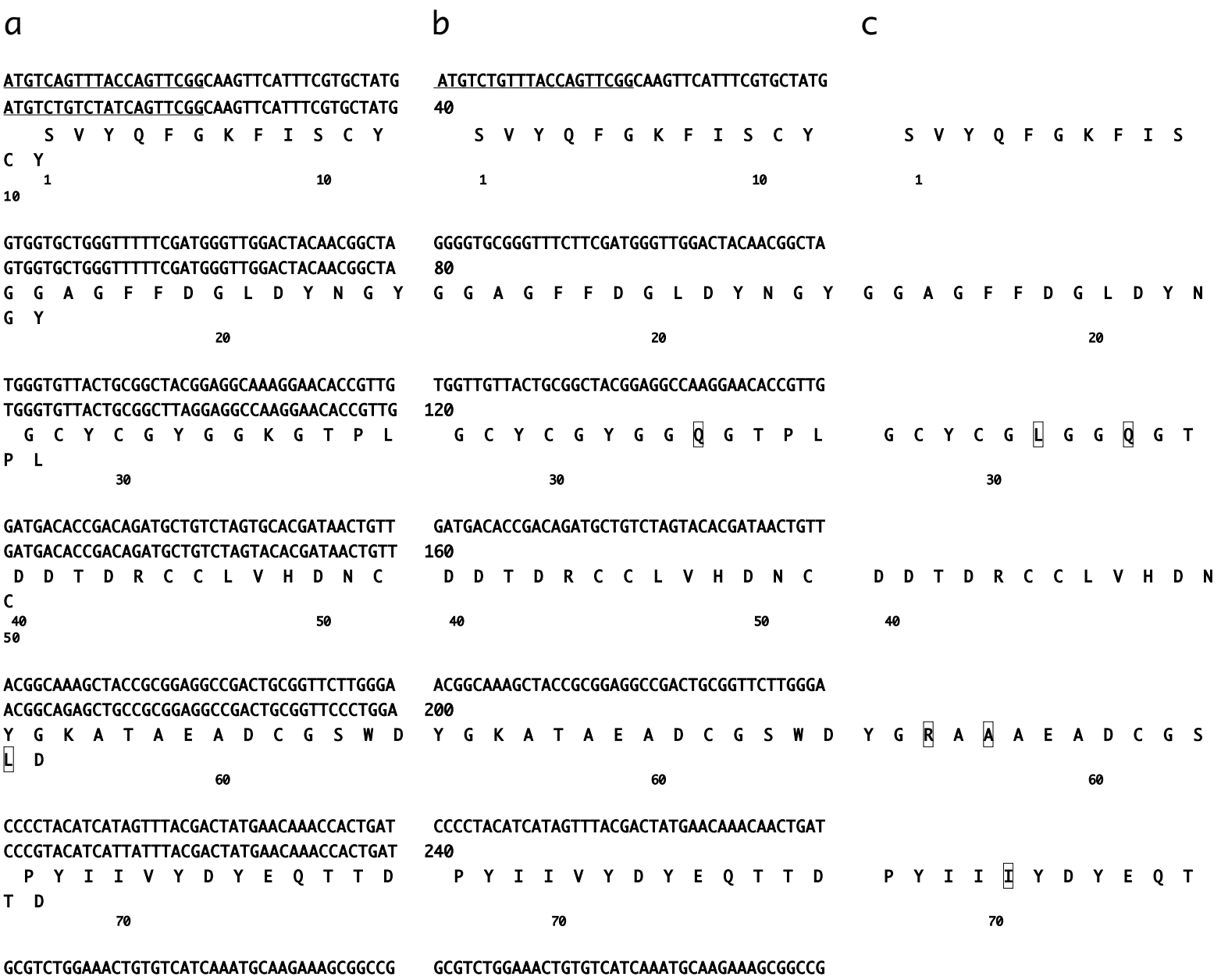
H. Kishimura

cDNA cloning of starfish phospholipase A_2


a
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c
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S V Y Q F G K F I S C Y
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G C Y C G Y G G K G T P L
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D D T D R C C L V H D N C
40
50
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Y G K A T A E A D C G S W D
L D
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P Y I I V Y D Y E Q T T D
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40
S V Y Q F G K F I S C Y
1
10
GGGGTGCGGGTTTCTTCGATGGGTTGGACTACAACGGCTA
80
G G A G F F D G L D Y N G Y
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G C Y C G Y G G Q G T P L
30
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D D T D R C C L V H D N C
40
50
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S V Y Q F G K F I S
1
G G A G F F D G L D Y N
20
G C Y C G L G G Q G T
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P Y I I I Y D Y E Q T
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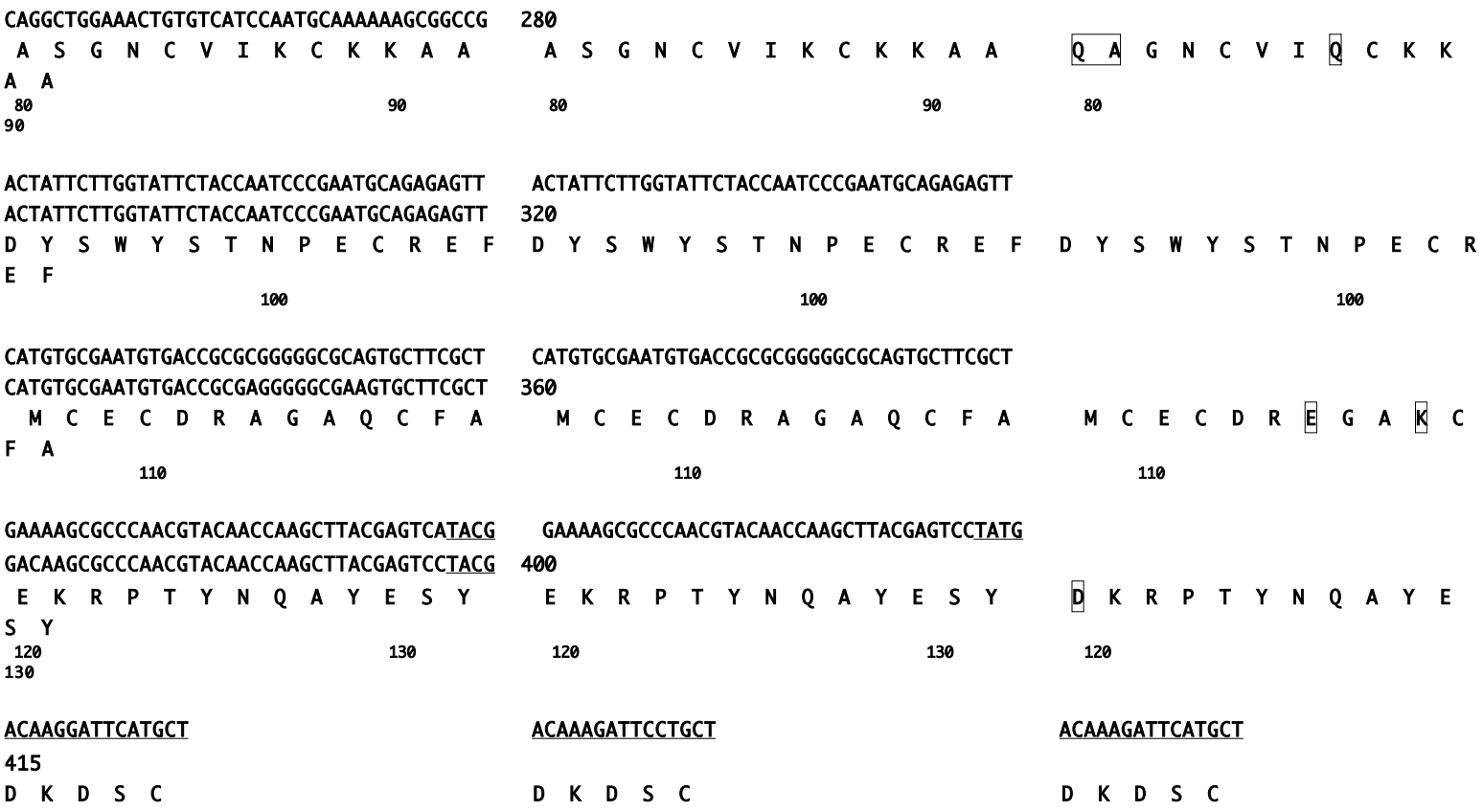
Fig. 2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D Y S W Y S T N P E C R E F
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E F
100
100
100
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M C E C D R A G A Q C F A
M C E C D R E G A K C
F A
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110
110
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E K R P T Y N Q A Y E S Y
D K R P T Y N Q A Y E
S Y
120
130
120
130
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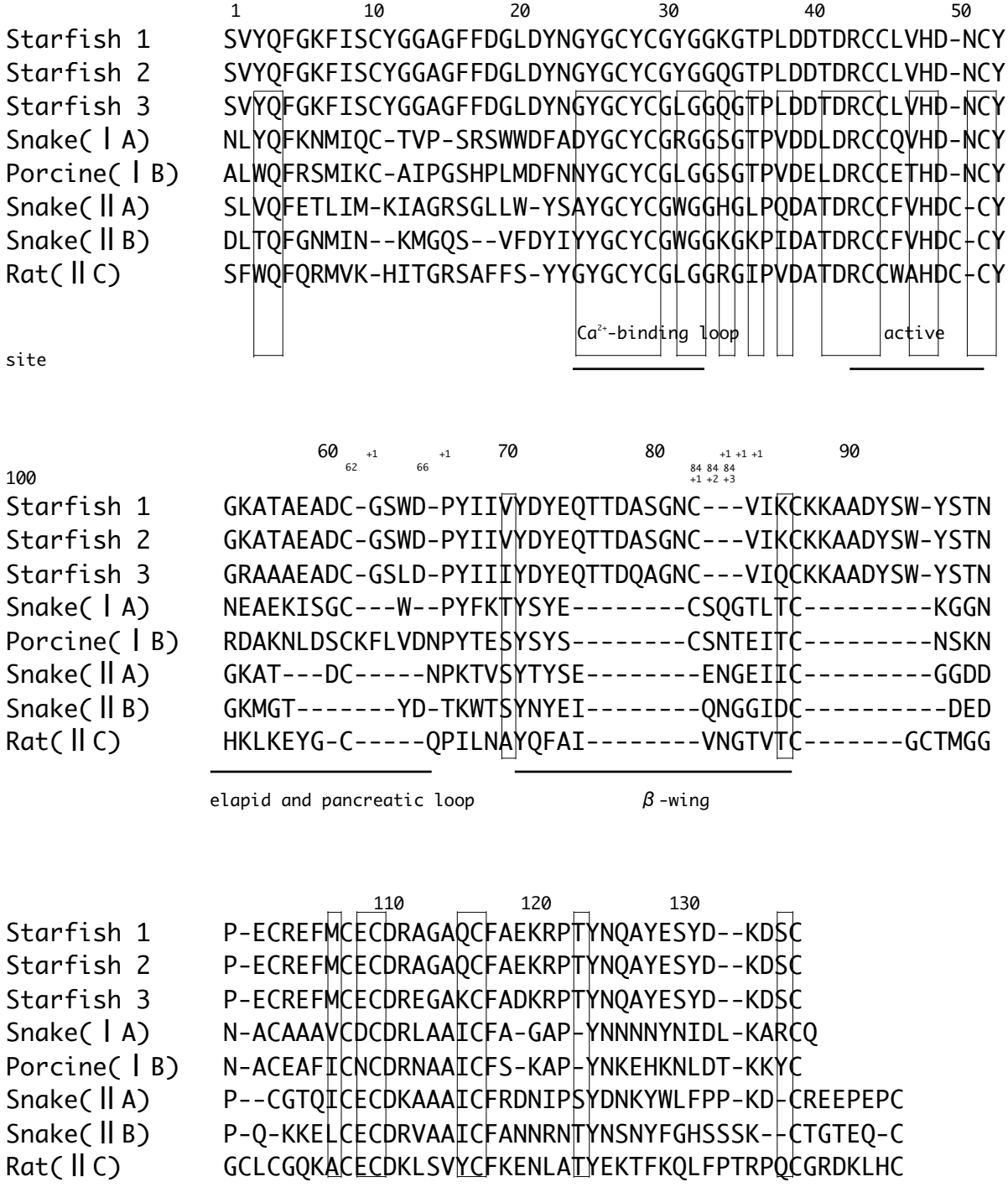
D K D S C
D K D S C
D K D S C

Fig.3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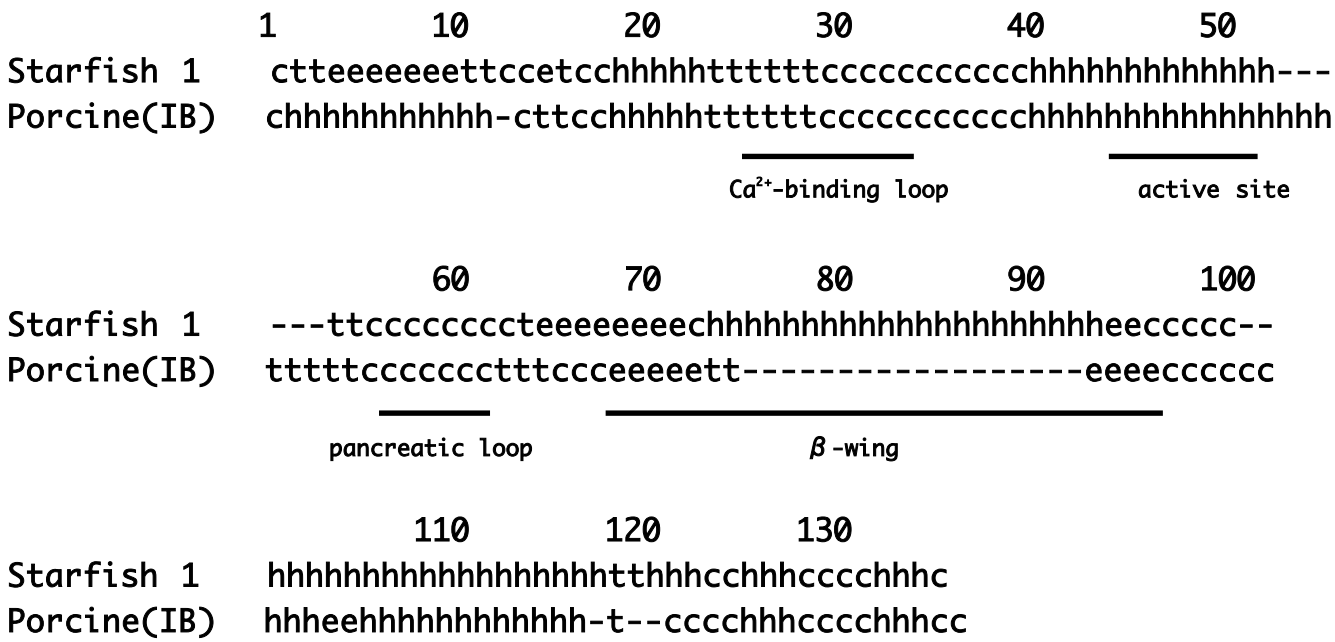
Starfish 3 SVYQFGKFISCYGGAGFFDGLDYNGYGCYCGLGGQGTPLDDTDRCCLVHD-NCY
Snake(Ⅰ A) NLYQFKNMIQC-TVP-SRSWWDFADYGCYCGRGGSGTPVDDLDRCCQVHD-NCY
Porcine(Ⅰ B) ALWQFRSMIKC-AIPGSHPLMDFNNYGCYCGLGGSGTPVDELDRCCETHD-NCY
Snake(Ⅱ A) SLVQFETLIM-KIAGRSGLLW-YSAYGCYCGWGGHGLPQDATDRCCFVHDC-CY
Snake(Ⅱ B) DLTQFGNMIN--KMGQS--VFDYIYYGCYCGWGGKGKPIDATDRCCFVHDC-CY
Rat(Ⅱ C) SFWQFQRMVK-HITGRSAFFS-YYGYGCYCGLGGRGIPVDATDRCCWAHDC-CY
Ca2+-binding loop
active site
60 62 +1 66 +1 70 80 84 84 84 +1 +1 +1 90 100
Starfish 1 GKATAEADC-GSWD-PYIIVYDYEQTTDASGNC---VIKCKKAADYSW-YSTN
Starfish 2 GKATAEADC-GSWD-PYIIVYDYEQTTDASGNC---VIKCKKAADYSW-YSTN
Starfish 3 GRAAAEADC-GSLD-PYIIIYDYEQTTDQAGNC---VIQCKKAADYSW-YSTN
Snake(Ⅰ A) NEAEKISGC---W--PYFKTYSYE--------CSQGTLTC---------KGGN
Porcine(Ⅰ B) RDAKNLDSCKFLVDNPYTESYSYS--------CSNTEITC---------NSKN
Snake(Ⅱ A) GKAT---DC-----NPKTVSYTYSE--------ENGEIIC---------GGDD
Snake(Ⅱ B) GKMGT-------YD-TKWTSYNYEI--------QNGGIDC----------DED
Rat(Ⅱ C) HKLKEYG-C-----QPILNAYQFAI--------VNGTVTC-------GCTMGG
elapid and pancreatic loop
β-wing
110 120 130
Starfish 1 P-ECREFMCECDRAGAQCFAEKRPTYNQAYESYD--KDSC
Starfish 2 P-ECREFMCECDRAGAQCFAEKRPTYNQAYESYD--KDSC
Starfish 3 P-ECREFMCECDREGAKCFADKRPTYNQAYESYD--KDSC
Snake(Ⅰ A) N-ACAAAVCDCDRLAAICFA-GAP-YNNNNYNIDL-KARCQ
Porcine(Ⅰ B) N-ACEAFICNCDRNAAICFS-KAP-YNKEHKNLDT-KKYC
Snake(Ⅱ A) P--CGTQICECDKAAAICFRDNIPSYDNKYWLFPP-KD-CREEPEPC
Snake(Ⅱ B) P-Q-KKELCECDRVAAICFANNRNTYNSNYFGHSSSK--CTGTEQ-C
Rat(Ⅱ C) GCLCGQKACECDKLSVYCFKENLATYEKTFKQLFPTRPQCGRDKLHC

Fig.4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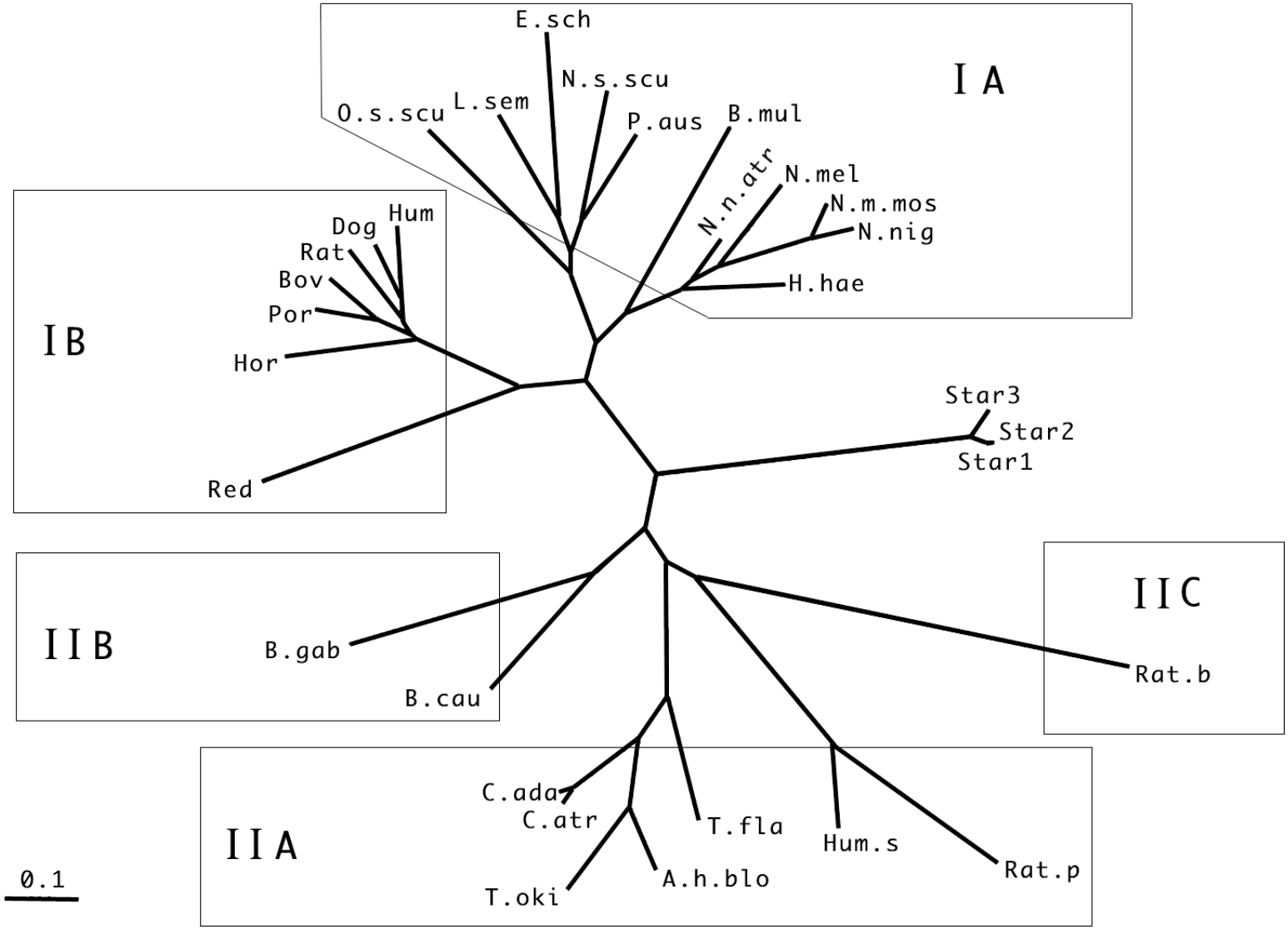
Ca2+-binding loop
active site
60 70 80 90 100
Starfish 1 ---ttccccccccteeeeeeeechhhhhhhhhhhhhhhhhhhhheeccccc--
Porcine(IB) tttttcccccccttttccceeeeett----------eeeeccccccc
pancreatic loop
β-wing
110 120 130
Starfish 1 hhhhhhhhhhhhhhhhhhhtthhhcchhhcccchhhc
Porcine(IB) hhheehhhhhhhhhhhh-t--cccchhhcccchhhcc

Fig.5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P.aus
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N.nig
H.hae
I A
Dog
Hum
Rat
Bov
Por
Hor
Red
I B
Star3
Star2
Star1
B.gab
B.cau
I I B
Rat.b
I I C
C.ada
C.atr
T.fla
Hum.s
Rat.p
T.oki
A.h.blo
I I A
0.1