

Islet Hormones from the African Bullfrog *Pyxicephalus adspersus* (Anura:Ranidae): Structural Characterization and Phylogenetic Implications

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The African bullfrog *Pyxicephalus adspersus* is generally classified along with frogs of the genus *Rana* in the subfamily *Raninae* of the family *Ranidae* but precise phylogenetic relationships between species are unclear. Pancreatic polypeptide (PP), insulin, and glucagon-like peptide (GLP-1) were isolated from an extract of *P. adspersus* pancreas and characterized structurally. A comparison of the amino acid sequence of *Pyxicephalus* PP (APSEPQ-HPGG¹⁰QATPEQLAQY²⁰YSDLYQYITF³⁰ITRPRF · NH₂) with those of the known amphibian PP molecules in a maximum parsimony analysis generates a single phylogenetic tree in which *Pyxicephalus* is the sister to the clade comprising the members of the genus *Rana*. The three orders of living amphibians form discrete clades with the representative of the Gymnophiona appearing as sister to the Caudata-Anura. In contrast, *Pyxicephalus* insulin (A chain, GIVEQC-CHSA¹⁰CSLYDLENYC²⁰N; B-chain, LANQHLCGSH¹⁰-LVEALYMVCG²⁰ERGFYYPKS³⁰) and and GLP-1 (HAEG-TFTSDM¹⁰TSYLEEKAAR²⁰EFVDWLIKGR³⁰PK) resemble more closely the corresponding peptides from the cane toad *Bufo marinus* than the peptides from any species of *Rana*. Cladistic analysis based upon the amino acid sequences of insulin produced a polyphyletic assemblage with the Gymnophiona nesting within an unresolved clade containing the non-ranid frogs. The data support the assertion that the

amino acid sequence of PP, but not those of the other islet hormones, is of value as a molecular marker for inferring phylogenetic relationships between early tetrapod species.

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Pancreatic polypeptide (PP) is a 36-amino acid residue C-terminally α -amidated peptide that is synthesized primarily in the F cell of the pancreatic islets of tetrapods. PP is a member of a family of homologous regulatory peptides that comprises, in addition to PP, neuropeptide tyrosine (NPY) synthesized in neurons of the central and peripheral nervous systems and in chromaffin cells of the adrenal medulla and peptide tyrosine tyrosine (PYY) localized primarily to endocrine cells in the lower intestinal tract (reviewed in Larhammar *et al.*, 1993; Larhammar, 1996). The structural similarity between the peptides suggests that the family has arisen from successive duplications of an ancestral gene that have taken place before, or concomitant with, the appearance of the amphibia (Conlon *et al.*, 1992; Larhammar, 1996). The physiological role of PP remains an enigma. A G-protein-coupled receptor (Y4/PP1) that selectively binds PP with high affinity has been identified in the human colon, small intestine, pancreas, and prostate (Lundell *et al.*, 1995) and infusions of PP inhibit pancreatic exocrine secretion and relax the gall bladder (Mannon and Taylor, 1994). More recently, transgenic mice overexpressing the PP gene in the pancreatic islets showed decreased

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food intake and a decreased rate of gastric emptying compared with control animals (Ueno *et al.*, 1999).

The interest of the authors' laboratories in PP lies in the potential value of the peptide as a molecular marker for inferring phylogenetic relationships between species (Platz and Conlon, 1997). In contrast to NPY and PYY (Wang *et al.*, 1999), evolutionary pressure to conserve the amino acid sequences of PP has been poor (Conlon *et al.*, 1998b). The marked structural diversity of PP in species of amphibia and reptiles has suggested to Larhammar (1996) that the molecule was in an a state of rapid evolutionary flux during the time when the early tetrapod species diverged from each other. PP sequences may thus be a useful tool with which to study the branching order in early tetrapod evolution. Among the amphibia, previous work from our and other laboratories has led to elucidation of the primary structure of PP for four species of Ranid frog [*Rana catesbeiana* (Pollock *et al.*, 1988), *Rana temporaria* (McKay *et al.*, 1990), *Rana sylvatica* (Conlon *et al.*, 1998b), *Rana ridibunda* (Conlon *et al.*, 1998b)], the cane toad *Bufo marinus* (Conlon *et al.*, 1998b), two salamanders [the three-toed amphiuma, *Amphiuma tridactylum* (Cavanaugh *et al.*, 1996), and the lesser siren, *Siren intermedia* (Conlon *et al.*, 1998b), Caudata], and the caecilian *Typhlonectes natans* (Gymnophiona) (Conlon *et al.*, 1998b).

The African bullfrog *Pyxicephalus adspersus* is a large, aggressive anuran found in sub-Saharan Africa that is best known for its impressive feats of gourmandism which include ingestion of rats, snakes, small chickens, and other frogs (Branch, 1976). The species has attracted interest as an animal model in which to study the effect of feeding and fasting upon adaptation of the gastrointestinal tract (Secor and Diamond, 1996). The animal fasts for several months during a period of underground dormancy during the dry season and then feeds voraciously after emerging during the rainy season. Feeding results in dramatic and rapid adaptive changes in the gastrointestinal tract such as increases in mucosal mass (3- to 4-fold), rates of intestinal nutrient uptake (5- to 10-fold), oxygen consumption rate (10-fold) and intestinal brush border oligopeptidase activity (2.5- to 4-fold). *Pyxicephalus* then down-regulates gut performance after digestion in order to conserve energy during periods of fasting.

This study was designed primarily to increase our understanding of the molecular evolution of the islet hormones within the Amphibia by purification and structural characterization of PP, insulin, and glucagon-like peptide-1 (GLP-1) from the African bullfrog *P. adspersus*. An assessment of the relative value of the amino acid sequences of these peptides as phylogenetic markers is made.

MATERIALS AND METHODS

Tissue Extraction

Pancreatic tissue (0.7 g) from the African bullfrog *P. adspersus* (10 adult specimens of both sexes; 5 fasted) was homogenized with ethanol/0.7 M HCl (3/1 v/v; 30 ml) using a Waring blender and stirred for 2 h at 0°C as previously described (Cavanaugh *et al.*, 1996). After centrifugation (4000g for 30 min), ethanol was removed from the supernatant under reduced pressure. After a further centrifugation (4000g for 30 min), the extract was pumped at a flow rate of 2 ml/min through 4 Sep-Pak C-18 cartridges (Waters Associates, Milford, MA) connected in series. Bound material was eluted with acetonitrile/water/trifluoroacetic acid (70.0/29.9/0.1) and freeze-dried.

Radioimmunoassay

Insulin-like immunoreactivity was measured using an antiserum raised against pig insulin as previously described (Flatt and Bailey, 1981). PP-like immunoreactivity was measured by radioimmunoassay using antiserum PP221 directed against the COOH-terminal region of human PP in a procedure that has been described previously (O'Hare *et al.*, 1983). The antiserum requires the presence of an α -amidated COOH-terminal residue in PP for reactivity but shows <0.5% cross-reactivity with pig PYY and NPY.

Purification of the Peptides

The pancreatic extract, after partial purification on Sep-Pak cartridges, was redissolved in 0.1% (v/v) trifluoroacetic acid/water (2 ml) and injected onto a 1 × 25-cm Vydac 218TP510 C-18 reversed-phase HPLC

column (Separations Group, Hesperia, CA) equilibrated with 0.1% (v/v) trifluoroacetic acid/water at a flow rate of 2 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 21% over 10 min and to 49% over 60 min with linear gradients. Absorbance was measured at 214 and 280 nm and fractions (1 min) were collected. The fractions designated I (containing insulin-like immunoreactivity) and P (containing PP-like immunoreactivity) (Fig. 1) were rechromatographed on a 0.46×25 -cm Vydac 214TP54 C-4 reversed-phase HPLC column equilibrated with acetonitrile/water/trifluoroacetic acid (21.0/78.9/0.1) at a flow rate of 1.5 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 42% over 40 min using a linear gradient. The *Pyxicephalus* peptides were purified to near homogeneity, as assessed by peak symmetry, by chromatography on a 0.46×25 -cm Vydac 219TP54 phenyl column under the same conditions as those used for the C-4 column.

As a radioimmunoassay for GLP was not available in the laboratory, all fractions with retention times between 41 and 68 min from the semipreparative Vydac C-18 column (Fig. 1) were individually chromatographed on analytical Vydac C-4 and phenyl columns under the same experimental conditions as those used for the purification of *Pyxicephalus* insulin. Purified peptides that were isolated in relatively high abundance were subjected to electrospray mass spectrometry.

Structural Characterization

Pyxicephalus insulin (approximately 1 nmol) was incubated for 3 h at room temperature with dithiothreitol (2 mg) in 0.1 M Tris-HCl/6 M guanidine hydrochloride buffer, pH 7.5 (0.4 ml) under an atmosphere of argon. Cysteine residues were derivatized by addition of 4-vinylpyridine (3 μ l) and the pyridylethylated A- and B-chains of insulin were separated on a 0.46×25 -cm Vydac 218TP54 C-4 column under the conditions used for the purification of intact insulin (Fig. 2A).

The primary structures of the peptides were determined by automated Edman degradation using a Perkin-Elmer Model 491A sequenator. Electrospray mass spectrometry was carried out using a Perkin-Elmer Sciex API 150EX single quadrupole instrument. The accuracy of mass determinations was $\pm 0.02\%$.

Cladistic Analysis

PAUP (version 3.1.1) developed by Swofford (1993) was used in a maximum parsimony analysis using amino acid residues of PP and insulin as separate, unordered characters. Branch and bound methods were used in both analyses.

For PP tree construction, sequences for 10 terminal taxa were included [*R. catesbeiana* (Pollock *et al.*, 1988); *R. temporaria* (McKay *et al.*, 1990); *R. sylvatica*, *R. ridibunda*, *B. marinus*, *S. intermedia*, *T. natans* (Conlon *et al.*, 1998b); *A. tridactylum* (Cavanaugh *et al.*, 1996); and *Xenopus laevis* (J. M. Conlon, unpublished data)]. The amino acid sequences used are shown in Fig. 3. The sequence of the PYY from the frog, *R. ridibunda* (Conlon *et al.*, 1992), was used as the outgroup for rooting the phylogenetic tree.

For insulin tree construction, sequences for 10 terminal taxa were included [*R. catesbeiana*, *R. sylvatica*, *R. ridibunda* (Conlon *et al.*, 1998c); *B. marinus* (Conlon *et al.*, 1998a); *X. laevis* (Shuldiner *et al.*, 1989); *Ceratophrys ornata* (White *et al.*, 1999); *A. tridactylum* (Conlon *et al.*, 1996); *S. intermedia* (Conlon *et al.*, 1997); and *T. natans* (Conlon *et al.*, 1995)]. The amino acid sequences used are shown in Fig. 3. The sequence of insulin from the Australian lungfish *Neoceratodus forsteri* (Conlon *et al.*, 1999) was used as the outgroup. Although interrelationships within the sarcopterygian clade remain controversial, analyses based upon morphological characteristics, the fossil record, and nucleotide sequences generally favor the hypothesis that the Dipnoi (lungfishes) are the closest living relatives of the Tetrapoda (amniotes and lissamphibians) (Cloutier and Ahlberg, 1996).

RESULTS

Purification of Insulin

The pancreatic extract, after partial purification on Sep-Pak cartridges, was chromatographed on a semipreparative Vydac C-18 column and the elution profile is shown in Fig. 1. The prominent peak designated I was associated with insulin-like immunoreactivity. Rechromatography of this fraction on an analytical Vydac C-4 column (Fig. 2A) revealed that the insulin-like immunoreactivity was associated with the major

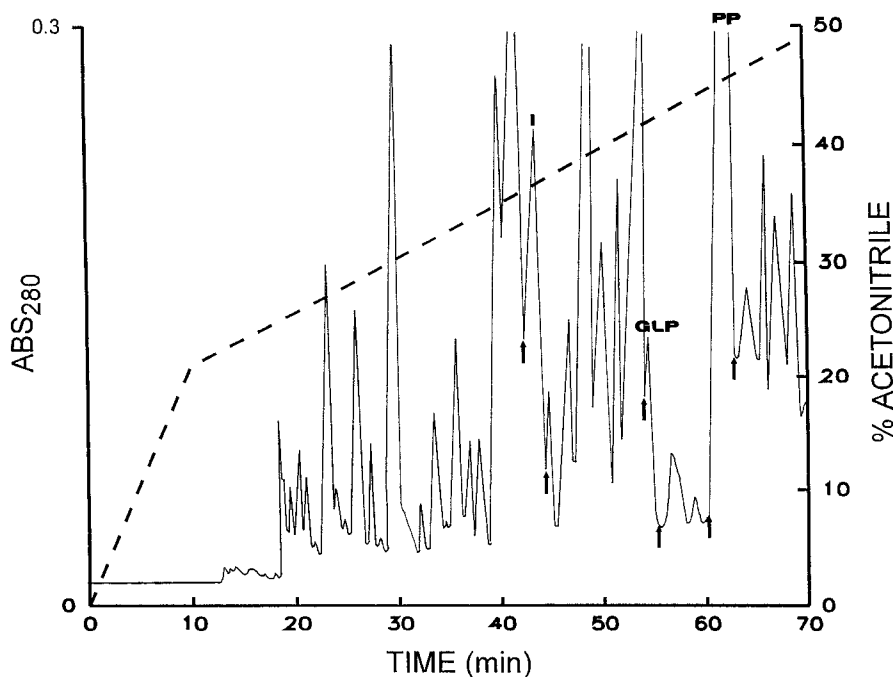


FIG. 1. Reversed-phase HPLC of an extract of the pancreas of *Pyxicephalus adspersus* after partial purification on Sep-Pak cartridges. I denotes the fraction containing insulin, GLP denotes the fraction containing glucagon-like peptide-1, and PP denotes the fraction containing pancreatic polypeptide. (---) The concentration of acetonitrile in the eluting solvent.

peak designated I and delineated by the arrows. *Pyxicephalus* insulin was purified to near homogeneity (as assessed by symmetrical peak shape) by a final chromatography on an analytical Vydac phenyl column. The final yield of the pure peptide was approximately 3 nmol.

Purification of PP

As shown in Fig. 1, PP-like immunoreactivity was eluted from the semipreparative Vydac C-18 column in the fraction corresponding to the major peak in the chromatogram designated PP. Rechromatography of this material on an analytical Vydac C-4 column resulted in complete separation of *Pyxicephalus* PP from the nonimmunoreactive material (Fig. 2B). Homogeneity of the peptide was confirmed by a further on an analytical Vydac phenyl column and the final yield of pure peptide was approximately 2 nmol.

Purification of GLP-1

As a radioimmunoassay for GLP was not available in the laboratory, the strategy employed to isolate

Pyxicephalus GLP-1 was based upon that used to isolate the corresponding peptide from an extract of *Ceratophrys* pancreas (White *et al.*, 1999) and involves purification to near homogeneity most of the components present in major abundance that were eluted from a semipreparative C-18 HPLC column with a retention time between 41 and 60 min (Fig. 1). The peptides were subjected to electrospray mass spectrometry and those peptides with a molecular mass between 3000 and 5000 Da were identified by amino acid sequence analysis. A total of 20 fractions (volume 2 ml) were individually chromatographed on an analytical Vydac C-4 column under the same conditions as those used for the purification of insulin. Peaks of major abundance (a total of 13) were rechromatographed on an analytical Vydac phenyl column and 11 peptides were isolated in pure form, as assessed by peak symmetry. Subsequent characterization of these peptides indicated that GLP-1 was eluted from the semipreparative C-18 column in the fraction designated GLP (Fig. 1). The purification of the peptide on the analytical C-4 column is shown in Fig. 2C. The final yield of pure *Pyxicephalus* GLP-1 was approximately 0.5 nmol. Attempts to identify peptides

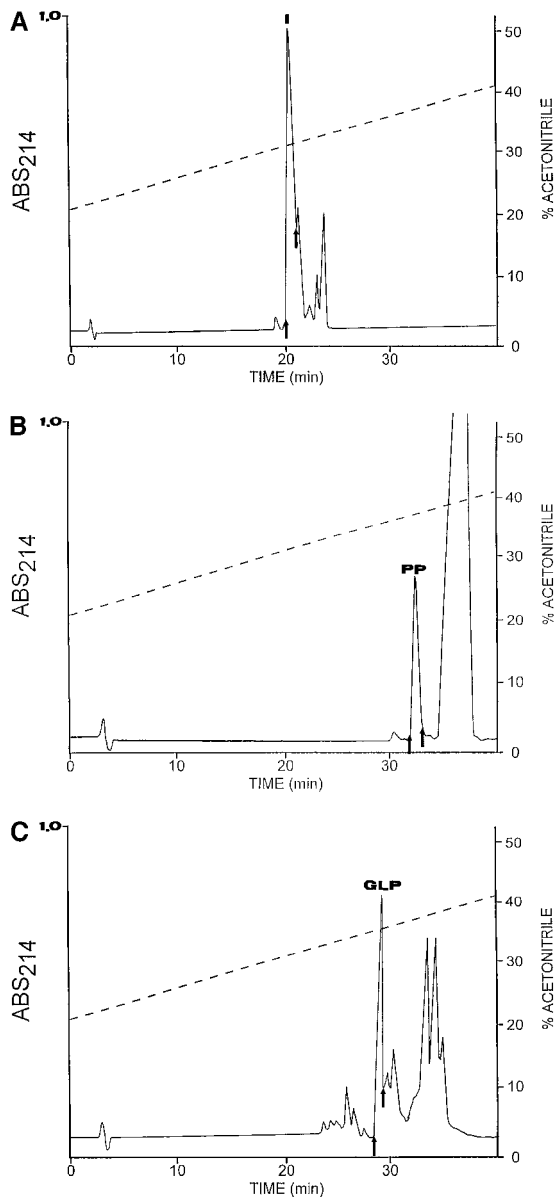


FIG. 2. Purification by reversed-phase HPLC on an analytical Vydac C-4 column of *Pyxicephalus* (A) insulin, (B) pancreatic polypeptide, and (C) glucagon-like peptide-1. The arrows show where peak collection began and ended.

with structural similarity to glucagon and GLP-2 were unsuccessful.

Structural Characterization

The primary structures of the pyridylethylated A-chains and B-chains of *Pyxicephalus* insulin were de-

termined by automated Edman degradation and the results are shown in Fig. 3. It was possible to identify without ambiguity phenylthiohydantoin-coupled amino acids for 21 cycles of operation of the sequenator during sequence analysis of the A-chain and for 30 cycles during analysis of the B-chain. The proposed amino acid sequence of *Pyxicephalus* insulin was confirmed by electrospray mass spectrometry. The observed molecular mass of the pyridylethylated A-chain was 2784.3 amu compared with a calculated average mass of 2784.6 amu and the observed molecular mass of the pyridylethylated B-chain was 3644.2 amu compared with a calculated average mass of 3644.0 amu.

The primary structures of *Pyxicephalus* GLP-1 and PP were determined without ambiguity by automated Edman degradation (Fig. 3). The proposed structures were confirmed by electrospray mass spectrometry (GLP-1, observed molecular mass 3685.9 amu, calculated average mass 3686.2 amu; PP, observed molecular mass 4171.4 amu, calculated average mass 4170.6 amu).

Cladistic Analysis

The branch and bound search technique used to identify the shortest phylogenetic trees based upon the amino acid sequences of PP resulted in a single most-parsimonious tree of 52 steps based upon 13 out of 36 (36%) informative sites in the peptide (Fig. 4). The retention index for the tree was 0.833 and the consistency index was 0.885, indicating a strong phylogenetic signal. Cladistic analysis of the data set comprising the insulin amino acid sequences generated two equally parsimonious trees of 41 steps each based upon 14 out of 58 (24%) informative sites in the molecule. The retention index for each tree was 0.757 and the consistency index was 0.780. Both trees shared the same general topology with the exception of the placement of *Ceratophrys* which clustered with *Bufo* in one tree and with *Pyxicephalus* in the other. The strict consensus cladogram of the two shortest trees is shown in Fig. 5.

DISCUSSION

The amino acid sequences of *Pyxicephalus* PP, insulin, and GLP-1 are compared with the corresponding

A-Chain

<i>Pyxicephalus adspersus</i>	GIVEQ	CCHSA	CSLYD	LENYC	N
<i>Rana catesbeiana</i>	KP	-----	---N-	-----	-
<i>Rana ridibunda</i>	KP	-----	---N-	-----	-
<i>Rana sylvatica</i>	KP	-----	---NM	-----	S
<i>Bufo marinus</i>	-----	----T	----E	-----	-
<i>Ceratophrys ornata</i>	-----	----T	----E	-----	-
<i>Xenopus laevis I</i>	-----	-----	--F-E	--S--	-
<i>Xenopus laevis II</i>	-----	-----	--F-E	-----	-
<i>Amphiuma tridactylum</i>	AR	-----	---NT	---NQ	-----
<i>Siren intermedia</i>	-----	---NT	---Q	-----	-
<i>Typhlonectes natans</i>	-----	K	--LST	----E	--S--
<i>Neoceratodus forsteri</i>	-----	---TP	----Q	-----	-ETE

B-Chain

<i>Pyxicephalus adspersus</i>	LANQH	LCGSH	LVEAL	YMVCG	ERFFF	YYPKS
<i>Rana catesbeiana</i>	FP--Y	-----	-----	-----	D-----	--S-R-
<i>Rana ridibunda</i>	FP--Y	-----	-----	-----	-----	--S-R-
<i>Rana sylvatica</i>	FP---	-----	--D--	-----	D-----	--S-R-
<i>Bufo marinus</i>	-----	---P-	-----	-L---	----Y	----V
<i>Ceratophrys ornata</i>	-----	---P-	-----	-L---	----Y	----V
<i>Xenopus laevis I</i>	-V--Y	-----	-----	-L---	D-----	----V
<i>Xenopus laevis II</i>	-A--Y	-----	-----	-L---	D-----	----I
<i>Amphiuma tridactylum</i>	IT--Y	-----	-----	-L---	D-----	--S--
<i>Siren intermedia</i>	VP-KP	---A-	---VM	-F---	D-----	--PSST
<i>Typhlonectes natans</i>	I----	-----	-----	-L--A	D-----	--T---
<i>Neoceratodus forsteri</i>	AAV---	-----	-----	-F---	-----	--L--G

FIG. 3. A comparison of the primary structures of insulin, pancreatic polypeptide, and glucagon-like peptide-1 from species of Amphibia. (-) Residue identity. For the purposes of cladistic analysis, the amino sequences of the amphibian insulins are compared with that of the Australian lungfish *Neoceratodus forsteri*.

sequences of the peptides from other amphibian species in Fig. 3. On the basis of morphological characters, *Pyxicephalus* is classified along with species of the genus *Rana* in the sub-family *Raninae* in the family *Ranidae* (Duellman and Trueb, 1994). *Pyxicephalus* PP

contains only two amino acid substitutions compared with PP from *R. ridibunda* and cladistic analysis based upon the amino acid sequences of PP (Fig. 4) supports the conventional assessment of a close phylogenetic relationship between *Pyxicephalus* and *Rana*. The rapid

Pancreatic Polypeptide

<i>P. adspersus</i>	APSEP QHPGG QATPE QLAQY YSDLY QYITF ITRPR F
<i>R. ridibunda</i>	----- ----D ----D ----- ----- ----- -
<i>R. catesbeiana</i>	----- H---D ----D ----- ----- ----- -
<i>R. temporaria</i>	----- H---D ---QD ----- ----- ----- V---- -
<i>R. sylvatica</i>	----- H---D ----D ----- ----- ----- -
<i>X. laevis I</i>	----- M---D --S-- ---K- -D-WW ----- ----- -
<i>X. laevis II</i>	----- M---D --S-- ---K- -E-WW ----- ----- -
<i>B. marinus</i>	T---- ----D --S-- ----- ---W ----- V---- -
<i>A. tridactylum</i>	--K-- E---D D-S-- --EK- -Q--F ---I- ----- Y
<i>S. intermedia</i>	----- E---D N-S-D E--K- ----W ----- VG--- Y
<i>T. natans</i>	G-T-- I---K D---- E-TK- ----- D---L VG-S- W

Glucagon-Like Peptide-1

<i>P. adspersus</i>	HAEGT FTSDM TSYLE EKA AK EFVDW LIKGR PK
<i>R. catesbeiana</i>	--D-- ----- S---- ----- ----- --
<i>B. marinus</i> GLP-32	----- ----- --F-- ----- ----- --
<i>B. marinus</i> GLP-37	----- Y-N-V -QF-- ----- --I-- -L--I --KQR LS
<i>C. ornata</i>	--D-- Y-N-V -QF-- ----- --I-- ----K --KQR LS
<i>X. laevis</i> GLP-1A	----- ----V -QQ-D ----- --I-- --N-G -SKEI I-
<i>X. laevis</i> GLP-1B	----- Y-N-V -E--- ----- --IE- ----K --
<i>X. laevis</i> GLP-1C	----- --N-- -N--- ----- --G- ----- --
<i>A. tridactylum</i>	--D-- L---I NKV-D KQ-T- --IA- -VS-- GRRQ

FIG. 3—Continued

frogs form a monophyletic assemblage in which *Pyxicephalus* is sister group to the clade comprising four frogs of the genus *Rana*. Bufonids are seen as sister to the ranids and the two PP sequences from the tetraploid *Xenopus* appear as the most primitive among those examined in this study. At the ordinal level, the representative of the Gymnophiona (*Typhlonectes*) appears in the cladogram as sister to the Caudata (amphiuma and siren) and Anura. At present, the relationship between the three orders of amphibians is not resolved (Gauthier *et al.*, 1988) but the growing consensus supports the Anura-Caudata sister relationship

(Pough *et al.*, 1998). Although cladistic analysis by Trueb and Cloutier (1991) using a data set based upon soft tissue morphology supported a Caudata-Gymnophiona sister relationship, a data set based upon osteological characters favored an Anura-Caudata sister relationship. Similarly, a comprehensive analysis by Milner (1993) concluded that the Gymnophiona were best treated as sister to the Anura-Caudata group. In contrast, a molecular cladistic study comparing mitochondrial DNA sequences of the 12S and 16S rRNA genes (Hedges *et al.*, 1993) supported a phylogeny in which the Caudata (represented by the amphiuma) is sister

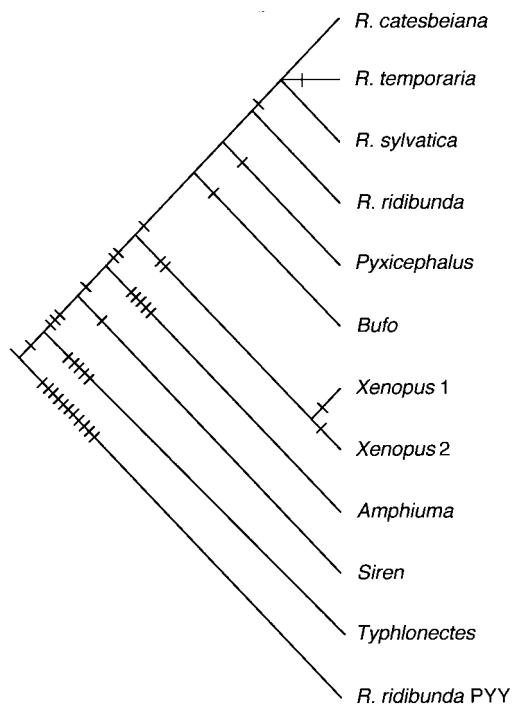


FIG. 4. Cladogram of the shortest phylogenetic tree obtained by analysis of the amino acid sequences of pancreatic polypeptide from representatives of the three living orders of amphibia. Hatch marks associated with ingroup taxa represent derived states for the various amino acid sites. Those lying on a line containing two or more taxa beyond the mark represent synapomorphies supporting a particular assemblage. Those that occur on single species branches are autapomorphies or amino acid replacements found only in that species. The amino acid sequence of PYY from the frog *Rana ridibunda* (YPPKPE¹⁰PGE¹⁰DASPEEMTKY²⁰LTALRHYINL³⁰VTRQRY) is used as outgroup to root the tree.

lineage to the Gymnophiona with the Anura (represented by *Xenopus*) as sister to this combined group. Although only 13 characters in the PP sequence are informative, each ordinal branch in our tree (Fig. 4) is supported by one, two, or three synapomorphies. The only unresolved portion of the cladogram is among three of the members of the genus *Rana* which might be expected as these taxa have diverged more recently.

Pyxicephalus insulin resembles most closely the common primary structure of the protein from *B. marinus* (Bufonidae) (Conlon *et al.*, 1998a) and *C. ornata* (Lepidodactylidae) (White *et al.*, 1999) with six amino acid substitutions (Fig. 3). The A-chain of *Pyxicephalus* insulin lacks the N-terminal dipeptide (Lys-Pro) extension found in A-chains of insulins from frogs of the genus *Rana* but the B-chain shares with the ranid frogs

the unusual substitution Leu → Met at position 17. The cladogram based upon the amino acid sequences of the amphibian insulins (Fig. 5) produced a consensus tree with membership at the ordinal level that is unsatisfactory. *Typhlonectes* appears as sister to an unresolved clade (polytomy) comprising *Pyxicephalus*, *Ceratophrys*, *Bufo*, and *Xenopus* with the ranid frogs forming a separate clade. Nesting of a member of the order Gymnophiona within the order Anura creates a polyphyletic assemblage. The entire subassembly of nonranid frogs and *Typhlonectes* is supported by the only synapomorphy obtained at the ordinal level. The fact that deeper branches in the rest of the tree lack any synapomorphies provides support for the assertion of other workers (Dores *et al.*, 1996) that the amino acid sequences of insulin are poor indicators of phylogenetic affinity.

A single molecular form of GLP-1 was isolated from the extract of *Pyxicephalus* pancreas that resembles most closely in primary structure the 32-amino acid form of GLP isolated from *B. marinus* pancreas (Con-

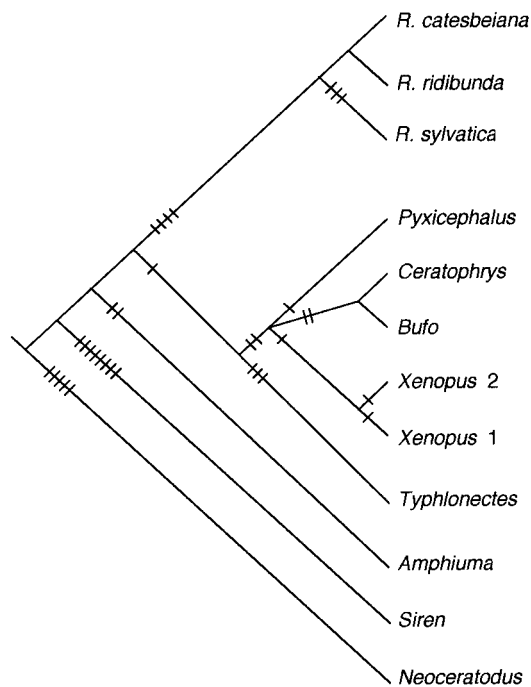


FIG. 5. Strict consensus cladogram of the two shortest phylogenetic trees obtained by analysis of the amino acid sequences of insulin from representatives of the three living orders of amphibia. The amino acid sequence of insulin from the lungfish *Neoceratodus forsteri* is used as outgroup to root the tree.

lon *et al.*, 1998a) with only one conservative amino acid substitution (Tyr¹³ → Phe). In *Pyxicephalus* pancreas, as in the pancreata of the *B. marinus*, *R. catesbeiana* (Pollock *et al.*, 1988), *C. ornata* (White *et al.*, 1999), and the amphiuma *A. tridactylum* (Cavanaugh *et al.*, 1996), GLP-1 is stored in the mature, fully processed form that corresponds to human intestinal GLP-1(7-37) (Orskov *et al.*, 1986). The amino acid sequences of vertebrate GLP-1 peptides, like those of PP, are highly variable with only three residues (Ala², Asp⁹, and Leu²⁶) being found in the same position in all known GLPs (Conlon *et al.*, 1994). On this basis, the peptide has the potential to be a valuable phylogenetic marker for studying branching order among the early tetrapods. Unfortunately, among the amphibia, the primary structure of GLP-1 had been determined for only four species of frog [*R. catesbeiana*, *C. ornata*, *B. marinus*, and *X. laevis* (Irwin *et al.*, 1997)] and for one salamander, *A. tridactylum*. Clearly, structural data from a much greater number of amphibian species are needed before a meaningful cladistic analysis can be carried out. Similarly, the use of GLP as a phylogenetic marker is complicated by the fact that the proglucagons in certain species, e.g., *X. laevis* (Irwin *et al.*, 1997), *B. marinus* (Conlon *et al.*, 1998a), and *Pipa pipa* (B. Matutte and J. M. Conlon, unpublished data) contain multiple GLP-1 peptides each with its own different amino acid sequence.

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