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IS THE AUSTRALIAN STRIPED BURROWING FROG *LITORIA ALBOGUTTATA* A MEMBER OF LITORIA OR CYCLORANA? A PROFILING STUDY USING SKIN PEPTIDES AS A PROBE

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Summary

The skin secretion of the Striped Burrowing Frog *Litoria alboguttata* contains the peptides named guttatins 1, 2 and 3. The sequences of these peptides are as follows:- guttatin 1 (GLLDSVL-NH_2); guttatin 2 (GLLDNVL-NH_2) and guttatin 3 (GLLDTVKGLN-NH_2). This peptide profile is different from those of congeners so far studied in that it contains neither neuropeptides nor antibiotic peptides. In contrast, the peptide profile of *L. alboguttata* is similar to that of *Cyclorana australis*. In particular, the two peptides in the skin secretion of *C. australis* correspond to guttatin 1 and GLLDGTVL-NH_2. Guttatin 1 is not present in the secretions of the other 22 *Litoria* species that we have investigated. Peptide profiling indicates that *Litoria alboguttata* is more closely related to the genus *Cyclorana* than *Litoria*.

KEY WORDS: *Cyclorana australis, Litoria alboguttata*, peptides, structure determination, mass spectrometry, classification.

ABBREVIATIONS: HPLC, high performance liquid chromatography; LC, liquid chromatography; MH^+ protonated molecule; MS, mass spectrometry; MS/MS, mass spectrometry/mass spectrometry (tandem mass spectrometry); *m/z*, mass to charge ratio; nNOS, neuronal nitric oxide synthase.

Introduction

Amphibians have rich chemical arsenals which form an integral part of their defence systems, and also assist with the regulation of dermal physiological action. In response to a variety of stimuli, host-defence compounds are secreted from special glands onto the dorsal surface and into the gut of the amphibian (Blevins & Zasloff 1990; Lazarus & Attila 1993; Erspamer 1994; Barra & Simmaco 1995). Many of these are neuropeptides; antimicrobial, anticancer, and antifungal active peptides or peptides which inhibit the formation of NO from neuronal nitric oxide synthase (nNOS).

During the last decade members of the Bowie/Tyler research group have identified some 200 active peptides from the glandular secretions of 35 species of Australian anurans from the genera *Crinia, Cyclorana, Limnodynastes, Litoria* and *Uperoleia*, recently reviewed by Apponyi *et al.* (2004). Most anurans of the genera *Crinia*, *Litoria* and *Uperoleia* have at least one neuropeptide, several antibiotic and anticancer active peptides, and at least one peptide which inhibits the formation of NO from nNOS (by complexing with, and changing the shape of the regulatory protein Ca^{2+} calmodulin). For example, the Green Tree Frog of Australia, *Litoria caerulea,* produces the potent neuropeptide caerulein, the hinged antibiotic caerin 1.1 and the nNOS inhibitor caerin 2.1 (see Table 1). There are several *Litoria* species which do not produce antibiotic peptides, instead, they secrete a variety of neuropeptides. For example, the Red Tree Frog *Litoria rubella* produces a number of neuropeptides, including tryptophyllin 1.4 (Table 1). In complete contrast, frogs of the genera *Cyclorana* [Gao, C.W., Bowie, J.H. and Tyler, M.J., unpublished work (with the exception of the current discussion of *C.australis*)] and *Limnodynastes* (Apponyi *et al.* 2004), produce small peptides which been shown to be inactive in our testing program. We do not know how these frogs protect themselves from predators, either large or small. As an example, *Limnodynastes interioris* produces the neutral peptide dynastin 1 (Table 1) (Apponyi *et al.* 2004). A number of tree frogs produce

inactive peptides analogous to dynastin 1, but these peptides co-occur in the glandular secretion with active peptides. For example *Litoria caerulea* produces caeridin 1 together with the neuropeptide caerulein, the antimicrobial peptide caerins 1.1, and the nNOS inhibitor caerin 2.1 (Table 1).

Table 1. Some amphibian peptides (Apponyi *et al.* 2004)

Name	Amino acid sequence	Activity
Caerulein	pEQDY(SO_3)TGWMDF-NH_2	a
Caerin 1.1	GLLSVLGSVAKHVLPHVVPVIAEHL-NH_2	b,c,d
Caerin 2.1	GLVSSIGRALGGLLADVVKSKGQPA-OH	e
Tryptophyllin 1.4	pEFPPWF-NH_2	f
Dynastin 1	GLVSNLGI-OH	g
Caeridin 1	GLLDGLLGTGL-NH_2	g

(a) Smooth muscle active neuropeptide (active at the nanomolar concentration).
(b) Antibiotic (micromolar).
(c) Anticancer active peptide (micromolar).
(d) Antifungal agent (micromolar).
(e) nNOS inhibitor (micromolar).
(f) Smooth muscle (active at the micromolar concentration)
(g) Inactive

In this paper we compare the peptide profiles of the Australian frogs *Litoria alboguttata* and *Cyclorana australis*. The reason why we have undertaken this study is explained by the background to the work. The relationship between the genera *Cyclorana* and *Litoria* (particularly the *L. aurea* group) is complex as observed by Burton (1996). All *Cyclorana* species have burrowing adaptations. Historically, *Cyclorana* was originally considered a member of the Australasian Leptodactylidae (now Myobatrachidae) (Parker 1940). Parker recognised two major groups, one of which comprised two species in which the outer metatarsal tubercle was not shovel shaped, characteristic of burrowing frogs. Subsequently, *C. inermis* was transferred to *Hyla* (now *Litoria*) by Straughan (1969), and *C. dahlii* to *Litoria* by Tyler *et al.* (1978). The latter species was classified as a member of the *L. aurea* group by Tyler and Davies (1978).

Cyclorana was considered to be a hylid genus by Tyler (1978) because of its possession of an apical element to the intermandibularis muscle characteristic of all Australopapuan hylids. *Cyclorana* may be distinguished from *Litoria* by the absence of intercalary cartilages. *Cyclorana alboguttata* was transferred to *Litoria* by Tyler (1974) because of its possession of intercalary cartilages which are characteristic of the genus *Litoria*. Chromosomal morphology led King (1981) to place this species in a monotypic group within *Litoria*, but subsequent work has favoured its return to *Cyclorana*: namely, immunological data (Hutchinson & Maxson 1987), cocoon formation (Withers & Richards 1995), hand musculature (Burton 1996) and sperm morphology (Meyer *et al.* 1997).

The comparison of the structures of active peptides in skin secretions of anurans (peptide profiling) together with morphological characteristics can indicate whether two species should be classified within one group, and also demonstrate the occurrence of different populations within a particular species. This is detailed in a recent review (Apponyi *et al.* 2004). Examples (taken from this review) of species classified within the same group are (i) *L. rubella* and *L. electrica*; (ii) *L. aurea* and *L. raniformis*; and (iii) *L. caerulea, L. splendida* and *L. gilleni*. Examples of species which peptide profiling indicates the occurrence of distinct and different populations within a particular species are *L. caerulea* and *L. rubella*.

The success of peptide profiling brings us to the present study. We report the structures of the peptides contained in the glandular secretion of *Litoria alboguttata*, and compare these structures with (i) those of peptides isolated from other frogs of the genus *Litoria*, and (ii) those produced by *Cyclorana australis*. Consideration of these data should indicate whether *alboguttata* has been correctly classified as a member of the genus *Litoria*.

Materials and Methods

The two female specimens of *Cyclorana australis* and *Litoria alboguttata* used in this study were collected (respectively) on the banks of the Adelaide River near Darwin in January 1992, and near Brisbane in March of 1999.

Preparation of Skin Secretions

Live *Cyclorana australis* or *Litoria alboguttata* was held by the back legs, the skin moistened with deionised water, and the granular dorsal glands were stimulated by means of a bipolar electrode of 21G platinum attached to a Palmer Student Model electrical stimulator. The electrode was rubbed gently in a circular manner on the dorsal surface of the animal, using 10 volts and a pulse duration of 3ms. (Tyler *et al.* 1992). The resulting secretion was washed from the frog with deionised water (50 mL), the mixture diluted with an equal volume of methanol, centrifuged, filtered through a Millex HV filter unit (0.45 mm), and reduced in volume to ca. 1 mL This procedure provided less than 1 mg of peptide material from each frog (estimated from HPLC separations), and does not injure the animal. The technique was approved by The University of Adelaide Animal Ethics Committee.

HPLC Separation of the Glandular Secretion

HPLC separation of the skin secretion was achieved using a VYDAC C18 HPLC column (5m, 300A, 4.6 x 250 mm) (Separations Group, Hesperia, CA., USA) equilibrated with 10% acetonitrile / aqueous 0.1% trifluoroacetic acid. Crude extract (150 µL) was injected into the column. The elution profile was generated using a linear gradient produced by an ICI DP 800 Data Station controlling two LC1100 HPLC pumps, increasing from 10-75% acetonitrile over a period of 30 min. at a flow rate of 1 mL/min. The eluant was monitored by ultraviolet absorbance at 214 nm using an ICI LC-1200 variable wavelength detector (ICI Australia, Melbourne, Australia). HPLC traces are shown in Fig. 2. Fractions were collected, concentrated and dried *in vacuo* for MS and Edman investigation. Purified fractions generally contain ≤ 25 µg of peptide material.

Sequence Determination of peptides using mass spectrometry

Electrospray MS/MS data were determined using a Micromass QTOF2 orthogonal acceleration time-of-flight mass spectrometer with a mass range to 10,000 Da. The QTOF2 is fitted with an electrospray source in an orthogonal configuration with the ZSPRAY interface. Samples were dissolved in acetonitrile/water (1:1) and infused into the electrospray source with a flow rate of 5 mL per minute. Conditions were as follows: capillary voltage 3.11 kV, source temperature 80ºC, desolvation temperature 150ºC and cone voltage 80-130V. MS/MS data were acquired with the argon collision gas energy set to ca. 90eV to give optimal fragmentation.

Edman degradation

Automated Edman sequencing of all peptides was performed by a standard procedure (Hunkapiller *et al.* 1983) using an Applied Biosystem 492 procise sequencer equipped with a 900A data analysis module. The best results were obtained using a disc of immobilon film treated with bioprene in ethanol, onto which the peptide was absorbed from aqueous acetonitrile (90%). The disc was pierced several times with a razor blade to aid the flow of solvent.

Discussion

The Striped Burrowing Frog *Litoria alboguttata* (Fig. 1A) has been classified as a member of the genus *Litoria* (Tyler 1974). However, *L. alboguttata* is the only species of *Litoria* to burrow and in this regard is similar to all species of *Cyclorana*. It is found in coastal regions of Queensland, northern New South Wales and the north east of the Northern Territory. Females of *L. alboguttata* can be up to 81 mm in length. The back is olive to pale green with brown patches and it has a pale yellow/green mid-vertebral stripe. The ventral surface is dull white with darker mottling. A distinctive feature is the dorso-lateral fold from the shoulder to the groin.

The Giant Frog *Cyclorana australis* (Fig. 1B) is the largest ground-dwelling frog in northern Australia, occurring from the north west of Western Australia through the Northern Territory to the eastern coastal region of Queensland. The female can reach 105 mm in length. The dorsum varies from pale grey to brown and green. Dark brown head stripes extend from the tip of the snout to the flanks. There is often a pale mid-dorsal stripe.

A

B

Figure 1. (A) *Litoria alboguttata* (olive green in colour), (B) *Cyclorana australis* (light brown in colour).
Colour versions of these pictures may be viewed at Frogs of Australia (ARC) website (http://frogs.org.au/frogs). See also (Barker *et al.* 1995).

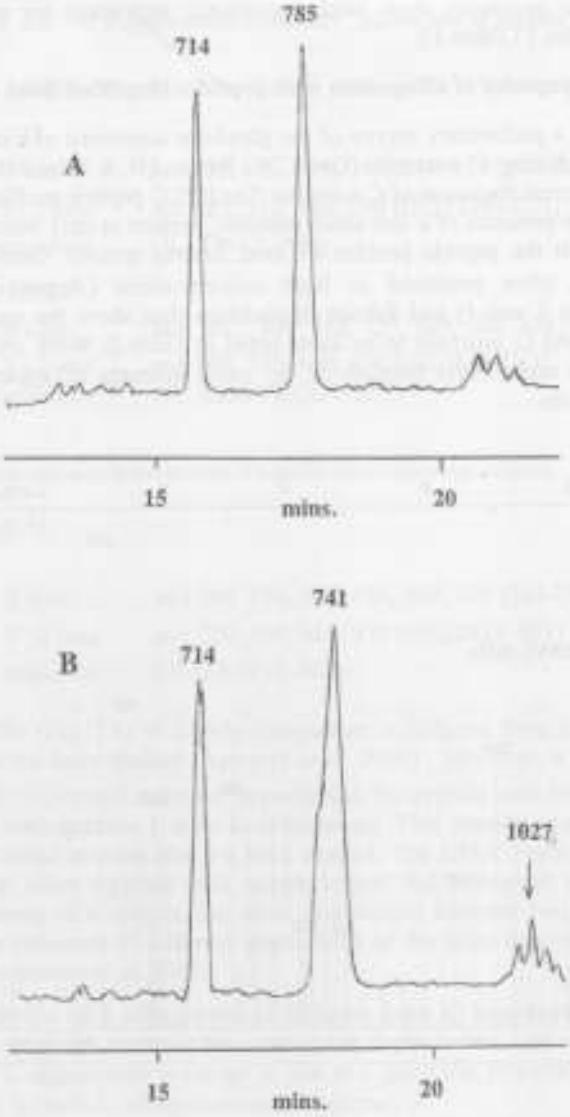

Figure 2. HPLC traces of peptide regions of the glandular secretions of (A) *Cyclorana australis*, and (B) *Litoria alboguttata*. Numbers above peaks are the molecular weights of the peptides.

Apart from colour differences, the two species *Litoria alboguttata* and *Cyclorana australis* look similar. Can peptide profiling provide any evidence as to the classification of *L. alboguttata* to a specific genus? The peptide regions of the HPLC profiles of the dorsal secretions of *L. alboguttata* and *C. australis* are shown in Fig. 2. The identified peptides are marked (by molecular weight) in Fig. 2. The amino acid sequences of the three peptides from *Litoria alboguttata*, named guttatins, together with those from *C. australis*, are shown in Table 2. The sequences were determined by a combination of positive ion electrospray mass spectrometry [see Table 2 and Fig. 3 (guttatin 1)] and automated Edman degradation (to differentiate between the isomeric residues Leu and Ile and to confirm the overall sequence).

We have not tested the peptides listed in Table 2 against micro-organisms. However amphibian peptides with similar sequences show neither antibiotic, anticancer nor neuropeptide activity [cf caeridin 1 and dynastin 1 (Table 1)].

Comparison of the peptides of *alboguttata* with peptides identified from *Cyclorana australis*

We have carried out a preliminary survey of the glandular secretions of a number of species of the genus *Cyclorana,* including *C. australis*. (Gao, C.W., Bowie, J.H. & Tyler, M.J., unpublished work, with the exception of the current discussion of *C.australis*). The HPLC peptide profiles (eg see Fig. 2B for *C. australis*) showed the presence of a few small peptides, present in only microgram amounts. This is to be contrasted with the peptide profiles of most *Litoria* species: these show the presence of numerous peptides, often produced in high concentrations (Apponyi *et al.* 2004)[1] Mass spectrometric (Tables 3 and 4) and Edman degradation data show the sequences of the peptides from L alboguttata and *C. australis* to be those listed in Table 2. Work on *Cyclorana* species was discontinued (in the mid 1990s) because of the small amounts of apparently inactive peptides present in the secretions.

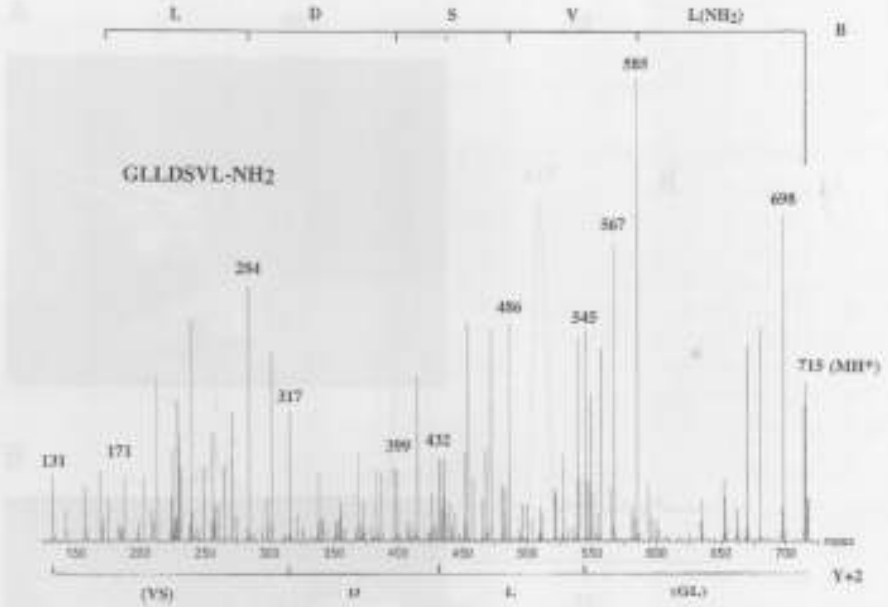

Figure 3. Electrospray mass spectrum (MS/MS) of guttatin 1. B and Y+2 fragmentations are shown schematically above and below the spectrum respectively [for a description of the positive ion fragmentations (and nomenclature) of peptide MH^+ ions, see (Biemann and Martin 1987).

Table 2. Sequences of peptides isolated from *Litoria alboguttata* and *Cyclorana australis*.

Name	Sequence	Molecular weight	Source
Guttatin 1	GLLDSVL-NH_2	**714**	*L.a* and *C.a*
Guttatin 2	GLLDNVL-NH_2	**741**	*L.a.*
Guttatin 3	GLLDTVKGLN-NH_2	**1027**	*L.a.*
Unnamed	GLLDGTVL-NH_2	**785**	*C.a*

Table 3. Mass spectrometric sequence determination of guttatins.

For a description of the B and Y+2 fragmentations of the MH^+ parent ions of peptides, see Biemann and Martin (1987).

Guttatin 1, MH^+, *m/z* 715 (See Fig. 3).

Guttatin 2, MH^+, *m/z* 742.

B ions	*m/z* 725, 612, 513, 399, 284, 171 [(GL)LDNVL-NH_2]
Y+2 ions	*m/z* 685, 572, 344, 230 [GL(LD)N-230]
sequence	GLLDNVL-NH_2

Guttatin 3, MH^+, *m/z* 1028

B ions	*m/z* 1011, 897, 784, 727, 599, 500, 399 [399-TVKGL-NH2]
Y+2 ions	*m/z* 858, 745, 630, 430 [(GL)LD(TV)]
sequence	(GL)LDTVKGL-NH_2

Table 4. Mass spectrometric sequence determination of peptides from *Cyclorana australis*.

MH^+ *m/z* 715 (See Fig. 3).

MH^+ *m/z* 786

B ions	*m/z* 769, 656, 557, 456, 399, 284 [284-DGTVL-NH_2]
Y+2 ions	*m/z* 729, 503, 388, 331 [GLLDGT-331]
sequence	GLLDGTVL-NH_2

The skin peptide profile (Fig. 2A) of *Litoria alboguttata* is different from those of other species of the genus *Litoria* that we have studied (Apponyi *et al.* 2004)[1]. However, it shows similarity to the skin peptide profile of *Cyclorana australis*. In particular, the peptide with MH^+ = 715 (Fig. 3) of *C. australis* is identical with guttatin 1 from *L. alboguttata*. This peptide is not produced by the 33 species of other Australian anurans that we have studied. The HPLC peptide profile of a frog is a sensitive probe (when taken together with morphological and biological characterics) which can determine the uniqueness of a species, the close relationship between two (or more) species, and even demonstrate the existence of different populations of the same species (Apponyi *et al.* 2004, Stone *et al.* 1993, Steinborner *et al.* 1996).

The HPLC peptide profile of *L. alboguttata* is different from all congeners so far studied, in that there are neither neuropeptides nor antibiotics present in the secretion. In contrast, the appearance of the peptide profile of *L. alboguttata* is similar to that of *C.australis*. In particular, the peptide named guttatin 1 is produced by both *L. alboguttata* and *C. australis*.[2]

In conclusion, the evidence from peptide profiling clearly shows that *Litoria alboguttata* is better classified as a member of the genus *Cyclorana* than *Litoria*. This is in agreement with immunological data (Hutchinson & Maxson 1987), cocoon formation (Withers & Richards 1995), hand musculature (Burton 1996) and sperm morphology (Meyer *et al.* 1997). However, the fact remains that *Litoria alboguttata* possesses intercalary structures that are characteristic of *Litoria* but not *Cyclorana*. Assuming that this classification (Tyler 1974) holds in all cases, it may be that *Litoria alboguttata*, rather than being returned to the genus *Cyclorana*, is better placed in a monotypic genus separate from *Litoria* and *Cyclorana*. We have not taken this action here pending the completion of revisionary studies of Australian hylids.

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1. There is a member of the genus *Litoria* that we have studied (*L. lesueuri*) which contains neither standard antibiotic nor smooth muscle active peptides. However this species does contain an nNOS inhibitor (lesueurin GLLDILKKVGKVA-NH_2) (Doyle *et al.* 2002).

2. Members of different genera do not usually produce the same peptide types in their skin secretions. There is an exception to this generalisation, in that the common neuropeptide caerulein (see Table 1 for the sequence of caerulein) has been detected in species of *Litoria* in Australia (Apponyi *et al*. 2004), together with the African frog *Xenopus laevis* and the South American leptodactylic frog *Leptodactylus labyrinthicus* (Erspamer 1994).

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