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Beta-Keratins of Reptilian Scales Share a Central Amino Acid Sequence Termed Core-Box

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Abstract: Beta-keratins determine most of mechanical resistance of reptilian scales. The amino acid sequence of these proteins is not known for most reptiles. An antibody directed against a known 20-amino acid sequence (core-box) of avian beta-keratins has been utilized to detect whether this epitope is also present in reptilian beta-keratins. The epidermis of most tested species (lepidosaurians, tuatara, chelonians andcrocodilians) shows immunoreactivity in the beta-layer. In immunoblots, specific immunolabeled bands are seen, mainly within 10-25 kDa (beta-keratin range). Bidimensional gel electrophoresis shows that most beta-keratins are basic proteins and contain the epitope for the presence of a core-box. The core-box is conserved among reptile and bird beta-keratins and seems linked to their structural properties in the formation of hard keratin packets. The only exception is represented by the soft shelled-turtle (Tryonix spiniferus). The latter species probably lacks the core-box in its beta-keratin and therefore cannot accumulate beta-keratin packets in the corneous layer explaining the softness of its shell. The molecular analysis on some known beta-keratin sequences suggests that the core-box epitope is centrally located in beta-keratins.

Key words: Reptiles, epidermis, beta keratins, electrophoresis, immunoblotting, immunocytochemistry

INTRODUCTION

Reptilian scales synthesize both soft (alpha-) keratins and hard (beta-) keratins (Baden and Maderson, 1970; Fraser *et al*., 1972; Maderson, 1985). Beta-keratins in particular form the hard corneous material of reptilian and avian scales and of feathers (Fig. 1A, B). Differently from most reptiles, in the soft-shelled turtle (Fig. 1C-C1) no hard scales are formed although beta-keratin filaments have been detected (Alibardi and Toni, 2006b). The molecular nature and the number of beta-keratins in different reptiles are not well known. It is known that these small proteins have a molecular mass ranging between 10-25 kDa. They polymerize into larger polymers to build betakeratin filaments (Alibardi and Toni, 2006a; Holmer *et al*., 2001; Rizzo *et al*., 2005; Sawyer *et al*., 2000; Thorpe and Ginnings, 1981; Wyld and Brush, 1979, 1983). The composition in amino acids and the electrophoresis patterns of reptilian proteins suggest that beta-keratins have a different composition (Alibardi, 2004; Alibardi *et al*., 2004a, b; Alibardi and Toni, 2004, 2005, 2006a; Baden *et al*., 1974; Gillespie *et al*., 1982; Inglis *et al*., 1987; Marshall and Gillespie, 1982). Beta-keratins of 13-16 kDa replace most of cytokeratin filaments in mature corneocytes of scales. These small proteins produce mechanically resistant scales since they probably pack into dense filaments that eventually merge into a compact mass of corneous material (Fraser *et al*., 1972; Fraser and Parry, 1996; Landmann, 1986).

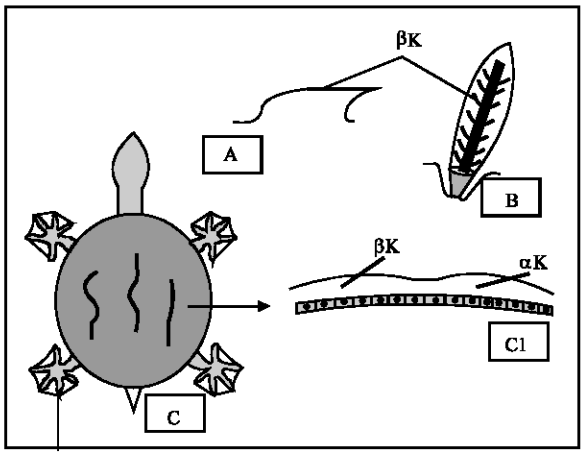

Fig. 1: Schematic drawing of hard reptilian/avian scales (A) and feather (B) where beta-keratins are present. In the soft-shelled turtle (C, the shell is roundish) little beta-keratin (®k) mixed with alpha-keratin (‹k) are present in the relatively soft horny layer or the epidermis (C1 ; b, basal layer)

Previous immunocytochemical and immunoblotting studies, using a broad-spectrum, cross- reactive antibody against a chicken scale keratin, showed that common epitopes are present in beta-keratins of reptiles and birds (Alibardi and Sawyer, 2002; Carver and Sawyer, 1987; Sawyer *et al*., 2000). A specific antibody, termed Universal, has been generated against a known amino

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acid sequence found in birds and in the alligator keratins (Sawyer *et al*., 2000, 2005). Although a recent sequencing of a lizard scale beta-keratin has shown that a similar amino acid sequence is present (Dalla Valle *et al*., 2005; Alibardi *et al*., 2006), it is not known whether this epitope is present in the other reptilian groups (lepidosaurians and chelonians). The presence of common epitopes shared among beta-keratins would indicate that the sequence presents a fundamental structural role of these proteins during corneification of reptilian epidermis.

In order to clarify whether beta-keratins of all reptilian groups share this sequence we have carried on the present immunological study using the universal beta-keratin antibody. We have extracted epidermal proteins from the epidermis or molts of various species of lizards, snakes, the tuatara and chelonians, separated these keratins by electrophoresis andidentified the cross-reactive protein bands by immunoblotting. The localization of a protein containing this epitope has been also evaluated by immunocytochemistry on skin sections for light and electron microscopy. Results have been completed by a literature search for similar sequences present in avian and reptilian beta-keratins. Results from this study address the future sequentiation of beta-keratins in reptilian epidermis, as it has been so far done for lizard epidermis (Dalla Valle *et al*., 2005).

MATERIALS AND METHODS

Immunocytochemistry: For immunocytochemistry and immonoblotting experiments, the epidermis from different reptilian species has been used (Table 1 and 2). Aside fresh epidermis, also skin molts collected from numerous species was also used (Table 2).

The molts from the rare tuatara (Sphenodon punctatus) for biochemical analysis were kindly supplied from Dr. Brian Gill (Auckland Museum, Auckland, NZ), Dr. N. Nelson (Victoria University, Wellington, NZ) under NZ DOC Permit n. WE/122/RES. Skin pieces from different reptiles (Table 1) were collected and immediately fixed. Skin pieces from the tail of five captive juvenile specimens of the tuatara (Sphenodon punctatus) were used. The tissues were kindly supplied from Mr. M. Fox at Otorohanga Kiwi House (Otorohanga, Waikato, New Zealand) under NZ DOC Permit n. WE/122/RES.

Pieces of scales (3-5 mm) were fixed in 4% paraformaldehyde in phosphate buffer for 3-4 h. The tissues were rinsed in buffer, dehydrated until 80% ethanol, infiltrated in a 1:1 mix of ethanol and Bioacryl resin for 1-2 h, immersed in pure resin for about 2 h andembedded in Bioacryl resin, made using the single chemical components as reported in Scala *et al*. (1992). The resin was polymerized at 0-4°C under UV-light for 2-3 days. Light microscopy immunocytochemistry was done on 2-4 μm thick sections collected on gelatin-chromoallume coated slides. Sections were pre-incubated for 20 min in 0.05 M Tris buffer at pH 7.6 containing 2% bovine serum albumin and 5% normal goat serum andthen incubated overnight with a rabbit polyclonal universal scale-keratin antibody at a dilution of 1:200 with the buffer. The antibody is directed against a specific epitope of 20 amino acids (SRVVIQPSPVVVTLPGPILS), indicated as universal sequence since it has been found in many avian and in the alligator beta-keratins (Sawyer *et al*., 2000, 2005). Sections were rinsed in buffer andincubated with a secondary antibody (IgG anti-rabbit fluorescein isotiocyanate conjugated (Sigma, St Louis, MI, USA) dilutes 1:40 in the buffer) for 1 h. Sections were observed and photographed using a fluorescence microscope equipped with a fluorescein filter (Zeiss, Jena, Germany).

For ultrastructural immunocytchemistry (Alibardi and Sawyer, 2002) thin sections (40-90 nm thick) were collected on nickel grids, incubated overnight in the primary antibody at 4°C, diluted 1:200 in TBS with 1% cold water fish gelatin. In controls for light and electron microscopy immunocytochemistry, the primary antibody was omitted. Grids were rinsed in buffer andincubated with the secondary antibody (10 nm gold conjugated IgG anti-rabbit, diluted 1:40) for 1 h at room temperature. Grids were rinsed in buffer, distilled water andthe sections were stained for 5 min in 2% uranyl acetate, dried andobserved with a CM-100 Philips electron microscope (Philips, Eindhoven, The Netherlands).

Protein extraction: For electrophoresis and western blot experiments, proteins were extracted from fresh tissue or from molts (Table 2). The skin was incubated in 5 mM EDTA in Phosphate Buffer Saline (PBS) for 5 min at 50°C and 2-4 min in cold buffer. The epidermis was separated from the dermis by dissection under a stereomicroscope using thin tweezers. Molts were cleaned in SDS 1% and abundantly rinsed with double distilled water. Molts and skin samples were homogenized in lysis buffer (8M urea, 50 mM Tris-HCl, pH 7.6, 0.1 M 2-mercaptoethanol, 1 mM dithiothreithol, 1 mM phenylmethylsulphonyl fluoride) to extract keratins (Sybert *et al*., 1985) as previously detailed (Alibardi *et al*., 2004a, b). The particulate was removed by centrifugation at 10,000 g for 10 min and the protein concentration was determined by the Lowry methods (Lowry *et al*., 1951). To calculate the protein concentration the O.D. was compared to a standard curve built by using BSA serial dilutions in the same extraction buffer used for protein homogenization.

Electrophoresis and immunoblotting: For mono-dimensional electrophoresis experiments, 50 μg of each sample denaturated in the sample buffer were loaded in each lane and separated in 15% SDS-polyacrylamide gels (SDS-PAGE) or in pre-casted gradient gels (10-20% BioRad, Hercule, CA, USA) according to Laemmli (1970).

For bidimensional electrophoresis experiments, the Ettan IPGphor III IEF System (GE Healthcare, Chalfont St Giles, UK) was used f or the Isoelectrofocusing (IEF). A 150 μg protein sample containing 2% CHAPS (Sigma, St. Louis, MI, USA.) and 1% carrier ampholyte mixture, pH 3, 5-10 (GE Healthcare, Chalfont St Giles, UK) was loaded on a 7 cm (pH 3-10) or on a 13 cm (pH 6-11) strip (GE Healthcare, Chalfont St Giles, UK), depending on the experiment. Application of the strips and running procedure was carried out as described by the manufacturer. The following protocol was used. Rehydratation was performed for 12 h at room temperature and was followed by the IEF, step by step, from 1/2 h 500 V, 1/2 h 1000 V, 1/2 h 5000 V (gradually).

In the last step was the IEF for 1 h at 5000 V. Strips were kept at 50 V until loaded on the second dimension. Before starting the second dimension, the strips were equilibrated for 10 min in 6 M urea, 30% glycerol, 50 mM Tris pH 6.8 and 2% DTT (Sigma, St. Louis, MI, USA). Afterward, strips were briefly rinsed with double distilled water and equilibrated in 6 M urea, 30% glycerol, 50 mM Tris pH 6.8 and 2.5% iodoacetamide (Sigma, St. Louis, MI, U.SA) for an additional 10 min. The second dimension was carried out in a MiniProtean III electrophoresis apparatus (Biorad, Hercule, CA, USA) in 15% SDS-polyacrylamide gels. Successively, mono-and bi-dimensional gels were immunoblotted on nitrocellulose paper (Hybond C+ Extra, GE Healthcare, Chalfont St Giles, UK).

In electrophoresis experiments Wide Range (M.W. 6,500-205,00) (Sigma, St. Louis, Missouri, USA) or Precision Plus Protein Standard (10-250 kDa) (Bio-Rad, Hercule, CA, USA) molecular weight markers were used. After western blot, the protein transfer to the nitrocellulose membrane was checked using a 15 min staining in 0.2% w v^{-1} Ponceau red (SIGMA, USA) in 3% w/v trichloroacetic acid (MERK, Germany) and then incubated with Beta universal primary antibody (dilution 1:2000) and secondary antibodies HRP-conjugated (Sigma, St. Louis, Missouri, USA) (dilution 1:1000) diluted in TBS-TWEEN + 5% non-fat milk powder. Detection was performed by using the enhanced chemiluminescence's procedure developed by GE Healthcare (ECL, GE Healthcare, Chalfont St Giles, UK).

RESULTS

Immunocytochemistry: The beta-universal antibody stained the superficial beta-layer of the epidermis of different reptilian species andthe scutate scale and feather of the chick and zebrafinch (Fig. 2 and Table 1). The beta-layer represented the more external and compact corneous layer of the studied epidermis. In lizards and snakes the beta-layer was thin (Fig. 2a-e) but the beta-layer comprised the whole thick corneous layer of scales of chelonians, crocodile and chick skin (Fig. 2f-i). Feathers used as positive control showed a green immunofluorescence in elongating cells that were forming barbs and barbules (Fig. 2 j).

Beneath the beta layer, the alpha layer of lizard and snake scales andthe suprabasal and basal layers of the epidermis of reptilian and avian epidermis were not reactive (Fig. 2a-i). The I mmunofluorescence marked the whole thickness of the beta-layer of scales: This varied from the thin layer of lizards and snakes to the thick beta-layer of alligator and especially turtle carapace and plastron. The negative controls were not labeled.

The ultrastructural examination showed that in the lizard Podarcis muralis, gold particles decorated only the keratin packets or larger filaments within beta-keratin cells andvery weakly the packets present in oberhautchen cells (Fig. 3a). The cytoplasm, nucleus and other cell organelles within beta-cells remained unlabeled.

A diffuse but specific labeling was also present over the beta-layer of the epidermis of the gecko Hemidactylus turcicus from which pointed spinulae were present externally. No labeling was present in alpha-cells such as clear and mesos cells (including their keratin bundles) localized underneath the beta-layer. No labeling was seen in negative controls. In the snake Natrix natrix also the tangled keratin bundles present in differentiating beta-cells appeared decorated with gold particles while the remaining cytoplasm

Table 1: Immunofluorescence positivity of the epidermis (beta-cells) in different species. The asterisks indicate the species where the immunopositivity has been check at the electron microscopy

P. muralis (wall lizard)
P. sicula (wall lizard)*
T. mauritanica (mediterranean gecko)
H. turcicus (gecko) *
L. fuscus (python snake)
N. natrix (colubrid snake) *
C. picta (freshwater turtle) *
T. hermanni (land tortoise) *
A. mississippiensis (American alligator) *
C. porosus (salwater crocodile)
G. gallus (chick)
T. castanotis (zebrafinch)

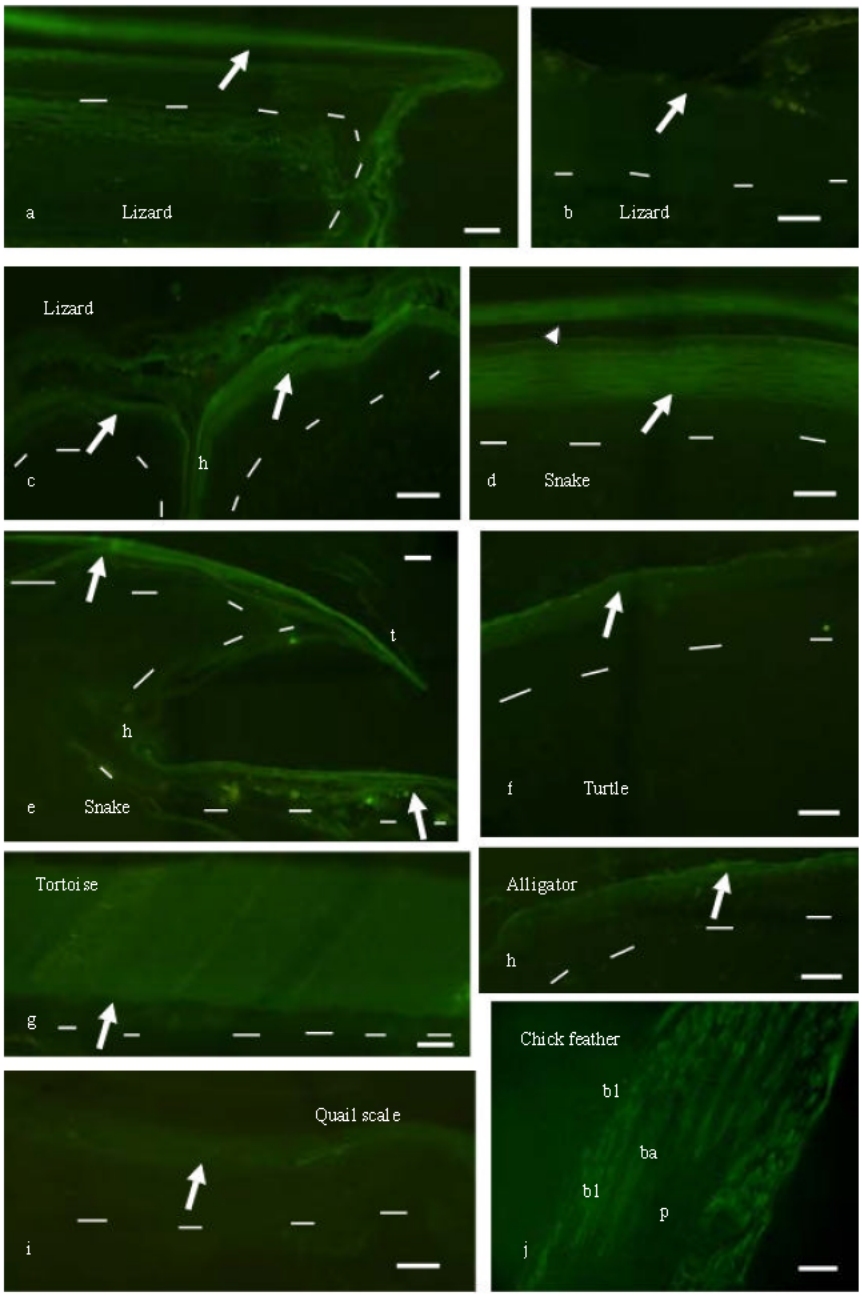

Fig. 2: Immunofluorescence labeling using the beta-universal antibody on the epidermis of different species. a, longitudinal section of lizard scale (*Podarcis sicula*) with reactive, cornified beta-layer (arrow). b, Negative control of *P. sicula* scale (arrow points the position of the betalayer). c, Cross section of lizard scale (*P. sicula*) with labeled beta-layer (arrows). h, hinge region. d, snake scale (*Natrix natrix*) showing the labeled outer (arrowhead) and inner (arrow) betalayers. e, snake scale (*N. natrix*) with immunolabeled beta-layer (arrows). h, hinge region among scales; t, tip of the scale. f, turtle (*Pelomedusa subrufa*). The arrow indicates the corneous layer.; g, turtle carapace (*Testudo hermanni*). The arrow indicates the corneous layer; h, alligator (Alligator mississippiensis). The arrow indicates the corneous layer; i, Quail (*Coturnix japonica*). The arrow indicates the corneous layer; j, feather of chick (*Gallus gallus*). ba, barbs; bl, barbules; p, pulp. Dashes in all images underlie the basal layer. Bar in all images, 10 μm

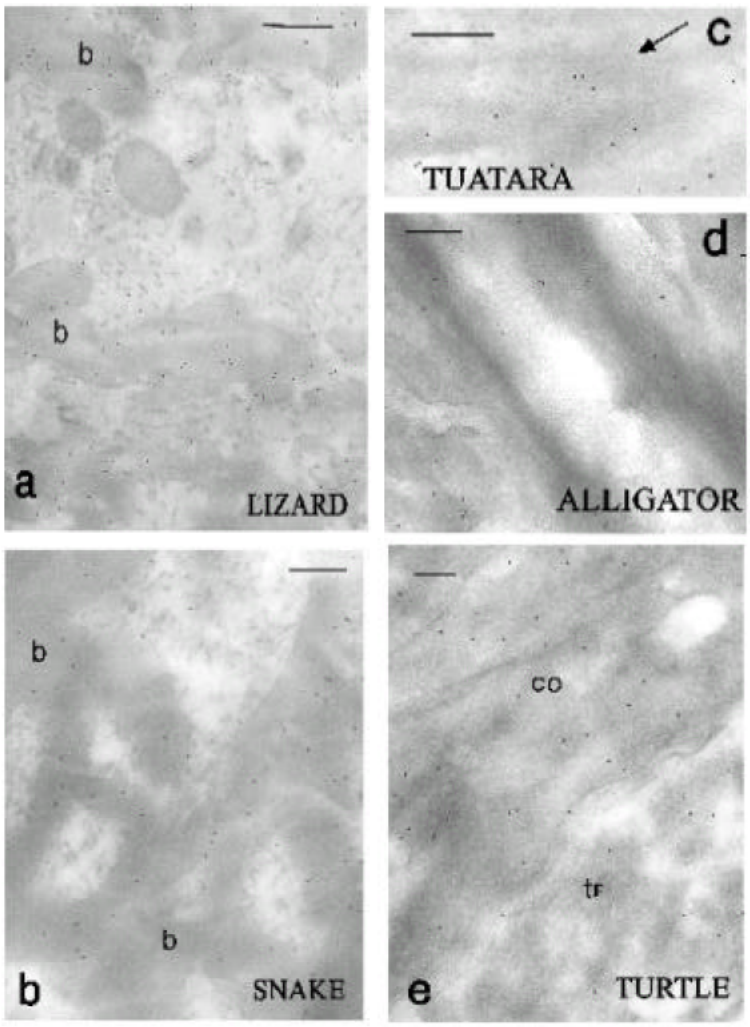

Fig. 3: Ultrastructural immunolabeling of lizard epidermis. a, immunolabeled beta-keratin filaments (b) of differentiating beta-cells among which few desmosomes are present (d). Bar, 500 nm b, merging immunolabeled beta-filaments (b) in differentiating beta-cell of the snake *Natrix natrix*. Bar, 250 nm. c, detail of diffusely immunolabeled keratin bundles (arrow) in differentiating beta cells of *S. punctatus*. Bar, 250 nm. d, diffusely immunolabeled corneous layer of *A. mississippiensis* epidermis. Bar, 250 nm. e, Ultrastructural view of the epidermis of carapace of the turtle *Chrysemys picta*. While precorneous transitional (tr) cells show a diffuse labeling of keratin bundles, the labeling increases in the compact corneous material of the stratum corneum (co). Bar 100 nm

remained unlabeled (Fig. 3b). Like in lizard epidermis, also in snake epidermis, the labeling was more diffuse in oberhautchen cells contacting the immunonegative clear layer.

A diffuse but specific labeling was present in the corneous beta-layer of the epidermis of the tuatara (Sphenodon punctatus) (Fig. 3c) as well as in that of the alligator (Alligator mississippiensis) (Fig. 3d). Both electron-dense and electron-clear areas of the compact corneous material of the stratum corneum were immunolabeled. Finally, an even labeling was only observed in dense keratin bundles of the pre-corneous and especially in the corneous layer of the carapace of the turtle Chrysemys picta (Fig. 3e). No other cells or their organelles of epidermis of turtles were immunolabeled, as well as the negative controls.

Immunoblotting: After blotting and incubation with the beta-universal antibody, immunopositive bands produced different patterns in the various studied species

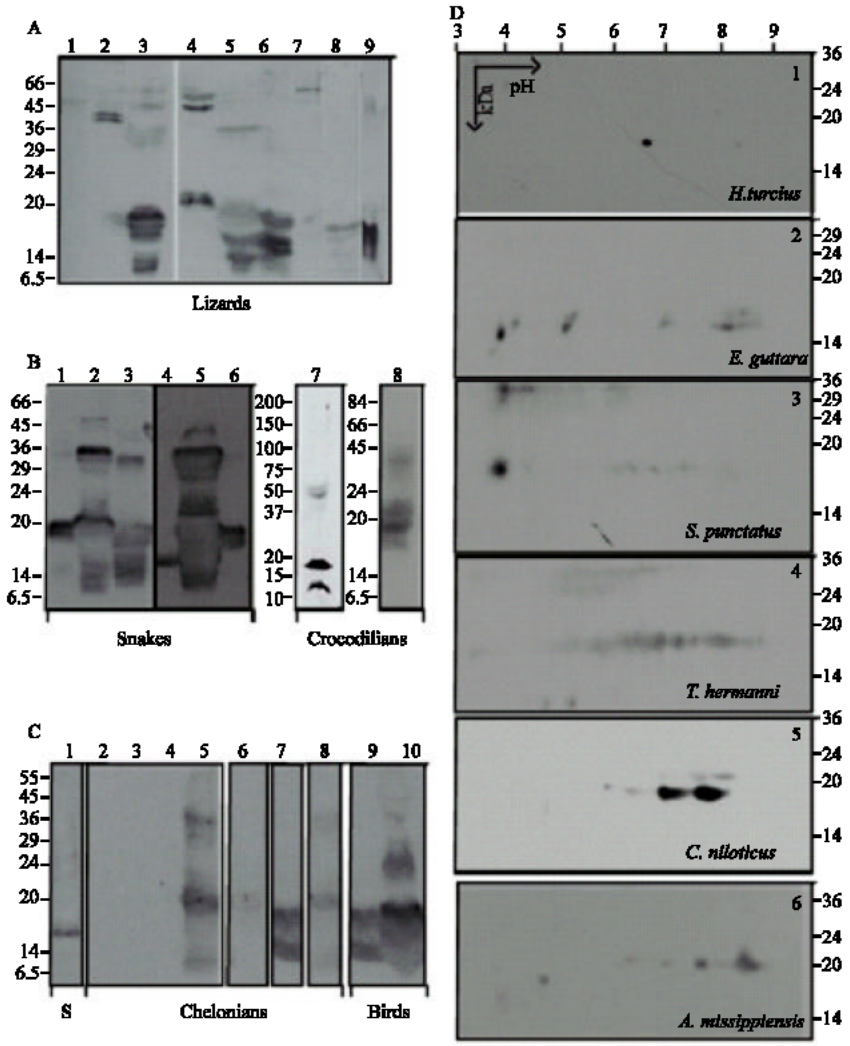

Fig. 4: Comparative protein pattern immunostained by beta universal antibody. Panels A-C, mono-dimensional analysis; panel D, bi-dimensional analysis. Panel A: Lizards (*Pogona vitticeps* (1), *Chamaeleo calyptratus* (2), *Iguana iguana* (3), *Podarcis muralis* (4), *Lialis burtoni*(5), *Trachydosaurus rugosus* (6), *Tiliqua scincoides* (7), *Varanus timorensis* (8), *Tarentola mauritanica* (9)); Panel B: Snakes (*Liasis childreni* (1), *Elaphe situla* (2), *Naja naja* (3), *Crotalus atrox* (4), *Trimeresurum albolensis* (5), *Cerastes cerastes* (6)) and crocodilians (*Alligator mississippiensis* (7), *Crocodylus niloticus* (8) ; Panel C: *Sphenodon punctatus* (1), chelonians (*Tryonix spiniferus* (2, soft epidermis; 3, carapace; 4, plastron), *Chrysemys picta* (5 carapace, 6 soft epidermis, 7 moult), *Testudo hermanni* (8)) and Birds (*Taeniopygia castanotis* (9), *Gallus gallus* (10)). Panel D: *H. turcicus* epidermis (1), *E. guttata's* moult (2), *S. punctatus'* moult (3), *T. hermanni's* carapace (4); *C. niloticus* epidermis (5), *A. mississippiensis* (6). 15% polyacrylamide gels were used, except for experiments shown in Panel B 7-8 in which 10-20% polyacrylamide gradient gel were used

(Table 2 and Fig. 4A). In lizard epidermis or in molts, more commonly bands at low molecular weight (8-20 kDa) resulted immunolabeled. In some species of lizards some labeling was seen in the 36-60 kDa. In snakes most labeled bands were seen at 8-18 kDa, some cases also at 29-30 kDa, but none above 42 kDa. Sphenodon punctatus showed a labeled band at 16 kDa. In chelonians, the stronger bands were seen at 17-18 kDa. Only the epidermis of the shell of the soft-shelled turtle Tryonix spiniferus did not show labeled proteins. Bands at 13 and 18 kDa were seen in the alligator epidermis. More intense bands at 16-18 kDa were seen for avian epidermis.

After bidimensional separation from the epidermis of some reptilian species (Fig. 4B), the beta-universal antibody stained some spots at 16-17 kDa with a pI of 6.6 in *H. turcicus*, at 15 kDa with a pI of 5.4, 7 and 8

Table 2: Species analyzed by western blotting. Bold characters indicate fresh tissues; normal characters indicate molts

Species	Common name	Families	Lane in Fig. 4
Alligator mississippiensis (4)	Mississippi alligator	Crocodylidae	B7, D6
Cerastes cerastes (1)	Desert horned viper	Viperidae	B6
Chamaeleo calyptratus(3)	Veiled Chameleon	Chamaeleonidae	A2
Chrysemys picta (2)	Eastern painted turtle		C5
		Emydidae	C6
			C7
Crocodylus niloticus	Crocodile	Crocodylidae	B8, D5
Crotalus atrox (2)	Western diamond-backed rattlesnake	Viperidae	B4
Elaphe situla (2)	Leopard snake	Colubridae	E2
Elaphe guttata	Red snake	Colubridae	D2
Gallus gallus	Chicken	C10	
Iguana iguana (4)	Common green iguana	Iguanidae	A3
Hemidactylus turcicus (2)		Gekkonide	D1
Lialis burtonis (1)	Burton's snake-lizard	Pygopodidae	A5
Liasis childreni (1)	Children python	Boidae	B1
Naja naja (2)	Indian cobra	Elapidae	E3
Podarcis muralis (5)	Common wall lizard	Lacertidae	A4
Pogona vitticeps (3)	Central bearded dragon	Agamidae	A1
Sphenodon punctatus (3)	Tuatara	Sphenodontidae	C1, D3
Taeniopygia castanotis	Zebrafinch	Passeriformes	C9
Tarentola mauritanica (4)	Common wall gecko	Gekkonidae	A9
Testudo hermanni (2)	Hermann's tortoise	Testudinidae	C8, D4
Tiliqua scincoides (1)	Common bluetongue	Scincidae	A7
Trachydosaurus rugosus (1)	Australian scingleback lizard	Scincidae	A6
Trimeresurum albolensis (1)	African bamboo viper	Viperidae	B5
Tryonix spiniferus (2)		Softshell Turtle	C2 (soft)
		Trionychidae	C3 (carapace)
			C4 (plastron)
Varanus timorensis (1)	Spotted tree monitor	Varanidae	A8

in *E. guttata*, at 18 kDa with a pI of 4, 6, 6.4, 6.8, 7.4 in *S. punctatus*, at 17-19 kDa with a pI of 6.7, 7.2, 7.4, 8 and 8.5 in *T. hermanni*, at 17-19 kDa with a pI of 7 and 7,8 in *C. niloticus* (Fig. 4D).

DISCUSSION

Distribution of the Beta-universal epitope in sauropsid epidermis The present immunological study shows that a specific epitope recognized by the beta-universal antibody is likely present in most reptilian species. The epitope corresponds to a known sequence of 20 amino acids presents in beta-keratins of birds and alligator (Sawyer *et al*., 2000, 2005) and lizards (Dalla Valle *et al*., 2005, 2007). This indicates that the epitope is present in beta- keratins of most lepidosaurian (lizards, snakes andSphenodon), chelonian and crocodilian scales. The ultrastructural analysis confirms that the antibody recognizes an epitope present among the tangled bundles of dense keratin present in beta-cells.

Keratin bundles in oberhautchen cells of lizard and snake appear less immunoreactive, suggesting that they also contain other proteins. In the other species (Sphenodon, turtle and alligator) the epitopes is detectable only in dense or packing bundles of keratin in the pre-corneous or corneous layers. The presence of this sequence or of similar amino acid sequences suggests that the epitope is important for the structural role of beta-keratins in the epidermis of most sauropsids. The epitope comprises an amino acid region with predicted secondary beta-fold or beta-sheet conformation andis probably utilized for polymerization of beta-keratin monomers to build the framework of beta-keratin (Fig. 5A-A2) (Fraser and Parry, 1996). The latter process gives origin to beta-keratin packets and eventually to the tangled beta-filaments that accumulate in beta cells forming scales and feathers (Fig. 5A3-A6). Aside scale keratin in alligator and several avianspecies (Sawyer *et al*., 2000) an homologous sequence is also present in beta-keratins of some lizard species and a snake (Dalla Valle *et al*., 2005, 2007). The recent molecular biology studies on the sequencing of lizard beta-keratins have identified a sequence of amino acids with high homology (60-80%) with the 20-amino acid epitope, indicated as core-box (Fig. 5).

Only the epidermis of the soft turtle, *Tryonix spiniferus*, does not appear to contain this epitope, although the epidermis of this specie is reactive for other beta-keratin antibodies (Alibardi and Toni, 2006b). We hypothesized that it is the lack of this epitope in the beta-keratin of the softer corneous layer of *T. spiniferus* that determines the resulting soft corneous phenotype. In fact, as presented in Fig. 5B-B5, in this species beta-keratins probably cannot form a beta-keratin framework as the monomers remain separated andtherefore no beta-keratin

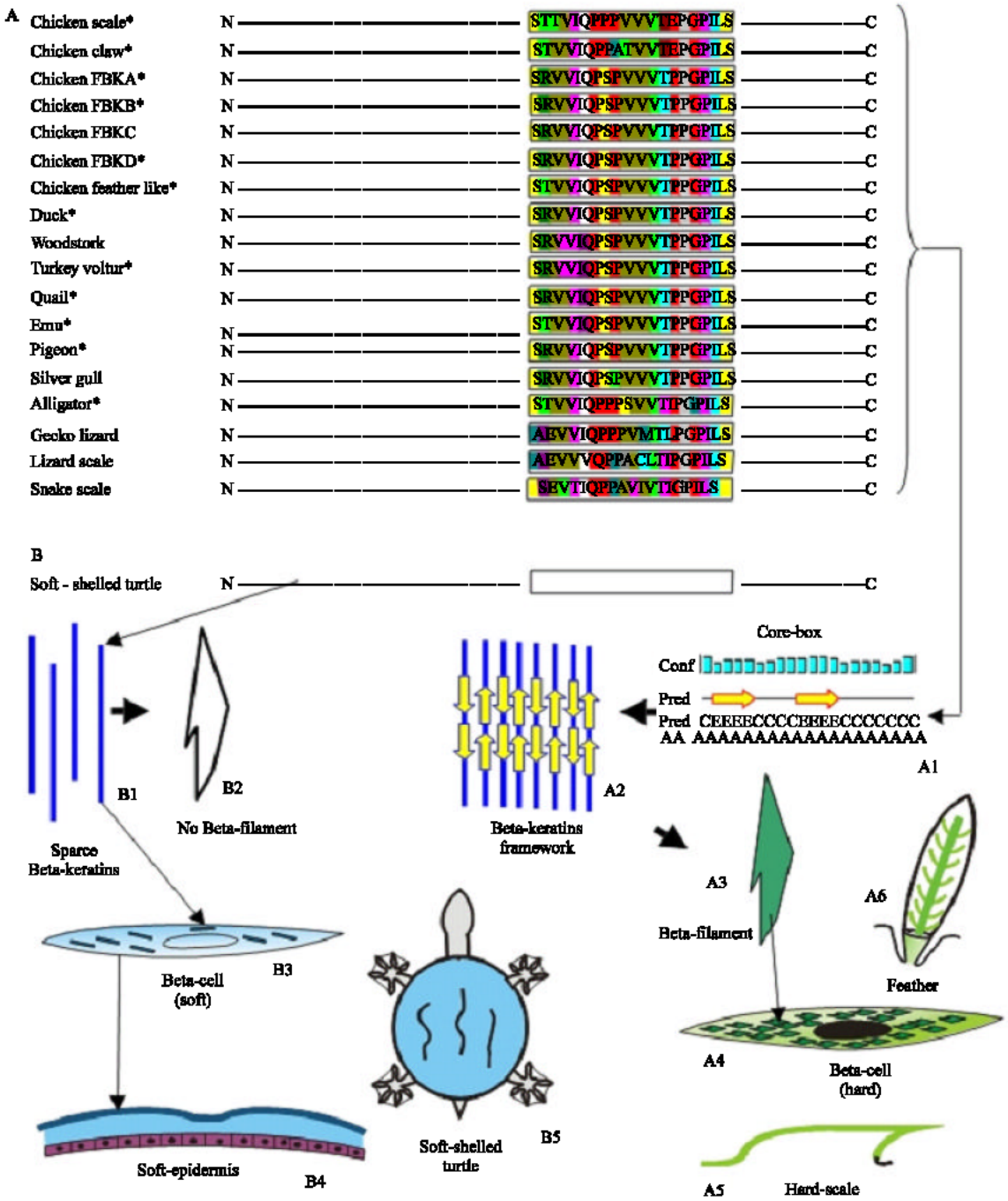

Fig. 5: A, amino acid sequences of the central region of beta keratins (core box) in different species of reptiles and birds. The prediction of the secondary structure of the 20 amino acids of the core box in different species (performed by using the PSIPRED Protein Structure Prediction Server at http://www2.ebi.ac.uk/clustalw/ (McGuffin, 2000) shows a beta conformation (the two yellow arrows in A1). This suggests that the core box is necessary for the formation of the betakeratin framework (A2) and for the origin of beta-filaments (A3) that accumulate in beta cells (A4). This packing determines the production of the hard keratin of scales (A5) and feathers (A6). B, beta-keratin in Trionix spiniferus likely lacks the core box (B) and the resulting protein is not aggregated into a beta-keratin framework (B1), does not form beta-filaments (B2) in beta-cells (B3) of the soft epidermis (B4). The resulting corneous layer of this turtle is soft (B5)

packets or filaments are formed in corneocytes of this species (Fig. 5B-B2) (Alibardi and Toni, 2006b). This hypothesis however awaits the nucleotide and amino acid sequentiation of beta-keratins in this species.

The immunoblotting study shows that the epitope is mainly present in beta-keratins in the range of 8-18 kDa. The low reactivity observed in protein bands at 30-33 or 38-45 kDa may be due to physiological or even to artifactual polymerization of beta-keratins into dimers or into higher molecular weigh polymers that have occurred during the extraction process or alteration of betakeratins in molts.

The present study reveals the presence of immunopositive protein spots in acidic, neutral and basic range of pH. It is suggested that the conserved region recognized by the beta-universal antibody is implicated in the specific folding and polymerization of the protein, which is somehow connected with the beta-keratin pattern observed after X-ray analysis and ultrastructural study (Fraser *et al*., 1972; Fraser and Parry, 1996). The presence of a similar epitope in different betakeratins can be exploited in future studies to design specific oligonucleotides probes for the selection of mRNAs of specific beta-keratins present in the epidermis of other species of reptiles (Dalla Valle *et al*., 2005, 2007).

Beta-keratins are glycine-proline-rich proteins: As opposed to cytokeratins that are ubiquitarious, beta-keratins are only present in reptiles and birds (Baden and Maderson, 1970; Landmann, 1986; Maderson, 1985). In the latter amniotes beta-keratins are present in hard structures such as scales, scutes, claws, beak, feathers, are accumulated in the pre-corneous layers of the epidermis but are absent in basal and suprabasal layers. This fact suggests that these small proteins represent specialized proteins produced in terminally differentiating beta-cells of the corneous layer of reptiles andonly in the beta-layer of lepidosaurians (Alibardi and Toni, 2006a). Reptilian beta-keratins appear to associate to the cytokeratin framework as matrix or cytokeratin-associated protein for the formation of a dense and hard corneous material. This information derives from the ultrastructural study on the modality of deposition of beta-keratins in beta-cells andon the molecular weight and amino acid sequences obtained so far for few of these proteins (Alibardi *et al*., 2006; Dalla Valle *et al*., 2005; Inglis *et al*., 1987). Therefore, as typical cytokeratins, also beta-keratins form filaments and bundles of corneous material within corneocytes.

Also mammalian hard-keratin associated proteins (sulfur-rich, ultra-sulfur-rich and tyrosine-rich) are present in specialized structures andbasically appear in differentiating, pre-corneous and corneous layers (Gillespie, 1991; Powell and Rogers, 1986, 1994). Therefore, both reptilian beta-keratins and mammalian keratin-associated proteins (for claws, hoof, horns, beak, feathers, scales, scutes etc) have small molecular weights (7-12 kDa) and are rich in glycine sequences. The richness in glycine-sequences found in hard-keratins may indicate a common protein precursor for both sauropsids (extant reptiles and birds) and therapsids (extant mammals). Smaller glycine-rich sequences can form dense aggregations and produce a higher mechanical resistance (Fraser *et al*., 1972; Gillespie, 1991). However the secondary conformation of mammalian proteins only origins an alpha-keratin pattern not a beta-keratin pattern like in reptiles and birds. This suggests that glycine-rich proteins in reptiles and birds have probably evolved differently than those of mammals andthat their amino acidic composition has shifted their secondary structure in order to produce beta-keratin pattern (in sauropsids) and alpha-keratin pattern (in therapsids). During evolution hard and glycine-rich small keratin-associated proteins might have acquired different amino acidic composition in sauropsids versus therapsids (Alibardi, 2006; Alibardi and Toni, 2006a).

In conclusion the present study shows that scales of modern reptilians (lizards and snakes) and birds share a 20 amino acid epitope with beta-keratins of ancient reptilian species such as *S. punctatus*, turtles and crocodilians. These proteins of 10-16 kDa might have derived from a common alpha-keratin present in ancient reptiles (cotylosaurians) andmight have diversified in the derived reptiles. It is very likely that each reptilian group possess its specific set of glycineglycine-rich hard keratins andthat in archosaurians the smaller type of these hard-keratins has been used to make feathers (feather keratin).

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