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Phylogenetic position of turtles among reptiles: evidence from immunological comparisons of eggshell matrices

Makiko NISHI[1], Isao SARASHINA[1], Minoru IIJIMA[1], Ren HIRAYAMA[2] and Kazuyoshi ENDO[1]

Abstract

The phylogenetic affiliation of turtles among major groups of reptiles is a controversial issue. Since turtles have no temporal fenestra unlike other reptiles and birds, turtles have been traditionally considered as the only survivors of anapsids, which have no fenestra, and as the most basal reptiles. Recent molecular studies, however, posit an archosaurian (crocodiles and birds) affinity of turtles and the earliest branching of squamates (lizards and snakes) instead of turtles. To address this issue, we performed enzyme linked immunosorbent assays (ELISA) on the extracts from eggshells of a total of 25 reptiles and birds. Antiserum raised against eggshell extracts from chicken (*Gallus gallus domesticus*) reacted with those from crocodiles and turtles stronger than those from squamates, and the other antiserum raised against eggshell extracts from a soft-shelled turtle (*Pelodiscus sinensis*) reacted with those from birds and crocodiles stronger than those from squamates. These results support the archosaurian affinity of turtles and the scheme of recent molecular phylogeny.

Key words: Anapsids, eggshells, ELISA, *Pelodiscus sinensis*, Turtles

Introduction

Reptiles have been conventionally classified mainly based on the presence or absence and the types of temporal fenestration of the skull (Günther, 1867; Willingston, 1917). Reptiles that have a completely roofed skull and no fenestra are called anapsids, which include turtles, whereas those with two fenestrae in the temporal region of the skull are known as diapsids, which include squamates (lizards and snakes), tuataras, crocodiles and birds. Among the diapsids, based on the paleontological and molphological evidence, squamates and tuataras have been put into one group, or the Lepidosauria, and the crocodiles and birds have been grouped together to form the Archosauria (Gauthier *et al.*, 1988; deBraga and Rieppel, 1997).

Turtles, which date back to Late Triassic (Li *et al.*, 2008), have been considered as the only surviving representatives of anapsids. Turtles have been grouped with pareiasaurs (Gregory, 1946; Lee, 1997) or procolophorids (Laurin and Reisz, 1995), both of which are extinct anapsid parareptiles lived in Permian and Triassic (Reisz, 1997). On the other hand, diapsids are generally regarded as more derived reptiles based on fossil record and comparative morphology (Benton, 1990). Therefore, turtles have been regarded as basal reptiles that branched off earlier than the divergence in the clade of diapsids (Fig. 1A: Romer, 1966; Carrol, 1988; but see for example Rieppel and de Braga, 1998 and Rieppel and Reisz, 1999 for arguments for a diapsid origin of turtles from the viewpoint of comparative morphology).

Although molecular phylogeny was expected to resolve the relationships of turtles to other reptiles, molecular data have often been equivocal (Hedge *et al.*, 1990; Marshall, 1992; Eernisse and Kluge, 1993; Van de Peer *et al.*, 1993; Caspers *et al.*, 1996; Fushitani *et al.*, 1996; Mannen *et al.*, 1997; Platz and Conlon, 1997; Gorr *et al.*, 1998; Grishin, 1999; Mannen and Li, 1999; Hedges and Poling, 1999; Cao *et al.*, 2000). These analyses used relatively small data sets and the results of them appeared to be highly sensitive to taxon sampling and the algorithms of tree reconstruction. In recent years, the complete mitochondrial genome sequences of the African side-necked turtle and the Green turtle were determined (Kumazawa and Nishida, 1999; Zardoya and Meyer, 1998). Phylogenetic analysis using these sequences supported the sister-group relationship between turtles and archosaurids, and rejected the hypothesis that turtles are the most basal living reptiles (Fig. 1B). Iwabe *et al.* (2005) conducted phylogenetic analysis using two nuclear genes which are considered to be single copy in vertebrates, excluding the possibility of paralogous comparison. Their

[1] Doctoral Program in Earth Evolution Sciences, Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba 305-8572, Japan
[2] School of International Liberal Studies, Waseda University, Tokyo 169-0051, Japan
* Corresponding author.
Kazuyoshi Endo Phone: +81-29-853-4427
FAX: +81-29-853-7887
E-mail: endo@geol.tsukuba.ac.jp

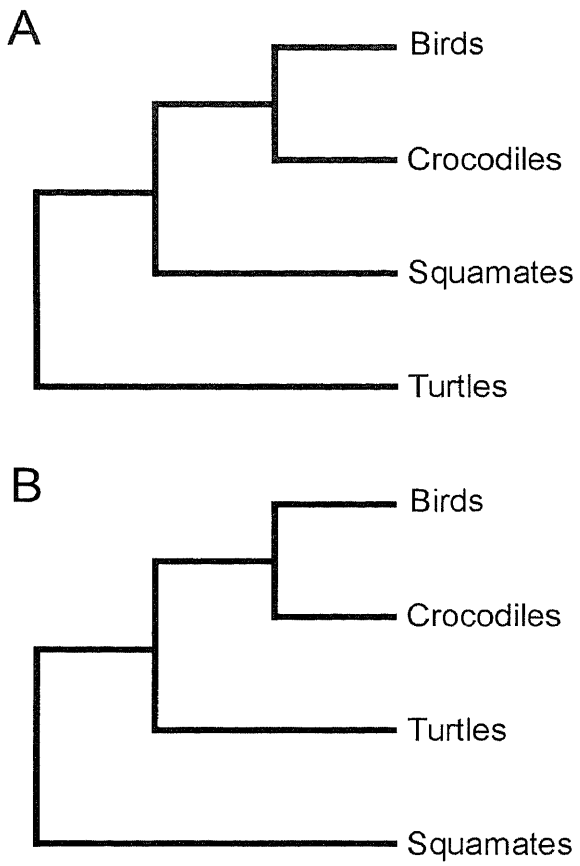

Fig. 1 Alternative hypotheses explaining the phylogenetic position of turtles within reptiles and birds. A: Traditional phylogenetic hypothesis mainly based on morphological and paleontological data. Turtles are the most basal reptiles. Squamates form a sister group with crocodiles and birds. B: Conflicting phylogenetic hypothesis mainly based on recent molecular data. Squamates are the most basal reptiles. Turtles form a sister group with crocodiles and birds.

order	suborder	family	species	
Squamata	Serpentes	Colubridae	*Elaphe climacophora*	Squamates
	Sauria	Gekkonidae	*Uroplatus phantasticus*	
Galliformes		Phasianidae	*Gallus gallus domesticus*	
			Coturnix japonica	
Struthioniformes		Struthionidae	*Struthio camelus*	Birds
Psittaciformes		Psittacidae	*Ara macao*	
Gruiformes		Gruidae	*Grus japonensis*	
Crocodilia	Eusuchia	Crocodylidae	*Crocodylus niloticus*	
			Crocodylus moreletii	
			Crocodylus rhombifer	
			Crocodylus siamensis	Crocodiles
			Osteolaemus tetraspis	
			Tomistoma schlegeli	
		Alligatoridae	*Caiman latirostros*	
			Caiman yacare	
Testudines	Pleurodira	Chelidae	*Chelodina siebenrocki*	
			Emydura subglobosa	
			Phrynops hilarii	
			Phrynops geoffroanus	
	Cryptodira	Trionychoidea	*Pelodiscus sinensis*	
		Testudinoidea	*Heosemys spinosa*	Turtles
			Hieremys annandalei	
		Kinosternidae	*Kinosternon scorpioides*	
		Testudinidae	*Dipsochelys dussumieri*	
		Chelidridae	*Chelydra serpentina*	

Fig. 2 List of specimens used in this study.

results also showed an archosaurian affinity of turtles. However, the phylogenetic position of turtles among the major groups of reptiles appears yet to be settled down.

Many reptiles and birds lay eggs with mineralized shells. That is one of the main characters which distinguish them from amphibians and most mammals. The mineralized eggshells contain organic matrix including proteins. These proteins are expected to contain phylogenetic information, and overall similarity of organic matrices probably reflects phylogenetic relationships. In this paper, we performed immunological assays on the extracts from 25 reptilian and avian eggshells (Fig. 2), using antisera raised against eggshell extracts from the chicken *Gallus gallus domesticus* and from the soft-shelled turtle *Pelodiscus sinensis*. We provide new evidence to clarify the issue, and discuss the phylogenetic position of turtles among major groups of reptiles.

Material and Methods

Eggs

Fresh eggs of *Gallus gallus domesticus* and *Coturnix japonica* were purchased from local markets. Fresh eggs of *Struthio camelus* were purchased from the ostrich farm, Dacho-Okoku (Ishioka, Ibaraki, Japan). Eggshells of *Crocodylus niloticus*, *Cr. moreletii*, *Cr. rhombifer*, *Cr. siamensis*, *Osteolaemus tetraspis*, *Tomistoma schlegeli*, *Caiman latirostros*, *Ca. yacare* were given from Atagawa Tropical & Alligater Garden (Atagawa Onsen, Shizuoka, Japan). Eggshells of *Elaphe climacophora* were given from The Japan Snake Institute (Ohta, Gunma, Japan). Eggshells of *Ara macao*, *Grus japonensis*, *Chelodina siebenrocki*, *Emydura subglobosa*, *Phrynops hilarii*, *Ph. geoffroanus*, *Heosemys spinosa*, *Hieremys annandalei*, *Kinosternon scorpioides*, *Geochelone gigantea* and *Chelydra serpentine* were given from the aquarium of turtles, Izu Andyland (Kawazu, Shizuoka, Japan). Eggshells of *Pelodiscus sinensis* were given from Riken Center for Developmental Biology (Kobe, Hyogo, Japan). Eggshells of *Pogona vitticeps* and *Uroplatus*

phantasticus were laid by reared individuals originally collected from Australia and Madagascar, respectively.

Extraction of soluble organic materials

Shell membranes were removed mechanically from the inner surfaces of eggshells. The eggshells were incubated in a 5% (v/v) aqueous solution of bleach with gentry shaking at room temperature for three hours to destroy surface contaminants. After thorough washing with ultrapure water, the shells were crashed to fine fragments. Organic materials were extracted by dissolution of 0.1 g of the shell fragments in 10 ml of 0.5 M ethylenediaminetetraacetate (EDTA), pH 8.0, with agitating from a magnetic stirrer at room temperature for 24 hours. These sample solutions were used for enzyme linked immunosorbent assay (ELISA). The solutions were lyophilized when used as antigen for antiserum preparation.

Antiserum preparation

Rabbit antisera were prepared against lyophilized extracts from eggshells of *G. gallus domesticus* and *Pe. sinensis*, respectively. One rabbit was injected with 1.0 mg of the lyophilized extract with Freund's complete adjuvant (FCA) as an immunoactivator, followed by the injections of 0.5 mg of the extract three times at interval of two weeks with Freund's incomplete Adjuvant (FIA) as an immunoactivator. Serum was collected one week after the last injection.

Enzyme linked immunosorbent assay (ELISA)

An aliquot (100 µl) of the sample solution described above were incubated at 37°C for 90 min in each well on a multiwell plate. After the wells were emptied, they were washed with 0.2% (v/v) Tween 20 in TBS (TBS/Tween) (TBS; 0.8% (w/v) NaCl, 0.2% (w/v) KCl, 25 mM Tris, pH 7.4) three times. The wells were blocked by incubation with 100 µl of 1% (w/v) gelatin in TBS at 37°C for 30 min. After the wells were emptied, 100 µl of rabbit antisera diluted appropriately (1/10–1/2621440) by 0.1% (w/v) gelatin in TBS/Tween (gelatin/TBS/Tween) were added to each well and incubated at 37°C for 90 min, followed by the TBS/Tween wash as above to remove unbound antibodies. Then 100 µl of 0.05% (v/v) Anti-rabbit IgG alkaline phosphatase conjugate (A8025, Sigma; St Louis, MO, USA) in gelatin/TBS/Tween was added to each well and incubated at 37°C for 90 min. After the TBS/Tween wash to remove unbounded second antibodies, 100 µl of 1% (w/v) 4-nitrophenyl phosphate (pNPP) disodium salt hexahydrate (S0942, Sigma) in 1 M diethanolamine, pH 9.8, with 0.5 M $MgCl_2$, was added to each well and incubated in dark at 37°C for 30 min, followed by addition of 100 µl of 1N NaOH to each well to stop the staining reaction. The color intensity was measured spectrophotometrically at 405 nm using a micro plate reader (MPR-A4i II; TOSOH, Tokyo). All assays were carried out in duplicate.

Quantification of immunological reactivity

In order to estimate the extent of immunological reactivity, we define the concentration factor (CF), which denotes the value of how much more concentrated antiserum is required to obtain the same level of reactivity, or the color intensity (an absorbance value of 0.1 at 405 nm), for a sample as the positive control (the reaction with the chicken antigen when using the chicken antiserum or the reaction with the soft-shelled turtle antigen when using the soft-shelled antiserum). CF of species A with chicken antiserum (CF^G_A) is given by $CF^G_A = C_A/C_G$, where C_A and C_G are the values of antiserum concentration that gave the absorbance value of 0.1 at 405 nm for species A and for the checken antigens, respectively. Similarly, CF of species A with soft-shelled turtle antiserum (CF^P_A) is given by $CF^P_A = C_A/C_P$, where C_P is the absorbance value for soft-shelled turtle.

Immunological distances (ID) from chicken and from soft-shelled turtle to species A are given by $ID^G_A = \log_{10} CF^G_A$ and $ID^P_A = \log_{10} CF^P_A$, respectively. We used these distances from chicken and from soft-shelled turtle to calculate the operational immunological distances (OID) that give the distances among the species compared in this study. OID between species A and B is given by $OID_{AB} = \sqrt{(ID^G_A - ID^G_B)^2 + (ID^P_A - ID^P_B)^2}$.

Results and discussion

Comparison of the intensities of the immunological reactions

We conducted serial dilution assays of ELISA and drew immunological binding curves for the reactions with the extracts from 25 reptilian and avian eggshells and EDTA as a negative control, using antisera raised against extracts from eggshells of *Gallus gallus domesticus* and *Pelodiscus sinensis*, respectively. Fig. 3 shows examples of binding curves. In order to estimate the intensities of immunological reactions, we calculated the concentration factor (CF). At the value of 0.1 in the absorbance at 405 nm in Fig. 3A, the antiserum concentration for *Pe. sinensis* (positive control) is $1/(6 \times 10^5)$ (= C_P; arrow (1) in Fig. 3A) and that for *Phrynops hilarii*, for example, is $1/(2 \times 10^3)$ (= C_{PH}; arrow (2) in Fig. 3A), then $CF_{PH} = C_{PH}/C_P = (6 \times 10^5)/(2 \times 10^3) = 300$. When the antibody raised against the

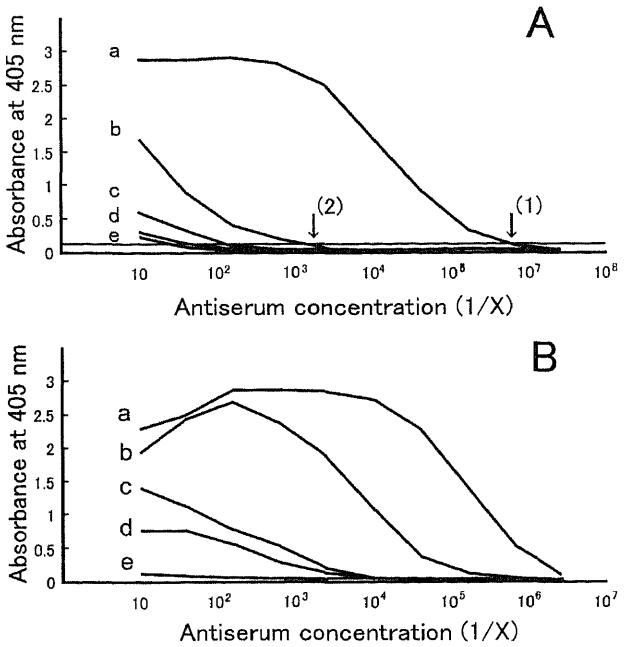

Fig. 3 Immunological binding curves for the extracts from eggshells. A: Results of ELISA using antiserum raised against the eggshell extract from the soft-shelled turtle *Pelodiscus sinensis* for eggshell extracts from (a) *Pelodiscus sinensis*, (b) *Phrynops hilarii*, (c) *Ara macao*, (d) *Crocodylus siamensi*, (e) *Elaphe climacophora*. B: Results of ELISA using antiserum raised against the eggshell extract from the chicken *Gallus gallus domesticus* for eggshell extracts from (a) *Gallus gallus domesticus*, (b) *Coturnix japonica*, (c) *Crocodylus rhombifer*, (d) *Chelydra serpentina*, (e) *Elaphe climacophora*. As to arrows, refer to the text.

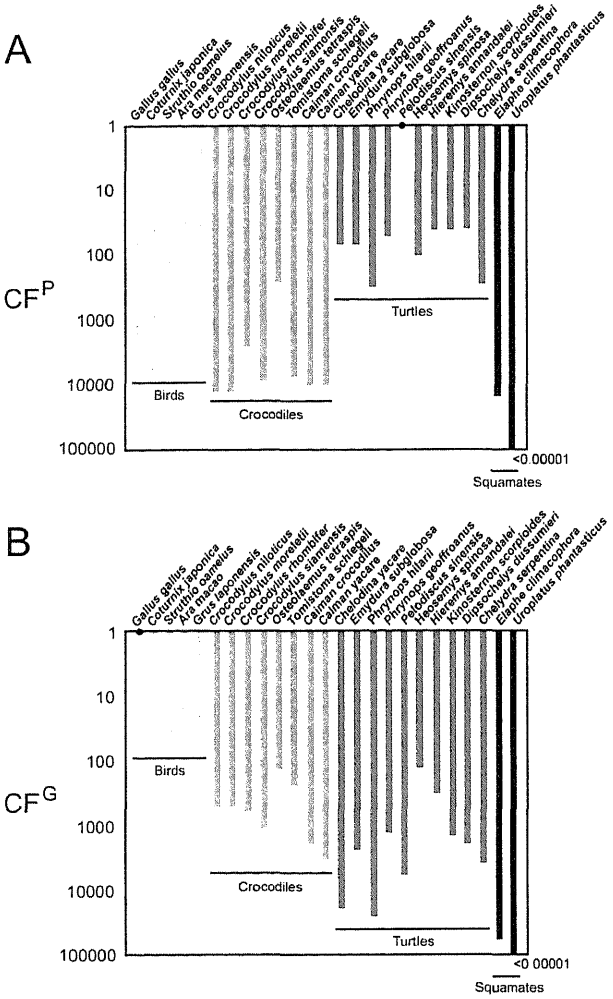

Fig. 4 Immunological reactivity represented by concentration factor (CF) for eggshell extracts from 25 reptiles and birds using antisera raised against the eggshell extracts from the soft-shelled turtle *Pelodiscus sinensi* (A) and the chicken *Gallus gallus domesticus* (B).

extract from the soft-shelled turtle *Pe. sinensis* was used (Fig. 4A), eggshell matrices extracted from turtles showed the highest reaction intensities in general, suggesting that the antiserum properly reacted with antigens. The reactions with crocodiles and birds were relatively high and approximately at the same level with each other, and the reactions with squamates were the lowest. On the other hand, when the antibody raised against the extract from the chicken *Ga. gallus domesticus* was used (Fig. 4B), birds showed the highest reaction intensities, suggesting that the antiserum was properly absorbed with antigens. The reactions with crocodiles were lower than those with birds, and the reactions with turtles were even lower than those with crocodiles. The reactions with squamates were the lowest as in the case with the soft-shelled turtle antiserum. The reaction intensities correlate with similarities of the component of eggshell extracts, and the similarities probably reflect the evolutionary relationships. These results, therefore, suggested that turtles rather than squamates are more closely related to archosaurids (birds and crocodiles).

Relationships among the major groups of reptiles and birds

In order to determine the relationships among the major groups of reptiles and birds, we calculated the operational immunological distances (OID). Figure 5 shows the matrix of OID among 25 reptiles and birds. The values of OID within each of the three major taxonomic groups (crocodiles, birds and turtles) were low (mostly below 1.5). Because the smaller value of OID means the evolutionarily closer relationship, the results

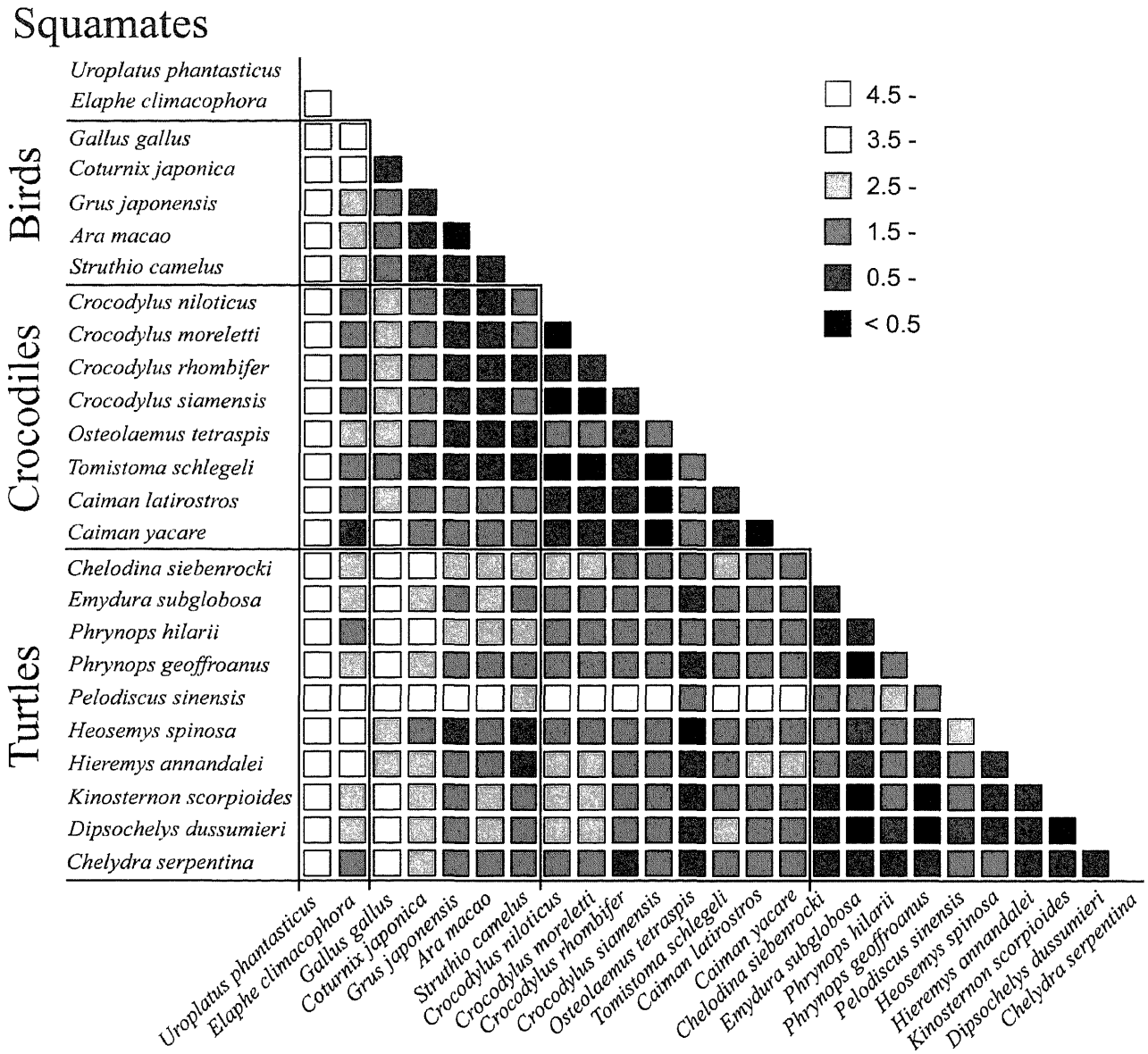

Fig. 5 Matrix of operational immunological distances (OID) among 25 reptiles and birds. The smaller value of OID means the evolutionarily closer relationship.

probably reflect that they form monophyletic groups. The values of OID between crocodiles and birds are relatively small (mostly 0.5–2.5). The values of OID between turtles and birds and those between turtles and crocodiles were approximately at the same level with each other, and they were larger than those between crocodiles and birds (mostly 1.5–3.5). These results suggest a sister group relationship between crocodiles and birds. The OID between turtles and crocodiles, or turtles and birds, were smaller than those between squamates and other major groups (mostly over 2.5). These results indicate that archosaurs (crocodiles and birds) are closer to turtles than to squamates. Because the OID values between squamates and other groups were the largest, squamates are probably the most basal reptiles.

Results based upon immunological assays on eggshell matrices, therefore, support an archosaurian affinity of turtles. Temporal fenestrae of the skulls of turtles were likely to have been secondarily closed. Eggshell matrices seem to be useful for phylogenetic analyses of reptiles and birds, because antisera raised against eggshell extracts from a turtle or a bird reacted strongest with those from turtles or birds, respectively. These facts indicate that the similarities of the components of eggshell matrices reflect evolutionary relation-

ships. To date, several avian eggshell matrix proteins have been identified (Castagnola *et al.*, 1991; Gautron *et al.*, 2007; Hincke *et al.*, 1999; Lakshminarayanan *et al.*, 2002, 2003; Mann and Siedler, 1999, 2006; Mann *et al.*, 2006, 2007; Reyes-Grajeda *et al.*, 2004). On the other hand, there is no report of reptilian eggshell matrix proteins except for pelovaterin, which was identified from the eggshells of the soft-shelled turtle (Lakshminarayanan *et al.*, 2005, 2008). Therefore, as of now, we are not able to use DNA or amino acid sequences of eggshell matrix proteins for the phylogenetic analyses. In order to have sufficient resolution as to the phylogenetic problems, it is required to identify more eggshell matrix proteins of reptiles and to determine and compare their DNA or amino acid sequences.

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