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Comparison of Serum Phospholipase A_2 Activities of All Known Extant Crocodylian Species

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Abstract

Serum samples from all 23 extant crocodilian species were tested for phospholipase A_2 (PLA_2) activity against nine different bacterial species. The data were used to generate a PLA_2 activity profile for each crocodilian species, and the data were used to compare the activities of the three main lineages (Alligatoridae, Crocodylidae, and Gavialidae), the seven different genera, and to compare all of the 23 individual species. The data revealed that the three lineages of crocodilians (Alligatoridae, Crocodylidae, and Gavialidae) exhibited PLA_2 activities toward nine species of bacteria that were statistically distinguishable. In addition, the PLA_2 activities of crocodilians in a specific genus tended to be more similar to other members in their genus than to members of other crocodilian genera.

Keywords

Antibacterial, Antimicrobial, Crocodilian, Crocodylia, Crocodylidae, Gavialidae, Innate Immunity, Phylogeny, PLA_2

1. Introduction

Phospholipase A_2 (PLA_2) is a ubiquitous intracellular enzyme that functions in the excision of fatty acids from the sn-2 position of structural membrane lipids. This enzyme plays an important role in the degradation and metabolism of fatty acids. Another significant function of this enzyme activity is to supply arachidonic acid, which is stored in the intracellular side of membrane phospholipids, for the

synthesis of eicosanoids. However, a soluble, circulating serum form of this enzyme ($sPLA_2$) has been identified [1], which is thought to impart extensive immune function [2] [3] [4]. A role for $sPLA_2$ has been implicated in innate immunity [2] and soluble PLA_2 has been identified as a potent antibacterial component of tears in mammalian systems [3] [5]. The $sPLA_2$ in the peripheral blood is thought to cleave fatty acids from the membranes of microbes, thus compromising pathogen membrane integrity.

There are currently 23 extant members of the family Crocodylia. Four genera (Alligator, Caiman, Paleosuchus, and Melanosuchus), including eight species, comprise the Alligatoridae. The Crocodylidae are represented by three genera (Crocodylus, Osteoleamus, and Mecistops), which include 13 species. A third clade, Gavialidae, has two monophyletic members (*Gavialis gangeticus* and *Tomistoma schlegelii*). Phylogenetic divergence of these taxa is evident in molecular data (Dessauer *et al.*, 2002) [6] and morphological data [7]. Temporally, the Alligatoridae is thought to have diverged from Crocodylidae, approximately 80 million years ago [8] [9]. Recent studies in our laboratory showed that the antibacterial properties of serum of the 23 members of Crocodylia exhibited distinctive differences among broad phylogenetic lineages (Merchant *et al.*, 2006) [10].

This study was conducted to compare the differences in PLA_2 activities of all 23 extant crocodilian species against nine species of bacteria, with the hypothesis that PLA_2 activity is more similar among more closely related taxa. It should be noted that when this study was conducted, *Crocodylus suchus* had not been split from *Crocodylus nitolticus*, and thus the results of this study do not reflect this relatively new development in crocodilian species descriptions.

2. Materials and Methods

Chemicals and biochemicals—4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-sindacene-3-hexadecanoic acid (BODIPY™ FL C16) was purchased from Invitrogen (Carlsbad, CA, USA). Calcium chloride and trizma HCl were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Treatment of animals–Blood was collected from the spinal vein [11] [12] using six-mL syringes and 3.81 cm 21 ga needles. All animals were feeding normally and were apparently healthy upon inspection. The samples were allowed to clot overnight at ambient temperature. The serum was separated and stored at −20˚C in poly propylene tubes until ready for use. The PLA_2 activities are stable for at least three months at −20˚C when stored in a non frost-free freezer (data not shown).

Bacterial cultures—One mL cultures of each bacterial species were grown overnight at 37˚C in nutrient broth. Each culture was used to inoculate 100 mL of sterile nutrient broth. These cultures were incubated for 24 h in the presence of 100 µg of BODIPY™ FL C16 (dissolved in 100 µL of DMSO). The bacteria were centrifuged at 8000 ×g for 15 min at ambient temperature, the cultures

were decanted, and the bacteria were resuspended in 10 mL of assay buffer (1 mM Ca^{2+}, 100 mM tris-HCl, pH 7.4).

PLA_2 assay—The serum from each crocodilian species was combined such that a single value for each species could be obtained. However, prior to combination, the activity of each individual was determined to ensure that fluctuations in individual PLA_2 activities were not radically altering the average for a species. Fifty μL of serum from each crocodilian species was incubated with 600 μL of assay buffer and 100 μL of each bacterial species (BODIPY-labeled) for 60 min at ambient temperature. The reactions were centrifuged at 16,000 ×g for 5 min and the clear supernatants were removed to one mL plastic cuvettes. The fluorescent intensity of each reaction supernatant was measured at an excitation λ of 500 nm and an emission λ of 510 nm (excitation and emission slits = 1 nm) in a Horiba Jobin Yvon Fluoromax™-4 fluorimeter. The PLA_2 activities of each crocodilian species were measured using a single bacterial preparation for each microbial species so that the relative activities were directly comparable without standardization. Previous results from our laboratory have shown that the production of fluorescent product is asymptotic with respect to time when 50 μL of serum is used in 750 μL total assay volume [13].

Statistics and controls—Each sample was analyzed in at least duplicate. The result from each crocodilian species' activity against each bacterial species was compared to all others using Pearson's correlation, thus generating a similarity index for each comparison [14]. In addition, each crocodilian genus was compared to all others using a Pearson correlation. The immune function of the Alligatoroidae, Crocodylidae and Gavialidae were compared via ANOVA using Duncan's post hoc comparisons to obtain the statistical level of significance for each comparison [15].

3. Results

Analysis of the PLA_2 innate immunological data collected from each crocodilian species indicated the similarities between members of the three extant clades of crocodilians (**Figure 1**). PLA_2 activity towards different bacteria species differed among crocodilians at the family level (Alligatorids, Crocodylids, and Gavialids). Based on the PLA_2 activities of the sera of each crocodilian species, Duncan's multiple range comparisons revealed a statistical difference between the Alligatoroidae and Crocodylidae ($p < 0.001$). Likewise, the Gavialidae were also distinguishable from the Alligatoridae ($p < 0.01$). However, there were no statistically discernable differences between the Gavialidae and Crocodylidae ($p > 0.05$). The relationships based on these PLA_2 activities reflect similar associations observed when innate immune activity was used as an indicator (Merchant *et al.*, 2006) [10], and are very similar to the relations noted by other investigators using genetic similarity matrices [16] [17] and albumin protein sequence analyses [18].

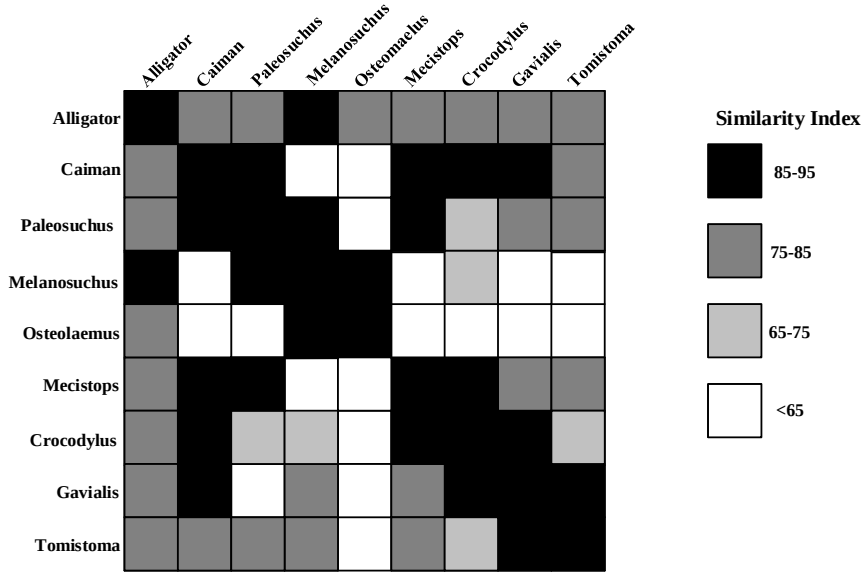

Figure 1. Pearson's correlation comparing the phospholipase A_2 activity of all eight genera of extant crocodylians. These results highlight the similarities of PLA_2 activities of the alligatorids, the differences between the alligatoroids and crocodylids, and the strong similarities of the gavialids.

PLA_2 activities were very similar among genera of the Alligatoroidae. The only aberrant association among this lineage was the low correlation of PLA_2 activities between *Melanosuchus* and *Caiman* (p = 61.3). In addition, member of the genus *Crocodylus* shared similar PLA_2 activities with the *Mecistops cataphractus*, and *Gavialis gangeticus*, and moderately high similarity with *Tomistoma shlegelii*. Of interest was low similarity in PLA_2 activity between the dwarf crocodile (*Osteolaemus*) and Crocodylids. The PLA_2 activities of *Osteolaemus* were very divergent from nearly every other crocodilian species, with few exceptions (**Table 1**, **Figure 1** and **Figure 2**), which is very similar to results previously reported when antibacterial studies of all crocodilian species were compared [10]. This is a result that was not predicted and mimicked by its sister species, *Mecistops cataphractus*. Additionally, serum PLA_2 activity of *Tomistoma* showed a much higher correlation with *Gavialis* and two *Caiman* than with Crocodylidae. Comparisons in PLA_2 activities of all 23 extant crocodilian species are displayed in **Figure 2**.

4. Discussion

Several recent studies have highlighted various components of the innate immune systems of crocodilians. For instance, serum complement activities have been described for the American alligator [19], the freshwater and saltwater crocodiles [20], the broad-snouted caiman [21], and the American crocodile [22]. In addition, dipeptidyl peptidase IV activity has been characterized in the American alligator [23] and the American crocodile [24]. Furthermore, several crocodilian species have been shown to express serum PLA_2 activities (Merchant

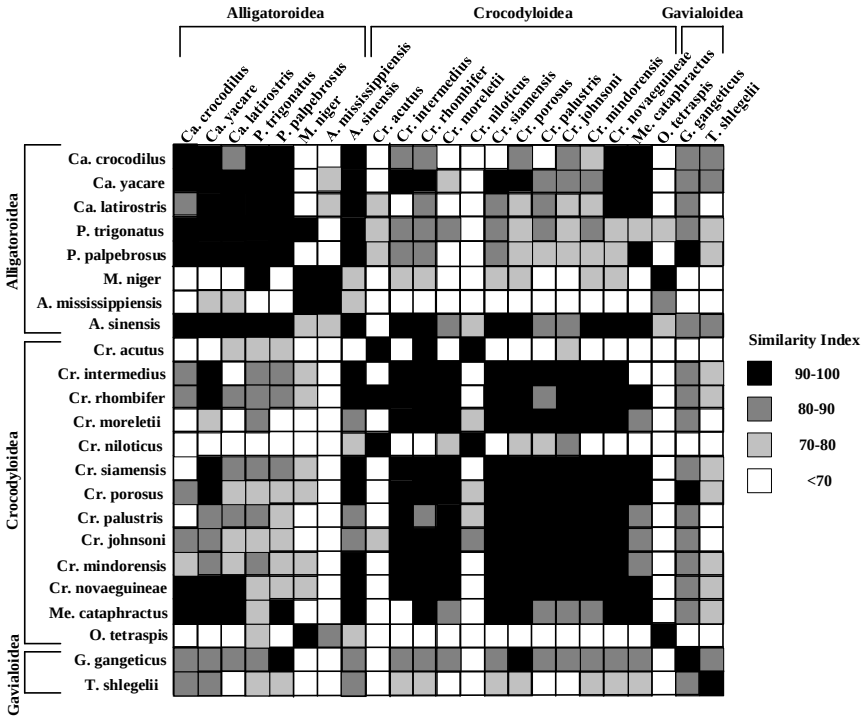

Figure 2. Pearson's correlation comparing the phospholipase A_2 activity of all 23 species of extant crocodylians toward 10 species of diverse bacteria. The correlations highlight the relations of phospholipase A_2 activity specificities toward different bacterial species between the individual species of crocodylians.

et al., 2008, Merchant *et al.*, 2009c, Nevalainen *et al.*, 2009) [25] [26] [27] [28]. Merchant *et al.* [10] showed that the antibacterial activities of all 23 species of crocodilian correlated with molecular and morphological hypotheses of crocodilian diversification. In this study, we have employed the PLA_2 assay to determine the sn-2 fatty acid excision activities of all extant crocodilian species against nine different bacterial species.

The results from our analyses indicate that the three families of crocodilians are distinguishable by their PLA_2 activity profiles (**Figure 1**). In general, the PLA_2 activities among members of the Family Crocodylidae were more disparate compared to those within the Alligatoridae and Gavialidae (**Figure 1**). This was also noted when serum complement immune activities were compared by Merchant *et al.* [10]. This observation, potentially, is an artifact of relatively high species richness and biogeographic breadth within Crocodylidae compared to Alligatoridae and Gavialidae, allowing or necessitating greater diversification in enzymatic activity. The members of the Family Crocodylidae are more diverse, being comprised of 14 species compared to eight Alligatoridae and two Gavialidae. The Crocodylidae are also found in a much wider geographical distribution, living on five continents, compared to three for the Alligatoridae and two for the Gavialidae), occupy more types of habitats (broad range of salinities, environments, etc.), and thus are potentially exposed to a broader spectrum of microbial

Table 1. PLA_2 activities of serum from all 23 species of extant crocodilian species were measured against nine different bacterial species. A = *Escherichia coliform*, B = *Providencia stuartii*, C = *Staphylococcus aureus*, D = *Streptococcus pyrogens*, E = *Streptococcus faecalis*, F = *Shigella flexneri*, G = *Salmonella typhimurium*, H = *Enterobacter cloacae*, I = *Klebsiella oxytoca*. The PLA_2 activities are expressed as 0 - 10^6 = +, 1 × 10^6 - 2 × 10^6 = ++, 2 × 10^6 - 3 × 10^6 = +++, 3 × 10^6 - 4 × 10^6 = ++++, 4 × 10^6 - 5 × 10^6 = +++++, and 5 × 10^6 - 6 × 10^6 = ++++++.

ALLIGATORIDAE	A	B	C	D	E	F	G	H	I
Alligator mississippiensis (N = 6)	+++	++++	++	++	++	++	++	+++	++++
Alligator sinensis (N = 4)	+++	++++	++	++	+++++	+++	++	++++	+++++
Caiman yacare (N = 5)	++	+++	++	+	++++	++	++	+++	++++
Caiman latirostris (N = 3)	++	++	++	+	++++	++	+	+++	++++
Caiman crocodylus (N = 5)	++	+++	++	+	++++	+	+	++	++++
Paleosuchus palpebrosus (N = 4)	+++	+++	++	+	++++	++	++	+++	++++
Paleosuchus trigonatus (N = 4)	++	+++	++	+	++++	++	++	+++	++++
Melanosuchus niger (N = 2)	++	++++	++	++	++	++	++	+++	++++
Osteolaemus tetraspis (N = 3)	+++	++++	+++	++	++	+++	++	++	++++
CROCODYLIDAE									
Crocodylus niloticus (N = 3)	++	++	+++++	++	+++++	+++	++	++++	++++++
Crocodylus moreletti (N = 4)	++	++++	++	++	+++++	+++	+++	+++++	+++++++
Crocodylus johnstoni (N = 2)	++	+++	+++	++	++++	++	++	++++	++++++
Crocodylus novaeguineae (N = 2)	++	++++	++	++	+++++	++	++	++++	++++++
Crocodylus rhombifer (N = 3)	++	++++	+++	++	+++++	++	+++	++++	++++++
Crocodylus mindorensis (N = 2)	++	++++	+++	++	++++	++	++	+++++	+++++
Mecistops cataphractus (N = 2)	++	++++	+++	++	+++++	++	++	+++	+++++
Crocodylus porosus (N = 4)	++	++++	++	++	+++++	++	++	++++	++++++
Crocodylus intermedius (N = 1)	++	++++	++	++	++++	++	++	++++	+++++
Crocodylus siamensis (N = 2)	++	++++	+++	++	++++	++	++	++++	+++++
Crocodylus acutus (N = 5)	++	++	++++	++	+++++	++	++	+++	++++
Crocodylus palustris (N = 2)	+	+++	++	++	+++++	++	++	++++	+++++
GAVIALIDAE									
Tomistoma schlegelii (N = 2)	+++	+++	++	+	+++	+	++	++	+++
Gavialis gangeticus (N = 1)	++	++	++	+	+++	+	++	+++	+++

Table 2. Amino acid sequence identities between representatives of the three families of crocodylians, Alligatoridae, Crocodylidae, and Gavialidae.

	American alligator	Estuarine crocodile	Indian gharial
American alligator *Alligator mississippiensis*	100 (143)	91.9%	86.2%
Estuarine crocodile *Crocodylus porosus*	91.9%	100 (147)	91.2%
Indian gharial *Gavialis gangeticus*	86.2%	91.2%	100 (149)

flora. Therefore, the contrasting range of PLA_2 activities in the Crocodylidae, compared to the Alligatoridae and Gavialidae, is not surprising.

The PLA_2 activities are more similar among species within the same family than between species in different families (**Table 1**, **Figure 2**). The same general trend is true at the level of *genus* aside from two notable aberrations: *Melanosuchus* and *Oesteolamus*. Interestingly, *Osteolaemus* exhibited PLA_2 activities that were more similar to those of many of the Alligatoridae than the Crocodylidae (**Table 1**, **Figure 2**). Likewise, although *Mecistops* PLA_2 activities correlate strongly with *C. rhombifer*, *C. siamensis*, and *C. novaguinae*, they also exhibit high similarity with members of the Family Alligatoridae (*Ca. yacare*, *latirostris*, and *crocodilus*, and *P. palpebrosus* and *A. sinensis*, **Table 1**, **Figure 2**). Additionally, and with regards to the similarities between PLA_2 activity presented here and existing phylogenies, PLA_2 activity in *Tomistoma* more closely resembles *Gavilais* than it does any genus within Crocodylidae. This finding is consistent with morphological [7] and molecular [29] phylogenetic hypotheses recovering a sister relationship between *Tomistoma* and *Gavialis*. However, the molecular phylogeny of Brochu and Densmore [30] did not recover this sister relationship. Therefore, the PLA_2 activity presented here could be homologous between *Tomistoma* and *Gavialis*. In addition, this conclusion supports the findings of phylogenetic linkages of antibacterial immunological activities [10] and amino acid sequences of, and immunoreactivity to, specific proteins [18]. Furthermore, the PLA_2 activities of *C. niloticus* are also interesting from an evolutionary perspective. One may hypothesize that PLA_2 activity in *C. niloticus* would resemble its sister clade (New World *Crocodylus*) [16] [29]. However, the PLA_2 activities of *C. niloticus* are very unique among most members of its genus, and do not resemble neither New World nor Old World *Crocodylus*.

The positive relationship between species-specific PLA_2 activities and the evolutionary relationships among those taxa indicates potential for immunological homology. Such an interpretation should be approached with caution, given the complex ability of the immune system to acclimatize via exposure, and other ecological influences on activity that mask relevant relatedness by descent of immunological traits. In defense of this notion, PLA_2 acts as an antimicrobial component in innate immunity, defined solely by the transcription of coding genes and limiting the ecological modification of activity from exposure history. Applicable is the work of Nakashima *et al.* [31], whose analysis recovered rapid evolution in the nucleotide sequence of protein-coding regions of PLA_2 isozyme genes between the venom glands of two closely related viper species (*Trimeresurus*). Therefore, the taxonomic resemblance of PLA_2 activity is likely more evident of immunological homology than the general serum antimicrobial activity among members of Crocodylia [10]. Nevertheless, the PLA_2 activity documented here reflects phylogenetic lineage relatedness to a great degree, indicating the potential for lineage-specific conservation in PLA_2 function based on coded structure. The amino acid sequences of the $sPLA_2$ for representatives (American alligator, estuarine croco-

dile, and gharial) of the three crocodylian Families (Alligatoridae, Crocodylidae, and Gavialidae), are quite divergent compared to other protein sequences that we have analyzed between these groups (Table 2). The sequence identities of 86.2 to 91.9% are dissimilar enough to provide different PLA_2 activity profiles toward the phospholipids of different bacterial species. In comparison, these same crocodilian species share 95.5% - 97.0% amino acid identity in their serum complement C3 proteins (Merchant *et al.*, 2016), and 96.5% - 97.8% amino acid identity in nuclear factor kB transcription factor (Merchant, unpublished data) are much higher than in the more divergent PLA_2 proteins. The diversity in $sPLA_2$ amino acid sequences between these crocodilians may reflect plasticity in the evolution of genes that code for proteins with important roles in immunological defenses. The optimization of immunological traits on existing phylogenies to explore immunological character evolution may be a worthy endeavor.

5. Conclusion

In conclusion, PLA_2 activity among extant crocodilians shows high taxonomic similarity based on Pearson correlation indices. Such results are likely indicative of relation by descent in the genetic underpinning of enzymatic operation. Additionally, regardless of phylogenetic hypotheses, PLA_2 activity appears to show a fair amount of convergence and independent evolution that makes for an interesting exercise in character evolution on proposed phylogenetic trees. More work need to be conducted concerning lineage-dependent shifts in PLA_2 activity. This would elucidate the "aberrations" found here and help decipher whether mutation or selective processes may account for activities that diverge from phylogenetic hypotheses.

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