

Comparisons of innate immune activity of all known living crocodylian species

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Abstract

Serum samples from all twenty-three known living members of the Crocodylia were tested for antibacterial activity against eight bacterial species. These data were used to generate an immune profile for each crocodylian species. Statistical analyses revealed that the three living lineages of crocodylians, Alligatoroidea, Crocodyloidea, and Gavialoidea, were distinguishable by their immunological activities. For instance, species within the Alligatoroidea and Crocodyloidea exhibited remarkable immune activity similarities to others in their own lineages. Comparisons of the members of the different lineages, however, revealed substantial differences in immune profiles. Furthermore, species that are in the same genus were shown to exhibit more immune similarities to each other than to members of other genera within the same family. Finally, our immunological analyses reveal that *Tomistoma schlegelii* aligns more closely with the Gavialoidea than the Crocodyloidea.

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1. Introduction

Many members of the Crocodylia are territorial animals that are frequently injured during both intraspecies and interspecies aggression. These disputes can lead to serious injuries, often involving loss of limbs. However, despite living in environments rich in potentially pathogenic microorganisms, these wounds often heal without infection. Several species of crocodylians have been shown to be resistant to disease. For instance, Madsen et al. (1998) found a variety of *Salmonella* serotypes in the cloacae of healthy Nile crocodiles (*Crocodylus niloticus*). Manolis et al. (1991) found a high incidence of *Salmonella* in farmed *Crocodylus johnsoni* and *Crocodylus porosus*, while Scott and Foster (1997) described the isolation of *Salmonellae* from both farmed and wild American alligators (*Alligator mississippiensis*). In addition, Williams et al. (1990) found 23 different species of bacteria in the gular and paracloacal glands of healthy American alligators.

The 23 extant members of the Crocodylia (Ross, 1998) represent three distinct lineages (see Brochu, 2003). The Alligatoroidea include eight species in four genera (*Alligator*, *Caiman*, *Melanosuchus*, and *Paleosuchus*). The Crocodyloidea comprises 12 recognized species of the genus *Crocodylus* in addition to the monotypic genus *Osteolaemus*. A third clade, the Gavialoidea, includes the Indian gharial (*Gavialis gangeticus*). The proper phylogenetic placement of the Malay gharial (*Tomistoma schlegelii*) is unsatisfactorily resolved and still a matter of great debate. Morphological information from paleontological and comparative anatomical studies clearly place *Tomistoma* within the Crocodyloidea (Norell, 1989; Poe, 1996; Salisbury and Willis, 1996; Brochu, 1997, 1999; Buscalioni et al., 2001). Findings from molecular and genetic data sets (Densmore, 1983; Densmore and Owen, 1989; Densmore and White, 1991; Hass et al., 1992; Gatesy and Amato, 1992; Gatesy et al., 1993, 2003; Aggarwal et al., 1994; Poe, 1996; Brochu, 1997; White and Densmore, 2001; Ray et al., 2001; Gatesy et al., 2003; Harshman et al., 2003), however, invariably identify *Tomistoma* as a sister taxon with *Gavialis*.

This study was conducted to serve as a brief comparison of innate immune activities of all 23 known crocodylian species.

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To our knowledge, this is the first study comparing the immune system of all living crocodylians.

2. Materials and methods

2.1. Bacterial species

All bacterial cultures were obtained from Remel (Lenexa, KS, USA). The following American Type Culture Collection (ATCC) bacterial strains were used for these studies: *Escherichia coli* (35218), *Shigella flexneri* (12022), *Enterobacter cloacae* (23355), *Klebsiella oxytoca* (33496), *Citrobacter freundii* (C109820), *Salmonella poona* (4840), *Providencia stuartii* (33672), and *Pseudomonas aeruginosa* (27853).

2.2. Treatment of animals

Blood samples were drawn from the spinal vein using a 3.81 cm 21 gauge needle and a 6 mL syringe (Olson et al., 1977; Zippel et al., 2003) and transferred to serum Vacutainer® tubes. The serum was separated, shipped on dry ice, and then stored at -80°C in polypropylene tubes.

2.3. Bacterial cultures

Bacteria were maintained on nutrient agar slants at 4°C . The day before an experiment, a 2-mL nutrient broth liquid culture was inoculated from the slant with a sterile cotton swab. The bacteria were allowed to incubate at 37°C overnight to obtain a log-phase culture.

2.4. Antibacterial assay

Nutrient agar was prepared and 14-mL aliquots were autoclaved in 150×25 mm tubes. The aliquots were stored at 4°C until used. On the day of the antibacterial assays, the 14-mL nutrient agar aliquots were boiled for 5 min and placed in a 42°C water bath. Each nutrient agar aliquot was inoculated with 100 μL of a log-phase bacterial culture. The inoculated agar sample was immediately transferred to a 150 mm Petri dish and the agar allowed to solidify. Wells of approximately 3 mm were cut into the agar using a sterile Pasteur pipette connected to a vacuum pump. Four wells were cut into the agar for each crocodylian serum sample to be tested. Five microliters of each serum sample was transferred aseptically into each of four wells. The serum samples were allowed to diffuse into the agar for 3 h at ambient temperature. After incubation, a 14 mL sample of top agar was poured onto each plate and allowed to solidify. The plates were stored inverted in a 37°C incubator overnight. The zones of inhibition were clearly visible and were measured manually.

2.5. Statistics and controls

Each sample was analyzed in at least triplicate. The result from each crocodylian species' activity against each bacterial species was compared to all others using Pearson's correlation,

thus generating a similarity index for each comparison (Kirk, 1995a). In addition, each crocodylian genus was compared to all others using a Pearson correlation. The immune function of the Alligatoroidea, Crocodyloidea and Gavialoidea were compared via ANOVA using Duncan's post hoc comparisons to obtain the statistical level of significance for each comparison (Kirk, 1995b).

3. Results

The three extant lineages of the Crocodylia were discernable by differences in immunological activities (Table 1). Analysis of variance showed a statistically significant difference in the mean innate immune activity of the families ($p < 0.0005$) with Duncan's multiple range, revealing that the mean in the Alligatoroidea was significantly higher than that of the Crocodyloidea or Gavialoidea. However, the Duncan's multiple range comparisons grouped the Crocodyloidea and Gavialoidea together ($p = 0.696$), showing that the gharials exhibit more similar innate immune activity profiles to the crocodiles than the alligatoroids. The immunological relationships observed in these three lineages are similar to those observed by other investigators using genetic identity matrices (Gatesy and Amato, 1992; Aggarwal et al., 1994) and albumin

Table 1
Immune system profiles for all 23 known crocodylian species

	A	B	C	D	E	F	G	H
<i>Alligatoroidea</i>								
<i>A. mississippiensis</i>	++	++	+++	-	++	-	+++	+++
<i>A. sinensis</i>	+++	++	++++	-	-	-	++	++
<i>Ca. crocodilus</i>	++	++	+++	++	++	++	+++	+++
<i>Ca. latirostris</i>	+++	+++	+++++	-	+++	++	+++	+++
<i>Ca. yacare</i>	+++	++	+++	-	++	++	++++	+++
<i>M. niger</i>	+++	++	++++	-	++	++	++++	+++
<i>P. palpebrosus</i>	++	++	+++	-	++	++	+++	++
<i>P. trigonatus</i>	++++	++	++++	-	+++	+++	+++	++++
<i>Crocodyloidea</i>								
<i>Cr. acutus</i>	++	-	+++	-	-	+++	+++	+++
<i>Cr. cataphractus</i>	+++	++	+++	++	-	-	+++	++++
<i>Cr. intermedius</i>	+++	-	+++	-	-	++	+++	++
<i>Cr. johnsoni</i>	+++	-	+++	-	-	++	+++	+++
<i>Cr. mindorensis</i>	+++	-	++++	-	-	++	+++	-
<i>Cr. moreletii</i>	++	-	+++	-	-	-	+++	-
<i>Cr. niloticus</i>	+++	-	+++	-	-	-	+++	-
<i>Cr. novaeguineae</i>	+++	-	++++	-	-	++	+++	-
<i>Cr. palustris</i>	++	-	++	-	++	+++	++++	-
<i>Cr. porosus</i>	+++	-	++++	+++	-	+++	+++	-
<i>Cr. rhombifer</i>	+++	-	+++	++	-	+++	++++	-
<i>Cr. siamensis</i>	++	-	-	+	-	+++	++++	-
<i>O. tetraspis</i>	++++	-	-	-	++++	++++	++++	++++
<i>Gavialoidea</i>								
<i>G. gangeticus</i>	++++	+	++++	-	-	-	++++	-
<i>T. schlegelii</i>	+++	++	-	-	-	-	++++	+

Innate immune activity of the serum from crocodylian species was tested against eight different bacterial species: A = *Salmonella poona*, B = *Citrobacter freundii*, C = *Escherichia coli*, D = *Pseudomonas aeruginosa*, E = *Enterobacter cloacae*, F = *Providencia stuartii*, G = *Shigella flexneri*, H = *Klebsiella oxytoca*. The zones of inhibition are expressed as: 0 mm = -, 0–2 mm = +, 2–4 mm = ++, 4–6 mm = ++++, >6 mm = +++++.

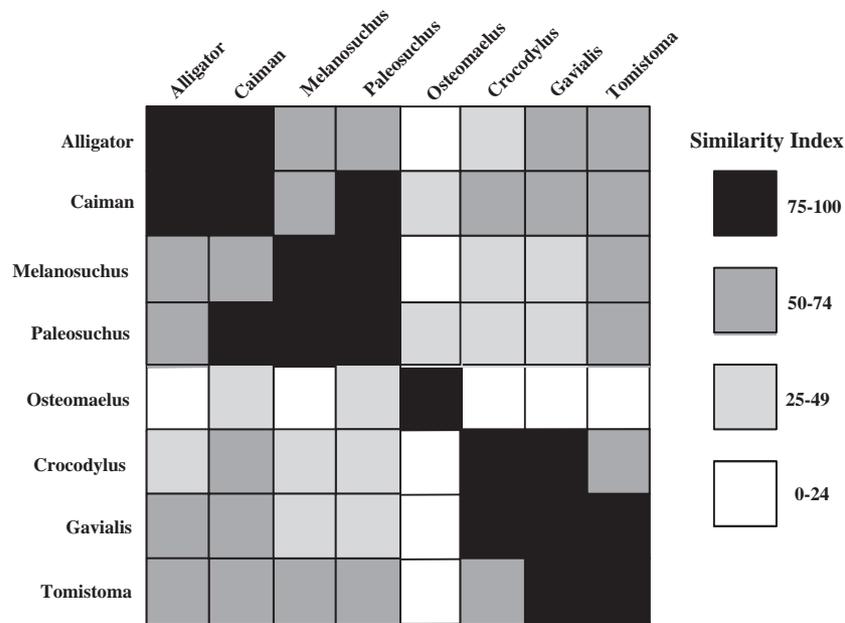


Fig. 1. Pearson's correlation of the immunological profiles of the eight genera of extant crocodylians. Immunological activities of the members of each genera were compared to those of each other crocodylian genera. These data highlight the similarities between the members of the alligatoroids, and the differences between the alligatoroid and crocodyloid lineages. These data also illustrate strong similarities in the innate immune activities of the *Gavialis* and *Tomistoma*.

immunodiffusion, starch gel electrophoresis, and globin peptide fragment analyses (Densmore, 1983). The overall antibacterial activities for the members of the Alligatoroidea appear to be higher than those of the Crocodyloidea and Gavialoidea. However, the numbers may be biased because the species of bacteria chosen to study were based on our previous studies of the alligatoroid *Alligator mississippiensis*.

Comparisons of the immunological indices of the different genera provided further evidence of taxonomic relationships. For instance, application of Pearson's correlation of the genus *Alligator* immunological profiles with *Caiman* (0.87), *Melanosuchus* (0.62), or *Paleosuchus* (0.72) resulted in moderately high relations (Fig. 1). However, comparison of any of the alligatoroid genera with the genus *Crocodylus* results in low correlation indexes (0.49). Comparison of the crocodyloid *Osteolaemus tetrapis* with any genus, including *Crocodylus*, results in poor correlation of immunological activities. In general, comparison of all crocodylian genera (except *Osteolaemus*) with *Gavialis* and *Tomistoma* result in moderate correlations. Finally, the monotypic gavialoid genera *Gavialis* and *Tomistoma* (0.92) exhibit very high similarity of innate immune profiles.

4. Discussion

Many authors have attempted to assess natural affinities of living crocodylians and organize these animals according to modern systematics. Early attempts to categorize crocodylians into phylogenetic categories were based on morphological characteristics (Kälin, 1955; Steele, 1973). Other studies attempted to distinguish crocodylians by karyotypic analyses (Cohen and Gans, 1970). Densmore (1983) used electrophoretic comparisons of 18 blood protein peptide fingerprints and

immunoprecipitation techniques to compare albumin and transferrin of living crocodylian species.

The tissues of several different crocodylian species have been shown to exhibit both potent and broad-acting antimicrobial properties. For instance, Shaharbanay et al. (1999) showed that tissue extracts from the Nile crocodile (*Crocodylus niloticus*) demonstrated antibacterial activities. Studies from our laboratory showed that serum from the American alligator (*A. mississippiensis*) exhibit antibacterial (Merchant et al., 2003, 2005b), amoebacidal (Merchant et al., 2004), and antiviral (Merchant et al., 2005a) activities. Other studies have shown similar antimicrobial properties of serum from *Cr. siamensis* (Siuntawineti et al., 2004) and *Ca. latirostris* (Pablo Siroski, personal communication). This study was designed to serve as a simple comparison of the innate immune activities of all 23 known living crocodylian species. We were surprised to find that the activities were generally split among accepted taxonomical relations. We are not suggesting that immunological methods as crude as the one reported in this study be used to align and compare taxonomical groups, but are simply reporting the amazing amount of taxonomical correlation that was found within the extant members of this relatively small order.

Results from this study show that the three major crocodylian lineages are discernable by the immunological profiles that they exhibit (Table 1). The immunological profiles between the collective activities of the members of the three clades were compared and assessed. Multivariate analyses revealed that the innate immune activities of the Alligatoroidea were statistically different from those of the Crocodyloidea and Gavialoidea. However, the statistical analysis also showed that the members of the Gavialoidea were more closely related to those of Crocodyloidea. Furthermore, the genera display characteristic activities that can be distinguished from other

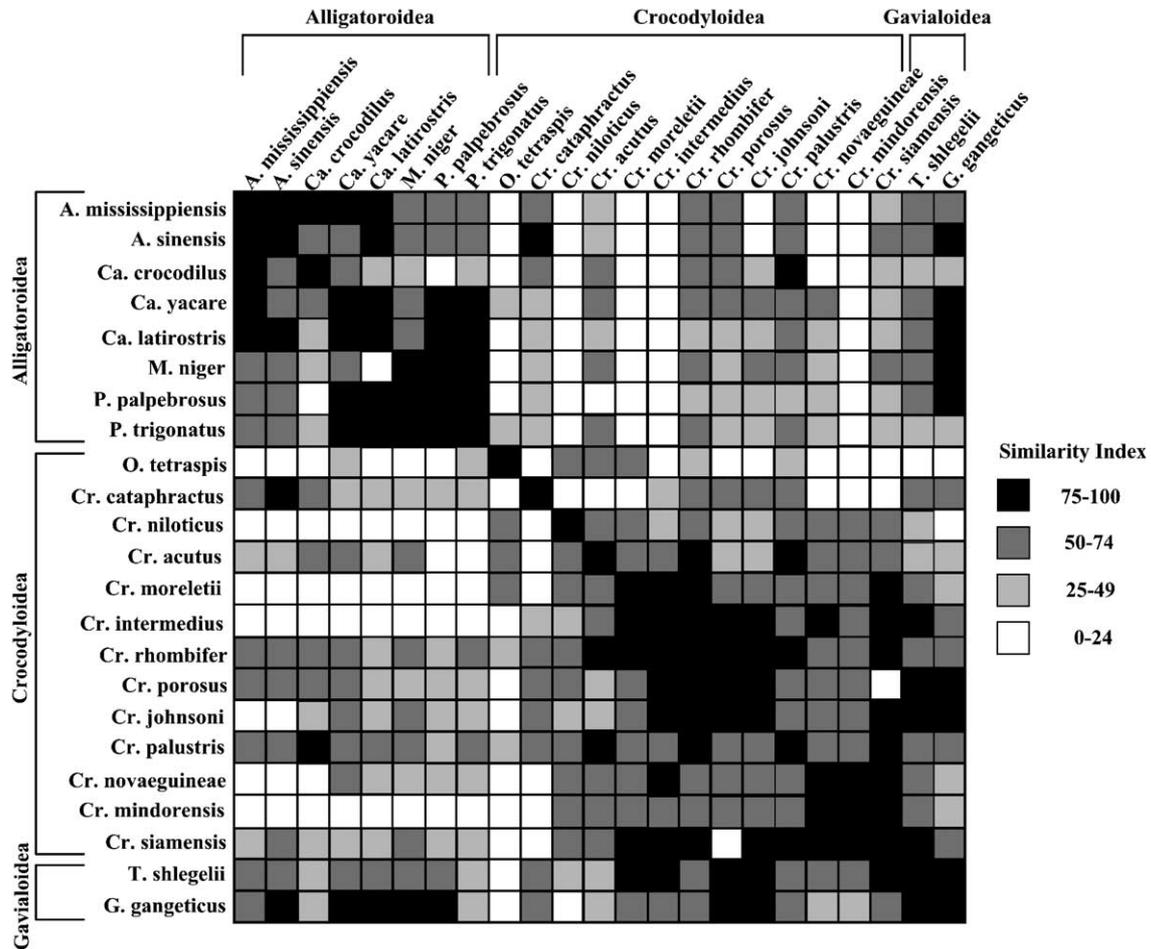


Fig. 2. Pearson's correlation of the immunological profiles of the 23 species of extant crocodylians. Immunological profiles were generated by comparing innate immunity to eight selected bacterial species. The profiles highlight the taxonomic relationships between the individual members of each crocodylian taxa.

genera within the same family. For instance, the immunological activities of the two species of *Alligator*, *A. mississippiensis* and *A. sinensis*, are almost identical (Table 1) and thus exhibit a high Pearson's correlation index (illustrated in Fig. 1). *Alligator sinensis* is geographically disjunct from all other living alligatoroid species. These data support previous studies showing that these two species are more closely related than any other two living crocodylians (Densmore, 1983). These two *Alligator* species shared a common ancestor sometime in the late Tertiary (Brochu, 1999), within the last 18 to 25 million years. In addition, *Alligator* immune activities are similar to those of the other members of the Alligatoroidea (the caiman genera *Caiman*, *Paleosuchus*, and *Melanosuchus*). However, the immune activities of the two *Alligator* species are more similar to each other than to the other members of the Alligatoridae. Immune activity similarities are easily observed in Fig. 1. These differences support the fossil evidence that suggests that the alligatorine and caimanine lineages split at least by the early Paleocene (Brochu, 1999). The immune similarity index shows the similarities in immunological activities between all of the alligatoroid genera. It also clearly shows the dissimilarities between the genera of the Alligatoroidea and Crocodyloidea.

The immunological data for the 12 species of the Crocodyloidea are more divergent than for those of the Alligatoroidea. This contradicts the conclusions of Densmore (1983) that members of the genus *Crocodylus* exhibit high protein sequence similarities and thus are closely related. The immunological profile for *Osteolaemus tetraspis* seems to be highly divergent from almost every other species, with few exceptions (Fig. 2). Interestingly, the immunological profile of the Malay gharial (*Tomistoma schlegelii*) is more closely aligned with the Indian gharial (*Gavialis gangeticus*) than to members of the Crocodyloidea (Fig. 2). These data thus support numerous other recent studies that place *T. schlegelii* within to the Gavialoidea rather than the Crocodyloidea (Densmore and Dessauer, 1984; Gatesy and Amato, 1992; Harshman et al., 2003). The immunological data that we have presented here correspond more with the molecular, rather than the morphological, approach to taxonomical alignment within the living Crocodylia.

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