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Multiple paternity in the nurse shark, *Ginglymostoma cirratum*

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Synopsis

For over a decade, we have been studying the reproductive behavior of the nurse shark, *Ginglymostoma cirratum*, in the Dry Torugas off the Florida Keys, an important mating and nursery ground for this species. In the course of these studies, we have used a variety of tags and tagging protocols to monitor individual animals. Here we report the use of molecular methods for the genetic analysis of nurse sharks. Specifically we have analyzed genetic variation at the MHC II α locus using the polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analysis of the amplified products. We found this technique to be a relatively rapid and reliable method for identifying genetic differences between individual sharks. Applying this method to a family of sharks consisting of a mother and 32 pups, we demonstrate that at least four fathers must have fathered this brood. Multiple paternity in the nurse shark suggests a mechanism by which populations of this species may maximize genetic variability. This seems especially valuable for philopatric species whose migratory movement, and thus potential for genetic diversity, is limited.

Introduction

For more than ten years, the reproductive biology of nurse sharks, *Ginglymostoma cirratum*, has been systematically studied in animals from an isolated group of islands in the Dry Tortugas in the westernmost Florida Keys (Carrier et al. 1994, Pratt & Carrier 2001) and has been further elucidated by the work of Castro (2000) for a wider distribution of nurse sharks. Individuals of this species show little apparent migratory movement and have a small home range during all intervals of life (Kohler et al. 1998, Carrier 1985). Frequent recaptures of tagged animals provide reliable information regarding growth (Carrier & Luer 1990) and permit sustained observations of reproductive behaviors by animals who faithfully return to the mating area at predictable times. The mating area in the Dry Tortugas has been recognized as an important mating and nursery grounds for nurse sharks and recent actions have been taken by the United States National Park Service to protect the area during the most active time of the year for mating in nurse sharks (Carrier & Pratt 1998). We continue to study the animals returning to this area in order to further our understanding of the reproductive biology of this species as well as to continue our conservation efforts.

Because animals can be relied upon to return to the area, a variety of tags and tagging protocols have been evaluated (Kohler 2001). Recently, the study of this nurse shark population has been expanded to include the routine sampling of blood and tissue for purposes of monitoring blood chemistry, including profiles of reproductive steroids in animals in the study area (Carrier & Pratt unpublished). Here, we report the development of molecular markers with which to conduct genetic analyses of nurse sharks, and describe

the application of these markers to address the question of whether multiple paternity can occur in this species. Our interest in multiple paternity arose from field observations of mating events in which individual females were photographically documented to copulate with more than one male during the limited time over which mating has been observed to occur (Carrier et al. 1994, Pratt & Carrier 2001). Since neither the precise number of male partners, the number of successful matings, nor the existence or extent of sperm competition could be determined from these observations, we have applied molecular genetic methods to begin to address these questions. Specifically, we have applied PCR-RFLP analysis to identify sequence variation at the major histocompatibility II alpha (MHC II α) locus. This approach is based on a similar method applied to assess sequence variation at HLA loci in humans (Maeda et al. 1990), and was adapted for use in the nurse shark using MHC II α sequences published by Kasahara et al. (1993).

Materials and methods

Shark tissue, consisting of a litter of 32 deceased pups and a vertebra and associated tissue from the mother of these pups was provided by J. Castro. Genomic DNA was isolated from tissue samples using commercial DNA isolation kits (Qiagen). PCR reactions were performed using 5 ul of 50 ul resulting from the DNA isolation procedure. Two primer combinations were used for PCR. The first combination was as published by Kasahara et al. (1993), and included primers GC78 (TCTCTCTSTCYCTSTCTCWYWCAGATCT) and GC74 (ACCTTTCGGTTCCGGGGTCCC), where Y = T or C, W = A or T, and S = G or C. GC78 and GC74 correspond to intron sequences flanking exon 2 of the MHC II α locus. A third primer designated GC 2946 (TGTATTTTGTGCAGCAGC) was used in combination with GC74 for some PCR reactions. Primer GC 2946 corresponds to nucleotides 29 through 46 of the published exon 2 sequence, a region completely conserved in all MHC II α alleles sequenced to date (Kasahara et al. 1993). PCR reactions were carried out using the *Taq* PCR core kit (Qiagen) and consisted of the following components: 5 ul genomic DNA, 2 mM $MgCl_2$, 100 uM dNTP mix, 0.3 uM of each primer, 0.5 units *Taq* polymerase, and 1X reaction buffer in a total volume of 100 ul, overlayed with 50 ul mineral oil. Assembled reactions were heated to 94°C for three minutes followed by 40 cycles of 94°C for one minute, 55°C for 30 seconds, and 72°C for 30 seconds. Following the 40th cycle, reactions were incubated at 72°C for an additional ten minutes. Negative controls lacking template DNA were performed in parallel with each set of PCR reactions. PCR products were subjected to electrophoresis through 1.5% agarose gels containing 5 ug ml^{-1} ethidium bromide, and were visualized and photographed using an ultraviolet light transilluminator. The approximate sizes of PCR products were determined by comparison to a 1 kb DNA size standard (Life Technologies).

For PCR-RFLP analyses, potential restriction site polymorphisms between MHC II α alleles were identified by analysis of published sequences (Kasahara et al. 1993) using the Gene Construction Kit II (GCK II) computer program (TextCo). Restriction digests of PCR products were carried out according to the manufacturers specifications (New England Biolabs), and were analyzed by agarose gel electrophoresis using 4% NuSieve 3 : 1 agarose gels (FMC BioProducts).

Results

In order to determine if multiple paternity occurs in the nurse shark, we analyzed DNA sequence polymorphisms within the major histocompatibility class II alpha (MHC II α) locus, which had been previously shown to exhibit a high degree of sequence polymorphism in this species (Kasahara et al. 1992, Kasahara et al. 1993, Ohta et al. 2000). To do this, DNA corresponding to exon 2 of the MHC II α locus (Kasahara et al. 1993) was amplified by PCR, then analyzed by RFLP analysis. The positions of the PCR primers and informative restriction sites in the MHC II α sequence are shown in Figure 1a. Typical PCR results using primers GC74 and GC78 are shown in Figure 1b. Amplification with these primers results in a prominent band consistent in size with that expected from the published sequence (275 base pairs). In many cases, a lower band was also observed. This band is probably due to the formation of primer dimers and did not interfere with subsequent restriction analysis.

PCR products were subjected to restriction digestion and agarose gel electrophoresis. The results of this analysis are summarized in Table 1. Each PCR product contained zero or one restriction site for each enzyme used. Thus, for a given restriction enzyme each PCR product would produce one band (if an allele did not

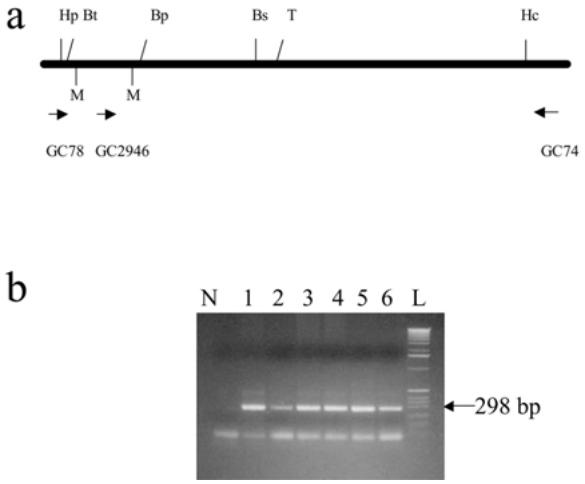

Figure 1. a – A map of polymorphic restriction sites used for RFLP analysis of PCR products. Arrows indicate the approximate position of PCR primers GC74, GC78, and GC2946. Abbreviations are as follows: Bs = Bsi EI, Bt = Bst NI, Hc = Hinc II, Hp = Hph I, M = Msl I, T = Tfi I. b – Representative PCR amplification products using primers GC74 and GC78 to amplify MHC II α DNA from nurse shark tissue samples. Lane N = no template DNA control reaction, lanes 1–6 = PCR amplification products from six different pups. The prominent band seen in lanes 1–6 runs just below the 298 kb band (arrow) of the DNA size marker (lane L), consistent with the mobility expected for the 275 bp fragment expected to be amplified using these primers. The lower molecular weight band is a PCR artifact seen to varying degrees in all amplification reactions with this primer set.

contain a restriction site for that enzyme), two bands (if an allele did contain a particular restriction site), or three bands (if the shark was heterozygous for two alleles, one that was cut and one that was not). Thus, each shark was assigned a 1, 2 or 3 for each enzyme used, corresponding to the number of bands generated by that enzyme. Combining the results for several enzymes results in a numerical code for each shark. Each distinct numerical code corresponds to a distinct genotype.

Using this approach, the mother's genotype is represented as 3,1,3,3,3,3. Amongst the 32 pups, we identified fourteen distinct genotypes (designated 'a' through 'm' in Table 1). A mating between this heterozygous female with only one heterozygous male would yield a maximum of four genotypes amongst the offspring. Thus, because there were greater than twelve genotypes represented amongst the pups analyzed, our results suggest there must have been more than three fathers for this litter.

Discussion

While our understanding of the reproductive biology of the nurse shark is incomplete, our extensive observations of mating activity have conclusively shown that females mate with more than one male in a given mating period. These observations led us to investigate whether more than one male could attain reproductive success with an individual female. Our results suggest that a minimum of four males successfully fertilized ova of one female.

A possible alternative interpretation of our results is that the PCR amplified paralogous sequences from the shark genome, leading to multiple restriction patterns amongst the sharks we examined. Consistent with this idea, Kasahara et al. (1993) suggested there may be two pseudoallelic MHC II α loci, which they referred to as Gici DAA and Gici DBA. In our analysis, we found several enzymes that could be used to distinguish DAA from DBA alleles. For example, Bst NI recognizes sequences present only in DAA alleles, while Fok I recognizes only DBA alleles. All of the PCR products analyzed in this study were digested with Bst NI, but not Fok I (Table 1). Thus, all of the amplified sequences in our study were found to contain only DAA alleles. We conclude that multiple paternity is the most likely explanation for the multiple restriction patterns observed.

Consistent with our results, Ohta et al. (2000) report multiple paternity in the nurse shark as a result of their investigation of linkage relationships between MHC class I and class II genes. Our results complement and extend this analysis. In addition to demonstrating a further example of multiple paternity, the PCR-RFLP approach complements the traditional RFLP analysis and DNA sequencing used by Ohta et al. (2000). This PCR-RFLP approach is more informative than traditional RFLP in that more alleles can be identified. At the same time it is less expensive and labor intensive than sequencing individual alleles from each shark as it doesn't require cloning of individual alleles prior to analysis.

The development of molecular techniques with which to answer questions of paternity allows us to expand on our field investigations of nurse shark mating behavior. Specifically, we have confirmed that females do, indeed, mate with multiple males, and, further, that several males can successfully fertilize the eggs of a single female. Multiple paternity in the nurse shark suggests a mechanism by which this species may maximize

Table 1. Observed restriction patterns for family 2 (mother and 32 offspring). The left-most column indicates the shark analyzed: M = mother, numbers 1–32 = individual pups. The restriction enzymes used are listed in the top row. The numbers in subsequent rows indicate the number of bands generated for each pup and enzyme combination. In the right column, each letter designates a different restriction pattern.

	Bpm I	Bsi E1	Fok I	Hinc II	Hph I	Msl I	Tfi I	Bst NI	Pattern
M	3	1	1	3	3	3	3	2	a
1	3	3	1	3	3	3	3	2	c
2	2	1	1	3	1	1	1	2	d
3	3	1	1	3	3	3	3	2	a
4	3	1	1	3	3	3	3	2	a
5	2	3	1	2	1	1	1	2	e
6	3	1	1	3	3	3	3	2	a
7	3	1	1	3	3	3	3	2	a
8	3	1	1	3	3	3	2	2	b
9	1	1	1	1	2	2	3	2	f
10	2	3	1	2	1	1	1	2	e
11	3	3	1	3	1	1	1	2	g
12	3	3	1	3	3	3	2	2	h
13	3	3	1	1	3	3	3	2	I
14	3	3	1	3	1	1	1	2	g
15	3	1	1	3	1	1	1	2	j
16	3	1	1	1	3	3	1	2	k
17	3	1	1	2	1	1	1	2	L
18	3	1	1	3	3	3	3	2	a
19	3	3	1	3	1	1	3	2	c
20	3	3	1	3	3	3	3	2	c
21	3	3	1	3	3	3	1	2	g
22	3	1	1	3	3	3	3	2	a
23	2	3	1	2	1	1	1	2	e
24	3	3	1	3	1	3	3	2	m
25	3	3	1	3	1	3	3	2	m
26	3	3	1	3	1	1	1	2	g
27	3	3	1	3	1	1	1	2	g
28	3	3	1	3	1	1	1	2	g
29	1	1	1	3	3	3	1	2	n
30	3	3	1	3	3	3	3	2	c
31	3	3	1	3	1	1	3	2	m
32	3	3	1	3	1	1	1	2	g

genetic variability. This seems especially valuable for a species whose migratory movement, and thus potential for genetic diversity, is limited. Multiple paternity also suggests a role for sperm competition in the nurse shark; however, direct evidence for sperm competition and its molecular mechanism in this species remain unresolved.

Finally, the methods described here may be extended to address more general questions regarding the genetic structure of nurse shark populations. There have been a limited number of population genetic studies of sharks in general, and none of the nurse shark in particular (reviewed by Heist 1999). Population genetic studies that have been done in sharks have relied on allozyme analysis, RFLP analysis of mitochondrial DNA, or microsatellite analysis (Heist 1999). However, none of these tools has been developed for the genetic analysis of the nurse shark. Molecular techniques, including the analysis of sequence variation at MHC loci have increasingly been used to assess population variation for purposes of conservation in a number of species (reviewed by Haig 1998). It is our intent to continue to analyze the MHC II α locus used here, in conjunction with additional MHC loci to provide powerful tools for future genetic analyses and conservation efforts of nurse shark populations.

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