

**DEVELOPING A PROCEDURE FOR ESTABLISHING
PATERNITY IN *Misumenoides formosipes*
USING RAPD PCR**

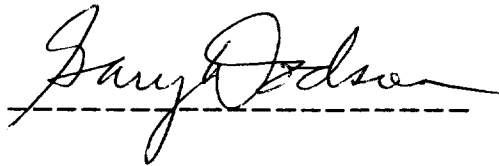
An Honors Thesis (HONRS 499)

by

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ABSTRACT

The topics of sperm competition and sexual selection are currently at the forefront of discussion among evolutionary and behavioral biologists. However, there is a great need for empirical testing of the emerging hypothesis. New methods are needed to quickly and accurately assess sperm competition as it relates to male reproductive success. Specimens of the crab spider species *Misumenoides formosipes* were collected from a single population. Virgin adult females were mated to two adult males on separate days. Several protocols of the Random Amplified Polymorphic DNA application of the Polymerase Chain Reaction (RAPD PCR) were then developed and optimized to assess the paternity patterns of the species. These data can then be used as an aid in interpretation of the male reproductive behavior of this species.

INTRODUCTION

A review of the current literature reveals that the concept of sperm competition in human and nonhuman animals has been a primary focal point of evolutionary biology in recent years (reviews in: Smith 1984; Birkhead and Moller 1992; also see most any recent issue of *Animal Behaviour*). Because it impacts sexual reproduction in such a fundamental manner, many levels of morphology and behavior are affected. In certain taxa empirical testing of theory is limited. Specifically in spiders, on the basis of such limited testing it is assumed that spider species generally exhibit behaviors and reproductive morphologies that support the hypothesis that the first male to mate with the female fertilizes the most eggs. By contrast most other invertebrates studied have been shown to exhibit sperm use patterns in which the last male to mate with the female fertilizes the majority of the eggs. This fact alone establishes the need to investigate further the credibility of the apparently different invertebrate mating systems.

In this preliminary investigation a technique has been partially developed that should ultimately generate paternity data for the species *Misumenoides formosipes*. This technique is also being developed for use as a tool in the analyses of sperm priority in other invertebrate species with the hope that an easier technique will result in sufficient data to reliably reveal overall sperm priority patterns. To this end Random Amplified Polymorphic DNA (RAPD) markers were used in the Polymerase Chain Reaction (PCR) to generate a genetic fingerprint for each individual. Preliminary investigations were then performed to assess the degree of differentiation that can be obtained using this technique with the ultimate goal of devising a protocol through which the degree of differential fertilization of an egg clutch with regard to two known potential sires can be determined quickly and easily.

REVIEW OF LITERATURE

Sperm Selection and Priority Patterns

Evolution arises due to many different factors, many known, some still yet to be discovered. One of the most fascinating of these factors is sexual selection. The "goal" of this oversimplified game of life for both males and females of any species is to pass on their genetic information in the form of gametic DNA to as many quality offspring as possible. For males this often means maximizing the number of eggs fertilized. For females this goal is realized by acquiring the most fit male to fertilize the eggs that she has available. These males are determined through such sexually selected traits as fighting behaviors and size.

In polygamous species where the gametes of more than one male may be present in the reproductive tract of a female simultaneously, competition between the males and even the sperm of the males is practically inevitable. Many strategies have evolved in response to that competition ranging from polymorphic sperm, which attack and destroy the sperm of other males (Sivinski

1980, Harcourt 1991), to the development of genitalia which scrape or flush the sperm of previous matings from the reproductive tract of the female (Waage 1979, Eberhard 1985).

It is assumed that when a female used the stored sperm from consecutive matings in a predictable manner, male behavioral patterns will arise that exploit those patterns. For example, when the sperm from the last male to mate with a female fertilizes the most eggs (last-male sperm priority) males would be expected to exhibit post-copulatory mate guarding to prevent other males from mating with her (Parker 1970). Post-copulatory mate guarding occurs in many insects that are known to have a last-male sperm priority pattern (Thornhill and Alcock 1983).

Conversely, when the sperm from the first male to mate with the female fertilizes the most eggs (first-male sperm priority), pre-copulatory mate guarding (guarding a female before mating) would be expected. First-male sperm priority has been demonstrated for several arachnid species (Jackson 1980, Vollrath 1980, Austad 1982, Christensen and Cohn 1988, Watson 1991, Eberhard et al. manuscript).

Females of the crab spider species *Misumenoides formosipes* possess a sperm storage organ, or spermatheca, with separate insemination and fertilization ducts, a characteristic of all members of the

large spider group Entelegynae. Austad (1984) theorized that this morphology predisposes these species to a first-male sperm priority pattern. Consistent with this prediction, male *M. formosipes* exhibits a first-male sperm priority pattern whereby the sperm from the first male to mate with the female fertilizes the most eggs. A primary goal of the current research was to develop a procedure to test this assumption.

The Polymerase Chain Reaction and Random Amplified Polymorphic DNA

The Polymerase Chain Reaction (PCR) has been established as a very powerful tool with a broad range of applications. By cycling a "cocktail" of deoxyribonucleotides (dNTP's; in excess), buffer solution *Taq* DNA polymerase (a thermostable enzyme), oligonucleotide primers and template DNA through certain temperature parameters, theoretically even genetic material from a single strand of DNA can be infinitely amplified. The thermal cycling parameters include three steps: denaturation, annealing and extension. In the denaturation step high temperatures (above 90° C) break the weak hydrogen bonds in the center of the double stranded DNA molecule, creating two single strands of DNA which can be used as templates for synthesis. At the annealing temperatures (between 35° C and 50° C) the primers bind to the

template DNA. Finally, at the extension temperatures of approximately 70 - 75° C, the new strands of DNA are synthesized by the *Taq* DNA polymerase. These parameters should then be cycles 30-40 times. Because the new strands that are synthesized can be used as new DNA templates, growth occurs at an exponential rate (Mullis and Faloona 1987).

Whereas standard PCR uses primers that have a sequence complimentary to a known sequence on the template DNA to amplify a prespecified region, Random Amplified Polymorphic DNA (RAPD) primers are shorter than standard primers (usually about 10 base pairs), of arbitrary sequence and the data that they provide may be used in a separate arena than that of conventional PCR products, DNA fingerprinting. Because the sequences of RAPD primers are arbitrary, they bind to the template DNA at several loci and with different affinities producing amplified fragments of different lengths and at different concentrations. The RAPD PCR products can then be run on an electrophoresed agarose gel which, when stained with ethidium bromide and viewed on an ultraviolet transilluminator, generates a genetic fingerprint for that individual (Welsh and McClelland 1991).

In theory, by analyzing the genetic fingerprints produced by a female, two potential male sires and their

offspring, the differential paternity pattern in
Misumenoides formosipes can be determined.

MATERIALS AND METHODS

Specimen Collection and Preservation

Adult males and immature females were collected primarily from the flower-tops of black-eyed susan (*Rudbeckia hirta*) and Queen Anne's lace (*Daucus carota*) on the grounds of Ball State University's Cooper Area in Muncie, Indiana. All specimens were collected in separate vials. Spiders were kept in an environmental chamber held at a humidity of 75%, with a light/temperature cycle of 14h/26° C day followed by a 10h/18° C night. Females were fed live house flies (*Musca domestica*) every two days. Males were not fed. Prey consumption and molts were recorded.

Mating Protocols

Matings were performed on cuttings of black-eyed susan in the laboratory. Adult, virgin females were introduced onto a flower and allowed to settle. A male was then introduced and allowed to mate with the female. Following copulation both spider were returned to their dishes. The total time the male spent on the

flower and the total time spent in the copulatory position were recorded. A mating was considered unsuccessful if the copulatory position was not assumed or only maintained for less than one minute. The same females were mated with different male no less than two days following the initial mating using the same protocol.

Specimen Storage

Following mating and egg laying the males, females and egg clutches were stored in a solution of 50% ethanol and 50% phosphate buffered saline solution in large Eppendorf tubes. It was later found that the storage solution may inhibit RAPD PCR so its use was discontinued. The subsequent specimens were stored whole in large Eppendorf tubes with no storage solution. Some egg clutches were allowed to hatch and the spiderlings were also preserved whole without storage solution. All specimens were stored at -80°C .

DNA Isolation and Storage

Several different procedures were used to isolate the genomic DNA from *M. formosipes* to be used in RAPD PCR.

Protocol #1

The storage solution containing the specimens was brought to a final volume of $400\ \mu\text{l}$ with 1X TE buffer (10

mM Tris, 1 mM EDTA) and gently boiled for 5 minutes. The specimen was then ground in the Eppendorf tube to produce a homogenous mixture. The tube was centrifuged and the resulting supernatant was poured off. Less than 1 μ l of RNase A (5490 units / ml) was added and the solution was incubated at 37° C for 30 minutes. Precipitation of the DNA was achieved by adding 7.5 M ammonium acetate (200 μ l) and 1200 μ l of cold ethanol and incubating overnight at 20° C. The tubes were then centrifuged for 10 minutes and the DNA pellet was resuspended in 100 μ l of water.

This protocol was also used with a sodium acetate:ethanol precipitation instead of ammonium acetate:ethanol. The resulting pellet was washed with 70% ethanol and resuspended in 50 μ l of 1X TE for some trials.

Protocol #2 (Detergent Prep)

Specimens were removed from their storage solutions and incubated for 1 hour in a 400 μ l solution of 70 mM NaCl, 10 mM Tris-HCl (pH 8.0), 25 mM EDTA, 0.9 % SDS, and 6 μ g/ml Proteinase K. Cold isopropanol (600 μ l) was added and the DNA was precipitated overnight at 20° C the tubes were then centrifuged and resuspended in 50 μ l of 1X TE.

Protocol #3 (Modified Doyle and Doyle)

Specimens were removed from the storage solution (if they had been preserved in one) and dried thoroughly. They were then added to a preheated (65° C) solution of 2% CTAB buffer [2% (w/v) cetyltrimmonium bromide (CTAB), 0.2% (v/v) β-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0)], macerated with a sterile pipet tip, and incubated at 65° C for 1.5 hours in a large Eppendorf tube vented with a 23 gauge sterile needle. After incubation the tube was filled with 400 μl of chloroform:isoamyl alcohol (24:1) and inverted several times. The resulting mixture was centrifuged for several minutes. The aqueous phase (≈400μl) was removed and added to 330μl cold isopropanol. This mixture was precipitated at -20° C overnight. The tubes were spun in the centrifuge for another 10 minutes. The resulting pellet was washed with cold 70% ethanol, air dried and resuspended in 25 μl of 1X TE.

Grinding the dried specimens in liquid nitrogen and sterile sand prior to incubation in CTAB solution was also tried with this procedure but was found to be of no extra benefit.

All prepared DNA samples were stored at -80° C in large Eppendorf tubes.

Random Amplified Polymorphic DNA

The optimized RAPD PCR conditions were obtained by altering MgCl₂, dNTP, primer and template DNA fingerprints with numerous discrete bands are produced.

Gel Electrophoresis

DNA isolates were visualized on a 0.7% Agarose gel in a 1X TBE buffer [89 mM Tris base, 89 mM boric acid, 2 mM EDTA (pH 7.6)]. These gels were electrophoresed at 50 V for 1 hour. The gels were then stained in a 25 $\mu\text{g}/\mu\text{l}$ ethidium bromide solution for 5 minutes and photographed on an ultraviolet transilluminator.

PCR products were visualized on a 1.5% Agarose gel in a 1X TBE buffer. The gels were stained in a 25 $\mu\text{g}/\text{ml}$ ethidium bromide solution for 5 minutes prior to electrophoresis. These gels were then run at 25 V for 3-4 hours and photographed on an ultraviolet transilluminator.

RESULTS

DNA Isolation and Storage

While all DNA isolation protocols generated approximately the same amount of DNA ($\approx 40 \mu\text{g}/\mu\text{l}$), the modified Doyle and Doyle protocol (Protocol #3) was the only preparation which generated a DNA fingerprint using RAPD PCR on *Misumenoides formosipes* specimens. Genomic DNA was isolated successfully from adult males, females and spiderlings (spiders just hatched from the egg) using this procedure.

RAPD PCR Conditions

The optimized RAPD PCR conditions for *Misumenoides formosipes* DNA entailed reducing MgCl_2 concentrations, elevating dNTP concentration and lowering the annealing temperature and time from the standard RAPD PCR conditions used in our lab as set forth by (Ehrlich 1989). A final reaction volume of $50 \mu\text{l}$ was used consisting of the following:

10X Reaction buffer	500 mM KCl, 100 mM Tris-HCl, 1% Triton X 100 (pH 9.0)	5 μ l
MgCl ₂	200 mM	4 μ l
dNTP's	250 μ M (each)	1.25 μ l (each)
Primer	500 nM	2.5 μ l
<i>Taq</i> DNA Polymerase	2 Units	0.5 μ l
Template DNA	--	1 μ l

Optimized thermal cycling parameters are shown in Figure 2.

DNA Fingerprints

The presence or absence of bands on agarose gels in each photograph generated were scored and compared to establish individuality. Figure 1 shows an agarose gel displaying differences between two individual spiders (one adult female and one spiderling). Scoring of the amplified sequence bands (plus indicates presence of a band and minus indicates absence of a band) for each individual are provided in Table 1.

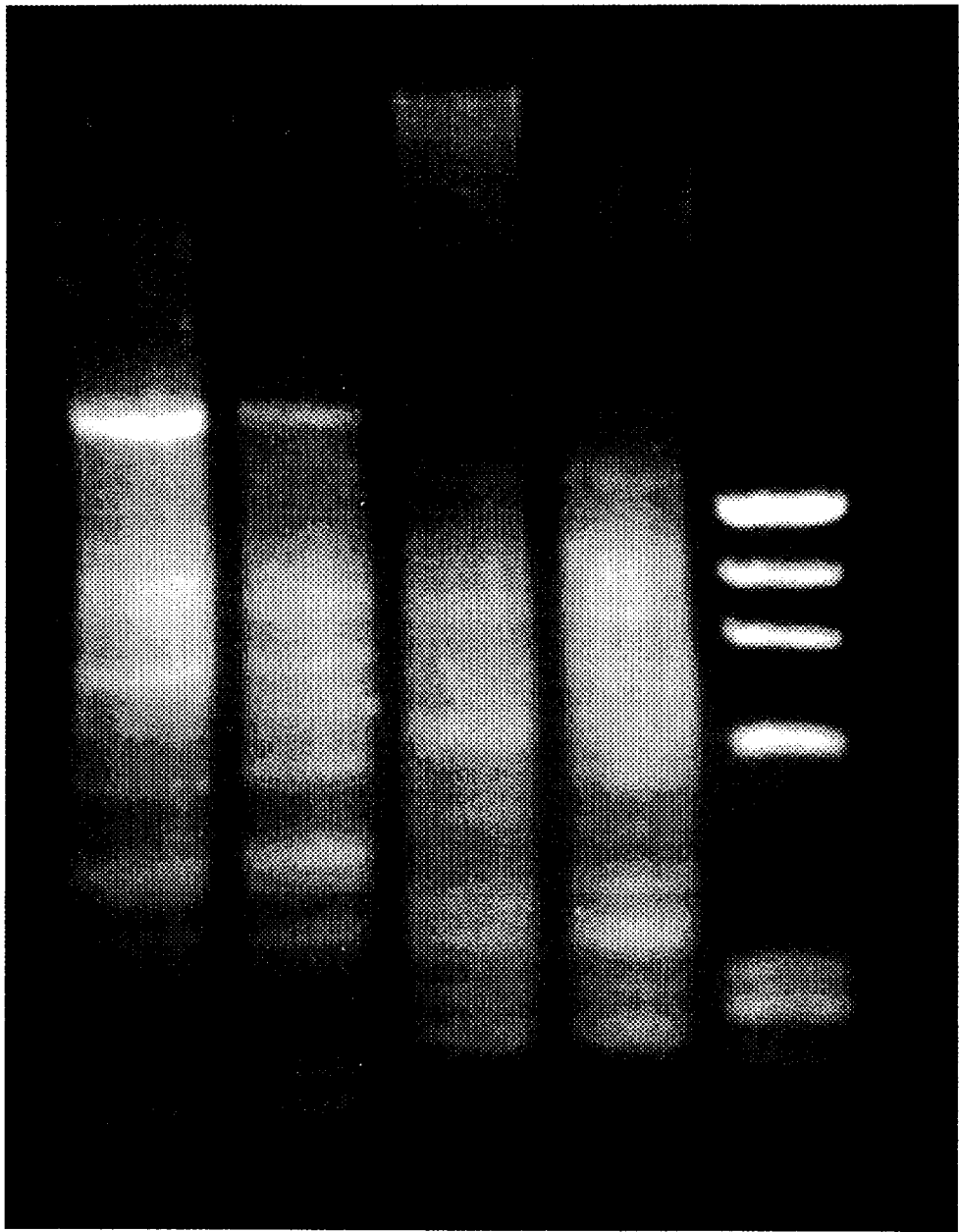


Figure 1

Lane	1234
1	++00
2	+000
3	0+++
4	+0++
5	+000
6	0+++
7	+000
8	++++
9	000+
10	++0+
11	00++
12	++0+
13	00++
14	++00
15	++0+
16	00+0
17	++++
18	00++
19	00++
20	++00
21	00++

Lane 1 Spiderling Xa Primer OPA 13
 Lane 2 Adult Female Xd Primer OPA 13
 Lane 3 Spiderling Xa Primer OPA 15
 Lane 4 Spiderling Xd Primer OPA 15

Table 1

Step (#of Cycles)	Type of Step	Temperature (in °C)	Duration of Step (in Minutes)
1. (X1)	Hot Start (Initial Denaturation)	94	5
2. (X35-40)	De-naturation	94	1
	Elongation	37	1
	Annealing	72	2
3. (X1)	Final Annealing	72	10
4. (X2)	Final Reaction Storage	4	240

Figure 2

DISCUSSION

Now that it has been established that individuality in *Misumenoides formosipes* can be discerned by using RAPD PCR, it remains to apply these techniques to the analysis of paternity. First, spiderling fingerprints must be compared to those of the related adults to test if banding patterns from adults are conserved in their offspring. Fingerprint analyses can then be performed on an individual level. The next step would be to isolate the DNA of an entire egg clutch (prior to hatching) to test if differential paternity can be established through the analysis of the relative intensities of sire-specific bands as read on a densitometer. This would be a potentially quick and easy method for analyzing arachnid paternity.

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