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Molecular evidence for variation in polyandry among praying mantids (Mantodea: *Ciulfina*)

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Abstract

Estimating paternity patterns provides insights into the importance of competing evolutionary forces on mating systems. The number of sires contributing to a female's offspring is mostly influenced by her relative promiscuity. However, in a postcopulatory context, it will also be affected by sperm competition and cryptic female choice. Here, we describe the paternity patterns of two species of praying mantis from the genus *Ciulfina*, the agile praying mantid. This study is the first to describe patterns of paternity in the Mantodea. We found a variation in paternity in these two closely related species. *Ciulfina rentzi* exhibited single paternity, with a single male siring all offspring within a clutch. By contrast, *Ciulfina klassi* displayed multiple paternity, with the minimum number of fathers contributing to a clutch ranging from one to four. Differences in copulation duration and reproductive output between these two species may help to explain these paternity patterns.

Introduction

Female promiscuity (polyandry) is a powerful evolutionary driver. It has significant implications for processes from gene flow to complex behaviours, and is common in a wide range of animals (see Petrie, Doums & Moller, 1998*b*; Simmons, 2001*a*; Griffith, Owens & Thuman, 2002 for reviews). Several authors have highlighted both the costs (Rowe, 1994; Chapman *et al*., 1998; Crudgington & Siva-Jothy, 2000) and the benefits (Zeh & Zeh, 1996; Arnqvist & Nilsson, 2000; Jennions & Petrie, 2000; Simmons, 2001*b*, 2003, 2005; Tregenza & Wedell, 2002) to females that mate multiply. Recently, with the use of molecular tools in genotyping parents and offspring, it has become apparent that polyandry is very common in many taxa (Bretman & Tregenza, 2005; Good, Ross & Markow, 2006; Simmons, Beveridge & Kennington, 2007). For example, before the advent of molecular genotyping technologies, it was widely accepted that most passerine birds were monogamous, with little evidence of polyandry. Molecular tools have subsequently revealed that 86% of passerines are polyandrous (Griffith *et al*., 2002). The extent to which polyandry affects the paternity of clutches depends on the relative importance of two postcopulatory phenomena: (1) sperm competition, which may result in single or multiple paternity (Simmons, 2003); (2) female choice (cryptic or otherwise), whereby a female may actively or passively choose the best sperm for fertilization and thus lay clutches biased towards the preferred male (Eberhard, 1996).

Accurate estimation of the degree of multiple mating and how it relates to multiple paternity is rarely carried out but is vital in fully understanding mating systems as they occur in nature (Simmons, 2001*a*; Bretman & Tregenza, 2005). Most of the evidence for the costs and benefits of polyandry comes from laboratory experiments, where females are either paired with a number of males sequentially or are confronted with a number of males simultaneously (Boomsma, Fjerdingstad & Frydenberg, 1999; Simmons, 2001*a*). For example, Fisher *et al*. (2006) presented female antechinus with (1) three different males; (2) the same male three times and found that the offspring of a polyandrous female had greater survival. However, whether these treatments reflect the natural availability of mates is unclear. As the laboratory environment may not adequately simulate conditions in the field, estimating true levels of paternity from these kinds of experiments is problematic in several ways. Firstly, the number of mates a female would naturally have is often unknown. Arbitrarily choosing a polyandrous treatment of a particular number, in many cases two mates, will be inappropriate for species that encounter more or less than two mates per reproductive event. Secondly, how frequently females encounter males in nature is also often unknown. If females naturally encounter mates only every few days, then paternity outcomes of mating trials a few hours apart are

biologically irrelevant because sperm may not normally occur contemporaneously in a female's genital tract. These limitations are a fundamental barrier to fully understanding the evolution of polyandry and its influences on paternity.

Studies of paternity in natural settings are vital to understanding mating systems as they occur in the wild. Using molecular tools to determine patterns of paternity within clutches from wild-caught females is powerful because the data represent the outcomes of naturally occurring reproductive behaviour. They result from mating events that occurred under natural circumstances and thus arise under appropriate environmental constraints. Patterns emerging from field studies of paternity can therefore provide an insight into the selective forces acting on a population (Petrie, Doums & Moller, 1998*a*). However, few studies have investigated the patterns of paternity in wild-sired insect clutches (a notable exception is the social insects (Boomsma *et al*., 1999; Hammond, Bourke & Bruford, 2001; Fernandez-Escudero, Pamilo &Seppa, 2002; Sumner *et al*., 2004). The insects most intensively examined using this approach are flies of the genus *Drosophila* (Ochando, Reyes & Ayala, 1996; Harshman & Clark, 1998; Imhof *et al*., 1998; Good *et al*., 2006). This bias may be largely due to logistical constraints including difficulty in observing copulations in the wild, large clutches and restricted access to parental genotypes (Lopez-Leon *et al*., 1995; Corley, Blankenship & Moore, 2001; Emery *et al*., 2001; Bretman & Tregenza, 2005). One approach to overcome these challenges is to collect gravid females from the wild, allow them to lay their eggs in captivity and genotype their offspring using molecular tools such as microsatellite markers (Simmons, 2001*a*).

Our aim was to discover the paternity patterns in two species of *Ciulfina* praying mantids. Previous studies have shown that these species differ in habitat ecology, where *Ciulfina rentzi* lives in a relatively benign rainforest habitat and *Ciulfina klassi* lives in more exposed sclerophyll woodland. Also, they differ in copulation duration; *C. rentzi* mate for up to three times longer than *C. klassi*. Accurate estimates of paternity in the field for these mantids will shed light on the potential selective pressures on mating strategies, that is sperm competition and female choice. *Ciulfina* mantids are non-sexually cannibalistic, with an extensive distribution across Australian tropical and subtropical regions (Holwell, 2007). Studying the reproductive behaviour of *Ciulfina* mantids provides an interesting comparison with species of praying mantids from temperate habitat that are frequently studied due to the sexual cannibalistic behaviour in females (Maxwell, 2000; Barry, Holwell & Herberstein, 2008).

Materials and methods

Sample collection and housing

Ciulfina klassi and *C. rentzi* occur in far north Queensland and are distributed between Townsville and Cairns, but in different habitats. *Ciulfina klassi* inhabit eucalypt woodland while *C. rentzi* are found in tropical rainforest. Adult female mantids were collected in March and April 2006, from Big Crystal Creek, Paluma (18°59″0.732′ 146°14″11.642′), and Flecker Botanical Gardens, Cairns (16°53″58.448′ 145°44″50.792′). We chose to collect females in March and April because this is late in the breeding season for both species (G. I. Holwell & J. C. O'Hanlon, unpubl. data). Doing so ensured as far as possible that females from both species were of the same age. All mantids were housed individually in upturned plastic cups (15 cm) with the plastic base replaced by gauze for airflow. All mantids were exposed the same conditions in the laboratory: *ad libitum* food (*Drosophila* sp.) and water; temperature of 25 °C; a diurnal period of 8–10 light hours per day.

Adult female mantids began laying oothecae from the time of capture. Within 2 days of being laid, oothecae were removed from the females' enclosures and placed in individual jars. Each ootheca remained separate and undisturbed until hatching. Nymphs were collected up to 48 h after hatching, asphyxiated with CO_2 and stored individually in 70% EtOH. Mothers were maintained in the laboratory to lay further oothecae. Only some females of *C. rentzi* also laid second oothecae before they died and paternity analyses showed no difference in the paternity patterns between the first and the second ootheca. Therefore, we only present paternity data from first oothecae (thus one ootheca per female) here. As the mothers eventually died, they were stored in 70% ethanol.

Microsatellite genotyping

DNA extractions were carried out using half of a mothers' thorax including a raptorial limb and from whole nymphs. DNA was extracted using a salting-out protocol (Sunnucks & Hales, 1996). Pellets were eluted in 30 μL of TE and a subsample of extractions was run on a 2% agarose gel at 100 W for 1 h [5 μL DNA with 5 μL loading dye and 100 base pairs (bp) marker]. From this, the DNA concentration was estimated and diluted accordingly (*c* 50 ng μL^{-1}; dilutions 1:20).

Cross-amplification of *Ciulfina* microsatellite loci was successful for *C. rentzi* and *C. klassi* (Attard *et al*., 2009). Two loci for each species amplified consistently and were sufficiently polymorphic for paternity analysis in *C. klassi and C. rentzi* (see Kellogg *et al*., 1995; Kelly, Godin & Wright, 1999 for discussion on the exclusionary power of polymorphic microsatellite loci). Forward primers for each locus were -21M13 tagged and complementary -21M13 fluorochromes (PET and FAM) were used in PCR reactions. Tagging, rather than direct attachment of fluorochrome to the primer, was chosen in an attempt to increase the specificity of amplification (Schuelke, 2000). Reactions of 10 μL contained *c*. 50 ng genomic DNA, 10 × Taq buffer, 2 mM $MgCl_2$, 200 μM of each dNTP, 1 μM forward and reverse primers, 2 μM -21M13 fluorochrome and 1 unit of Taq DNA polymerase.

PCR amplification was achieved using a PCT-100 thermocycler (MJ Research, Minneapolis, MN, USA). The

touchdown profile used was as follows: 90 °C for 3 min; 60 °C for 30 s; 72 °C for 45 s; 94 °C for 30 s; 58 °C for 30 s; 72 °C for 45 s; 94 °C for 30 s; 56 °C for 30 s; 72 °C for 45 s; 94 °C for 30 s; 54 °C for 30 s; 72 °C for 45 s; 94 °C for 30 s; 52 °C for 30 s; 72 °C for 45 s, followed by 35 cycles of 94 °C for 30 s; 50 °C for 30 s; 72 °C for 45 s; a final extension period at 72 °C for 10 min.

PCR products (5 μL) were multiplexed – fragments were of two different size classes ($\geq$200 bp; $\leq$ 170 bp) and two different-coloured fluorochromes were used (PET-red and FAM-blue). For electrophoresis, 1 μL of pooled PCR product was run. If the product was too strong, a 1:2 dilute was used for electrophoresis. Gels were run on an automated sequencer and analysed using GENEMAPPER software (Applied Biosystems, Foster City, CA, USA). To confirm data and for failed reactions, new dilutes were made from stock DNA, a new PCR reaction was run and then the new product was run on an electrophoresis gel.

Paternity analysis

Mother–progeny arrays were genotyped at two loci for *C. rentzi* and *C. klassi* populations (mean number of nymphs per clutch $\pm$ SD: *C. rentzi* = 11.88 $\pm$ 2.23, range: 10–14; *C. klassi* = 12.24 $\pm$ 2.27 range: 7–23). Complete progeny arrays were used due to the relatively small average number of offspring per clutch. This enabled us to estimate paternity across whole clutches, logistically difficult in many species due to large clutch sizes (Simmons, 2001*a*)

We used the program GERUD 2.0 to estimate the minimum number of fathers contributing to progeny arrays. In addition, GERUD 2.0 calculated the most likely paternal genotypes and the probable contribution of each paternal genotype to the progeny array (Jones, 2005). Upon identifying the maternal alleles in a set of progeny genotypes, GERUD 2.0 then subtracts all maternal alleles and, using a stepwise function, determines the most probable paternal genotype-based and independent sample of population-wide allele frequencies (Jones, 2005). These data were also inspected by identifying maternal alleles and counting all additional (paternal) alleles.

Summary genetic data

To estimate the genetic parameters of each population, individuals from these locations, but independent of the mother–progeny arrays, were genotyped. Thirty-two *C. rentzi* and 16 *C. klassi* individuals were typed at the same loci as the mother–progeny arrays. Data were analysed using the genetics software program GenAlEx (Peakall & Smouse, 2006).

Reproductive output

The number of oothecae produced by each female was recorded, as was the number of nymphs that hatched from each ootheca. From these data, the average number of oothecae per female and the average number of nymphs per first ootheca were compared using a student's *t*-test.

Results

Summary genetic data

Both loci were variable in both species. The number of alleles at each locus varied from three to nine (Table 1). A significant departure from Hardy–Weinberg equilibrium owing to a homozygote excess was observed for locus 6 in *C. rentzi* (Table 1). Inspection of mother–offspring genotypes suggests that this result is most likely explained by the presence of null alleles. Despite lower exclusionary probability in *C. rentzi*, this is unlikely to account for our observations, given that the probability of not detecting multiple paternity is 0.01.

Paternity analysis

Ciulfina rentzi exhibit single paternity, with a single male siring all offspring within a clutch (mean number of fathers $\pm$ SD = 1 $\pm$ 0; mean number of nymphs per clutch $\pm$ SD = 9.00 $\pm$ 3.46; total number of clutches = 8). By contrast, *C. klassi* displayed multiple paternity, with the minimum number of fathers contributing to a progeny array ranging from one to four (mean fathers = 2.14 $\pm$ 1.21; mean number of nymphs per clutch = 11.88 $\pm$ 2.23; total number of clutches = 7) (Fig. 1).

In *C. klassi* clutches with multiple sires, each sire contributed unequally. The percentage of progeny sired by each male skewed towards a single male (Fig. 2). In the clutch with two fathers, one male sired around 80% of the progeny, and the other only around 20%. The clutches with three sires showed a similar pattern; one male sired *c.* 60% while both the others sired around 20% of the progeny. Finally, in the clutch with four sires one male sired 40% and the other three males each sired 20% of the progeny (Fig. 2) The difference in paternity between the clutches cannot be explained by variation in number of offspring (one-tailed

Table 1 Summary of genetic data

	Ciulfina rentzi		*Ciulfina klassi*	
Locus	58	6	58	47
Total exclusionary power	0.51		0.75	
Number of alleles	3	7	9	4
Allelic richness	3.00	7.00	8.62	4.00
Expected heterozygosity (H_E)	0.54	0.57	0.70	0.67
Observed heterozygosity (H_O)	0.42	0.27	0.75	0.93
Hardy–Weinberg equilibrium				
Probability (*P*)	0.08	0.01	1.00	0.11
Significance	NS	*	NS	NS

The significant homozygote excess for *C. rentzi* at locus 6 was most likely due to the presence of null alleles. Despite this, sufficient alleles were available to ensure that multiple fathers could have been detected. At locus 58, *C. rentzi* and *C. klassi* populations showed significant genetic deviation (F_{ST} = 0.31; $P \leq 0.02$) (*$P < 0.05$; NS, $P > 0.05$).

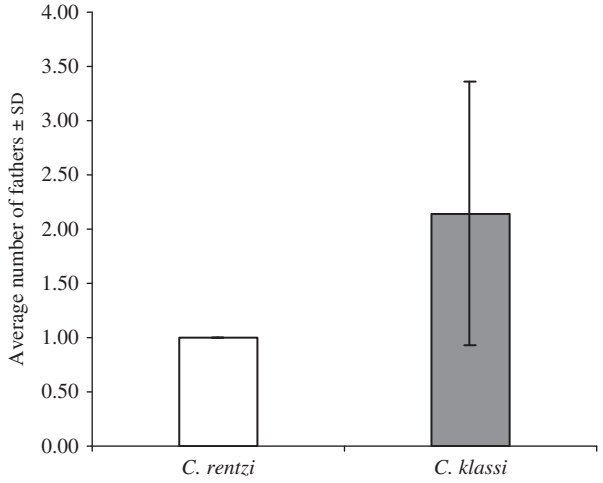

Figure 1 Average minimum number of sires in *Ciulfina rentzi* and *Ciulfina klassi* based on clutches produced by females collected in the field.

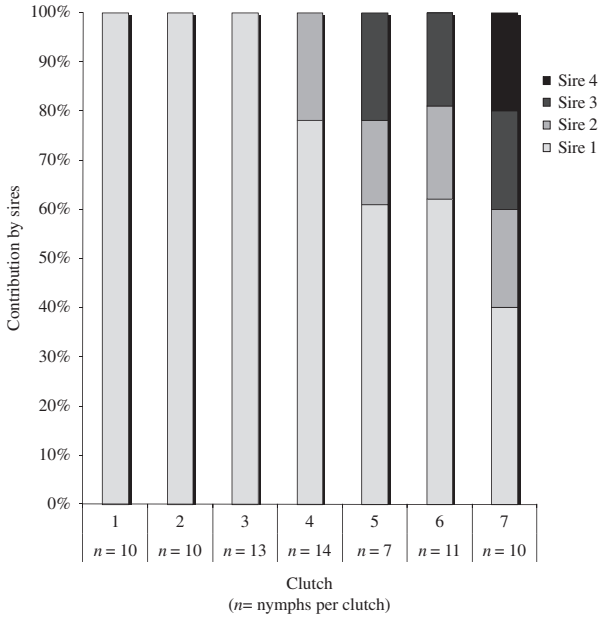

Figure 2 Sires contributed unequally to *Ciulfina klassi* clutches. Three clutches had a single sire, one clutch had two sires, two clutches had three sires and one clutch had four sires.

z-test: mean number of nymphs per clutch ± SD = 12.24 ± 2.27 $z = 0.5$, d.f. = 6; $P \gg 0.05$).

Reproductive output

The time from laying until hatching ranged from 5 to 8 weeks and did not seem to vary with the species. The number of progeny from the first ootheca only was compared as few females laid more than one. On average, *C. rentzi* mantids had fewer nymphs per first ootheca than *C. klassi* (two-tailed *t*-test: $t_{1,\ 14} = 1.97$, $P = 0.07$). Conversely, the average number of oothecae per mother for *C. rentzi* tended to be greater than that for *C. klassi* (two-tailed *t*-test: $t_{1,\ 14} = 1.47$, $P = 0.17$).

Discussion

Patterns of paternity in *Ciulfina*

This study is the first to describe patterns of paternity in mantids. We found a variation in paternity in two closely related species. *Ciulfina klassi* exhibited multiple paternity, with up to four sires per clutch, whereas only a single paternity was detected in *C. rentzi*. Reproductive output also varied somewhat between these species. *Ciulfina klassi* laid fewer larger clutches, while *C. rentzi* often laid more smaller clutches (Hopper, 1999).

We have recently started to gain a better understanding of the true extent of female promiscuity (reviewed in Simmons, 2005). The results for *C. klassi* in this study add to the pool of field-based evidence for female promiscuity as one of a few studies of field-based paternity patterns in non-social invertebrates. Bretman & Tregenza (2005) report patterns of paternity for seven mother–progeny arrays of field crickets *Gryllus bimaculatus*. Using microsatellite analysis, they found an average minimum of 2.4 fathers, with a range of one to six, contributing to each clutch. Good *et al.* (2006) similarly found 3.1 sires per brood, with a range of two to six sires over 20 mother–progeny arrays in *Drosophila mojavensis*. Simmons *et al.* (2007) found nine clutches with 2.8 sires on average with a range of two to four in the tettigoniid, *Requena verticalis*. These results are comparable to our results for *C. klassi*, which exhibited an average minimum of 2.14, with a range of one to four sires.

Single paternity in *Ciulfina rentzi*

Unusually, only a single sire was detected in *C. rentzi* for each clutch despite sufficient exclusionary power. This result may represent monandry. With the growing molecular evidence for polyandry among insects in wild populations, this result was unexpected. Although multiple mating may have benefits, there is growing evidence for substantial costs associated with multiple mating, particularly in insects (Arnqvist & Rowe, 2005). Considering these apparent costs, there are many potential benefits to monandry including reduced risks of disease transmission, predation and male-imposed harm (Thornhill & Alcock, 1983; Gwynne, 1989; Arnqvist & Nilsson, 2000; Arnqvist & Rowe, 2005).

Alternatively, as the number of sires contributing to a clutch does not necessarily reflect the number of mating events, strong sperm competition could also explain single paternity in *C. rentzi*. *Ciulfina rentzi* may have pronounced sperm precedence, where the sperm of one male fertilizes all of the female's eggs (Simmons, 2001*a*). Paternity patterns such as those indicating total sperm precedence are often linked to spermathecal morphology, which is likely to be very similar, in this species (Winnick, Holwell & Herberstein, 2009). Some species with spherical spermathecae are known to have mixed paternity, while spermathecae with a

different shape tend to generate last male sperm precedence (Simmons, 2001*a*). In species with spherical spermathecae, sperm stratification is likely to determine male fertilization success (Simmons, 2001*a*). The degree to which sperm is stratified within a spermatheca can be the result of copulation duration (Simmons, 2001*a*). While the copulation duration of *C. rentzi* (*c.* 120 min) is much greater than that of *C. klassi* (*c.* 40 min), it is unknown whether greater numbers of sperm are transferred by male *C. rentzi* and whether this may explain the paternity patterns we have observed (Holwell & Herberstein, in press).

The most comprehensive study of an insect system that describes monandry is that of the green-veined white butterfly *Pieris napi* (Wedell, Wiklund & Cook, 2002; Valimaki & Kaitala, 2006; Valimaki *et al.*, 2006). Wedell *et al.* (2002) reported a genetic basis for both monandry and polyandry in *P. napi*. They suggest that environmental conditions have perpetuated the persistence of an early-emerging, environmentally vulnerable polyandrous phenotype from southern distributions and a late-emerging, environmentally robust monandrous phenotype from more northern distributions (Wedell *et al.*, 2002; Valimaki & Kaitala, 2006). *Ciulfina rentzi* and *C. klassi* live in different forest types; hence, it is possible that *C. rentzi* mantis lay many smaller clutches fathered by sequential males whereas *C. klassi* lay larger clutches but use sperm from several males to offset potential environmental stochasticity. Exactly how environmental factors might influence the paternity patterns in *C. klassi* and *C. rentzi*, remains uncertain.

Differences paternity and reproductive output between *C. klassi* and *C. rentzi*

The similarities in precopulatory behaviour, genital morphology, spermathecal morphology and population density between *C. klassi* and *C. rentzi* suggest that none of these factors are likely to explain differences in the paternity patterns found here (Holwell, 2007; Holwell, Ginn & Herberstein, 2007; Holwell & Herberstein, in press). However, there are a few major differences in the natural history of these species that may explain their species-specific paternity patterns. These include reproductive output, habitat and copulation duration (Holwell, 2007). *Ciulfina rentzi* copulates for approximately three times longer than *C. klassi*. The longer copulations of *C. rentzi* may allow males to displace the ejaculates of prior mates, leading to a strong skew in paternity. Conversely, the longer copulations of *C. rentzi* may be more costly for females, leading to greater reluctance to mate again, similarly resulting in single male paternity. Lastly, to account for environmental stochasticity, females may be using different risk spreading strategies, with single clutches sired by many males in *C. rentzi* compared with many clutches sired by single males in *C. klassi* (Hopper, 1999). These potential explanations for the variation between *C. klassi* and *C. rentzi* are also not mutually exclusive, and require further investigation to tease apart the mechanisms behind our observed patterns.

Our study discovered different patterns of paternity in two closely related mantids. It reinforces the importance of collecting data from populations under natural conditions. Further, it highlights the requirement for laboratory experiments on the evolution of polyandry to be firmly rooted in field-based data.

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