Conspecific sperm precedence in flour beetles

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Two related species may mate readily yet rarely form hybrid zygotes. Such cryptic reproductive isolation may occur as a result of conspecific sperm precedence, suggesting that postmating sexual selection is a key process in speciation. However, demonstrating conspecific sperm precedence is nontrivial, and several methodological problems may confound the results of such studies. By mating females to conspecific and heterospecific males of varying degree of relatedness, we established the existence of conspecific sperm precedence in flour beetles, *Tribolium* spp. Postmating incompatibilities seem to accumulate rapidly in this group of insects, and we discuss the implications of our findings for the influence of postmating sexual selection on speciation.

The evolution of reproductive isolation is key in the formation of new species. In the absence of behavioural isolation, partial reproductive isolation may be cryptic. That is, conspecific male gametes are used preferentially for the fertilization of eggs when females mate with both conspecific and heterospecific males (Howard 1999; Eady 2001). Such conspecific sperm precedence (CSP) may be widespread and can in theory be efficient in limiting gene exchange between incipient or closely related species. More generally, the prevalence of CSP between closely related species suggests that cryptic divergence evolves rapidly and that postmating sexual selection, involving behavioural and physiological traits, may be central to animal speciation (Arnqvist et al. 2000; Eady 2001; Price et al. 2001; Howard et al. 2002).

Gilchrist & Partridge (1997) exposed a methodological problem that may bias the view of CSP. They pointed out that, in intraspecific studies, differential juvenile survival will affect estimates of sperm precedence when these are based simply on the proportion of adult offspring produced by, for example, discrete male genotypes. Variance in zygote to adult survival will seriously contaminate such measures of sperm precedence. In studies involving different species or incipient species, this can be an even more serious problem. Genetic incompatibilities between types often reduce the hatching rate of eggs or increase juvenile mortality in hybrid compared with nonhybrid offspring. This fact alone can cause apparent CSP, even if gamete utilization is random with respect to species. Thus, measures of postmating prezygotic isolation risk being

confounded by partial postzygotic isolation (see also Price 1997; Howard et al. 1998). An additional methodological problem is the fact that mating behaviour is sometimes ignored. Rather than directly recording matings, males and females are simply held together for a given period in some studies of CSP. In such cases, differential offspring production by females after matings may be due to elevated mating rates with conspecifics rather than a per-mating fertilization advantage for conspecific males (see also Nilsson et al. 2003).

It is imperative to assess all aspects of zygote and juvenile survival, to observe mating behaviour and to control mating rates in studies of CSP. However, many studies have failed to do so. For example, *Tribolium* flour beetles are often cited as a classic example of a system where CSP has been demonstrated (e.g. Price 1997; Howard 1999; Jennions & Petrie 2000; Eady 2001; Servedio 2001; Simmons 2001), although no study on CSP in this group has simultaneously controlled for differential hybrid/nonhybrid offspring survival and for differences in female mating rates with heterospecific and conspecific males (Robinson et al. 1994; Wade et al. 1994). By controlling for such confounding factors, we assessed whether CSP is truly a potential isolating mechanism in flour beetles. We also assessed the rate at which such postmating prezygotic isolation evolves by using an experimental protocol in which females were mated to males of varying degrees of relatedness.

METHODS

We chose the standard wild type strain Georgia (G) of *T. castaneum* as a reference form, and all matings involved

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females of this strain. Each female then mated twice, once with a marker male and once with a focal male belonging to one of seven types, either to a conspecific population (Georgia (G), CR-1 (C), Abidjan (A), NDG-2 (N), Tiw-1 (T)) or to another species (*T. freemani* or *T. madens*). All beetles were provided by the *Tribolium* stock centre at the U.S. Grain Marketing Research Laboratory in Manhattan, Kansas, U.S.A. (http://bru.usgmrl.ksu.edu/proj/tribolium/index.asp). The relative relatedness of the populations and species used was established by genetic sequence data on retrotransposon insertion sites (Beeman et al. 1996) combined with the molecular phylogeny presented by Ugarkovic et al. (1996). *Tribolium castaneum* and *T. freemani* are closely related sibling species, which readily produce viable but infertile hybrid offspring (e.g. Wade et al. 1994; Ugarkovic et al. 1996).

Virgin G females (10–15 days posteclosion) each mated with two males on 2 consecutive days, once with a focal male (see above) and once with a conspecific male from a phenotypic marker strain (homozygous for a semidominant autosomal mutation causing black body coloration; Sokoloff et al. 1960). We measured male sperm defensive ability (P1) and sperm offensive ability (P2) in two series of mating trials, where females were mated with black males as either second or first mates, allowing us to estimate independently the proportion of offspring fathered by the focal male when mated as first (P1) or second (P2) male. We observed behavioural interactions and recorded all matings. Only copulations lasting longer than 35 s were included in the analysis (Edvardsson & Arnqvist 2000; Nilsson et al. 2003).

Beetles were kept at $30 \pm 0.5\,^\circ$C and $60 \pm 10\%$ RH in darkness. After the second mating, and for each of the 14 treatment groups (7 forms × 2 mating orders), five females were transferred to and allowed to oviposit in a 9-cm petri dish with 12 g of fine sifted standard medium. Four such replicates were set up for each group, and each replicate thus represents the average sperm utilization pattern of five individual females. Females were transferred daily into a fresh petri dish during 14 days, allowing an analysis of temporal trends in sperm precedence (Simmons 2001). All petri dishes were incubated for 6 days, after which the medium was sifted with a fine meshed sieve (300 μm). All larvae were counted and transferred back into a dish with 12 g of fresh standard medium, and unhatched eggs were preserved in 70% ethanol and subsequently counted under a dissecting microscope. Dishes with larvae were incubated for another 4 weeks, after which we counted the adult offspring. This protocol allowed us to assess differences in both egg hatching rate and larval survival across treatments. Paternity was determined by the body coloration of adult offspring.

We analysed effects of male type on egg hatchability, larval–adult mortality, P1 and P2 (i.e. the proportion of offspring fathered by the focal male) using generalized linear models with binomial errors and a logit link function. To compensate for overdispersion, we implemented the method of Williams (1982) before statistical inference.

RESULTS

There was no significant effect of focal male type on female fecundity, defined as the total sum of eggs and larvae divided by the number of females in each replicate in either the P2 mating order replicates ($F_{6,21} = 1.675$, $P = 0.176$) or the P1 mating order replicates ($F_{6,22} = 2.413$, $P = 0.060$). There was also no significant effect of male type on egg hatchability during the first ($\chi^2_6 = 5.13$, $P = 0.527$) or second week ($\chi^2_6 = 6.63$, $P = 0.356$), or over both weeks ($\chi^2_6 = 6.06$, $P = 0.417$). This result did not change when we tested for effects separately for the two mating orders. Egg hatchability was, on average, 84.7%. Similarly, male type did not significantly affect larval–adult survival during either the first week ($\chi^2_6 = 1.73$, $P = 0.943$), the second week ($\chi^2_6 = 3.74$, $P = 0.712$) or over both weeks ($\chi^2_6 = 1.97$, $P = 0.922$). Again, this conclusion remained unaltered when we tested for effects separately for the two mating orders. Larval–adult survival was, on average, 96.8%.

In the absence of any effects of male type on fecundity, egg hatching rate and juvenile survival, patterns of sperm utilization can be deduced from data on adult offspring production (Gilchrist & Partridge 1997). The pattern of female sperm utilization (i.e. the proportion of offspring fathered by the focal male) during the first week after mating was highly positively correlated with that of the second week across all replicates (Spearman rank correlation: $r_S = 0.91$, $P < 0.001$). Data for the 2 weeks were thus pooled for the following analyses. Male type significantly affected the female sperm utilization pattern, both in cases where focal males were mated as first mates (P1: $\chi^2_6 = 15.25$, $P = 0.018$) and when mated as second mates (P2: $\chi^2_6 = 27.68$, $P = 0.0001$). In general, sperm precedence was highest when females mated with males of the same type (Fig. 1). Within species (*T. castaneum*), the observed sperm utilization pattern decreased significantly with increased phylogenetic distance across the five
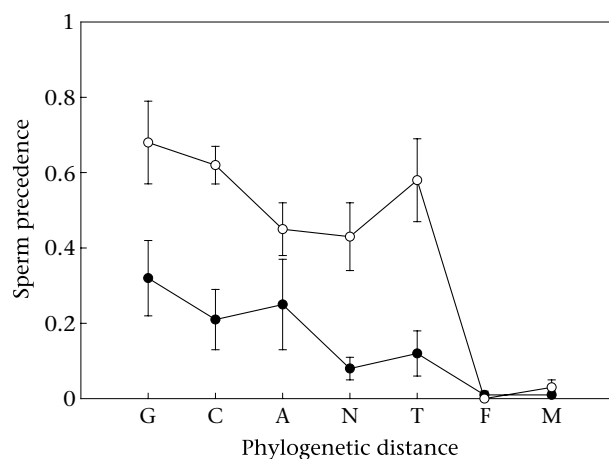

Figure 1. Mean ± SE sperm precedence in focal females (*T. castaneum* [G]) measured as either P1 (●) or P2 (○), in relation to the phylogenetic distance of their mate. G–T: five conspecific populations of *T. castaneum*; F: *T. freemani*; M: *T. madens*. Position along the abscissa indicates increased relative rather than absolute phylogenetic distance between *T. castaneum* (G) and the male type.

genotypes for data on sperm defence (P1, Spearman rank correlation: $r_S = -0.50$, $N = 20$, $P < 0.05$) but not for data on sperm offence (P2: $r_S = -0.14$, $N = 20$, $P > 0.5$; Nilsson et al. 2003).

DISCUSSION

Our results indicate that conspecific sperm precedence is almost complete in *T. castaneum*. This finding is in line with those of earlier studies of CSP in flour beetles (Robinson et al. 1994; Wade et al. 1994), and verifies that this form of cryptic homogamy may be an important isolating mechanism in the absence of behavioural isolation in this group of insects (Wade et al. 1994). More generally, our results support the suggestion that hetero-specific matings may commonly not be very costly to females (Veen et al. 2001), and that postmating prezygotic events may be an important factor in the evolution of reproductive isolation (Howard 1999; Eady 2001).

Variance in sperm precedence within flour beetle species is influenced by both males (e.g. Arnaud et al. 2001) and females (e.g. Edvardsson & Arnqvist 2000). Although theory predicts that primarily female adaptations should cause CSP (Price 1997), because hybridization is more costly for females (Parker & Partridge 1998), the relative roles of the sexes in generating CSP in flour beetles is unknown.

The fact that CSP has been observed even between closely related species (Howard 1999; Eady 2001) suggests that the evolution of CSP is rapid (but see Dixon et al. 2003). This suggestion is strengthened by recent in-traspecific studies of insects showing that female sperm utilization depends on whether males come from the same or a different allopatric population (e.g. Clark & Begun 1998; Clark et al. 1999; Brown & Eady 2001; Hosken et al. 2002; Nilsson et al. 2003). If CSP is generally important in maintaining the genetic integrity of species, then a key question is what processes are responsible for this rapid accumulation of postmating prezygotic in-compatibilities. Although all forms of postmating sexual selection can in theory contribute to the evolution of CSP (Howard 1999; Eady 2001), sexually antagonistic coevo-lution generated by conflicts between the sexes is now recognized as a particularly potent mechanism for the generation of postmating divergence (Clark & Begun 1998; Rice 1998; Arnqvist et al. 2000; Civetta & Clark 2000; Eady 2001; Howard et al. 2002; Chapman et al. 2003; Dixon et al. 2003). Parker & Partridge (1998) suggested that if sexually antagonistic coevolution is responsible, females should sometimes lack resistance to males with which they have not coevolved. They might therefore prefer to mate with males from a closely related but different population than with males from the same population, or may preferentially use the sperm of males from a different rather than the same population to fertilize their eggs (see also Andrés & Arnqvist 2001; Hosken et al. 2002). Although this suggestion is not straightforward (see Brown & Eady 2001; Chapman et al. 2003), several studies have documented such a pattern in *Tribolium* (Nilsson et al. 2002, 2003) suggesting that CSP

in *Tribolium* may be an incidental side effect of divergent sexually antagonistic coevolution within allopatric pop-ulations/species.

In conclusion, by using an experimental protocol that avoids some of the problems of earlier studies, we have verified the occurrence of strong CSP in flour beetles. The results also showed that the accumulation of postmating prezygotic reproductive incompatibilities is rapid (Fig. 1), considering that all conspecific types included share a common habitat and food source and have spread over much of their range during relatively recent times as the storage of grain and flour became established in human populations (Sokoloff 1974). Our results are thus in line with those of other studies of flour beetles documenting rapid evolution of male postmating 'signals' and female reproductive responses to these (Lewis & Austad 1990; Robinson et al. 1994; Wade et al. 1994; Nilsson et al. 2002, 2003).

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