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SHORT COMMUNICATION

Production of all female progeny: evidence for the presence of the male sex determination factor on the Y chromosome

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ABSTRACT

The red flour beetle, *Tribolium castaneum*, follows an XX (female) and XY (male) sex determination system. Maternal supply of the protein Transformer (Tra) is required for XX insects to follow the female pathway. The nature and source of the signal that regulates male sex determination in XY beetles are not known. Parental RNAi-aided knockdown in expression of *tra* masculinizes genetic females (XX) that are fertile. The virgin females mated with these masculinized genetic females produced all female progeny. We present the genetic evidence to show that the factor responsible for male sex determination is present on the Y chromosome. These data also suggest that the Y chromosome in *T. castaneum* is not required for male fertility.

KEY WORDS: Transformer, Doublesex, RNAi, Alternative splicing, *Tribolium*

INTRODUCTION

Insects are the most diverse group of the animal kingdom. Multiple sex determination mechanisms are found among different insect species. Comparison of these mechanisms suggests the existence of a common downstream regulatory pathway, i.e. Transformer (Tra)/Transformer-2 (Tra-2) regulation of *doublesex* (*dsx*) splicing (Verhulst et al., 2010). The coleopteran model insect *Tribolium castaneum* (Herbst 1797) follows an XX (female) and XY (male) sex determination system (Pease and Hahn, 2012). Maternal supply of *tra* is required to initiate the female sex determination cascade in XX embryos because parental RNAi of *tra* results in the production of all male progeny, which includes genetic males (XY) as well as masculinized genetic females (XX males) (Shukla and Palli, 2012). It has been hypothesized that an inhibitory signal from the Y chromosome curtails the establishment of the female sex determination cascade in XY embryos, resulting in the splicing of *Tctra* and *Tcdsx* pre-mRNAs into the male mode (Shukla and Palli, 2012). However, the experimental proof that the Y chromosome harbors the male sex determination signal in *T. castaneum* is lacking.

We hypothesized that a cross between masculinized genetic females (XX progeny of *Tctra* RNAi females) and virgin females (XX) would produce all female progeny if the male sex determination signal lies on the Y chromosome, or both male and female progeny if this signal is not located on the Y chromosome. To test this hypothesis, we crossed virgin females with masculinized genetic females. The data obtained from these crosses suggest that the male sex determination signal is present on the Y chromosome in *T. castaneum*. In addition, our experiments also showed that, unlike in the fruit fly *Drosophila melanogaster* and in humans, the Y chromosome in *T. castaneum* is not required for male fertility.

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RESULTS AND DISCUSSION

We previously reported production of all male progeny when *Tctra*-dsRNA-injected females were mated with normal virgin males (Shukla and Palli, 2012). We took advantage of this observation to determine whether male sex determination genes are present on the Y chromosome. *Tctra*- or *malE*-dsRNA-injected females were mated with untreated virgin males and all progeny (males) resulting from this cross were mated with virgin females (Fig. 1). In the first experiment, 135 larvae (72 females and 63 males) were produced when five control *malE*-dsRNA-injected females were mated with five untreated virgin males. In contrast, mating of five *Tctra*-dsRNA-injected females with five untreated males produced only 80 larvae that developed into male pupae and adults. In the second experiment, 213 larvae (103 females and 110 males) were produced when five control *malE*-dsRNA-injected females were mated with five untreated virgin males. In contrast, mating of five *Tctra*-dsRNA-injected females with five untreated males produced only 108 larvae that developed into male pupae and adults. These data showed that mating of control beetles produced the expected 1:1 male to female ratio. In contrast, mating of *Tctra*-dsRNA-injected females with untreated males produced only male progeny, confirming our previous report (Shukla and Palli, 2012). Next, all progeny (males) developed from the eggs produced by *TcTra* RNAi females in both the experiments were crossed with untreated virgin females. In the first experiment, out of 80 pairs mated, two pairs produced all female progeny. In the second experiment, out of 110 pairs mated, three pairs produced all female progeny. In the first experiment, the average number of progeny detected in crosses that produced both males and females was 63 per female compared with 60 per female detected in crosses that produced only females. In the second experiment, the average number of progeny detected in crosses that produced both males and females was 55 per female compared with 49 per female detected in crosses that produced only females. These data showed that all females produced a similar number of progeny and approximately 2.5% of crosses produced only female progeny. Why did only 2.5% of crosses produce all female progeny? As explained previously (Shukla and Palli, 2012), it is possible that most of the XX progeny developed from the eggs produced by *Tctra* RNAi females died during early embryonic development, most likely because of misregulation of complex dosage-compensation genes. The presence of TcTra above the threshold levels required to inhibit the activation of dosage compensation pathway in a few XX individuals may have helped them to escape zygotic death. However, the TcTra in these individuals may not have reached levels required to execute the splicing of *Tcdsx* pre-mRNA into the female mode, resulting in the production of masculinized females.

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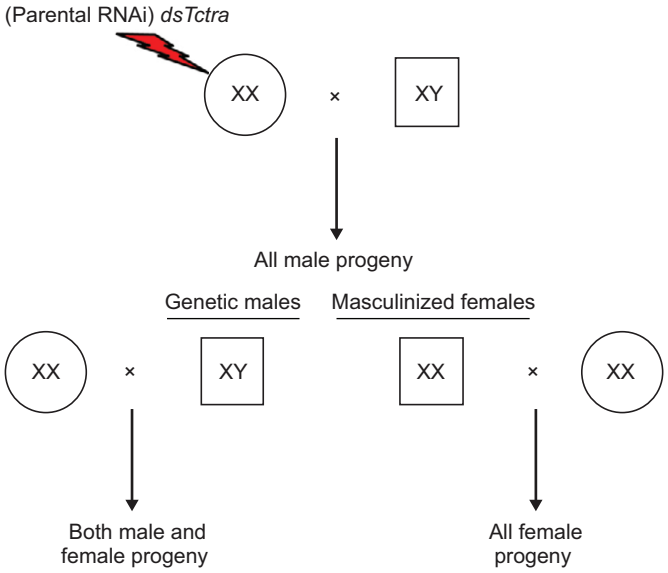

Fig. 1. Schematic diagram summarizing the experimental setup and results obtained.

We used Y-chromosome-specific and non-Y-chromosome-specific markers to determine the genetic makeup of male parents that produced all female progeny. In the first experiment, neither of the two masculinized females showed a Y-chromosome-specific marker (Fig. 2A). Similarly, in the second experiment, none of the three masculinized females showed a Y-chromosome-specific marker (Fig. 2B). These data showed that the crosses that produced all female progeny had a masculinized female (XX) as the male parent.

In insects, an amazing diversity of sex chromosome systems exists: male heterogamety, e.g. *D. melanogaster* (XY males), female heterogamety, e.g. butterflies and moths (ZW females), and haplodiploidy, e.g. bees, ants and wasps. Sex chromosome variations among different groups are further aided by different level of sex chromosome differentiation (reviewed in Kaiser and Bachtrog, 2010). *Tribolium castaneum* is an excellent model for the order Coleoptera, which follows an XY sex determination system. The maternally supplied *tra* homolog *Tctra* serves as a female sex determining signal in XX embryos of *T. castaneum* (Shukla and Palli, 2012). Maternal *Tctra* is inhibited in XY embryos by an unknown mechanism, forcing them to follow the male determination pathway. Parental RNAi of *Tctra* results in all male progeny, the majority of which are XY males. A few XX individuals are also converted to males as a result of *Tctra* parental RNAi.

According to Rice's hypothesis, genes with sex-biased or sex-specific expression are overrepresented on the X chromosome (Rice, 1984). Similarly, because the Y chromosome is never under selection in females, it is assumed to accumulate the genes that are beneficial to males (Kaiser and Bachtrog, 2010). Consistent with this, most of the genes on the Y chromosome in *D. melanogaster* are required for sperm motility and, in turn, male fertility (reviewed in Bernardo Carvalho et al., 2009). Our data suggest that the masculinized genetic females (XX) are fully fertile, as the number of progeny produced by the virgin females mated with these males is almost equal to the number of progeny in crosses between control beetles. In other words, it appears that the Y chromosome in *T. castaneum* does not contain any male fertility genes. Also, all the progeny produced by virgin females mated with XX males were females, suggesting that a factor responsible for male sex determination is present on the Y chromosome of *T. castaneum*. This is in contrast to *D. melanogaster*, where the Y chromosome has no role in male sex determination: sex in *D. melanogaster* is governed by the X:A ratio, i.e. the number of X chromosomes relative to the number of autosomes (Cline, 1993). The chromosomal position (Y-linked, autosome-linked or even X-linked) of the maleness factor varies in different populations of *Musca domestica* (Hamm and Scott, 2009). In *Megaselia scalaris*, the male determining factor lies within a transposable element and hence is present at different locations (Traut and Willhoeft, 1990). Interestingly, the Y chromosome in humans harbors a gene that regulates male sex determination as well as the genes required for male fertility (Skaletsky et al., 2003).

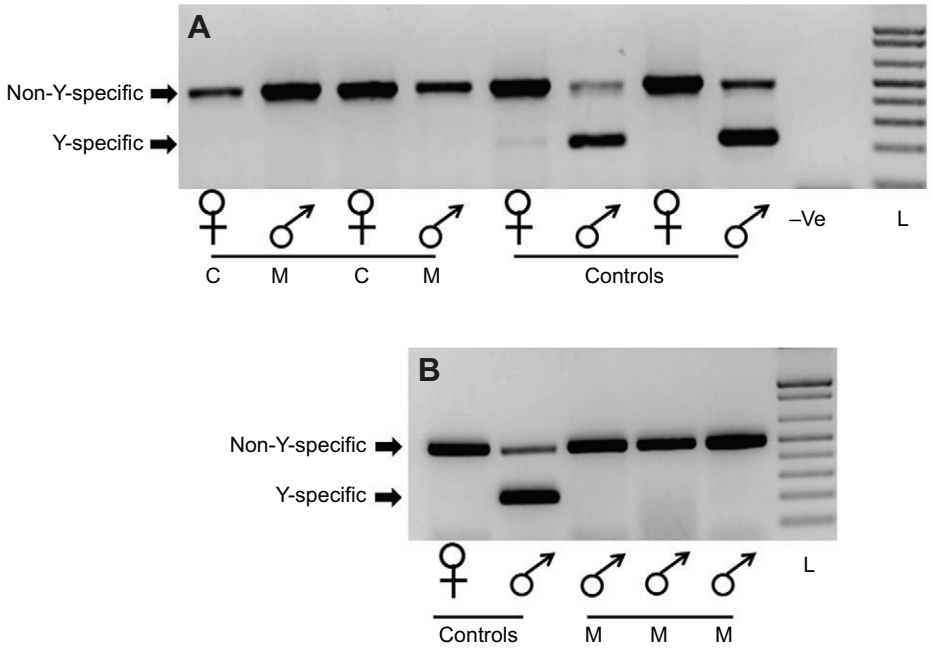

Fig. 2. Multiplex PCR with Y-specific and non-Y-specific primers (Lagisz et al., 2010) amplifies two bands in males but only one band in females. No Y-specific amplification was detected in DNA isolated from masculinized females that supported production of all female progeny. C, normal females; M, masculinized females; –Ve, negative control; L, 1 kb DNA ladder.

The data included in this communication provide genetic evidence for the presence of the male sex determination gene on the Y chromosome of *T. castaneum*. The initial characterization of Y sequences (1.5 MB of 5 MB) in *T. castaneum* suggests an active transcription of some genes, but none of them have been shown to be required for male sex determination (N. Grubbs, F.-C. Chu, M. Robinson, D. Bopp and M. D. Lorenzen, unpublished data). Therefore, the identity of the male sex determination factor remains elusive. Further studies on additional Y chromosome genes will help in the identification of the male sex determination factor in *T. castaneum*. Also, the mechanism by which the factor that regulates male sex determination inhibits the maternal *Tctra* in XY embryos needs further investigation.

MATERIALS AND METHODS

For parental RNAi, 6-day-old virgin females (n=5) were injected with *Tctra* or *malE* dsRNA (control *malE* dsRNA was prepared using a fragment of the *malE* gene from *Escherichia coli*) and reared at room temperature. After 24 h, the dsRNA-injected females were transferred to standard rearing conditions and crossed with virgin males of the same age [see Shukla and Palli (Shukla and Palli, 2012) for details]. At 20 days after initiation of mating, the number of larvae was counted and the larvae were further reared to separate them according to sex during the pupal and adult stages. Sexing of pupae and adults was carried out as described previously (Shukla and Palli, 2012). Fig. 1 shows the schematic diagram of the experimental setup.

Each individual adult developed from the progeny of *Tctra* parental RNAi was crossed with a virgin female. After 10 days of mating, parents from each cross were transferred to corresponding numbered tubes and stored at −80°C in a freezer for future use. The larvae from these crosses were maintained under standard rearing conditions. After they developed to pupal and adult stages, the sex of each individual was recorded. DNA was isolated from parent beetles using the DNeasy Blood & Tissue Kit (Qiagen) and the genetic sex of male and female parents was determined using multiplex PCR employing Y-specific and non-Y-specific primers (Lagisz et al., 2010).

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Competing interests

The authors declare no competing financial interests.

Author contributions

J.S. designed and conducted the research and wrote the paper. S.R.P. designed the research and wrote the paper.

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