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A Z-linked sterility locus causes sexual abstinence in hybrid females and facilitates speciation in *Spodoptera frugiperda*

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In the fall armyworm, *Spodoptera frugiperda* (Lepidoptera, Noctuidae), two sympatric strains have been recognized that have been termed corn strain (C) and rice strain (R), referring to their most common host plants. Both strains are reproductively isolated via a distinct prezygotic barrier as well as via an intriguing postzygotic phenomenon: when R females have mated with C males, the resulting RC hybrid females exhibit dramatically reduced fertility independent of their mating partner. Here, we demonstrate that the reduced fertility is caused by the fact that these females refrain from mating, that is, females are behaviorally sterile. We identified a Z-chromosomally linked sterility locus that is most likely incompatible with yet to be identified autosomal (or cytoplasmic) factors, leading to the observed sexual abstinence. Within-chromosome mapping revealed the sterility locus to be located in an area of strongly reduced interstrain recombination.

KEY WORDS: **Behavioral sterility, hybrid incompatibility, sex chromosome, sex chromosomal–autosomal incompatibility, unidirectional sterility.**

Speciation is fundamentally driven by the evolution of reproductive isolation barriers that reduce or prevent gene flow between diverging populations (Coyne and Orr 2004). Among the repertory of reproductive barriers (Futuyma 1998), intrinsic postzygotic isolation is developmentally mediated and expressed by a reduction in hybrid fertility or viability. Interestingly, intrinsic isolation can manifest itself as behavioral sterility when courtship behavior is disrupted. Extrinsic behavioral sterility on the other hand can be attributed to intermediate courtship behaviors of the hybrids (Coyne and Orr 2004). In general, behavioral sterility has been described in several species, for example, grasshoppers (Gottsberger and Mayer 2007), spiders (Stratton and Uetz 1986), butterflies (Davies et al. 1997), sticklebacks (Hatfield and Schluter 1999), and parasitoid wasps (Clark et al. 2010), but its molecular basis is only poorly understood and not much investigated, except in *Drosophila* (Noor 1997).

Genetically, hybrid sterility and inviability (commonly termed hybrid incompatibilities, HIs) can arise from chromosomal rearrangements, such as gene transpositions or duplications, chromosomal inversions or translocations, as well as from polyploidy (Lynch and Force 2000; Masly 2006; Kirkpatrick and Barton 2007). HI can also be caused by sexually transmitted microbial agents (Somerson et al. 1984), or by the mismatch between gut bacteria and the host genome (Brucker and Bordenstein 2013). However, genetic incompatibilities seem to be the most important cause of HI (Orr 1995; Orr and Turelli 2001; Coyne and Orr 2004). As described by the Bateson–Dobzhansky–Muller (BDM) model, two (or more) mutations that are individually adaptive or

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(nearly) neutral in their respective genomic backgrounds can be functionally incompatible in hybrids. It is worth pointing out that the underlying mutations and chromosomal rearrangements are not mutually exclusive. Within the last years, several HI genes and gene pairs following the BDM model have been identified (Johnson 2010; Presgraves 2010; Wolf et al. 2010), mainly in well-established model organisms such as *Drosophila*. Interestingly, hybrid inviability and sterility have often been observed to unequally affect reciprocal crosses, and usually the heterogametic sex, which is known as Haldane's rule (Haldane 1922; Orr et al. 1997). Such asymmetry requires the involvement of uniparentally inherited factors, for example, mitochondria, chloroplasts or sex chromosomes, or asymmetrically expressed genes. Turelli and Moyle (2007) furthermore modeled BDM incompatibilities and identified specific factors to affect the asymmetry, such as the number of genetic incompatibilities or the fitness effect of individual loci.

The fall armyworm, *Spodoptera frugiperda* (Lepidoptera, Noctuidae), is a highly polyphagous moth native to North and South America (Sparks 1979; Casmuz et al. 2010) that exhibits complementing pre- and postzygotic isolation barriers (Groot et al. 2010). Within this species, two sympatric strains have been recognized that have been termed corn strain (C) and rice strain (R), referring to the host plants on which larvae are usually found (Pashley 1986, 1989; Pashley et al. 1985). However, the association between strain and host plant is not absolute (Juárez et al. 2012, 2014). The two strains differ in their sex pheromone blend (Groot et al. 2008; Lima and McNeil 2009), but as these differences appear too weak to cause assortative mating (Unbehend et al. 2013), this does not seem a potent prezygotic barrier. By contrast, the corn strain mates significantly earlier in the night than the rice strain (Pashley et al. 1992; Schöfl et al. 2009), an isolating behavior that is likely to be under the control of the circadian clock (Hänniger 2015).

Postzygotic isolation between R and C individuals has been found as well, although reported results are partially contradictory. Pashley and Martin (1987) and Dumas et al. (2015) found that R females that were crossed with C males produced viable offspring, but not vice versa. However, in three other studies, R and C individuals were crossed successfully in both directions (Whitford et al. 1988; Quisenberry 1991; Groot et al. 2010). Considering backcrosses and interhybrid crosses, unidirectional HI has been observed repeatedly (Pashley and Martin 1987; Whitford et al. 1988; Groot et al. 2010). Hybrid females with an R mother and a C father (RC hybrid females) mated only rarely, if at all. Such fertility impairments were not found in female offspring originating from the reciprocal cross (CR), or in any type of hybrid males.

Despite the repeated description of RC hybrid female sterility, this postzygotic isolation barrier has never been characterized further, so that its role in the speciation process of *S. frugiperda* is still unclear. In this study, we aimed to answer two major questions: (1) what are the phenotypic causes leading to RC hybrid female sterility, and (2) what is the genetic basis of this phenomenon? We hypothesized that the RC hybrid female sterility is due to behavioral sterility. In addition, because only RC but not CR females are impaired in their fertility, we hypothesized that the genetic basis of this phenomenon is located on the uniparentally inherited Z-chromosome.

Methods

Spodoptera frugiperda POPULATIONS AND INSECT REARING

Four laboratory populations of *S. frugiperda* were established based on field-collected larvae, representing two distinct geographic origins: East and South, referring to the two main migratory routes of this species (Nagoshi and Meagher 2008). The eastern population overwinters in southern Florida and/or Puerto Rico and seems to migrate northward to the Ohio Valley and Maryland. The southern population overwinters in southern Texas and appears to move northward into Oklahoma and northeasterly into the Mississippi river valley. From the East, about 300 corn strain (C) larvae were collected in April 2010 in two corn fields in Santa Isabel, Puerto Rico (corn field 1: $+17°59'0.93''/-66°23'29.88''$; corn field 2: $+17°57'30.65''/-66°23'32.43''$). This population will subsequently be called "eastern corn." Regarding the rice strain (R) population, around 300 larvae were collected in May 2010 in a grass field at the Graham Farm in Moore Haven, Glades County, Florida ($+20°53'3.04''/-81°7'21.17''$). This population will be referred to as "eastern rice." From the South, about 250 C larvae for the corn strain population were collected in August 2008 in a corn field in Stoneville, Mississippi ($+33°25'23.86''/-90°53'32.68''$). This population will be referred to as "southern corn." Around 250 R larvae were collected in August 2008 in a grass field in Raymond, Mississippi ($+32°16'44.12''/-90°22'48.35''$). The respective population will be referred to as "southern rice."

Larvae of all four populations were shipped to the Max Planck Institute for Chemical Ecology and reared until adulthood on artificial pinto bean diet. To confirm their strain identity, all adults were screened for the well-established *COI* (cytochrome oxidase I) markers (Lu and Adang 1996; Levy et al. 2002; Meagher and Gallo-Meagher 2003; Nagoshi et al. 2006a) before population establishment. All populations were reared in climate chambers under a reversed light (L): dark (D) cycle and 14-h L: 10-h D photoperiod at 26°C and 70% relative humidity. Adults were fed with a 10% honey–water solution and the populations were maintained by means of random single pair matings, avoiding sib mating to minimize inbreeding.

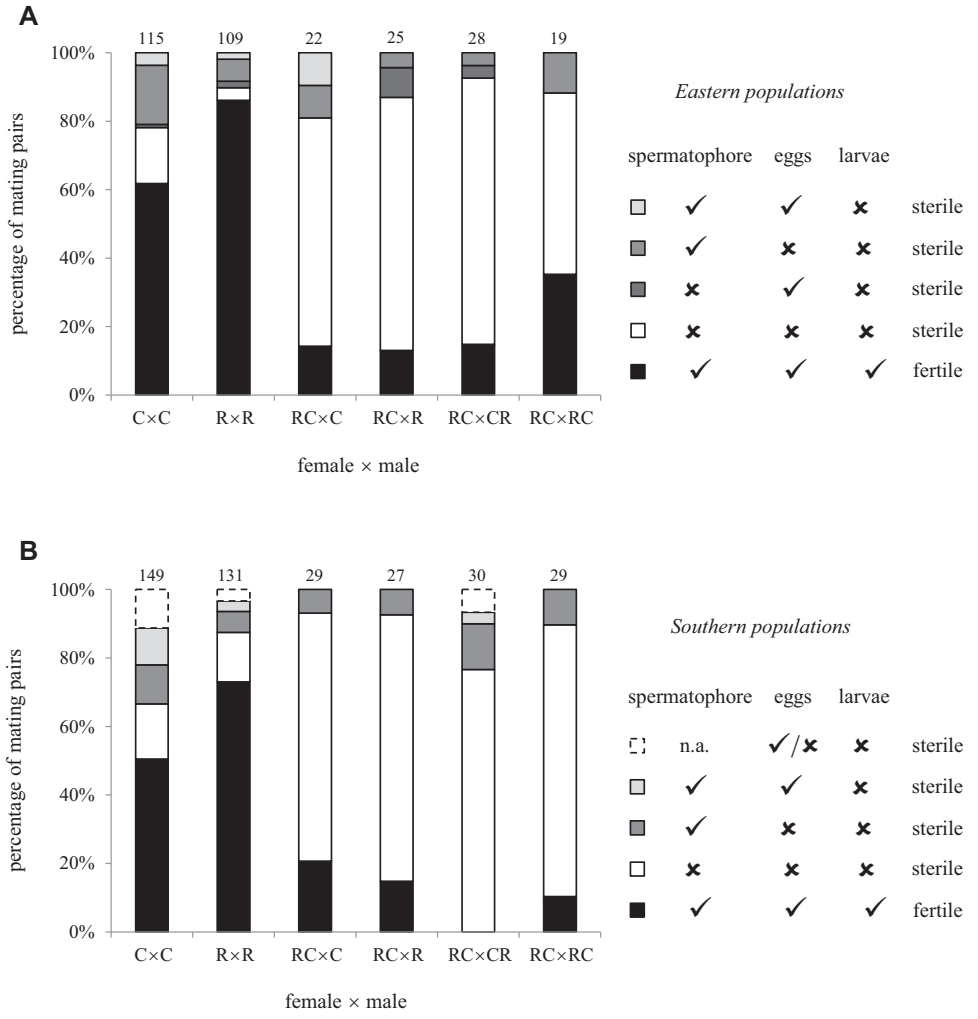

Figure 1. Percentage of mating pairs (using the *eastern* and *southern* populations), resulting from different types of crosses, that is characterized by a certain combination of three traits (presence of a spermatophore in the female, females' ability to lay eggs, and capability to produce viable larvae). ✓ = presence of a trait; ✗ = absence of a trait; n.a. = trait not analyzed. In cases where the trait was not analyzed (dashed section in B), this was due to the fact that dissection was not possible. C = corn strain and R = rice strain of *Spodoptera frugiperda*; RC = hybrid individuals having an R mother and a C father; CR = hybrid individuals having a C mother and an R father. Numbers of pairs tested (*n*) are depicted above the respective bars.

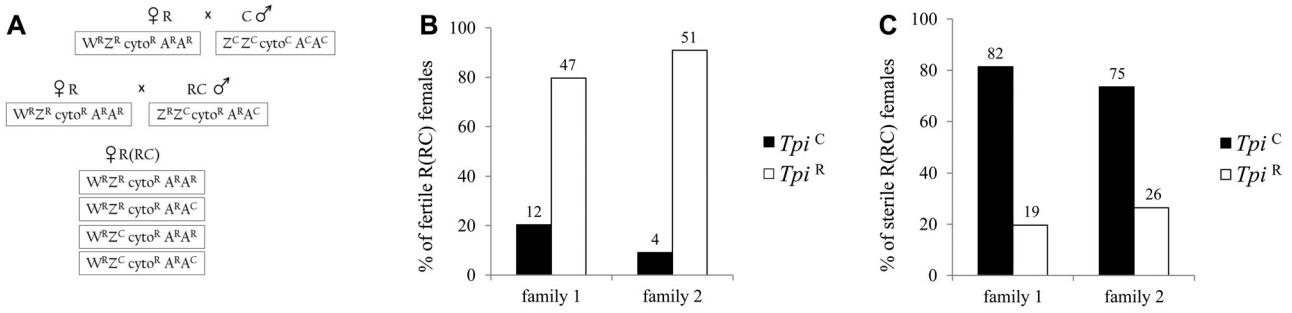

Figure 2. (A) Crossing scheme illustrating the route to produce R(RC) backcross females. R = rice strain and C = corn strain of *Spodoptera frugiperda.* Framed are the potential genotypes of the respective individuals for any particular locus with "W" and "Z" being the sex chromosomes and "cyto" signifying cytoplasmically inherited factors, such as, for example, mitochondria. A^R and A^C indicate the respective origin of the diploid autosomes. (B) Percentage of fertile and (C) percentage of sterile R(RC) females that carry the *Tpi* allele from C (TPI^C) or from R (TPI^R) separate for two families. *Tpi* = triose phosphate isomerase gene. Numbers of pairs tested (*n*) are depicted above the respective bars.

FERTILITY MEASUREMENT OF DIFFERENT CROSSES

All crosses were set up as single pair matings using one- to four-day-old adults. Mating pairs were kept until either the female or both partners died and subsequently frozen at −80°C. Eggs of the respective crosses were retained until larvae hatched or egg clutches desiccated (indicating that the eggs had not been fertilized). Lifetime fertility was measured as the ability of a respective mating pair to produce viable offspring. At the beginning of the experiments, the colonies were in their sixth (*eastern corn*), fifth (*eastern rice*), 21st (*southern corn*), and 19th (*southern rice*) laboratory generation. Crosses were replicated as depicted in Figures 1, 2, and S1. All crosses were set up only within regions (e.g. *East* or *South*) with the female always mentioned first. To statistically compare the proportions of fertile pairs between the different crosses, a Bonferroni-corrected *G*-test of independence was performed. Comparisons were conducted pairwise with the focal cross type tested against the sum of all other crosses, respectively.

MATING ACTIVITY ASSESSMENT

To test the hypothesis that RC hybrid females are behaviorally sterile, we determined their mating status. All females that failed to produce viable offspring were dissected to assess the presence of a spermatophore, a proteinaceous structure that is formed in the females during copulation containing the sperm (Takeuchi and Miyashita 1975; Amaldoss and Hsue 1989; Seth et al. 2002). Frozen females were thawed, their abdomen was ventrally opened from the distal end, and the genital pouch (bursa copulatrix) was freed to check for the presence of a spermatophore.

Z-CHROMOSOMAL LINKAGE AND WITHIN-CHROMOSOME MAPPING OF THE STERILITY LOCUS

To test for Z-chromosomal linkage and to conduct within-chromosome mapping of the sterility locus, two separate mapping populations were generated by backcrossing an RC hybrid male to an R female (Fig. 2A). At the beginning of the experiment, the *eastern corn* population was in its 13th and the *eastern rice* population in its 12th laboratory generation. To have genetically similar individuals for both mapping populations, RC hybrid brothers were crossed with R sisters. This approach resulted in 160 and 157 R(RC) females, respectively, which were phenotyped (tested for fertile eggs as described above), checked for the presence of a spermatophore, and genotyped. Genotyping comprised scoring polymorphisms of the triose phosphate isomerase (*Tpi*) gene and eight additional marker genes that are all located on the Z-chromosome. The scoring revealed whether a certain female carried the R- or C-allele of these nine markers. Because in the pool of R(RC) females the Z-chromosome and thus the Z-linked markers segregate (Fig. 2A), a cosegregation of sterility with these markers demonstrates the Z-linkage of the phenotype. Due to the fact that in Lepidoptera crossing over occurs only in males, recombination will occur between the two Z-chromosomes present in the RC hybrid males (Fig. 2A). As a consequence, the daughters of these males (the R(RC) backcross females) can be used to map the sterility locus on the Z-chromosome.

For the genotyping, DNA was extracted using a classical CTAB protocol as described by Unbehend et al. (2013). After a PCR-based approach (where PCR is polymerase chain reaction) with subsequent Exo-SAP cleanup (Werle et al. 1994), direct Sanger sequencing was performed to check whether the R(RC) females carried the corn- or rice-allele of a respective marker. Whenever possible, strain-specific size differences of the respective PCR products were used directly for the scoring. Primers and scoring strategies for all nine markers are depicted in. Table S1.

Recombination frequencies among all markers were calculated based on the combined data from both mapping populations using the software package Mapmaker (Copyright 1992 Whitehead Institute for Biomedical Research; Lander et al. 1987). To statistically test for the correlation of the sterility phenotype and thus the sterility locus with the respective markers, individual χ^2-tests were performed.

WITHIN-STRAIN MAPPING OF Z-CHROMOSOMAL MARKERS

To check for the recombination frequencies of four Z-chromosomal marker genes in the pure strains, intrastrain matings were set up using the *eastern corn* (25th–26th laboratory generation) and the *eastern rice* (24th–25th laboratory generation) populations, respectively. Both mating types were replicated thrice with nonrelated individuals, resulting in 62 C and 38 R females for the genotyping. Genotyping followed the procedure described above, using the primer combinations listed in Table S1. In the C females, the markers *tan*, *2087*, and *637* were scored; in the R females, the markers *acj6*, *tan*, and *637* were scored. Because the *637* primer combination listed in Table S1 did not reveal any within-strain SNPs for C, alternative primers were used (5′-AGA TGT CTG CGT GGA CGT C-3′/5′-CTT GGG CAT TAT TTC GTG G-3′).

VISUALIZATION OF Z-CHROMOSOMAL MARKERS IN PURE STRAINS

To visualize the physical order of six Z-chromosomal marker genes in the corn as well as in the rice strain, fluorescence in situ hybridization (FISH) with bacterial artificial chromosome (BAC) probes was performed, following Yoshido et al. (2014). Probes were generated based on six individual BAC clones (from the library Sfr-B-SFB MC provided by the Centre National de Ressources Génomiques Végétales in Toulouse, France), each containing the respective *S. frugiperda* ortholog of the

Bombyx mori Z-linked genes *tan*, *2087*, *acj6*, *kettin*, *637,* or *Tpi*. Chromosome preparations were made from ovaries of three- to five-day-old female pupae. Because the BAC containing *acj6* labeled the whole W-part of the WZ bivalent and moreover detected repetitive sequences throughout the entire chromosome preparation, chromosome preparations were also made from testes of 5th instar male larvae and the amount of competitor DNA was increased to block unspecific binding of the probe. In the male chromosome preparations, *acj6* was visualized together with *tan*, *2087*, and *kettin* as reference genes, to be able to merge the data obtained from both sexes.

Results

Intrastrain crosses, using *eastern* C and R individuals, resulted in 48 and 85% fertile mating pairs, respectively (Fig. S1A). This means that on average 67% of these pure strain pairs produced fertilized eggs from which viable larvae hatched. Bidirectional interstrain crosses (RxC and CxR) led to 60 and 33% fertile mating pairs, respectively (Fig. S1A). The proportion of RC hybrid females producing viable offspring in backcrosses was significantly lower (14 and 12% fertile pairs) than the overall proportion of fertile pairs (Fig. S1A). In contrast, the mating success of CR hybrid females was not statistically different in backcrosses (48 and 69% fertile pairs) compared to the overall proportion of fertile pairs (Fig. S1A). The latter holds true for all backcrosses involving RC or CR hybrid males, where between 37 and 65% fertile pairs were observed (Fig. S1A). One F_2 cross type involving RC hybrid females produced a significantly lower proportion of fertile pairs (14%) compared to the rest of the crosses, while this was not the case for the second F_2 cross type involving RC hybrid females (32% fertile pairs) and both F_2 crosses involving CR hybrid females (63 and 58% fertile pairs; Fig. S1A). Combining backcross and F_2 data, only 18% of the RC hybrid females produced viable offspring, while about 60% CR hybrid females were fertile (Fig. S1A). Similar results were obtained using *southern* C and R populations (Fig. S1B).

To test the hypothesis that the dramatically reduced fertility of RC hybrid females can be attributed to the fact that they simply do not mate, all fertile and sterile females were examined for the presence of a spermatophore. As expected, all fertile pure strain as well as fertile RC hybrid females possessed a spermatophore

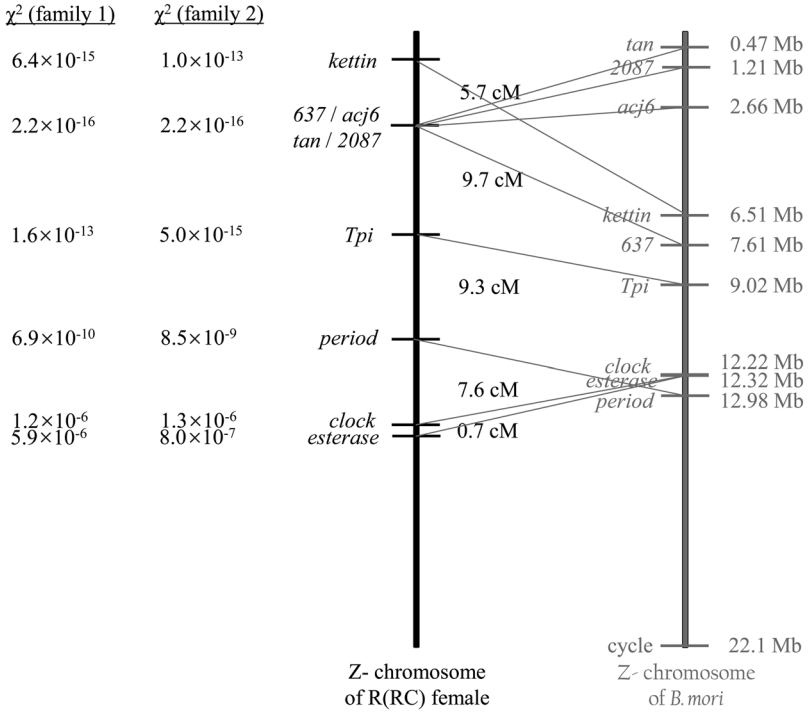

Figure 3. Z-chromosomal linkage map of R(RC) females. R(RC) = individuals resulting from backcrosses of RC hybrid males with R females, where C = corn strain and R = rice strain of *Spodoptera frugiperda* (using the *eastern* populations); *kettin* = homolog to *Bombyx mori* gene BMgn000622; *637* = homolog to *B. mori* gene BMgn000637 (Iroquois-like protein); *acj6* = homolog to *B. mori* gene BMgn002017 (putative inhibitory POU domain carrying protein); *tan* = homolog to *B. mori* gene BMgn002077; *2087* = homolog to *B. mori* gene BMgn002087 (macrophage migration inhibitory factor); *Tpi* = triosephosphate isomerase gene; *esterase* = homolog to *B. mori* gene BMgn000729 (carboxylesterase-like protein); cM = centi-Morgan. Results of χ^2 tests for cosegregation of the sterility phenotype with each marker gene are depicted next to the respective markers separately for two families. Z-chromosomal linkage map of *B. mori* is put for direct comparison of gene orders. Mb = mega base pairs.

(Fig. 1, black bars). Regarding the sterile crosses, the vast majority of RC hybrid females did not contain a spermatophore, while the percentage without spermatophore was low in matings involving C and R females (Fig. 1, white and dark gray bars). The percentage of mating pairs with a spermatophore, but without eggs and/or larvae, was comparably low in crosses involving C, R, or RC females (Fig. 1, medium and light gray bars). The distribution of spermatophores was similar for fertile and sterile females originating from the East and the South.

To determine whether the sex-chromosome (Z) contributes to RC hybrid female sterility, we checked for cosegregation of sterility with strain-specific alleles of the Z-linked *Tpi* gene in R(RC) backcross females (Fig. 2B and C). Considering the two separate backcross families, 20 and 10% of the fertile R(RC) females carried the *Tpi* allele from C, while the remaining 80 and 90% of these females possessed the *Tpi* allele from R (Fig. 2B). For the sterile R(RC) females of the two backcross families, the opposite picture emerged (Fig. 2C): 81 and 73% of the sterile females carried the *Tpi* allele from C, while 19 and 26% carried the *Tpi* allele from R.

Within-chromosome mapping of the sterility locus based on R(RC) backcross females showed that the nine chosen Z-chromosomal markers cover a region of 33 cM, with *kettin* being the most distal and *esterase* the most central gene (Fig. 3). Flanked by *kettin* and *Tpi*, four genes—namely *637*, *acj6*, *tan*, and *2087*—are located in a region that did not show any recombination (Fig. 3). This region mapped 5.7 cM away from *kettin* and 9.7 cM away from *Tpi*. Interestingly, this is exactly the region where the sterility locus maps. Accordingly, the sterility locus is most tightly linked with the nonrecombining markers *637*, *acj6*, *tan*, and *2087*.

Both families showed an overrepresentation of C alleles for all markers scored on the Z chromosome (Fig. S2). This could be due to segregation distortion during meiosis in their RC hybrid fathers, viability differences among sperm bearing a Z-chromosome from C versus R, or viability differences after zygotes have been formed. The magnitude of the skew followed a gradient along the Z-chromosome, becoming most pronounced for *kettin* where the C:R ratio was nearly 2:1 (Fig. S2). The genetic factor responsible for this skew may be distinct from the sterility locus, because the skew was smaller for the block of four nonrecombining markers, although not significantly so (Fig. S2).

Within-strain mapping revealed recombination between *tan*, *2087*, and *637* in the corn strain, and between *tan*, *acj6*, and *637* in the rice strain (Fig. S3). Direct visualization of six Z-chromosomal markers via BAC–FISH revealed identical gene orders in the *eastern* corn and rice strain. In females of both strains, *tan* was the most distal gene, followed by *2087*, *kettin*, and *637*, and finally *Tpi* being located close to the center of the chromosome (Fig. 4A–E). In males of both strains, *tan* was the most distal gene, followed by *2087*, *acj6*, and *kettin* (Fig. 4F–J). Combining the data obtained from both sexes, the physical order in both strains is *tan*, *2087*, *acj6*, *kettin*, *637*, and *Tpi* (Fig. 4K).

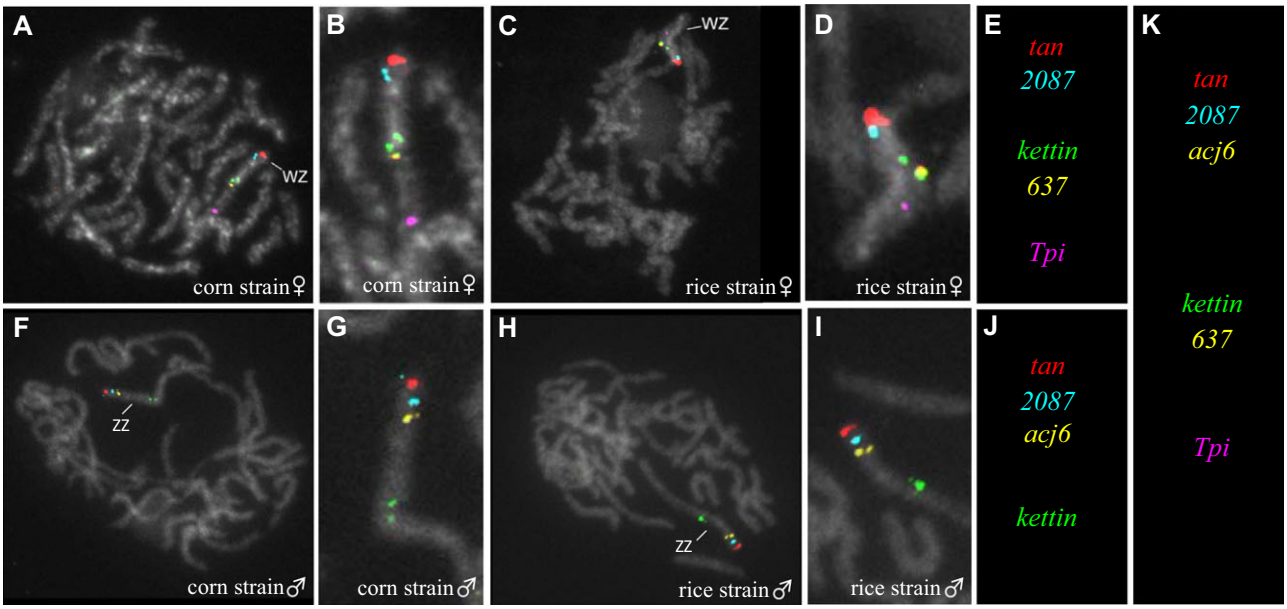

Figure 4. **Visualization of different Z-chromosomal marker genes via BAC-FISH mapping. (A) and (C) Pachytene complements of corn and rice strain females. (F) and (H) Pachytene complements of corn and rice strain males. (B), (D), (G), and (I) enlarged details of the respective left images. (E) and (J) Physical order of the marker genes in females and males. (K) Merged physical order of marker genes in *Spodoptera frugiperda*. *tan* = homolog to *B. mori* gene BMgn002077; *2087* = homolog to *Bombyx mori* gene BMgn002087 (macrophage migration inhibitory factor). *acj6* = homolog to *B. mori* gene BMgn002017 (putative inhibitory POU domain carrying protein); *kettin* = homolog to *B. mori* gene BMgn000622; *637* = homolog to *B. mori* gene BMgn000637 (Iroquois-like protein); *Tpi* = triose phosphate isomerase gene.**

Discussion

In this study, we demonstrate that RC hybrid females are behaviorally sterile and that a genetic incompatibility involving a Z-chromosomally linked sterility locus accounts for the observed sexual abstinence.

We found markedly reduced fertility specifically among RC hybrid females from two geographic regions, which confirms the observations by Pashley and Martin (1987), Whitford et al. (1988), and Groot et al. (2010) and strongly suggest a genetic basis. RC females have a W-chromosome, mitochondria, and cytoplasm from the R-strain, a Z-chromosome from the C-strain, and one set each of R and C autosomes. R(RC) backcross females likewise have their W-chromosome and cytoplasm from the R-strain and a single Z-chromosome (R, C, or recombinant). About half of the R(RC) females possess autosomes with two R copies and the remaining individuals autosomes with one R and one C copy. Therefore, the reduction in fertility of RC females and some R(RC) females cannot be due solely to a Z-linked sterility factor from the C-strain, because C females are fertile; it must be due to an incompatibility between such a Z-linked factor from C and other factors from R. The fertility of CR females shows that this incompatibility is unidirectional. Our experimental design does not permit us to discriminate among the R-derived W-chromosome, cytoplasm, or autosomes as the contributing partner(s) to the RC incompatibility. However, a comparable pattern of unidirectional hybrid sterility has been found among two races of *Heliconius melpomene*, where additional crosses ruled out incompatibilities due to the W-chromosome and cytoplasm and implicated autosomal factors in the genome of the other race (Jiggins et al. 2001).

Considering the sterility of RC hybrid females in *S. frugiperda*, the vast majority did not contain a spermatophore, which explicitly demonstrates that these females did not mate successfully. Only during successful copulation is a spermatophore formed inside the female's genital pouch, the *bursa copulatrix* (Takeuchi and Miyashita 1975; Amaldoss and Hsue 1989; Seth et al. 2002). As the spermatophore persists after the sperm has been transported to the *spermatheca* (the final sperm storage organ in the female; Takeuchi and Miyashita 1975; Seth et al. 2002), absence of a spermatophore cannot be due to resorption. The finding that most RC hybrid females did not mate successfully confirms the behavioral observations of Schöfl et al. (2009), who found that RC hybrid females call, copulate, and oviposit less often than C, R, or CR females. Taken together, both studies demonstrate that eggs of RC hybrid females are not fertilized because the females hardly attempt to mate.

The hybrid females' behavior might seem to be a prezygotic mating barrier from an individuals' perspective. However, regarding the entire population, the sterility or rather nonmating of hybrid females represents a postzygotic isolation barrier, because all isolation barriers that reduce the fitness of hybrid zygotes (in our case RC hybrid female zygotes) are by definition postzygotic (Futuyma 1998). Intriguingly, in *S. frugiperda* hybrid sterility represents an unusual form of intrinsic behavioral sterility rather than impairments in development (such as in the *Heliconius* system [Naisbit et al. 2002]) or impairments in metabolism without behavioral consequences (such as described, e.g., for hybrids between *Saccharomyces cerevisae* and *S. bayanus* [Lee et al. 2008] that are defective in respiration and sporulation). Behavioral sterility has been reported for *Drosophila*, where male hybrids between *D. pseudoobscura* and *D. persimilis* are characterized by anomalously weak courtship intensity, which was found to result from an interaction between the *D. persimilis* X-chromosome and the *D. pseudoobscura* autosomes or Y-chromosome (Noor 1997). Our results are in line with this study, because in *Drosophila* as well as in *Spodoptera* the fertility of the heterogametic sexes is affected, as predicted by Haldane's rule.

A tempting hypothesis regarding the RC hybrid females' sexual abstinence in *Spodoptera* is that they are deficient in knowing or sensing time and thus lack the trigger for initiating mating activities. This hypothesis nicely links the observed behavioral sterility with the major reproductive isolation barrier between the two strains, namely the difference in timing of mating activities throughout the night. Once the genetic basis underlying the temporal difference has been identified (Hänniger 2015), the RC hybrid females could be tested in this regard.

Apart from the behavioral cause of RC hybrid female sterility, females might be sterile even though they contained a spermatophore, for example, by a misplacement or malformation of the spermatophore within the *bursa copulatrix*, such that the sperm cannot get out and thus is not available for egg fertilization (Navon and Marcus 1982). Alternatively, the couple might not be able to separate after copulation, a scenario that we observed in one *southern* pair. Finally there might be a failure of egg or embryo development, but these cases were rare (9 and 5.2% for the *eastern* and *southern* population) and we found this similarly frequently in C females (20 and 11% for the *eastern* and *southern* population) and R females (8.2 and 4.6% for the *eastern* and *southern* population).

The segregation pattern of sterility and the C-derived *Tpi* allele was significantly but not absolutely correlated among R(RC) backcross females. Because (1) *Tpi* is located on the Z-chromosome and (2) R(RC) females carry a possibly recombined Z-chromosome, this result clearly supports the hypothesis that a factor affecting RC hybrid female sterility maps to the Z-chromosome and indicates the sterility locus to be linked with the *Tpi* locus. Linkage of sterility to *Tpi* has also been described for *Heliconius* (Jiggins et al. 2001; Naisbit et al. 2002), based on 16 genotyped individuals. Interestingly, this not only indicates synteny between *Spodoptera* and *Heliconius*, but also suggests that

related genetic regions lead to different sterility characteristics—namely behavioral or developmental impairments—in different genera. The observations that (3) markers from C were overrepresented over that from R on the Z-chromosome, and (4) this overrepresentation was skewed near *kettin* argue for yet another Z-chromosome-linked locus that affects chromosomal segregation or viability.

Within-chromosome mapping on the Z-chromosome revealed a highly significant correlation of the sterility locus with the four nonrecombining markers *637*, *acj6*, *tan*, and *2087*, which was 100 times higher than for the markers *kettin* or *Tpi*. The lack of recombination between *637*, *acj6*, *tan*, and *2087* might be attributed to close spacing on the chromosome so that no crossing over and thus no recombination is observed with the given sample sizes. However, preliminary mapping of these markers within pure strains revealed recombination between *tan*, *2087*, and *637* in the corn strain, and between *tan*, *acj6*, and *637* in the rice strain. Another possible explanation is a chromosomal inversion. Inversions typically give rise to different gene orders on the respective homologous chromosomes, which can prevent correct pairing of, and hence inhibit crossing over and recombination, between the involved loci during meiosis. However, direct visualization of *tan*, *2087*, *acj6*, *kettin*, *637*, and *Tpi* in C and R revealed identical gene orders of the respective genes on the Z-chromosomes originating from both strains. Alternatively, the lack of recombination might be caused by a rearrangement between *acj6* and *kettin* (based on BAC-FISH results), as recombination suppression can extend beyond the limits of the inverted region (Noor 1997). Finally, the recombination rate might be so low that we were not able to detect it even with our moderately large mapping populations.

Surprisingly, the within-chromosome mapping revealed a fundamentally different Z-chromosomal gene order than the direct visualization approach. This discrepancy could be explained by a localized translocation event, in which only the *kettin* gene has moved to the distal part of the chromosome, leaving its flanking sequences behind. In this scenario, the respective BAC might detect the flanking sequences and thus the original location of the *kettin* gene rather than the *kettin* sequence itself. However, the within-chromosome mapping relies on the *kettin* sequence and would thus detect the gene at its new position. According to the direct visualization approach, the Z-chromosomal gene order of *S. frugiperda* reflects that of *B. mori*, while based on the within-chromosome mapping in *Spodoptera*, the gene order would be different between the two species. Even though the actual location of *kettin* could not yet be conclusively determined, different gene orders between the two species would be consistent with local genome rearrangements between *B. mori*, *S. frugiperda*, and *Helicoverpa armigera*, as well as between *B. mori* and *Bicyclus anynana*, despite the high degree of overall synteny conservation (Beldade et al. 2009; d'Alençon et al. 2010).

In the field, the observed HI likely significantly reduces gene flow between the two strains, as 56–66% of all hybrids found in nature were shown to be RC hybrids (Nagoshi and Meagher 2003; Prowell et al. 2004; Nagoshi et al. 2006b). This postzygotic isolation barrier likely plays an important role in the divergence of the two strains of *S. frugiperda*, because the prezygotic isolation barriers appear to be weak (Schöfl et al. 2009; Juárez et al. 2012; Unbehend et al. 2013). This is in contrast, for example, to host races of other species, such as *Ostrinia nubilalis* or *Rhagoletis pomonella*, where the proportion of assortative mating due to host plant usage was found to be as high as 95% (Feder et al. 1994; Malausa et al. 2005). The fact that we have identified behavioral sterility to be the basis of RC hybrid female sterility, with this locus mapping to the Z-chromosome, is not only of considerable importance in elucidating the process of speciation in *S. frugiperda*, it also significantly contributes to the understanding of intrinsic postzygotic isolation due to behavioral impairments.

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DATA ARCHIVING

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Primers used to generate PCR products for the scoring of marker genes in R(RC) females.
Figure S1. Percentage of fertile mating pairs resulting from different types of crosses using the eastern (A) and the southern (B) populations.
Figure S2. Distribution of the corn (C) and rice (R) allele of the different marker genes in R(RC) females belonging to two families.
Figure S3. Recombination frequencies (%) of different marker genes in the corn (A) and the rice strain (B) of Spodoptera frugiperda.