

The maternal-effect, selfish genetic element *Medea* is associated with a composite *Tc1* transposon

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Maternal-Effect Dominant Embryonic Arrest (“*Medea*”) factors are selfish nuclear elements that combine maternal-lethal and zygotic-rescue activities to gain a postzygotic survival advantage. We show that *Medea*¹ activity in *Tribolium castaneum* is associated with a composite *Tc1* transposon inserted just downstream of the neurotransmitter reuptake symporter *bloated tubules* (*blot*), whose *Drosophila* ortholog has both maternal and zygotic functions. The 21.5-kb insertion contains defective copies of *elongation initiation factor-3*, *ATP synthase subunit C*, and an *RNaseD*-related gene, as well as a potentially intact copy of a prokaryotic *DUF1703* gene. Sequence comparisons suggest that the current distribution of *Medea*¹ reflects global emanation after a single transpositional event in recent evolutionary time. The *Medea* system in *Tribolium* represents an unusual type of intragenomic conflict and could provide a useful vehicle for driving desirable genes into populations.

postzygotic | selfish gene | *Tribolium* | gene driver

Propagation of selfish elements nonessential for host survival can be mediated either cytoplasmically or chromosomally. Many cytoplasmically inherited *Wolbachia* or microsporidia enhance their own propagation by feminizing, sterilizing, or killing infected males or male gametes, thereby redirecting investment toward production of (infected) females and ensuring a more rapid spread of the pathogen than could be achieved solely by passive transmission in the cytoplasm (1). In other cases such a transmission advantage is achieved by sperm from *Wolbachia*-infected males conferring inviability on eggs laid by uninfected mates (2). Chromosomally inherited *Segregation Distorter* (*SD*) and *sex-ratio* meiotic drive alleles in *Drosophila* create a prezygotic transmission advantage in heterozygous males by conferring inviability on rival spermatids bearing the alternative alleles (3–5).

We discovered the existence of a novel category of selfish genetic elements that act postzygotically and include a maternal and a zygotic component, both of which are required for expression of selfish behavior (6, 7). These factors, which we termed *Medea* (*M*) elements, are widespread in natural populations of *Tribolium* flour beetles but are otherwise unknown in the invertebrate world (8). Our initial discovery (6) was followed by two reports describing similar phenomena in mice. The first involved a spontaneous chromosome 8 mutation that occurred only once in a single laboratory strain of *Mus musculus* and is associated with a maternally conferred autoimmune disease, *severe combined anemia and thrombocytopenia* (*scat*) (9). A second example, also from *M. musculus*, involves the homogeneously staining region (HSR) on chromosome 1, which consists of variable numbers of tandem copies of a 100-kb repeat. Like *Medea* and *scat*, some variants of HSR impart maternal lethality to late embryos, which is prevented by a zygotic copy of the HSR chromosome inherited from either parent (10–12). The mechanisms of postzygotic self-selection in these systems are still unknown.

Because no molecular mechanism has yet been reported for any maternal selfish gene, we decided to identify the molecular lesion associated with the *Medea* factor *M*¹ on the third linkage group of *Tribolium castaneum*. Our findings indicate that insertion of a very large, composite *Tc1*/*mariner* transposon is tightly associated with *M*¹ activity and is the probable cause of the maternally controlled selfish behavior of this locus. This insertion appears to have occurred only once, followed by worldwide spread of the affected chromosome.

Results

Positional Cloning of *Medea*¹. We positionally cloned a region encompassing the site of the *M*¹ locus on the third linkage group of *T. castaneum* by performing a chromosome walk in a non-M BAC library. The walk spanned a total of >700 kb and was oriented and monitored by high-resolution recombinational mapping using “left”-flanking (centromere-proximal) markers only, because no right-flanking, visible mutant markers were available. This mapping yielded 107 recombination events in the 182-kb segment bounded by the recessive visible *aureate* mutation and the estimated position of the target *M*¹ locus (Fig. 1A). Thus, the mean distance between recombination events was 1.7 kb. This process confined the probable site of the *M*¹ trait to a small region flanked by the 3' ends of the *Tribolium* orthologs of the *Drosophila* genes *highwire* (*hiw*) (13) and *bloated tubules* (*blot*) (14), closely juxtaposed in tail-to-tail orientation (Fig. 1A).

***M*¹ Colocalizes with a Large Insertion.** The corresponding region of an *M*¹ strain was obtained by screening an *M*¹ BAC library with a *hiw*-specific probe. A 129-kb BAC clone was identified and confirmed to contain the region of interest by *hiw*- and *blot*-specific PCR, as well as PCR specific for regions upstream of *blot*. The clone was completely sequenced and provided ≈40 kb of *M*¹ sequence upstream (telomeric) of *blot* as well as ≈60 kb

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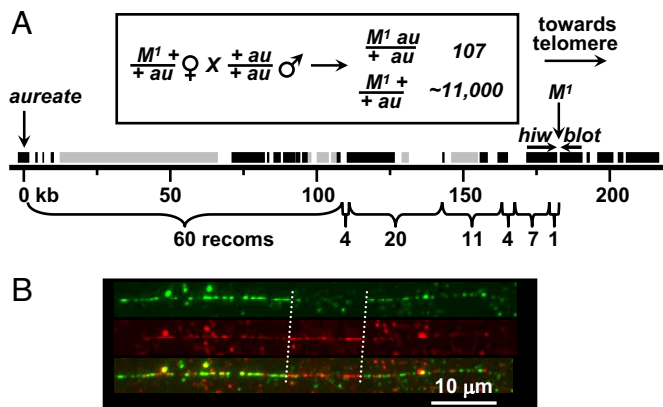


Fig. 1. M^1 is associated with an insertion. (A) *aureate*-to-*Medea*¹ region of the chromosome walk in the non- M^1 GA2 strain. Brackets below the chromosome show the distribution of 107 *aureate* (*au*)-*Medea*¹ (M^1) recombination events among seven segments constituting the 182-kb region. The recombinants were found among $\approx 11,000$ progeny of the indicated backcross (boxed) of the transheterozygote to the double recessive. Bars above the chromosome indicate transcribed regions predicted by GLEAN (25) with support from ESTs, cDNAs, or tiling array data (black bars) or without such support (gray bars). The position of the M^1 locus just downstream of the *blot* gene in the non- M^1 strain was predicted from the distribution and average spacing (1.7 kb) of recombination events. Directions of transcription of *hiw* and *blot* are indicated by horizontal arrows. The site of the *au* mutation is set at “zero” on the kb scale. (B) Cytological visualization of the M^1 lesion. The panel depicts a single M^1 chromosome fiber (24). Fibers were hybridized to a non-*Medea* (GA2-strain) BAC clone (green fluorescence, g) and an M^1 BAC clone (red fluorescence, r). Both BAC clones include the M^1 insertion point and >30 kb of flanking sequence on each side. The digital overlay (o) of the red and green images clearly indicates the presence of a large insertion in the M^1 fiber (delimited by vertical dashed lines), which does not hybridize with the GA2-derived probe. For the red-filtered image, note the relative enhancement of red fluorescence in the insertion region, in which there is no competition for green-labeled probe.

upstream (centromeric) of *hiw*. Inspection of the M^1 sequence revealed one striking difference between M^1 and non- M^1 strains, namely the presence in the M^1 strain of a 21.5-kb insertion of exogenous sequence at precisely the calculated position of the M^1 locus as determined by high-resolution genetic mapping. To verify the presence of the M^1 insertion by direct visualization, we performed fluorescence *in situ* hybridization to M^1 chromosome fibers (fiber-FISH). The presence of a large insertion of exogenous DNA was indeed confirmed (Fig. 1B).

The M^1 Insertion Is a Composite *Tc1* Transposon. Sequence analysis indicated that the entire 21.5-kb insertion comprises a large, composite *Tc1* transposon, i.e., a transposon within a transposon (Fig. 2). The *hiw*-to-*blot* intergenic region of the non- M strain GA2 is depicted in Fig. 2A. The region spans 776 nt flanked by the 3' UTRs of *hiw* and *blot*. The insertion point of the 21.5-kb segment is approximately midway between the *hiw* and *blot* polyadenylation signals. The centromere-proximal end of the insertion consists of a full-length (1.4-kb) *Tc1* element encoding a nearly intact, 346-residue transposase [Fig. 2B and supporting information (SI) Figs. S1 and S2]. A single-base transition, C \rightarrow T, which converts R to a stop codon at residue 182, is the only apparent defect. The 50 nt at the telomere-proximal end of the 21.5-kb insertion consists of an intact, *Tc1* right terminus including a full, 35-nt inverted terminal repeat (ITR). DNA transposons of the *Tc1*/*mariner*/*IS630* superfamily are the most plentiful found in the *Tribolium* genome, being distributed among 30 subfamilies (15).

M^1 -Associated *Tc1* Transposon Family Members Are Nondegenerate. A total of five closely related copies of the M^1 -associated *Tc1* element are present in the GA2 genome assembly. All five are

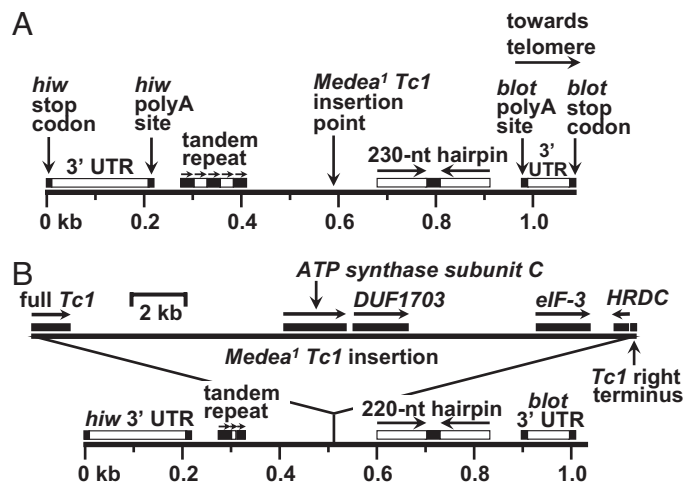


Fig. 2. The M^1 -associated insertion is a 21.5-kb composite *Tc1* element. (A) Map of *hiw*-to-*blot* region of a wild-type chromosome. The two genes (tail-to-tail orientation) are separated by a span of 1.1 kb (stop codon to stop codon). Vertical arrows indicate stop codons, polyA addition sites, and *Tc1* insertion site. The insertion point is flanked by five copies of a 27-nt, imperfect tandem direct repeat, as well as a 230-nt hairpin consisting of a 20-nt central loop flanked by a pair of 105-nt, imperfectly palindromic stems. Horizontal arrows indicate the tandem direct repeats, hairpin stems, and orientation of the entire segment on the chromosome. Horizontal bars above insertion target chromosome denote main features of region flanking the M^1 insertion target. (B) Map of *hiw*-to-*blot* region of an M^1 chromosome. Solid bars above the 21.5-kb composite *Tc1* insertion (top part of B) denote genes or gene fragments within the insertion. Horizontal arrows indicate directions of transcription. Of these genes (*Tc1* transposase, *ATP synthase subunit C*, *DUF1703*, *eIF-3*, and *HRDC*) only *DUF1703* is thought to be potentially functional (see Fig. S3). The isolated *Tc1* right terminus is not shown to scale. Note that the tandem repeat and hairpin are shorter in the M^1 strain than in wild type (compare A and B). Also note the different scales for insertion vs. flanking regions.

full-length, and two of the five have intact coding sequences and potentially encode functional transposase (Fig. S2). The five are members of the *Tc1*-DD34E subclass (16). In view of the dearth of accumulated mutational defects in members of this subclass, it is likely that this element invaded the *Tribolium* genome in recent evolutionary time, quite possibly since the speciation of *castaneum*. We could find no examples of highly degenerate members of this *Tc1* subclass or of any large, composite *Tc1* transposons in the GA2 genome.

The M^1 Insertion Contains Defective Copies of Vital Genes. We determined that no well conserved copy of the 20-kb central segment (forming the “cargo” of the composite *Tc1* element) exists in the non-*Medea* (GA2) genome but that there are regions of extensive similarity on three other linkage groups (LG), namely 6, 8, and 10. This central segment contains incomplete or defective copies of at least three genes, namely *ATP synthase subunit C* (*ATPsynC*), *elongation initiation factor 3* (*eIF3*), and a gene encoding a helicase RNaseD C-terminal domain (HRDC) (Fig. 2B). Orthologs of *ATPsynC* and *eIF3* are vital in *Drosophila*, and intact copies occur elsewhere in the GA2 genome (Fig. S3).

The M^1 Insertion Contains a Bacterial Gene. In addition to the defective *ATPsynC*, *eIF3*, and HRDC-motif genes, there is a fourth gene, previously known only from bacteria. Sequence analysis indicates that this is the only potentially functional gene present in the central cargo segment of the composite *Tc1* insertion. The protein encoded by this intronless gene shows no similarity to any known or predicted animal protein but is related to a group of proteins containing the bacterial domain of unknown function *DUF1703* (pfam08011) (Fig. S4). *DUF1703*

proteins have recently been proposed to be members of the PD-(D/E)XK nuclease superfamily (17). Before our analysis, *DUF1703* genes were known only in prokaryotes (<http://pfam.sanger.ac.uk/family?entry=pf08011&type=Family>), the sole exception being a single, highly truncated fragment found in the bacterivorous amoebozoan *Hartmannella vermiformis* (18). The (non-*Medea*) *Tribolium* genome assembly contains four apparently intact copies of the *DUF1703* gene that are closely related to the *M¹* copy, showing ≈90% sequence identity over almost 700 amino acid residues. At least two of these are transcriptionally active, both on LG6. There are also four truncated copies that are largely identical to corresponding regions of the *M¹* copy and ≈10 additional copies more distantly related (20–30% identical at the amino acid level). These more distantly related copies are clearly also members of the bacterial *DUF1703* family (Fig. S4). We are unaware of other examples of lateral transfer of a prokaryotic gene into an animal genome followed by expansion in copy number of the invading gene. The grouping of the *Tribolium* *DUF1703* proteins into a distinct clade suggests either a recent gene family expansion in the beetle's genome or evolutionary constraints imposed on a functionally related group.

The *M¹* Insertion Occurred Once, Then Spread Globally. Sequence comparisons among geographically diverse strains of *T. castaneum* indicate a single origin of *M¹* followed by global radiation. We compared sequences flanking the site of the *M¹*-associated *Tc1* insertion from 23 strains of *T. castaneum* collected in 15 countries worldwide and previously categorized as *M¹* (11 strains) or non-*M¹* (12 strains) based on an *in vivo* genetic test for maternal lethality (see *Materials and Methods*). Sequence analysis confirmed earlier diagnoses of *Medea* genotypes (8). Fig. 3 shows the results of an unrooted cluster analysis of sequence relatedness in the region of the *Tc1* insertion point. A striking observation that emerges from this comparison is that *M¹* strains are all nearly identical in sequence and constitute a single, distinct clade despite their widespread global distribution, spanning Central America, South America, Africa, East Asia, and Southeast Asia. Non-*M¹* strains of equally diverse origins constitute a second, more highly variable clade. This suggests a single origin of the *M¹* insertion followed by worldwide spread in recent evolutionary time, as has been suggested for organophosphate insecticide resistance in *Culex pipiens* (19). Sequence variation among non-*M¹* strains, as well as differences between *M¹* and non-*M¹*, rapidly diminish >300 nt from the insertion point on either side (Fig. S5). The clade differentiation between *M¹* and non-*M¹* is not apparent in the 3' UTRs of the flanking *blot* and *hiw* genes.

***M¹* Is Sandwiched Between Two Genes with Both Maternal and Zygotic Components.** The *blot* gene, like *Medea* itself, has both maternal and zygotic functions, at least in *Drosophila* (14). The *Drosophila* Highwire protein negatively regulates synaptic proliferation at neuromuscular junctions by binding to the Smad protein *Medea* (Med, no apparent relation to *Medea* in *Tribolium* despite the coincidence of names). In *Drosophila*, Med mediates synaptic proliferation via the BMP signaling cascade. Although *Drosophila* *highwire* is apparently a strictly zygotic gene, its binding partner Med is required both maternally and zygotically (20). However, there is no obvious mechanism that connects the mixed maternal/zygotic attributes of either *blot* or *hiw* with those of the closely juxtaposed *Medea* locus.

Discussion

Chen *et al.* (21) constructed a synthetic *Medea* factor in *Drosophila* based on our conception of a maternal poison acting in concert with a tightly linked zygotic antidote. The lethal component in this design was a maternally expressed microRNA

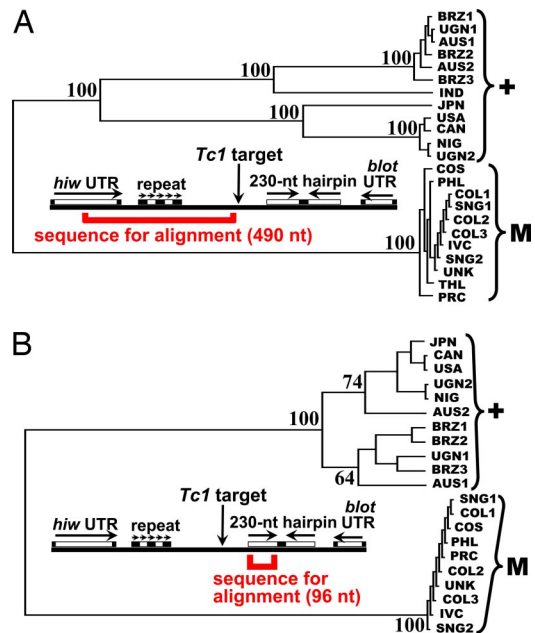


Fig. 3. Phylogenetic analysis of insertion-site sequences. Shown are unrooted phylogenetic trees based on multiple alignments of sequences flanking both sides of the *Tc1* insertion point. Trees are shown in Phylip format, with bootstrap values indicated. Data in *A* are based on "left"-flanking sequences (centromeric side of insertion), and data in *B* are based on right-flanking sequences (telomeric side of insertion). Segment lengths refer to the non-*Medea* GA2 (USA) strain. Countries of origin include Brazil, Uganda, Australia, India, Japan, the United States, Canada, Nigeria, Costa Rica, the Philippines, Colombia, Ivory Coast, Singapore, Thailand, and Peoples' Republic of China, abbreviated BRZ, UGN, AUS, IND, JPN, USA, CAN, NIG, COS, PHL, COL, IVC, SNG, THL, and PRC, respectively. UNK was collected by Alexander Sokoloff before 1970 and is of unknown origin. The USA (GA2) strain, which was the source of the whole-genome sequence, was collected in 1983. All other strains were collected in 1988. Brackets to the right indicate grouping of *M¹* ("M") and non-*M¹* ("+") strains, as determined by genetic testing (see *Materials and Methods*).

(miRNA) that silenced the maternally required *myd88* gene. The rescue component was a zygotically expressed variant of *myd88* that carried a deletion rendering it insensitive to the miRNA toxin. The (early) zygotic expression of the *myd88* antidote proved effective in fulfilling a function that is normally provided maternally. As predicted (7), this artificial *Medea* chromosome supplanted its nonselfish homolog in population cage studies. It is unknown whether *Medea* in *Tribolium* functions in a similar manner or by some still-unforeseen mechanism. Although there are several pseudogenes and one potentially intact gene within the *M¹* insertion, the transcriptional activity of these regions has not been examined.

One way to gain insight into the *Medea* mechanism might be through molecular analysis of *Medea* revertants. We have isolated five such mutations after γ -irradiation of *M¹* males. Each revertant chromosome has lost maternal-lethality while retaining zygotic rescue (unpublished observations). Identification of the underlying molecular lesions and sequence analysis of the insertion regions of revertant chromosomes could reveal motifs required for *M*-related lethality.

In addition to *M¹* there is at least one other *Medea* factor, namely *M⁴*, that could be targeted for positional cloning. The sequence of the *M⁴* locus is unknown, but it is the most prevalent *Medea* factor found in natural populations of *Tribolium* worldwide (8). Like *M¹*, it maps near one extreme end of the third linkage group, but on the opposite chromosome arm. These two factors display identical maternal-lethal and zygotic-rescue behavior but do not cross-

rescue: a zygotic copy of M^1 does not prevent the maternal-lethal action of M^4 , and vice versa (8). Interestingly, although they function independently and can easily be isolated from double-*Medea* strains by genetic segregation, M^1 has never been found in nature in the absence of M^4 (8).

Our data provide very strong evidence for a single origin of M^1 followed by global dispersal. The current, patchy distribution of M^1 (and perhaps also M^4) might exist as a remnant of the original patterns of global spread, or it could reflect regional differences in the outcome of conflict between *Medea* and non-*Medea* genotypes. One evolutionary hypothesis is that each *Medea* element arose as a single mutational (insertion) event within a population and then spread. An alternative hypothesis is that the *Medea* type sequence was fixed in a species or population where it had no effect, but then a hybridization event occurred, perhaps even across species, and in the new genetic background it expressed its selfish behavior. In this scenario, one can imagine that the *Medea* type species carried a version of chromosome 3 that was fixed for both M^1 and M^4 and that the current distribution of M strains, in which M^1 is confined to a subset of the regions where M^4 is fixed, derived from secondary loss of M^1 in some areas after the selfish M^1 M^4 chromosome had spread widely.

A complete understanding of the *Medea* syndrome will not be attained without additional studies of the hybrid incompatibility factor “*H*” on the ninth linkage group (22). This factor, naturally occurring in several *Tribolium* strains from India where *Medea* is notably absent, is so named because of its incompatibility with either of the third linkage group *Medea* factors M^1 or M^4 (23). Hybrid beetles carrying one copy of *H* and one copy of either M^1 or M^4 die as larvae. In the presence of the *H* factor, the lethal effect of M is zygotic rather than maternal, and there is no zygotic protection. The discovery of *H*-incompatibility, the positional cloning and molecular characterization of the M^1 locus, and the isolation of several M^1 knockout mutations coupled with the completion of the genome sequence, the availability of expression microarrays, the development of efficient germ-line transformation protocols, and the power of RNA interference in *Tribolium* at long last provide the tools required to examine the underlying mechanisms for this unique and fascinating phenomenon.

Materials and Methods

BAC Library Construction and Positional Cloning. A genomic BAC library was prepared from the highly inbred, non-*M* strain GA2. High-molecular-weight DNA was partially digested by using a combination of EcoRI and EcoRV methylase, then size-fractionated by pulsed-field gel electrophoresis. DNA fragments were cloned into the pBACe3.6 vector between the two EcoRI sites. A sequence tagged site ≈ 1 cm from the target was used to initiate the bidirectional walk. For each step the library was screened with overgo probes derived from BAC end sequences. The BACs were ordered and oriented by PCR of end sequences, and the walk was oriented by analysis of recombination events occurring within the BAC contig (Fig. 1A). After identifying the correct orientation, the walk was continued unidirectionally. The walk hit an obstruction in the telomere-proximal direction (toward the target M^1 locus) in the form of a highly repetitive region that could not be crossed in the EcoRI library. This obstacle was circumvented by taking one step in a HindIII library, after which the walk resumed in the EcoRI library, proceeding to a point beyond the target M^1 locus. A second genomic BAC library was prepared by Amplicon Express,

from the Singaporean M^1 strain in the pECBAC1 cloning vector. Genomic DNA was partially digested with MboI and ligated into the BamHI cloning site.

Sequence Analysis. The M^1 BAC was sequenced and assembled by the Human Genome Sequencing Center at Baylor College of Medicine. The 21.5-kb insertion was compared with the sequenced *T. castaneum* genome (BLASTN), and the insertion sequence, as well as the corresponding GA2 regions, were analyzed for the presence of known genes, gene predictions, or other potential coding regions by tBLASTN and BLASTX. DNA templates obtained from world strains were sequenced by using an ABI 373A DNA sequencer (College of Veterinary Medicine, Kansas State University).

Fiber Fluorescence in Situ Hybridization (Fiber-FISH). Chromosome fibers were prepared from M^1 -strain, whole fifth-instar larval nuclei using the method of Jackson *et al.* (24). Fibers were hybridized to the biotin-conjugated (green fluorescent) GA2-strain (non-*Medea*) BAC clone 40D19 (GenBank accession no. AC154135), which includes the empty site of the M^1 -associated insertion, and digoxigenin-conjugated (red fluorescent) M^1 -strain BAC clone 22J23 (GenBank accession no. AC205539), which contains the insertion.

Phylogenetic (Cluster) Analysis of Insertion-Site Sequences. Multiple sequence alignments (AlignX; Invitrogen, followed by manual adjustment) were used to construct unrooted phylogenetic trees using TreeTop (www.genebee.msu.edu/services/phtree-reduced.html) with the following settings (extra tree format: Phylip; picture format: Unrooted). Separate alignments were made based on the 490-nt segment extending from the middle of the *hiw* 3' UTR to the *Tc1* insertion point (centromeric side of insertion) and on the 96-nt segment that comprises the left stem of the 230-nt hairpin (telomeric side of insertion). Sequences were derived from 12 non- M^1 and 11 M^1 strains collected in 15 countries.

Correlation Between *Tc1* Insertion and *Medea* Genotype in Wild-Caught *T. castaneum*. Twenty-three strains from 15 countries worldwide were tested for *Medea*¹-associated maternal-lethal activity, then subjected to sequence analysis in the vicinity of the insertion site. Maternal-lethal activity was tested by “marker exclusion” as follows: Crosses were established in triplicate between females from each test strain and non- M^1 *3P2/au*¹⁴ males (one female and two males for each cross), where *3P2* represents a dominantly marked, second linkage group balancer tightly linked to M^1 , and *au*¹⁴ is a lethal balanced by *3P2*. Females were allowed to mate and oviposit for 10 days, after which time genomic DNA was isolated from each female for PCR-sequence analysis (see below). F₁ female *3P2*^{*} progeny were collected from each cross, where * indicates the test chromosome (M^1 or non- M^1). The females were test-crossed in small groups to non- M^1 , *mas p au* males (five of each sex). At least 100 progeny from each test cross were collected and scored for the dominant *3P2* phenotype. Non-*Medea* lines were expected to produce $\approx 50\%$ *3P2*-bearing progeny, whereas *Medea*¹ lines were expected to produce no *3P2* progeny, because of M^1 -induced maternal lethality to zygotes bearing this non-*Medea* chromosome.

After the above-described genetic diagnoses, females were tested for the presence of the *Tc1* insertion by PCR, and sequences flanking the insertion sites or insertion junctions were determined. PCR was done in two ways: in the first experiment, the PCR mixture had three primers, namely a forward *hiw* primer common to M^1 and non- M^1 , a reverse primer specific for M^1 , and another reverse primer specific for non- M^1 . In this way heterozygotes could be differentiated from either homozygote in a single reaction. In addition, we performed separate PCRs specific for M^1 and non- M^1 . All experiments were double-blind. See the Fig. 3 legend for strain names and geographic origins.

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