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The Normal Function of a Speciation Gene, Odysseus, and Its Hybrid Sterility Effect

Sha Sun, *et al.*

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The Normal Function of a Speciation Gene, *Odysseus*, and Its Hybrid Sterility Effect

Sha Sun,[1]* Chau-Ti Ting,[2] Chung-I Wu[1]†

To understand how postmating isolation is connected to the normal process of species divergence and why hybrid male sterility is often the first sign of speciation, we analyzed the *Odysseus* (*OdsH*) gene of hybrid male sterility in *Drosophila*. We carried out expression analysis, transgenic study, and gene knockout. The combined evidence suggests that the sterility phenotype represents a novel manifestation of the gene function rather than the reduction or loss of the normal one. The gene knockout experiment identified the normal function of *OdsH* as a modest enhancement of sperm production in young males. The implication of a weak effect of *OdsH* on the normal phenotype but a strong influence on hybrid male sterility is discussed in light of Haldane's rule of postmating isolation.

The evolution of reproductive isolation is an intriguing process. Obviously, there is no advantage for a gene to induce inviability or sterility. Postmating isolation mechanisms such as hybrid sterility must be a by-product of species divergence or, more specifically, functional divergence of the underlying genes (*1*). Therefore, to understand the molecular mechanisms of speciation, we need to study the individual genes and ask the two following questions: (i) what are the normal functions of genes of reproductive isolation? and (ii) what is the connection between the normal function in the pure species and the incompatibility effect in the hybrids?

We address these questions by studying the hybrid male sterility gene, *OdsH* (*Odysseus*), in *Drosophila*. *OdsH* is a fast-evolving homeobox gene that causes male sterility in the *D. simulans* background when co-introgressed with closely linked factors from *D. mauritiana* (*2*, *3*). Divergence in *OdsH* among closely related species has been observed for both DNA sequences and expression patterns. *OdsH* has been evolving away from the ancestral embryonic function toward a predominantly spermatogenic expression (*4*). We used three approaches, gene expression assay, gene transformation, and gene knockout, to link the hybrid sterility effect of *OdsH* to its normal function in reproduction. The expression analysis was carried out with RNA in situ hybridization in the testes of fertile and sterile hybrids (Fig. 1). The fertile and sterile lines we used differ by only 3 kb, which spans the exons 3 and 4 of *OdsH* (fig. S1) (*2*), but are otherwise genetically identical. Figure 1A shows the expression of *OdsH* in the sterile introgression line. The striking observation is the strong accumulation of the *OdsH* transcript near the apical tip of the testis. This distinct pattern is not observed in the fertile line (Fig. 1B), nor in any of the pure species assayed, including *D. simulans* and *D. mauritiana* (*4*). The apical region of the testis contains primarily premeiotic cells that have not yet entered the rapid growth phase when transcription becomes highly active (*5*). Apparently, the testicular expression of *OdsH* is misregulated in the sterile introgression line. We reproduced this expression pattern for a comparison with the expression of *unc-4*, the duplicate homolog of *OdsH* (fig. S2). The expression of *unc-4* shows no transcript accumulation at the apical end and is comparable between the sterile (fig. S2C) and the fertile (fig. S2D) lines.

In addition to the misexpression of *OdsH*, we tested the sterility effect from *OdsH* coding sequence using transgenic analysis. *OdsH* full-length cDNA was driven by the testis-specific β*2-tubulin* promoter (*6*, *7*). We used *OdsH* alleles from *D. simulans* ($OdsH^{sim}$) and *D. mauritiana* ($OdsH^{mau}$). The P-element constructs, $P\{w^+$, β*2-tubulin*::$OdsH^{sim}\}$ and $P\{w^+$, β*2-tubulin*::$OdsH^{mau}\}$, were each injected into *D. simulans*, and the expression of each transgene was confirmed by reverse transcription polymerase chain reaction. We observed no sterility effect with the transgenes (heterozygous or homozygous) in the pure *D. simulans* background. This observa-

[1]Department of Ecology and Evolution, University of Chicago, Chicago, IL 60637, USA. [2]Department of Life Science, National Tsing Hua University, Hsinchu, Taiwan 300, ROC.

*Present address: Department of Molecular and Cell Biology, University of California, Berkeley, 16 Barker Hall, MC 3204, Berkeley, CA 94720, USA.
†To whom correspondence should be addressed. E-mail: ciwu@uchicago.edu

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tion corroborates the genetic evidence that a complex of genetic interactions often underlies hybrid incompatibility (*3*, *8*). In the sensitized genetic background carrying an appropriate fertile introgression from *D. mauritiana* (*3*) (fig. S1), the transgenes (heterozygous or homozygous) indeed lowered male fertility. This fertile introgression line is the same one used in Fig. 1B and is itself free of the sterility effect (*3*). The sterility effect induced by β*2-tubulin*::*OdsH*^{mau} is significantly more intense than that induced by β*2-tubulin*::*OdsH*^{sim} (Table 1). Thus, the agreement between the transgenic study and the earlier genetic assays is as expected (*2*, *3*). Furthermore, that the *OdsH*^{mau} transgene can cause sterility despite the presence of the native *OdsH*^{sim} allele suggests the dominant sterility effect of *OdsH*.

If the observations on the sterility effect of *OdsH* imply a direct link between such a phenotype and the gene's normal function in the pure species, one might expect *OdsH* to be an essential component of spermatogenesis. On the other hand, two observations suggest that the sterility effect of *OdsH* might not be connected to its normal function. First, the expression pattern of *OdsH* in the testis of the sterile line is distinct from that of the fertile line or any of the pure species. Second, no sterility effect was observed with the testis-specifically expressed transgene in the pure *D. simulans* background. We therefore ask: Does *OdsH* play a role in normal spermatogenesis? How is this role, if any, related to the hybrid sterility effect? To answer these questions, we created a null mutant of *OdsH* by gene knockout.

The knockout procedure used is the "gene targeting" technique (*9*, *10*). A null mutant *OdsH*^{0} was obtained in *D. melanogaster* by a two-step scheme shown in Fig. 2 (*11*). The mutant allele carries three consecutive stop codons in the three reading frames at the beginning of exon 2, upstream of the homeobox domain (*2*). In all the measurements taken below, flies bearing the null allele, *OdsH*^{0}, were compared with flies bearing a normal *OdsH*$^{+}$. The latter allele was obtained by the same procedure as *OdsH*^{0} and hence came from an identical genetic background.

OdsH^{0} flies showed no observable defects in morphology, viability, or fertility in the absence of rigorous experimentation. Microscopic inspection of spermatogenesis and sperm motility (*3*, *5*, *12*) also revealed no discernable defects. The *OdsH* gene is thus "dispensable" according to these crude phenotypic assays. To measure the subtle fertility effect of *OdsH* more precisely, we carried out sperm-exhaustion experiments on young males, which are more susceptible to sperm exhaustion than are older males. Each male was given three virgin females when it was 2 days old and on every day afterward (*11*). As shown in Fig. 3, the fertility reduction in *OdsH*^{0} males is 61%, 45%, and 13% as males age from 2 to 3 to 4 days. Statistical analysis based on a linear mixed-effects model was carried out for the contributing effects from genotype, mating date, and their interaction, on the fertility of these males (*11*). The results indicate that *OdsH*^{0} significantly reduces male fertility ($P = 0.0015$). The reduction effect of *OdsH*^{0} is strongest on Day 2 (Fig. 3) ($P < 0.0001$). The *OdsH*^{0} effect on Day 3 is also significant ($P = 0.0085$). However, while the average number of sons from *OdsH*$^{+}$ males decreases significantly on Day 4 compared with Day 2 ($P = 0.0004$), the corresponding number from *OdsH*^{0} males increases on Day 4 ($P = 0.0446$). Thus, the effect of *OdsH*^{0} on Day 4 is not significant ($P = 0.5639$). Subsequent experiments have also shown that the fertility effect of *OdsH* on males more than 5 days old is weak or nonexistent (*13*). In summary, the function of *OdsH* is probably the enhancement of the fertility of very young males, perhaps by accelerating sperm maturation during the larval or pupal stages.

The knockout experiment was carried out in *D. melanogaster*, whereas the hybrid sterility was observed between its sibling species, *D. simulans* and *D. mauritiana*. Because the *OdsH* expression in testis is strongest in *D. melanogaster* among the sibling species (*4*), we infer that the effect of *OdsH*^{0} on male fertility in *D. melanogaster* would be as large as its possible effect in other species, for which gene targeting is still not feasible. The observation that *OdsH* is a fast-evolving gene does not contradict this inference, as most rapidly evolving genes are indeed dispensable for viability and fertility (*14*). If this inference is correct, the very weak sterility phenotype of the *OdsH*^{0} males is intriguing, given the strong role *OdsH* plays in the hybrid sterility between *D. simulans* and *D. mauritiana*.

In general, hybrid incompatibility results from a complex web of genetic interactions among loci of the two parental species. That a gene whose absence has only a mild effect on male fertility can play a

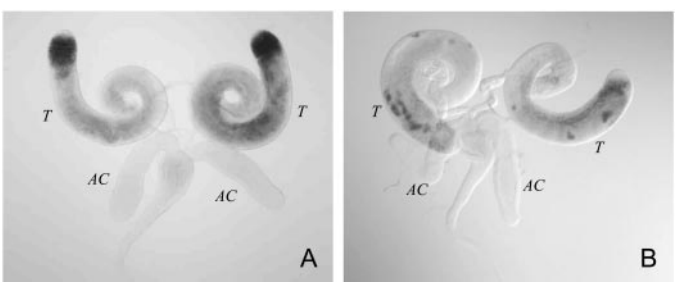


Fig. 1. *OdsH* gene expression in the male reproductive tissues of the sterile (**A**) and the fertile (**B**) introgression lines (see fig. S1). T, testis; AC, accessory gland. The pattern reported is robust and reproducible, as seen in multiple preparations; another example is shown in fig. S2.

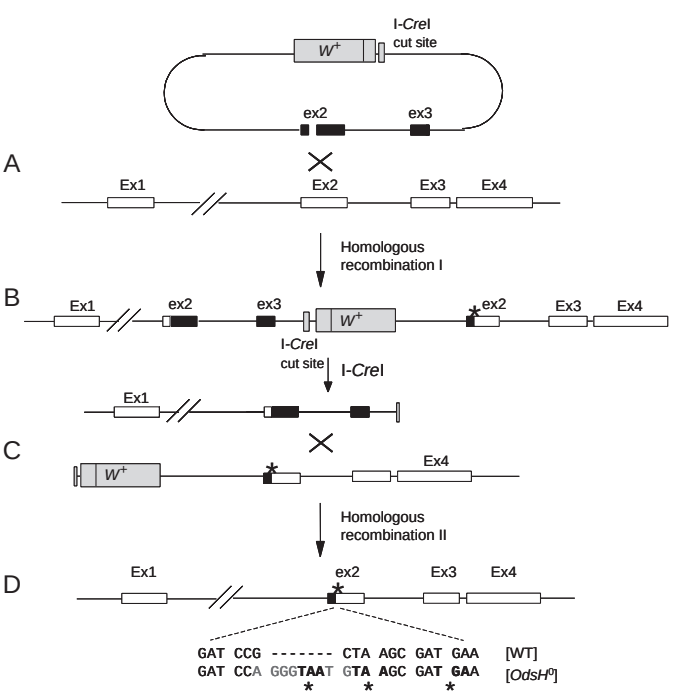


Fig. 2. The two-step "gene targeting" scheme for *OdsH* knockout [see (*11*) for details]. The asterisk indicates the frameshift in exon 2. The first-step homologous recombination (**A**) results in a site-specific insertion (**B**); the second homologous recombination was activated by the I-*Cre*I generated double-strand-break (**C**). The final product (**D**) is a null *OdsH*^{0} allele with stop codons in exon 2. These stop codons, in all three reading frames, are indicated.

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major role in sterilizing interspecific hybrids has far-reaching implications on the genetics and evolution of postmating isolation. It is most relevant to a long-standing question about reproductive isolation—why is there so much hybrid male sterility, often the first mechanism of postmating isolation to evolve during speciation (*15*)? Indeed, the number of genes contributing to hybrid male sterility between closely related species is large (*16*–*18*), whereas the number of hybrid inviability or female sterility genes is likely to be an order of magnitude smaller (*19*). This phenomenon is one of several components of Haldane's rule of postmating isolation (*15*, *20*). In species hybridization, this so-called "fast male evolution" plays a large role in restricting gene flow across species during the incipient phase of speciation (*19*, *21*, *22*).

A possible explanation for the rapid evolution of hybrid male sterility is sexual selection (*8*, *15*, *23*). Because genes pertaining to male reproduction have been shown to evolve rapidly in a diverse array of organisms, the explanation has gained considerable support (*24*–*27*). However, it is perplexing that spermatogenesis but not other aspects of male reproduction (accessory glands, for example) is preferentially involved in postmating isolation. In this respect, an alternative explanation may also have strong merit. In this explanation, spermiogenesis is particularly sensitive to perturbation in gene expression, perhaps due to the lack of postmeiotic transcription regulation (*15*, *28*). Slight misregulation of a few loci, which are buffered in other developmental processes without ill effect, may have drastic consequences on sperm formation. If any gene that plays even a minor role in normal spermatogenesis can be a major factor in sterilizing hybrids, many genes could potentially be involved in hybrid male sterility. [We should also note that the genetics underlying hybrid male sterility and hybrid inviability may be very different (*14*), as the contrast between detailed studies of the two aspects has revealed (*17*, *29*).] Therefore, the genetics and molecular biology of spermatogenesis uniquely underlie the phenomenon of "fast male evolution."

The evolution of postmating isolation remains one of the most intriguing processes in biology. Although it is central to the formation of biological species, the phenotypes (hybrid sterility or inviability) themselves offer no hint of their evolution. Furthermore, the observable phenotypes (e.g., hybrid male sterility) and the underlying genetic processes (e.g., spermatogenesis) may only be weakly coupled. For these reasons, an understanding of the evolution of postmating isolation would probably require an explicit knowledge of the underlying genes and their molecular functions. *OdsH* may provide a window to observe the connection between the normal function and its unpredictable manifestation in the hybrids.

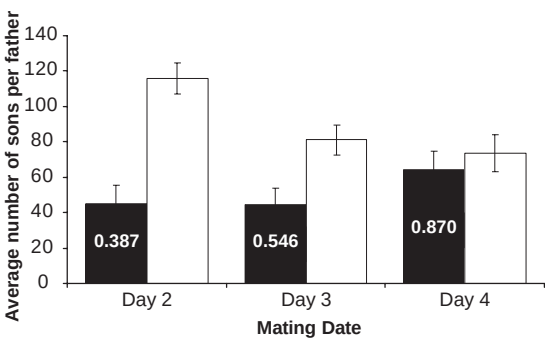


Fig. 3. Fertility comparison between $OdsH^0$ (black bar) and $OdsH^+$ (white bar) males at the young-adult stage under sperm-exhaustion conditions. Each male was given three virgin females each day, at ages 2, 3, and 4 days, consecutively. Offspring produced by those females were counted in accordance with the date of mating. The average number of sons per father (sample size, $N_{OdsH}{}^0 = 18$; $N_{OdsH}{}^+ = 20$) is shown for each mating date. As *OdsH* is on the X chromosome, the sons would have the identical genotype, regardless of the paternal genotype. [These sons all have the $(OdsH^+)$/Y genotype, where $(OdsH^+)$ denotes the wild-type $OdsH^+$ allele from the mother.] The ratio (the value shown in the black bar) of the numbers of sons from the two paternal genotypes is thus indicative of paternal fertility differences.

Table 1. The transgenic effect on male fertility. P-element containing the $OdsH^{sim}$ or $OdsH^{mau}$ cDNA, driven by the β2-*tubulin* promoter, was introduced into *D. simulans*. Male fertility was categorized by the quantity of motile sperm present in the seminal vesicles. The effects of $OdsH^{sim}$ and $OdsH^{mau}$ were compared in either the pure *D. simulans* background or in the fertile introgression background (*3*) (see fig. S1 for illustration). The *P* value was obtained with Fisher's exact test.

Genetic background	Transgene*	Motile sperm quality		Total no. of males	Transgene effect
		Normal†	Deficient†		
Pure *D. simulans*	$OdsH^{sim}$	8	1	9	None
	$OdsH^{mau}$	8	0	8	
Fertile introgression	$OdsH^{sim}$	36 (83%)	8 (18%)	44	$P < 10^{-6}$
	$OdsH^{mau}$	14 (27%)	38 (73%)	52	

*In the pure *D. simulans* background, the introduced gene is in double dose (homozygous), whereas in the background of fertile introgression, the introduced gene is in either single dose (heterozygous) or double dose. †The normal class is for males with an abundance of motile sperm in their seminal vesicles, whereas the deficient class is for males with few or no motile sperm. Few tested males were ambiguous by this classification.

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