

Review

Beetling around the genome

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Summary

The red flour beetle, *Tribolium castaneum*, has been selected for whole genome shotgun sequencing in the next year. In this minireview, we discuss some of the genetic and genomic tools and biological properties of *Tribolium* that have established its importance as an organism for agricultural and biomedical research as well as for studies of development and evolution. A *Tribolium* genomic database, Beetlebase, is being constructed to integrate genetic, genomic and biological data as it becomes available.

1. Studies in *Tribolium* will contribute to many areas of biological research

Beetles (the Coleoptera) comprise the largest and most diverse of all eukaryotic orders. They include many beneficial and deleterious species, the latter associated with billions of dollars' worth of agricultural losses annually. There are several compelling reasons to study the red flour beetle, *Tribolium castaneum*. First and foremost, *Tribolium* is one of the most sophisticated genetic model organisms among all higher eukaryotes. Among arthropods, only *Drosophila* offers greater power and flexibility of genetic manipulation. Second, *Tribolium* might prove to be invaluable in linking genome sequence information from *Drosophila*, honeybee and *Anopheles* with vertebrate gene annotation. Third, as a member of the most primitive order of holometabolous insects, the Coleoptera, it is in a key phylogenetic position to inform us about the genetic innovations that accompanied the evolution of higher forms with more complex development. Fourth, *Tribolium* offers the only genetic model for the profusion of medically and agriculturally important coleopteran species. Finally, many genetic and genomic tools have been developed for *Tribolium*, and both forward and reverse genetic approaches are available to facilitate functional genetic analysis.

Tribolium species host a large variety of protozoan and bacterial parasites and/or symbionts that provide

fertile material for the study of host–pathogen interactions. Like many arthropods, certain *Tribolium* species are infected with *Wolbachia*, a *Rickettsia*-like organism. Strains of some *Tribolium* species (e.g. *Tribolium confusum*) harbor *Wolbachia* as obligate intracellular symbionts or parasites, and show classic incompatibility syndromes when mated with *Wolbachia*-deficient strains (Wade & Stevens, 1985). Other species, such as *Tribolium madens*, are devoid of *Wolbachia* and develop lethal infections when artificially inoculated (Fialho & Stevens, 2000). Still others, including *T. castaneum*, appear to be refractory to *Wolbachia*, in that they are immune to infection and are host-incompetent. *Wolbachia* infection is being examined for potential use in reducing the population density of insect vectors of human disease, either directly or as a vehicle to drive vector incompetence or other favorable traits through populations (Brownstein *et al.*, 2003; Dobson, 2003; Rascon *et al.*, 2003). Similar approaches might be efficacious with respect to insect pests of agriculture.

As a major global pest of stored grain and cereal products, peanuts and many other dried and stored commodities for human consumption, *Tribolium* has a long history of exposure to pesticides. It has proved to be readily adaptable to all classes of insecticides and fumigants, having developed resistance via oxidative and hydrolytic metabolism, target insensitivity, and other mechanisms (Andreev *et al.*, 1994; Beeman & Nanis, 1986; Beeman & Stuart, 1990). These features,

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in combination with excellent genetic tractability, recommend *Tribolium* as an ideal subject for the identification of new pesticide targets through knowledge of resistance mechanisms (Andreev *et al.*, 1994; Beeman *et al.*, 1992b).

Bioactive agents with potential impact on human health and biology have long been sought in the plant and fungal biodiversity present in the tropical rain forests. Beetles, with their unparalleled species and habitat diversity, might be a major untapped source of antibiotics and biopharmaceuticals. In *Tribolium*, *p*-benzoquinones, aliphatic hydrocarbons and other potential repellents, irritants, toxicants and antifungal or antibacterial components are produced in large 'stink' glands (Blum, 1981). Naturally occurring polymorphism in antibiotic potency has been demonstrated in at least two *Tribolium* species (Prendeville & Stevens, 2002), and several mutations have been identified that affect the biosynthesis and secretion of stink gland components (Hoy & Sokoloff, 1965; Englert, 1966; Beeman *et al.*, 1996). *T. castaneum* was the first animal species ever reported to produce prostaglandin synthetase inhibitors (Howard *et al.*, 1986). Given the importance of anti-inflammatory drugs acting through the prostaglandin pathway, the detection of this class of inhibitory compounds in *Tribolium* demonstrates the potential for drug discovery via mining the *Tribolium* genome.

The first maternal effect selfish (*Medea*) genes were discovered in *Tribolium* (Beeman *et al.*, 1992a), and similar mechanisms of maternal selection of selfish genes have subsequently been discovered in mammals (Hurst, 1993; Peters & Barker, 1993).

2. Studies in *Tribolium* contribute to our understanding of evolution and development

As a coleopteran, *Tribolium* is in a key phylogenetic position to inform us about the genetic innovations that accompanied the evolution of higher insects. Among winged insects, the relatively more primitive orders are placed in the hemimetabola. These insects do not develop morphologically distinct larval and adult forms but rather undergo a series of molts in which earlier stages (nymphs) resemble miniature adults. By contrast, higher insects, which undergo complete metamorphosis, comprise the holometabola. Larval forms are worm-like, then transform into pupae within which develop adult insects of much different appearance. Comparative genomics promises to improve our understanding of the evolutionary changes that accompanied the rise of the holometabola, as well as other morphological specializations. The Coleoptera occupy a basal position among the holometabola. In comparison, the Diptera (which include *Drosophila melanogaster*, the most highly characterized insect) is one of the most advanced orders. Beetles and flies

diverged close to 300 million years ago (Kristensen, 1999). One of their more conspicuous differences is embryonic segmentation, which occurs simultaneously along the anterior–posterior axis in *Drosophila* (long-germ development) but sequentially from anterior to posterior in more primitive insects (and vertebrates) (Davis & Patel, 2002). Insect larvae typically have heads derived from several segments including gnathocephalic segments with appendages that function in feeding. During *Drosophila* embryogenesis, all of these segments move through the presumptive anterior opening of the digestive tract to occupy internal positions, where they elaborate evolutionarily novel structures. The resulting larva is essentially headless and also lacks the thoracic limbs characteristic of almost all insects. By contrast, related developmental events in *Tribolium* are much more generalized. Larvae have typical head and gnathocephalic segments, and bear thoracic limbs. Because head segments remain in a linear order, studies in *Tribolium* will contribute to a comprehensive understanding of head and, ultimately, brain development. *Tribolium* head development is probably very similar to that in hemimetabolous insects, including grasshoppers, in which brain development has been very carefully described (Watson & Schurmann, 2002), but in which functional approaches are limited compared with *Tribolium*.

Advances in cellular, developmental and neurobiology are predominantly gained by studies of model organisms amenable to a range of genetic and biochemical approaches. Many of the intricacies of embryonic patterning have been elucidated by genetic and molecular studies in *Drosophila* (for review see Driever, 1993; Martinez Arias, 1993; Pankratz & Jäckle, 1993; Sprenger & Nüsslein-Volhard, 1993). However, many aspects of *Drosophila* development are highly derived, and evidence suggests that the underlying genetic regulation is equally derived. Comparative studies in several insects have provided insight into many developmental processes. *Tribolium* has figured prominently in these contributions, as highlighted below.

The establishment of anterior–posterior and dorsal–ventral coordinates in early embryos is one area in which *Drosophila* has been shown to display highly specialized regulatory mechanisms. Rapid (24 h) embryonic development in *Drosophila* is facilitated by the maternal contribution of factors that define the egg coordinates. However, there is mounting evidence that the gene encoding the anterior morphogen Bicoid evolved within the dipteran lineage by gene duplication and divergence (Brown *et al.*, 2001; Stauber *et al.*, 2002). In fact, recent analysis indicates that *hunchback* and *orthodenticle* pattern the anterior region of the *Tribolium* embryo and might be part of an ancestral patterning system (Schroder, 2003). The dorsal–ventral axis in *Tribolium* appears to be

patterned by gradients of Toll receptor and Dorsal protein centered on the ventral midline (Maxton-Kuchenmeister *et al.*, 1999; Chen *et al.*, 2000), which regulate *twist* (Sommer & Tautz, 1994) and *decapentaelegic (dpp)* (Sanchez-Salazar *et al.*, 1996) expression, as in *Drosophila*. However, these gradients are formed by zygotic regulation of transcription in *Tribolium*, implying that the local activation of maternally supplied Toll receptor and ventrally limited nuclear localization of Dorsal are derived features of dorsal–ventral patterning in *Drosophila*. Expression of *caudal*, *tailless* and *forkhead* homologs in *Tribolium* suggest that posterior, but not anterior, aspects of the terminal patterning system are conserved (Schulz *et al.*, 1998; Schroder *et al.*, 2000).

Comparative analysis of segmentation in *Tribolium* has shown that gap and pair-rule genes are expressed in conserved patterns (Sommer & Tautz, 1993; Wolff *et al.*, 1995; Brown & Denell, 1996) but forward and reverse genetic approaches suggest that conserved expression patterns do not always indicate conserved function. For example, a deletion that removes the pair-rule gene *fushi tarazu* does not result in a pair-rule phenotype in *Tribolium* (Brown *et al.*, 1994a). These results emphasize the importance of comparative studies using genetically tractable insects such as *Tribolium*. However, it is likely that segment boundaries are formed by an ancient mechanism, as evidenced by the highly conserved expression patterns of the segment polarity genes *wingless* and *engrailed* (Nagy & Carroll, 1994; Brown *et al.*, 1994b) as well as functional studies (Oppenheimer *et al.*, 1999). Genetic analysis of homeotic mutants in *Tribolium* provided the first evidence for a contiguous cluster of Hox genes (Beeman, 1987), as later observed in vertebrates. Genetic and molecular analysis of *Tribolium* homeotic genes and mutants continues to correlate changes in regulatory gene interactions and morphological evolution, and shows that some Hox gene functions in *Drosophila* are highly specialized for dipteran-specific adaptation and are not representative of most taxa (Brown *et al.*, 2000, 2002b; Lewis *et al.*, 2000; DeCamillis *et al.*, 2001).

Formation of limb fields requires expression of *dpp* in *Drosophila* but not in *Tribolium* (Jockusch *et al.*, 2000). Although several genes required for imaginal leg development in *Drosophila* are also expressed in developing embryonic legs in *Tribolium*, their relative expression patterns indicate that there are significant differences in the genetic regulation of leg development (Beermann *et al.*, 2001; Prpic *et al.*, 2001). The expression patterns observed in *Tribolium* are similar to other insects, whereas those in *Drosophila* are specialized, perhaps reflecting development from imaginal discs.

The organization of the central nervous system (CNS) is highly conserved in arthropods. In

Drosophila, the segmentation and homeotic genes are expressed in the developing CNS and are required for proper neuronal differentiation. In *Tribolium*, segmentation and homeotic genes are also expressed in the developing CNS in segmentally reiterated patterns that closely resemble those of their *Drosophila* counterparts. Recently, sequence analysis of bacterial artificial chromosome (BAC) clones containing proneural genes in *Tribolium* has provided insight into the evolution of the achaete/scute complex (Wheeler *et al.*, 2003). Comparison of the function of the single *Tribolium* *achaete/scute* homolog with that of the *Drosophila* proneural *ac/sc* genes suggests that the *Drosophila* *ac/sc* genes acquired new developmental roles in specifying the fate of neural precursors while maintaining an ancestral function in their formation. Further analysis indicates that *ventral neurons defective (vnd)*, *intermediate neurons defective (ind)* and *muscle segment homeobox (msh)* are expressed in *Tribolium* in patterns largely similar to those of their *Drosophila* homologs. However, gaps in the expression of *vnd* indicate that some neurons in the *Tribolium* CNS must be patterned by different genes (Wheeler *et al.*, 2003).

3. Many genetic and genomic tools enhance *Tribolium* research

Tribolium has been raised for more than four decades in the laboratory and thrives on a simple diet of wheat flour supplemented with 5% yeast. It is easily manipulated, and tolerates crowding and inbreeding. The generation time is flexible (3–8 weeks, depending on the rearing temperature), and adults have long reproductive lives. *Tribolium* eggs are approximately twice the size of *Drosophila* eggs, and most protocols developed for experimental manipulation in *Drosophila*, including *in situ* hybridization and immunohistochemistry, work well in *Tribolium*. *Tribolium* lacks polytene chromosomes but mitotic and meiotic spreads are easily obtained (Stuart & Mocelin, 1995). Nine autosomes and X/Y sex chromosomes compose the chromosomal complement in *Tribolium castaneum*, and recombination occurs in both sexes.

Among beetles, sophisticated genetic manipulations are possible only in *Tribolium*. Genetic screens in *Tribolium* have produced a wealth of morphological, physiological and developmental mutants. At the genetic stock center in Manhattan, Kansas, there are over 300 mutant strains that are easily maintained at room temperature by subculturing every 3–4 months. Gain-of-function mutations have been reverted by mutagenesis to reveal null phenotypes (Stuart *et al.*, 1993; Brown *et al.*, 2000; Shippy *et al.*, 2000). Mutant alleles of homeotic genes not previously identified by mutation were identified in screens designed to saturate the region of the HOMC uncovered by deficiencies (Brown

et al., 2000). For several regions, chromosomal rearrangements have been induced to generate balancer chromosomes (Beeman *et al.*, 1986), which facilitate stock maintenance and genetic manipulation.

Tribolium is likely to be an important resource for genome annotation. Analysis of the recently published *Anopheles* genomic sequence indicates that mosquito and fly genes are markedly less similar than expected for two taxa separated for about 200 million to 250 million years (De Gregorio & Lemaitre, 2002). Furthermore, when *Tribolium*, *Drosophila* and human sequences are compared, the *Tribolium* sequences are often more similar to their human counterparts than are their *Drosophila* homologs (D. Tautz, personal communication; R. Beeman, unpublished). In fact, some *Drosophila* gene sequences have diverged to the point that BLAST analysis of certain honeybee expressed sequence tags (ESTs) found better matches among chordates than in *Drosophila* (Whitfield *et al.*, 2002). For most of these, *Drosophila* homologs could be identified but, in some cases, the *Apis* coding sequences have no match in the *Drosophila* genome, indicating that these genes were lost in the *Drosophila* lineage. These results indicate that data from other insect orders will be required to link human genes to their *Drosophila* homologs. In fact, the relationship between the *Drosophila zen* and human *HOX3* genes was elucidated by comparisons including data from *Schistocerca* and *Tribolium* (Falciani *et al.*, 1996). More recently, in a chromosomal walk to clone several *Tribolium* genes positionally, we identified the insect ortholog of a human muscular dystrophy gene that was not identified by direct comparison with *Drosophila* sequence (R. Beeman, unpublished). Thus, analysis of the *Tribolium* genome will provide informative comparisons for the identification of insect homologs of human genes. Moreover, *Tribolium* is the most efficient system in which to perform functional analysis of those genes lost in the *Drosophila* lineage but conserved in other insects.

We have developed several genetic tools that will allow the new sequence information to be used effectively. Tools for reverse genetic analysis in *Tribolium* include RNA interference and transformation. Maternal and/or zygotic mRNAs can be depleted in *Tribolium* by injecting double-stranded RNA into the abdomen of female pupae (Bucher *et al.*, 2002) or freshly laid eggs (0–2 h old) (Brown *et al.*, 1999). Thus, *Tribolium* is well suited to high-throughput genome-wide RNA interference screens, as in *Caenorhabditis elegans* (Maeda *et al.*, 2001; Kamath *et al.*, 2003). Such screens will identify embryonic and maternal genes, as well as genes with functions relevant to basic cell biology that produce phenotypes affecting oogenesis.

Using the piggyBac vector and eye-specific transformation markers, it is now possible to introduce

DNA into the *Tribolium* genome (Berghammer *et al.*, 1999; Lorenzen *et al.*, 2002, 2003). Several researchers are designing vector constructs to induce tissue- and stage-specific expression of introduced genes and double-stranded RNA constructs. In addition, several labs are preparing to perform genome-wide screens using transposon-mediated mutagenesis.

The *Tribolium* genome is approximately 0.2 pg or 200 Mb, based on hybridization kinetics (Cot analysis) (Brown *et al.*, 1990) and microdensitometric quantitation of Feulgen-stained spermatids (Alvarez-fuster *et al.*, 1991). Unique sequences compose more than 60% of the genome and repetitive DNA displays a long-period interspersed pattern. Several satellite DNA sequences have been identified that are conserved between *Tribolium* species (Juan *et al.*, 1993) and are clustered in putative centromeric regions (Plohl *et al.*, 1993). A strain of *T. castaneum* that had been inbred by single-pair matings of full siblings for 20 consecutive generations (S. Thomson, University of Wisconsin, Parkside, unpublished) was the source of DNA for construction of BAC libraries, as well as molecular and physical maps. This strain is currently maintained in several laboratories in the USA and will be used as the source of DNA for whole genome shotgun sequencing.

The linkage groups of the original recombination map were identified by morphological mutations, whereas physiological and biochemical markers have since been added. A higher resolution recombination map based on molecular markers is under construction. This map contains over 400 markers from BAC end sequences, ESTs and developmentally important genes identified by individual researchers. In addition, we are constructing a physical map based on *HindIII* digests of the more than 27,000 clones in a BAC library (Exelixis Pharmaceutical, South San Francisco). The molecular and physical maps will be integrated by including BAC end sequences in the molecular map. Together, these resources provide a high-resolution scaffold on which to assemble the genome sequence. Several BACs sequenced by shotgun methods were assembled with no difficulty (Brown *et al.*, 2002a; Wheeler *et al.*, 2003).

It will be a major undertaking to annotate the *Tribolium* genome sequence. There are several programs available to automate most of the process, and we have several sources of cDNA sequence data available to train a gene-finding model specifically for *Tribolium*. Researchers at Exelixis Pharmaceutical have sequenced more than 8800 ESTs derived from adult tissue and assembled them into more than 4600 contigs. More than 2000 additional ESTs from embryonic tissue have been sequenced and assembled into a minimum of 586 non-redundant clones (D. Tautz, personal communication). In addition, cDNAs from developmental studies by individual researchers are

also available for annotation purposes. It is likely that a significant amount of annotation will be facilitated by comparison with other insect genomes. In addition, the sequence of the *Tribolium castaneum* mitochondrial genome has recently been published (Friedrich & Muqim, 2003). Clearly, the rationale to sequence the *Tribolium* genome is strongly supported by the plethora of genomic resources already available to facilitate the assembly and analysis of the *Tribolium* genome sequence. To make this information available to the scientific research community, we are constructing a *Tribolium* genomic database, Beetlebase, that will integrate genetic, genomic and biological data as it becomes available.

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