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Relationships among pest flour beetles of the genus *Tribolium* (Tenebrionidae) inferred from multiple molecular markers

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Abstract

Model species often provide initial hypotheses and tools for studies of development, genetics, and molecular evolution in closely related species. Flour beetles of the genus *Tribolium* MacLeay (1825) are one group with potential for such comparative studies. *Tribolium castaneum* (Herbst 1797) is an increasingly useful developmental genetic system. The convenience with which congeneric and other species of tenebrionid flour beetles can be reared in the laboratory makes this group attractive for comparative studies on a small phylogenetic scale. Here we present the results of phylogenetic analyses of relationships among the major pest species of *Tribolium* based on two mitochondrial and three nuclear markers (*cytochrome oxidase 1, 16S ribosomal DNA, wingless, 28S ribosomal DNA, histone H3*). The utility of partitioning the dataset in a manner informed by biological structure and function is demonstrated by comparing various partitioning strategies. In parsimony and partitioned Bayesian analyses of the combined dataset, the *castaneum* and *confusum* species groups are supported as monophyletic and as each other's closest relatives. However, a sister group relationship between this clade and *Tribolium brevicornis* (Leconte 1859) is not supported. Therefore, we suggest transferring *brevicornis* group species to the genus *Aphanotus* Leconte (1862). The inferred phylogeny provides an evolutionary framework for comparative studies using flour beetles.

Keywords

Tribolium; *Aphanotus*; Bayesian phylogenetics; data partitioning; RNA doublet modeling

Introduction

Studies of developmental evolution have increasingly focused on small phylogenetic scales. In part, this has been driven by technical advances, such as RNA interference (Brown et al., 1999) and promiscuous transposon-based transgenic methods (Berghammer et al., 1999; Pavlopoulos et al., 2004), which allow investigators to more easily study genetic functions in related non-model species. The availability of whole genome sequence data has also facilitated the isolation and study of orthologous genes from non-model species. Such comparative studies have revealed the evolutionary flexibility of developmental mechanisms at the species level (e.g. Raff et al., 1999; Kopp et al., 2000; Gompel and Carroll, 2003; Hoekstra and Nachman, 2003; Voss and Smith, 2005; Kronforst et al., 2006; Protas et al., 2006), allowing insights into the evolution of novel phenotypes (e.g. Wittkopp et al., 2002) as well as the conservation of ontogenetic functions (e.g. Abouheif and Wray, 2002).

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Many of these studies have explored genera in which one species is an established developmental genetic model organism. Comparative studies of development in drosophilids (e.g. Kopp and True, 2002) and teleosts related to the zebrafish (Parichy, 2006) have benefited from the genetic models and experimental methods transferable from the model species *Drosophila melanogaster* and *Danio rerio*, respectively. In these and other groups, congeneric species often possess divergent phenotypes, which are experimentally accessible by developmental genetics, morphometrics, or other methods. A phylogenetic framework facilitates work in groups of closely related species because it identifies species at various evolutionary distances to the model species and provides information about the polarity of evolutionary changes.

Here we present a phylogenetic study of pest flour beetle species in the genus *Tribolium* MacLeay (1825), based on five markers from the mitochondrion and nucleus totaling 3106 bp. *Tribolium castaneum* (Herbst 1797) is a promising genetic model organism with a genome sequencing project currently underway (Brown et al., 2003) and amenable to sophisticated methods of genetic manipulation. The genetic and developmental methods applicable to *T. castaneum* provide a starting point for examination of related species. The genus *Tribolium* includes 36 described species (Table 1), many of which are easily cultured in the lab. A robust phylogenetic framework will inform future comparative studies employing additional members of this genus.

Tribolium as an experimental organism

The red flour beetle *Tribolium castaneum* is an increasingly versatile model organism. Classically, this species has been used in population genetic studies (e.g. Park et al., 1964; Wade, 1976) and studies of mutagenesis (e.g. Sokoloff et al., 1963; Sulston and Anderson, 1996). In recent decades, this knowledge of *Tribolium* biology and genetics has enabled studies of population differentiation (DeMuth and Wade, 2007a; 2007b), comparative reverse genetic studies (Brown et al., 1994; Tomoyasu et al., 2005; Ober and Jockusch, 2006; Savard et al., 2006), and sophisticated transgenic experiments (Eckert et al., 2004; Lorenzen et al., 2007). The experimental potential of *T. castaneum* is likely to expand rapidly, due to sequencing and annotation of the genome now underway (Brown, et al., 2003) and the recent publication of a fine-scale physical and molecular map of the genome (Lorenzen et al., 2005).

The beetles (Coleoptera) are the most species-rich order of eukaryotes, with more than 350,000 described species occupying a wide range of ecological niches (Daly et al., 1998). Coleoptera are thought to have diverged from other holometabolous insect lineages relatively early, and to retain many primitive features of the Holometabola (Kjer, 2004; Grimaldi and Engel, 2005). *Tribolium castaneum* is arguably the most experimentally tractable of the Coleoptera. As such, developmental and genetic data from *T. castaneum* are often used in macroevolutionary comparisons to other common model insect species, especially *Drosophila melanogaster* (e.g. Jockusch et al., 2004; Angelini and Kaufman, 2005). For example, the larvae of *T. castaneum* and other coleopterans possess robust ventral appendages, and adult appendages develop from epidermal precursor cells in the larval limbs. This contrasts with the cyclorrhaphous Diptera, such as *D. melanogaster*, in which larvae lack appendages and adult limbs develop from internal imaginal discs. Moreover, *Tribolium* are economically important as destructive cosmopolitan pests of stored flour, corn, peanuts, and other dried agricultural products (Sokoloff, 1972; Throne et al., 2003).

Habits and diversity of *Tribolium* species

The genus *Tribolium* includes 36 described species (Table 1). Ten have become pests in dry, stored agricultural products (Nakakita, 1983), and some, including *T. castaneum* and *T. confusum* Jacquelin du Val (1868), are now cosmopolitan due the international shipment of

infested grain and flour. Very little is known about the biology of *Tribolium* species that are not synanthropic or about species in their ancestral habitats. Outside of human foodstuffs, *Tribolium* have been found under the bark of trees (Blair, 1930;Magis, 1954), where they are thought to feed on fungi (Sokoloff, 1972), and in the nests of domesticated and wild bees (Magis, 1954;Neboiss, 1962) and ants (Lea, 1904), where adults have been observed feeding on pollen. These conditions likely represent the ancestral habitats of the genus (Sokoloff, 1972). Tenebrionids are adapted to arid environments, with features for increased water retention (Duncan, 2003). These features have presumably pre-adapted *Tribolium* species, as well as other tenebrionids, for the invasion of agricultural products, such as flour, where water is extremely limited.

Hinton's (1948) synopsis of the genus *Tribolium*, which placed the species into five species groups (Table 1) based on geographic distributions and a few morphological characters, is the most complete taxonomic treatment thus far. The *castaneum* group includes 10 species distributed across southern and southeastern Asia. The *myrmecophilum* group, known from Australia, includes only two species with dorsoventrally flattened antennae. These two groups are characterized by antennae in which the three distal segments are enlarged into a club, while all other species of the genus have a less prominent club including the five distal segments. The *confusum* group contains 14 species from Africa, including the pests *T. confusum* and *T. destructor* Uyttenboogaart (1934). Three species endemic to Madagascar comprise the *alcine* group. Finally, the *brevicornis* group is found in North and South America and includes seven species that are much larger than others in the genus.

Proposed relationships among *Tribolium* species

Phylogenetic relationships within *Tribolium* remain unclear. The relationships among species of *Tribolium* were loosely considered by Hinton (1948), based on their geographic distributions and morphological characters such as body size, the number of enlarged antennal segments forming the club, and the form of margins on the vertex and pronotum. Hinton proposed the *castaneum* and *myrmecophilum* groups as sisters, with the basal split separating the *brevicornis* group from the rest of the genus. Some new species have been described in recent decades (Kaszab, 1982; Grimm, 2001). However, since their description by Hinton, nearly 60 years ago, most species have not been reported in the literature, presumably because many species are rare or endemic to remote areas. Nevertheless, in an attempt to understand the relationships and evolutionary history of this economically important group, several studies have considered the relationships among pest species.

Previous phylogenetic studies have presented conflicting phylogenetic hypotheses. A chemotaxonomic study based on the secreted organic chemical compounds produced by eight *Tribolium* species (those pests used in the present study) concluded that the *castaneum* and *confusum* species groups were well supported but also that *T. brevicornis* was sister to the *castaneum* group (Howard, 1987). Alternatively, isozyme data have suggested a closer relationship between *T. confusum* and *T. brevicornis*, with *T. castaneum* basal to this pair and to the *confusum* group species *T. destructor* (Wool, 1982). These relationships were also weakly supported when the chemotaxonomic evidence (Howard, 1987) was combined with satellite DNA data (Juan et al., 1993).

Several lines of evidence have supported monophyly of the *castaneum* species group. A study of satellite sequences and orientation in *T. confusum, T. castaneum, Tribolium freemani* Hinton (1948), and *Tribolium madens* (Charpentier 1825) grouped the latter three, and suggested that *T. freemani* was sister to *T. castaneum* (Ugarković et al., 1996b). Two other species of the *castaneum* group, *T. madens* and *Tribolium audax* Halstead (1969), are morphologically very similar and were not recognized as distinct until 1969 (Halstead, 1969). Examination of chromosome number and banding also provides support for the *confusum* species group.

Tribolium confusum and *T. destructor* share a large autosomal translocation to the X chromosome relative to the karyotype of *T. castaneum* and other tenebrionids (Smith, 1952a).

Meštrović et al. (2006) inferred phylogenies based on *cox1* and *16S rDNA* sequences from eight *Tribolium* species (those pests used in the present study). Their results from parsimony and Bayesian analyses conflicted on the placement of *T. brevicornis* but supported monophyly of the *castaneum* and *confusum* species groups. This study used different outgroup species than our present study, contributing to important differences in the conclusions regarding the monophyly of *Tribolium*.

In the present study, the relationships of *Tribolium* species are clarified with DNA sequence data from two mitochondrial and three nuclear markers using several phylogenetic inference methods, including parsimony, likelihood, and Bayesian inference using various partitioning strategies. Within the limits of taxon sampling, monophyly of the *castaneum* and *confusum* species groups is strongly supported, and the combined analyses strongly support a sister group relationship between these lineages. However, *Tribolium* is not resolved as monophyletic in these analyses because of the placement of *T. brevicornis*. Inclusion of *T. brevicornis* in *Tribolium* is also rejected in individual analyses of four of five markers. By comparing random partitioning strategies to those informed by sequence structure and function, we conclude that the confidence of phylogenetic inference is significantly improved through the use of informed partitioning strategies.

Materials and Methods

Taxon sampling

Tribolium castaneum adults were obtained from Carolina Biological Supply and kept as a breeding laboratory culture. Cultures of *T. freemani, T. madens, T. confusum*, and *T. brevicornis* were obtained from the USDA Grain Marketing Research Center in Manhattan, Kansas. Additionally, *cox1* and *16S* rDNA sequences were obtained from GenBank for three other *Tribolium* pest species: *T. audax* (**AJ438086, AJ438151**), *Tribolium anaphe* Hinton (1948) (**AJ438083, AJ438150**) and *T. destructor* (**AJ438089, AJ438147**). Taxon sampling was limited to economically detrimental pest species because material from other species was unavailable. We included three additional tenebrionid outgroup species. Selection of these species was guided by hypothesized tribal relationships, since a comprehensive molecular phylogenetic analysis of the group is not yet available. Tenebrionidae includes roughly 20,000 species in 16 tribes (Daly, et al., 1998). Currently, *Tribolium* is placed in the tribe Triboliini (Doyen, 1985), along with *Latheticus* and *Palorus*, genera also including agricultural pests, as well as *Aesymnus, Lyphia, Metulosonia, Mycotrogus, Tharsus*, and *Ulosonia*. We included *Latheticus oryzae* Waterhouse (1880), a species of pest Triboliini, in this study. Until recently, *Tribolium* was placed in the tribe Ulomini (Horn, 1870; Sokoloff, 1972), from which we included *Gnathocerus cornutus* (Fabricius 1798). Finally, we also included the mealworm beetle *Tenebrio molitor* Linnaeus (1758) (Tenebrionini), which has been considered a close ally of the Ulomini (Kwieton, 1982) and Triboliini (Doyen, 1985). *Gnathocerus cornutus* and *T. molitor* are also common pest beetles that are easily kept in lab culture.

Greater taxonomic sampling was included in a separate analysis of additional tenebrionid *28S* rDNA sequences from GenBank (**AY310661; AY310668; AY310671; PBO565954; PGR565947; PCO565971; TSC565974**). This marker was chosen because it provided the most extensive available sampling of tenebrionid taxa.

Isolation of DNA sequences

Genomic DNA was isolated from the larvae of laboratory beetle stocks using the NucleoSpin DNA extraction kit (Clontech). Because the genus *Tribolium* is globally distributed it has traditionally been assumed that its major lineages radiated in the Middle Cretaceous (Hinton, 1948). Therefore we chose five markers that span a range of evolutionary rates. Mitochondrial *cytochrome oxidase subunit I* (*cox1*) and *16S* ribosomal DNA, as well as the nuclear loci *wingless* (*wg*), *histone H3*, and *28S* ribosomal DNA were amplified using a combination of previously published and novel primers (Table 2) using Titanium Taq DNA polymerase (Clontech). In most cases, this provided an amplicon that could be sequenced directly using a dye-terminator mix with the ABI3100 capillary electrophoresis system. For some templates and primer pairs, PCR did not yield a single band. These PCR products were excised from an agarose gel and cloned into the Topo4-TA vector (Invitrogen). Multiple clones were sequenced and polymorphic positions, which were rare, were coded as missing data. Chromatogram base-calls were inspected by eye and edited as necessary in Sequencher 4.1 (Gene Codes Corporation). Sequences obtained for this study have been deposited in GenBank (accession numbers: **EU048277-EU048316**).

Sequence alignment and model selection

Alignment of orthologous sequences was done manually for most genes. For the expansion region of *28S* rDNA, alignment was assisted by ClustalX (Higgins et al., 1996), using a transition-transversion weight ratio of 0.5 and gap penalties of 10 for opening and 0.2 for extension. The aligned combined dataset is available in TreeBASE (accession number **SN3489**). For each data partition, the best-fitting model of nucleotide evolution was determined using the Akaike information criterion as implemented in Modeltest 3.7 (Posada and Crandall, 1998). Because there was no variation in the second codon position of *histone H3*, the Jukes-Cantor method was arbitrarily selected to model these data when partitioned individually. For ribosomal stem regions, we also tested the usefulness of a nucleotide doublet model (Ronquist and Huelsenbeck, 2003). The evolutionary model selected for each data partition is listed in Table 3.

Data partitioning and evaluation of its effectiveness

Large differences in nucleotide divergence among markers (Table 3) suggested the need for data partitioning. Within markers, we also tested the effects of partitioning protein-coding genes by codon position. For ribosomal RNA genes, each sequence was partitioned into putative stem and loop regions, as determined by FoldAlign (Havgaard et al., 2005).

Using the same dataset, separate Bayesian analyses using different partitioning strategies or evolutionary models may be compared by Bayes factors. Bayes factor (BF) can be approximated as the ratio of harmonic mean of likelihoods in the stationary phase of the null model analysis (L_0) and the analysis of a second model of interest (L_1) (Brandley et al., 2005). Better fit of a model is reflected in a positive value of the ln Bayes factor. The evidence against the null hypothesis is considered to be strong when $2\ln BF > 10$. Evidence against the null hypothesis is weaker when $0 < 2\ln BF < 10$, and the null hypothesis is preferred when $2\ln BF < 0$ (Kass and Raftery, 1995). Several potential partitioning strategies were tested with the combined dataset: 1) no partitioning, 2) partitioning by marker, 3) partitioning by marker and by codon position or stem-loop structures within markers; and 4) random partitions (Table 4). Because of high variation in base composition at third codon positions of *wg* and *H3*, we also conducted analyses in which these partitions were excluded.

Phylogenetic analyses

Phylogenies were inferred using a number of methods. Parsimony and maximum likelihood analyses were conducted in PAUP* 4.0beta10 (Swofford, 2003). Heuristic maximum likelihood (ML) searches included 10 random addition replicates and 100 bootstrapping iterations each with a single random addition replicate. Parsimony analyses used the branch and bound algorithm, and gaps were treated as missing data. The program TreeRot (Sorenson, 1999) was used for the calculation of partitioned decay indices (Baker and DeSalle, 1997). In ML searches, values of parameters for rate matrices, site heterogeneity and invariance were estimated by Modeltest on a neighbor-joining tree. MrBayes 3.1 was used for Bayesian phylogenetic inference (Ronquist and Huelsenbeck, 2003). In most analyses, four Markov chains were allowed to sample parameters and tree topologies every 1000 generations for 1,100,000 generations. Inspecting logged parameter values by eye with Tracer (Rambaut and Drummond, 2004) demonstrated that values typically stabilized after 3000 to 4000 generations, and the first 100,000 were discarded as a conservative burn-in. For each Bayesian analysis, Tracer was also used to verify that autocorrelation between samples was limited enough to produce effective sample sizes greater than 100 for each parameter. In highly partitioned analyses making use of doublet RNA models, it became necessary to run analyses longer (2.5×10^7 generations) to ensure adequate effective sample sizes for doublet parameters. In all analyses, *Tenebrio molitor* was used to root trees.

Results and Discussion

Sequence data were obtained from two mitochondrial genes and three nuclear loci from five *Tribolium* species and three other tenebrionids. These data were supplemented with orthologous mitochondrial sequences of three additional *Tribolium* species from GenBank. Thus, the combined dataset includes 11 taxa and 3106 aligned nucleotide positions. Selected models and corrected divergence levels between *T. castaneum* and several other species are listed in Table 3 for each data partition. Between species groups, coding sequences appear to be saturated at third codon positions.

As illustrated by nucleotide divergence (Table 3), the evolutionary rates of individual markers and partitions vary greatly, suggesting that analyses would benefit from a method of data partitioning. MrBayes 3.1 currently implements data partitioning in a Bayesian phylogenetic framework (Ronquist and Huelsenbeck, 2003). Bayesian methods also have the advantage of sampling many trees through a multi-chain Monte Carlo (MCMC) process, and the posterior distribution of topologies and model parameters provides estimations of support for particular branches and parameter values. By testing various partitioning strategies, we determined that a highly partitioned, combined analysis was preferred over unpartitioned analysis (Δ 2ln BF = 2799.0; Table 4; Fig. 1) and over partitioning by gene alone (Δ 2ln BF = 2031.1). After discussing the phylogenies inferred using the results of combined analyses, we will address methodological questions related to individual markers and data partitioning.

Monophyly is strongly supported for the *castaneum* and *confusum* species groups

The consensus phylogram from the partitioned Bayesian analysis using the complete, combined dataset is presented in Figure 1. The *castaneum* and *confusum* species groups are recovered as monophyletic in all the sampled trees and also in all ML bootstrap replicates. Within the *castaneum* group, relationships are resolved, with *T. castaneum* + *T. freemani* sister to *T. madens* + *T. audax*. A close relationship between *T. castaneum* and *T. freemani* has been suggested by their ability to produce hybrid offspring (Nakakita et al., 1981;Wade and Johnson, 1994b). *Tribolium madens* and *T. audax* are morphologically quite similar, and a close relationship has been suggested by Halstead (1969). Relationships among the three *confusum* group species are less certain. The consensus topology unites *T. confusum* and *T.*

anaphe to the exclusion of *T. destructor* but this relationship is weakly supported (Bayesian posterior support: 0.52; ML bootstrapping: 54%), with the other two possible arrangements receiving roughly equal support in the Bayesian posterior trees. All combined Bayesian and ML analyses provide moderate to high support for a close relationship between the *castaneum* and *confusum* species groups relative to other species in this study. The monophyly of species groups was also recovered using parsimony (Fig. 2). Bootstrap support and decay indices for the relationships within the *castaneum* species group are stronger than within the *confusum* group (Fig. 2). The most parsimonious tree in combined analyses groups *T. anaphe* and *T. destructor*, but the decay index for this branch is low and comes exclusively from *cox1* (Fig. 2).

These results support the relationships suggested by Hinton (1948), and contradict the phylogenetic hypotheses of later studies based on chemotaxonomy (Howard, 1987), isozyme cladistics (Wool, 1982), and satellite DNAs (Juan, et al., 1993). The mitochondrial phylogeny of Meštrović et al. (2006) also strongly supports the monophyly of the *castaneum* and *confusum* species groups, although using parsimony and Bayesian methods they obtain conflicting species relationships within the *confusum* group.

The genus *Tribolium* is not monophyletic

Multiple analyses suggest that *Tribolium* is not monophyletic (Table 4; Fig. 1, 2, 3). In the combined Bayesian analysis both *L. oryzae* and *G. cornutus* are inferred to be more closely related to the *castaneum* + *confusum* species groups than is *T. brevicornis*. Although neither of these relationships is strongly supported (<0.83 posterior probability), monophyly of *Tribolium* had a very low posterior probability (0.052). Maximum likelihood analysis in PAUP is equivocal on the relations of *L. oryzae, G. cornutus*, *T. brevicornis*, and other *Tribolium* species (which are grouped as in Fig. 1). The ML tree unifies *Tribolium* (−lnL = 13705.2; Table 4); however, the second best tree (−lnL = 13705.4) places both *L. oryzae* and *G. cornutus* as more closely related to the *castaneum* + *confusum* groups, as in the Bayesian analysis. Only five of the ten best ML trees (−lnL < 13709.5) support the monophyly of *Tribolium*. Moreover, monophyly is supported in only 37% of bootstrap trees. Parsimony also places *L. oryzae* as sister to the clade of *castaneum* + *confusum* species groups (Fig. 2). Although the decay index is low for this branch, support comes from multiple markers.

Other evidence is consistent with the hypothesis that the *brevicornis* group is not sister to the other *Tribolium* species. For more than 80 years, *brevicornis* group species were regarded as a distinct genus, *Aphanotus* (Leconte, 1862; Casey, 1890), until they were subsumed into *Tribolium* by Hinton (1948). Despite this merger, exceptions for *brevicornis* group morphology are made in the *Tribolium* diagnosis with respect to the form of the prosternal and pronotal apices. The *brevicornis* group species were regarded as primitive in these characters relative to other *Tribolium* species (Hinton, 1948). Hinton also conceded that the *brevicornis* group might merit recognition at the genus level because of similarity between other *Tribolium* groups and the genus *Lyphia*. While other *Tribolium* species are native to the tropical Old World, *brevicornis* group species are distributed in the New World and occur in more temperate climates (Table 1). Furthermore, a recent analysis of satellite DNA sequences from *T. brevicornis* has identified satellites in this species that are unrelated to others in the genus *Tribolium* (Mravinac et al., 2005).

In an effort to further examine the relationship of *T. brevicornis* to other species of *Tribolium*, we conducted analyses using sequence data from additional tenebrionids. A previous mitochondrial phylogeny of *Tribolium* has suggested monophyly of the genus, but this is likely due to the fact that fewer and different outgroup (non-*Tribolium*) species were included (Meštrović, et al., 2006). Here we sought to include as broad a sampling of tenebrionids as possible. We used *28S* rDNA sequences available from GenBank, yielding a

total of 15 tenebrionid species representing four tenebrionid tribes and three subfamilies, rooted with a sequence from the allied family Zopheridae. The relationships between *T. castaneum, T. freemani, T. madens*, and *T. confusum* are consistent in these analyses (Fig. 3) and the multigene analyses (Fig. 1, 2; Table 4). Most basal relationships could not be resolved. However, the monophyly of *Tribolium* was again recovered with very low probability (0.029) from the Bayesian posterior sample. Instead, *Tentyria schaumi* Kraatz (1865) (Tentyrini) is supported as the closest relative of the *castaneum + confusum* species groups (Fig. 3A; 0.94 posterior probability). *Tribolium brevicornis* appears as sister to this larger clade with modest support (0.80). Parsimony also groups *Tentyria schaumi* as sister to the *castaneum + confusum* species groups (Fig. 3B), although this branch does not receive strong support (40% bootstrap support; decay index of 1). While it is clear that greater taxon sampling is needed to definitively assign relationships among Tenebrionidae, these results reinforce the conclusion that *T. brevicornis* is not the closest relative of other *Tribolium* species groups.

Transfer of *brevicornis* group species from *Tribolium* MacLeay (1825) to *Aphanotus* Leconte (1862)

Considering the results of this study and the morphological observations of Hinton (1948), it is appropriate that *brevicornis* group species be removed from *Tribolium*. The genus *Aphanotus* Leconte (1862) previously contained *T. brevicornis* and *T. parallelus* (Casey 1890) before being subsumed by Hinton (1948) into *Tribolium*. This taxon is currently available, containing no species. Therefore, all *brevicornis* group species are here transferred to *Aphanotus* in order to reflect their independent evolutionary history. Thus, the genus *Aphanotus* contains seven known species: *Aphanotus brevicornis* (Leconte, 1859), *Aphanotus carinatum* (Hinton, 1948) n. com., *Aphanotus gebieni* (Uyttenboogaart, 1934) n. com., *Aphanotus linsleyi* (Hinton, 1948) n. com., *Aphanotus parallelus* (Casey, 1890), *Aphanotus setosum* (Triplehorn, 1978) n. com., and *Aphanotus uezumii* (Nakane, 1963) n. com. The descriptions of *Aphanotus* given by Leconte (1859; 1862) are very brief. Therefore, Hinton's (1948) description of the *brevicornis* species group remains the best description of the genus *Aphanotus*. This change does not affect the use of Hinton's key for the identification of species.

Problems with dating divergences among *Tribolium*

The dates of lineage splits separating *T. castaneum* from its relatives remain obscure. It was Hinton's supposition that *Tribolium* species groups were very old due to their wide geographic distributions. However, the fact that *T. castaneum* and *T. freemani* as well as *T. madens* and *T. audax* are capable of hybridization may suggest more recent origins. The topologies of all analyses in this study were not clock-like, preventing a reliable estimate of divergence times from either a fixed or relaxed clock model. Branch lengths are most consistent among the *castaneum* and *confusum* group species where a clock might be applicable. Using various mitochondrial molecular clock estimates (Venanzetti et al., 1993; Farrell, 2001), divergence times for *T. castaneum* and *T. freemani* range from 11.6 to 47.0 million years ago (Mya), and for *T. castaneum* and *T. confusum* between 13.9 and 60.7 Mya. While fossil data on various beetle families could be used to constrain a relaxed clock analysis, the extreme rate variation among lineages would still make such an undertaking problematic. Therefore, a reliable estimate of divergence dates within *Tribolium* will ultimately require calibration information specific to this lineage.

Conflict among individual markers and combined analyses

The five markers used in this study present a moderate degree of conflict (Fig. 4; Table 4). Conflict among characters or multiple loci is a frequent problem which has been considered extensively (e.g. Olmstead and Sweere, 1994;Mason-Gamer and Kellogg, 1996;Nixon and Carpenter, 1996;Gatesy et al., 1999;Kjer et al., 2001;Funk and Omland, 2003). Partitions

including *cox1, 16S,* and first and second codon positions of *histone H3* contribute negative decay indices for at least one branch in the combined parsimony analysis (Fig. 2). In the Bayesian analyses of this study, nuclear and mitochondrial markers differ mostly in their placement of *L. oryzae*, with mitochondrial markers weakly supporting this species as sister to the *castaneum* group (0.57), and nuclear markers supporting a clade containing *L. oryzae* and *G. cornutus* (0.67 posterior probability; 0.95 probability of *castaneum + confusum* groups).

The monophyly of *Tribolium* is recovered only in analyses of *28S* rDNA alone (Fig. 4D, 0.82 Bayesian posterior probability; Fig. 4I, 63% bootstrap support, decay index of 3). However, in the expanded *28S* analyses, *Tribolium* is rendered paraphyletic by *Tentyria schaumi* (Fig. 3). Partitioned decay indices reveal that most support for the topology in combined parsimony analysis comes from *cox1* and somewhat from *28S* (Fig. 2). Thus, while single marker analyses are not sufficient to resolve relationships among *Tribolium* species groups, together they provide strong support for the main conclusions of this study: that the *castaneum* and *confusum* species groups are monophyletic and together form a clade, and that *T. brevicornis* is not included with other *Tribolium* species.

In Bayesian analyses, most individual markers suggest the monophyly of *Tribolium* species groups (Fig. 4A–E). The only exception is *wingless*. When trees are rooted with *Tenebrio molitor*, the *castaneum* species group is rendered paraphyletic by a clade containing all other species, and the pattern of branch lengths suggests that the tree is improperly rooted (Fig. 4C). If the tree is instead rooted with *G. cornutus* or *L. oryzae*, then the *castaneum* group is instead rendered paraphyletic by the inclusion of *Tenebrio molitor*. However, this placement of *Tenebrio molitor* appears with posterior support of less than 0.0002 in all combined Bayesian analyses. One possible explanation of this surprising result is a shared base composition bias. *Tenebrio molitor* and *T. castaneum* share high G+C content in third codon positions of the *wingless* gene (85.5% and 75.3%, respectively) relative to other species in this study (31.0% for *L. oryzae* to 65.6% for *T. brevicornis*). Excluding third codon positions reduces support for *Tenebrio molitor + T. castaneum* to a posterior probability of 0.051 (Fig. 4C'). Similarly, base composition differs at third codon positions of *histone H3*, where G+C content is low in *L. oryzae* (17.2%) and *T. confusum* (27.3%), relative to other taxa (37.3% for *T. freemani* to 66.4% for *Tenebrio molitor*). With the exclusion of *H3* third codon positions, monophyly of the *castaneum + confusum* species groups is supported in Bayesian analysis (Table 4). Therefore, it is possible that selection on G+C content or codon bias has led to nucleotide convergence in synonymous nuclear sites of some species. Exclusion of third codon positions of both *wg* and *H3* from the combined five-marker Bayesian analysis, does not change the consensus topology (Table 4), however, support is increased for basal branches of the tree (indicated parenthetically in Fig. 1). Inclusion of the two partitions with heterogeneous base composition alters phylogenetic relationships in parsimony grouping (Table 4).

Data partitioning

A major advantage of phylogenetic studies incorporating relatively few taxa is that it becomes feasible to explore a variety of analytical methods. Here, we have examined the merit of a wide range of data partitioning strategies in combined Bayesian analyses. As implemented in MrBayes, separate data partitions allow individual models of nucleotide evolution, and parameters, such as rate matrices, base frequencies, and rate heterogeneity, to be estimated independently for each partition.

Data are often partitioned in a manner informed by biological structure and function, for example by gene or by codon position within protein-coding sequences. However, if a dataset contains characters with an effectively multimodal distribution of model parameters, any partitioning strategy, including random partitioning, has the potential to isolate these conflicting characters and improve performance. Therefore we compared the effects of

biologically informed partitioning strategies to those of several different strategies for randomly assigning nucleotide characters to partitions. For single genes, either two (ribosomal DNA) or three (protein-coding) random partitions were created. Because the biologically informed, partitioned analyses resulted in 5 (by gene) or 12 (by codon position and stem-loop structure) partitions, combined analyses were performed with 5 or 12 partitions to which characters where randomly assigned. In these analyses, the length of partitions was set equal to their biologically based counterparts. An additional five-partition analysis was conducted in which the length of each partition was also randomly determined.

In 6 of 8 analyses, random partitions improved model fit relative to unpartitioned data, but the improvements were small, especially compared to the improvement from biologically-informed partitioning (Table 4). This improved fit is likely due to the random partitions capturing some of the otherwise un-modeled complexity in the dataset. However, in two cases, the full dataset with 12 partitions and *cox1*, random partitioning was highly detrimental. The length of partitions did not have a strong effect, but the Bayes factor became significantly more negative in the more highly partitioned analysis. Furthermore, increased partitioning may not be effective when the data contains little or no additional structure. For example, when partitions consisting of first, second, and third codon positions from *cox1* were each further randomly partitioned in two, the resulting analysis was not favored over partitioning by codon position alone (2ln BF = −2.4). These results suggest that random partitioning is not an effective method to reliably improve model fit. Instead, comparison to partitions based on sequence structure and function emphasize the importance of biological relevance to partitioning.

Given the limited utility of random partitioning, to what extent is biologically informed partitioning useful? Partitioning of the combined dataset by marker or by structure within markers resulted in significantly improved fit relative to unpartitioned analysis (2ln BF = 767.8 and 2782.1, respectively). In analyses of single markers, partitioning by codon position or stem-loop structure was highly favored (2ln BF between 36.8 and 542.4). However, increased partitioning was not always favored. For each of the three protein-coding markers used in this study, we tested partitioning third codon positions and each position individually compared to an unpartitioned analysis. For *cox1* and *histone H3*, partitioning each codon individually was a significantly better fit than combining first and second positions (2ln BF = 46.3 and 86.9, respectively). However, for *wingless* partitioning each codon position did not produce as good a fit as when first and second positions were combined (2ln BF = −28.7). Therefore, it is not universally true that greater biologically-based partitioning is consistently beneficial to analyses (Brandley, et al., 2005).

RNA doublet modeling significantly improves the performance of rDNA partitions

In addition to testing partitions within protein-coding markers, the effect of partitioning ribosomal DNA sequences based on their predicted RNA secondary structure was also examined. The complement of nucleotides in predicted RNA stem structures was used to specify nucleotide doublet pairs in MrBayes. Doublet models account for the fact that mutation in one nucleotide of a pair increases the likelihood of complementary substitution at the apposing site (Schoniger and Von Haeseler, 1994), effectively reducing branch lengths.

Partitioning of predicted stem and loop regions significantly improved the performance of analyses for *16S* (2ln BF = 36.8) and *28S* rDNA (2ln BF = 112.9). However, a drastic gain in fit was obtained by applying the doublet model to stem regions. The difference in 2ln BF was 176.6 for *16S* and 569.0 for *28S*. Therefore, stem-loop partitioning and stem doublet modeling appear to be beneficial to parameter fit in combined Bayesian analyses. While it seems likely that partitions based on very accurate stem-loop predictions, such as those of nuclease-protection assays or x-ray crystallography would perform still better, a relatively simple prediction method such as FoldAlign is highly effective.

Conclusions

Through the use of multiple evolutionary models in data partitions based on sequence structure and function in combined Bayesian analyses, we have inferred a well-supported phylogeny for pest species of *Tribolium* (Fig. 1). This is also supported by combined parsimony analysis (Fig. 2). The developmental genetic model species *T. castaneum* appears to be highly nested within *Tribolium*. Thus, other species of the genus provide a range of phylogenetic distances for comparison to *T. castaneum*. The monophyly of the *castaneum* and *confusum* species groups is recovered with high support. These groups also appear to be each other's closest relatives. Significantly, the genus *Tribolium* as previously recognized is not recovered as monophyletic. *Tribolium brevicornis* is more distantly related to *Tribolium* sensu stricto than are representatives of other tenebrionid genera in analyses of the combined dataset and four of five single markers. Based on this and other evidence, *brevicornis* group species are removed to the available genus *Aphanotus*. Thus, derived characters shared by *Aphanotus* and *Tribolium* likely provide examples of convergence. These conclusions should provide an evolutionary framework for comparative studies of *Tribolium* and other flour beetles, utilizing the genetic and genomic tools of *T. castaneum* in related, phenotypically distinct species.

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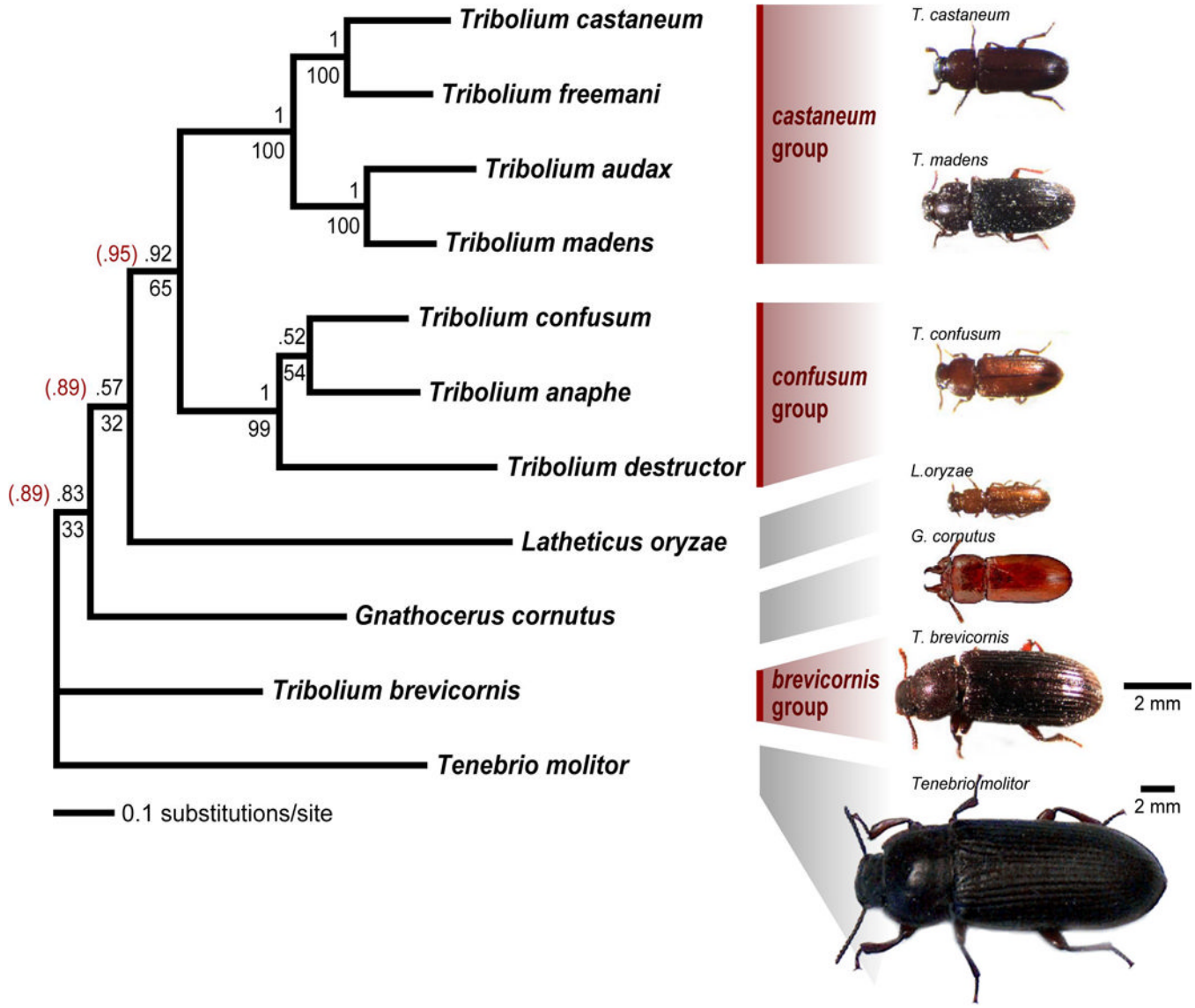

Figure 1.

Consensus phylogram from the complete combined partitioned Bayesian analysis. Bayesian posterior probabilities (above the branch) and bootstrap values > 50% in the combined ML analysis are shown (below the branch). Posterior probabilities given in parentheses result from exclusion of partitions in which base composition is not homogeneous across taxa (third codon positions of *wingless* and *histone H3*). Adults of selected species are shown to scale, with the exception of *Tenebrio molitor*.

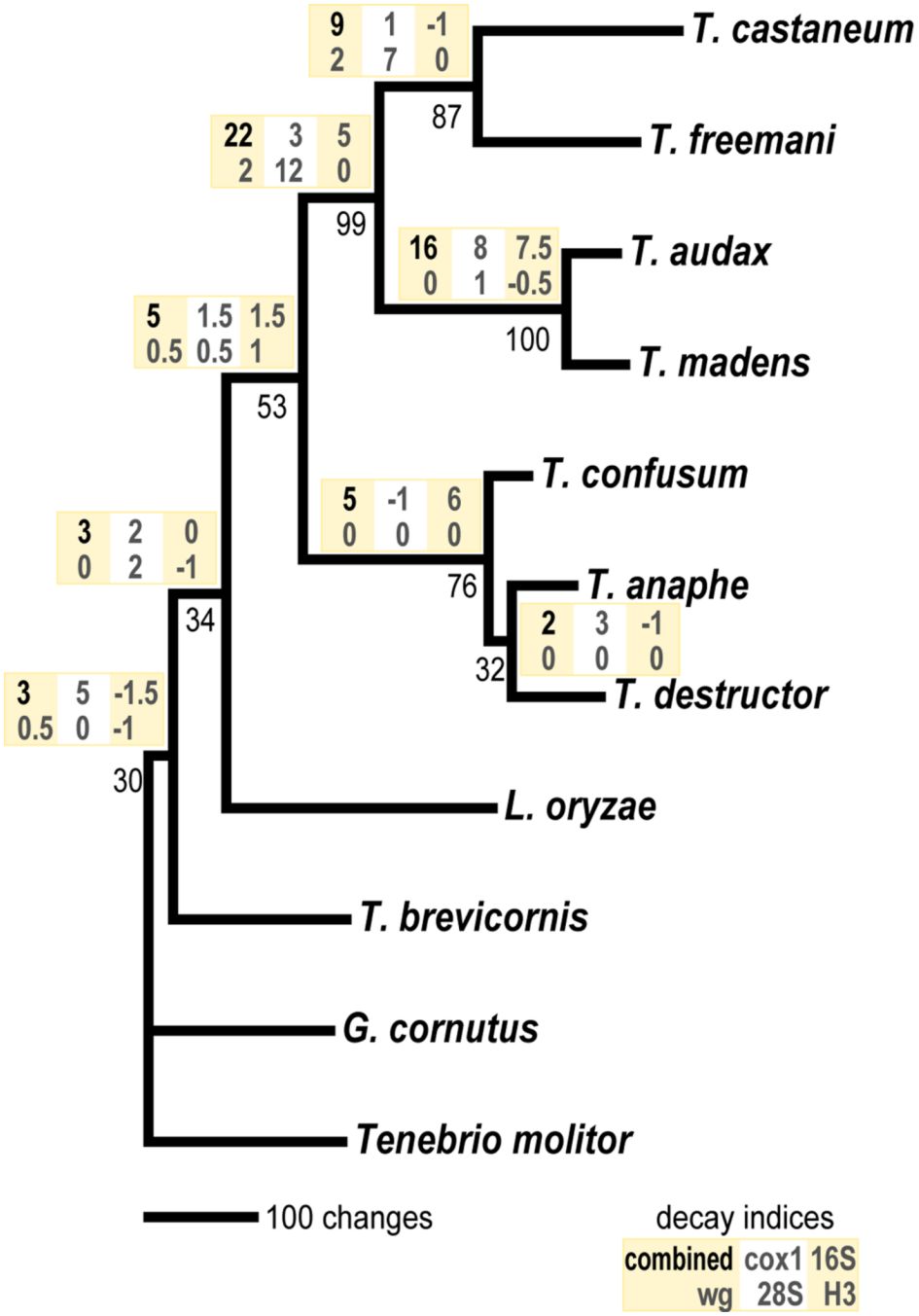

Figure 2.

Most parsimonious tree based on combined analysis of *cox1, 16S* rDNA, *wg, 28S* rDNA, and *histone H3*. This analysis excluded codon third positions from *wg* and *histone H3* based on failure of the base homogeneity test ($P \ll 0.01$). Decay indices based on the combined dataset and each partition are given above each branch. Bootstrap values are given below branches.

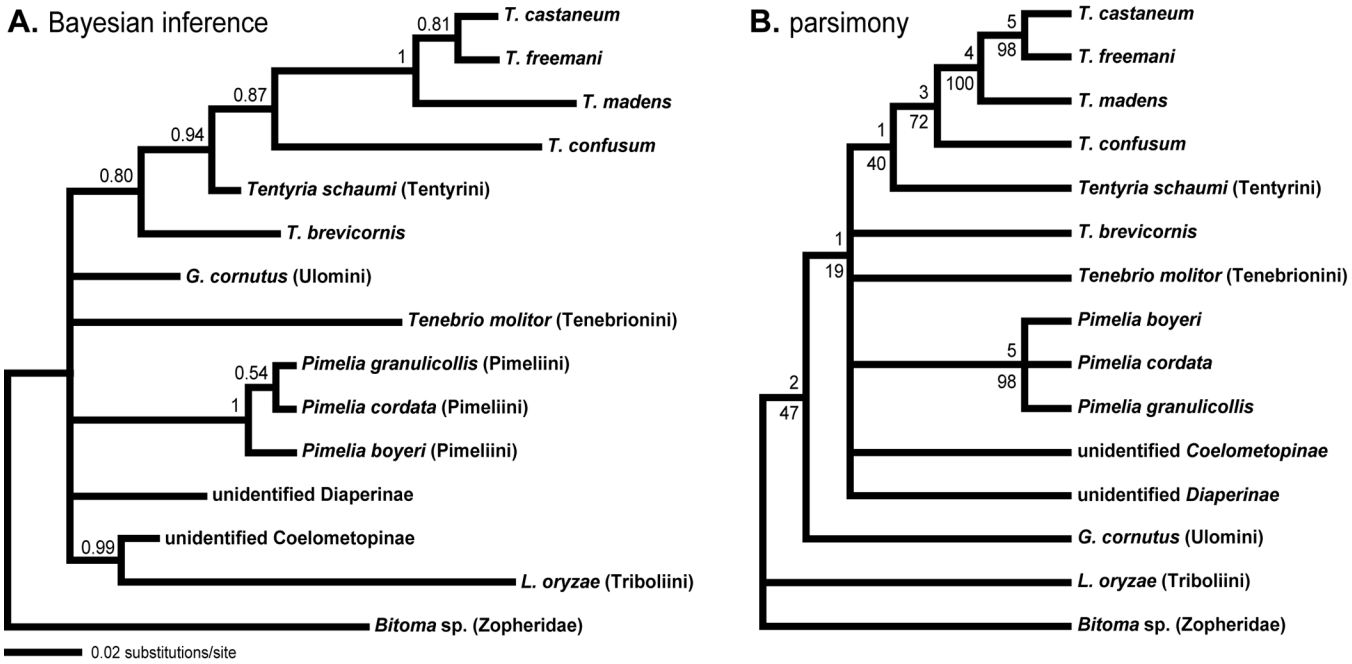

Figure 3.

Trees produced from analyses of tenebrionid *28S* rDNA sequences. (A) Majority consensus phylogram from Bayesian inference. Posterior probabilities are given above each branch. (B) Consensus cladogram of six equally most parsimonious trees. Decay indices are shown above branches. Bootstrap values are given below each branch. In both trees, taxa are poorly resolved at the subfamilial and tribal levels. Notably *Tribolium* is rendered polyphyletic in these analyses.

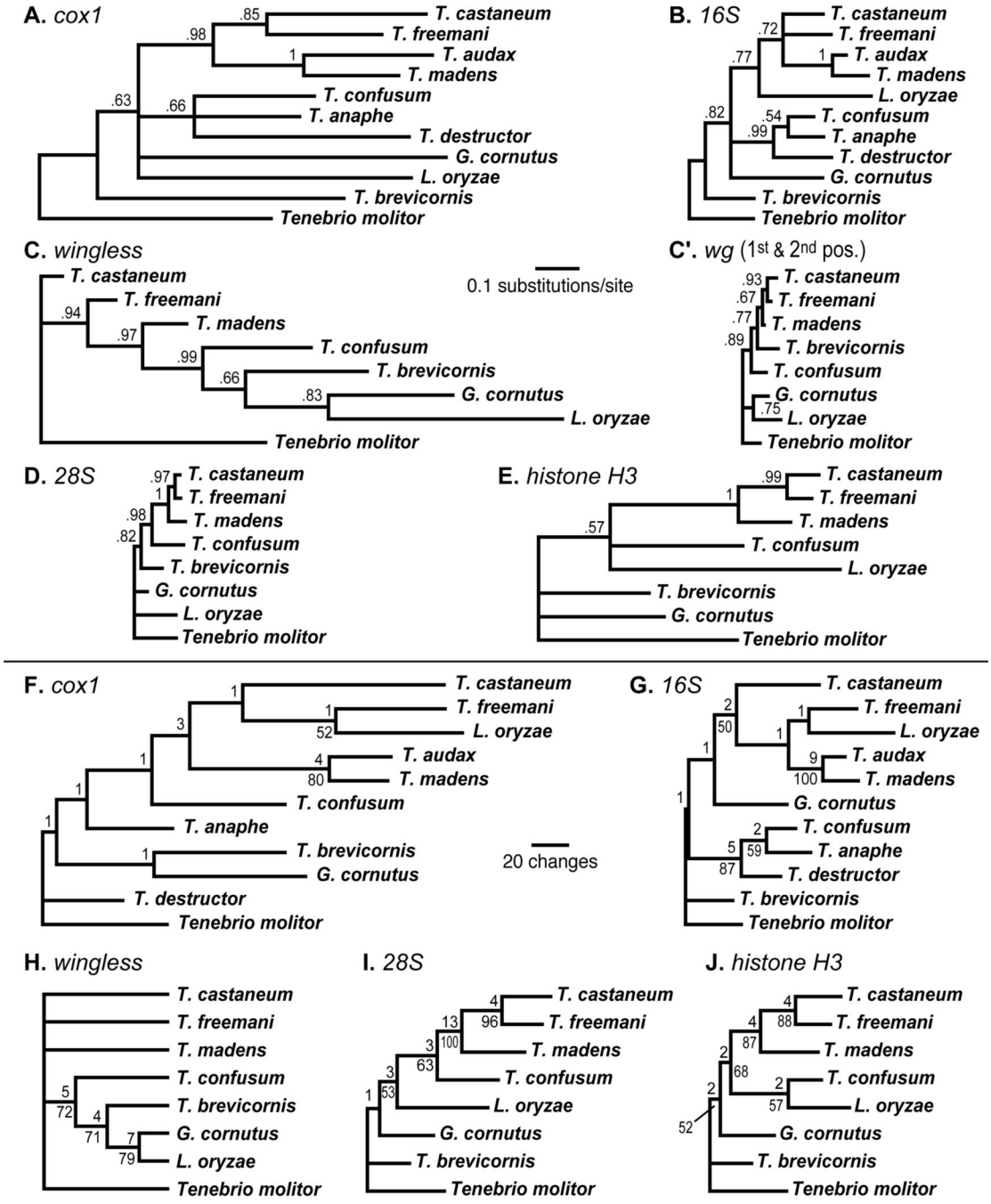

Figure 4.

Trees from single marker analyses. The scale of branch lengths is comparable among Bayesian (A–E) and parsimony (F–J) analyses. In Bayesian consensus phylograms posterior probabilities are given above each branch. A single most parsimonious tree was obtained for most markers (F,G,I,J). However analysis of *wg* yielded two equally parsimonious trees. The consensus cladogram from this analysis is shown (H); note that branch lengths are arbitrary in this tree. Decay indices for parsimony trees is given above each branch, and bootstrap values > 50% are shown below branches.

Table 1

A list of known *Tribolium* species, the relative degree to which they are agricultural pests, and their described distributions.

Tribolium **MacLeay (1825) species**	**pest**	**known distribution**
brevicornis group		North and South America
T. brevicornis (Leconte 1859)	++	California
T. carinatum Hinton (1948)		Argentina
T. gebieni Uyttenboogaart (1934)		Paraguay
T. linsleyi Hinton (1948)		Mexico
T. parallelus (Casey 1890)	+	western North America
T. setosum Triplehorn (1978)		Arizona
T. uezumii Nakane (1963)[a]		Honshu (Japan)*
confusum group		Africa
T. anaphe Hinton (1948)	+	central Africa
T. arndti Grimm (2001)		South Africa
T. beccarii Gridelli (1950)[a]		Aïr (Niger)
T. bremeri Grimm (2001)		South Africa
T. confusum Jacquelin du Val (1868)	+++	cosmopolitan*
T. destructor Uyttenboogaart (1934)	+++	cosmopolitan*
T. downesi Hinton (1948)		Mali, Sudan, Chad
T. ferreri Grimm (2001)		Gambia
T. indicum Blair (1930)		central Africa, Saudi Arabia*, Iran*, India*
T. risbeci Lepesme (1943)		Senegal
T. semele Hinton (1948)		Mali, Mauritania, Sudan, Chad
T. semicostata (Gebien 1910)		Kenya
T. sulmo Hinton (1948)		Ethiopia, Gambia, Ghana
T. thusa Hinton (1948)	+	Chad, South Africa, Botswana, Namibia
alcine group		Madagascar
T. alcine Hinton (1948)		Madagascar
T. ceto Hinton (1948)		Madagascar
T. quadricollis (Fairmaire 1902)		Madagascar
castaneum group		South and Southeast Asia
T. apiculum Neboiss (1962)		Australia
T. audax Halstead (1969)	++	North America*
T. caledonicum Kaszab (1982)		Lifou (New Caledonia)
T. castaneum (Herbst, 1797)	+++	cosmopolitan*
T. cylindricum Hinton (1948)		Malay peninsula, Indonesia, Borneo, Philippines
T. freemani Hinton (1948)	+	Kashmir, Japan, Brazil**
T. madens (Charpentier 1825)	+++	cosmopolitan*
T. parki Hinton (1948)		Bali, Larat (Tanimbar archipelago, Indonesia)
T. politum Hinton (1948)		Doerian (Indonesia)
T. waterhousei Hinton (1948)		Queensland, New South Wales
myrmecophilum group		Australia
T. antennatum Hinton (1948)		Queensland
T. myrmecophilum Lea (1904)		Victoria

* Possibly introduced by human activity.

[a] *T. uezumii* may be synonymous with *T. carinatum*, and *T. beccarii* may be a subspecies of *T. downesi* (Halstead, 1967).

Table 2

Primers used to amplify genomic sequence data.

gene	amplicon	primer name	sequence	reference
COI	820 bp	C1-J-2195	TTGATTTTTTGGTCATCCAGAAGT	Simon et al., 1994
		TL2-N-3014	TCCAATGCACTAATCTGCCATATTA	
16S rRNA	512 bp	16Sbr	CCGGTCTGAACTCAGATCACGT	Simon et al., 1994
		16Sar	CGCCTGTTTAACAAAAACAT	
wingless	468 bp	wg1MP-F3	GArTGyAArTGyCAyGGCATGTCsGG	M. Polihronakis, unpubl.
		wg1MP-R3	ACyICGCArCACCArTGGAAIGTGCA	
	472 bp	Tsp'wg-f1	ACnAThAArACnTGyTGGATGCGnCT	this study
		Tsp'wg-r1	CrCArCACCArTGrAAnGTrCArAT	
28S rRNA	1044 bp	D2-3665F (Bel28S)	AGAGAGAGTTCAAGAGTACGTG	Hancock et al., 1988
		D5-4749R (28SD4rev)	GTTACACACTCCTTAGCGGA	
histone H3	330 bp	HexAF	ATGGCTACCAAGCAGACGGC	Ogden & Whiting, 2003
		HexAR	ATATCCTTGGGCATGATGGTGAC	

Table 3

Information on sequence data and partitions.

partition	length (bp)	parsimony informative sites		model used in MrBayes	maximum likelihood divergence from *T. castaneum*			
					T. freemani	*T. confusum*	*T. brevicornis*	*L. oryzae*
combined	3106	653	(21.0%)	GTR+I+Γ	8.3%	13.6%	14.6%	16.6%
COI	813	234	(28.8%)	GTR+I+Γ	16.2%	17.6%	20.9%	20.0%
pos. 1	271	47	(17.3%)	GTR+Γ	7.4%	10.7%	12.0%	13.0%
pos. 2	271	10	(3.7%)	GTR+Γ	2.2%	3.7%	3.8%	3.7%
pos. 3	271	177	(65.3%)	HKY+Γ	39.1%	38.4%	47.0%	43.5%
16S rRNA	451	115	(25.5%)	GTR+I+Γ	13.1%	16.6%	15.4%	17.2%
stem	130	17	(13.1%)	HKY+Γ	5.4%	10.8%	6.2%	6.9%
loop	321	98	(30.5%)	GTR+I+Γ	16.3%	19.0%	19.5%	21.6%
wingless	468	121	(25.9%)	GTR+Γ	7.6%	15.5%	16.5%	26.2%
pos. 1	156	11	(7.1%)	GTR+Γ	2.6%	7.4%	6.5%	8.5%
pos. 2	156	6	(3.8%)	HKY+I	0.6%	2.0%	5.2%	4.5%
pos. 3	156	104	(66.7%)	HKY+Γ	19.6%	38.9%	37.7%	65.9%
28S rRNA	1044	97	(9.3%)	GTR+I+Γ	1.6%	7.1%	6.5%	9.0%
stem	394	46	(11.7%)	GTR+Γ	1.8%	9.1%	7.1%	11.2%
loop	650	51	(7.8%)	GTR+I+Γ	1.6%	5.9%	6.1%	7.6%
histone H3	330	86	(26.1%)	GTR+I+Γ	4.5%	17.6%	21.1%	18.2%
pos. 1	110	8	(7.3%)	GTR+Γ	0.9%	2.7%	5.5%	6.0%
pos. 2	110	0		JC+I	0	0	0	0
pos. 3	110	78	(70.9%)	HKY+Γ	12.7%	50.0%	58.3%	48.4%

Table 4

A summary of phylogenetic analyses performed on various data subsets. Inclusion of data from each marker is denoted by the shaded boxes at left. The partitioning strategy with markers is indicated within the box. Combination of all characters of a marker is denoted by "+". Coding sequence partitioning based on codon positions is denoted by "1/2/3", etc. Additional random partitioning within codon position partitions is denoted "11/22/33". Partitioning by stem-loop structure is denoted "S/L". Doublet modeling in stem regions is denoted "Sd/L". Tree scores for maximum likelihood (ML) analyses represent the log likelihood of the best tree according to the model. For Bayesian inference (BI) analyses, tree scores are given as the harmonic mean of the log likelihood. Tree scores from maximum parsimony (MP) analyses are given in the number of tree steps. For Bayesian analyses topologies reflect majority consensus trees. Topologies listed for ML analyses are from 50% bootstrap consensus trees. For MP analyses the most parsimonious tree or strict consensus of equally most parsimonious trees is shown. Tree topologies are described by abbreviating species and species groups as follows: CST, *castaneum* species group with internal topology as in Fig. 1; CNF, *confusum* species group; cnf, *T. confusum*; ana, *T. anaphe*; dst, *T. destructor*; brv, *T. brevicornis*; Lth, *Latheticus oryzae*; Gnt, *Gnathocerus cornutus*; Ten, *Tenebrio molitor*.

dataset					method	tree score	$2(\ln L_0 - \ln L_1)$	topology	Fig.
cox1	**16S**	**wg**	**28S**	**H3**					
1/2/3	Sd/L	12/3	Sd/L	1/2/3	BI	12332.8	2799.0	((((CST,CNF),Lth),Gnt),brv),Ten	1
+	+	+	+	+	BI	13348.3	767.8	((CST,CNF),(Gnt,Lth)),brv),Ten	
12 random partitions of set length					BI	13749.0	−33.4	((((CST,CNF),Lth),Gnt),brv),Ten	
5 random partitions of set length					BI	13728.8	6.9	((CST,CNF),(Gnt,Lth)),brv),Ten	
5 random partitions of random length					BI	13729.9	4.6	((CST,CNF),(Gnt,Lth)),brv),Ten	
combined					BI	13732.2	(L_0)	(CST,CNF),brv,(Gnt,Lth),Ten	
combined					ML	13705.2		(CST,CNF),brv,(Gnt,Lth),Ten	
combined					MP	2272		(((CST,(CNF,Lth)),brv),Gnt),Ten	
1/2/3	Sd/L	12	Sd/L	1/2/3	BI	11154.2		((((CST,CNF),Lth),Gnt),brv),Ten	
1/2/3	**Sd/L**	**12**	**Sd/L**	**1/2**	BI	10260.7		((((CST,CNF),Lth),Gnt),brv),Ten	(1)
combined (w/o *wg&H3* 3rd pos)					MP	1726		((((CST,CNF),Lth),brv),Gnt),Ten	2
1/2	Sd/L	12	Sd/L	1/2	BI	7700.3		((((CST,CNF),Lth),Gnt),brv),Ten	
combined (only 3rd positions)					BI	4284.8		(((CST,(cnf,Lth)),brv),Gnt),Ten	
1/2/3	Sd/L				BI	6390.4	1055.2	((((CST,Lth),CNF),Gnt),brv),Ten	
combined					BI	6918.0	(L_0)	(((CST,Lth),CNF,Gnt),brv),Ten	
combined					ML	7699.1		((Lth w/in CST),(CNF,brv),Gnt),Ten	
combined					MP	1321		((CST,Lth),Gnt),(CNF,brv),Ten	
		12/3	Sd/L	1/2/3	BI	5902.7	1318.1	(CST,cnf),brv,(Gnt,Lth),Ten	
		combined			BI	6561.8	(L_0)	((CST,cnf),brv),(Gnt,Lth),Ten	
		combined			ML	6542.1		(CST,cnf),brv,(Gnt,Lth),Ten	
		combined			MP	937		(((CST,(cnf,Lth)),brv),Gnt),Ten	
		12	Sd/L	1/2/3	BI	4724.7	939.1	resolves only (CST,cnf)	
		combined (w/o wg pos3)			BI	5194.3	(L_0)	((CST,cnf),brv),(Gnt,Lth),Ten	
		12	Sd/L	1/2	BI	3765.6	751.0	((CST,cnf),brv),Gnt,Lth,Ten	
		combined (no pos3)			BI	4141.1	(L_0)	((CST,cnf),brv),(Gnt,Lth),Ten	
		combined (no pos3)			MP	390		((((CST,cnf),Lth),Gnt),brv),Ten	
11/22/33					BI	4193.4	540.0	((CST,CNF,Lth,Gnt),brv),Ten	

dataset					method	tree score	$2(\ln L_0 - \ln L_1)$	topology	Fig.
cox1	16S	wg	28S	H3					
1/2/3					BI	4192.2	542.4	((CST,CNF,Lth,Gnt),brv),Ten	4A
12/3					BI	4235.7	455.5	resolves only CST	
rand (3)					BI	4472.5	−18.0	resolves only CST and (cnf,dst)	
+					BI	4463.4	(L_0)	((CST,Lth,Gnt),(cnf,dest),ana,brv),Ten	
+					ML	4431.1		(((CST,Lth),Gnt),CNF),brv),Ten	
+					MP	897		(((((Lth w/in CST),cnf),ana),(brv,Gnt)),dst),Ten	4F
	Sd/L				BI	2187.5	213.4	((CST,Lth),CNF,Gnt),brv),Ten	4B
	S/L				BI	2275.7	36.8	((CST,Lth),CNF,Gnt),brv),Ten	
	rand				BI	2292.0	4.3	((CST,Lth),CNF,Gnt),brv),Ten	
	+				BI	2294.2	(L_0)	((CST,Lth),CNF,Gnt),brv),Ten	
	+				ML	2265.7		(((CST,Lth)(CNF,Gnt)),brv),Ten	
	+				MP	421		((((Lth w/in CST),Gnt),CNF),brv),Ten	4G
		1/2/3			BI	1985.4	214.1	(((Gnt,Lth),cnf),brv),(Ten w/in CST)	
		12/3			BI	1971.0	242.8	(((Gnt,Lth),brv),cnf),(Ten w/in CST)	4C
		rand			BI	2089.4	6.0	(((Gnt,Lth),cnf),brv),(Ten w/in CST)	
		+			BI	2092.4	(L_0)	(((Gnt,Lth),brv),cnf),(Ten w/in CST)	
		+			ML	2074.4		resolves only (Gnt,Lth) and (cst,Ten)	
		+			MP	361		(((Lth,Gnt),brv),cnf),mad,fre,cst,Ten	4H
		1/2			BI	752.0	3.0	(((CST,brv),cnf),(Gnt,Lth)),Ten	
		12			BI	753.5	(L_0)	(((CST,cnf),brv),(Gnt,Lth)),Ten	4C'
		12			MP	57		resolves only CST	
			Sd/L		BI	2614.5	681.9	((CST,cnf),brv),Lth,Gnt,Ten	4D
			S/L		BI	2899.0	112.9	((CST,cnf),brv),Lth,Gnt,Ten	
			rand		BI	2953.5	3.9	((CST,cnf),brv),Lth,Gnt,Ten	
			+		BI	2955.5	(L_0)	((CST,cnf),brv),Lth,Gnt,Ten	
			+		ML	2932.4		((CST,cnf),brv),Lth,Gnt,Ten	
			+		MP	312		((((CST,cnf),Lth),Gnt),brv),Ten	4I
				1/2/3	BI	1294.2	317.9	(CST,cnf,Lth),brv,Gnt,Ten	4E
				12/3	BI	1317.4	271.6	(CST,cnf,Lth),brv,Gnt,Ten	
				rand	BI	1446.2	13.9	(CST,cnf),Lth),brv),Gnt),Ten	
				+	BI	1453.1	(L_0)	(CST,cnf),brv,Lth,Gnt,Ten	
				+	ML	1426.0		((((CST,cnf),Lth),brv),Gnt),Ten	
				+	MP	247		(((CST,(cnf,Lth)),Gnt),brv),Ten	4J
				1/2	BI	407.8	25.1	((CST,cnf),brv,Gnt),Lth,Ten	
				12	BI	420.4	(L_0)	resolves only (cst,fre,mad,cnf)	
				12	MP	17		(((cst,mad),fre,(cnf,brv),Gnt),Lth),Ten	