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Draft genome of the scarab beetle *Oryctes borbonicus* on La Réunion Island

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Abstract

Beetles represent the largest insect order and they display extreme morphological, ecological and behavioral diversity, which makes them ideal models for evolutionary studies. Here, we present the draft genome of the scarab beetle *Oryctes borbonicus***, which has a more basal phylogenetic position than the two previously sequenced pest species** *Tribolium castaneum* **and** *Dendroctonus ponderosae* **providing the potential for sequence polarization.** *O. borbonicus* **is endemic to La Réunion, an island located in the Indian Ocean, and is the host of the nematode** *Pristionchus pacificus,* **a well-established model organism for integrative evolutionary biology. At 518 Mb, the** *O. borbonicus* **genome is substantially larger and encodes more genes than** *T. castaneum* **and** *D. ponderosae***. We found that only 25% of the predicted genes of** *O. borbonicus* **are conserved as single copy genes across the nine investigated insect genomes, suggesting substantial gene turnover within insects. Even within beetles, up to 21% of genes are restricted to only one species, while most other genes have undergone lineage specific duplications and losses. We illustrate lineage-specific duplications using detailed phylogenetic analysis of two gene families. This study serves as a reference point for insect/coleopteran genomics, although its original motivation was to find evidence for potential horizontal gene transfer (HGT) between** *O. borbonicus* **and** *P. pacificus***. The latter was previously shown to be the recipient of multiple horizontally transferred genes including some**

genes from insect donors. However, our study failed to provide any clear evidence for additional HGTs between the two species.

Introduction

Insects are one of the most diverse and successful animal groups on earth. More than one million species have been documented and there are probably ten times more waiting to be described (Scherkenbeck and Zdobinsky, 2009). Within insects, the Coleoptera (beetles) is the most evolutionarily successful order (Hunt *et al*., 2007) containing over 400,000 described species (Hammond, 1992) demonstrating extraordinary morphological, ecological and behavioral diversity (Crowson, 1960). However, despite the enormous size of this group, only few beetles have had their genomes sequenced so far, the red flour beetle *Tribolium castaneum* (Richards *et al*., 2008), the mountain pine beetle *Dendroctonus ponderosae*, a major forest pest (Keeling *et al*., 2013), the coffee berry borer *Hypothenemus hampei* (Vega *et al*., 2015) and the social beetle *Nicrophorus vespilloides* (Cunningham *et al*., 2015).

The Scarabaeoidea (=Lamellicornia) represent a major superfamily within the Coleoptera consisting mostly of phytophagous beetles. Some species are notorious agricultural pests such as the cockchafers, whereas others are considered useful because of their contribution to the decompositon of livestock manure (Scholtz and Grebennikov, 2005). Besides their enormous ecological significance, scarab beetles are also noted for their impressive phenotypic plasticity, which gives rise to a great variety of sexually dimorphic horns and antlers, whose size depends on the nutritional status of the animal (Moczek 2009, Lavine *et al*., 2015).

Within Scarabaeoidea, the genus *Oryctes*, family Dynastidae (Endrödi, 1985) comprises of 43 often tropical species (Bedford, 1976; Dechambre, 1983). Some species are pests of palm trees like the most widespread *Oryctes rhinoceros* (Ohler 1999), which can do considerable harm on coconut farms (Bedford, 1980). In contrast to *O. rhinoceros* and its close relative *O. monoceros*, the majority of *Oryctes* species have root-feeding grub stages and have undergone enormous speciation in Africa, Southeast Asia and the Indian Ocean (Dechambre, 1983). One such beetle species is *Oryctes borbonicus* (Dechambre, 1982), a species that is endemic to La Réunion Island, a biodiversity hotspot located in the Indian Ocean (Myers *et al*., 2000). *O. borbonicus* is included in the subgenus *Insuloryctes* placed together with endemics from Mauritius (*O. tarandus* & *O. chevrolati*i) and Rodriguez (*O. minor*) (Dechambre, 1982). The endemic scarab beetles of Mauritius might be extinguished due to the usage of a baculovirus to control the palm tree pest species *O. rhinoceros* and *O. monoceros*. Therefore,it has not been possible to get DNA of the putative sister species to construct a phylogeny of the genus. The biology of *O. borbonicus* is largely unknown. In its distribution area are no palm trees growing, so in contrast to many other *Oryctes* species, *O. borbonicus* does not use palms as food source on La Réunion. The larvae of *O. borbonicus* have not yet been found in nature, but all the area where *O. borbonicus* can be caught is covered with grassland. Therefore, we assume that grubs feed on roots of different grasses. Also, breeding experiments suggest that the development of the grubs can be estimated to be around three years (personal communication Jacques Rochat).

The nematode *Pristionchus pacificus* is known to be associated with *O. borbonicus* (Herrmann *et al*., 2006). *P. pacificus* is a nematode model organism used in evolutionary biology for studies that aim to integrate developmental biology, ecology and population genetics (Sommer & McGaughran, 2013; Sommer, 2015). *Pristionchus* was shown to have a necromenic association with scarab beetles. After invading the beetles as developmentally arrested dauer stages, dauer larvae wait for the death of the beetle to continue their development, feeding on the microbes growing on the carcass (Weller *et al*., 2010; Ragsdale *et al*., 2015). *P. pacificus* had its genome originally sequenced a decade ago, the results of which revealed a surprisingly complex genomic composition encoding more than 25,000 protein coding genes (Dieterich *et al*., 2008). Surprisingly, these studies revealed a substantial amount of horizontal gene transfer (HGT) into the nematode genome, most likely from different donors. For example, the *P. pacificus* genome contains seven cellulase genes that are originally of microbial origin but have duplicated and diversified within the genus *Pristionchus* (Dieterich *et al*., 2008; Mayer *et al*., 2011; Schuster and Sommer, 2012). It also contains diapausins, which are thought to be beetle-derived (Dieterich *et al*., 2008; Rödelsperger and Sommer, 2011). Further analysis based on various homology searches and codon usage suggested that insects might have been the donors of additional *Pristionchus* gene acquisitions by HGT, which could be confirmed by phylogenetic analysis for a family of retrotransposons (Rödelsperger & Sommer, 2011). HGT between insects and nematodes appears very likely, given the close entomophilic association of *Pristionchus* species and other genera of the family Diplogastridae (Kanzaki and Giblin-Davis, 2015). Furthermore, roughly one

third of the *P. pacificus* gene predictions do not have homologs in other nematodes indicating the presence of so-called orphan (or taxonomically restricted) genes (Borchert *et al*., 2010, Rödelsperger *et al*, 2013). Therefore, sequencing the genome of the scarab beetle *O. borbonicus* will provide the opportunity to search for HGT events, which could not be detected previously due to the scarcity of beetle genomes.

In this study, we present the draft genome of *O. borbonicus* as a resource for the insect genomics community. In a first general characterization of the *O. borbonicus* genome we show that, in spite of its basal phylogenetic position relative to the two other sequenced beetles, *O. borbonicus* has undergone a substantial amount of lineage-specific gene expansions and losses. We were able to further confirm this fact in a detailed phylogenetic analysis of two large gene families, the Glutathione S-Transferases (GST) and Cytochrome P450 (CYP) gene families, respectively. At the same time, the analysis of the *O. borbonicus* genome revealed no further evidence for HGT between beetles and *Pristionchus* nematodes.

Material and Methods

Genome sequencing

High molecular weight DNA extraction

One male *Oryctes borbonicus* beetle was dissected under sterile conditions. The thoracal flight muscles were prepared for DNA extraction with the Qiagen genomic DNA extraction kit and Qiagen genomic tip columns (Qiagen, Hamburg, Germany) following the manufacturer's instructions. DNA quality and integrity was determined with a NanoDrop ND 1000 spectrometer (PeqLab, Erlangen, Germany) and by gel electrophoresis. The high molecular weight DNA was used to generate mate-pair libraries. Paired-end libraries were prepared with DNA extracted from two legs of the same beetle with the epicenter DNA extraction kit (Epicentre, Madison, USA) following the manufacturer's instructions.

RNA extraction

Two legs from the same individual beetle were frozen in liquid nitrogen and ground to a fine powder using mortar and pestle. The powder was directly transferred into Tri Reagent® (Sigma-Aldrich, Munich, Germany) and RNA was extracted with the Direct-zol™ RNA MiniPrep (Zymo, Freiburg, Germany) following the manufacturer's protocol. RNA quality was determined with a NanoDrop ND 1000 spectrometer (PeqLab, Erlangen, Germany) and RNA integrity was checked with the Agilent RNA Nano Chip

Assay (Agilent, Santa Clara, USA). Only RNA samples of high quality (OD 260/280 > 2 and OD 260/230 > 1.8) and high integrity were used for library preparation.

DNA sequencing

Two paired-end libraries with an average insert size of 450 and 500 bp and four mate-pair libraries of 3, 5, 8 and 11 kb were prepared using Illumina's paired-end and Nextera mate-pair kits respectively (Illumina, Eindhoven, Netherlands) following the manufacturer's protocols. For the paired-end libraries a starting amount of 100 ng of DNA was sheared with a Covaris S2 ultrasonicator (Covaris Inc. Woburn, San Diego). The mate-pair libraries were prepared from 4 µg DNA. Both the paired-end libraries (2 plex) and the mate-pair libraries (5 plex) were each run on a single 101 bp paired-end flow cell lane of an Illumina HiSeq 2000 Sequencer (Illumina, Eindhoven, Netherlands).

Overlap library

The overlap library was created using the Illumina Nano kit (Illumina, Eindhoven, Netherlands) following the manufacturer's protocol. The average size of the library was approximately 450 bp. Subsequent sequencing was performed on the MiSeq platform with the reagent Kit v2, with 2 x 250 bp read length (Illumina, Eindhoven, Netherlands).

RNA sequencing

The high quality RNA obtained from two beetle legs was used to prepare two independent libraries following Illumina's TruSeq RNA library preparation protocol

(Illumina, Eindhoven, Netherlands). Both libraries (2 plex) were sequenced together on a single 101 bp pair-end flow cell lane of an Illumina HiSeq 2000 Sequencer.

Genome assembly

Genomic paired and mate-pair reads were quality trimmed and filtered as described in Rödelsperger *et al*. 2014. Genomic paired-end libraries were used to generate a first draft assembly with the Velvet assembler (Version 1.2.10) with a k-mer size of 41 (Zerbino and Birney, 2008). In a second step, we used SSPACE (version 2.0) with default settings for scaffolding using the mate-pair data (Boetzer *et al*., 2011). Intrascaffold gaps were closed using the Gapclosing module (version 1.10) of the SOAP package. For removal of contamination, we ran a blastn search against the NCBI nt database, searching for hits with sequence identity above 95% over a length of 100bp. After taxonomic inspection of significant hits, the contaminating contigs (mostly of fungal origin) were removed from the assembly. The final assembly comprised 150,243 scaffolds spanning 518 Mb (494Mb excluding gaps) with an N50 of 105kb. The largest scaffold spans 1.1Mb. The genome-wide GC content was 34.4%. To obtain independent estimates of genome size and repeat content we used the software jellyfish (version 1.1.4) to generate k-mer spectra of original raw sequencing data (Marçais and Kingsford, 2011).

Gene and repeat annotation

Repeats were identified using the RepeatModeler and RepeatMasker pipeline, which identified 154Mb (29.2%) as repetitive (~4.5% LINE elements, ~10% DNA elements, and ~14.4% unclassified). Transcriptomic reads were aligned to the *Oryctes borbonicus* assembly using TopHat (v2.0.3), and reference-guided transcriptome assemblies were generated using the software Cufflinks (v2.0.1). Transcriptome assemblies were used to train the gene finder AUGUSTUS (v2.6.1) (Stanke *et al*., 2006), which predicted 23,278 protein coding genes in the repeat masked assembly. To compare gene predictions with a purely evidence-based set of gene annotations, we made use of the MAKER2 pipeline (version 2.31.8; Holt and Yandell, 2011). MAKER2 was run once on the repeat masked genome using only de novo assembled transcripts (Trinity version: trinityrnaseq_r20140413p1; Grabherr et al., 2011) and protein homology data from *D. ponderosae* and *T. castaneum*. Thus, no implicit call of ab initio gene finders was done for this set of gene annotations.

Protein domains were annotated using the hmmsearch program of the HMMER3 package and the included PFAM profiles. For analysis of core eukaryotic genes, we downloaded a set of 458 profile HMMs, which were searched against the database of predicted proteins using hmmsearch (evalue < 0.001) (CEGMA database, Parra et al., 2007).

Comparative genomic data

We downloaded protein sequences of two more beetles and of seven representative species from seven different insect orders from Ensembl Metazoa release 25. This data set comprised 13,457 protein sequences from *Dendroctonus ponderosae* (Coleoptera), 16,526 sequences from *Tribolium castaneum* (Coleoptera), 30,362 protein sequences representing 13,918 genes from *Drosophila melanogaster* (Diptera), 15,314 sequences from *Apis mellifera* (Hymenoptera), 15,441 sequences from *Rhodnius prolixus* (Hemiptera), 12,829 sequences from *Heliconius melpomene* (Lepidoptera), 10,788 sequences from *Pediculus humanus* (Phthiraptera), 14,610 sequences from *Zootermopsis nevadensis* (Isoptera). In the case of *D. melanogaster*, we used the longest isoform per gene for further analysis. Conserved synteny between beetle genomes was detected by means of CYNTENATOR software (Rödelsperger and Dieterich 2010), which computes gene order alignments using a phylogenetic scoring function. This approach identified conserved syntenic blocks between *O. borbonicus* and the *T. castaneum* genome spanning the 42Mb of the *T. castaneum* genome and 39Mb of conserved syntenic blocks between *O. borbonicus* and D. *ponderosae.* Conserved synteny was used to identify contigs that are likely of X-chromososomal origin (Supplemental Figure 3). For the CYP family, manually curated sequences, including those from the moth *Bombyx mori*, were retrieved from the website of David Nelson (http://drnelson.uthsc.edu/biblioC.html), and experimentally characterized sequences of other beetles (*Ips paraconfusus, Phyllopertha diversa,* and *Leptinotarsa decemlineata*) were retrieved from Genbank. Artifactual fusions and fissions in protein predictions were manually corrected as described

previously (Markov et al., 2015), with further expert polishing by D. Nelson regarding CYPs, and are provided as a supp. dataset.

Ortholog clustering and phylogenetic analysis

In order to identify orthologous clusters we used orthomcl (Li *et al*, 2003) software with default settings. We identified 2,355 1:1 orthologs from 14,748 orthomcl clusters using a custom perl script. We generated multiple sequence alignments for each 1:1 ortholog cluster using Clustal Omega (Sievers *et al*, 2011). After model testing with ProtTest 3 (Darriba *et al*, 2011) 2,355 individual gene trees and one tree based on all concatenated alignments were constructed using RAxML (version 8.1.20, Stamatakis.A, 2014). Concatenation as well as looking at the most frequently reconstructed gene trees, resulted in the same species tree topology (Fig. 1A). The analysis of the GST and CYP gene families was done by aligning sequences using MUSCLE (Edgar, 2004). Conserved sites were manually selected using Seaview (Gouy *et al*., 2010). Phylogenetic trees were inferred using PhyML (Guindon and Gascuel, 2003) with the LG+G substitution model. Branch support values were assessed using the approximate likelihood ratio test (Anisimova and Gascuel, 2006).

Results

Draft genome assembly of *Oryctes borbonicus*

From a sampling trip to La Réunion island, of which the primary goal was to find more beetle associated nematode strains, we set two male beetle specimen aside that should provide enough material to sequence the genome and transcriptomes. Our original intention was to use only data from one specimen for the assembly, but since additional sampling trips were not an option, we collected a second individual as backup.

We used state-of-the-art sequencing technology including mate-pair, paired-end and overlap libraries to assemble a draft genome of *O. borbonicus.* We initially planned to generate an assembly using data from a single individual with the Allpath-LG assembler. However, without knowing the actual genome size, we found that the coverage of the overlap library was insufficient to generate an *ab initio* assembly by Allpath-LG (Supplemental Figure 1). We therefore followed an alternative approach, that used data from a single individual for the initial assembly and data from both individuals for scaffolding and gap-closing (see *Methods*). Our final assembly has a size of 518 Mb (Table 1), which is substantially larger than the two previously sequenced beetle genomes (*Tribolium castaneum* has 230 Mb and *Dendroctonus ponderosae* 208 Mb). Calculation of genome size based on the total amount of sequence data, the expected coverage and the size of the X-chromosomes (as inferred by coverage and synteny analysis), results in a similar estimate of roughly 500Mb. High quality reads without any uncalled bases were realigned against the final assembly in order to assess the completeness of the genome

assembly. This showed that more than 99% of reads could be placed onto the assembly, indicating that the assembly covers almost all of the raw sequencing data. However, we find evidence that unresolved repeats caused some problems in the assembly resulting in high rates of ambiguous base calls and a heterogenous coverage profile (Supplemental Figures 2-4). The fraction of repetitive sequences in the final assembly was estimated to be 29%, which matches the range of 17 – 34% found in the two previously sequenced beetle genomes (Keeling *et al*. 2013, Richards *et al*. 2008). In addition, analyzing the k-mer spectrum of raw sequence data (Supplemental Figure 5), we find that around 70% of sequence data is represented by k-mers that have at most the expected coverage (30X). 23,278 protein-coding genes were predicted using the AUGUSTUS gene finder after training with RNA-seq data. Again, this number of protein-coding genes is substantially larger than the values reported for the two previously sequenced beetle genomes (N=13,457 for *D. ponderosae* and N=16,526 for *T. castaneum*).

We used the MAKER2 pipeline (version 2.31.8; Holt and Yandell, 2011) to generate a set of 20,504 evidence-based gene annotations based on *de novo* assembled transcripts and protein sequences from other insects (PMID:21572440 and PMID: 22192575). These annotations were used to further evaluate the AUGUSTUS predictions showing that around 81% of evidence-based exons overlap predicted exons. Second, we tested for the representation of core eukaryotic genes in the predicted gene set. We used the definition of core eukaryotic genes as provided by the CEGMA database (Parra *et a*l., 2007) and found that 445 (97%) of the 458 core eukaryotic gene profiles are represented in the predicted proteome of *O. borbonicu*s and the best hit covered in median 92%

(interquartile range: 73-98%) of the query profile. Third, we generated our "own" core insect gene data set by assembling homologous sequences into orthologous clusters (Li *et al*, 2003) in order to identify genes that are presented as 1:1 orthologs in all three beetle-, as well as in the six non-beetle insect genomes (see *Method*s). This approach identified 2,355 genes that have a 1:1 relationship in all nine insect genomes. We used these orthologous clusters to test whether *O. borbonicus* genes show a tendency to represent incomplete or partial predictions by comparing their size with the size of the corresponding genes in the other eight insect genomes. As indicated in Supplementary Figures 6A and B, *O. borbonicu*s gene predictions show a similar size range relative to *Drosophil*a as the gene sets of the other sequenced insect genomes.

Next, to assess the degree of completely missing gene predictions, we counted outlier orthology clusters representing those genes that have a 1:1 orthology relationship in all but one genome (Supplementary Figure 6C). The number of outliers was in a range of a few dozen for all nine genomes, again supporting the notion that at least in highly conserved regions most if not all of the sequenced genomes are of comparable quality. In summary, these results underpin the high quality of gene predictions in *O. borbonicu*s relative to the eight other insect genomes (Supplementary Figure 6).

The considerable differences in genome size and gene number between *O. borbonicus* and the two previously sequenced beetle motivated us to investigate further to what extent these differences are manifest across different gene classes. We made the surprising finding that 41% of *O. borbonicus* gene predictions represent orphan singleton genes, i. e. genes that lack sequence similarity in any other of the tested insect genomes

as well as in the *O. borbonicus* genome itself (as opposed to orphans with intra-species paralogs) (Supplementary Figure 6 and Figure 1A,B). This is in strong contrast to the two previously sequenced beetle genomes, for which we found 17% of genes to be orphan singletons in *D. ponderosae* and 24% in *T. castaneum*. Genes can be classified as orphan genes due to multiple technical as well as biological reasons. Technical reasons are the lack of phylogenetic resolution of sampled genomes, but also artifactual gene predictions. Consistent with a previous analysis of orphan genes in the nematode *P. pacificus* (Wissler *et al*. 2013), orphan singletons are relatively short, their total coding sequence does not correspond to gene number (Figure 1B) and they show lesser evidence of expression (Supplementary Figure 7). Biologically, orphan genes may very well represent truly functional lineage-specific genes.

To further support that at least a fraction of identified orphan genes represent truly functional sequences, we investigated to what extent, orphan singletons show evidence of protein domains (PFAM, e-value < 0.001), weak homologies in other insects (BLASTP e-value<0.001), or have expression evidence (FPKM >1). In total, we found that 4057 (43%) of orphan singletons fulfill at least one of the three mentioned criteria suggesting that large portions of these predictions are real genes. However, further insight into the origins of orphan genes can only be gained on the basis of much broader phylogenetic sampling and also broader transcriptome data. As this is a future project, the remaining part of our analysis will focus on genes with homologs in other beetle and insect genomes - a gene set that might be of greatest value for current comparative genomic analyses.

Distribution of conserved, beetle-specific and orphan genes

We used the identified orthologous clusters to compare i) conserved genes, with detectable homologs across different insect orders, ii) beetle-specific genes that are found in at least two of the beetle genomes but not in any other insect, and iii) orphan genes, which are restricted to one specific lineage. In order to obtain more robust estimates of the relative size of different gene classes, we excluded orphan singletons from this analysis, as it is unclear to what extent this class includes artifactual gene predictions. Figure 1A and B shows the fine scale distribution of different homology classes across all nine insect genomes. As mentioned above, we originally identified 2,355 orthologous clusters, whose genes were predicted as 1:1 orthologs in all nine species. These 1:1 orthologs make up between 17 and 25% of the gene repertoire in these different insects, indicating that the majority of gene families have undergone lineage-specific gene birth and death events. The first three categories in Figure 1A and B define conserved genes that are found across all analyzed insect orders. It is important to note that this gene set is by far the largest class. Focusing only on the beetle genomes, the fraction of conserved genes ranges from 73% for *O. borbonicus* to 87% for *D. ponderosae.* At the same time, the second most abundant group of genes are orphan genes (orphan with paralogs), which constitute between 7% of genes in *D. ponderosae* and 21% in *O. borbonicus.* Finally, only a minor fraction of genes (6-8%) are specific to and conserved only within beetles (with homologs in at least one other beetle genome), suggesting that the generation of novel genes has not strongly contributed to the evolution of the order Coleoptera.

Lineage-specific patterns in beetle genome evolution

Our prior analyses indicated that a substantial fraction of genes are either conserved among insects or highly specific to individual lineages (Figure 1A and B). Therefore, one major benefit of the *O. borbonicus* genome is its position as an outgroup relative to the two previously sequenced beetle genomes (Hunt *et al*., 2007). In order to confirm previous analyses, we reconstructed phylogenetic trees based on the 2,355 orthologous gene clusters showing that the most frequently predicted tree topologies are fully consistent with the tree shown in Figure 1. Similarly, the reconstruction of a genome-wide species phylogeny based on the concatenation of the alignments (supermatrix approach) of all 2,355 orthologous clusters revealed that *O. borbonicus* indeed represents an outgroup to the two previously sequenced beetles.

To further characterize lineage-specific patterns in the evolution of beetle genomes, we first screened for drastic changes in the size of gene families. In the ideal case, detailed phylogenetic analyses (see below) would be necessary to reconstruct the exact evolutionary history of duplication and gene loss events. However, comparisons of approximate gene family sizes provide a proxy to prioritize candidate gene families for more detailed investigation. Here, we defined gene families based on the presence of a certain protein PFAM domain and performed all three pair-wise comparisons of the distributions of gene family sizes (Figure 1C-E). In the comparison between *T. castaneum* and *D. ponderosae,* reverse transcriptases (PF00078) and seven transmembrane proteins (PF02949 and PF08395) are present in much higher number in *T.*

castaneum (Figure 1C). In the absence of an outgroup it would remain unclear whether this result is caused by an expansion in the *T. castaneum* lineage or by a loss in the *D. ponderosae* lineage. The comparison with *O. borbonicus* as outgroup (Figure 1E) shows higher numbers for both gene families in *T. castaneum,* suggesting that these families have undergone a lineage-specific expansion in the branch leading to *T. castaneum*. It should be noted however, that the number of genes encoding reverse transcriptases is also moderately larger in *O. borbonicus* relative to *D. ponderosae* (Figure 1D). We interpret this finding as evidence that a second expansion in the lineage leading to *O. borbonicus* is the most likely scenario to explain this increase, because we are not aware of a mechanism that would lead to a lineage-specific loss of transposable elements. Nonetheless, detailed phylogenetic analyses are ultimately needed to confirm this second expansion. While expansions of reverse transcriptases and retroviral integrases (PF00665) in the *O. borbonicus* and *T. castaneum* lineages could point to recent activity of retroviruses and retrotransposons, the expansion of seven transmembrane proteins could be due to a variety of different events. Proteins of the seven transmembrane families are involved in many essential signaling pathways, for example as receptors for neuropeptides. These short proteins have been shown to be crucial not only for developmental processes, such as molting and diapause (Verlinden *et al*., 2015), but also play important roles in the maintenance of homeostasis of metabolites and proteins, in the regulation of water balance and in several behaviors (Scherkenbeck and Zdobinsky, 2009, van Hiel *et al*., 2010).

Accelerated gene turnover in the *D. ponderosae* lineage

To quantify the rate of gene turnover across the beetle phylogeny, we identified orthologous clusters with beetle-specific gene losses or expansions. In addition, we mapped these events onto specific branches in the beetle phylogeny and compared these numbers with the estimations of simulated gene losses and gains that were randomly placed onto the beetle phylogeny. For this analysis, we restricted ourselves to orthologous clusters with one member in *H. melpomene* and *D. melanogaster*, respectively, and screened for beetle-specific losses and gains. For example, the presence of a member of such an orthologous cluster in *O. borbonicus* but its absence in the two other beetles would suggest that the ortholog was lost in the common ancestor of *T. castaneum* and *D. ponderosae.* Following this methodology, we identified 320 clusters with *O. borbonicus*-specific losses and 220 with expansions, 281 losses and 459 expansions in *D. ponderosae*, 52 losses and 74 expansions in *T. castaneum*, and 48 losses and 34 expansions in the ancestor of *T. castaneum* and *D. ponderosae*, respectively.

We then simulated 10,000 evolutionary scenarios, randomly placing gene losses and gains onto branches of the phylogeny with probabilities proportional to the branch lengths as derived from our supermatrix tree. The most striking patterns discovered in these analyses, are the strong depletions of gene losses and gains in the *T. castaneum* lineage ($P<10^{-4}$) with a simultaneously increased number of duplications in the *D. ponderosae* lineage ($P<10^{-4}$). This result is particularly interesting, as *D. ponderosae* did not show any large expansions of specific gene families in our previous analysis based on protein domains (Figure 1C and D), suggesting that both approaches reveal rather

complementary patterns of gene family evolution. In summary, our analysis has shown lineage specific expansions and losses as a recurrent trend in beetles. However, more detailed phylogenetic analysis is needed to confirm these trends in individual gene families. We thus focus our final analysis on the GST and CYP gene families, which are important molecular players in insect physiology.

Oryctes-specific expansions in the ancient GST sigma and theta families

GSTs are members of an ancient gene family, present in both bacterial and eukaryotic organisms. Insect GSTs can be divided into two major groups, the cytosolic and the microsomal GST genes. The cytosolic group further divides into six classes, the Delta, Epsilon, Sigma, Omega, Theta, and Zeta classes, respectively (Friedman *et al.* 2011). The Delta and Epsilon classes are insect-specific, and comprise enzymes that metabolize pesticides (Che-Mendoza *et al*., 2009; Enayati *et al*., 2005). However, some of these GST genes are also regulating the metabolism of endogenous hormonal compounds (Enya *et al*., 2015), whereas others have non-enzymatic functions (Sheehan *et al*., 2001). Consistent with previous reports (Shi *et al*., 2012; Keeling *et al*., 2013), we find that the Delta/Epsilon GSTs are highly expanded in *T. castaneum* independent of *D. melanogaster* (Figure 2, Supplementary Figure 8). The overall number of cytosolic GSTs is similar across the three beetle species (36 in *T. castaneum*, 28 in *D. ponderosae*, 30 in *O. borbonicus*). However, the addition of the *O. borbonicus* genome suggests that only three out of the 30 cytosolic GSTs are 1:1 orthologs among beetles (highlighted in Fig. 2). Interestingly, the biggest expansion regarding *Oryctes* GSTs occurs in the Sigma

class, which comprises enzymes involved in the synthesis of prostaglandins in vertebrates and nematodes (Sheehan *et al*., 2001), and also in *Bombyx mori* (Yamamoto *et al*., 2013). Furthermore, our analysis suggests that four of the biggest expansions are located in the same genomic cluster. A second smaller *Oryctes*-specific expansion, limited to three paralogs, is found in the theta class and a third one concerns three genes in the Delta-Epsilon family.

Ten independent *Oryctes*-specific expansion events across the CYP family

The CYP family is present in most aerobic eukaryotes, and also in some bacteria (Nelson et al., 2013). In insects, the CYP family divides into four major clans, clan 2, clan 3, clan 4 and the mitochondrial or mito clan, respectively (Feyereisen, 2006). We found a total of 115 CYPs in *O. borbonicus*: 6 in clan 2, 62 in clan 3, 39 in clan 4, and 8 in the mito clan (Figure 3, Supplementary Figure 9). Interestingly, clan 2 and the mito clan are enriched in 1:1 orthologs across insects and many of these genes are suggested to be involved in the biosynthesis or the degradation of the molting hormone ecdysone. Also, one of them is involved in the final step of juvenile hormone synthesis. Interestingly, the mito clan also comprises the xenobiotic-metabolizing CYP12 gene from dipterans, which just like the *Oryctes*-specific expansion of Sigma GSTs, represents a case of lineage-specific expansion in a part of the tree that is otherwise conserved across insects.

Clan 4 comprises some fatty-acid hydroxylating enzymes (Nelson *et al*., 2013), but also enzymes involved in the synthesis or degradation of pheromones in other beetles (Qiu et al., *et al*., 2012). For example, individual sequences from another scarab beetle, *Phyllopertha diversa* (Maïbèche-Coisne et al., 2004) and the bark beetle *Ips paraconfusus* (Huber et al., 2007), indicate that duplication events are widespread among species and higher taxa, confirming the notion that pheromone-synthesizing genes evolve rapidly (Liénard *et al*., 2008). It is also interesting to note that clan 4 provides the most striking example of *Oryctes*-specific gene expansions. Specifically, in the group termed CYP4C3 (Supplementary Figure 9), comprising a single gene from *D. melanogaster* (CYP4C3), *A. mellifera* (CYP4AV1) and *T. castaneum* (CYP4BM1), *D. ponderosae* has two duplicates. In addition, the partial data for *I. paraconfusus* suggest the existence of at least four paralogs. In contrast, the number rises to 19 for *O. borbonicus*, suggesting extremely high gene birth, and potentially death rates, consistent with previous reports in insects (Feyereisen, 2006).

Clan 3 indicates an additional example of high numbers of lineage-specific clusters, including genes that are involved in insecticide degradation in at least two beetles (Zhu *et al*. 2010, Zimmer et al., 2010) and pheromone synthesis in bark beetles (Sandstrom *et al*., 2006; Song *et al*., 2014). Again, there are many *O. borbonicus*-specific amplifications including one with 16 genes (Supplementary Fig 9). Interestingly, the same pattern of lineage-specific expansions has also been observed in other insects (Richards *et al*., 2008).

Absence of additional HGT events from beetles to nematodes

Our initial motivation to study the genome of *O. borbonicus* goes back to previous studies of the *P. pacificus* genome, which characterized a number of horizontal gene transfers (HGTs) from different donor organisms including insects (Dieterich *et al*., 2008 *et al.*, Mayer *et al*. 2011, Rödelsperger and Sommer, 2011) and showed that roughly one third of *P. pacificus* genes, so called orphan genes, do not have homologs in other nematodes (Borchert *et al*., 2010, Rödelsperger *et al*, 2013). Thus, the analysis of the genome of the scarab beetle *O. borbonicus,* a well established host of *P. pacificus,* provided the unique opportunity to search for other HGTs that had so far missed detection due to the scarcity of beetle genomes.

We obtained previously identified candidate gene sets for HGT (Rödelsperger and Sommer, 2011) on the basis of BLAST analysis using nematode and insect data and used them to screen for homologs in *O. borbonicus*. However, based on blastp and tblastn searches, followed by multiple alignment and phylogenetic analysis, we could neither identify members of the Diapausin family in the *O. borbonicus* genome, which had been previously proposed to be transferred from beetles (Dieterich *et al*, 2008, Rödelsperger and Sommer, 2011), nor could we find any other convincing candidates for HGT from the beetle to the nematode. Similarly, previously identified horizontally transferred retrotransposons did not show higher similarity to *O. borbonicus* sequences than to sequences from Lepidopterans (Rödelsperger and Sommer, 2011). These results suggest that the horizontal transfer events could date back to a time before the *Pristionchus* – scarab beetle association.

Discussion

Beetles represent the largest insect order but in comparison to flies and ants they are largely underrepresented at the level of genome sequencing analysis (Drosophila 12 Genomes Consortium, 2007, Roux *et al*., 2014). In this study, we have sequenced and annotated the genome of the scarab beetle *O. borbonicus*, which was proposed to be a basal representative in comparison to two previously sequenced genomes of *T. castaneum* (Richards *et al*., 2008) and *D. ponderosae* (Keeling *et al*., 2013), and might therefore be of importance to polarize genomic patterns between the two pest species. Please note that during the last phase of finalizing this manuscript, the genomes of *Nicrophorus vespilloides* and *Hypothenemus hampei* were published (Cunningham *et al*., 2015; Vega *et al*., 2015), which unfortunately we were not able to include in our study without redoing all analyses.

The *O. borbonicus* genome is 518 Mb in size and thus substantially larger, encoding a higher number of predicted genes than other sequenced beetle genomes. While we find a higher ratio of orphan genes in *O. borbonicus* (genes that lack sequence homology in any of the eight other insect genomes that are analyzed in this study), further data will be needed to quantify, to what extent orphan singleton genes represents a true biological phenomenon. For our overall insect genome analysis, we have excluded singleton orphan genes to ensure that they do not affect any of the conclusions drawn from these analyses. Overall, our comparative genomic analyses show that while many gene families are conserved across multiple insect orders, some have undergone lineage-

specific gene duplications and losses. Even among beetles, various different approaches (comparing protein domain counts, screening for branches with increased rates of duplications in the beetle phylogeny, detailed phylogenetic analyses of individual gene families) show a common trend of substantial gene turnover among genomes. In this context it is important to note that even in gene families that do not show any striking differences in gene family size (Figure 1 C-E) we can detect numerous large gene expansions using detailed phylogenetic analysis. This highlights the importance of detailed phylogenetic analyses of manually curated data sets to ensure the robustness of duplication patterns in genome evolution (Markov *et al*. 2015). Furthermore, we would like to add, that although heterozygosity in the sequenced beetle individual can lead to nearly identical duplicates, our general analysis of genome quality (Supplementary Figure 4) suggests that the assembler rather has the tendency to overcompress and merge repetitive regions. Thus, we conclude that the identified gene expansions in the *O. borbonicus* genome are indeed real.

Despite the lack of further evidence for HGTs, the *O. borbonicus* genome is of particular interest because of the study of the association of *Pristionchus* nematodes with scarab beetles. Previous work has shown that *Pristionchus* species and *P. pacificus* strains show specificity in their response to the pheromones of their beetle hosts (Hong and Sommer 2006, McGaughran *et al*., 2013, Cinkornpumin *et al*., 2014). Non-hydroxylated fatty acid ester derivatives pheromones were identified in four species from the *Oryctes* genus, that all are coconut pest species (Said et al., 2015). If the same kind of molecules would be active as pheromones in *O. borbonicus*, some Cytochrome P450s

may be involved in their inactivation, as they do for molecules from other chemical classes in the scarab beetle *Phyllopertha diversa* (Maïbèche-Cosne et al. 2004). However, a detailed phenotypic interpretation of genomic patterns is hampered by the lack of functional data and also by the low phylogenetic resolution. Thus, more genomic and functional studies will be needed to better characterize the interaction between nematodes and beetles.

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Data availability

The genome assembly including evidence-based annotations has been submitted to NCBI Genbank under the accession LJIG00000000.1. The complete annotations are available at http://www.pristionchus.org/download/. Raw reads have been submitted to the NCBI sequence read archive under the study accession PRJNA293509. The raw RNA-seq reads are available in the NCBI sequence read archive **(SRX1458313).**.

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Figure legends

Figure 1. Conserved and lineage-specific patterns of gene content evolution.
(A) Schematic phylogeny of investigated insect genomes (Trautwein 2012, Hunt *et al.*, 2007) and distribution of genes in different orthology classes. (B) Amount of coding sequence in different orthology classes. (C-E) Protein domain (PFAM) count comparison between all three beetle genomes. Large protein domain families that show the most extreme differences in gene counts are labeled in each comparison.

Figure 2. A maximum-likelihood tree of beetle cytosolic GSTs.
The tree is rooted with sequences from *Drosophila* and *Apis*, and was calculated under the LG+G model. A linear version is available in Supplementary Figure 8.

Figure 3. A maximum-likelihood tree of insect CYPs.
The tree was calculated under the LG+G model, and is rooted by CYP51 sequences. A linear version of the tree is available in Supplementary Figure 9.

Supplementary Figure 1 – k-mer analysis of raw data. Raw reads were used to calculate k-mer histograms (k=17) for all three genomic libraries that were obtained from two *O. borbonicus* specimen (S1 and S3). The x-axis shows the coverage of a k-mer (number of perfect matches in the complete library) and the y-axis the number of k-mers at a given coverage. The huge peak at coverage values around 1 indicates sequencing errors, while the two smaller peaks in the coverage range 10-30 approximately denote the coverage in unique portions of the genome. The k-mer histogram for the overlap library exhibits lower coverage and higher sequencing error rates, which likely explains why we were unable to assemble the *O. borbonicus* genome with an alternative assembler such as AllPaths-LG.

Supplementary Figure 2 – Relationship between ambiguous basecalls and coverage.

To investigate the bimodal distribution of the principal coverage peak in the range 10-30X in greater detail, we compared the coverage after mapping to the genome with evidence for ambiguous basecalls. Ambiguous basecalls can arise through heterozygosity, recent duplication events, and assembly errors and may also be informative to infer scaffolds representing the sex chromosome. The gray histogram shows the coverage in 2kb windows across the whole genome. The red curve shows the coverage profile in a subset of windows without any ambiguous base call. Interestingly the red curves shows two distinct peaks at around 16X and 32X coverage, which supports that the lower peak is of X-chromosomal origin. However, it remains unclear, why the autosomal peak seems to be smaller. The fact that windows with many ambiguous basecalls peak between the two red peaks contradicts the assumption, that they may be duplication derived. In addition, we would also expect that truly heterozygous regions would peak at the same position as the autosomal peak, which supports the explanation that the intermediate coverage values are generated by assembly problems.

Supplementary Figure 3 – Coverage in X-linked and highly conserved regions

In order to confirm that the low coverage peak corresponds to X-chromosomal sequences, we inferred *O. borbonicus* regions that display conserved synteny with X-linked regions in *T. castaneum* and compared their coverage profile to high quality assembly regions as identified by genomic regions of one-to-one orthologs in all analysed insect genomes. X-linked genes clearly show a unimodal distribution with the peak corresponding to the low coverage peak. Interestingly in highly conserved genes, the autosomal peak now shows stronger signal.

Supplementary Figure 4 – Influence of unpaired reads in the coverage profile

To confirm that heterogenous coverage profile is due to assembly problems, we plotted the fraction of read pairs for which the second pair could not be aligned in proper orientation as a function of coverage in 2kb windows. Consistent with previous analysis, we see two high density clouds of high quality at coverage values 16X and 32X (almost all pairs are mapped in correct orientation). In addition, we see a strong drift from the

higher peak to the lower peak with increasing fraction of not properly aligned pairs, indicating that indeed misassembled regions are responsible for the heterogenous coverage profile.

Supplementary Figure 5 - Repetitive k-mers within the the raw sequence data
Based on k-mer counts from the raw data (genomic library from individual S1), we excluded k-mers that occurred less than four times as likely sequencing errors and plotted the cumulative sum of the product of coverage and frequency of all k-mers at different coverage scales (black:4X-100X, blue:4x:10,000X). The product between coverage and frequency is approximately proportional to the amount of sequence that is represented by k-mers of a given coverage. Thus, the cumulative distribution indicates that for example around 70% of sequence data is represented by k-mers that have at most coverage around 30X.

Supplementary Figure 6. Comparative assessment of gene annotation quality.
(A) median and interquartile range of gene length of one-to-one orthologs (across all tested insect genomes) relative to *D. melanogaster*. (B) gene length relative to median of all nine species. (C) Number of one-to-one orthologs in all but one genome. (D) Fraction of total genes that were clustered by orthoMCL.

Supplementary Figure 7. Distribution of expression levels among different orthology classes.
(A)The graph shows expression levels as quantified in FPKM from RNA-seq data in three different categories for various orthology classes. While orphan genes show a strong bias towards lack of expression (FPKM=0 category), we still find expression evidence for around 30% of orphan genes. Furthermore even up to 30% of conserved gene classes do not show expression in our transcriptome data, indicating developmentally or tissue-specific expression. (B) The graph shows the distribution of genes expression values for various gene classes.

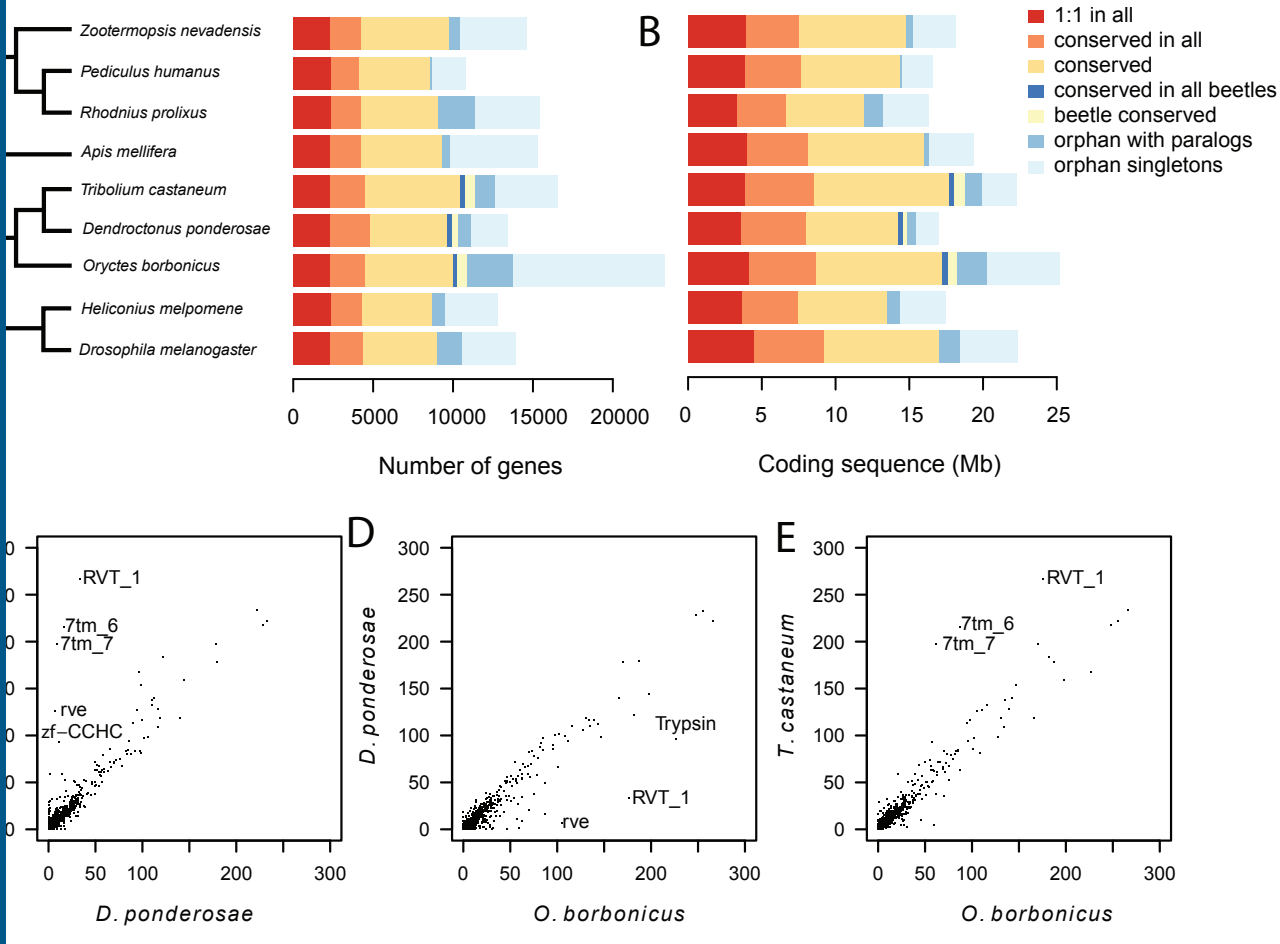
Supplementary Figure 8. A maximum-likelihood tree of cytosolic beetle GSTs.
The tree is rooted with sequences from *Drosophila* and *Apis*, and was calculated under the LG+G model.

Supplementary Figure 9. A maximum-likelihood tree of insect CYPs.
The tree was calculated under the LG+G model, and is rooted by CYP51 sequences.

Supplementary Dataset. Manually edited protein predictions from beetle GSTs and CYPs.

Table 1: Genome statistics. Assembly features such as size and number of sequences were collected for the raw Contig assembly, scaffolded genome, and three types of gene annotations. Please note that arbitrary minimum cutoffs were used by the different programs.

	Genome assembly		De novo transcriptome	Evidence-based annotation	Gene prediction
	Scaffolds	Contigs	Trinity	MAKER2	AUGUSTUS
Total size [Mb]	517.9	426.3	27.2	16.6	25.2
N sequences	150243	30471	18177	20504	23278
Largest [kb]	1101	457	16	32.4	41
Smallest [kb]	0.1	0.1	0.5	<0.1	<0.1
N50 [kb]	104.8	33.1	2.0	1.5	1.8
N sequences (length > N50)	1365	3590	3915	3168	3712


Zootermopsis nevadensis
Pediculus humanus
Rhodnius prolixus
Apis mellifera
Tribolium castaneum
Dendroctonus ponderosae
Oryctes borbonicus
Heliconius melpomene
Drosophila melanogaster
0
5000
10000
15000
20000
Number of genes
B
1:1 in all
conserved in all
conserved
conserved in all beetles
beetle conserved
orphan with paralogs
orphan singletons
0
5
10
15
20
25
Coding sequence (Mb)
RVT_1
7tm_6
7tm_7
rve
zf−CCHC
D. ponderosae
D
Trypsin
O. borbonicus
E
T. castaneum

Sigma

Theta

Omega

Zeta

Delta/Epsilon

0.5

D. melanogaster amplifications

T. castaneum amplifications

O. borbonicus amplifications

1:1 orthologs among beetles

D. melanogaster GSTs

O. borbonicus GSTs

D. ponderosae GSTs

T. castaneum GSTs

A. mellifera GSTs