The nuclear and mitochondrial genomes of the facultatively eusocial orchid bee *Euglossa dilemma.*

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Data availability

The *E. dilemma* genome assembly *Edil_v1.0*, the annotation, and the original gene set *Edil_OGS_v1.0* are available for download via NCBI [XXX], Beebase [XXX], the i5k NAL workspace [xxx], and the Ramirez Lab website [URL]. The raw reads are available at the NCBI Sequence Read Archive [XXX]. The published raw transcriptome sequence reads are available at the NCBI Sequence Read Archive [SRA: SRX765918].

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Abstract

Bees provide indispensable pollination services to both agricultural crops and wild plant populations, and several species of bees have become important models for the study of learning and memory, plant-insect interactions and social behavior. Orchid bees (Apidae: Euglossini) are especially important to the fields of pollination ecology, evolution, and species conservation. Here we report the nuclear and mitochondrial genome sequences of the orchid bee *Euglossa dilemma*. *Euglossa dilemma* was selected because it is widely distributed, highly abundant, and it was recently naturalized in the southeastern United States. We provide a high-quality assembly of the 3.3 giga-base genome, and an official gene set of 15,904 gene annotations. We find high conservation of gene synteny with the honey bee throughout 80 million years of divergence time. This genomic resource represents the first draft genome of the orchid bee genus *Euglossa,* and the first draft orchid bee mitochondrial genome, thus representing a valuable resource to the research community.

Introduction

Bees (Apoidea) are important models for the study of learning and memory (Menzel and Muller 1996), plant-insect interactions (Doetterl and Vereecken 2010) and the evolution of social behavior (Nowak *et al.* 2010; Woodard *et al.* 2011; Kapheim *et al.* 2015). Among the >20,000 bee species worldwide, lineages have evolved varied degrees of specialization on floral resources such as pollen, resins, and oils (Wcislo and Cane 2003; Michener 2007; Litman *et al.* 2011). These relationships are wide-ranging and have substantial impact on the health and function of natural and agricultural systems (Klein *et al.* 2007). Furthermore, several transitions from an ancestral solitary to a derived eusocial behavior have occurred within bees (Danforth 2002; Cardinal and Danforth 2011; Branstetter *et al.* 2017). Thus, bees provide unique opportunities to investigate the genetic underpinnings of multiple major ecological and evolutionary transitions. The repeated evolution of different behavioral phenotypes in bees, including foraging and social behavior, provides a natural experiment that allows for the determination of general as well as species-specific molecular genomic changes underlying phenotypic transitions. In order to capitalize on this potential, whole-genome sequences of a divergent array of bee species with different life histories are needed (Kapheim *et al.* 2015).

Orchid bees (Apidae; Euglossini) are among the most important pollinators of thousands of diverse neotropical plant species (Ramírez *et al.* 2002). While female orchid bees collect nectar, pollen and resin for nest construction and provisioning, male bees collect perfume compounds from floral and non-floral sources (Vogel 1966; Whitten *et al.* 1993; Eltz *et al.* 1999; Roubik and Hanson 2004). These volatile compounds are used to concoct a species-specific perfume blend that is subsequently used during courtship, presumably to attract conspecific females. This unique male scent-collecting behavior has recently been examined in a broad array of molecular ecological and evolutionary studies, focusing on phenotypic evolution,

chemical communication, plant-insect mutualisms, and speciation (Eltz *et al.* 2008; 2011; Ramírez *et al.* 2011; Brand *et al.* 2015).

While most of the approximately 220 species of orchid bees appear to be solitary, several species have transitioned to living in coordinated social groups (Garófalo 1985; Pech *et al.* 2008; Augusto and Garófalo 2009). Female *Euglossa dilemma* individuals, for example, can either form solitary nests and provision their own brood cells, or live in small groups where daughters remain in their natal nest and help their mother rear her offspring, instead of dispersing to found their own nest. The social *E. dilemma* nests (similar to the closely related *E. viridissima;* Pech *et al.* 2008) exhibit true division of labor, with subordinate daughters foraging for resources and the reproductively dominant mothers laying eggs (Saleh & Ramirez pers. obs.). This facultative eusocial behavior represents an early stage in social evolution and makes *E. dilemma* well-suited for studying the genomic changes underlying the transition from solitary to eusocial behavior. While other facultative eusocial species evolved throughout the bee lineage, orchid bees have a unique taxonomic position (Cardinal and Danforth 2011). Orchid bees are part of the corbiculate bees, together with the honey bees, bumblebees and stingless bees, three obligately eusocial bee lineages (Figure 1a). As the sister group to all other corbiculate bee lineages (Romiguier *et al.* 2015; Peters *et al.* 2017; Branstetter *et al.* 2017), orchid bees may provide key insights into the early stages of eusociality and the possible evolutionary trajectories that led to the obligate eusocial behavior exhibited by honey bees.

Here we present the draft genome of the orchid bee species *Euglossa dilemma*. Using a combined paired-end and mate-pair library sequencing approach, we assembled 18% of the predicted 3.3Gb genome, and annotated a high-quality gene set including 15,904 genes. In addition, we reconstructed three quarters of the mitochondrial genome with the help of transcriptome data, representing the first orchid bee mitogenome. These genomic resources will facilitate the genetic study of outstanding ecological and evolutionary questions, such as the evolution of resource preferences and the evolution of eusociality. Moreover, it provides an important genomic resource for an endangered group of crucial neotropical pollinators, that are of specific concern for conservation biologists (Zimmermann *et al.* 2011; Suni and Brosi 2012; Suni 2016; Soro *et al.* 2016).

Materials and Methods

Genome sequencing and assembly

Nuclear Genome. Sequencing of the *E. dilemma* genome was based on six haploid male individuals collected at Fern Forest Nature Center in Broward County, FL (26°13'46.3''N, 80°11'08.9''W) in February 2011. This population was chosen due to its low nucleotide diversity resulting from a bottleneck during a single introduction to Southern Florida about 15 years ago (Skov and Wiley 2005; Pemberton and Wheeler 2006; Zimmermann *et al.* 2011). DNA was extracted from each bee

independently and used for the construction of four paired-end (two 170bp and 500bp libraries, respectively) and four mate-pair (two 2kb and 5kb libraries, respectively) sequencing libraries. Next, the paired-end libraries were sequenced in 90 cycles and the mate-pair libraries for 49 cycles on an Illumina HiSeq2000. The resulting sequence data was run through fastuniq v1.1 (Xu *et al.* 2012) to remove PCR duplicates and quality trimmed using trim_galore v0.3.7 (Babraham Bioinformatics). Subsequently, reads were used for *de novo* assembly with ALLPATHS-LG v51750 (Gnerre *et al.* 2011) and Soap-denovo2 (Luo *et al.* 2012) with varying settings. Gaps within scaffolds were closed using GapCloser v1.12 (Luo *et al.* 2012) for each assembly. ALLPATHS-LG with default settings resulted in the highest-quality assembly, based on assessments of annotation completeness (see below). This assembly (*E. dilemma* genome assembly v1.0) was used for all subsequent analyses. All other assemblies were excluded from analysis, but are available upon request.

The pre-processed reads were used for k-mer based genome size estimates. We used ALLPATHS-LG to produce and analyze the k-mer frequency spectrum (k=25). Genome size was estimated on the basis of the consecutive length of all reads divided by the overall sequencing depth as $(N \times (L - K + 1) - B)/D = G$, where N is the total number of reads, L is the single-read length, K is the k-mer length, B is the total count of low-frequency (frequency ≤3) k-mers that are most likely due to sequencing errors, D is the k-mer depth estimated from the k-mer frequency spectrum, and G is the genome size. In addition, we used the ALLPATHS-LG k-mer frequency spectrum to predict the repetitive fraction of the genome.

The quality of the genome assembly was assessed using standard N statistics, and assembly completeness as measured by the CEGMA v2.5 (Parra *et al.* 2007) and BUSCO v1.1 (Simão *et al.* 2015) pipelines. CEGMA was run in default mode, whereas BUSCO was run with the arthropoda_odb9 OrthoDB database (Zdobnov *et al.* 2017) in genome mode.

We estimated the mean per-base genome coverage on the basis of the pre-processed reads and the estimated genome size as $\frac{\sum_{i=1}^{4}(R_i * L_i)}{G} = C$, where R is the number of reads and L the mean read length of sequence library i, G is the estimated genome size and C the resulting per-base coverage.

Mitogenome. Initial attempts to reconstruct the mitochondrial genome from our whole-genome shotgun sequencing reads including read subsampling and exclusion of rare variants were only partially successful, due to high sequence variability of sequencing reads with similarity to mitochondrial loci (data not shown). In addition, we have observed that the amplification of mitochondrial DNA in standard polymerase chain reactions (PCR) leads to a high level of polymorphic sites in *E. dilemma* and other orchid bees (Brand & Ramírez pers. obs.). Together, this suggests the presence of nuclear copies of the mitochondrial genome (NUMTs) that interfere with the assembly process and PCR amplification. Therefore, we used available *E.*

dilemma transcriptome assemblies in order to reconstruct the mitochondrial genome from cDNA (Brand *et al.* 2015). In order to find expressed mitochondrial genes represented in the transcriptome assembly of Brand et al. 2015, we used blastx with the honey bee mitochondrial genome as query (Crozier and Crozier 1993) and an E-value cutoff of 10E-12 (Altschul *et al.* 1990; Camacho *et al.* 2009). The contigs and scaffolds that were detected with this approach were annotated following Dietz *et al.* 2016. Briefly, we performed tblastn and tblastx searches with protein coding genes and rRNA genes of the honey bee mitochondrial genome, respectively. All hits were used for manual gene annotation using Geneious v8.0.5 (Biomatters Ltd. 2012). Since the recovered mitochondrial mRNA scaffolds contained more than one gene, we searched and annotated intergenic tRNAs using ARWEN 1.2.3 (Laslett and Canbäck 2008) and tRNAscan-SE 1.21 (Lowe and Eddy 1997).

Genome annotation

Gene annotation. Genes were annotated based on sequence homology and *de novo* gene predictions. The homology approach was based on the recently updated high-quality official gene set of the honey bee (OGS v3.2; Elsik *et al.* 2014). All honey bee original gene set (OGS) proteins were used in initial tblastn searches against all *E. dilemma* scaffolds with an E-value cutoff of 10E-4. Proteins with a hit covering ≥ 50% of the honeybee protein query were selected for accurate exon-intron boundary prediction for each scaffold using exonerate v2.42.1 (Slater and Birney 2005) with the minimum fraction of the possible optimal similarity per query set to 35%. This approach allowed the annotation of homologous genes of different length between the honey bee and *E. dilemma*. In a second round, genes not annotated under the previous settings were rerun with minimum similarity set to 15%. In the case of multiple hits of honey bee proteins to the same genomic region in *E. dilemma* leading to multiple independent annotations, we discarded all but one annotation with the best hit (based on completeness and similarity). This approach proved feasible due to the relatedness of *E. dilemma* and the honey bee. For *de novo* gene prediction we used Augustus (Stanke *et al.* 2008) and SNAP (Korf 2004) trained on the honey bee, with the *E. dilemma* genome masked for repetitive regions (See below) as input. Only genes predicted by both programs were taken into account. Gene predictions with ≥85% sequence similarity to each other were discarded, to prevent the inclusion of putative unmasked transposable element derived genes in the official gene set. *De novo* predictions were added to the *E. dilemma* OGS if not annotated by the homology-based approach.

Repetitive element annotation. Repetitive elements including tandem repeats, nuclear copies of the mitochondrial genome (NUMTs), and transposable elements (TEs) were annotated using multiple methods.

Tandem repeats. We searched for micro- and mini-satellites (1–6 bp and 7–1000 bp motif length, respectively) in all scaffolds using Phobos 3.3.12 (Mayer 2010). We performed two independent runs for each class of tandem repeats with Phobos

parameter settings following Leese et al. 2012 (gap score and mismatch score set to -4 and a minimum repeat score of 12; Leese *et al.* 2012).

NUMTs. We annotated NUMTs using blastn runs with the partial mitochondrial genome (see above) as query and an E-value cutoff of 10E-4 as used in NUMT analyses of other insect genomes (Pamilo *et al.* 2007). This approach allowed us to find NUMTs with medium to high similarity to the actual transcriptome-based mitochondrial genome.

TEs. In order to annotate TEs, we used Repeatmasker v4.0.5 (Smit et al. 2016) with Crossmatch v. 0.990329 as search engine in sensitive mode. We excluded low complexity regions and small RNA from the analysis (settings –nolow and -norna). This way Repeatmasker only searched for interspersed repeats. The Repbase invertebrate database v21.12 (Jurka 2000; Bao *et al.* 2015) was used as TE reference, due to the lack of a bee-specific database.

Genome structure

To analyze genome structure, we compared the genome wide gene synteny of *E. dilemma* and the honey bee. We used the genomic locations of homologous genes (as determined above) of the honey bee and *E. dilemma* scaffolds of at least 100kb length to build haplotype blocks with a minimum length of 1kb. Haplotype blocks included the entire gene span as well as intergenic regions whenever two or more adjacent genes were homologous in both species. We discarded gene annotations from downstream analysis that were recovered as homologous to multiple genomic locations in either species. Furthermore, we excluded *E. dilemma* genes that were recovered as homologous to honey bee scaffolds belonging to unknown linkage groups.

Data availability

The *E. dilemma* genome assembly *Edil_v1.0*, the annotation, and the original gene set *Edil_OGSv1.0* are available for download via NCBI [XXX], Beebase [XXX] (Elsik *et al.* 2016), the i5k NAL workspace [xxx] (i5K Consortium 2013), and the Ramirez Lab website [URL]. The raw reads are available at the NCBI Sequence Read Archive [XXX]. The published raw transcriptome sequence reads are available at the NCBI Sequence Read Archive [SRA: SRX765918] (Brand *et al.* 2015).

Results and Discussion

Whole-genome assembly

The *E. dilemma* genome assembly resulted in 22,698 scaffolds with an N50 scaffold length of 144Kb and a total length of 588Mb (Table 1). This represents 18% of the k-

mer based estimated genome size of 3.2Gb. Of all sequence reads, 68% aligned to the genome assembly, of which 56% aligned more than once. Further, the k-mer frequency spectrum based on all sequencing reads was strongly positively skewed indicating the presence of highly repetitive sequences in the read set (Figure 1b). Based on the k-mer frequency spectrum, 87.7% of the genome was estimated to be repetitive. This suggests that the genome of *E. dilemma* consists largely of highly repetitive sequences, explaining the low consecutive assembly length, the high assembly fragmentation, and the high fraction of sequence reads mapping multiple times to the assembly. The mean per-base coverage was estimated to be comparatively low in comparison to previous bee genome assemblies, with 19.7x based on the pre-processed reads and estimated genome size (Kocher *et al.* 2013 95.65x coverage; Kapheim *et al.* 2015 120x - 272.3x coverage). Total genomic GC content was 39.9%, and thus similar to previously sequenced bee genomes ranging between 32.7% and 41.5% (Table 1) (Kocher *et al.* 2013; Elsik *et al.* 2014; Kapheim *et al.* 2015).

Despite the fragmentation of the genome assembly representing less than 20% of the estimated genome size, CEGMA analysis revealed complete assemblies of 231 out of 248 core eukaryotic genes (93.2% completeness). Similarly, BUSCO analysis revealed that 1007 out of 1066 highly conserved arthropod genes were completely assembled (94.4% completeness). Our gene prediction approach generated a comprehensive official gene set including 15,904 protein-coding genes (Table 1). Of these gene models, 11,139 were derived from homology-based predictions, representing 73% of the 15,314 honey bee genes used for annotation. These annotations are well within or exceeding previous bee genome assemblies, and are similar to those reported for the other orchid bee genome available (Table 1) (Kocher *et al.* 2013; Elsik *et al.* 2014; Park *et al.* 2015; Sadd *et al.* 2015; Kapheim *et al.* 2015).

The CEGMA and BUSCO analysis and the gene annotation results suggest that the gene-coding fraction of the *E. dilemma* genome was properly assembled, despite the large estimated genome size and comparatively low per-base sequencing coverage. Fragmentation of the assembly is thus likely to be primarily the result of highly repetitive genomic elements, and less the result of low coverage. Overall, the results suggest that our approach was sufficient to produce a high quality official gene set. Due to the chosen homology-based approach, the majority of annotated genes in the official gene set has known homology to honey bee genes (Table S1), which will greatly facilitate genome-wide expression studies including gene ontology analyses and comparative gene set analyses among insects.

Mitochondrial Genome assembly

The recently published transcriptome assembly used for the reconstruction of the mitochondrial genome contained four scaffolds between 1,222bp and 4,188bp long with a total consecutive length of 11,128bp (Figure 2). This corresponds to about 75% of the estimated length of the mitochondrial genome, based on other corbiculate bee species (Crozier and Crozier 1993; Cha *et al.* 2007). The *E. dilemma*

mitogenome fragments contained 5 out of 22 tRNAs, 11 out of 13 protein coding genes of which two were only partially recovered, and the 16S rRNA gene. Within scaffolds all genes showed the known hymenopteran gene order and orientation, while the orientation of the 5 tRNAs detected was identical to those in the honey bee (Crozier and Crozier 1993; Cha *et al.* 2007). Attempts to complete the mitochondrial genome using the nuclear genome assembly yielded no improvement of the assembly (data not shown).

The high success in mitochondrial gene reconstruction is likely due to the nature of the analyzed transcriptome data. Short intergenic regions as well as polycistronic mitochondrial mRNA likely lead to the assembly of multiple genes into single scaffolds. The A-T rich region is completely missing as well as the ND2 and 12S rRNA genes flanking the region in insect mitogenomes. This unrecovered region also contains a high number of tRNAs in the honeybee, which could explain the low number of recovered tRNAs in *E. dilemma*. While the partial mitochondrial genome assembly is only 75% complete, it represents the first mitogenome for the group of orchid bees and will thus be a valuable resource for future phylogenetic analyses within the lineage and between more distantly related bee taxa.

Repetitive elements

Tandem Repeats. We detected 76,001 microsatellite loci with a consecutive length of 2,291,067 bp. Minisatellites with motif lengths from 7bp to 1000bp were less numerous in the genome (67,323 loci), totaling 13,343,515bp. Accordingly, tandem repeats represent 3.86% of the genome assembly, suggesting that they contribute only a small proportion to the overall genome size (Figure 1c).

NUMTs. We detected fragments with similarity to the draft mitochondrial genome on 129 scaffolds totaling a length of 150,670 bp. The fragments had a mean length of 764.8 bp and a mean similarity of 91.5% to the mitogenome. This suggests that these fragments are not derived from the mitochondrial genome and represent actual NUMTs. A total of 39 scaffolds carried multiple fragments with high similarity to the mitogenome with a concatenated length of up to 6566bp, suggesting that respective NUMTs might have originated from larger fragments of the mitogenome. In total, only 0.04% of the whole-genome assembly had hits to the mitogenome (Figure 1c). This is likely an underestimate, due to the incompleteness of the reconstructed mitochondrial genome. Nevertheless, NUMTs likely represent only a small fraction of the whole nuclear *E. dilemma* genome. Previous analyses have shown a high density of NUMTs in the honey bee in comparison to other insect genomes totaling about 0.1% of the overall genome size (Pamilo *et al.* 2007). Accordingly, given the NUMT content detected in *E. dilemma*, it is possible that a comparatively high NUMT density is a common feature of corbiculate bee genomes.

TEs. In our RepeatMasker analysis we detected 47,553 interspersed repeats with a cumulative length of 7,747,824bp. This corresponds to 1.82% of the genome assembly (Figure 1c). This low number is surprising, since large genome sizes as in

E. dilemma have been mainly associated with TE activity and content in genomes from unicellular eukaryotes to complex multicellular organisms including plants, invertebrates and vertebrates (Kidwell 2002). However, TEs are fast evolving and highly specific to their host lineages, which leads to large underestimates of genomic TE content in previously unstudied lineages (Chalopin *et al.* 2015; Platt *et al.* 2016). The only bee repeat content included in the Repbase database used for TE annotation is the honey bee, a species with a comparatively small genome (0.23Gb) and low TE diversity and content (Weinstock *et al.* 2006; Kapheim *et al.* 2015). Thus, the low percentage of TEs detected in the *E. dilemma* genome is very likely an underestimate of the actual density. *De novo* TE reconstructions using the genomic resources presented here should be performed in the future to provide a better estimate of the actual TE density in the *E. dilemma* genome.

Genome structure

Of the 22,698 *E. dilemma* scaffolds, 580 were at least 100kb in length and used for synteny analysis with the honey bee genome. A total of 356 of these scaffolds carried at least one gene annotation with known homology to the honey bee, and 329 of these *E. dilemma* scaffolds were homologous to honey bee scaffolds with known linkage group (LG) association (Table S1). Of these scaffolds, 272 (83%) showed ≥95% syntenic homology to a single honey bee LG (Figure 1d). Overall, the detected syntenic linkage blocks cover 222MB of scaffold length with homology to the honeybee, representing 85% of the 329 filtered scaffolds. Mean syntenic block length is 206,033bp for *E. dilemma* (min: 1017bp; max: 4,458,123bp; median: 47,154bp) and 143,221.4bp for the honey bee (min: 1029bp; max: 2,205,566bp; median: 41,726bp). This suggests that the genomic architecture is very similar between *E. dilemma* and the honey bee, representing a high level of conservation during the 80 million years since the two lineages diverged. Further, our results support a recent comparative analysis of the honey bee and the bumblebee genomes, which revealed high conservation of genomic synteny (Stolle *et al.* 2011). Together, these results support a general pattern of surprisingly slow evolution of gene synteny in corbiculate bees, independent of the fraction of repetitive genome content.

Conclusion

The genome assembly of the orchid bee *E. dilemma* that we present here is of high quality, despite its large genome size (estimated to be 3.3Gb). The 15,904 gene annotations provide a comprehensive set of genes with known homology to the honey bee, facilitating future gene ontology and functional genomic analyses. While we were unable to annotate the mostly repetitive majority of the genome assembly with our approach, the provided sequence reads will be useful for future analyses of repetitive genetic elements in the genome. The nuclear and mitochondrial draft genomes represent a valuable genomic resource for the community of bee geneticists. This genomic resource will likely prove valuable in genetic and functional genomic analyses dealing with the ecology, evolution, and conservation of

orchid bees. Furthermore, the genome of the facultatively eusocial *E. dilemma* will be helpful in the study of the evolution of eusociality, due to its taxonomic placement as the sister lineage to the three obligately eusocial corbiculate bee tribes including stingless bees, bumblebees, and honey bees.

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Figures

Figure 1. Genomic features. (A) Phylogeny of the four corbiculate bee tribes with orchid bees as sistergroup to honey bees, stingless bees, and bumblebees (Romiguer et al. 2015). **(B)** K-mer distribution spectrum (k=25) of genomic sequence reads. The positively skewed spectrum reveals a high abundance of a few k-mers, leading to an estimate of 87.7% repetitiveness of the *E. dilemma* genome. Red shows the k-mer spectrum before, and blue after error correction. **(C)** Genomic element density including genic and non-genic features as a fraction of the overall genome assembly length. Over 85% of the assembly could not be annotated with the selected methods. **(D)** Synteny between the *E. dilemma* and the honey bee (*Apis mellifera*) genome. In an analysis including *E. dilemma* scaffolds of ≥100kb length, 83% showed ≥95% synteny to a single honeybee scaffold. Photographs in **(A)** are reproduced from Wikimedia under the CC BY-SA 3.0 license.

Figure 2. Mitochondrial genome reconstruction. The structure of the honey bee mitochondrial genome and information of the homologous reconstructed parts of the *E. dilemma* mitochondrial genome. Non-reconstructed parts of incompletely reconstructed genes are hatched.

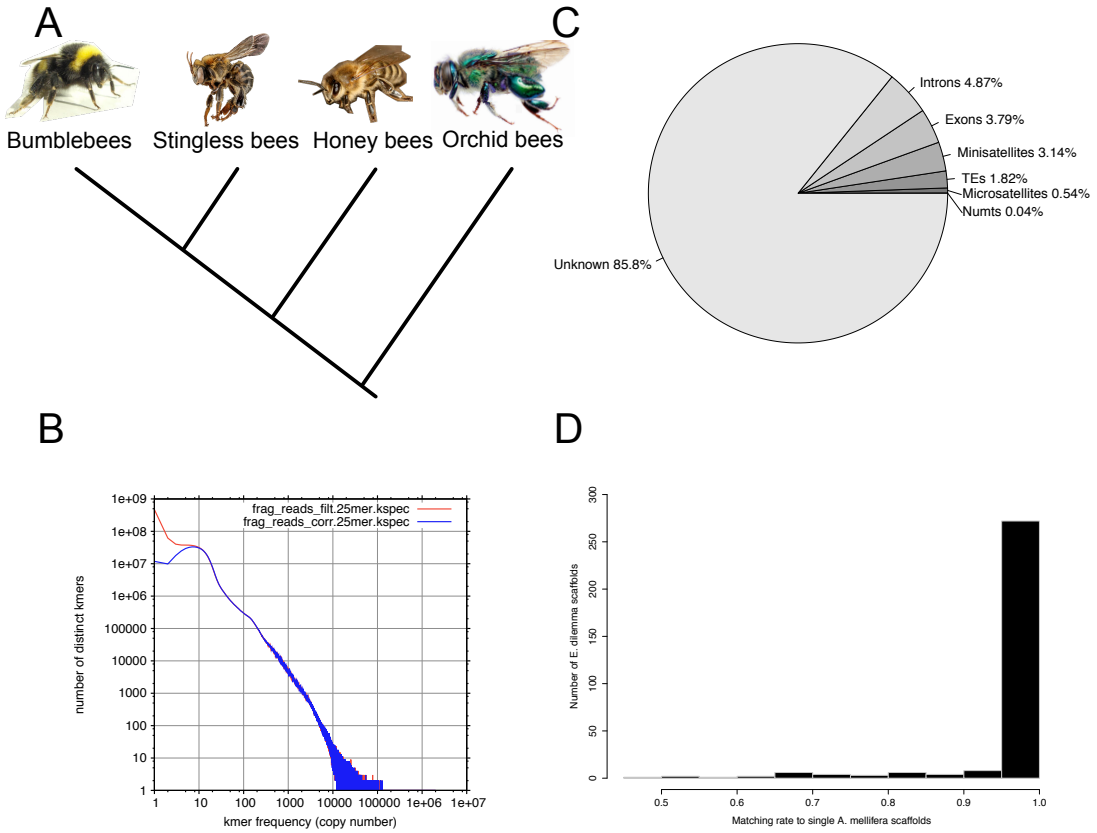

Figure 1

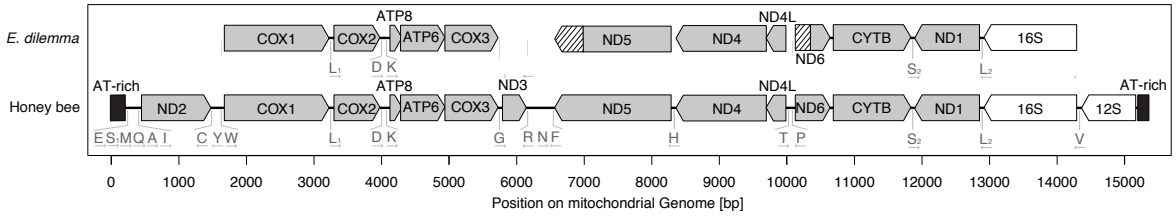

Figure 2

Tables

Table 1. *E. dilemma* genome assembly statistics in comparison to previously published bee genomes.

Table 1

Species	N50	N25	Longest scaffold	Scaffolds	Assembly length	%GC	Predicted genes	Ref.
Euglossa dilemma	143,590	1,417,006	10,108,120	22,698	588,199,720	39.94	15,904	1
Eufriesea mexicana	2,427	443,231	4,677,300	3,522,543	1,031,837,970	41.38	12,022	2

Apis mellifera	997,192	1,922,192	4,736,299	5,644	234,070,657	32.70	15,314	3
Melipona quadrifasciata	68,085	1,896,322	12,087,087	38,604	507,114,161	38.88	14,257	2
Bombus impatiens	1,399,493	2,389,513	5,466,090	5,559	249,185,056	37.75	15,896	4
Lasioglossum albipes	616,426	1,130,413	3,533,895	41,433	341,616,641	41.50	13,448	5

N50 and N25 indicate the length of the shortest scaffold of those including 50% and 25% of the base pairs in a genome assembly. References (Ref.): 1: This study, 2: Kapheim et al. 2015, 3: Elsik et al. 2014, 4: Sadd et al. 2015, 5: Kocher et al. 2012.