Genomic Assembly and Functional Analysis of Novel Date Palm Menace: Dubas Bug *(Ommatissus lybicus)*

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Abstract

The Dubas bug (*Ommatissus lybicus)* is a sap feeding insect found in the Middle East and North African regions. They are most known for sucking the sap from date palms which are culturally and economically important to countries in these regions and secreting honeydew that promotes growth of black sooty mold. The Dubas bug infests date palms by laying their eggs into holes of the tissue in date palm fronds which causes chlorosis. The high infestation levels have led to destruction of palm plantations and attempts for extermination or reduction of the Dubas bug population. Despite these countries' best efforts, their methods have caused negative environmental impacts to non-target species and human health. We have been given the genomic sequence of the Dubas bug.

Different bioinformatic tools were used to assemble and annotate this sequence for key genes that may play a role in insecticide resistance. This could also lead to tailoring of more suitable insecticides against the Dubas bug. By using command line bioinformatic tools such as FastQC and Trimmomatic PE, we were able to check the quality of our sequences and trim them accordingly. We were then able to create a hybrid assembly using SPAdes. To check the quality of our assembly we used BUSCO and to predict genes we used Augustus. For further understanding of the species, KEGG pathways was used for functional analysis. This newly found knowledge of the biological pathways and gene functions in the Dubas bug has many applications, such as combatting Dubas bug infestations in the Middle East through targeting of genes that promote insecticide resistance or enable adaptations to the organism's environment.

Introduction

The fruit of date palm is an essential crop that has a long history in Middle East countries. This crop that accounts for 70% of all date production worldwide, has been subjected to harsh exploitation with rapidly increasing human populations, domestic animals, and natural pests [1]. Nearly 30% of date palm tree production is lost to pests and disease.

Threats like the Dubas bug and red wheevil are two of the most predominant pests that affect quality and yield of dates from these Arabic countries. The Dubas bug (*Ommatissus lybicus)* is a sap feeding insect found to originate from the Tigris-Euphrates River Valley [2] now affecting in the Middle East and North African regions. They specifically live in the mountain wadi biomes and near fresh water [3]. They avoid extreme temperatures and direct sunlight. They are most known for sucking the sap from date palms and secreting honeydew that promotes growth of black sooty mold [3,4]. The growth of this mold hinders photosynthetic productivity of date palms. Infestations can result in premature fruit shedding, delay in fruit maturity, reduction of fruit shelf life, reduction in post-harvest storage periods, and changes in the fruit taste [5]. Additionally, *O. lybicus* infests date palms by laying eggs into holes of the tissue in date palm fronds which causes chlorosis [3]. Longer infestations have been reported to destroy whole palm plantations [5, 6].

Figure 1. *Ommatissus lybicus*, commonly known as the Dubas bug.

Figure 2. *O. lybicus* infestation on a date palm.

The devastating consequences of an infestation have been a driving force to develop methods of extermination or reduction of the pest's population. Various insecticides have been tested successfully in countries such as Israel and Iraq but must be used with reserves due to potential negative side effects and expensive costs [7]. Despite these countries' best efforts, their methods have caused negative environmental impacts to non-target species and human health at large costs in efforts to exterminate this pest at best [6]. In other countries like Oman, the *O. lybicus* population appears to be highly resistant to chemical measures and is consistently an antagonist to their agricultural industry [4]. Over a span of 20 years, Oman has spent $18.5 million on more than 500 tons of arial applications of insecticides with limited success [8].

In more recent years, genomic analysis has been very quickly developed to be better used as a powerful tool to investigate functionally significant genetic elements. The ability to characterize these genomes allows for more accurate insights into the molecular mechanisms underlying the biological processes of date palm menaces like *O. lybicus*. Annotated genomic sequences can help uncover essential genetic elements that have been key in *O. lybicus* resistance to current insecticides. With the development of second-generation sequencing technologies like Illumina, the quality of whole genome sequencing has greatly evolved [9, 10]. However, these short Illumina reads on their own still lead to poor *de novo* assembly. Hence the development of long-sequencing reads, Oxford Nanopore, which helps with analyzing highly repetitive elements [11, 12]. The purpose of assembling and annotating the genome sequence is to better understand why and how the *O. lybicus* infests the date palm and secretes honeydew.

The *Ommatissus* genus consists of two other species: *O. binotatus* and *O. lofouensis.* There is little to no information on the *O. lofouensis* species as well. *Tropiduchidae* is a family of plant hoppers and consists of 39 descendants [13, 14]. Unfortunately, there were no genes or

genome assemblies available for comparison in either the family or genus of *O. lybicus*. For this reason, any reference sequences we used were from the order Hemiptera, or more broadly the Arthropoda phylum. Typical insects with a moderately sized genome are expected to be around 504Mb. In hemipteran genomes, size is generally expected to be between 407 to 1230Mb [15].

With a genome of this size, usage of command line prompts and cluster servers is expected. Some key tools are Geneious Prime and SPAdes for assembly, QUAST for trimming analysis, and BUSCO for overall quality assessment [16]. Augustus serves as a tool to make *Ab-initio* homology-based gene prediction for eukaryotic genomic sequences [17]. With annotations from Augustus, KEGG metabolic maps can be reconstructed to better understand the functional qualities of the genome [18].

Understanding of the *O. lybicus* sequence is still limited to a few studies [3, 4, 8]. Additionally, there have been no studies that have used genomic hybrid *de novo* assembly techniques and annotation methods to better understand the *O. lybicus* species despite their detrimental effects on date palms. In this experiment, a combination of Illumina and Oxford Nanopore technologies was used to sequence the *O. lybicus* genome. The genomic sequence can then be further studied for establish important metabolic pathways for the species and understand its relationship with other organisms of close lineage. Developing a better understanding of the insect will be helpful in developing more tailored methods of controlling and managing *O. lybicus* infestations.

Methodology

Initial forward and inverse Illumina reads for *O. lybicus,* sequences were paired and trimmed accordingly through a Geneious Prime workflow (https://www.geneious.com/tutorials/de-novo-assembly/) [16]. Both Illumina-only assemblies and hybrid assemblies were created. These assemblies were compared to a previously prepared sample hybrid assembly to determine which assembly would better produce the best annotations. Raw Illumina forward and reverse reads of *O. lybicus* were submitted to FastQC, a quality assessment program, and quality histograms showed that trimming of the reads could be performed for a better assembly. These Illumina reads were then submitted to Trimmomatic, a pair-aware trimming program, and ran through FastQC again for comparison with the untrimmed reads. Comparing the untrimmed and trimmed reads for both the forward and reverse, approximately a gigabyte of each sequence had been trimmed. Once trimmed reads were good quality, genome assembly began.

Model workflows were considered from literary research on other genomic assemblies and analysis of novel insect sequences [19, 20]. After considering various programs, SPAdes was used for genomic assembly for the Illumina-only assembly. This was completed successfully using the UH HPE Sabine Cluster and a batch script, as attempts to assemble on the Mac Mini encountered repeated memory and storage issues. The resulting scaffolds file created by SPAdes was then checked for quality control and completeness through QUAST and BUSCO analysis, respectively. Hybrid assembly was built using both the Illumina and Nanopore reads on the Sabine Cluster, much like Illumina-only assembly. The same strategy used for the Illumina-only assembly was used for the hybrid assembly, albeit with the inclusion of Nanopore reads in SPAdes. Assembly with a scaffolds file that was successful.

After consideration between the Illumina-only assembly, the hybrid assembly with Nanopore reads, and the previously prepared hybrid assembly, QUAST statistics and overall assembly completeness determined it would be ideal to continue to annotation with the previously prepared assembly rather than the assemblies we had created.

Augustus was used to predict genes in the *O. lybicus* sequence. Taxonomical search determined that *A. pisum*, a species related on the order level (Hemiptera), was related most closely to our novel species of interest. *A. pisum* was used as the reference genome. After successfully predicting genes with Augustus, the contigs file was extracted and processed in BlastKOALA in three sections to accommodate file size issues. Sections from BlastKOALA were compiled and collectively processed in KEGG mapping to produce the reconstructive metabolic maps for the novel insect.

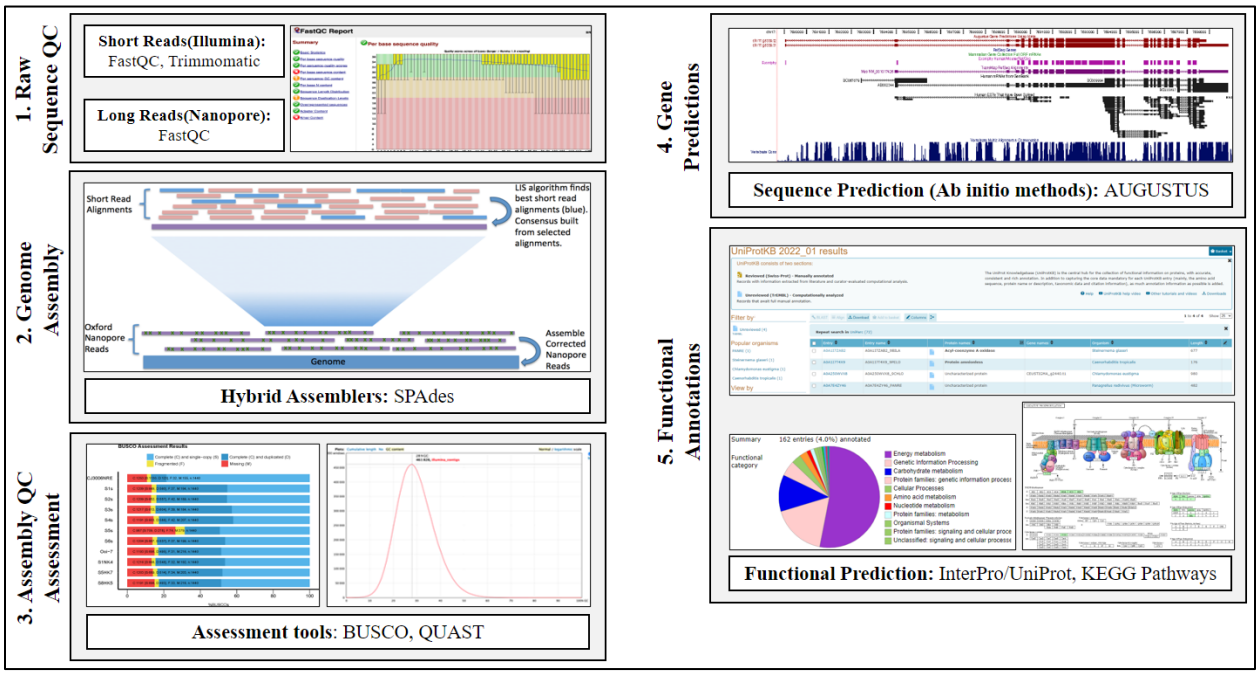

Figure 3. The workflow followed to conduct *O. lybicus* genome assembly and functional annotation.

Results

FastQC was used to compare the sequences before and after trimming with Trimmomatic. The following histograms were given as outputs from FastQC. Indication of good quality trimmed reads are those that are in the green section of the graph. As can be seen, the forward read was trimmed well but the reverse still had some in the orange. However, when looking at the amount of sequence that had been dropped, we noticed that a gigabyte had been dropped from both reads. We decided as a team not to trim any further because it could give skewed results; the trimmed sequences were then used for the assembly.

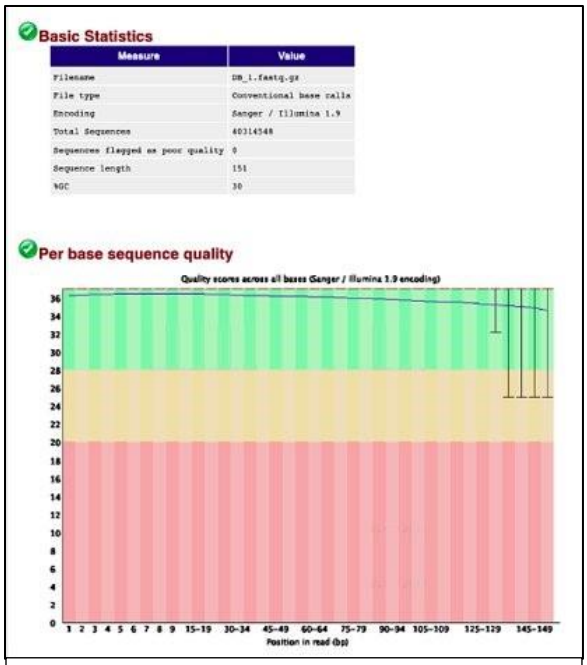

Figure 4a. Quality assessment of forward read before trimming.

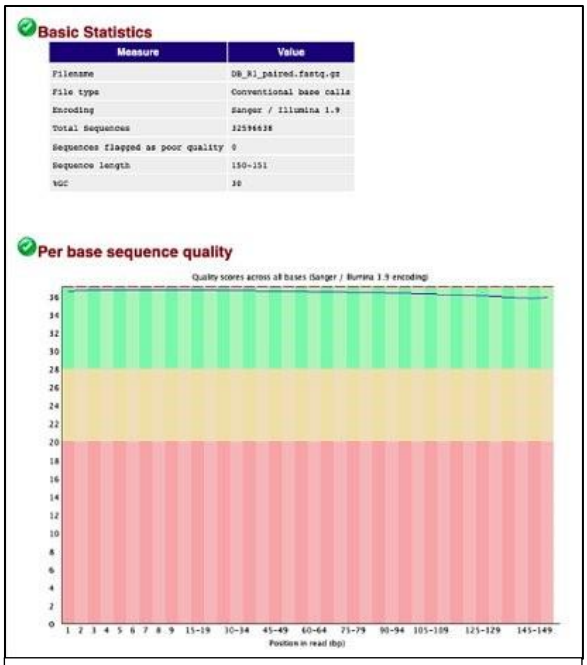

Figure 4b. Quality assessment of forward read after trimming.

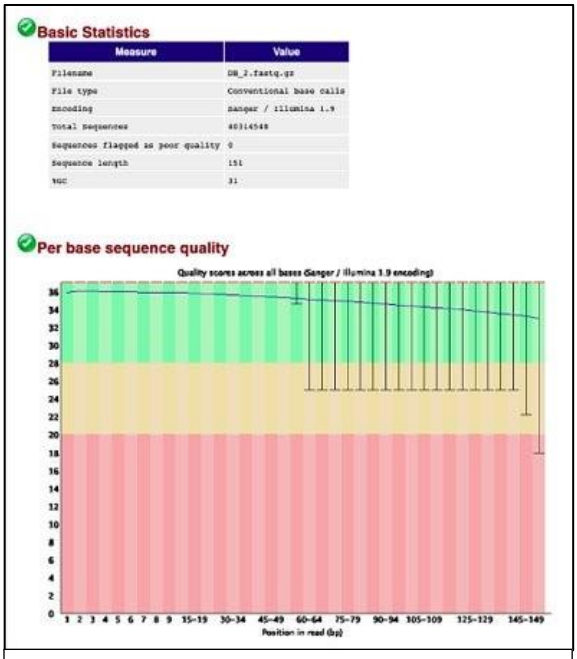

Figure 5a. Quality assessment of reverse read before trimming.

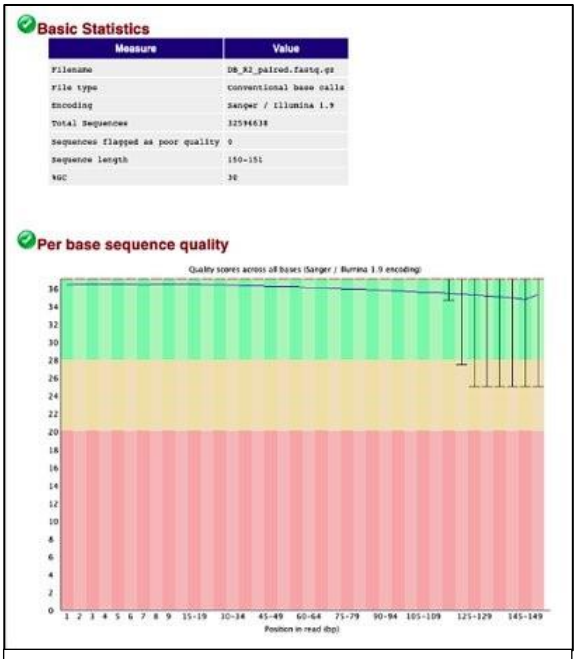

Figure 5b. Quality assessment of reverse read after trimming.

From the assembly workflow, a total of two assemblies were created: the Illumina-only assembly and the hybrid assembly. As mentioned, both assemblies were checked for quality using QUAST, followed by assessing completeness with BUSCO. A major red flag in our hybrid assembly was the high number of mismatches, specifically the presence of nearly 30,000 N's in the assembly. Another issue that occurred was the relatively low completeness of the Illumina-only and hybrid assembly; ideal completeness should be around 90-100%, but each assembly measured at only 31.5% and 0.1% respectively. After consideration between the Illumina-only assembly, the hybrid assembly with Nanopore reads, and the previously prepared hybrid assembly, QUAST statistics and overall assembly completeness indicated it would be ideal to continue to annotation with the previously prepared assembly.

Statistics without reference	contigs
# contigs	251 301
# contigs (>= 0 bp)	429 618
# contigs (>= 1000 bp)	215 544
# contigs (>= 5000 bp)	53 822
# contigs (>= 10000 bp)	12 160
# contigs (>= 25000 bp)	291
# contigs (>= 50000 bp)	18
Largest contig	371 075
Total length	881 123 220
Total length (>= 0 bp)	922 503 297
Total length (>= 1000 bp)	853 792 737
Total length (>= 5000 bp)	455 372 907
Total length (>= 10000 bp)	169 395 744
Total length (>= 25000 bp)	10 386 116
Total length (>= 50000 bp)	2 364 547
N50	5182
N90	1620
L50	50 913
L90	168 526
GC (%)	29.52
Mismatches	
# N's per 100 kbp	0
# N's	0

Statistics without reference	scaffolds
# contigs	3256
# contigs (>= 0 bp)	28 752
# contigs (>= 1000 bp)	2735
# contigs (>= 5000 bp)	1021
# contigs (>= 10000 bp)	321
# contigs (>= 25000 bp)	16
# contigs (>= 50000 bp)	2
Largest contig	112 795
Total length	14 221 540
Total length (>= 0 bp)	17 531 148
Total length (>= 1000 bp)	13 828 314
Total length (>= 5000 bp)	9 751 420
Total length (>= 10000 bp)	4 708 548
Total length (>= 25000 bp)	615 281
Total length (>= 50000 bp)	170 877
N50	7500
N90	1862
L50	599
L90	1991
GC (%)	32.13
Mismatches	
# N's per 100 kbp	215.74
# N's	30 681

Figure 6. Comparison of Illumina-only (left) and hybrid (right) assemblies using QUAST.

The Quast report for the previously prepared hybrid assembly used for annotations and proteomic analysis consisted of 19814 contigs at a total length of 90535597 base pairs. By using the predicted genes from Augustus to do KEGG annotation, we were able to identify what genes from the *O. lybicus* were associated with respective biosynthetic pathways provided by the reconstructive maps of KEGG mapping.

The proteins according to KEGG pathway involved were divided into five branches: metabolism, genetic information processing, and signaling and cellular processes. According to KEGG pathways analysis, there were a total of 151 pathways matched. Sixty-two matches for metabolic pathways and 21 matches for biosynthesis of secondary metabolites. Particularly interesting pathways with high numbers of matching contigs included carbon fixation, hypoxia-inducible factor 1 (HIF-1) signaling, thermogenesis, and salivary secretion. The largest number of matched contigs (total of 58) was for ribulose-bisphosphate carboxylase in carbon fixation.

Discussion

Among the matches determined through KEGG is ribulose-bisphosphate carboxylase which was strongly regulated for. This lyase is involved in catalyzing oxygen-dependent production of phosphoglycolate [21]. It is commonly found in the reductive pentose phosphate cycle (Calvin cycle) and photorespiration pathways. Under low temperature and high CO_2: O_2 ratio, rubisco uses CO_2 as the substrate to initiate Calvin cycle and produce sugar. In situations of high temperature and low CO_2: O_2 ratio, rubisco uses O_2 as the substrate to initiate photorespiration. The pathway begins when rubisco acts on oxygen instead of carbon dioxide. This is helpful in decreasing sugar synthesis in situational environments where climate is hot and dry [22].

Photorespiration is characteristic of the chlorophyll, typically found in autotrophic plants. However, studies have noted photorespiratory-like processes found in insects such as the aphid, which is related to the *O. lybicus* on an order level (Hemiptera) [22]. Aphids have been documented to have the ability to synthesize carotenoid pigments to maintain a health immune system, synthesize certain vitamins, and produce energy. It is possible the *O. lybicus* shares similar qualities given the strong indication that the *O. lybicus* genome contains a respectable quantity of photosynthesis proteins. However, further studies on potential photosynthesis-like systems in *O. lybicus* is necessary to affirm the function.

Ribulose-bisphosphate carboxylase is labeled as 4.1.1.39 in figure 7 below. Following the reconstructive map from KEGG, it converts D-Ribulose 1, 5-bisphosphate to 2, 3-Phospho-D-glycerate in the presence of carbon dioxide and water.

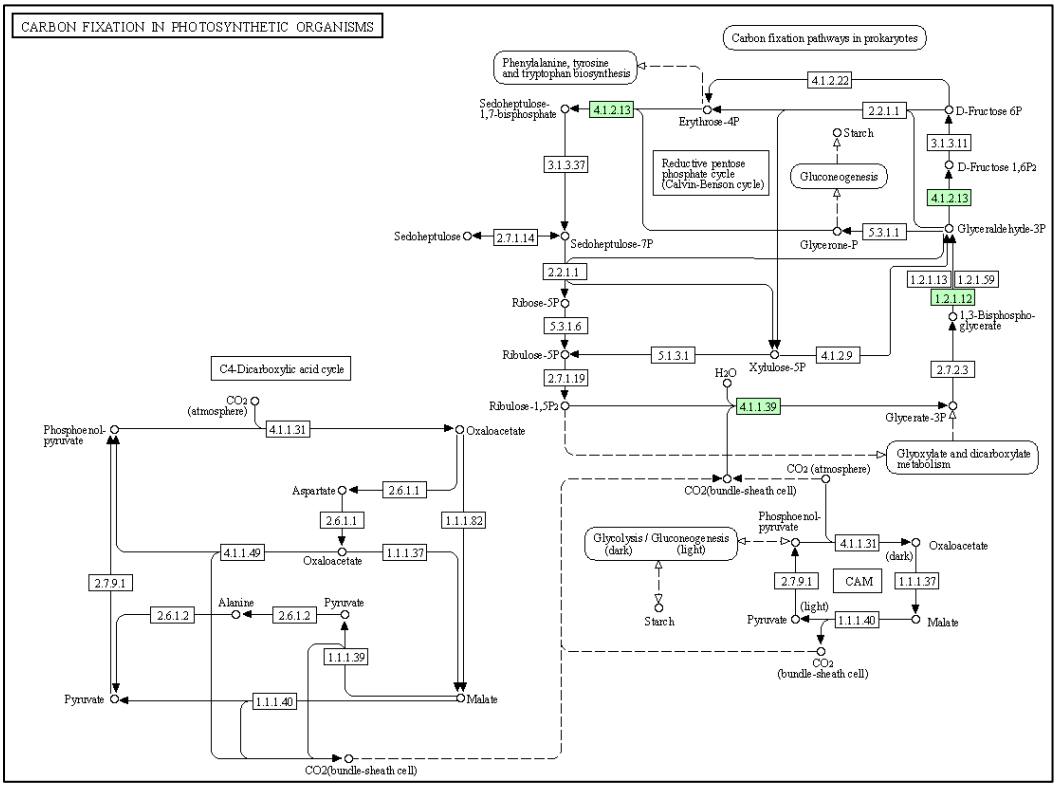

Figure 7. Reconstructive KEGG map of the carbon fixation pathway in photosynthetic organisms. [18]

A significant biological pathway in the *O. lybicus* is the hypoxia-inducible factor 1 (HIF-1) pathway that helps the bug thrive in the often-extreme conditions of the Middle East. Seven contigs correspond to the hypoxia-inducible factor 1 (HIF-1) pathway. Under normal conditions, HIF-1 alpha is a hallmark transcriptional regulator of hypoxia that undergoes hydroxylation at specific prolyl residues leading to immediate ubiquitination followed by proteasomal degradation of the subunit. However, under hypoxia (oxygen starved), the alpha subunit becomes stable and interacts with coactivators like p300/CBP to modulate transcriptional activity through regulation of a collection of hypoxia-inducible genes [23]. This induces the expression of genes that

promote oxygen delivery to alleviate hypoxia, such as erythropoietin. The HIF-1 gene is also associated with cell proliferation and iron metabolism [24]. The target genes of HIF-1 encode proteins that increase O_2 delivery and mediate adaptive responses to O_2 deprivation, or lacking nitric oxide, or various growth factors [23].

Notice in figure 7 the proteins marked in green. PDH is pyruvate dehydrogenase E1 component alpha subunit, HK is a hexokinase, GAPDH is glyceraldehyde 3-phosphate dehydrogenase (phosphorylating), and ALDOA is fructose-bisphosphate aldolase, class I. Under conditions in which oxygen consumption is limited, these components of the sequence aid in promoting anaerobic metabolism. Due to the hot, dry nature of the Middle east, it is an adaptation that *O. lybicus* has developed to combat it. Elevated temperatures often induce oxidative stress and antioxidant response in insects like *O. lybicus* [25].

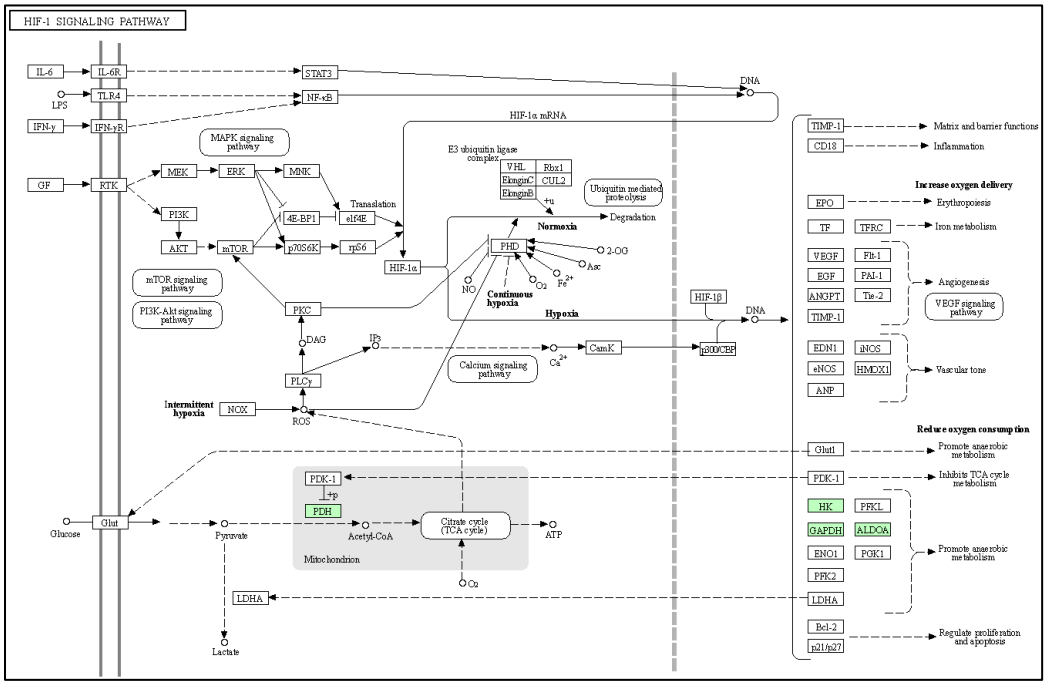

Figure 8. Reconstructive KEGG map of the Hypoxia-inducible factor 1 (HIF-1) signaling pathway of *O. lybicus*. [18]

O. lybicus also requires thermogenesis. Despite the *O. lybicus*, like many Arthropods, being ectothermic (cold-blooded), it hatch in two seasonal batches: once around late April and another time around late September. The most ideal temperature for these biological activities is around 27.5°C [4]. Notably this late April and September is when the species sucks the most sap from leaflets. Additionally, indirect damage is enhanced by the female Dubas bugs depositing eggs beneath the biaxial frond, inhibiting photosynthesis [26]. Thermogenesis in brown and beige adipose tissue is controlled by norepinephrine, which is released in response to cold or dietary stimuli. Signaling pathways such as the cAMP and MARK pathways help to produce norepinephrine, as well as the mitochondrial uncoupling protein 1 (UCP1) which participates in thermogenic respiration [27]. Because fat in insects is typically found as a large organ underneath the gut and reproductive organ, thermogenesis regulation in the adipocytes may correlate to successful egg production in Dubas bugs [28]. Further research on this biological pathway in the species is necessary to understand the role of thermogenesis in its lifecycle.

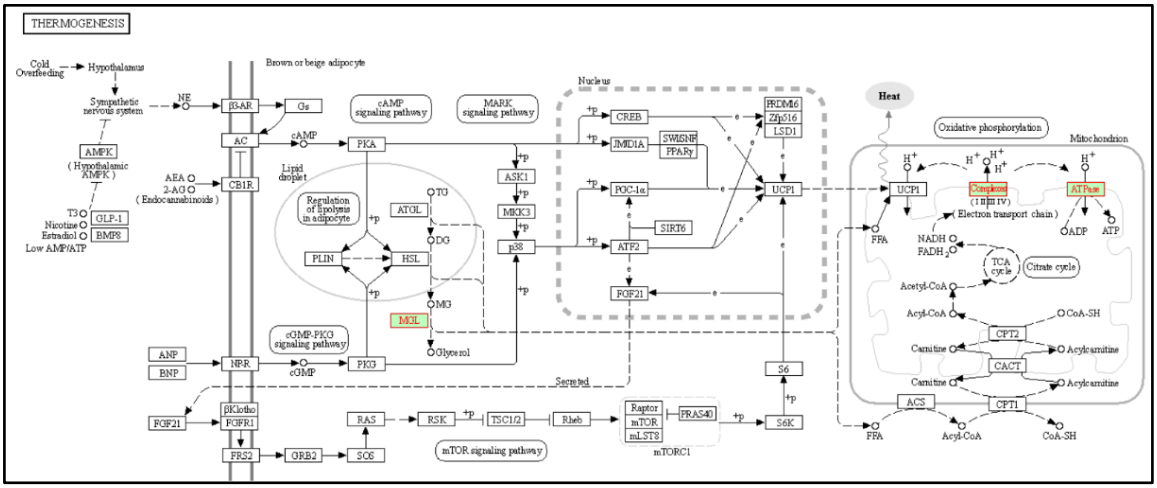

Figure 9. Reconstructive KEGG map of the thermogenesis pathway of *O. lybicus.* [18]

Saliva has a large variety of functions from maintaining the integrity of the oral tissues to tasting and ingesting food. Saliva production in *O. lybicus* is made possible by the protein exocytosis and fluid secretion pathways. Sympathetic stimulation, or stimulation in response to

stressors such as heightened emotions or exertion, activates adenylate cyclase and accumulates intracellular cAMP, which then stimulates production of mucin, leading to thicker saliva. On the other hand, parasympathetic stimulation activates phospholipase C, an enzyme that increases production of Ca^{2+}, resulting in increased fluid secretion.

The two matches for salivary secretion were DMBT1 (deleted in malignant brain tumors 1 protein) and ATP2B (P-type Ca^{2+} transporter type 2B). Modifications to the salivary secretion pathway may help combat how the pest sucks the sap from date palms.

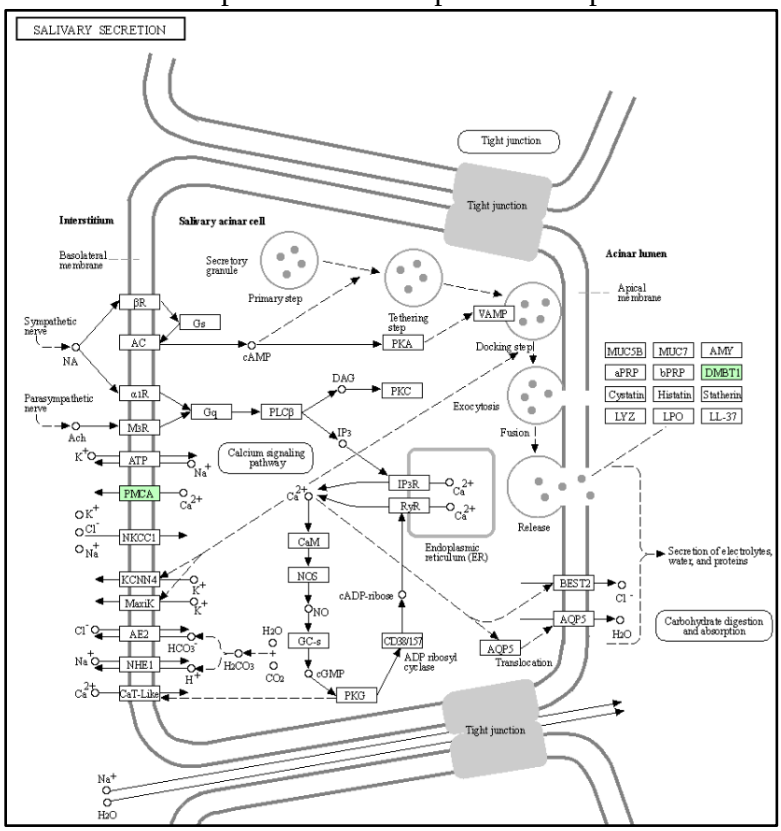

Figure 10. Reconstructive KEGG map of the salivary secretion pathway of *O. lybicus*. [18]

While feeding on date palms, the insect excretes honeydew that contains sugars and other waste contributing to the development of pathogenic infections [3]. Further studies of *O. lybicus* waste is necessary to pinpoint what is specifically responsible for the decay, but the genes associated to the proteins mentioned serve as a foundational understanding for which proteins are likely relevant and are most prolifically regulated in *O. lybicus.*

Conclusion

As a result of our research throughout the past few months we gained valuable insight into the intricate process of assembly and annotation of a genome. We also learned new information about *O. lybicus*. We were successful in assembling an Illumina and hybrid (Illumina + Nanopore) genome sequence. However, due to time constraints and computer storage errors, we were unable to obtain a high-quality hybrid assembly. An alternative hybrid assembly was used for annotation and functional analysis. By utilizing KEGG pathways, we were able to identify key genes in the *O. lybicus* that play a role in metabolism and pathways associated with producing waste known to be detrimental to date palm photosynthesis. By further studying these genes and associated pathways, we can better understand how this organism works metabolically and how it secretes honeydew promoting the growth of a black sooty mold.

In the future, this annotated genome could be used as a resource to isolate key functional components of the *O. lybicus* genome. Targeting the insect's immune response, metabolic pathways, or salivary secretion may be suitable methods to combat infestations and reinvigorate the date palm industry of impacted Middle East countries.

Limitations

Over the last few months, there were a few key limitations we faced during our research, one of the primary categories of which being technological limitations. For the majority of bioinformatics performed, an Intel Core i7 Mac Mini with 64 GB of RAM was sufficient. However, attempting assembly on the Mac Mini repeatedly threw errors regarding memory and storage, and it was decided that the UH Sabine Cluster would be used for both Illumina-only and hybrid assembly. An additional limiting factor was our own personal knowledge of genome

assembly and annotation. All three team members have only had about one semester worth of experience in genome assembly and annotation, vastly low compared to many professional bioinformaticians. As a result, our assembly and annotation could have undoubtedly been of higher quality if we had the experience to completely harness the power of the bioinformatic tools we used. Lastly, time was an overarching limitation during our research. Towards the end of the semester parts of our original research plan had to be omitted so that we could finish on time, although we do plan to continue our research on *O. lybicus* throughout the upcoming summer.

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