Biodiversity of Drosophila species at a new university campus in Kunshan, China

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Abstract. The project aimed to explore the biodiversity of Drosophila species in Kunshan using genomic approaches. DNA sequences, specifically the Cytochrome Oxidase I (COI) gene, were utilized as genetic "barcodes" to identify the organisms. Three wild-caught fruit flies and a D. melanogaster control were selected, and their DNA was isolated through PCR amplification of the COI gene. The PCR products were sequenced, and the results were used to create phylogenetic trees and uploaded to the Barcode of Life Database Student Data Portal (BOLD-SDP) alongside species photos. The results identified 10 Drosophila species, with 6 species remaining unchanged over two years and 3 new species discovered this year. One sample was identified as D. neohypocausta, a Chinese endemic species, which had appeared only in the last two years in Kunshan. However, its sample size was small, suggesting it may not be native to the area. Further research revealed significant genetic differentiation between different clades of D. immigrans, which likely explained the limited number of D. neohypocausta samples, as most D. immigrans species are confined to specific regions. To gain a comprehensive understanding of biogeographic, ecological, and evolutionary patterns across the Drosophila genus, expanded sampling and taxonomic revision are necessary, particularly for the large immigrans species groups that have been inferred as polyphyletic in some studies. This study underscores the importance of genomic approaches in taxon diagnosis and highlights the need for further exploration to uncover the intricate relationships within the Drosophila genus.

Keywords: Genomic approaches, Taxon diagnosis, Cytochrome Oxidase I (COI) gene, Drosophila species, Biodiversity exploration.

1. Introduction

Biodiversity encompasses various dimensions, and a fundamental step is to catalog the organisms present (Naeem et al., 2016). Environmental biodiversity can fluctuate across multiple scales, from diurnal to seasonal changes and shifts between wet and dry conditions (Bloch, Bar-Shai, Cytter, & Green, 2017; Dornelas et al., 2013). Over longer periods, factors like urbanization (Buczkowski & Richmond, 2012), fire recovery (Tingley, Ruiz-Gutiérrez, Wilkerson, Howell, & Siegel, 2016), and climate change (Casajus et al., 2016) can significantly impact the species composition. Fruit flies of the Drosophilae family have been utilized to study regional changes (Guruprasad & Padmaja, 2016). Past reports have cataloged the species diversity and distribution of wild-caught Drosophila in specific regions (McInnis, Schaffer, & Mettler, 1982), while multi-site collections demonstrated diversity changes over defined periods (Bombin & Reed, 2016).

A crucial aspect of these investigations involves identifying and documenting invasive species as they spread through regions (Ørsted, Ørsted, & Garcia, 2019). Species like Drosophila suzukii, also known as Spotted Wing Drosophila, and Zaprionus indianus, the "African Fig Fly," have invaded new territories, posing threats to local ecosystems and agriculture (Rota-Stabelli, Blaxter, & Anfora, 2013; Neelendra K. Joshi, David J. Biddinger, Kathleen Demchak, & Alan Deppen, 2014). Understanding the occurrence and timing of these invasions is essential for effective management.

Recent studies have highlighted the benefits of "Curricular-based Undergraduate Research Experiences" (CUREs), demonstrating improved engagement, retention, and enthusiasm among students compared to traditional laboratory courses. CUREs have explored various topics, including annotating Drosophila genomes, discovering new phage, and studying biodiversity (Shaffer et al., 2010; Hanauer et al., 2017; Hyman, 2016). Undergraduate courses offer an opportunity to address

large-scale problems by breaking them down into manageable tasks suitable for budding scientists (Corwin, Graham, & Dolan, 2015).

In this survey, students in an introductory cell and molecular biology class conducted a population study of Drosophila collected from a local site. Emphasizing diversity, students selected individuals that appeared distinct from each other. Through photography and DNA barcoding, students identified X different species, spanning a wide evolutionary range within Drosophilidae, including the pest species D. suzukii.

2. Methods

2.1. Collection

The fruit flies used were attracted to a 1L pitcher of mashed bananas and grapes on Duke Kunshan campus and were collected with a drosophila net and put into accompanying vials roughly daily from 3rd October to 1st Nov, 2021. The flies were then brought into the lab, chilled in a refrigerator. After the insects were immobile, they were dumped through a funnel into glass vials with 100% ethanol. Then we sealed the vials and stored them at room temperature until use.

2.2. Photography

After picking 4 flies (3 from the 100% EtOH vial, one from the D. melanogaster vial) under a Nikon SMZ1270 microscope and put them on the petri dish containing numbered paper, picked a focal plane by optical zoom and focused on the body, set exposure comp at +1 EV and the magnification at 0.63X (magnification of objective lens at 0.63X, magnification of eyepiece at 10X). Then captured the image at 500μm through NIS-Elements by Nikon and saved it.

2.3. DNA extraction

Numbered five 0.2μl PCR tubes as TB2.1-TB2.5 and correspondingly put in four dead fly. Mixed 6μl of prot K and 294μl of SB in another 1.5μl tube, then injected 50μl mixture into each PCR tube with a fly and 50μl of SB into tube 5. Stabbed the flies repeatedly for around 20 seconds with most of the liquid in the tip. After the liquid turned red, ejected the remaining solution into the tubes over the carcass and placed the tubes on ice. Then incubated the tubes for 30 minutes at 37°C and 2 minutes at 95°C in the PCR machine, and kept on ice when finished. Move 45μl liquid to a PCR strip with 6 labeled tubes (TB2.1-TB2.6), stored them at 4°C.

2.4. PCR

2.4.1. Setup PCR for COI gene

Mixed 63μl of nuclease-free water, 75μl of Platinum Green Hot Start PCR (Platinum Taq DNA polymerase, dNTPs, magnesium, two tracking dyes for the convenience of direct gel loading of PCR products), and 3μl of LCO1490 primer (sequence: 5'-GGTCAACAAATCATAAAGATATTGG-3', 10μM) and HCO2198 primer (sequence: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3', 10 μM) each. Labeled 5 PCR tubes as TB2.1(COI)-TB2.5(COI), mixed 24 μl of compounded liquid with 1μl of each DNA template into the corresponding PCR tube. Then put the tubes in the thermal cycler: 94°C for 3 minutes first. Then with 35 cycles of 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 2 minutes. Finally, 72°C for 7 minutes. After the program is finished, stored the samples at 4°C.

2.4.2. Gel electrophoresis of COI PCR Products

After getting 90 ml of 1X TBE buffer (0.13 M Tris, 45 mM Boric acid, 2.5 mM EDTA) in a cylinder and transferred to a 250mL flask, weighed out 0.9g agarose powder on a diagonally folded paper and added to the TBE buffer. Melted the agarose in a microwave for 3 minutes and then cooled it to 50°C. At the same time, put the gel tray into the gel caster and placed comb in slot at the end of the gel casting tray. Added and gently swirled 9 μl of SYBR safe nucleic acid stain (10000X concentrated) to the gel solution until it is evenly distributed. Poured all of the agarose solution into

the casting tray and waited about 20 minutes until the gel is solidified. Then removed the comb and placed the gel inside the apparatus with the wells near the negative electrode. Filled the apparatus with 1X TBE until the gel is completely submerged. Loaded 3 µl of 1kb Plus DNA Ladder in Ladder Lane (L), and 5 µl of COI PCR Reaction samples successively. After connecting the positive electrodes with the red wire, and the negative electrodes with the black wire, ran the gel at 150 volts for 25 minutes. Then removed the gel from the apparatus and visualized the DNA fragment through the Tanon gel imaging machine.

2.4.3 Exo-SAP of COI PCR product

Separately added 8 µl ExoSAP directly to the 20 µl PCR product in TB2.1(COI)-TB2.3(COI) and mixed the samples. Incubated at 37°C for 15 minutes, then inactivated ExoSAP by heating to 80°C for 15 minutes. Labeled 3 microfuge tubes as TB2.1-TB2.3 at the same time. Took out the samples from the thermal cycler after the running program is finished and then transferred all the solution into the corresponding 1.5 ml microfuge tubes. Wrapped them by parafilm before putting them into the Genewiz bag.

2.5. Sequencing and analysis

DNA sequencing determines the order of the four nucleotides that make up DNA. Through amplifying and sequencing the appropriate gene by PCR, we can identify the specific type of organism: animal, plant, fungi, etc. and compare to a known database. This technique requires the target DNA segment with primer, DNA nucleotides (dATP, dTTP, dGTP, and dCTP), DNA polymerase, and fluorescently labeled dideoxy triphosphate nucleotide (ddNTP). Due to the lack of 3'-OH group of ddNTP, it cannot bind to dNTP and form a phosphodiester bond. The primer pair LCO1490 (forward) and HCO2198 (reverse) are used to amplify a 658bp fragment of the COI gene (Folmer et al. 1994). After these ddNTPS labelled with different fluorescent dye are subsequently examined through gel electrophoresis, we removed primers and nucleotides from PCR reaction by Exo/SAP and sent the PCR products to GENEWIZ, Suzhou (a company in R&D genomics services that provides Sanger DNA sequencing) for DNA sequencing. Once the COI sequences had been given back from the gene-sequencing company, we analyzed results and determined species to make phylogenetic tree (s).

3. Results

3.1. Introduction to the Project

Genomic approaches to taxon diagnosis exploit diversity among DNA sequences to identify organisms (animal, plant, fungi, etc.) (Kurtzman 1994; Wilson 1995). These sequences can be viewed as genetic “barcodes” that are embedded in every cell. Through amplifying and sequencing the appropriate gene by PCR, we can compare it to a known database. Cytochrome Oxidase I (COI) is used for animals.

In order to find the biodiversity of Drosophila species in Kunshan, we first picked three wild-caught fruit flies and a D. melanogaster control from the collection and photographed them. Then we ground up each fly to isolate DNA (the squishing buffer prep) and performed PCR on the COI gene. The primer pair LCO1490 (59-GGTCAACAAATCATAAAGATATTGG-39) and HCO2198 (59-TAAACTTCAGGGTGACCAAAAAATCA-39) was used to amplify a 658bp fragment of the COI gene (Folmer et al. 1994). After the PCR products were examined through gel electrophoresis, we removed primers and nucleotides from PCR reaction by Exo/SAP and sent the PCR products to GENEWIZ for DNA sequencing. Once the COI sequences had been given back from the gene-sequencing company, we analyzed results and determined species to make phylogenetic tree(s). We also uploaded the nucleotide sequences to the Barcode of Life Database Student Data Portal (BOLD-SDP) database along with the species photos.

3.2. Images of Flies

As shown in figure 1, three fruit flies were picked up for the test group from the EtOH under a dissecting microscope and laid them on a patri dish with labeled numbers (1-3). These flies look different from each other in order to get a wider range of samples: number 1 is a large, yellowish species with dorsal midline of abdomen not dark (Werner et al., 2017). Number 2 is slimmer and longer than the first one both in shape and wings, and has black stripes on the back. Number 3 looks really tiny, but it has long wings (if observing it by the scale). Subsequently, a fly from the OreR strain was chosen to serve as the control group, which was then placed at position number 4. Notably, this fly exhibited a noticeably leaner physique and possessed wings of a lighter hue compared to the specimens in the test group.



Figure 1. Photographs of individual flies sampled show diverse appearance

The picture of four fruit flies with No.1-3 picked from the EtOH pool and No.4 from the OreR. No.1 is a fleshy fruit fly with a larger size and broader wings than average, and it seems to be female. No.2 is slimmer and longer than No.1 and has black stripes on the back. According to the size and tail, it is a male fruit fly. No.3 shows a size much smaller than average. However, it has long wings if observing it by the scale. No.4 is a male one, which is much slimmer. Its long wings are lighter in color than the previous three.

3.3. Picture of Tubes

The Proteinase K (10mg/ml) and squishing buffer (composed by 10mM Tris-HCl, pH 8.0, 1mM EDTA, and 25 mM NaCl) were used to do the DNA extraction and preparation of PCR. Once the four samples became mashed in distinct tubes with the liquid turning red, they were moved into the PCR machine. After getting the PCR results, we transferred the supernatant into other labeled PCR strips, and then preserved the DNA templates at 4°C for further usage.

The PCR strips No.1-4 contains the four corresponding drosophila DNA and No.5 contains water. The number of the strip corresponds to the number of the fruit flies. The supernatant of No.1-4 all turn to yellow with diminishing shades of color. No.1 has the darkest color because the first fly has the biggest size and is easier to be fully smashed with more DNA gained. No.3 and No.4 correspond to relatively small flies, and therefore they have lighter color. No.5 only contains Squishing Buffer and Proteinase K mixed solution, and thus has no visible changes. It serves as a blank control, so it is still colorless and transparent.



Figure 2. Tubes of DNA template display different levels of pigment.

As shown in figure 2, the picture of the PCR strip with No.1-4 containing the four drosophila DNA and No.5 is water. The number of the strip corresponds to the number of the fruit flies. The supernatant of No.1-4 all turn to yellow with diminishing shades of color. No.5 has no visible changes, it is still colorless and transparent.

3.4. Gel Electrophoresis Image

In this gel electrophoresis image (As shown in figure 3), three lanes labeled as L were filled with 3μl of 1kb Plus DNA Ladder as the reference criteria. Among these lanes, the first and third lanes appear relatively clear. Notably, the 1.5kb, 1kb, and 500bps markers exhibit higher intensity compared to the other bands in the ladder lanes.

Lanes COI.1-COI.4 were loaded with 5μl of PCR products of the COI gene from four different flies, namely, No.1-No.4. All four lanes exhibit DNA fragments of approximately 700bps in length. COI.1 displays a similar brightness to COI.2, while COI.3 shows a comparable intensity to COI.4. This observation suggests that the size and quantity of these four COI genes are similar, indicating a potential membership in the same species.

COI.5 was used as a negative control and displayed no detectable DNA sequence, remaining blank. The varying intensity of bands may be attributed to uneven primer concentration during dilution.

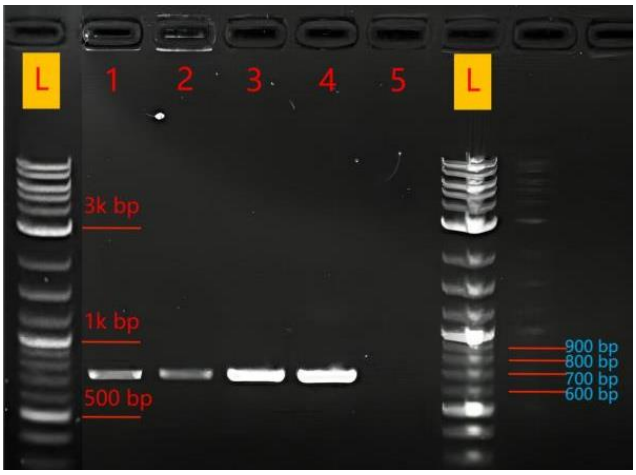


Figure 3. Gel electrophoresis shows single bands of the right size

The picture of COI PCR product in ladder lanes after gel electrophoresis. Two “L”-labeled lanes were filled with 3μl of 1kb Plus DNA Ladder. All four COI lanes show about 700bps long, with COI.1 having similar brightness as COI.2 and COI.3 having similar brightness as COI.4. TH.5 and COI.5 are blank as negative control.

3.5. DNA & Protein sequences

After amplification of the COI gene using the primer pair LCO1490 (forward) and HCO2198 (reverse), the final nucleotide sequence of the COI.1 sample is presented below:

AACTTTGTATTTTATTTTTGGAGCATGAGCCGGAATAGTTGGAACATCTTTAAGAAT
TTTAATTCGAGCAGAATTAGGTCACCCAGGAGCCTTAATTGGAGATGATCAAATTTATA
ATGTAATTGTTACAGCTCATGCTTTTATTATAATTTTTTTTATAGTTATACCTATTATAAT
TGGAGGATTTGGAAATTGATTAGTTCCTTTAATATTGGGAGCTCCAGATATGGCCTTTC
CTCGAATAAATAATATAAGTTTTTGACTTCTTCCTCCAGCTTTATCCCTTCTTCTAGTCA
GTAGTATAGTGGAAAATGGAGCCGGAACAGGGTGAACTGTTTACCCTCCTCTATCGTC
AGGAATTGCACATGGTGGGGCTTCTGTTGACTTAGCAATTTTCTCTTTACATTTAGCCG
GAATTTCTTCAATTTTAGGAGCTGTAAATTTTATTACAACTGTAATTAATATGCGATCAT
CAGGAATTACTTTAGATCGAATACCTTTATTTGTATGGTCAGTTGTAATTACAGCTTTAT
TATTACTTCTATCTTTACCCGTTTTAGCTGGGGCAATTACTATATTATTAACTGATCGAA
ATTTAAATACTTCATTCTT

This fly is identified as Drosophila neohypocausta through BOLD. The length of nucleotide sequence is 612bp with 170 A, 105 G, 96 C, 241 T. The length of amino acid sequence is 215 residues. There is no ambiguous character or stop codon. The quality of the forward primer LCO1490 is mediate and the reverse primer HCO2198 is high.

The successful sequencing of only the first COI PCR sample indicates that there might have been issues with the other samples, possibly due to various causes of failed DNA sequencing reactions. Some common reasons for unsuccessful sequencing include poor-quality DNA, loss of sequencing reaction products during clean-up, and degraded or faulty synthesis primers (Nucleics, 2020). Misoperations during the sequencing process might also have contributed to this outcome.

Additionally, the shorter length of both the nucleotide sequence (658bp) and amino acid sequence (233 residues) compared to the expected length suggests that the PCR sample may not have been of optimal quality. As a result, the sequencing machine likely trimmed off too much of the sequence during the analysis.

To address these issues and obtain more accurate sequencing results in future experiments, it is crucial to ensure the use of high-quality DNA samples and reliable synthesis primers. Proper handling and execution during the sequencing process should also be taken into consideration to minimize the risk of encountering similar challenges.



Figure 4. Nucleotide and Amino Acid sequence of samples

The picture of nucleotide and amino acid sequence of COI.1 sample (the only successfully sequenced one). This fly is identified as Drosophila neohypocausta through BOLD. The length of nucleotide sequence is 612bp with 170 A, 105 G, 96 C, 241 T. The length of amino acid sequence is 215 residues. There is no ambiguous character or stop codon. The quality of the forward primer is mediate and the reverse one is high.



Figure 5. Phylogenetic tree of course samples

Shown is a cladogram of the 10 species identified at DKU using a Neighbor-joining tree without distance correction

4. Discussion

After collecting and identifying wild fruit flies, the COI PCR sequencing at Duke Kunshan University revealed the presence of 10 fruit fly species. Among them, six species remained consistent over the past two years, including D. albomicans, D. bipectinata, D. melanogaster, D. simulans, D. suzukii, and Scaptodrosophila coracina. Interestingly, three new species were identified this year: D. parabipectinata, D. kohkoa, and Phortica speculum, indicating changes in biodiversity.

Notably, my fly sample was identified as D. neohypocausta, a species subgroup within the Drosophila immigrans group and endemic to China (Huang et al., 2002). Surprisingly, this species was absent in Durham over the past three years but appeared only in the last two years (2020-2021) in Kunshan. The limited sample size (3 samples in 2020 and 2 samples this year) suggests that D. neohypocausta may not be native to the area. Analyzing the proportion of certain species in wild-caught fruit flies allows us to explore biodiversity and the species' adaptability to the environment.

An interesting discovery was the discrepancy in the identified species between the initial BOLD result (D. albomicans) and the later re-check, which confirmed D. neohypocausta. Further research unveiled that both species belong to the immigrans species group, with D. albomicans belonging to the nasuta subgroup and D. neohypocausta to the hypocausta subgroup, representative species groups of Drosophila in East Asia (Zhou et al., 2012; Katoh et al., 2007). A recent study revealed significant genetic differentiation between two clades of D. immigrans, possibly splitting around 2 million years ago, with one clade consisting of flies from Taiwan and the other from Laos and the United States (Liu et al., 2015). This finding may explain the scarcity of D. neohypocausta samples, as most D. immigrans species are confined to the region extending from the African coast through Asia to the Pacific islands (Katoh et al., 2007).

To gain a comprehensive understanding of biogeographic, ecological, and evolutionary patterns within the Drosophila genus, expanded sampling is necessary. Additionally, the large immigrans species groups, which have been inferred as polyphyletic in some studies, require further taxon sampling and taxonomic revision (O'Grady et al., 2018). Conducting such investigations is vital to placing the various lineages that constitute the genus Drosophila and unraveling their complex relationships.

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