Research Activity Report
Supported by "Leading Graduate Program in Primatology and Wildlife Science"

2024. 11, 19

Affiliation/Position	Wildlife Research Center/M1
Name	Casey Mack

1. Country/location of visit

WRC, Kyoto

2. Research project

Genome sequencing field course

3. Date (departing from/returning to Japan)

2024/11/11-2024/11/18

4. Main host researcher and affiliation

Dr. Murayama, and Dr. Sato, Kyoto University WRC

5. Progress and results of your research/activity

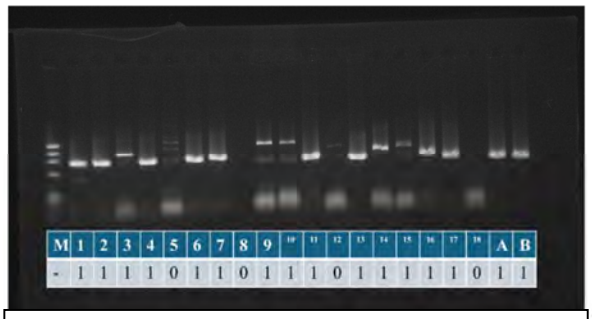
In this intensive course, we conducted species and sex identification with mitochondrial DNA collected from feather samples.

Day 1:
On the morning of the first day, we went to Kyoto-Gosho to collect feather samples. On the way there, we noticed there were a lot of egrets and cormorants in the Kamogawa, so we stopped there on the way back to collect some more samples. We collected 43 samples, which was too many to run in one day, so we started with 18, and added 2 samples provided to the lab by the zoo. Then, we extracted the DNA from the basal tip of these feathers. Next, we measured the DNA concentration, then completed PCR amplification using CO1-forward as the primer for species identification using mitochondrial DNA. We ran the PCR at the end of the day, and had the samples incubating in 10°C until we came back the next morning, as our PCR conditions were LA55C40, meaning 40 cycles of (95°C for 30s, then 55°C for 30s, then 74°C for 1 minute), followed by a 10-minute period of incubation at 74°C, then cooling at 10°C until we were ready for the next step.

[Figure 1: Collecting feathers at Kyoto-Gosho]

Day 2:
On the second day, we started with gel electrophoresis to check the success of our PCR. At this stage, the DNA is still double-stranded. (We also practiced making gels. We made 1.5% TBE gels, which was determined to be the best for our uses. I learned that increasing the amount of powder added increases the percentage/thickness of the gel, and thicker gels make the DNA flow across slower.) Before loading into the gel for electrophoresis, we mixed our DNA samples with loading buffer, which is heavier than TBE buffer and water, so this ensures our sample stays in the well. As you can see in Figure 2, we had 4 of these samples fail the electrophoresis, meaning there was not enough DNA present by this step. Thus, we were prompted to repeat this process later, after we finished with the successful samples. Next, we did purification of the PCR products. This step removes excess primers, and we end up with 'clean DNA'. After purification, we ran sequencing. The first part of this step is creating a sequencing reaction to create DNA fragments we select for with the primer (in our case, Bird F1 primer). We put this mix of primer, BigDye terminator, BigDye Buffer, and our PCR products back into the PCR machine to run the sequencing reaction (96°C for 10s, 50°C for 5s, 60°C for 1 min for 25 cycles), then incubate at 10°C until the next morning.

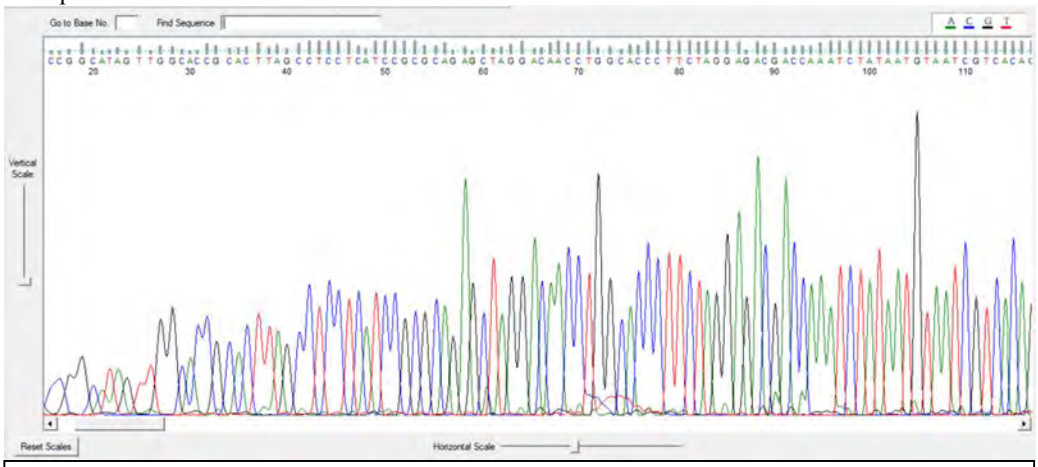
La Taq Enzyme
LA55C40
Tissue samples/feathers



[Figure 2: Gel electrophoresis results from our first set of species ID samples]

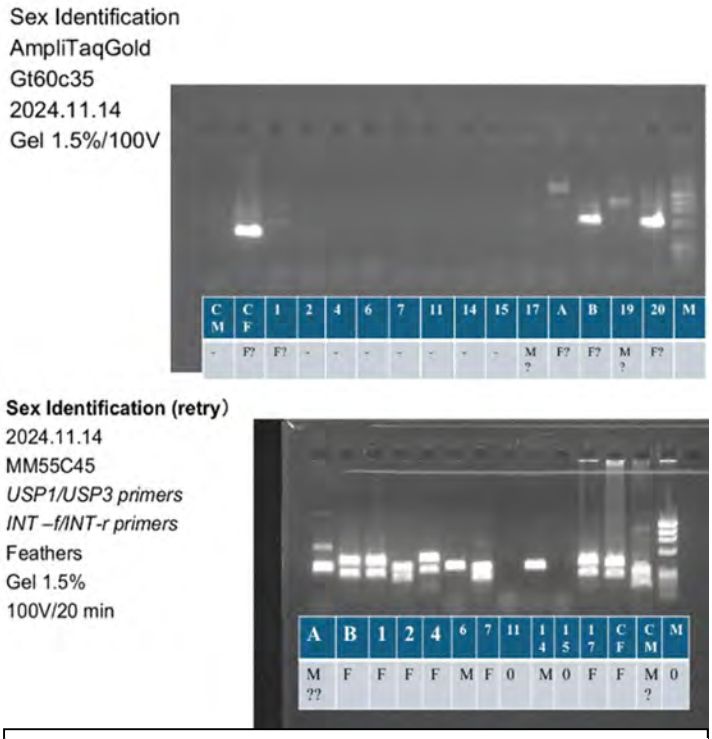
2024.11.12
Genome Training
Course 1
Liu Liu, Haruka, Casey

Day 3:
The first thing we did on the third day was continue the sequencing step from Day 2. In the next part of this step, we purified again with ethanol, to remove unused primers and excess DNA from the sequencing reaction before finally running the sequencing. This step was a little tricky, involving centrifuging our samples upside-down without the lids to get out excess liquid and leave our DNA at the bottom of the tube! Then, we ran sequencing! To prepare our samples for the sequencer, we added HDFA to our samples, then heat-denatured at 96°C for 2 minutes, then snap-cooled on ice to keep the single-stranded DNA created from the denaturing from going back into a double-helix. The sequencer machine (ABI3130xl) runs one cycle, consisting of 16 samples, for 90 minutes. If we have less than 16 samples, we must be sure to add HDFA to empty wells to make sure the machine doesn't get damaged from attempting to read empty wells. The data from the sequencing machine is provided in two forms: ab1 and seq files. We downloaded "FinchTV" software to view the ab1 files, which plotted the sequence of base pairs found in the fragments as shown in Figure 3. The higher peaks meant the percentage of fragments with the reported base pair (i.e. A, T, C or G) was higher, so we are more confident in the samples that have high peaks. We also are looking for sequences that have good peaks for more than 400 base pairs to accurately identify species. We identified species by using the seq files to copy the sequence of base pairs and paste into the nucleotide Blast function on BOLDSYSTEMS in the identify section. Then, the results would yield the list of species with the most similar order of base pairs. We considered 98% identical to be accurate for each sample. Of the first set of samples we processed, we successfully identified the species of 11 samples out of the 20 samples in the set. This is apparently a good turnout for using feathers, as they are a non-invasive type of sample.



[Figure 3: Base pair sequence of one of our samples displayed in FinchTV software]

Day 4:
On the fourth day, we simultaneously continued our extraction and species identification attempts for a new set of samples, including some that failed the first time, and started the sex identification procedure for a new set of samples. The sex identification procedure is shorter than species identification, because we don't need to sequence this DNA, we stop just after gel electrophoresis. In the electrophoresis results, we should be able to see the "ZZ" gene in males as one band, and "ZW" as two bands indicating females. W is a longer band than Z, which is why it stops before the Z gene when moving through the gel, resulting in two distinct bands, see Figure 4a for the results of this sex ID attempt.



[Figure 4a(top): Electrophoresis results of our first Sex ID attempt, which all failed, Figure 4b:results of sex ID retry]

Clearly, this was not the result we anticipated. We think this electrophoresis attempt failed due to the CHD primer that we used. This primer is usually good at sex ID in birds, but not pigeons, which most of our samples turned out to be. Also, we ran our PCR conditions at gt60c35, using AmpliTAQ gold instead of the LA taq we used for species identification. We believe these PCR conditions may not have been sufficient for noninvasive sample types, so when we re-attempted to identify sex, we changed to 55°C and 45 cycles. Figure 4b is showing our reattempt at sex identification, we had more success this time, though we still did not get absolute results for every sample. In the re-try, we changed our primers to USP-1 and USP-3, as well as Int-F and Int-R. We also used Multiplex enzyme instead of AmpliTAQ gold.

Day 5 and 6:
On the last two days, we repeated with our failed samples. On Day 5, we repeated the sex ID procedure with our samples (minus samples #18-19, provided by Fadel) and a control female and male. On Day 6, we repeated sequencing with our already-purified DNA samples (set 2). To do this, we first ran gel electrophoresis to make sure DNA was still present. Actually, one of our samples failed this stage, so we did not continue with that one. Then, we ran the sequencing reaction again with the BigDye reagents and CO1 primer. Next, we repeated ethanol participation and gene sequencing with our 8 samples. This time, we tried using 1microliter of our DNA sample in one column, then 3microliters of our same samples in the next column, to see make sure we had enough DNA for the sequencing not to fail this time!

Discussion:
Overall, we identified 6 different species from our samples (not including the samples provided by the zoo). A lot of our samples turned out to be pigeons, these are common birds, and perhaps pigeon DNA is easier to amplify? We expected to identify a lot of crows, as they were seen frequently when we collected our samples. There are two species of crows that are found at Kyoto-Gosho, the large-billed crow and carrion crow. We identified at least one sample for each of these species. One of the samples provided to us from the zoo (the samples with letters instead of numbers) was incorrectly identified as a pigeon, even though the zoo informed us the sample was of a snowy owl. We believe this sample may have been contaminated at some point in our process, and this must have overpowered the snowy owl DNA, or the snowy owl DNA was lost completely at some point. Many of our successfully identified samples came from Kyoto-Gosho, while a lot of samples that came from Kamogawa, including all of our potential egret samples, failed DNA extraction, perhaps because they were wet and DNA was washed away from the basal tip.

Please see the table on the next page for the list of all samples we successfully identified the species for.

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Name	Date	Place	Condition	Nakamura- san's guess	Electro-phoresis	[DNA] (ng/ug)	Purity	Identification	Common Name
A	11/11/24	Zoo	small	Unknown	SUCCESS	2.6	1.56	Strix uralensis	Fukuro (Owl)
B	11/11/24	Zoo	Small	Unknown	SUCCESS	10.8	1.78	Phoeniconaias minor	flamingo
1	11/11/24	Gosho	Small	Mejiro	SUCCESS	3.9	2.77	treron sieboldii	Aobato (pigeon)
2	11/11/24	Gosho	good	Unknown	SUCCESS	7.1	2.09	Streptopelia orientalis	Kijibato (Turtle dove)
4	11/11/24	Gosho	good	Unknown	SUCCESS	1.8	1.58	Streptopelia orientalis	Kijibato
6	11/11/24	Gosho	small	Kijbato	SUCCESS	2	1.99	Streptopelia orientalis	kijibato
7	11/11/24	Gosho	good	Kawarabato	SUCCESS	6.2	1.37	Streptopelia orientalis	kijibato
11	11/11/24	Kamogawa	wet	Unknown	SUCCESS	1.6	1.17	Columba livia	kawarabato
13	11/11/24	Tadasunomori	small	Unknown	SUCCESS	2.3	2.05	Streptopelia orientalis	Kijibato
14	11/11/24	Kamogawa	wet	Unknown	SUCCESS	0.7	-2.56	Columba livia	kawarabato
15	11/11/24	Tadasunomori	small	Tobi	SUCCESS	0.6	1.8	Milvus migrans	tobi (Black Kite)
16	11/11/24	Kamogawa	good	Unknown	SUCCESS	1.4	1.97	columba livia	Kawarabato
17	11/11/24	Tadasunomori	good	Unknown	SUCCESS	4.6	1.28	Columba livia	kawarabato ??
19	11/11/24	Gosho	old;broken	Crow	SUCCESS?	3.4	53.37	Corvus corone	carrion crow (Hashiboso-garasu)
25	11/11/24	Gosho	dirty	Unknown	SUCCESS	2.8	32.31	Streptopelia orientalis	Kijibato (Turtle dove)
27	11/11/24	Gosho	small feather	pigeon	SUCCESS?	1	-0.68	Corvus macrorhynchos	Large-billed crow (Hashibuto-garasu)
C		zoo		owl	SUCCESS?	69.7	1.99	Streptopelia orientalis	Kijibato (Turtle dove)

6. Others

I would like to thank Sato-sensei and Murayama-sensei for their guidance and insight in this course. They were very patient with us, as this was the first time my course mates and I completed this process. Then, I would like to thank Mohamed-san and Fadel-san for their assistance and guidance throughout the course. I am especially grateful for my course mates, Haruka-san and Liu Liu-san for their teamwork, as well as Nakamura-san for his guesses for species identification and Xorlali-san for his help.