Moran Lab Notebook

8/4/2011

Notes on aphid and psyllid dissections:

Thinking about methods for extracting DNA/RNA from bacteriomes. I spoke with Allison Hansen and her undergrad Adam about how she approached this for her aphids. Here’s a general run down:

Put aphid in chilled Buffer A (about 500 ul; I believe the description is in her PNAS paper). Stab in the head with one insect pin, then rip open body with second pin spilling contents into buffer (note that Adam prefers to first cut off legs with a razor blade, then cut off bottom of abdomen and squeeze contents of aphid). This is all done in a 9-well glass dissecting dish. As quickly as possible, pipette up bacteriocytes and put them into “RNA Protect”. Bacteriocytes are distinguished by their “pearly” appearance. Bright white and almost sparkly. Distinguished from some other tissue which is almost pale. The bacteriocytes do not hold together in any coherent bacteriome organ structure, so they are largely gathered individually. Put rest of body minus the embryos into separate RNA Protect container. This can be kept at room temp a little while until being frozen.

I tried a few dissections on some extra aphids Kim had lying around (pea aphids I assume). Then I tried doing it on some psyllid nymphs. These were from galls brought in by Nancy on Aug. 1, 2011. They were collected from a hackberry (species?) near her house. They have been kept at 4 C since then, although I have had them out a few time, some times for at least a couple hours to look at them. Most of the galls are very prominent and are probably what is called hackberry nipple gall. There was also a set of much flatter galls that also contained psyllid nymps (extremely similar to my eye). I don’t know if these are from a different species or just represent aborted or not yet fully developed galls.

Important difference between psyllid and aphid bacteriomes. Psyllid bacteriomes are distinctly yellowish-orange. They are vaguely visible through the exoskeleton from the ventral side. They also are much more structurally coherent than in the aphids such that, at least sometimes, they seem to hold together as one organ. The psyllid nymphs (which I believe are sexual and dioecious) also lack the huge developing embryos found inside the aphids.

Observations on technique:

--I could not find a way to gently “dissect” the psyllid nymphs. It more came down to using pins to rip them open from the top or the bottom or both and squeeze out the contents.

--There seemed to be a lot of variance in how intact the bacteriome was after extrusion. I seemed that larger nymphs produced a less intact bacteriome (yellow cells interspersed more with the abundant white cells that are also extruded. It might also be that squeezing out the head side was worse than the butt side. I had one come out very cleanly when I ripped from both end simulataneously and opened up a fissure near the middle, but I had a tough time replicating this.

8/8/11

Notes on dissections of aphids from witch hazel cone galls. I collected some galled leaves from a plant growing in Edgewood park (on trail that starts just past the bridge on north side when heading west on Edgewood Ave.—about half way in on left). They were collected on 8/6/11 and have been kept on a moist paper towel at 4 C since then. There were multiple trees in the area. I did not pay close attention to which specific tree I collected from. Stored at 4 C in Ziploc with moist paper towel.

Based on quick internet search, these appear to be Hormaphis (there are a couple different species; I made no attempt to distinguish).

Galls contained dozens of aphids of variable size. Reddish/purplish in color. Covered in fluffy white material (crystalized honeydew?).

Upon, dissection of aphids, they released numerous aphid embryos as well as what I think are bacteriocytes based on the pearly/iridescent description given by Adam and Allison for the pea aphid. If I am correct in my bacteriocytes identication, they did not seem to be as plentiful as what I remember in the pea aphids. Sometimes, I could only identify one or two (other times there seemed to be more like 10 or so).

I took some images through the dissecting scope of the galls and aphids

8/9/11

Making up 500 ml of “Buffer A” as described in Hansen and Moran 2011 PNAS:

25 mM KCl

35 mM Tris-HCl (pH not specified)

100 mM EDTA

250 mM sucrose

In Autoclaved dH2O

KCl:

Make 100 ml of 1 M stock: 74.55 g/mole \* 1 mol/L \* .1 L = 7.455 g

🡪 Autoclave

Use 12.5 ml of stock

Tris-HCl:

17.5 ml of 1 M stock, pH 7.4 (American Bioanalytical)

EDTA:

100 ml of 0.5 M stock (American Bioanalytical)

sucrose:

342.3 g/mol \* 0.25 mol/L \* 0.5 L = 42.8 g

Note that sucrose was added after autoclaving (to avoid hydrolysis of disaccharides, although I have no idea whether this matters) 🡪 Not sterile.

Started dissecting out bacteriomes from the hackberry nipple gall psyllids (see 8/4/11). These will be used for a test DNA extraction. I will also extract from body tissue.

I only selected the prominent galls, not the other flat morphology, which may be aborted galls from the same psyllid or another psyllid species.

I took some images of the galls, psyllids, and an extracted bacteriome.

Basic process:

1. Slice open gall with razor blade (on a couple of occasion I damaged the psyllid—there is only one per gall—doing this. In one severe case I did not proceed).

2. Put psyllid in well of glass dissecting dish. Add 200 ul of ice cold Buffer A (see above).

3. “Dissect” 🡪 attempt to tear open the base of the nymph’s abdomen and squeeze out contents. Look for a bright yellow structure (the bacteriome). When things went well it was readily identifiable, but I think it was often damaged, becoming intermingled with other tissues. With P20 pipettor, transfer bacteriome to an eppendorf on ice. Then transfer remaining body tissue to a second tube, also on ice. Bacteriome and body samples from multiple dissections all pooled into one tube

4. Centrifuged tubes for 5 min at 10,000 rpm and 4 C in microfuge. This seemed to cleanly pellet the bacteriomes. But the bodies loosened up when I tried to aspitate the sup. So I re-spun the bodies for 5 min at 13,200 rpm and 4 C. Supernatants were removed and the pellets were stored at -20 C. The bacteriome pellet was VERY small but visible with naked eye.

I dissected a total of 13 galls but only ended up pooling tissue from 7. Here’s what happened to the other 6:

--two dead on arrival. These were smaller, sickly looking galls.

--one was mangled when I sliced into the gall

--one gall seemed to be empty

--one had a bacteriome that was so mangled after dissection that it couldn’t be collected.

--one produced a good bacteriome, but it stuck to the inside of the pipet wall and I couldn’t extract it (stay away from the P1000 for transferring).

Issues and concerns:

--Time. This was a slow process. Probably spent 10-15 min per gall. I’m sure I will speed up, but if sitting on ice is a problem, I may need to make adjustments. The earliest samples spent hours on ice.

--I felt pretty good that my bacteriome sample was fairly pure. I’m sure there are some stray cells mixed in, but I think it’s probably pretty good. I can’t say the same for the purity of the body sample. I think in many cases the bacteriome was disrupted, so that I was able to identify and pull and nice chunk of it, but other bacteriocytes are interspersed with the body tisse. This may present an issue when I want to do RNA-seq of bacteriome vs. body. For now, I was just collecting bodies for practice. I don’t intend to anything with the body DNA sample.

--The internal contents of the psyllid become widely dispersed throughout the buffer. I made an effort to pipet up some, but a good deal is definitely left behind. I wonder if certain cell types would be preferentially excluded because of this.

--I had some difficulty with tissue sticking to the inside of pipet tips. I may need to look into a solution for this.

8/10/11

Continuing dissections of hackberry nipple gall psyllid. I separated bacteriome and body tissue for 13 more psyllids (see yesterday). To get that many, I actually cut open 25 galls, but a number failed somewhere along the way (i.e., psyllid was dead on arrival, was severely damaged during gall dissection or did not yield a clean bacteriome extraction).

It took about 3 hours to go through that many galls. I also didn’t have time to centrifuge the samples before lab meeting, so the earliest extractions stayed on ice for a total of about 5 hours.

I then centrifuged the samples at 13,200 rpm and 4 C in the microfuge, aspirated the supernatant, and stored the pellets at -20 C.

Began DNA extractions from isolated psyllid gall tissues using QiaAmp DNA Micro Kit.

Three extractions:

1. Isolated bacteriomes (~20 pooled from yesterday and today)
2. Isolated body tissue (~20 pooled from yesterday and today)
3. A single whole, live, psyllid nymph freshly pulled from one of the hackberry nipple galls.

Following tissue extraction protocol in the manual:

Added 180 ul of buffer ATL to each (or 90+90 for the samples that needed to be pooled from multiple days). Used a pestle to hand grind the whole nymph and the bodies. No grinding of the bacteriomes was performed. Incubated overnight (starting at 5:00 pm) at 56 C and 300 rpm on shaking heat block thing.

8/11/11

Continued with the Qiagen DNA extraction protocol.

It was apparent that the exoskeletons in the pooled body samples were not broken down the process. They were still clearly visible.

Added buffer AL and EtOH as instructed. Adding AL generated a noticeable precipitate that would not dissolve with vortexing. But after adding EtOH, it dissolved.

When transferring lysate to columns, I tried to exclude exoskeletons in the pooled body sample (weakly pelleted after a quick centrifuge spin), but at least a small amount did get transferred onto the column.

After the wash spins, I noticed a small bead of liquid on the outside of the rubber rim about the membrane. I tried rotating the tubes 180 degrees and spinning for 30 more seconds, but there was still liquid visible. I used a P20 to remove as much of it as I could (P10 would have been better, I guess), but there was probably at least a smidge of residual EtOH going into elution.

Eluted in two rounds:

Round 1: 25 ul buffer AE (with ~7 minutes of room temp incubation time)

Round 2: 50 ul buffer AE (essentially no incubation time)

Samples stored at -20 C. Labelled as Psyllid (Whole|Bodies|Bacteriomes) DNA (1|2)

…with the last part referring to round 1 vs. round 2 extraction.

Quantified with Qubit High Sensitivity (HS) dsDNA kit:

|  |  |
| --- | --- |
| **Sample** | **DNA Concentration (ng/ul)** |
| Whole 1 | 6.54 |
| Whole 2 | 2.61 |
| Bodies 1 | 88.80 |
| Bodies 2 | 39.70 |
| Bacteriomes 1 | 3.47 |
| Bacteriomes 2 | 1.35 |

|  |  |  |
| --- | --- | --- |
| **Sample** | **DNA Concentration (ng/ul)** | **Total Yield (in ng, assuming full vol. recovery)** |
| Whole 1 | 6.54 | 164 |
| Whole 2 | 2.61 | 131 |
| Bodies 1 | 88.80 | 2220 |
| Bodies 2 | 39.70 | 1985 |
| Bacteriomes 1 | 3.47 | 87 |
| Bacteriomes 2 | 1.35 | 68 |

Running gel:

1% agarose. Small gel. 110 V for 45 min. Stained for 20 min in lab EtBr stock. Destained for a few minutes.

Loading order.

1. 1 kb plus ladder (1/10x) - 5 ul
2. Whole 1 – 5 ul
3. Whole 2 – 5 ul
4. Bodies 1 – 1 ul
5. Bodies 2 – 1 ul
6. Blank
7. Bacteriomes 1 – 5 ul
8. Bacteriomes 2 – 5 ul
9. Blank
10. 1 kb plus ladder (1/10x) – 5 ul

Image taken (see PhotosAndGelPics directory on my computer, 20110811\_DNAextractions.TIF)

Results: Relative concentrations look to be in line with estimates from Qubit. There is a lot of of degradation in the round 1 samples, but there is still a strong band above the ladder. The round 2 samples also show some evidence of degradation, but much less so. I suspect the small fragments dissociate faster from the membrane so they are highly enriched in the first fraction. I suspect I will be able to proceed with whole genome amplification on this sample if I want to go ahead and sequence its genome.

8/14/11

Collected more witch hazel cone galls from the same location as on 8/6/11 (see description of 8/8/11). I collected a large number (~40?) of galled leaves (usually 1-3 galls per leaf) from a single tree (or perhaps two as there were two trunks emerging from the ground very close to each another). This was first witch hazel I came to when walking into the park from Edgewood Ave (as described on 8/8/11). The tree is on left about 60 m past the spot with four wooden benches. It is between the 8th and 9th wooden posts after those benches. I’m fairly sure this is NOT the same tree that I collected leaves from on 8/6/11.

Leaves were stored at 4 C in a Ziploc with a moist paper towel.

8/15/11

Tried whole genome amplification of DNA extractions from 8/10 and 8/11. Used the Qiagen REPLI g Mini Kit to perform multiple displacement amplification (MDA). This is what is left over in the lab from Eva and Zakee’s work.

I followed the standard Qiagen protocol using the 5 ul template options.

I ran 3 reactions:

Bacteriomes elution 2 (8/11/11)

Bodies elution 2 (8/11/11)

Neg Control (dH2O)

Incubated overnight in 30 C H2O bath, starting at 5:30 pm.

8/16/11

Stopped amplification reaction at 9:30 am (16 hours). 3 min at 65 C on heat block to kill the enzyme.

Ran gel to evaluate amplification. 1% agarose. 40 min @ 110 V

Lanes:

1. 1. 1 kb plus ladder (1/10x) - 5 ul
2. Bacteriome amplification – 1 ul
3. Unamplified DNA from psyllid “whole” elution 2 (8/11/11) – 1 ul. Note that this wasn’t the sample used for amplification but it is fairly comparable to the bacteriome sample used for amplification so it is a useful unamplified comparison.
4. Bodies amplification – 1 ul
5. Skipped lane (air bubble)
6. Neg control amplification -1 ul

See image: 20110816\_MDA.TIF

Results: Clear amplification in all 3 MDA samples (even the negative control!). The product from the “body” amplification looks a little bit stronger, but the bacteriome and negative control samples are very similar, which is somewhat disconcerting. I talked to Eva and Zakee. They do not normally run a negative control. The Qiagen manual troubleshooting guys notes that this can occur with no template controls and that it should not have an effect in the actual samples.

EtOH precipitate DNA to clean it up….

Making 3 M NaOAc stock for EtOH precipitation:

24.61 g of NaOAc in dH2O to a total volume of 100 ml.

Autoclaved (but I used it today prior to autoclaving).

49 ul of MDA reactions remaining

Add 0.1 volumes (4.9 ul) of 3 M NaOAc (see above). Vortex

Add 2.5 volumes (122.5 ul) of 100% EtOH. Vortex

Precipitate at -20 C for 1 hour.

10 min spin at 4 C, max speed.

Aspirate supernatant and wash in 500 ul 70% EtOH (from -20 C)

5 min spin at 4 C, max speed.

Aspirate supernatant and dry in speed vac for 5 min.

Dissolved pellet in 50 ul buffer AE from Qiagen kit.

Quantified with Qubit dsDNA BR kit.

|  |  |  |
| --- | --- | --- |
| **Sample** | **Concentration (ng/ul)** | **Total Yield (ug)** |
| Bacteriome MDA | 17.5 | 0.875 |
| Body MDA | 27.9 | 1.395 |
| Neg Control MDA | 17 | 0.85 |

Two concerns: 1) Yields are not as high as they are supposed to be and 2) similarity between negative control and bacteriome sample.

Set up repeat run of whole genome amplification.

Same as above, but used new water (though I don’t think this was the problem). Three different reactions: Bacteriome DNA elutions 1 and 2 (8/11/11) and dH2O negative control.

Started 30 C incubation at 5:45 pm.

8/17/11

Completed and killed MDA reaction at 9:45am (16 hours).

Ran gel with MDA products as well as some psyllid genomic templates from previous work by Nancy, MyLo Thao, Paul Baumann, etc. DNAs had been stored in the horizontal -80 C in rm 214 (top of sleeve near back right of freezer). The box is labeled “Psyllid DNA from P. Baumann”.

I went through the tubes and summarized rough amount, color, and concentration (as reported on tube) for each sample. For amount (i.e., volume in tube), I roughly approximated and binned things into 3 categories. #1: < 20 ul. #2: 20-50 ul. #3: >50 ul. For color, the following code applies. C: clear. Y: light yellowish brown. B: darker yellowish brown.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Genus | Species | GenBank Accession from Thao et al. (2000 Appl. Env. Microbiol.) | Amount | Concentration on tube (ng/ul) | Color | Gel 20110817 Loading Order |
| Bemisia | tabaci | ? | 2 | ? | C | 1 |
| Ctenarytaina | eucalypti | AF211133/AF231385 | 3 | 500 | Y | 2 |
| Ctenarytaina | spatulata | AF211135/AF231384 | 2 | 640 | C | 3 |
| Boreioglycaspis | melaleucae | AF211128/AF231383 | 2 | 465 | C | 4 |
| Glycaspis | brimblecombei | AF211137/AF231381 | 3 | 193 | C | 5 |
| Pachypsylla | celtidis | AF211141/AF231379 | 3 | 471 | C | 6 |
| Pachypsylla | venusta | AF211143/AF231378 | 3 | 640 | C | 7 |
| Acizzia | uncatoides(2) | AF211124/AF231366 | 3 | 898 | Y | 8 |
| Psylla | buxi | AF211146/AF231371 | 2 | 213 | C | 9 |
| Cacopsylla | peregrina | AF211130/AF231373 | 1 | 246 | C | 10 |
| Heteropsylla | texana | AF211139/AF231375 | 3 | 376 | C | 11 |
| Heteropsylla | cubana | AF211138/AF231376 | 3 | 883 | Y | 12 |
| Neotriozella | hirsuta | AF211140/AF231363 | 2 | 100 | C | 13 |
| Trioza | urticae | AF211152/AF231364 | 2 | 105 | Y | 14 |
| Trioza | eugeniae | AF211151/AF231362 | 3 | 636 | Y | 15 |
| Calophya | schini | AF211132/AF231369 | 3 | 513 | Y | 16 |
| Diaphorina | lycii | ? | 2 | 260 | Y | 17 |
| Neophyllura | arbuti | ? | 2 | 285 | C | 18 |
| Bactericera | cockerelli | AF211126 | 1 | 228 | Y | 19 |
| Aphalara | longicaudata | AF243137 | 2 | 159 | C | 20 |
| Arytaina | genistae | AF243136 | 3 | 1150 | B |  |
| Aphalaroida | inermis | AF211125 | 1 | 380 | C |  |
| Acizzia | uncatoides(1) | AF211123/AF231367 | 1 | 74 | C |  |
| Blastopsylla | occidentalis | AF211127/AF231382 | 1 | 375 | B |  |
| Cacopsylla | brunneipennis | AF243138 | 1 | 220 | C |  |
| Cacopsylla | myrthi | AF211129/AF231368 | 1 | 653 | B |  |
| Cacopsylla | pyri | AF211131/AF231372 | 2 | 383 | B |  |
| Cardiospina | albitextura | ? | 2 | 1060 | B |  |
| Ctenarytaina | longicauda | AF211134/AF231386 | 1 | 540 | C |  |
| Diaphorina | citri | AF211136/AF231365 | 1 | 570 | C |  |
| Livia | junci | ? | 1 | 143 | C |  |
| Pachypsylla | pallida | AF211142/AF231377 | 1 | 586 | C |  |
| Panisopelma | fulvescens | AF211144 | 1 | 228 | Y |  |
| Panisopelma | sp. | AF211145/AF231387 | 1 | 355 | B |  |
| Psylla | sp. | AF211147/AF231389 | NotFound |  |  |  |
| Russelliana | intermedia | AF211148/AF231388 | 0 |  |  |  |
| Spanioneura | fonscolombii | AF211149/AF231374 | 0 |  |  |  |
| Tainarys | sordida | AF211150/AF231380 | 1 | 256 | C |  |

I ran a 1% agarose gel for 45 minutes at 110 V.

Loading order:

1. 1 kb plus DNA ladder (1/10x) – 5 ul
2. Bacteriome MDA #1 - 1 ul
3. Bacteriome MDA #2 – 1 ul
4. Neg Control MDA – 1 ul
5. Whole psyllid DNA extraction elution #2 (8/11/11) – 1 ul
6. 1 kb plus DNA ladder (1/10x) – 5 ul

7-26. Twenty genomic DNA samples from different psyllids (see above for loading order, note that the numbering starts at 1 not 7) – 1 ul each

27. 1 kb plus DNA ladder (1/10x) – 5 ul

See image: 20110817\_MDAandBaumannDNA\_highexp.TIF and 20110817\_MDAandBaumannDNA\_lowexp.TIF

Results:

For MDA, significant amplification for both samples as well as negative control. Unlike yesterday, the bacteriome samples do look stronger than the negative control (but there is definitely a lot of HMW DNA in the negative control).

For old psyllid DNAs, the preps do appear to contain substantial quantities of DNA, although they all seem to have a lot of degraded and very low MW material. However, most also have a significant HMW fraction.

EtOH precipitations of new MDA preps. Dissolved dried pellets in 50 ul buffer AE from Qiagen kit.

Qubit dsDNA BR kit to quantify MDA EtOH precipitations and a sample of Baumann psyllid DNAs.

Bacteriome MDA 1: 23.7 ng/ul

Bacteriome MDA 2: 7.0 ng/ul

Neg Control MDA: 10.8 ng/ul

Bemisia tabaci: 11.8 ng/ul

Ctenarytaina eucalypti: 200 ng/ul

Ctenarytaina spatulata: 355 ng/ul

Boreioglycaspis melaleucae: 116 ng/ul

It looks like I might have had differential sample loss from the MDA EtOH precipitations. Qubit values don’t correlate with band intensity of the pre-preciptiation gel.

Mix 40 ul of Bacteriome MDA 1 (from today) with the 40 ul of the Bacteriome MDA sample from yesterday. This should be about ~20 ng/ul and serve as material for submission for Illumina library prep.

Prepare Ctenarytaina eucalypti sample for submission for Illumina library prep. The sample is yellowish in color. Try running it through a Qiagen minElute column to clean it up. Used QiaAmp DNA Micro Kit (Genomic DNA clean up protocol). Started with 50 ul (should be about 10 ug, albeit with a large low MW fraction). Eluted with buffer AE in 3 rounds: 25 ul (with 1 min incubation). 50 ul (with 5 min incubation). 50 ul (with 1 min incubation). I was hoping this might enrich for high MW DNA in 2nd and 3rd elutions.

Quantifying and characterizing samples (qubit, nanodrop and gel):

Qubit dsDNA broad range.

Bacteriome MDA pool: 62.0 ng/ul

Ctenarytaina eucalypti elution 1: 65.4 ng/ul

Ctenarytaina eucalypti elution 2: 12.2 ng/ul

Ctenarytaina eucalypti elution 3: 43.6 ng/ul

MDA pool is 3x times higher than expected. Previous qubit measurements on both components of the pool were ~20 ng/ul.

Tough to make sense of the concentration distribution of the three C. euc. Elutions.

Ran Nanodrop:

|  |  |  |  |
| --- | --- | --- | --- |
|  | Concentration (ng/ul) | 260/280 | 260/230 |
| Bacteriome MDA--pool | 220.8 | 1.62 | 2.41 |
| Cten. euc. elute1 | 914.7 | 1.99 | 2.28 |
| Cten. euc. elute2 | 109.5 | 1.99 | 2.16 |
| Cten. euc. elute3 | 306.3 | 1.9 | 1.83 |

I don’t know why these are so high, but I don’t think the concentration estimates can be believed. 260/280 value might be informative. The relative values are consistent with Qubit except they suggest bacteriome MDA is overestimate which would be consistent with original qubit. Note that the 260/280 ratio is low for the MDA pool.

Run 1% agarose gel for 30 min @ 120 V.

Loading order:

1. 1 kb plus DNA ladder (1/10x) – 5 ul
2. Bacteriome MDA pool - 1 ul
3. Cten. Euc. Elution 1 – 1 ul
4. Cten. Euc. Elution 2 – 1 ul
5. Cten. Euc. Elution 3 – 1 ul
6. Whole psyllid DNA 2 (8/11/11) – 1ul
7. 1 kb plus DNA ladder (1/10x) – 5 ul

See image: 20110817\_submissionSamples.TIF

Results: In line with qubit except suggest that MDA pool was overestimated. Otherwise, I bet the last qubit was more or less on target. Lots of degradation in Cten. euc. samples.

8/18/11

Poooled 41 ul of Cten. euc. elution 3 and 20 ul of Cten. euc. elution 1 for submission for Illumina sequencing (see yesterday).

Ran one more qubit dsDNA BR (values have quite variable; ran two replicates of each):

Bacteriome MDA pool: 78.0 and 61.5 ng/ul.

Cten euc pool: 86.8 and 87.3 ng/ul.

Both of these sets of values are higher than I would have expected (I was thinking 20 ng/ul for the MDA pool and 50 ng/ul for Cten euc). Oh well. Clearly I need a more repeatable quantification method. But the gels, qubit, and nanodrop all point to having substantial amounts of DNA (> 1ug per 50 ul). So I will take a rough average of the qubit readings and report that if they ask for a specific concentration (probably 30 and 60 ng/ul to be on the conservative side).

Submitting samples to Yale Center for Genome Analysis (YCGA). These will be used for standard Illumina paired-end library construction with the center’s multiplex tags. They will be included in a single lane Hi-Seq run with a total of 13 samples (mine plus 11 other genomes/fosmids from the Moran Lab).

Name: DBS001

Description: Isolated bacteriomes from Pachypsylla spp.

Concentration: 30 ng/ul

Total amount: 1.8 ug (in 60 ul)

Name: DBS002

Description: Ctenarytaina eucalypti

Concentration: 60 ng/ul

Total amount: 3.6 ug (in 60 ul)

\*\*Note this sample is very degraded but there is still a substantial amount of HMW DNA.

8/23/11

Trying to isolate RNA from psyllid bacteriomes and body samples. Nancy brought in some more hackberry leaves from her yard covered in nipple galls on 8/17/11. They have been stored at 4 C wrapped in a damp paper towel since then.

**DISSECTIONS**

I extracted a total of 18 psyllid nymphs from galls (I opened up a few more galls than that, but lost some nymphs b/c I mangled them with the razor blade or they were dead on arrival). I found that the best way to open the galls was to make a radial vertical slice with a razor blade and then pry the gall in half with the side of the blade. This seemed to minimize the chance of slicing through the psyllid while still being quick.

I collected all 18 nymphs at one and kept them in a petri dish on ice. These were dissected in the 6-well glass dissecting dishes. I was not super careful about RNase-free techniques (seemed unnecessary when still working with live tissue which is surely full of RNases). I kept three dissecting plates on ice and took one off at a time to do a single nymph dissection. I rotated through, so each one would only spend a couple of minutes off ice during one dissection. Because of this, they built up condensation, so I would wipe down each well with a Kimwipe prior to each dissection (potential concern for sterility/contamination/RNase/etc). I added 100 ul of Buffer A to a well immediately prior to each dissection. The buffer (which is not RNase-free; see 8/9/11) was kept on.

Nymphs were dissected with two insect pins. One used to puncture the nymph and/or hold it steady and the other used to rip it open. I think having the nymphs cold improved the extent to which the distinctly yellow bacteriome could be isolated as a single entity. I separated out the bacteriome (or pieces thereof) from other tissue as best I could and then pipetted it up with a P200. I tried to minimize any additional cells that were taken up with the pipettor. In general, I think this was fairly clean, but there definitely was a small amount of free floating cells that was transferrd as well.

I pipetted the bacteriomes into a tube containing 500 ul of Qiagen RNAprotect Bacteria reagent. With a clean pipette, I then tried to transfer as much of the remaining body tissue and cells into one of two additional tubes (body 1 and body 2) containing 1 ml of RNAprotect. I put the bodies for the first 9 nymphs in body 1 and the second 9 nymphs in body 2. I tried to keep the volumes small for the body transfers (this was not an issue for the small, discrete bacteriomes). The total ration of RNA protect to buffer should be 5:1. I may have ended up slightly below that for the body samples. Initially, I was keeping the RNA protect tubes on ice, but that resulted in rapid formatting of precipitant, so kept them at room temp after only a couple dissections.

Of the 18 dissections, I only successfully got bacteriomes from 12. One was lost because it irrecoverably stuck to the side of the pipette tip. The other 5 just didn’t yield a discrete bacteriome upon ripping open of the nymph (presumably b/c the bacteriome was too badly disrupted). If I couldn’t get a bacteriome, I didn’t keep the body tissue either. In many cases where I could isolate clear pieces of the bacteriome, there may also have been disrupted pieces left with the body tissue (i.e., the body samples are unlikely to be bacteriome free). Only in a few cases was a fairly confident that I was getting the entire organ in one piece.

Once I finished dissections (took about an hour and 15 minutes to go through all 18), I centrifuged the tubes at max speed for 5 minutes (room temp) and then stored them for a few hours at -20 C.

**RNA EXTRACTIONS**

I thawed the 3 tubes (bacteriome, body 1 and body 2) from the above dissections and spun them once again at max speed (room temp). I pipetted off most of the RNA protect supernatant, but the pellets weren’t tight so I probably ended up leaving ~20 ul.

I then ran through a fairly standard Trizol extraction as follows:

1. Add 500 ul of Trizol. Grind body samples with pestle followed by vortexing. Mix bacteriome samples by pipetting and vortexing.
2. Incubate at room temperature for 10 minutes.
3. Add 100 ul chloroform, vortex for 20 seconds.
4. Incubate at room temperature for 10 minutes.
5. Centrifuge at 12,000g for 15 minutes at 4°C.
6. Transfer upper aqueous phase to a new centrifuge tube containing 250 ul isopropanol.
7. Mix thoroughly by inversion 50-100x.
8. Incubate at room temperature for 10 minutes.
9. Centrifuge at max speed for 20 minutes at 4°C.
10. Pipet off supernatant, add 500 ul 80% EtOH.
11. Wash pellet thoroughly by inversion.
12. Centrifuge at 7,500g for 5 minutes at 4°C.
13. Pipet off supernatant.
14. Centrifuge briefly, pipette off remaining supernatant.
15. Air dry 5 minutes, until the edges of the pellet become translucent (protocol said 10-20 minutes but my pellets were small and seemed to dry quickly).
16. Add 20 ul RNase-free water.
17. Incubate at 65°C for 10 minutes, until pellet is fully in solution.

I could visibly see a whitish pellet for the two body samples but there was nothing visible for the bacteriome sample. I may have let the body samples dry too long. They went completely transparent/invisible in only a few minutes.

Nanodropped to get a sense of quantity/purity (remember that this machine was yielding estimates that were probably an order of magnitude too high for my earlier DNA work).

|  |  |  |  |
| --- | --- | --- | --- |
| **Sample** | **Concentration (ng/ul)** | **260/280** | **260/230** |
| Bacteriome | 63.8 | 1.81 | 0.97 |
| Body 1 | 391 | 1.93 | 0.95 |
| Body 2 | 344 | 1.86 | 1.89 |

I am dubious about the high magnitude of these estimates. I will try to run an agilent to confirm. The 260/280 ratios are a little low and the 260/230 ratios are really bad for bacteriome and body 1, so there may be some purity issues.

8/24/11

Run 1% agarose gel to get a crude sense of RNA size distribution from yesterday’s extractions. 120 V for 25 min.

Loading order:

1. 1 kb plus DNA ladder (1/10x) – 5 ul
2. Bacteriome RNA - 8 ul
3. Body 1 RNA – 8 ul
4. Body 2 RNA -8 ul
5. 1 kb plus DNA ladder (1/10x) – 5 ul

See image: 20110824\_RNA.TIF

Results: Both the ladder and the samples appeared to have a tough time getting out of the wells (might want to use comb with thicker teeth in the future or running at a slightly lower voltage). There does appear to be a lot of RNA in the body samples but note that I loaded 8 ul of each sample. I can make out what appear to be the large and small rRNA subunits bands in these samples. But there is A LOT of low MW material. More than I would have expected, so this probably indicates degradation. I can also pick up a little bit of higher MW signal so this might indicate DNA contamination.

The bacteriome sample is tougher to interpret because the lane ran poorly. It is clear, however, that, as expected, there is much less RNA in this sample.

Next up, run these samples on the Agilent Bioanalyzer, which should provide better size and quantity data.

Agilent Bioanalyzer Run: RNA 6000 Nano Kit. Using filtered gel prepared by Adam on 6/28/11 (it is supposed to be used within one month but this is all we have left).

I loaded samples in the following order (I did not enter the samples names, so these correspond to sample1-12 in the output files). Note that I loaded duplicates of all samples/dilutions

1. Bacteriome RNA 1x rep 1
2. Body 1 RNA 1x rep 1
3. Body 2 RNA 1x rep 1
4. Bacteriome RNA 1/10x rep 1
5. Body 1 RNA 1/10x rep 1
6. Body 2 RNA 1x rep 1
7. Bacteriome RNA 1x rep 2
8. Body 1 RNA 1x rep 2
9. Body 2 RNA 1x rep 2
10. Bacteriome RNA 1/10x rep 2
11. Body 1 RNA 1/10x rep 2
12. Body 2 RNA 1x rep 2

Output: see AgilentRuns/2011-08-24

Results: Quantifications are actually pretty closely in line with the nanodrop estimates. Using the concentrations estimates from the first 3 samples,

I get the following.

|  |  |  |
| --- | --- | --- |
|  | **Concetrnation (ng/ul)** | **Total Yield (ng)** |
| Bacteriome | 52.8 | 1056 |
| Body 1 | 306.9 | 6138 |
| Body 2 | 257.7 | 5154 |

There are multiple dominant peaks in the expected vicinity of rRNAs. Three in the body sample, 4 in the bacteriome sample. There is also a higher MW peak (much more pronounced in the bacteriome sample, but faintly noticeable in the the body samples. I recall reading in some online troubleshooting forum that contaminating DNA can run as a band slightly above your rRNAs (though I don’t understand why it would be so small, it’s also unclear why the bacteriome sample show have so much more DNA contamination as a proportion of the total sample). It is also possible that the 4th bacteriome “rRNA” peak is present in the body as well but drowned out by higher background and higher intensity of the other peaks.

The RNA doesn’t look terrible, but there definitely seems to be significant degradation. I believe the rule of thumb is that intact RNA should have LSU rRNA peaks that are 2x the size of SSU rRNA peaks. Although I need to sort out which peak is which as I have multiple apparent rRNA peaks, it looks like the LSU peaks are of similar height or even lower than the SSU peaks.

I spoke to Allison Hansen about her results from her pea aphid RNA extractions. She normally found 4 very distinct peaks in bacteriocyte samples presumably representing the rRNAs for both aphid and Buchnera.

In looking at her runs, it looks like the 23S rRNA is **very** large, close to 4kb. So one alternative is that my RNA is **extremely** degraded, especially from the body samples and that the little peak near 4000 bp is all that’s left of the LSU rRNA ☹

Things to try:

--Do a trizol extraction directly on whole live psyllids. I would expect (or at least hope for) minimal degradation here, so the RNA size profile would be informative. I could also do this with one of the RNeasy kits floating around the lab to compare the two different methods.

--Dissect in RNAprotect? Allison recommends against this because she finds that she gets rapid cell lysis. But might worth at least seeing what happens if I try with pyllids.

--Keep RNAprotect on ice during dissections (in spite of the precipitant)

--Do not freeze dissected samples. Proceed immediately to RNA extraction.

--Make an RNase free batch of buffer A, and be generally more picky about RNase when doing the dissections.

8/29/11

Making a 100 ml stock of Buffer A (see 8/9/11). This should be closer to “RNase-Free” than the original stock I prepared. However, I am not going all out (e.g., I am using the original KCl stock which was prepared with regular milli-Q dH2O, and I am not chloroforming or baking glassware).

250 mM (8.56 g) sucrose

25 mM KCl (2.5 ml of 1 M stock 8/9/11)

35 mM Tris-HCl pH 7.4 (3.5 ml of 1 M stock from American Bioanalytical)

100 mM EDTA (20 ml 0.5 M stock from American Bioanalytical)

RNase-free dH2O to a total volume of 100 ml

Autoclaved on 20 min liquid cycle (note that there will probably be some breakdown of the sucrose b/c of this; not sure if that will have any effect).

I am going to try out some different RNA preps to try to optimize my results from last week.

First I want do extraction on whole, live nymphs in the hopes that these will be less degraded and could serve as a good basis of comparison. I will do this with both a Qiagen kit and a Trizol based extraction.

For the Qiagen extraction, I am using an RNeasy Mini Plus Kit. It looks on the old side (manual is dated 2005), but I don’t think it was ever used. I made a stock of buffer RLT plus by adding 50 ul of Beta-Mercaptoethanol to 5 ml of the Qiagen reagent. This is stored in a 5 ml falcon tube and should be good for 1 month. I also added the EtOH to the buffer RPE concentrate.

I collected 5 nymphs from the hackberry nipple galls from Nancy (see 8/23/11). These were pooled into one eppendorf and kept on ice. I added 350 ul of buffer RLT plus and squished the nymphs against the side wall with a pestle. I then homogenized with Qiashredder column (stolen from plant DNA extraction kit) and followed the standard protocol. I did the optional 1 min max speed spin at the end to dry the column. I eluted in two rounds of RNase-Free dH2O: 30 ul, then 50 ul .

Stored samples at -20 C.

Next, I did a Trizol extraction on a similar collection of 5 nymphs. Followed basic protocol from 8/23/11 except I doubled the Trizol, chloroform and alcohol volumes. Eluted in 20 ul RNase-free dH2O (from Qiagen kit). Note that I ended up leaving the sample precipitating in isopropanol longer than anticipated (about 17 minutes rather than 10 min).

Stored sample at -20 C

8/30/11

Continuing RNA extractions. I did two sets of extractions of dissected psyllid nymph bacteriomes and remaining whole body samples.

First, I tried dissecting samples in 100 ul of RNAprotect Bacterial Reagent. I then used a pipet to transfer the bacteriomes in the smallest volumes possible to another tube (already containing an additional 200 ul of RNAprotect). I then pipetted up the entire remaining quantity of RNAprotect and dissected body tissue and transferred it to a separate tube. I kept the separated samples on ice while I processed the nymphs (which causes a precipitate to form).

Second, I tried dissecting in 100 ul Buffer A (cleaner version; see yesterday’s notes). Again I transferred dissected bacteriomes in the minimal possible volume to a tube with 200 ul RNAprotect. But I did not transfer the entire remaining volume – only about 30-50 ul or so, trying to get the major pieces of the body and highest concentrations of cells. The body samples were transferred into one of two tubes containing 1 ml RNAprotect. The buffer A was kept on ice and added to each nymph individually and immediately before dissection. But I did not keep the 9-well glass dissecting dishes on ice as I did last time. I kept separated samples at room temp while I processed other nymphs. I noticed that contents of the nymph would sometimes “explode” out into the buffer upon puncturing the nymph. This seemed more dramatic than I remember, making me wonder if there is anything different about this new batch of buffer.

For both approaches, I dissected from a total of 12 nymphs from galls collected by Nancy (see 8/23/11 notes). I collected the nymphs for each batch before hand. I put them all in a small plastic petri dish. I kept moving this from ice to room temp. I figured keeping them on ice would keep them alive longer, but condensation would start to build up and I worries about them drowning. It would be nice to check how long they survive at room temp on a simple plastic surface. It might also be worth keeping them with some of the gall tissue. I imagine that would let them survive for plenty of time and it would not introduce cold stress effects. I pooled all 12 bacteriomes into one tube and split the body samples into two tubes of 6.

For RNA extractions, I followed the same basic Trizol procedure that is described on 8/23/11 except that I scaled up to a 1 ml Trizol volume (with corresponding increases in chloroform and alcohol volumes). I eluted in 20 ul RNase-free dH2O from the Qiagen kit. I proceeded immediately to the Trizol extraction after each set of 12 nymphs described above (i.e., I did two different rounds of extraction). There was no freezing of the samples in RNAprotect in between. I centrifuged each samples for 5 min at max speed and aspirated as much of the RNAprotect off as possible. There was a little bit left for the body samples.

Ran 1% agarose gel at 90 V for 30 minutes.

Loading order:

1. 1 kb plus ladder (1/10x) – 5 ul
2. Whole insect Qiagen RNA elution 1 (8/29/11) – 8 ul
3. Whole insect Qiagen RNA elution 2 (8/29/11) – 8 ul
4. Whole insect Trizol RNA (8/29/11) – 8 ul
5. Bacteriome RNAprotect RNA (8/30/11) – 8 ul
6. Body 1 RNAprotect RNA (8/30/11) – 8 ul
7. Body 2 RNAprotect RNA (8/30/11) – 8 ul
8. Bacteriome Buffer A RNA (8/30/11) – 8 ul
9. Body 1 Buffer A RNA (8/30/11) – 8 ul
10. Body 2 Buffer RNA (8/30/11) – 8 ul
11. Body 2 RNA (8/23/11) – 8 ul
12. 1 kb plus ladder (1/10x) – 5 ul

See images: 20110830\_RNA.TIF and 20110830\_RNA\_lowexp.TIF

Results: There is a fairly dramatic difference between the Qiagen prep and all the Trizol preps in both quantity and degradation. The Qiagen yield appears to be much lower, but also a lot less degraded. Because one of these Trizol preps was performed on whole, live nymphs, it does not appear that the dissection process if the major source of degradation. Instead, I have to figure out why I am getting so much RNA breakdown in the trizol process. It would be nice to get this optimized so that I could take advantage of the higher yields and not have to use Qiagen. It also appears that the Trizol preps suffer from a good deal of DNA contamination. I believe Allison has a commercial product designed to form a more discrete barrier between the aqueous and organic phases in the trizol prep. That might help with this issue because I think the genomic DNA is supposed to accumulate at the interface.

8/31/11

Ran RNA 6000 nano chip on Agilent Bioanalyzer 2100.

Sample order:

1. Whole insect Qiagen RNA elution 1 (8/29/11)
2. Whole insect Trizol RNA (8/29/11)
3. Bacteriome RNAprotect RNA (8/30/11)
4. Body 1 RNAprotect RNA (8/30/11)
5. Body 2 RNAprotect RNA (8/30/11)
6. Bacteriome Buffer A RNA (8/30/11)
7. Body 1 Buffer A RNA (8/30/11)
8. Body 2 Buffer RNA (8/30/11)
9. Whole insect Qiagen RNA elution 1 (8/29/11) - duplicate
10. Whole insect Trizol RNA (8/29/11) - duplicate
11. Bacteriome RNAprotect RNA (8/30/11) - duplicate
12. Bacteriome Buffer A RNA (8/30/11) - duplicate

Output: see “AgilentRuns/2011-08-31”

Results: Still confusing. I am becoming more confident that the peak around 4 kb (absent or nearly so in many samples) is actually the full-length psyllid 28S rRNA. I think the larger of the two fairly clear bands from yesterday’s gel for the Qiagen sample corresponds to this putative 28S rRNA. The relative intensity of this band looks even worse than it did on the gel. The smaller of the two Qiagen bands appears to be a doublet on the bioanalyzer. What’s weird is that all the Trizol samples appear to have a triplet instead of a doublet here. The bacteriome samples also have the same additional peak at ~1800 nt as in the first Agilent run.

If I am right about the 28S rRNA peak, it suggests even the Qiagen extraction is really low quality with lots of degradation of these larger seqs.

I think I should try some extractions from pea aphids along side the psyllids. Then I can compare against Allison’s results.

Other things to consider about the poor quality:

--Grind under N2 instead of in buffer?

--Do more things on ice? Cut out the 65 C step?

--Cut pelleting centrifuge speed to 12000 g (I have doing at max speed up until now)

--Add beta-mercaptoethanol to Trizol (may be totally unnecessary; I know it is added in some RNA extraction/lysis buffers).

Nanodrop:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Concentration (ng/ul) | |  |  |
|  | nanodrop | agilent (see above) | 260/280 | 260/230 |
| Whole insect Qiagen RNA elution 1 (8/29/11) | 22.9 | 20.5 | 2.19 | 0.41 |
| Whole insect Trizol RNA (8/29/11) | 587.8 | 451.6 | 1.87 | 1.94 |
| Bacteriome RNAprotect RNA (8/30/11) | 42.7 | 17.55 | 1.71 | 1.33 |
| Body 1 RNAprotect RNA (8/30/11) | 369.2 | 344.8 | 1.85 | 2.07 |
| Body 2 RNAprotect RNA (8/30/11) | 461.5 | 380.2 | 1.88 | 2.04 |
| Bacteriome Buffer A RNA (8/30/11) | 50.45 | 16.4 | 1.69 | 0.26 |
| Body 1 Buffer A RNA (8/30/11) | 406.9 | 348.8 | 1.88 | 1.83 |
| Body 2 Buffer RNA (8/30/11) | 331.6 | 280.9 | 1.81 | 2.16 |

9/1/11

Trying a Qiagen RNA extraction in search of more intact RNA….

Two samples:

1. 7 (smallish) psyllid nymphs dissected from hackberry nipple galls as described in the previous two days
2. 3 large pea aphids (leftover in the fridge from 8/22/11 Tuc7 line).

Both psyllids and aphids were kept at 4 C previous to this. I made sure they were all alive before pooling them into an eppendorf tube and keeping them on ice.

Ground samples under liquid nitrogen (no special RNase-free tubes or pestles at this stage). This seemed to go smoother with the aphids than the psyllids. I did the psyllids first and couldn’t get the pestle to the bottom of the tube. So I switched to a “pestle tube” for the aphids.

After grinding and adding 350 ul buffer RLT (with B-ME, stock from 8/29/11), I homogenized with a QiaShredder column (taken from a plant DNeasy kit). 3 minute spin instead of two minute spin. I then put the sup directly on the gDNA eliminator rather than spinning again. I did not do the optional drying spin (trying to reduce handling time in both these cases). Eluted only once in 50 ul RNase-free dH2O. Stored at -20 C.

Ran 1% agarose gel at 90 V for 40 minutes.

Loading order:

1. 1 kb plus ladder (1/10x) – 5 ul
2. aphid RNA (today) – 5 ul
3. psyllid RNA (today) – 5 ul
4. psyllid Qiagen RNA elution 2 (8/29/11) – 8 ul
5. psyllid Trizol RNA (8/29/11) – 2 ul
6. 1 kb plus ladder (1/10x) – 5 ul

Image: see 20110901\_RNA.TIF

Results: A lot more RNA in the aphid sample and noticeably more low MW stuff. Not sure how much of that is more breakdown vs. higher overall concentration. Liquid N2 does not appear to have dramatically improved integrity. The relative intensity of the two rRNA bands is similar between today’s psyllid Qiagen prep and the one from a few days ago (perhaps a little bit better today, but that might reflect freeze/thaw degradation in the older sample. Despite all the low MW stuff going on, the aphid sample does seem to have at least as much 28S as 18S. The aphid results do suggest that the higher MW band in the psyllid samples in the 28S.

Two more preps, this time with a modified version of the Trizol protocol from the last few days…

Two samples:

1. 5 psyllid nymphs (exactly as above)
2. 1 large aphid (exactly as above)

Ground samples under liquid nitrogen.

1. Add 1 ml of Trizol.
2. Incubate at room temperature for 5 minutes.
3. Add 200 ul chloroform, mix by shaking for 15 sec.
4. Incubate at room temperature for 3 minutes.
5. Centrifuge at 12,000g for 15 minutes at 4°C.
6. Transfer 250 ul of upper aqueous phase to a new centrifuge tube containing 250 ul isopropanol.
7. Mix thoroughly by inversion.
8. Incubate at room temperature for 10 minutes.
9. Centrifuge at 12,000xg for 10 minutes at 4°C.
10. Pipet off supernatant, add 1 ml 80% EtOH.
11. Wash pellet thoroughly by inversion.
12. Centrifuge at 7,500g for 5 minutes at 4°C.
13. Pipet off supernatant.
14. Centrifuge briefly, pipette off remaining supernatant.
15. Air dry until pellet went clear (probably should have gotten to it quicker).
16. Add 20 ul RNase-free water. Mixed by pipetting and flicking. Stored at -20 C.

Some of the key changes here include: Liquid N2 grinding, shorter incubation times both before and after chloroform addition, take much less than max amount to avoid interphase contamination, pelleting for less time and at lower speed, and no 65 C step at the end.

Ran another 1% agarose gel. 90 V for 50 minutes.

Loading order:

1. 1 kb plus ladder (1/10x) – 5 ul
2. aphid Trizol RNA (today) – 7 ul
3. aphid Trizol RNA (today) – 1 ul
4. aphid Qiagen RNA (today) – 8 ul
5. psyllid Trizol RNA (today) – 7 ul
6. psyllid Trizol RNA (today) – 1 ul
7. psyllid Qiagen RNA (today) – 8 ul
8. psyllid RNAprotect Body 2 RNA (8/30/11) – 2 ul
9. 1 kb plus ladder (1/10x) – 5 ul

Image: see 20110901\_RNA2.TIF

Results: No noticeable improvement in psyllid RNA quality based on changes in protocol. New aphid RNA prep has similar degradation issues as the Qiagen prep from earlier today but the 28S/18S ratio looks decent. The greater 28S rRNA degradation in the psyllid relative to the aphid is striking.

Things to try:

--Dissect whole psyllids in RNAprotect (just as sham to simulate the dissection process). Collect bodies. Grind in liquid nitrogen. Extract with Qiagen kit. Perhaps if this yields the same Qiagen level quality that I am getting for the whole psyllids, it could work (though it is still not great quality and it would probably require A LOT of bacteriomes to get sufficient RNA quantity).

--Try a Trizol/Qiagen hybrid. This seems popular. It’s not clear that it would help here, but at least it might help pinpoint where my degradation is occurring.

9/2/11

New RNA extraction using RNAprotect and Qiagen kit. 5 psyllid nymphs collected as done in the last few days. Each was “dissected” in 100 ul of RNA protect in 9-well glass dissecting dish, i.e., ripped apart with two insect pins. I then transferred the all tissue and buffer into a single pooled tube, kept at room temp. Each dissection was done within a minute or so. Then I let the tube sit for an additional 10 minutes to simulate the delays associated with dissection.

Centrifuged pooled sample at max speed in the old eppendorf 30-sample centrifuge (note that this is a wider radius and correspondingly greater rcf thatn the 18-sample unit I have used in the past. I did not that ths unit had gotten pretty hot by the end of the 5 minutes). The pellet did seem a little firmer with this higher g spin. Maybe I should use the refrigerated 30-sample centrifuge in the future to keep the temp near room temp. Removed sup (didn’t really have to leave any behind as I have in the past.

Qiagen extraction following yesterday’s protocol. Eluted in 50 ul RNase-free dH2O. Stored at -20C.

Trying a hybrid Trizol/Qiagen extraction.

Collected 5 psyllid nymphs as done in the last few days. Liquid N2 grinding. Most recent Trizol protocol up through phase separation step. The transferred 350 ul of aqueous phase to a tube containing 350 ul of 70% EtOH. Applied to Qiagen RNeasy column and picked up the Qiagen protocol from their (see above). Eluted in 50 ul RNase-free dH2O. Stored at -20 C.

Ran RNA 6000 Nano chip on Agilent Bioanalyzer 2100.

Sample loading order:

1. Aphid Trizol (9/1/11)
2. Aphid Qiagen (9/1/11)
3. Psyllid Trizol (9/1/11)
4. Psyllid Qiagen (9/1/11)
5. Psyllid RNAprotect Qiagen (9/2/11)
6. Psyllid Trizol/Qiagen hybrid (9/2/11)
7. Bacteriome RNA (8/23/11)
8. Aphid Qiagen (9/1/11) – duplicate
9. Psyllid Trizol (9/1/11) – duplicate
10. Psyllid Qiagen (9/1/11) – duplicate
11. Psyllid RNAprotect Qiagen (9/2/11) – duplicate
12. Psyllid Trizol/Qiagen hybrid (9/2/11) – duplicate

Output: AgilentRuns/2011-09-02

Results: The aphid samples look a bit better on the agilent than they did on the gel. The intermediate MW material does not look as pronounced. The rRNA ratios for these samples are not perfect, but they compare favorably to what Allison showed me from her work where the two peaks were of about equal height. The putative Buchnera rRNA peaks are clearly detectable in the aphid sample.

The new Qiagen prep done after sham dissection in RNAprotect looks to more or less the same as the Qiagen prep done of whole, live individuals. That’s encouraging as it suggests I’m not getting much degradation as part of the dissection itself.

The hybrid Trizol/Qiagen prep produced RNA that looks like a Trizol prep, not a Qiagen prep, suggesting that the greater degradation observed in Trizol vs. Qiagen preps is occurring in the initial Trizol/chloroform/phase separation steps and not in later precipitation/washes/dissolving steps.

9/7/11

It appears that Nancy left some more Pachypsylla galls for me at 4 C on this date. But I didn’t discover them until 10/11/11. They still appear to be viable although getting a tad moldy.

10/10/11

Howard brought in some hackberry galls that he and Nancy collected on a recent trip to Arizona. He said they were from the same site as the original Carsonella genome collection, but I will have to get more info. Many of these are petiole galls (P. venusta), but some of the leaves from the same trees have blister galls, and there is a whole separate collection with some fuzzy galls (Pachypylla pallida?). These were put in plastic bags and kept at 4 C.

🡪 Note it appears that at least some of the “fuzzy” galls are completely grown over and dead. No sign of life inside.

I opened up a couple of the petiole galls. The first one did not appear to contain any viable nymphs (at least one dead one was present), but instead had 3 fat larvae, which I would guess are parasitoid wasps in the family Torymidae. These look similar to ones I have occasionally seen in nipple galls. Note that Nancy had previously said that parisitoids are usually unable to puncture the large, thick walls of the petiole galls.

10/11/11

Did Trizol RNA extraction on 1 large pea aphid (left over from K. Hammond in fridge—Tuc7 line) and a pool of 3 P. venusta nymphs (from petiole galls provided by howard yesterday). 9/1/11 protocol. Except I added a 3 min incubation at 56 C at the end. Nymphs still appear to be in an early instar. Minimal wing bud development.

10/12/11

Qiagen RNA extractions. I used 1 large Tuc7 pea aphid and 4 P. venusta nymphs from petiole galls (see 10/11/11). Followed 9/1/11 protocol for Qiagen extractions.

Run 1% agarose gel at 100 V for 30 minutes

Sample loading order:

1. 1 kb plus ladder (1/10x) – 5 ul
2. P. venusta Trizol RNA (yesterday) – 3 ul
3. P. venusta Qiagen RNA (today) – 8 ul
4. Old psyllid Trizol RNA (9/1/11) – 3 ul
5. Old psyllid Qiagen RNA (9/1/11) – 8 ul
6. aphid Trizol RNA (yesterday) – 3 ul
7. aphid Qiagen RNA (today) – 8 ul
8. old aphid Trizol RNA (9/1/11) – 3 ul
9. 1 kb plus ladder (1/10x) – 5 ul

RESULTS: Looks bad. Same patterns of trizol and Qiagen degradation in P. venusta samples as I have seen previously with other psyllid samples.

10/15/2011

I gave Adam Hejmowski a couple galls with P. venusta nymphs (see 10/10/11) to try his RNA extraction protocol in parallel with his aphid samples.

See his e-mail pasted below:

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

Hi Dan,

Attached are the results of the 3 trials I ran for the psyllids.

First off, you gave me an unlucky gall...it only contained about 4 psyllids.

Second, I ran 3 trials:

   a) 4 psyllids crushed in RNA Protect (500uL)

   b) 4 psyllids crushed directly in Trizol (500uL)

   c) The head of a psyllid only

Following my procedure, I obtained 50 uL of large RNA (>200 bp) and small RNA (<200 bp).  The large RNA came out well I believe, but I do not know if the right peaks were obtained.  If you are looking into smaller RNA fragments, more psyllids would have to be "sacrificed."

Let me know if these results are similar to the ones you obtained previously, or not at all indicative of what is expected.

Thanks,

Adam Hejmowski

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

In looking at the traces he provided, the peaks look very similar to what I see except that RNAprotect sample looks even more degraded. What now….?

10/25/11

I found a new batch of hackberry petiole galls in the refrigerator. I believe Nancy dropped these off recently. They would have been collected on the same trip as the 10/10/11 samples but I don’t know if they are from the exact same site. I will continue to store these in a Ziploc at 4 C.

Doing Qiagen QiaAmp DNA Micro Kit extraction on psyllid nymphs (for future PCR or what have you. I collected 8 Pachypsylla venusta nymphs from two petiole galls (see 10/10/11) and ~10 Pachypsylla pallida nymphs from multiple fuzzy twig galls (see 10/10/11; I was able to find some viable individuals in that sample). Nymphs from both species appeared to be relatively early stage, but I can’t say which instar. The pallida nypmhs were extremely small, yellow and sickly looking.

Following tissue protocol for the QiaAmp kit

I ground the samples in buffer ATL with a plastic pestle. After grinding the venusta sample was very thick and sticky, so I added a second 180 ul of buffer ATL and split the sample into two tubes.

Started 56 C incubation (600 rpm heated shaker) at 3:00 pm. Let it go overnight.

10/26/11

Continued with Qiagen DNA extractions. Standard tissue protocol. I manually pipetted off residual wash buffer trapped around the rubber seal after the long drying spin. I eluted each prep in 100 ul buffer AE and then pooled the two P. venusta preps. Stored at -20 C. Will run on the next gel I set up.

10/27/11

Isolating bacteriomes and extracting RNA from Pachypsylla venusta nymphs.

I left 8 hackberry petiole galls out at room temp overnight (starting yesterday) in hopes that the psyllids would “wake up”, resulting in more normal metabolic activity and RNA expression. See 10/10/11 for collection info.

I isolated bacteriomes from a total of 24 nymphs (I also dissected 5 additional nymphs that didn’t yield a clean bacteriome isolation—these were discarded). I would guess that most of the nymphs were 2nd or 3rd instar based on the drawings of other Pachypsylla species in Moser 1965. But there was one nymph that was clearly more developed and was probably 5th instar (assuming it was P. venusta!!). This individual was part of the body pool #1 (see below).

Each nymph was pulled from the gall and dissected in 100 ul of Qiagen RNAprotect bacteria reagent. I ripped apart the nymph with two pins and then teased the noticablly yellow bacteriome away from other body contents. I then pipetted this into a separate tube with 400 ul of RNAprotect (kept on ice, resulting in a lot of precipitate). The remaining RNAprotect and body contents were pipetted up and put in separate “body” tubes. First 9 nymphes were in “body 1”, second 9 nymphs were in “body 2”, and the last 6 nymphs were in “body 3”. It took about 2.5 hours to do the dissections.

I then let the samples re-equilibrate to room temp, so that the RNAprotect precipitate to re-dissolve. I then spun the tubes at room temp, max speed for 5 min and removed supernatant (note this looked like it included a lot of white cellular material in the body samples, so some cell types may have been lost. I then did a Trizol RNA extraction using 5 Primer Phase Lock Heavy gel columns to improve separation between aqueous and organic phases.

1. 1 ml of Trizol added to each sample. Bacteriome sample was pipetted up and down and shaken vigorously to break up cell pellet. For body samples 500 ul of trizol was added followed by pestle grinding and then the other 500 ul. Bacteriome sample was processed first and spent ~15 min in Trizol. Last body sample only ~5 min.
2. Transfer to phase-lock tube (pre-spun for 3min at 1500g)
3. 200 ul chloroform. Vigorous shaking. 3 min at room temp
4. 15 min at 12000g, 4C
5. Transfer sup to tube with 500 ul isopropanol. Mix by pipetting/inversion
6. 10 min at 12000g, 4C
7. Wash pellet with 1 ml 80% EtOH.
8. 5 min at 7500g, 4C. Remove sup
9. Air dry pellet.
10. Dissolve in 40 ul RNase-free dH2O just before pellet went completely transparent.
11. ~3 min at 55 C.
12. store at -80 C following nanodrop and bioanalyzer

Nanodrop

|  |  |  |  |
| --- | --- | --- | --- |
|  | Conc (ng/ul) | 260/280 | 260/230 |
| Bacteriome | 82.5 | 1.84 | 2.26 |
| Body1 | 762.5 | 1.98 | 2.09 |
| Body2 | 770 | 1.97 | 2.22 |
| Body3 | 307.6 | 1.87 | 1.46 |

Agilent Bioanalyzer 2100 RNA Nano 6000 chip

Loading order (not 7-11 are duplicates of 2-6).

1. P. venusta bacteriomes (today)
2. P. venusta body 1 (today)
3. P. venusta body 2 (today)
4. P. venusta body 3 (today)
5. Old aphid trizol (9/1/11?)
6. Old aphid Qiagen (9/1/11?)
7. P. venusta body 1 (today)
8. P. venusta body 2 (today)
9. P. venusta body 3 (today)
10. Old aphid trizol (9/1/11?)
11. Old aphid Qiagen (9/1/11?)
12. Blank

Output: see /Users/drt\_lab/Documents/Yale/MoranLab/AgilentRuns/2011-10-27

Set aside one 30 ul sample of bacteriome RNA (=~1.4 ug according to Agilent) for Illumina library construction. 🡪 library code DBS003

10/31/11

Setting up whole genome amplification (MDA) with Qiagen Repli G mini kit (purchased in August but not opened until now).

Followed standard protocol to amplify from 5 ul of template.

2 reactions: Psyllid (P. celtidismamma) bacteriome DNA elution 1 and 2 from 8/11/11.

Started 30 C incubation in H2O bath at 6:00 pm.

11/1/11

Killed MDA reactions. 3 min @ 65 C. Stored @ -20 C.

11/2/11

EtOH precipitation of yesterday’s MDA reactions. 5 ul 3M NaOAc. 125 ul EtOH. 1 hour at -20C. 10 min at max speed and 4 C. Wash with 500 ul 70% EtOH (from freezer). Air dry pellets. Dissolve each in 35 ul Qiagen Buffer AE.

Qubit (BR dsDNA) samples for eventual Illumina sequencing. Four DNA from Baumann collection (see 8/17/11). Two MDA samples from yesterday.

Heteropsylla texana: 90 ng/ul

Heteropsylla cubana: 299 ng/ul

Ctenarytaina eucalypti: 209 ng/ul

Ctenarytaina spatulata: 583 ng/ul

Pachypsylla celtidismamma MDA 1: 74 ng/ul

Pachypsylla celtidismamma MDA 2: 93 ng/ul

Run 1% agarose gel at ~110 V for 45 minutes.

Loading order:

1. 1 kb plus ladder 1/10x, 5 ul
2. Heteropsylla texana, 1 ul
3. Heteropsylla cubana, 1 ul
4. Ctenarytaina eucalypti, 1 ul
5. Ctenarytaina spatulata, 1 ul
6. Pachypsylla celtidismamma MDA 1, 1 ul
7. Pachypsylla celtidismamma MDA 2: 1 ul
8. MDA Neg control from 8/15/11, 1ul
9. Pachypsylla venusta (10/25/11), 1 ul
10. Pachypsylla pallida (10/25/11), 1 ul

Image taken (see PhotosAndGelPics directory on my computer, 20111102\_DNA.TIF)

Results: Lots of degradation in the old samples (especially C. spatulata), but no major surprises. Decent looking yields for the P. venusta and P. pallida Qiagen preps from 10/25 and 10/26 days ago.

Preparing aliquots to submit to YCGA for Illumina library construction and sequencing.

Heteropsylla cubana: 25 ul (not sure I’ll actually submit this one, but I prepared it just in case)

Heteropsylla texana: 50 ul 🡪 DBS004

Ctenarytaina eucalypti: 40 ul 🡪 DBS005

Ctenarytaina spatulata: 25 ul 🡪 DBS006

Pachypsylla celtidismamma MDA 1: 30 ul 🡪 DBS007

The above volumes were chosen in an attempt to roughly get into the range of 1-5 ug of HMW DNA, taking into account the concentration estimates from Qubit and the degradation observed on the gel.

11/3/11

Submitted samples to YCGA for library construction:

DBS003: One RNA sample for polyA selection and cDNA library construction, no normalization (see 10/27/11).

DBS004-DBS007: Four gDNA samples for PE library construction (see yesterday)

Looking for potential Bemisia tabaci DNA to use for Illumina sequencing to assemble the *Portiera* genome.

Patrick Degnan did some extractions from B. tabaci while at Arizona getting samples from Molly Hunter’s lab (simple Qiagen extractions). He also did more involved filtration based preps using multiple grams of whiteflies in an attempt to isolate Hamiltonella. These preps were done with colonies kept in the Moran lab at the time. 454 Sequencing was performed on one of these colonies. See the following summary e-mail from Patrick:

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

Dan,

Based on a first pass screen of the initial 454 generated assembly there were ~137 contigs putatively assigned to Portiera. This comprised 4385 reads, for a total of ~202 kb at 8X coverage and with a genome GC% of 27%.

From my notes an entire 3.35ug sample was prepared and sent to Joe Jones at South Carolina for sequencing.  How this sample compares in purity to the other gDNAs I do not know.

The sff file on the lacie harddisk is:

FVFUGUP01.sff

The summary statistics table I generated based on initial screening is pasted below.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| H. defensa str. BTAB 454 1/2 run | | |  |  |  |
| 202,642 | reads |  |  |  |  |
| 70,167,183 | bases |  |  |  |  |
| 346 | avg. read size | |  |  |  |
|  |  |  |  |  |  |
| FVFUGUP01 |  |  |  |  |  |
|  | length | reads | % total reads | coverage | G+C% |
| H. defensa | 501,257 | 6,889 | 3% | 5.2 | 39.9 |
| putative H. defensa | 5,313 | 96 | 0.05% | 6.2 | 48.1 |
| mtDNA | 16,202 | 4,271 | 2% | 70.8 | 28.5 |
| Portiera | 201,654 | 4,385 | 2% | 7.8 | 27.3 |
| Rickettsia | 1,006,302 | 27,778 | 14% | 11.7 | 32.2 |
|  |  |  |  |  |  |
| unknown | 7,365,548 | 123,648 | 61% | 7.6 | 39.3 |
| unassembled | – | 35,575 | 18% | – | – |

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

Ran Qubit dsDNA BR (1 ul each) on 4 qiagen preps and two “filtered” preps that Nancy found in the freezer, labeled as follows:

B. tabaci AZ B-1 gDNA 06170[last digit illegible] 🡪 too low (<2 ng/ul)

Btab AZ-B dneasy-III 1 010606 🡪 too low (<2 ng/ul)

Btab AZ-B dneasy-IV 1 010606 🡪 too low (<2 ng/ul)

Btab DN-V 011407 🡪 16.8 ng/ul

Btab gFiltered 021108, 1ul 🡪 14.5 ng/ul

Btab filter gDNA 3/15, 1ul 🡪 69.6 ng/ul

Ran 1% agarose gel. ~110 for 45 min.

1. 1 kb plus ladder 1/10x, 5 ul
2. B. tabaci AZ B-1 gDNA 06170[last digit illegible], 1ul
3. Btab AZ-B dneasy-III 1 010606, 1 ul
4. Btab AZ-B dneasy-IV 1 010606, 1ul
5. Btab DN-V 011407, 1ul
6. Btab gFiltered 021108, 1ul
7. Btab filter gDNA 3/15, 1ul
8. MDA Neg control from 8/15/11, 1ul (for comparison) , 1ul

Results: Confirmation of qubit values. First 3 are not visible. Other samples all have substantial HMW material but also some degradation. These look like they would be marginal at best for Illumina sequencing.

11/8/11

Repeating P. venusta RNA extractions from 10/27/11 so that I can replicate RNA-seq libraries.

Late yesterday afternoon, I took 12 hackberry petiole galls out of the refrigerator. These are from the second Arizona collection (see 10/25/11), so they are not from the same collection as the 10/27 extraction.

I followed essentially the exact same protocol as on 10/27. I pooled bacteriomes from 24 nymphs (from 7 galls—also dissected 6 additional nymphs but they were excluded because of poor bacteriome isolation) and divided the remaining body tissue into 3 pools of 8 psyllids. I did notice that some (not all) of the bacteriomes looked more orange and almost dried out. There was also an increase in the number of apparently late stage nymphs. I only saw one of these on 10/27, but I saw a total of 5 today (4 in body pool 1 and 1 in body pool 2).

Final RNA pellets were dissolved in 40 ul RNase-Free dH2O (from Qiagen kit) and stored at -80 C.

Upon pelleting of the precipitated RNA, I could not see a visible pellet in the bacteriome sample (whereas I have always been able to in the past. Nanodrop supported a much lower yield for the bacteriome sample than I’ve seen in the past, especially because the poor 260/280 and 260/230 ratios suggest that the estimate is inflated.

Nanodrop

|  |  |  |  |
| --- | --- | --- | --- |
|  | Conc (ng/ul) | 260/280 | 260/230 |
| Bacteriome | 10.6 | 1.67 | 1.50 |
| Body1 | 561.5 | 1.87 | 2.06 |
| Body2 | 434.6 | 1.84 | 2.02 |
| Body3 | 362.4 | 1.84 | 1.97 |

I don’t have any good explanation for the marked reduction in bacteriome RNA yield compared to early rounds…

1. I didn’t vigorously break up the bacteriome pellet after adding trizol. Seems unlikely that it failed to lyse, but maybe.
2. Lower quality tissue? Perhaps the psyllids are getting old and weak at 4 C. Maybe the orange color in some of them is a sign of stress
3. Longer sitting time. With breaks, it probably took a full three hours between initiating the dissections and beginning the RNA extractions. Perhaps excess waiting time leads to sample degradation and loss?
4. Sunspots?

Try repeating with 40 nymphs, making sure to break up final pellet.

11/10/11

Repeating Pachypsylla venusta bacteriome isolations and RNA extractions. Had 12 large galls at room temp. Some of these were left over from the 10/8 preps others were taken out after the 10/8/11 prep, so they have spent either 2 or 3 nights at room temp. They looked somewhat dried out, so keep in mind whether there might have been an effect of being out for so long (I had intended to do this yesterday).

Basically repeated everything from 10/8/11 except I isolated a total of 40 bacteriomes. One bacteriome pool. 5 pools of 8 bodies each.

Breakdown of early vs. late instar nymph numbers in each pool:

Pool 1: 2 early, 6 late

Pool 2: 0 early, 8 late

Pool 3: 5 early, 3 late

Pool 4: 6 early, 2 late

Pool 5: 2 early, 6 late

Did isolations in the afternoon and stored them at -20 C overnight.

11/11/11

Performd Trizol RNA extractions on 6 frozen samples from yesterday (1 P. venusta bacteriome pool and 5 body pools).

10/27/11 extraction protocol. Dissolved pellets in 40 ul dH2O. Stored in -80 C. For bacteriome sample, I stored a separate 30 ul aliquot which could be used as an Illumina library submission.

Nanodrop for the 6 RNA samples:

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Concentration (ng/ul)** | **260/280** | **260/230** |
| **Bacteriome** | 80 | 1.81 | 1.98 |
| **Body 1** | 257 | 1.80 | 2.09 |
| **Body 2** | 558 | 1.80 | 2.43 |
| **Body 3** | 190 | 1.82 | 2.08 |
| **Body 4** | 428 | 1.84 | 2.08 |
| **Body 5** | 470 | 1.85 | 1.97 |

Bemisia tabaci DNA extractions:

Kevin Vogel shipped a sample of whiteflies (Bemisia tabaci) from Molly Hunter’s lab colony (University of Arizona). They were collected last week and stored in EtOH at 4 C. They were shipped at ambient temp, and then put back at 4 C upon arrival. After settling in EtOH, they occupy about 2 ml of volume in a 15 ml falcon tube. They are extremely small so this represents thousand of insects.

Qiagen Blood and Tissue DNeast Kit extractions. For future Illumina sequencing (in hopes of getting the primary symbiont Portiera).

I am trying 4 extractions each with different quantities of whiteflies.

#1: Pipete about 200 ul with a P1000 with cutoff tip from the settled mass of whiteflies. They don’t come up well at all so I was pulling a large amount of ethanol. Tried to keep the tip moving.

#2: About 400ul

#3: About 800 ul

#4: About 1600 ul

Even the smallest samples was still >100 whiteflies.

Followed Qiagen manual for animal tissue. Buffer ATL lysis at 56 C for 3 hours. I added some 600rpm shaking for the first half of the incubation.

Eluted in two rounds of buffer AE, 100 ul each.

Tubes labeled as follows:

Bemisia tabaci 1, 2, 3 , or 4 for first elution

Bemisia tabaci 1.2, 2.2, 3.2 , or 4.2 for second elution

Qubit dsDNA BR quanitifcation of Bemisia preps:

|  |  |
| --- | --- |
|  | **Concentration (ng/ul)** |
| **Extraction 1** | 31.8 |
| **Extraction 2** | 27.4 |
| **Extraction 3** | 30.4 |
| **Extraction 4** | 13 |
| **Extraction 1.2** | 6.9 |
| **Extraction 2.2** | 6.7 |
| **Extraction 3.2** | 7.8 |
| **Extraction 4.2** | 6.5 |

Ran 1% agarose gel for 45 min at ~100 V

Loading order

1. 1 kb plus ladder 1/10x, 5 ul
2. B. tabaci extraction 1, 5 ul
3. B. tabaci extraction 2, 5 ul
4. B. tabaci extraction 3, 5 ul
5. B. tabaci extraction 4, 5 ul
6. B. tabaci extraction 1.2, 5 ul
7. B. tabaci extraction 2.2, 5 ul
8. B. tabaci extraction 3.2, 5 ul
9. B. tabaci extraction 4.2, 5 ul
10. P. venusta Body 3 RNA (10/8/11), 2 ul

Results: DNA extractions look decent but there is a huge amount of RNA contamination. John Overton says not to worry about it and just submit the DNA sample for illumina sequencing. I ran the RNA sample to see if there was a ton of DNA contamination. Doesn’t look too bad. No obvious HMW DNA band.

On Monday (11/14/11), I will submit two more samples to YCGA for gDNA paired-end Illumina library prep.

DBS008 – Heteropsylla cubana 25 ul (see 11/2/11)

DBS009 – Bemisia tabaci extraction 1, 60 ul (see above).

11/15/11

Doing another round of RNA extraction from Pachpyslla venusta bacteriome and body samples. 40 nymphs taken from 6 large galls left out overnight at room temp after extended period at 4 C (see 10/25/11 notes). See 10/11/11 notes and references therein for protocol description.

1 bacteriome pool. 5 body pools of 8 bodies each. Breakdown of “early” vs “late” instar counts in each body pool:

Pool 1: 3 early, 5 late

Pool 2: 6 early, 2 late

Pool 3: 6 early, 2 late

Pool 4: 7 early, 1 late

Pool 5: 6 early, 2 late

Stored isolated tissue samples in RNA protect at -20 C overnight.

Took some pictures of galls, nymphs (both early and late stage), and bacteriome isolation (see PhotosAndGelPics

11/16/11

Performd Trizol RNA extractions on 6 frozen samples from yesterday (1 P. venusta bacteriome pool and 5 body pools).

10/27/11 extraction protocol. Dissolved pellets in 40 ul dH2O. Stored in -80 C. For bacteriome sample, I stored a separate 30 ul aliquot which could be used as an Illumina library submission.

Nanodrop for the 6 RNA samples:

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Concentration (ng/ul)** | **260/280** | **260/230** |
| **Bacteriome** | 97 | 1.83 | 2.54 |
| **Body 1** | 1069 | 1.92 | 2.10 |
| **Body 2** | 592 | 1.83 | 2.32 |
| **Body 3** | 921 | 1.90 | 2.18 |
| **Body 4** | 383 | 1.82 | 1.87 |
| **Body 5** | 1286 | 1.91 | 2.33 |

Ran RNA 6000 nano chip on Agilent Bioanalyzer 2100.

Include today’s 6 samples and 6 samples from 11/11/11

Sample order:

1. Pachypsylla venusta bacteriome RNA (11/11/11)
2. Pachypsylla venusta body 1 RNA (11/11/11)
3. Pachypsylla venusta body 2 RNA (11/11/11)
4. Pachypsylla venusta body 3 RNA (11/11/11)
5. Pachypsylla venusta body 4 RNA (11/11/11)
6. Pachypsylla venusta body 5 RNA (11/11/11)
7. Pachypsylla venusta bacteriome RNA (11/16/11)
8. Pachypsylla venusta body 1 RNA (11/16/11)
9. Pachypsylla venusta body 2 RNA (11/16/11)
10. Pachypsylla venusta body 3 RNA (11/16/11)
11. Pachypsylla venusta body 4 RNA (11/16/11)
12. Pachypsylla venusta body 5 RNA (11/16/11)

Output: see “AgilentRuns/2011-11-16”

11/17/11

Another round of Pachypsylla venusta RNA extractions. Exact same approach as above (11/15 to 11/16).

I ended up dissecting bacteriomes from 33 nymphs from a total of 10 galls that had been at room temp for either two or three nights (see 10/25/11 for collection info).

1 bacteriome pool and 4 body pools

Breakdown of “early” vs “late” instar counts in each body pool (note that the last pool has 9 nymphs):

Pool 1: 0 early, 8 late

Pool 2: 4 early, 4 late

Pool 3: 6 early, 2 late

Pool 4: 3 early, 6 late

Stored dissected samples overnight in RNA protect at -20 C.

11/18/11

Continuing RNA extractions, using Trizol method as in previous round. 40 ul elutions in RNase-free dH2O. Stored at -80 C.

Stored a 30 ul aliquot of the bacteriome sample in separate tube.

Nanodrop:

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Concentration (ng/ul)** | **260/280** | **260/230** |
| **Bacteriome** | 53 | 1.89 | 2.16 |
| **Body 1** | 777 | 1.83 | 2.34 |
| **Body 2** | 329 | 1.85 | 1.82 |
| **Body 3** | 596 | 1.84 | 1.85 |
| **Body 4** | 1245 | 1.90 | 2.18 |

Ran RNA 6000 nano chip on Agilent Bioanalyzer 2100.

Include today’s 5 RNA samples plus 3 for Eli

Sample order:

1. Bacteriome
2. Body1
3. Body2
4. Body3
5. Body4
6. Eli’s sample 1
7. Eli’s sample 2
8. Eli’s sample 3
9. Empty
10. Empty
11. Empty
12. Empty

Output: see “AgilentRuns/2011-11-18”

11/21/11

Nancy brought back a new batch of Pachpyslla venusta petiole galls from around Tucson, AZ. They are stored at 4 C.

12/26/11

Used Qubit dsDNA BR kit to quantify Pachypsylla venusta and Pachypsylla pallida DNA preps from 10/25/11:

P. venusta: 17.4 ng/ul

P. pallida: 6.6 ng/ul

\*\*\*\*\*\*\*\*

Qiagen Blood and Tissue DNeast Kit extractions from individual Pachypsylla venusta nymphs brought back by Nancy (see 11/21/11). Doing 3 extractions from one nymph each. The nymphs have large abdomens and appear to be relatively late stage, but I don’t know which instar they would be classified as.

Extractions #2 and #3 are from nymphs pulled from the same gall today. Extraction #1 is from a nymph from a different gall that I pulled out on 12/23/11. It has been in an eppendorf tube at 4 C since that point. All three nymphs were alive (still moving when at room temp).

Following animal tissue lysis protocol in manual that came with kit. Hand ground live nymphs in buffer ATL with plastic pestle. Buffer ATL/prot K incubation for 4 hours at 56 C. The first three hours were done without any movement. For the last hour, I turned on shaker block at 700 rpm. After incubation, added 4 ul of RNase A solution (Qiagen #158922) to each reaction. Incubated at room temp for ~10 min.

Eluted in two round of 100 ul Buffer AE (denoted by .1 and .2 below. On the tubes, the first round just has the sample number, while the second round has the sample number followed by .2).

Quantify with Qubit dsDNS BR kit:

|  |  |
| --- | --- |
| **Sample** | **Concentration (ng/ul)** |
| 1.1 | 11.2 |
| 1.2 | 5.9 |
| 2.1 | 9.9 |
| 2.2 | 5.0 |
| 3.1 | 10.8 |
| 3.2 | 4.7 |

Looks like I got ~1.5 ug total per prep (~1 in the first elution and ~0.5 in the second).

I will run a gel to assess the amount of degradation.

12/27/11

Run gel with yesterday’s DNA extractions…

1% agarose TBE gel. 100 V for 40 minutes.

Loading order (note the different loading volumes):

1. 1 kb plus ladder. 1/10x. 5ul
2. P. venusta extraction 1.1. 1ul
3. P. venusta extraction 2.1. 3ul
4. P. venusta extraction 3.1. 3ul
5. P. venusta extraction 1.2. 1ul
6. P. venusta extraction 2.2. 3ul
7. P. venusta extraction 3.2. 3ul
8. 1 kb plus ladder. 1/10x. 5ul

Results: DNA looks good. Some degradation but not a lot.

12/28/11

Doing more Qiagen extractions on Pachypsylla venusta nymphs. Same basic protocol as above. 8 nymphys. 1 nymph per extraction. The first seven (labeled #s 4-10) are from the same gall. The eighth (#11) is a bit smaller than the rest and was the only nymph in a separate gall. Both galls were from the 11/21/11 collection.

Buffer ATL/prot K incubation: 56 C, 700rpm on shaker block. 3.5 hours

4 ul of Qiagen RNase A (#158922) for 10 min at room temp.

Standard Qiagen protocol. Elute in two rounds (denoted by .1 and .2 below and on the tubes) of 100 ul Buffer AE each--PRE-WARMED TO 60 C. The pre-warming is a change from last time.

Used Qubit dsDNA BR kit to quantify:

|  |  |
| --- | --- |
| **Sample** | **Concentration (ng/ul)** |
| 4.1 | 16.1 |
| 4.2 | 7.2 |
| 5.1 | 6.3 |
| 5.2 | 3.1 |
| 6.1 | 4.3 |
| 6.2 | 2.5 |
| 7.1 | 8.4 |
| 7.2 | 3.3 |
| 8.1 | 7.9 |
| 8.2 | 4.2 |
| 9.1 | 16.9 |
| 9.2 | 8.2 |
| 10.1 | 6.5 |
| 10.2 | 3.9 |
| 11.1 | 11.1 |
| 11.2 | 6.8 |

12/29/11

Prepping more Pachypsylla venusta DNA extractions. Pulling nymphs out of galls. 12 nymphs total. From 11/21/11 collection.

Groups from the same gall:

#12-16

#17-20

#21

#22-23

Note that #22 might be wounded. Check DNA quantity/quality before pooling.

I’m feeling sick. Left nymphs at 4 C in eppendorf tubes and went home.

12/31/11

Picking up where I left off with latest DNA extractions. 12 nymphs at 4 C. Qiagen extractions as above. 1 nymph per extraction.

This time, I pre-mixed the Buffer ATL and prot K.

56 C incubation for only 2 hrs.

+4 ul of RNase A for 10 minutes at room temp before adding buffer AL and EtOH.

Pre-warmed Buffer AE to 56 C.

Eluted in two rounds of buffer AE. 100 ul. Then 200 ul.

1/3/12

Organizing RNA samples for submission to YCGA for cDNA library construction and Illumina sequencing. These are Pachypsylla venusta samples to be used to look for differential gene expression between the bacteriome and whole body tissue. They were prepped in late October to Mid-November 2011 and have been in the -80 C since.

Here is a summary of the RNA concentration in each sample as estimated by Agilent Bioanalyzer (earlier nanodrop values recorded in this notebook are roughly similar but a bit higher).

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | Bacteriome | Body 1 | Body 2 | Body 3 | Body 4 | Body 5 | Avg. Body |
| 10/27/11 | 46.9 | 582.8 | 575.5 | 240.2 | x | x | 466.2 |
| 11/11/11 | 57.2 | 607.9 | 454.4 | 572.9 | 490.8 | 835.4 | 592.3 |
| 11/16/11 | 65.9 | 487.7 | 448.5 | 713.3 | 220.7 | 916.2 | 557.3 |
| 11/18/11 | 39.1 | 336.9 | 243.7 | 338.4 | 759.5 | x | 419.6 |

The 10/27/11 bacteriome sample was submitted previously (DBS003). The other 3 bacteriome samples all have a 30 ul aliquot that was set aside at the time of prep to be submitted.

For the body samples from each date, I will generate an equal volume mixture by a combining 5 ul from each tube. For each of the 4 pools, generate a 30 ul 1/10x dilution (3 ul of pooled RNA in 27 ul RNase-free dH2O).

Store samples in -80 C and submit the 30 ul aliquots/dilutions tomorrow:

DBS010 – 10/27/11 body pool

DBS011 – 11/11/11 bacteriome

DBS012 – 11/11/11 body pool

DBS013 – 11/16/11 bacteriome

DBS014 – 11/16/11 body pool

DBS015 – 11/18/11 bacteriome

DBS016 – 11/18/11 body pool

1/5/12

Quantifying yield from DNA extractions performed on 12/31/11. Qubit dsDNA BR kit…

|  |  |
| --- | --- |
| **Sample** | **Conc (ng/ul)** |
| 12.1 | 14.7 |
| 13.1 | 9.4 |
| 14.1 | 7.7 |
| 15.1 | 7.6 |
| 16.1 | 7.5 |
| 17.1 | 8.1 |
| 18.1 | 9.3 |
| 19.1 | 8.5 |
| 20.1 | 10.4 |
| 21.1 | 9.8 |
| 22.1 | 10.4 |
| 23.1 | 8.6 |
| 12.2 | 3.9 |
| 13.2 | 2.6 |
| 14.2 | 3.0 |
| 15.2 | 2.7 |
| 16.2 | 3.0 |
| 17.2 | 3.4 |
| 18.2 | 2.9 |
| 19.2 | 2.4 |
| 20.2 | 3.4 |
| 22.2 | 3.8 |

\*note that 21.2 and 23.2 were not measure (ran out of tubes).

Run a couple samples from each of the two recent rounds of DNA extraction to make sure nothing screwy happened with the DNA quality.

1% agarose TBE gel at 100V for 40 minutes.

Loading order:

1. 1 kb plus ladder 1/10x, 5 ul
2. Pachypsylla venusta gDNA #4.1 (12/28/11), 2ul
3. Pachypsylla venusta gDNA #5.1 (12/28/11), 2ul
4. Pachypsylla venusta gDNA #22.1 (12/31/11), 2ul
5. Pachypsylla venusta gDNA #23.1 (12/31/11), 2ul
6. Pachypsylla venusta gDNA #4.2 (12/28/11), 2ul
7. Pachypsylla venusta gDNA #5.2 (12/28/11), 2ul
8. Pachypsylla venusta gDNA #22.2 (12/31/11), 2ul
9. Pachypsylla venusta gDNA #23.2 (12/31/11), 2ul

Image taken. See PhotosAndGelPics directory.

Results: Look fine. Some degradation, but nothing too bad. The first sample was expected to be the most concentrated, but the difference in intensity exceeds what I would have expected based on the qubit values??

Trying out some EtOH precipitations to ensure that I don’t get too much sample loss. Using samples that are not part of a big groups from a single gall.

5 precipitations:

1. P. ven 11.1 (12/28/11)
2. P. ven 11.2 (12/28/11)
3. P. ven 22.1 (12/31/11)
4. P. ven 22.2 (12/31/11)
5. Pooled P. ven 23.1 and 23.2

The original nominal volumes of these 5 samples was 100, 100, 100, 200, 300 ul. I will use approximations of 90, 190, and 290 ul for the three different levels.

EtOH preciptitations will be performed with 0.1 volumes of 3 M NaOAc and 2.5 volumes of 100% EtOH.

|  |  |  |
| --- | --- | --- |
| **Approx. Sample Vol (ul)** | **3M NaOAc vol (ul)** | **EtOH vol (ul)** |
| 90 | 9 | 225 |
| 190 | 19 | 475 |
| 290 | 29 | 725 |

Samples allowed to precipitate overnight at -20 C.

1/6/12

Continuing EtOH precipitations from yesterday.

Spun at max speed for 10 min, 4 C

Poured off sup.

Washed in 70% EtOH (cold)

Spun at max speed for 5 min, 4 C.

Fully aspirated supernatant (with extra quick spin to collect remaining liquid)

Air dried pellets

Dissolved each pellet in 10 ul Qiagen buffer AE.

Dissolving included lots of pipetting up and down and one quick vortex followed by spinning back down. Might want to check the effect of this on DNA quality/shearing. Overall it was tough to get the pellet fully back into solution.

Qubit dsDNA BR quantification:

Sample 11.1: 73.3 ng/ul

Sample 11.2 :19.6 ng/ul

Sample 22.1: 74.5 ng/ul

Sample 22.2: 18.8 ng/ul

Sample 23: 80.9 ng/ul

\*\*\*NOTE\*\*\*

I now believe that my earlier pooled RNA extractions from hackberry psyllid galls contain a mixture of two different psyllid species: Pachypsylla venusta and some other unidentified psyllid. I had previously noticed morphological differences among the nymphs in these galls but attributed to differences in developmental stage (see 10/27/11). However, in seeing the nymphs used in the more recent DNA extractions, it is apparent that the late instar nymphs of P. venusta are not the same as what I was previously referring to as late-instar nymphs. So far, the most recent collection (see 11/21/11) does not appear to contain any of the other species.

I do not know the identity of the other species. I assume it as acting as some sort of kleptoparasite. I have few nymphs of both species preserved in EtOH. These are from one of the earlier pooled RNA extractions, but I don’t know which one. I just labeled the eppendorf tube with today’s date. They are just at room temp (currently on my office desk). The RNA-seq data (DBS003), supports a low level of contamination from some other psyllid species. I found evidence of low abundance mtDNA fragments that are significantly divergence from the published P. venusta mt genome, but still clearly from a psyllid. They look to be functional (indels typically occurring only in non-coding regions, low dN/dS from one cox3 fragment that I checked). There is very little in the way of psyllid mtDNA sequence available on GenBank, but one 12 rRNA fragment returned a number of non-Pachypsylla psyllid hits with higher sequence identity than P. venusta (see D. Percy 2003 Evolution), suggesting that the other species is not a close relative within the genus.

This means that all four pooled RNA samples are contaminated with material from this other species—although some of the subpools for the body samples should be OK. Therefore, I cancelled the Illumina RNA-seq runs planned for these samples (DBS010 to DBS016). Unfortunately, it will probably have to wait until next October/November to get enough individuals to repeat the extractions ☹.

1/12/12

More Pachypsylla venusta DNA and RNA extractions for use in a potential i5k genome project.

I opened up the 4 remaining galls from Nancy’s 11/21/11 collection. The first two galls the insects appeared to be dead or just on the verge of death (occasional leg twitches). The larger of these galls had 10 nymphs (probably 5th instar). I took 8 of these and did Qiagen DNA extractions as before. These were labeled samples #24-31, continuing the series as above.

Extraction notes:

--pre-mixed ATL and prot K

--ca. 4 hour incubation at 56 C.

--Eluted in two rounds of Buffer AE, 100 ul then 200 ul.

--Did not pre-warm the elution buffer

The quality of these DNA samples should be checked given that the nymphs appeared to be dead or close to it. Stored at -20 C.

The last two galls I opened contained nymphs that were significantly more lively. The larger of these contained 10 nymphs. I took four of these (probably 5th instar) for individual RNA extractions: 2 Trizol (see 10/27/11) and 2 Qiagen (see 8/29/11).

For both extraction types, I eluted/dissolved in 50 ul of RNase-free dH2O.

Preps are labeled P. ven Whole RNA.

Tri 1 and Tri 2 or Q1 and Q2, for Trizol and Qiagen respectively.

Stored the 4 RNA preps at -80 C. For each one, I took a 3 ul aliquot and stored it in a separate tube (simply labeled Tri1, Tri2, Q1, or Q2 with today’s date). This can be used for future quantification and QC w/o thawing the main sample).

I did not do any quantification or QC for either the DNA or RNA extractions. I will check them out if it turns out I need them for the i5k genome project.

\*\*\*\*\*

Preserving the last of the psyllids from the 11/21/11 collection. Freeze some (-80 C). Store some in acetone. Store some in ethanol. Nymphs are probably 5th instar.

Gall 1 : Corresponds to the one used for DNA extractions today. There were two more individuals from this gall. Stored in two separate eppendorfs at -80 C. Labeled P. ven 1.1 and P. ven 1.2.

Gall 2: Corresponds to the second gall from today (6 nymphs showing only minimal movements). Stored in 1 ml of 100% acetone. Labeled P. ven 2

Gall 3: Corresponds to the gall used for today’s RNA extractions. Frozen at -80 C in individual eppendors (6). Labeled P. ven 3.1-3.6

Gall 4: Corresponds to 4th gall from today. 5 lively nymphs. Stored in 1 ml of 100% EtOH. Labeled P. ven 4.

Gall 5: 5 nymphs taken from a single gall on 1/5/12 (I was looking if I could distinguish genders). They have been at 4 C up until now. Frozen at -80 C in one tube labeled P. ven 5.

Gall pool 6: 6 nymphs taken from multiple different galls on 1/6/12 (still looking at gender. They have been at 4 C up until now. Stored in 1 ml of 100% EtOH. Labeled P. ven 4.

For now, I am keeping the EtOH and acetone samples at room temp. Ryu Koga suggested replacing the EtOH/acetone after about two weeks. I also just replaced the EtOH in the earlier mixed sample of two different psyllids from hackberry petiole galls (see 1/6/12).

1/20/12

Preparing dilutions of psyllid genomic DNA for future PCR work. 5 samples total:

4 DNAs from the Baumann collection corresponding to those submitted for Illumina sequencing (libraries DBS004, DBS005, DBS006, and DBS008):

Hte: Heteropsylla texana

Hcu: Heteropsylla cubana

Ceu: Ctenarytaina eucalypti

Csp: Ctenarytaina spatulata

One MDA sample from Pachypsylla celtidis (spp?). MDA #1 from 10/31/11. This corresponds to the DBS007 library submitted for Illumina sequencing.

For each of the 5 samples, I made two serial 10-fold dilutions (1 ul DNA into 9 ul dH2O).

1/23/12

Doing PCR for gap closing of Carsonella genomes…

Using standard Moran lab PCR reagents:

--NEB Taq DNA Polymerase w/ ThermoPol buffer (M0267)

--10 mM dNTPs (= 2.5 mM each).

--Thermal Cyclers: Eppendorf Master Cycler pro S

|  |  |  |  |
| --- | --- | --- | --- |
| **Fwd Primer** | **Rev Primer** | **Templates** | **# rxns** |
| DBS006\_gap1F | DBS006\_gap1R | Ctenarytaina spatulata 1/10x (1/20/12); Ctenarytaina spatulata 1/100x (1/20/12); dH2O | 3 |
| DBS006\_gap2F | DBS006\_gap2R | Ctenarytaina spatulata 1/10x (1/20/12); Ctenarytaina spatulata 1/100x (1/20/12); dH2O | 3 |
| DBS007\_gapF | DBS007\_gapR | Pachypsylla celtidis spp. 1/10x (1/20/12); Pachypsylla celtidis spp. 1/100x (1/20/12); dH2O | 3 |
| DBS004\_tandemF | DBS004\_tandemR | Heteropsylla texana 1/10x (1/20/12); Heteropsylla texana 1/100x (1/20/12); dH2O | 3 |
| DBS008\_tandemF | DBS008\_tandemR | Heteropsylla cubana 1/10x (1/20/12); Heteropsylla cubana 1/100x (1/20/12); dH2O | 3 |

|  |  |  |
| --- | --- | --- |
|  |  | **Mult Fact** |
| **Reagent** | **Vol per rxn (ul)** | **22** |
| Template | 1 |  |
| 10x Buffer | 1.5 | 33 |
| 25 mM MgCl2 | 0 | 0 |
| 2.5 mM dNTPs | 1.2 | 26.4 |
| Fwd Primer (10 uM) | 0.75 |  |
| Rev Primer (10 uM) | 0.75 |  |
| Taq (5 U/ul) (5 U/ul) | 0.15 | 3.3 |
| dH2O | 9.65 | 212.3 |
| **Total Vol** | **15** | **275** |
|  |  |  |
| Cocktail Vol (per rxn) | Mult Factor |  |
| 12.5 | 4 | 50 |
| Volume per primer | 3 |  |

For first 3 primers pairs:

|  |  |  |
| --- | --- | --- |
| **Temp** | **Time** | **Cycle #** |
| 94 deg | 2 min |  |
| 94 deg | 15 sec |  |
| 51 deg | 15 sec | 40 |
| 72 deg | 1 min |  |
| 72 deg | 5 min |  |
| 4 deg | Hold |  |

For last 2 primer pairs

|  |  |  |
| --- | --- | --- |
| **Temp** | **Time** | **Cycle #** |
| 94 deg | 2 min |  |
| 94 deg | 15 sec |  |
| 51 deg | 15 sec | 40 |
| 72 deg | 3 min |  |
| 72 deg | 5 min |  |
| 4 deg | Hold |  |

1/24/12

Ran PCR samples from yesterday on a 1% agarose TBE gel. 100 V for 30 min.

Loading order:

Top tier: 5 ul of 1/10x 1 kb plus ladder, 3 ul of each of the first 9 PCR samples.

Bottom tier: 5 ul of 1/10x 1 kb plus ladder, 3 ul of each of the last 6 PCR samples.

See PhotosAndGelPics dir for gel image.

Results: It looks like I got at least some amplification for each of the first 3 primer pairs. Yield looks to be as good or better for the 1/100x dilution in each case. Based on the current genome assembly coordinates, the predicted fragment sizes are as follows:

DBS006\_gap1: 651 bp

DBS006\_gap2: 384 bp

DBS007\_gap: 616 bp.

The product sizes look to be somewhat larger than this, but that might be expected if the gap size is larger than the sequences of 10 Ns representing the gaps at each appropriate spot in the genome. Certainly this is the case in DBS006\_gap2 where the gap is definitely bigger.

For DBS006\_gap1, the product is very faint and only really apparent in the 1/100x sample.

For DBS006\_gap2, the product looks fairly clean, especially the 1/100x sample.

For DBS007\_gap, there is a competing higher MW band.

For the two tandem repeat regions, there doesn’t appear to be any amplification whatsoever. These were large (>1kb) even with a single copy of the putative repeat. Given that they might have many copies, amplifying these large AT rich regions might be challenging. Consider designing two primers within the putative repeat region, but pointing away from each other rather than towards each other. Also look for clusters of outward facing PE conflicts in this region to confirm existence of tandem repeats.

1/30/12

Preparing reagents for cloning of PCR products.

Pouring LB-amp plates.

Made 1 L of LB

5 g Bacto yeast extract (BD 212750)

10 g Tryptone (Fisher BP1421-500)

10 g NaCl (Fisher S271-3)

🡪 dissolved in dH2O to a final volume of 1 L.

Poured over 15 g of bacteriological agar (American Bioanalytical AB01185-00500).

Autoclaved on liquid cycle (Philipp brought it up to the autoclave, so I am not sure of the exact cycle).

Temper in incubator to ~55 C

Add ampicillin to 1 concentration of 100 ug/ml (1000-fold dilution from Eli Powell’s 100 ug/ul lab stock).

Poured plates in laminar flow hood, and stored upside down at 4 C.

I will try out cloning with only one PCR sample: DBS006 gap2 1/10x template (see 1/23/12 for PCR details). Since it’s been a few days, I will reincubate with Taq to make sure the 3’ A overhangs are intact.

Added ~0.1 ul Taq. Incubated at 72 C for 5 min.

Now perform EtOH precipitation to reduce primer dimer.

11 ul PCR product, 1.1 ul 3 M NaOAc, 27.5 ul EtOH. Incubate at -20 C for ~1 hr. Spin at max speed, the wash with 200 ul cold 70% EtOH.

Dissolved in 10 ul TE made freshly today (10 mM Tris-HCl ph 8.0, American Bioanalytical borrowed from Philipp’s stock; 1 mM EDTA, American Bioanalytical stock; in HyClone dH2O).

Cloning reaction with the Promega pGEM-T Easy Vector Kit: <http://www.promega.com/~/media/Files/Resources/Protocols/Technical%20Manuals/0/pGEM-T%20and%20pGEM-T%20Easy%20Vector%20Systems%20Protocol.ashx>

Setting up 2 parallel reactions (one with the above cleaned PCR sample; the other with control insert DNA provided with the kit). Reagents added in the following order followed by mixing by pipetting.

5 ul of 2x ligation buffer

1 ul dH2O

1 ul pGEM-T Easy Vector

2 ul PCR product (see above) or control DNA

1 ul T4 ligase

Incubated overnight at 4 C, starting at 6 pm.

Set up PCR to amplify Heteropsylla texana tandem repeat region with “inverted” primers (goal here is to determine the repeating unit).

|  |  |  |  |
| --- | --- | --- | --- |
| **Fwd Primer** | **Rev Primer** | **Templates** | **# rxns** |
| DBS004\_invF | DBS004\_invR | Heteropsylla texana 1/10x (1/20/12); Heteropsylla texana 1/100x (1/20/12); dH2O | 3 |

|  |  |  |
| --- | --- | --- |
|  |  | **Mult Fact** |
| **Reagent** | **Vol per rxn (ul)** | **4** |
| Template | 1 |  |
| 10x Buffer | 1.5 | 6 |
| 25 mM MgCl2 | 0 | 0 |
| 2.5 mM dNTPs | 1.2 | 4.8 |
| Fwd Primer (10 uM) | 0.75 | 3 |
| Rev Primer (10 uM) | 0.75 | 3 |
| Taq (5 U/ul) (5 U/ul) | 0.15 | 0.6 |
| dH2O | 9.65 | 38.6 |
| **Total Vol** | **15** | **56** |

|  |  |  |
| --- | --- | --- |
| **Temp** | **Time** | **Cycle #** |
| 94 deg | 2 min |  |
| 94 deg | 15 sec |  |
| 51 deg | 15 sec | 40 |
| 72 deg | 1 min |  |
| 72 deg | 5 min |  |
| 4 deg | Hold |  |

1/31/12

Continuing with cloning from yesterday, starting at 10 am.

Prepared a total of 4 LB-Amp plates (see yesterday) , by adding 100 ul of 100 mM lab stock of IPTG and 50 ul of 20 mg/ml lab stock of x-gal and spreading over the plate (done in flow hood). Plates were incubated for 30 min at 37 C to absorb these volumes and then left out at room temp to equilibrate.

Added 2 ul of ligation reactions from yesterday to 1.5 ml tubes.

Thawed 50 ul aliquot of Invitrogen DH5α in ice bath for ~5 min. Pipeted into 1.5 ml tube containing plasmid ligation. Stirred gently with pipet tip and very gently flicked tubes. Incubated on ice for 20 minutes.

45 sec heat shock in 42 C water bath.

Returned to ice for 2 min.

Added 950 ul SOC to each tube.

Incubated for 1.5 hours at 37 C, 215 rpm shaking (protocol says ~150, but the table was already being used at this speed).

Plate each transformation at two different volumes, 10 ul and 100 ul. For the 10 ul sample, dilute first in 90 ul SOC to provide sufficient volume for spreading.

Incubate at 37 C overnight (starting at 12:40 pm).

2/1/12

Low colony counts on plates

DBS006 gap2 100ul: 4 white colonies (including a doublet), 2 blue colonies

DBS006 gap2 10ul: 0 colonies

+ Control 100 ul: 1 white colony, 1 blue colony

+ Control 10 ul: 1 white colony

Try spinning down the rest of the culture volumes (~900 ul; note these have been at 4 C overnight). 10 min at 1000g, 4 C. Remove all nut ~100 ul of supernatant. Resuspend cell pellet by pipetting. Plate entire volume on an LB/Amp/IPTG/x-gal plate prepared in the same fashion as yesterday.

Incubate overnight at 37 C, starting at noon.

Try colony PCR on the small number of colonies on the DBS006 gap2 100 ul plate to see if any of them actually have the insert:

|  |  |  |  |
| --- | --- | --- | --- |
| **Fwd Primer** | **Rev Primer** | **Templates** | **# rxns** |
| T7 | SP6 | colony1, colony2, colony3, colony4 (blue), surface control, control plate colony1, Ctenarytaina spatulata 1/100x (1/20/12); dH2O | 8 |
| DBS006\_gap2F | DBS006\_gap2R | colony1, colony2, colony3, colony4 (blue), surface control, control plate colony1, Ctenarytaina spatulata 1/100x (1/20/12); dH2O | 8 |

Note that colony 2 is one side of a doublet. I did my best to get it from the side. The marking “2” on the under side of the plate is written on the side that I aimed for.

|  |  |  |
| --- | --- | --- |
|  |  | **Mult Fact** |
| **Reagent** | **Vol per rxn (ul)** | **20** |
| Template | 1 |  |
| 10x Buffer | 1.5 | 30 |
| 25 mM MgCl2 | 0 | 0 |
| 2.5 mM dNTPs | 1.2 | 24 |
| Fwd Primer (10 uM) | 0.75 |  |
| Rev Primer (10 uM) | 0.75 |  |
| Taq (5 U/ul) (5 U/ul) | 0.15 | 3 |
| dH2O | 9.65 | 193 |
| **Total Vol** | **15** | **250** |
|  |  |  |
| Cocktail Vol (per rxn) | Mult Factor |  |
| 12.5 | 9 | 112.5 |
| Volume per primer | 6.75 |  |

|  |  |  |
| --- | --- | --- |
| **Temp** | **Time** | **Cycle #** |
| 94 deg | 2 min |  |
| 94 deg | 15 sec |  |
| 51 deg | 15 sec | 40 |
| 72 deg | 1 min |  |
| 72 deg | 5 min |  |
| 4 deg | Hold |  |

Run 1% Agarose TBE gel with the 16 PCR samples from today and the 3 samples from 1/30/12. 100 V for 30 min.

Loading order:

1. 1/10x 1 kb plus ladder – 5ul

2-17. PCR samples 1-16 from today

18-20. PCR samples 1-3 from 1/30/12

21. 1/10x 1 kb plus ladded – 5ul

See PhotosAndGelPics directory for gel image.

Results: Looks like the white colonies do actually contain the desired insert. It’s a little tough to tell b/c the surface control also came up positive (but somewhat more weakly) with the original primer pair. But colonies 1 and 3 both show an SP6/T7 product that looks like the right size. This reaction failed for the colony 2, but I bet it might work it if was repeated. All PCRs look messy (continuing upping the annealing temp for future runs). But it is probably worth trying to sequence the SP6/T7 PCR products from colonies 1 and 3 with the internal (i.e., original) primers. That internal primers should hopefully take care of any competing products.

The 1/30/12 PCRs appear to to have worked. Both template concentrations produced similar results (stronger signal from 1/100x). The product is smeared upward, but that makes sense given the putative tandem repeat structure. Try direct sanger sequencing. If the repeats are clean and there isn’t any structural polymorphism, this may work even though there are multiple products of different sizes.

2/2/12

Checked plates from yesterday (i.e., the rest of the cells from the previous days cloning). As expected, the colony counts were much higher b/c I plated ~900 ul of culture (after spinning down and resuspending in a smaller culture). I will proceed with the few colonies from yesterday. For now, just store these plates at 4 C.

Repeat cloning for the other two successful PCRs from 1/24/12:

DBS006 gap1; 1/100x template

DBS007 gap; 1/100x template

First, transfer 9 ul (leaving a tiny bit in case I want to reamplify) of each to new PCR tubes and spike in some extra Taq (~0.1 ul, but might be more than that given pipetting inaccuracies). Inubate for 5 min at 72 C.

EtOH precipitate PCR products. I didn’t see as clear a pellet as I would have expected. Quantify DNA before proceeding with cloningLeave as dried pellets at -20 C through the weekend.

2/6/12

Dissolve pellets in 5 ul TE. Quantify 1 ul of each PCR product with Qubit dsDNA BR kit.

DBS006 gap1 🡪 4 ng/ul

DBS007 gap 🡪 17 ng/ul

Same protocol as before for setting up pGEM ligation reactions except use 3 ul of product (and no dH2O) for DBS006 gap1 and 1 ul of product (and 2ul dH2O) for DBS007 gap.

5 ul of 2x ligation buffer

2 (or 0) ul dH2O

1 ul pGEM-T Easy Vector

1 (or 3) ul PCR product or control DNA

1 ul T4 ligase

Incubate overnight at 4 C starting at 5pm.

2/7/12

Continued with cloning from yesterday. Same protocol as 1/31/12 except I had to use the Groisman lab shaking incubator which was set at 250 rpm.

Plated 3 volumes of cells: 10 ul (diluted up to 100 ul with SOC); 100 ul; and the rest (~900 ul spun down and resuspended in ~100 ul SOC) on LB/Amp/IPTG/x-gal plates. Incubate overnight at 37 C.

2/8/12

Plates from yesterday’s transformations look good. Much higher efficiency than last time the 900ul plates have hundreds of colonies. The 100 ul plates have ~100 colonies for DBS007 and a few dozen for DBS006. The 10 ul plates each have a few colonies. The white/blue ratio looks very good. Only a small percentage of blue colonies.

PCR Screen sample of colonies looking for insert… (try raising annealing temp to 55 to improve on cleanliness of rxns).

Made new 1/100x dilutions of Ceu, Csp, and Pcel DNA templates from the 1/20/12 1/10x dilutions.

|  |  |  |  |
| --- | --- | --- | --- |
| **Fwd Primer** | **Rev Primer** | **Templates** | **# rxns** |
| DBS006\_gap1F | DBS006\_gap1R | DBS006 gap1 cloning 10 ul plate colonies 1-5 + surface control, Ctenarytaina spatulata (1/100x; 2/8/12), dH2O | 8 |
| T7 | SP6 | DBS006 gap1 cloning 10 ul plate colonies 1-5 + surface control, Ctenarytaina spatulata (1/100x; 2/8/12), dH2O | 8 |
| DBS007\_gapF | DBS007\_gapR | DBS007 gap cloning 100 ul plate colonies 1-5 + surface control, Pachypsylla celtidis (1/100x; 2/8/12), dH2O | 8 |
| T7 | SP6 | DBS007 gap cloning 100 ul plate colonies 1-5 + surface control, Pachypsylla celtidis (1/100x; 2/8/12), dH2O | 8 |

\*\*Used left over master mix to run one separate reaction with Ingrid’s 16S\_fwd and 16S\_rev primers using Ceu 1/100x as template.

|  |  |  |
| --- | --- | --- |
|  |  | **Mult Fact** |
| **Reagent** | **Vol per rxn (ul)** | **38** |
| Template | 1 |  |
| 10x Buffer | 1.5 | 57 |
| 25 mM MgCl2 | 0 | 0 |
| 2.5 mM dNTPs | 1.2 | 45.6 |
| Fwd Primer (10 uM) | 0.75 |  |
| Rev Primer (10 uM) | 0.75 |  |
| Taq (5 U/ul) (5 U/ul) | 0.15 | 5.7 |
| dH2O | 9.65 | 366.7 |
| **Total Vol** | **15** | **475** |
|  |  |  |
| Cocktail Vol (per rxn) | Mult Factor |  |
| 12.5 | 9 | 112.5 |
| Volume per primer | 6.75 |  |

|  |  |  |
| --- | --- | --- |
| **Temp** | **Time** | **Cycle #** |
| 94 deg | 2 min |  |
| 94 deg | 15 sec |  |
| 55 deg | 15 sec | 40 |
| 72 deg | 1 min |  |
| 72 deg | 5 min |  |
| 4 deg | Hold |  |

\*\*\*Note, I used a PCR plate as opposed to strip tubes for the first time in the lab with the plastic seal. Upon placing the samples on the heated block and pressing down on the sealing film, I saw some weird bubbling around the rims of each well. I had inverted the plate earlier (my normal method for sealing the film by pressing it against the bench top), but I didn’t see any sign that the samples had slipped.

Ran 1% agarose TBE gel at 100 V for 25 min.

Loading order:

Top tier:

1. 1 kb plus ladder 1/10x – 5 ul

2-17. Reactions 1-16 from above (DBS006 samples) – 3 ul

18. 1 kb plus ladder 1/10x – 5ul

Bottom tier:

1. 1 kb plus ladder 1/10x – 5 ul

2-17. Reactions 17-32 from above (DBS007 samples) – 3 ul

18. 1 kb plus ladder 1/10x – 5ul

19. 16S PCR reaction (see note above)

See PhotosAndGelPics dir for gel image.

Results: Clear amplification for almost all samples (SP6/T7 primers failed for the first 2 DBS006 gap1 colonies). Surface negative control gave a positive result for DBS007 gap plate (albeit at a weaker level than most colonies). There is a second competing higher MW band in the samples, especially in SP6/T7 reactions. This is somewhat disconcerting because the orginal DBS007 also produced a competing band in this size range. Contamination of multiple clones? Some weird second order reaction that occurs in this region? Something unrelated that is produced by these primers? Try sequencing the SP6/T7 reactions with the original PCR primers. Hopefully that will confer some specificity.

2/9/12

Setting up “Exo-CIP” reactions to clean PCR samples for submission to the

Set up the following reactions:

3 ul PCR product

8.3 ul dH2O

0.1 ul ExonucleaseI (ExoI). 20 U/ul NEB M0293L

0.1 ul Calf intestine phosphatase (CIP). 10 U/ul NEB #M0290S

0.5 ul 10 uM primer (not added until after reaction!!)

|  |  |  |  |
| --- | --- | --- | --- |
| **ID** | **Primer** | **Template** | **Template Date** |
| DBS001 | DBS006\_gap1F | T7/SP6 Colony PCR of DBS006 gap1 cloning, well 2.4 | 2/8/12 |
| DBS002 | DBS006\_gap1R | T7/SP6 Colony PCR of DBS006 gap1 cloning, well 2.4 | 2/8/12 |
| DBS003 | DBS006\_gap2F | T7/SP6 Colony PCR of DBS006 gap2 cloning, well 1.1 | 2/1/12 |
| DBS004 | DBS006\_gap2R | T7/SP6 Colony PCR of DBS006 gap2 cloning, well 1.1 | 2/1/12 |
| DBS005 | DBS007\_gapF | T7/SP6 Colony PCR of DBS007 gap cloning, well 4.2 | 2/8/12 |
| DBS006 | DBS007\_gapR | T7/SP6 Colony PCR of DBS007 gap cloning, well 4.2 | 2/8/12 |
| DBS007 | DBS004\_invF | Heteropsylla texana 1/100x, PCR well 2 | 1/30/12 |
| DBS008 | DBS004\_invR | Heteropsylla texana 1/100x, PCR well 2 | 1/30/12 |

Submitted to Yale Science Hill sequencing facility under Philipp Engel’s user account (they are still setting up my account). Order #137117

2/13/12

Making dilutions of psyllid DNAs (Thao/Baumann samples from -80 C). Two serial ten-fold dilutions to generate 1/10x and 1/100x stocks.

Dilution 1: 2 ul sample + 18 ul TE

Dilution 2: 5 ul of dilution 1 + 45 ul PCR dH2O

Stored at -20 C. Each tube labeled with first letter of genus name and first two letters of species name (e.g. Psylla buxi = Pbu).

List of species used for dilutions…

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Genus** | **Species** | **GenBank Accession from Thao et al. (2000 Appl. Env. Microbiol.)** | **Amount** | **Concentration on tube (ng/ul)** | **Color** | **Notes** |
| Acizzia | uncatoides(2) | AF211124/AF231366 | 3 | 898 | Y |  |
| Aphalara | longicaudata | AF243137 | 2 | 159 | C |  |
| Aphalaroida | inermis | AF211125 | 1 | 380 | C |  |
| Arytaina | genistae | AF243136 | 3 | 1150 | B |  |
| Bactericera | cockerelli | AF211126 | 1 | 228 | Y |  |
| Bemisia | tabaci |  |  |  |  | Whitefly; My Qiagen extraction. #1 on 11/11/11 |
| Blastopsylla | occidentalis | AF211127/AF231382 | 1 | 375 | B |  |
| Boreioglycaspis | melaleucae | AF211128/AF231383 | 2 | 465 | C |  |
| Cacopsylla | peregrina | AF211130/AF231373 | 1 | 246 | C |  |
| Cacopsylla | brunneipennis | AF243138 | 1 | 220 | C |  |
| Cacopsylla | pyri | AF211131/AF231372 | 2 | 383 | B |  |
| Calophya | schini | AF211132/AF231369 | 3 | 513 | Y |  |
| Cardiospina | albitextura | ? | 2 | 1060 | B |  |
| Ctenarytaina | eucalypti | AF211133/AF231385 | 3 | 500 | Y |  |
| Ctenarytaina | spatulata | AF211135/AF231384 | 2 | 640 | C |  |
| Ctenarytaina | longicauda | AF211134/AF231386 | 1 | 540 | C |  |
| Diaphorina | lycii | ? | 2 | 260 | Y |  |
| Diaphorina | citri | AF211136/AF231365 | 1 | 570 | C |  |
| Glycaspis | brimblecombei | AF211137/AF231381 | 3 | 193 | C |  |
| Heteropsylla | texana | AF211139/AF231375 | 3 | 376 | C |  |
| Heteropsylla | cubana | AF211138/AF231376 | 3 | 883 | Y |  |
| Neophyllura | arbuti | ? | 2 | 285 | C |  |
| Neotriozella | hirsuta | AF211140/AF231363 | 2 | 100 | C |  |
| Pachypsylla | celtidis | AF211141/AF231379 | 3 | 471 | C |  |
| Pachypsylla | venusta | AF211143/AF231378 | 3 | 640 | C | Main stock; There’s another diluted stock in the box. |
| Pachypsylla | pallida | AF211142/AF231377 | 1 | 586 | C |  |
| Panisopelma | fulvescens | AF211144 | 1 | 228 | Y |  |
| Psylla | buxi | AF211146/AF231371 | 2 | 213 | C |  |
| Tainarys | sordida | AF211150/AF231380 | 1 | 256 | C |  |
| Trioza | urticae | AF211152/AF231364 | 2 | 105 | Y |  |
| Trioza | eugeniae | AF211151/AF231362 | 3 | 636 | Y |  |

2/14/12

Checking DNA quantity and quality for a sample of the extractions from 1/12/12. Just a subset to see if they are similar to previous rounds.

Qubit dsDNA BR kit. 1 ul per sample…

Samp. Concentration (ng/ul)

#24.1 7.0

#25.1 11.3

#30.1 6.4

#31.1 6.3

#24.2 2.9

#31.2 undetectable

Run 3 ul of each of the above on a 1% agarose gel. 100 V for 45 min.

See PhotosAndGelPics dir for gel image.

Results:

Looks fine. Some degradation but not too bad. Intensities appear to roughly correlate with qubit estimates.

Trying a EtOH precipitations to concentrate DNA samples. Only do a set of 4 just to make sure nothing screwy is going on before processing all the samples.

Pooled the first and second elution for each of sample #4, 5, 6, and 7 from 12/28/11.

Assumed approx. volume for each is 190 ul.

🡪 add 19 ul 3M NaOAc

🡪 add 475 ul EtOH

invert numerous times and incubate overnight at -20 C.

2/15/12

Processing the EtOH precipitations from yesterday.

10 min spin ay max speed, 4 C. Wash with 500 ul cold 70% EtOH. 5 min re-spin.

Elute in 21 ul Qiagen buffer AE.

Qubit. 1ul sample from each dsDNA BR kit.

Smpl Conc (ng/ul)

#4 60.4

#5 15.8

#6 16.6

#7 24.6

Proceed with EtOH precipitations of the rest of the Pachypsylla venusta. Precipitate first and second round elutions separately. Do not precipitate sample #31 (round 1 or 2). That will be left for future work in the lab here.

100 ul elutions estimated as 90 ul volumes. 200 ul elutions estimated as 190 ul volumes. Added 2.6 volumes of a 25:1 EtOH:NaOAc(3M) solution. Inverted many times and stored overnight at -20 C.

2/16/12

Proceeding with EtOH precipitations from yesterday.

The general plan here is to produce 10 individual DNA samples (ideally the most abundant ones) and pool the rest. Individual samples are dissolved in 21 ul (with 1 ul being used for Qubit, leaving ~20 ul). For the pooled sample, I will use 20 ul to dissolve each sample, but I will reuse the same volumes transferring them from tube to tube. I will use a total of 5 volumes 🡪 ~100 ul (although all the pellet volumes should add a little to the volume.

Resuspensions are in Qiagen buffer AE.

The individual ones will be sample numbers:

4, 11, 22, 23 (all previously generated)

Note that 11 and 22 need their first and second elutions combined. Volumes in each tube were ca. 8.5 ul. Combined the two elutions for 11 and for 22 and added 3 ul Qiagen Buffer AE to each of the combined volumes to bring it up to ~20 ul. Added 11.5 ul buffer AE to sample 23 (which already is combination of two elutions

Note that pellets from today’s precipitations didn’t come back into solution very well (I’ve noticed this before too). I took some time in mechanically disrupting them with pipetting and a few quick vortexes. Still some debris left over.

For the pooled sample, I did a quick spin taking it up to 9000 rpm (and then immediately back down). To pellet some of the macroscopic debris. I then pipeted off ~108 ul of supernatant to save as the pooled sample. I then added 20 ul buffer AE to the pellet (there was also a few ul left over). This sample is labled P. ven “left over”.

Qubit dsDNA BR kit…

Sample #9: 64.8 ng/ul

Sample #12: 48.2 ng/ul

Sample #1: 49.1 ng/ul

Sample #3: 50.2 ng/ul

Sample #20: 53.9 ng/ul

Sample #2: 54.2 ng/ul

\*\*\*Note that the above 6 samples were quanitified in a separate batch that appeared to be trending 10-15% high based on comparison to the previous and following calibration. Might be safer to assume quanties are only ~90% of what’s reported above.

Pooled sample: 157 ng/ul

Leftover sample: 22 ng/ul

Ran 1% agarose gel for 1 hour at 100 V.

Loaded 5 ul 1 kb plus ladded (1/10x) and 1 ul of the pooled P. venusta sample from above.

See gel image in PhotosAngGelPics dir

Results: It looks like the increased handling with all these precipitations and pooling has caused more degradation. Most of the DNA is still HMW but there is a substantial smear.

Summary of what I have for submission to i5k genome project. We’ll have to see if it’s enough…

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Concentration (ng/ul) | Volume (ul) | Total yield (ug) | Gall | Notes |
| Pooled sample (20 nymphs) | 157 | 105 | 16.5 | many |  |
| Sample #1 | 44 | 20 | 0.9 | a | calculated based on 90% of qubit reading |
| Sample #2 | 49 | 20 | 1.0 | b | calculated based on 90% of qubit reading |
| Sample #3 | 45 | 20 | 0.9 | b | calculated based on 90% of qubit reading |
| Sample #4 | 60 | 20 | 1.2 | c |  |
| Sample #9 | 58 | 20 | 1.2 | c | calculated based on 90% of qubit reading |
| Sample #11 | 40 | 20 | 0.8 | d | Calculated based on ~85% volume retention |
| Sample #12 | 43 | 20 | 0.9 | e | calculated based on 90% of qubit reading |
| Sample #20 | 49 | 20 | 1.0 | f | calculated based on 90% of qubit reading |
| Sample #22 | 40 | 20 | 0.8 | g | Calculated based on ~85% volume retention |
| Sample #23 | 34 | 20 | 0.7 | g | Calculated based on ~85% volume retention |

Note that the gall letters listed above do not correspond to any previous notes or nomenclature, they just indicate which individuals came from the same or different galls.

3/5/12

Doing some QC on the Pachypsylla venusta RNA samples from 1/12/12 (2 Qiagen preps and 2 Trizol preps). I left separate 3 ul aliquots from each of these for this purpose.

Nanodrop

|  |  |  |  |
| --- | --- | --- | --- |
| **Sample** | **Concentration (ng/ul)** | **A260/A280** | **A260/A230** |
| Qiagen 1 | 10.1 | 2.12 | 4.20 |
| Qiagen 2 | 10.9 | 1.95 | 4.80 |
| Trizol 1 | 193.4 | 1.77 | 2.14 |
| Trizol 2 | 224.4 | 1.83 | 2.22 |

Ran the same 4 samples (in same order) on Agilent Bioanalyzer 2100 RNA nano 6000 chip. See AgilentRuns/2012-03-05

Concentration estimates are consistent but lower than above…

Qiagen1 3.6 ng/ul

Qiagen2 1.5 ng/ul

Trizol1 143.9 ng/ul

Trizol2 157.6 ng/ul

Overall it looks like the Qiagen extractions were a dud. But the trizol preps look ok in terms of quantity. The elutions were in 50 ul, so I should have ~7 ug. The Bioanalyzer traces show the typical patterns of Trizol degradation that I’ve seen in the past. Almost no LSU rRNA peak left. But that’s probably the best we’re going to be able to do right now.

3/7/12

Got some information from Molly Hunter about the source of their colony from which Bemisia tabaci samples were collected (see 11/11/11).

“The Bemisia is the B biotype, collected in October of 2009 in Maricopa County, AZ, and maintained in culture on cowpeas (Vigna unguiculata) since then.”

“You might also like to know that the whiteflies were collected in the field from cotton.”

3/12/12

Shipped Pachypsylla venusta DNA and RNA to Baylor for the i5k genome sequencing project.

11 DNA samples (1 pool + 10 individuals).

1 RNA sample (Trizol #2 from 1/12/12).

All samples were thawed and transferred to 2D barcode tubes provide by Baylor. Capped with rubber friction-fit seals and then wrapped in parafilm.

See pachypsylla\_i5k\_sampleSheet.xls for tube placement in rack.

I sent all the entire volumes from each tube. But I did save the tubes in case we get really desperate to try amplifying any residual volume in the tube.

Shipped on dry ice to:

ATTN: Sandy Lee

Human Genome Sequencing Center

Baylor College of Medicine

1 Baylor Plaza, N-1505 Alkek Bldg., MS 226

Houston, Texas 77030

FedEx Tracking #798156448514

FedEx Pickup Confirmation #HVNA114

3/29/12

Setting up PCR in an attempt to sequence the 16S rRNA from the Ctenarytaina spatulata S-symbiont. This had very low representation in the Illumina dataset (which was biased against GC rich seqs). I’m using primers that should exclude Carsonella, but they could pick up other bacteria if they are present in high abundance.

|  |  |  |  |
| --- | --- | --- | --- |
| **Fwd Primer** | **Rev Primer** | **Templates** | **# rxns** |
| Csp\_16S\_F | Csp\_16S\_R1 | Ctenarytaina spatulata 1/100x (2/8/12); Ctenarytaina eucalypti 1/100x freshly diluted in dH2O from 1/20/12 1/10x sample; dH2O | 3 |
| Csp\_16S\_F | Csp\_16S\_R2 | Ctenarytaina spatulata 1/100x (2/8/12); Ctenarytaina eucalypti 1/100x freshly diluted in dH2O from 1/20/12 1/10x sample; dH2O | 3 |

|  |  |  |
| --- | --- | --- |
|  |  | **Mult Fact** |
| **Reagent** | **Vol per rxn (ul)** | **8** |
| Template | 1 |  |
| 10x Buffer | 2.5 | 20 |
| 25 mM MgCl2 | 0 | 0 |
| 2.5 mM dNTPs | 2 | 16 |
| Fwd Primer (10 uM) | 1.25 |  |
| Rev Primer (10 uM) | 1.25 |  |
| Taq (5 U/ul) (5 U/ul) | 0.25 | 2 |
| dH2O | 16.75 | 134 |
| **Total Vol** | **25** | **172** |
|  |  |  |
| Cocktail Vol (per rxn) | Mult Factor |  |
| 21.5 | 3.5 | 75.25 |
| Volume per primer | 4.375 |  |

|  |  |  |
| --- | --- | --- |
| **Temp** | **Time** | **Cycle #** |
| 94 deg | 2 min |  |
| 94 deg | 15 sec |  |
| 51 deg | 15 sec | 40 |
| 72 deg | 2 min |  |
| 72 deg | 10 min |  |
| 4 deg | Hold |  |

4/2/12

Ran above PCR out on a 1% agarose TBE gel. 30 min @ 100V.

3 ul of each PCR product

5 ul of 1 kb plus ladder (1/10x) on each side.

15 min staining in EtBr. Note that upon removing the gel from the stain I saw that the level of the stain solution was pretty low and barely covering the gel. As a result the gel had become lodged on the bottom and wasn’t moving around much. I did a quick dunk in the destain and took a picture. The staining quality appears poor (presumably because of the conditions notes immediately above), but it was still clear enough.

See photosAndGelPics directory for the picture.

Amplification looked pretty good for both templates and both primer pairs. Try sequencing the Ctenarytaina spatulata F/R1 sample with the original PCR primers as well as 16S\_fwd and 16S\_rev.

Ingrid Rochon included these four reactions with a sequencing order submitted this afternoon.

5/15/12

Nancy brought in some insect samples collected from in and around her house. Both were collected in Tupperware.

Cacopsylla buxi (psyllid). Collected from Boxwood. May 11, 2012. New Haven.

Aphis spiraecola (aphid). Collected from Spiraea. May 11, 2012. New Haven.

Both tubs were stored at 4 C.

5/16/12

Ingrid came in and tried dissecting some of the Cacopsylla buxi adults (see above). She didn’t save any of her dissections, but she stored the remaining adults in 95% EtOH at -20 C.

I did one dissection and was able to separate out the bacteriome (obvious bright yellow color) from the rest of the body. Dissection was done in EtOH. Stored the bacteriome and remaining body tissue in separate eppendorfs and gave to Ingrid. She put them in -20 C with the rest of the adults.

5/19/12

Note from Nancy:

XXXXXXXXXXXXX

Dan,

The Pachypsylla are out and ovipositing into the hackberry leaves. I took a couple of pictures of them. You can see the tip of the abdomen at the leaf surface.

On the earlier leaves, the galls are enlarged, though the larvae are still very small.

I collected some adults, will bring them in on Monday. And I can bring some more of the Cacopsylla busi (that name should be checked, I'm not sure it is current).

Nancy

XXXXXXXXXXXXXX





5/21/12

Nancy brought in the Cacopsylla busi (= Psylla buxi?) and Pachypsylla mentioned in her e-mail above.

There are numerous Cacopsylla busi, and Nancy mentioned that males could be distinguished based on their clasping parts at the end of their abdomens. I’ve read similar comments about other species.

She also speculated that there were multiple species of Pachypsylla based on their different sizes. This would make sense given that their should at least be nipple and blister gall makers. Perhaps also multiple types of nipple gall makers? And an inquiline species? But I am not sure when the latter would be ovipositing relative to the true gall makers.

I took a quick look at some of these under the dissecting scope and took some pictures. See 20120521\_Pachypyslla\_and\_Cacopsylla\_busi directory in my MoranLab photos collection.

Nancy had portioned the Pachypsylla individuals into 3 bins. One bin clearly had larger individuals, with perhaps a medium and a small bin, but the difference there wasn’t clear.

I refer to these three bins as 1, 2, and 3 in increasing order in size.

Pachypsylla #3. 4 individuals. 2 Stored to in EtOH. 2 Stored in acetone. In eppendorf tubes (2 insects in each tube). About 1 ml each

Pachypsylla #2. 12 individuals. 4 Stored to in EtOH. 4 Stored in acetone. In eppendorf tubes (4 insects in each tube). About 1 ml each. Also 4 stored dry at -80 C.

Pachypsylla #1. 7 individuals. 3 Stored to in EtOH. 2 Stored in acetone. In eppendorf tubes. About 1 ml each. Also 2 stored dry at -80 C.

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

Cacopsylla buxi. Lots of individuals. I made a single eppendorf for each of three different storage methods (EtOH, acetone, dry @ -80 C). About 10 individual per. After this there were dozens of individuals left. I put these in a few mls of EtOH and stored them in a 15 ml conical.

Note that some individuals from all these groups might have been DOA. Most seemed to be doing OK, however.

All EtOH and acetone samples stored at -20 C. Eppendorfs in “DBS” box. 15 ml conical on top shelf of “bubbles” in Styrofoam rack. I have made a note to change out solutions in two weeks.

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

Note that in the pictures taken today, I took only one individual from each of the three Pachypsylla size classes and it might not have been representative. In particular the class 1 individual looked a bit abnormal with upturned wings.

In the last C. busi picture, there are two different individual which I believe represent the two different sexes, the male being the one with upturned abdomen tip.

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

Nancy suggested there may be a hackberry near Whitney and Armory(?). Near Eli Whitney Park. Across the street from big silver building. Something to check out since I’ve had little luck spotting hackberries while just keeping my eye out.

5/22/12

I am going to try to PCR amplify a chorismate mutase gene from Pachypsylla venusta genomic DNA. RNA seq data from P. venusta bacteriome tissue suggests that this could be an example of endosymbiotic gene transfer. It is tempting to hypothesize that the psyllids have partitioned the phenylalanine biosynthesis pathway between this (nuclear encoded?) gene and the divergent prephenate dehydratase that appears to be retained in the genome. But it appears to belong to the AroQ-gamma (check that?) family of chorismate mutases and therefore might be involved in host plant interactions (as in root knot and cyst nematodes).

Using two different forward and reverse primers in all 4 possible combinations. Plus a bacterial rRNA primer pair as a positive control. Two different P. ven templates.

|  |  |  |  |
| --- | --- | --- | --- |
| **Fwd Primer** | **Rev Primer** | **Templates** | **# rxns** |
| CM\_F1 | CM\_R1 | Pachypyslla venusta 10/26/11; Pachypsylla venusta 31.1 1/12/12; dH2O | 3 |
| CM\_F1 | CM\_R2 | Pachypyslla venusta 10/26/11; Pachypsylla venusta 31.1 1/12/12; dH2O | 3 |
| CM\_F2 | CM\_R1 | Pachypyslla venusta 10/26/11; Pachypsylla venusta 31.1 1/12/12; dH2O | 3 |
| CM\_F2 | CM\_R2 | Pachypyslla venusta 10/26/11; Pachypsylla venusta 31.1 1/12/12; dH2O | 3 |
| 16S\_fwd | 16S\_rev | Pachypyslla venusta 10/26/11; Pachypsylla venusta 31.1 1/12/12; dH2O | 3 |

|  |  |  |
| --- | --- | --- |
|  |  | **Mult Fact** |
| **Reagent** | **Vol per rxn (ul)** | **20** |
| Template | 1 |  |
| 10x Buffer | 1.5 | 30 |
| 25 mM MgCl2 | 0 | 0 |
| 2.5 mM dNTPs | 1.2 | 24 |
| Fwd Primer (10 uM) | 0.75 |  |
| Rev Primer (10 uM) | 0.75 |  |
| Taq (5 U/ul) | 0.15 | 3 |
| dH2O | 9.65 | 193 |
| **Total Vol** | **15** | **250** |
|  |  |  |
| Cocktail Vol (per rxn) | Mult Factor |  |
| 12.5 | 3.5 | 43.75 |
| Volume per primer | 2.625 |  |

|  |  |  |
| --- | --- | --- |
| **Temp** | **Time** | **Cycle #** |
| 94 deg | 2 min |  |
| 94 deg | 15 sec |  |
| 51 deg | 15 sec | 40 |
| 72 deg | 1 min |  |
| 72 deg | 5 min |  |
| 4 deg | Hold |  |

Ran 1% agarose TBE gel. 100 V for 25 min.

Loading order:

Top tier. Ladder (1 kb plus 1/10x, 5 ul), Samples 1-9 (3 ul each).

Bottom tier. Ladder. Samples 10-15. Ladder

Results: See 20120522\_PCR.TIF in photosAndGelPics directory.

Amplification from all primer pairs. Reactions involving primer CM\_F2 seem to have expected product size around 300 bp. In contrast, reactions involving CM\_F1 have strongest band of ~2.5 kb. Much larger than expectation of ~550 bp. But there are also some other bands (more so in the first P. venusta samples which is a pool of 8 individuals. Try repeating CM\_F1/CM\_R1 PCR with longer extension time, higher annealing temp and larger volume (in case I need many reactions for sequencing.

|  |  |  |  |
| --- | --- | --- | --- |
| **Fwd Primer** | **Rev Primer** | **Templates** | **# rxns** |
| CM\_F1 | CM\_R1 | Pachypyslla venusta 10/26/11; Pachypsylla venusta 31.1 1/12/12; dH2O | 3 |

|  |  |  |
| --- | --- | --- |
|  |  | **Mult Fact** |
| **Reagent** | **Vol per rxn (ul)** | **4** |
| Template | 1 |  |
| 10x Buffer | 2.5 | 10 |
| 25 mM MgCl2 | 0 | 0 |
| 2.5 mM dNTPs | 2 | 8 |
| Fwd Primer (10 uM) | 1.25 | 5 |
| Rev Primer (10 uM) | 1.25 | 5 |
| Taq (5 U/ul) | 0.25 | 1 |
| dH2O | 16.75 | 67 |
| **Total Vol** | **25** | **96** |

|  |  |  |
| --- | --- | --- |
| **Temp** | **Time** | **Cycle #** |
| 94 deg | 2 min |  |
| 94 deg | 15 sec |  |
| 56 deg | 15 sec | 40 |
| 72 deg | 2 min |  |
| 72 deg | 5 min |  |
| 4 deg | Hold |  |

Note that all the “normal” thermal cyclers (Eppendorf Master Cycler pro S) were taken, so I had to run this one on one of the older MasterCycler models.

5/23/12

Run out second batch of PCR from yesterday. 1% agarose TBE gel. 100 V for 30 min.

Loading order:

1. Ladder 1 kb plus (1/10x) – 5 ul
2. PCR sample 1 – 4ul
3. PCR sample 2 – 4ul
4. PCR sample 3 – 4ul
5. PCR sample 1 from yesterday’s first batch for comparison – 4ul

See gel image: 20120523\_PCR.TIF

Results: Increased annealing temp seems to have cleaned up the first template. Two clear bands around 2.5 and 3.5 kb. Other faint bands seem to have disappeared. But the other template (31.1 single individual), which was cleaner last time is completely gone.

Try amplifying 31.1 one more time with 53 degree annealing temp. Also include other reactions for P. celtidis and P. pallida. To conserve 31.1 DNA, I will only use it for the first reaction. I will use the 10/26/11 P. venusta samples for the other 3 reactions (as a positive control).

|  |  |  |  |
| --- | --- | --- | --- |
| **Fwd Primer** | **Rev Primer** | **Templates** | **# rxns** |
| CM\_F1 | CM\_R1 | Pachypsylla venusta 31.1, Pachypsylla pallida (10/26/11), Pachypsylla celtidis? (whole #2; 8/11/11), dH2O | 4 |
| CM\_F1 | CM\_R2 | Pachypsylla venusta (10/26/12), Pachypsylla pallida (10/26/11), Pachypsylla celtidis? (whole #2; 8/11/11), dH2O | 4 |
| CM\_F2 | CM\_R1 | Pachypsylla venusta (10/26/12), Pachypsylla pallida (10/26/11), Pachypsylla celtidis? (whole #2; 8/11/11), dH2O | 4 |
| CM\_F2 | CM\_R2 | Pachypsylla venusta (10/26/12), Pachypsylla pallida (10/26/11), Pachypsylla celtidis? (whole #2; 8/11/11), dH2O | 4 |

|  |  |  |
| --- | --- | --- |
|  |  | **Mult Fact** |
| **Reagent** | **Vol per rxn (ul)** | **20** |
| Template | 1 |  |
| 10x Buffer | 2.5 | 50 |
| 25 mM MgCl2 | 0 | 0 |
| 2.5 mM dNTPs | 2 | 40 |
| Fwd Primer (10 uM) | 1.25 |  |
| Rev Primer (10 uM) | 1.25 |  |
| Taq (5 U/ul) | 0.25 | 5 |
| dH2O | 16.75 | 335 |
| **Total Vol** | **25** | **430** |
|  |  |  |
| Cocktail Vol (per rxn) | Mult Factor |  |
| 21.5 | 4.5 | 96.75 |
| Volume per primer | 5.625 |  |

|  |  |  |
| --- | --- | --- |
| **Temp** | **Time** | **Cycle #** |
| 94 deg | 2 min |  |
| 94 deg | 15 sec |  |
| 53 deg | 15 sec | 40 |
| 72 deg | 2 min |  |
| 72 deg | 5 min |  |
| 4 deg | Hold |  |

Run gel with above PCR products. 1% TBE agarose. 100 V for 30 min.

Loading order:

Top tier. Ladder (5ul). Samples 1-8 (4ul)

Bottom tier. Ladder (5 ul). Samples 9-15 (4 ul). Skipped the last neg control (sample 16), b/c bottom comb only had 8 wells.

Results:

Consistent amplification for both P. venusta and P. pallida, but no amplication for P. celtidis (no positive control that this DNA works with anything).

Large double bands for both P. venusta primer pair 2 and P. pallida primer pair 1 (remember that the 10/26/11 preps were pooled from multiple individuals.

Try sequencing the large products (or parts thereof)…

|  |  |
| --- | --- |
| Primer | Template |
| CM\_F1 | Pachypsylla venusta 31.1 CM\_F1/CM\_R1 well 1 |
| CM\_R1 | Pachypsylla venusta 31.1 CM\_F1/CM\_R1 well 1 |
| CM\_F2 | Pachypsylla venusta 31.1 CM\_F1/CM\_R1 well 1 |
| CM\_R2 | Pachypsylla venusta 31.1 CM\_F1/CM\_R1 well 1 |
| CM\_F1 | Pachypsylla pallida CM\_F1/CM\_R2 well 6 |
| CM\_F2 | Pachypsylla pallida CM\_F1/CM\_R2 well 6 |
| CM\_R2 | Pachypsylla pallida CM\_F1/CM\_R2 well 6 |
| CM\_F2 | Pachypsylla pallida CM\_F1/CM\_R1 well 2 |

\*\* can’t use CM\_\_R1 on the pallida CM\_F1/CM\_R2 product, so try to get this end with CM\_F2 against the CM\_F1/CM\_R1 product (two bands in that one).

6/4/12

Replaced acetone and EtOH in the preserved psyllid specimens from 5/21/12.

6/14/12

Performing PCR on Bemisia tabaci to address a couple issue involving assembly ambiguities and potential recombination in the Portiera genome assembly.

|  |  |  |  |
| --- | --- | --- | --- |
| **Fwd Primer** | **Rev Primer** | **Templates** | **# rxns** |
| prfA\_F | prfA\_R | Bemisia tabaci #1 (11/11/11) | 1 |
| prfA\_F | prfB\_R | Bemisia tabaci #1 (11/11/11) | 1 |
| prfB\_F | prfA\_R | Bemisia tabaci #1 (11/11/11) | 1 |
| prfB\_F | prfB\_R | Bemisia tabaci #1 (11/11/11) | 1 |
| Port\_rep\_F1 | Port\_rep\_R1 | Bemisia tabaci #1 (11/11/11) | 1 |
| Port\_rep\_F1 | Port\_rep\_R2 | Bemisia tabaci #1 (11/11/11) | 1 |
| Port\_rep\_F2 | Port\_rep\_R1 | Bemisia tabaci #1 (11/11/11) | 1 |
| Port\_rep\_F2 | Port\_rep\_R2 | Bemisia tabaci #1 (11/11/11) | 1 |

|  |  |  |
| --- | --- | --- |
|  |  | **Mult Fact** |
| **Reagent** | **Vol per rxn (ul)** | **10** |
| Template | 0.1 | 1 |
| 10x Buffer | 1.5 | 15 |
| 25 mM MgCl2 | 0 | 0 |
| 2.5 mM dNTPs | 1.2 | 12 |
| Fwd Primer (10 uM) | 0.75 |  |
| Rev Primer (10 uM) | 0.75 |  |
| Taq (5 U/ul) | 0.15 | 1.5 |
| dH2O | 10.55 | 105.5 |
| **Total Vol** | **15** | **135** |

|  |  |  |
| --- | --- | --- |
| **Temp** | **Time** | **Cycle #** |
| 94 deg | 2 min |  |
| 94 deg | 15 sec |  |
| 54 deg | 15 sec | 40 |
| 72 deg | 1 min |  |
| 72 deg | 5 min |  |
| 4 deg | Hold |  |

Note: Something funny happened with this run. I started it on the thermal cycler before heading to lab meeting. But when I returned 1.5 hours later, it said it had only started ~20 min ago, and it was only on cycle 7. While the initial batch continued to run, I just mixed up a new set of the same reactions. The corresponding strip tubes were labeled with a number 2 on the first tube. I will run both sets out on a gel.

1% agarose TBE gel. 120V for 30 min.

Loading order.

1. Ladder. 1/10x 1 kb plus. 5 ul

2. PCR samples 1-16 (two duplicate patches of 8. See above).

3. Ladder. 1/10x 1 kb plus. 5 ul

Gel image saved in photosAndGelPics directory.

Note that gel image quality is not great. I somehow managed to save the wrong image on the first take, so it had to be pulled from the gel graveyard and re-imaged 30 min later.

Results: amplification for all loci. Both sets, although the first set looks to have more non-specific product. Product sizes look to be around expectations, ~400 bp for prfA/B and ~600 for Port\_rep. Try sequencing each pair.

6/15/12

Setting up sequencing reactions for PCR products from yesterday. Only use batch 2, because, there was less non-specific product.

16 reactions (= each forward and reverse for each of the 8 PCR products).

3 ul of PCR product for each template. 12 ul total volume. Exo-CIP clean up (see 2/9/12).

|  |  |  |  |
| --- | --- | --- | --- |
| Position | Template | Size (bps) | Primer |
| 1 | Bta\_prfA\_F\_prfA\_R | 500 | prfA\_F |
| 2 | Bta\_prfA\_F\_prfB\_R | 500 | prfA\_F |
| 3 | Bta\_prfB\_F\_prfA\_R | 500 | prfB\_F |
| 4 | Bta\_prfB\_F\_prfB\_R | 500 | prfB\_F |
| 5 | Bta\_Port\_rep\_F1\_R1 | 500 | Port\_rep\_F1 |
| 6 | Bta\_Port\_rep\_F1\_R2 | 500 | Port\_rep\_F1 |
| 7 | Bta\_Port\_rep\_F2\_R1 | 500 | Port\_rep\_F2 |
| 8 | Bta\_Port\_rep\_F2\_R2 | 500 | Port\_rep\_F2 |
| 9 | Bta\_prfA\_F\_prfA\_R | 700 | prfA\_R |
| 10 | Bta\_prfA\_F\_prfB\_R | 700 | prfB\_R |
| 11 | Bta\_prfB\_F\_prfA\_R | 700 | prfA\_R |
| 12 | Bta\_prfB\_F\_prfB\_R | 700 | prfB\_R |
| 13 | Bta\_Port\_rep\_F1\_R1 | 700 | Port\_rep\_R1 |
| 14 | Bta\_Port\_rep\_F1\_R2 | 700 | Port\_rep\_R2 |
| 15 | Bta\_Port\_rep\_F2\_R1 | 700 | Port\_rep\_R1 |
| 16 | Bta\_Port\_rep\_F2\_R2 | 700 | Port\_rep\_R2 |

Submitted for Sanger sequencing to Yale Science Hill facility. Order #140778.

6/20/2012

Went to the Yale main campus greenhouse (“Marsh Botanic Gardens”) with Kim Hammond to collect whiteflies in hopes of finding the “greenhouse whitefly”, Trialeurodes vaporariorum. They have an outbreak of whiteflies, but the species is currently unknown.

We collected adults with a simple mouth aspirator (a glass pipette connected to plastic tubing with plastic mesh as the connecting point to keep insects in the pipette). We simply sucked them off the leaves and blew them into 15 ml conicals through a small hole melted in the cap. Adults were collected into 4 different tubes off multiple different host plants: (labeled as Duranta “sapphire swirl”, Lantana hybrid, Gossypium sp., Clerodendrum speciosissimum, and one heavily infected species that was unlabeled). Tubes were not necessarily restricted to one host plant.

We also collected some leaves with eggs and nymphs that Kim is going to try to get growing in “culture” (i.e., on contained plants) in the lab.

The tubes were kept in a cooler on cold packs for transport. In the lab, I immediately filled two with EtOH and kept the other two on ice.

Looked at whiteflies under dissecting scope. We expect that the most likely species to find in the greenhouse would be Bemisia tabaci or Trialeurodes vaporariorum.

Resources for identification.

USDA Whitefly Knowledge Base. See ID Key:

<http://entnemdept.ufl.edu/fasulo/whiteflies/wfly0002.htm>

<http://www.ipm.ucdavis.edu/PMG/C783/m783bpwhitefly.html>

My best guess is that these are Bemisia and not Trialeurodes based on the following:

--I can’t see a fork in the wing venation as expected for Trialeurodes

--Wings are often angled up in “tent-like” fashion over the body, and it is often possible to see the body between the wings. This seems to be more characteristic of Bemisia than Trialeurodes.

--There is a good deal of color variation, but there is a clear yellow color to most bodies. Again this is more consistent with Bemisia than Trialeurodes, which should be more white.

Nancy all took a look at these. She saw some that appear to have a branch in the wing vein. But felt the other characeristics were more Bemisia like. Also, she didn’t see a big difference between individuals with branched vs. unbranched vein.

I pooled all four tubes in a single 15 ml conical. Stored in EtOH at -20 C. Top shelf of “Bubbles” in Styrofoam tube rack.

6/21/12

Qiagen Blood and Tissue DNeasy Kit extractions from whiteflies collected yesterday (probably Bemisia?).

Two preps. One with many insects taken by drawing up 200 ul of from the settled mass at bottom of tube in EtOH. The rate of pullup isn’t great, so there was still lots of EtOH. Very roughly, there may be around 100 whiteflies (adults). The other prep is from a single individual. I washed it once in 1 ml EtOH, but keep in mind that it was taken from a larger EtOH pool of individuals. For both samples, I aspirated off as much samples as possible before adding buffer ATL.

Followed standard Qiagen protocol for animal tissue. 1.5 hr incubation with buffer ATL. I did not include the optional RNase step. Eluted the sample with one individual in 50 ul Buffer AE and the sample with many individuals in 100 ul. But I forgot to change the centrifuge back down from max to 6000g for the final spin. I think that’s fine. But just in case, I did a second elution with 50 ul each. These are labeled as “Temp Single 2” and “Temp Multi 2”.

6/25/12

Try cloning and sequencing chorismate mutase PCR amplifications from Pachypsylla venusta and P. pallida (see 5/22/13 to 5/23/12). I did a previous batch of sequencing for some of these (see 5/23/12). These didn’t work well but did provide some evidence of a spliceosomal intron. The problem may have been a mixture of competing templates and low template concentration.

I will use the following two PCR samples. I am also doing this in parallel with Ingrid, who is processing one of her own samples.

P. venusta 31.1 CM\_F1/CM\_R1. 5/22/12 (batch 1) reaction #2.

P. pallida CM\_F1/CM\_Rq. 5/23/12 PCR, reaction #2 (note that this has two strong bands, ~1.5kb and ~2.5 kb).

Incubate 10 ul of each PCR volumes at 72 C for 5 min with 0.1 ul of additional Taq polymerase.

EtOH preicipitate samples

Add 1 ul 3 M NaOAC and 25 ul EtOH. In freezer from 11:15am to 12:35pm.

Spun at max speed and 4 C for 10 min. Washed in 500 ul cold EtOH. Spun again at max speed for 5 min. Removed supernantant and air dried. Note that I really couldn’t see much of a pellet.

Dissolved pellets in 4 ul TE. Quantified with Qubit dsDNA BR kit.

P. venusta CM PCR: 20.3 ng/ul

P. pallida CM PCR: 11.5 ng/ul

Ingrid made LB + 100 ug/ml Amp plates following standard lab recipe (see 1/30/12). Left at room temp to dry overnight.

Will continue with cloning tomorrow.

6/26/12

Continuing with cloning from yesterday. pGEM-T Easy cloning kit (Promega).

Set up two ligation reactions (one for each of the processed PCR products from yesterday)

5 ul 2x Buffer

1 ul pGEM-T Easy vector

1 ul dH2O

2 ul PCR product (see yesterday)

1 ul T4 Ligase

Incubate overnight at 4 C, starting at 9:30 am.

Aliquoted Invitrogen DH5-alpha chemically competent cells for transformation tomorrow. Thawed one tube on ice. Then transferred 50 ul aliquots to eppendorf tubes that were pre-chilled in an EtOH-dry ice path. Quickly returned to bath and then transferred to -80 C.

PCR to eventually sequence and identify whiteflies collected in Yale greenhouse on 6/20/12.

|  |  |  |  |
| --- | --- | --- | --- |
| **Fwd Primer** | **Rev Primer** | **Templates** | **# rxns** |
| wtfly\_COI\_F | wtfly\_COI\_R | Unknown Whitefly Multi (6/21/12); Unknown Whitefly Single (6/21/12); B. tabaci #2 (11/11/11); dH2O | 4 |

|  |  |  |
| --- | --- | --- |
|  |  | **Mult Fact** |
| **Reagent** | **Vol per rxn (ul)** | **5** |
| Template | 0.1 |  |
| 10x Buffer | 1.5 | 7.5 |
| 25 mM MgCl2 | 0 | 0 |
| 2.5 mM dNTPs | 1.2 | 6 |
| Fwd Primer (10 uM) | 0.75 | 3.75 |
| Rev Primer (10 uM) | 0.75 | 3.75 |
| Taq (5 U/ul) | 0.15 | 0.75 |
| dH2O | 10.55 | 52.75 |
| **Total Vol** | **15** | **74.5** |

|  |  |  |
| --- | --- | --- |
| **Temp** | **Time** | **Cycle #** |
| 94 deg | 2 min |  |
| 94 deg | 15 sec |  |
| 54 deg | 15 sec | 40 |
| 72 deg | 1 min |  |
| 72 deg | 5 min |  |
| 4 deg | Hold |  |

Ran 1% agarose TBE gel. 110 V for ~45 min (not sure of exact time).

Loading order:

1. 1 kb plus ladder 1/10x – 5 ul

2-5. PCR samples 1-4. 3 ul each

6-8. Genomic DNA samples corresponding to templates for PCR samples 1-3.

9. 1 kb plus ladder 1/10x – 5 ul

Results: Amplification for all 3 templates. A little bit of a higher MW band, but looks pretty good. DNA prep from single insect is almost undetectable on gel, but clearly it was enough for PCR. RNA contamination in DNA preps is substantial (as expected).

Proceed with sequencing of COI from the two unknown samples:

* 1. ul dH2O

3 ul PCR product

* 1. ul ExoI
  2. ul CIP

Incubate for 15 min at 37 and 15 min at 80.

Add 0.5 ul of primer.

|  |  |  |  |
| --- | --- | --- | --- |
| Position | Template | Size (bps) | Primer |
| 1 | unk\_multi | 600 | wtfly\_COI\_F |
| 2 | unk\_multi | 600 | wtfly\_COI\_R |
| 3 | unk\_single | 600 | wtfly\_COI\_F |
| 4 | unk\_single | 600 | wtfly\_COI\_R |

Submitted as order # 141174 to Yale sequencing center at Science Hill.

6/27/12

Continuing with PCR cloning from yesterday. Following same protocol as before. See 1/31/12.

LB-Amp plates from Ingrid’s batch 6/25/12. Coated with 100 ul IPTG (100 mM) and 50 ul of x-gal (20 mg/ml).

2 ul of yesterday’s ligations + 50 ul of yesterday’s cell aliquots (thawed in ice bath). Gently mixed followed by 20 min on ice. 45 sec heat shock at 42 C. 2 min on ice. Add 950 ul SOC.

Incubate for 1.5 hrs on shaker. 37 C. 150 rpm.

Plated two different volumes (20 ul and 200 ul) onto prepared plates and incubated overnight at 37 C starting around noon. Remaining culture volume stored temporarily at 4 C in case we need to plate more.

6/28/12

Checked on cloning plates from yesterday. Lots of colonies. Maybe 100-200 on the 20 ul plates and >1000 on the 200 ul plates. The % of blue colonies is very high (easily over 50%), but there are still plenty of white colonies to screen.

|  |  |  |  |
| --- | --- | --- | --- |
| **Fwd Primer** | **Rev Primer** | **Templates** | **# rxns** |
| SP6 | T7 | Chorismate mutase P. venusta colonies 1-7 + surface control; Chorismate mutase P. pallida colonies 1-7 + dH2O control; | 16 |

Set up colony PCR:

|  |  |  |
| --- | --- | --- |
|  |  | **Mult Fact** |
| **Reagent** | **Vol per rxn (ul)** | **18** |
| Template | 0 |  |
| 10x Buffer | 2.5 | 45 |
| 25 mM MgCl2 | 0 | 0 |
| 2.5 mM dNTPs | 2 | 36 |
| Fwd Primer (10 uM) | 1.25 | 22.5 |
| Rev Primer (10 uM) | 1.25 | 22.5 |
| Taq (5 U/ul) | 0.25 | 4.5 |
| dH2O | 17.75 | 319.5 |
| **Total Vol** | **25** | **450** |

|  |  |  |
| --- | --- | --- |
| **Temp** | **Time** | **Cycle #** |
| 94 deg | 2 min |  |
| 94 deg | 15 sec |  |
| 51 deg | 15 sec | 40 |
| 72 deg | 2 min 30 sec |  |
| 72 deg | 10 min |  |
| 4 deg | Hold |  |

Run 1% agarose TBE gel. 110 V for 50 minutes.

Loading order: 16 PCR products (3 ul each) in order (see table above) with 5 ul 1/10x 1 kb ladder on either side.

Results: see 20120628\_PCR.TIF in PhotosAndGelPics directory. Strong amplification from all colonies. But the sizes are generally too small. For P. venusta, all the amplicons are way too small. There is one around 220 bp in 6 of 7 colonies (actual insert or just closed vector? I should check the positions of the primers). The other (#5) is around 500 bp. Clearly an insert, but way short of expected size, which was > 2 kb. In all cases, each band has a higher MW competing band. I’m not sure how to explain this, but I’ve seen it before. In the past, using internal primers has worked out fine for sequencing, but I should investigate to see what’s goin on here.

For P. pallida, 3 of the colonies (#3,4,5) have very small amplicons. Colonies 1, 2, 6, and 7 appear to have an insert around 1.5-2kb. I imagine this is the smaller of the doublet, see in the original PCR gel. This could be sequenced, but there seems to be significant discrimination against the large (2+ kb) products. Perhaps, I will screen a substantially larger number of colonies.

7/3/12

PCR to amplify and sequence carotenoid genes in Trialeurodes vaporarium. The unknown whitefly DNA extracted (and COI sequenced) turned out to be the common greenhouse whitefly T. vaporarium, not B. tabaci. See 6/21/12.

|  |  |  |  |
| --- | --- | --- | --- |
| **Fwd Primer** | **Rev Primer** | **Templates** | **# rxns** |
| Ta\_crtI\_F | Ta\_crtY\_R1 | unknown whitefly single (6/21/12); unknown whitefly multi (6/21/12); dH2O | 3 |
| Ta\_crtI\_F | Ta\_crtY\_R2 | unknown whitefly single (6/21/12); unknown whitefly multi (6/21/12); dH2O | 3 |

|  |  |  |
| --- | --- | --- |
|  |  | **Mult Fact** |
| **Reagent** | **Vol per rxn (ul)** | **8** |
| Template | 0.1 |  |
| 10x Buffer | 2.5 | 20 |
| 25 mM MgCl2 | 0 | 0 |
| 2.5 mM dNTPs | 2 | 16 |
| Fwd Primer (10 uM) | 1.25 |  |
| Rev Primer (10 uM) | 1.25 |  |
| Taq (5 U/ul) | 0.25 | 2 |
| dH2O | 17.65 | 141.2 |
| **Total Vol** | **25** | **179.2** |
|  |  |  |
| Cocktail Vol (per rxn) | Mult Factor |  |
| 22.4 | 3.5 | 78.4 |
| Volume per primer | 4.375 |  |

|  |  |  |
| --- | --- | --- |
| **Temp** | **Time** | **Cycle #** |
| 94 deg | 2 min |  |
| 94 deg | 15 sec |  |
| 51 deg | 15 sec | 40 |
| 72 deg | 3 min 30 sec |  |
| 72 deg | 10 min |  |
| 4 deg | Hold |  |

Run 1% agarose TBE gel at 120 V for 40 min.

6 Samples loaded in order indicated by above reaction table (3 ul of each PCR product), plus 5 ul of 1/10x 1 kb plus ladder on either side.

Results:

See 20120703\_PCR.TIF in photosAndGelPics directory.

Gel image quality is not good. Even ladder bands are faint relative to background. Getting this image required opening the aperature up fully and increasing exposure to 8 secs. I’m not sure what happened here.

Regardless, faint bands just above 2kb are visible in the F-R1 primer pair reactions. Much fainter with the single template, but the multi-template has a long smear surrounding the band on both sides. Nothing besides primer dimer for the F-R2 pair.

I need to double check, but I believe the expected product size (assuming no large deletions relative to Bemisia) would be >3 kb. So it is not clear that this is the target product.

Next up, try amplifying with a shorter extension time (2 min). Use the single PCR product as template for re-amplification. Also amplify from original DNAs again, and try a couple reactions with internal primers.

|  |  |  |  |
| --- | --- | --- | --- |
| **Fwd Primer** | **Rev Primer** | **Templates** | **# rxns** |
| Ta\_crtI\_F | Ta\_crtY\_R1 | Single F-R1 PCR product from yesterday; unknown whitefly single (6/21/12); unknown whitefly multi (6/21/12); dH2O | 4 |
| Ta\_crtB\_F | Ta\_crtY\_R1 | Single F-R1 PCR product from yesterday; unknown whitefly single (6/21/12); unknown whitefly multi (6/21/12); dH2O | 4 |
| Ta\_crtB\_F | Ta\_crtY\_R2 | Single F-R1 PCR product from yesterday; unknown whitefly single (6/21/12); unknown whitefly multi (6/21/12); dH2O | 4 |

7/4/12

New PCR following up on yesterday

|  |  |  |
| --- | --- | --- |
|  |  | **Mult Fact** |
| **Reagent** | **Vol per rxn (ul)** | **15** |
| Template | 0.1 |  |
| 10x Buffer | 2.5 | 37.5 |
| 25 mM MgCl2 | 0 | 0 |
| 2.5 mM dNTPs | 2 | 30 |
| Fwd Primer (10 uM) | 1.25 |  |
| Rev Primer (10 uM) | 1.25 |  |
| Taq (5 U/ul) | 0.25 | 3.75 |
| dH2O | 17.65 | 264.75 |
| **Total Vol** | **25** | **336** |
|  |  |  |
| Cocktail Vol (per rxn) | Mult Factor |  |
| 22.4 | 4.5 | 100.8 |
| Volume per primer | 5.625 |  |

|  |  |  |
| --- | --- | --- |
| **Temp** | **Time** | **Cycle #** |
| 94 deg | 2 min |  |
| 94 deg | 15 sec |  |
| 51 deg | 15 sec | 40 |
| 72 deg | 2 min |  |
| 72 deg | 5 min |  |
| 4 deg | Hold |  |

7/5/12

Run gel of PCR products. 1% agarose TBE. 100 V for 30 min.

Loading order.

Top tier: ladder (5 ul 1/10x 1 kb plus), first 8 PCR products (3 ul each), ladder

Bottom tier: last 4 PCR products, ladder, PCR #15 from 6/28/12 (as control for gel image quality because of problems experienced with last gel).

Results: Initial gel staining looked like previous one—very poor signal—so I spiked in more EtBr to the bath, which made a huge difference.

Not much amplification. The multi sample has a strong band around 2 kb for the F-R1 pair, but also some lower MW, faint bands. The single sample has two bands corresponding to the 2 kb and one of the faint bands in the multi sample. Other reactions failed.

Try Sanger sequencing the “unknown” whitefly (= Trialeurodes vaporarium) multi F-R1 PCR product. With 5 different primers. The original PCR primers might have problems with the competing faint bands. And it’s not clear that this is the right product given its small size. Plus polymorphic indels could be present given the pooled source of the DNA sample. So, all in all, these 5 seqs might not be destined for success. But it seems like the cheapest/quickest way to assess wheterh we have the correct product.

|  |  |  |  |
| --- | --- | --- | --- |
| Position | Template | Size (bps) | Primer |
| 1 | Tvap\_multi\_F-R1 | 2000 | Ta\_crtB\_F |
| 2 | Tvap\_multi\_F-R1 | 2000 | Ta\_crtB\_R |
| 3 | Tvap\_multi\_F-R1 | 2000 | Ta\_crtI\_F |
| 4 | Tvap\_multi\_F-R1 | 2000 | Ta\_crtY\_R1 |
| 5 | Tvap\_multi\_F-R1 | 2000 | Ta\_crtY\_R3 |

Submitted to Science Hill DNA sequencing facility: Order #141468

7/9/12

Kim Hammond, Ingrid Rochon, and I Collected whiteflies that were living in our West Campus greenhouse, feeding on fava beans and alfalfa. They potentially are Bemisia tabaci, but this will need to be checked by sequencing. I stored mine (perhaps around 30 individuals) in EtOH at -20 C for future identification/diagnostic sequencing. The rest were used to found a colony in the lab growth chamber. I will get more details from Kim on growing conditions, host plants, etc.

Trying out reverse transcription and qPCR on psyllid RNA samples. I will use two primers pairs to target the chorismate mutase gene, identified by RNA-seq, as well as rpl7 as a reference gene. Rahul Raghavan and Dan Phillips also provided me with some E. coli RNA (DNased and nanodropped at 290 ng/ul) and corresponding primers (groS F and R) to use as a positive control.

Reverse transcription:

iScript Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad).

20 ul reactions:

4 ul iScript reaction mix

1 ul iScript reverse transcriptase

10 ul RNA (diluted in some cases; see below)

5 ul RNase free dH2O (comes with kit)

3 reactions:

--E. coli positive control: Dilute 3 ul of RNA in RNase-free dH2O to a total volume of 10 ul.

--Pachypsylla venusta bacteriome RNA. Sample DBS013 (see 1/3/12). 10 ul undiluted.

--Pachypsylla venusta body RNA. Sample DBS014 (see 1/3/12). 10 ul undiluted (although note this sample was previously diluted 10x).

Master mix (3.5 reaction volumes)

17.5 ul dH2O

14 ul iScript reaction mix

3.5 ul reverse transcriptase

I am not doing conventional RT- controls b/c Rahul informed me that you run out of reaction buffer that way. Instead, I will due a crude version, by just diluting the RNA down to the same extent in dH2O and using that as a template for qPCR. For P. venusta samples, this means a 2-fold dilution (I will do 5 ul RNA + 5 ul dH2O). I will also save 1.5 ul of RNA undiluted in case I want to do QC on the RNA later on without thawing the main tube. For the E. coli RNA, I will dilute 3 ul RNA in 17 ul dH2O.

Incubated actual RT reactions on thermal cycler (but not the pseudo RT- controls):

42 C for 30 min

85 C for 5 min

hold at 4 C.

Note that I did not do a hot start so the samples sat on a unheated block for a couple minutes while the lit heated. After the reaction, I transferred the three cDNA samples to individual eppendorf tubes and stored at -20 C along with the RNA dilutions and small aliquots. I returned the main RNA stocks to the -80 C.

7/10/12

Plan for testing qPCR:

KAPA SYBR FAST qPCR Kit.

20 ul reactions:

10 ul 2x Master mix

0.4 ul F primer (10 uM)

0.4 ul R primer (10 uM)

1 ul template DNA

8.2 ul dH2O

A total of 25 reactions with 4 different primers pairs. Template cDNAs and RNAs are all from yesterday.

|  |  |  |  |
| --- | --- | --- | --- |
| **Fwd Primer** | **Rev Primer** | **Templates** | **# rxns** |
| CM\_qF1 | CM\_qR1 | P. ven bact cDNA rep1; P. ven bact cDNA rep2; P. ven bod cDNA rep1; P. ven bod cDNA rep2; P. ven bact RNA(no RT); P. ven bod RNA(no RT); dH2O | 7 |
| CM\_qF2 | CM\_qR2 | P. ven bact cDNA rep1; P. ven bact cDNA rep2; P. ven bod cDNA rep1; P. ven bod cDNA rep2; P. ven bact RNA(no RT); P. ven bod RNA(no RT); dH2O | 7 |
| rpl7\_qF | rpl7\_qR | P. ven bact cDNA rep1; P. ven bact cDNA rep2; P. ven bod cDNA rep1; P. ven bod cDNA rep2; P. ven bact RNA(no RT); P. ven bod RNA(no RT); dH2O | 7 |
| groS F | groS R | E. coli cDNA rep1; E. coli cDNA rep2; E. coli RNA (no RT); dH2O | 4 |

Make 28x master mix:

--280 ul of 2x master mix

--229.6 ul dH2O

\*\*\*\*\*\*Note I accidentally reversed these two volumes. They are written properly above, but I added 229.6 of the master mix and 280 ul of dH2O.

Submaster mixes

For Pachypsylla primer pairs:

--136.5 ul of above master mix

--3 ul of each primer

For E. coli primer pair

--81.9 ul of above master mix

--1.8 ul of each primer

Pipet 19 ul of appropriate submaster mixes into wells and add 1 ul of the appropriate template.

Plate: Eppendorf Twin-Tec RT PCR Plates

Seal: Dot Scientific #T393

Seal firmly with paint roller and by hand. Briefly centrifuge (@300 rpm in Groisman lab large eppendorf centrifuge) and then reseal by hand.

Cycling conditions for Eppendorf realplex2 thermal cycler.

--95 C for 3 min

--40 Cycles:

--95 C for 3 sec

--60 C for 20 sec (joint annealing and extension)

+ Melt curve at the end: 60 C to 95 C

Assay program saved in DBS folder on the computer connected to thermal cycler as 20120710\_CM

Plate loading order:

Primer pair 1 (CM\_qF1-CM\_qR1): Row A

Primer pair 2 (CM\_qF2-CM\_qR2): Row B

Primer pair 3 (rpl7\_qF-rpl7\_qR): Row C

Primer pair 4 (groS\_F-groS\_R): Row D

Skipped column 1 because others in the lab have reported problems with reading from those wells. So, the primer pair 1 samples are loaded in wells A2-A8, primer pair 2 samples are loaded in wells B2-B8, etc. Loading order within each row is as indicated in the table above.

Results Summary: Overall, looks to show solid evidence for preferential expression of chorismate mutase in bacteriome. Both primer pairs for this gene show 6 or 7 cycle difference in CT values between bacteriome and body RNA sample. In contrast, reference gene (rpl7) is 1-2 cycles in the opposite direction (all of these are based on threshold of 10000 fluorescence units; see below). Negative controls look pretty good. They come up much later if at all. There is clearly some genomic DNA contamination in the RNA. Notably, the first CM primer pair does not show much amplification in the no RT control, presumably b/c it spans an intron. Melt curves for the main sample all look good with one clear, dominant peak. My only concern is that, for my 3 primer pairs, there is a slight initial bump/upward trend in the curve before it becomes clearly sigmoidal. This is not the case with the E. coli primers, which produce a cleaner S-shape. Because of this there is some noise introduced to the CT estimates at the automatically generated threshold. To reduce this noise, I manually set the threshold to 10000. With this change, all replicate CT values are within 0.5 of each other. Output CT values and the .asy file are saved in the following directory of my local machine:

/Users/drt\_lab/Documents/Yale/MoranLab/projects/psyllidTranscriptomes/Pachypsylla\_venusta/qPCR/runs/20120710\_CM

7/20/12

Nancy brought in a collection of psyllid galls from a large hackberry in Austin, TX. These include petiole galls, leaf blister galls, and leaf star galls. Stored in Ziploc bag at 4 C.

7/23/12

Took a couple pictures under the dissecting scope of the star galls that Nancy brought in (see above) since I hadn’t seen this type before. Nancy also mentioned that their nymphs appear to have more yellow/orange pigment in their bodies than other species. I agree, but this trait also may be plastic. I also remember the Pachypsylla pallida nymphs being quite yellow.

See photosAndGelPics/20120723\_hackberryLeafStarGalls directory for the gall pics

Collecting samples for DNA extractions.

Trialeurodes vaporarium—

Collected ca. 30 adult whiteflies from a colony set up with individuals collected from Marsh greenhouse on 6/20/12. This colony has been growing on fava bean plants in the walk in growth chamber (kept at 20 C).

Hackberry petiole gall psyllids—

Collected psyllids from hackberry petiole galls collected in Austin by Nancy (see 7/20/12). I opened up two galls. Most of the nymphs were very small (probably 2nd instar). But a couple were dramatically larger. Given my previous observations of multiple species inhabiting these galls, I wonder if these are the different species. So I collected 10 small nymphs and 2 large nymphs, which will be extracted in separate pools.

New whiteflies from west campus greenhouse (species unknown)—

See 7/9/12. I will do extractions from a single individual as well as the remaining pool of ~30 individuals that have been stored in EtOH since initial collection.

DNA extractions with Qiagen Blood and Tissue DNeasy kit. Following animal tissue protocol. Added 4 ul of Qiagen RNase for the Trialeurodes sample only. Did not include optional RNase step for other 4 samples. Incubated at 56 C for 1 hour with a couple of vortexes to mix up the samples during this time.

Eluted in 60 ul buffer AE. Did a second elution for the the T. vaporarium sample only in 100 ul.

Sample labels:

Trialuerodes vaporarium 🡪 T. vap (elute 1 or elute 2) DNA

Petiole galls 🡪 HPGP (big or small) DNA

West campus greenhouse whiteflies 🡪 WC wtfly (multi or single) DNA

Qubit dsDNA BR kit to quantify these extractions:

T. vap elute 1: 16.8 ng/ul

T. vap elute 2: 7.6 ng/ul

HPGP small: 10.9 ng/ul

HPGP big: 7.6 ng/ul

WC wtfly multi: 27.6 ng/ul

WC wtfly single: below detection threshold

PCR to check identities on this round of DNA extractions

|  |  |  |  |
| --- | --- | --- | --- |
| **Fwd Primer** | **Rev Primer** | **Templates** | **# rxns** |
| Pv\_COI\_F | Pv\_COI\_R | HPGP small; HPGP big; dH2O | 3 |
| wtfly\_COI\_F | wtfly\_COI\_R | T. vap elute2; WC wtfly multi; WC wtfly single; dH2O | 4 |

|  |  |  |
| --- | --- | --- |
|  |  | **Mult Fact** |
| **Reagent** | **Vol per rxn (ul)** | **10** |
| Template | 0.1 |  |
| 10x Buffer | 1.5 | 15 |
| 25 mM MgCl2 | 0 | 0 |
| 2.5 mM dNTPs | 1.2 | 12 |
| Fwd Primer (10 uM) | 0.75 |  |
| Rev Primer (10 uM) | 0.75 |  |
| Taq (5 U/ul) | 0.15 | 1.5 |
| dH2O | 10.55 | 105.5 |
| **Total Vol** | **15** | **134** |
|  |  |  |
| Cocktail Vol (per rxn) | Mult Factor |  |
| 13.4 | 4.5 | 60.3 |
| Volume per primer | 3.375 |  |

|  |  |  |
| --- | --- | --- |
| **Temp** | **Time** | **Cycle #** |
| 94 deg | 2 min |  |
| 94 deg | 15 sec |  |
| 54 deg | 15 sec | 40 |
| 72 deg | 1 min |  |
| 72 deg | 5 min |  |
| 4 deg | Hold |  |

Run DNA extractions and PCR products on a 1% agarose TBE gel. 110 V for 30 min.

Loading order:

Top tier: 1 kb plus ladder 1/10 x – 5ul, PCR products 1-7 ordered as indicated in above table (3 ul each)

Bottom tier: 1 kb plus ladder 1/10 x – 5 ul, DNA extractions (T. vap elute1, T. vap elute2, HPGP small, HPGP big, WC wtfly multi, WC wtfly multi), 2 ul each.

Results: COI amplification worked for all samples except the WC wtfly single extraction. I probably just didn’t get enough DNA from that one. I can’t see anything on the gel and it was undetectable with Qubit. The DNA extractions otherwise look OK. There is definitely a good deal of degradation in the T. vap sample. In fact, the second elution appears to have similar concentration of HMW DNA but is missing a lot of the LMW stuff that was preferentially eluted in the first pass.

Proceed with COI sequencing to check identities…

Exo-CIP each sample (see 2/9/12) and submit to Science hill sequencing facility (order # 142116).

|  |  |  |  |
| --- | --- | --- | --- |
| Position | Template | Size (bps) | Primer |
| 1 | HPGPsmall | 700 | Pv\_COI\_F |
| 2 | HPGPsmall | 700 | Pv\_COI\_R |
| 3 | HPGPbig | 700 | Pv\_COI\_F |
| 4 | HPGPbig | 700 | Pv\_COI\_R |
| 5 | Tvap | 700 | wtfly\_COI\_F |
| 6 | Tvap | 700 | wtfly\_COI\_R |
| 7 | WC\_wtfly\_multi | 700 | wtfly\_COI\_F |
| 8 | WC\_wtfly\_multi | 700 | wtfly\_COI\_R |

7/31/12

Preparting Trialeurodes vaporarium DNA sample for submission to YCGA for Illumina sequencing. The sample was extracted on 7/23/12. Subsequent COI sequencing (above) confirmed the species identity and did not show any evidence of contamination from other species (e.g., Bemisia).

I combined 50 ul of the “elute 1” sample with 10 ul of the “elute 2” sample to produce ca. 1 ug of DNA in 60 ul. This represented the entirety of the “elute 1” sample.

Sample was submitted as DBS017 for library construction and sequencing in 1/6th of multiplexed 2x75bp HiSeq lane. I provided the following notes/instructions with the submission (#RQ0871):

Per earlier correspondence with John Overton, this sample is to be run in 1/6th of a lane (pooled with samples from other users).

Please target a paired-end insert size of 400+ bp during library construction.

To the extent possible, please minimize the number of PCR cycles in library construction and use a polymerase that is less prone to GC-content bias (e.g., KAPA Bio HiFi).

I am providing 1 ug of DNA in 60 ul (16 ng/ul) and would anticipate the entire sample being used for library construction.

8/10/12

Kim Hammond is retiring the original Trialeurodes vaporarium culture grown on fava beans (two new cultures have been subsequently founded from this colony on a different host plant—alfalfa I believe, but I need to check with Kim on that). She collected a large number of adults from the fava bean culture. I stored these in EtOH at -20 C.

9/18/12

Collected Pachypsylla-galled hackberry leaves (Celtis occidentalis?) from three adjacent trees at the intersection of Whitney and Armory near the border of New Haven and Hamden, CT. All leaves put in one large ziplock bag (not differentiated by tree) and stored at 4 C.

I also e-mailed Quinn McFrederick in Austin, TX. He is going to try to collect and ship Pachypsylla venusta hackberry petiole galls from a tree Nancy found.

Macintosh HD:Users:drt_lab:Desktop:AustinHackberryTree.pdf

9/21/12

Received shipment of hackberry petiole galls from Quinn McFrederick in Austin, TX (see above). Stored in ziploc bag at 4 C.

Preparing for RNA extraction tomorrow, I left a handful of hackberry leaves with nipple galls (collected in New Haven, CT on 9/18/12) in the walk-in growth chamber at 20 C. I will leave these overnight in hopes that gene expression will reflect more natural metabolic conditions.

9/22/12

Performing bacteriome dissections and RNA extractions from the hackberry nipple galls left overnight at 20 C (see above).

I dissected bacteriomes from 24 nymphs (all probably fifth instar) from nipple galls. I only included galls with a single nymph in them. As I understand it, P. celtidismamma should only have one individuals for gall. Presence of multiple individuals suggests presence of the related kleptoparasite. Nevertheless, there was noticeable color variation among individuals. The most common coloration was green with orange wings. But there also individuals that had a more whitish yellow coloring and some that were noticeable pinkish/orange. I will try some DNA extractions from a range of the different color patterns and do some mtDNA sequencing to get a sense of genetic variation.

Dissections were each performed in 100 ul Qiagen RNA Protect Bacterial reagent. Bacteriomes were all pooled into one tube (kept on ice), while the remaining bodies were split into three batches of 8.

RNA extractions were performed as follows:

1. Let samples equilibrate to room temp to eliminate precipitate from RNA Protect.
2. Centrifuge dissected samples in RNA Protect at max speed at room temp for 5 min and remove supernatant.
3. Add 1 ml of Trizol to each sample. This was pipetted directly to bacteriome sample followed by vortexing. For body samples, 0.5 ml of Trizol was added followed by grinding with plastic pestle. I then added the rest of the Trizol and vortexed. All samples were then incubated for 5 min at room temp.
4. Centrifuge for 10 min 2 12000g, 4C.
5. Transfer sample to 5 Prime Phase Lock Heavy Gel tube (pre-spun for 3min at 1500g)
6. Add 200 ul chloroform. Shake vigorously by hand. Incubate 3 min at room temp.
7. Centrifuge for 15 min at 12000g, 4C.
8. Add 1 ul of 5ug/ul glycogen solution (Ambion)
9. Transfer supernatant to clean tube with equal volume (~500 ul) isopropanol. Mix by pipetting/inversion. Incubate at room temp for 10 minutes.
10. Centrifuge for 10 min at 12000g, 4C.
11. Discard supernatant and wash pellet with 1 ml 80% EtOH.
12. Centrifuge for 5 min at 7500g, 4C. Discard supernatant
13. Air dry pellet.
14. Dissolve in 40 ul RNase-free dH2O
15. Incubate on heat block for 5 min at 55 C.
16. Store at -80 C.

Note that the 10 min spin before adding chloroform is new for me. I saw this recommended in some Trizol product literature if you do not want to save the sample for subsequent DNA extraction. Also the addition of glycogen as a carrier is new. I thought it might be helpful with the low concentrations in the bacteriome sample. I also noticed a yellow coloration to the aqueous phase of each of the three body samples. I do not remember this from previous preps. I stored the samples in two separate tubes (35 ul and 5 ul), with smaller one to be used for QC. Tubes are labeled with today’s date as follows:

Bact or Bod1/2/3 Pc [Samp]

9/23/12

Collected more leaves/galls from the same New Haven hackberry trees as the collection on 9/18/12. Stored in ziplock bag at 4 C.

9/24/12

Estimating concentration from RNA extractions on 9/22/12

I used the nanodrop to estimate total nucleic acid concentration (set at RNA-40). I also used to qubit dsDNA BR kit to measure contamination from dsDNA (this assay should not be affected by the presence of RNA). I used 1 ul of sample for both the nanodrop and the qubit tests. I also tried a water sample (40 ul) with 5 ug of glycogen on the nanodrop to see if the presence of glycogen would inflate concentration estimates. But it had very little aborbance (A260 corresponded to less than 1 ng/ul).

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Nanodrop** | | |
| **Qubit (dsDNA ng/ul)** | **Concentration (ng/ul)** | **A260/280** | **A260/230** |
| Undetectable | 121 | 1.83 | 2.55 |
| 5.7 | 410 | 1.85 | 1.79 |
| 8.6 | 719 | 1.99 | 2.06 |
| 7.1 | 589 | 1.93 | 2.09 |

Performing DNA extractions on individual nymphs taken from hackberry nipple galls. Nipple gals were dissected from samples collected on 9/18/12. I am trying to sample some of the color variation that went into the pooled RNA extractions from 9/22/12. Note, however, that I am not including any nymphs that were entirely pink/orange. They were rare in 9/22 the pooled sample but there was one or two of them. As indicated below, some of the nymphs were alone in the gall whereas others were in a gall with two nymphs. I also found some galls with 3 or 4 nymphs but none of those are included in this sample (all nymphs in the 9/22 pooled sample were from single-occupied galls).

12 extractions with Qiagen DNeasy blood and tissue kit. Samples lableled as “HNGP” with a number 1-12, corresponding to the following:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Number** | **Body Color** | **Wing Color** | **Nymphs Per Gall** | **Gall mates** |
| HNGP 1 | white | black/brown | 1 | - |
| HNGP 2 | green | orange | 2 | HNGP 3 |
| HNGP 3 | green | white | 2 | HNGP 2 |
| HNGP 4 | green | orange | 2 | HNGP 5 |
| HNGP 5 | green | brown (dark orange?) | 2 | HNGP 4 |
| HNGP 6 | white | black/brown | 2 | HNGP 7 |
| HNGP 7 | green | orange | 2 | HNGP 6 |
| HNGP 8 | white | black/brown | 1 | - |
| HNGP 9 | lt. green | orange (brownish) | 1 | - |
| HNGP 10 | white | black/brown | 1 | - |
| HNGP 11 | green | orange | 1 | - |
| HNGP 12 | lt. green | orange | 1 | - |

See the following directory for images taken at 32x magnification with dissecting score of HNGP1 and HNGP2: /Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/1\_Photos/20120924\_hackberryNippleGalls

Note that all samples were initially placed in EtOH during removal from galls. Extractions performed following the animal tissue protocol. Tissue ground with pestle in buffer ATL at room temp after removing the ethanol. No liquid nitrogen. No RNase. 2hr ATL incubation. Eluted each sample in 100 ul buffer AE. Stored at -20 C.

9/25/12

Trying a round of bacteriome dissections and RNA extractions with the Pachypsylla venusta samples from Quinn McFrederick (9/21/12). I left the leaves/galls in a sealed ziplock in the 20 C walk in growth chamber.

I dissected a total of 24 nymphs from 5 galls. I pooled the bacteriomes into a single RNA extraction and divided the bodies into three extractions of 8 individuals each. Dissection and extraction protocol followed the one described on 9/22/12. Tubes labeled as Bact or Bod1/2/3 Pv. I stored a separate 5 ul aliquot labeled the same way plus the word “samp”.

Gall 1: Individuals 1-8

Gall 2: Individuals 9-13

Gall 3: Individuals 14-18

Gall 4: Individuals 19-19

Gall 5: Individuals 20-24

Note that many (or all?) of the nymphs had dark spots on the underside of their abdomen, positioned distally on each segment. Some of them also appeared to have some dark spots on top of their heads between their eyes. This was not as extreme as the kleptoparasite I found in some hackberry petiole galls last year. It was much more developed and with much darker melanization in multiple places. But I am still worried and should try sequencing mtDNA from one of these individuals.

Nanodrop quantification of today’s RNA extractions.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Concentration (ng/ul)** | **A260/280** | **A260/230** |
| Bacteriomes | 47 | 1.87 | 1.89 |
| Body 1 | 179 | 1.78 | 2.01 |
| Body 2 | 134 | 1.79 | 1.78 |
| Body 3 | 509 | 1.86 | 2.20 |

9/26/12

DNA extractions from two nymphs from hackberry petiole galls (9/21/12). Nymphs were taken from two different galls. The first has the lateral abdominal spots and the darkened spots between the eyes that were described yesterday. The second has the lateral spots but no obvious eye spots. It also appears to be less melanized overall. Qiagen DNeasy Blood and Tissue kit. Same protocol as 9/24/12. Samples labeled as HPGP 1 and HPGP 2.

PCR and subsequent sequencing to check identity of psyllids used for recent RNA extractions.

|  |  |  |  |
| --- | --- | --- | --- |
| **Fwd Primer** | **Rev Primer** | **Templates** | **# rxns** |
| Pv\_COI\_F | Pv\_COI\_R | HPGP 1 and 2 (9/26/12); Pachypsylla venusta 31.2 (1/12/12); dH2O | 4 |
| Pc\_COI\_F | Pc\_COI\_R | HNGP 1 through 12 (9/24/12); Pachypsylla venusta 31.1 1/12/12; dH2O | 14 |

|  |  |  |
| --- | --- | --- |
|  |  | **Mult Fact** |
| **Reagent** | **Vol per rxn (ul)** | **20** |
| Template | 1 |  |
| 10x Buffer | 1.5 | 30 |
| 25 mM MgCl2 | 0 | 0 |
| 2.5 mM dNTPs | 1.2 | 24 |
| Fwd Primer (10 uM) | 0.75 |  |
| Rev Primer (10 uM) | 0.75 |  |
| Taq (5 U/ul) | 0.15 | 3 |
| dH2O | 9.65 | 193 |
| **Total Vol** | **15** | **250** |
|  |  |  |
| Cocktail Vol (per rxn) | Mult Factor |  |
| 12.5 | 4.5 | 56.25 |
| Volume per primer | 3.375 |  |
|  |  |  |
| Cocktail Vol (per rxn) | Mult Factor |  |
| 12.5 | 14.5 | 181.25 |
| Volume per primer | 10.875 |  |

|  |  |  |
| --- | --- | --- |
| **Temp** | **Time** | **Cycle #** |
| 94 deg | 2 min |  |
| 94 deg | 15 sec |  |
| 51 deg | 15 sec | 40 |
| 72 deg | 1 min |  |
| 72 deg | 5 min |  |
| 4 deg | Hold |  |

Run 1% agarose TBE gel. 100 V for 30 min.

Loading order:

Top tier: 5ul 1/10x 1 kb ladder; Pv PCR samples 1-4 and Pc PCR samples 1-5.

Bottom tier: 5ul 1/10x 1 kb ladder; Pc PCR samples 6-14.

See 20120926\_PCR.TIF in PhotosAndGelPics directory

Results: Looks good. Successful amplification for all templates except negative controls.

9/27/12

Proceed with Sanger sequencing of PCR products from yesterday. Use only forward primer. Sequence first two Pv samples and first 12 Pc samples.

Exo-CIP clean up (see 2/9/12) and submit samples to science hill sequencing facility (order #144036).

|  |  |  |  |
| --- | --- | --- | --- |
| Position | Template | Size (bps) | Primer |
| 1 | HPGP1 | 600 | Pv\_COI\_F |
| 2 | HPGP2 | 600 | Pv\_COI\_F |
| 3 | HNGP1 | 600 | Pc\_COI\_F |
| 4 | HNGP2 | 600 | Pc\_COI\_F |
| 5 | HNGP3 | 600 | Pc\_COI\_F |
| 6 | HNGP4 | 600 | Pc\_COI\_F |
| 7 | HNGP5 | 600 | Pc\_COI\_F |
| 8 | HNGP6 | 600 | Pc\_COI\_F |
| 9 | HNGP7 | 600 | Pc\_COI\_F |
| 10 | HNGP8 | 600 | Pc\_COI\_F |
| 11 | HNGP9 | 600 | Pc\_COI\_F |
| 12 | HNGP10 | 600 | Pc\_COI\_F |
| 13 | HNGP11 | 600 | Pc\_COI\_F |
| 14 | HNGP12 | 600 | Pc\_COI\_F |

Ran Agilent Bioanalyzer RNA 6000 Nano chip to assess quality of the two recent rounds of RNA extractions (9/22/12 and 9/25/12). The samples were loaded in the following order (concentration estimates noted):

1. Nipple gall bacteriomes (9/22/12) 🡪 148 ng/ul
2. Nipple gall bodies 1 (9/22/12) 🡪 533 ng/ul
3. Nipple gall bodies 2 (9/22/12) 🡪 744 ng/ul
4. Nipple gall bodies 3 (9/22/12) 🡪 656 ng/ul
5. Petiole gall bacteriomes (9/22/12) 🡪 59 ng/ul
6. Petiole gall bodies 1 (9/22/12) 🡪 199 ng/ul
7. Petiole gall bodies 2 (9/22/12) 🡪 154 ng/ul
8. Petiole gall bodies 3 (9/22/12) 🡪 546 ng/ul

Files are saved in the following directory:

/Users/drt\_lab/Documents/Yale/MoranLab/AgilentRuns/2012-09-27

\*\*\*\*\*\*\*\*\*\*\*

I opened up a hackberry petiole gall (9/21/12) and pulled out three nymphs to take pictures under the dissecting scope and get a sense of their developmental stage.

See the following directory: /Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/1\_Photos/20120927\_hackberryPetioleGalls

Note that “nymhp1” and “nymph3” are the same individual. I also took an image on a grid background to give a sense of size (1mm = 1 square).

Based on comparisons with drawings of other Pachypsylla species in Moser 1965, it appears that these nymphs are probably instar 3 or 4.

On one of them (nymph2) the lateral spots were much less noticeable. I stored this nymph and nymph1 (I think) in separate EtOH tubes, labeled as “no spots” and “spots” with today’s date. Stored at 4 C in case I want to go back and do extractions.

9/30/12

Collected more leaves/galls from the same New Haven hackberry trees as the collection on 9/18/12. Stored in ziplock bag at 4 C.

10/1/12

Received COI seqs back from Sanger facility (see 9/27/12).

The two petiole gall samples were almost identical (only one SNP) and they only had two fixed differences relative to the published Pachypsylla venusta mitochondrial genome seq. 🡪 Proceed with petiole gall extractions, using all individuals as it appears that they are all P. venusta.

The nipple galls appear to be a more confusing story. Nine of 12 samples appear to have an identical COI sequence. But HNGP #3, 4, and 7 differ substantially from the other nine. 3 and 4 are identical to each other. 7 is different than everybody else and is probably a numt based on the presence of two frameshift indels that indicate that it’s a pseudogene. Oddly, the COI haplotypes do not bare any obvious relationship to the different morphologies I noted (9/24/12). The only pattern I see is that all single-nymph galls have the common haplotype. And all double nymphs galls have one common haplotype and one rare haplotype. Assuming there was no sample mix-ups, this suggests that the morphological variation I was observing is largely independent of “species”, at least defined by mtDNA similarity. It has been reported that Pachypsylla celtidismamma exclusively forms single-occupied nipple galls and the presence of multiple nymphs in a gall is an indication of kleptoparasite invasion (Yang et al 2001. Zoologica Scripta). That would be consistent with my findings. I should probably proceed by screening a large number of single-occupied nipple galls to see if they all share the same mtDNA haplotype.

10/2/12

Dissecting bacteriomes and extracting RNA from Pachypsylla venusta nymphs from hackberry petiole galls (see 9/21/12). Everything was done in the same fashion as on 9/25/12, except that I did two separate batches of 24 nymphs each. I dissected all the bacteriomes and then did all 8 RNA extractions (2 bacteriomes samples and 6 body samples) at once. The nymphs came from a total of 8 galls—3 in the first bacth of 24 nymphs and 5 in the second batch. I did the dissections from ~9am to 1pm. The RNA extraction followed shortly thereafter. Samples were all kept on ice after dissections.

Gall 1: individuals 1-8

Gall 2: individuals 9-16

Gall 3: individuals 17-24

Gall 4: individuals 25-34

Gall 5: individuals 35-41

Gall 6: individuals 42-45

Gall 7: individuals 46-47

Gall 8: individuals 48-48

Samples were labeled as “A” and “B”, corresponding to the two different batches. For example, Pv Bact A, Pv Bact B, Pv Bod A 1, Pv Bod A 2, etc…

10/3/12

Did another batch of P. venusta dissections and RNA extractions. Exactly as yesterday (two sets of 24 nymphs). I did the first set of dissections in the morning and the second set in the afternoon after lab meeting. Then all RNA extractions were done at once. Labeled as above except as “C” and “D”.

5 galls for batch “C”, and 7 galls for batch “D”.

Gall 1: individuals 1-2

Gall 2: individuals 3-7

Gall 3: individuals 8-11

Gall 4: individuals 12-18

Gall 5: individuals 19-24

Gall 6: individuals 25-30

Gall 7: individuals 31-32

Gall 8: individuals 33-37

Gall 9: individuals 38-40

Gall 10: individuals 41-43

Gall 11: individuals 44-46

Gall 12: individuals 47-48

Note that I’m pretty sure that I lost the bacteriome RNA pellet for batch D (in the wash step). Argh!

Nanodrop from the 4 sets of extractions in the last two days:

|  |  |  |  |
| --- | --- | --- | --- |
| **Sample** | **Concentration(ng/ul)** | **260/280** | **260/230** |
| Bact A | 103 | 1.86 | 2.26 |
| Bact B | 122 | 1.88 | 2.23 |
| Bod A 1 | 432 | 1.82 | 2.22 |
| Bod A 2 | 558 | 1.81 | 2.18 |
| Bod A 3 | 612 | 1.83 | 2.15 |
| Bod B 1 | 581 | 1.82 | 2.34 |
| Bod B 2 | 532 | 1.85 | 1.99 |
| Bod B 3 | 911 | 1.94 | 2.28 |
| Bact C | 114 | 1.86 | 2.37 |
| Bact D | 10.2 | 1.68 | 0.78 |
| Bod C 1 | 764 | 1.96 | 2.28 |
| Bod C 2 | 375 | 1.84 | 2.12 |
| Bod C 3 | 1384 | 1.99 | 2.19 |
| Bod D 1 | 658 | 1.96 | 2.24 |
| Bod D 2 | 796 | 1.96 | 2.15 |
| Bod D 3 | 629 | 1.93 | 2.07 |

Looks good except for the lost pellet in bacteriome D sample.

10/4/12

Estimated dsDNA contamination in a sample of the recent RNA extractions from Pachypsylla venusta. dsDNA BR Kit

|  |  |
| --- | --- |
| **Sample** | **Concentration (ng/ul)** |
| Bact A | 2.8 |
| Bact B | 3.5 |
| Bact C | 3.1 |
| Bod A 1 | 8.4 |
| Bod B 3 | 13.8 |
| Bod C 3 | 23.6 |

There is definitely a detactable amount of DNA contamination in the RNA, but it app ears to be small compared to the corresponding RNA concentration estimates from nanodrop.

I also tried a Bioanalyzer RNA nano 6000 run for the “A”, “B”, and “C” batches of RNA extractions. But there was some sort of reagent or equipment failure. Everything, including the ladder, looked like total garbage. High background. Inconsistent baseline. Eli Powell subsequently tried a run with completely new reagents. He got somewhat similar problems, but not as extreme. So I tried a test run with the following samples, using the same new reagents as Eli.

1. Blank
2. Bact D
3. Bod D 1
4. Bod D 2
5. Bod D 3
6. Ladder (run in sample well)
7. Blank
8. Bact D
9. Bod D 1
10. Bod D 2
11. Bod D 3
12. Blank

This run looks fine. I’m not sure what went awry with our earlier runs, but I will retry my actual run tomorrow. Note that I did not save either of today’s runs on my local machine.

10/5/12

Now doing a real Bioanalyzer run with the following samples from recent rounds of Pachypsylla venusta RNA extractions. RNA Nano 6000 kit. Estimated RNA concentrations from the run are listed next to each sample.

1. Bact A – 81.6 ng/ul
2. Bod A 1 – 342.8 ng/ul
3. Bod A 2 – 411.7 ng/ul
4. Bod A 3 – 346.2 ng/ul
5. Bact B – 85.7 ng/ul
6. Bod B 1 – 425.3 ng/ul
7. Bod B 2 – 425.0 ng/ul
8. Bod B 3 – 586.7 ng/ul
9. Bact C – 78.2 ng/ul
10. Bod C 1 – 595.8 ng/ul
11. Bod C 2 – 327.0 ng/ul
12. Bod C 3 – 904.8 ng/ul

See the following directory for run files: /Users/drt\_lab/Documents/Yale/MoranLab/AgilentRuns/2012-10-05

The traces look consistent across samples, with same type of differences between bacteriome and body samples that I am used to seeing.

10/8/12

Two more batches of P. venusta bacteriome dissections and RNA extractions. The same gall collection, protocol, and sample size as 10/2/12 except that in leaving the galls out overnight, I just put them on the lab bench at room temp in a Ziploc bag. The walk in growth chamber was not available because of power issues.

The batches were labeled as “E” and “F”. Batch E was dissected in the morning. Batch F was dissected in the later morning/early afternoon with a break for lunch. All RNA extractions were done together (samples were kept on ice after dissection).

Gall 1: individuals 1-3

Gall 2: individuals 4-11

Gall 3: individuals 12-18

Gall 4: individuals 19-23

Gall 5: individuals 24-24

Gall 6: individuals 25-26

Gall 7: individuals 27-32

Gall 8: individuals 33-39

Gall 9: individuals 40-44

Gall 10: individuals 45-48

Note that gall 9 had a suspiciously wide range of variation in development. Two of nymphs appeared to be instars 3/4 --typical of what I’ve generally seen from this batch. But there were two nymphs that were noticeably smaller (probably instar 2). And one that looked more developed (probably instar 5 although it wasn’t particularly large). I included all of these. One of the small individuals went in the body pool 2 for batch F. The rest went in body pool 3.

10/9/12

Switching over to hackberry nipple galls (Pachypsylla celtidis\*) for bacteriome dissections and RNA extractions.

A handful of galled leaves from the 9/23/12 collection were left overnight in a Ziploc bag in the walk in growth chamber (20 C).

I dissected 24 nymphs. All appeared to be 5th instar. Bacteriomes from all 24 were pooled into a single sample, while bodies were split into 3 groups of 8. Dissections were performed in 100 ul Qiagen RNA protect bacterial reagent. Samples were kept on ice after dissection.

I restricted sampling to individuals in singly occupied galls. Any galls with more than one nymph likely contain related kleptoparasites. For this batch, I did not otherwise distinguish based on phenotype, as my earlier comparisons of different color morphs did not find a clear associations with mtDNA sequence. Most of the nymphs had greenish bodies and orange wings. There were two individuals (both in body pool #2) that had white bodies—one with brown/black wings, the other almost clear wings.

In dissecting these individuals, I found that it was more effective to “rip off their butts” (last segment or so from posterior end) and squeeze out the bacteriome. This contrasts with the approach I used for the P. venusta extraction, where I general tried to rip of their head/thorax and squeeze the bacteriome out that end.

\*\*\*Important. I believe my bacteriome sample may include tissue that is not just bacteriomes. I found that a subset of the individuals I dissected (roughly half?) yielded additional lobed structures that were brightly pigmented with the same yellow coloration I typically see in the bacteriome. I included these with my bacteriome samples throughout. But I quickly came to believe that these are not bacteriomes. Could they be ovaries, explaining why they are present in only some of the individuals? Or maybe they are only pigmented/noticeable in some individuals, but they are present in all? Were they absent altogether in P. venusta. Those nymphs were at an earlier developmental stage. But I also dissected in a different fashion, so maybe the structure aren’t seen as easily with that approach. I will look into this more tomorrow or in the neat future. Note that I remember seeing these same structures in the RNA extractions done on 9/22/12, so that bacteriome sample should also contain this tissue.

RNA samples were labeled as follows

Pc Bact G

Pc Bod G 1/2/3

The “G” refers to the lettering scheme that I initiated with P. venusta. I didn’t want to introduce confusion by starting over at A, so I just picked up with G.

10/11/12

Preparing Pachypsylla venusta bacteriome and body RNA samples for submission to YCGA for Illumina mRNA-seq library construction and sequencing.

Each submitted sample will have 1 ug of RNA in 40 ul of RNase-free dH2O, as calculated from the concentration estimates from the Bioanalyzer run. For the body pools, I will combine 1 ug from each of the three subpools (i.e., I will normalize based on differences in yield from each of the body extractions) to produce a 3 ug sample (in 120 ul), but only submit 1/3 of it.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **ID** | **Sample** | **Conc (ng/ul)** | **Vol for 1 ug (ul)** | **Added dH2O (ul)** |
| DBS017 | Bact A | 81.6 | 12.25 | 27.75 |
| DBS019 | Bact B | 85.7 | 11.67 | 28.33 |
| DBS021 | Bact C | 78.2 | 12.79 | 27.21 |
|  | Bod A 1 | 342.8 | 2.92 |  |
| DBS018 | Bod A 2 | 411.7 | 2.43 | 111.77 |
|  | Bod A 3 | 346.2 | 2.89 |  |
|  | Bod B 1 | 425.3 | 2.35 |  |
| DBS020 | Bod B 2 | 425 | 2.35 | 113.59 |
|  | Bod B 3 | 586.7 | 1.70 |  |
|  | Bod C 1 | 595.8 | 1.68 |  |
| DBS022 | Bod C 2 | 327 | 3.06 | 114.16 |
|  | Bod C 3 | 904.8 | 1.11 |  |

The A and B samples are from 10/2/12, while the C samples are from 10/3/12.

10/12/12

Submitted the above samples (DBS017-DBS022) to YCGA for Illumina mRNA-seq library construction and sequencing (submission ID RQ1023).

I instructed facility to use polyA selection for library construction and to multiplex the 6 samples into a single 2x75 Illumina HiSeq lane.

\*\*\*NOTE: After submission of these samples, I realized that I duplicated the DBS017 ID. That same name was also used for the previous Trialeurodes gDNA library.

10/17/12

After conversations with Gordon Bennett and a little lit searching, I have come to the conclusion that the additional yellow pigmented strucutres found in roughly have of the hackberry nipple gall psyllid nymphs are testes. This is consistent with literature descriptions in other psyllid species (e.g., Carter RD, 1961. Distinguishing sexes in nymphs of the tomato psyllid. Annals Entomological Society of America) and the presence of what appear to be developing ovaries in at least some of the individuals that lack these yellow structures. The bacteriome also appears to be smaller in putative males than females THEREFORE, THE PREVIOUS BATCHES OF NIPPLE GALL PSYLLID BACTERIOME EXTRACTIONS CONTAIN RNA FROM TESTES.

Continuing with another batch of bacteriome dissections and RNA extractions. Same general protocol as recent batches. Galled leaves from 9/23/12 left out overnight at 20 C. I tried to exclusively take nymphs (all 5th instar) from galls containing only a single individual. I also avoided a couple individuals that were mainly white/yellowish in body color with black/brown wings (even though it was not apparent from COI sequencing that these pigmentation differences were at all related to species differences).

More notes on pigmentation: The nymphs bodies generally looked to be greenish—sometimes fairly strongly. This appeared to a blue-greenish color surrounding(?) the white (fat?) cells filling most of the abdomen. The wings and tissue neighboring the wings and legs (musculature?) was sometimes yellow or orangish. The pigments had a noticeable effect on the color of the Trizol, changing the body samples to a more orangish color. The aqueous phase was noticeably yellow, in the body but not the bacteriome samples.

24 nymphs. One bacteriome pool. Three body pools.

Labeled samples as Pc Bact H and Pc Bod H 1/2/3. Also included 5ul aliquots in “samp”

Pool 1: 9 individuals (4 male)

Pool 2: 7 individuals (5 male)

Pool 3: 8 individuals (2 male)

10/17/12

Check quantity and quality on some of the recent round of RNA extractions (but not for the Pc “G” batch, as that has a mixture of testes and bacteriome in the sample—see above).

Nanodrop and Agilent Bioanalyzer RNA nano 6000 chip. Loading order is as indicated by the table.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  |  | **NanoDrop** |  | **Bioanalyzer** |
| **Species** | **Sample** | **Conc (ng/ul)** | **A260/280** | **A260/230** | **Conc (ng/ul)** |
| Pv | Bact E | 112 | 1.78 | 2.35 | 80 |
| Pv | Bact F | 130 | 1.80 | 2.38 | 88 |
| Pv | Bod E 1 | 472 | 1.77 | 2.11 | 359 |
| Pv | Bod E 2 | 290 | 1.74 | 1.97 | 215 |
| Pv | Bod E 3 | 572 | 1.74 | 2.27 | 438 |
| Pv | Bod F 1 | 889 | 1.79 | 2.15 | 549 |
| Pv | Bod F 2 | 1268 | 1.87 | 2.24 | 716 |
| Pv | Bod F 3 | 488 | 1.85 | 2.00 | 325 |
| Pc | Bact H | 154 | 1.90 | 2.09 | 103 |
| Pc | Bod H 1 | 1173 | 1.85 | 2.31 | 935 |
| Pc | Bod H 2 | 1527 | 1.87 | 2.33 | 885 |
| Pc | Bod H 3 | 1420 | 1.89 | 2.33 | 845 |

See the following directory for Bioanalyzer output: /Users/drt\_lab/Documents/Yale/MoranLab/AgilentRuns/2012-10-17

10/18/12

Another round of bacteriome dissections and RNA extractions from hackberry nipple gall psyllid nymphs. Galled leaves from 9/23/12 left out overnight at 20 C.

Bacteriomes pooled from 24 nymphs. Body samples split into three pools of 8 individuals each. Pools 1, 2, and 3 had 3, 5, and 4 males respectively.

Note that, although I tried to restrict samples to nymphs from singly-occupied galls, I believe one of the individuals in pool 1 was from a two-nymph gall. I only noticed the side gall after dissection of the first nymph (which did appear to be in the larger of the chambers in the gall).

Labeled samples as Pc Bact I and Pc Bod I 1/2/3.

10/22/12

Two more batches of hackberry nipple gall psyllid nymph bacteriome dissections and RNA extractions. Everything was essentially the same as above, except…

--Galls were from the 9/30/12 collection

--Two batches of 24 instead of 1 (batches “J” and “K”)

--I did not exclude nymphs that lacked the green body/orange wing pigmentation, although these were still the clear majority.

--Dissolved pellet in 60 ul dH2O instead of 40 ul (no real reason. I just forgot to reset my pipette on the first sample, so I just kept the larger volume for all of them).

In total, 6 pools of 8 bodies. Male counts in each of those 6 pools:

J #1: 5 males

J #2: 2 males

J #3: 7 males

K #1: 4 males

K #2: 5 males

K #3: 1 male

10/25/12

QC on recent RNA extractions (batches I, J, and K). Nanodrop and Bioanalyzer RNA Nano 6000 Chip.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  |  |  | **NanoDrop** |  | **Bioanalyzer** |
| **Species** | **Sample** | **Conc (ng/ul)** | **A260/280** | **A260/230** | **Conc (ng/ul)** |
| Pc | Bact I | 82.7 | 1.78 | 2.33 | 52 |
| Pc | Bact J | 60.4 | 1.82 | 2.15 | 41 |
| Pc | Bact K | 73.6 | 1.90 | 2.20 | 48 |
| Pc | Bod I 1 | 1237 | 1.83 | 2.34 | 698 |
| Pc | Bod I 2 | 764 | 1.76 | 2.33 | 475 |
| Pc | Bod I 3 | 1241 | 1.88 | 2.29 | 696 |
| Pc | Bod J 1 | 736 | Not Record. | Not Record. | 445 |
| Pc | Bod J 2 | 795 | 1.85 | 2.32 | 463 |
| Pc | Bod J 3 | 624 | 1.81 | 2.25 | 446 |
| Pc | Bod K 1 | 755 | 1.79 | 2.34 | 466 |
| Pc | Bod K 2 | 963 | 1.88 | 2.35 | 564 |
| Pc | Bod K 3 | 1001 | 1.89 | 2.32 | 606 |

Note that I forgot to write down the nanodrop ratios for the Bod J 1 sample. Also, remember that batches J and K were dissolved in 60 ul of dH2O instead of the usual 40.

See the following directory for Bioanalyzer output: /Users/drt\_lab/Documents/Yale/MoranLab/AgilentRuns/2012-10-25

Note that the Bact J sample has some anomalous peaks that could be rRNA from some unknown organism—in which case this sample might be OK for qPCR, but would be dubious for RNA-seq.

10/30/12

Nancy collected more hackberry petiole galls from the same tree in Austin, TX (see 9/18/12 and 9/21/12). Stored samples at 4 C.

10/31/12

DNA extractions from individual hackberry nipple gall nymphs (9/30/12 collection). I’m trying to assess differences between closely related species that may inhabit these galls. My current hypothesis based on the literature (Yang et al 2001. Zoologica Scripta) and the last round of COI sequencing (10/1/12) is that one species (Pachypsylla celtidismamma?) inhabits most of the galls, which normally only contain a single nymph. But in some cases a second kleptoparasitic species can also colonize these galls in which case multiple nymphs are found. I removed nymphs from galls, some with a single individual and some with two individuals. I then set up Qiagen DNeasy Blood and Tissue kit extractions with each nymph. Set up buffer ATL lysis and incubated overnight at 56 C. No RNase included.

|  |  |  |  |
| --- | --- | --- | --- |
| **Sample** | **Gall Occupancy** | **Body/Wing color** | **Notes** |
| 1 | Single | light green/orange |  |
| 2 | Single | green/orange | almost dead |
| 3 | Single | green/orange | wounded |
| 4 | Single | pinkish green/orange |  |
| 5 | Single | green/orange |  |
| 6 | Single | green/orange |  |
| 7 | Single | green/orange |  |
| 8 | Single | green/orange |  |
| 9 | Double | green/orange | same gall as #10 |
| 10 | Double | green/orange | same gall as #9 |
| 11 | Double | green/orange | same gall as #12. Larger nymph. Main chamber? |
| 12 | Double | green/orange | same gall as #11. Smaller nymph. Side chamber? |

11/1/12

Completed DNA extractions from yesterday. Eluted each sample in 200 ul buffer AE. Labeled each sample as HNGP2 plus the above sample number (the “2” distinguishes this batch from the last batch of hackberry nipple gall psyllid extractions).

Setting up PCR of COI for subsequent Sanger sequencing:

|  |  |  |  |
| --- | --- | --- | --- |
| **Fwd Primer** | **Rev Primer** | **Templates** | **# rxns** |
| Pc\_COI\_F | Pc\_COI\_R | HNGP2 1-12 (11/1/12); HNGP 1 (9/26/12); dH2O | 14 |

|  |  |  |
| --- | --- | --- |
|  |  | **Mult Fact** |
| **Reagent** | **Vol per rxn (ul)** | **15** |
| Template | 1 |  |
| 10x Buffer | 1.5 | 22.5 |
| 25 mM MgCl2 | 0 | 0 |
| 2.5 mM dNTPs | 1.2 | 18 |
| Fwd Primer (10 uM) | 0.75 | 11.25 |
| Rev Primer (10 uM) | 0.75 | 11.25 |
| Taq (5 U/ul) | 0.15 | 2.25 |
| dH2O | 9.65 | 144.75 |
| **Total Vol** | **15** | **210** |

|  |  |  |
| --- | --- | --- |
| **Temp** | **Time** | **Cycle #** |
| 94 deg | 2 min |  |
| 94 deg | 15 sec |  |
| 51 deg | 15 sec | 40 |
| 72 deg | 1 min |  |
| 72 deg | 5 min |  |
| 4 deg | Hold |  |

Ingrid Rochon ran samples on a gel for me. Good product (~600 bp) from each except negative control. I did not save a picture of the gel, but she should have it if needed.

11/2/12

Prepared PCR products from 12 new DNA extractions for Sanger sequencing (see above). Forward primer only. 3 ul PCR product. Exo-CIP treatment. Submitted to Science Hill facility (order #144945).

|  |  |  |  |
| --- | --- | --- | --- |
| Position | Template | Size (bps) | Primer |
| 1 | HNGP2-1 | 600 | Pc\_COI\_F |
| 2 | HNGP2-2 | 600 | Pc\_COI\_F |
| 3 | HNGP2-3 | 600 | Pc\_COI\_F |
| 4 | HNGP2-4 | 600 | Pc\_COI\_F |
| 5 | HNGP2-5 | 600 | Pc\_COI\_F |
| 6 | HNGP2-6 | 600 | Pc\_COI\_F |
| 7 | HNGP2-7 | 600 | Pc\_COI\_F |
| 8 | HNGP2-8 | 600 | Pc\_COI\_F |
| 9 | HNGP2-9 | 600 | Pc\_COI\_F |
| 10 | HNGP2-10 | 600 | Pc\_COI\_F |
| 11 | HNGP2-11 | 600 | Pc\_COI\_F |
| 12 | HNGP2-12 | 600 | Pc\_COI\_F |

11/19/12

RNA extractions from entire Pachypsylla venusta nymphs from 10/30/12 collections (all from a single gall). Straight from 4 C.

Very large nymphs 🡪 5th instar

I first dissected each nymph in 50 ul Qiagen RNA Protect bacterial reagent to distinguish between what I believe are males and females. If I am correct about my gender IDs, the testis are much larger than the ovaries. I took a few pics to give a rough sense of size. See the following directory: /Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/1\_Photos/20121119\_Pachypsylla\_venusta

Trizol RNA extractions from 4 samples: 2 males and 2 females.

Transferred entire dissected sample in all 50 ul of RNA protect to an eppendorf tube.

Ground in 1 ml Trizol. Mixed by vortexing

Incubated for 10 min at room temp.

Spun for 10 min at 12000g, 4 C

Transferred supernatant to clean tube.

Added 200 ul chloroform. Mixed by shaking

Spun for 15 min at 12000g, 4 C

Transferred 500 ul of aqueous phase to clean tube with 500 ul isopropanol. Mixed by inversion.

Incubated at room temp for 10 min.

Spun for 10 min at 12000g, 4 C

Washed with 1 ml cold 80% EtOH. Vortexed.

Spun for 5 min at 7500g, 4 C

Air dried pellets. Dissolved in 40 ul RNase-Free dH2O

Heated at 55 C for 3 min.

Transferred 5 ul aliquot to a separate tube labeled “samp”.

Note that the above is more or less the standard Trizol protocol I’ve been using recently except that I did not use the phase-lock tubes, and I did not add glycogen.

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

I would be interested in collecting adult P. venusta psyllids for RNA samples. Any chance I can get them to emerge from one of the collected gall samples? Probably a longshot. But I put a couple of petiole galls from the 10/30/12 collection into small individual Ziploc bags. I’ll keep an eye on them.

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

DNA extraction from Bemisia tabaci sample. Collected ~100 individuals from our lab culture.

Qiagen DNeasy Blood and Tissue Kit. Animal tissue extraction protocol.

Ground individuals after liquid nitrogen freezing.

1 hour incubation at 56 C in Buffer ATL/prot K

Added 4 ul RNase with 3 min incubation at room temp prior to adding buffer AL and EtOH.

Eluted in 2 rounds of 100 ul Buffer AE (collected separately and labeled 1 and 2).

11/20/12

Qubit dsDNA BR quantification of yesterday’s DNA prep:

Elution1: 18.4 ng/ul

Elution2: 6.1 ng/ul

Ran 4 ul of each elution on a 1% agarose TBE gel with 5 ul of 1/10x 1 kb plus ladder on either side.

Image taken. See 20121120\_DNAextractions.TIF in PhotosAndGelPics directory.

Results: DNA looks decent but not great. There is definitely some degradation. Try a CTAB based protocol to improve yield and quality.

11/26/12

Trying CTAB extraction from Bemisia tabaci.

CTAB Buffer (from Eli Powell):

100 ml 1 M Tris-HCl

280 ml 5 M NaCl

40 ml of 0.5 M EDTA

20 g of CTAB

Bring total vol to 1 L with ddH2O

1. Collected B. tabaci adults from lab culture. I collected significantly more than individuals than last time. Perhaps a few hundred.
2. Add 4 ul of beta-mercaptoethanol to 600 ul of CTAB
3. Freeze with liquid nitrogen and grind with pestle.
4. Add CTAB w/ B-ME to ground tissue
5. Add 20 ul of proteinase K solution from Qiagen kit
6. Mix by inversion
7. Incubate at 55 C for 1.5 hour, inverting occasionally
8. 5 min @ 37 C
9. Centrifuged for 1 min at max speed, 4 C. But this failed to pellet debris. Do not do in the future.
10. Add 10 ul of Qiagen RNase A stock. 10 min @ 37 C
11. Add 600 ul of phenol: chloroform: isoamyl alcohol (25:24:1)
12. Mix thoroughly by inversion. 2 minutes on ice.
13. Spin @ max speed, 4 C, 10 min.
14. Transfer aqueous phase (~500 ul) to new tube.
15. Add equal volume chloroform:isoamyl alchohol (24:1).
16. Mix thoroughly by inversion. 2 minutes on ice.
17. Spin @ max speed, 4 C, 10 min.
18. Transfer aqueous phase (~425 ul) to new tube.
19. Add equal volume isopropanol.
20. Mix by inversion
21. 10 min on ice
22. Spin @ max speed, 4 C, 10 min.
23. Remove supernatant and wash with 1 ml cold 70% EtOH
24. Spin @ max speed, 4 C, 3 min.
25. Remove supernatant and air dry pellet.
26. Dissolve in 50 ul Qiagen Buffer AE.

Notes: Aqueous phases and (to lesser extent) final pellet had a noticeable yellow color to them.

Qubit dsDNA BR quantification of sample: 517 ng/ul.

Run 1% agarose TBE gel at 100 V for 50 min.

Loading order:

1. 1 kb plus ladder 1/10x – 5 ul
2. Bemisia tabaci CTAB extraction (from today) – 1 ul
3. Bemisia tabaci Qiagen extraction elution 2 (11/20/12) – 3 ul
4. 1 kb plus ladder 1/10x – 5 ul

Image taken. See /Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20121126\_DNAextractions.TIF

Results:

There is some HMW DNA including some trapped in the well, but there is also very strong signal at <1 kb in size. Unless this is partially degraded RNA, I think I’m in trouble.

Try small experiment. Three 2-ul aliquots of the CTAB DNA extraction. Treat one overnight at room temp with 1 ul Qiagen RNase solution. Leave the second overnight at room temp without any RNase. Leave the third at -20 C overnight. Run them out on a gel tomorrow to assess the effect of RNase.

11/27/12

Ran 1% agarose TBE gel at 100 V for 70 min with samples from the overnight RNase experiment.

Ran full 2 ul of each treatment plus 5ul 1/10x 1 kb plus ladder in the following order.

1. Ladder
2. RNase treatment
3. Room temp control
4. Freezer control

Image taken. See /Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20121127\_DNA.TIF

Results:

Looks pretty good. The RNase (but not the other treatments) does appear to have removed most of the LMW signal. Looks like it was partially degraded RNA. The DNA isn’t perfect, there is definitely some streaking down the gel. But that might have been exacerbated by the long overnight incubation. Hopefully ramping up the RNase treatment will do the trick for future preps. For this prep, I may try doing an RNase treatment with a fresh round of phenol chloroform extraction.

11/28/12

Repeating CTAB extraction with minor modifcations including more intensive RNase treatment.

1. Collected B. tabaci adults from lab culture. Perhaps a few hundred individuals
2. Add 8 ul of beta-mercaptoethanol to 1000 ul of CTAB buffer (10/26/12)
3. Freeze with liquid nitrogen and grind with pestle.
4. Add CTAB w/ B-ME to ground tissue
5. Add 40 ul of proteinase K solution 20 mg/ml solution from Vince Martinson. Add 10 ul Qiagen RNase A solution.
6. Mix by inversion
7. Split into two 500 ul samples
8. Incubate at 55 C for 2.75 hours, inverting occasionally
9. 1 min @ 37 C
10. Add 5 ul of Qiagen RNase A stock to each sample. 5 min @ 37 C
11. Add 500 ul of phenol: chloroform: isoamyl alcohol (25:24:1) to each sample
12. Mix thoroughly by inversion. 2 minutes on ice.
13. Spin @ max speed, 4 C, 10 min.
14. Transfer aqueous phase (450 ul) to new tubes.
15. Add equal volume chloroform:isoamyl alchohol (24:1).
16. Mix thoroughly by inversion. 2 minutes on ice.
17. Spin @ max speed, 4 C, 10 min.
18. Transfer aqueous phase (400 ul) to new tubes.
19. Add equal volume isopropanol.
20. Mix by inversion
21. 10 min on ice
22. Spin @ max speed, 4 C, 10 min.
23. Remove supernatants and wash with 1 ml cold 70% EtOH
24. Spin @ max speed, 4 C, 3 min.
25. Remove supernatant and air dry pellets.
26. Dissolve each prep in 50 ul Qiagen Buffer AE.

Qubit dsDNA BR quantification:

Prep 1: 87 ng/ul

Prep 2: 91 ng/ul

Run 1% agarose TBE gel. 100 V for 50 min.

Loading order:

1. 5 ul 1/10x 1 kb plus ladder
2. 2 ul DNA prep 1
3. 2 ul DNA prep 2

Image taken. See /Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20121128\_DNAextractions.TIF

Results: Doesn’t look good. Same problem with LMW signal despite changes in RNase treatment. Perhaps the presence of the proteinase K is just overwhelming the RNase. Try treating each of my samples with RNase and then doing a fresh phenol:chloroform extraction.

11/29/12

Treating the two recent rounds of Bemisia tabaci CTAB extractions with RNase in an attempt to remove the low molecular weight signal I am seeing on a gel.

Combined the two samples from yesterday (11/28). Left the sample from 11/26 separate.

Added 4 ul of Qiagen RNase stock to each sample. Incubated at 37 C for 40 min.

Brought volume of each sample up to 500 ul with Qiagne Buffer AE.

Did phenol:chloroform extraction as yesterday except that I added 1/10 volume of NaOAc to the isopropanol step (because the samples were not in a salt-containing buffer like yesterday).

Dissolved pellets in 50 ul each of Qiagen buffer AE.

Qubit dsDNA BR concentration estimates:

11/26 sample: 382 ng/ul

11/28 sample: 287 ng/ul

\*note that the 11/28 concentrations went up substantially more than expected (with perfect yield it should have doubled). Possible measurement error either before or now?

Ran 1% agarose TBE gel for 1 hour at 100 V

Loading order:

1. 5 ul 1/10 x 1 kb plus ladder
2. 2 ul 11/26 B. tabaci DNA sample (after new RNasing)
3. 2 ul 11/28 B. tabaci DNA sample (after new RNasing)

Image taken. See /Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20121127\_DNA.TIF

Results: Looks good. LMW signal (probably RNA) appears to have been removed. There is some weird streaking that appears to be an artifact of the gel and something hung up in the wells. But I think these samples are good enough to proceed with Southerns. First, I should verify taxonomic identity of samples with PCR and sequencing.

12/3/12

Doing CTAB extractions from single Bemisia tabaci B-biotype whiteflies. Taken from our lab cultures. Aspirate individuals into single eppendorf tubes. I can’t guarantee that there wasn’t any contamination (for example of whitefly scales) during the aspiration process

Modified CTAB protocol for small samples. Main differences are highlighted below.

1. Added 10 ul of B-ME to 1.2 ml of CTAB buffer.
2. Ground each individual in 200 ul of above with pestle. No liquid nitrogen freezing.
3. Added 6 ul of proteinase K (20 mg/ml) from Vince Martinson to each sample. [No RNase]
4. Mixed by vortexing
5. Incubated at 55 C for 1 hour
6. Extract with equal volumes P:C:I (200 ul) and C:I (175 ul). Mix by vigorous shaking (not worried about shearing DNA)
7. Add 2 ul (=10 ug) of glycogen and precipitate for 1 hr at -20 C with equal volumes isopropanol (150 ul).
8. Spin and wash with 500 ul 70% EtOH.
9. Air dry pellet and dissolve in 25 ul Qiagen buffer AE.

Note that the interface did not hold very well for sample 4, 5, and 6 at the chloroform extraction step, so I ended up with less volume from each of those.

PCR to confirm identity/purity of bulk B. tabaci preps (see 11/29/12) and to test for the presence of multiple Portiera conformations in DNA extracted from a single host individual (with the caveat that PCR mediated recombination could cause false positives). Use old (11/11/11) B. tabaci extraction as a positive control. Use Qiagen buffer AE as a negative control instead of dH2O in case there has been any contamination of the Qiagen buffer.

|  |  |  |  |
| --- | --- | --- | --- |
| **Fwd Primer** | **Rev Primer** | **Templates** | **# rxns** |
| prfA\_F | prfA\_R | 7 Bemisia tabaci samples: 1/10x #1 (11/11/11); 1/100x 11/26 (11/29/12); 1/100x 11/28 (11/29/12); Individuals 1-4 from today; Qiagen Buffer AE | 8 |
| prfA\_F | prfB\_R | 7 Bemisia tabaci samples: 1/10x #1 (11/11/11); 1/100x 11/26 (11/29/12); 1/100x 11/28 (11/29/12); Individuals 1-4 from today; Qiagen Buffer AE | 8 |
| prfB\_F | prfA\_R | 7 Bemisia tabaci samples: 1/10x #1 (11/11/11); 1/100x 11/26 (11/29/12); 1/100x 11/28 (11/29/12); Individuals 1-4 from today; Qiagen Buffer AE | 8 |
| prfB\_F | prfB\_R | 7 Bemisia tabaci samples: 1/10x #1 (11/11/11); 1/100x 11/26 (11/29/12); 1/100x 11/28 (11/29/12); Individuals 1-4 from today; Qiagen Buffer AE | 8 |
| Port\_rep\_F1 | Port\_rep\_R1 | 7 Bemisia tabaci samples: 1/10x #1 (11/11/11); 1/100x 11/26 (11/29/12); 1/100x 11/28 (11/29/12); Individuals 1-4 from today; Qiagen Buffer AE | 8 |
| Port\_rep\_F1 | Port\_rep\_R2 | 7 Bemisia tabaci samples: 1/10x #1 (11/11/11); 1/100x 11/26 (11/29/12); 1/100x 11/28 (11/29/12); Individuals 1-4 from today; Qiagen Buffer AE | 8 |
| Port\_rep\_F2 | Port\_rep\_R1 | 7 Bemisia tabaci samples: 1/10x #1 (11/11/11); 1/100x 11/26 (11/29/12); 1/100x 11/28 (11/29/12); Individuals 1-4 from today; Qiagen Buffer AE | 8 |
| Port\_rep\_F2 | Port\_rep\_R2 | 7 Bemisia tabaci samples: 1/10x #1 (11/11/11); 1/100x 11/26 (11/29/12); 1/100x 11/28 (11/29/12); Individuals 1-4 from today; Qiagen Buffer AE | 8 |
| wtfly\_COI\_F | wtfly\_COI\_R | 7 Bemisia tabaci samples: 1/10x #1 (11/11/11); 1/100x 11/26 (11/29/12); 1/100x 11/28 (11/29/12); Individuals 1-4 from today; Qiagen Buffer AE | 8 |

|  |  |  |
| --- | --- | --- |
|  |  | **Mult Fact** |
| **Reagent** | **Vol per rxn (ul)** | **80** |
| Template | 1 |  |
| 10x Buffer | 1.5 | 120 |
| 25 mM MgCl2 | 0 | 0 |
| 2.5 mM dNTPs | 1.2 | 96 |
| Fwd Primer (10 uM) | 0.75 |  |
| Rev Primer (10 uM) | 0.75 |  |
| Taq (5 U/ul) | 0.15 | 12 |
| dH2O | 9.65 | 772 |
| **Total Vol** | **15** | **1000** |
|  |  |  |
| Cocktail Vol (per rxn) | Mult Factor |  |
| 12.5 | 8.5 | 106.25 |
| Volume per primer | 6.375 |  |

|  |  |  |
| --- | --- | --- |
| **Temp** | **Time** | **Cycle #** |
| 94 deg | 2 min |  |
| 94 deg | 15 sec |  |
| 54 deg | 15 sec | 40 |
| 72 deg | 1 min |  |
| 72 deg | 5 min |  |
| 4 deg | Hold |  |

Ran PCR samples on 1% agarose TBE gel. 100V for 35 min. 3 ul of each PCR product and 5 ul of 1/10x 1 kb plus ladder.

I ran the samples grouped by DNA template rather than primer pair. So the gel is layed out in groups of 9 PCR lanes each followed by a ladder lane. The 9 PCR products are in the order of reactions indicated in the table above. And the template groups are also in the order indicated in the table (with the first 4 templates on the top tier of the gel and the last 4 on the bottom.

Image taken. See /Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20121202\_PCR.TIF

Results: Looks good. Expected amplification for most of the prf and 6kb repeat reactions. In a few cases the prfA\_F/prfB\_R product is weak or absent. But that might be a more tempremental reaction because one of the failed reactions is in the positive control. Conclusion: PCR-based evidence of alternative genome conformations even within single individuals (remember the caveat about PCR-mediated recombination).

The COI reaction wasn’t as robust. Little or no visible product for the individual preps. Weak product for the 3 pooled preps. Try Sanger sequencing COI from the two CTAB extractions.

Used standard Sanger prep protocol for sending sample to Keck Science Hill facility except that I used 5 ul of template in each reaction instead of 3 ul.

Submitted to Science Hill as order number 145747

|  |  |  |  |
| --- | --- | --- | --- |
| Position | Template | Size (bps) | Primer |
| 1 | Btab\_1026 | 600 | wtfly\_COI\_F |
| 2 | Btab\_1026 | 600 | wtfly\_COI\_R |
| 3 | Btab\_1028 | 600 | wtfly\_COI\_F |
| 4 | Btab\_1028 | 600 | wtfly\_COI\_R |

Results [12/6/12]: Both sequences confirm Bemisia tabaci biotype B. Only one site with obvious polymorphism in the pooled samples.

12/10/12

|  |  |  |  |
| --- | --- | --- | --- |
| **Fwd Primer** | **Rev Primer** | **Templates** | **# rxns** |
| PABT\_blot\_F | PABT\_blot\_R | Duplicate reactions of Bemisia tabaci 11/26 (11/29/12) 1/100x and Bemisia tabaci 11/28 (11/29/12) 1/100x; dH2O | 5 |

Running PCR to amplify probe for southern blot of Portiera genome in Bemisia tabaci. The goal is assess whether alternative structural forms co-exist associated with the 6kb circle.

|  |  |  |
| --- | --- | --- |
|  |  | **Mult Fact** |
| **Reagent** | **Vol per rxn (ul)** | **5** |
| Template | 2 |  |
| 10x Buffer | 3 | 15 |
| 25 mM MgCl2 | 0 | 0 |
| 2.5 mM dNTPs | 2.4 | 12 |
| Fwd Primer (10 uM) | 1.5 | 7.5 |
| Rev Primer (10 uM) | 1.5 | 7.5 |
| Taq (5 U/ul) | 0.3 | 1.5 |
| dH2O | 19.3 | 96.5 |
| **Total Vol** | **30** | **140** |

|  |  |  |
| --- | --- | --- |
| **Temp** | **Time** | **Cycle #** |
| 94 deg | 2 min |  |
| 94 deg | 15 sec |  |
| 55 deg | 15 sec | 40 |
| 72 deg | 90 sec |  |
| 72 deg | 5 min |  |
| 4 deg | Hold |  |

12/11/12

Run 1% agarose TBE gel with PCR products from yesterday. 2 ul of each and 5 ul of 1 kb plus ladder. 100 V for ~30 min.

Image taken. See. /Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20121211\_PCR.TIF

Results:

Strong amplification across all 4 samples. In addition to the expected band around 850 bp, there is a faint band around 1700 bp. Contaminating product or some kind of amplification doublet? Proceed with use of these products for Southern blotting, but be aware of the possibility of contamination.

Clean PCR products with Qiagen Qiaquick PCR extraction kit. Pooled duplicate pairs of PCR products, so I had ~50 ul each for the 11/26 sample and the 11/28 sample. Eluted samples in 40 ul buffer EB. Note that I got pulled away in the middle of this prep and the samples set in elution buffer on the column for ~1 hour.

Run gel as above with cleaned PCR products. Loading order.

1. Ladder (5 ul)
2. Cleaned PCR 11/26 (1.6 ul)
3. Cleaned PCR 11/28 (1.6 ul)
4. Original PCR 11/26 (2 ul)

Loading volumes are such that perfect yield for clean up should yield bands of equal intensity compared to original PCR.

Results: Looks good. Yield appears to be high.

/Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20121211\_PCR\_cleaned.TIF

Qubit dsDNA BR concentration estimates for the two samples are:

11/26 sample 🡪 49 ng/ul

11/28 sample 🡪 44 ng/ul

Performing DIG (digoxigenin) labeling of the 11/26 PCR sample (above) to use as a probe in Southern blots.

Using the following kit:

Roche DIG High Prime DNA Labeling and Detection Starter Kit II. Version 12.

Cat # 11 585 614 910

Took 16 ul sample of cleaned PCR product (= ca. 800 ng) and incubated on 95 C heat block for 10 min. Transferred immediately to an ice water bath for approx. 1-2 minutes.

Vortexed tube of DIG-High primer (vial 1) to mix and added 4 ul to the denatured PCR product. Vortexed to mix and then briefly spun down sample.

Incubated at 37 C overnight starting at 4 pm.

\*\*\*\*\*\*\*\*\*\*

Preparing 20x SSC (saline-sodium citrate buffer) for later use in southern blotting.

175.3 g NaCl (= 3 M)

88.2 g Trisodium Citrate (= 300 mM)

in 800 ml ddH2O

Adjusted pH to 7.0 with drops of 1 M HCl

Brought up final volume to 1 L with ddH2O

Autoclaved on 20 minute liquid cycle. Stored at room temp.

12/12/12

Continuing with testing of DIG-labeled probe for Southern blots. Following protocol from Roche kit: /Users/drt\_lab/Documents/Yale/MoranLab/projects/PortieraTV/Southerns/protocols/11585614910\_en\_12.pdf

I stopped the DIG labeling reaction at 9:00 AM (17 hrs) by adding 2 ul of 200 mM EDTA, vortexing, and heating on block at 65 C for 10 min. Sample was then spun down briefly. I kept it on ice while preparing buffers and dilutions for today’s dot blot work, and then stored it at -20 C.

Preparing 1x buffers for Southern blots from 10x stocks in the Roche DIG Wash and Block Buffer Set (#11 585 762 001). This kit was purchased by Eva Novakova (date unknown), and stored at 4 C. But the components are stable at room temp, so we have been keeping on the bench for the last few days.

Made the following volumes of 1x buffers. All were diluted 10-fold from 10x stocks in autoclaved MilliQ water and put in autoclaved glassware. All will be stored at room temp.

500 ml Maleic Acid Buffer

500 ml Wash Buffer (mixed 10x stock by shaking prior to diluting—contains tween)

100 ml Detection Buffer

Prepared 25 ml of 1x Block Solution by adding 2.5 ml of 10x stock to 22.5 ml of 1x Maleic Acid Buffer (above). Note that Eva Novakova’s buffer set contains a large bottle of the 10x blocking solution, but it is supposed to be stored at -20 after opening, so I used one of the bottles in the our new DIG labeling/detection kit. Made 13 ml aliquots and stored at -20 C.

This 1x block solution was prepared in the morning and kept on ice until dot blots were made in the afternoon.

Prepared dilution series of control DIG-labeled DNA from Roche kit and my labeled probe. All dilutions performed with DNA dilution buffer from Roche kit. Control DNA was diluted 5-fold (1 ul sample + 4 ul dilution buffer) to generate sample with 1 ng/ul concentration. Based on information provided in Roche protocol, I predicted that my labeled probe should have a concentration of roughly 100 ng/ul, so I initiated the dilution series with a 100-fold dilution of the probe (1 ul probe + 99 ul dilution buffer). Starting with each of these “1 ng/ul” samples, I performed the following dilution series. This produces the same concentrations as described in the Roche manual, but the details of the actual dilutions are altered to save on reagents and make it less confusing. Samples were prepared in the morning and kept on ice until needed in the afternoon.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Sample # | Concentration (pg/ul) | Transfer Vol (ul) | Dilution Buffer Vol (ul) | Dilution factor |
| 1 | 1000 |  |  |  |
| 2 | 10 | 1 | 99 | 100 |
| 3 | 3 | 3 | 7 | 3.3 |
| 4 | 1 | 3 | 6 | 3 |
| 5 | 0.3 | 3 | 7 | 3.3 |
| 6 | 0.1 | 3 | 6 | 3 |
| 7 | 0.03 | 3 | 7 | 3.3 |
| 8 | 0.01 | 3 | 6 | 3 |
| 9 | 0 | 0 | 6 |  |

Centrifuged antibody stock from Roche kit at 10,000 RPM, 4C for 5 min and added 1 ul to 10 ml of 1x block solution to make antibody solution. Made in the afternoon immediately before beginning blotting.

Cut a ca. 2” x 2” square piece of Roche positively-charged nylon membrane (#11 209 272 001) and pipetted 1 ul each from samples 2 through 9 from the control and probe dilution series directly onto the membrane. I cut off the bottom-right corner of the membrane to distinguish which side of the blot had the DNA. I also made dots with a sharpie along the edge of the membrane noting the rough position of the grid onto which the samples were spotted. I made 4 rows of 4 spots, with the top two rows containing the control dilution series and the bottom two rows containing the probe dilution series. I marked the 1st and 3rd rows with a “C” and a “P” to indicate this.

Cross-linked DNA to membrane using Stratagene UV Stratalinker 1800. I wasn’t sure about the proper settings. Notes from Andy Alverson said 1200 uJoules in 30 seconds (I think he meant 120,000 uJoules; the units for energy on the instrument are uJoules x 100). And it took a lot longer than 30 secs to reach that level. I let it run for 60 seconds on the DNA side (~100,000 uJoules) and then flipped it over for 30 more seconds (~50,000 uJoules). In the future, I think it would be better to just give it the full 120,000 uJoules on each side, as total energy is probably more important than the time in which it is delivered. Cross-linking was performed on top of a dry piece of filter paper, which itself was on top of a plastic petri dish.

Series of incubations and washes all done at room temp in clean plastic pertri dishes on VWR OS-500 orbital shaker at a low speed setting (2). Membrane was transferred from one dish to next with blot forceps, dabbing off excess liquid on the side of the old dish before transferring. Forceps were washed in house dH2O and dried with a Kimwipe between transfers.

1. 2 min in 20 ml Maleic Acid Buffer
2. 30 min in 10 ml Blocking Solution
3. 30 min in 10 ml Antibody Solution
4. 2x15 min in 10 ml Washing Buffer
5. 4 min in 10 ml Detection Buffer

Transferred blot to inside of heat-sealable clear plastic pouch (Kapack/Scotchpach Polyester barrier film, quart size; stock #404). The sides of the envelope tend to curl up after cutting, which made the membrane placement awkward. In the future it might be helpful to weight down the corners when placing the blot in the envelope.

Added 6 drops of CSPD “ready-to-use” substrate (~150 ul) to the membrane and immediately covered with top sheet of envelope. Removed air bubbles with paint roller.

Incubated at room temp for 5 minutes then squeezed out excess liquid volume and air (not much of either) with paint roller and sealed with heat sealer.

Took images on Groisman Lab ImageQuant LAS 4000 more or less immediately after the 5 minute incubation time.

Imager settings:

Chemiluminescence

Tray position 1

Exposure: Precision, Manual (one image taken at 2min, another at 20min)

Resolution: High

Image files:

/Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20121212\_dotBlot\_2min.tif

/Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20121212\_dotBlot\_20min.tif

Results: Looks good. Clear signal with low background. On the 20 minute exposure, I can see a dot for all of the control dilutions except the lowest concentrations 0.01 ng/ul (maybe a very faint one). The probe dilutions were slightly less intense and were generally one dilution behind the control series, suggesting ~3-fold lower concentration in the starting sample. Therefore, I would estimate the labeled probe concentration to be ~30 ng/ul.

12/13/12

Trying digestion of Bemisia tabacia genomic DNA in preparation for Southern Blots.

2 samples 11/26 and 11/28 (see RNase clean up and concentration summary on 11/29/12).

4 digest treatments

* No Digest
* EcoRV-HF (NEB 20 U/ul)
* SpeI-HF (NEB 20 U/ul)
* EcoRV-HF/SpeI-HF double digest

10 ul reaction volumes:

4.6 ul of DNA (= 1.33 ug\*)

3.8 ul dH2O

1 ul NEB 10x buffer #4

0.1 ul NEB 100x BSA

0.25 ul per enzyme (replacing buffer EB for enzyme in single digests).

\*Note that the 11/26 sample was originally at a higher concentration, so it was first diluted with buffer EB to the same concentrations as 11/28 sample)

Incubatd in 37 C H2O bath for 1 hr then spiked in an additional 0.25 ul of each enzyme (or buffer EB), bring volume to 10.5 ul and incubating at 37 C for another 75 min.

Heat inactivated at 80 C for 20 min.

For the no digest treatment, and equal amount of DNA (1.33 ug) was diluted to a total volume of 10.5 ul in buffer EB.

Running gel in large rig (20 cm long gel cast) to test out for future Southern use. 1% agarose TBE gel. 44 V overnight. Starting at 5:30pm.

Ran 2.5 ul samples of each digest treatment (should be about 300 ng, leaving 1 ug for actual blotting. 5 ul 1 kb plus ladder (1/10x).

Used a loading dye from Qiagen, which I believe includes bromophenol blue and xylene cyanol in addition to the orange dye we typically use in the lab. 1 ul for sample. Plus I ran 5 ul of dye (by itself) in a lane off to the side. I want to know how strong the loading dye signal will be in the morning.

Loading order

12/14/12

Stopped gel at 9:15am (15 hrs and 45 min). The loading dye was barely visible even in the lane with 5 ul, so if I want to use color as an indicator during depurination, it would be better add some fresh into an extra well near the end of the run and let it migrate into the gel without diffusing too much.

Image taken. /Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20121213\_Digests.TIF

Results: looks pretty good. The digests are long smears as expected. Faints hints of banding at some places, which could represent high copy elements (mtDNA, rDNA, etc). The 300 bp ladder fragment is right at the end of the gel, so I didn’t lose much of the end. There is some degradation in the uncut sample but not too bad. Tough to tell, but I think the digests are relatively complete. Almost all DNA hung up in the well in the uncut sample is gone in the digested samples. And there is a clear downward shift in the top most band.

For the actual blot run, I should probably switch to a lower % agarose (maybe 0.8%) to get a little bit better resolution, but other than that I think I can give it a go.

12/17/12

Setting up gel for Portiera Southern blot to run overnight.

0.8% agarose TBE gel. Poured in long 20 cm cast. Run at 44 V (~22 mA). Starting at 11:15pm.

Loading order. Loading full remaining volume of each digest (~1 ug of DNA each)

Well #

1. Blank
2. 2 ul Roche DNA Molecular Weight Marker III, DIG-labeled
3. Blank
4. Uncut 11/26
5. Uncut 11/28
6. SpeI 11/26
7. SpeI 11/28
8. EcoRV 11/26
9. EcoRV 11/28
10. SpeI+EcoRV 11/26
11. SpeI+EcoRV 11/28
12. Blank
13. 8 ul Roche DNA Molecular Weight Marker III, DIG-labeled
14. Blank
15. PCR Control 1/10x
16. PCR Control 1x
17. PCR Control 10x
18. Blank
19. Blank (14 ul loading dye to be able to see in the morning)
20. Blank (14 ul loading dye to be able to see in the morning)
21. Blank
22. Blank
23. 12 ul Roche DNA Molecular Weight Marker III, DIG-labeled
24. 5 ul Invitrogen 1kb plus ladder (1/10x)

The two ladders at the end will be cut off the gel and stained with ethidium to provide improved size estimation for the blot (there is a huge gap in the DIG labeled ladder from 5 kb to 21 kb).

The PCR controls are diluted PCR products matching the probe to serve as a positive control. The 1x control should be diluted down to match the expected amount of target in 1 ug total cellular DNA.

PCR dilution:

1 ug DNA

\* 0.03 (=fraction of Portiera DNA in total insect DNA based on Illumina data)

\*0.0025 (=fraction of Portiera genome covered by probe)

\_\_\_\_\_\_\_\_\_\_

0.07 ng

Use 11/26 purified PCR product (49 ng/ul; see 12/11/12).

70x dilution 🡪 0.7 ng/ul (load 1 ul for 10x control)

700x dilution 🡪 0.07 ng/ul (load 1ul for 1x control)

7000x dilution 🡪 0.007 ng/ul (load 1ul for 1/10x control)

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

Prepared and autoclaved buffers for Southern blot work…

1 L 20x SSC (see above for recipe)

2 L 5 M NaCl

6 L dH2O

12/18/12

More buffer preparation.

400 ml of 250 mM HCl

Diluted HCl from concentrated stock in autoclaved dH2O. Stock is at 36.5-38% purity. Assume 12.1 M.

🡪 8.3 ml conc. HCl + 391.7 ml sterile ddH2O = 400 ml of 250 mM HCl

1 L of denaturation solution (0.5 M NaOH, 1.5 M NaCl)

🡪 20 g NaOH pellets

🡪 300 ml 5 M NaCl (from yesterday)

🡪 sterile ddH2O to 1 L

800 ml of neutralization solution (0.5 M Tris-HCl pH 7.5; 1.5 M NaCl)

🡪 400 ml 1 M Tris-HCl pH 7.4 (close as I have to 7.5)

🡪 240 ml 5 M NaCl

🡪 160 ml sterile ddH2O

At 12:45 pm (13.5 hrs), I momentarily stopped the gel and spiked in 5 ul of loading dye containing bromophenyl blue into lane 1. This will be used as a color marker during depurination.

At 2:45 (15.5 hrs). I stopped the gel for good.

Split the gel after lane 18. The right half of the gel (two ladders) was stained in EtBR, imaged, and discarded.

See image: /Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20121218\_ladders.TIF

Results: Looks like I got good separation. The gel ends somewhere between the 500 bp and 650 bp markers in the 1 kb ladder. The relative positions of the ladder will be valuable for interpolating between the big size gaps in the DIG-labeled ladder.

The left half of the gel was used for a series of washes in various buffers to depurinate, denature, and neutralize the DNA. All washes were performed in a long rectangular 76 oz. plastic container (Ziploc knockoff from Target) with a depth sufficient to submerge the gel (~300 ml). Buffers were simply poured off at the end of each step, and the next step was performed in the same container. All incubations were down at room temp on VWR orbital shaker at lowest speed setting.

1. 250 mM HCl (until bromophenyl blue band turns yellow 🡪 6 min)
2. ddH2O. ~1 min
3. Denaturation solution. 15 min
4. Denaturation solution. 15 min
5. ddH2O. ~1 min
6. Neutralization solution. 15 min
7. Neutralization solution. 15 min
8. 20x SSC. >10 min (went about 25 min in this case)

Assembled blot sandwich in the same large gel rig that was used to run the gel.

Inverted gel tray and placed in the middle of the reservoir to act as a pedestal for the sandwich. Filled gel reservoirs with 20x SSC (~2 L).

From bottom to top…

--3 long strips of Whatman 3mm chromatography paper. These were wider than the width of my gel portion and long enough to reach into the both ends of my gel rig reservoirs. These were pre-soaked in the 20x SSC buffer and layered immediately on top of each other. Used a serological pipette as a roller to squeeze out air bubbles between them.

--Gel, upside down. Trimmed off top with razor blade to right above the wells. Trimmed off a couple centimeters from bottom to match size of membrane and glass pane. Trimmed edge of gel near lane 1 b/c it was trapping air bubbles. Rolled out air bubbles with pippete.

--Saran wrap covering entire rig. Cut out a hole in saran wrap around the gel with razor bladed. This is to prevent evaporation and changes in buffer concentrations.

--Roche positively-charged nylon membrane. Cut to be a bit larger than the gel (although not much larger in the vertical axes, as I wanted to trim the gel as little as possible). Placed down gently over gel to avoid air bubbles and movements after placement. Rolled out air bubbles with pipette.

--A dry piece of Whatman 3mm chromatography paper cut to the size of the membrane. Laid on gently until it started absorbing moisture, but I didn’t try to get a tight air-free seal.

--Paper towels. Start with ~20 as fully expanded single sheets cut to match size of membrane. Then put to folded stacks side by side for the rest of paper towel tower. ~3 or 4 inches high.

--Glass pane

--500 ml bottle water (as weight)

Assembled sandwich and let transfer overnight starting at 5pm.

Thoughts for modification in future runs:

--Either don’t run out the gel quite as far or don’t be constrained by the size of the glass pane. Any, flat rigid object should suffice.

--Pre-cut membrane, top piece of chromatography paper, and paper towels to be better fit to gel.

--Slice off tips of wells to avoid air bubbles right at the top of the gel?

12/19/12

Continuing with Southern blot.

Prepared pre-hybridization and hybridization buffers from Roche Hyb Granules (bottle 7 from Roche DIG kit).

Added 64 ml sterile ddH2O to granules. Dissolved.

Pre-hybridization buffer: 20 ml of above 🡪 pre-warm in 37 C H2O bath

Hybridization buffer: 7 ml of above + 7 ul of denature probe (see below) 🡪 pre-warmed in 37 C H2O bath

Denaturing probe. Probe from 12/12/12. Denature for 10 min at 96 C. Immediately transfer to ice bath. Spin down briefly and add to pre-warmed (37 C) buffer.

Chose low (37 C) hybridization temp because of the high AT content of my probe. Guidelines for choosing hybridization temps can be found in DIG kit manual.

Disassembled blot sandwich at ~1:30pm (20.5 hrs). Cut a notch in bottom right corner of membrane to distinguish top side from bottom. Made small pencil marks at top corners of gel and on top of wells 1, 5, 9, 13, and 17.

Placed membrane on top of the soaked bridge filter paper from the blot. And cross-linked on both sides with 120,000 uJoules in UV Stratalinker 1800.

Then let the membrane air dry (accidentally skipped the ddH2O wash in Andy Alverson’s protocol).

Incubated membrane in 25 ml of pre-heated (65 C) 0.1% SDS, 0.1x SSC in Roche hybridization bag at 65 C on VWR orbital shaker at speed setting 2. Shaker was placed in drying oven to reach temperature. This is supposed to reduce background according to Andy’s protocol, but most protocols don’t include.

Remove blot from bag (discard bag). And wash in 2x SSC for ~1 min

Transfer membrane to new hybridization bag and added 20 ml of pre-warmed (37 C) pre-hybridization buffer. This was a disaster. Getting the membrane into a bag when it was already wet was unwieldy. The membrane dried out some during the process. It also wrinkled/folded at times, and I ended up grabbing it with my gloved hands multiple times. There ended up being lots of bubbles.

Incubated for ~1 hour at 37 C on VWR orbital shaker (speed 2). I used a second shaker b/c I didn’t think the first would cool down fast enough.

Drained pre-hyb buffer (stored for re-use at -20 C). Added 7 ml of pre-warmed (37 C) hybridization buffer. Eliminated as many air bubbles as possible. Incubated overnight at 37 C on VWR orbital shaker (speed 2). Starting at 5 pm

Thoughts for next time...

--Skip the 65 C incubation step. It made the transfer of the membrane extremely awkward

--Consider diluting probe before 96 C denaturation. A lot of evaporation within tube resulted in very small volume at end of incubation.

--Smaller gel/membrane?

--Larger pre-hyb and hyb volumes?

12/20/12

Continuing with Southern blot: washing and detection, generally following instructions from Roche DIG kit

Starting at 12:30 pm (20.5 hrs of hybridization).

Poured off probe-containing hybridization buffer and stored at -20 C for future re-use.

Low stringency wash in 100 ml of 2x SSC 0.1% SDS at room temp. Setting 5 on VWR orbital shaker. 5 min.

Repeat above except I extended the time to 15 in to allow the buffer for the next step to warm up to 60 C (should have been pre-warmed).

2x15 min high stringency washes in 60 C drying over on VWR orbital shaker (setting 4). 100 ml 0.5x SSC 0.1% SDS In the first wash, the shaker was acting a bit finicky, so I had to open the door a few times to turn it back on.

Ca. 5 min wash in 100 ml 1x Washing buffer. Room temp. Orbital shaker (setting 2).

30 min in 200 ml 1x blocking solution. Room temp. Orbital shaker (setting 2).

30 min in 50 ml antibody solution. Room temp. Orbital shaker (setting 2).

2x15 min in 150 ml 1x washing buffer. Room temp. Orbital shaker (setting 2).

4 min in ca. 40 ml of 1x detection buffer.

Laid membrane gently onto cut open heat-sealed pouch (using small flasks to weigh down the corners.

Dripped on 2 ml of CSPD ready-to-use. Gently closed pouch avoiding air bubbles.

Incubated for 5 min at room temp.

Eliminated excess liquid, and sealed pouch with heat sealer.

Incubated at 37 C for 10 min.

Took pictures on Groisman lab LAS 4000 imager. Chemisluminecent setting. Tray position 3. Two exposure times. 5 min, 30 min.

Image files are in photosAndGelPics directory (two different formats)

20121220\_1555\_5min.tif

20121220\_1556\_5min.gel

20121220\_1639\_30min.tif

20121220\_1638\_30min.gel

Results: Doesn’t look half-bad for a first run through. A fair amount of background, some of which may be alleviated by better handling techniques not that I’ve been through the protocol once.

The EcoRV and SpeI/EcoRV digests match expectations perfectly with a strong linearized 6.5 kb band corresponding to the “subcircle” conformation and weaker 12.9 (EcoRV) and 2.5 (SpeI/EcoRV) kb corresponding to the master circle conformation. The SpeI also has a weak 7.6 kb band matching expectaions for the master circle conformation. What is unexpected, however, is there is no evidence of a 6.5 kb circular molecule (super-coiled or relaxed) in either the SpeI samples (does not contain site within the 6.5kb sequence) or the uncut samples. Instead, all the signal in these lanes is at much higher molecular weights.

Both uncut and SpeI have two VERY high molecular weight bands way above the 21 kb marker. The uncut sample also has a noticeable quantity of DNA stuck in the well. Most of the signal for these samples however is around where the bulk of the DNA preps runs (see earlier test gel with ethidium stain). The uncut sample also has a distinct band slightly below this at around 21 kb. In contrast, the SpeI has at least 3 bands up in this vicinity, which could conceivably correspond to the 14.1, 20.7, and 27.2 kb fragments predicted if you had 2x, 3x, and 4x concatamers embedded WITHIN the main genome.

Conclusions:

--The Portiera genome coexists in multiple conformations.

--One of these is consistent with the published form of the genome containing one integrated copy of the 6 kb sequence.

--The most abundant form of the 6kb sequence, however, is in something that MAPS as a circle, producing a linear full-length piece when cut with an enzyme that cuts once within the 6 kb sequence.

--The actual molecule, however, is NOT a circle based on the absence of a signal at the corresponding size in the SpeI. Instead, it is present as head-to-tail concatamers.

--The most abundant form of this 6 kb region cannot be a “pure” concatamer. Because SpeI produces a different pattern than the uncut sample.

Caveats to keep in mind:

--These DNA sample are not from the same insects as the genome sequence

--The uncut control was not incubated at 37 C for 2 hours or heat killed, so the difference between SpeI and uncut isn’t necessarily caused by restriction sites.

One possible model:

--Genome consists of large circles of various size differing in the number of tandem copies of the 6 kb repeat. The number can range from 0 to 4+. Most molecules have 0, but there are enough molecules with multiple tandem copies that the average copy number is close to one. The total number of copies of 6kb repeats found in molecules with >1 copy greatly exceeds the total in molecules with one copy.

--What this would explain: Circular mapping but lack of actual. Alternative structures. 3 bands in SpeI digest. Difference between uncut and SpeI. Roughly equal coverage in Illumina data across genome.

--What this would not explain: 2 very high MW bands in uncut and SpeI (branched recombination products that get hung up??). ~20 kb band in uncut (I have no clue)

An alternative model:

--The 6kb concatamer region does exist as a separate molecule, but attached to some unknown sequence that contains SpeI sites and this concatamer can vary in length

--What this would explain: Pretty much everything explained by the above model

--What this would not explain: Same as above, plus where this unknown flanking sequence is and why I didn’t notice it in the original assembly process (low abundance relative to repeat sequence?)

Next steps:

--To test the above model, I could probe with a sequence just outside the 6 kb sequence to estimate the relative abundance of molecules with 0 copies vs molecules with >1 copies. Prediction would be the 0 copies are stoichiometrically dominant.

--Cut with enzymes other than SpeI that also do not have a cut site in the 6 kb sequence. The above model would predict that the fragments should shift up based on proximity of cut sites. Include IceuI (only cuts once in genome, and David Williams has some)

--Run on pulsed-field gel to get better size separation of bands near 20 kb in SpeI sample.

--Check alternative PE connections from fragments with one end mapping to 6 kb circle.

--qPCR to estimate relative abundance of 6kb sequence to the rest of the genome (this will require and absolute quantification method).

--On a related note, I should probe with something to detect recombination across the short repeat in the prfA and prfB genes.

--It would also be nice to try a digest on DNA from a single individual to see if I can pick up direct evidence of polymorphism within an individual.

12/26/12

Doing Bemisia tabaci DNA extractions to generate more DNA for Southern blots. All from live insects in out lab culture (growing on cow pea, Vigna unguiculata; I finally got around to asking Kim what plant she was growing our whitefly pops on).

4 extractions:

#1 Single individual

#2 Single individual

#3 Pooled individuals (>100)

#4 Pooled individuals (> probably at least 2-fold more than sample #3).

1.6 ml CTAB stock buffer + 12 ul B-Mercaptoethanol + 48 ul Proteinase K 20 mg/ml (from Vince Martinson; note that prot K was not added until after grinding).

100 ul of buffer for samples 1 and 2. 600 ul buffer for samples 3 and 4.

Grind whiteflies after freezing with liquid nitrogen. Mix by inversion.

1.5 hr incubation at 55 C with occasional inversion.

Standard phenol:chloroform 🡪 chloroform 🡪 isopropanol clean-up protocol, except that I added a 30 min incubation at 37 C with RNase A (Qiagen stock; 1 ul for samples 1 and 2; 4 ul of samples 3 and 4) to the aqueous phase from the chloroform step. Then I went back and started at the beginning with a new phenol:chloroform. I did this because previous attempts to do RNase treatment at other stages failed and required a separate treatment and repurifcation. I’m hoping this will save some time by only requiring on isopropanol precipitation.

See notes on 11/26/12 for spin speeds/times/temps etc.

I added 2 ul of glycogen (5 ng/ul) to prior to precipitating samples 1 and 2.

Dissolved final pellets in 10 ul Qiagen Buffer AE for samples 1 and 2 and 30 ul for samples 3 and 4.

Qubit dsDNA BR quantification:

1. 8 ng/ul
2. 32 ng/ul
3. 348 ng/ul
4. 134 ng/ul

Ran 1 % agarose TBE gel. 20 min at 130 V.

Samples:

1. 5 ul of 1/10x 1 kb plus ladder
2. 1 ul of DNA extraction #3
3. 1 ul of DNA extraction #4

Image taken. See photosAndGelPics directory.

Results: Not good. Size/degradation looks OK, but there is a huge RNA signal. Clearly my RNase treatment was insufficient.

12/27/12

Continuing with DNA extractions from yesterday (stored overnight at -20 C).

Add 1 ul of Qiagen RNase A to samples 1 and 2 and 3 ul to samples 3 and 4. Incubate at 37 C for 40 min.

Increased sample volumes to 200 ul with buffer AE, then cleaned up with another round of phenol:chloroform -> chloroform -> isopropanol precipitation. For the precipitation I added 1/10 volume 3 M NaOAc to the isopropanol. 30 min at -20 C.

Dissolved final pellets in 10 ul (sample 1 and 2) or 30 ul (sample 3 and 4) buffer AE.

Qubit dsDNA BR kit:

1. 7 ng/ul
2. 3 ng/ul
3. 3 ng/ul
4. 21 ng/ul

Where did all the DNA go? Much lower yields than expected, especially for sample 3, where I clearly saw a pellet.

Ran 1 % agarose TBE gel. 40 min at 100 V.

Samples:

1. 5 ul of 1/10x 1 kb plus ladder
2. 1 ul of DNA extraction #3
3. 1 ul of DNA extraction #4

Image taken. See /Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20121227\_DNAextractions.TIF

Results: confirms qubit. Yield appears low in sample 4 and essentially absent from sample 3. RNA signal is gone, however. In taking a second look at yesterday’s gel, it is possible that the original qubit estimates were grossly inflated. It is supposed to be insensitive to RNA contamination, but the DNA bands in that gel do not look likw >>100 ng/ul.

How to proceed? Maybe I can squeeze out what I need from the old 11/26 and 11/28 samples (perhaps using less DNA per digest). I can also try all of sample 1 in a single digest to see if I can pick up a signal of alternative genome conformations in a single individual.

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

I have been keeping a couple hackberry petiole galls (see 10/30/12) at room temp in a small Ziploc bag since mid November. About a week ago, I cut one of these open and left it in the bag. Today I noticed a couple adult psyllids. I think there is one male and one female of Pachypyslla venusta. The wing markings differ markedly. One of them has the tip of its abdomen upturned (the reason why I think it’s a male). I put these in eppendorfs and stored at -80 C. They were alive (barely?) when I froze them.

Took pictures:

/Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/1\_Photos/20121227\_Pachypsylla\_venusta\_adults

1/7/13

PCR amplification for new Portiera (Bemisia tabaci) probes.

|  |  |  |  |
| --- | --- | --- | --- |
| **Fwd Primer** | **Rev Primer** | **Templates** | **# rxns** |
| prfB\_blot\_F | prfB\_blot\_R | Duplicate reactions of Bemisia tabaci 11/26 (11/29/12) 1/100x | 2 |
| 6kb\_flank\_F | 6kb\_flank\_R | Duplicate reactions of Bemisia tabaci 11/26 (11/29/12) 1/100x | 2 |

|  |  |  |
| --- | --- | --- |
|  |  | **Mult Fact** |
| **Reagent** | **Vol per rxn (ul)** | **6** |
| Template | 2 |  |
| 10x Buffer | 3 | 18 |
| 25 mM MgCl2 | 0 | 0 |
| 2.5 mM dNTPs | 2.4 | 14.4 |
| Fwd Primer (10 uM) | 1.5 |  |
| Rev Primer (10 uM) | 1.5 |  |
| Taq (5 U/ul) | 0.3 | 1.8 |
| dH2O | 19.3 | 115.8 |
| **Total Vol** | **30** | **150** |
|  |  |  |
| Cocktail Vol (per rxn) | Mult Factor |  |
| 25 | 2.5 | 62.5 |
| Volume per primer | 3.75 |  |

|  |  |  |
| --- | --- | --- |
| **Temp** | **Time** | **Cycle #** |
| 94 deg | 2 min |  |
| 94 deg | 15 sec |  |
| 55 deg | 15 sec | 40 |
| 72 deg | 1 min |  |
| 72 deg | 5 min |  |
| 4 deg | Hold |  |

Combined each pair of reactions and cleaned with Qiagen Qiaquick PCR extraction kit. Eluted each product in 35 ul Buffer EB.

Qubit dsDNA BR quanitification of cleaned PCR products, Very low concentrations:

prfB 🡪 3 ng/ul

6kb \_flank 🡪 2 ng/ul

Ran 1% agarose TBE gel. 120 V for 30 min.

Loading order:

1. 5 ul 1 kb plus ladder
2. 2 ul prfB PCR product (cleaned)
3. 2 ul 6k\_flank PCR product (cleaned)

Image taken. See /Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20130107\_PCR\_highexp.TIF

Results: The prf reaction appears to have failed completely—only primer dimer dimer. Some blunder along the way, or perhaps a difference in sequence between the current DNA samples and those used for the initial genome sequence. The 6kb\_flank reaction produced a product of expected size but it is very faint.

Try repeating both PCRs with the following changes.

--make up new working stocks of the first two primers

--reduce annealing temps to 51 C

--increase template volume.

--include old primer pair as a positive control

--do a single reaction (60 ul) with a longer initial melting temp

|  |  |  |  |
| --- | --- | --- | --- |
| **Fwd Primer** | **Rev Primer** | **Templates** | **# rxns** |
| prfB\_blot\_F | prfB\_blot\_R | Bemisia tabaci 11/26 (11/29/12) 1/100x | 1 |
| 6kb\_flank\_F | 6kb\_flank\_R | Bemisia tabaci 11/26 (11/29/12) 1/100x | 1 |
| PABT\_blot\_F | PABT\_blot\_R | Bemisia tabaci 11/26 (11/29/12) 1/100x | 1 |

\*\*\*\*NOTE: When loading the reaction I accidentally added the wrong primers, so the adjusted order of reactions is:

1. prfb\_F/R
2. prb\_R only (worthless reaction)
3. 6kb\_flank\_F/R
4. PABT\_blot\_F/R (20 ul only)

|  |  |  |
| --- | --- | --- |
|  |  | **Mult Fact** |
| **Reagent** | **Vol per rxn (ul)** | **3.5** |
| Template | 10 | 35 |
| 10x Buffer | 6 | 21 |
| 25 mM MgCl2 | 0 | 0 |
| 2.5 mM dNTPs | 4.8 | 16.8 |
| Fwd Primer (10 uM) | 3 |  |
| Rev Primer (10 uM) | 3 |  |
| Taq (5 U/ul) | 0.6 | 2.1 |
| dH2O | 32.6 | 114.1 |
| **Total Vol** | **60** | **189** |

|  |  |  |
| --- | --- | --- |
| **Temp** | **Time** | **Cycle #** |
| 94 deg | 3 min |  |
| 94 deg | 15 sec |  |
| 51 deg | 15 sec | 40 |
| 72 deg | 1 min |  |
| 72 deg | 5 min |  |
| 4 deg | Hold |  |

Ran 1% agarose TBE gel. 140 V for 25 min.

Loading order (note the extra sample—see above)

1. 5 ul 1 kb plus ladder (1/10x)
2. 2 ul prfb\_F/R PCR product
3. 2 ul 6kb\_flank\_F/R PCR product
4. 2 ul PABT\_blot\_F/R PCR product
5. skip a lane
6. 2 ul prfb\_R only PCR product (worthless reaction)

Image taken. /Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20130107\_PCR2.TIF

Results: Once again, the prfB product completely failed. The 6kb\_flank product worked but it is noticeably fainter than the original probe product. Next up… order new primers for prfB and do another 6kb\_flank amplification so that two of these can be pooled into a single PCR clean up rxn.

\*\*\*\*\*\*\*\*\*

Made buffers for future Southern Blot work.

1 L 20x SSC

--see 12/11/12 protocol

1 L 1 M Tris-HCl pH 7.5

--121.1 g Tris Base in 800 ml ddH2O

--Added concentrated HCl until I reached a pH of 7.5

Autoclaved both SSC and Tris-HCl (20 min liquid cycle). Also autoclaved ~6 L of ddH2O for future use.

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

1/14/13

Continuing with PCR for southern blot probes in Portiera-BT

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Fwd Primer** | **Rev Primer** | | | **Templates** | | **# rxns** |
| prfB\_blot\_F | prfB\_blot\_R | | | Bemisia tabaci 11/26 (11/29/12) 1/100x | | 1 |
| prfB\_blot\_F | prfB\_blot\_R2 | | | Bemisia tabaci 11/26 (11/29/12) 1/100x | | 1 |
| prfB\_blot\_F2 | prfB\_blot\_R | | | Bemisia tabaci 11/26 (11/29/12) 1/100x | | 1 |
| prfB\_blot\_F2 | prfB\_blot\_R2 | | | Bemisia tabaci 11/26 (11/29/12) 1/100x | | 1 |
| 6kb\_flank\_F | 6kb\_flank\_R | | | Bemisia tabaci 11/26 (11/29/12) 1/100x | | 1 |
| PABT\_blot\_F | PABT\_blot\_R | | | Bemisia tabaci 11/26 (11/29/12) 1/100x | | 1 |
|  | |  | **Mult Fact** | |
| **Reagent** | | **Vol per rxn (ul)** | **6.5** | |
| Template | | 4 | 26 | |
| 10x Buffer | | 6 | 39 | |
| 25 mM MgCl2 | | 0 | 0 | |
| 2.5 mM dNTPs | | 4.8 | 31.2 | |
| Fwd Primer (10 uM) | | 3 |  | |
| Rev Primer (10 uM) | | 3 |  | |
| Taq (5 U/ul) | | 0.6 | 3.9 | |
| dH2O | | 38.6 | 250.9 | |
| **Total Vol** | | **60** | **351** | |

|  |  |  |
| --- | --- | --- |
| **Temp** | **Time** | **Cycle #** |
| 94 deg | 3 min |  |
| 94 deg | 15 sec |  |
| 51 deg | 15 sec | 40 |
| 72 deg | 1 min |  |
| 72 deg | 5 min |  |
| 4 deg | Hold |  |

Run 1% agarose TBE gel at 140 V for 20 minutes.

Loading order: 5 ul of 1/10x 1 kb plus ladder, 2 ul each of PCR products in order indicated by table.

Image taken: /Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20130114\_PCR.TIF

Results: Both reactions involving prfB\_F failed, but the others all worked, suggesting that there is something wrong with that primer (mismatch to this population? Synthesis problem?). The intensity of the successful prfB reactions appears comparable to that of the PABT\_blot reaction, which is noticeably stronger than the weak 6kb\_flank amplification.

Proceed with PCR purification with Qiagen Qiaquick PCR extraction kit.

Two clean-ups:

1. Today’s prfB\_blot\_F2/R2 reaction (#4)
2. Pool of today’s 6kb\_flank reaction (#5) and the 6kb\_flank reaction from the second batch of PCR on 1/7/13 (tube #3—but it’s listed as reaction #2 in the table)

Elute each reaction in 30 ul Buffer EB.

Qubit dsDNA BR kit:

prfB: 90 ng/ul

6kb\_flank: 17 ng/ul

\*\*\*\*\*\*\*\*

Perform overnight labeling of cleaned PCR products with Roche DIG kit (see 12/11/12).

20 ul total reaction volumes.

prfB: 1 ug in 16 ul Buffer EB (11.1 ul cleaned PCR product + 4.9 ul buffer EB)

6kb\_flank: 272 ng in 16 ul Buffer EB (16 ul cleaned PCR product)

Denature each at 95 C for 10 min. Transfer immediately to ice bath. Add 4 ul of Roche DIG-high prime (pre-mixed vial by vortexing).

Vortexed and spun down each sample.

Incubated overnight at 37 C starting at 3:30 pm.

1/15/13

Continuing with DIG labeling of probes from yesterday.

Stopped labeling reactions at 10:30am (19 hrs) with 1 ul of 200 mM EDTA and 10 min at 65 C.

Tested labeling efficiency as described previously. See 12/12/12. Once again, I assumed a yield of ~2 ug for each probe (despite the differences in initial template concentration. So I started with a 100x dilution in Roche DNA dilution buffer to generate the “1 ng/ul” sample. I made fresh dilution series (see 12/12/12 notes) for both probes as well as the control DNA sample. I also made a dilution series for a probe from Eli Powell that he wanted checked. Dotted 1 ul samples onto membrane in 4 rows. Top row is control, followed by 6kb\_flank, prfB and Eli’s sample. Cross-linked the membrane on both sides with Statalinker 1800 autocrosslink setting (120,000 uJoules on each side). Proceeded with efficiency test, using same protocol as 12/12/12.

In developing this blot, I accidentally applied the developing reagent to the wrong side (the membrane is close enough to being square that my notch in the bottom right corner could be misinterpreted).

I didn’t realize this until I took the picture. So I ended up taking a second picture of the correct side. Despite the screw up, it appears to have turned out OK. My new probes appear to be one or two dilutions behind the control DNA (suggesting an actual concentration of 10-30 ng/ul).

Images taken on Groisman lab LAS 4000. 20 min exposure. Changed image quality setting from high to super (somehow this seems to have made the quality worse). Otherwise all settings are the same as before. (the second one is properly oriented).

/Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20130115\_1410\_20min.tif

/Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20130115\_1440\_20min\_top.tif

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*

Proceeding with two blots:

1. Re-probe the blot from 12/20/12 with 6kb\_flank probe. This should test the prediction that most copies of the Portiera BT genome have zero copies of the 6 kb tandem repeat sequence.
2. Generate a new blot with the old PABT\_blot probe. This will include some new restriction enzymes as well one DNA sample taken from a single whitefly to test some of my predictions about Portiera BT genome structure.

Reprobing

--Pre-warmed DIG easy-hyb buffer to 37 C for pre-hybridization solution. I mostly re-used the pre-hyb solution from last time, but I topped it off to 20 ml with fresh buffer.

--Incubcated old blot in pre-hyb buffer at 37 C (drying oven) on low speed orbital shaker for 30 min. In heat sealed pouch. I didn’t get a very even seal initially, so there were a large amount of bubbles.

--Pre-warmed 7 ml of fresh DIG easy-hyb solution at 37 C.

--Denatured 10 ul of 6kb\_flank probe (see above) by heating at 96 C for 10 min and immediately transferring to ice. Added probe to pre-warmed solution.

--Poured off pre-hyb solution (saved). Added probe-containing hyb solution to bag. Incubated at 37 C (drying oven) on low speed orbital shaker overnight, started at 5:30 pm.

New Blot

Restriction digest treatments for Portiera BT samples (11/26 and 11/28):

--uncut (no-enzyme, but otherwise treated exactly like the other samples)

--XbaI

--SpeI-HF

--EcoRV-HF

--BamHI

Additional samples digested with EcoRV:

--single whitefly #1 (12/27/12)

--left over 11/26 and 11/28 samples. Pooled to form approximately one full reaction.

8 ul Digest Volumes

4.88 ul dH2O

0.8 ul NEB Buffer #4

0.08 100x BSA

0.5 ul Enzyme (or dH2O for uncut)

1.74 ul DNA (= 0.5 ug; a portion of the 11/26 sample was diluted to have same starting concentration as 11/28)

Enzymes were added in two stages. 0.25 ul initially. And then spiked in another 0.25 ul after 1 hour.

SpeI-HF and EcoRV-HF are the same batch I ordered and used previously. XbaI (NEB 20 U/ul) was new recently purchased by David Williams. I borrowed a little from him. The BamHI is old (expired in 2007). Also note that BamHI is not shipped with buffer #4, but the NEB site lists 100% activity in this buffer.

Heat killed enzymes (although BamHI is not supposed to be susceptible to heat inactivation) for 20 min at 80 C.

Run 0.8% agarose TBE gel (20 cm length) overnight at 33 V (starting at 6:15pm).

Full volumes of each digest loaded.

Loading order.

Lane:

1. blank
2. Roche DNA Molecular Weight Marker III, DIG-labeled (4 ul)
3. Uncut 11/26
4. Uncut 11/28
5. XbaI 11/26
6. XbaI 11/28
7. SpeI 11/26
8. SpeI 11/28
9. EcoRV 11/26
10. EcoRV 11/28
11. Blank
12. EcoRV single whitefly
13. Blank
14. Roche DNA Molecular Weight Marker III, DIG-labeled (2 ul)
15. BamHI 11/26
16. BamHI 11/28

I then skipped the rest of the lanes and, on other side of the gel, loaded 5 ul of 1 kb plus ladder and EcoRV digest of the pooled left-over DNA. This part of the gel will EtBr stained and imaged to check digest and electrophoresis quality.

1/16/13

Continuing with two blots (at different stages) from yesterday.

First, the reprobing of the old blot. Stopped hybridization at 9am (15.5 hrs). Followed washing and detection protocol described on 12/20/12. Did two different exposures on LAS 4000 (10 min and 1 hr).

/Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20130116\_1159\_10min.tif

/Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20130116\_1404\_1hr.tif

Results: Looks terrible. Dark splotchy background. Ladder is still fairly clear and there is some HMW signal in each lane, but there doesn’t seem to be any biological information to glean from this.

\*\*\*\*\*\*\*\*\*

New blot . Continuing with overnight gel run from yesterday.

At 11 am, added 8 ul of 6x loading dye containing bromophenyl blue as a future marker for depurination.

Stopped gel for good at 1 pm (18.75 hrs). Followed protocol for depurination and setting up blot from 12/18/12.

Depurinated in 250 mM HCl for a full 10 minutes and the bromophenyl blue band was still not totally yellow, but I still stopped at that point (last time it turned yellow in only 6 min.

Set up capillary blot using the portion of the gel with first ~17 wells (not all contain samples). Began transfer at 2:30 pm. Note that the gel broke in two pieces when I was placing it atop the pre-soaked filter paper. But I think I was more or less able to repair such that there will be little disruption in the transfer process.

Took picture of the other half of gel with 1 kb plus ladder and test EcoRV digest. Both looked good.

/Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20130116\_digest.TIF

1/17/13

Continuing with blot from yesterday. Stopped transfer at 10:30am (20 hours).

Cross-linked wet membrane with Stratalinker 1800 w/ 120,000 uJoules on each side. On top of soaked filter paper from transfer.

Rinsed membrane briefly in sterile ddH2O and then air dried.

Incubated in sealed hybridization pouch for 30 min at 37 C with 20 ml of Easy-Hyb buffer.

Prepared 7 ml of Easy-hyb buffer pre-warmed to 37 C and added 7 ul of denatured PABT\_blot DIG-labelled probe (12/12/12).

Drained off pre-hybridization solution and incubated in above hybridization solution overnight, starting at noon.

Ethidium stained and imaged the gel after transfer. Looks good. No sign of DNA that failed to transfer. The image also shows where the gel broke, which may be helpful for interpreting the results of hybridization later.

\*\*\*\*\*

New single-whitefly DNA extraction for potential use in future blots.

Four DNA preps each from a single Bemisia tabaci individual taken from our lab culture, growing on cowpea.

Ground under liquid nitrogen.

Added 100 ul CTAB buffer (see earlier) with 1 ul of Beta-Mercaptoethanol

Added 4 ul of 20 mg/ml Proteinase K

Incubated at 55 C for 45 min

Add 100 ul of phenol: chloroform: isoamyl alcohol (25:24:1)

Mix thoroughly by inversion. 2 minutes on ice.

Spin @ max speed, 4 C, 10 min.

Transfer aqueous phase to new tube.

Add equal volume chloroform:isoamyl alchohol (24:1).

Mix thoroughly by inversion. 2 minutes on ice.

Spin @ max speed, 4 C, 10 min.

Transfer aqueous phase to new tube.

Add 2 ul (=10 ug) glycogen

Add equal volume isopropanol.

Mix by inversion

30 min at -20 C

Spin @ max speed, 4 C, 10 min.

Remove supernatant and wash with 1 ml cold 70% EtOH

Spin @ max speed, 4 C, 3 min.

Remove supernatant and air dry pellet. Left dried pellets at room temp overnight.

\*\*Note that I had a very difficult time seeing pellets in samples 2 and 3 after wash. They may have been lost.

1/18/13

Continuing with Southern blot from yesterday.

First, the reprobing of the old blot. Stopped hybridization at 8:30am (20.5 hrs). Followed washing and detection protocol described on 12/20/12.

30 min exposure of LAS 4000.

Results: Disaster. Massive background like the last re-probed blot. Maybe the problems had nothing to do with re-probing because this is a brand new blot. I can’t think of any obvious causes that differentiate the first successful run vs. the last two. A few miscellaneous changes (grasping as straws here).

--I used Roche hybridization bags for the hyb and pre-hyb steps the first time. Since then, I have used heat sealable pouches.

--I have been re-using the large plastic tubs with simple ddH2O rinses between each step. They would have been brand new for the first blot.

--I did a 65 C wash before hybridization the first time, but not this time (does not really apply to the issue of re-probing)

--I may not have had the orbital shaker all the way down to 2 for pre-hyb and hyb in these last two rounds.

--For development, I believe I used the smaller type of pouch for the first blot and the larger pouches for the last two.

I will probably try to move forward with a new blot—very small and simple to (look at prfB probe?). The hope will try to do everything as similar as possible to my first pass through.

\*\*\*\*\*\*\*\*

Continuing with DNA extraction from yesterday. Dissolved each pellet in 10 ul Qiagen buffer AE.

Add 1 ul of Qiagen RNase A to each sample and incubated at 37 C for 1 hour.

Re-purified with EtOH precipitation (no phenol/chloroform). Dissolved pellets in 10 ul Qiagen Buffer AE.

Measured concentrations with Qubit dsDNA BR kit:

#1: 3.6 ng/ul

#2: Below detection limit

#3: Below detection limit

#4: 3.2 ng/ul

Labeled as B. tab 1 through 4. Stored at -20 C.

1/21/13

PCR to use as a positive control for Southern blots.

|  |  |  |  |
| --- | --- | --- | --- |
| **Fwd Primer** | **Rev Primer** | **Templates** | **# rxns** |
| prfB\_blot\_F2 | prfB\_blot\_R2 | Bemisia tabaci 11/26 (11/29/12) 1/100x | 1 |
| 6kb\_flank\_F | 6kb\_flank\_R | Bemisia tabaci 11/26 (11/29/12) 1/100x | 1 |

|  |  |  |
| --- | --- | --- |
|  |  | **Mult Fact** |
| **Reagent** | **Vol per rxn (ul)** | **2.5** |
| Template | 1 | 2.5 |
| 10x Buffer | 1.5 | 3.75 |
| 25 mM MgCl2 | 0 | 0 |
| 2.5 mM dNTPs | 1.2 | 3 |
| Fwd Primer (10 uM) | 0.75 |  |
| Rev Primer (10 uM) | 0.75 |  |
| Taq (5 U/ul) | 0.15 | 0.375 |
| dH2O | 9.65 | 24.125 |
| **Total Vol** | **15** | **33.75** |

|  |  |  |
| --- | --- | --- |
| **Temp** | **Time** | **Cycle #** |
| 94 deg | 2 min |  |
| 94 deg | 15 sec |  |
| 51 deg | 15 sec | 40 |
| 72 deg | 1 min |  |
| 72 deg | 5 min |  |
| 4 deg | Hold |  |

\*\*\*\*\*\*\*\*

New Bemisia tabaci DNA extractions. CTAB protocol.

6 samples (all from our lab B. tabaci culture):

4 single individuals

2 pools of >100 individuals.

Master mix of 1.7 ml CTAB buffer + 14 ul B-Mercaptoethanol.

Ground samples after freezing in liquid nitrogen. Add 600 ul of buffer for pools and 100 ul for singles. Added 20 ul of prot K (20 mg/ml) to pools and 4 ul to singles.

Incubated at 55 C for 1 hr 15 min.

Phenol and Chloroform extractions

Isopropanol precipitation. (added 2 ul of 5 ug/ul glycogen to the single samples).

Dissolved pellets in 10 ul (singles) or 50 ul (pools) Qiagen buffer AE.

Proceeded with RNase A digestion for the pooled samples only (single samples will still have RNA but I don’t want to lose DNA unneccesarily).

Add 4 ul of Qiagen RNase A stock to each pooled sample. Incubated at 37 C for 1 hour. Another round of phenol/chloroform clean up and isopropanol precipitation (added 0.1x volume of 3 M NaOAc and 2 ul of 5 ug/ul glycogen to each sample). Dissolved pellets in 50 ul Qiagen Buffer AE.

Samples labeled as B. tab S1-S4 (for single samples) and P1-P2 (for pooled samples)

Qubit dsDNA BR quantifications:

S1 8 ng/ul

S2 4 ng/ul

S3 5 ng/ul

S4 undetectable

P1 26 ng/ul

P2 29 ng/ul

Restriction Digests of B. tabaci DNA for Southern blots

To be probed with prfB:

EcoRI—2 samples (11/26 and 11/28, 1 ug each)

EcoRV—3 samples (11/26 and 11/28, 1 ug each; Today’s S1 sample, 8.4 ul, ~60 ng)

To be probed with 6kb\_flank:

EcoRV/SpeI double digest—3 samples (11/26 and 11/28, 1 ug each; Today’s S2 sample, 8.4 ul, ~35 ng)

10 ul reactions

8.4 ul DNA + ddH2O

1 ul 10x Buffer #4

0.1 ul 100x BSA

0.5 ul Each enzyme (total rxn volume for double digest was 10.5 ul)

Added the half the enzyme(s) and incubated for 1 hour. Then added other half and incubated for another hour.

Ran 0.8% agarose TBE gel. Medium gel tray/rig. 11x16 cm gel cast. 125 V for 1 hour.

30 well comb. Loading order:

1. Roche DNA Molecular Weight Marker III, DIG-labeled (4 ul)
2. EcoRI: 11/26
3. EcoRI: 11/28
4. EcoRV: 11/26
5. EcoRV: 11/28
6. EcoRV: S1
7. Roche DNA Molecular Weight Marker III, DIG-labeled (2 ul)
8. Blank
9. prfB PCR product from today. 1 ul of 200x dilution.
10. Blank
11. Blank
12. Blank
13. Roche DNA Molecular Weight Marker III, DIG-labeled (4 ul)
14. SpeI/EcoRV: 11/26
15. SpeI/EcoRV: 11/28
16. Blank
17. SpeI/EcoRV: S2
18. Blank
19. Roche DNA Molecular Weight Marker III, DIG-labeled (2 ul)
20. Blank
21. 6kb\_flank PCR product from today. 1 ul of 200x dilution.
22. Blank
23. Blank
24. Blank
25. Blank
26. 1 kb plus ladder (1/10x) – 5 ul
27. P1 DNA extraction from today – 1 ul
28. P2 DNA extraction from today – 1 ul
29. prfB PCR product from today – 3 ul
30. 6kb\_flank PCR product from today – 3ul

Cut off everything after lane 22 and ethidium stained it. Image taken:

/Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20130121\_extractionsAndPCR.TIF

Results: DNA preps look good (no major degradation). Amplification in both PCRs. The 6kb\_flank is on the weak side, as usual.

Set up the rest of the gel for transfer to nylon membrane. See 12/18/12 protocol. Transfer to single membrane that will be subsequently split at lane 11 into section that will be probed with either prfB (lanes 1-10) or 6kb\_flank (lanes 12-22).

Depurination took the full 10 minutes to get bromophenyl blue band to turn yellow

Started transfer at 6:30pm.

1/22/13

Stopped transfer at 9:30am (15 hrs). The paper towels were already wet through to the top in some places—particularly what will correspond to the bottom portion of the prfB blot. This has not happened before (and is to be avoided). In the future I should either use more paper towels or a lighter weight (1 used the same 500 ml squeeze bottle as I had been using in the past) with these smaller gels.

Generally followed 12/19/12 protocol for hybridization. I did include the 65 C incubation. I also remembered the ddH2O wash that I forgot on 12/19.

I divided the membrane in two at lane 11. I cut off the bottom right corner of each membrane for orientation. The cut-off corner in the 6kb\_flank membrane (lanes 12-22) was made larger to distinguish between the two membranes. VWR orbital shaker, setting 2.

65 C wash was done in 25 ml (each membrane) of 0.1x SSC and 0.1% SDS. 20 min

Pre-hybiridization in 10 ml of easy-hyb buffer at 37 C for 35-40 min. VWR orbital shaker, setting 2.

Hybridization in 5 ml of easy hyb-buffer with 10 ul of the corresponding denatured probe (8 min at 99 C on thermal cycler and immediately transferred to ice). 37 C starting at 11:30am. VWR orbital shaker, setting 2.

These steps were all performed in Roche hybridization bags (not heat sealable pouches like the last two blots).

1/23/13

Stoppped hybridization at 8:15 am (20 hrs, 45 min) and proceeded with washing and detection of Southern blots. Generally following protocol from 12/20/12. Note the following changes. Both blots were processed in parallel (but always in separate washing containers)

Low and high stringency washes done in 125 ml volumes. Both low stringency washes were done for 10 minutes.

110 ml of blocking solution

20 ml of antibody solution

20 ml of detection buffer

1 ml of CSPD

No 37 C incubation after heat sealing pouch.

In general, I tried to return to the protocol I used for the 12/20/12 blot protocol that was relatively successful (in contrast to the last two). This included the following changes relative to more recent runs:

--hybridization in Roche hybridization bags rather than the heat-seal pouches

--development in the smaller (green package) heat seal pouches

--make sure to keep low orbital shaker speed (setting 2) during pre-hyb and hyb steps

--I didn’t get new tupperwares, but I did wash them with alconox between each step, rather than just a ddH2O rinse.

Took images of the two blots (both blots are included in each image) with Groisman lab LAS 4000.

/Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20130123\_1125\_10min.tif

/Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20130123\_1502\_30min.tif

/Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20130123\_1528\_10min\_2.tif

The first image (10 min exposure) was taken essentially right after sealing (remember there was no 37 C treatment). The other two pictures were taken about 3 hours later when the signal intensity had increased.

Results: Looks a lot better than the last two rounds. The terrible background problems appear to have abated. But the bands are not as sharp as my first blot and there is more non-specific hybridization within the lanes. I would guess that the fuzzy signal reflects one or both of the following: 1) moisture reached the top of the paper towel stack during transfer, and 2) I ran the gel for a short time at high voltage (125 V for 1 hr).

The prfB blot appears to confirm the following predictions, with main fragment clearly stronger than the secondary fragment:

EcoRI

main fragment: 4483 bp

recombinant: 2020 bp

EcoRV:

main fragment: 8824 bp

recombinant: 6853 bp

There are also some other weak but detectable bands in the prfB blot.

The 6kb\_flank blot also seems to confirm the following prediction:

EcoRV/SpeI double digest

--ca. 1180 bp (dominant, 0 copies of 6 kb seq)

--ca. 2510 bp (rare, 1 or more copies of 6 kb seq)

In this blot, there are even more (and stronger) unexpected bands, plus a lot of HMW signal from 5 to 20+ kb. Remember that this probe was especially short and AT rich, so specificity may be an issue with it.

The single insect samples in each blot were two weak to provide any evidence of structural variation within an individual.

How to proceed: More or less repeat the new blot design started on 1/15/13. Include multiple single individual samples (EcoRV) side by side.

1/24/13

Took another image of the blots from yesterday with a 5 minute exposure time. The overnight wait appears to have cleaned up the signal somewhat (and changed the relative intensity of some of the band—perhaps the substrate concentration has been reduced in some places?)

20130124\_0926\_5min.gel

20130124\_0927\_5min.tif

\*\*\*\*\*\*\*

Doing extractions on more single Bemisia tabaci whitefly individuals for next Southern blot. From our lab colony. 6 samples. Same basic protocol as 1/21/13.

Labeled tubes B. tab S5-S10 (continuing number scheme from last batch).

Qubit dsDNA BR quantification:

S5 9 ng/ul

S6 9 ng/ul

S7 3 ng/ul

S8 14 ng/ul

S9 12 ng/ul

S10 5 ng/ul

\*\*\*\*\*\*\*\*\*\*\*\*

I’m running low on my original DIG labeled probe and will need some more for my next planned blot….

Repeated DIG labeling from 12/11/12, using most of the remaining cleaned PCR product (exact same sample as used on 12/11/12). Same protocol. Started overnight incubation at 3:45pm.

1/25/13

Stopped labeling reaction at 8:45am (17 hours) by adding 2 ul 200 mM EDTA and incubating at 65 C for 10 min. Stored at -20 C.

Setting up restriction digests for a new Southern Blot.

Planned gel layout:

1. EcoRV - Single sample S6 (1/24/13)
2. EcoRV - Single sample S8 (1/24/13)
3. EcoRV - Single sample S9 (1/24/13)
4. Blank
5. Roche DNA Molecular Weight Marker III, DIG-labeled (1 ul)
6. Uncut – 11/26 sample (11/29/12)
7. Uncut – P1 sample (1/21/13)
8. Uncut – P2 sample (1/21/13)
9. XbaI – 11/26 sample (11/29/12)
10. XbaI – P1 sample (1/21/13)
11. XbaI – P2 sample (1/21/13)
12. SpeI – 11/26 sample (11/29/12)
13. SpeI – P1 sample (1/21/13)
14. SpeI – P2 sample (1/21/13)
15. EcoRV – 11/26 sample (11/29/12)
16. EcoRV – P1 sample (1/21/13)
17. EcoRV – P2 sample (1/21/13)
18. BamHI – 11/26 sample (11/29/12)
19. BamHI– P1 sample (1/21/13)
20. BamHI – P2 sample (1/21/13)
21. Roche DNA Molecular Weight Marker III, DIG-labeled (4 ul)

The P1 and P2 samples are much less concentrated than the 11/26 sample (more than an order of magnitude difference according to the original Qubit measurements on each). But I re-ran a Qubit dsDNA BR quantification on a 10x dilution of the 11/26 sample, and got a value of 17 ng/ul (only about half of what I expected). So I am going to dilute the 11/26 sample 5-fold and use it at equal volumes with the (undiluted) P1 and P2 samples

10 ul reaction volumes

8.4 ul DNA

1 ul NEB 10x Buffer 4

0.1 ul NEB 100x BSA

0.5 ul enzyme

Starting with 0.25 ul of each enzyme for 40 min incubation at 37 C. Then add a second 0.25 ul of enzyme to each reaction and incubate for another 30 min (plus 10 min handling time associated with adding the second dose of enzyme.

Note that the uncut sample will be processed with the digestions (including all heating steps), just with ddH2O in place of enzyme.

NOTE: I screwed up when I added the second round of enzyme, and accidentally added XbaI to the three “uncut” samples. So, I pulled these three samples out and will run them along side the main samples. I will ethidium stain them to get a sense of digest quality (even though they were only digested for ca. 30 min) I replaced them with new uncut DNA samples. Unfortunately, there are two limitations. First, the samples only experienced the last ~20 min at 37, so they are no longer well matched control. Second, I did not hae enough DNA left of the P1 and P2 samples, so they only included about half the DNA (roughly 4 ul each).

Inactivated enzymes with 20 min at 80 C.

Ran large (20 cm; 36-tooth comb) gel with the loading order indicated above. On far the right side of the gel. I added the following 5 samples:

1. DIG labeled ladder – 5 ul
2. 1 kb plus ladded – 5ul
3. uncut + accidental XbaI 11/26 sample
4. uncut + accidental XbaI P1 sample
5. uncut + accidental XbaI P2 sample

All samples loaded with standard lab “OJ” loading dye. But I added 5 ul of blue dye to the blank lane (#4) to try to gauge migration distance.

0.8% agarose TBE gel. Run at 44 V starting at 3:15pm.

1/26/13

Stopped gel at 10:45a,=m (19.5 hrs). Did depurination, denaturation, and transfer setup as described on 12/18/12.

Transferred portion of gel corresponding to lanes 1-22. Cut off ~2 inches off bottom of gel.

Transfer started at 12:30.

Ethidium stained and imaged the 5 right-most lanes on the gel.

/Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20130126\_digests.TIF

Results: Looks good. Gel retains fragments of a little over 1 kb and above. But for the transfer, I cut off the bottom at roughly 2 kb. The relative intensity of the DNA samples appears to vary, with the 11/26 sample noticeably weaker than the other two.

1/27/13

Stopped transfer after ca. 24 hours. Followed protocol from 12/19/12 for cross-linking, 65 C pre-wash, pre-hyb and hybridization. (I did include the ddH2O wash forgotten on 12/19/12). Used 30 ml of pre-hyb solution and 10 ml of hybridization solution with 10 ul of PABT probe. The probe was the last 5 ul of the original 12/11/12 labeling and 5 ul from the new batch from 1/24/13. Note that the latter has not been measured for labeling efficieny. I am just assuming it’s similar to the original labeling reaction.

Started hybridization (37 C) at 2 pm. Left overnight.

Ethidium stained gel post-transfer. Did not see any signal from residual DNA (no image saved).

1/28/13

Stopped hybridization at 9:00am (19 hrs). Essentially followed 12/20/12 protocol for washing and detection, with slight modifications:

-10 min each for low stringency washes.

-Also, I didn’t precisely measure out the volumes for most of the wash/incubation steps, so they are not exactly the values from 12/20/12. They tend to be slightly above those values.

-No, 10 min 37 C incubation after adding development reagent.

Took multiple images on Groisman Lab LAS 4000.

--15 min exposure: right away

--1 hour exposure: immediately after 15 min exposure

--1 hour exposure: 3.5 hrs after development

--20 min exposure: immediately after 2nd 1 hour exposure

I also plan to take a 40 minute exposure tomorrow morning.

All images can be found in the following directory: /Users/drt\_lab/Documents/Yale/MoranLab/projects/PortieraTV/Southerns/exposures/20130128

Results: The signal is weak relative to background. The background problems aren’t as extreme as my 2nd and 3rd blots, but it’s pretty ugly. Part of the problem might be the lower quantities of DNA that were loaded (less signal, requiring longer exposures).

But there is still some interpretable signal…

--First, it is clear that the single DNA samples contain two bands confirming that variation exists within a single individual. This is most clear in the second of the three individual samples loaded. If I wanted to go out on a limb and overinterpret the data, a case could be made that there is variance in the relative intensity of the two bands, with the HMW band being weakest in sample 1 and strongest (relative to the LMW band) in sample 3.

--XbaI has similar HMW banding to SpeI, but it lacks the 7.65 kb band. This make general sense given the model of tandem duplications. This is generally consistent with the tandem duplication model. The only thing I’m uncertain about is that XbaI bands should be slightly lower MW than the SpeI bands (e.g., 13 kb vs 14 kb), and I don’t see evidence of this. They look very similar, so one alternative interpretations is that the those high MW bands represent some sort of alternative conformation (circle or circular multimer in different supercoiled vs relaxed states??). This could be tested with another enzyme(s) that generate a smallest predicted band <<14 kb or >>14 kb. But I don’t think this is necessary. The banding does not show up in the uncut sample, so I think the tandem repeat interpretation is well supported

--BamHI shares the dominant 6.5 kb band with EcoRV as expected (one cut site in the 6 kb “circle”). It’s tough to tell if it is has the expected faint band 42 kb, but there’s a bit of a smudge.

--SpeI and EcoRV show expected banding pattern consistent with the first blot

--In the first blot, there was an interesting double band at very HMW—just out of the wells. The bands were present in uncut and SpeI samples, but absent in samples any samples with EcoRV. This made me wonder if there was some sort of complex branched structure (replication related?) in the 6 kb tandem repeat region that was eliminated whenever you cut inside it (EcoRV). If that were the case, I would have expected to see the same bands in XbaI samples but not in BamHI samples. Unfortunately, the image quality is too poor to get any sense of whether these bands are present or absent in any of the samples, so I don’t think I can shed any light on this question.

\*\*\*\*\*\*\*\*\*\*\*

I have been keeping some split-open hackberry petiole galls at room temp in very small Ziploc bags on my dest (I think since 12/27/12, but it might have been since 1/7/13). Adults have been emerging sporadically for a while. Some have died, but I went through today and collected live ones. Eight individuals in total. I distinguished what I believe are males based on their upturned abdomens. I dissected two individuals (and took pictures). I stored the other 6 in different ways: 2 in EtOH at -20 C. 2 in RNA protect at -20 C. 2 at 80 C.

1. Female – dissected
2. Male – dissected
3. Male – RNA protect
4. Female – EtOH (looked immature compared to others. Wings not well developed.
5. Female – EtOH
6. Female – RNA protect
7. Male – 80 C
8. Female – 80 C

Dissection supported my gender identification. The putative male contained two very large, bright yellow/orange structures, which I believe to be testis. The female contained white lobed structures, which I believe to be ovaries.

Images were taken of individuals 1 and 2, including shots post-dissection. The images are titled Pv1\_X and Pv2\_X for individuals 1 and 2, respectively. They can be found in the following directory:

/Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/1\_Photos/20130128\_Pachypsylla\_venusta\_adults

Looking at the images, I see that the wing markings between the male and female show the same differences that I commented on earlier (12/12/27). Only two of each gender inspected but this could be an easy way to distinguish genders.

1/29/13

After taking a final 40 min exposure of yesterday’s blot, I stripped the membrane as follows:

Rinse in ddH2O

2x15 min in ~50 ml of 0.2 M NaOH at 37 C, orbital shaker, setting 2

5 min in 100 ml 2x SSC. Room temp. orbital shaker, setting 2

Aired dried and stored at room temp between 2 pieces of filter paper

2/7/13

Based on the early returns from sequencing and assembly, it sounds like I may need more DNA for the i5K Pachypsylla venusta genome project.

I still have live nymphs at 4 C from Nancy’s 10/30/12 gall collection in Austin, TX. But they are near their limit after 3+ months in the fridge, so I removed a total of 44 (still living) 5th instar nymphs from 9 galls, and stored them in individual eppendorf tubes at -80 C. Each nymph was briefly rinsed in EtOH and dried on a kim-wipe before being put in the eppendorf tubes.

The tubes were labeled with only two numbers corresponding to gall and nymph #.

For example, 4.3 would mean the third nymph from gall 4. They were not dated or labeled with any other identifying information. The (square, plastic) freezer box is labeled “DBS 2/7/13 Pachypsylla venusta Nymphs”.

2/27/13

I pulled some more (barely) live nymphs from the hackberry petiole galls collected in Austin on 10/30/12 for a round of DNA extractions today.

Eight nymphs (5th instar) from two galls. Number 1-6 were from the same gall, while numbers 7 and 8 were from a second gall. All individuals showed at least slight signs of life (twitching legs). Each was briefly dipped in EtOH to surface sterilize and then dried on a kimwipe. Put in individual eppendorf tubes and stored on dry ice in -80 C for 1 hour (we are out of liquid nitrogen, so this my attempt at deep freezing).

CTAB buffer: 4.5 ml of stock + 30 ul B-ME

Ground each nymph with pestle (pre-cooled at -80 C).

Incubated at 55 C in 500 ul CTAB buffer for 2 hours. After 2 hours, I realized I never added proteinase K, so I added 10 ul of 20 mg/ml (from Vince Martinsen) to each tube and continued incubation overnight.

2/28/13

Continuing with DNA extractions.

Added 500 ul PCI. Mix by inversion. Settle for a couple minutes

Spin 10 min at max speed, 4 C

Transfer aqueous phase to tube with 450 ul CI. Mix by inversion and let settle.

Spin 10 min at max speed, 4 C

Transfer aqueous phase to tube with 400 ul isopropanol. Mix by inversion. 1 hr at -20 C

Spin 10 min at max speed, 4 C

Wash pellet in 500 ul cold 70% EtOH.

Spin 5 min at max speed, 4 C

Dry pellet and dissolve in 100 ul Qiagen buffer AE.

Add 4ul of Qiagen RNase A stock to each tube. Mix by inversion. 30 min at 37 C.

EtOH Precipitation (0.1 vol 3 M NaOAc, 2.5 vol EtoH, ~30 min at -20 C). Spin 10 min at 4 C, max speed. Wash in 500 ul cold 70% EtOH. Spin 5 more min. Remove sups. Dry pellets and dissolve each in 10 ul Qiagen buffer AE.

Remove 1.5 ul from each tube and dilute 1:1 with dH2O.

Qubit dsDNA BR quantification:

|  |  |  |  |
| --- | --- | --- | --- |
| **Sample** | **Qubit Reading (ng/ul)** | **Undiluted Conc (ng/ul)** | **Total Remaining Yield (ng)** |
| 1 | 20 | 40 | 340 |
| 2 | 42 | 84 | 714 |
| 3 | 25 | 50 | 425 |
| 4 | 61 | 122 | 1037 |
| 5 | 66 | 132 | 1122 |
| 6 | 50 | 100 | 850 |
| 7 | 41 | 82 | 697 |
| 8 | 69 | 138 | 1173 |

Ran 0.5x TBE gel with 5 ul of 1/10 x 1 kb plus ladder and 1.5 ul of (2-fold diluted) DNA from each sample. 120 V for 40 min. Gordon Bennett took picture for me because I had to leave early.

Image:

/Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20130228\_extractions.TIF

Results: Looks OK. Mostly HMW DNA but there is also some streaking.

3/4/13

Took some pictures at max zoom on dissecting scope of Bemisia tabaci adults from our lab colony growing on cow pea.

/Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/1\_Photos/20130304\_Bemisia\_tabaci.

3/7/13

Qiagen Blood and Tissue DNA extraction kit preps from Pachypsylla venusta nymphs from the 10/30/12 collection in Austin, TX. Twelve extractions (1 5th instar nymph each). From two different galls. Tubes labeled Pv 1-1 to 1-6 and 2-1 to 2-6.

ca. 4hr incubation step at 56 C

Included optional RNase digestion.

Eluted in 50 ul Buffer AE. For half of the samples (all those ending in even number, e.g., 1-2, 1-4, 2-2, etc.). I took the eluate and added it back to the column and did a second centrifugation. Based on qubit values, this appears to have roughly doubled yield.

Qubit dsDNA BR quantification.

|  |  |
| --- | --- |
| **Sample** | **Conc (ng/ul)** |
| 1-1 | 5.4 |
| 1-2 | 12.9 |
| 1-3 | 8.8 |
| 1-4 | 12.3 |
| 1-5 | 9.8 |
| 1-6 | 16.1 |
| 2-1 | 6.6 |
| 2-2 | 15.2 |
| 2-3 | 4.8 |
| 2-4 | 13.9 |
| 2-5 | 9.2 |
| 2-6 | 18.9 |

Stored at -20 C.

Ran 1% agarose TBE gel with each DNA sample from above. 1 ul for the double elution samples, and 2 ul for the single elution samples. 5 ul of 1 kb plus Invitrogen ladder. Ran at 100 V. I’m not sure how long. I asked Katherine Urban-Mead to stop it and take a picture for me.

Image: /Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20130308\_extractions.TIF

Results: Looks like there is some degradation, but it’s tough to tell because the image is underexposed.

3/25/13

More Pachypsylla venusta DNA extractions. As above, except I did 4 cycles of elutions (kept passing the same 50 ul volume of buffer AE over the column to increase yield).

Two galls: 12 samples.

Labled 1-1 to 1-7 and 2-1 to 2-5. All were clearly alive except the last two (2-4 and 2-5), which did not show signs of movement.

Stored at -20 C.

Qubit dsDNA BR kit:

|  |  |
| --- | --- |
| **Sample** | **Concentration (ng/ul)** |
| 1-1 | 24.9 |
| 1-2 | 21.2 |
| 1-3 | 33.9 |
| 1-4 | 21.1 |
| 1-5 | 32.9 |
| 1-6 | 31.0 |
| 1-7 | 28.3 |
| 2-1 | 25.0 |
| 2-2 | 21.3 |
| 2-3 | 20.6 |
| 2-4 | 31.3 |
| 2-5 | 18.7 |

5/21/13

Doing a couple of RNA extractions from adult Pachypsylla venusta specimens that have been stored at -80 C.

Two samples:

P. venusta #7 male. 1/28/13

P. venusta #8 female. 1/28/13

1. Remove frozen psyllids from -80 C.
2. Add 1 ml of Trizol added to each sample.
3. Grind with pre-cooled pestles (-80 C).
4. Vortex.
5. 10 min at room temp.
6. Add 200 ul chloroform. Vigorous shaking. 3 min at room temp
7. 15 min at 12000g, 4C
8. Transfer 550 ul sup to new tube
9. Add 1 ul (5 ug) glycogen
10. Add 550 ul isopropanol.
11. Mix by inversion
12. 10 min at 12000g, 4C
13. Wash pellet with 1 ml 80% EtOH.
14. 5 min at 7500g, 4C. Remove sup
15. Air dry pellet.
16. Dissolve in 40 ul RNase-free dH2O just before pellet went completely transparent.
17. ~3 min at 55 C.

🡪 Remove 1.5 ul samples for subsequent Agilent analysis.

1. Treat each sample with Ambion DNA-free kit. Add 4 ul 10x DNase Buffer and 1 ul rDNase I.
2. 30 min @ 37 C
3. Add 4.5 ul DNase inactivation reagent
4. 2-3 min at room temp with occasional mixing
5. 1.5 min @10,000g, 4 C.
6. Transfer sup to new tube. Add 120 ul cold EtOH and 1.5 ul 3M NaOAc. Mix by inversion
7. 1.5 hours at -20 C
8. Spin 10 min, 12000g, 4 C
9. Wash in 500 ul cold 80% EtOH
10. Air dry pellet.
11. Dissolve in 21 ul RNase-free dH2O
12. ~3 min at 55 C.

Stored samples at -80 C

Ran Agilent Bioanlyzer 2100 RNA Nano 6000 chip.

4 samples:

#1. Pven 7 male, pre-DNase

#2. Pven 8 female, pre-DNase

#3. Pven 7 male, post-DNase

#4. Pven 8 female, post-DNase

See results here:

/Users/drt\_lab/Documents/Yale/MoranLab/AgilentRuns/2013-05-21

Concentration estimates for samples 1-4 are as follows:

1. 40.4 ng/ul
2. 143.1 ng/ul
3. 26.8 ng/ul
4. 214.4 ng/ul

Consistent with very small (almost invisible pellet for the male). Looks like I have 0.5 ug for the male and 4 ug for the female.

No evidence of major degradation (typical funny rRNA size pattern).

5/27/13

Running 1% agarose TBE gel to check out DNA quality from recent rounds of Pachypyslla venusta genomic extractions. 100V for 1 hour.

Loading order:

1. Invitrogen 1 kb plus ladder. 1/10x. 5 ul
2. Pv 1-1 (3/7/13) – 2 ul
3. Pv 1-2 (3/7/13) – 1 ul
4. Pv 1-3 (3/7/13) – 2 ul
5. Pv 1-4 (3/7/13) – 1 ul
6. Pv 1-1 (3/25/13) – 1 ul
7. Pv 1-2 (3/25/13) – 1 ul

Image taken: /Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20130527\_DNAextractions.TIF

Also retook image with fresh ethidium:

/Users/drt\_lab/Documents/Yale/MoranLab/PhotosAndGelPics/20130527\_DNAextractions2.TIF

Results: Looks fine. Some degradation, but nothing more than normal. No obvious difference in quality between samples with two passes over the Qiagen column vs. those with a single pass.

7/1/13

Shipped RNA samples from adult Pachypsylla venusta males and females to Baylor HGSC to be used for RNA-seq libraries for annotation in i5k project.

Samples from 5/21/13 and labeled:

Pven\_male

Pven\_female

Shipped standard overnight on dry ice.

8/9/13

I was running on Shields St. in Fort Collins and I saw some hackberries with nipple galls on the leaves. I’m just recording this in case I want to go back to them. The trees were planted in the Sping Creek Medical Park. [On subsequent walks around town, I’ve also seen hackberries on the south side of Drake between Dunbar and Shields, and on CSU campus – I believe it was on East Dr. in front of the Natural and Environmental Sciences Building].