Protocol 1: *B. anynana* Crossing Scheme and Genomic DNA Extraction

I. Crossing Scheme (Figures 1 and 2)

A. Produce recombinant, backcrossed offspring between *Spotty* and Wt individuals using a wild-type (Wt) *B. anynana* adult mated with a Spotty (*S/S*) *B. anynana* adult.

B. Cross a heterozygous Spotty female resulting from the initial pair cross with a homozygote Spotty male to produce heterozygote Spotty offspring with an identical Wt allele (note: there is no recombination in female Lepidoptera).

C. Set up single-pair matings with heterozygote Spotty offspring to produce the next generation of offspring, collect within 24 hours of emergence, and store at -20C.

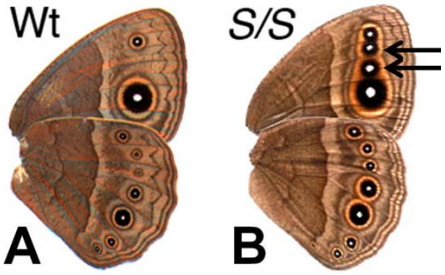

Figure 1: The *Spotty* phenotype in *B. anynana*. **A)** Wild-type (Wt) forewing and hindwing. **B)** A *Spotty* mutant (S/S) with extra eyespots (black arrows) on the ventral forewing.

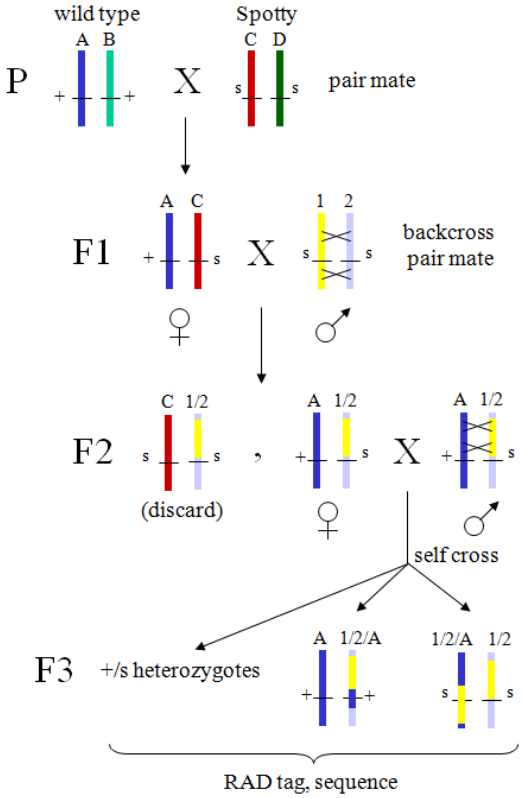

Figure 2: Crossing Scheme between Wt and Spotty

II. Genomic DNA Extraction

A. This protocol has been modified from the DNeasy Blood and Tissue Kit (Qiagen #69506) and Purification of Total DNA from Insects (Qiagen Supplementary Protocol).

B. Tissue Disruption

1. Prepare Proteinase K/Buffer AL mastermix in a Falcon tube (20uL Proteinase K and 200uL Buffer AL per sample, mixed thoroughly) with an extra 20uL Proteinase K and 200uL AL so there is enough for all animals (i.e. 5200uL AL with 520uL Proteinase K will be enough for 24 samples). Mix well before aliquoting below.
2. Add 75uL of mastermix to eppendorf tubes with 3 scoops of 0.9-2.0mm Stainless Steel beads (Next Advance #SSB14B) (have tubes ready to add tissue after dissection).
3. Dissect thorax tissue (without legs, wing fragments, etc.) under a dissection scope with microdissection tools (Fine Science Tools) by cutting off head and abdomen. Let thorax air-dry for several minutes and then add thorax to tubes after completely dry of EtOH. Clean tools with both water and 100% EtOH after each dissection.
4. Add to Bullet Blender Homogenizer/Tissue Disrupter (Next Advance), blend twice for 5 minutes at 10 speed, and spin down (13,200rpm for 1 min) to make sure

thorax is disrupted before continuing to the next step. If not, reblend and spin down until disruption occurs (Figure 3).

Figure 3: Bullet Blender (Next Advance) was used to disrupt butterfly thoracic tissue to isolate genomic DNA in the Monteiro lab.

C. Tissue Incubation and RNase Treatment

1. Add 145uL (remainder of 220uL) to tubes and then place tubes in a 56C tube Thermomixer (eppendorf) for 20 minutes at 500rpm (Figure 4).
2. Spin down tissue and beads (13,200rpm for 1 minute). Add supernatant (not the precipitate on the side of the tube) to a fresh tube.
3. Add 4uL RNAse A (Qiagen #158922). Flick tube and vortex, then spin on tabletop centrifuge briefly to make sure RNAse is incorporated well. Incubate at RT for 2 minutes.
4. Add 200uL EtOH to tubes and mix thoroughly by vortexing.

Figure 4: A Thermomixer (eppendorf) was also used to isolate genomic DNA in the Monteiro lab, in preparation for RAD-library construction in the Hoekstra lab.

D. Genomic DNA Collection, Elution, and Quantification

1. Pipet solution into DNeasy Mini spin column in a collection tube and centrifuge at 8000rpm for 1 min.
2. Discard collection tube, add new collection tube, and add 500uL Buffer AW1. Spin for 1 min at 8000rpm.
3. Discard collection tube, add new collection tube, and add 500uL Buffer AW2. Spin for 1 min at 8000rpm.
4. Discard collection tube, add new collection tube, and spin for another 1 min at 8000rpm.
5. Place spin column in a clean, labeled eppendorf tube and add 50uL Buffer AE directly to filter membrane (can elute up to 200uL but if high concentration of genomic DNA is needed, 50uL works well). Let tubes sit at RT for at least 1 minute.
6. Spin columns at 8000rpm for 1 minute.
7. Check concentration on both a Nanodrop spectrophotometer (Thermo Scientific) and via fluorimetric analysis on a microplate reader (Tecan) with Quant-IT PicoGreen dsDNA reagent and kit (Life Technologies #P11496) and then store at -20C.