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Habitat and Genetic Variation as Drivers of Diversity in the Tiger Moth Genus *Virbia* Walker

Introduction

Tiger moths are a diverse group within Lepidoptera in terms of behavior and morphology. Within this lineage, there are over 11,000 described species with substantial variations in color and size. Originally, tiger moths were classified under the family Arctiidae but were moved into the family Erebidae based on molecular phylogenetics (Zahiri et al. 2012).

Virbia Walker is a genus of moths within Erebidae that is known to have irregular morphological characteristics. Within some of the species there are several different sets of scale variations on the wings. Some of these variations happen on the same section of wing where a spot, series of spots, or solid coloration are shown. These characteristics do not hold true for the entire species, just individuals, making it difficult to identify and study them (Zaspel JM, Weller SJ 2006). Thus far, phenotypes have proven to have small amounts of variation between species. Since the observable characteristics can vary the habitat boundaries can be used to help identify specimens.

An accumulation of data was undertaken by Zaspel and Weller (2006) to pool together all known data about *Virbia*. This publication listed and described the differences in phylogeny of several *Virbia* species. Later, a monographic study was organized to research species-level morphological variation within this group (Zaspel, J.M., Weller S.J., & Cardé, R.T. 2008). This paper will take these known records and attempt to directly compare the phylogeny to habitat restrictions in order to find out if habitat types directly influenced the relatedness between the species.

Procedures and Techniques

Mapping

Seventeen museums sent excel documents containing label data for fourteen *Virbia* species: *V. aurantiaca, V. costata, V. fergusoni, V. ferruginosa, V. fragilis, V. immaculata, V. laeta, V. lamae, V. marginata, V. nigricans, V. opella, V. ostenta, V. rindgei,* and *V. rubicundaria*. There were a total of 12,418 total labels. The data was then sorted by museum, collected location, and collection date. All duplicate labels were removed, so all labels that contained those same three pieces of information were reduced to one. This left 2,509 unique specimen localities. The unique specimen localities were separated into new excels documents based on species and finally used to generate maps by using QGIS Essen v2.1.4. A shape file was made so the specimen markers would be show within the appropriate areas in North America north of Mexico (NAnM). Once a limited text layer was added to the under layer, the coordinates were uploaded. Maps were then edited for aesthetic appeal.

Molecular Data

Several steps were taken to extract the DNA from the insect tissues. The first was to remove the thoracic segment from the physical specimen and let them dry for a few minutes. After the thorax was dry it was added to 180uL ATC, and ground up with a mortar and pestle. Once the thorax was ground up 20uL of proteinase was added. It was shortly mixed in a fixed speed vortex before being incubated at 56°C for 24 hours in a digital dry bath. These steps are directly followed from the manufacturing protocol.

Once the samples had been incubated for a full 24 hours they were removed and mixed on the vortex mixer, and 4uL of RNase A were added then left to sit for two minutes. After the two minutes, 200uL of buffer AL. The samples were then mixed and 200uL of 95% ETOH was quickly added. All materials were transferred to a DNeasy mini spin column and then centrifuged at 8000 rpm for one

minute. The flow through was discarded and the collection tubes were replaced. Then, 500uL of buffer AW1 was added and centrifuge at 8000 rpm for one minute again. Once the collection tubes were replaced and the flow through was discarded, 500uL of buffer AW2 was added. They were then centrifuged at 13200 rpm for three minutes. The tubes were then replaced with a micro centrifuge tube and 100uL of buffer AE was added. The tubes were closed and sat for one minute. They were then centrifuged at 8000 rpm for one minute. The tubes were not replaced and another 100uL of buffer AE were added. Once the closed tubes had sat for one minute they were centrifuged for a final one minute at 8000 rpm. Finally, 40uL of the DNA extraction were put into 5 test tubes.

After DNA purification, the DNA was quantified using a Nanodrop. For the Nanodrop, 4uL of buffer AE were put onto the Nanodrop 2000 slab and ran as a blank. Once the blank was set 4uL of each DNA sample were tested; the testing area was wiped with a chemwipe between each test. The blank was reset after every ten tests. If satisfying amounts of DNA were extracted then the process of PCR can start.

Three genetic markers were used for PCR, so all listed steps were repeated for each marker: CO1, RPs5, and cytb. The PCR tubes were lined up with sides A on the same side and then labeled based on sample voucher labels. A master mix was made from 90 uL nuclease free and 5uL of both forward and reverse primers. To create the mixture that goes into the PCR machine 9 uL RO water was mixed with 12.5 uL accuzyme, 1 uL DNA, and 2 uL of the master mix. The last component of the mixture was 0.5 uL of Taq polymerase; this enzyme is sensitive so it was kept in the freezer as long and as much as possible. It was mixed gently to get rid of bubbles and tubes were then closed. The tubes were checked again to make sure they were secured in the machine and the lid was tightened. The primer used determined the cycle profile. The profiles were created using a series of temperatures that allow DNA to separate, anneal to primers, separate again, and reanneal to more primers. This caused specific proteins in the DNA to replicate quickly. Once everything had been double checked the cycle was started.

The gels were made by mixing 60 mL of TBE buffer and 0.6 grams of agarose powder. The mixture was microwaved in order to dissolve the agarose. Once the solution was heated and the color was near clear, it was poured into the mold. Combs were put into the gel while it was still a liquid to create the wells for the DNA mixture. It took about one hour for the gel to fully cool. Once it is, the combs were carefully removed without breaking the gel or tearing the wells.

The gel then needed to be put into the electrophoresis tank. The tank was filled with tank buffer so that it went over the gel up to the max fill line. A sheet of black paper was placed under the tank so the wells are more visible. Carefully, 12 uL were added into each of the wells; the 12 uL was a mixture of 4 uL of loading buffer and 8 uL of the PCR mixture. When filling the wells, well number one should be the ladder. The ladder was 4 uL of RO water, 2 uL of ladder, and 1 uL of dye. The next well should be skipped; well number three should contain the control. After that, well five would be the first to contain the samples and no more wells needed to be skipped.

After the gel was made, the tank filled with buffer, and the wells filled with all needed mixtures then the machine can be started. The lid needed to be secured with the black plugs (negative charge) on the same side and red (positive end) on the other. The wells were placed on the negative end because DNA is negatively charged. This means the DNA will travel down the gel towards the positive end. The voltage was kept between 100-110V.

Once the gels had been run, they needed to be imaged. The gels needed to be put in a dark area and submerged in imaging solvent for 12 minutes. The room needed to be kept dark so the chemical used to view the bands does not degrade. The gels were carefully placed in the imaging machine and the computer set up to run the GelRed protocol. This will image the gel and save it as a picture. The picture can be edited to better see the bands. Then the purified contents that made successful bands were sent to a facility to be sequenced.

Once all sequences were returned and aligned using Geneious v8.1 (Kearse *et* al. 2012), they were combined with the 58 morphological characteristics found in the Zaspel and Weller (2006) publication. Bayesian analysis was done with MrBayes v3 (Ronquist and Huelsenbeck 2003). Again, this was with all 14 NAnM *Virbia* species and the one outgroup. All outputs were edited with FigTree v1 (Rambaut and Drummond 2007).

Habitat Characterization

A phylogeny versus biomes chart was created by directly comparing habitat ranges and restrictions to the population boundaries of the species used. The phylogenetic tree was used to list the species based on direct relatedness. A table was inserted to show the species names and the habitat, or habitats, in which their populations are in. Habitat ranges were taken from the biome map in Figure 4 (Coniferous Forest Biome Map 2009).

Results

The results are split into three sections. The first section is based on the maps generated from QGIS Essen v2.1.4. The second will be from the combined genetic and morphological data that created the phylogenetic tree. The final section is interpreting the environmental data.

A total of fifteen maps was regenerated; one was made for each species and a final one was an accumulation of all unique species labels. The maps were checked for outliers and possible miss identification just on known population areas. Thirteen were found and marked in the original excel documents as a comparison for later review but not removed from maps. Figure 1 shows four of the generated maps. The population boundaries demonstrate the diversity in habitat that this genus can survive in.
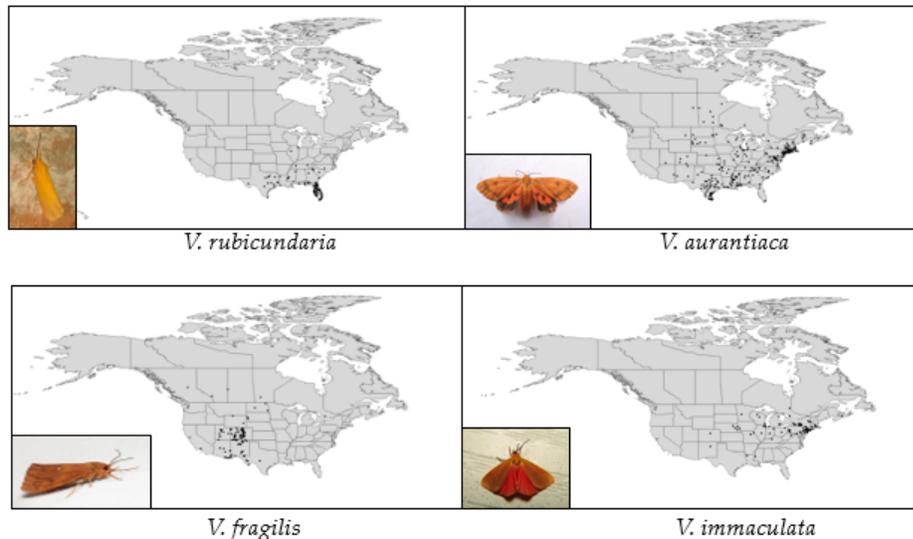

Figure 1: Four of the fifteen generated maps for the species in the genus *Virbia*. Sowing the species *V. rubicundaria, V. aurantiaca, V. fragilis,* and *V. immaculatae*.

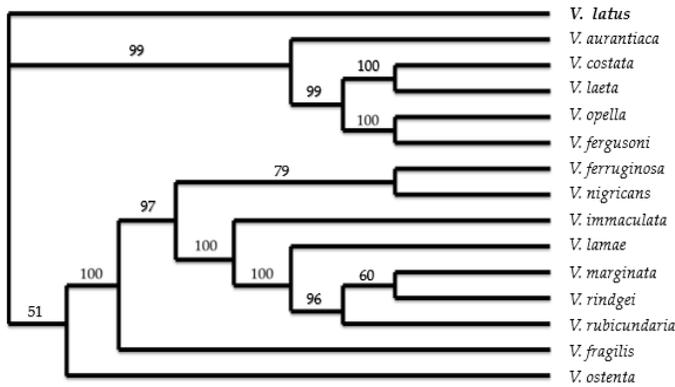V. rubicundaria

V. aurantiaca

V. fragilis

V. immaculata

Figure 2: Reconstructed phylogenetic tree. Bolded species name is the Cuban endemic out group.

Using the genetic and morphological data listed in the materials and methods section, a phylogenetic tree was reconstructed (Figure 2). Along with the fourteen species used to generate the maps, a fifteenth species was chosen to be an out group. *V. latus* is a Cuban endemic species and has a far different lineage then the species in NAnM. In figure 2, the tree shows *V. latus* in a clade of its own which gives higher support to the tree. The high posterior probability numbers confirm the likeliness of the accuracy given the data sets used.

Figures 3 and 4 are the end results from the habitat characterization methods. They provide an easier way to view the different habitat boundaries, and are viewed using the phylogentic genetic tree. The tree was used here to show the similarities and differences in habitat of closely related species.
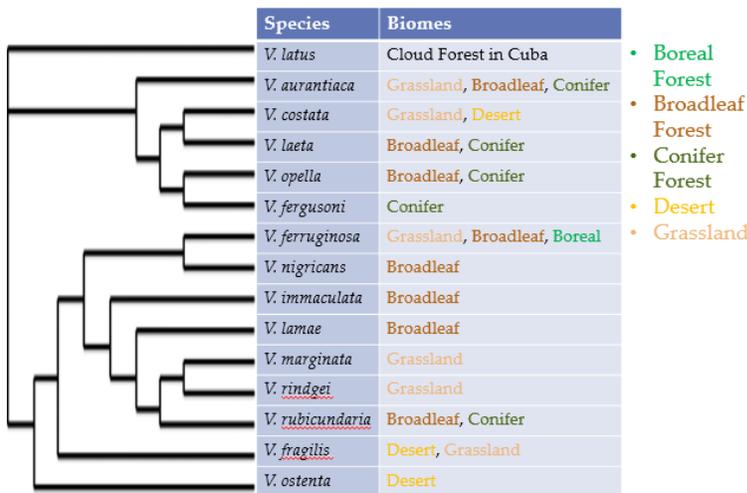

Figure 3: Species and the biomes they inhabit; organized by species relatedness based on the reconstructed phylogenetic tree.
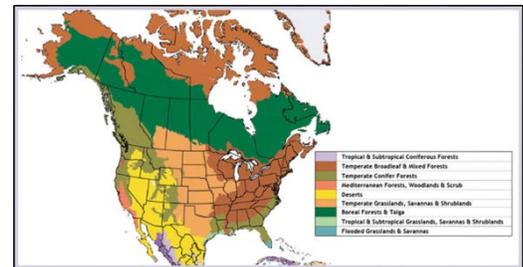

Figure 4: Biome map from (Coniferous Forest Biome Map 2009)

Discussion

By looking at Figure 3 some connections can be seen between clades and biome. Species that are closely related seem to share similar biomes. Figure 5 takes Figure 4 and separates it into its two main clades. When looking closer at clade 1, there seems to be some similarities. For example, *V. fergusoni* and *V. opella* both inhabit conifer forests, but *V. costata* and *V. laeta* share no common biome ranges.

Clade 2 shows many more similar biomes between closely related species but few are completely the same of follow a pattern with others in close proximity within the clade. For example, *V. marginata* and *V. rindgei* both are only found in the grassland biome and *V. lamae* and *V. immaculata* both inhabit only the broadleaf forest biome. There seem to be many more similarities in clade 2 then in clade 1, but in the clade 2 there are some areas unlike anything near it. The only appearance of the boreal forest and a singular conifer forest is shown.

Species	Biomes
V. latus	Cloud Forest in Cuba
V. aurantiaca	Grassland, Broadleaf, Conifer
V. costata	Grassland, Desert
V. laeta	Broadleaf, Conifer
V. opella	Broadleaf, Conifer
V. fergusoni	Conifer

(a)

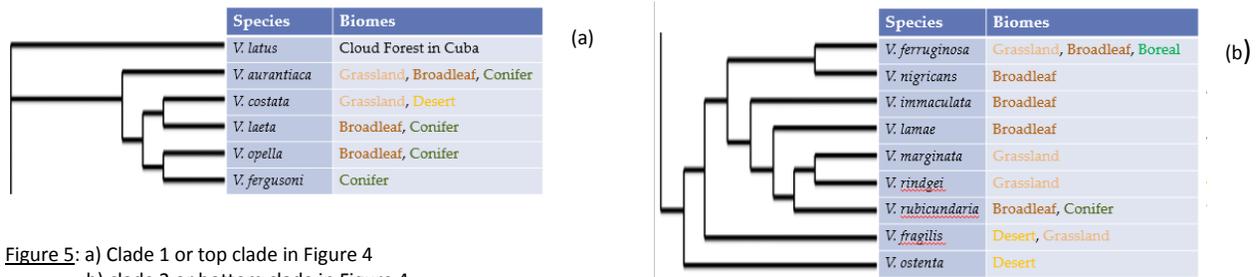| Species	Biomes
V. ferruginosa	Grassland, Broadleaf, Boreal
V. nigricans	Broadleaf
V. immaculata	Broadleaf
V. lamae	Broadleaf
V. marginata	Grassland
V. rindgei	Grassland
V. rubicundaria	Broadleaf, Conifer
V. fragilis	Desert, Grassland
V. ostenta	Desert

(b)

Figure 5: a) Clade 1 or top clade in Figure 4
b) clade 2 or bottom clade in Figure 4

Even though there are some similarities between the species and clades, it is an overall random pattern. Even though the pattern is seemingly random, it could show that some species habitat drives diversity more than others.

Other possible reasons could be a lack of collecting. There are few species found in the far western states and some central states are under sampled. With more collecting and label information, the maps could be added to, and possibly different biomes could be added for some species in Figure 3.

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