Next-Generation Sequencing of *Coleomyia* (Diptera: Asilidae)

by
Chris Cohen

A PROJECT

submitted to

Oregon State University

University Honors College

in partial fulfillment of
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degree of

Honors Baccalaureate of Science in Zoology
(Honors Associate)

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Abstract approved:

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David Maddison

Coleomyia (Diptera: Asilidae) is a small genus of robber flies found in western North America, totaling eight described species. The goal of this project was to sequence the transcriptome and genome of *Coleomyia*. In the most recent phylogenetic analysis, it could not be placed in a recognized subfamily. Furthermore, there are only a few sequenced transcriptomes and no sequenced genomes for the family. Lastly, adult asilids are known to possess venom, a unique condition among Diptera. However, this venom has not been characterized, and the venom genes are unknown. Fresh specimens of *Coleomyia setigera* were collected and prepared for transcriptomic and genomic extraction. Libraries were subsequently prepared for Illumina sequencing. Raw sequences (reads) were assembled into contigs, then annotated using various programs. The assembled transcriptome was used, in conjunction with other sequence data, to infer the phylogeny of Asilidae. Assembly revealed that the genome was severely incomplete, while the transcriptome was mostly complete. The annotation identified 14 putative venom genes, although their role as venom in Asilidae is doubtful. The robber-fly phylogeny was unable to resolve the position of *Coleomyia* with strong support. The sequences produced in this study will aid future work in robber-fly systematics and venom characterization.

Key Words: Diptera, Asilidae, Genome, Transcriptome, Systematics

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Chris Cohen, Author

Introduction

Coleomyia Wilcox and Martin (1935) (Diptera: Asilidae) is a small genus of robber flies found in western North America, totaling eight described species (e.g. Fig. 1). The genus is generally found in mid- to high-elevation pine forests where they typically perch on the ground or on fallen logs. Not much else is known about their natural history.

Figure 1. *Coleomyia setigera*. Lateral profile.

Coleomyia was selected for transcriptomic and genomic analysis because it can be found locally. Also, in the most recent morphological phylogenetic analysis, the genus could not be placed in a recognized subfamily (Dikow 2009a), making it *incertae sedis* (of uncertain placement). It was not included in a subsequent molecular phylogeny (Dikow 2009b). Furthermore, little next-generation sequencing has been done in Asilidae, with 1KITE (see Misof et al 2014) transcriptomes for *Zosteria rosevillensis* and *Elegopogon bifidus* being the sole examples. Next-gen data are important because they allow for studies of the genetics and systematics of Asilidae not otherwise possible. Finally, robber flies are the only adult dipterans known to possess venom (Dikow, pers. comm.), which they use to subdue and externally digest prey (Wood 1981). Little work has been done on robber-fly venom, but studies have shown that there is variation in toxicity between species and that venom is apparently produced in the salivary gland (Kahan 1964; Musso, Garnier, and Legier 1978). However, the

venom has not been characterized, nor have possible venom genes been identified. Sequencing *Coleomyia*'s genome and transcriptome would be the first step towards understanding this phenomenon, while also developing knowledge and skills necessary for PhD work.

This project will consist of two sections. The first is a basic exploration of the *Coleomyia* genome and transcriptome. This will involve collecting specimens, preparing libraries, and sequencing these libraries. The next step requires assembling the sequence reads produced by the sequencing machine into longer contigs. After this, it becomes possible to annotate the sequences with known or suspected genes, including putative venom genes. The second section of the project will extract genes from the transcriptome, and use these to infer a phylogeny of Asilidae in combination with genes from a published phylogeny (Dikow 2009b). The goal here is to hypothesize the subfamily placement of *Coleomyia* using molecular data, something that has not yet been done.

Materials & Methods

Collection, Extraction, Library Preparation, and Sequencing

Coleomyia setigera was collected from Elk Meadow, Mt. Hood National Forest, OR in September and specimens were stored in 95% ethanol. DNA for genomic sequencing was extracted using the Qiagen DNeasy kit. *Coleomyia setigera* was also collected from McDonald-Dunn Research Forest, Corvallis, OR in August. A single specimen was stored in RNA*later*® immediately after death and kept in a -80 °C freezer until ready for extraction. RNA extraction was done using TRIzol® and the Qiagen RNeasy kit, following a modified protocol (Ambion 2010). Library preparation for the transcriptome and genome utilized the NEBNext Ultra RNA and DNA prep kits. The

transcriptome and genome libraries were multiplexed with other samples (1/12 and 1/5 of a lane respectively) on a 100 bp paired-end run on an Illumina Hi-Seq 2000.

Assembly/Annotation/Exploration

The genome and transcriptome were initially assembled in CLC Genomics Workbench 7. CEGMA (Parra, Bradnam, and Korf 2007), via Virtual Box, was used to estimate the completeness of both the genome and transcriptome. The program Jellyfish (Marcais and Kingsford 2011) was used to construct k-mer curves with the intent of estimating the genome size of *Coleomyia*. MAKER (Cantarel et al. 2008), a genome annotator, was run on a computing cluster. These programs were chosen because they were readily available and had been used successfully in the past. The Trinotate pipeline (Grabherr et al. 2011) was used to annotate the transcriptome. Trinotate is an annotation suite that utilizes programs that search for homology among databased sequences, that identify protein domains, and that predict signal peptide and transmembrane domains. Trinotate outputted the annotations as an excel spreadsheet, which was then searched for annotations including the word "venom" or "toxin."

Phylogeny of Asilidae w/ *Coleomyia*

The full dataset from Dikow's 2009 molecular phylogeny (5 genes: CAD, AATS, 18S, 28S, and EF1-alpha) was downloaded from GenBank as a large FASTA file. This data was imported into Geneious (Kearse et al. 2012), where an exemplar for each gene was BLASTed (Altschul et al. 1990) against the *Coleomyia* transcriptome. Putative orthologs of each of the Dikow genes were found in *Coleomyia* and added to the FASTA files, except for CAD which was incomplete. CAD was excluded pending a more thorough analysis of the putative BLAST matches.

These sequences were then partitioned by gene and edited so that they could be downloaded into Mesquite (Maddison and Maddison 2015) as a single taxon block. MAFFT (Katoh and Standley 2013) was used to align the gene sequences, but manual editing with the alignment tools was required to remove gaps and poorly aligned regions. The aligned and edited sequences were then combined into a concatenated data matrix. RAxML v8.1.20 (Stamatakis 2006) was then run on a computing cluster, searching for a Maximum Likelihood tree with 150 search replicates. The model GTR + Γ was used, although ideally jModelTest (Darriba et al. 2012; Guindon and Gascuel 2003) would be used to choose the optimal model. The bootstrap support values for each node, calculated in RAxML using 500 bootstrap replicates, were mapped onto the ML tree.

Results

Assembly and Exploration

The results of the sequencing and assembly of the *Coleomyia* genome and transcriptome are summarized in Table 1. Jellyfish failed to produce a k-mer curve suitable for estimating the genome size. However, the assembly length of the *Coleomyia* genome in CLC was about 200 mb. MAKER was run twice on the cluster, but both times ran to completion without actually annotating the genome - no specific errors were given. A possible reason for this failure and that of Jellyfish is described in the discussion.

Table 1. Sequencing and Assembly results

	# Reads	N50	# Contigs	Partial CEGs	Complete CEGS
genome	77.7 mil.	397	452,429	35.08%	6.05%
transcriptome	25.7 mil.	1,251	34, 695	85.48%	78.23%

Transcriptome Annotation

Trinotate annotated at least 10,000 contigs and identified 14 unique genes (some with several copies) that are classified as venoms in other animals (Table 2).

Table 2. Venom annotations from Trinotate (duplicate results excluded) with the UniProt code and source organism included.

Code	Gene/Protein	Organism
B2D0J5	Venom carboxylesterase-6	Hymenoptera: Apidae: *Apis mellifera*
P35786	Venom allergen 5	Hymenoptera: Vespidae: *Vespula squamosa*
P35779	Venom allergen 3	Hymenoptera: Formicidae: *Solenopsis richteri*
B1A4F7	Venom dipeptidyl peptidase 4	Hymenoptera: Vespidae: *Vespula vulgaris*
Q5BLY5	Venom acid phosphatase Acph-1	Hymenoptera: Apidae: *Apis mellifera*
C9WMM5	Venom serine carboxypeptidase	Hymenoptera: Apidae: *Apis mellifera*
B5AJT4	Venom metalloproteinase 3	Hymenoptera: Eulophidae: *Eulophus pennicornis*
H2CYP1	Venom peptide Pc	Scorpiones: Scorpionidae: *Pandinus cavimanus*
B5U2W0	Venom serine protease Bi-VSP	Hymenoptera: Apidae: *Bombus ignites*
Q8MQS8	Venom serine protease 34	Hymenoptera: Apidae: *Apis mellifera*
P10736	Venom allergen 5.01	Hymenoptera: Vespidae: *Dolichovespula*
Q8QG86	Snake venom serine protease BITS01A	Squamata: Viperidae: *Bothrops insularis*
Q9PTL3	Snake venom serine protease salmonase	Squamata: Viperidae: *Gloydius brevicaudus*
Q5Y4U4	U8-agatoxin-Ao1a	Araneae: Agelenidae: *Agelena orientalis*

The following information on venom proteins was taken from the respective protein profiles on the UniProtKB online database (www.uniprot.org) unless otherwise noted. Many of the venom proteins are rich in disulfide bonds, and all are produced in the venom gland or venom duct of the source organism.

Venom allergen 3, Venom allergen 5, and Venom allergen 5.01 all produce allergic reactions in humans. The following are also known to cause allergic reactions in addition to their other functions: Venom carboxylesterase-6 catalyzes the breakdown of carboxylic esters. Venom dipeptidyl peptidase 4 may "*process venom proteins into their active forms and/or modulate the chemotactic activity of immune cells after the insect sting.*" Venom acid phosphatase Acph-1 catalyzes the breakdown of phosphate monoesters. Venom serine carboxypeptidase catalyzes the release of a C-terminal amino acid. Venom metalloproteinase 3 has been experimentally shown to have gelatinase

activity, to cause insect mortality, and to retard development and growth. The precise function of venom serine protease 34 does not appear to be known.

The following proteins are not known to produce allergic reactions in humans, but may have other effects. Venom peptide Pc is produced by the venom gland and acts as a toxin. Venom serine protease Bi-VSP is a multifunctional protein produced in the venom gland with different effects depending on the organism injected. In arthropods, it "*induces lethal melanization response in target insects by modulating the innate immune response."* Snake venom serine protease BITS01A is produced in the venom gland and may impair the hemostasis of prey (prevents coagulation/causes bleeding). Snake venom serine protease salmonase may have a similar function. While its biological function is not known, U8-agatoxin-Ao1a may perform a similar function to other agatoxins which interfere with ions channels, causing paralysis (Adams 2004).

Five-gene Phylogeny w/ *Coleomyia*

All five genes used by Dikow (2009b) were recovered in the *Coleomyia* transcriptome. The ML phylogeny (Figure 2) and consensus bootstrap tree (Figure 3) were inferred as follows.

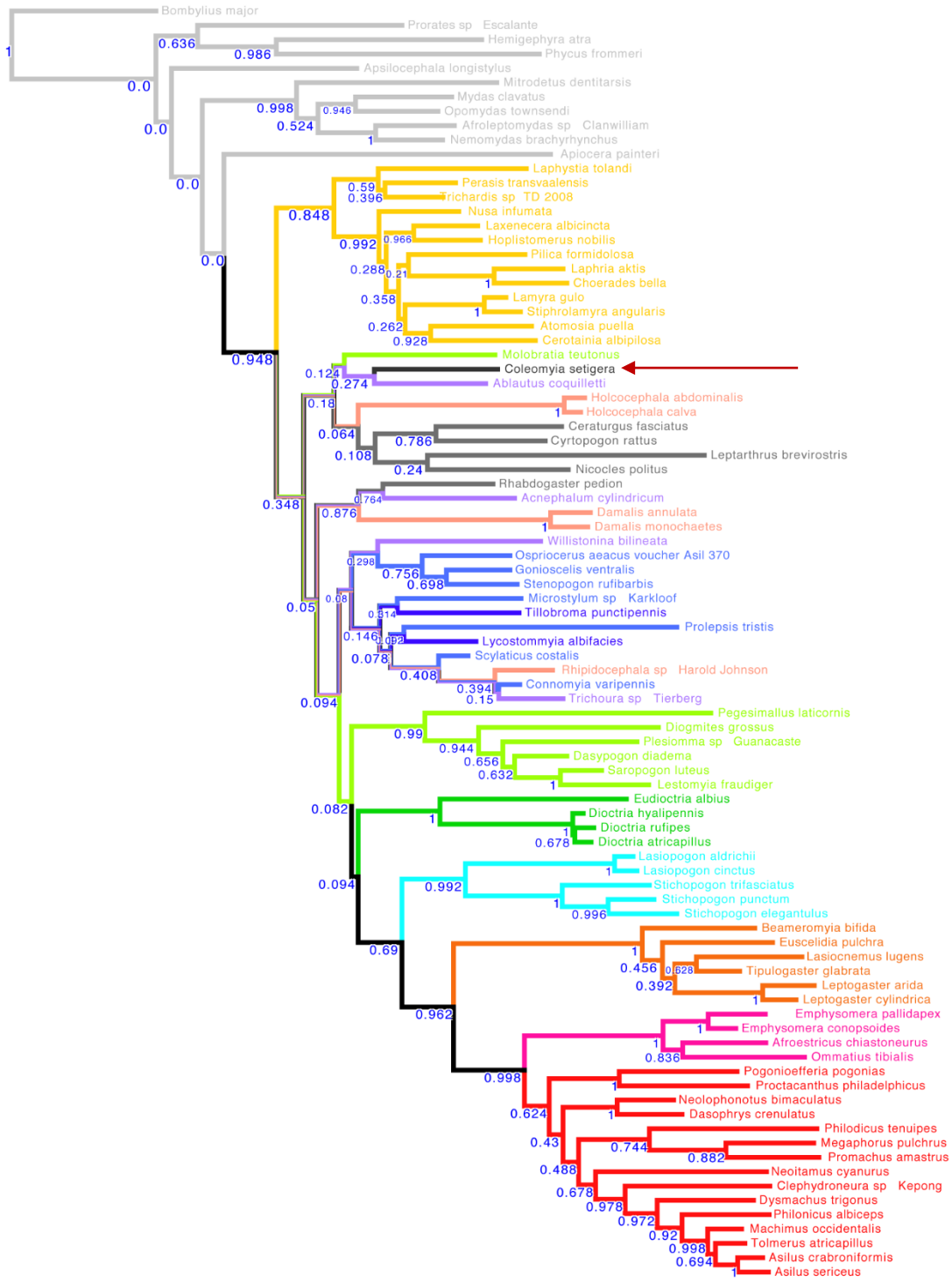

Figure 2. Maximum Likelihood tree of the family Asilidae and outgroup taxa inferred in RAxML. Bootstrap values are mapped for each node. Branch lengths represent degree of molecular divergence. Colors denote expected subfamily classification *sensu* Dikow 2009a. Arrow indicates position of *Coleomyia*.

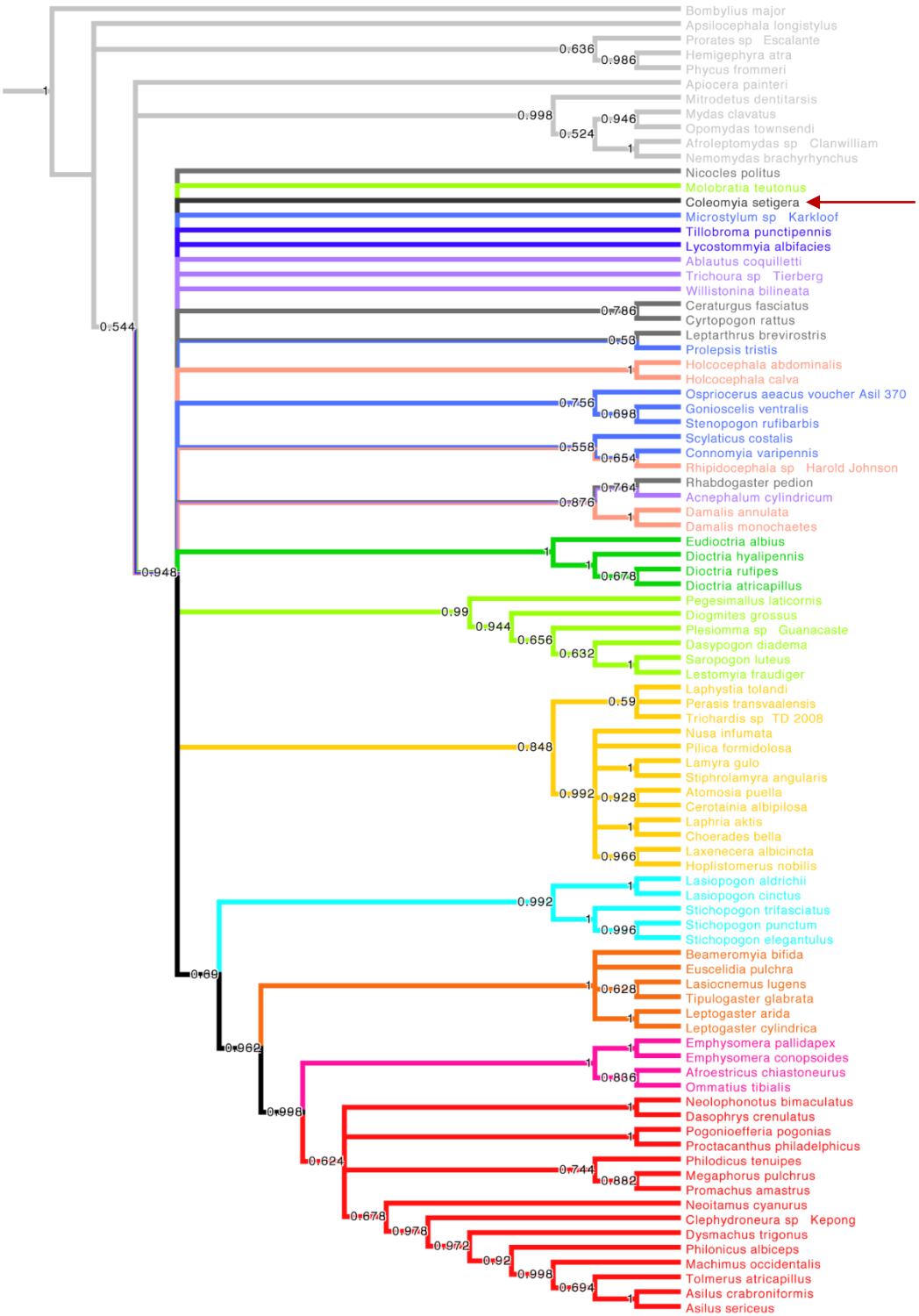

Figure 3. Maximum consensus bootstrap tree of the family Asilidae and outgroup taxa inferred in RAxML. Bootstrap values greater than 0.5 are mapped for each node. Branch lengths are equal. Colors denote expected subfamily classification *sensu* Dikow 2009a. Arrow indicates position of *Coleomyia*.

Discussion

Assembly

With an N50 of 397 and with only 6.05% complete CEGs, the genome coverage is quite low. It would seem the total read count of about 77.7 million was insufficient to provide adequate coverage, given the likely size of the genome. The minimum size of the genome is probably about 200 mb, based on the CLC assembly. Assuming no systematic errors in the assembly, this would suggest that the *Coleomyia* genome is larger than *Drosophila melanogaster* at about 120 mb (Adams et al. 2000), but perhaps smaller than the recently sequenced genome of the house fly *Musca domestica* at about 690 mb (Scott et al. 2014). However, because of the incompleteness of the genome, Jellyfish could not be used to provide a more accurate estimate of genome size. The genome annotator software MAKER could not successfully annotate the genome, presumably for the same reason. The transcriptome N50 of 1,250 is about average for *Bembidion* transcriptomes (Sproul, pers. comm.). While also incomplete (78.23% complete CEGs), it is much less so than the genome.

Annotation

Fourteen putative venom genes/proteins have been identified from the *Coleomyia* transcriptome. These include known allergens, hemostasis inhibitors, and a potential neurotoxin. Venom dipeptidyl peptidase 4, venom metalloproteinase 3, and U8 agatoxin Ao1a, when BLASTed against the non-venomous *Anopheles gambiae* and *Drosophila melanogaster* genomes, all had hits below an arbitrary E value threshold of 4e-25. This value appeared to be low enough to exclude obviously poor BLAST hits (e.g. small fragments) while also allowing for hits that were not close matches. In addition to these

BLAST searches, most putative venom genes have been annotated in other, non-venomous insects, including variously the dipterans *Ceratitis capitata*, *Bactrocera dorsalis* (Geib et al. 2014), *Bactrocera cucurbitae* (Sim et al. 2015), and *Musca domestica* (Table 3). In the absence of more sophisticated methods (discussed later), there is insufficient evidence to conclude that these genes do in fact code for venomous proteins in *Coleomyia*.

Table 3. GenBank records showing nonvenomous insects with venom gene annotations. Only one example is included for each gene, with a bias towards Diptera.

Gene/Protein	Annotated Organism	Accession #
Venom carboxylesterase-6	*Ceratitis capitata* (Tephritidae)	XM_012302017
Venom allergen 5	*Bactrocera dorsalis* (Tephritidae)	XM_011209048
Venom allergen 3	*Ceratitis capitata* (Tephritidae)	XM_004536051
Venom dipeptidyl peptidase 4	*Ceratitis capitata* (Tephritidae)	XM_012302867
Venom acid phosphatase Acph-1	*Ceratitis capitata* (Tephritidae)	XM_004534209
Venom serine carboxypeptidase	*Ceratitis capitata* (Tephritidae)	XM_004537138
Venom metalloproteinase 3	No nonvenomous records	NA
Venom peptide Pc	*Musca domestica* (Muscidae)	XM_005175227
Venom serine protease Bi-VSP	*Diaphorina citri* (Psyllidae)	XM_008486004
Venom serine protease 34	*Musca domestica* (Muscidae)	XM_005178810
Venom allergen 5.01	*Bactrocera cucurbitae* (Tephritidae)	JAC98046
Snake venom serine protease BITS01A	No nonvenomous records	NA
Snake venom serine protease salmonase	No nonvenomous records	NA
U8-agatoxin-Ao1a	*Bactrocera cucurbitae* (Tephritidae)	JAD05153

However, it is important to note that there are several shortcomings in this approach. Firstly, identifying gene function by BLASTing a database requires that relevant, homologous genes have already been described. If no identified, homologous venom genes have been uploaded to the database, then no amount of BLAST searches will recover the homologs in the transcriptome/genome. This is especially an issue with unique, derived taxa that have little representation in sequence databases. This is the case for Asilidae, in which few sequences are available from Asilidae or related families.

Furthermore, as robber flies are the only adult dipterans to possess venom, the condition must be independently derived, meaning that no homologous venom genes

exist from outside the family (although *non*venomous homologs certainly might). Therefore the best that can be hoped for is that BLAST searches and similar methods pick up on protein motifs that are common to venoms regardless of ancestry. However, even with close matches, these gene annotations are just hypotheses. Experimentation is required to show that a putative venom gene (from another organism) is actually expressed in the robber-fly venom gland, and that it is used as a toxin and not for some other function.

Future research in and characterization of asilid venom will require much more sophisticated methods. One approach will be to determine the amino acid composition of the venom itself, using HPLC for example. The venom could be extracted from the salivary glands or perhaps milked from the fly using electrical shocks. A complementary approach will be to sequence the transcriptome of the salivary gland, reducing the amount of gene candidates that must be sifted through. These two methods can be combined to together using advanced technology like MudPIT (Motoyama et al. 2006) to identify the venom genes. This platform utilizes 2-D liquid chromatography and mass spectroscopy to separate and characterize peptides. Once this has been accomplished for venom, studies can be undertaken to ascertain how these proteins evolved in Asilidae, examine the variation across the family, and determine how this variation itself evolved.

Five-gene Phylogeny w/ *Coleomyia*

The phylogeny, while including the same dataset and taxa (88 plus *Coleomyia*) as Dikow (2009b), does not have the same topology as his molecular-only analysis. This is partly due to different methods, as Dikow used Parsimony in POY (Varon et al. 2008) while this tree was inferred using Maximum Likelihood in RAxML. However, it should

be noted that in an unpublished phylogeny by Dikow (personal comm.) with an expanded dataset (6 genes, ~218 taxa), the topologies are very similar, although differing in several respects. This can be at least partially attributed to inference method, as this unpublished phylogeny is a ML tree.

Coleomyia does not group with a recognized subfamily, but instead with several rogue taxa. However, the 12.4% and 27.4% bootstrap values for these relationships are very low, and thus *Coleomyia*'s position within Asilidae is best considered unresolved. In fact many subfamilies in this analysis are paraphyletic (as was the case in Dikow's molecular phylogeny), and bootstrap support for many nodes is quite poor, suggesting that more taxa and genes will be required to resolve the subfamily classification in Asilidae.

Conclusion

Through this project the genome and transcriptome of *Coleomyia setigera* were sequenced and assembled, and the transcriptome annotated. The next step will be to extract orthologous genes from this transcriptome and those from other flies, in order to infer a next-gen phylogeny of Diptera. In the bigger picture, this project is the first step that will eventually allow unprecedented insight into the physiology and genetics of Asilidae, and will also serve as the first step in designing a probe set for hybrid enrichment (Gnirke et al. 2009). This phylogenomic method will allow for the simultaneous sequencing of hundreds of genes from dozens or hundreds of taxa. Such vast data will hopefully provide robust support for both deep and shallow nodes throughout the robber fly phylogeny.

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