De-novo transcriptome assembly and gene expression quantification using RNASeq in non-model species.

Vasiliki Mantzana Oikonomaki[1], Ariel Rodriguez[1], Heike Proehl[1]

1. University of Veterinary Medicine of Hannover

In short.

A. *de novo* transcriptome assembly of *Oophaga granulifera*

B. Identification of coloration and alkaloid associated gene expression profiles of red and green *Oophaga pumilio* and *O. granulifera*.

C. Differences in colour and alkaloid related gene expression in red and green colour morphs of two different species of poison frogs.

1. Introduction

Animals that are toxic, unpalatable or otherwise unsuitable as prey items, often advertise their unprofitability to potential predators through bright, conspicuous colours, a phenomenon known as aposematism. Aposematism has been observed across many species, including invertebrates, amphibians, reptiles and birds. Conspicuous colouration will be favoured because noxious prey will be recognized (memorized) as such by experienced predators, and thus will be avoided, whereas the costs of 'educating' the predator will have been shared amongst the whole population of conspicuous prey. The hypothesis of aposematism further predicts that the evolution of toxicity will occur before, or in tandem with, the evolution of bright coloration.

Opposite to aposematism and brightly coloured unpalatable animals, cryptic animals, that are usually non-toxic, use cryptic colours to blend in with their natural background and hide from predators. Consequently, in addition to colouration and toxicity, aposematic and cryptic animals may also differ in behavioural aspects. Conspicuous and chemically defended prey species tend to be diurnal, travel and forage more

openly and flee when threatened while cryptic animals are usually less active during foraging, and stay still to camouflage and avoid predation.

The monophyletic family of Neotropical poison frogs of the family Dendrobatidae are a known and well-studied system for unravelling the coevolution between the components of colouration and defensive strategies. Specifically, the strawberry poison frog, *Oophaga pumilio*, is a great species for further studying colour variation and its connection to toxicity and aposematism. At the Bocas del Toro Archipelago in Panama, populations are diverged into more cryptic (green, blue or brown) and more aposematic (orange, red, yellow) morphs, while populations in Costa Rica and Nicaragua are all aposematically red.

Even though animal colouration has been receiving more attention over the past years, identifying the underlying genes and genetic structure of variation in colour traits has not been studied intensively in non-model species and in the wild. High-throughput sequencing technologies allow us to overcome previous limitations, and open new avenues to study the genetic basis of animal coloration in a broad number of species. Specifically, differentially expressed genes related to pigment production in the skin have been found in different colour morphs in *O. pumilio*..

In vertebrates, there are three main categories of pigments that underly skin colouration, known as melanins, carotenoids and pteridines. Melanin-based colouration is linked to darker or lighter colouration through the interaction of melanocortin-1 receptor (MC1R) and Agout-signalling protein (ASIP). Not as much is known for pteridine and carotenoid based colouration, however, all aspects of carotenoid and pteridine coloration are likely under genetic control (Eriksson et al., 2008).

Oophaga granulifera is distributed on the Pacific lowlands of Costa Rica and displays red colour morphs in the south, green colour morphs in the north and intermediate (orange, bronze) colour morphs in between. Surprisingly, an inverted aposematic pattern is observed as toxicity is stronger in the cryptic green morph than in the conspicuous red morph frogs. The reverse relationship between conspicuousness and toxicity is contrasting to the pattern found in *O. pumilio* and other aposematic animal groups, and might be mediated by selection posed by a variety of predator groups. The contrasting relationship between conspicuousness in *O. pumilio* (high

conspicuousness – high toxicity) and *O. granulifera* (high conspicuousness – low toxicity) offers the exciting possibility to investigate whether coloration and toxicity genes are oppositely regulated in both species and how this is related to the presence of pigments and alkaloids.

The overall objective of this project is to connect the phenotype of the frogs (coloration, pigments and skin morphology) to its underlying molecular basis (gene expression in candidate genes involved in toxicity and coloration). Using RNA extracts from skin and liver, in combination with morphological and anatomical data, we will compare gene expression, colouration and pigments in the skin between aposematic red colour morphs with more cryptic green colour morphs in two species of *Oophaga*: *O. pumilio* and *O. granulifera* from Panama and Costa Rica. Expected results will uncover the mechanisms underlying differences between cryptic and aposematic phenotypes and/or species in poison frogs.

2. Methods

The reference transcriptome of *O. pumilio* and newly assembled transcriptomes of the other two target species will be used to estimate transcript abundance per sample by pseudo-aligning the quality-trimmed reads to the respective transcriptome reference sequences with Kallisto software. Differential expression analyses will be later conducted with the Sleuth R-package. A gene-level aggregation of statistical results will be obtained after clustering de-novo assembled transcripts into genes using Corset software or by resourcing to genome guided methods in the case of *O. pumilio* (as in Rodríguez et al., 2020).

Given that these analyses often produce a large number of statistically significant genes, we plan to focus our biological interpretations on sets of candidate genes associated to pigmentation and alkaloid sequestering. This candidate set approach relies on the conservatism of metabolic pathways across vertebrates, an assumption that might not hold true for all the underlying metabolic pathways under study. Therefore, we will also seek to identify gene modules de-novo using Weighted Gene Correlation Network Analysis (WGCNA), a powerful method to untangle regulatory mechanisms and pathways.

In addition to the within species WGCNA, a cross species analysis of co-expression will be conducted to examine the extent of expression conservatism and divergence

across species. Orthologous genes between species will be identified from the three reference transcriptome assemblies. Expression estimates will then be associated with the obtained orthogroups and a cross species WGCNA will be performed. For this later step, we will use the recently developed GeneBridge approach to calculate cross species Gene-Module Association Determination (G-MAD), aiding the identification of gene function, and Module-Module Association Determination (M-MAD) allowing cross-species connectivity estimations among gene modules.

References

Bray, N. L., Pimentel, H., Melsted, P., & Pachter, L. (2016). Near-optimal probabilistic RNA-seq quantification. *Nature Biotechnology*, *34* (5), 525–527. DOI: doi.org/10.1038/nbt.3519

Davidson, N. M., & Oshlack, A. (2014). Corset: enabling differential gene expression analysis for de novo assembled transcriptomes. *Genome Biology*, *15* (7), 410. DOI: doi.org/10.1186/s13059-014-0410-6

Langfelder, P., & Horvath, S. (2008). WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics*, *9* (1), 559. DOI: doi.org/10.1186/1471-2105-9-559

Maan, M. E., & Cummings, M. E. (2012a). Poison Frog Colors Are Honest Signals of Toxicity, Particularly for Bird Predators. *The American Naturalist*, *179* (1), E1–E14. DOI: doi.org/10.1086/663197

Maan, M. E., & Cummings, M. E. (2012b). Poison Frog Colors Are Honest Signals of Toxicity, Particularly for Bird Predators. *The American Naturalist*, *179* (1), E1–E14. DOI: https://doi.org/10.1086/663197

Mundy, N. I. (2005). A window on the genetics of evolution: MC1R and plumage colouration in birds. *Proceedings. Biological Sciences*, *272* (1573), 1633–1640. DOI: doi.org/10.1098/rspb.2005.3107

Mundy, N. I., Stapley, J., Bennison, C., Tucker, R., Twyman, H., Kim, K.-W., Burke, T., Birkhead, T. R., Andersson, S., & Slate, J. (2016). Red Carotenoid Coloration in the Zebra Finch Is Controlled by a Cytochrome P450 Gene Cluster. *Current Biology*, *26*(11), 1435–1440. DOI: doi.org/https://doi.org/10.1016/j.cub.2016.04.047

Pimentel, H., Bray, N. L., Puente, S., Melsted, P., & Pachter, L. (2017a). Differential analysis of RNA-seq incorporating quantification uncertainty. *Nature Methods*, *14*(7), 687–690. DOI: doi.org/10.1038/nmeth.4324

Pröhl, H., & Ostrowski, T. (2011). Behavioural elements reflect phenotypic colour divergence in a poison frog. *Evol. Ecol.*, *25*, 993–1015. DOI: doi.org/10.1007/s10682-010-9455-5

Rodríguez, A., Mundy, N. I., Ibáñez, R., & Pröhl, H. (2020). Being red, blue and green: the genetic basis of coloration differences in the strawberry poison frog (*Oophaga pumilio*). *BMC Genomics*, *21* (1), 301. DOI: doi.org/10.1186/s12864-020-6719-5

Ruxton, G. D., Sherratt, T. N., & Speed, M. P. (2004). *Avoiding Attack: The Evolutionary Ecology of Crypsis, Warning Signals and Mimicry*.

San-Jose, L. M., & Roulin, A. (2017). Genomics of coloration in natural animal populations. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *372* (1724), 20160337. DOI: doi.org/10.1098/rstb.2016.0337

San-Jose, L. M., & Roulin, A. (2017). Genomics of coloration in natural animal populations. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *372* (1724), 20160337. DOI: doi.org/10.1098/rstb.2016.0337

Sanchez, E., Rodríguez, A., Grau, J. H., Lötters, S., Künzel, S., Saporito, R. A., Ringler, E., Schulz, S., Wollenberg Valero, K. C., & Vences, M. (2019). Transcriptomic Signatures of Experimental Alkaloid Consumption in a Poison Frog. *Genes*, *10* (10). DOI: doi.org/10.3390/genes10100733

Summers, K. (2003). Convergent evolution of bright coloration and toxicity in frogs. *Proceedings of the National Academy of Sciences*, *100* (22), 12533–12534. DOI: doi.org/10.1073/pnas.2335928100

Summers, K., & Clough, M. E. (2001). The evolution of coloration and toxicity in the poison frog family (Dendrobatidae). *Proceedings of the National Academy of Sciences*, *98* (11), 6227–6232. DOI: doi.org/10.1073/pnas.101134898

Wang, I. J. (2011). Inversely related aposematic traits: reduced conspicuousness evolves with increased toxicity in a polymorphic poison-dart frog. *Evolution*, *65* (6), 1637–1649. DOI: doi.org/10.1111/j.1558-5646.2011.01257.x