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| Project Title: | **You can’t protect what you can’t see: harnessing next generation sequencing to identify unique lineages within the cryptic and threatened Cascade torrent salamander, *Rhyacotriton cascadae*** |
| Principal Investigator (PI) name:  Job title:  Institution:  Address:  Phone:  Email address: | Christopher Cousins  PhD Graduate Student  Oregon State University  Nash Hall, 2820 SW Campus Way, Corvallis, OR, 97331  5417374531  Christopher.Cousins@oregonstate.edu |
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| Amount of Grant Request: | **$3977** |
| Collaborator name:  Job title:  Institution:  Address:  Phone:  Email address: | Dr. Tiffany Garcia  Professor  Oregon State University  Nash Hall, 2820 SW Campus Way, Corvallis, OR, 97331  5417374531  Tiffany.Garcia@oregonstate.edu |
| Collaborator name:  Job title:  Institution:  Address:  Phone:  Email address: | Dr. Brian Sidlauskas  Professor  Oregon State University  Nash Hall, 2820 SW Campus Way, Corvallis, OR, 97331  5417374531  Brian.Sidlauskas@oregonstate.edu |

Type of project *(circle all that apply):*

Education Conservation Research

Abstract *(no more than 250 words):*

This project aims to reveal the evolutionary history and clarify the taxonomy of *Rhyacotriton cascadae,* an endemic salamander of the Pacific Northwest currently under consideration for listing under the US Endangered Species Act. In 2022, as part of a study with the goal of identifying genetically distinct populations within *R. cascadae,* we sampled across the range of the species and used generation sequencing techniques to assemble genetic markers. Our preliminary results putatively identified four populations, one of which showed an unexpected and surprising amount of differentiation relative to the others. Further investigation using phylogenetic tree building methods, and inclusion of congeners, has failed to explain the patterns we are seeing within the currently accepted taxonomy of Rhyacotritonidae. To better understand the evolutionary history of *R. cascadae* and Rhyacotritonidae, we will assemble a dataset of all four extant species of the family, as well as individual representatives of six other families within the order Caudata. This dataset will enable both a fine and broad scale examination of lineage diversification and speciation events within Rhyacotritonidae, critical for successful conservation of evolutionarily significant units. Including other families in our analyses will allow us to root our network, adding a temporal dimension to our study, as well as the ability to infer the biogeographic history of the populations. This research will also contribute to the taxonomical placement of Rhyacotritonidae within Caudata, an area of active research.

**1. Background**

This project will support conservation of *Rhyacotriton cascadae* and further taxonomic research in amphibians by examining speciation events in Rhyacotritonidae. Due to its high sensitivity to environmental change, and predicted loss of habitat in future climate scenarios, the Cascade torrent salamander (*R. cascadae*), endemic to the western slopes of the Cascade Range of Oregon and Washington, is currently being assessed by the United States Fish and Wildlife Service (USFWS) for listing under the federal Endangered Species Act (ESA).The USFWS uses the concept of ‘distinct population segments’ (DPS’s) to spatially allocate conservation efforts, protecting unique evolutionary lineages identified by genetic studies. In 2022, we performed the first range wide population genomics study for *R. cascadae;* to support conservation efforts and inform the species assessment. Our preliminary results tentatively identified four to five distinct populations. Unexpectedly, one population showed markedly elevated levels of differentiation from the others. Creating an additional dataset that includes all 4 extant species of Rhyacotritonidae in addition to representatives of six other salamander families will allow us to know the sequence of diversification, vicariance, and dispersal events in the radiation of this genus. Our analyses of this dataset will identify the ancestral population of not just *R. cascadae,* but all Rhyacotritonidae. Our research is a priority as our preliminary results suggest that currently accepted species boundaries may be incorrect, which may lead to a misallocation of conservation efforts for *R. cascadae.*

**2. Project Description**

Our phylogenetic project has its beginnings in unexpected preliminary results of an ongoing population genomics project study of *R. cascadae*. The goal of our ongoing study is to identify genetically distinct populations and reservoirs of genetic diversity within the species. As the impacts of climate change accelerate, populations with higher genetic diversity, and thus, higher adaptive potential, will have higher likelihoods of persistence. This necessitates identification of these unique evolutionary lineages, which in turn helps to identify DPS’s, the units used by the USFWS to allocate conservation efforts for ESA species. To examine population structure across the species, we collected a total of 144 tail tips from individuals at 28 sites across the range of *R. cascadae* to perform a population genomics study using double digest restriction-site associated DNA sequencing (ddRADseq) to assemble markers across the genome.

As salamanders have genomes which are larger than most other vertebrates by an order of magnitude, we increased the amount of starting DNA per sample and used a narrow fragment size selection window to ensure that we would have sufficient sequencing depth and coverage. We submitted tissue to the Center for Quantitative Life Sciences (CQLS) at Oregon State University (OSU) for DNA extraction, library preparation, and next-generation sequencing on an Illumina NextSeq 2000 using a 150 base pair paired end P2 flow cell. We chose to use ddRADseq to identify genetic markers, as multiple studies have found that this method produces higher numbers of markers than microsatellites, mitochondrial DNA, and other molecular techniques, allowing for detection of genetic differentiation between populations that might go otherwise undetected. We also used these methods with previously collected tissue of ESA candidate species *R. kezeri* and *R. variegatus* (two of the other three species within the monogenetic family). We performed quality control and filtering using the STACKS pipeline, identifying the optimal parameters by examining the total number of single nucleotide polymorphisms (SNPs), total number of loci within 80% of all samples, individual heterozygosity, and cumulative variance of PCA’s within subsets of the dataset ran with varying parameters. We used optimal parameters to assemble a dataset from a subset of samples (112 samples and twenty sites) for preliminary analyses.

Our preliminary results have putatively identified 4-5 distinct populations within the species, and evidence of little to no gene flow between populations. Unexpectedly, we found surprising levels of genetic differentiation between the two identified populations at the northern extent of the species range boundary. When including both *R. kezeri* and *R. variegatus* along with *R. cascadae* in RAXML and other phylogenetic analyses, results suggest that the divergences between these populations could be at similar magnitudes to those between the congeneric species. To better understand the phylogenetic structure within the family, we will construct a dataset with *R. olympicus*, in addition to representatives of six other families within the order Caudata. This dataset will contain all current species of Rhyacotritonidae, and seven of the ten currently recognized families of salamanders (Rhyacotritonidae, Ambystomatidae, Cryptobranchidae, Dicamptodontidae, Plethodontidae, Proteidae, and Sirenidae). We have intentionally chosen families that (with our current taxonomical knowledge) are either close relatives to, or sufficiently distant from Rhyacotritonidae, to allow for a broad temporal scale of diversification within the family. The breadth of the dataset will allow us to compare interspecific levels of divergence within *R. cascadae* to interfamilial levels of divergence within Rhyacotritonidae, and levels of divergence and speciation within Caudata, resolving ongoing debate about its placement within the order.

To ensure that we have sufficient genetic markers to allow for examining phylogenetic relationships within the species, we will use library preparation and sequencing methods proven with our population genomics study, as well as tailored loci filtering methods. This study will contribute to the efforts of our ongoing population genomics work to identify distinct population segments within R. cascadae, adding a broader lens to our investigations. Our work will elucidate the evolutionary history and speciation events within Rhyacotritonidae and ensure that future conservation efforts are able to take distinct genetic lineages into account. As the USFWS is currently considering listing *R. cascadae* under the ESA, timely and accurate delimitation of the species and genetically distinct populations is critical for supporting efforts to ensure the long-term persistence of the species. These results will also contribute to the active research in amphibian taxonomy. The evolutionary history of salamander families within the order Caudata is an area of debate, and our study will help support efforts to trace the evolutionary history of Caudata.

**3. If request involves a multi-year project, please outline the funding strategy for subsequent years**

N/A

**4. Have all the necessary permits or letters of approval by the appropriate regulatory agencies or recovery team been obtained for the project? (Y/N)**

Yes

**Have Institutional Animal Care and Use Committee (IACUC) approvals been obtained, if necessary? (Y/N)**

Yes

**If yes to either question, attach copies of related permits and approvals to this proposal. Proposals cannot be considered unless proof of permits or support letters from the permitting authority is provided.**

**5. Project timeline:**

**We will complete the data collection and analysis for this project in 2023, with a draft manuscript completed by early 2024.**

**6. Is the project underway? (Y/N)**

Yes, we have sequenced all the *Rhyacotriton cascadae* samples that we need, as well as those of *R. kezeri* and *R. cascadae.* We have successfully requested tissue from the Florida Museum of Natural History’s Genetic Resources Repository, which are sending us samples the week of January 16th. Collaborators with the Washington Department of Fisheries and Wildlife have also committed to sending samples, as has a collaborator at the Indiana University of Pennsylvania. We are currently in talks with St. Louis Zoo to obtain samples and will obtain the remaining samples needed from the Oregon State University Herpetology Collection. We have been working with both the USGS and USFWS on our population genomics work and will be sharing these results with them as well.

**C) PROJECT BUDGET FOR ONE YEAR**

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| **Budget Category** | **Item/Amount** | **Amount Requested from FCSal** | **Other Funding Sources;**  **Identify Pending, In-Kind, or Other Support** |
| Sequencing | **Nextseq 2000 150 bp x 2 PE P2 Sequencing: $3977** | **$3977** | **0** |
| Sequencing | **Library Preparation: $1288** | **0** | **$1288, In-Kind** |
| Sequencing | **Fragment Size Selection: $176** | **0** | **$176, In-Kind** |
| Sequencing | **qPCR: $34** | **0** | **$34, In-Kind** |
|  |  |  |  |
| **Total** | **$5475.00** | **$3977** | **$1498, In-Kind** |

**D) ATTACHMENTS (digital submissions only)**

1. **Letters of support from the CEO/Director of the Primary Investigator(s) and collaborating institution(s)**

Attached letter of support from the lab head of the PI

1. **Copies of appropriate permits and/or IACUC approvals**

Attached collection permits for Oregon and Washington, and IACUC approval

**3. *Curriculum vitae* of Primary Investigator(s); maximum one page per investigator**

Attached 1 page CV of the PI