Using Environmental DNA to Delineate the Distribution of the Ouachita Streambed Salamander

Project Summary:
The Ouachita Streambed Salamander (*Eurycea subfluvicola*) is a newly described species from the Ouachita Mountains of Arkansas, and is currently known from only a single locality (Steffen et al. 2014). This paedomorphic species retains the morphological and ecological characteristics of aquatic larvae as sexually mature adults, and is only seasonally found on the surface. *Eurycea subfluvicola* co-occurs with the larvae of the Many-Ribbed Salamander (*E. multiplicata*), which can make it difficult to visually distinguish between the two species in the field. These aspects of the ecology and life history of *E. subfluvicola* are in part likely why this species remained undetected until now. There is an immediate need to determine the extent of the distribution of *E. subfluvicola* to develop appropriate conservation measures or actions. We propose to assess the distribution of *E. subfluvicola* employing environmental DNA (eDNA) and visual encounter surveys in streams of the Caddo and Trap mountain subranges of the greater Ouachita Mountain ecoregion. We will further document and analyze stream ecological parameters to identify factors that dictate the distributional limits of this species.

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Project Budget:
SWG amount requested: $53,971
Match amount provided: $29,119
Total amount of project: $83,090

Project Statement

Need:

The newly described Ouachita Streambed Salamander (*Eurycea subfluvicola*) is currently known from only a single locality in the southeastern Ouachita Mountains of Arkansas (Steffen et al. 2014). This species currently has the smallest known distribution of any amphibian species in the United States. *Eurycea subfluvicola* retains the morphological and ecological characteristics of aquatic larvae throughout life and are therefore restricted to living in streams. This species is only seasonally found on the surface and presumably spends dry months in subsurface waters beneath the streambed. *Eurycea subfluvicola* co-occurs with the larvae of the Many-Ribbed Salamander (*E. multiplicata*), and it can be difficult to visually distinguish between the two in the field. These aspects of the ecology and life history of *E. subfluvicola* are in part likely why this species remained undetected until recently. This newly described species with an extremely small known distribution warrants assigning the Ouachita Streambed Salamander as a species of greatest conservation need (SGCN). There is an immediate need to determine the extent of the distribution of *E. subfluvicola* to develop appropriate conservation measures and/or actions to ensure the long-term survival of the species.

Purpose and Objectives:

Purpose: The purpose of this project is to expand our understanding of the distribution and ecological limits of the Ouachita Streambed Salamander. This project will also determine the limits of detectability for the environmental DNA (eDNA) sampling technique employed here.

Objectives: The objectives of this project will be to sample ~50 streams for the presence of the Ouachita Streambed Salamander and measure habitat parameters that are associated with its presence/absence. Environmental DNA sampling and analyses at the known *E. subfluvicola* site will determine the downstream detectability limits of this species. This assessment will be essential for interpreting eDNA data collected from across the region.

Location:

Stream surveys will be conducted in the Caddo and Trap mountain subranges of the Ouachita Mountain ecoregion Garland, Hot Spring, and Montgomery Counties). Genetic and ecological analyses of water sample and stream data will be performed at the University of Tulsa, Tulsa, Oklahoma.

Approach:

eDNA surveys: eDNA has become an established method for the detection of aquatic species (e.g., fish, aquatic salamanders) and has been used to provide evidence of the presence of a species where other sampling techniques have failed. This technique is based upon the collection and analysis of shed genetic material (i.e., dermal cells) in water samples. Up to six 1-liter bottles of stream water will be collected per "site". A "site" is a single point on a stream, and the number of "sites" sampled per stream will be based on eDNA detectability limits (i.e., downstream distance from known salamander location) at the type locality of *E. subfluvicola*. The type locality is a first order stream within Lake Catherine State Park. To date, salamanders have only been found within an ~50 meter segment of this stream, with the largest concentration in a pool in the uppermost 10 meters. Water samples will be taken starting at the "pool" thence downstream at 10, 20, 50, 75, 100, 150, and 200 m. Analysis of these samples will enable us to determine the eDNA detectability distance from a known point.

We will then sample ~50 other streams within the Caddo and Trap Mountain subranges of the Ouachita Mountain ecoregion. Streams will be selected based on similar geological signatures as those observed at the type locality. If eDNA surveys at the type locality show that *E. subfluvicola* is only detectable within ≤50 m then samples will be taken from several "sites"; particularly for streams that contain favorable habitat over long distances. If *E. subfluvicola* eDNA is detectable over long distances (up to 200 m) then samples will be taken from one or two sites along a stream. This would enable us to expand our sampling to include additional streams. A 1-liter sample of distilled water will be used as a laboratory control. Wilcoxon signed rank tests will be used to test for significant differences in the detectability of *E. subfluvicola* eDNA among sampling sites.

Environmental DNA analyses: Water samples for eDNA will be filtered through cellulose nitrate filter paper using a vacuum pump, and filters will be stored in 95% ethanol in a -20^{o}C freezer. eDNA will be extracted from filter paper using Qiagen DNA extraction kits. The presence of *E. subfluvicola* will be tested using quantitative Polymerase Chain Reactions (qPCR), which will specifically amplify the DNA of *E. subfluvicola* when present in a water sample. We will design this assay for the mitochondrial DNA gene *cytochrome b*. This gene is highly variable between species and has many distinct mutations that allow us to identify *E. subfluvicola* from the common Many-Ribbed Salamander, which occurs in the same streams. We already have many *cytochrome b* sequences for both of these species, which will expedite gene assay design. The assay will utilize ABI Taqman chemistry (Applied Biosystems Inc.) and will be run on an ABI StepONE quantitative PCR machine. Three qPCR reactions will be performed for each 1-liter sample. A total of 18 qPCR reactions (six 1-liter bottles x 3 replicates of each sample) will be run for each site. *Eurycea subfluvicola* DNA will be run as a positive control and extractions from filtered 1-liter bottled water, and unfiltered sterilized water, will be used as negative controls. Our lab has extensive experience in designing qPCR assays for eDNA detection (quantification) as well as gene expression (e.g. Aran et al. 2014).

Relationships of new populations: If new localities of *E. subfluvicola* are detected it will be important to determine how genetically different they are from the extant population. Such information will enable us to assess the genetic diversity within *E. subfluvicola* and evaluate the potential for additional undescribed paedomorphic species in the Ouachita Mountains. Representative individuals or positive eDNA samples (in the absence of live specimens) will be sequenced for the mitochondrial gene *cytochrome b* on an ABI capillary sequencer. Spatial population genetic analyses will be used to assess the genetic differences among *E. subfluvicola* from different sites (Emel and Bonett 2011).

Visual encounter surveys and ecological data: Fieldwork to conduct visual encounter surveys and ecological data collection will take place during the winter and spring months (February through May) of 2016 and 2017. First and second order streams, focusing on those originating in the Arkansas Novaculite Formation flowing into alluvial valleys on the Stanley Shale, will be located using GIS maps to aid in identifying potential habitat. We estimate that we will be able to survey 15 to 25 locations per year, for a total of 40 to 50 locations over two years. Visual encounter surveys will be conducted by day and at night, and consist of moving surface cover in lotic habitat or spotting salamanders active on the stream bottom. Stream characteristic data such as stream size, water temperature (collected via iButton data loggers), substrate type, substrate embeddedness, and canopy cover will be compiled for each sampling station. GIS software will be used to plot the presence/absence distribution of *E. subfluvicola* based on survey results.

Expected Time Line:
Fall 2015: Develop eDNA assay.
Winter/Spring 2016: Collect eDNA water samples and stream characteristics data at type locality and additional sites.
Summer/Fall 2016: Analyze eDNA samples
Winter/Spring 2017: Continue eDNA sample and stream characteristics data collection.
Summer/Fall 2017: Analyze eDNA and ecological data, write-up results, and prepare report.

Expected Results and Benefits:
The recently described Ouachita Streambed Salamander is an Arkansas endemic, known only from the type locality, and as such, is a species of conservation concern. The proposed study would produce detailed locality information and maps of the known distribution of *E. subfluvicola*. Results of stream characteristic analyses will provide expected habitat requirements and limits of Ouachita Streambed Salamanders, and serve as a guide for future population monitoring and habitat management efforts. This information is fundamental for making informed conservation and management decisions.

Budget:

	Year One 10/01/2015 - 9/30/2016			**Year Two 10/01/2016 - 9/30/2017**		
	Sponsor	TU*	Total	Sponsor	TU*	Total
Graduate Assistant** 7.5 mos Year 1, 4.5 mos Year 2	$13,125	$0	$13,125	$7,875	$0	$7,875
Fringe Benefits - Grad. Student (8%)	$1,050	$0	$1,050	$630	$0	$630
Total Salaries/Wages	**$14,175**	**$0**	**$14,175**	**$8,505**	**$0**	**$8,505**
Total Domestic Travel	**$4,212**	**$0**	**$4,212**	**$4,212**	**$0**	**$4,212**
Other Direct Costs						
Materials/Supplies	$9,960	$0	$9,960	$8,000	$0	$8,000
Other						
GA Tuition	$0	$8,300	$8,300	$0	$6,100	$6,100
Total Direct Costs	**$28,347**	**$8,300**	**$36,647**	**$20,717**	**$6,100**	**$26,817**
MTDC Base	**$28,347**	**$0**	**$28,347**	**$20,717**	**$0**	**$20,717**
Indirect Costs (MTDC) @ 10% (TU Rate 40%)	$2,835	$8,504	$11,339	$2,072	$6,215	$8,287
Total Costs	**$31,182**	**$16,804**	**$47,986**	**$22,789**	**$12,315**	**$35,104**

	Sponsor	**TU**	**Total**
Cumulative Year 1 + Year 2	**$53,971**	**$29,119**	**$83,090**

*The University of Tulsa will match up to the amounts indicated for GRA tuition and unrecovered indirect costs in order to meet the 35% match requirement.

Qualifications

Dr. Ronald M. Bonett is an Associate Professor in the Department of Biological Science at the University of Tulsa (2007 -present). Dr. Bonett has worked on the ecology, evolution, and biodiversity of amphibians for more than 17 years, with a particular emphasis on salamanders of the Family Plethodontidae in North America. This group of salamanders includes the Ouachita Streambed Salamander, which was recently described by Dr. Bonett and colleagues. Dr. Bonett's active research program employs ecological and genetic techniques to understand the distribution and biodiversity of salamanders in the Ozark Plateau and Ouachita Mountains.

Kelly J. Irwin has a M.S. in Wildlife & Fisheries Science from Texas A&M University (1997). Kelly has worked on amphibian and reptile conservation and management projects as AGFC herpetologist for 15 years. Kelly and Michael Steffen were the co-discoverers of the Ouachita Streambed Salamander.

Michael A. Steffen is a doctoral student in the Department of Biological Science at the University of Tulsa. Michael studies the ecology, evolution, and biodiversity of salamander in eastern North America, including the brook salamanders in the Ouachita Mountains. Michael and Kelly Irwin were the co-discoverers of the Ouachita Streambed Salamander.