ISOLATION AND CHARACTERIZATION OF POLYMORPHIC MICROSATELLITE LOCI IN THE GREEN SALAMANDER, \textit{Aneides aeneus}

Key Terms: Green salamander (\textit{Aneides aeneus}); Microsatellites; PCR primers; Population genetics; Conservation genetics

INTRODUCTION
The global decline of amphibians as a result of rapid growth of human populations and their expansive impact on natural environments is well documented (Petranka 1998; Collins and Storfer 2003; Becker et al. 2007), with many species becoming extinct, endangered, or threatened (Pechmann and Wake 1997; Alford and Richards 1999; Stuart et al. 2004). Furthermore, amphibians have in recent decades become more threatened than birds or mammals (Stuart 2004). It has been found that direct environmental alteration (i.e. deforestation, habitat fragmentation and timber harvesting) is the primary cause of North American amphibian declines (Petranka 1998; Becker et al. 2007). In these human modified environments, patterns of dispersal and gene flow among populations can be significantly altered (Coulon et al. 2006; Cushman 2006; Zeilmer and Knowles 2009; Apodaca 2012). Consequently, landscape-genetic studies that estimate levels of gene flow and identify levels of population connectivity are crucial to the conservation and management of imperiled species.

Populations of the Green salamander (\textit{Aneides aeneus}) have been documented to be declining at alarming rates, possibly due to overcollecting, habitat loss, and climate change (Corser 1991; Corser 2001). Consequently, the species is now protected in every state in which they occur (Amphibiaweb). Furthermore, the species is listed as Vulnerable by NatureServe, as they are habitat specialists threatened by habitat loss and fragmentation, with patchily distributed populations of which the most isolated are declining (naturereserves.org). In the 1970’s, Green salamander populations collapsed in the Blue Ridge Escarpment (BRE), a disjunct portion of their range (Snyder 1983; Corser 1991; Snyder 1991). Those within their main range however remained stable (Snyder 1991). Although several reports were made in which these populations were speculated to have recovered (Snyder 1991; Petranka 1998), Corser (2001) studied population trends at several Green salamander populations in North Carolina throughout the 1990s and found a 98% decline in relative abundance since 1970.

In his 2001 publication, Corser found that a dramatic decline such as this was likely to be explained by lack of reproduction. Furthermore, he noted a lack of evidence for successful recolonization, the probability of which is significantly lowered when habitat loss is considered (Tilman et al., 1994). If Corser’s claim, that successful migrations between subpopulations of \textit{A. aeneus}, both within the BRE, and between the BRE and main range of the species, has not been sufficient to reverse these declines (Corser, 2001), then it is of crucial conservation concern that studies are conducted to determine the genetic stability of this isolated population and how the landscape influences migration between these populations.

It has been found that maintenance of genetic diversity is crucial to the long-term survival and recovery of threatened species (Avise 2004; Morgan et al. 2008). Several
publications (Gilpin and Hanski 1991; Harrison 1991; Swindell and Bouzat 2005) have shown that genetic diversity and population viability is maintained primarily by gene flow among local populations. In a healthy state, this transference of genes contributes to population persistence, adaptive response, and release from inbreeding depression (Frankham et al. 2002). Likewise, if genetic factors are ignored, extinction risk will be underestimated (Frankham 2005), and conservationists may develop inappropriate recovery strategies. Genetic markers have frequently been used to investigate population genetic structure, produce estimates of gene flow, and augment conservation management for at risk species (Croshaw et al. 2005; Noël et al. 2007; Apodaca et al. 2012). Microsatellite loci (also known as simple sequence repeats; SSRs) are one of the most useful forms of genetic markers for addressing conservation genetic questions, and have long been used as important tools in population genetic studies because of their versatility (Bebee 2005). In this study, I intend to develop ten polymorphic microsatellite loci in A. aeneus for later use in projects investigating the viability of the disjunct population of A. aeneus in the Blue Ridge Escarpment.

METHODS

The initial stage of this project is planned take place during the later half of March at the Savannah River Ecology Laboratory. While there, I will develop the microsatellite loci using the direct “Seq-to-SSR” protocol developed by Castoe et al. 2012. This is an approach to identifying potentially amplifiable SSR (microsatellite) loci that uses Illumina paired-end sequence data to identify reads containing SSR loci (Castoe et al. 2012). This is followed by an identification of flanking sequences appropriate for PCR primer sites. Once completed, these produce what are called a “potentially amplifiable locus” (PAL). Such methods are capable of identifying and characterizing thousands of potentially amplifiable SSR loci in a cost efficient, reliable way (Castoe et al. 2012).

Specifically, total DNA will be extracted using standard methods with the use of the DNeasy tissue kit and protocol (Qiagen, Valencia, CA). An Illumina paired-end (PE) shotgun library of about 325 bp will then be prepared using the extracted DNA using the protocol established by Gibbons et al. 2009. SSR loci will then be identified using a Perl script developed by Castoe et al. which defines SSRs as simple repeats of at least 12 bp long for 2-4mers or at least 3 repeats for 5mers and 6mers (Castoe et al. 2012). Reads containing SSR loci will then be screened to identify flanking regions which have high-quality PCR priming sites, as these newly developed SSR loci will only be useful if primers may be designed for use in PCR amplification (Castoe et al. 2012).

Once developed, these primer pairs will then be tested for amplification and polymorphism using DNA obtained from A. aeneus samples which we currently possess (Lance et al. 2009). Once shown to amplify, variability in these loci will be tested in all specimens, estimating number of alleles per locus (k) and observed and expected heterozygosity (\(H_o\) and \(H_e\)) (Lance et al. 2009). Additionally, tests for deviations from the Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium will be conducted.
Key Literature Citations Supporting Research:

AmphibiaWeb - *Aneides aeneus* [WWW Document].


Comprehensive Report Species - *Aneides aeneus* [WWW Document].


Snyder, David H, 1983. The apparent crash and possible extinction of the green salamander, Aneides aeneus, in the Carolinas. Association of Southeastern Biologists Bulletin 30, 82.


ISOLATION AND CHARACTERIZATION OF POLYMORPHIC MICROSATELLITE LOCI IN THE GREEN SALAMANDER, *Aneides aeneus*

PROPOSED BUDGET

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**Total Price** $3067.00

**Asking Price** $567.00

With my goal of developing ten polymorphic microsatellite loci in mind, costs will well exceed that which is available to me within the scope of this grant. To accommodate this, I ask only for $567. This sum will allow me to purchase the DNeasy Blood and Tissue kit to be used in the extraction of DNA from samples as well as the Taq polymerase to be used in PCR amplification of genetic samples. This will then provide me the means necessary to develop polymorphic microsatellite loci in *A. aeneus* to be used in projects investigating the viability of the disjunct population of *A. aeneus* in the Blue Ridge Escarpment. I am not requesting funds to cover the costs of lab consumables which include consumable plastics, such as microcentrifuge tubes, pipette tips, etc. This category has thus been left general. The Pugh Endowed Fund for Undergraduate Research will be covering $2000 of these funds, with an additional $500 to be covered by J.J. Apodaca's personal grant resources.
April, 11, 2013

Synergistic Qualities of Goldenseal, *Hydrastis canadensis*,
versus Berberine Isolate in Vitro

**Introduction**

Goldenseal, *Hydrastis Canadensis*, has been used by Native Americans for thousands of years. Traditionally, the Cherokee employed goldenseal topically for skin and eye disease and the Iroquois used goldenseal for gastrointestinal complaints (Blumenthal, 2003). Today, goldenseal is recommended as a treatment for an array of ailments such as viral and bacterial infections and as an antidiarrheal (Upton, 2001). In 2006, Goldenseal was ranked among the 20 most popular herbal supplements used worldwide (Herbal Supplements, 2006). The medicinal qualities of goldenseal can perhaps be attributed to its most abundant alkaloid berberine, (Heon, 2005) which has shown to possess antimicrobial activity in vitro. Berberine has also shown promising effects in vivo to treat severe diarrhea and eye infections caused by *Chlamydia trachomati* (Purohit, 1969).

However, when researchers explore botanical medicines, they confront a formidable challenge. Due to an array of chemically diverse aspects of whole plant preparations, it is difficult to determine its medicinal effects. For instance, potentiation, one type of synergy, occurs when a compound yields no activity on its own, but enhances the activity of another compound. Hivas Junio asserts, “Their [botanicals’] components interact synergistically; the combined effect is greater than the sum of the parts,” (Junio, 2012).

Therefore, to untangle the complexity of botanical medicines and compare their integral bioactive constituents would allow more accurate quality control and replicable clinical trials. One step towards this goal is to determine whether the active constituents of goldenseal are more effective in the phytochemical matrix than when isolated. In the following proposal, goldenseal extract will be compared to isolated berberine in vitro to measure the inhibition of bacteria such as *Shigella* (Joshi, 2011), *Staphylococcus aureus* (Ettefagh, 2011), *Neisseria gonorrhoeae* (Cybul ska, 2011) and *Enterococcus faecalis* (Parhi, 2012).

**Methods**

Goldenseal extract tinctures will be obtained from the local commercial supplier, Gaia herbs. The extracts of berberine will be obtained from Sigma Chemicals.

Goldenseal extracts will be tested using HPLC coupled with UV/VIS detector according to Brown and Roman’s study in 2008. The extracts will be diluted to promote more interaction with the stationary phase which help provide more separation and resolution in the chromatograms. The berberine extract will then be diluted until its concentrations matches those in the commercial goldenseal ethanol extracts.
## Budget

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ANALYSIS OF BERBERINE AND HYDRASTINE IN RHIZOMES OF
GOLDENSEAL (**Hydrastis canadensis** L.) GROWN IN VARIED LEVELS OF
NITROGENATED SOIL

April 12, 2013

INTRODUCTION:

Goldenseal (**Hydrastis canadensis** L.) is a slow-growing, native herbaceous perennial that occurs in hardwood forests throughout the eastern United States. This plant, a member of the Ranunculaceae or buttercup family has been gathered and utilized for generations to treat a myriad of disorders ranging from cancer and sore throats to influenza. (Davis, 1993; Cecil et. al, 2011) This plant is also a popular herbal remedy for treating skin infections as two of the main compounds within its rhizomes are well known for having anti-microbial properties. (Cech et al., 2012) Hydrastine and berberine (see Figure 1) are the most well known of the alkaloids present in this understory woodland plant and it is these compounds which are often attributed to displaying healing properties. (Brown et. al, 2008)

Wild goldenseal populations are currently threatened by overharvesting due to high market demand of this plant material. In North Carolina, goldenseal has even achieved endangered species status therefore rendering wild harvest of this plant illegal. (Davis, 1993) In an effort to cut down on the pressure that continued illegal harvest wreaks on wild populations, many have turned to propagating goldenseal in forest agricultural settings. The question now has become, how can the efficiency of growing these plants be improved? One way that has been speculated is through increasing the concentration of the target compounds they are prized for. In this way, plants could be grown with more medicinal potency so that less biomass would be needed to achieve the same medicinal and dollar values for growers.

High Performance Liquid Chromatography or "HPLC" is the method that is most often utilized by those who analyze raw goldenseal material for medicinal potency. (Dawes, 2011, Brown et. al, 2008) Once concentrations of the target compounds have been identified, extraction and production of supplements, tinctures, and herbal powders can be made and put out into the market for sale. Canadine is an example of another popular alkaloid that is sometimes analyzed in conjunction with hydrastine and berberine however these other alkaloids are often found in such low concentrations that it is
nutrient conditions (specifically soil Nitrogen levels) can significantly increase the concentration of the target compounds that goldenseal is prized for. This variable has been chosen because nitrogen deficiency has been shown to increase secondary metabolites in plants. (Ibrahim et. al, 2011, Knight, 2012) Although it is known that the medicinal action of this plant is due to more than just the action of berberine and hydrastine alone, this study will focus on these two alkaloids due to the limitations that a one-year, undergraduate study inherently presents.

METHODS:

This study will utilize 56 goldenseal plants that were propagated from rhizome divisions in the fall of 2011. Rhizome propagation is a fairly simple technique used to create new plants from existing stock. (Davis, 1995) The rhizomes used in this planting came from a cultivated plot in the Warren Wilson College Forest where plants were dug, rhizomes were cut and separated (each containing at least one viable bud), and then replanted in pots.

In the spring of 2013, these 56 goldenseal plants were transplanted into a semi-sterile growing medium consisting of a 1:1 ratio of peat to vermiculite and randomly assigned to four treatment groups. In their new pots, the plants were then randomly arranged onto a series of pallets in a section of forest on the Warren Wilson College land. Once the plants leaf out, four different recipes of Hoagland's nutrient solution will be made up in order to have a control and three experimental levels with varying quantities of nitrogen in them. Although the nitrogen levels will vary in these treatments, all other variables will be held constant. A 1:4 dilution of these solutions will be used for each nutrient watering which will take place once in the first week of every month from May through October. Other basic nutrient-free watering will occur as needed throughout the season but will be the same for all plants. Following the trends used by goldenseal growers, plants will then be harvested in the late fall to make root extracts that will be used to determine the present concentrations of the target compounds. (Persons et. al, 2005) Using an HPLC method that was developed by Paula Brown & Mark Roman, the extracts will be analyzed. (Dawes, 2011, Brown et. al, 2008) The detector for the specific HPLC unit that will be used is UV/VIS Spectroscopy. Standards from Sigma Aldrich will
ANALYSIS OF BERBERINE AND HYDRASTINE IN RHIZOMES OF GOLDEN SEAL (*Hydrastis canadensis*) GROWN IN VARIED LEVELS OF NITROGENATED SOIL

**PROPOSED BUDGET**

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THE ESTIMATED TOTAL COST OF THE PROJECT WILL BE $39.00