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Determining the Genetic Distances between sub-populations of *Aneides aeneus* in the Westvāco Wildlife and Ecological Research Forest

Thesis submitted to
The Graduate College of
Marshall University

In partial fulfillment of the
Requirements for the degree of
Master of Science
Biological Sciences

by

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November 21, 2002

Determining the Genetic Distances between sub-populations of *Aneides aeneus* in the Westvāco Wildlife and Ecological Research Forest.

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Aneides aeneus, the Green Salamander, is a plethodontid species ranging from southern Pennsylvania to central Alabama and from eastern Mississippi to Maryland. Populations of Green Salamanders most often inhabit cliffs and rocky outcrops which are moist but not wet and shaded. The animal is listed as a species of special concern by the West Virginia Division of Natural Resources Wildlife Diversity Program. Genetic and reproductive isolation due to strict habitat preferences of the Green Salamander could be a factor in the decline of the species. Seventeen tissue samples were retrieved from five sites within the Westvāco Wildlife and Ecosystem Research Forest (WWERF) in Randolph County, West Virginia for the purposes of determining the extent of gene flow between those 5 separate populations. The polymerase chain reaction was used to amplify approximately 940 base pairs of the ND4 NADH dehydrogenase region of mitochondrial DNA (mtDNA). These amplicons were sequenced and then analyzed using distance matrix phylograms, maximum parsimony cladograms, and linear regression analysis. Tree topologies, rooted by an outgroup Green Salamander specimen from North Carolina, showed no consistent grouping by individual populations. The North Carolina specimen consistently branched away from the West Virginia specimens. However, linear regression analysis showed a significant correlation ($R=0.645$, $P=0.008$) between genetic distances and geographical distances of the samples both within the WWERF and extending to the North Carolina specimen. This correlation was not significant for populations which were within one kilometer of each other. Linear regressions of protein genetic distances and geographic distances proved to have no significant correlation for populations inside the WWERF. We were unable to demonstrate, through phylogenetic methods or linear regressions, that populations within one kilometer are genetically isolated. This work suggests that gene flow can occur within a one kilometer radius, but is reduced or absent at three kilometers for *A. aeneus*. Continued monitoring of these populations and examination of the ND4 region of mtDNA from other populations is important for the Green Salamander. Knowledge of the extent of species migration is important to provide proper protective measures for this species.

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Chapter 1

Literature Review

Species Description

Aneides aeneus is a plethodontid salamander which can be characterized by the absence of lungs. Both skin and mouths are used for gas exchange (Green and Pauley 1987). Wake considered the genus *Aneides* to be closely related to the genus *Plethodon* and to be the most advanced of all the plethodontids (1966). The common name for *Aneides* is the climbing salamander, which alludes to the arboreal and climbing tendencies of several species of the group (Conant 1998). *Aneides aeneus* is the only species of the genus to be found east of the Mississippi River.

Like all *Aneides*, the Green Salamander has a flattened body, long legs, a prehensile tail, and expanded, square toe tips which are all adaptations for living in rock crevices. The Green Salamander is identifiable by its greenish/yellowish mottling on the dorsal side (Figs. 3 and 4) and a light colored and unmarked ventral surface (Petranka 1998). The specific epithet *aeneus* (Latin *aeneus*, of bronze or copper) refers to the bronze, lichen-like patches on the dorsum of the salamander (Gordon 1967).

Aneides aeneus (Cope and Packard) ranges from southern Pennsylvania to central Alabama and from eastern Mississippi to Maryland (Fig. 1). There is also a disjunct population in the Blue Ridge Escarpment of North Carolina. In West Virginia (Fig. 2), the salamander inhabits the central counties of the Allegheny Plateau, from Monongalia and Preston counties southwest to the Big Sandy River (Green and Pauley 1987). The

Green Salamander has been found between 139 meters and 1333 meters in elevation across its range (Gordon 1967).

In West Virginia, *A. aeneus'* egg laying occurs in the first two weeks of June, shortly after mating (Canterbury and Pauley 1994). Females attach eggs in clusters to the top or sides of rock crevices and then brood the eggs and hatchlings for several months. Canterbury and Pauley (1994) estimated that the duration of the incubation period is between 82-90 days.

Initially the salamander's habitat was described as dead or fallen trees. This was later changed to include rock crevices of all formations (Netting and Richmond 1932; Walker and Goodpaster 1941; Gordon and Smith 1949). The movement of Green Salamanders from rock outcrop to rock outcrop is of special importance to this study.

Species Habitat and Migration

Green Salamanders prefer crevices which are moist, but not wet, and well shaded from weather and sunlight (Gordon 1952). The organisms most often inhabit cliffs and rocky outcrops which are moist and shaded (Petranka 1998). These rock faces provide crevices, cracks, and fissures which provide the salamanders with their preferred habitat. Due to its rigid habitat requirements, individual Green Salamanders seem to spend most of their lives on a single rock formation.

The extent to which individuals disperse from one rock outcrop to another is unclear (Petranka 1998). In 1952, Gordon noted, "The sedentary nature of the species, plus the selection of a more or less specialized habitat, points to a colonial existence over its range, probably with little or no gene flow between colonies." Rarely are Green

Salamanders found on the forest floor or in surrounding areas of inhabited rock outcrops (Snyder 1991).

Gordon writes that the effect of man's activities may have been the cause of the unique habitat and distribution of the animal (1952). The following account comes from a personal correspondence between Gordon and N. B. Green of Marshall College in 1950. Green told Gordon the following:

> *"on the assumption that Aneides must have been here before the Ohio River, I have explored the area immediately west of the river here (Huntington, WV) in Ohio and was able to obtain some specimens from rock crevices in a shallow cave near the Ohio River in Lawrence County. The history of southeastern Ohio is tied in with the scarcity of records, I believe, in this manner: At the close of the last century and earlier, the wooded slopes of southeastern Ohio were cleared to provide fuel for the smelting furnaces. This destroyed the habitat of Aneides and only those that were fortunate to find deep rock crevices and caves survived. For this reason its distribution is somewhat spotty."*

There have been a few occasions where *Aneides aeneus* has been witnessed crossing roads during the summer (Cupp 1991; Williams and Gordon 1961). In a recent field study, Waldron set up drift fences and pitfall arrays around three rock outcrops containing *A. aeneus* populations in the Westvāco Wildlife and Ecosystem Research Forest (WWERF), located 2.9 miles southwest of Adolph, West Virginia in Randolph County (Fig. 5). Waldron was unable to capture a single Green Salamander on the forest floor. Yet, during a year-long mark and recapture study she found that a single individual from a population of 38 marked salamanders had migrated from one outcrop to another (2000).

Canterbury (1991) also found several individuals had moved between nearby outcrops (1991). His mark and recapture study involved monitoring 53 rock outcrops and

212 marked salamanders within a 1.5 hectare area. Ten individuals, consisting of males, females, and sub-adults, were found to have dispersed to different rock outcrops over the course of the two-year study. These migrations ranged from 6.4 meters to 46.3 meters in distance.

Green Salamanders have been seen climbing trees and living in the bark of upright, dead trees, and rotting logs (Petranka 1998). Although these accounts show that Green Salamanders can leave their outcrops, they seem insufficient to explain the amount of gene flow necessary to sustain a viable species. The West Virginia Division of Natural Resources Wildlife Diversity Program lists *Aneides aeneus* as a species of special concern. This is probably due to the patchy and discontinuous nature of its populations. In the 1970's, the disjunct population located in the Blue Ridge Escarpment suffered a major decline. There have been many theories that attempt to explain the decline. One theory proposed that an infection of a *Batrachochytrium*-like fungus was the cause (Longcore et al., 1999). Over-collecting, climate change, and habitat loss are probably all main contributors to the problem (Corser 2001). Narrowly distributed montane specialists like Green Salamanders would be most affected by climate change (Donelly and Crump 1998). Corser also points out that in the case of habitat loss, salamanders would no longer be able to migrate through the safety of old growth forests to recolonize populations that were wiped out. My study attempts to answer the question of how much gene flow exists between rock outcrop populations of *Aneides aeneus* in a small area in Randolph County, West Virginia.

Gene Flow

Understanding the extent of the salamander's ability to migrate and its mechanism of gene flow is important to understand how *Aneides aeneus* survives. Gene flow is the migration of any individual and the incorporation of their genes into another population (Storfer 1999). Restriction of gene flow could result in local differentiation among populations and perhaps even speciation (Mayr 1963; Slatkin 1985). Genetic drift, occurs as a result of low levels of gene flow, can account for genetic divergence of a population (Wright 1931). Liebherr (1988) states that these relative levels of gene flow can determine whether a species will cohere or fragment genetically. The ability of a species to cohere genetically is the result of how effective populations are at dispersing and coming in contact with other populations. The amount of gene flow that is evolutionarily important to an individual species depends on other selective pressures within that species (Slatkin 1985).

In computerized, simulated models studying the levels of genetic drift and gene flow, Slatkin (1981, 1985, 1987) provided evidence that gene flow levels can vary dramatically between species. His studies yielded a procedure that can be used to categorize the amount of gene flow and migration of a species. Slatkin showed that there is a linear correlation between the number of individuals migrating into new populations and the overall genetic likeness of those populations. More migration leads to the loss of genetic diversity within a population.

Salamanders, especially plethodontids, have been found to have low levels of gene flow (Larson et al. 1984; Routman 1993). Larson et al. (1984) found that the maximum rates of migration in some plethodontid populations were higher than the

inferred rate of mutation for the groups. They used the procedure derived from Slatkin's computer model to estimate the amount of gene flow using the results of an electrophoretic protein analysis. They concluded that a majority of the 22 species of plethodons in their study contained populations which were completely isolated from genetic exchange. This fragmented metapopulation structure (Corser 2001) could result in the weakening of a plethodontid species and leave them susceptible to destruction by a localized environmental disruptions.

It would seem, given the fragmented nature of Green Salamander habitat and the apparent reluctance of the organism to leave its habitat, that *Aneides aeneus* would show some form of genetic divergence at each outcrop. This divergence would not necessarily manifest itself as a phenotypic difference, but possibly subtle genotypic differences could be detected.

Molecular Phylogeography

Phylogeography is the evolutionary history of a species or group of related species and how that can change throughout the animals' range (Campbell 1993). The use of mitochondrial deoxyribonucleic acid (DNA) sequences for the purpose of examining intra-specific and inter-specific relationships has become a popular tool in phylogeography. Mitochondrial DNA mutates at a rate approximately ten times faster than that of nuclear DNA, allowing researchers to estimate genetic distance among organisms (Wiesner et al. 1991; Patarnello et al. 1994). This fast mutation rate is especially important to studies that examine gene flow between proximal populations.

The nicotinamide adenine dinucleotide dehydrogenase subunit 4 mitochondrial gene (ND4) was chosen for this study because of its ease of amplification and its previous use to resolve both broad and narrow phylogenetic questions (Arévelo et al. 1994; Mahoney 2001a; Janzen et al, 2002; Gorrochotegui-escalante et al, 2002). The primer pair chosen for this study (Table 1) amplify a region of approximately 950 base pairs which include most of the ND4 region and transfer RNA's for Histine, Leucine, and Serine.

Mahoney (2001a) used the ND4 region to determine the relationship of eastern *Plethodon*, western *Plethodon*, and *Aneides* (2001). She employed the ND4 region to resolve the genetic history of an entire taxanomic family of salamanders. Therefore, this region of mtDNA was demonstrated to be conserved enough to resolve relatively distant phylogenetic relationships.

Janzen et al. (2002) used the ND4 region along with ND2 and cytochrome *b* to examine regional genetic variances of the common garter snake, *Thamnophis sirtalis*. They found that genetic relationships of the species were not concordant with the already designated morphologically based subspecies. Three biogeographically distinct clades of *T. sirtalis* were evident from their results. They were also able to detect intra-population genetic differences within the range of the garter snake.

Gorrochotegui-Escalante et al. (2002) examined local patterns of gene flow within the mosquito, *Aedes aegypti*, with a 387 base pair region of ND4. They found that in one region of the mosquito's range, gene flow decreased as geographic distance increased. However, this was not true for the species throughout its range. Extensive gene flow, not restricted by geographical distance, was reported for other regions of the mosquito's

range. Both Janzen et al. (2002) and Gorrochotequi-Escalante et al. (2002) show that the ND4 region can be used to resolve more detail than what is needed for family level examinations along with the ability to resolve recent phylogenetic events.

Chapter 2

Methods

Study Site Description

Salamander tissue samples were collected from 6 sites within the Westvāco Wildlife and Ecological Research Forest (WWERF). The WWERF comprises 8433 acres of experimental forest and is located 2.9 miles southwest of the town of Adolph, Randolph County, West Virginia (Fig. 5). All roads are gated in the WWERF to dissuade public use of the land which can interfere with ongoing research. The area often receives more than 160 cm of precipitation during the year (http://www.mwerf.org). The majority of the forest overstory is characterized by a northern hardwood-Allegheny hardwood type of *Fagus grandifolia*, *Betula alleghaniensis*, *Prunus nigra*, *Magnolia fraseri*, *Acer rubrum* and *Acer saccharum* occurring at elevations above 850 meters. All six sites (Fig. 6) were chosen because of historical accounts of Green Salamander populations. For latitude and longitude at each site, refer to Table 2.

The Kittle Creek site was located at the top of a ridge running from southwest to northeast at about 912 meters in elevation. The forest on the top of this ridge has not been logged for 84 years (Westvāco Corporation 2002). This site contained the largest rock outcrop of all the sites and is also the farthest from all of the other sites. There is a clearcut area that lies approximately 200 meters down the southern slope of the ridge.

The Clearcut site was named because of its proximity to a clearcut hillside which is now in its third year of hardwood regeneration. The rock outcrop is located on the south-facing slope of the hill approximately 20 meters from the clearcut in a patch of forest that has not been logged for 74 years (Westvāco Corporation 2002). It was surrounded by vegetation on all other sides. Its altitude is approximately 924 meters. It lies on the eastern adjacent ridge to Rockyrun 1 and 3, "64", and Cubbyhole sites.

The Cubbyhole site is on a west-facing slope. It lies on the same ridge as "64" and the Rockyrun sites at about 936 meters in altitude. The Cubbyhole site was named for a peculiar formation in the outcrop that forms a shaded pocket within the rock. The forest in this area has not been logged for 72 years (Westvāco Corporation 2002).

The two Rockyrun sites (Rockyrun 1 and Rockyrun 3) lie on a west-facing slope at about 876 meters in elevation. Waldron's study (2000) of the vertical stratification of salamanders utilized these two sites along with a third rock in the vicicnity. These two sites are 26 meters apart and are situated in a part of the forest that has not been logged for 74 years (Westvāco Corporation 2002).

Site "64" is characterized by dense rhododendron growth on a west-facing slope. It lies on the same ridge as the Rockyrun sites and Cubbyhole. The elevation of site "64" is approximately 966 meters. The forest in this area has not been logged for 72 years (Westvāco Corporation 2002).

Tissue Sample Retrieval

Salamanders were usually captured at night when they were more active. Animals found on the rock face were captured by hand. Salamanders in crevices were

forced out with the use of a blunt wire which was bent to fit into the crevice. Salamander tails were collected because the tail can be regenerated and removing part of the tail causes relatively little stress to the animal. Because hatchlings and sub-adults may not handle the stress of tail clipping as well as adults (Fig. 7), only adult salamanders were included in the study.

Tail clips (3-10 mm) were taken from the posterior end of the tail and stored in 95% ethanol. Scissors used to clip tissue samples were rinsed with ethanol and dried before and after each use. Salamanders were then returned to the crevice where they were found. Tail samples were placed in a 2 ml Eppendorf tube with approximately 1.5 ml of 95% ethanol. Samples were brought back to the lab and stored at –70°C until used.

DNA Isolation

All tools used for DNA isolation were first cleaned in sterile water and then stored in 95% ethanol. All procedures were carried out under a laminar flow hood. Tissue samples were removed from the freezer and allowed to thaw. Tail samples were retrieved from their tubes with sterile tweezers. Sterile scissors were used to cut approximately 0.5 mm cross-section of tail tissue so that it could be placed into a sterile 1.5 ml Eppendorf tube. Chelex (100 µl, appendix A) and 1 µl of Proteinase K (appendix A) were added to the tube. Tubes were placed in a dry bath at 56°C overnight. Following overnight incubation, tubes were boiled for 8 minutes, immediately centrifuged for 5 minutes at 16,200 × *g*, and stored at -20°C.

Amplification

The ND4 region of the NADH dehydrogenase from mitochondrial DNA was chosen for amplification using primers Leu and ND4 (Table 1, Aravelo et al. 1994, Mahoney 2001). Polymerase Chain Reaction (PCR) amplifications were prepared with PCR Core Kits from Boerhinger Mannheim (Germany) according to the manufacturers instructions. Taq DNA polymerase was added last, and only after the tubes had been heated to 94°C for at least one minute. Cycle One was five minutes at 94°C to allow for the addition of the Taq polymerase. Cycle Two consisted of one minute at 92°C for denaturation of the DNA strands, one minute at 45°C to allow the primers to anneal, and 1.5 minutes at 72°C for primer extension. Cycle Two was repeated 30 times. Cycle Three consisted of one minute at 92°C for denaturation, one minute at 45°C for annealing, and 7 minutes at 72°C for final extension. Thermal cycling was done in a Bio-Rad Gene Cycler. After complete amplification, vials were stored at -20°C.

Agarose gel (appendix A) electrophoresis was used to determine the presence or absence of the amplicon. Ten □l of PCR product was mixed with 2 µl of blue/orange 6× loading dye (Promega, Madison, WI) and then transferred to a lane on a 1% agarose gel. Amplicon size was determined by comparison to a 1 kilobase DNA ladder (Promega). Agarose gel electrophoresis was run using Bio-Rad Mini-Sub® Cell GT with 100 volt Bio-Rad Power Pac Junior power source. Gels were then viewed under 302 nanometers ultraviolet light and photographed using a Polaroid Photo-Documentation camera with Polaroid type 667, 3000 ISO black and white film.

Cloning

Amplicons were cloned into the pCR® II vector using a TA Cloning® Kit (Invitrogen, address). The cloning procedure was adapted from the protocol supplied by Invitrogen. Amplified salamander DNA (1 □l), 1µl of 10X ligation buffer, 1µl of pCR® II vector (25ng/µl), 6.5 µl of sterile water, and 0.5 µl of T4 DNA ligase were added to a sterile 1 ml Eppendorf tube. Ligation reactions were incubated in a dry bath at 14°C overnight and then stored at -20°C until transformation.

Ligation mixtures (2 µl) were placed into 1.5 ml Eppendorf tubes with 25 µl of *Escherichia coli* One Shot ® INVαF′ competent cells (Invitrogen). The mixture was stirred gently with a pipette tip and then incubated on ice for 30 min. After incubation the transformation reactions were heat shocked for 30 seconds at 42°C and then placed on ice. SOC medium (250 □l, room temperature) was added and the tubes were shaken horizontally at 37°C for one hour at 225 RPM in a shaking incubator. Aliquots (100 µl) of each transformation reaction were plated onto Luria-Bertani (LB) medium (appendix A) plus 50 µg/ml of ampicillin. In addition, 40 µl of 40 mg/ml of the chromogenic substrate X-Gal was spread onto each plate before plating the competent cells. Plates were allowed to incubate upside-down at 37°C for at least 24 hours.

Escherichia coli One Shot ® INVαF′ cells carry the *lac*Z□ gene at the cloning site. The intact *lac*Zα gene produces a subunit of □-galactosidase that allows the cell to metabolize X-Gal. A colony that contains an uninterrupted *lac*Zα gene will turn blue. A colony that contains a *lac*Zα gene that is interrupted by the insertion of an amplicon, is white.

White colonies were replicated onto fresh LB plates with 50 mg/ml ampicillin. To determine if the white colonies carried the insert, they were cracked using an SDS based cracking buffer (appendix A, Maniatis et al. 1982). Sterile toothpicks were used to pick small amounts of white colonies and place them into 0.5 ml Eppendorf tubes along with 25 µl of cracking buffer. Tubes were placed into a dry bath at 68°C for 1-2 hours. Stop buffer (25 □l, appendix A) was added to the vials. The cracked cell mixture was put onto a 1% agarose gel for electrophoresis. Presence/absence of plasmid and plasmid size were determined by comparison to a 1kb DNA ladder (Promega, Madison, WI). Gels were then viewed under 302 nanometers ultraviolet light and photographed using a Polaroid Photo-Documentation camera with Polaroid type 667, 3000 ISO black and white film.

All white colonies with inserts of appropriate size were placed in 50 ml of TSB (appendix A) with 50 µg/ml of ampicillin and shaken overnight at 37°C. Aliquots (5 ml) of these cultures were mixed with an equal volume of sterile 24% glycerol and frozen at -70°C.

Glycerol stocks (5 ml) of each colony were added to 150 ml of 2× YT (appendix A) plus 50µg/ml of ampicillin and the cultures were shaken overnight at 37°C. Wizard® Plus Maxipreps DNA Purification Systems (Promega, Madison, WI) were used to extract and purify plasmids from each culture. Cells were centrifuged (15,000 × *g*) and resuspended in buffer (15 ml) before cell lysis solution (15 ml) was added. Lysis was halted by the addition of neutralization buffer (15 ml) and the tube was centrifuged for 15 minutes at 14,000 × *g*. The supernatant was filtered with a coffee filter and one-half volume of

isopropanol was added. The solution was centrifuged and the pellet was suspended in 2 ml of TE buffer (appendix A). Purification resin (10 ml) was added and the mixture was placed into a vacuum Maxicolumn. The column was washed with column wash solution and 80% ethanol. The column was then placed into a 50 ml screw-cap bottle and centrifuged at 1,300 × *g*. After the resin dried, warm water (1.5 ml) was added to elute the DNA. The DNA was spun out of the column at 1,300 × *g* and filtered (0.2 μm syringe filter) before it was stored at -20°C.

DNA Sequencing and Analysis

Purified plasmids were sent to the Marshall University DNA Corp Facility (Huntington, WV) for sequencing. M13 reverse and forward primers were used for the sequencing of the insert. Sequencing was done with LI-COR (Lincoln, NE) automated, dye primer, simultaneous bi-directional sequencer. Sequence information was manually edited to remove vector sequence and resolve ambiguous base.

Sequences were aligned with ClustalX (Thompson et al. 1994), and phylogenetic analyses were performed using DNAPars and DNADist programs in the PHYLIP software package (Felsenstein 1989). Multiple distance matrix trees were produced by bootstrap analyses to determine the confidence level of retrieved tree topology. Additional statistical analyses were done using Microsoft Excel Data Analysis Toolpak.

Chapter 3

Results

Approximately 945 base pairs of mitochondrial DNA were sequenced from 17 *Aneides aeneus* (see Appendix B). GenBank Accession numbers can be found in Table 3. Along with NADH Dehydrogenase subunit 4, three RNA's were sequenced, Leucine, Histine, and Serine. Clustal alignments showed high conservation for the DNA sequence and the protein translation of the ND4 region.

Distance matrix and maximum parsimony analysis were performed on both DNA and protein sequences. The tree topologies show that this region was useful in separating intra-specific genetic differences (Figs 8, 9, and 10). The outgroup, Mahoney, was named for Meredith Mahoney who used this specimen in her genetic analysis of plethodontid salamanders. It consistently branched away from West Virginia specimens. Kittle Creek specimens clustered more consistently and showed the most genetic divergence among West Virginia specimens. Phylogenetic analyses based on protein sequences showed very similar results with the exception of a clustering of Cubbyhole 3 and Cubbyhole 2. Regardless of whether DNA or protein sequences were used, none of the tree topologies showed exclusive clustering by sample location, and no two specimens grouped together consistently.

Genetic distance ranged from 0 to 0.0781 substitutions per residue for all DNA sequences, including Mahoney. Within West Virginia populations, genetic distance ranged from 0 to 0.0086.

Protein distances ranged from 0 to 0.0578 substitutions per amino acid residue for all specimens. In West Virginia, the range was 0 to 0.0169 substitutions per position.

Linear regression analysis of genetic distance versus geographic distance (Fig. 11) indicated that the correlation of genetic variance and geographic distance was highly significant within the West Virginia populations ($R=0.645$, $P=0.008$). Regression analysis of protein distance versus geographic distance proved not to be significant ($R=0.511$, $P=0.051$; Fig. 12). However, when the Kittle Creek site was removed from the analysis of WWERF specimens, the relationship between geographic and genetic distances was no longer significant ($R=0.201$, $P=0.57$; Fig. 13). When sequence data from the North Carolina specimen (Mahoney) was added to the statistical analysis, the correlation was highly significant ($R=0.992$, $P<0.001$; Fig. 14).

Chapter 4

Discussion

The purpose of this study was to determine the extent to which *A. aeneus* has become genetically isolated due to habitat preference using the ND4 region of mitochondrial DNA as a tool for monitoring gene flow. The study concentrated on the amount of gene flow among 5 populations of Green Salamanders. These populations were all within 3 kilometers of each other and located in the Westvāco Wildlife and Ecological Research Forest (WWERF).

Two types of analyses were performed on the sequence data recovered from 17 specimens. The first analysis used the PHYLIP software package (Felsenstein 1989) to create distance matrix phylograms and parsimony-based cladograms (Figs. 8, 9, and 10). The second type of analysis was a linear regression to determine the relationships between genetic and geographic distances, discussed below.

None of the phylograms or cladograms (Figs. 8, 9, and 10) consistently cluster salamander populations by site of specimen recovery. Lack of clustering by site indicates that genetic distances were not sufficiently different between specimens from separate sites to allow discrimination by these methods. Trees constructed by maximum parsimony analysis showed similar results to distance matrix trees. Using these observations, there seems to be little evidence of restricted gene flow between sites. In all cases, however, the sequence of the North Carolina specimen clearly branches away

from all of the West Virginia specimens, indicating that geographically isolated groups do experience significant genetic drift.

Kittle Creek sites showed the most consistent grouping of all of the populations. Two Kittle Creek specimens clustered together in both mtDNA and protein analyses. The Kittle Creek site is farthest from all other sites and specimens from that site could be expected to experience the most genetic drift. In the mtDNA analysis, the Kittle Creek 2 and Kittle Creek 4 specimens grouped together and showed the most genetic divergence from other salamanders. In the analysis of the ND4 translation, Kittle Creek 3 and Kittle Creek 5 specimens showed the most genetic divergence and grouped together. Although the 2 trees were not the same, grouping Kittle Creek sites within both trees suggests a relationship between genetic and geographic distances.

Linear regression analyses were used to test the relationship between geographic and genetic distances suggested by the phylogenetic trees (Fig.11). Linear regressions were performed on both mtDNA sequences and the protein sequences. Genetic distance was calculated with distance matrix programs (Dnadist and Protdist) in the PHYLIP software package (Felsenstein 1989). Results were recorded as the number of substitutions per site (either nucleotide or amino acid) corrected for multiple substitutions. A regression analysis was completed with the Excel Analysis Toolpak from Microsoft. A highly significant P value ($P=0.008$) was obtained for the mtDNA plot of genetic distance versus geographic distance between the WWERF sites (Fig. 11). When the plot was extended to include the North Carolina sample (named Mahoney), the relationship was also significant ($R=0.992$, $P<0.001$; Fig 14). However, specimens

collected from intermediate distances (between 3 and 448 kilometers) are needed to verify the latter observation.

Regression analyses using genetic distances calculated from protein sequences were less clear. The plot of geographic versus protein genetic distance within the WWERF did not show a statistically significant relationship (R=0.512, P=0.051; Fig 12).

DNA regression analyses within the WWERF indicate that genetic drift occurrs even over the relatively small physical distances represented at the Westvāco site. Genetic distances reported in this study are too small to be detected as genetic isolation by phylogeny methods, but clearly indicate that distance is a significant factor in the gene flow between Green Salamander populations at the WWERF. The lack of a significant correlation using protein sequences may be due to the redundancy of the genetic code. That is, DNA sequence changes do not always lead to changes in amino acid sequence. Since there is reduced selection on nucleotide changes that do not result in amino acid substitutions, these mutations can accumulate within recent gene homologues before significant amino acid change occurs.

The regression analyses support the use of the ND4 gene as a molecular marker for gene flow. Early results of this study pointed to a flaw in the choice of this 941 base pair region of mtDNA. The only source of confidence for our study was the negative control from North Carolina, which grouped away from the West Virginia populations. Otherwise, this region of mtDNA did not seem to resolve intra-specific differences by population location.

The linear regression analyses suggest that this region can show evidence of genetic drift if the analysis tools are sufficiently sensitive. Because there is a highly

significant correlation between genetic and geographic distance in the regression analyses, this gene could be used to examine gene flow of populations over a larger region.

This study also suggests that these salamanders may not be as sedentary as previously believed. When the Kittle Creek data are removed from the DNA linear regression analysis, the correlation between physical and genetic distance is not significant (R=0.201, P=0.576; Fig. 13). Therefore, specimens taken within a one kilometer radius are not significantly different. There is also evidence presented in Figs 8, 9, and 10 that these salamanders are migrate and move in and out of populations, at least between closely grouped rock outcrops. It is important to note the obstacles of gene flow that are in place between each site. This includes distance, elevation change, clearcuts, roads, and streams (Table 4). If gene flow did not cease after the removal of old growth forests, obstacles presented today for salamanders do not seem insurmountable.

The mechanism for this gene flow remains unclear. Although, Green Salamanders have been found away from their rock outcrop (Cupp 1991; Williams and Gordon 1961, Canterbury 1991; Waldron 2000), mass migrations or consistent movement of salamanders from the habitat have not been seen.

Aneides genus includes many arboreal species, but *Aneides aeneus* is considered the least arboreal of the genus (Petranka, 1998). Recently, *A. aeneus* has been witnessed at heights in trees (pers. comm. Jayme Waldron). It is possible that the extent to which the Green Salamander is an arboreal species has been overlooked. Waldron, during nocturnal surveys, witnessed salamanders as high in trees as her light source could

illuminate. This does not agree with the assumption of a weakly arboreal species. Although nests were not located in trees, it is possible that an arboreal aggregation could lead to the courtship and nesting of salamanders in this habitat. Nests have been found in the loose bark of dead trees (Pope 1928). It is possible that the bark of living trees or upright, dead trees could provide the requirements for a successful nest site. The existence of Green Salamanders in trees may give a possible explanation for the gene flow between local populations of Green Salamanders. If they are an arboreal species, clearcuts may be the only serious obstacles of gene flow for the animal. Cupp (1991) and Williams and Gordon (1961) have witnessed the salamander crossing roads. Waldron has witnessed the salamander living in fallen trees which cross streams. Distance between habitats would no longer be an obstacle of gene flow if the salamanders' habitat includes the forest canopy.

This species could have been strongly arboreal in the 19th century when old growth forests still existed in abundance. It has been speculated that the Green Salamander was forced to retreat to rock outcrop habitats after the harvesting of old growth forests (Green and Gordon personal correspondence, 1950; Gordon 1952). It is possible that we allow the organism some "windows of opportunity" for gene flow. These "windows" come when we manage forests and allow them to reach an age which could benefit the salamander. It may provide enough interaction between populations to allow appropriate levels of migration.

Another possibility is that these salamanders simply do not need the amount of gene flow that allows other organisms to maintain healthy populations. In fact, all salamanders tend to have low levels of gene flow, but remain healthy (Larson et al. 1984;

Routman 1993). Andrew Storfer (1999) contends that amphibians in general are important in the study of gene flow relative to environmental degradation. He makes an especially valid point for the study of Green Salamanders, ". . . knowledge of natural levels of gene flow will help determine the potential for extant amphibians to migrate to new breeding sites."

This study intended to uncover evidence of about species in decline because of low levels of gene flow due to fragmentation of its habitat. Although the salamander is still a species of concern, I could find no evidence that restricted gene flow is a culprit in the failing of populations.

Chapter 5

Conclusion

This study arrived at some important conclusions dealing with both the dynamics of a small population of *Aneides aeneus* and with the use of the ND4 NADH dehydrogenase region of mitochondrial DNA. I was unable to demonstrate, through phylogenetic methods or linear regressions, that populations within one kilometer are genetically isolated. It is, therefore, possible that Green Salamanders are more capable of migration between rocky outcrops than has been previously recognized. Regression analyses indicate that genetic distance is correlated to geographic distance for populations that are separated by 3 kilometers. These data indicate that populations separated by more than one kilometer have accumulated significant genetic drift and, therefore, reduced gene flow. Clearly, geographic distances in the hundreds of kilometers act as barriers to gene flow. This work suggests that gene flow can occur within a one kilometer radius, but is reduced or absent at 3 kilometers. Further work is needed to clarify the limitations of Green Salamander gene flow, and the types of barriers that could cause this organism to become reproductively isolated.

It will be important to monitor Green Salamanders in the coming years. Research should continue on the mitochondrial DNA of this species for the purpose of comparisons with this study. In addition, studies on the arboreal nature of the species should continue in West Virginia. I believe there is much to be learned about this segment of the salamander's habitat. Currently Meade/Westvāco has begun a clearcut of the forest that has already started within yards of the Rocky Run sites. Although this is personally

upsetting, it will provide a unique opportunity for research. There is already population and gene flow data for these healthy sites dating back to 1999. The WWERF sites should continue to be monitored for evidence of migration after the trees have been harvested.

The region of mitochondrial DNA chosen for this study proved to be a valuable tool. However, it may have a more useful application if the inquiry examines a larger area of land. I do not believe that the ND4 region is sensitive enough for the accurate measurement of gene flow for populations within a local area. But, this region of mtDNA could be valuable for studies involving possible sub-species or known meta-populations.

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_____ and _____. 2002. Aneides aeneus isolate Aaen4 NADH dehydrogenase subunit 4 gene, partial cds; tRNA-His gene, complete sequence; tRNA-Ser gene, complete sequence; tRNA-Leu gene, complete sequence; mitochondrial genes for mitochondrial products. (specimen Cubbyhole#2) LAT 38.42'18'N LONG 80.04'16'W. Genbank Accession no. AF489618.

_____ and _____. 2002. Aneides aeneus isolate Aaen4 NADH dehydrogenase subunit 4 gene, partial cds; tRNA-His gene, complete sequence; tRNA-Ser gene, complete sequence; tRNA-Leu gene, complete sequence; mitochondrial genes for mitochondrial products. (specimen Rockyrun1#1) LAT 38.42'19'N LONG 80.04'27'W. Genbank Accession no. AF489619.

_____ and _____. 2002. Aneides aeneus isolate Aaen4 NADH dehydrogenase subunit 4 gene, partial cds; tRNA-His gene, complete sequence; tRNA-Ser gene, complete sequence; tRNA-Leu gene, complete sequence; mitochondrial genes for mitochondrial products. (specimen Rockyrun1#2) LAT 38.42'19'N LONG 80.04'27'W. Genbank Accession no. AF489620.

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_____ and _____. 2002. Aneides aeneus isolate Aaen4 NADH dehydrogenase subunit 4 gene, partial cds; tRNA-His gene, complete sequence; tRNA-Ser gene, complete sequence; tRNA-Leu gene, complete sequence; mitochondrial genes for mitochondrial products. (specimen Kittle Creek#1) LAT 38.43'19'N LONG 80.04'27'W. Genbank Accession no. AF489622.

_____ and _____. 2002. Aneides aeneus isolate Aaen4 NADH dehydrogenase subunit 4 gene, partial cds; tRNA-His gene, complete sequence; tRNA-Ser gene, complete sequence; tRNA-Leu gene, complete sequence; mitochondrial genes for mitochondrial products. (specimen Kittle Creek#5) LAT 38.43'19'N LONG80.02'58'W. Genbank Accession no. AF489623.

_____ and _____. 2002. Aneides aeneus isolate Aaen4 NADH dehydrogenase subunit 4 gene, partial cds; tRNA-His gene, complete sequence; tRNA-Ser gene, complete sequence; tRNA-Leu gene, complete sequence; mitochondrial genes for mitochondrial products. (specimen Cubbyhole#1) LAT 38.42'18'N LONG 80.04'16'W. Genbank Accession no. AF489624.

_____ and _____. 2002. Aneides aeneus isolate Aaen4 NADH dehydrogenase subunit 4 gene, partial cds; tRNA-His gene, complete sequence; tRNA-Ser gene, complete sequence; tRNA-Leu gene, complete sequence; mitochondrial genes for mitochondrial products. (specimen Clearcut#3) LAT 38.42'43'N LONG 80.04'44'W. Genbank Accession no. AF489625.

_____ and _____. 2002. Aneides aeneus isolate Aaen4 NADH dehydrogenase subunit 4 gene, partial cds; tRNA-His gene, complete sequence; tRNA-Ser gene, complete sequence; tRNA-Leu gene, complete sequence; mitochondrial genes for mitochondrial products. (specimen Cubbyhole#3) LAT 38.42'18'N LONG 80.04'16'W. Genbank Accession no. AF489626.

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Figure 1. Range of *Aneides aeneus* in the United States (Petranka 1988).

Figure 2. Range of *Aneides aeneus* throughout West Virginia (Green and Pauley 1987).

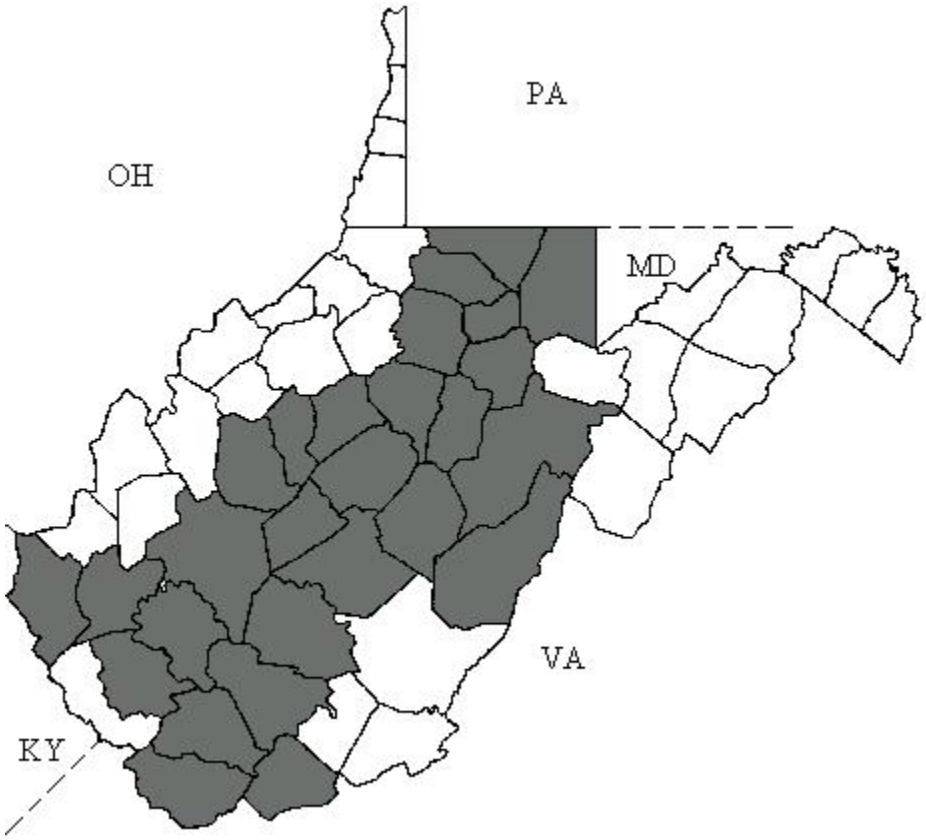


Figure 3. *Aneides aeneus* on an oak leaf. Photo courtesy of Mizuki Takahashi.

Figure 4. *Aneides aeneus* on sandstone within the Westvāco Wildlife and Ecological Research Forest. Photo courtesy of Zach Felix.

Figure 5. Location of the Westvāco Wildlife and Ecological Research Forest in West Virginia.

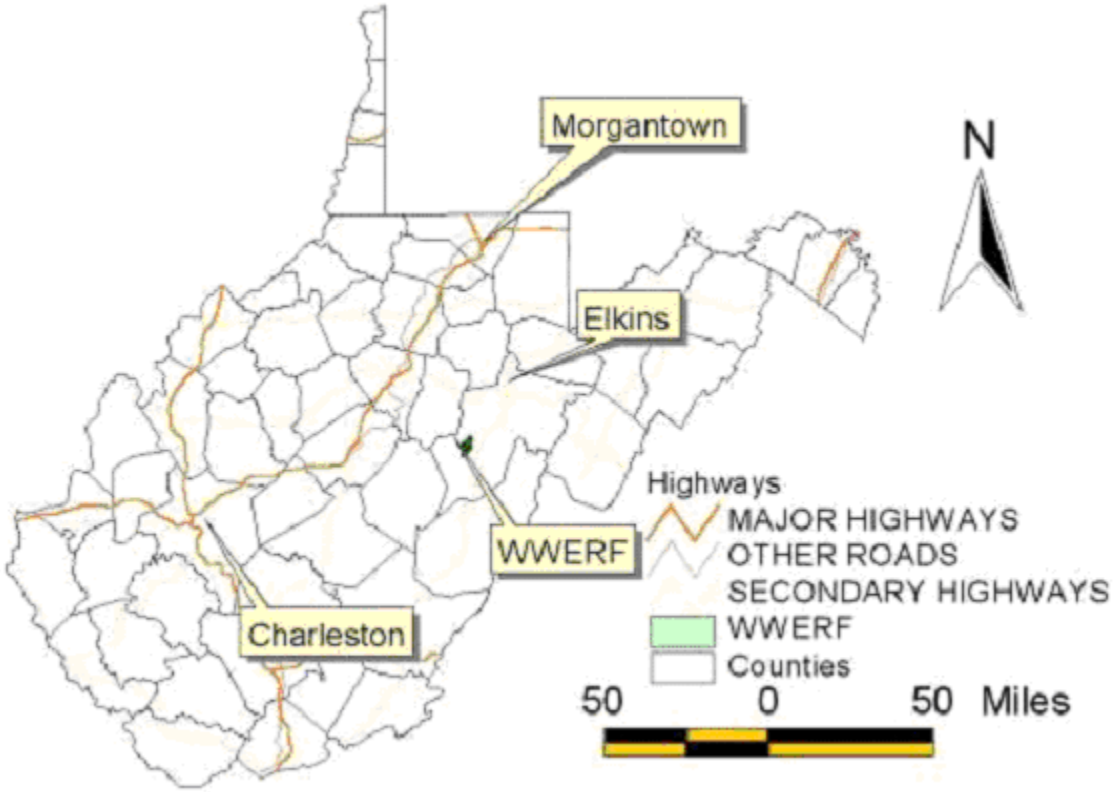


Figure 6. Location of the sample specimen retrieval sites in the Westvāco Wildlife and Ecological Research Forest.

Scale: 1 kilometer = ----------

Contour interval 40 feet

N

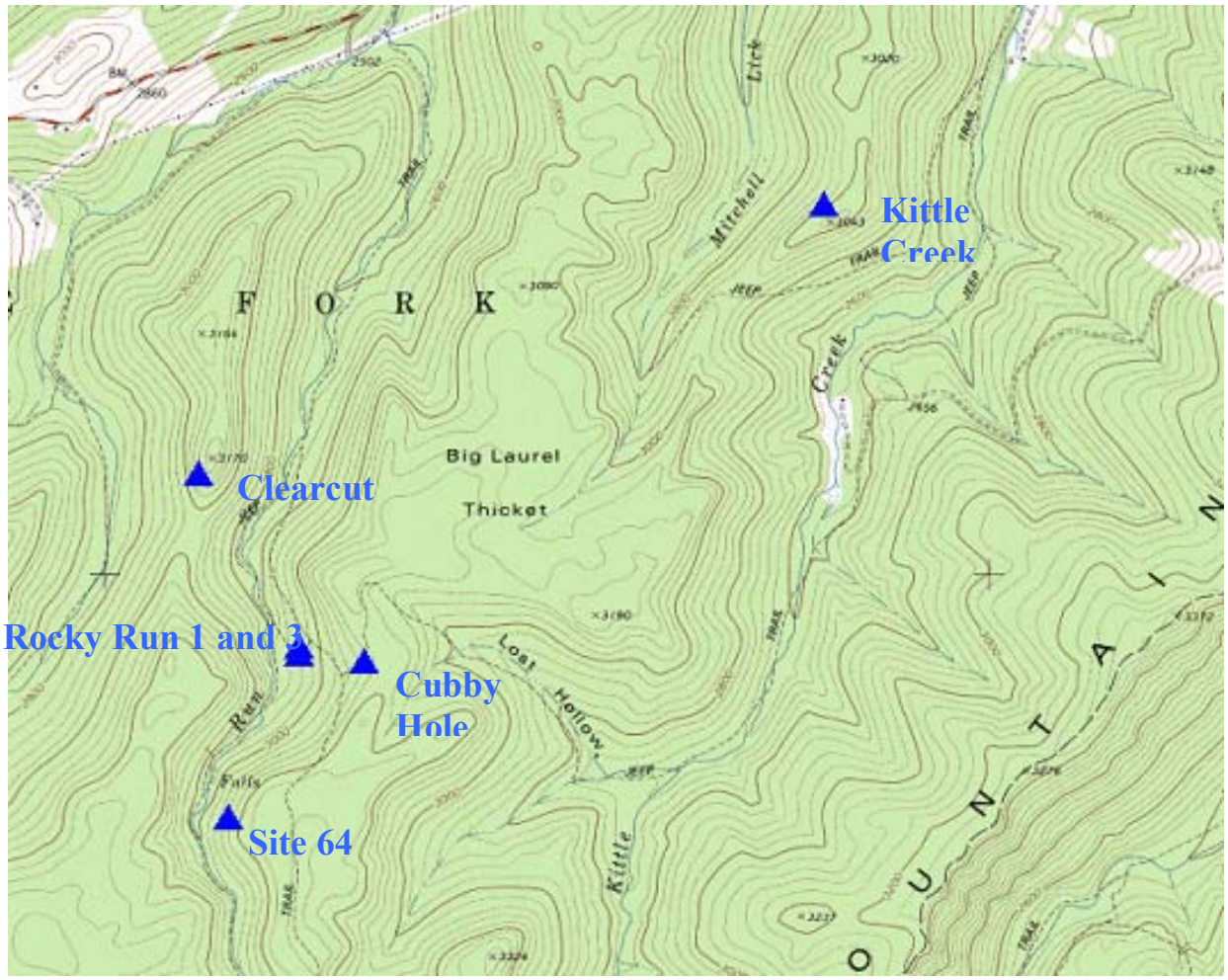


Figure 7. Size classes of *Aneides aeneus*. Photo courtesy of Jeff Humphries.

Figure 8. A distance matrix phylogram based on sequence data. "Mahoney" serves as the root and the out group of the phylogram.

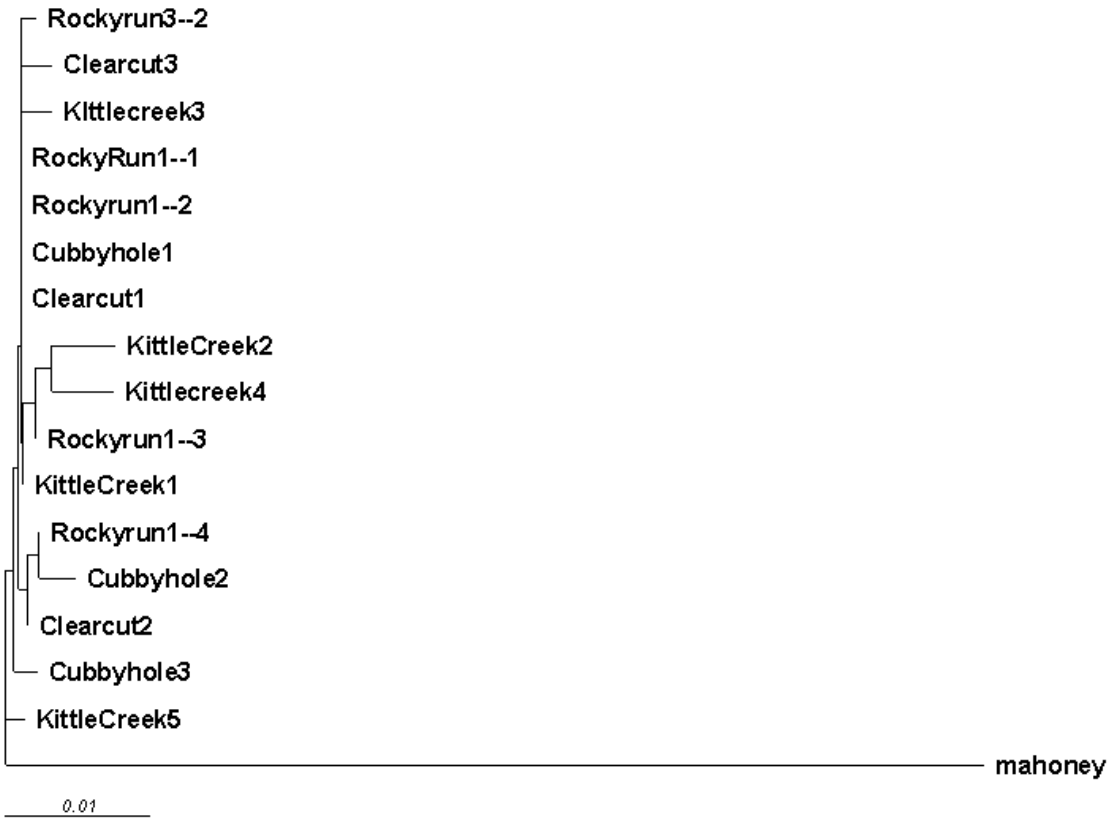


Figure 9. A cladogram based on sequence data shows clearer grouping of sample sites. "Mahoney" serves as the root and the out group of the cladogram.

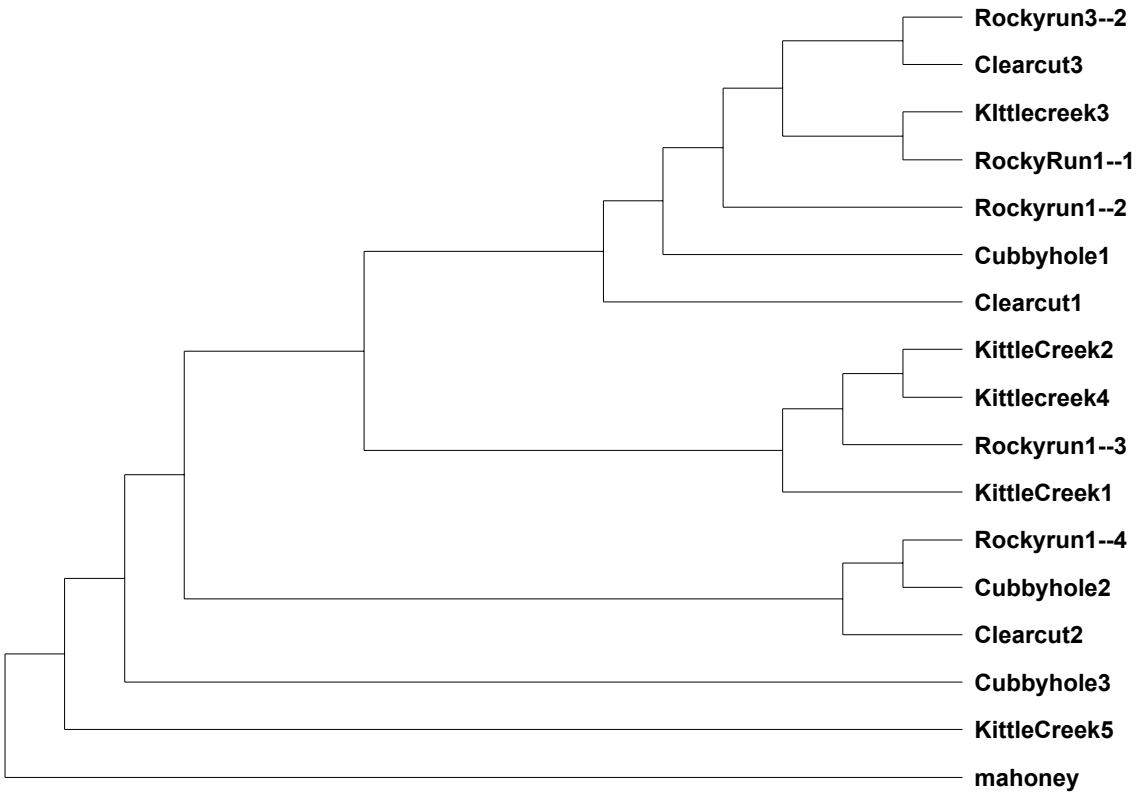


Figure 10. Distance matrix phylogram of 245 amino acids transcribed from the ND4 region.

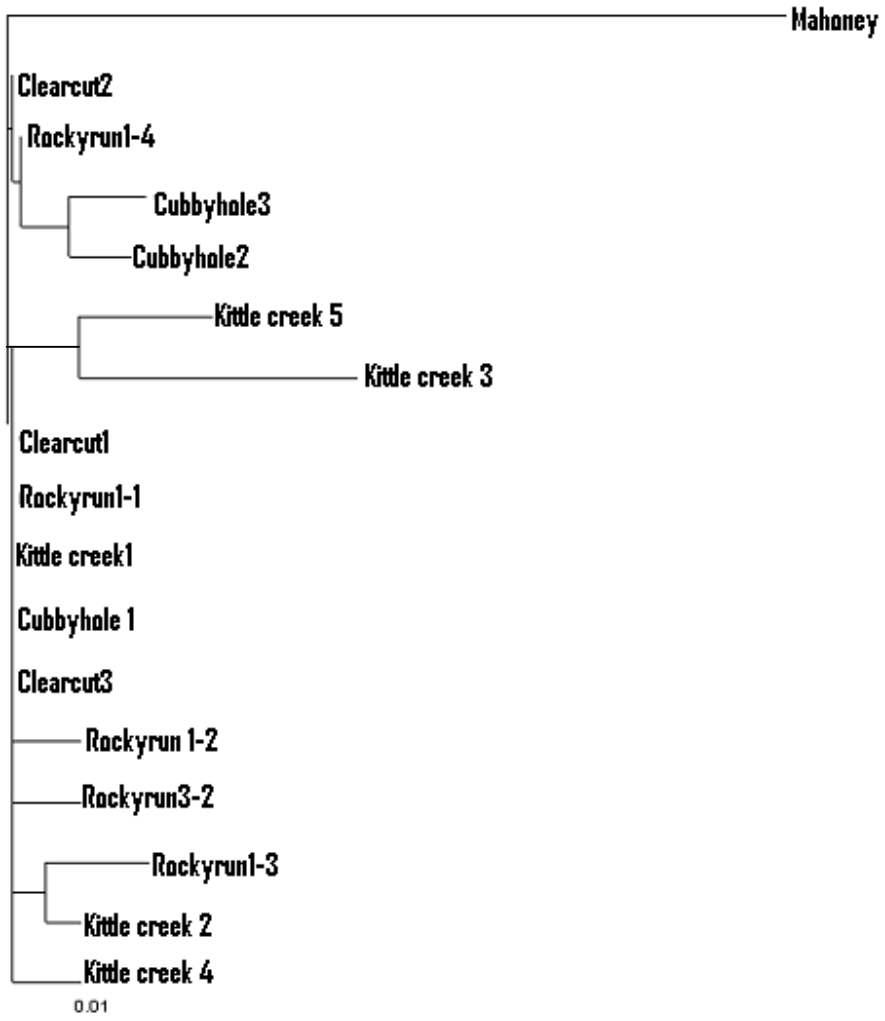


Figure 11. Regression analysis of genetic distance versus geographic distance for specimens within the WWERF.

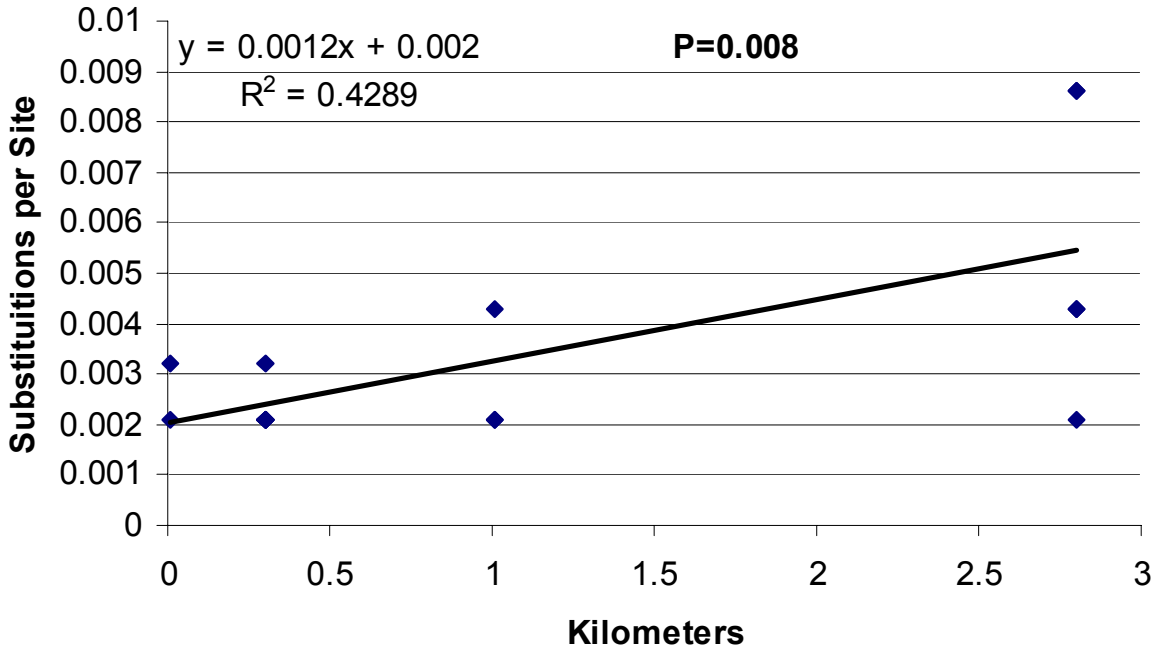


Figure 12. Regression analysis of protein distance versus geographical distance between specimens in the WWERF.

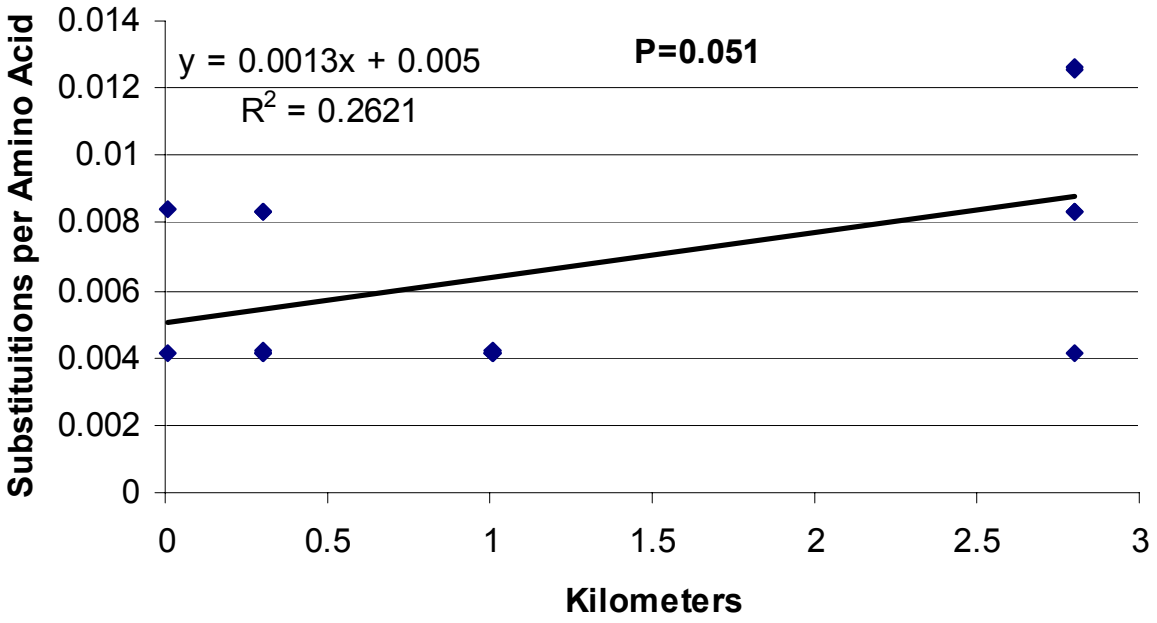


Figure 13. Regression analysis of genetic distance versus geographic distance for specimens within the WWERF, excluding the Kittle Creek site.

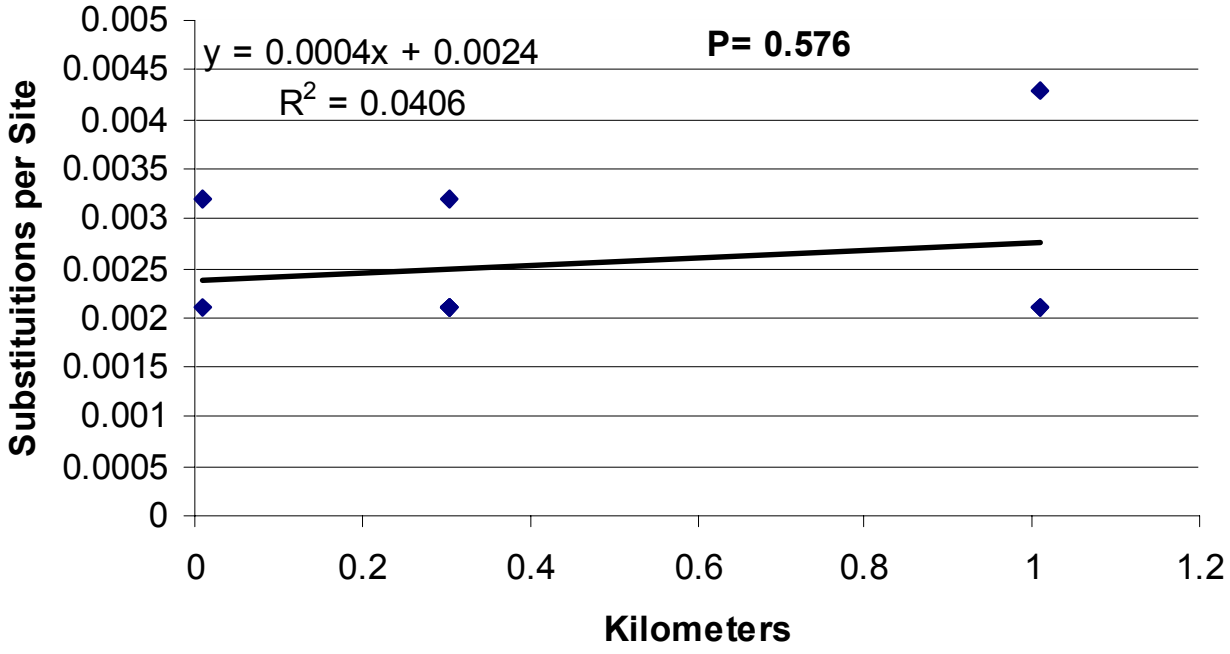


Figure 14. Regression analysis of genetic distance versus geographic distance for all specimens including Mahoney.

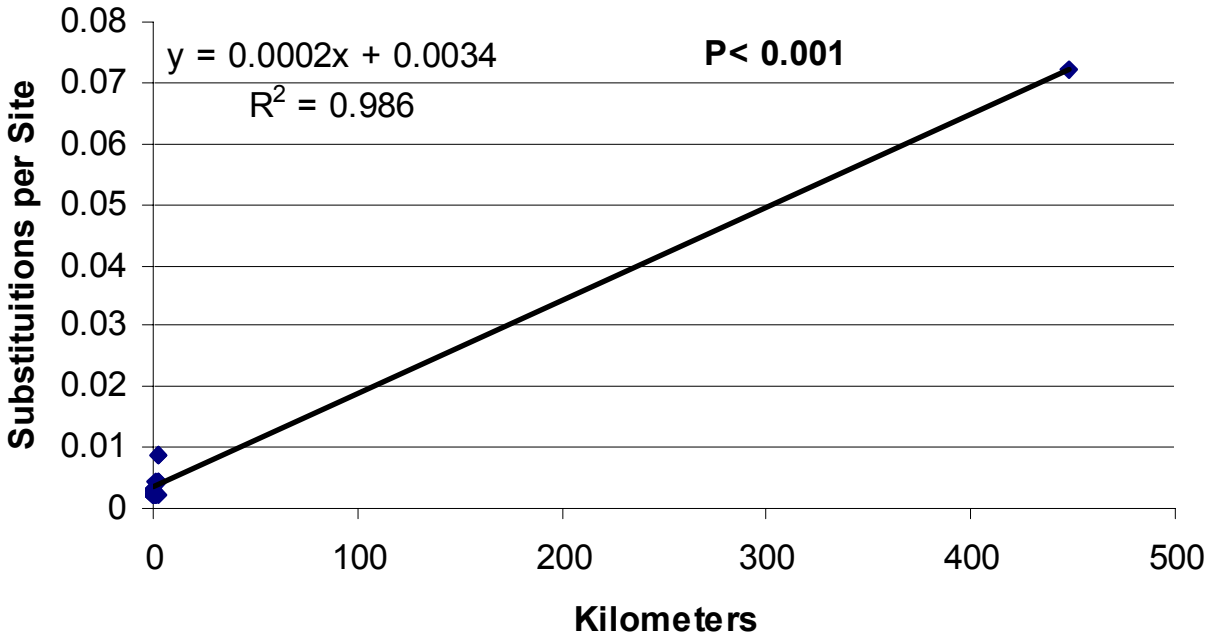


Table 1. Oligonucleotide Primers. Oligonucleotide analysis data calculated using OLIGO 4.0 Primer Analysis Software (NBI).

Name of Primer	Sequence	Oligonucleotide Analysis			
LEU	CATTACTTTTACTTGGATTTGCACCA	Td	= 68.5º (nearest neighbor method)		
		Tm	=69.7º (%GC Method)		
		Tm	=70.0º (2*(A+T) + 4*(GC))		
		Nmol/OD	=4.17 (nearest neighbor extn. coeff.)		
		µg/OD	=33.2		
		Composition	=	A+T 17	(65.4%)
				C+G 9	(34.6%)
ND4	CACCTATGACTACCAAAAGCTCATGTAGAAGC	Td	=74.7º (nearest neighbor method)		
		Tm	=78.3º (%GC Method)		
		Tm	=92.0º (2*(A+T) + 4*(GC))		
		Nmol/OD	=3.17 (nearest neighbor extn. coeff.)		
		µg/OD	=31.2		
		Composition	=	A+T 18	(56.2%)
				C+G 14	(43.8%)

Table 2. Exact locations of sampling sites within the WWERF.

Site	Latitude	Longitude
Rockyrun 1	38.42'19" N	80.04'27" W
64	38.42'43" N	80.04'44" W
Cubby Hole	38.42'18" N	80.04'16" W
Kittle Creek	38.43'19" N	80.02'58" W
Clearcut	38.42'43" N	80.04'44" W
Rockyrun 3	38.42'20" N	80.04'27" W

Note: Mahoney specimen was collected 0.7 miles WNW of Bat Cave in Henderson County, North Carolina. No Lat/Long data was available for that specimen.

Table 3. Genbank Sequence Information.

DNA Sequence	Genbank Accession Number	Authors
Mahoney	AF329322	Meredith Mahoney
Clearcut 2	AF489614	Johnson, A. N. and Somerville, C. C.
Rockyrun 3-2	AF489615	Johnson, A. N. and Somerville, C. C.
Clearcut 1	AF489616	Johnson, A. N. and Somerville, C. C.
Rockyrun 1-4	AF489617	Johnson, A. N. and Somerville, C. C.
Cubbyhole 2	AF489618	Johnson, A. N. and Somerville, C. C.
Rockyrun 1-1	AF489619	Johnson, A. N. and Somerville, C. C.
Rockyrun 1-2	AF489620	Johnson, A. N. and Somerville, C. C.
Kittle Creek 3	AF489621	Johnson, A. N. and Somerville, C. C.
Kittle Creek 1	AF489622	Johnson, A. N. and Somerville, C. C.
Kittle Creek 5	AF489623	Johnson, A. N. and Somerville, C. C.
Cubbyhole 1	AF489624	Johnson, A. N. and Somerville, C. C.
Clearcut 3	AF489625	Johnson, A. N. and Somerville, C. C.
Cubbyhole 3	AF489626	Johnson, A. N. and Somerville, C. C.
Kittle Creek 4	AF489627	Johnson, A. N. and Somerville, C. C.
Rockyrun 1-3	AF489628	Johnson, A. N. and Somerville, C. C.
Kittle Creek 2	AF489629	Johnson, A. N. and Somerville, C. C.

Table 4. Obstacles for salamander migration between outcrops.

Sites	Distance between sites (meters)	Stream Crossings	Road Crossings	Elevation Change (meters)	Clearcut Crossings
64 x Rockyrun	960	0	1	132	0
64 x Cubby Hole	1040	0	0	48	0
64 x Kittle Creek	3600	1	3	348	1
64 x Clearcut	1680	1	2	312	1
Rockyrun x Cubby Hole	304	0	1	72	0
Rockyrun x Kittle Creek	2900	1	4	312	1
Rockyrun x Clearcut	784	1	1	132	1
Cubby Hole x Kittle Creek	2800	1	3	264	1
Cubby Hole x Clearcut	1008	1	2	216	1
Kittle Creek x Clearcut	2900	2	4	576	1

Table 5. Genetic distance between all *Aneides aeneus* specimen collected.

	Rocky 1-4	Cubby ho2	Rocky 1-3	Kittle Creek 2	Kittle Creek 4	Rocky 3-2	Clear cut1	Clear cut3	Cubby ho1	Kittle Creek 1	Kittle Creek 3	Rocky 1-2	Rocky 1-1	Kittle Creek 5	Clear cut2	Cubby ho3	Mahoney
Rockyrun1-4	0	0.0011	0.0011	0.0065	0.0064	0.0011	0	0.0021	0	0	0.0021	0	0	0.0021	0	0.0021	0.0708
Cubbyhole2	0.0011	0	0.0021	0.0075	0.0075	0.0021	0.0011	0.0032	0.0011	0.0011	0.0032	0.0011	0.0011	0.0032	0.0011	0.0032	0.0722
Rockyrun1-3	0.0011	0.0021	0	0.0065	0.0054	0.0021	0.0011	0.0032	0.0011	0.0011	0.0032	0.0011	0.0011	0.0032	0.0011	0.0021	0.0725
Kittle Creek 2	0.0065	0.0075	0.0065	0	0.0086	0.0075	0.0064	0.0086	0.0064	0.0064	0.0086	0.0064	0.0064	0.0086	0.0065	0.0086	0.0781
Kittle Creek 4	0.0064	0.0075	0.0054	0.0086	0	0.0075	0.0064	0.0086	0.0064	0.0054	0.0086	0.0064	0.0064	0.0086	0.0065	0.0086	0.0781
Rockyrun3-2	0.0011	0.0021	0.0021	0.0075	0.0075	0	0.0011	0.0032	0.0011	0.0011	0.0032	0.0011	0.0011	0.0032	0.0011	0.0032	0.0722
Clearcut1	0	0.0011	0.0011	0.0064	0.0064	0.0011	0	0.0021	0	0	0.0021	0	0	0.0021	0	0.0021	0.0708
Clearcut3	0.0021	0.0032	0.0032	0.0086	0.0086	0.0032	0.0021	0	0.0021	0.0021	0.0043	0.0021	0.0021	0.0043	0.0021	0.0043	0.0736
Cubbyhole1	0	0.0011	0.0011	0.0064	0.0064	0.0011	0	0.0021	0	0	0.0021	0	0	0.0021	0	0.0021	0.0708
Kittle Creek 1	0	0.0011	0.0011	0.0064	0.0054	0.0011	0	0.0021	0	0	0.0021	0	0	0.0021	0	0.0021	0.0708
Kittle Creek 3	0.0021	0.0032	0.0032	0.0086	0.0086	0.0032	0.0021	0.0043	0.0021	0.0021	0	0.0021	0.0021	0.0043	0.0021	0.0043	0.0737
Rockyrun1-2	0	0.0011	0.0011	0.0064	0.0064	0.0011	0	0.0021	0	0	0.0021	0	0	0.0021	0	0.0021	0.0708
Rockyrun1-1	0	0.0011	0.0011	0.0064	0.0064	0.0011	0	0.0021	0	0	0.0021	0	0	0.0021	0	0.0021	0.0708
Kittle Creek 5	0.0021	0.0032	0.0032	0.0086	0.0086	0.0032	0.0021	0.0043	0.0021	0.0021	0.0043	0.0021	0.0021	0	0.0021	0.0043	0.0722
Clearcut2	0	0.0011	0.0011	0.0065	0.0065	0.0011	0	0.0021	0	0	0.0021	0	0	0.0021	0	0.0021	0.0708
Cubbyhole3	0.0021	0.0032	0.0021	0.0086	0.0086	0.0032	0.0021	0.0043	0.0021	0.0021	0.0043	0.0021	0.0021	0.0043	0.0021	0	0.0722
Mahoney	0.0708	0.0722	0.0725	0.0781	0.0781	0.0722	0.0708	0.0736	0.0708	0.0708	0.0737	0.0708	0.0708	0.0722	0.0708	0.0722	0

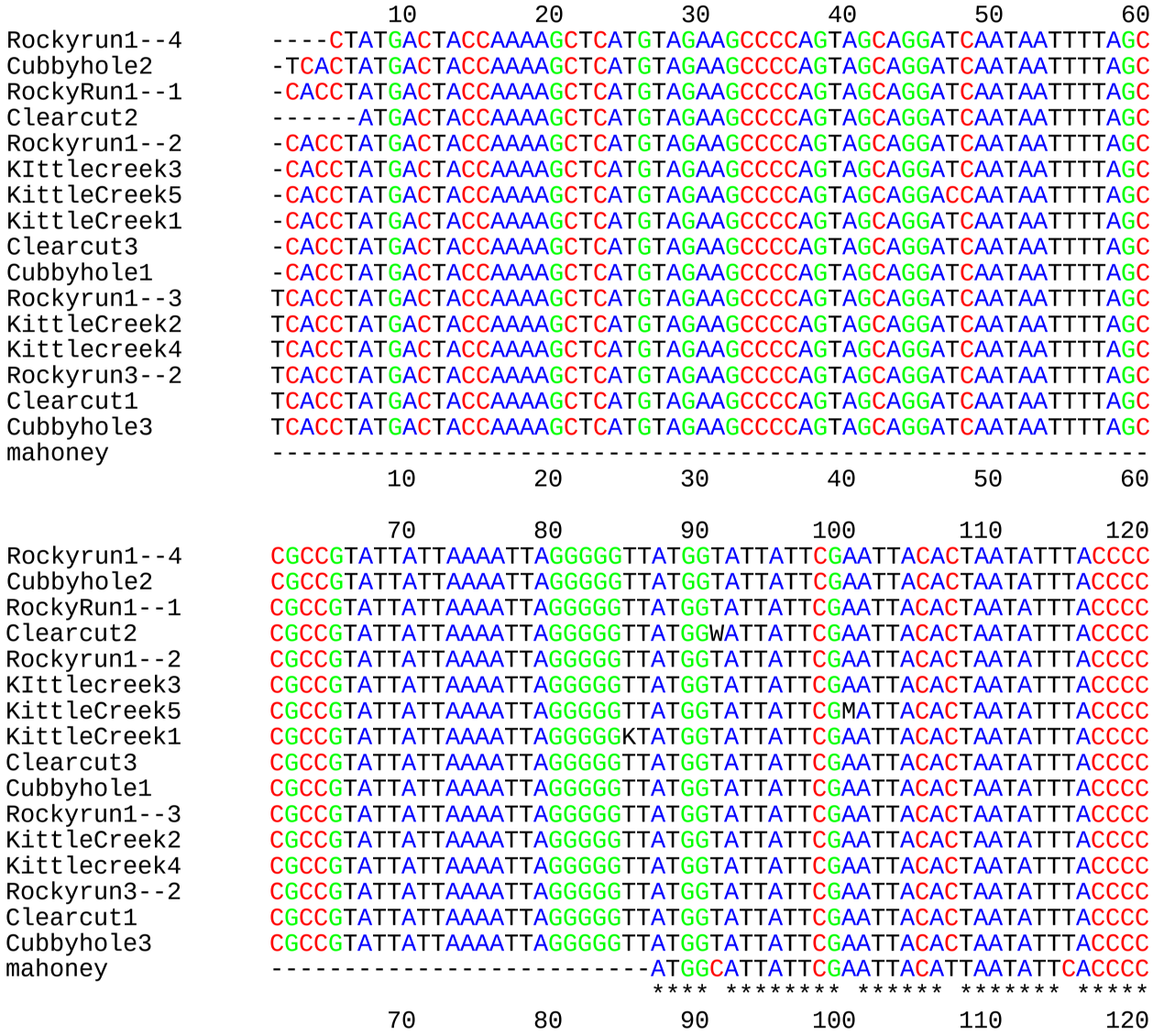
Table 6. Protein sequence distance between all *Aneides aeneus* specimen collected.

	Cubbyho3	Cubbyho2	Rocky1_4	Clearcut2	Rocky1_3	KC2	Rocky1_2	Rocky3_2	KC4	Clearcut1	Rocky1_1	KC1	Cubbyho1	Clearcut3	KC5	KC3	Mahoney
Cubbyho3	0	0.0084	0.00417	0.00418	0.00834	0.00835	0.00416	0.00832	0.00832	0.00415	0.00415	0.0042	0.00415	0.00415	0.01255	0.0127	0.0525
Cubbyho2	0.0084	0	0	0	0.00843	0.00843	0.0042	0.0084	0.0084	0.00419	0.00419	0.0042	0.00419	0.00419	0.0127	0.0128	0.0476
Rocky1_4	0.0042	0	0	0	0.00415	0.00416	0	0.00414	0.00414	0	0	0	0	0	0.00835	0.0084	0.0473
Clercut2	0.0042	0	0	0	0.00416	0.00417	0	0.00415	0.00415	0	0	0	0	0	0.00837	0.0084	0.0473
Rocky1_3	0.0083	0.0084	0.00415	0.00416	0	0.00414	0.00415	0.00829	0.00829	0.00414	0.00414	0.0041	0.00414	0.00414	0.01254	0.0126	0.0523
KC2	0.0084	0.0084	0.00416	0.00417	0.00414	0	0.00415	0.0083	0.0083	0.00414	0.00414	0.0041	0.00414	0.00414	0.01255	0.0126	0.0523
Rocky1_2	0.0042	0.0042	0	0	0.00415	0.00415	0	0.00414	0.00414	0	0	0	0	0	0.00834	0.0084	0.0474
Rocky3_2	0.0083	0.0084	0.00414	0.00415	0.00829	0.0083	0.00414	0	0.00827	0.00413	0.00413	0.0041	0.00413	0.00413	0.0125	0.0126	0.0521
KC4	0.0083	0.0084	0.00414	0.00415	0.00829	0.0083	0.00414	0.00827	0	0.00413	0.00413	0.0041	0.00413	0.00413	0.0125	0.0126	0.0521
Clercut1	0.0042	0.0042	0	0	0.00414	0.00414	0	0.00413	0.00413	0	0	0	0	0	0.00832	0.0084	0.0473
Rocky1_1	0.0042	0.0042	0	0	0.00414	0.00414	0	0.00413	0.00413	0	0	0	0	0	0.00832	0.0084	0.0473
KC1	0.0042	0.0042	0	0	0.00414	0.00414	0	0.00413	0.00413	0	0	0	0	0	0.00832	0.0084	0.0473
Cubbyho1	0.0042	0.0042	0	0	0.00414	0.00414	0	0.00413	0.00413	0	0	0	0	0	0.00832	0.0084	0.0473
Clercut3	0.0042	0.0042	0	0	0.00414	0.00414	0	0.00413	0.00413	0	0	0	0	0	0.00832	0.0084	0.0473
KC5	0.0126	0.0127	0.00835	0.00837	0.01254	0.01255	0.00834	0.0125	0.0125	0.00832	0.00832	0.0083	0.00832	0.00832	0	0.0169	0.0524
KC3	0.0127	0.0128	0.0084	0.00842	0.01262	0.01263	0.00839	0.01258	0.01258	0.00837	0.00837	0.0084	0.00837	0.00837	0.01686	0	0.0578
Mahoney	0.0525	0.0476	0.04727	0.00473	0.0523	0.05234	0.0474	0.05211	0.05211	0.04727	0.04727	0.0473	0.04727	0.04727	0.05236	0.0578	0

Appendix A: Composition for all reagents which were not ordered commercially.

Reagent	Composition
Chelex	5% chelex in water
Proteinase K	20 mg/ml in water
1× Tris-acetate (TAE) Buffer	0.04 M Tris-acetate 0.001 M EDTA
Agarose gel	50 ml of 1× TAE buffer 0.75g Agarose 2 µl Ethidium Bromide (1%)
Cracking Buffer	50 µl 10N stock of NaOH 100 µl 0.5M EDTA 200 µl of 25% SDS 250 µl 1%Bromocresol green 9.4 ml of distilled water
Stop Buffer	100% Glycerol 0.7 ml 0.5 MEDTA (pH 8.0) 0.3 ml Bromphenol Blue for color
Tryptic Soy Broth	30 g trypticase soy broth 1 liter distilled water 50 µg/ml Ampicillin Autoclave @125C for 20 min
Tris-EDTA (TE) Buffer	10mM Tris·CL (pH 7.4) 1mM EDTA (pH 8.0)
2× YT	16 g Bacto-tryptone 10 g Yeast Extract 5 g NaCl adjust pH to 7.6 Autoclave @125C for 20 min

APPENDIX B: Sequence alignment of mitochondrial ND4 Nadh Dehydrogenase, tRNA-His, tRNA-Ser, and tRNA-Leu regions of Aneides aeneus located within the Westvāco Wildlife and Ecological Research Forest. Conserved base pairs throughout all individuals are indicated by *. "Mahoney" sequence was taken from Genbank (accession number AF329322).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Cubbyhole2 -TCACTATGACTACCAAAAGCTCATGTAGAAGCCCCAGTAGCAGGATCAATAATTTTAGC
RockyRun1--1 -CACCTATGACTACCAAAAGCTCATGTAGAAGCCCCAGTAGCAGGATCAATAATTTTAGC
Clearcut2 ------ATGACTACCAAAAGCTCATGTAGAAGCCCCAGTAGCAGGATCAATAATTTTAGC
Rockyrun1--2 -CACCTATGACTACCAAAAGCTCATGTAGAAGCCCCAGTAGCAGGATCAATAATTTTAGC
KIttlecreek3 -CACCTATGACTACCAAAAGCTCATGTAGAAGCCCCAGTAGCAGGATCAATAATTTTAGC
KittleCreek5 -CACCTATGACTACCAAAAGCTCATGTAGAAGCCCCAGTAGCAGGACCAATAATTTTAGC
KittleCreek1 -CACCTATGACTACCAAAAGCTCATGTAGAAGCCCCAGTAGCAGGATCAATAATTTTAGC
Clearcut3 -CACCTATGACTACCAAAAGCTCATGTAGAAGCCCCAGTAGCAGGATCAATAATTTTAGC
Cubbyhole1 -CACCTATGACTACCAAAAGCTCATGTAGAAGCCCCAGTAGCAGGATCAATAATTTTAGC
Rockyrun1--3 TCACCTATGACTACCAAAAGCTCATGTAGAAGCCCCAGTAGCAGGATCAATAATTTTAGC
KittleCreek2 TCACCTATGACTACCAAAAGCTCATGTAGAAGCCCCAGTAGCAGGATCAATAATTTTAGC
Kittlecreek4 TCACCTATGACTACCAAAAGCTCATGTAGAAGCCCCAGTAGCAGGATCAATAATTTTAGC
Rockyrun3--2 TCACCTATGACTACCAAAAGCTCATGTAGAAGCCCCAGTAGCAGGATCAATAATTTTAGC
Clearcut1 TCACCTATGACTACCAAAAGCTCATGTAGAAGCCCCAGTAGCAGGATCAATAATTTTAGC
Cubbyhole3 TCACCTATGACTACCAAAAGCTCATGTAGAAGCCCCAGTAGCAGGATCAATAATTTTAGC
mahoney ----------
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70 80 90 100 110 120
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Cubbyhole2 CGCCGTATTATTAAAATTAGGGGGTTATGGTATTATTCGAATTACACTAATATTTACCCC
RockyRun1--1 CGCCGTATTATTAAAATTAGGGGGTTATGGTATTATTCGAATTACACTAATATTTACCCC
Clearcut2 CGCCGTATTATTAAAATTAGGGGGTTATGGWATTATTCGAATTACACTAATATTTACCCC
Rockyrun1--2 CGCCGTATTATTAAAATTAGGGGGTTATGGTATTATTCGAATTACACTAATATTTACCCC
KIttlecreek3 CGCCGTATTATTAAAATTAGGGGGTTATGGTATTATTCGAATTACACTAATATTTACCCC
KittleCreek5 CGCCGTATTATTAAAATTAGGGGGTTATGGTATTATTCGMATTACACTAATATTTACCCC
KittleCreek1 CGCCGTATTATTAAAATTAGGGGGKTATGGTATTATTCGAATTACACTAATATTTACCCC
Clearcut3 CGCCGTATTATTAAAATTAGGGGGTTATGGTATTATTCGAATTACACTAATATTTACCCC
Cubbyhole1 CGCCGTATTATTAAAATTAGGGGGTTATGGTATTATTCGAATTACACTAATATTTACCCC
Rockyrun1--3 CGCCGTATTATTAAAATTAGGGGGTTATGGTATTATTCGAATTACACTAATATTTACCCC
KittleCreek2 CGCCGTATTATTAAAATTAGGGGGTTATGGTATTATTCGAATTACACTAATATTTACCCC
Kittlecreek4 CGCCGTATTATTAAAATTAGGGGGTTATGGTATTATTCGAATTACACTAATATTTACCCC
Rockyrun3--2 CGCCGTATTATTAAAATTAGGGGGTTATGGTATTATTCGAATTACACTAATATTTACCCC
Clearcut1 CGCCGTATTATTAAAATTAGGGGGTTATGGTATTATTCGAATTACACTAATATTTACCCC
Cubbyhole3 CGCCGTATTATTAAAATTAGGGGGTTATGGTATTATTCGAATTACACTAATATTTACCCC
mahoney ----------ATGGCATTATTCGAATTACATTAATATTCACCCC
**** ******** ****** ******* *****
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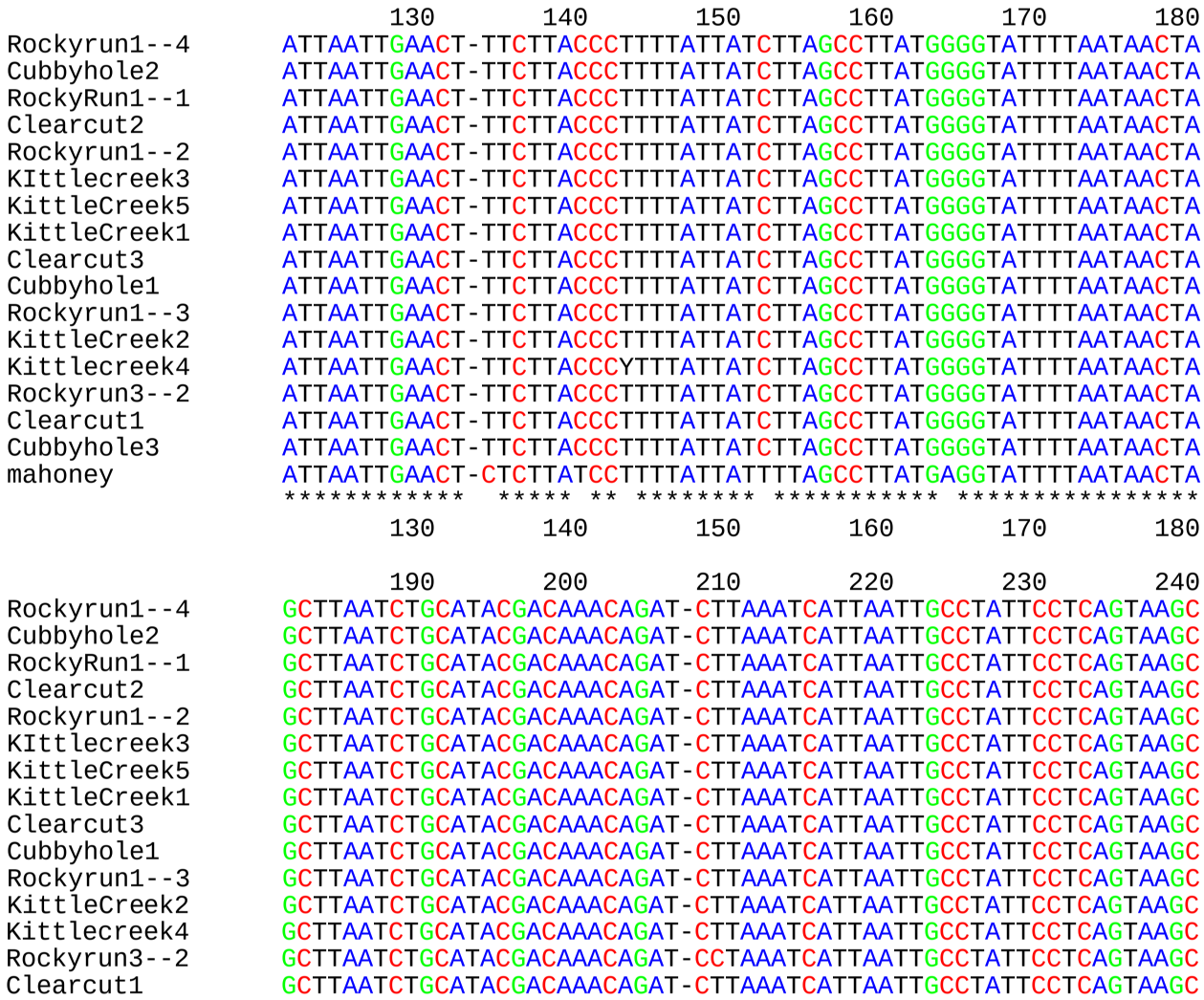
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Cubbyhole2 ATTAATTGAACT-TTCTTACCCTTTTATTATCTTAGCCTTATGGGGTATTTTAATAACTA
RockyRun1--1 ATTAATTGAACT-TTCTTACCCTTTTATTATCTTAGCCTTATGGGGTATTTTAATAACTA
Clearcut2 ATTAATTGAACT-TTCTTACCCTTTTATTATCTTAGCCTTATGGGGTATTTTAATAACTA
Rockyrun1--2 ATTAATTGAACT-TTCTTACCCTTTTATTATCTTAGCCTTATGGGGTATTTTAATAACTA
KIttlecreek3 ATTAATTGAACT-TTCTTACCCTTTTATTATCTTAGCCTTATGGGGTATTTTAATAACTA
KittleCreek5 ATTAATTGAACT-TTCTTACCCTTTTATTATCTTAGCCTTATGGGGTATTTTAATAACTA
KittleCreek1 ATTAATTGAACT-TTCTTACCCTTTTATTATCTTAGCCTTATGGGGTATTTTAATAACTA
Clearcut3 ATTAATTGAACT-TTCTTACCCTTTTATTATCTTAGCCTTATGGGGTATTTTAATAACTA
Cubbyhole1 ATTAATTGAACT-TTCTTACCCTTTTATTATCTTAGCCTTATGGGGTATTTTAATAACTA
Rockyrun1--3 ATTAATTGAACT-TTCTTACCCTTTTATTATCTTAGCCTTATGGGGTATTTTAATAACTA
KittleCreek2 ATTAATTGAACT-TTCTTACCCTTTTATTATCTTAGCCTTATGGGGTATTTTAATAACTA
Kittlecreek4 ATTAATTGAACT-TTCTTACCCYTTTATTATCTTAGCCTTATGGGGTATTTTAATAACTA
Rockyrun3--2 ATTAATTGAACT-TTCTTACCCTTTTATTATCTTAGCCTTATGGGGTATTTTAATAACTA
Clearcut1 ATTAATTGAACT-TTCTTACCCTTTTATTATCTTAGCCTTATGGGGTATTTTAATAACTA
Cubbyhole3 ATTAATTGAACT-TTCTTACCCTTTTATTATCTTAGCCTTATGGGGTATTTTAATAACTA
mahoney ATTAATTGAACT-CTCTTATCCTTTTATTATTTTAGCCTTATGAGGTATTTTAATAACTA
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190 200 210 220 230 240
Rockyrun1--4 GCTTAATCTGCATACGACAAACAGAT-CTTAAATCATTAATTGCCTATTCCTCAGTAAGC
Cubbyhole2 GCTTAATCTGCATACGACAAACAGAT-CTTAAATCATTAATTGCCTATTCCTCAGTAAGC
RockyRun1--1 GCTTAATCTGCATACGACAAACAGAT-CTTAAATCATTAATTGCCTATTCCTCAGTAAGC
Clearcut2 GCTTAATCTGCATACGACAAACAGAT-CTTAAATCATTAATTGCCTATTCCTCAGTAAGC
Rockyrun1--2 GCTTAATCTGCATACGACAAACAGAT-CTTAAATCATTAATTGCCTATTCCTCAGTAAGC
KIttlecreek3 GCTTAATCTGCATACGACAAACAGAT-CTTAAATCATTAATTGCCTATTCCTCAGTAAGC
KittleCreek5 GCTTAATCTGCATACGACAAACAGAT-CTTAAATCATTAATTGCCTATTCCTCAGTAAGC
KittleCreek1 GCTTAATCTGCATACGACAAACAGAT-CTTAAATCATTAATTGCCTATTCCTCAGTAAGC
Clearcut3 GCTTAATCTGCATACGACAAACAGAT-CTTAAATCATTAATTGCCTATTCCTCAGTAAGC
Cubbyhole1 GCTTAATCTGCATACGACAAACAGAT-CTTAAATCATTAATTGCCTATTCCTCAGTAAGC
Rockyrun1--3 GCTTAATCTGCATACGACAAACAGAT-CTTAAATCATTAATTGCCTATTCCTCAGTAAGC
KittleCreek2 GCTTAATCTGCATACGACAAACAGAT-CTTAAATCATTAATTGCCTATTCCTCAGTAAGC
Kittlecreek4 GCTTAATCTGCATACGACAAACAGAT-CTTAAATCATTAATTGCCTATTCCTCAGTAAGC
Rockyrun3--2 GCTTAATCTGCATACGACAAACAGAT-CCTAAATCATTAATTGCCTATTCCTCAGTAAGC
Clearcut1 GCTTAATCTGCATACGACAAACAGAT-CTTAAATCATTAATTGCCTATTCCTCAGTAAGC

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Cubbyhole3      GCTTAATCTGCATACGACAAACAGAT-CTTAAATCATTAATTGCCTATTCCTCAGTAAGC
mahoney         GTTTAATCTGTATACGACAAACAGAT-CTTAAATCATTAATTGCCTATTCCTCAGTAAGC
                * ******** *************** * ******************************
                       190       200       210       220       230       240

                       250       260       270       280       290       300
Rockyrun1--4    CATATAGGACTTGTAATTGCCGCTACTCTTATCCAAACCCCATGAAGTCTTACAGGAGCA
Cubbyhole2      CATATAGGACTTGTAATTGCCGCTACTCTTATCCAAACCCCATGAAGTCTTACAGGAGCA
RockyRun1--1    CATATAGGACTTGTAATTGCCGCTACTCTTATCCAAACCCCATGAAGTCTTACAGGAGCA
Clearcut2       CATATAGGACTTGTAATTGCCGCTACTCTTATCCAAACCCCATGAAGTCTTACAGGAGCA
Rockyrun1--2    CATATAGGACTTGTAATTGCCGCTACTCTTATCCAAACCCCATGAAGTCTTACAGGAGCA
KIttlecreek3    CATATAGGACTTGTAATTRCCGCTACTTTTATCCAAACCCCATGAAGTCTTACAGGAGCA
KittleCreek5    CATATAGGACTTGTAATTGCCGCTACTCTTATCCAAACCCCATGAAGTCTTACAGGAGCA
KittleCreek1    CATATAGGACTTGTAATTGCCGCTACTCTTATCCAAACCCCATGAAGTCTTACAGGAGCA
Clearcut3       CATATAGGACTTGTAATTGCCGCTACTCTTATCCAAACCCCATGAAGTCTTACAGGAGCA
Cubbyhole1      CATATAGGACTTGTAATTGCCGCTACTCTTATCCAAACCCCATGAAGTCTTACAGGAGCA
Rockyrun1--3    CATATAGGACTTGTAATTGCCGCTACTCTTATCCAAACCCCATGAAGTCTTACAGGAGCA
KittleCreek2    CATATAGGRCTTGTAATTGCCGCTACTCTTATCCAAACCCCATGAAGTCTTACAGGAGCA
Kittlecreek4    CATATAGGACTTGTAATTGCCGCTACTCTTATCCAAACCCCATGAAGTCTTACAGGAGCA
Rockyrun3--2    CATATAGGACTTGTAATTGCCGCTACTCTTATCCAAACCCCATGAAGTCTTACAGGAGCA
Clearcut1       CATATAGGACTTGTAATTGCCGCTACTCTTATCCAAACCCCATGAAGTCTTACAGGAGCA
Cubbyhole3      CATATAGGACTTGTAATTGCCGCTACTCTTGTCCAAACCCCATGAAGTCTTACAGGAGCA
mahoney         CATATAGGACTTGTAATTGCCGCCACCCTCATCCAAACCCCATGAAGTATTACAGGAGCA
                ******** ********* **** **   *  ***************** ***********
                       250       260       270       280       290       300

                       310       320       330       340       350       360
Rockyrun1--4    ATTATTTTAA-TAGTTTCACACGGCTTAATTTCCTCAACCCTTTTTTGTTTAGCAAATAT
Cubbyhole2      ATTATTTTAA-TAGTTTCACACGGCTTAATTTCCTCAACCCTTTTTTGTTTAGCAAATAT
RockyRun1--1    ATTATTTTAA-TAGTTTCACACGGCTTAATTTCCTCAACCCTTTTTTGTTTAGCAAATAT
Clearcut2       ATTATTTTAA-TAGTTTCACACGGCTTAATTTCCTCAACCCTTTTTTGTTTAGCAAATAT
Rockyrun1--2    ATTATTTTAA-TAGTTTCACACGGCTTAATTTCCTCAACCCTTTTTTGTTTAGCAAATAT
KIttlecreek3    ATTATTTWAA-WAGTTTCACACGGCTTAATTTCCTCAACCCTTTTTTGTTTAGCAAATAT
KittleCreek5    ATTATTTTMA-TAGTTTCACACGGCTTAATTTCCTCAACCCTTTTTTGTTTAGCAAATAT
KittleCreek1    ATTATTTTAA-TAGTTTCACACGGCTTAATTTCCTCAACCCTTTTTTGTTTAGCAAATAT
Clearcut3       ATTATTTTAA-TAGTTTCACACGGCTTAATTTCCTCAACCCTTTTTTGTTTAGCAAATAT
Cubbyhole1      ATTATTTTAA-TAGTTTCACACGGCTTAATTTCCTCAACCCTTTTTTGTTTAGCAAATAT
Rockyrun1--3    ATTATTTTAA-TAGTTTCACACGGCTTAATTTCCTCAACCCTTTTTTGTTTAGCAAATAT
KittleCreek2    ATTATTTTAA-TAGTTTCACACGGCTTAATTTCCTCAACCCTTTTTTGTTTAGCAAATAT
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Kittlecreek4      ATTATTTTAA-TAGTTTCACACGGCTTAATTTCCTCAACCCCTTTTTGTTTAGCAAATAT
Rockyrun3--2      ATTATTTTAA-TAGTTTCACACGGCTTAATTTCCTCAACCCTTTTTTGTTTAGCAAATAT
Clearcut1         ATTATTTTAA-TAGTTTCACACGGCTTAATTTCCTCAACCCTTTTTTGTTTAGCAAATAT
Cubbyhole3        ATTATTTTWAATAGTTTCACACGGCTTAATTTCCTCAACCCTTTTTTGTTTAGCAAATAT
mahoney           ATTATTTTGA-TAGTTTCACACGGCTTAATTTCTTCAACCCTTTTCTGTTTAGCAAATAT
                  ******* *  ********************** ******* *** **************
                         310       320       330       340       350       360

                         370       380       390       400       410       420
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Cubbyhole2        AAATTATGAACGAACACACAGCCGAACTATATTATTAGCCCGAGGACTTCAATCCATACT
RockyRun1--1      AAATTATGAACGAACACACAGCCGAACTATATTATTAGCCCGAGGACTTCAATCCATACT
Clearcut2         AAATTATGAACGAACACACAGCCGAACTATATTATTAGCCCGAGGACTTCAATCCATACT
Rockyrun1--2      AAATTATGAACGAACACACAGCCGAACTATATTATTAGCCCGAGGACTTCAATCCATACT
KIttlecreek3      AAATTATGAACGAACACACAGCCGAACTATATTATTAGCCCGAGGACTTCAATCCATACT
KittleCreek5      AAATTATGAACGAACACACAGCCGAACTATATTATTAGCCCGAGGACTTCAATCCATACT
KittleCreek1      AAATTATGAACGAACACACAGCCGAACTATATTATTAGCCCGAGGRCTTCAATCCATACT
Clearcut3         AAATTATGAACGAACACACAGCCGAACTATATTATTAGCCCGAGGACTTCAATCCATACT
Cubbyhole1        AAATTATGAACGAACACACAGCCGAACTATATTATTAGCCCGAGGACTTCAATCCATACT
Rockyrun1--3      AAATTATGWWCGNACACACAGCCGVACTATATTATTAGCCCGAGGRCTTCAATCCATACT
KittleCreek2      AAATTATGGACGGACACACAGCCGRACTATATTATTAGCCCGGGGRCTTCAATCCATACT
Kittlecreek4      AAATTATGAACGAACACACAGCCGGACTATATTATTAGCCCGAGGGCTTCAATCCATACT
Rockyrun3--2      AAATTATGAACGAACACACAGCCGAACTATATTATTAGCCCGAGGACTTCAATCCATACT
Clearcut1         AAATTATGAACGAACACACAGCCGAACTATATTATTAGCCCGAGGACTTCAATCCATACT
Cubbyhole3        AAATTATGAACGAACACACAGCCGAACTATATTATTAGCCCGAGGACTTCAATCCATACT
mahoney           AAATTATGAACGAACACACAGCCGAACTATACTATTAGCCCGAGGACTTCAATCTATACT
                  ******** ** *********** ****** ********** ** ******** *****
                         370       380       390       400       410       420

                         430       440       450       460       470       480
Rockyrun1--4      ACCATTAATAGCTGCTTGATGATTAATCACTAATTTATTTAATATAGCTTTACCACCAAC
Cubbyhole2        ACCATTAATAGCTGCTTGATGATTAATCACTAATTTATTCAATATAGCTTTACCACCAAC
RockyRun1--1      ACCATTAATAGCTGCTTGATGATTAATCACTAATTTATTTAATATAGCTTTACCACCAAC
Clearcut2         ACCATTAATAGCTGCTTGATGATTAATCACTAATTTATTTAATATAGCTTTACCACCAAC
Rockyrun1--2      ACCATTAATAGCTGCTTGATGATTAATCACTAATTTATTTAATATAGCTTTACCACCAAC
KIttlecreek3      ACCATTAATAGCTGCTTGATGATTAATCACTAATTTATTTAATATAGCTTTACCACCAAC
KittleCreek5      ACCATTAATAGCTGCTTGATGATTAATCACTAATTTATTTAATATAGCTTTACCACCAAC
KittleCreek1      ACCATTAATAGCTGCTTGATGATTAATCACTAATTTATTTAATATAGCTTTACCACCAAC
Clearcut3         ACCATTAATAGCTGCTTGATGATTAATCACTAATTTATTTAATATAGCTTTACCACCAAC
Cubbyhole1        ACCATTAATAGCTGCTTGATGATTAATCACTAATTTATTTAATATAGCTTTACCACCAAC
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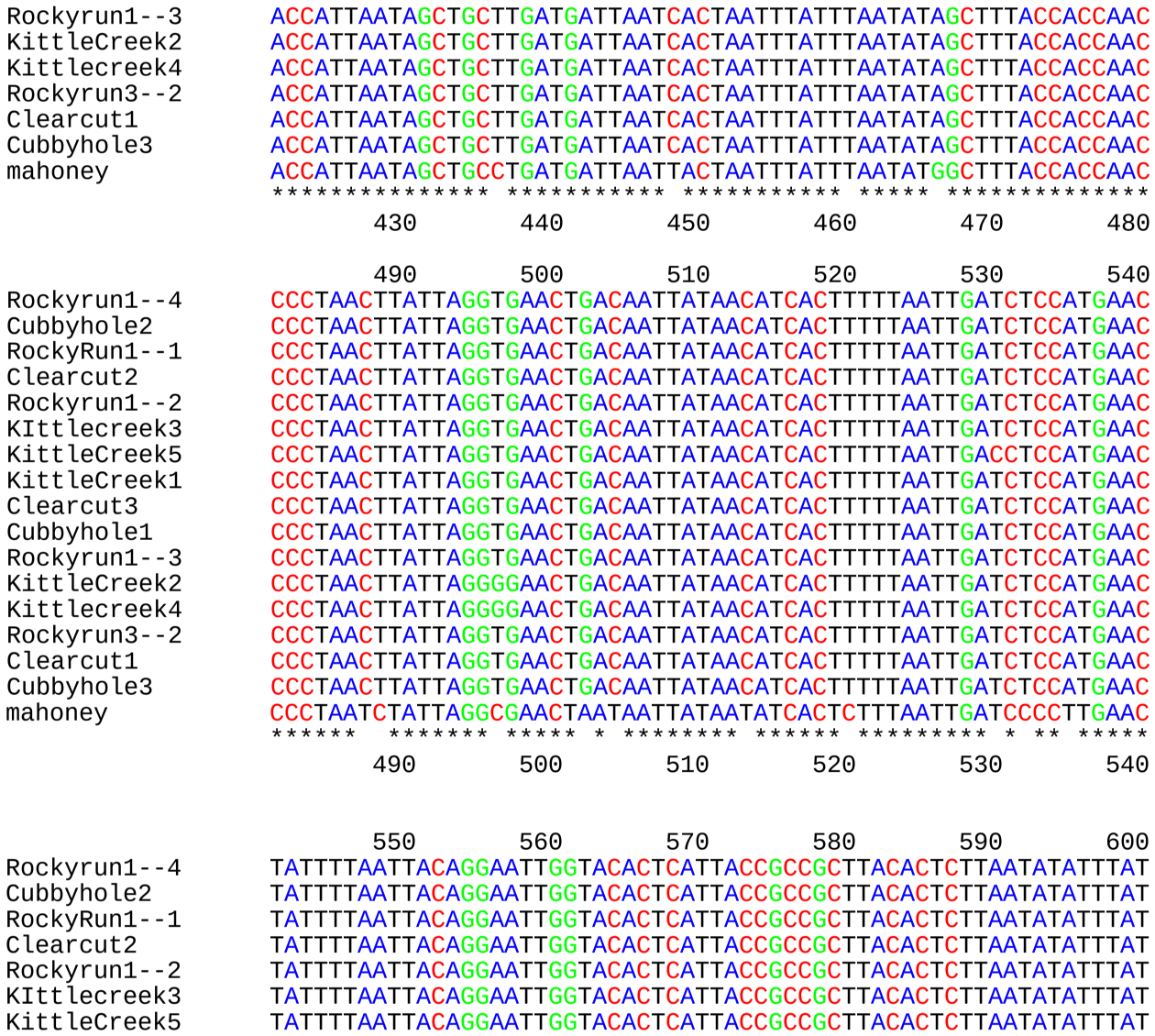
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KittleCreek2 ACCATTAATAGCTGCTTGATGATTAATCACTAATTTATTTAATATAGCTTTACCACCAAC
Kittlecreek4 ACCATTAATAGCTGCTTGATGATTAATCACTAATTTATTTAATATAGCTTTACCACCAAC
Rockyrun3--2 ACCATTAATAGCTGCTTGATGATTAATCACTAATTTATTTAATATAGCTTTACCACCAAC
Clearcut1 ACCATTAATAGCTGCTTGATGATTAATCACTAATTTATTTAATATAGCTTTACCACCAAC
Cubbyhole3 ACCATTAATAGCTGCTTGATGATTAATCACTAATTTATTTAATATAGCTTTACCACCAAC
mahoney ACCATTAATAGCTGCCTGATGATTAATTACTAATTTATTTAATATGGCTTTACCACCAAC
430 440 450 460 470 480
490 500 510 520 530 540
Rockyrun1--4 CCCTAACTTATTAGGTGAACTGACAATTATAACATCACTTTTTAATTGATCTCCATGAAC
Cubbyhole2 CCCTAACTTATTAGGTGAACTGACAATTATAACATCACTTTTTAATTGATCTCCATGAAC
RockyRun1--1 CCCTAACTTATTAGGTGAACTGACAATTATAACATCACTTTTTAATTGATCTCCATGAAC
Clearcut2 CCCTAACTTATTAGGTGAACTGACAATTATAACATCACTTTTTAATTGATCTCCATGAAC
Rockyrun1--2 CCCTAACTTATTAGGTGAACTGACAATTATAACATCACTTTTTAATTGATCTCCATGAAC
KIttlecreek3 CCCTAACTTATTAGGTGAACTGACAATTATAACATCACTTTTTAATTGATCTCCATGAAC
KittleCreek5 CCCTAACTTATTAGGTGAACTGACAATTATAACATCACTTTTTAATTGACCTCCATGAAC
KittleCreek1 CCCTAACTTATTAGGTGAACTGACAATTATAACATCACTTTTTAATTGATCTCCATGAAC
Clearcut3 CCCTAACTTATTAGGTGAACTGACAATTATAACATCACTTTTTAATTGATCTCCATGAAC
Cubbyhole1 CCCTAACTTATTAGGTGAACTGACAATTATAACATCACTTTTTAATTGATCTCCATGAAC
Rockyrun1--3 CCCTAACTTATTAGGTGAACTGACAATTATAACATCACTTTTTAATTGATCTCCATGAAC
KittleCreek2 CCCTAACTTATTAGGGGAACTGACAATTATAACATCACTTTTTAATTGATCTCCATGAAC
Kittlecreek4 CCCTAACTTATTAGGGGAACTGACAATTATAACATCACTTTTTAATTGATCTCCATGAAC
Rockyrun3--2 CCCTAACTTATTAGGTGAACTGACAATTATAACATCACTTTTTAATTGATCTCCATGAAC
Clearcut1 CCCTAACTTATTAGGTGAACTGACAATTATAACATCACTTTTTAATTGATCTCCATGAAC
Cubbyhole3 CCCTAACTTATTAGGTGAACTGACAATTATAACATCACTTTTTAATTGATCTCCATGAAC
mahoney CCCTAATCTATTAGGCGAACTAATAATTATAATATCACTCTTTAATTGATCCCCTTGAAC
490 500 510 520 530 540
550 560 570 580 590 600
Rockyrun1--4 TATTTTAATTACAGGAATTGGTACACTCATTACCGCCGCTTACACTCTTAATATATTTAT
Cubbyhole2 TATTTTAATTACAGGAATTGGTACACTCATTACCGCCGCTTACACTCTTAATATATTTAT
RockyRun1--1 TATTTTAATTACAGGAATTGGTACACTCATTACCGCCGCTTACACTCTTAATATATTTAT
Clearcut2 TATTTTAATTACAGGAATTGGTACACTCATTACCGCCGCTTACACTCTTAATATATTTAT
Rockyrun1--2 TATTTTAATTACAGGAATTGGTACACTCATTACCGCCGCTTACACTCTTAATATATTTAT
KIttlecreek3 TATTTTAATTACAGGAATTGGTACACTCATTACCGCCGCTTACACTCTTAATATATTTAT
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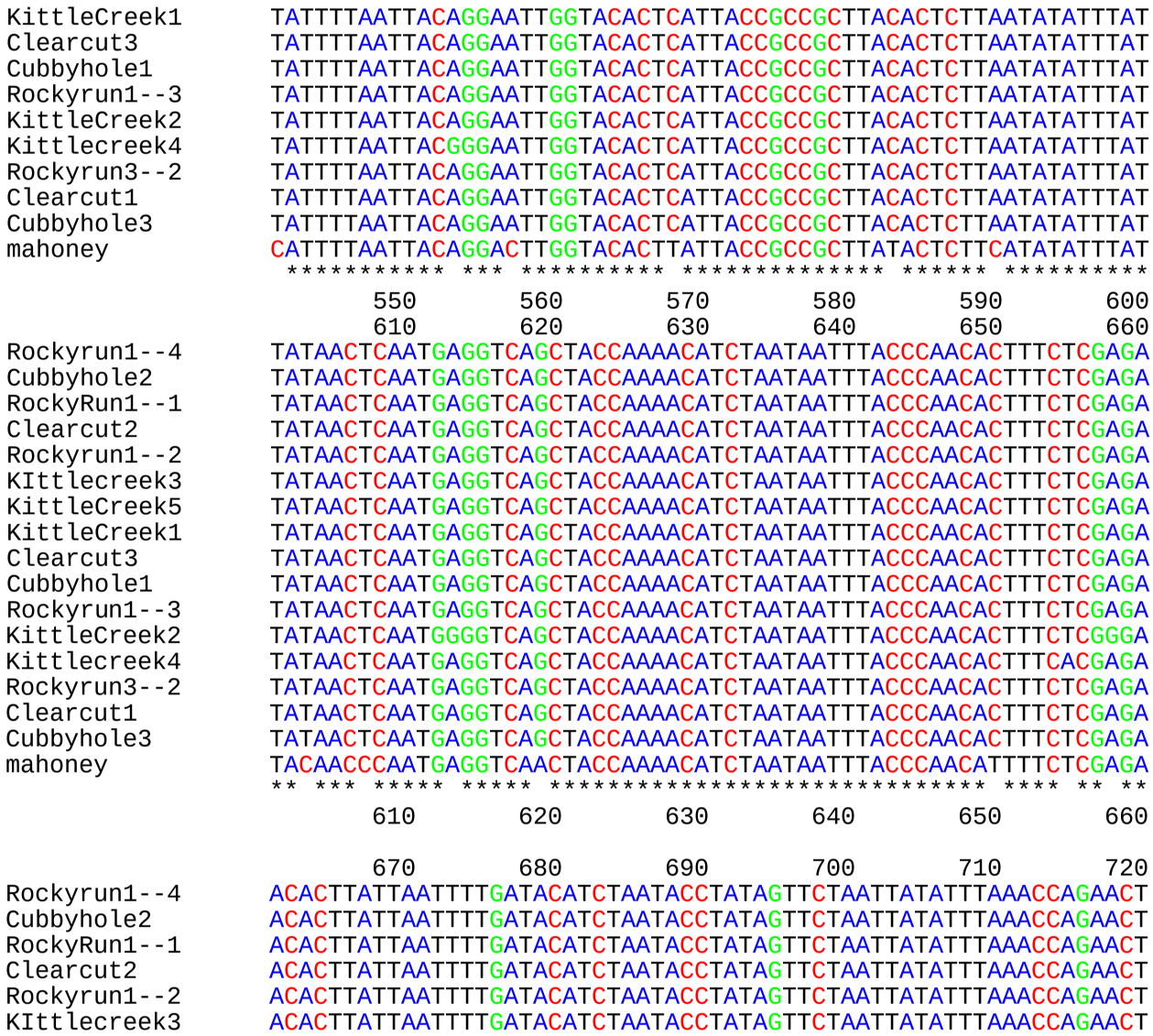
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Cubbyhole1 TATTTTAATTACAGGAATTGGTACACTCATTACCGCCGCTTACACTCTTAATATATTTAT
Rockyrun1--3 TATTTTAATTACAGGAATTGGTACACTCATTACCGCCGCTTACACTCTTAATATATTTAT
KittleCreek2 TATTTTAATTACAGGAATTGGTACACTCATTACCGCCGCTTACACTCTTAATATATTTAT
Kittlecreek4 TATTTTAATTACGGGAATTGGTACACTCATTACCGCCGCTTACACTCTTAATATATTTAT
Rockyrun3--2 TATTTTAATTACAGGAATTGGTACACTCATTACCGCCGCTTACACTCTTAATATATTTAT
Clearcut1 TATTTTAATTACAGGAATTGGTACACTCATTACCGCCGCTTACACTCTTAATATATTTAT
Cubbyhole3 TATTTTAATTACAGGAATTGGTACACTCATTACCGCCGCTTACACTCTTAATATATTTAT
mahoney CATTTTAATTACAGGACTTGGTACACTTATTACCGCCGCTTATACTCTTCATATATTTAT
550 560 570 580 590 600
610 620 630 640 650 660
Rockyrun1--4 TATAACTCAATGAGGTCAGCTACCAAAACATCTAATAATTTACCCAACACTTTCTCGAGA
Cubbyhole2 TATAACTCAATGAGGTCAGCTACCAAAACATCTAATAATTTACCCAACACTTTCTCGAGA
RockyRun1--1 TATAACTCAATGAGGTCAGCTACCAAAACATCTAATAATTTACCCAACACTTTCTCGAGA
Clearcut2 TATAACTCAATGAGGTCAGCTACCAAAACATCTAATAATTTACCCAACACTTTCTCGAGA
Rockyrun1--2 TATAACTCAATGAGGTCAGCTACCAAAACATCTAATAATTTACCCAACACTTTCTCGAGA
KIttlecreek3 TATAACTCAATGAGGTCAGCTACCAAAACATCTAATAATTTACCCAACACTTTCTCGAGA
KittleCreek5 TATAACTCAATGAGGTCAGCTACCAAAACATCTAATAATTTACCCAACACTTTCTCGAGA
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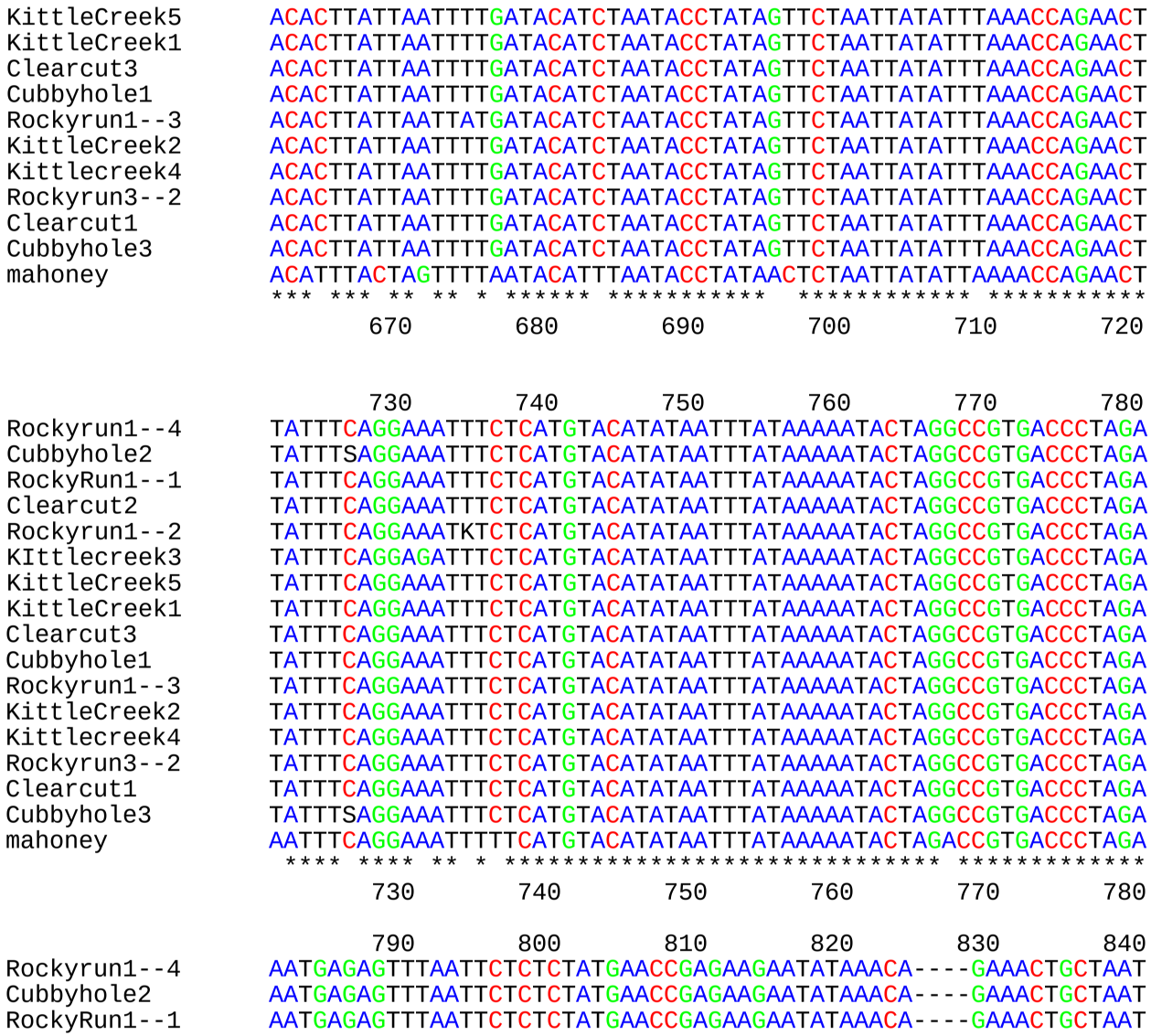
Clearcut3 TATAACTCAATGAGGTCAGCTACCAAAACATCTAATAATTTACCCAACACTTTCTCGAGA
Cubbyhole1 TATAACTCAATGAGGTCAGCTACCAAAACATCTAATAATTTACCCAACACTTTCTCGAGA
Rockyrun1--3 TATAACTCAATGAGGTCAGCTACCAAAACATCTAATAATTTACCCAACACTTTCTCGAGA
KittleCreek2 TATAACTCAATGGGGTCAGCTACCAAAACATCTAATAATTTACCCAACACTTTCTCGGGA
Kittlecreek4 TATAACTCAATGAGGTCAGCTACCAAAACATCTAATAATTTACCCAACACTTTCACGAGA
Rockyrun3--2 TATAACTCAATGAGGTCAGCTACCAAAACATCTAATAATTTACCCAACACTTTCTCGAGA
Clearcut1 TATAACTCAATGAGGTCAGCTACCAAAACATCTAATAATTTACCCAACACTTTCTCGAGA
Cubbyhole3 TATAACTCAATGAGGTCAGCTACCAAAACATCTAATAATTTACCCAACACTTTCTCGAGA
mahoney TACAACCCAATGAGGTCAACTACCAAAACATCTAATAATTTACCCAACATTTTCTCGAGA
610 620 630 640 650 660
670 680 690 700 710 720
Rockyrun1--4 ACACTTATTAATTTTGATACATCTAATACCTATAGTTCTAATTATATTTAAACCAGAACT
Cubbyhole2 ACACTTATTAATTTTGATACATCTAATACCTATAGTTCTAATTATATTTAAACCAGAACT
RockyRun1--1 ACACTTATTAATTTTGATACATCTAATACCTATAGTTCTAATTATATTTAAACCAGAACT
Clearcut2 ACACTTATTAATTTTGATACATCTAATACCTATAGTTCTAATTATATTTAAACCAGAACT
Rockyrun1--2 ACACTTATTAATTTTGATACATCTAATACCTATAGTTCTAATTATATTTAAACCAGAACT
KIttlecreek3 ACACTTATTAATTTTGATACATCTAATACCTATAGTTCTAATTATATTTAAACCAGAACT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Clearcut3 ACACTTATTAATTTTGATACATCTAATACCTATAGTTCTAATTATATTTAAACCAGAACT
Cubbyhole1 ACACTTATTAATTTTGATACATCTAATACCTATAGTTCTAATTATATTTAAACCAGAACT
Rockyrun1--3 ACACTTATTAATTATGATACATCTAATACCTATAGTTCTAATTATATTTAAACCAGAACT
KittleCreek2 ACACTTATTAATTTTGATACATCTAATACCTATAGTTCTAATTATATTTAAACCAGAACT
Kittlecreek4 ACACTTATTAATTTTGATACATCTAATACCTATAGTTCTAATTATATTTAAACCAGAACT
Rockyrun3--2 ACACTTATTAATTTTGATACATCTAATACCTATAGTTCTAATTATATTTAAACCAGAACT
Clearcut1 ACACTTATTAATTTTGATACATCTAATACCTATAGTTCTAATTATATTTAAACCAGAACT
Cubbyhole3 ACACTTATTAATTTTGATACATCTAATACCTATAGTTCTAATTATATTTAAACCAGAACT
mahoney ACATTTACTAGTTTTAATACATTTAATACCTATAACTCTAATTATATTAAAACCAGAACT
670 680 690 700 710 720
730 740 750 760 770 780
Rockyrun1--4 TATTTCAGGAAATTTCTCATGTACATATAATTTATAAAAATACTAGGCCGTGACCCTAGA
Cubbyhole2 TATTTSAGGAAATTTCTCATGTACATATAATTTATAAAAATACTAGGCCGTGACCCTAGA
RockyRun1--1 TATTTCAGGAAATTTCTCATGTACATATAATTTATAAAAATACTAGGCCGTGACCCTAGA
Clearcut2 TATTTCAGGAAATTTCTCATGTACATATAATTTATAAAAATACTAGGCCGTGACCCTAGA
Rockyrun1--2 TATTTCAGGAAATKTCTCATGTACATATAATTTATAAAAATACTAGGCCGTGACCCTAGA
KIttlecreek3 TATTTCAGGAGATTTCTCATGTACATATAATTTATAAAAATACTAGGCCGTGACCCTAGA
KittleCreek5 TATTTCAGGAAATTTCTCATGTACATATAATTTATAAAAATACTAGGCCGTGACCCTAGA
KittleCreek1 TATTTCAGGAAATTTCTCATGTACATATAATTTATAAAAATACTAGGCCGTGACCCTAGA
Clearcut3 TATTTCAGGAAATTTCTCATGTACATATAATTTATAAAAATACTAGGCCGTGACCCTAGA
Cubbyhole1 TATTTCAGGAAATTTCTCATGTACATATAATTTATAAAAATACTAGGCCGTGACCCTAGA
Rockyrun1--3 TATTTCAGGAAATTTCTCATGTACATATAATTTATAAAAATACTAGGCCGTGACCCTAGA
KittleCreek2 TATTTCAGGAAATTTCTCATGTACATATAATTTATAAAAATACTAGGCCGTGACCCTAGA
Kittlecreek4 TATTTCAGGAAATTTCTCATGTACATATAATTTATAAAAATACTAGGCCGTGACCCTAGA
Rockyrun3--2 TATTTCAGGAAATTTCTCATGTACATATAATTTATAAAAATACTAGGCCGTGACCCTAGA
Clearcut1 TATTTCAGGAAATTTCTCATGTACATATAATTTATAAAAATACTAGGCCGTGACCCTAGA
Cubbyhole3 TATTTSAGGAAATTTCTCATGTACATATAATTTATAAAAATACTAGGCCGTGACCCTAGA
mahoney AATTTCAGGAAATTTTTCATGTACATATAATTTATAAAAATACTAGACCGTGACCCTAGA
730 740 750 760 770 780
790 800 810 820 830 840
Rockyrun1--4 AATGAGAGTTTAATTCTCTCTATGAACCGAGAAGAATATAAACA----GAAACTGCTAAT
Cubbyhole2 AATGAGAGTTTAATTCTCTCTATGAACCGAGAAGAATATAAACA----GAAACTGCTAAT
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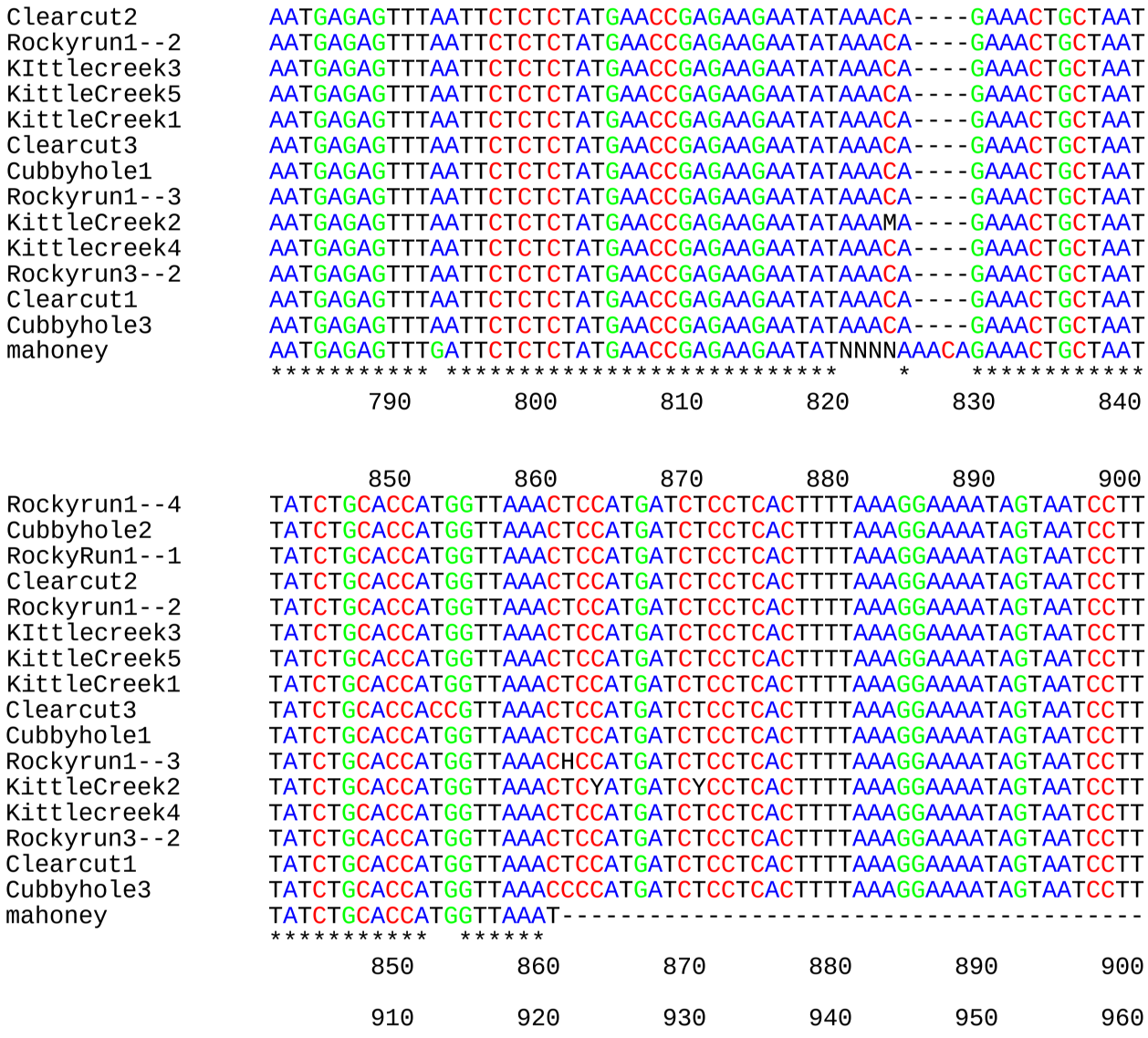
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Rockyrun1--2 AATGAGAGTTTAATTCTCTCTATGAACCGAGAAGAATATAAACA----GAAACTGCTAAT
KIttlecreek3 AATGAGAGTTTAATTCTCTCTATGAACCGAGAAGAATATAAACA----GAAACTGCTAAT
KittleCreek5 AATGAGAGTTTAATTCTCTCTATGAACCGAGAAGAATATAAACA----GAAACTGCTAAT
KittleCreek1 AATGAGAGTTTAATTCTCTCTATGAACCGAGAAGAATATAAACA----GAAACTGCTAAT
Clearcut3 AATGAGAGTTTAATTCTCTCTATGAACCGAGAAGAATATAAACA----GAAACTGCTAAT
Cubbyhole1 AATGAGAGTTTAATTCTCTCTATGAACCGAGAAGAATATAAACA----GAAACTGCTAAT
Rockyrun1--3 AATGAGAGTTTAATTCTCTCTATGAACCGAGAAGAATATAAACA----GAAACTGCTAAT
KittleCreek2 AATGAGAGTTTAATTCTCTCTATGAACCGAGAAGAATATAAAMA----GAAACTGCTAAT
Kittlecreek4 AATGAGAGTTTAATTCTCTCTATGAACCGAGAAGAATATAAACA----GAAACTGCTAAT
Rockyrun3--2 AATGAGAGTTTAATTCTCTCTATGAACCGAGAAGAATATAAACA----GAAACTGCTAAT
Clearcut1 AATGAGAGTTTAATTCTCTCTATGAACCGAGAAGAATATAAACA----GAAACTGCTAAT
Cubbyhole3 AATGAGAGTTTAATTCTCTCTATGAACCGAGAAGAATATAAACA----GAAACTGCTAAT
mahoney AATGAGAGTTTGATTCTCTCTATGAACCGAGAAGAATATNNNNAAACAGAAACTGCTAAT
790 800 810 820 830 840
850 860 870 880 890 900
Rockyrun1--4 TATCTGCACCATGGTTAAACTCCATGATCTCCTCACTTTTAAAGGAAAATAGTAATCCTT
Cubbyhole2 TATCTGCACCATGGTTAAACTCCATGATCTCCTCACTTTTAAAGGAAAATAGTAATCCTT
RockyRun1--1 TATCTGCACCATGGTTAAACTCCATGATCTCCTCACTTTTAAAGGAAAATAGTAATCCTT
Clearcut2 TATCTGCACCATGGTTAAACTCCATGATCTCCTCACTTTTAAAGGAAAATAGTAATCCTT
Rockyrun1--2 TATCTGCACCATGGTTAAACTCCATGATCTCCTCACTTTTAAAGGAAAATAGTAATCCTT
KIttlecreek3 TATCTGCACCATGGTTAAACTCCATGATCTCCTCACTTTTAAAGGAAAATAGTAATCCTT
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Clearcut3 TATCTGCACCACCGTTAAACTCCATGATCTCCTCACTTTTAAAGGAAAATAGTAATCCTT
Cubbyhole1 TATCTGCACCATGGTTAAACTCCATGATCTCCTCACTTTTAAAGGAAAATAGTAATCCTT
Rockyrun1--3 TATCTGCACCATGGTTAAACHCCATGATCTCCTCACTTTTAAAGGAAAATAGTAATCCTT
KittleCreek2 TATCTGCACCATGGTTAAACTCYATGATCYCCTCACTTTTAAAGGAAAATAGTAATCCTT
Kittlecreek4 TATCTGCACCATGGTTAAACTCCATGATCTCCTCACTTTTAAAGGAAAATAGTAATCCTT
Rockyrun3--2 TATCTGCACCATGGTTAAACTCCATGATCTCCTCACTTTTAAAGGAAAATAGTAATCCTT
Clearcut1 TATCTGCACCATGGTTAAACTCCATGATCTCCTCACTTTTAAAGGAAAATAGTAATCCTT
Cubbyhole3 TATCTGCACCATGGTTAAACCCCATGATCTCCTCACTTTTAAAGGAAAATAGTAATCCTT
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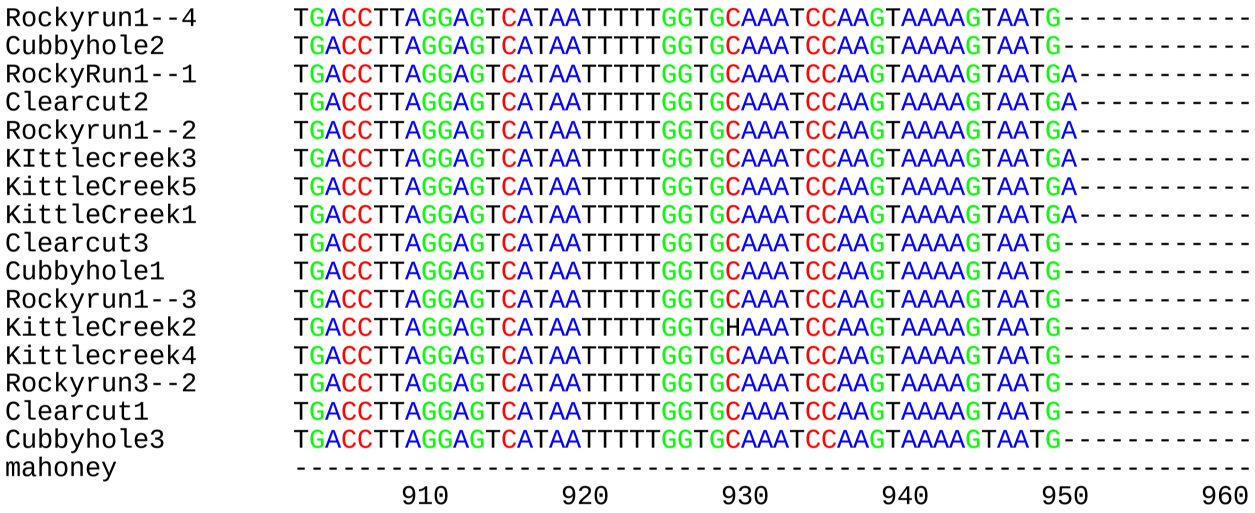
850 860 870 880 890 900
910 920 930 940 950 960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Cubbyhole2 TGACCTTAGGAGTCATAATTTTTGGTGCAAATCCAAGTAAAAGTAATG------------
RockyRun1--1 TGACCTTAGGAGTCATAATTTTTGGTGCAAATCCAAGTAAAAGTAATGA-----------
Clearcut2 TGACCTTAGGAGTCATAATTTTTGGTGCAAATCCAAGTAAAAGTAATGA-----------
Rockyrun1--2 TGACCTTAGGAGTCATAATTTTTGGTGCAAATCCAAGTAAAAGTAATGA-----------
KIttlecreek3 TGACCTTAGGAGTCATAATTTTTGGTGCAAATCCAAGTAAAAGTAATGA-----------
KittleCreek5 TGACCTTAGGAGTCATAATTTTTGGTGCAAATCCAAGTAAAAGTAATGA-----------
KittleCreek1 TGACCTTAGGAGTCATAATTTTTGGTGCAAATCCAAGTAAAAGTAATGA-----------
Clearcut3 TGACCTTAGGAGTCATAATTTTTGGTGCAAATCCAAGTAAAAGTAATG------------
Cubbyhole1 TGACCTTAGGAGTCATAATTTTTGGTGCAAATCCAAGTAAAAGTAATG------------
Rockyrun1--3 TGACCTTAGGAGTCATAATTTTTGGTGCAAATCCAAGTAAAAGTAATG------------
KittleCreek2 TGACCTTAGGAGTCATAATTTTTGGTGHAAATCCAAGTAAAAGTAATG------------
Kittlecreek4 TGACCTTAGGAGTCATAATTTTTGGTGCAAATCCAAGTAAAAGTAATG------------
Rockyrun3--2 TGACCTTAGGAGTCATAATTTTTGGTGCAAATCCAAGTAAAAGTAATG------------
Clearcut1 TGACCTTAGGAGTCATAATTTTTGGTGCAAATCCAAGTAAAAGTAATG------------
Cubbyhole3 TGACCTTAGGAGTCATAATTTTTGGTGCAAATCCAAGTAAAAGTAATG------------
mahoney --
910 920 930 940 950 960