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Inferring Dispersal of Aquatic Invertebrates from Genetic Variation: A Comparative Study of an Amphipod (Talitridae Hyalella azteca) and Mayfly (Baetidae Callibaetis americanus) in Great Basin Springs

Heather Lynn Stutz
Brigham Young University - Provo

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Heather Lynn Stutz

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Dennis K. Shiozawa, Chair
Russell B. Rader
Keith A. Crandall

Department of Biology

Brigham Young University

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ABSTRACT

Inferring dispersal of aquatic invertebrates from genetic variation: a comparative study of an amphipod (Talitridae *Hyalella azteca*) and mayfly (Baetidae *Callibaetis americanus*) in Great Basin springs

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Whether active or passive, dispersal accompanied by gene flow shapes the population genetics and evolutionary divergence of species. Indirect methods which use genetic markers have the ability to assess effective dispersal—that which resulted in gene flow. My objective was to see if an aquatic insect and an obligate aquatic invertebrate show similar phylogeographic patterns and genetic uniqueness. *Hyalella azteca* and *Callibaetis americanus* were collected from 4-5 springs in each of six basins in the Great Basin of western North America. No dispersal or genetic studies of *C. americanus* have been conducted to date. However, several studies focusing on mtDNA diversity of *H. azteca* have revealed a tremendous degree of cryptic diversity in the desert springs of the Great Basin. Nested clade phylogeographical analysis (NCPA), F_{ST} values, AMOVA, and Mantel tests were used to examine geographical associations. I also used traditional phylogenetic approaches including maximum parsimony (MP) and likelihood (ML) analyses using cytochrome c oxidase subunit I (COI), 28S, and 16S as genetic markers. The mitochondrial COI sequence divergences in *C. americanus* were higher than *H. azteca* COI divergences within springs but lower among springs. F_{ST} values were very high in *H. azteca* reaching near fixation for certain alleles. *C. americanus* F_{ST} values were lower suggesting greater gene flow and, consequently, greater dispersal rates. Even though Mantel tests did not detect significant isolation by distance when evaluating all haplotypes together, nested clade analysis was able to examine smaller networks of related haplotypes and detect significant isolation by distance. Whereas the genetic structure in *C. americanus* was dominated by restricted gene flow with isolation by distance, *H. azteca* was characterized more by gradual range expansion followed by fragmentation. Mayflies likely showed more gene flow than amphipods because of their flight capabilities, but movement was still restricted by long distances between isolated springs.

Keywords: aquatic invertebrates, dispersal, *Hyalella azteca*, *Callibaetis americanus*, Great Basin, nested clade phylogeographical analysis

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Introduction

Aquatic invertebrates can either disperse actively through adult flight and overland migration or passively by wind, drift, and animal vectors (Müller 1982, Cáseres and Soluk 2002, Figuerola and Green 2002). However, not all dispersal takes place in the present. One aspect of dispersal that receives less attention is the role of historical events. These events may be rare but biologically significant. Whether active or passive, dispersal accompanied by gene flow shapes the genetic makeup of populations and evolutionary divergence of species. Direct methods of studying dispersal can be difficult to implement. In addition, they are often anecdotal, assess short distances, do not detect rare dispersal events, cannot assess frequency of dispersal, and cannot get sufficient numbers needed for mark-recapture studies (see Bilton et al. 2001 for review). Indirect methods which use genetic markers, however, have the ability to assess effective dispersal—that which resulted in gene flow (Bilton et al. 2001).

Population genetic analyses have been used on a myriad of species to estimate dispersal from stoneflies to fish to deer (Kauwe et al. 2004, Gutiérrez-Rodríguez et al. 2008, Skog et al. 2009). Specifically, nested clade phylogeographical analysis (NCPA, Templeton et al. 1995) has greater power than traditional F-statistics in detecting geographical associations (Templeton 1998). It uses temporal as well as spatial patterns to differentiate between ongoing and historical gene flow. It does this by inferring restricted gene flow under isolation by distance or historical events such as fragmentation and range expansion. It can infer multiple patterns as well since these events are not always mutually exclusive.

Recent papers have tested the statistical rigor of NCPA and claimed that it results in too many false positives especially in inferring isolation by distance (Knowles and Madison 2002, Panchal and Beaumont 2007). Arguments have followed advocating either the complete abandonment of the analysis or continued validation of the much needed analysis (Garrick et al. 2008, Knowles 2008, Petit 2008). However, errors in the simulations have since been identified and additional validation given for NCPA (Templeton 2008, Templeton 2009). In order to cautiously decrease inference errors, I used multiple DNA regions to cross-validate (Templeton 2004) and also relied on results from multiple tests (e.g. F_{ST}, AMOVA, Mantel).

In this study, the dispersal of two spring invertebrates was evaluated by examining genetic variation. The mayfly, *Callibaetis americanus*, was chosen to represent a good disperser, and *Hyalella azteca*, the amphipod, represented a poor disperser. No dispersal or genetic studies of *C. americanus* has been conducted in the desert springs of the Great Basin to date. Most studies have focused on recruitment in streams. Hughes et al. (2000) found the greatest genetic differentiation among mayflies in Australia at the reach scale and concluded that dispersal shapes genetic structure on the large spatial scale while mating shapes it on the small-scale. However, insect studies in North America showed contrasting results with the greatest genetic differentiation occurring at the stream scale (Hughes et al. 1999, Hughes et al. 2003).

Zickovich and Bohonak (2007) compared dispersal ability and genetic structure of *H. azteca* with the mayfly, *Fallceon quilleri*. In intermittent streams, amphipods experienced frequent bottlenecks due to their poor dispersal ability resulting in low intrapopulation genetic diversity. However, noticeable differentiation existed between

populations in upper and lower catchments. *F. quilleri*, on the other hand, showed very little divergence between populations but higher overall diversity, which was hypothesized to be due to their high dispersal ability.

Several studies have focused on mtDNA diversity of *H. azteca* in the springs of the Great Basin. Analyses have revealed a tremendous degree of cryptic diversity and endemism despite *H. azteca* being morphologically recognized as a single species (Witt et al. 2006). Since then, very high as well as heterogeneous rates of cytochrome c oxidase subunit I (COI) evolution among lineages have been documented (Witt et al. 2008). These differing rates may cause confusion when trying to decipher relative contributions of dispersal and vicariance in forming biogeographic patterns. Their phylogenetic relationships did not suggest vicariance signifying that dispersal has played a more important role in shaping today's patterns. Another poor disperser showing similar cryptic speciation is the springsnail, *Pyrgulopsis*, found throughout western America. Genetic studies show many springsnail species are unique to only a single spring (Liu et al. 2003, Hurt 2004).

My objective was to test if aquatic insects, which have relaxed dispersal restraints, show the same phylogeographic patterns and genetic uniqueness as obligate aquatic invertebrates. Aquatic insects, such as mayflies, are capable of dispersing and colonizing springs in a short period of time and may, thereby, cloud the genetic signal associated with their geographic origin. On the other hand, obligate aquatic invertebrates, such as amphipods, are more likely to be isolated, have low gene flow and, consequently, more time to diverge from other populations. Amphipod populations should be genetically differentiated amongst springs, while the mayflies should be more genetically

homogeneous amongst springs. If evidence of gene flow due to dispersal does exist, more dispersal should occur within a basin than between basins. However, if dispersal occurs between basins, I expect greater dispersal among basins north and south of each other rather than east to west because the geographic barriers in the Great Basin tend to be oriented north to south.

Methods

Sampling

Samples were collected from 4-5 springs in each of six basins totaling 27 sites (Table 1, Fig. 1). *H. azteca* was found in 19 of those sites, and *C. americanus* in 21. Sampling was conducted during the summers of 2006 and 2007. Invertebrates were collected with aquatic nets, stored in 95% ethanol, and identified in the lab.

The northern Great Basin had Pleistocene connections with the Columbia and Snake river drainages while the southern basins had connections with the Colorado River (Polhemus and Polhemus 2002). An east/west band of magmatic intrusion divides the northern and southern basins within the Great Basin and acts as a biological barrier as reflected in the distribution of aquatic insects (Polhemus and Polhemus 2002). Therefore, the six basins sampled were in the northern Great Basin to insure commonality of species among sites. The six basins include Spring, Snake, Steptoe, Goshute, Butte, and Antelope valleys and are located in White Pine County, Nevada and Millard County, Utah. Spring Valley is neighbored by Snake Valley on the east and Steptoe Valley on the west. North

of Steptoe is Goshute Valley, which is neighbored by Butte Valley on the west and Antelope Valley to the east.

DNA amplification

DNA was isolated from up to 50 individuals of each population using the DNeasy protocol (Qiagen, Valencia, California). Universal primer sequences LCO and HCO for COI were used to amplify a 710-bp fragment of the COI gene from *H. azteca* and *C. americanus* when possible (Folmer et al. 1994, see Table 2 for primer sequences). COI is being widely utilized for DNA barcoding of taxa (Hebert et al. 2003). Internal primers were created based on the sequences of previously sequenced individuals of both species. These internal primers were used when amplification proved difficult. Primer combinations for *H. azteca* were 5587R with HCO and 5587F with LCO (Table 1). *C. americanus* primer combinations were NR with HCO and NF with LCO (Table 1). Final consensus sequences were trimmed to 369-bp in order to include representatives from each spring. Amplifications were completed in 40-50 µl total reaction volumes containing 2.0 µl of DNA template, 5.0 µl of 10x buffer containing 15 mM $MgCl_2$, 5.0 µl 1.25 mM dNTPs, 2 µl of each primer (0.00001 µM), 0.30 µl *Taq* polymerase, and the remainder volume of sterile water. The program for polymerase chain reactions (PCR) consisted of 36 cycles of 30 seconds at 95ºC, 30 seconds at 48ºC, 1 minute at 72ºC, and a 7 minute extension step at 72ºC.

The large ribosomal subunit, 28S rDNA, was selected as a second genetic marker in *H. azteca*. It is much more conserved than COI (Witt et al. 2006). This provides a

conservative method of validating the phylogenetic signal from COI. Primer sequences 3311F and 4434R (Witt et al. 2006) were paired with internal primers Rnest and Fnest, respectively, to amplify a 959-bp fragment. Internal primers were again developed to help amplify troublesome individuals. Amplifications were completed in 50 µl total reaction volumes containing 1-2 µl DNA template, 5 µl 10x buffer containing 15 mM $MgCl_2$, 5.0 µl 1.25 mM dNTPs, 2-4 µl of each primer (0.00001 µM), 0.30 µl *Taq* polymerase, and the remaining volume of sterile water. The PCR program consisted of a 1 minute denaturation step at 94ºC, 39 cycles of 1 minute at 94ºC, 1 minute at 51 ºC, 1 minute at 72 ºC, and a 5 minute extension step at 72 ºC.

I was unable to amplify 28S from *C. americanus*, so the 16S ribosomal subunit from the mitochondrial genome was amplified as a second marker for validation with the COI mayfly dataset. Primer sequences S2 and 16Sar were used to obtain a 564-bp fragment of the 16S gene (Simon et al. 1994, Gieβler et al. 1999). Amplifications were performed in 40-50 µl total reaction volumes containing 1-2 µl DNA template, 5 µl 10x buffer containing 15 mM $MgCl_2$, 5.0 µl 1.25 mM dNTPs, 2 µl of each primer (0.00001 µM), 0.30 µl *Taq* polymerase, and the remaining volume of sterile water. The PCR program consisted of a 1.5 minute denaturation step at 93 ºC, followed by 41 cycles of 1 minute at 93 ºC, 1 minute at 55 ºC, and 2 minutes at 72 ºC.

Amplified DNA was confirmed on a 1.5% agarose gel stained with ethidium bromide. DNA was cycle sequenced using ABI Big Dye terminator protocol. The reactions were completed in 10 µl total volumes containing 2 µl template, 1 µl primer, 0.5 µl Big Dye, and 6.5 µl sterile water. Big Dye products were cleaned over Sephadex columns and dehydrated in the appropriate well of the sample plate. Sequences were

obtained from the BYU DNA Sequencing Center using either a Perkin-Elmer ABI Prism 377 automated sequencer or an ABI 3100 automated sequencer. Sequences were edited and aligned using SEQUENCHER™ v4.8 (Gene Codes Corp., Ann Arbor, MI). They included no insertions or deletions. *Gammarus lacustris* sequences obtained from Genbank were used as outgroups for COI and 28S *H. azteca* datasets. *Baetis vernus* and *Baetis tricaudatus* sequences from Genbank were used as outgroups for COI and 16S *C. americanus* datasets, respectively.

Phylogenetic analyses

Haplotypes of each dataset were analyzed in PAUP* v4.0b10 (Swofford 2002) using traditional phylogenetic approaches including maximum parsimony (MP) and maximum likelihood (ML) analyses. MP analyses were run using the heuristic algorithm, tree bisection-reconnection (TBR) branch-swapping, and 1000 random addition sequence replicates. Strict consensuses were constructed to take into account equally parsimonious trees. 1000 bootstrap replicates were run at each node for support (Felsenstein 1985). ML analyses were also run under a heuristic search with the best fit models selected in Modeltest 3.0 under the Akaike Information Criterion (AIC, Posada and Crandall 1998). Nodal support was obtained with 1000 bootstrap replicates.

Population genetic analyses

Pair-wise sequence divergences among haplotypes were calculated in SEQUENCHER™ v4.8 and used to find intrapopulation and interpopulation divergences. The Mantel and Analysis of Molecular Variance (AMOVA) tests were run in GENALEX (Peakall and Smouse 2006). Mantel tests for isolation by distance through a regression of genetic and geographic distances. AMOVA partitions total genetic variation into percent variation among basins, among spring populations, and within springs. F_{ST} values were calculated among springs using Arlequin v2.000 (Schneider et al. 2000). F_{ST} values give information on the degree of gene flow among populations. As the F_{ST} value approaches one, the alleles move toward fixation. Values approaching zero indicate more gene flow and mixing of alleles.

Nested Clade Phylogeographical Analysis (NCPA) gives further insight on the degree of genetic variation among basins and springs and was utilized to generate inferences on the degree of historical versus modern movement between springs. Nested clade analysis was implemented using two software programs, TCS v1.21 (Templeton et al. 1992, Clement et al. 2000) and GEODIS v2.5 (Posada et al. 2000). TCS uses statistical parsimony which allows haplotypes with low divergences to be organized into cladograms, or networks, according to the numbers of mutations that may be present among them. I allowed up to 20 mutational steps between haplotypes to increase haplotype network connectivity. Ambiguities (a single haplotype connected to at least two other haplotypes which form a loop) in the haplotype network were resolved based on the topology, frequency, and geography criteria (Pfenninger and Posada 2002). The

haplotype networks were organized into a series of nested clades (Templeton 1998). GEODIS then measured the statistical relationships of haplotypes and their geography using the distances between populations based on GPS coordinates. Distance measures calculated include average clade distance (D_c), nested clade distance (D_n), and interior-tip distances (I-T). The statistically significant associations at α=0.5 were run through the inference key available in GEODIS testing the null hypothesis of no association between genetic variation and geography.

Results

H. azteca

Phylogenetic analyses.—Five of the 43 COI haplotypes detected among *H. azteca* occurred in multiple springs. Maximum parsimony analysis for COI showed that most haplotypes grouped together by basin (Fig. 2). However, Snake Valley showed associations with Spring, Steptoe, and Antelope valleys. A polytomy among basins made it unclear which basin contained the most basal lineages. Twelve of 27 nodes were well supported (92-100). Five nodes were moderately supported (72-85). Ten of 27 nodes had low bootstrap support (64 and below).

The model of molecular evolution chosen for maximum likelihood analysis was K81uf+G for the *H. azteca* COI dataset. The analysis gave results similar to maximum parsimony analyses in that most haplotypes grouped together by basin (Fig. 2). However, maximum likelihood analyses gave evidence that Snake Valley contained the

most basal lineages. In addition, Steptoe and Antelope valleys contained the most derived lineages. Snake Valley continued to show associations with Spring, Steptoe, and Antelope valleys. Seven of thirty nodes were well supported (90-100). Four nodes were moderately supported (75-86). Nineteen of thirty nodes had low bootstrap support (66 and below).

The thirteen 28S haplotypes were shared among springs more often than were the haplotypes in the COI dataset. Maximum parsimony analysis of the 28S sequences did not resolve the basins well (Fig. 3). Two of four nodes were well supported (99-100). One node had moderate support (80), and one had low support (67). The model of molecular evolution chosen for maximum likelihood analysis of the 28S dataset was TVM+I. The likelihood analysis resolved the dataset better than did the maximum parsimony analysis, but basins did not group well together (Fig. 3). It suggested that Antelope Valley lineages were more basal, and Spring and Snake Valley lineages were more derived. Four of seven nodes were well supported (93-100). Three nodes had low support (62 and below).

Population genetic analyses.—Pairwise sequence divergences among *H. azteca* COI haplotypes ranged from 0.3-15.0% within springs with an average divergence of 3.7% (Table 3). Sequence divergences among springs ranged from 0.3-22.6% with an average divergence of 12.5%. Pairwise sequence divergences among *H. azteca* 28S haplotypes ranged from 0.0-0.1% within springs with an average divergence of 0.02% (Table 4). Sequence divergences among springs ranged from 0.0-2.9% with an average divergence of 1.3%. The average pairwise F_{ST} values among *H. azteca* springs were 0.81

for COI and 0.91 for 28S (Table 5, 6). Mantel tests revealed no significant relationships between genetic distance and geographic distance among *H. azteca* COI or 28S haplotypes (R^2=0.0823 and 0.0462, respectively). According to the AMOVA test, 42% of the total COI genetic variation in *H. azteca* could be explained by among spring population variation (Fig. 4a). 39% of the variation was due to among region or basin variation, and 19% of the total variation was within spring populations. 99% of the total 28S genetic variation could be explained by among spring population variation (Fig. 4b). None of the variation was due to among region or basin variation, and only 1% of the total variation was within spring populations.

NCPA.—COI haplotypes formed six different haplotype networks (A, B, C, D, E, F) separated from each other by at least 20 mutational steps. After running statistically significant clades through the inference key, clades 4-3 and 5-1 of network A were characterized by restricted gene flow but with some long distance dispersal (Fig. 5, Appendix A). This network consisted of haplotypes from Leland Harris Spring in Snake Valley and unnamed springs by Cleeve Creek and Rosenlund Ranch in Spring Valley. All other clades in network A could not reject the null hypothesis that clades are randomly distributed across geographic locations. Network B contained two loops that were resolved according to topology and frequency criteria. Network B had an inconclusive outcome and consisted only of haplotypes from Leland Harris Spring in Snake Valley (Fig. 6). Clade 3-1 in network C was characterized by range expansion, and all other internal clades could not reject the null hypothesis of no association between genetic variation and geography. Network C consisted of Flat Spring from Steptoe Valley, Caine

Spring from Snake Valley, and Chin and Tippett Springs from Antelope Valley (Fig. 7). All clades in networks D and E showed no association between genetic variation and geography (Fig. 9, 10). Network D consisted of haplotypes from Indian Spring in Steptoe Valley and Caine Spring in Snake Valley. Network E contained haplotypes from Quilici Spring and an unnamed ("S") spring in Butte Valley. Clade 2-1 in network F had an inconclusive outcome and all other clades showed no geographical association among haplotypes. Network F consisted of haplotypes from Twin Springs and an unnamed ("S") spring in Butte Valley (Fig. 8).

28S haplotypes formed two haplotype networks separated from each other by at least 20 mutational steps. After running significant clades through the inference key, clades 1-1, 2-1, 3-1 and 4-1 of network A were characterized by past gradual range expansion followed by fragmentation, while all other clades showed no geographical association among haplotypes (Fig. 11). Network A consisted of Twin and Quilici springs in Butte Valley, Flat and Indian springs in Steptoe Valley, Horse, Big, Currie and Twin springs in Goshute Valley, unnamed springs by Cleeve Creek and Rosenlund Ranch in Spring Valley, and Tippett and Stockade springs and an unnamed spring by Chin Creek in Antelope Valley. Clade 1-1 in network B had insufficient genetic resolution to distinguish between range expansion and restricted gene flow but with some long distance dispersal (Fig. 12). However, clades 2-1 and 3-1 gave evidence for past gradual range expansion followed by fragmentation.

C. americanus

Phylogenetic analyses.—Fourteen of the 55 *C. americanus* COI haplotypes were shared among springs. Maximum parsimony analysis for COI showed no monophyly among basins (Fig. 13) but generated three clades. The most basal clade consisted of haplotypes from Antelope, Snake, Butte, and Steptoe valleys with the majority from Antelope Valley. The other two clades were equally derived. One clade consisted of haplotypes from all six basins. The third clade consisted of haplotypes from Steptoe, Antelope, Spring, and Butte valleys. It was a large polytomy making fine-scale relationships among haplotypes in these valleys unclear. Four of the 16 nodes were well supported (95-99). Four nodes were moderately supported (73-88), and eight nodes had low support (64 and below). The model of molecular evolution selected with Modeltest for maximum likelihood analysis was HKY+I+G for the *C. americanus* COI dataset. The likelihood analysis showed similar results to the maximum parsimony analyses in that there were no monophyletic basins (Fig. 13). There was more resolution, however, giving further evidence for the same three haplotype clades. One of the 33 nodes was well supported (92). Three nodes were moderately supported (71-89), and 29 nodes had low support (68 and below).

The *C. americanus* 16S dataset had 43 haplotypes. Maximum parsimony analysis for 16S had several polytomies, but there was evidence for three clades similar to the COI clades. In addition to these three clades, 16S suggested a fourth clade of Spring Valley haplotypes (Fig. 14). The most basal clade contained haplotypes from Antelope, Snake, Butte, Spring, and Steptoe valleys with Antelope and Snake being most prominent. The

Spring Valley clade was ancestral to the Steptoe, Antelope, Spring, and Butte valleys clade. The most derived clade contained haplotypes from all basins. Seven of fifteen nodes were well supported (91-100). One node had moderate support (75), and seven had low support (65 and below). The best fit models of molecular evolution selected for maximum likelihood analysis were HKY+I+G for the *C. americanus* COI dataset and GTR+I+G for the 16S dataset. The likelihood analysis was very similar to maximum parsimony analysis and shared the same four haplotype clades (Fig. 14). One of nineteen nodes was well supported (92). Two nodes had moderate support (88-89), and sixteen had low support (68 and below).

Population genetic analyses.—Pairwise sequence divergences among *C. americanus* COI haplotypes ranged from 0.4-17.1% within springs with an average divergence of 5.8% (Table 7). Sequence divergences among springs ranged from 1.0-16.4% with an average divergence of 9.7%. Pairwise sequence divergences among *C. americanus* 16S haplotypes ranged from 0.0-15.0% within springs with an average divergence of 3.6% (Table 8). Sequence divergences among springs ranged from 1.0-14.5% with an average divergence of 6.0%. The average pairwise F_{ST} values among *C. americanus* springs were 0.44 for COI and 0.52 for 16S (Table 9, 10). Mantel tests revealed no significant relationships between genetic distance and geographic distance among *C. americanus* COI or 16S haplotypes (R^2=0.0947 and 0.0234, respectively). According to the AMOVA test, 45% of the total COI genetic variation in *C. americanus* could be explained by within spring population variation (Fig. 4c). Thirty-eight percent of the variation was due to among spring population variation, and 17% of the total

variation was among regions or basins. Thirty-six percent of the total 16S genetic variation could be explained by within spring population variation (Fig. 4d). Thirty-three percent of the 16S variation was due to among region or basin variation, and 31% of the total variation was among spring populations.

NCPA.—C. americanus COI haplotypes formed four different haplotype networks (A, B, C, D) separated by at least 20 mutational steps. Network A contained haplotypes from Caine Spring in Snake Valley, Flat Spring in Steptoe Valley, and Blind, Perkins, and Stockade Springs in Antelope Valley (Fig. 15). After running statistically significant clades through the inference key, all clades showed no geographical association of haplotypes.

Network B contained three ambiguous loops that were resolved according to topology, geography, and frequency criteria (Fig. 16). Clades 2-7 and 3-1 in network B gave evidence for restricted gene flow with isolation by distance. Clades 1-1, 1-3, 1-4, 1-13, 2-1, 2-2, 3-3, 3-4, and 4-1 could not reject the null hypothesis. Network B contained haplotypes from Twin and Big Springs in Goshute Valley, Quilici and unnamed ("Sound of Music") Springs from Butte Valley, Blind Spring from Antelope Valley, Caine Spring from Snake Valley, Cress Spring in Steptoe Valley, and Rock Spring and spring by Cleeve Creek in Spring Valley. Clade 3-1 in network C showed no association between genetic variation and geography. It contained haplotypes from an unnamed ("Sound of Music") spring in Butte Valley and Perkins Spring in Antelope Valley (Fig. 17).

Network D had 12 ambiguous loops that were resolved according to topology, geography, and frequency criteria (Fig. 18). Clades 1-3, 2-2, and 3-2 were characterized

by restricted gene flow with isolation by distance. Clades 2-5 and 3-1 were characterized by restricted gene flow but with some long distance dispersal. Clade 1-5 gave evidence for allopatric fragmentation. Clades 1-1, 1-2, 1-6, 1-10, 1-11, 2-3, and 2-4 could not reject the null hypothesis, and clades 2-1 and 4-1 had inconclusive outcomes. Network D contained haplotypes from Flat, Cress, and Becky springs in Steptoe Valley, Perkins, Chin and Stockade springs in Antelope Valley, unnamed spring by Rosenlund Ranch in Spring Valley, and an unnamed ("Sound of Music") spring and Quilici Spring in Butte Valley.

The 16S haplotypes formed three networks (A, B, C) and two unconnected haplotypes. Network A had two loops that were resolved based on geography and frequency criteria (Fig. 19). Clades 1-13 and 2-5 had insufficient genetic resolution to discriminate between range expansion and restricted gene flow. Clade 2-1 gave evidence for restricted gene flow with isolation by distance. Clades 1-1, 1-2, 1-11, 1-12 and 3-2 could not reject the null hypothesis, and clade 4-1 was inconclusive. Network A consisted of haplotypes from Twin, Knoll, and Caine Springs in Snake Valley, Stockade, Blind, Perkins, and Tippett springs in Antelope Valley, unnamed springs by Cleeve Creek and Rosenlund Ranch and Rock and Millick springs in Spring Valley, Twin, Mustang, and Big springs in Goshute Valley, and an unnamed ("Sound of Music") spring and Quilici Spring in Butte Valley.

Clades 1-1 and 4-1 in network B were characterized by restricted gene flow with isolation by distance (Fig. 20). Clade 1-5 had an inconclusive outcome, and clades 2-1, 2-2, 2-3, 3-1, and 3-2 showed no association between genetic variation and geography. Network B contained haplotypes from Caine, Twin and Knoll springs in Snake Valley,

Flat Spring in Steptoe Valley, Perkins, Blind and Stockade springs in Antelope Valley, spring by Spring Creek in Spring Valley, and an unnamed spring ("Sound of Music") in Butte Valley. Network C consisted of two haplotypes from Rock Spring in Spring Valley. Since there was no geographical variation, it could not be run through the inference key. Lone haplotypes not connected to networks were from Rock Spring in Spring Valley and Twin Springs in Goshute Valley.

Discussion

Genetic diversity

Although mitochondrial sequence divergences based on COI among *H. azteca* may appear high, they are consistent with reported values. Witt et al. (2006) reported an average of 3.75% intrapopulation divergence for Great Basin *Hyalella*, which is comparable to my 3.7%. They also reported 4.4-29.9% sequence divergences among provisional species. I did not try to delineate provisional species, but I calculated a range of 0.3-22.6% among spring populations. The nuclear gene, 28S, does not show equally high divergence rates among haplotypes due to its slower mutational rate.

The average within spring mitochondrial COI sequence divergences (5.8%) in *C. americanus* were higher than that recorded in the *H. azteca* COI divergences but lower among springs (9.8%). The mitochondrial 16S divergences in *C. americanus* were lower than COI divergences within springs (3.6%) and among springs (6.0%). Although exact sequence divergences have not been reported for *C. americanus*, COI sequence

divergences in leptophlebiid mayflies have been shown to be as high as 9.9-18.7% among phylogenetic lineages, which consisted of mayflies from one or more river catchments (Baker et al. 2004).

F_{ST} values were very high in *H. azteca* reaching near fixation for certain alleles. *C. americanus* F_{ST} values were lower suggesting higher gene flow and, consequently, dispersal rates. AMOVA tests showed that *H. azteca* has more among population genetic variation consistent with more isolated populations, and *C. americanus* has more within population genetic variation also consistent with higher dispersal abilities. Miller et al. (2002) demonstrated that the majority of genetic variation for aquatic insects was within sites (>90%) with a small amount among sites (~1%). The mayfly, *Baetis* (Baetidae), demonstrated significant genetic differentiation at small spatial scales within streams and smaller genetic structure at larger spatial scales (Bunn and Hughes 1997). New colonists introduce diversity into spring populations, thus increasing genetic differentiation within springs and decreasing differentiation among springs (Slatkin 1985, Miller et al. 2002). For poor dispersers who remain isolated, genetic differentiation is greater among springs than within.

Dispersal inferences

H. azteca COI haplotype networks showed isolation of Butte, Spring, Steptoe and Antelope valleys. Snake Valley also had an isolated lineage but, in addition, relationships were shown between Snake and Spring, Snake and Steptoe, and Snake and Antelope valleys. Current genetic relationships were formed primarily from historic range

expansion as well as restricted gene flow but with some long distance dispersal. Inferences from *H. azteca* 28S haplotype networks further showed that these relationships were formed by past gradual range expansion followed by fragmentation. Snake Valley may have been the valley from which range expansion began, although phylogenetic analyses were discordant on whether Snake or Antelope Valley was most ancestral.

Mitochondrial-based phylogenetic analyses of *H. azteca* showed evidence of divergence among basins and subsequent isolation of spring populations, while the more conserved nuclear region could not. Witt et al. (2008) found the rates of COI evolution to be heterogeneous among amphipod lineages, while the exact rate of 28S evolution is unknown. However, it is well known that nuclear genes are slower evolving than mitochondrial genes (Brown et al. 1979, 1982). Witt et al.'s (2008) phylogenetic relationships using COI suggested that dispersal events explained amphipod relationships. However, my two datasets appear to show two different points in evolutionary history where COI suggests more recent vicariance and 28S suggests historic dispersal. Relict dace, *Relictus solitarius*, occur in the Steptoe, Butte, Goshute, and Ruby valley regions. A phylogeographic study of pikeminnow in western North America indicated that *R. solitarius* split from the Colorado pikeminnow/Utah chub-roundtail chub (*Gila atraria-Gila robusta*) lineage in the early to mid-Miocene or about 9.2 million years ago (Houston et al. 2009 in review). This gives insight on when amphipods may have entered those basins. They would have subsequently been isolated in their separate basins.

Mitochondrial-based phylogenetic analyses of *C. americanus* showed evidence of divergence among combinations of basins. If these associations give evidence for

possible dispersal routes, then they prove my hypothesis false that dispersal would only occur among basins north and south of each other. One of the mayfly clades represented haplotypes from all basins in this study giving further evidence for mayflies' higher dispersal ability in comparison to the poor dispersing amphipods. The mountain ranges do not appear to be geographic barriers for the good dispersers.

C. americanus COI haplotype networks resolved the same three clades as the phylogenetic trees. Network A consisted of Antelope, Snake and Steptoe valleys as did the first clade. However, Butte Valley was at least 20 mutational steps away from this network while it was included in the first clade. Network B and the second clade consisted of all valleys. Network D and the third clade included Steptoe, Antelope, Spring, and Butte valleys. The valleys' close relationships were represented as a large polytomy in the maximum parsimony tree and by many ambiguous loops in the haplotype network. Genetic relationships were formed by restricted gene flow with isolation by distance, restricted gene flow but with some long distance dispersal, and allopatric fragmentation.

C. americanus 16S haplotype networks resolved the same three clades as COI in addition to a Spring Valley network. These 16S networks were consistent with the clades from the 16S phylogenetic trees. Network B and the first clade contained Antelope, Snake, and Steptoe valleys. Network A and the second clade contained consisting of all valleys, which was separated by 15 mutational steps from the third clade consisting of Antelope, Steptoe, Butte, and Spring valleys. These networks inferred restricted gene flow with isolation by distance. Although the Mantel tests did not detect significant isolation by distance when evaluating all haplotypes together, nested clade analysis

examined smaller networks of related haplotypes and was able to detect isolation by distance (Hughes et al. 2003).

Whereas the genetic structure in *C. americanus* was dominated by restricted gene flow with isolation by distance, *H. azteca* was characterized by gradual range expansion followed by fragmentation. This is consistent with their differences in dispersal abilities. *Callibaetis* showed more gene flow than *Hyalella* because of their flight capabilities, but their movement was still restricted by long distances between isolated springs.

It is suggested that a nuclear gene for *C. americanus* be analyzed in the future and compared with the mitochondrial results. For example, allozyme analyses on Baetidae in the Rocky Mountains have revealed contrasting genetic patterns with COI (Hughes et al. 2003). COI showed greatest genetic differentiation among streams, and allozyme variation was greatest among sites within a stream. Nuclear DNA analyses on *C. americanus* will provide greater insight into their historical dispersal ability as it has with *H. azteca*.

Application

The largest threat to the survival of aquatic species is habitat loss, destruction, and fragmentation (Dudgeon et al. 2006). Today's Great Basin species are facing this in the form of groundwater development (Appendix B). Two basins, Snake and Spring valleys, in this study are part of groundwater development plans. As wells are being built to pump the aquifers, the fates of the desert springs and their residents are in question. The persistence of spring invertebrates despite habitat loss and degradation will largely rely

on their ability to disperse. Good dispersers are more easily capable of increasing their geographic range and preventing extinction of the species. Dispersal studies have typically focused on stream dwelling invertebrates (Tronstad et al. 2007, Chaput-Bardy et al. 2008, James et al. 2008). However, spring invertebrates occur in discrete sites often distantly isolated from other water sources. They cannot rely on a colonization cycle for persistence where upstream dispersal compensates for downstream drift (Müller 1954). Populations are not discrete units in that they exist through time as well as space, and limiting their dispersal could threaten their future persistence.

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Figures

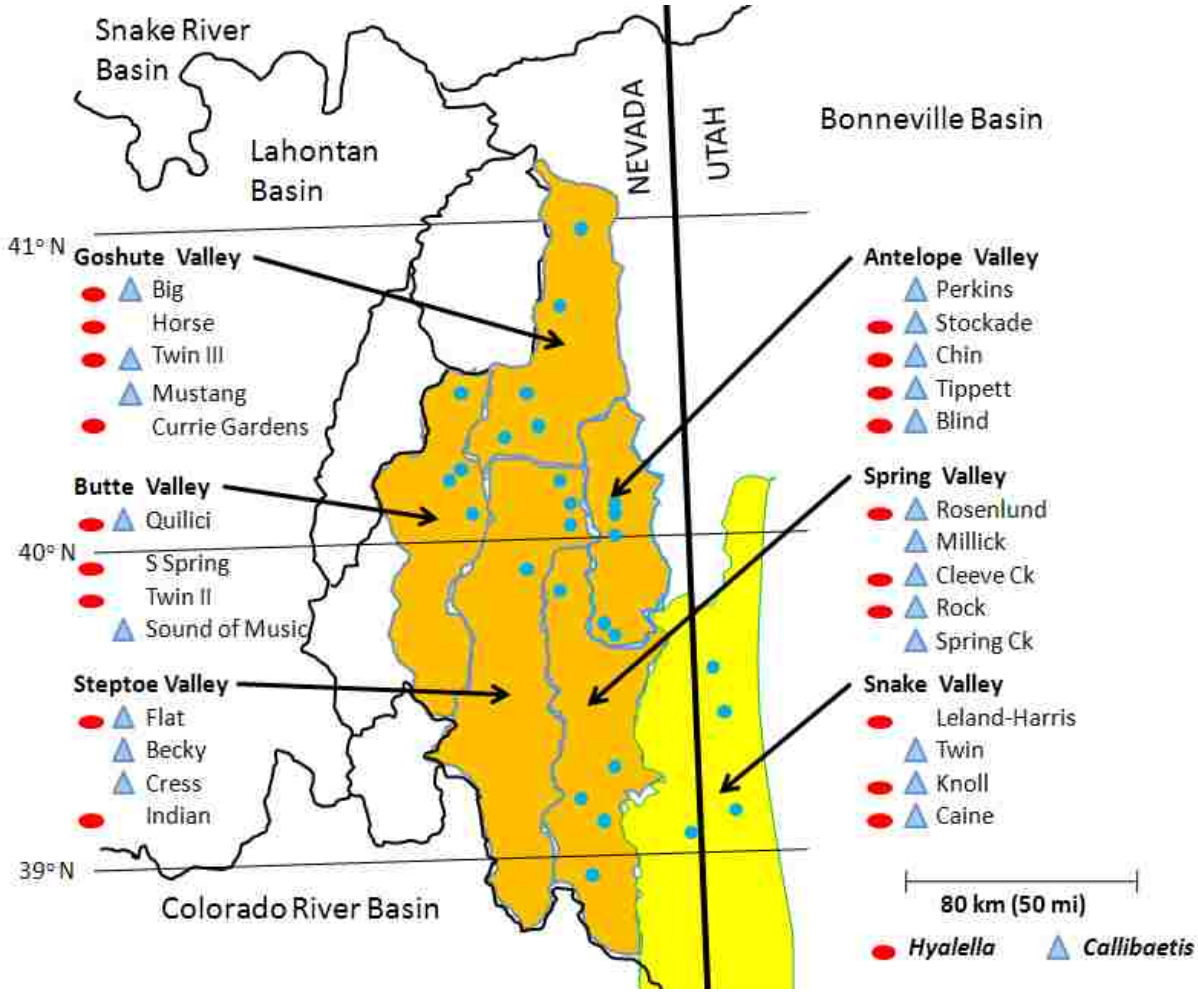

FIG. 1.

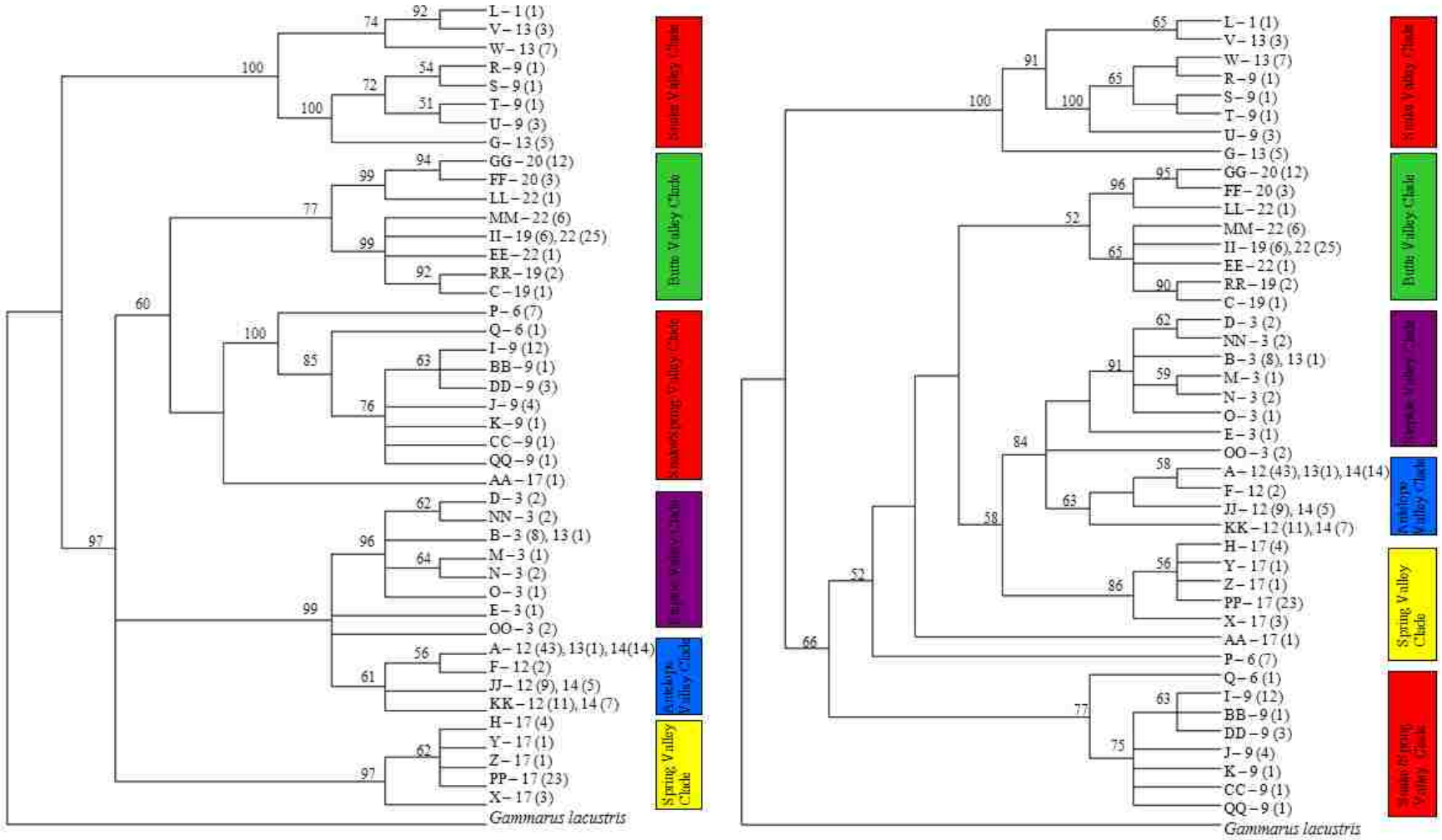
L – 1 (1)
V – 13 (3)
W – 13 (7)
R – 9 (1)
S – 9 (1)
T – 9 (1)
U – 9 (3)
G – 13 (5)
GG – 20 (12)
FF – 20 (3)
LL – 22 (1)
MM – 22 (6)
II – 19 (6), 22 (25)
EE – 22 (1)
RR – 19 (2)
C – 19 (1)
P – 6 (7)
Q – 6 (1)
I – 9 (12)
BB – 9 (1)
DD – 9 (3)
J – 9 (4)
K – 9 (1)
CC – 9 (1)
QQ – 9 (1)
AA – 17 (1)
D – 3 (2)
NN – 3 (2)
B – 3 (8), 13 (1)
M – 3 (1)
N – 3 (2)
O – 3 (1)
E – 3 (1)
OO – 3 (2)
A – 12 (43), 13 (1), 14 (14)
F – 12 (2)
JJ – 12 (9), 14 (5)
KK – 12 (11), 14 (7)
H – 17 (4)
Y – 17 (1)
Z – 17 (1)
PP – 17 (23)
X – 17 (3)
Gammarus lacustris
Swales Valley Clade
Butte Valley Clade
Snakespring Valley Clade
Aspen Valley Clade
Antelope Valley Clade
Spring Valley Clade

FIG. 2

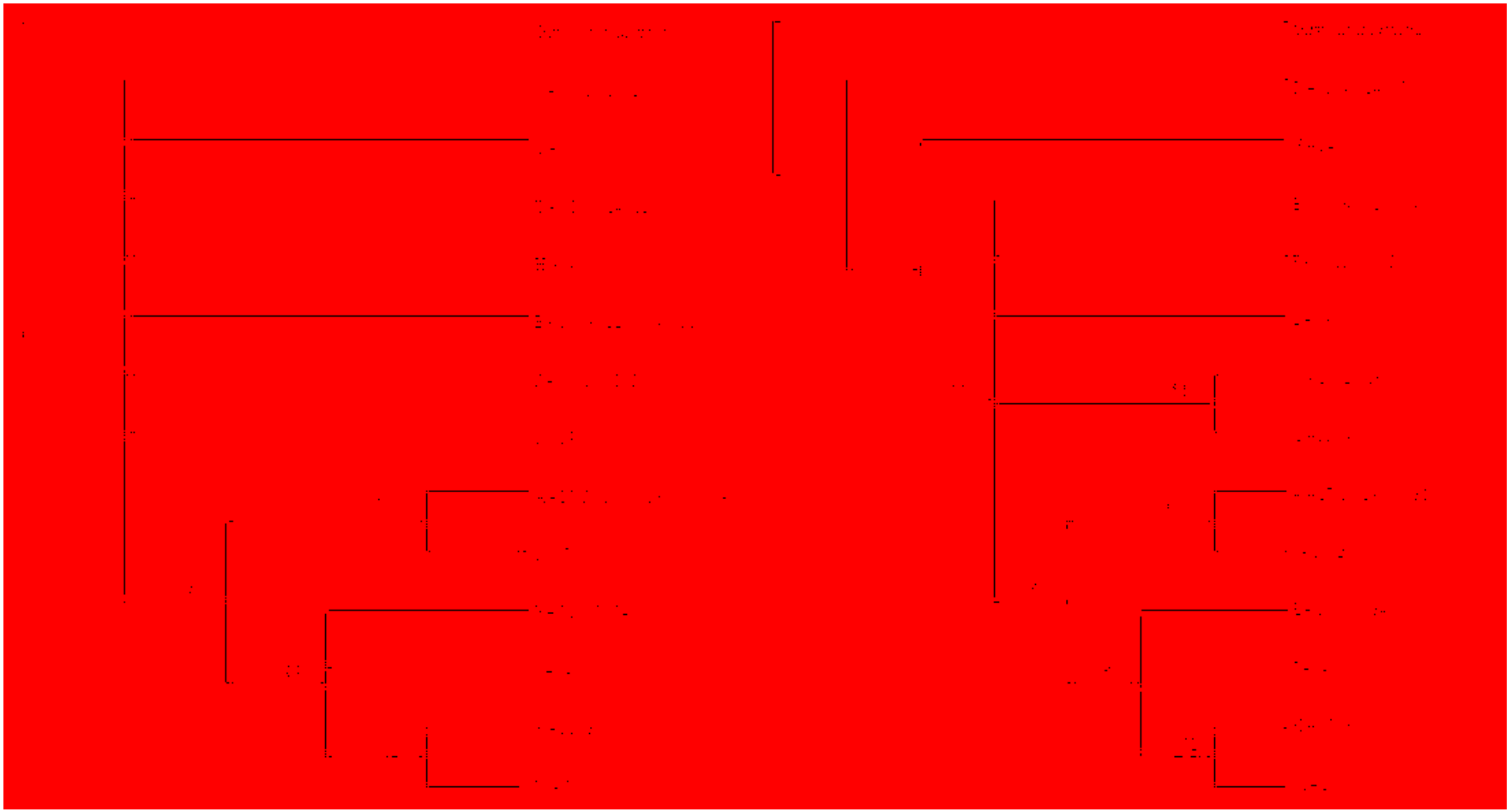

FIG. 3

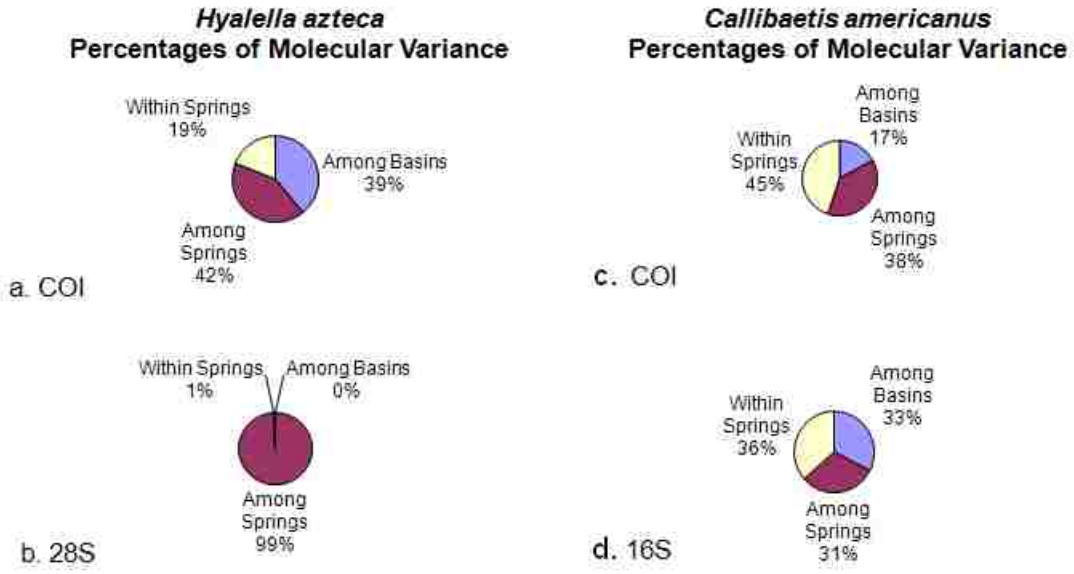
Hyalella azteca
Percentages of Molecular Variance
Within Springs 19%
Among Basins 39%
Among Springs 42%
a. COI
Within Springs 1%
Among Basins 0%
Among Springs 99%
b. 28S
Callibaetis americanus
Percentages of Molecular Variance
Among Basins 17%
Within Springs 45%
Among Springs 38%
c. COI
Among Basins 33%
Within Springs 36%
Among Springs 31%
d. 16S

FIG. 4

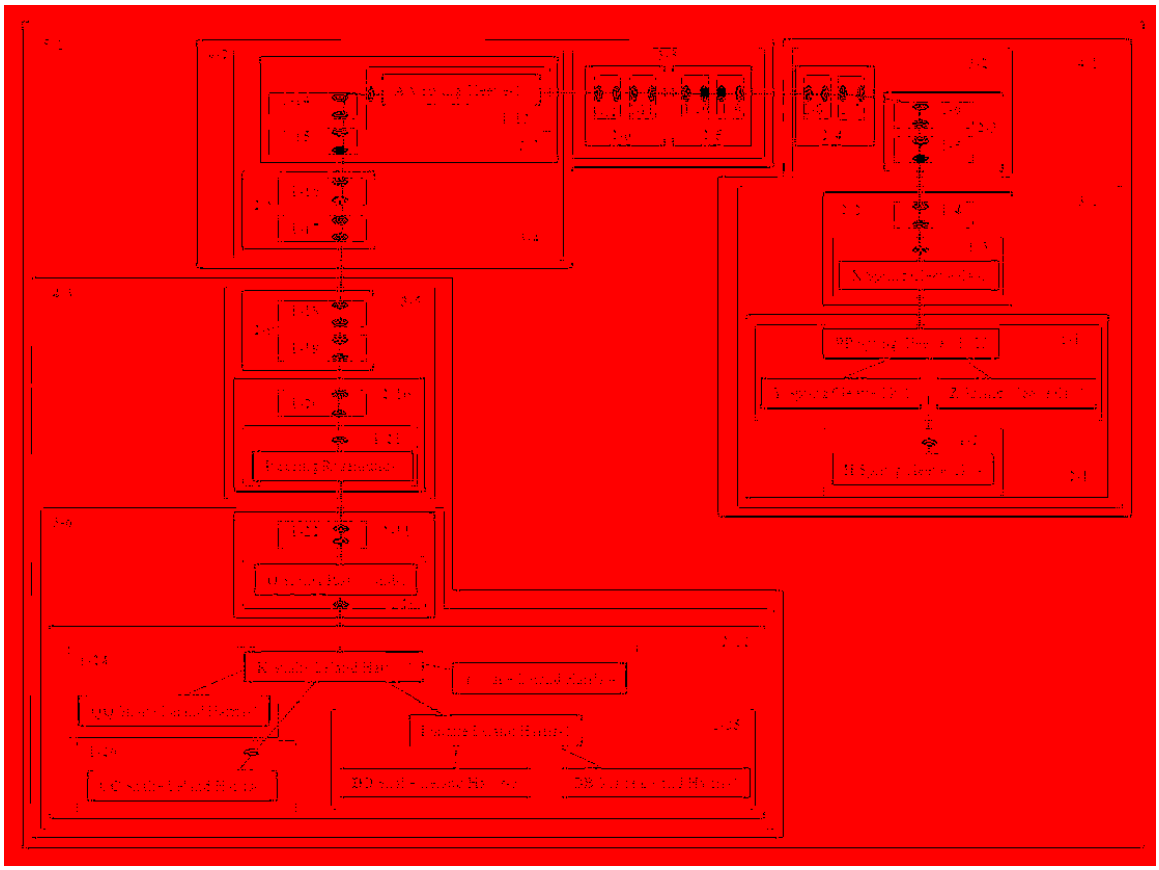

FIG. 5

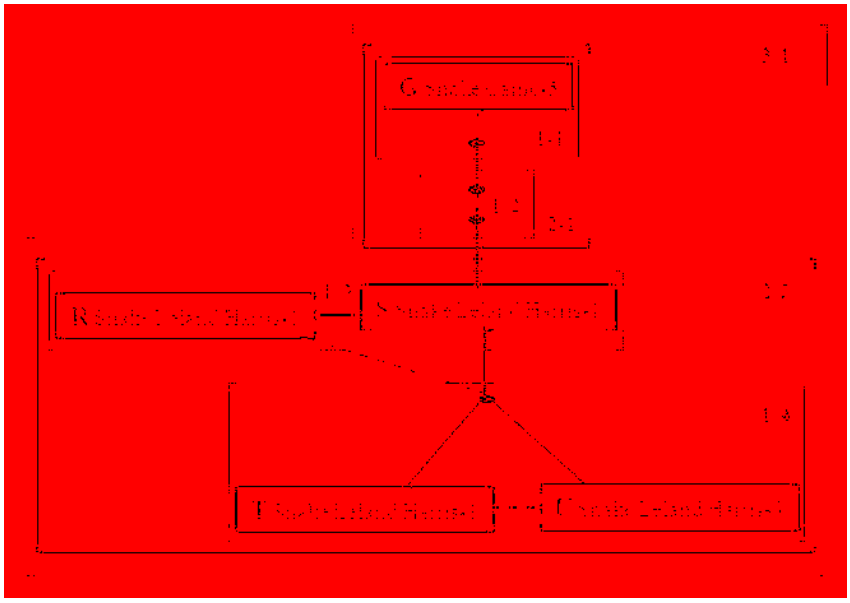

FIG. 6

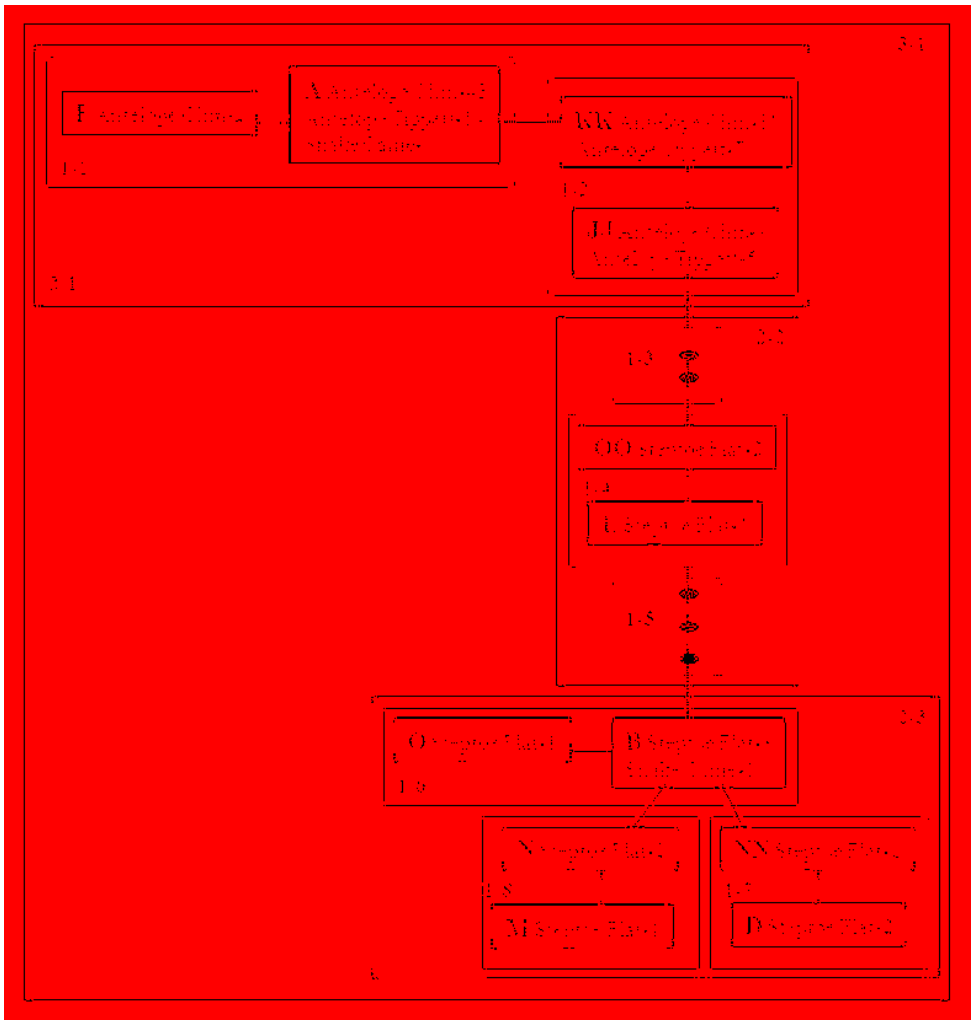

FIG. 7

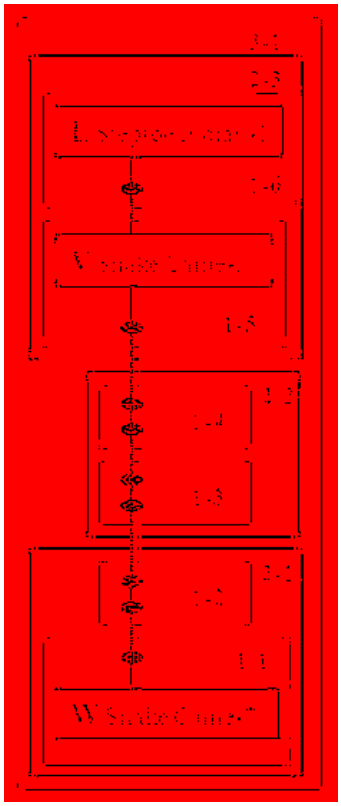

FIG. 8

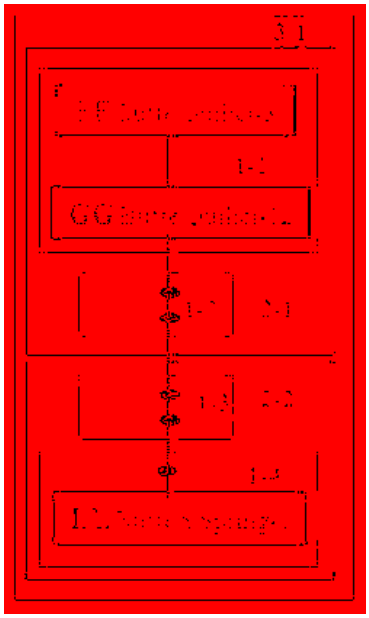

FIG. 9

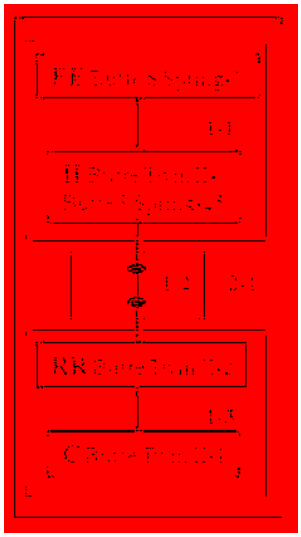

FIG. 10

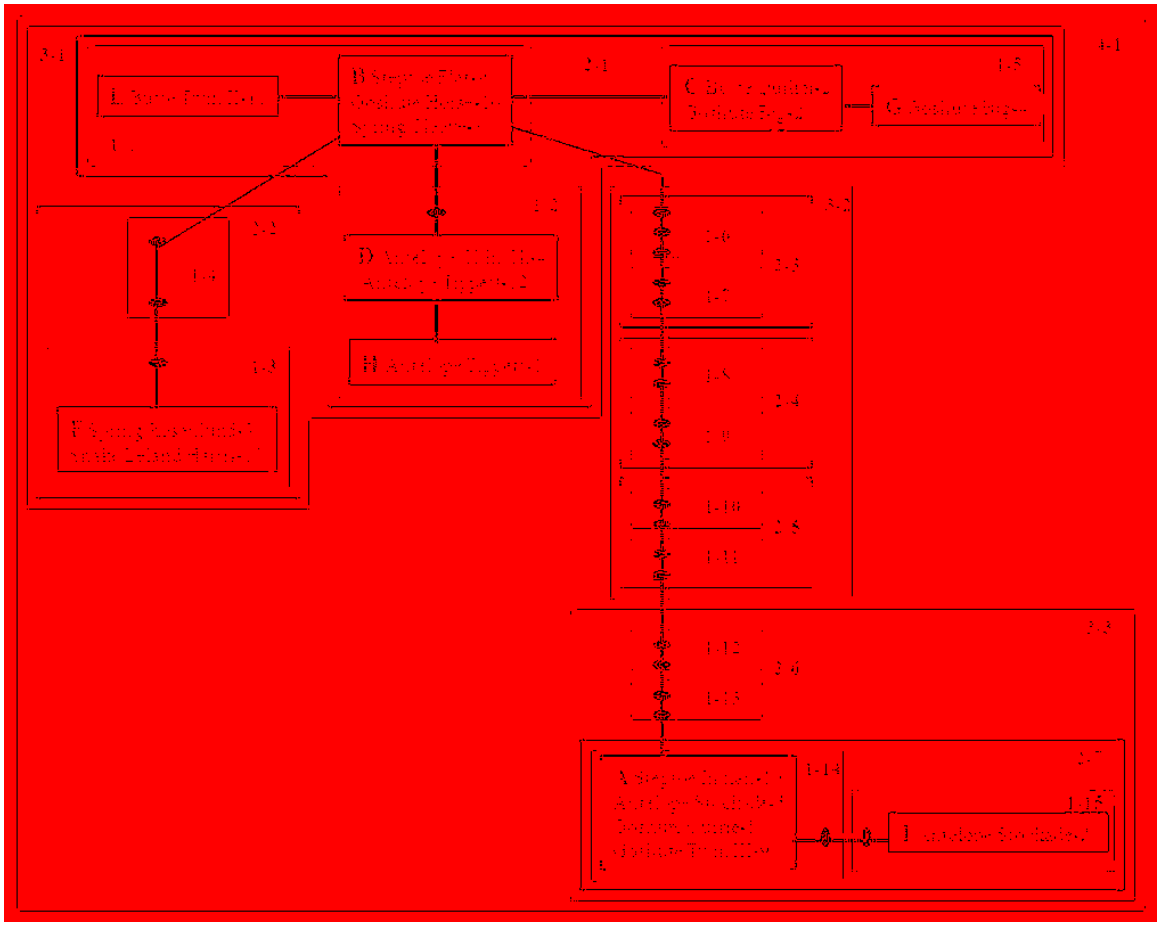

FIG. 11

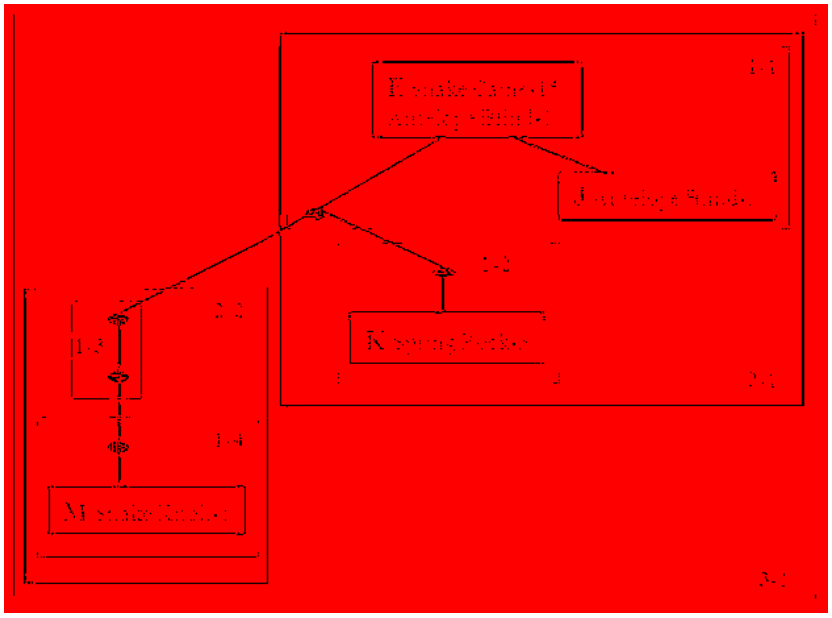

FIG. 12

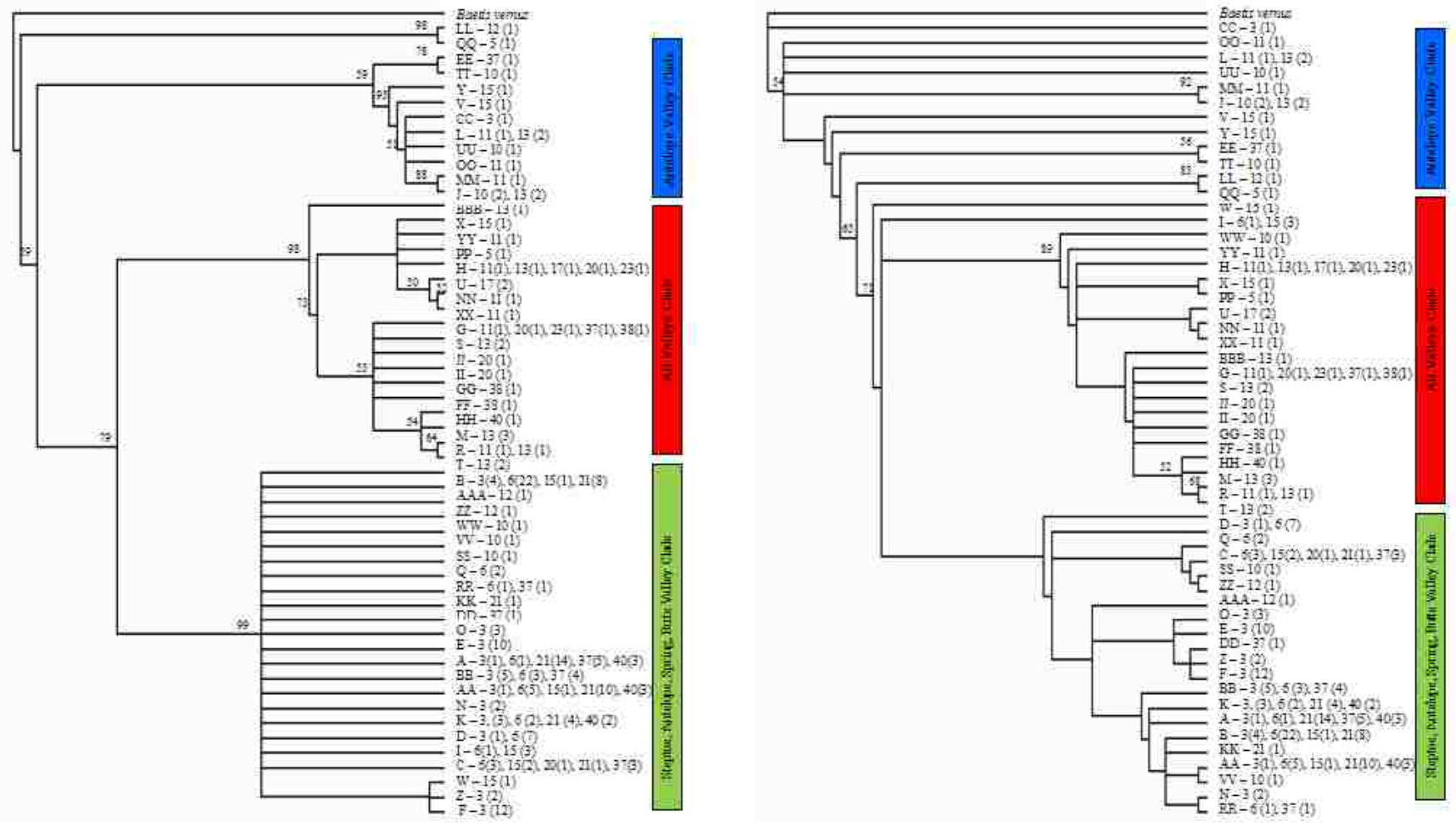
Beetle Valley Clade
All Valleys Clade
Steptoe, Kittitas, Spring, Butte Valley Clade

FIG. 13

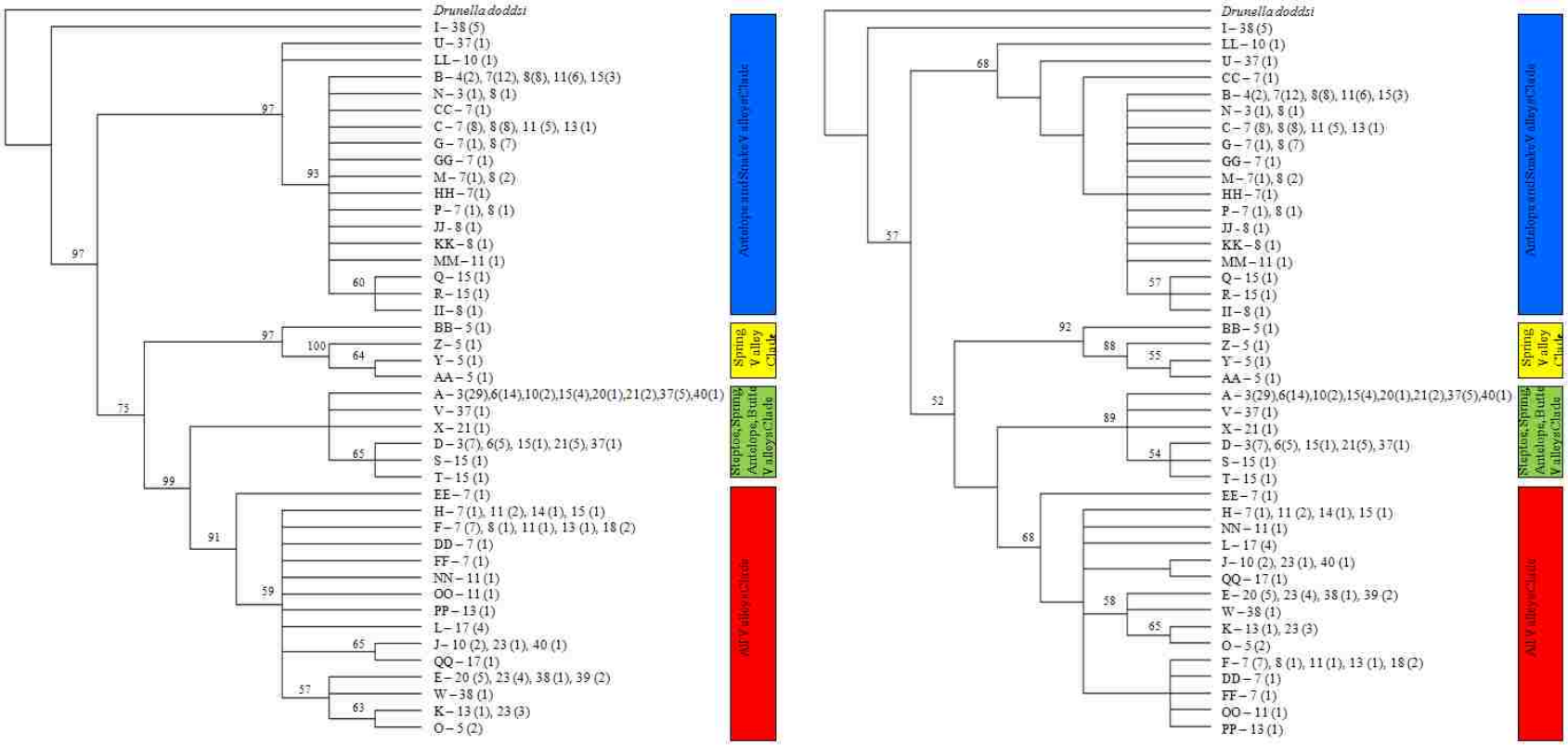
Drunella doddsi
I – 38 (5)
U – 37 (1)
LL – 10 (1)
B – 4(2), 7(12), 8(8), 11(6), 15(3)
N – 3 (1), 8 (1)
CC – 7 (1)
C – 7 (8), 8 (8), 11 (5), 13 (1)
G – 7 (1), 8 (7)
GG – 7 (1)
M – 7(1), 8 (2)
HH – 7(1)
P – 7 (1), 8 (1)
JJ - 8 (1)
KK – 8 (1)
MM – 11 (1)
Q – 15 (1)
R – 15 (1)
II – 8 (1)
BB – 5 (1)
Z – 5 (1)
Y – 5 (1)
AA – 5 (1)
A – 3(29),6(14),10(2),15(4),20(1),21(2),37(5),40(1)
V – 37 (1)
X – 21 (1)
D – 3(7), 6(5), 15(1), 21(5), 37(1)
S – 15 (1)
T – 15 (1)
EE – 7 (1)
H – 7 (1), 11 (2), 14 (1), 15 (1)
F – 7 (7), 8 (1), 11 (1), 13 (1), 18 (2)
DD – 7 (1)
FF – 7 (1)
NN – 11 (1)
OO – 11 (1)
PP – 13 (1)
L – 17 (4)
J – 10 (2), 23 (1), 40 (1)
QQ – 17 (1)
E – 20 (5), 23 (4), 38 (1), 39 (2)
W – 38 (1)
K – 13 (1), 23 (3)
O – 5 (2)
Antelope and Snake Valleys Clade
Spring Valley Clade
Steptoe, Spring, Antelope, Butte Valleys Clade
All Valleys Clade
Drunella doddsi
I – 38 (5)
LL – 10 (1)
U – 37 (1)
CC – 7 (1)
B – 4(2), 7(12), 8(8), 11(6), 15(3)
N – 3 (1), 8 (1)
C – 7 (8), 8 (8), 11 (5), 13 (1)
G – 7 (1), 8 (7)
GG – 7 (1)
M – 7(1), 8 (2)
HH – 7(1)
P – 7 (1), 8 (1)
JJ - 8 (1)
KK – 8 (1)
MM – 11 (1)
Q – 15 (1)
R – 15 (1)
II – 8 (1)
BB – 5 (1)
Z – 5 (1)
Y – 5 (1)
AA – 5 (1)
A – 3(29),6(14),10(2),15(4),20(1),21(2),37(5),40(1)
V – 37 (1)
X – 21 (1)
D – 3(7), 6(5), 15(1), 21(5), 37(1)
S – 15 (1)
T – 15 (1)
EE – 7 (1)
H – 7 (1), 11 (2), 14 (1), 15 (1)
NN – 11 (1)
L – 17 (4)
J – 10 (2), 23 (1), 40 (1)
QQ – 17 (1)
E – 20 (5), 23 (4), 38 (1), 39 (2)
W – 38 (1)
K – 13 (1), 23 (3)
O – 5 (2)
F – 7 (7), 8 (1), 11 (1), 13 (1), 18 (2)
DD – 7 (1)
FF – 7 (1)
OO – 11 (1)
PP – 13 (1)
Antelope and Snake Valleys Clade
Spring Valley Clade
Steptoe, Spring, Antelope, Butte Valleys Clade
All Valleys Clade

FIG. 14

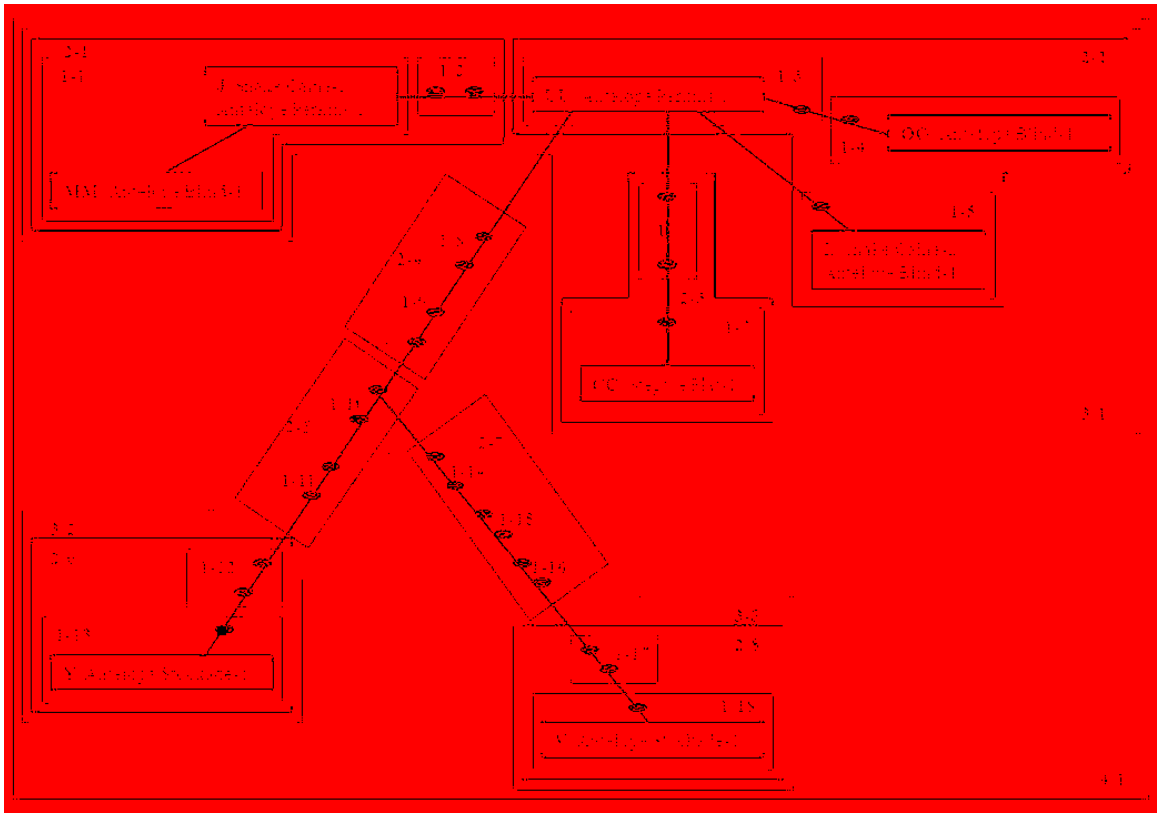

FIG. 15

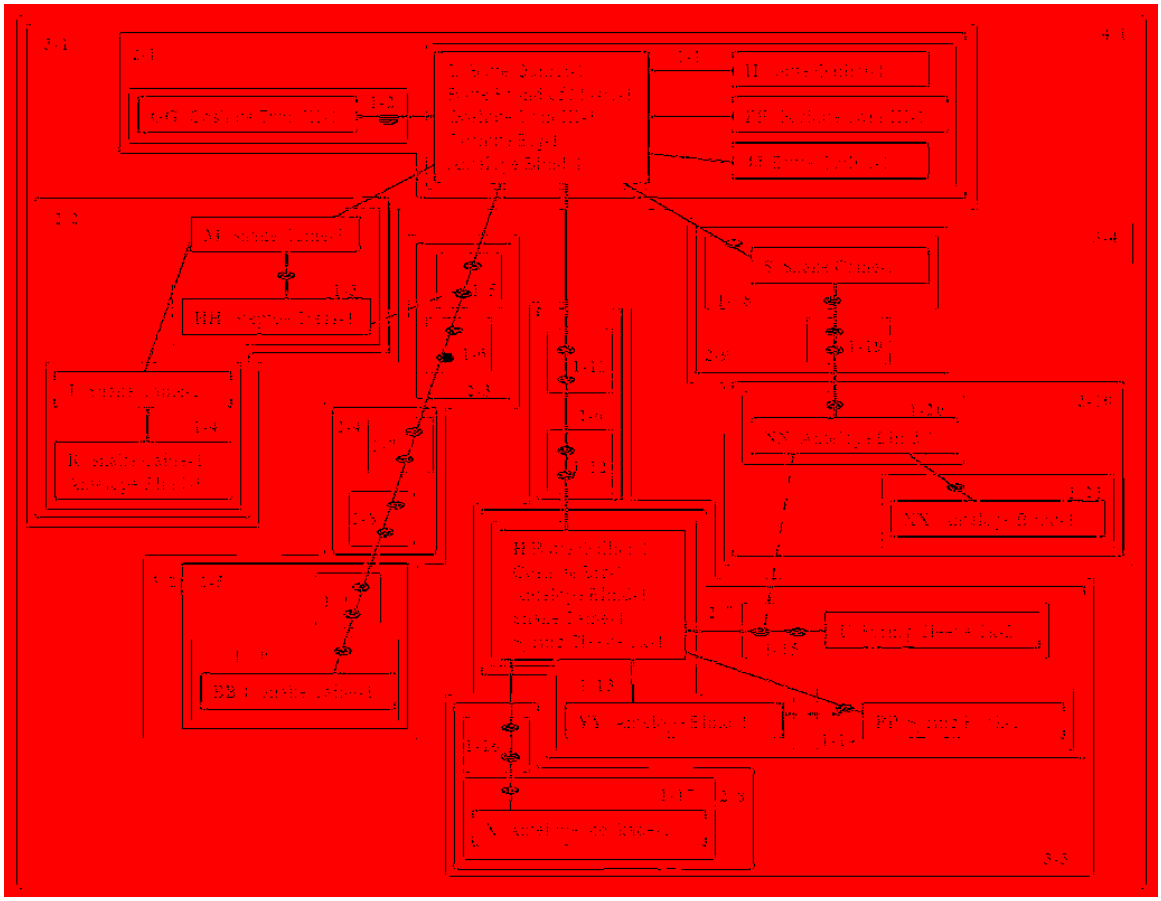

FIG. 16

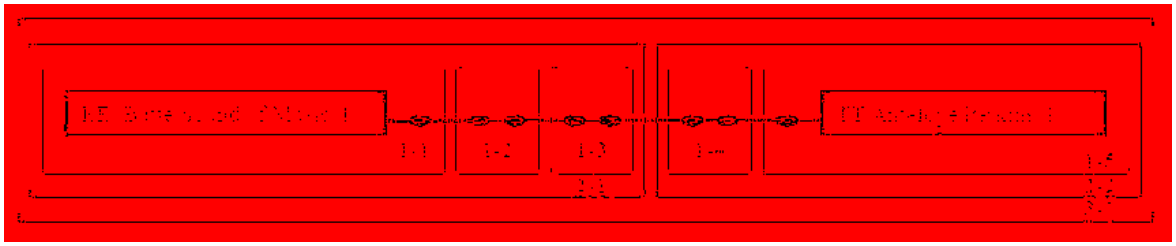

FIG. 17

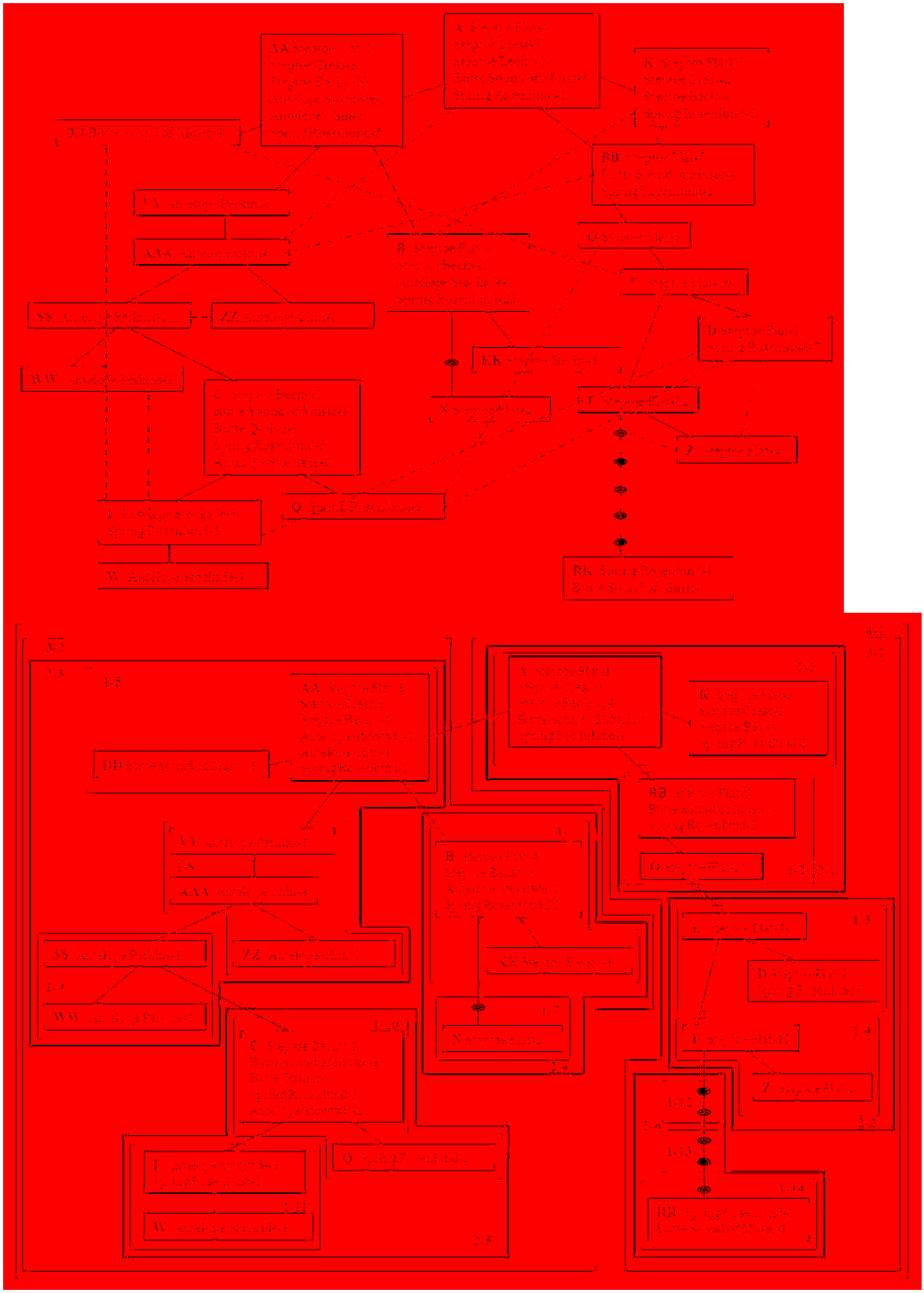

FIG. 18

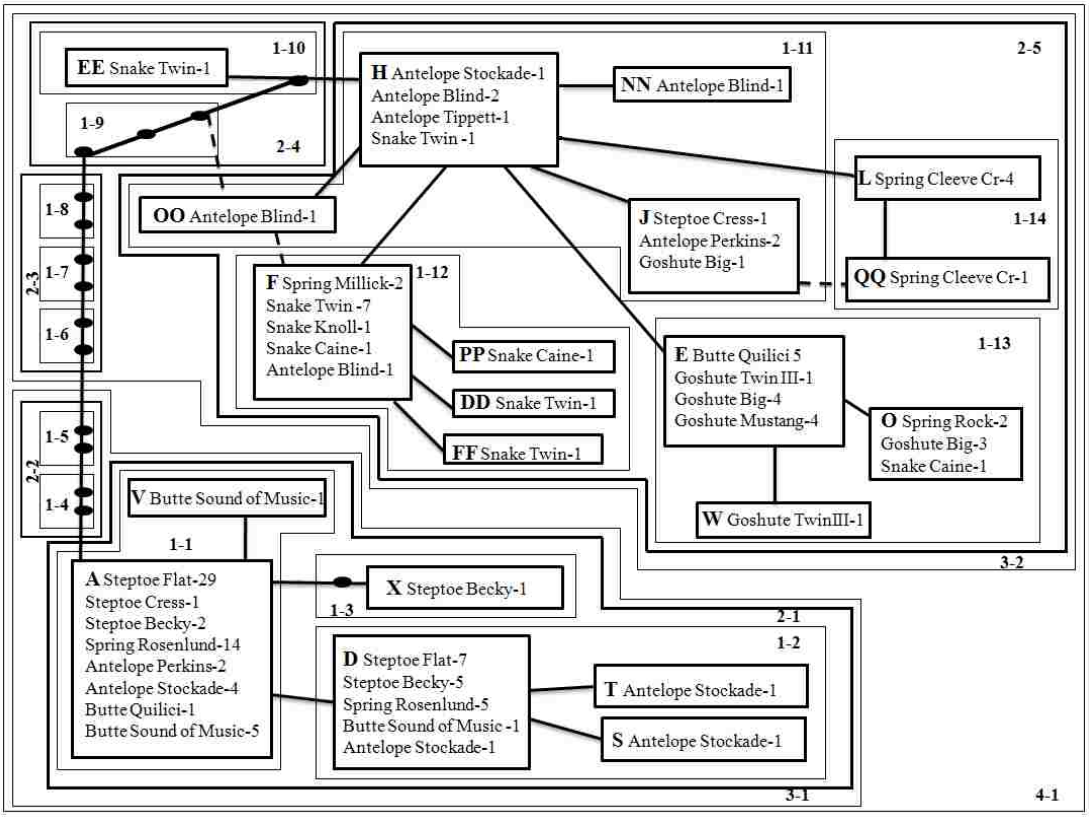
EE Snake Twin-1
1-10
1-9
2-4
H Antelope Stockade-1
Antelope Blind-2
Antelope Tippett-1
Snake Twin -1
1-11
NN Antelope Blind-1
2-5
L Spring Cleeve Cr-4
1-14
QQ Spring Cleeve Cr-1
1-8
1-7
1-6
2-3
OO Antelope Blind-1
J Steptoe Cress-1
Antelope Perkins-2
Goshute Big-1
F Spring Millick-2
Snake Twin -7
Snake Knoll-1
Snake Caine-1
Antelope Blind-1
1-12
PP Snake Caine-1
DD Snake Twin-1
FF Snake Twin-1
E Butte Quilici 5
Goshute Twin III-1
Goshute Big-4
Goshute Mustang-4
1-13
O Spring Rock-2
Goshute Big-3
Snake Caine-1
W Goshute TwinIII-1
3-2
1-5
1-4
2-2
V Butte Sound of Music-1
1-1
A Steptoe Flat-29
Steptoe Cress-1
Steptoe Becky-2
Spring Rosenlund-14
Antelope Perkins-2
Antelope Stockade-4
Butte Quilici-1
Butte Sound of Music-5
X Steptoe Becky-1
1-3
2-1
1-2
D Steptoe Flat-7
Steptoe Becky-5
Spring Rosenlund-5
Butte Sound of Music -1
Antelope Stockade-1
T Antelope Stockade-1
S Antelope Stockade-1
3-1
4-1

FIG. 19

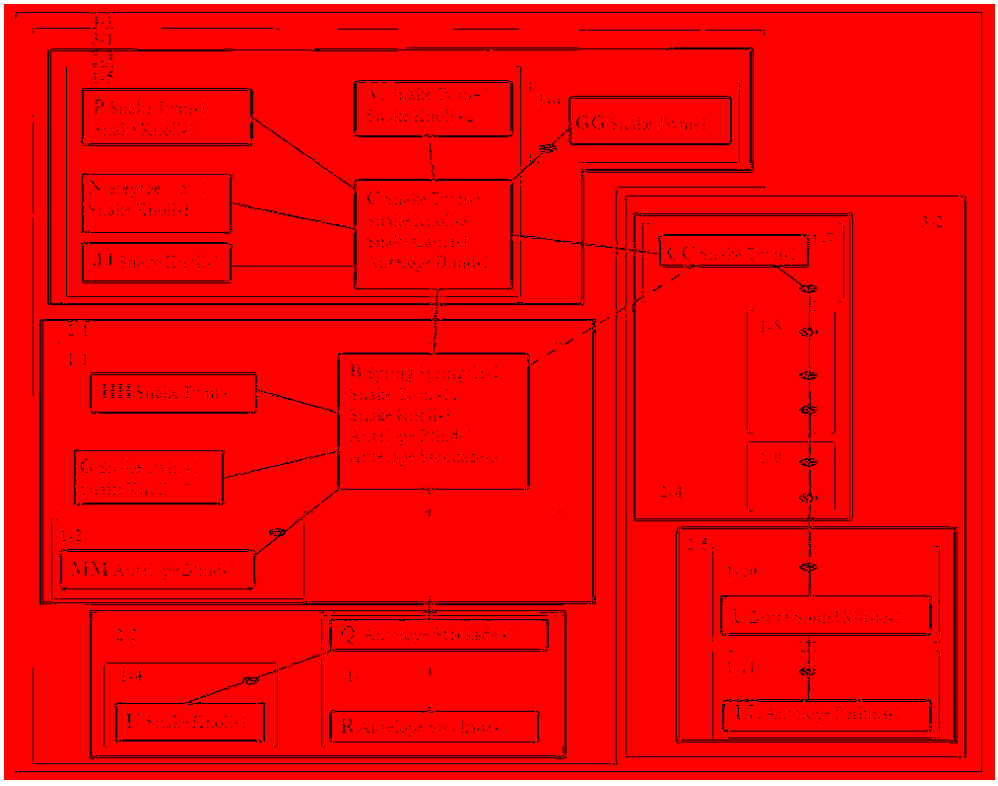

FIG. 20

Tables

TABLE 1.

Hydrographic Basin	Population ID	Spring	GPS coordinates
Snake Valley	7	Twin Springs I	39º24'12.5"N 113º51'46.5"W
	8	Knoll Springs	39º14'26.7"N 113º52'41.4"W
	9/33	Leland Harris Springs	39º33'11.5"N 113º53'47.6"W
	13/31	Caine Spring	39º08'19.4"N 114º02'55.6"W
	16/26	Warm Springs	39º27'35.7"N 114º02'13.5"W
Spring Valley	4	Unnamed spring by Spring Ck	38º57'28.0"N 114º24'32.1"W
	5/35	Rock Spring	39º10'51.9"N 114º22'29.9"W
	6	Unnamed spring by Rosenlund Ranch	39º50'15.5"N 114º33'42.4"W
	17/32	Unnamed spring east of Cleeve Ck	39º12'11.9"N 114º27'44.6"W
	18	North Millick Spring	39º18'09.4"N 114º23'19.3"W
Steptoe	1/29	Indian Springs	39º54'17.0"N 114º40'59.4"W
Valley	3/30	Flat Spring	40º04'00.2"N 114º28'51.1"W
	21	Becky Spring	40º03'49.3"N 114º35'08.1"W
	40	Cress Spring	40°03'12.3"N 114°29'29.1"W
Antelope	10	Perkins Spring	40º07'20.6"N 114º24'47.3"W
Valley	11/34	Blind Spring	39º50'24.9"N 114º22'16.7"W
	12/25	Unnamed Spring by Chin Ck	40º00'55.7"N 114º22'50.0"W
	14/24	Tippett Spring	39º52'37.0"N 114º22'23.5"W
	15/27	Stockade Spring	40º03'33.1"N 114º23'38.4"W
Goshute	23	Big Spring	40º59'03.7"N 114º30'16.3"W
Valley	36	Currie Gardens	40º15'19.5"N 114º 49'02.4"W
	38	Twin Springs III	40º21'08.5"N 114º49'45.3"W
	39	Mustang Spring	40º17'42.4"N 114º45'30.0"W
	41	Unnamed ("Horse") spring	40º38'37.9"N 114º51'50.9"W
Butte Valley	19	Twin Springs II	40°10'10.5"N 114°59'23.0"W
	20	Quilici Spring	40°19'51.5"N 115°03'33.4"W
	22	Unnamed ("S") spring	40°11'06.6"N 114°59'18.9"W
	37	Unnamed ("Sound of Music") spring	40°01'49.4"N 114°53'54.2"W

TABLE 2.

Species	Gene	Primer Sequence
H. azteca	COI	LCO: 5'-GGTCAACAAATCATAAAGATATTGG-3'
		HCO: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'
	28S	5587R: 5'-TAGCGCAGTCATTCGATCGGAGTT-3'
		5587F: 5'-GCCCCAGCCAAATGCAAAGAAAAA-3'
		4434R: 5'-CCAGCTATCCTGAGGGAAACTTCG-3'
		3311F: 5'-GGGACTACCCCCTGAATTTAAGCAT-3'
		Rnest: 5'-ATGCTATACTCCTTGGCCCGTGTT-3'
		Fnest: 5'-ACCGTGAAACCGCTCAGAGTACAA-3'
C. americanus	COI	NR: 5'-AAGATTGTTAATTCGAGCTGAATT-3'
		NF: 5'-ATACCAGCTAAGTGTAATGAAAAG-3'
	16S	S2: 5'-GGAGCTCCGGTTTGAACTCAGATC-3'
		16Sar: 5'-CGCCTGTTTAACAAAAACAT-3'

TABLE 3.

Pop. ID	1	3	6	9	12	13	14	17	19	20	22
1	0.00000										
3	0.21334	0.00908									
6	0.21862	0.10627	0.00820								
9	0.17897	0.15619	0.09825	0.12863							
12	0.21048	0.02085	0.10383	0.15216	0.00455						
13	0.08685	0.13092	0.17274	0.16179	0.12732	0.14985					
14	0.21139	0.01948	0.10291	0.15141	0.00319	0.12732	0.00364				
17	0.20595	0.07169	0.09768	0.15038	0.06648	0.15023	0.06694	0.02332			
19	0.19941	0.11402	0.11430	0.16747	0.11840	0.17146	0.11840	0.10155	0.00729		
20	0.22591	0.07978	0.09722	0.15501	0.08625	0.16904	0.08625	0.10040	0.07710	0.00273	
22	0.20730	0.10278	0.10236	0.15871	0.10820	0.16900	0.10820	0.09919	0.02327	0.05894	0.03520

TABLE 4.

Pop. ID	6	8	12	19	20	23	24	27	29	30	31	32	33	34	35	36	38	41
6	0.00000																	
8	0.02629	0.00000																
12	0.00449	0.02638	0.00000															
19	0.00336	0.02522	0.00336	0.00000														
20	0.00336	0.02522	0.00336	0.00224	0.00000													
23	0.00392	0.02580	0.00393	0.00280	0.00056	0.00112												
24	0.00505	0.02696	0.00056	0.00393	0.00393	0.00449	0.00112											
27	0.01477	0.02852	0.01478	0.01364	0.01365	0.01423	0.01535	0.00112										
29	0.01477	0.02852	0.01477	0.01364	0.01364	0.01422	0.01535	0.00056	0.00000									
30	0.00224	0.02405	0.00224	0.00112	0.00112	0.00168	0.00280	0.01250	0.01250	0.00000								
31	0.02165	0.00447	0.02172	0.02056	0.02057	0.02115	0.02230	0.02443	0.02385	0.01941	0.00000							
32	0.00224	0.02405	0.00224	0.00112	0.00112	0.00168	0.00280	0.01250	0.01250	0.00000	0.01941	0.00000						
33	0.00000	0.02629	0.00449	0.00336	0.00336	0.00392	0.00505	0.01477	0.01477	0.00224	0.02165	0.00224	0.00000					
34	0.02224	0.00503	0.02230	0.02115	0.02115	0.02173	0.01367	0.02501	0.02443	0.01999	0.00056	0.01999	0.02224	0.00111				
35	0.02512	0.00558	0.02519	0.02404	0.02404	0.02462	0.02578	0.02792	0.02734	0.02287	0.00334	0.02287	0.02512	0.00390	0.00000			
36	0.01477	0.02852	0.01477	0.01364	0.01364	0.01422	0.01535	0.00056	0.00000	0.01250	0.02385	0.01250	0.01477	0.02443	0.02734	0.00000		
38	0.01477	0.02852	0.01477	0.01364	0.01364	0.01422	0.01535	0.00056	0.00000	0.01250	0.02385	0.01250	0.01477	0.02443	0.02734	0.00000	0.00000	
41	0.00224	0.02405	0.00224	0.00112	0.00112	0.00168	0.00280	0.01250	0.01250	0.00000	0.01941	0.00000	0.00224	0.01999	0.02287	0.01250	0.01250	0.00000

TABLE 5.

Pop. ID	1	3	6	9	13	12	14	17	19	20	22
1	0										
3	0.9709	0									
6	0.9909	0.9508	0								
9	0.5425	0.6002	0.1694	0							
13	0*	0.7649	0.7335	0.5601	0						
12	0.9908	0.8507	0.9801	0.7480	0.8686	0					
14	0.9895	0.8001	0.9778	0.6330	0.7941	0.0090	0				
17	0.9747	0.9154	0.9536	0.6562	0.8072	0.9499	0.9361	0			
19	0.9775	0.9466	0.9679	0.5551	0.7259	0.9800	0.9751	0.9489	0		
20	0.9960	0.9494	0.9861	0.5648	0.7789	0.9791	0.9791	0.9603	0.9685	0	
22	0.9796	0.9524	0.9628	0.6695	0.8132	0.9754	0.9696	0.9517	0.1041	0.9535	0

TABLE 6.

Pop. ID	6	8	12	19	20	23	14	27	29	30	31	32	33	34	35	36	38	41
6	0																	
8	1	0																
12	1	1	0															
19	1	1	1	0														
20	1	1	1	1	0													
23	0.9719	0.9855	0.9101	0.9270	0.4000	0												
14	0.9873	0.9959	0*	0.9715	0.9543	0.9279	0											
27	0.9787	0.9546	0.9541	0.9706	0.9381	0.9496	0.9717	0										
29	1	1	1	1	1	0.9903	0.9956	0.2350	0									
30	1	1	1	1	1	0.8735	0.9558	0.9669	1	0								
31	1	1	1	1	1	0.9956	0.9978	0.9838	1	1	0							
32	1	1	1	1	1	0.8640	0.9536	0.9644	1	0	1	0						
33	0	1	1	1	1	0.9479	0.9811	0.9622	1	1	1	1	0					
34	0.9967	0.9130	0.9924	0.9961	0.9882	0.9839	0.9932	0.9594	0.9955	0.9956	0.8614	0.9951	0.9930	0				
35	1	1	1	1	1	0.9890	0.9962	0.9642	1	1	1	1	1	0.9205	0			
36	1	1	1	1	1	0.9729	0.9923	0*	0	1	1	1	1	0.9820	1	0		
38	1	1	1	1	1	0.9896	0.9954	0.2145	0	1	1	1	1	0.9951	1	0	0	
41	1	1	1	1	1	0.8817	0.9579	0.9691	1	0	1	0	1	0.9959	1	1	1	0

TABLE 7.

Pop. ID	3	5	6	10	11	12	13	15	17	20	21	23	37	38	40
3	0.02085														
5	0.13808	0.17084													
6	0.02626	0.13540	0.00360												
10	0.10267	0.16385	0.08471	0.11505											
11	0.14181	0.13009	0.12830	0.13088	0.09724										
12	0.07053	0.13589	0.12759	0.11083	0.14809	0.08899									
13	0.14202	0.12998	0.12759	0.13342	0.08374	0.14603	0.09209								
15	0.07630	0.14665	0.05688	0.09948	0.12564	0.08917	0.12709	0.09402							
17	0.12318	0.09247	0.10986	0.14555	0.06937	0.13212	0.06311	0.11965	0.00822						
20	0.11002	0.10816	0.09409	0.13131	0.07899	0.11767	0.06967	0.10922	0.03436	0.05322					
21	0.04309	0.13894	0.00598	0.08538	0.13003	0.04844	0.12860	0.05813	0.11321	0.09556	0.00569				
23	0.12552	0.09736	0.11158	0.14140	0.06511	0.13194	0.05493	0.11920	0.01102	0.02921	0.11353	0.01375			
37	0.05125	0.13782	0.03030	0.09401	0.12652	0.06576	0.12412	0.07096	0.10897	0.09468	0.03045	0.10806	0.05137		
38	0.13141	0.10588	0.11647	0.14124	0.06618	0.13516	0.12211	0.12211	0.02073	0.03024	0.11703	0.00963	0.11079	0.00547	
40	0.04837	0.13477	0.05529	0.09525	0.11663	0.06225	0.11115	0.06924	0.09585	0.08076	0.02529	0.09180	0.04310	0.06877	0.04560

TABLE 8.

Pop. ID	3	4	5	6	7	8	10	11	13	14	15	17	18	20	21	23	37	38	39	40
3	0.06721																			
4	0.07065	0.00000																		
5	0.10843	0.12477	0.06000																	
6	0.03391	0.10412	0.10048	0.00185																
7	0.06453	0.04075	0.11299	0.07592	0.03402															
8	0.06861	0.01251	0.12241	0.09620	0.04447	0.02383														
10	0.05452	0.07433	0.10378	0.04468	0.06391	0.07250	0.07805													
11	0.06169	0.05909	0.10663	0.06245	0.05546	0.05942	0.05783	0.06074												
13	0.05896	0.07723	0.10041	0.04982	0.05982	0.07364	0.05325	0.05025	0.05472											
14	0.05383	0.10306	0.09158	0.02921	0.06577	0.09465	0.04368	0.04587	0.02861	0.00000										
15	0.05152	0.06644	0.11010	0.04442	0.06297	0.06603	0.05718	0.06063	0.05896	0.05477	0.06273									
17	0.05462	0.10337	0.09110	0.03024	0.06633	0.09503	0.04415	0.04621	0.02939	0.00093	0.05553	0.00185								
18	0.05440	0.10093	0.09110	0.03114	0.06431	0.09254	0.04423	0.04522	0.02762	0.00185	0.05517	0.00278	0.00000							
20	0.04398	0.10200	0.09510	0.01603	0.07004	0.09404	0.04467	0.05373	0.03869	0.01505	0.04965	0.01603	0.01694	0.03017						
21	0.03444	0.10449	0.10073	0.00154	0.07603	0.09650	0.04480	0.06242	0.04967	0.02889	0.04491	0.02991	0.03081	0.01603	0.00247					
23	0.05531	0.10236	0.09105	0.03178	0.06629	0.09427	0.04589	0.04699	0.02937	0.00247	0.05610	0.00278	0.00433	0.01633	0.03146	0.00371				
37	0.04457	0.08204	0.10649	0.02718	0.06965	0.07869	0.05142	0.06368	0.05839	0.04844	0.05180	0.04956	0.04935	0.03855	0.02766	0.05108	0.05311			
38	0.11362	0.14213	0.14487	0.09936	0.11842	0.13721	0.10552	0.10525	0.09361	0.07516	0.11360	0.07559	0.07557	0.08695	0.09899	0.07571	0.11159	0.14974		
39	0.05441	0.10094	0.09079	0.03114	0.06517	0.09292	0.04564	0.04602	0.02855	0.00185	0.05517	0.00278	0.00371	0.01509	0.03082	0.00185	0.05042	0.07476	0.00000	
40	0.04434	0.10306	0.09483	0.01603	0.07069	0.09500	0.04405	0.05419	0.03942	0.01505	0.05005	0.01510	0.01694	0.01601	0.01602	0.01695	0.03856	0.08735	0.01694	0.03017

TABLE 9.

Pop. ID	3	5	6	10	11	12	13	15	17	20	21	23	37	38	40
3	0														
5	0.8242	0													
6	0.1480	0.8935	0												
10	0.6118	0.2393	0.6952	0											
11	0.7462	0.0929	0.8021	0.2018	0										
12	0.3940	0.1481	0.4784	0.1434	0.3647	0									
13	0.7415	0.2126	0.7860	0.2745	0*	0.4247	0								
15	0.2334	0.3388	0.3111	0.1200	0.3387	0*	0.3962	0							
17	0.8816	0.2814	0.9379	0.4900	0.0901	0.5958	0.1636	0.5277	0						
20	0.7967	0.1734	0.8648	0.3705	0.0214	0.4072	0.0333	0.3772	0.0796	0					
21	0.3350	0.9235	0.1891	0.7114	0.8129	0.5172	0.7902	0.3743	0.9685	0.8966	0				
23	0.8772	0.0469	0.9347	0.3938	0*	0.4988	0*	0.4729	0.3077	0*	0.9660	0			
37	0.1603	0.6059	0.1566	0.3503	0.5344	0.0438	0.5502	0.0344	0.7366	0.5753	0.1667	0.7087	0		
38	0.8882	0.3684	0.9397	0.4713	0.0533	0.6067	0.0088	0.5443	0.7500	0*	0.9687	0.1000	0.7406	0	
40	0.2312	0.6551	0.1614	0.3648	0.5286	0.0744	0.5403	0.0758	0.8287	0.6210	0.0795	0.7989	0*	0.8272	0

TABLE 10.

Pop. ID	3	4	5	6	7	8	10	11	13	14	15	17	18	20	21	23	37	38	39	40
3	0																			
4	0.9431	0																		
5	0.7995	0.5516	0																	
6	0*	0.9927	0.7812	0																
7	0.6805	0*	0.5140	0.6559	0															
8	0.9246	0*	0.8251	0.9411	0.1782	0														
10	0.4574	0.5384	0.2970	0.4963	0.3258	0.8011	0													
11	0.7477	0*	0.4908	0.7228	0*	0.214	0.3087	0												
13	0.7270	0.5017	0.2733	0.7663	0.2377	0.7900	0*	0.2222	0											
14	0.8106	1	0.0776	0.9732	0.3864	0.9099	0*	0.3694	0*	0										
15	0.4138	0.2985	0.3739	0.3866	0.1915	0.5871	0*	0.1701	0.0554	0*	0									
17	0.8805	0.9981	0.7441	0.9838	0.6295	0.9434	0.5355	0.6978	0.5112	0.8947	0.5577	0								
18	0.8327	1	0.3186	0.9763	0.4659	0.9129	0*	0.4679	0*	1	0.2201	0.9495	0							
20	0.7586	0.9160	0.4773	0.8816	0.5179	0.9062	0.0996	0.5364	0.0584	0*	0.2961	0.6028	0.1241	0						
21	0.0180	0.9832	0.6423	0.1586	0.5983	0.9252	0.2969	0.6336	0.5962	0.9342	0.2585	0.9771	0.9464	0.7958	0					
23	0.8488	0.9828	0.5729	0.9646	0.5571	0.9239	0.3172	0.5931	0.2001	0.2468	0.4169	0.8313	0.6439	0.0544	0.9395	0				
37	0.0428	0.7594	0.4978	0.1100	0.4854	0.8556	0.0198	0.4939	0.3060	0.3185	0.0789	0.7835	0.4811	0.4692	0.0288	0.6570	0			
38	0.8603	0.5495	0.5406	0.8223	0.6659	0.8418	0.5105	0.6277	0.4832	0.3276	0.5726	0.8	0.4749	0.6196	0.7069	0.6795	0.6367	0		
39	0.8327	1	0.3107	0.9763	0.4720	0.9130	0*	0.4701	0*	1	0.2201	0.9495	1	0*	0.9464	0*	0.4866	0.4799	0	
40	0.4657	0.8491	0.2664	0.752	0.4460	0.8975	0*	0.4367	0*	0*	0*	0.8246	0.1111	0.0328	0.5429	0.5723	0*	0.4619	0.1111	0

Appendix A

Nested clade phylogeographical analyses of *H. azteca* and *C. americanus* networks with average clade distances (D_c) and nested clade distances (D_n) for each haplotype and interior-tip distances (I-T) for each clade. Significantly large and small distances at the α=0.05 level are denoted (^{L}) and (^{S}), respectively.

Species	Dataset	Network	Clade	Topology	Haplotype	D_c	D_n
H. azteca	COI	A	3-6	Interior	2-11	0	57.8
				Tip	2-12	0	7.24
					I-T	0	50.56
			4-3	Interior	3-5	0^S	32.49^S
				Tip	3-6	12.85^S	32.54^L
					I-T	-12.85^S	-0.05^S
			5-1	Tip	4-1	0^S	38.83^L
				Interior	4-2	0	38.83
				Tip	4-3	32.52^S	37.82
					I-T	-12.51^L	0.39
		B	3-1	Tip	2-1	0^S	23.95^L
				Tip	2-2	0^S	23.94^S
		C	1-1	Interior	A	39.41	39.16
				Tip	F	0	33.98
					I-T	39.41	5.17
			1-2	Interior	JJ	7.51	7.46
				Interior	KK	7.31	7.36
			1-6	Interior	B	54.36	54.64
				Tip	O	0	56.22
					I-T	54.36	-1.58
			2-1	Tip	1-1	39.07	35.84
				Interior	1-2	7.4	17.44
					I-T	-31.66	-18.40
			2-3	Interior	1-6	54.73	55.14
				Tip	1-7	0	40.79
				Tip	1-8	0	40.79
					I-T	54.73	14.36
			3-1	Tip	2-1	30.18	30.68^S
				Interior	2-2	0	35.21
				Tip	2-3	54.20^L	49.81
					I-T	-33.46	1.55
		D	2-3	Interior	1-5	0	3.43
				Tip	1-6	0	97.55
					I-T	0	-94.12
			3-1	Tip	2-1	0	1.05
				Tip	2-3	6.62	4.41
		E	3-1	Tip	2-1	0	8.63
				Tip	2-2	0	8.63
		F	1-1	Tip	FF	0	0.7
				Interior	II	0.85	0.84

Species	Dataset	Network	Clade	Topology	Haplotype	D_c	D_n
					I-T	0.85	0.15
			2-1	Tip	1-1	0.83^S	0.87
				Tip	1-3	0	0.87
	28S	A	1-1	Interior	E	26.46^S	41.35^L
				Tip	J	0	41.33^S
					I-T	26.46	0.02^L
			2-1	Interior	1-1	41.34^L	42.21^L
				Tip	1-2	0	24.7^S
					I-T	41.34^L	17.51^L
			3-1	Tip	2-1	36.37^L	36.21^L
				Tip	2-2	0	27.77
		B	1-1	Interior	B	65.15^L	67.35^L
				Tip	L	0^S	35.19^S
					I-T	65.15^L	32.15^L
			1-2	Interior	D	7.7	7.71
				Tip	H	0	7.71
					I-T	7.7	-0
			1-5	Interior	C	32.38	43.18
				Tip	G	0	43.13
					I-T	32.38	0.05
			2-1	Interior	1-1	59.31^L	58.83
				Tip	1-2	7.71^S	30.95^S
				Tip	1-5	43.16	67.44
					I-T	40.25^L	16.21^L
			2-7	Interior	1-14	1305.14	1275.9
				Tip	1-15	0	824.26
					I-T	1305.14	451.64
			3-1	Tip	2-1	54.01	53.85
				Tip	2-2	32.52^S	52.15
			4-1	Tip	3-1	53.51	237.71
				Tip	3-3	1243.64	949.34
C. americanus	COI	A	1-1	Interior	J	55.66	55.11
				Tip	MM	0	14.23
					I-T	55.66	40.89
			2-2	Interior	1-3	0	51.76
				Tip	1-4	0	20.77
				Tip	1-5	39.69	45.50
					I-T	-29.77	12.44
			3-1	Tip	2-1	46.03	43.54
				Interior	2-2	41.40	36.67
				Tip	2-3	0	33.17
					I-T	3.04	-5.15
			4-1	Tip	3-1	38.37	35.92
				Interior	3-2	0	24.15
				Tip	3-3	0	24.15

Species	Dataset	Network	Clade	Topology	Haplotype	D_c	D_n
					I-T	-35.17	-10.79
		B	1-1	Interior	G	40.16	39.58
				Tip	FF	0	3.88
				Tip	II	0	19.45
				Tip	JJ	0	19.45
					I-T	40.16	25.32
			1-3	Interior	M	0	83.42
				Tip	HH	0	25.01
					I-T	0	58.41
			1-4	Interior	T	0	37.60
				Tip	R	38.76	42.28
					I-T	-38.76	-4.68
			1-13	Interior	H	82.79	82.99
				Tip	YY	0	33.00
					I-T	82.79	49.99
			2-1	Interior	1-1	32.46	32.45
				Tip	1-2	0	3.50
					I-T	32.46	28.95
			2-2	Interior	1-3	38.49	46.15
				Tip	1-4	37.97	49.32
					I-T	0.52	-3.17
			2-7	Interior	1-13	77.50	85.10^{L}
				Tip	1-14	0	50.40
				Tip	1-15	0	47.33
					I-T	77.50^{L}	36.75
			3-1	Interior	2-1	29.63^{S}	40.55
				Tip	2-2	46.99	53.69
					I-T	-17.36	-13.15
			3-3	Interior	2-7	66.29	70.05
				Tip	2-8	0	36.64
					I-T	66.29	33.41
			3-4	Interior	2-9	0	51.69
				Tip	2-10	0	31.00
					I-T	0	20.69
			4-1	Interior	3-1	45.03	52.65
				Tip	3-2	0	96.32
				Tip	3-3	62.06	65.36
				Tip	3-4	38.76	46.75
					I-T	-6.68	-9.81
		C	3-1	Tip	2-1	0	21.26
				Tip	2-2	0	21.25
		D	1-1	Interior	A	13.37	12.35
				Tip	K	5367	9.80
					I-T	7.70	2.56
			1-2	Interior	BB	17.02	17.97
				Interior	O	0	19.76
			1-3	Interior	E	0	14.13^{L}
				Tip	D	6.62^{S}	12.50^{S}

Species	Dataset	Network	Clade	Topology	Haplotype	D_c	D_n
					I-T	-6.62	1.63^{L}
			1-5	Interior	AA	13.02	12.27
				Tip	DD	0	35.45^{L}
					I-T	12.43	-23.11^{S}
			1-6	Interior	B	13.53	13.55
				Tip	KK	0	13.10
					I-T	13.53	0.45
			1-8	Interior	VV	0	21.04
				Interior	AAA	0	10.52
				Tip	ZZ	0	10.52
					I-T	0	5.26
			1-10	Interior	C	24.80	25.25
				Tip	Q	0	49.95
					I-T	24.80	-24.71
			1-11	Interior	I	2.95	5.62
				Tip	W	0	1.19
					I-T	2.95	1.43
			2-1	Interior	1-1	11.61	12.66^{S}
				Interior	1-2	18.23^{L}	18.98^{L}
			2-2	Interior	1-3	11.90^{L}	10.62^{L}
				Interior	1-4	0^{S}	4.95^{S}
			2-3	Interior	1-5	13.54	14.42
				Interior	1-8	0	18.56
				Interior	1-9	0	14.03
			2-4	Interior	1-6	13.54	13.58
				Tip	1-7	0	12.95
					I-T	13.54	0.62
			2-5	Interior	1-10	25.92	29.92^{S}
				Tip	1-11	2.27^{S}	34.64
					I-T	23.64^{L}	-4.72
			3-1	Interior	2-1	14.20	13.58
				Interior	2-2	8.04^{S}	13.08
				Tip	2-6	12.78	24.37^{L}
					I-T	-1.01^{S}	-10.99^{S}
			3-2	Interior	2-3	15.26	18.16
				Tip	2-4	13.55	14.68^{S}
				Tip	2-5	31.08	34.02
					I-T	-3.71	-2.49
			4-1	Tip	3-1	13.77	12.97^{S}
				Tip	3-2	23.07	23.36
	16S	A	1-1	Interior	A	16.65	16.95
				Tip	V	0	28.00
					I-T	16.65	-11.05
			1-2	Interior	D	12.28	12.37
				Tip	S	0	11.39

Species	Dataset	Network	Clade	Topology	Haplotype	D_c	D_n
				Tip	T	0	11.39
					I-T	12.28	0.99
			1-11	Interior	H	8.68	15.92
				Tip	J	20.16	24.33
				Tip	NN	0	16.10
					I-T	-7.44	-6.77
			1-12	Interior	F	24.49	24.63
				Tip	DD	0	21.12
				Tip	FF	0	21.12
				Tip	OO	0	62.86
				Tip	PP	0	20.33
					I-T	24.49	-6.72
			1-13	Interior	E	26.11^{S}	44.39^{S}
				Tip	O	66.34	107.04^{L}
				Tip	W	0	32.28
					I-T	-30.75^{S}	-51.98^{S}
			2-1	Interior	1-1	17.28^{L}	16.96^{L}
				Tip	1-2	12.12	12.34
				Tip	1-3	0	4.02
					I-T	5.71	4.99
			2-5	Interior	1-11	19.01^{S}	33.63^{S}
				Tip	1-12	25.79^{S}	56.08
				Tip	1-13	65.78	85.26
				Tip	1-14	0^{S}	56.33
					I-T	-22.55^{S}	-36.34^{S}
			3-2	Interior	2-4	0	56.85
				Tip	2-5	61.66	61.65
					I-T	-61.66	-4.80
			4-1	Tip	3-1	15.41	30.11
				Tip	3-2	61.62	62.60
		B	1-1	Interior	B	51.0	50.91
				Tip	G	4.30^{S}	37.94
				Tip	HH	0	32.18
					I-T	47.18^{L}	13.61
			1-5	Interior	C	18.67	40.76
				Tip	M	8.33	36.32
				Tip	N	6.78	64.63^{L}
				Tip	P	9.04	34.42
				Tip	JJ	0	40.35
					I-T	11.59	-2.67
			2-1	Interior	1-1	49.63	49.72
				Tip	1-2	0	42.40
					I-T	49.63	7.32
			2-2	Tip	1-3	0	8.01
				Tip	1-4	0	93.03
			2-3	Tip	1-5	48.39	48.32

Species	Dataset	Network	Clade	Topology	Haplotype	D_c	D_n
				Tip	1-6	0	27.92
			2-5	Interior	1-10	0	21.26
				Tip	1-11	0	21.25
					I-T	0	0.01
			3-1	Interior	2-1	49.46	49.47
				Tip	2-2	14.76	59.74
				Tip	2-3	48.06	48.92
					I-T	4.17	-0.36
			3-2	Interior	2-4	0	96.95
				Tip	2-5	21.25	21.32
					I-T	-21.25	77.63
			4-1	Tip	3-1	49.87^S	51.49^S
				Tip	3-2	22.79	59.62

Appendix B

The Great Basin is a semi-arid region lying between the Sierra Nevadas on the west and the Wasatch Range on the east with the Colorado River bordering the south and the Columbia plateau to the north. It is characterized by isolated basins of broad sagebrush plains numbering around 90 and separated by about 160 juniper covered mountain ranges (Blackwelder 1948). The mountains seen today formed during the Pleistocene epoch, and rivers flowing from the ends of late Pleistocene glaciers formed lakes in the valleys. At the end of the last glacial age, temperatures rose and these lakes dried up (5500-500BC). Today's aquatic environments consist of broken streams and rivers, isolated springs, playas, and salt lakes. However, evidence exists for early pluvial connections between the following basins: Steptoe and Goshute, Steptoe and Butte, Steptoe and Antelope, and possibly Steptoe and Spring (Hubbs and Miller 1948).

Many endemic species exist in the desert springs and wetlands of the Great Basin (least chub, Perkins et al. 1998; springsnails, Hershler and Sada 2002; hemipterans, Polhemus and Polhemus 2002; pupfish, Smith et al. 2002). In addition to endemics, many cryptic species also exist in these springs (Witt et al. 2006), and all are potentially threatened by groundwater exploitation. For almost the past two decades, the Southern Nevada Water Authority (SNWA) has been claiming water rights to deep water in the carbonate aquifers of southeastern and central Nevada. These claims were based on the presumption that the deep water in the Great Basin represented untapped water resources from the Pleistocene that could be mined. This assumption was based on work by the U.S. Geological Survey and others hypothesizing that the underlying limestone of Nevada is fractured and that these fractures allow water to flow between isolated surface

basins of the Great Basin (Winograd and Eakin 1965, Harrill 1986). They further assumed that this deep water had no connection to surface waters, and thus removal of the deep water could be accomplished with no impact on existing surface water rights. These data do not necessarily support the hypothesis that spring discharge is controlled by local recharge and interbasin transfers rather than recharge from deep aquifers.

To the contrary, a study of deuterium (δD) and oxygen ($\delta^{18}O$) isotopes in spring discharge and of the bedrock structure in Death Valley shows that neither modern local recharge nor interbasin flow can account for observed spring fluxes (Anderson et al. 2006). Instead, the springs have a large component of old water, suggesting that recharge occurred during a pluvial period. If this pattern reflects aquifers elsewhere in the Great Basin, then surface waters are intermingling with deep waters. Mining of deep water could therefore impact water table levels. In addition, effects of this groundwater pumping from Snake and Spring valleys were simulated, and a groundwater decline of greater than 100 feet was predicted in addition to a possible reversal of local potentiometric gradients (Schaeffer and Harrill 1995). Therefore, discharge at existing springs may be reduced or go dry, and this poses many negative consequences to the native organisms living in the natural springs and wetlands of these areas. Recognized species as well as cryptic species found nowhere else may be lost due to the loss of their habitat.

The invertebrates studied were partly from two basins directly impacted from groundwater development including Snake and Spring valleys. The four neighboring basins, Steptoe, Antelope, Butte, and Goshute valleys, could offer immigrants. If the wetlands affected by groundwater pumping dry out, the poor invertebrate dispersers are

more likely to be lost. If wetland loss is recognized and redevelopment of the wetlands and springs is allowed, good dispersers have the greater potential to recolonize from either within a basin or from neighboring basins. I have attempted to identify genetically differentiated invertebrate populations in 6 basins of the Great Basin, make predictions of invertebrates that will be lost if wetlands dry out, and make predictions of invertebrates that will colonize dried wetlands from neighboring basins.