An investigation of the phylogenetic standing of *Cottus bairdi* (Cottidae) in Montana.

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INTRODUCTION

Cottus bairdii (Brown1971) is the only recognized cottid in the upper Missouri River drainage in Montana. Its presence in this region is not surprising since the species is abundant in both the eastern and western United States and the range of the western form of *Cottus bairdii* occurs in the Snake River Drainages adjacent to the southwest border of the Missouri headwaters. In addition, the eastern form of *Cottus bairdii* occurs as far west as Minnesota, and glacial periods could have easily provided access of the eastern form into the Missouri River headwaters. Robins (1954) suggested that *Cottus bairdii* in the upper Missouri River Drainage were the eastern form. Yet the status of *Cottus bairdii* is known to be complicated (Bailey and Bond 1963, Bisson and Bond 1971) and Jenkins and Burkhead (1993) specifically questioned the taxonomic standing of the *Cottus bairdii* of the upper Missouri system.

Cottids are difficult to identify. They have few consistent and easily recognized morphological characters. For that reason cottids are often field classified to the genus level, or if field identified, their species designation is based on their similarity to known local species. Even when specimens are taken to the laboratory for more careful examination, a high degree of variation in taxonomic characters, both within and between populations of the same species can complicate identification. Studies attempting to separate various species have often recognized that the informative characters are only useful at the regional level (e.g. Bailey and Bond 1963, Bisson and Bond 1971, Maughan 1978). Molecular techniques provide additional characters for separating species through genetic similarity/dissimilarity with allozymes (Strauss 1980, Grover 1992, Kinziger, Raesly, and Neely 2000) or with DNA based markers (Neely 2003, Crowley 2004, Kinziger and Wood 2003, Kinziger et al. 2005). In 1991 25 described species comprised the North American members of the genus *Cottus* (Committee on Names of Fishes1991). This number is now at approximately 42 species (Kinziger and Wood 2003).

STATEMENT OF PROBLEM

In a study of *Cottus bairdii* in the Basin and Range province of North America (see Crowley 2004), we examined a population of *C. bairdii* from the Judith River and cottids from the Yellowstone River, both in the upper Missouri River drainages of Montana. This study examined a portion of the ND-4 region of the mitochondrial genome. When the sequences were compared phylogenetically, the Montana specimens were clearly separated from the western *Cottus bairdii* clade.

Montana's Missouri River *Cottus bairdii* was not included in the major review of the genus *Cottus* provided by Kinziger et al (2005). Therefore the phylogenetic affiliations between it, other western *Cottus* species, and Eastern cottids of the *bairdii*, *cognatus*, and *carolinae* groups are unclear. Neely (2003) was the first to identify the upper Missouri River *Cottus bairdii* as a separate species and his study sahowed it most closely related to *Cottus rhotheus* and the western *Cottus bairdii*. Crowley (2004) also included the Missouri River *Cottus bairdii* in his thesis work and while his inference was based on a single mtDNA region (partial sequence of ND4) and the taxa diversity was great enough to insure that all pertinent phylogenetic relationships

were included, it clearly corroborates the findings of Neely (2003).

OBJECTIVES

This study focuses on two areas. First examining the potential sister taxa of Montana's Missouri River *Cottus bairdii* and second, examining the genetic structure within the Missouri River cottid populations. This project compared populations classified as *Cottus bairdii* in the headwaters of the Missouri River system with various *Cottus* species from the Columbia River drainage in western Montana and Idaho, the Colorado River Basin, several closed basins of the Great Basin, and cottids from the east-central United States. The taxa from the East-Central United States represent *Cottus cognatus*, *Cottus carolinae*, and the eastern form of *Cottus bairdii*. Western species represented *Cottus bairdii*, *Cottus beldingii*, *Cottus rhotheus*, and *Cottus confusus*. By including taxa from adjacent drainages both east and west of the Missouri River headwaters we hoped to be able to more completely clarify relationships among these taxa.

In addition, associations among populations of Montana's Missouri River *Cottus bairdii* are unknown. These cottids may have originated from a relatively recent dispersal event (late Pleistocene), and thus may show little structure or isolation between populations, or they could be relicts from older dispersal events and considerable genetic structure may exist between the populations. Standard population genetics (F-statistics) will be examined, and haplotype networks (e.g. TCS networks; Clement et al. 2000) can be analyzed with nested clade analysis (Templeton 1998, Posada et al. 2000) which tests discrete hypotheses about dispersal mechanisms.

MATERIALS AND METHODS

Locations of sampling sites

Since *Cottus bairdii* is reported from the entire upper Missouri River basin in Montana we examined fish from a broad cross section of the drainage (Table 1). Our objective was to insure that the same genetic lineage occurs in most of the basin. Therefore we did not examine high numbers of streams within any single Missouri River subbasin. The sampling risked missing some potential information, if multiple species of cottids exist in the system, especially if they have distinct microhabitat segregation. For example, in Utah, *Cottus beldingii* are most likely to be found in rapidly flowing freestone riffles, while *Cottus bairdii* are found in a much wider range of habitat (Shiozawa unpublished data). However since no evidence exists for more than one species in the upper Missouri region of Montana, the sampling was not designed for detecting additional species, since it would require a much higher sampling density.

Upper Missouri River cottid samples included the Jefferson, Madison, Musselshell, Milk, Marias, Judith, and Yellowstone river basins. Most of the samples were collected in 2004. We also obtained collections of cottids from the Kootenai (5 populations) and Clark Fork (2 populations) rivers of western Montana. These two systems are part of the Columbia River Basin (Table 2).

Table 1. Sampled populations from the upper Missouri River Basin in Montana.

Pop.#	Location	Basin	Collection date	BYU #	*Cottus* sp.
3229	Big Hole River- Pool, MT	Jefferson	20Oct2004	068341-068364	*C. bairdii* MT
3230	Big Hole River- Riffle, MT	Jefferson	20Oct2004	068285-068307	*C. bairdii* MT
3130	Duck Creek, MT	Madison	25May2004	059199-059238	*C. bairdii* MT
3147	Flatwillow Creek, S. Fk, MT	Musselshell	29Jun2004	059417-059449	*C. bairdii* MT
3227	Midvale Cr, MT, Two Medicine	Marias	11Aug2004	068396-068424	*C. bairdii* MT
3231	Milk River, S. Fork, MT	Milk	23Sep2004	068365-068395	*C. bairdii* MT
3157	Spring Creek, MT	Judith	11Sep2003	059544-059569	*C. bairdii* MT
3224	St. Mary River, MT	Milk	18May2004	068249-068253	*C. bairdii* MT
3225	St. Mary River, MT	Milk	11Aug2004	068263-068284	*C. bairdii* MT
3226	St. Mary River, MT	Milk	04Aug2004	068254-068262	*C. bairdii* MT
3228	Teton River, MT	Marias	18Oct2004	068308-068340	*C. bairdii* MT
3266	Yellowstone River, MT	Yellowstone	30Nov2004	068720-068749	*C. bairdii* MT
2805	Yellowstone River, MT	Yellowstone	08Sep2002	058084-058085	*C. bairdii* MT

Table 2. Sampled populations from the upper Columbia River Basin.

Pop.#	Location	Basin	Collection date	BYU #	
2914	Skalaho Cr. MT	Clark Fork	08Sep2003	062473-062495	
3119	Rock Creek, MT	Clark Fork	04May2004	059041-059088	
3170	Libby Creek (upper), MT	Kootenai	07Jul2004	059658-059693	*C. bairdii* MT?
3171	Libby Creek (lower), MT	Kootenai	11Aug2004	059694-059729	*C. rhotheus*
3172	Yaak River, W. Fork, MT	Kootenai	25Aug2004	059730-059775	*C. bairdii* MT?
3173	Pipe Creek, MT	Kootenai	06Jul2004	059776-059814	*C. bairdii* MT?
3174	Pleasant Valley Fisher River, MT	Kootenai	07Jul2 004	059815-059853	*C. rhotheus*

We have found that the interpretation of phylogenetic relationships is often clarified if multiple populations from a given taxa are included in the analysis. For that reason we expanded the set of cottids to include representatives of not only a number of western and eastern taxa but also, when possible, multiple populations for each taxon. The western taxa included *C. bairdii*, *C. rhotheus*, *C. confusus*, and *C. beldingii* of the Snake River and lower Columbia River basins (Table3), and *C. bairdii* and *C. beldingii* of the Great Basin (Lahontan and Bonneville basins; Table 4) and the Colorado River Basin (Table 4). We also examined cottids from eastern North America (Table 5). These included *C. bairdii* and *C. cognatus* from Wisconsin (both the Mississippi River and Lake Superior basins) and *C. carolinae* from Alabama. These represent populations which have access to the Mississippi River basin, and thus which may have had access to the upper Missouri River Basin during the Pleistocene.

The following individuals obtained the specimens examined in this study. **Upper Missouri River drainages:** Jim Magee and Tracy Elam (Population #3229, 3230); Chad Taber and Travis Lohrenz (Population #3130); Jim Boyd and Dave Stearns (Population #3147); Robin Wagner (Population #3227, 3231, 3224, 3225, 3226); Anne Tews, Jim Boyd, and Kate McLaughlin (Population #3157); David Moser and Adam Strainer (Population #3228); P. Byorth and S. Opitz (Population #3266); Dennis Shiozawa (Population#2805). **Upper Columbia River Basin:** Chris Clancy (Population #2914); Jim Olsen (Population #311); Mike Hensler (Population

Table 3. Sampled populations from the Snake River Basin.

Pop.#	Location	Basin	Collection date	BYU #	*Cottus* sp.
3766	Medicine Lodge Creek, ID	Snake	2002	142409-142428	*C. bairdii* MT
1571	Weiser River, ID	Snake	Fall 2001	060164-060173	*C. bairdii*
2693	Toponce Creek, ID	Snake	26Sep2000	060805-060812	*C. bairdii*
3031	Fall River, ID	Snake	17Jul2002	061385-061454	*C. bairdii*
3251	Boise River, South Fork, ID	Snake	04Aug2002	058078, 80, 81	
0692	Raft River, ID	Snake	17Oct1990	089543-089550	*C. bairdii*
3686	Portneuf River, ID	Snake	16Aug2002	068527-068593	*C. beldingii*
1682	Big Gulch Creek, ID	Snake	01Oct2001	060309-060318	*C. confusus*
1684	Eighteen Mile Creek,ID	Snake	15Oct2001	060329-060338	*C. confusus*
1685	Wildhorse Cr, Big Lost River/ID	Snake	2001	060339-060348	*C. confusus*
2803	Boise River, South Fork, ID	Snake	04Aug2002	061182-061184	*C. confusus*
2804	South Fork, Boise River, ID	Snake	04Aug2002	058073-058079	*C. confusus*
4870	Upper Salmon -Stanley, ID	Salmon	07Jul2004	069174-069178	*C. confusus*
1687	Pahsimeroi River, ID	Salmon	2001	060359-060368	*C. confusus*
3166	Ferguson Creek, OR	Willamette	1997	137478-137480	*C. rhotheus*
3167	Muddy Creek, OR	Willamette	1997	137481-137482	*C. rhotheus*
3168	Wynoochee River, WA	Chehalis	1997	137483-137485	*C. rhotheus*

Table 4. Sampled populations from the Great Basin and the Colorado River Basin.

Pop.#	Location	Basin	Collection date	BYU #	*Cottus* sp.
1290	Yampa River, CO	Colorado	02Aug2001	079684-079709	*C. beldingii*
3196	Deep Creek,UT	Bonneville	07May2004	068129-068132	*C. beldingii*
3245	Lake Creek, UT	Bonneville	04Dec2004	068495-068518	*C. bairdii*
3175	Green River, WY	Colorado	14Sep2004	059936-059950	*C. bairdii*
3205	Blue Cr., UT	Bonneville	29Sep2003	068248	*C. bairdii*
3267	Truckee River,NV	Lahontan	07Aug2002	068814-068821	*C. beldingii*
1316	Dolores River, CO	Colorado	20Sep2001	057139-057169	*C. beldingii*
1643	Bear Lake, UT	Bonneville	26Jul2001	060412-060444	*C. extensus*

Table 5. Sampled populations from eastern drainages.

Pop.#	Location	Basin	Collection date	BYU #	*Cottus* sp.
3120	Willow Creek, WI	Mississippi	05May2004	059089-059114	*C. bairdii* East
3133	Blueberry Creek, WI	Lake Superior	01Oct2003	059239-059270	*C. bairdii* East
3134	Little Bois Brule River, WI	Lake Superior	01Oct2003	059271-059296	*C. cognatus*
3123	Seas Branch, WI	Mississippi	05May2004	059127-059152	*C. cognatus*
3127	Mud Creek, AL	Mississippi	10Apr2004	059192-059197	*C. carolinae*
3125	W. Branch Raccoon Cr, WI	Mississippi	13May2004	059179-059188	*C. cognatus*
3124	Legler School Branch, WI	Mississippi	13May2004	059153-059178	*C. bairdii* East
3122	Sleighton Creek, WI	Mississippi	05May2004	059127-059152	*C. cognatus*

#3170, 3171, 3172, 3173, 3174). **Snake River Basin and Lower Columbia River:** Bart Gammett (Population# 3766 1682, 1684,1685,1687); Jared Crowley and Derek Houston (Population# 2803, 2804, 3251); Mike McGee (Population# 1571); Univ. of Wyoming (Population# 2693); USGS (Oregon) (Population#3166, 3167,3168); Dan Christensen (Population# 4870); Dennis Shiozawa Clayton Nii, Roger Haga Population#3031)Dennis Shiozawa Clayton Nii Population# 3686) Dennis Shiozawa Population# 0692). **Great Basin**

and the Colorado River Basin: Matt McKell, Keoni Kauwe, Erin Linton, Emily McLaughlin (Population# 1290); R. Spall, Don Duff (Population# 3196); Peter Cavalli (Population# 3175); Scott Tolentino, Bryce Nelson, Michael Mills (Population# 1643); Dennis Shiozawa, Paul Evans, Becky Miller, Andrew Moffit, Gretchen Baker (Population# 3245); Dennis Shiozawa, Paul Evans , Paul Thompson (Population#3205). **Eastern drainages:** John Lyons (Population#3120,3122, 3123,3124,3125); Dennis Pratt (Population# 3133, 3134); B. J. Weibell (Population# 3127).

Laboratory processing

All fish were individually tagged with a BYU museum number and each discrete collection was given a population number and formally accessioned as vouchers into the collection at the Monte L. Bean Life Science Museum at Brigham Young University. A fin sample was taken at the time of accessioning and was later used for DNA extraction. The voucher specimens are kept so that DNA findings can be traced back to the individual from which the sample was taken. We focused on mtDNA in this study because it lacks the complications of recombination.

DNA extraction

DNA was isolated from each fin clip using the PureGene isolation kit (Gentera Systems, Minn., MN). Successful DNA isolations were determined by gel electrophoresis on a 1% agarose gel. DNA will be obtained using standard total DNA extraction procedures. Purified DNA was stored at 4 C until analyzed.

DNA amplification

Aliquots of the purified DNA were amplified using the polymerase chain reaction. Amplification conditions include 100 ng of DNA, 8 pmol of each primer, 4 µl of 10X reaction buffer, and 0.1 µl of Taq polymerase. The thermal-cycler temperature settings at 50 seconds denaturing at 94ºC, 55 seconds annealing at 55ºC, and 2.5 minutes elongation at 72ºC. Thirty-eight amplification cycles were run. Primers for both the ND4 region (developed in the Evans-Shiozawa lab) and Cytochrome B (Schmidt and Gold 1993) were be utilized. An internal primer, developed by Kinziger and Wood (2003), will be used to insure complete amplification of the Cytochrome B region.

PCR products were sequenced using ABI Prism BigDye Terminator Cycle Sequencing chemistry on an ABI 377 automated sequencer. Sequence alignment and editing was performed using Sequencher 3.0 (Gene Codes Corp., Ann Arbor, MI).

mtDNA

MtDNA is a maternally inherited DNA genome located within the mitochondria of the cell. It is distinct from the nuclear genome, and therefore does not undergo the recombinant events that typify nuclear genes. Analysis of mtDNA results in discrete predictions of maternal lineages. We are not running nuclear markers for this study. However, we have examined one nuclear marker and, while it has less resolution than the mtDNA markers, it has confirmed associations

seen among several of the cottid species examined in this study.

Analysis

Trimmed DNA sequences for ND-4 and the D-loop were aligned with the program Sequencher™. Phylogenetic analyses were performed using the program PAUP*, version 4.0 (Swofford, 1998). The Montana populations were compared with cottid species and populations from the western part of the state and from outside of the state, in particular from the eastern *bairdii*, *carolinae*, and *cognatus* groups and from states to the south of Montana. The outgroup is *Leptocottus armatus*, which we have archived in our collection at BYU. The ND-4 data set was also analyzed with TCS to produce a haplotype network. This network allows a visual examination of base pair differences between individual haplotypes. Presumed intermediate changes in base pairs are generated by the program.

RESULTS and DISCUSSION

This is an interim report. The DNA isolation and sequencing has been completed for the ATP-ase, ND4, and the D-loop of the mitochondrial genome. We are in the process of compiling the full data set for phylogenetic and population genetic analyses. Here we discuss preliminary analyses based on reduced data sets so that general patterns can be seen. The analyses will included all haplotypes in the final report and the additional ATA-ase dataset. We therefore expect to see additional adjustments in the phylogenies and haplotype networks discussed below.

Phlyogenetic Analyses

Phylogenetic tree reconstruction was completed using maximum likelihood procedures (Figure 1). The consensus tree was rooted with *Leptocottus armatus* as the outgroup. *Cottus carolinae* was unresolved in this analysis. Both *C. beldingii* and *C. confusus* are more interior than *C. carolinae*, in contrast to the results in Kinzieger et al. (2005), based on ATA-ase and cytochrome b. The signal in the D-loop may be too rapidly evolving to accurately depict deeper phylogentic associations and Kinzieger et al. (2005) showed *C. carolinae* to be part of the most divergent Uranidea clade. Our data supports a western *Cottus bairdii* clade. The western forms, including specimens from the Bonneville Basin, Colorado River, Snake River, and Salmon River basins are in a single well supported clade. The Bear Lake sculpin, *Cottus extensus*, is also a member of this clade and shows its closest association with *Cottus bairdii* from the Bonneville Basin. This is not unexpected since Bear Lake is a part of the northeastern Bonneville Basin.

The second clade includes *Cottus bairdii* from Wisconsin. This group is basal to both *Cottus rhotheus*, from Oregon and several locations in the Kootenai River Basin of the upper Columbia River, and the upper Missouri River cottids from Montana. The upper Missouri River *Cottus bairdii* form a single clade, indicating that the same species occurs throughout the basin. However at this time the relationships within the upper Missouri River Basin are not resolved by the phylogenetic analysis. A second group, from the upper Libby Creek, the Yaak River, and Pipe Creek in the Kootenai River Basin falls between *Cottus rhotheus* and the upper Missouri River *Cottus bairdii*. The group appears to be distinct enough to be a separate species

TCS Haplotype Network

The TCS network (Figure 2) allows a visualization of associations between haplotypes. Each box represents a haplotype which, in this case, is based on ly on ND4 sequence data. Linkages between haplotypes are designated by lines and presumed mutational steps between haplotypes are represented by the dots on the lines. The TCS network was generated with a 90% connection limit since that level allowed inclusion of *Cottus cognatus* in the network. However two loops (multiple paths to a haplotype) exist in the network, one being at the species level, and the other within the upper Missouri River *Cottus bairdii*. These loops can not be resolved with the data utilized in this analysis, but a full data set should allow resolution in the

Leptocottus armatus, *C. carolinae, C. beldingii* and *C. confusus* were too divergent to link with the taxa of interest in the TCS network and therefore are not shown in the figure. The western *Cottus bairdii* again form a single grouping. The Snake River form appears to be the most closely related to the other cottid species in the network while *Cottus bairdii* in the Colorado River Basin and *Cottus extensus* of Bear Lake appear to be the most divergent within the western *bairdii* clade.

Cottus cognatus and the eastern *Cottus bairdii*, while both very divergent from other cottids in the network, are most closely associated with the western *Cottus bairdii.* *Cottus rhotheus* forms a separate lineage as well, with the haplotypes examined all being identical. The upper Missouri River *Cottus bairdii* form their own group. The six haplotypes included in this report show mostly one step base changes between one another. The presence of multiple haplotypes indicates that we should be able to gain more insight about the genetic structuring of this taxon once the full data set is analyzed with a nested clade analysis.

The Pipe, Yaak, and upper Libby samples from the Kootenai River of western Montana are distinct from the other cottids in the network. They show as many steps divergence from the upper Missouri River *Cottus bairdii* as *Cottus cognatus* shows from the western *Cottus bairdii*. These represent a separate species and additional investigation into their geographical distribution is needed.

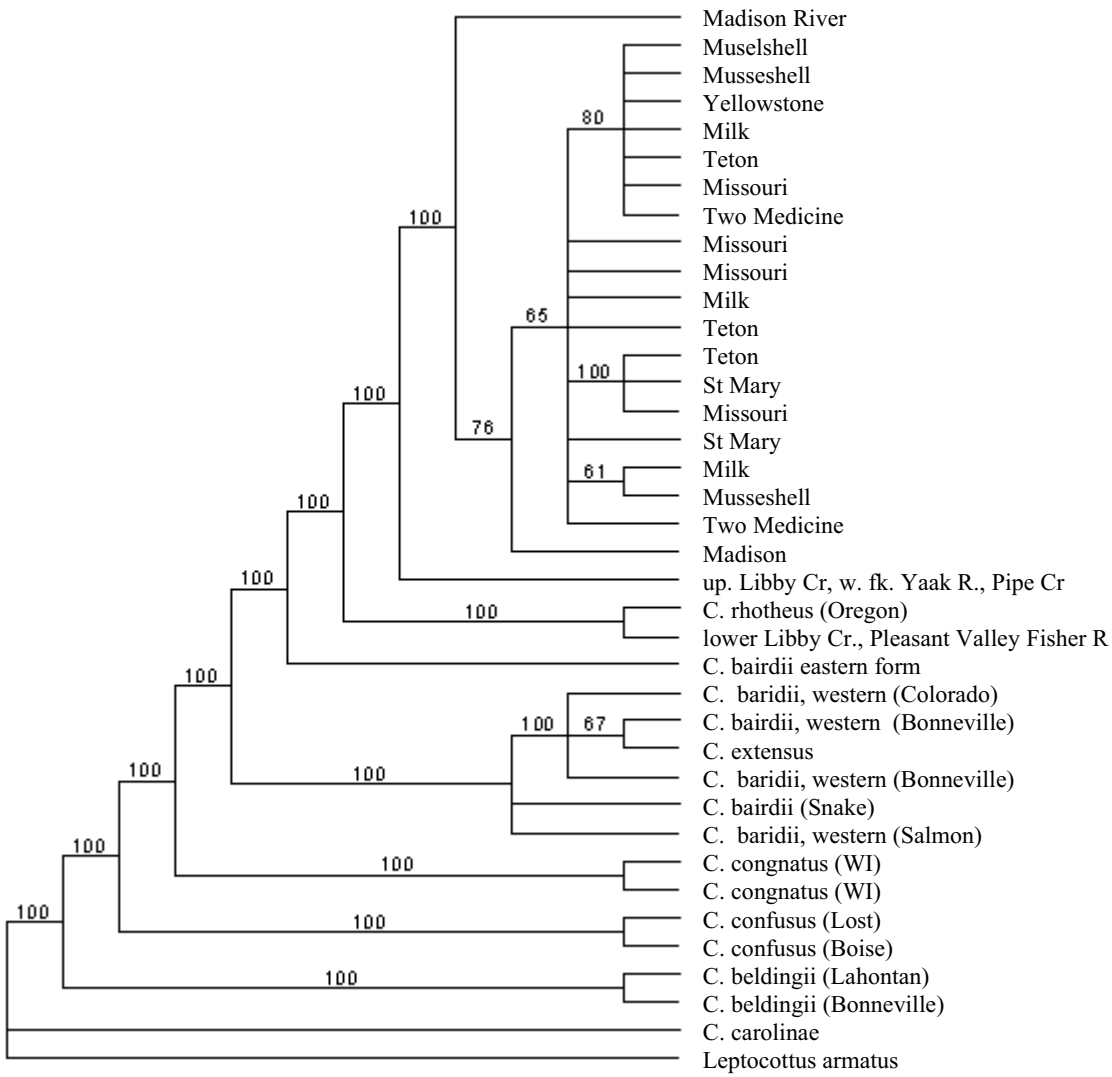

Figure 1. Phylogeny based on ND4/D-Loop of 24 populations and 238 individuals. Maximum Likelihood Consensus.

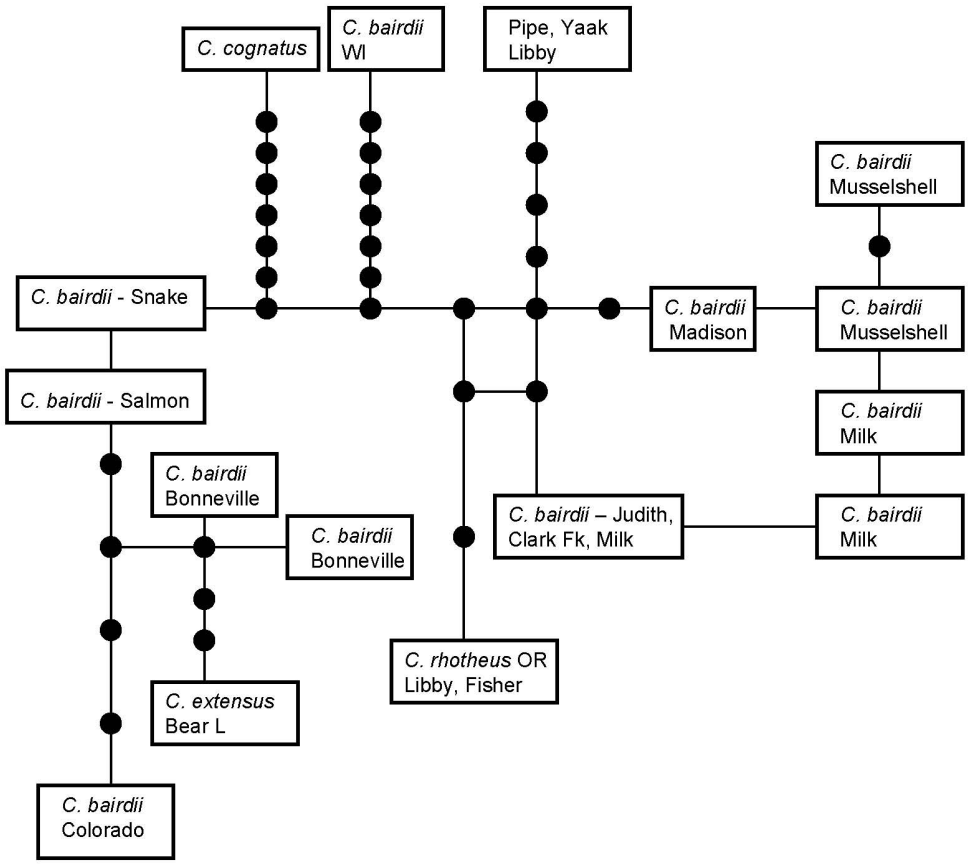

Figure 2. TCS network based on 313 base pairs from the ND-2 mitochondrial gene.

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