MOLECULAR PHYLOGENETICS OF THE FRESHWATER GASTROPOD GENUS *JUGA* (CERITHIOIDEA: SEMISULCOSPIRIDAE)

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ABSTRACT--The taxonomy of the genus *Juga* (Semisulcospiridae), as in many freshwater mollusks, poses several challenges. Only a few species have been studied genetically or anatomically. We analyzed a portion of the *cox1* gene for 103 specimens, emphasizing the upper Sacramento – Pit system but including all recognizable named species and subspecies from across the geographic range. The *rrnL* gene was also sequenced for 35 specimens and showed similar patterns, but was less informative. None of the three extant subgenera appear monophyletic and additional clades of similar rank exist. Several currently recognized taxa such as *J. silicula*, *J. nigrina*, *J. hemphilli*, *J. bulbosa*, and *J. acutifilosa* are polyphyletic. In contrast to previous classifications, the clades found in our study correspond closely to geography. Some geographically and genetically distinctive populations do not fit in any named species.

Keywords: paralog; *Juga*; Semisulcospiridae; biogeography; freshwater mollusks

1. Introduction

The western USA endemic semisulcospirid genus *Juga* H. and A. Adams, 1858 ranges from central California north to Washington (Figure 1). Historical reports from southwestern Canada are doubtful (Clarke, 1981). Some East Asian species have been assigned to *Juga* (e.g., Bogatov and Zatravkin, 1990), but more recently have been reassigned to other genera (Starobogatov *et al.*, 2004). The long geologic history of *Juga* (Eocene-Recent: Henderson, 1935) and the drainage shifts caused by major late Tertiary volcanic and tectonic events along an active and evolving continental margin (Pease, 1965) have given ample opportunity for dispersal and isolation. Many populations occur only in springs, spring runs, small coastal streams, or other discontinuous habitats, providing great potential for genetic isolation. Because the isolation is primarily by distance and genetic drift, speciation might not always be accompanied by external morphological differentiation. On the other hand, Hannibal (1912) and Goodrich (1942) posited high

levels of ecophenotypic variation in *Juga*. Not all of their ideas are generally accepted, but the degree of ecophenotypic variation remains unknown. Species concepts in *Juga* currently rely almost exclusively on shell form, so the possibilities of cryptic variation or ecophenotypic variation remain largely untested. Our goal was to test current classification in the genus *Juga* and to test the level of differentiation between populations with distinctive shell morphologies (including shape, sculpture, and color), using molecular data.

Juga appears most closely related to Asian genera such as *Koreoleptoxis*, *Semisulcospira*, and *Hua* based on anatomical features (Strong and Frest, 2007; Strong and Köhler, 2009). Although *Juga* is difficult to separate from genera such as *Elimia* based on shell characters alone, there are consistent anatomical and reproductive differences. *Juga* and *Semisulcospira* possess a seminal receptacle, unlike eastern North American Pleuroceridae (Prozorova, 1990; Strong, 2005). Different reproductive anatomy leads to differences in egg masses. All *Juga* egg masses that we observed were large, internally disordered, elongate, gelatinous strings or masses with hundreds of eggs, also unlike Pleuroceridae. Molecular data agree with the anatomical patterns, placing *Juga* closest to *Semisulcospira*, *Hua*, and similar Asian taxa in Semisulcospiridae (Strong and Köhler, 2009 and references therein). Previous molecular analyses included no more than four species of *Juga* in each, leaving many populations unstudied.

As for most freshwater snails, many nomenclatural problems exist (Burch, 1988). Several named species of *Juga* are based on unfigured or poorly-figured types with vague or inaccurate locality data (Supplement 1). Different authors have ranged from lumping to splitting, often with little or no justification of the decisions. The most current classifications (Turgeon *et al.* (1998), and Johnson *et al.* (2013)) recognized 12 or fewer species and subspecies. Four subgenera were recognized in *Juga* by Taylor (1966) based on early teleoconch sculpture (Figures 1-2): *Juga s.s.* (with strongly plicate sculpture), *Calibasis* (with strongly lirate sculpture), *Idabasis* (with cancellate sculpture; fossil only), and *Oreobasis* (with weak to no sculpture). Sculpture on the early whorls may become reduced or absent on later whorls, making the line between *Oreobasis* and other subgenera problematic. Many specimens, including many types, have lost or eroded early whorls, adding to the challenge of applying this character. Strong and Frest (2007) discussed the anatomy of the types of the three extant subgenera, finding little difference between *Juga s.s.* and *J. (Oreobasis)* but more distinguishing features for *Calibasis*.

Because several species of *Juga* have been reported as wide-ranging, we tried to sample topotypic populations for all recognizable species to compare them with our detailed sampling in the Pit – upper Sacramento region (Supplements 1-2, Supplementary Figures 1-2). Currently recognized subgenera overlap widely in range (Figure 1). However, morphologically distinct, geographically isolated populations are often found, suggesting that actual distributions may be much narrower. Conversely, alteration of regional drainages by geologically recent changes such as glaciation, volcanism, and earthquakes may make modern isolation less meaningful. Other snails with similar habitat preferences and distribution have very high diversity with many cryptic species and often small ranges (Hershler *et al.*, 2003, 2007), but some freshwater snail genera have high morphological variation and only a few, widely-ranging species (Walther *et al.*, 2006; Lee *et al.*, 2007). Based on our sampling, many populations and morphologically distinctive forms of *Juga*

have narrow ranges, but as currently defined, most recognized species range across multiple drainages and wide geographic areas.

We typically found *Juga* in clear, permanent springs, creeks and rivers, especially on solid substrates. They appear to be intolerant of excessive sedimentation and absent from impoundments and other still water types such as ponds. The combination of localized distribution and sensitivity to disturbance make these taxa especially vulnerable to human impact. However, uncertainty about the number of species and their identification has made it very difficult to accurately assess both their distribution and their conservation status. Although the upper Sacramento drainage retains comparatively good habitat in many areas, large areas of stream and spring habitat have been strongly impacted by pollution, diversion, impoundment, irrigation, surface and groundwater withdrawal, *etc.* (e.g., Mailliard, 1923). Hence, many formerly suitable sites no longer have *Juga*, such as the Smith and lower Shasta Rivers in northern California, which have been dewatered by intensive water withdrawals (*pers. obs.*).

In light of the taxonomic uncertainties, we sought to generate molecular data to establish general patterns of mitochondrial genetic variation in the genus and to examine particular populations of interest in the upper Sacramento and Pit River drainages. Problematic alleles of mitochondrial genes are known for Asian semisulcospirids (Lee *et al.*, 2007, Miura *et al.*, 2013), so we also looked for any anomalous sequences that might represent a similar pattern.

2. Materials and Methods

2.1. Collection and preservation

Our sampling emphasized isolated populations, which commonly showed conchological differences in addition to the geographic separation. Because many species have been reported from wide geographic ranges, we included all of the topotypic populations for named species that we could locate and access. If the type locality could not be accessed or no longer had *Juga*, the closest morphologically similar populations were sampled. For species with poor (e.g. “Oregon”) or no locality data in the description, we chose populations that were morphologically most similar to the types, from sites visited by the original collector, if that was known. Supplement 1 lists all nominal extant species of *Juga*, their type localities, and the best match that we could find. Although *J. bulbosa* has been widely reported in literature (in part as a presumed senior synonym of *J. newberryi* [Strong and Frest, 2007]), no populations precisely matching the types have been found, so we could not analyze it. Also, the paralectotype lot in the MCZ contains at least three shell morphs. GenBank has a sequence listed under *J. bulbosa*, but this is incorrect (Holznagel and Lydeard, 2000).

Specimens were collected by hand or dipnet and either frozen or preserved in 95% ethanol. If any variation was observed in color or sculpture within a locality, the forms were sorted into different lots. Due to difficulties in accessing some localities, extraction was attempted from some older lots that were initially fixed in 5% formalin and then transferred to 70% ethanol.

2.2. DNA extraction and sequencing

DNA was extracted using a standard CTAB-phenyl-chloroform technique (Winnepeinckx *et al.*, 1993). Initially, we chose the *cox1* (COI) mitochondrial gene. Several studies have sequenced it for pleurocerids and semisulcospirids, beginning with Minton and Lydeard (2003). Although some studies found anomalously divergent

sequences, other sequences appear normal and yield plausible biogeographic patterns (Whelan and Strong, 2015). As more molecular studies reported unusual results, we began supplementing the *cox1* data with *rrnL* (16S) for the same individual that amplified for *cox1*. Like *cox1*, there are several studies using it, beginning with Lydeard *et al.* (1997). As for *cox1*, results of these studies are mixed, with some anomalous sequences standing out from others that yielded a more normal pattern (Whelan and Strong, 2015). Whelan (2013) makes the case for these anomalous sequences being nuclear paralogs. In particular, the unusual *cox1* sequences have high levels of nonsynonymous substitution. Whelan and Strong (2015) argued that they were not nuclear paralogs; however, as they only tested one allele by genomic sequencing, the question needs more investigation.

Although *cox1* and *rrnL* are inherited together as mitochondrial genes, they are separated by several genes and are on opposite strands in *Semisulcospira* and other cerithioideans (Kojima, 2010; Zeng *et al.*, 2014). The nature of these anomalous genes remains unknown, but this distance may decrease the likelihood of both genes being affected by a single event. Apart from Dillon and Frankis (2004) and Whelan and Strong, (2015), published studies have not provided any comparison between results from the two genes in pleurocerids or semisulcospirids. The only nuclear gene that has been studied extensively in these families is 28S ribosomal RNA, which also sometimes shows anomalous patterns; apart from the anomalous sequences, there is low variation between closely related species (Lee *et al.*, 2007 and personal observations). Whelan and Strong (2015) likewise found low variation in histone H3. We attempted using ITS1 as a nuclear marker, but resulting sequences were unreadable for *Juga* and all but one *Elimia* attempted, suggesting multiple divergent sequences in a single individual (a problem for this DNA region that has been noted in several other taxa [Armbruster and Korte, 2006]). Although analyzing nuclear genes would be desirable, gastropod genomics remain too poorly known to identify suitable targets.

Standard PCR and sequencing techniques were used (Campbell *et al.*, 2005). Table 1 lists the primers used. We targeted the 658 base pair (bp) region between the Folmer primers, and this was the only region used in phylogenetic analyses. If the Folmer primers did not work, other primers were used. Sequencing additional individuals, sometimes requiring new collections, usually eventually yielded complete sequences. Only *J. orickensis* and three populations of *J. nigrina* were represented by partial sequences in the phylogenetic analyses (Supplement 2). Ten short sequences (indicated in Supplement 2) representing only the last 1/3 of the Folmer region were generated. These were not included in the phylogenetic analyses, but were compared to the more complete sequences as a check for individual variation and possible anomalous sequences. Primers 16F and 16R amplified 407 bp of the *rrnL* gene. Towards the end of the study, we amplified a larger region by also using primers 16SF and ND1R. Using 16F and ND1R for PCR and then 16R and 16SF as internal sequencing primers worked well. Only the 16F-16R region was analyzed, but the additional sequence amplified by other primers includes variable regions that promise to be informative for future studies. One or two additional specimens from some lots were sequenced. In all cases the second sequence differed by no more than 4 bases (0.6%). Multiple haplotypes are identified with a lowercase letter after the species. For reference, we identify distinctive lineages not corresponding to previously named taxa as OTUs (operational taxonomic units); these range from slightly to highly differentiated

and no doubt include intraspecific and interspecific variations. Definition of OTUs reflected molecular results and morphological differences.

2.3. Outgroup selection

Based on previous analyses (Strong *et al.*, 2011 and references therein), outgroups included representatives of Eurasian Melanopsidae, eastern Asian Semisulcospiridae and eastern North American Pleuroceridae (Supplement 2). For *cox1*, we eliminated all possible outgroups that had more than 4 nonsynonymous substitutions. For both genes, we eliminated possible outgroups that formed particularly long branches. For example, sequences from the Miura *et al.* (2013) study that placed in the large clade with relatively long branches, including an odd mix of Japanese and Korean sequences, were not used in this study. New sequences are assigned GenBank accession numbers EF586887-EF587015 and HM016243-HM016256 (Supplement 2). Reference specimens are in the Field Museum of Natural History (FMNH 342623-342878), Deixis Consultants, and Invertebrate Identification collections (database searchable on request). Most lots were divided among all three institutions. All lots are represented at the Field Museum of Natural History, but the other material may be examined by consulting the authors. DNA extraction often left little of the specimen, so the lots are paravouchers sensu Groenenberg *et al.* (2011). The remaining tissue and (generally broken) shell material from the analyzed specimens is included in the vouchers, but if multiple sequences were attempted the individual specimens are not separately identified. As no other snail in the area resembles *Juga*, there is no risk of confusion with other genera. In the few instances with more than one *Juga* species present in a single area, they had clear morphological differences in addition to genetic differentiation. Therefore, we believe that the sequenced specimens accurately represent the reference lots.

2.4. Data analysis

CLUSTAL W (as implemented in BioEdit 7.0.5.3, Hall (1999)) was used for preliminary alignment, followed by manual alignment. If a sequence is missing an end, CLUSTAL often matches some of the terminal bases incorrectly, inserting unnecessary indels. Also, if a sequence does contain indels, like the 16S, CLUSTAL can align identical sequences differently. For example, if one sequence contains an extra base, the corresponding gap is not always inserted in the same place in all the other sequences. DNA data were analyzed in PAUP*4.0a141 (Swofford, 2002), TNT 1.1 (Goloboff *et al.*, 2008), and MrBayes3.2 (Ronquist *et al.*, 2012). Identical duplicate sequences were eliminated from the parsimony and Bayesian analyses, as they can cause problems in both. Although both analyzed genes are mitochondrial and thus expected to show similar patterns, the significant differences in taxon representation made combined analysis impractical.

The parsimony searches used 500 replicates, with all of the "new technology" methods implemented in TNT (Goloboff *et al.*, 2008). Parsimony bootstrapping in TNT used 500 replicates, each replicate being a random-addition heuristic search with 10 replicates. To test whether our results differed significantly from current classification, analyses of *cox1* data were also run constraining the subgenera of *Juga* as monophyletic. Because the *Calibasis* group seemed especially distinctive in anatomy (Strong and Frest, 2007) and genetically, another constraint was run in which a few weakly-sculptured populations were transferred from *Oreobasis* to *Calibasis* (*J. interioris*, *J. laurae*, and *J. (C.)* OTU 3, 4, and 7) to determine if, with the removal of those species and OTUs, *Juga s.s.* and *Oreobasis* could be monophyletic. The resulting trees were contrasted using the

approximately unbiased (AU) test (Shimodaira, 2002) as implemented in PAUP*. To focus on the impact on *Juga*, these tests of monophyly included only a single *Semisulcospira* as an outgroup. MrModeltest 2.2 (Nylander, J. A. A., 2004, Evolutionary Biology Centre, Uppsala University, Sweden) was used to select a maximum likelihood model using hLRT and AIC (GTR+I+G for *cox1*, HKY+I+G for *rrnL*) that was then input into MrBayes 3.2 (Ronquist *et al.*, 2012). The Bayesian analyses used 20,000,000 generations and 8 chains, with revmat, shape, pinvar, and statefreq unlinked. Burnin was identified by examining when the average standard deviation of split frequencies stayed below 0.01 (Ronquist *et al.*, 2011). The Bayesian analyses on *rrnL* data treated gaps as unknown, but there were no indels in the *cox1* data.

3. Results

Of 658 bases sequenced in the Folmer region of *cox1* (excluding primers), 243 were parsimony-informative. Only three samples failed to amplify the first 1/3 of the region (*nigrina* Sac333, *nigrina* Sac073, *orickensis* 5004). Using the invertebrate mitochondrial code in BioEdit 7.0.5.3 (Hall, 1999) to translate the sequences, no stop codons and few nonsynonymous substitutions were present in our sequences for *Juga*. For the *rrnL* gene, about 407 bases (some indels) were sequenced using the Krebs primers and 62 were informative. No exceptionally long branches were present, and none of the sequences were particularly unusual, so no obvious pseudogenes were present. A total of 75 nonsynonymous substitutions were observed, 0.36% of the 20760 amino acids analyzed. Of these, 24 specimens had A instead of V at position 139 and 42 specimens had A instead of V at position 106. Except for one specimen, these common nonsynonymous substitutions were characteristic of entire clades, and the amino acids in question are chemically similar. Thus, these two substitutions probably represent actual amino acid variation. *Juga acutifilosa* 1287 had two of the remaining nine substitutions; seven other specimens each had one rare nonsynonymous substitution. In contrast, there were 3521 synonymous substitutions, out of 62419 bases (5.6%).

Supplement 2 lists the populations sequenced and locality information. Up to 9 identical sequences were obtained from different nearby populations. Figures 2-3 show the current results. In the test for subgenus monophyly, the unconstrained tree had a length of 559. Constraining subgenus monophyly raised the length to 657, an increase of 17.5%. If the weakly-sculptured taxa that placed in clades A, B, and C are transferred to *Calibasis*, constraining the revised subgenera still increased the length to 595, a 6.4% increase. In both cases, the AU test indicated that the maximum parsimony trees were significantly more likely than the constrained trees, with a probability of " ~ 0 ", *i.e.*, well below 0.05. About 70% bootstrap support is generally considered strong support for a clade, though this reflects several assumptions about the evolutionary patterns that may not apply to a given data set (Swofford *et al.*, 1996). Bayesian analysis tends to slightly overestimate support for clades, in contrast to the parsimony bootstrap, but it is less sensitive to some factors that can affect parsimony (Simmons *et al.*, 2004). Thus, a posterior probability of 0.95 is an approximate cutoff for significance in Bayesian results. Nine clades are labeled A-I on the figures to facilitate reference to various groups of taxa.

The percent differences in the *cox1* sequences are summarized in Supplement 3. Full details can be requested from the first author. Major clades (clades D through I and the clade including A, B, and C) differed from each other by over 5%, and members of other genera differed from *Juga* by over 14%. Variation is not uniformly distributed across the

cox1 gene. Often, the short sequences had lower percent differences than the complete sequences, but this was not consistent.

4. Discussion

All analyses support the separation of Semisulcospiridae and Pleuroceridae. In most analyses, *Juga* is monophyletic, with modest bootstrap support, but the *cox1* Bayesian analysis anomalously placed *Semisulcospira* as a long branch within clade H of *Juga*. The *rrnL* analyses recovered a large polytomy for Semisulcospiridae including the Asian semisulcospirids and a clade of *Juga*. Because *rrnL* had less resolution and fewer taxa represented, the discussion primarily reflects the *cox1* data, noting any differences with *rrnL*.

Several recent studies have found anomalous sequences in Semisulcospiridae and Pleuroceridae, first noticed by Lee *et al.* (2007). Whelan (2013) made a strong case for the anomalous mitochondrial sequences being nuclear paralogs, but Whelan and Strong (2015) argued that they were probably rare mitochondrial alleles. Nuclear genes have similar issues (Simpson *et al.*, 2005; Lee *et al.*, 2007), suggesting that gene duplication may be widespread in gastropods. The anomalous sequences are characterized by high divergence from other sequences, by showing anomalous phylogenetic and geographic patterns, and by features such as high levels of nonsynonymous substitutions, long branches, or stop codons. We did not find any unusually high numbers of nonsynonymous substitutions, premature stop codons, exceptionally long branches, or other indicators of unusual sequences in our new sequences for *Juga*. Our sequences show closest affinity with other sequences from the same or nearby drainages, unlike the pattern found for anomalous sequences by Lee *et al.* (2007) and Miura *et al.* (2013). Either anomalous sequences are rare in *Juga* for this gene, or our protocol favored one version of the genes, if the anomalous sequences are paralogs. We do not yet have enough data to rule out the possibility that one of our sequences for *J. (O.)* OTU 4 may be moderately aberrant, but if so it is not divergent to the degree seen in other genera. Although *cox1* and *rrnL* sequences for this taxon placed differently in the trees, the difference was not strongly supported. Whelan and Strong (2015) recommend large sample size to test for anomalous sequences. Our sampling across the geographic range of *Juga* provides the most comprehensive sampling for a single genus in these families; however, delimiting species will require more intensive sampling of individual populations to test for rarer alleles.

Within *Juga*, the molecular analyses suggest that the current classification, at both the subgeneric and species levels, requires modification. All species with spiral sculpture ("*Calibasis*") place within a single large clade (the clade including clades A, B, and C), but a few populations with little to no sculpture ("*Oreobasis*") also place here. Species with axial sculpture ("*Juga*" s.s.) and weakly to unsculptured forms make up the remaining clades, but many clades contain both more and less-sculptured forms. As no firm line divides a weakly sculptured *Calibasis* or *Juga* s.s. from *Oreobasis*, it is unsurprising that *Oreobasis* appears highly polyphyletic, being represented in all but two major clades. However, three of the lettered clades (G, H, I) consist exclusively of *Oreobasis* morphologies, and most of the clades found in our analyses are dominated by a single nominal subgenus, so there is some phylogenetic signal in the trait. Several monophyletic clades (D, E, H, I), evident in Figures 2-3, are of similar rank to the clades containing the presently recognized subgeneric types (F, G, and ABC). This suggests that recognition of subgenera is not very useful at this time. Strong and Frest (2009) recommended

synonymizing *Oreobasis* with *Juga* s.s. Although we also recommend synonymizing the subgenera, they are used herein as morphological tags for the populations that were not definitely assigned to known species.

Although a few regions have more than one clade present, each clade shows a coherent geographic distribution. This contrasts with current classification, in which several species are reported to range patchily across multiple drainages. In particular, *J. acutifilosa* is reported from the Great Basin, much of the Pit River system, and the Klamath system; *J. nigrina* is used for all weakly sculptured forms from the Pit and Sacramento system, sometimes also including the Great Basin or drainages north and west of the Sacramento system; and *J. silicula* is used for various populations ranging from Puget Sound drainages through northern coastal California, sometimes synonymized with *J. plicifera* in the Columbia system. However, our data indicate that each of these broad species concepts represent populations from multiple major clades.

The upper Sacramento River system is very diverse with multiple clades (Figure 4), similar to the diversity observed in *Fluminicola* (Hershler *et al.*, 2007). Often, genetic variation in *Juga* has similar distributions to *Fluminicola* species in this system. In both cases, representatives of multiple clades occur in the Upper Sacramento River system, so that all OTUs in this river do not form a monophyletic clade. However, within each major clade, the OTUs in this system are often a monophyletic group. The complex geologic history of this region (Pease, 1965) and the restriction of populations to cool, flowing water habitats accounts for the presence of multiple OTUs within drainages currently connected by the Pit River.

Clade A is found in the central Pit system, although morphologically similar forms were reported from the central and lower Sacramento in the 1800's. Clade B includes the upper Pit and nearby parts of the Great Basin, and is the only clade known from the Great Basin. Clade C occurs in the upper Sacramento (above Shasta Lake) and in the upper Klamath. Together, these three clades include all populations assigned to *Calibasis* and several weakly sculptured to smooth populations that would be called *J. nigrina* under most classifications. However, topotypic *J. nigrina* is quite different genetically. Among sculptured forms, the stouter morphs identified as *J. occata* place in clade A. The taller morphs, identified as *J. acutifilosa*, include populations from each of the three clades. Topotypic *J. acutifilosa*, from Willow Creek in the Honey Lake drainage (Great Basin), near the southern headwaters of the Pit system, places in clade B. Within clade A, most *J. acutifilosa*-like populations showed little genetic difference from typical *J. occata*. Populations from Sac113 and Sac373 formed a distinct group (*J. (C.)* OTU 3 and 4). These were the most upstream samples in clade A. The other significantly divergent population in clade A was from Sac329, in Hat Creek, a southern tributary of the Pit River (*J. (C.)* OTU 5). Hat Creek is one of the longer tributary creeks and flows past the Lassen volcanic region, providing a high likelihood of drainage rearrangements and isolation. It also contains a distinctive population of *J. (O.)* OTU 3, which is otherwise known from central Sacramento tributaries.

Clade B is the sister clade of Clade A. The most genetically divergent population (*J. (C.)* OTU 1) is from upper Willow Creek, a tributary of Ash Creek in the upper Pit system. (other Willow Creeks in the area include the Honey Lake tributary that is the type location for *J. acutifilosa*.) The remaining populations in this clade have relatively low genetic differentiation. However, they show significant morphological variation. The

Nevada populations have smooth shells whereas the Honey Lake and upper Pit populations have strong sculpture. The Nevada populations include topotypic *J. interioris* and *J. laurae*, and the California populations include topotypic *J. acutifilosa* and morphologically similar forms from the upper Pit. Although these nominal species have also been reported from other areas, neither our samples from those other areas nor the published sequence attributed to *J. interioris* by Lee *et al.* (2006) belong to clade B. In describing *J. interioris* and *J. laurae*, Goodrich (1944) assigned multiple populations to each species but speculated that they could have independently arrived at similar morphologies.

Clade C is sister to the combined clade of A and B, and is found in the upper Sacramento and middle Klamath systems. Clade E also crosses the divide between these rivers, though it occurs lower in both systems and also extends into coastal drainages north and south of the Klamath system. Like the other *Calibasis* clades, Clade C includes both sculptured and smooth forms. The upper Sacramento populations (*J. (C.)* OTU 7) are smooth, but some Klamath populations (*J. (C.)* OTU 6) are more sculptured and have been identified as *J. acutifilosa*. Despite the morphological variation, genetic differences are low and the two were not mutually monophyletic; they appear to be conspecific.

Clades D and G are confined to the middle Columbia River and the Deschutes River system (Figure 4). (This is the Deschutes River in central Oregon, not the Deschutes River tributary to Puget Sound.) Clade D has strong axial sculpture, like *Juga s.s.*, whereas clade G is smooth. Thus, although the two do co-occur, they are easily distinguished. Clade D includes *J. bairdiana* and *J. hemphilli maupinensis*, both treated as subjective synonyms of other taxa in current classification. However, in our analyses they appear quite distinctive and are not closely related to any other sampled species. Clade G is only known from the Deschutes system. It includes *J. newberryi* and a form from Oak Springs, Tuskan, Oregon (*J. (O.)* OTU 1). As *J. newberryi* is the type of *Oreobasis*, the consistent though weakly-supported placement of clade G as the sister taxon of clade F, containing the type of *Juga*, supports the synonymization of the two subgenera. Although *J. bulbosa* is widely reported in the literature, we could not locate populations that matched the type material. Johnson *et al.* (2013) alluded to *J. bulbosa* as possibly extinct in the text, but the final status rank was endangered. *Juga newberryi* is widely considered a junior synonym of *J. bulbosa*, but the two are distinct, though possibly related (Strong and Frest, 2007). The sequence listed on GenBank as *J. bulbosa* was corrected by Strong and Köhler, 2009 to *J. newberryi*, but the original publication (Holznagel and Lydeard, 2000) listed it as *J. nigrina*. The specimen came from the upper Umpqua River system, distant from the type drainages for all three names, and belongs in clade E instead.

Clade E primarily occurs in smaller coastal drainages, at least from southern Oregon to northern California (Figure 4). It includes both axially sculptured and largely smooth forms. The clade also reaches the upper Sacramento system and the lower Klamath (including the Trinity River system). Although *J. nigrina* has been reported widely, with one author or another treating all weakly sculpted forms as synonyms of it, topotypic populations are closely related only to other populations from the Clear Creek system and the upper Sacramento and McCloud drainages. Specimens with and without color bands showed no genetic separation. It is fairly distinct from the other members of clade E, but the genetic distance between the sampled populations of other nominal species in the Klamath and various smaller coastal drainages is small. These populations include axially sculptured forms such as *J. coquillensis*, *J. silicula shastaensis*, *J. y. obscura*, and *J. y.*

yrekaensis and weakly sculptured forms such as *J. chacei* and *J. orickensis*. Despite the current classification, *J. shastaensis* belongs to a different clade from *J. silicula*. Unstudied *Juga* populations occur in Pacific slope drainages north and south of these, and they should be compared with this clade.

All of Clade F has strong axial sculpture, at least early in ontogeny. It ranges from the Puget Sound region to the middle Columbia River and into the Willamette River, but was not found in other southern tributaries of the Columbia such as the Deschutes or John Day system (Figure 4). This clade includes the type of *Juga*, *J. silicula*, along with *J. draytonii*, *J. h. hemphilli*, *J. h. dallesensis*, *J. plicifera*, *J. p. oregonensis*, *J. (J.) OTU 1*, and *J. (J.) OTU 2*. *Juga h. dallesensis* and *J. p. oregonensis* do not seem genetically different from the nominate forms in our data, and the relatively low morphological differences may be merely individual variation. Similarly, *J. bulimoides* is not clearly different from *J. plicifera*, and has generally been synonymized on morphological grounds as well. Overall, genetic distances within clade F were quite small, and many may be conspecific. However, the large genetic distance between *J. s. silicula* and *J. (J.) OTU 1*, despite their close geographic proximity along Puget Sound, suggests the possibility that they are separate taxa, especially in contrast to the modest genetic distance between *J. s. silicula* and the Columbia River system members of clade F. The specimen used for molecular analyses identified as *J. hemphilli dallesensis* in Lee *et al.* (2006) was mislabeled material from Florida (Ó Foighil *et al.*, 2009). The specimen identified as *J. plicifera* in Lee *et al.* (2006) is from the type locality for *J. bulimoides* as restricted by Graf (2001). It matches our sequence of *J. plicifera* from its type locality and supports the synonymy of *J. bulimoides* with *J. plicifera*. The sequence identified as *J. silicula* in Holznagel and Lydeard (2000) was from the middle Willamette River drainage, whereas the *J. silicula* of Lee *et al.* (2006) came from the lower Willamette River. Both of these are members of clade F but not identical to the sequences obtained in the present study for *J. silicula* and other members of the *Juga* clade; they are also far from the type area for *J. silicula* and may be better assigned to *J. plicifera*.

Clade H has weak sculpture. Members of clade H have been identified as *J. nigrina* or *J. interioris*, but the clade does not include topotypes of any named species and instead apparently contains at least two undescribed species. It ranges from the central and southern Pit system into eastern tributaries of the central and southern Sacramento system (Figure 4). *Juga (O.) OTU 2* and *3* formed a consistently but weakly supported clade H, not closely related to topotypic populations of *J. nigrina*. *Juga (O.) OTU 2* comes from the central and lower Pit River drainage. It was widespread in this system, with a similar range to *J. occata*. Populations with a modest degree of genetic differentiation from the standard *J. (O.) OTU 2* were confined to the Burney Creek system and nearby areas in the central Pit River. This probably reflects genetic structure within the species, possibly due to Pit River Falls. The other group in clade H, *J. (O.) OTU 3*, includes four populations: two from Hat Creek (a southern tributary of the central Pit River); one (with relatively high genetic differentiation) from Battle Creek, a central Sacramento River tributary; and one from an upper tributary of the Feather River system. The headwaters of these three creeks are adjacent in an area affected by Holocene volcanic activity around Mt. Lassen (which last erupted in 1917, directly impacting Hat Creek), suggesting that a formerly continuous distribution may have been disrupted in the recent geologic past. The Lee *et al.* (2006) sequence for *J. interioris* represents our *J. (O.) OTU 3*, from the same region of central

California, although they do not identify which stream it came from. *Juga interioris*, *J. nigrina*, and *J. (O.)* OTU 3 have relatively featureless shells and could readily be confused.

The last clade, clade I, also has weak sculpture. It only includes *J. (O.)* OTU 4, from the upper Middle Fork John Day River system. Like clade H, the entire clade is undescribed. Morrison (1954) identified this population as *J. bulbosa*, but it does not match the types. The type locality for *J. bulbosa* is probably the Deschutes River, not the John Day River. *Juga (O.)* OTU 4 was genetically distant from the rest of the genus, not surprising for the easternmost and geographically most isolated sampled population.

The numerous undescribed OTUs and high local endemism found in this study suggest that many previous records of particular *Juga* species are incorrect. Taylor (1975) estimated that half of the Neogene and Recent freshwater mollusk records for western North America were incorrect, though that included records from many non-malacological papers. Nevertheless, all reports of *Juga* species away from their type regions require careful scrutiny, and many species names in *Juga* are used too widely. The problems in applying current nomenclature for *Juga* species to actual specimens is illustrated by previous molecular analyses. Except for the specimens identified as *J. plicifera* by Holznagel and Lydeard (2000) and Lee *et al.* (2006) and one *J. hemphilli dallesensis* sequence in Ó Foighil *et al.* (2009), none of the published sequences exactly matched those from our topotypic specimens. Of course, review of species-level taxonomy within *Juga* was outside the scope of all of those studies. Except for *J. h. dallesensis*, no previous molecular sequence for *Juga* came from the type locality of the respective species.

The major clades found in this study show distinct biogeographic patterns, not evident from the current taxonomy of the genus (contrast Figure 1 with Figure 4). As currently defined, subgenera and species range widely across multiple drainages, whereas our data suggest a high level of local and regional endemism, especially in the Upper Sacramento drainage (Figure 4). In general, the geographic patterns in our results match well with those observed in fish (Minckley *et al.*, 1986) and truncatelloideans (Hershler *et al.*, 2003, 2007). Thus, the distribution of *Juga* OTUs and clades of OTUs ties closely with drainage systems, as expected for freshwater taxa with limited dispersal ability. As *Juga* species place within geographically restricted clades, additional unsampled drainages may contain endemic species and should receive attention.

These geographic patterns indicate that the previous reports of supposedly wide-ranging species are incorrect. Most forms from the Pit-Sacramento system are endemic; a few extend into adjacent parts of the Klamath or Great Basin, but no further.

The present study represents a first pass at surveying the diversity of *Juga*. We are continuing with anatomical studies and hope that others will also be able to use and expand on these data. Overall our data suggest that *Juga* includes additional species with narrower ranges than current classification would suggest. Even with the current broad definitions of the species, Johnson *et al.* (2013) listed one species of *Juga* as stable and six as endangered, with the remaining ones either threatened or vulnerable. If additional data confirm that many species of *Juga* have narrow ranges, this would generally increase conservation concerns. Protection of streams and springs with extant populations of the rarest species needs to become a conservation priority for the region, especially with ongoing climate change. This requires not only protection of physical habitat, but of surrounding groundwater and surface water quality.

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TABLE 1. Primers used and sources.

Name	Sequence	Source
Folmer L	GGTCAACAAATCATAAAGATATTGG	Folmer <i>et al.</i> , 1994
Folmer H	TTAACTTCAGGGTGACCAAAAAATCA	Folmer <i>et al.</i> , 1994
gasCOI L	GGAATATTATTATCTCATTCTAAAGC	Minton and Lydeard, 2003
gasCOI H	TTTATTACTACTATTTTAAATATACG	Minton and Lydeard, 2003
HCOout	CCAGGTAAAATTAAAATATAAACTTC	Carpenter and Wheeler, 1999
LCO1731	GCTCCTGATATRGCTTTYCC	Latiolais, 2003*
HCO2004	CTCAAACGTATACCACGYCATC	Latiolais, 2003*
16F	CGCCTGTTTAACAAAAACAT	Krebs <i>et al.</i> , 2003
16R	CCGGTTTGAAC TCAGATCATGT	Krebs <i>et al.</i> , 2003
16SF	TTGYGACCTCGATGTTGGA	Rawlings <i>et al.</i> , 2003
ND1R	WGCATCHGCAATAGGYTG	Rawlings <i>et al.</i> , 2003

*=slightly modified

FIGURE 1--Geographic distribution of currently recognized subgenera in *Juga*.

FIGURE 2--Strict consensus phylogram of 33 trees, length 1670, maximum parsimony analysis of *cox1* data. Outgroups are not listed (Supplement 2). Numbers are bootstrap percentages over 50%, followed by posterior probability if over 80%. *=100% or posterior probability of 1.00; - = < 50% bootstrap support or < 0.8 posterior probability. Abbreviations: *J*=*Juga*, *C*=*Calibasis*, *O*=*Oreobasis*. Multiple identical sequences are indicated by multiple locality numbers, by a number in parentheses after the locality, or by †: *Juga* (*C.*) *acutifilosa* 1286(2)† also includes localities Sac364 and Sac487; *Juga* (*C.*) *occata* Sac111† also includes Sac164, Sac418, Sac 514, Sac532, Sac548, and Sac549; *Juga* (*O.*) *nigrina* Sac071(2)† also includes Sac024 and Sac112; *Juga* (*O.*) OTU 2 Sac036† also includes Sac040, Sac321, Sac395, Sac405 (2 sequences), Sac420, Sac560, Sac584, and Sac585. Locality key: C = Columbia (and tributaries), GB = Great Basin, K = Klamath, M = McCloud, P = Pit, PS = Puget Sound, S = Sacramento, W = western coastal drainages (Umpqua, Coquille, Rogue, Smith, Redwood Creek). Larger systems modified with l = lower, m = middle, s = south (tributary), u = upper. Also illustrated are shells representing the extant subgenera of *Juga*. A. *Juga* (*Calibasis*) *acutifilosa*, Willow Creek (Honey Lake basin), Lassen Co., California (clade B). B. *Juga* (*Oreobasis*) *nigrina*, Clear Creek, Shasta Co., California (clade E). C. *Juga* (*Juga*) *silicula*, McAllister Creek, Pierce Co., Washington (clade F). Scale bar=1 mm.

FIGURE 3--Phylogram, strict consensus of 151 trees of length 654, rrnL data. Numbers are bootstrap percentages over 50%. Published *Juga* sequence sources: Lyd = Lydeard *et al.*, 1997; HL = Holznagel and Lydeard, 2000; Lee = Lee *et al.*, 2006. Alphanumeric codes in outgroups derive from the published source. Locality and subgenus abbreviations as in Figure 5. Following Strong and Köhler (2009), "*Parajuga*" is used in quotes to indicate that the genus is not properly described. *Juga* (*J.*) *silicula* 5709† also includes *J. (J.) silicula* 6454 and 6457, *J. (J.) plicifera* HL, Lee, Whelan (both), 1477, and 5310, and *J. (J.)* OTU 2 5312.

FIGURE 4--Geographic distribution of major clades detected in the present study. Apart from D and G (which share a distribution primarily in the Deschutes River system) and A and H (which overlap in the middle and lower Pit River system), all clades are allopatric.

Figure 1

Figure 2

Figure 3

Figure 4