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SHORT COMMUNICATION

Three deeply divided lineages of the freshwater mussel genus *Anodonta* in western North America

Jer Pin Chong · Jayne C. Brim Box · Jeanette K. Howard · David Wolf · Terry L. Myers · Karen E. Mock

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Abstract The surprising diversity and recent dramatic decline of freshwater mussels in North America have been well documented, although inventory efforts to date have been concentrated in the eastern United States. Unlike their eastern counterparts, western freshwater mussels have received comparatively little attention. The accurate identity of western lineages is a necessary component for future inventory, monitoring, and ecological work involving these taxa. Here we initiate a study involving the most speciose genus (*Anodonta*) in western North America, incorporating information about type localities and type specimen morphology and describing the discovery of three highly divergent lineages among four western *Anodonta* species. In a limited phylogenetic analysis, we find (1) that *A. californiensis/nuttalliana* and *A.oregonensis/kennerlyi* are distinct, highly divergent clades, and (2) that *A. beringiana* is more closely allied with *A. woodiana*, an Asian species, than either of the other two western North American clades. We were largely unable to resolve the placement of these three clades with respect to other anodontines, and suggest the need for a broader phylogenetic framework. We recommend, however, that the existence of these three deeply divergent groups be considered in the development of regional monitoring, conservation and research plans despite the taxonomic uncertainty.

Keywords *Anodonta* · Floater · Freshwater mussel · Mitochondrial DNA · North America · Unionid

J. P. Chong · K. E. Mock (✉)
Wildland Resources Department, Utah State University, Logan, UT 84322-5230, USA
e-mail: karen.mock@usu.edu

J. C. Brim Box · J. K. Howard · D. Wolf
Confederated Tribes of the Umatilla Indian Reservation, Department of Natural Resources, Pendleton, OR 97801, USA

T. L. Myers
Apache-Sitgreaves National Forests, Springerville, AZ 85938, USA

Introduction

The advent of molecular tools over the past few decades has led to an enormous wave of genus- and species-level taxonomic revisions in nearly all groups of organisms. This is particularly true in molluscan taxa, many of which were designated in the 19th and early 20th centuries based on conchological variance, which we now know to be influenced by convergent evolution as well as environmental and developmental factors (e.g. Lydeard and Lindberg 2003; Minton and Lydeard 2003; Campbell et al. 2005). Appropriate taxonomy is critically important to the effective protection, management, and monitoring of imperiled species, but high-level taxonomic revisions often require extensive sample acquisition efforts and phylogenetic analyses that proceed slowly and are subject to subsequent revision as molecular tools and analytical approaches evolve. For species on a rapidly declining trajectory, it is often important to recognize the existence of regional, genetically divergent groups worthy of independent management prior to the development of a full phylogeny and formal set of revisions involving many other taxa. Here we describe such a "bottom-up" approach using topotypic material to define genetically distinct groups in the freshwater mussels of western North America.

Freshwater mussels (Order Unionoida) are important members of riverine and lacustrine ecosystems, and the decline of freshwater mussels has been recognized as a global phenomenon of growing concern (Bogan 1993; Neves et al. 1997; Master et al. 2000; Lydeard et al. 2004). North America has the greatest freshwater mussel diversity (approximately 300 species) in the world (Stansbery 1971), and most of these species are found in the southeastern US (Burch 1973; Lydeard and Mayden 1995). In the western US and western Canada, species diversity is markedly lower, with only eight species and three genera (*Anodonta, Margaritifera* and *Gonidea*) currently recognized (Turgeon et al. 1998). Among these western genera, *Anodonta* is the most species-rich, with 6 species: *A. beringiana* Middendorff (1851); *A. californiensis* Lea (1852); *A. dejecta* Lewis (1875) (possibly extinct); *A. kennerlyi* Lea (1860); *A. nuttalliana* Lea (1838); *A. oregonensis* Lea (1838) (Turgeon et al. 1998). However, western *Anodonta* has undergone many taxonomic revisions (Carpenter 1856; Call 1884; Henderson 1929; Kat 1983), and species ranges and boundaries continue to be unclear due to morphological similarity among species and variation among locations (e.g. Zanatta et al. 2007).

To date, taxonomic designations in western *Anodonta* have been made almost entirely on the basis of variation in conchological features, which can be quickly identified by field personnel on live animals or shells, and provide an intuitive sense of taxonomic subdivision. However, conchological characteristics can be unreliable indicators of evolutionary divergence patterns (Hoeh 1990; Williams and Mulvey 1997; Lydeard et al. 2004). The objective of our study was to determine whether patterns of genetic divergence among *Anodonta* species in the western US and western Canada are reflected in current taxonomic designations. Our sampling strategy was designed to include as much topotypic material as possible, to facilitate future taxonomic revisions and to establish a framework for additional survey, morphometric, and phylogeographic studies in these organisms.

Methods

We collected material from five of the currently recognized *Anodonta* species in the western US and Canada. The earliest type locality for *Anodonta* in western Canada and the western US is the confluence of the Willamette and Columbia Rivers in Oregon, where three species were originally described: *A. nuttalliana, A. oregonensis* and *A. wahlametensis* (Lea 1838). Subsequently, *A. wahlametensis* was placed in the synonymy of *A. nuttalliana* by Call (1884) and remains there (Turgeon et al. 1998), although many biologists continue to recognize it as a distinct form. None of these designations have been assessed genetically. Because taxonomic revisions follow the International Laws of Zoological Nomenclature and are based, in part, on the earliest nomenclature, we chose this type location as an appropriate place to initiate a broad review of genetic divergence among western *Anodonta* species. We collected live adult *Anodonta* specimens in August 2004 from two areas near the Columbia/Willamette confluence: (Bybee Lake (ABB); $n = 11$) and the Columbia Slough (ACC); $n = 8$) (Fig. 1). Two distinct morphotypes were evident in both of these locations (Fig. 2), and both were included in our collections.

In addition, we collected specimens representing *A. kennerlyi* from Chilliwack Lake, British Columbia (AKCR; $n = 7$), *A. californiensis* from the East Fork Black River, Arizona (ABR; $n = 5$), and *A. beringiana* from Waldron Lake in Anchorage, Alaska (ABW; $n = 5$). Chilliwack Lake, B. C. is near the type locality for *A. kennerlyi* (Lea 1860). The Black River, Arizona, is in the Colorado River drainage, and the population of *Anodonta* in this locality is thought to be representative of *A. californiensis*, which was originally described from the lower Colorado River (Lea 1852). The Black River in Arizona

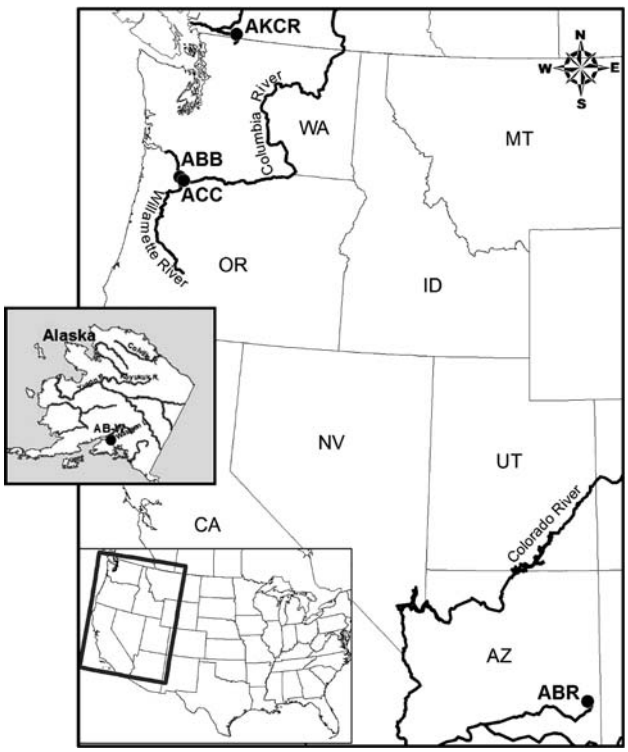

Fig. 1 *Anodonta* sample locations: Bybee Lake, Oregon (ABB) and Colombia Slough, Oregon (ACC) (*A. oregonensis* and *nuttalliana* morphs); Lake Chilliwack, British Columbia (AKCR) (*A. kennerlyi* morph), the East Fork of the Black River, Arizona (ABR) (putatively *A. californiensis*), and Waldron Lake, Alaska (ABW) (putatively *A. beringiana*)

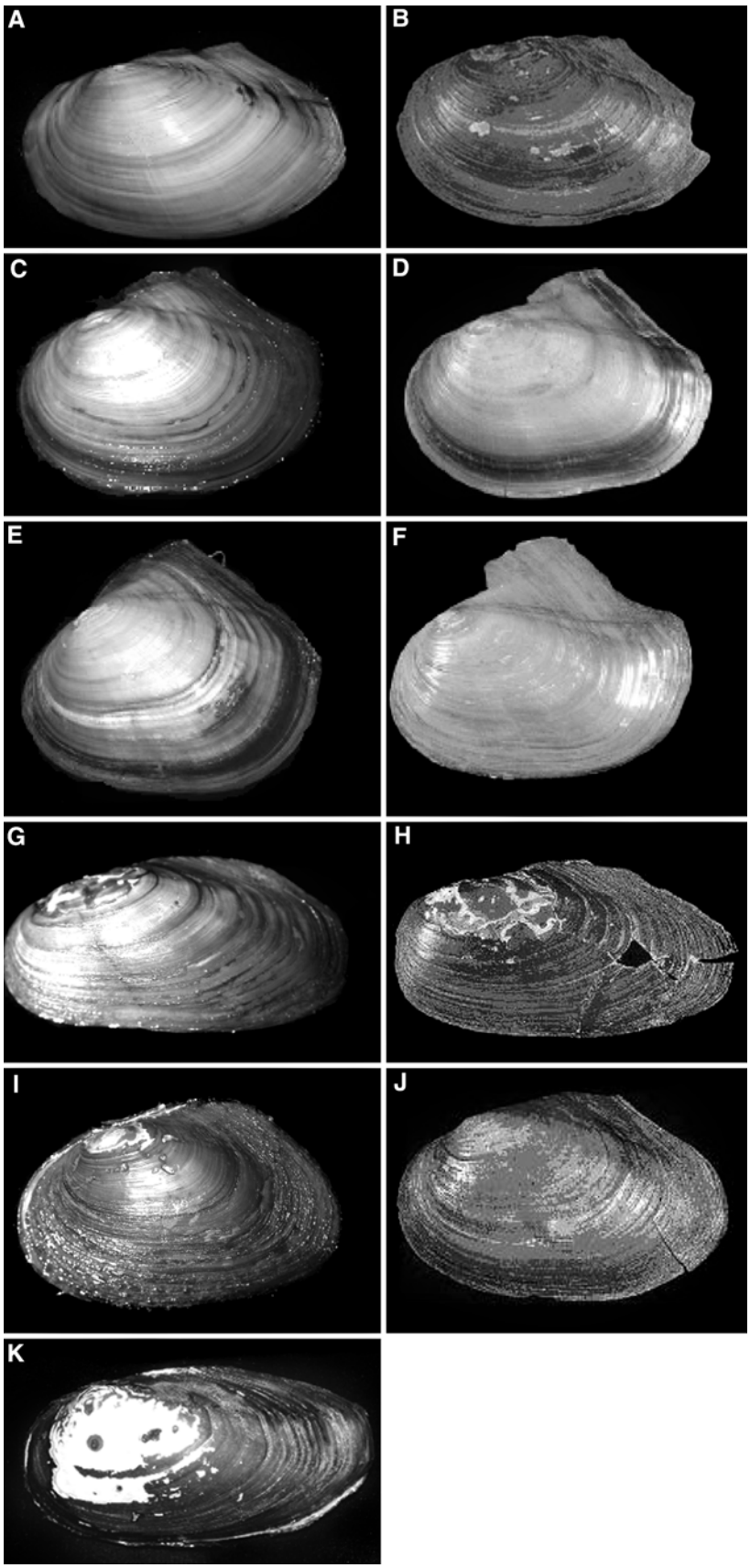

Fig. 2 *Anodonta* specimens from western North America: (**a**) contemporary *A. oregonensis* morphotype from the confluence of the Willamette and Columbia Rivers (length = 8.1 cm); (**b**) *A. oregonensis* figured specimen (USNM 86432; length 8.1 cm); (**c**, **e**) contemporary *A. nuttalliana* morphotypes from the confluence of the Willamette and Columbia Rivers (lengths = 8.5 cm, 8.3 cm respectively); (**d**) *A. nuttalliana* figured specimen (USNM 86391; length 6.0 cm); (**f**) *A. wahlametensis* figured specimen (USNM 86363; length 6.3 cm); (**g**) contemporary *A. kennerlyi* morphotype from the Lake Chilliwack (length = 5.0 cm); (**h**) *A. kennerlyi* figured specimen (USNM 86533; length 6.5 cm); (**i**) Contemporary *A. californiensis* morphotype from the East Fork Black River (length = 7.1 cm); (**j**) *A. californiensis* figured specimen (USNM 86393; length 5.8 cm); (**k**) contemporary *A. beringiana* morphotype from Lake Waldron, Alaska (figured specimen not available). Photographs **b**, **d**, **f**, **h** and **j** are copyrighted by Sheila Nadimi and appear courtesy of the Confederated Tribes of the Umatilla Indian Reservation

contains one of the only known remaining populations of *A. californiensis* in the entire Colorado River system. Although Middendorf (1851) did not designate a type locality for *A. beringiana*, his original collections were made from eastern Siberia and Alaska. Specimens figured by Lea (i.e., *A. oregonensis*, *A. nuttalliana*, *A. kennerlyi*, and *A. californiensis*) and currently housed in the USNM type collection were photographed in order to provide a morphological comparison with the contemporary specimens collected in this study.

Live specimens were preserved by severing adductor muscles and placing in a large volume (>5× specimen volume) of 95% ethanol. A sample of mantle or foot tissue (approximately 2 mm^3) from each field-collected specimen was dissected, and genomic DNA extracted using a salt chloroform protocol (Mullenbach et al. 1989) or Qiagen DNeasy® Tissue kit. An approximately 650 bp region of the mitochondrial F-lineage cytochrome c oxidase subunit I (COI) gene was amplified using the primers LCO1490 and HCO2198 (Folmer et al 1994; King et al 1999). We designed two internal primers LCO1550 (5′-ATTCGAGCT GAGTTGGGTCAA) and HCO2100 (5′-GTAATAGCACC AGCTAAAACT) and used them in various combinations with LCO1490 and HCO2198 to generate bidirectional sequences of this region (GenBank accessions EU327350–EU327357). PCR reactions consisted of 0.20mM dNTPs, 1× PCR buffer, 2.5 mM $MgCl_2$, 0.5 μM primers and 1U Taq polymerase in a total volume of 50 μl. Reactions were initiated with a denaturation step at 94°C for 4 min, followed by 31 cycles of 92°C for 30 s, 50°C for 1 min and 72°C for 90 s, and a final 72°C extension for 10 min. Reaction products were purified using Microcon-PCR® spin columns (Millipore), followed by a sequencing reaction using the Applied Biosystems, Inc. (ABI) BigDye Terminator Kit v3.1. Sequencing reaction products were separated and visualized using an ABI PRISM® 3730 Genetic Analyzer. Contiguous sequences for each individual were constructed and aligned using SEQMAN and MEGALIGN software (DNAStar). We constructed a 516-bp alignment of our unique haplotypes with 13 other Anodontinae COI sequences available in GenBank, and included a member of the Unioninae (*Unio tumidis*) as an outgroup. Using MEGA 3 software (Kumar et al. 2004) we constructed a minimum evolution tree using the Kimura 2-parameter model of substitution (Kimura 1980) following the recommendations of Nei and Kumar (2000), and obtained 1000× bootstrap values to assess nodal support.

In order to assess the possibility of mitochondrial introgression where multiple lineages were found in sympatry, we performed an amplified fragment length polymorphism (AFLP) analysis (Vos et al. 1995), following the protocols described in Mock et al. (2004) with the selective primers *Eco*RI-ACG (FAM-labeled) and *Mse*I-AC. For this analysis we included all samples collected from the ACC population, where two of the major lineages were found in sympatry (see results).

Results

The two morphotypes collected from ABB and ACC we tentatively designated *A. oregonensis* and *A. nuttalliana*, based on comparisons to our photographs of the specimens figured by Lea (1838), as well as Lea's original descriptions. According to Lea (1838), *A. wahlametensis* resembled *A. nuttalliana*, but differed from the latter with respect to outline, the degree of inflation, and the prominence of the wing. While specimens bearing some similarities to Lea's description of *A. wahlametensis* were found and collected at ABB and ACC, shell morphology varied considerably, and there was no clear distinction between these morphotypes and specimens designated *A. nuttalliana*. Therefore, these specimens were included in a group designated as the *A. nuttalliana* morphotype. The morphology of specimens collected from AKCR and ABR were consistent with the original descriptions for *A. kennerlyi* and *A. californiensis*, respectively.

Analysis of the western *Anodonta* sequences revealed the existence of three deeply divergent, well-supported groups: a clade containing the *A. oregonensis* & *A. kennerlyi* morphs, a clade containing the *A. californiensis* and *A. nuttalliana* morphs, and *A. beringiana* (Fig. 3). Using the Kimura 2-parameter model of substitution, the *A. oregonensis/kennerlyi* clade differed from the *A. californiensis/nuttalliana* clade by 13.2% and from the *A. beringiana* clade by 17.5%. The *A. californiensis/nuttalliana* clade differed from the *A. beringiana* clade by 15.8%. Divergence within these clades was minimal (0.4–1.2%). Clade membership at the Columbia/Willamette type locality (haplotypes D-H vs. haplotype AA) was entirely congruent with the division between the two observed morphotypes (*A. oregonensis* vs. *A. nuttalliana*) (Fig. 3). However, the minimum evolution dendrogram failed to resolve relationships between the three North American clades and other anodontine species, with the exception of an affiliation between *A. beringiana* and *A. woodiana* (an Asian species), which was moderately well supported. The topology and nodal strength depicted in the minimum evolution tree did not change significantly with the use of different substitution models and optimality criteria.

AFLP profiles for the specimens from the Columbia/Willamette type locality (ACC) were consistent with the deep mitochondrial subdivision observed between mitochondrial clades, suggesting reproductive isolation. Two distinct classes of AFLP profiles were evident. Only nine bands between 50 and 300 bp were present in all samples,

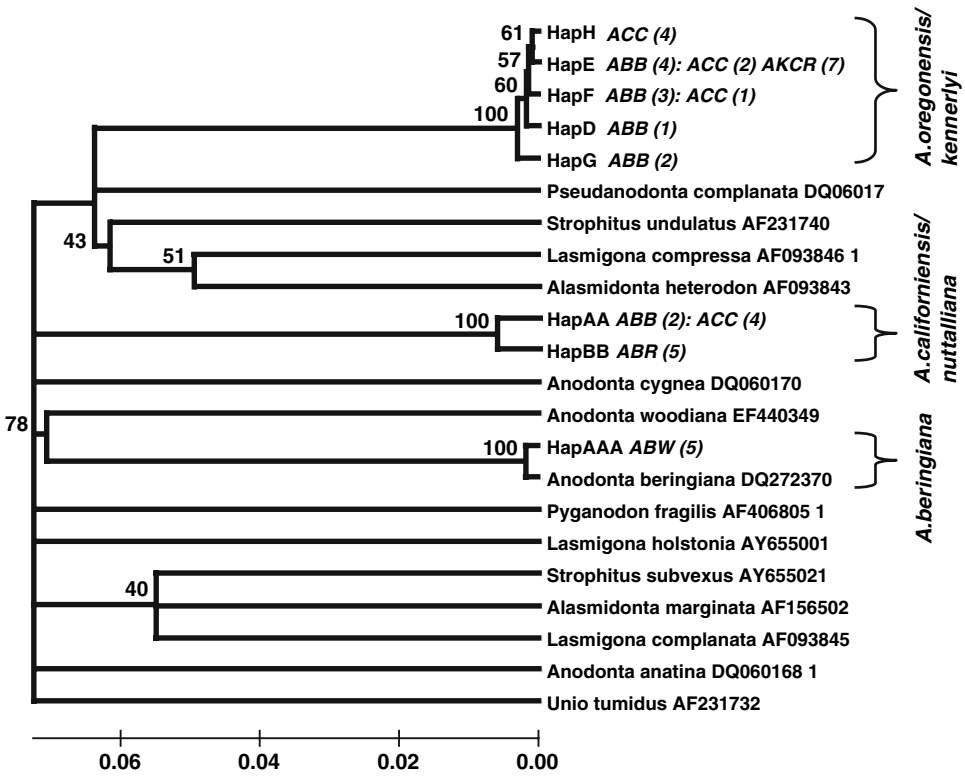

Fig. 3 Minimum evolution dendrogram of western North American *Anodonta* and other anodontine mitochondrial COI sequences using Kimura's 2-parameter model of substitution (Kimura 1980). Specimens from Bybee Lake, Washington (ABB), the Columbia Slough, Washington (ACC), Lake Chilliwack, British Columbia (AKCR), and the Black River, Arizona (ABR) are included, and numbers of individuals represented by each haplotype are provided in parens. Among the comparative anodontine taxa, *A. cygnea*, *P. complanata*, and *A. anatina* are European taxa, *A. woodiana* is an Asian taxon, and the remainders are eastern North American taxa. *Unio tumidis* was used as an outgroup. Numbers at nodes represent the proportion of 1000 bootstrap replicates supporting each node. Bootstrap values less than 40% are not shown

while 46 bands were consistently present in one clade and absent in the other (i.e. diagnostic).

Discussion

Our findings indicate the existence of three deeply divided lineages within western North American *Anodonta*: one clade including *A. oregonensis* and *A. kennerlyi*, one clade including *A. californiensis* and *A. nuttalliana*, and one clade including *A. beringiana*. These patterns are not well represented by the current taxonomy or morphological similarity of these animals (Fig. 2). Specimens representing the first two clades were found in sympatry at the confluence of the Willamette and Columbia rivers, which is the type locality for both *A. oregonensis* and *A. nuttalliana* Lea (1838). Thus, both *A. oregonensis* and *A. nuttalliana* Lea (1838) appear to be distinct and valid species names, and represent the oldest names affiliated with western Anodonta. In comparison, the third species described by Lea (1838) from this type locality, *A. wahlametensis*, was synonymized under *A. nuttalliana* (Turgeon et al. 1998), and our molecular and morphological data supported this synonymy. However, it is possible that a distinct taxon once existed in this location and has since been extirpated or was not sampled in our surveys.

The grouping of *A. kennerlyi* with *A. oregonensis* and *A. californiensis* with *A. nuttalliana* was surprising, because *A. oregonensis* and *A. californiensis* are morphologically similar (Fig. 2). By contrast, the *A. kennerlyi* and *A. oregonensis* morphs, generally considered to be quite distinct, shared haplotype E, the most common haplotype in the *A. kennerlyi/oregonensis* clade. The divergence between the *A. kennerlyi/oregonensis* and *A. californiensis/nuttalliana* clades (13.2%) was comparable to or greater than the divergence between different genera of other anodontines (e.g. *Alasmidonta marginata* vs. *Strophitus subvexus* 10.8% divergence; *Lasmigona compressa* vs. *Pseudanodonta complanata* 12.5% divergence). Divergence of the *A. beringiana* clade from the other two western clades was even more pronounced.

Our phylogenetic analyses, including sequence data from a limited number of publicly available anodontine species, indicated an alliance between *A. beringiana* and *A. woodiana*, an Asian taxon. Such a biogeographical link between western North America and Asia has also been observed in the freshwater gastropods (Lydeard et al. 2002). Relationships of the three western *Anodonta* clades with other anodontines were not resolved by our phylogenetic analysis (Fig. 3), and will require a much more extensive dataset, both in terms of sequence data and taxa represented. In addition, the surprising depth of the divergence among these clades suggests the need for revisiting these phylogenetic relationships at the genus level, as has been recently undertaken for the North American amblemines (Campbell et al. 2005).

Until these genus-level relationships can be resolved, we suggest that the three clades of western *Anodonta* be carefully considered in the development of regional management and monitoring plans. For example, if the goal of conservation planning for freshwater mussels is to preserve divergent lineages, then the boundary between *A. californiensis* and *A. oregonensis* is much more important than that between *A. oregonensis* and *A. kennerlyi*. In addition, research programs investigating host fish relationships, ecological associations, and phylogeography should recognize the evolutionary depth separating these three clades, and the relative shallowness of divergence within clades. Additional genetic, morphological, and ecological data will be required to assess the validity of current taxonomic differences within the *A. californiensis/nuttalliana* clade and the *A. kennerlyi/oregonensis* clade.

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