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REGULAR ARTICLE

A COMPARISON OF GLOCHIDIAL SHELLS OF THE FRESHWATER MUSSELS *ANODONTA CALIFORNIENSIS*, *ANODONTA KENNERLYI*, *ANODONTA NUTTALLIANA*, AND *ANODONTA OREGONENSIS*

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ABSTRACT

Only recently have substantial efforts been made to understand phylogenetic relationships among freshwater mussels of the western United States and Canada. Genetic studies show the existence of two divergent clades in western *Anodonta*, one containing *Anodonta californiensis* and *Anodonta nuttalliana*, and another containing *Anodonta oregonensis* and *Anodonta kennerlyi*, but relationships within these two clades remain unclear. For example, some authors have placed *A. californiensis* in the synonymy of *A. nuttalliana*, but additional taxonomic information is needed to resolve these issues. We examined glochidial shell size and fine structure of these four species to assess the taxonomic utility of these characters. Glochidia of *A. oregonensis* and *A. kennerlyi* were similar in size and fine structure, which supports their proposed close relationship. Glochidia of *A. californiensis* and *A. nuttalliana* were smaller in all dimensions than *A. oregonensis* and *A. kennerlyi*, which supports the existence of two divergent clades. However, shell size and fine structure also differed between *A. californiensis* and *A. nuttalliana*, which supports the distinctiveness of these two taxa. Glochidial characters may help to clarify evolutionary relationships among western U.S. *Anodonta* and other problematic groups.

KEY WORDS: glochidia, *Anodonta*, Anodontinae, scanning electron microscopy

INTRODUCTION

Until recently, six species of freshwater mussels in the genus *Anodonta* were recognized from western North America (Turgeon et al. 1998): Yukon Floater, *Anodonta beringiana* A. Middendorf, 1851; California Floater, *Anodonta californiensis* I. Lea, 1852; Woebegone Floater, *Anodonta dejecta* Lewis, 1875; Western Floater, *Anodonta kennerlyi* I. Lea, 1860; Winged Floater, *Anodonta nuttalliana* I. Lea, 1838; and Oregon Floater, *Anodonta oregonensis* I. Lea, 1838. A recent taxonomic revision reassigned *A. beringiana* to the genus *Sinanodonta* based on its closer genetic relationship to Asian *Sinanodonta woodiana* Lea, 1834, than to North American *Anodonta* (Chong et al. 2008; Lopes-Lima et al. 2017). Williams et al. (2017) also synonymized *A. dejecta* under *A. californiensis* based primarily on adult shell morphology (see Bequaert and Miller 1973; AZGFD 2017).

Genetic studies show that the remaining four species represent two highly divergent clades: *A. oregonensis*/*kennerlyi* and *A. californiensis*/*nuttalliana* (Chong et al. 2008; Mock et al. 2010). Both species within each clade are genetically similar, and their distinctiveness is unclear. Blevins et al. (2017) suggested synonymizing *A. californiensis* under *A. nuttalliana* based on overlapping adult shell morphology. However, adult shell morphology in western *Anodonta* is highly variable, and additional characters are needed to evaluate the status of these taxa (Mock et al. 2010).

Gross glochidial shell morphology has been used to inform mussel taxonomy since the early 1900s (LeFevre and Curtis 1910; Surber 1912). More recently, glochidial fine structure as revealed by scanning electron microscopy (SEM) has been

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used to inform phylogenetic hypotheses (Sayenko et al. 2005; Pimpão et al. 2012; Sayenko 2016a, Sayenko 2016b). We examined glochidial shell morphology of *A. californiensis*, *A. kennerlyi*, *A. nuttalliana*, and *A. oregonensis* to assess whether these characters may be useful for better understanding relationships among these taxa.

METHODS

We collected two to four gravid females of each species from the following locations (Fig. 1): *A. californiensis*, Wildhorse Creek, tributary of the Umatilla River, Oregon (*A. californiensis* is extirpated at the type locality); *A. kennerlyi*, Lake Chilliwack, British Columbia (type locality); *A. nuttalliana*, Columbia Slough, near Portland, Oregon (type locality); and *A. oregonensis*, Walla Walla River, Washington (*A. oregonensis* was unavailable at the type locality). The specimens of *A. kennerlyi* and *A. nuttalliana* we used for this project were the same specimens whose identification was described by Chong et al. (2008) based on mitochondrial cytochrome c oxidase subunit I (COI) sequencing. We verified our specimens of *A. californiensis* and *A. oregonensis* based on adult shell shape and examination of mitochondrial COI sequences that allow assignment to the clades described by Chong et al. (2008).

Upon collection, we examined the gills of each mussel to assess gravidity; gravid gills were identified as having a puffy or swollen appearance. We transported gravid mussels to the laboratory where we collected glochidia by rupturing the gill and flushing out glochidia with a wash bottle filled with water. We used only fully mature glochidia for analysis. Maturity was determined by introducing several grains of salt into a subsample of glochidia; fully mature glochidia snapped shut after exposure to salt (Zale and Neves 1982).

We preserved and examined glochidia in two ways. We preserved one subsample of glochidia from each female in 70% ethanol and measured the size of 20 glochidia from each subsample. For each glochidium, we measured the following shell dimensions under a light dissecting microscope using ImageJ image analysis software (NIH 2004): height (the widest point from the dorsal to ventral shell edge), length (the widest point from the anterior to posterior shell edge), and hinge length (Fig. 2).

We preserved a second subsample of glochidia from each female for examination of shell fine structure with SEM. We removed glochidial tissue by soaking glochidia in a 5% sodium hypochlorite solution for 2 min, followed by five rinses in tap water and preservation in 70% ethanol (Kennedy et al. 1991; O'Brien et al. 2003). Glochidial shell samples were shipped to the Interdisciplinary Center for Biotechnology Research at the University of Florida, Gainesville, for SEM, where several hundred shells of each species were mounted on double-sided carbon tape, air dried for 15 min, and coated with gold. Photos were taken of the exterior and interior valve; the flange region, a flattened area along the ventral margin of the glochidial valve; and shell sculpture.

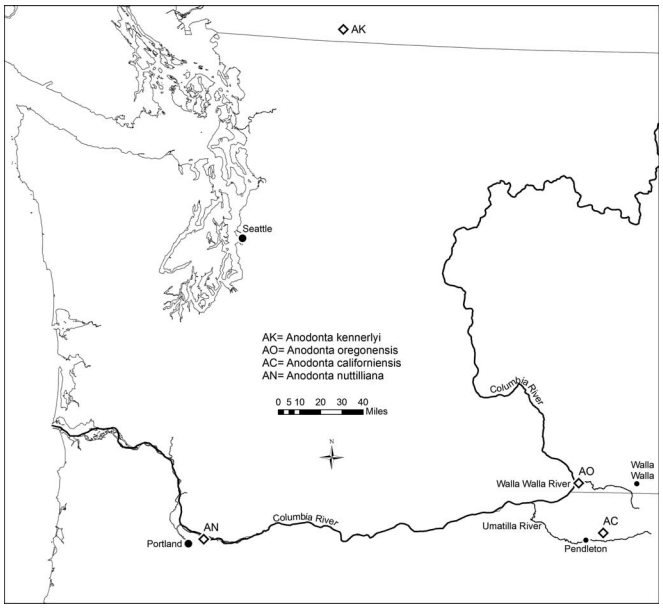

Figure 1. Map of Oregon and Washington, USA, and southern British Columbia, Canada, showing sites where mussels were collected for this study.

We examined the following fine structures: the styliform hook, the projection from the ventral edge of the valve; microstylets, larger (>1.0 μm) toothlike projections located on the styliform hook; micropoints, smaller points (<1.0 μm) located along the ventral valve edge; and exterior shell sculpture, the fine surface texture on the valve (Fig. 3; see Clarke 1981; Hoggarth 1999).

We used multivariate analysis of variance (MANOVA) to examine how glochidia size varied among the four species. All linear combinations of the dependent variables were approximately normally distributed based on examination of scatter

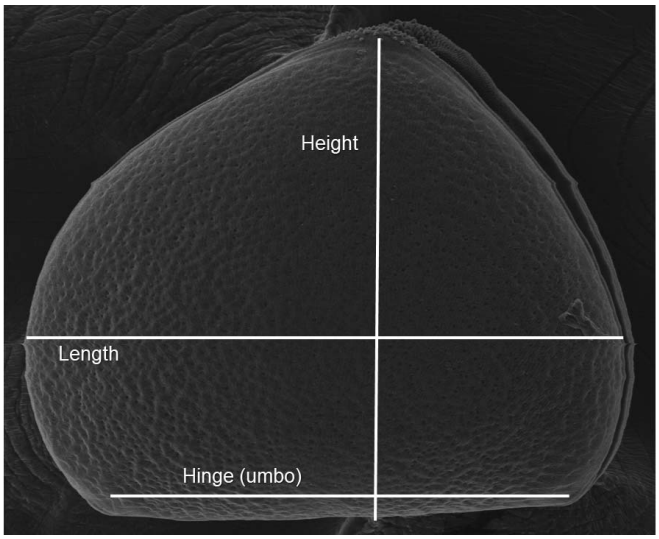

Figure 2. Scanning electron micrograph of an anodontine glochidium showing size dimensions used in this study. Photo by K. Backer-Kelley.

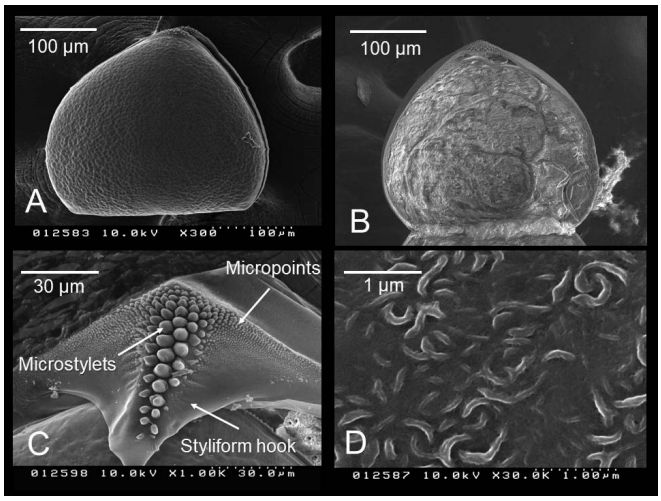

Figure 3. Scanning electron micrographs of *Anodonta californiensis* glochidia. A, exterior of valve (300×); B, interior of valve (300×); C, flange region with styliform hook and associated structures (1,000×); D, exterior valve sculpture (30,000×). Photos by K. Backer-Kelley.

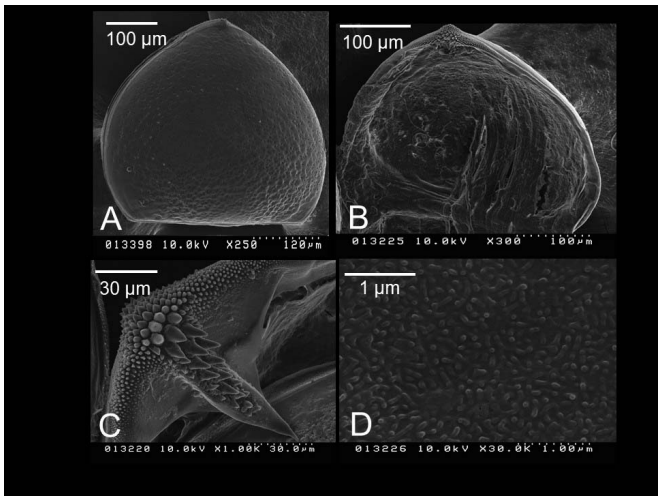

Figure 4. Scanning electron micrographs of *Anodonta kennerlyi* glochidia. See Fig. 3 for details. Photos by K. Backer-Kelley.

plots, and there were no departures from normality or homogeneity of variance (Shapiro-Wilks test; Bartlett's test for homogeneity of variance). Because the MANOVA overall F test was significant, we examined the three size variables separately using ANOVA and Tukey's HSD post-hoc tests. All analyses were conducted with JMP 11 (SAS Institute, Cary, NC, USA).

We made qualitative comparisons of fine structure morphology among species.

RESULTS

There were significant overall differences in shell size among the four species (Wilks $\lambda = 0.05$, $F_{177,81} = 45.3$, $P < 0.001$). Shell length, height, and hinge length each differed significantly among the four species ($F_{3,78} = 94.76$, 143.37, 167.03, respectively; $P < 0.0001$ for all tests; Table 1). Shell length was greatest in *A. kennerlyi* and smallest in *A. californiensis*, and length differed among all four species. Shell height and hinge length were not significantly different between *A. kennerlyi* and *A. oregonensis* but were significantly larger than in *A. nuttalliana* or *A. californiensis*. Shell height and hinge length differed significantly between *A. nuttalliana* and *A. californiensis*, with *A. californiensis* having the smallest values.

Microstylet morphology was similar within each clade, but it differed between the two clades. However, microstylet arrangement was similar between the *A. oregonensis/kennerlyi* clade and *A. nuttalliana*, but it differed in *A. californiensis*. Shell sculpture differed between the *A. californiensis/nuttalliana* and *A. oregonensis/kennerlyi* clades, but this character was similar within clades (Table 2; Figs. 3–6). Shell sculpture of *A. oregonensis* and *A. kennerlyi* was intermediate between two previously described sculpture patterns, beaded and loose-looped (Hoggarth 1999). Shell shape and micropoint morphology did not provide consistent discrimination of clades or species.

Table 1. Glochidial shell measurements of four western North American *Anodonta*. Values are means ± standard deviation (µm) and are based on univariate ANOVA for each size variable. Values within a column with different superscripted letters are significantly different ($P < 0.05$, Tukey's HSD post-hoc tests).

Species	Length	Height	Hinge Length
A. californiensis	252.6 ± 10.2[a]	230.5 ± 16.4[a]	162.3 ± 13.6[a]
A. nuttalliana	265.0 ± 10.5[b]	260.4 ± 10.1[b]	204.1 ± 10.2[b]
A. oregonensis	299.6 ± 13.6[c]	301.1 ± 14.4[c]	234.9 ± 12.1[c]
A. kennerlyi	317.7 ± 19.2[d]	312.5 ± 14.8[c]	242.9 ± 14.5[c]

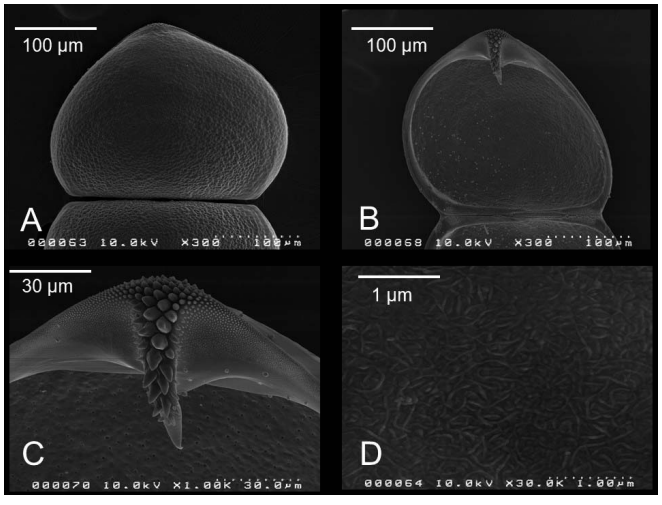

Figure 5. Scanning electron micrographs of *Anodonta nuttalliana* glochidia. See Fig. 3 for details. Photos by K. Backer-Kelley.

Table 2. Glochidial fine structure characters of four western North American *Anodonta*.

Species	Shape	Microstylets	Micropoints	Shell sculpture
A. californiensis	Subtriangular	Blunt; in rows	Numerous; along ventral rim and microstyle	vermiculate
A. nuttalliana	Subtriangular	Blunt; in broken rows	Few; along ventral rim and microstyle	vermiculate
A. oregonensis	Subtriangular	Sharply pointed; in broken rows	Few; along ventral rim and microstyle	intermediate beaded/ loose-looped
A. kennerlyi	Subtriangular	Sharply pointed; in broken rows	Numerous; along ventral rim	intermediate beaded/ loose-looped

DISCUSSION

The similar glochidial size of *A. oregonensis* and *A. kennerlyi* supports the close genetic relationship between these species (Chong et al. 2008). The smaller size of *A. californiensis* and *A. nuttalliana* potentially supports the close relationship between these two species and their distinctiveness from the *A. oregonensis/A. kennerlyi* clade. However, the consistent and marked differences in size between *A. californiensis* and *A. nuttalliana* do not support placement of *A. californiensis* in the synonymy of *A. nuttalliana* (Blevins et al. 2017).

Patterns of glochidial shell fine structure among the four species were similar in most respects to patterns of size. *Anodonta oregonensis* and *A. kennerlyi* had similar patterns of shell sculpture, which supports their close genetic relationship (Chong et al. 2008). This pattern, which was intermediate between beaded and loose-looped sculpture, has been described in one other North American anodontine, *Utterbackiana implicata* (Hoggarth 1999), and in an Asian species, *Kunashiria haconensis* (Sayenko 2016a). Shell sculpture also was similar between *A. californiensis* and *A. nuttalliana*, which supports their close genetic relationship, and the difference in sculpture between these species and *A. oregonensis/kennerlyi* supports the existence of two divergent clades in western *Anodonta* as proposed by Chong et al. (2008). Sculpture similar to that of *A. californiensis* and *A. nuttalliana* also is present in North American *Utterbackiana suborbiculata* (Hoggarth 1999) and Asian *Anodonta cygnea* and *Cristaria tuberculata* (Sayenko 2016a, 2016b). Microstylet morphology also supported differences between the two clades, but microstylet arrangement (continuous versus broken rows) of *A. californiensis* differed markedly from *A. nuttalliana*, which does not support the placement of *A. californiensis* in the synonymy of *A. nuttalliana* as proposed by Blevins et al. (2017). However, microstylet arrangement of *A. nuttalliana* was more similar to the more distantly related *A. oregonensis/kennerlyi* than to its apparent close relative, *A. californiensis*. Micropoint morphology did not appear to be useful for diagnosing clades or species.

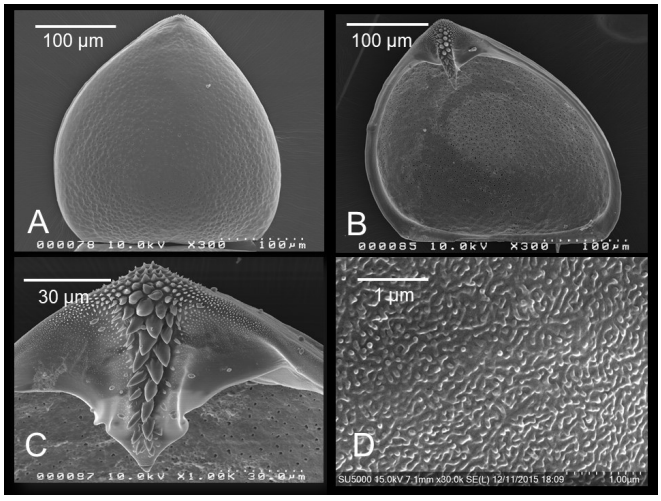

Figure 6. Scanning electron micrographs of *Anodonta oregonensis* glochidia. See Fig. 3 for details. Photos by K. Backer-Kelley.

Patterns of glochidia shell size and fine structure among these four species of western *Anodonta* largely support proposed phylogenetic relationships based on genetic data (Chong et al. 2008; Mock et al. 2010), but they provide additional information about the potential distinctiveness of *A. californiensis* and *A. nuttalliana*. Glochidial shell features provide less ambiguous and less variable characters than notoriously vague and highly variable adult shell characters, which can be influenced to a large extent by environmental factors. Our conclusions are based on glochidia from a single population for each species. Within-population variation in glochidial size generally is low, but little is known about among-population variation (Kennedy and Haag 2005), and our data do not reflect this latter potential source of variation. Nevertheless, use of glochidial characters in conjunction with genetic data, adult anatomical characters, reproductive traits, and other data may help to clarify evolutionary relationships among western North American *Anodonta* and other problematic groups.

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