

## A DNA-based Identification Key to Pacific Northwest Freshwater Mussel Glochidia: Importance to Salmonid and Mussel Conservation

### Abstract

Glochidia larvae of unionacean mussels native to Pacific coast drainages are temporary obligate parasites on the gills or fins of fish, including juvenile Pacific salmonids. This parasitic condition in fishes has been termed glochidiosis, and may have a detrimental impact on both long- and short-term survival and growth in juvenile salmonids, particularly in hatcheries. Here we report diagnostic differences in the nucleotide sequences of a 710-bp fragment of the mitochondrial cytochrome *c* oxidase subunit I gene (*COI*) from five unionacean freshwater mussels native to the Pacific Northwest: the western pearlshell *Margaritifera falcata*; the western ridged mussel *Gonidea angulata*; the Yukon floater *Anodonta beringiana*; and two additional species of the genus *Anodonta* (tentatively, the winged floater *A. nuttalliana* and the Oregon floater *A. oregonensis*). Digestion of polymerase chain reaction products for the *COI* gene with the restriction endonuclease *AluI* produced species-specific differences in size and number of mitochondrial DNA fragments. These DNA fragment patterns were diagnostic for glochidia that were sampled from the gills of juvenile Pacific salmon. We anticipate that this molecular-identification tool for mussel glochidia will be useful in both ecological studies of natural glochidia-host relationships and in salmonid hatchery situations where specific glochidia pathogens require identification.

### Introduction

Freshwater mussels (Unionacea) are among the most imperiled fauna in North America with over 70% of the nearly 300 recognized species being classified as either endangered, threatened, or of special concern (Williams et al. 1993, Strayer et al. 2004). Until recently, few studies of any kind had been conducted on unionacean mussels in the Pacific Coastal Region of North America (Johnson 1980), which contains, arguably, eight species: the western pearlshell *Margaritifera falcata* (Gould, 1850); the western ridged mussel *Gonidea angulata* (Lea, 1838); the winged floater *Anodonta nuttalliana* Lea, 1838; the Willamette papershell *A. wahlamatisensis* Lea, 1838; the Oregon floater *A. oregonensis* Lea, 1838; the Yukon floater *A. beringiana* Middendorff, 1851; the California floater *Anodonta californiensis* Lea, 1852; and the western floater *A. kennebecensis* Lea, 1860 (Henderson 1929, 1936; Ingram 1948; Turgeon et al. 1998; Nadeau et al. 2005). Although *M. falcata* and *G. angulata* are well-recognized species, the current taxonomic status of the *Anodonta* species in this region is problematic (Mock et al. 2004, Nadeau et al. 2005), and rigorous genetic work on this group has only just begun (Mock et al. 2004).

Although undoubtedly reduced in numbers and distribution by anthropogenic activities, the conservation status of native freshwater mussels in the Pacific Northwest was, until recently, largely unknown (Williams et al. 1993). However, since the status assessment of Williams et al. (1993), *G. angulata* has been designated a species of "special concern" in western Canada (COSEWIC 2003); *A. californiensis* has been designated a State Candidate Species in Washington (Larsen et al. 1995); and Frest and Johannes (1995) have advocated for federal listing of *A. wahlamatisensis* and *A. californiensis* under the U.S. Endangered Species Act (ESA) and sensitive species status for *G. angulata* and *M. falcata* by federal land-management agencies.

Unionacean mussels were once, and in some locations still are, an important component of freshwater-salmonid ecosystems throughout the Pacific Northwest and were a traditional source of food and shells for Native Americans (Lyman 1984, Toy 1998). These mussels depend on salmonids, and potentially other fishes, to host their parasitic larval phase, the glochidium (Clarke 1981, McMahon and Bogan 2001).

Glochidia larvae of unionaceans are normally found encysted as ecto-parasites on the gills and/or fins of a "host" fish. Some mussel species have a narrow host range, successfully parasitizing

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a particular species or group of fish, whereas others tolerate a wide range of fish hosts (Kat 1984). Glochidia that attach to unsuitable fish are quickly shed and fail to develop (Young and Williams 1984, Bauer and Vogel 1987). Depending on the mussel species, the glochidia may or may not grow during encystment and stay attached to their host for varying lengths of time (from a few days to several months) prior to metamorphosis. Some glochidia require nutrition from the host to complete development (Arey 1932, Kat 1984), and all unionacean mussels use the obligatory parasitic glochidial stage as a means of dispersal into favorable habitats (Kat 1984). Salmonids may also benefit from the mussels, which maintain water quality (Pusch et al. 2001), reduce suspended particles (Ziuganov et al. 1994, McMahon and Bogan 2001), and control nutrient and plankton levels in lakes and rivers (Green 1980, Nalepa et al. 1991, Welker and Walz 1998) through filter-feeding. Furthermore, mussel beds provide habitat for benthic organisms that juvenile salmonids feed upon (Ziuganov et al. 1994, 2001) and provide water-flow refugia for salmonid juveniles (Ziuganov et al. 2001, Hastie and Young 2003). Therefore, understanding parasite-host relationships is vital to mussel, and potentially salmonid, conservation efforts.

Although parasitic glochidia are sometimes reported not to harm wild fish (Nezlin et al. 1994, Hastie and Young 2003), many studies have reported that glochidial infestation or “glochidiosis” has a detrimental impact on both long- and short-term growth and survival in juvenile salmonids in both wild and hatchery situations (Murphy 1942; Meyers and Millemann 1977; Karna and Millemann 1978; Moles 1980, 1983; Cunjak and McGladery 1991; Toy 1998; Hastie and Young 2001). Despite the documented harm that glochidia have on juveniles of economically important salmon, little is known about host-parasite relationships between native fish and the eight putative unionacean species indigenous to the Pacific Coastal Region of North America.

Correct species identification of glochidia on host fishes is vital, both in ecological studies of natural glochidia-host relationships and in salmonid hatcheries where diagnosis of the species causing glochidiosis may be required. However, identification of glochidia to species is difficult. Traditional methods to identify host-glochidia associations have involved either artificial in-

oculation of potential host fishes with glochidia obtained from gravid female mussels (Haag and Warren 2003) or morphological identification of glochidia attached to wild-caught fishes (Rand and Wiles 1982, Waller et al. 1988, Pekkarinen and Englund 1995). The first method may take months and requires laboratory fish culture, because hosts may acquire immunity after a single successful wild infestation (Bauer and Vogel 1987, Rogers and Dimock 2003). Following glochidial challenge, fish are usually classified as suitable hosts if they harbor glochidia that successfully metamorphose; however, this approach may result in conclusions that differ from actual glochidia-host relationships in the wild (see Murphy 1942, White et al. 1996). Morphological identification of glochidia attached to wild-caught fishes sometimes requires expensive and time-consuming scanning electron microscopy SEM (Rand and Wiles 1982, Waller et al. 1988, Pekkarinen and Englund 1995).

To overcome the problems with these traditional methods, White et al. (1994, 1996) and Gerke and Tiedemann (2001) produced molecular-genetic keys to adult mussels that could be used to identify glochidia larvae on wild hosts in French Creek, Pennsylvania and Europe, respectively. These authors used restriction fragment length polymorphism (RFLP) analysis of the first internal transcribed spacer (ITS-1) region of nuclear ribosomal DNA (rDNA) to identify species-specific differences in the number and sizes of rDNA fragments in adult mussels and glochidia. However, other investigators working with freshwater gastropods (see references in Jones et al. 1999), have found substantial ITS sequence variation in both populations and individuals (Jones et al. 1999), limiting the usefulness of rDNA as a species-specific diagnostic tool. For these reasons we did not use the rDNA ITS-1 region in this study. Instead, we used a 710-base pair (bp) fragment of the mitochondrial cytochrome *c* oxidase subunit I gene (*COI*) to produce a diagnostic suite of restriction sites (see Baldwin et al. 1996) for each of the sampled mussel species in the Pacific Coastal Region. We then used these markers to identify glochidia encysted on juvenile Pacific salmonids.

## Materials and Methods

Unionacean mussels can inherit two forms of mitochondria, a female-type (F-type) and a male-

type (M-type); a pattern of inheritance that has been called “double uniparental inheritance” (DUI) (Hoeh et al. 1996, Liu et al. 1996). Male mussels possess both M-type (present only in the male gonad) and F-type mitochondria (present in the somatic tissues), whereas female mussels possess only the F-type. In the course of DUI, female mussels pass their F-type mitochondria to offspring of both sexes, whereas males pass on their M-type mitochondria to their male offspring alone. Since the F-type is present in somatic tissue of both sexes we chose to develop a diagnostic suite of restriction sites for *COI* based only on the F-type mitochondria (Krebs 2004).

### Sample Collection and DNA Extraction

Adult mussels were obtained under permit from 13 sites in Washington (Washington Department of Fish and Wildlife permits 01-244, 02-320, and 03-217), and from Bureau of Land Management, Olympic National Park and Katmai National Park personnel in Oregon, Washington and Alaska, respectively (Table 1, Figure 1). Organism voucher specimens were preserved in 95% ethanol. Whole genomic DNA was extracted from approximately 1 mm<sup>3</sup> of adductor muscle or mantle tissue of representative samples from each location using a Qiagen DNeasy 96 Tissue Kit (Qiagen Sciences, Germantown, Maryland) following the

manufacturer’s protocol. No gonadal tissue was extracted because we were primarily interested in obtaining the F-type *COI* sequences (Krebs 2004). This resulted in a product ranging from 15 to 30 ng DNA/μl for each sample. Total DNA was re-suspended in 100-150 μl of the manufacturer provided buffer.

We obtained fish-attached glochidia samples preserved in 95% ethanol from infested steelhead smolt (*Oncorhynchus mykiss*) obtained from the Washington Department of Fish and Wildlife Tokul Creek Hatchery, located in the Snoqualmie River Basin in Washington State. Total DNA was extracted (as above) from whole glochidia while still encysted in host tissue, because host tissue was found not to amplify with our *COI* primers.

### DNA Amplification and Sequencing

Protocols for polymerase chain reaction (PCR) amplification of a 710-bp fragment of the mitochondrial cytochrome *c* oxidase subunit I gene (*COI*) were those of Folmer et al. (1994) and Baldwin et al. (1996). Each DNA isolate was PCR-amplified using the primer pair: LCO1490/HCO2198b. These *COI* primers have been shown to reliably amplify mtDNA from unionacean bivalves (Hoeh et al. 1996, 1998; Mock et al. 2004). Non-unionacean mtDNA, as found in host-fish tissue samples, did not amplify with these PCR primers.

TABLE 1. Collection data for adult mussels.

Sample number and location	Drainage	Collection Date	method	Specimens	
				Putative species	sequenced
1. Ozette Lake	Ozette River	Dec 1999	Snorkel	<i>Anodonta</i> cf. <i>oregonensis</i>	2
				<i>Margaritifera falcata</i>	1
2. Lake Sammamish	Lake Washington	Jul 2001	Snorkel	<i>Anodonta</i> cf. <i>oregonensis</i>	3
3. Bear Creek	Lake Washington	Aug 2001	Wading	<i>Margaritifera falcata</i>	4
4. Sand Point	Lake Washington	Sep 2001	SCUBA	<i>Anodonta</i> cf. <i>oregonensis</i>	2
5. Enatai Beach	Lake Washington	Nov 2001	SCUBA	<i>Anodonta</i> cf. <i>oregonensis</i>	4
6. Marsh Park	Lake Washington	Jan 2002	SCUBA	<i>Anodonta</i> cf. <i>oregonensis</i>	3
7. Pleasure Point	Lake Washington	Jan 2002	Wading	<i>Anodonta</i> cf. <i>oregonensis</i>	1
8. Seward Park	Lake Washington	Mar 2002	SCUBA	<i>Anodonta</i> cf. <i>oregonensis</i>	3
9. Pilchuck Creek	Stillaguamish River	May 2003	Wading	<i>Margaritifera falcata</i>	3
10. Kalama, Washington	Columbia River	Aug 2003	Snorkel	<i>Anodonta</i> cf. <i>nuttalliana</i>	8
				<i>Anodonta</i> cf. <i>oregonensis</i>	1
11. Upper Yakima River	Columbia River	Aug 2003	Snorkel	<i>Margaritifera falcata</i>	7
12. Lewis River/Cedar Creek	Columbia River	Aug 2003	Wading	<i>Margaritifera falcata</i>	6
13. Wenatchee River/Nason Creek	Columbia River	Sep 2003	Wading	<i>Margaritifera falcata</i>	2
14. Owyhee River/Greeley Bar	Columbia River	Sep 2003	Wading	<i>Gonidea falcata</i>	8
15. Vantage, Washington	Columbia River	Sep 2003	Snorkel	<i>Anodonta</i> cf. <i>oregonensis</i>	4
16. Jo-Jo and Grosvenor Lakes, Alaska	Naknek River	Aug 2004	Wading	<i>Anodonta beringiana</i>	4

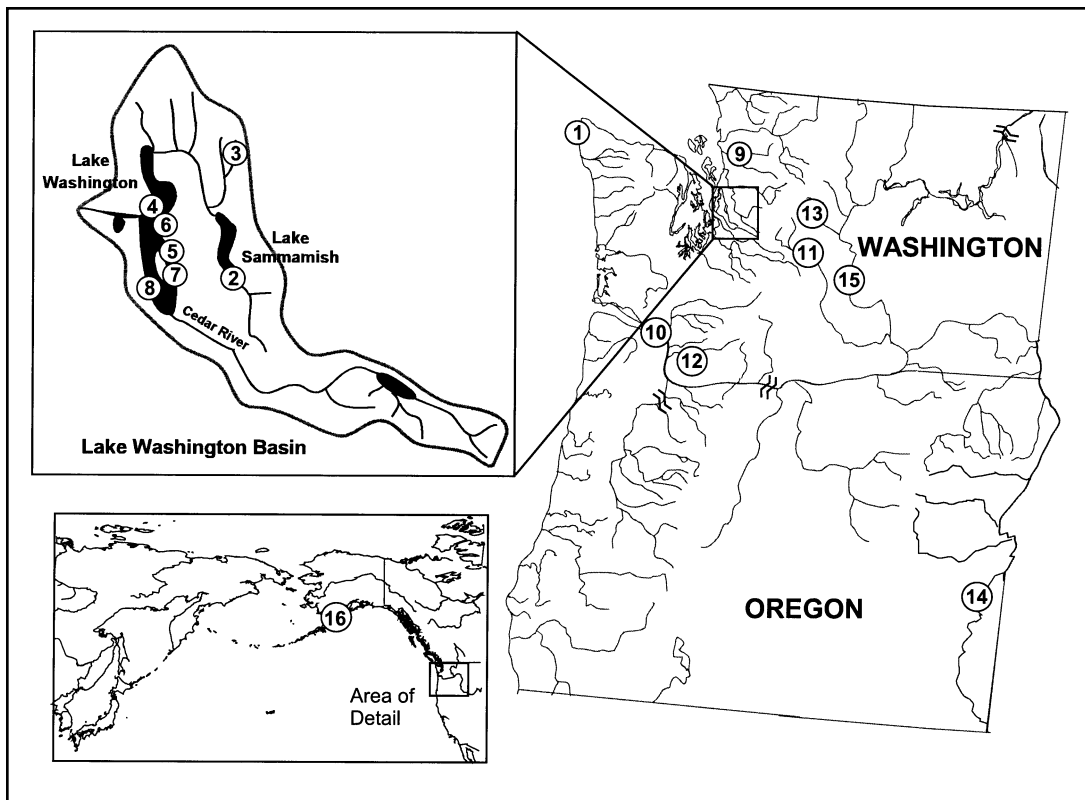


Figure 1. Locations of mussel collections.

PCRs were performed on DNA samples using a 10  $\mu$ l reaction mix containing 3  $\mu$ l of isolated template DNA, 0.4  $\mu$ M forward primer, 0.4  $\mu$ M reverse primer, 0.2 mM each dNTP (Promega, Madison, Wisconsin), 2.0 mM MgCl<sub>2</sub>, 0.5 units Taq DNA polymerase (Promega), 1X manufacturer's supplied buffer (Promega), and 1X bovine serum albumin (BSA) (New England Biolabs, Beverly, Massachusetts). Genomic DNA was put through a DNA-denaturing step at 95°C for 2 min followed by 33 thermal cycles using the following profile: 94°C for 40 s, 49°C for 40 s, and 72°C for 40 s, and a final extension cycle of 72°C for 5 min.

Double-stranded PCR products were purified using Microcon PCR spin columns (Millipore, Billerica, Massachusetts). Sequencing reactions were performed from both ends of the amplified product using an ABI BigDye Kit (Applied Biosystems, Foster City, California) with the same primers. PCR products were purified using CleanSEQ (Agencourt Bioscience Corporation,

Beverly, Massachusetts) eluted in purified distilled water and analyzed on an ABI 3100 automated sequencer, yielding sequences of up to 710 bp. All F-type *COI* sequences were aligned with *Gene Runner* (version 3.05; Hastings Software, Inc., 1994), using published unionacean sequences as a template (Mock et al. 2004). The 25 unique *COI* sequences generated from this project were deposited in the GenBank data base under accession numbers DQ272359 to DQ272383. Phylogenetic analyses of *COI* sequences were conducted using MEGA version 3.1 (Kumar et al. 2004).

#### Restriction Endonuclease Digestion

Sequence differences between mussel species were also analyzed with *Gene Runner* to determine one or more restriction endonucleases that could produce diagnostic *COI* fragment profiles among the target mussel species. To perform the restriction analysis of the amplified fragment of *COI* we used the restriction endonuclease *AluI* (New England Biolabs). For each sample,

15 µl of PCR- amplified product was combined with 1 µl of *AluI*, 2 µl of buffer (supplied by the manufacturer with the enzyme), 2 µl of sterile distilled water, and 1 µl of 10X acetylated BSA, and the reaction was run for 16 hours at 37°C. The entire reaction volume was then run on a 1.1% SYNERGEL (Diversified Biotech, Boston, Massachusetts) and 0.8% agarose gel for four hours. The gel was stained with ethidium bromide and DNA bands were visualized within the gels with UV light.

*COI* PCR-product was obtained from glochidia larvae encysted on the gills of juvenile steelhead from the Tokul Creek Hatchery. The PCR product was digested with *AluI*, as with adult mussels, and the resulting DNA bands were visualized on ethidium bromide stained gels.

**Results**

A minimum of 669 bp of *COI* nucleotide sequence was resolved for 66 individuals from the 16 collection sites (Table 1, Figure 1). These 66 samples yielded 25 haplotypes (GenBank accession numbers DQ272359 to DQ272383) that were interpreted to represent five putative species of unionacean bivalves (Table 2, Figure 2). Based on conchological features and habitat characteristics at the site of collection, we identified three of these species as *Margaritifera falcata*, *Gonidea angulata*, and *Anodonta beringiana*. Likewise, we identified the other two species as *A. oregonensis* and *A. nuttalliana*, although these latter two identifications should be considered tentative, because systematics of the genus *Anodonta* in the Pacific Coastal Region is currently unresolved (Frest and Johannes 1995, Nadeau et al. 2005) and conchological features in this genus are sometimes misleading (Hoeh 1990, Mock et al. 2004). Intraspecific sequence variation for this

gene fragment was not detected among specimens of *A. beringiana* but ranged from 0.0–0.3% in *A. cf. oregonensis*, 0.1–0.8% in *A. cf. nuttalliana*, 0.1–0.5% in *G. angulata*, and 0.1–1.4% in *M. falcata*. Based on the consensus sequences of these five putative species (Figure 3), we performed a computer search with *Gene Runner* for one or more restriction endonucleases that could produce diagnostic *COI*-fragment profiles among the putative species. These analyses revealed that the 4-base (AG/CT) restriction endonuclease *AluI* could potentially produce diagnostic fragments of the *COI* PCR-product. A direct test with this restriction enzyme produced diagnostic profiles for species, as illustrated in Figure 4. Because none of the observed intraspecific sequence variation involved the AG/CT recognition site, the DNA fragment patterns were always the same for individuals within a species.

Digestion of *COI* PCR-product with the restriction endonuclease *AluI* resulted in two fragments in both *Margaritifera falcata* and *Gonidea angulata*, but the fragment base pair sizes were diagnostic (399 + 271 bp and 616 + 54 bp, respectively) (Figure 4). Six fragments were obtained in *Anodonta cf. nuttalliana* (233 + 207 + 93 + 75 + 33 + 19 bp), nine fragments in *Anodonta cf. oregonensis* (237 + 104 + 99 + 63 + 53 + 45 + 33 + 21 + 15 bp), and six fragments in *Anodonta beringiana* (301 + 158 + 117 + 55 + 52 + 7 bp). The summed fragment lengths for each taxon do not always add up to the expected 710 bp, because of estimation errors associated with fragment size determination and because small fragments cannot be identified. In practice, major fragments larger than 100 bp in size and minor fragments less than 100 bp in size were difficult to visualize together on a single gel; however, fragment patterns of the major bands provide diagnostic profiles on a single gel for all

TABLE 2. Dichotomous identification key to Pacific Northwest unionacean mussels based on molecular features. *COI* PCR products digested with restriction enzyme *AluI*.

1. Digestion results in 2 or 3 DNA fragments.....	2
Digestion results in many DNA fragments, all less than 240 bp .....	3
2. Digestion results in 2 DNA fragments (616 and 54 bp) .....	<i>Gonidea angulata</i>
Digestion results in 3 DNA fragments (399, 271, and 38 bp) .....	<i>Margaritifera falcata</i>
3. Digestion results in many small DNA fragments less than 105 bp in length and	
1 fragment over 200 bp (237 bp) .....	<i>Anodonta cf. oregonensis</i>
Digestion results in many small DNA fragments less than 120 bp in length and 2 fragments over 120 bp .....	4
4. Two DNA fragments over 120 bp (233 and 207 bp).....	<i>Anodonta cf. nuttalliana</i>
Two DNA fragments over 120 bp (301 and 158 bp).....	<i>Anodonta beringiana</i>

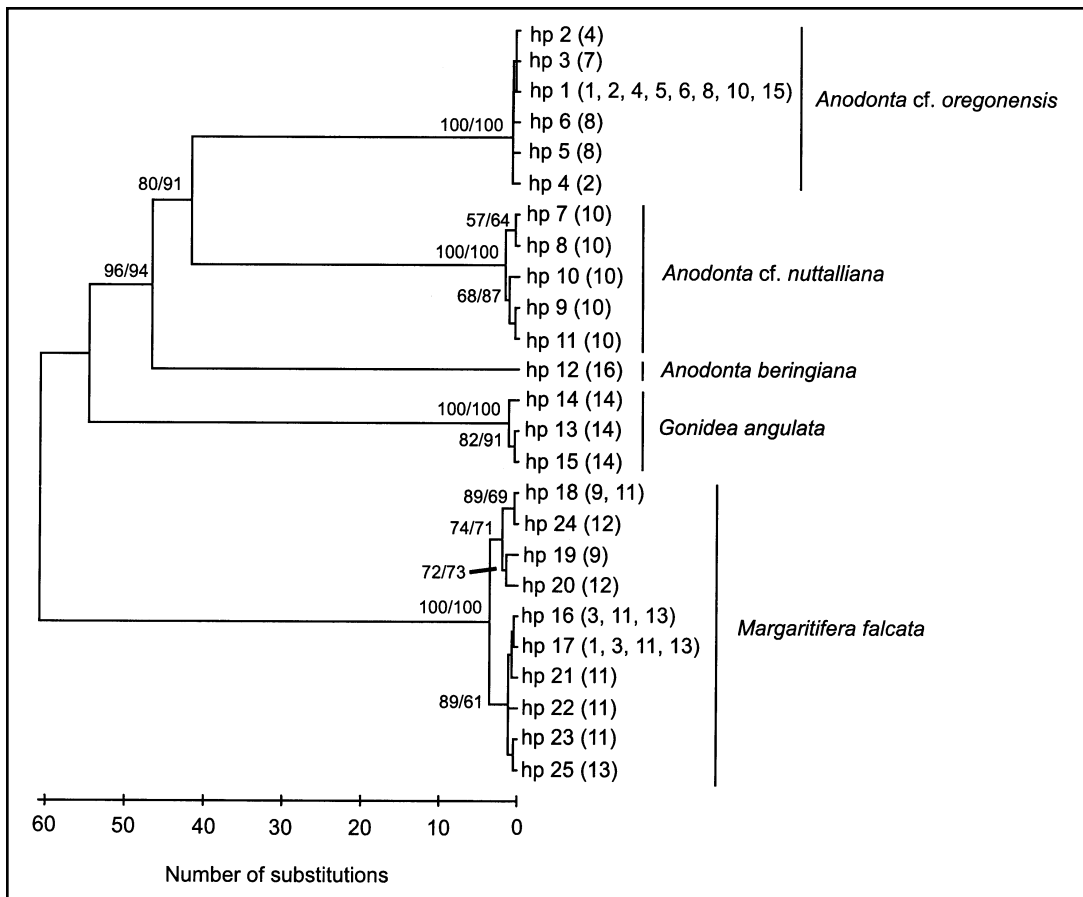


Figure 2. Neighbor-joining tree of relationships among all 25 resolved haplotypes (hp) among the 5 sampled freshwater unionacean mussels based on the total number of substitutions. Tree constructed using MEGA version 3.1 (Kumar et al. 2004). The numbers at internal branches indicate percentage bootstrap support greater than 50% for neighbor-joining/maximum parsimony analyses (NJ/MP). Numbers in parentheses indicate locations where the haplotypes were detected as described in Table 1.

species, as presented in Figure 4. A dichotomous molecular-identification key using the fragment patterns of the major bands for each species is provided in Table 2. Analysis of RFLP patterns from glochidia attached to steelhead trout at the Tokul Creek Hatchery indicated that these glochidia were *M. falcata* (Figure 4).

## Discussion

This study is the first to evaluate genetic variation among Pacific Northwest unionacean mussels and has demonstrated the utility of using RFLPs of the mitochondrial *COI* gene for species identification of glochidia attached to juvenile Pacific salmonids and other fishes. Previously, RFLPs

based on *COI* mtDNA sequences have been used as a diagnostic tool for morphologically indistinct larval (Baldwin et al. 1996, Claxton and Boulding 1998), early juvenile (Claxton et al. 1997), and adult (Lee and Kim 2003) bivalve molluscs. Previous studies on unionacean mussels have used RFLP analysis of the ITS-1 region of nuclear ribosomal DNA to identify glochidia larvae of 25 and 6 unionid species encysted on wild host fishes in French Creek, Pennsylvania (White et al. 1996) and in Europe (Gerke and Tiedemann 2001), respectively.

Determination of natural interactions between host fish and glochidia of a particular mussel species is vital information for mussel propagation

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Mf ATTGGGACTT TATATTTGTT GTTGGCCTTG TGATCTGGCC TTATTGGTTT GGCTTTGAGG 60
Ga .....T..AC .G.....T....G.....GT .A.....G.. A.....A
Ao .....T.... .A..A..T....G.....TT .A.....G.. A.....
An .....T.... .C..AC.T..T..TC.. .G.....AT .G.....G.. A.....
Ab .....T.... .A.....T....G.....TT .A.....G.. A.....A..C

TTGTTGATTC GTGCTGAGTT GGGTCAACCT GGTAGTTTGT TGGGGGATGA TCAGTTGTAT 120
.....A.... .G..G..... A..A..... .A..A..A.. .A..T..... ..AC.T...
C.T..... .A..... .A.....A..A..G... .A..... .A.....C
C.....A.... .A..... .A.....A..A..A.. .A..... .A.....A..C
.....A.... .A..A.... A.....G... .A..GC.A.. .....C.. .C.A...

AACGTTATTG TTACGGCTCA TGCTTTTATA ATAATTTTCT TTTTGGTTAT GCCAATAATA 180
..T..A.... .A..A.. ..... .A.....A.....
..T..G.... .A..... .....T.. .C.....A.. A.....G..G
..T..A.... .C.....C..G .....T.. .C.....A.. A.....G..G
..T....C. .... .C.....A.....

ATTGGGGGTT TTGGTAATTG ACTTATTCCT CTTATGATTG GGGCTCCCGA TATGGCTTTT 240
.....T.... .G.....G.....T.....C.....A..T...
.....T..G. ....G.....T..A..A... .T.....T.. .A.....
.....T..A.. .C.....T..G.....T..G..A... .T.....T.. .....
.....T..A.. .A.....G.....T..G..A..C.. .....T.. .....C...

CCCCGCCTTA ACAATTTGAG GTTTTGATTG CTTGTGCCTG CTCTTTTCTT GTTATGAGT 300
..T..T..G. .T.....A.....G.....A..G.....T.. A.....A..G
..T..AT..A. .T.....A.....G.....A..A.. .T..A..T.. .C..G..A..G
..T..GT..A. .T.....A.....A.....A..A.. .T..G..T.. .....A..C
..T..AT..A. .T.....A.....GC.. .A..A.. .AT..A..T.. ..G..A..A

TCCTTCGTTAG TGGAGAGTGG TGTTGGTACT GGTGGACTG TTTATCCCCC TTTGTC AAGG 360
.....A.... .A.....G.....G.....A..G.....G... G.....TG..
..A..A..G. .A.....G.....A.....A..A..A..A..C..T.. ..TG..A
.....A.... .G.....A.....A.....A..A.. .A.....A.. ..A..TG..A
.....T..G. .A.....G..C.....G.....A..A.....A.. ..TG..

AATGTTTCAC ATTCTGGGGC TTCTGTAGAT TTAGCTATTT TTTCTTTGCA CCTTGCTGGT 420
.....G..T. ....G..T..C..G..... .C..T..T.....G...
..G.....G..T. ....G..G...G..C... .C.....A..T.....C...
.....G..T. ....G.....G..C... .A.....T.....
.....GGT. ....C..T.. ..G.....C.. ..A.....T..A.....

GCGTCTTCTA TTTTGGGGGC TATTAATTTT ATTTCGACTG TTGGTAATAT GCGCTCCCCT 480
..T..... .T.....T.....G..G.....T..T...
..T..C..A. .A..T...C..... .C..T.....G.....A.....A
..T.....A.. .C.....T.....A..C... ..A..T...
..T..A..A. .... .T.....A..A.....A..A..T..A

GGCGTAGTTG CTGAACGAAT TCCATTGTTT GTGTGAGCCG TCACTGTGAC GGCTATTTTG 540
..GT..... .G..G...T.....T...G..T..G..... ..AG...A
..TT..C...G.....T.....T..T.....T..T..A...A...G..G...A
..TT..G..C...G.....T..A..T..T..G..T..TG..C..T..A...G..G..A
..TT.....G.....T..A..T..A..G..T..G.....A..A...G...A

TTAGTGGCGG CATTGCCTGT TTTGGCAGGT GCTATTACTA TGTTGTTGAC TGATCGCAAT 600
..G..T..A..A.....A..A.....T.....G..A..AC..T... ..T...
..T..T..T..T..A..A..A..A..T... ..G..A..C..T... ..T...
..T..T..T..T..A..A..A..A..T... ..A.....A..C.....G.....T...
C..G..T..T..T.....T.....T.....A.....C..CC..T.....

TTAAATACAT CTTTTTTTGA TCCAAC TGGG GGTGGTGACC CTATTTTGT A TATGCACTTG 660
C..T.....G.....T.....T.....G..G..T..A.....C..... ..A..T..A
C..G..C..T..G.....C..T..G...A.....T.....T.....A..T...
.....C..T..A.....C..T..A...A..G..T..... ..A..T...
C..G.....T.....C.....C..T.....A.....T.....

TTTTGATTT 669
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.....

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Figure 3. Consensus nucleotide sequences of cytochrome *c* oxidase subunit I from five indigenous freshwater mussels of the Pacific Northwest: Mf, *Margaritifera falcata*, Ga, *Gonidea angulata*, Ao, *Anodonta cf. oregonensis*; An, *A. cf. nuttalliana*, Ab, *A. beringiana*.

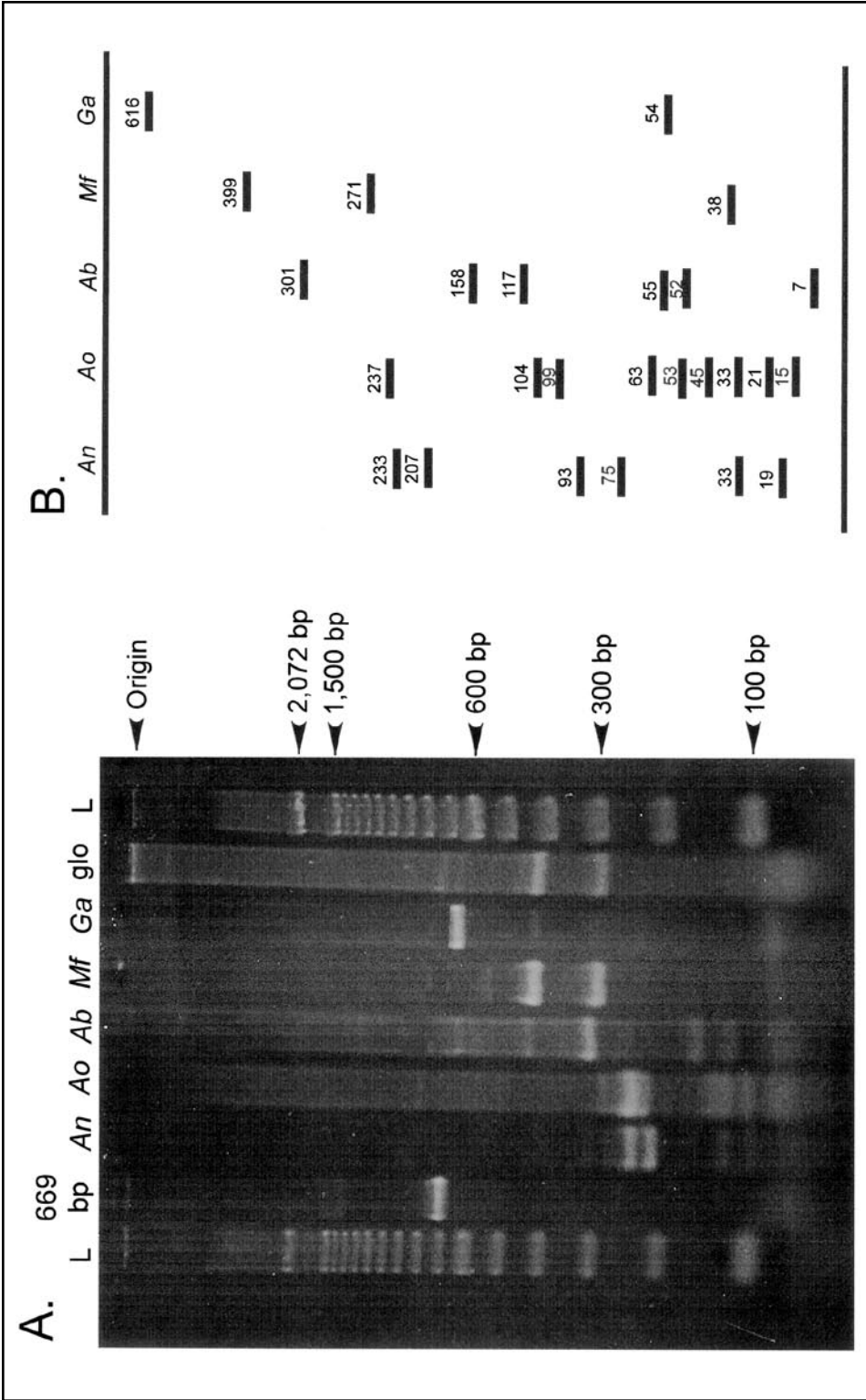


Figure 4. Products of RFLP analyses. (A) Ethidium bromide-stained gel of restriction fragments of the *COI* mitochondrial gene of adult mussels and glochidia sample after application of the restriction endonuclease *AatII*. Lane L, Ready-Load DNA base pair ladders (Invitrogen, Carlsbad, California); 669 bp, total PCR product prior to enzyme restriction; An, *Anodonta cf. nuttalliana*; Ao, *A. cf. oregonensis*; Ab, *A. beringiana*; Mf, *Margaritifera falcata*; Ga, *Gonidea angulata*; glo, glochidia from Tokul Creek Hatchery steelhead. Small DNA fragments less than 100 bp in length are not resolved in this gel. (B) Idealized schematic of fragment banding patterns of the *COI* mitochondrial gene of adult mussels after application of the restriction endonuclease *AatII*. Numbers represent base pair sizes. Note that the y-axis is nonlinear. Abbreviations as above.

and restoration efforts. The PCR-RFLP tool outlined in the current study can be used to identify the species of glochidia infesting wild-caught fishes. To date, the natural fish host(s) have not been identified for *G. angulata*, *A. nuttalliana*, *A. wahlamatensis*, or *A. californiensis* (Clarke 1981, COSEWIC 2003); however, more is known about fish-host relationships for *A. beringiana*, *A. oregonensis*, *A. kennerlyi*, and *M. falcata*. Because taxonomy of the species of *Anodonta* in the Pacific Coastal Region is in flux, species designations for this genus in the following studies should be considered as tentative. Cope (1959) reported glochidia of *A. beringiana* on Chinook (*Oncorhynchus tshawytscha*) and sockeye salmon (*O. nerka*) juveniles and on threespine sticklebacks (*Gasterosteus aculeatus*) in an Alaskan stream. Similarly, Moles (1980, 1983) reported glochidia of *A. oregonensis* encysted on wild coho salmon fry from an Alaskan lake, as well as on threespine sticklebacks (Moles 1982). Martel and Lauzon-Guay (2005) recently verified successful metamorphosis of *A. kennerlyi* glochidia on the prickly sculpin (*Cottus asper*) and the threespine stickleback in three lakes on Vancouver Island, British Columbia. Low numbers of *A. kennerlyi* glochidia were also observed on wild Dolly Varden (*Salvelinus malma*) and cutthroat trout (*O. clarki*) (Martel and Lauzon-Guay 2005).

Pearl mussels in the family Margaritiferidae and salmonid fishes have reportedly co-evolved since the Eocene (Ziuganov et al. 1994) and many species in this family, including *M. falcata*, would likely be incapable of completing their life cycle without salmonid fishes (Vannote and Minshall 1982, Bauer and Vogel 1987, Ziuganov et al. 1994, Bauer 1997, Kondo et al. 2000). The natural fish-hosts of *M. falcata* reportedly include threespine sticklebacks, steelhead/rainbow trout (*O. mykiss*), coastal cutthroat trout (*O. clarki clarki*), and Chinook and coho salmon (Murphy 1942, Karna and Millemann 1978, Trotter 1996). Additional salmonids, as well as various other fishes, have been experimentally infected with glochidia of *M. falcata* (Meyers and Millemann 1977, Murphy 1942, Trotter 1996). Recent information indicates that salmonids are the preferred glochidial hosts for most species in the genus *Margaritifera* (Bauer and Vogel 1987, Ziuganov et al. 1994, Bauer 1997). In each of the above studies, there was prior evidence that only one mussel occurred in the study area, simplifying the identification of

the glochidial source species.

It is likely that glochidial presence in wild juvenile Pacific salmonids commonly goes unnoticed, since it occurs seasonally (spring-summer), is of temporary duration (weeks-months), and involves encystment of glochidia less than 1 mm in size (Hare and Frantsi 1974). However, when glochidia are present, a large percentage of any single wild salmonid population can be impacted. Karna and Millemann (1978) found that 97% of wild Chinook, 86% of wild coho, and 94% of wild steelhead juveniles were infested with glochidia in the Siletz River, Oregon in May and June of 1971. In experimental culture conditions, high levels of glochidial infestation in both juvenile Chinook salmon and rainbow trout may kill the fish (Murphy 1942, Karna and Millemann 1978), and experimentally infected coho salmon fry have shown reduced growth, regardless of the level of parasite load (Moles 1983). In the wild, coho juveniles infested with 15 or more glochidia weighed significantly less than un-infested juveniles (Moles 1983). These results led Moles (1983) to predict that coho juveniles surviving infestation would be smaller and more susceptible to pollutants and other stress factors in the environment than uninfested fish (Moles 1980, 1983).

Bauer (1997) has argued that glochidia of *Margaritifera* should be classified as benign parasites, because under natural conditions, dense populations of these mussels occur together with large healthy populations of salmonids. However, the detrimental effects of glochidiosis in wild fish are likely exacerbated at high mussel density and low host density (Kat 1984). This is precisely the situation that obtains in river basins in the Pacific Northwest that still support large populations of the long-lived western pearl mussel, *M. falcata*. Although size and density of native pearl mussel populations have reportedly decreased in many Pacific Northwest streams, average density in patches of *M. falcata* were over 400 mussels/m<sup>2</sup> in the Siletz River, Oregon (Karna 1973); 192 mussels/m<sup>2</sup> in the Salmon River, Idaho (Vannote and Minshall 1982); and 56, 80, and 120 mussels/m<sup>2</sup> in Bear Creek, Battle Creek (Toy 1998) and Cedar Creek (Stone et al. 2004), in western Washington. In addition, the 90–110+ yr life span of *M. falcata*, (Vannote and Minshall 1982, Toy 1998) exceeds the generation time of its salmonid hosts (3–7 yr) by a factor of between 10 and 20 (Bauer 1997). The long-term persistence of dense populations

of *M. falcata* (capable of releasing large numbers of glochidia) together with the drastically reduced abundance of obligate salmonid hosts (as indicated by the recent listing of over 25 Evolutionarily Significant Units of anadromous Pacific salmon under the U.S. ESA) (NOAA Fisheries 2004), suggests that in some cases glochidial infestation could be a significant risk to Pacific salmon populations. Likewise, freshwater mussels that depend on fish hosts to complete their larval development have been adversely affected by recent declines in abundance of anadromous salmonid populations in California (Howard and Cuffey 2003) and the Pacific Northwest.

To date, species-specific relationships between host salmonids and mussel glochidia are relatively unknown in the Pacific Northwest. We anticipate that future uses of the molecular key to glochidia species described in this paper (Figure 4) will include evaluation of species-specific parasite-host relationships in hatchery salmonids experiencing glochidiosis, as well as descriptions of natural parasite/host relationships among unionacean mussels and juvenile Pacific salmonids and other fishes in the Pacific Northwest.

Our species identifications, based on conchological features, matched the molecular data for *M. falcata* and *G. angulata*; however, morphologically diverse specimens of *Anodonta* were in many cases genetically identical, particularly among the specimens tentatively referred to as *A. oregonensis* (Figure 2). Currently, we are analyzing *COI* sequence data from additional populations to build on the assessment of genetic diversity in the genus *Anodonta* in the Pacific Coastal Region that was begun by Mock et al. (2004).

The present PCR-based technique provides

an attractive, alternative approach for the identification of freshly collected, frozen, or ethanol preserved glochidia and is reasonably cost-effective compared with traditional methods (Rand and Wiles 1982, Waller et al. 1988, Pekkarinen and Englund 1995, Haag and Warren 2003). This genetic approach should also be easier to automate and transfer to field stations lacking SEM or fish rearing facilities. It should be noted that the yield of PCR product from individual glochidia, and hence the intensity of bands on the RFLP gel, was much less than that observed from the adults or from pooled samples of glochidia. In these cases, reamplification from gel purified product may be necessary to provide sufficient material for RFLP analysis.

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