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Taxonomic identity of two amnicolid gastropods of conservation concern in lakes of the Pacific Northwest of the USA

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ABSTRACT

In this paper we attempt to clarify the identity of two purportedly new species of amnicolid snails in Pacific Northwest lakes that were vaguely described in grey literature and recently petitioned for federal listing. As currently understood the western American amnicolid fauna consists of the endemic genus *Colligyrus* (three species) and *Amnicola limosa*, which is distributed in a single site in western Montana (and also throughout much of eastern North America). The 'Washington duskysnail' was proposed for the Montana population of *A. limosa* and two recently discovered populations in northern Washington. The 'masked duskysnail' is a small amnicolid of uncertain generic status from two lakes in northern Washington. Our assessment of these putative species was based on genetic and morphological study of specimens from previously reported sites and recently discovered localities in Montana (both snails) and Washington (the first only). Molecular analyses (based on the mtCOI gene) resolved the western American populations of *Amnicola* as a weakly supported subunit of a clade that also contained eastern *A. limosa* and *A. dalli*; the western *Amnicola* differed from eastern *A. limosa* and *A. dalli* by 2.1% mean sequence divergence. We also found that western *Amnicola* and eastern *A. limosa* do not differ in body pigmentation as previously postulated and that these snails are closely similar in all other morphological details. We conclude that the Washington duskysnail is not a distinct species and that all of the western populations of *Amnicola* are *A. limosa*. This finding extends the range of *A. limosa* westward almost to the Pacific margin. Our molecular phylogenetic analyses and study of the female reproductive anatomy of the masked duskysnail congruently indicated that this snail, which was previously compared with other western amnicolids, belongs to the genus *Lyogyrus*, which is otherwise restricted to eastern North America. We were unable to resolve the taxonomic status of the masked duskysnail further, owing to the paucity of pertinent data for the poorly known eastern *Lyogyrus* fauna. We recommend that the masked duskysnail be treated as '*Lyogyrus* sp.' pending further study of this genus.

INTRODUCTION

The Amnicolidae are a medium-sized (150–200 species; Strong *et al*., 2008) family of freshwater caenogastropods distributed in temperate Asia, Europe and North America (Wilke *et al*., 2013). As currently understood, the western North American amnicolid fauna is composed of the three species in the genus *Colligyrus*, which live in springs and streams in portions of the northwestern United States (Hershler, 1999; Hershler *et al*., 2003); and *Amnicola limosa* (Say, 1817), which is distributed in a single lake in Montana (Taylor & Bright, 1987: 252) and also throughout much of eastern North America. A second western population of *A. limosa* (in Utah Lake) is apparently extinct (Taylor & Bright, 1987; Oliver & Bosworth, 1999). During the past few decades several putatively undescribed western amnicolids have been identified in grey literature (i.e. unpublished contract reports) and subsequently become focal points of attention of state and federal land management agencies and conservation organizations. This is the second of two papers in which we evaluate the taxonomic status of these purported new species. Previously we showed that the 'Columbia duskysnail', distributed in springs in the lower Columbia River basin, is a broadly disjunct subunit of *C. greggi* (Liu, Hershler & Roessell, 2015). The present study focuses on two amnicolids of conservation concern in Pacific Northwest lakes.

The vernacular name 'Washington duskysnail' was used for the first reported western population of *A. limosa* and two recently discovered populations in northern Washington (Frest & Johannes, 1995; WDNR, 2007). Frest & Johannes (1995) vaguely

differentiated this supposedly new species from eastern North American *A. limosa* by several details of body pigmentation. There have been no subsequent studies of the Washington duskysnail, which was recently petitioned for addition to the Federal List of Endangered and Threatened Wildlife (USFWS, 2009).

The 'masked duskysnail' is a small amnicolid from two sites in northern Washington that is currently protected under the Survey and Manage Program of the Northwest Forest Plan (USDA & USDI, 2007) and was recently petitioned for federal listing (USFWS, 2012). Clarke (1976: 5) reported the discovery of this "apparently new species of *Lyogyrus*" in Fish Lake (Chelan County) without additional comment. *Lyogyrus*, as currently constituted, is restricted to eastern North America (Thompson, 1968; Thompson & Hershler, 1991). Frest & Johannes (1995: 185) stated that the masked duskysnail is a new species of *Lyogyrus* whose "shell shape and pigment pattern are distinctive as compared to previously described forms," but did not elaborate further. In a subsequent field guide aiming to 'survey and manage' freshwater molluscs, Frest & Johannes (1999) figured the headfoot and several shells of the masked duskysnail and differentiated this supposedly new species from other western amnicolids (in a key) by its elongate shell and 'mask' of pigment around the eyes. The masked duskysnail was recently assigned to *Colligyrus*, without explanation, in an unpublished report (Johannes, 2011); this classification was subsequently followed by the USFWS (2012).

At present there is no well corroborated evidence supporting recognition of either the Washington duskysnail or the masked duskysnail as new species. The USFWS (2012: 57925) cited the absence of this information in ruling that the masked duskysnail does not constitute a listable entity under the Endangered Species Act. Indeed, the scant morphological description of the masked duskysnail is not sufficient to determine whether it belongs to *Colligyrus* or *Lyogyrus*, closely similar genera that are differentiated by details of female reproductive anatomy (Hershler, 1999). Here we attempt to clarify the identity of these two purportedly new species based on morphological study and analysis of mt COI sequences. We also briefly discuss our findings as they relate to the systematics of North American Amnicolidae.

MATERIAL AND METHODS

For the molecular component of the project we sequenced specimens of *Amnicola limosa* from the previously reported site in Montana (McWennegar Slough) and seven recently discovered localities (lakes) in western Montana and northern Washington (herein we refer to these populations collectively as 'western *Amnicola*'). Specimens of the masked duskysnail were sequenced from the two previously reported sites in northern Washington and two recently discovered localities (lakes) in western Montana. The locations of these sites are shown in Figure 1. The sequenced specimens were either recently (2014) collected by us (and preserved in 90% ethanol in the field) or obtained from samples in the Smithsonian Institution's National Museum of Natural History (USNM) collection. In order to facilitate comparisons we also sequenced specimens of the type species of *Lyogyrus* (there are no previously published sequences for this genus in GenBank) and three *A. limosa* populations from eastern

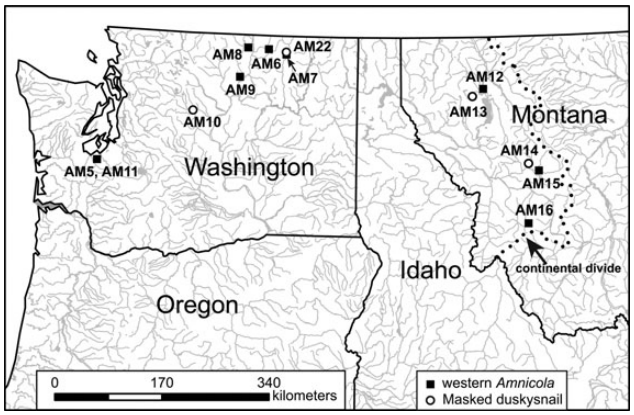

Figure 1. Map of the northwestern United States showing the collecting localities for western *Amnicola* and Masked duskysnail samples used in the molecular analysis. Specimen codes are from Table 1.

Table 1. Samples that were newly sequenced (for COI) for this study, with codes (used in Figs 1 and 2), locality and voucher details, and GenBank accession numbers.

Taxon	Code	Locality (voucher material)	GenBank accession number
Western *Amnicola*	AM5, AM11	Pattison Lake, east side at Washington Department of Fish and Wildlife boat ramp, Thurston Co., WA (USNM 1163822, USNM 1258916)	KU991117, KU991123
	AM6	Bonaparte Lake, south end at campground, Okanogan Co., WA (USNM 1256490)	KU991118, KU991119
	AM7	Curlew Lake at state park west of Hwy 21, Ferry Co., WA (USNM 1256491)	KU991120
	AM8	Spectacle Lake, south side, near resort, Okanogan Co., WA (USNM 1258913)	KU991121
	AM9	Leader Lake, north side near dam, Okanogan Co., WA (USNM 1258914)	KU991122
	AM12	McWennegar Slough, south side near Hwy 35, Flathead Co., MT (USNM 1263000)	KU991124, KU991125
	AM15	Brown's Lake, cutoff pond along northeast side, Powell Co., MT (USNM 1263003)	KU991126, KU991127
	AM16	Georgetown Lake, east side north of Stuart Campground, Granite Co., MT (USNM 1253360)	KU991128, KU991129
Amnicola limosa	AM2	Clinton River at Elizabeth Lake Road bridge, Oakland Co., MI (USNM 1022333)	KU991113, KU991114, KU991115
	PK09A	Promised Land Lake, just above spillway along Hwy 390, Pike Co., PA (USNM 1282837)	KU991130
	AM1	San Marcos Springs (Spring Lake), Hays Co., TX (USNM 1146330)	KU991112
Amnicola dalli	AM3	Natural Bridge Spring, Leon Co., FL (UF 278973)	KU991116
Masked duskysnail	AM10	Fish Lake, south side at Cove Resort, Chelan Co., WA (USNM 1258915)	KU991131
	AM22	Curlew Lake at Camp Curlew Road terminus, Ferry Co., WA (USNM 1299472)	KU991131
	AM13	Smith Lake, south side at public access boat ramp, Flathead Co., MT (USNM 1253002)	KU991132, KU991133, KU991134
	AM14	Upsata Lake, east side near outlet, Powell Co., MT (USNM 1262996)	KU991134, KU991136
Lyogyrus pupoides	AM21	Pond along Mill Brook north of Willowdale Road, Scarborough, Cumberland Co., ME (USNM 1296761)	KU991137

North America, including near topotypes from the Delaware River basin (Pennsylvania).

Genomic DNA was extracted from entire snails (1–4 specimens per sample) using a CTAB protocol (Bucklin, 1992); each specimen was analysed for mtDNA individually. Primers LCO1490 (Folmer *et al*., 1994) and COH743 (5′GGT AAA ATT AAA ATA TAT ACT T3′) were used to amplify a 720 base pair (bp) fragment of COI. Amplification conditions and sequencing of amplified PCR product followed Liu, Hershler & Clift (2003). Sequences were determined for both strands and then edited and aligned using SEQUENCHER v. 5.0.1. The 63 newly sequenced specimens were analysed together with sequences obtained from GenBank for *A. limosa*, a second species of *Amnicola* (*A. dalli*), the three species of *Colligyrus* and outgroups consisting of representatives of other amnicolid genera from Asia (*Baicalia*, *Erhaia*, *Maackia*, *Moria*), Europe (*Marstoniopsis*) and North America (*Antroselates*), and the closely related (western North American) genus *Taylorconcha*. A species of *Baicalia* was used as the root in each analysis. One example of each haplotype detected in a given sample was used in the analyses. The new haplotypes from each sampling locality were deposited in GenBank. Sample information and GenBank accession numbers for specimens that were newly sequenced for this study are given in Table 1.

MRMODELTEST v. 2.3 (Nylander, 2004) was used to obtain an appropriate substitution model (using the Akaike Information Criterion) and parameter values for the molecular phylogenetic analyses. This program selected HKY + I + G model parameters as the best fit model for the COI dataset. Phylogenetic analyses were performed using four different methodologies—distance, maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference. The distance, MP and ML analyses were performed using PAUP* v. 4.0b10 (Swofford, 2002) and the Bayesian analyses were conducted using MRBAYES v. 3.2.3 (Ronquist & Huelsenbeck, 2003). For the distance analyses, HKY distance was used to generate a neighbour-joining (NJ) tree (Saitou & Nei, 1987). The MP analyses were conducted with equal weighting, using the heuristic search option with tree bisection reconnection branch-swapping and 100 random additions. The ML analyses were performed using the HKY + I + G model; a HKY distance based NJ tree was used as the initial topology for branch-swapping. Nodal support was evaluated by 10,000 bootstrap pseudoreplicates except for the ML analysis, for which support values were based on 1000 replications. For the

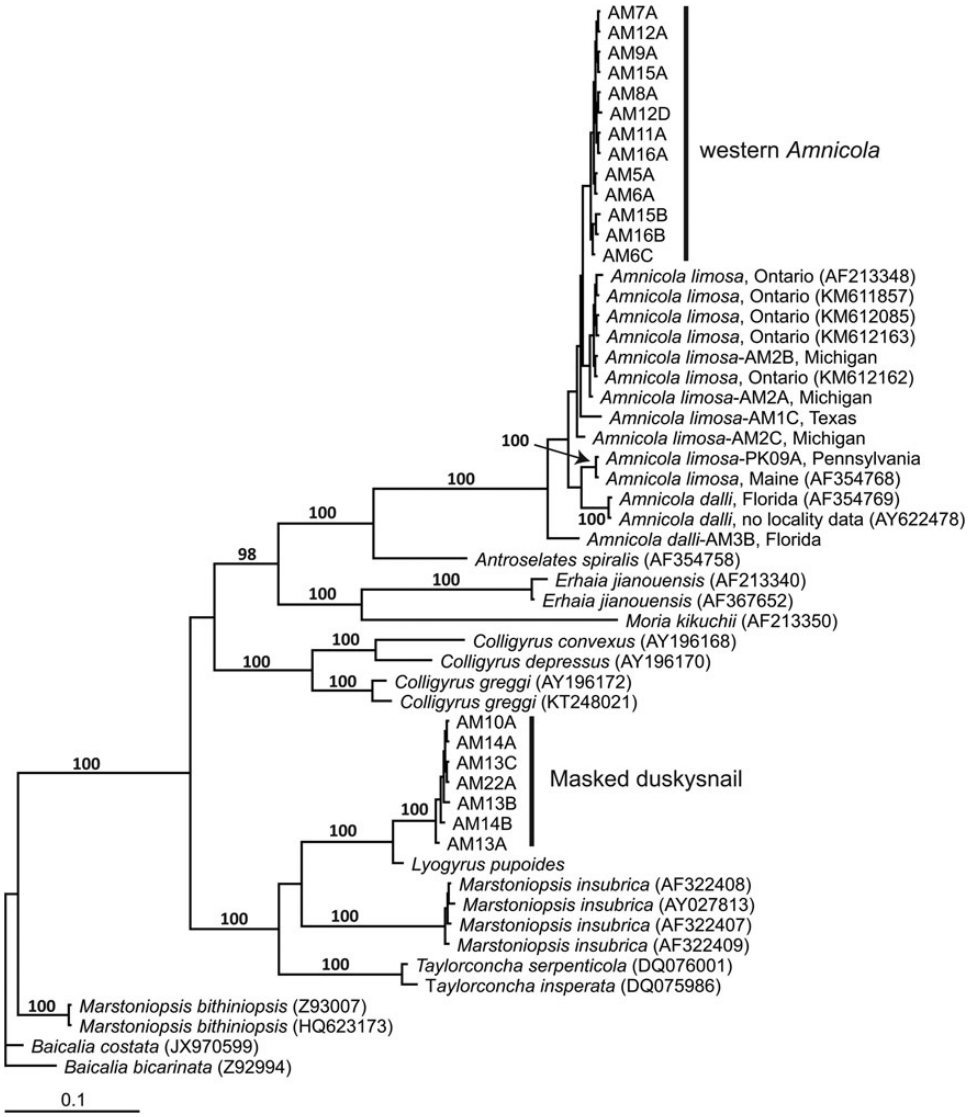

Figure 2. Bayesian tree based on the COI dataset. Nodes having posterior probabilities ≥ 95% are shown. Specimen codes are from Table 1.

Bayesian analyses Metropolis-coupled Markov chain Monte Carlo simulations were run with four chains (using the model selected by MRMODELTEST) for 5,000,000 generations. Markov chains were sampled at intervals of 10 generations to obtain 500,000 sample points. We used the default settings for the priors on topologies and the HKY + I model parameters selected by MRMODELTEST as the best fit model for both analyses. At the end of the analyses, the average standard deviation of split frequencies was 0.002 and the potential scale reduction factor was 1, indicating that the runs had reached convergence. The sampled trees with branch lengths were used to generate a 50% majority rule consensus tree, with the first 25% of the samples removed to ensure that the chain sampled a stationary portion.

Genetic distances within and between samples were calculated using MEGA6 (Tamura *et al*., 2013), with standard errors estimated by 1,000 bootstrap replications with pairwise deletion of missing data. Since MEGA does not contain the HKY model that was selected by MRMODELTEST, we used the Tajima-Nei distance, which is the nearest model.

Pigmentation patterns on the headfoot and pallial roof were studied using recently collected specimens that had been relaxed with menthol crystals prior to fixation; photographs were taken using a Coolpix 990 mounted on an Olympus SZX12 dissecting microscope. Other morphologic methods were routine. Pertinent material in the USNM and University of Minnesota Bell Museum of Natural History (BellMNH) collections was examined (see Supplementary Material).

RESULTS

The COI haplotypes of *Amnicola limosa* and *A. dalli* formed a strongly supported clade (sister to *Antroselates spiralis*) in all of the trees (the Bayesian topology is shown in Fig. 2); western *Amnicola* was resolved as a weak to moderately supported subunit of this clade in all of the analyses. The sequences of western *Amnicola* differed from each other by 0.1% (mean sequence divergence) and from those of eastern American *A. limosa* and *A. dalli* by 2.1% (Table 2). The masked duskysnail sequences were resolved as a strongly supported clade sister to *Lyogyrus pupoides* (Fig. 2) in all of the resulting trees. The sequences of these two snails differed by 3.9% on average (Table 2). Variation among masked duskysnail specimens was slight (0.3%).

We were unable to confirm the purportedly distinctive body pigmentation of western *Amnicola* reported by Frest & Johannes (1995). All of the specimens that we examined had longitudinal pigment stripes along the outer edges of the cephalic tentacles and a pigment streak extending along the anterior edge and left side of the pallial roof; these features are characteristic of *A. limosa* (Hershler & Thompson, 1988: fig. 4a). Photographs of representative western and eastern American specimens are shown in Figure 3A–D to illustrate this point. A previously published drawing of a specimen from McWennegar Slough (Taylor & Bright, 1987: fig. 8) incorrectly depicted the pigment stripes on the cephalic tentacles as mid-dorsally positioned, which may have prompted Frest & Johannes (1995: 158) to suggest that these snails differ from eastern *A. limosa* in this feature. Western *Amnicola* also closely conform to *A. limosa* in shell size (height ranging up to 4.76 mm) and shape (Fig. 4), reproductive anatomy (Fig. 5) and all other features (see Berry, 1943; Hershler & Thompson, 1988).

The female reproductive anatomy of the masked duskysnail is shown in Figure 6A. The presence of a single sperm pouch confirms our molecular phylogenetic finding that this snail belongs to the genus *Lyogyrus* (as defined by Hershler, 1999). Note that *Colligyrus*, in contrast, has three sperm pouches (e.g. *C. greggi*, Hershler, 1999: fig. 2A, B). The narrowly conical shell of the masked duskysnail (Fig. 7A, B) has a spire that is about 1.5 times the height of the aperture, highly convex teleoconch whorls and a rather small aperture. These shells closely resemble

Table 2. Mean COI sequence divergence (Tajima-Nei distance) among amnicolid lineages.

	Eastern *Amnicola limosa* + A. *dalli*	Western *Amnicola*
Eastern *A. limosa* + *A. dalli*	2.3 ± 0.4 (0.0–4.8)	
Western *Amnicola*	2.1 ± 0.4% (0.8–4.2)	0.1 ± 0.1% (0.0–0.6)
	Lyogyrus pupoides	Masked duskysnail
Masked duskysnail	3.9 ± 0.7	0.3 ± 0.1

Values are percentage ± standard deviation with ranges given in parentheses.

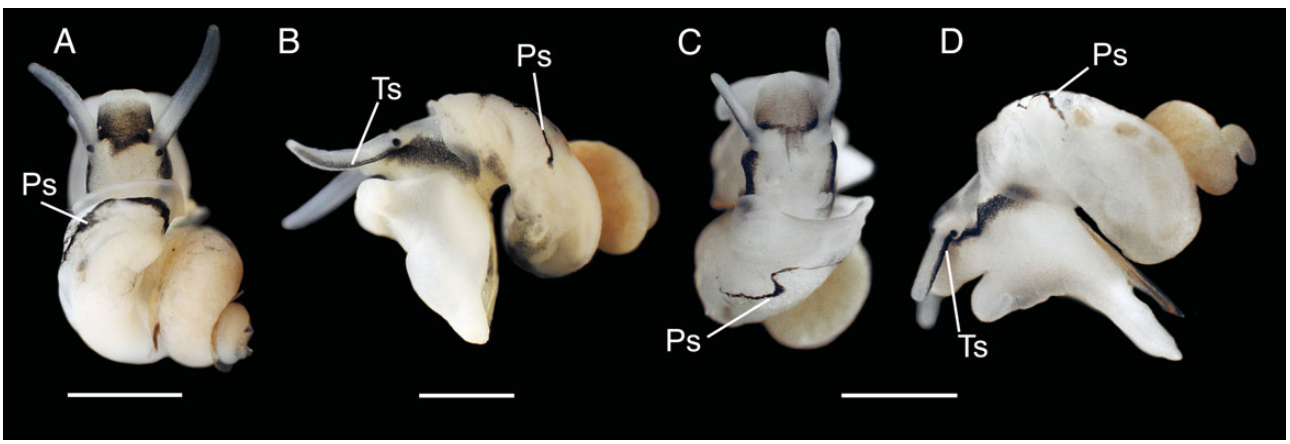

Figure 3. Photographs of specimens of western *Amnicola* and eastern American *A. limosa*, showing closely similar pigmentation. **A, B.** USNM 1258916, Pattison Lake, Washington. **C, D.** USNM 1291110, Pohick Bay, Virginia. Abbreviations: Ps, pigment streak on pallial roof; Ts, pigment bar on outer edge of cephalic tentacle. Scale bar = 1.0 mm.

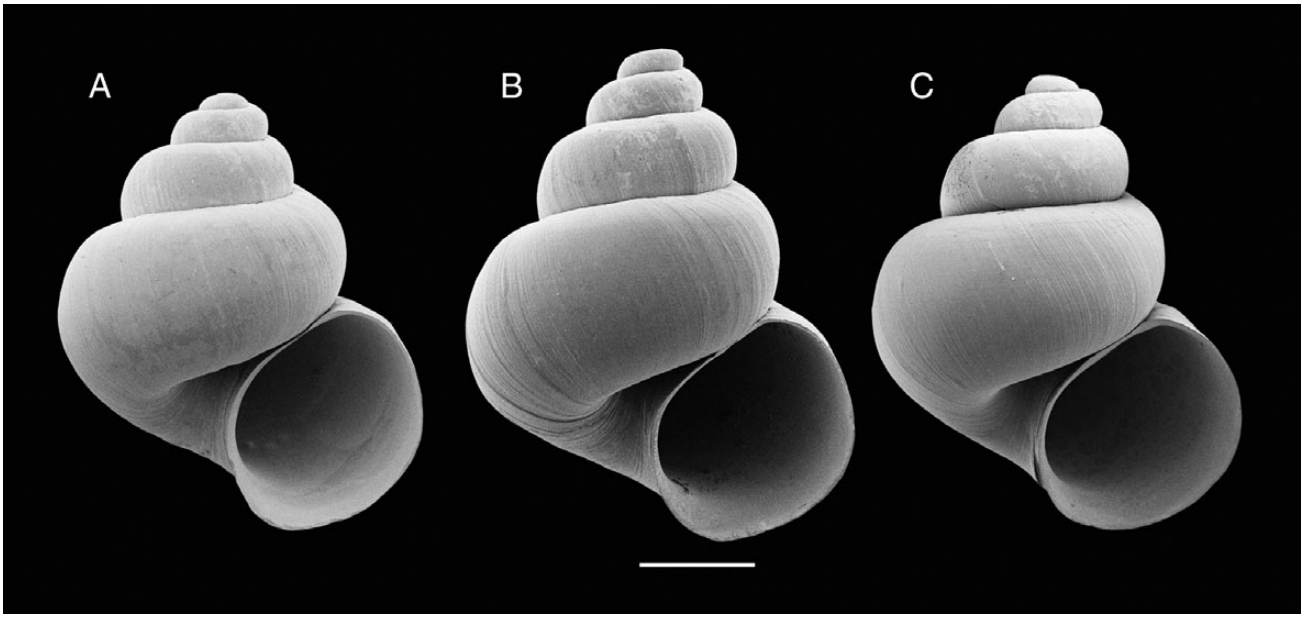

Figure 4. Scanning electron micrographs of shells of western *Amnicola*. **A.** USNM 1263000, McWennegar Slough, Montana. **B.** USNM 1263003, Brown's Lake, Montana. **C.** USNM 1256491, Curlew Lake, Washington. Scale bar = 1.0 mm.

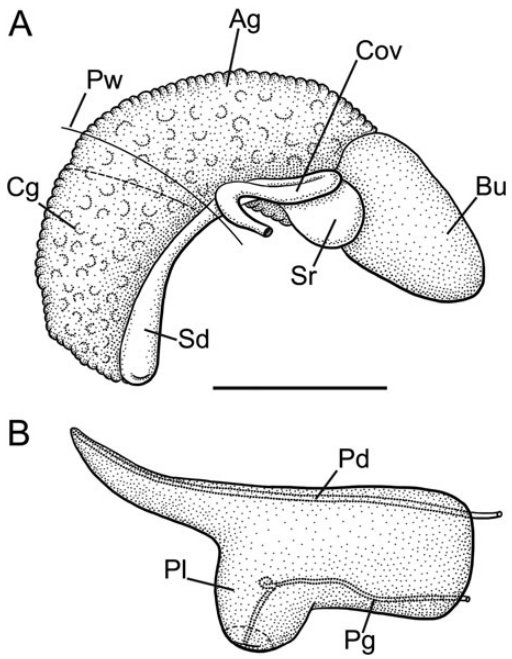

Figure 5. Reproductive anatomy of western *Amnicola*. **A.** Female glandular oviduct and associated structures (viewed from left side; duct to albumen gland not shown). USNM 1163822. **B.** Penis, dorsal surface. USNM 1258914. Abbreviations: Ag, albumen gland; Bu, bursa copulatrix; Cg, capsule gland; Cov, coiled oviduct; Pd, penial duct; Pg, penial gland; Pl, penial lobe; Pw; posterior wall of pallial cavity; Sd, spermathecal duct; Sr, seminal receptacle. Scale bar = 1.0 mm.

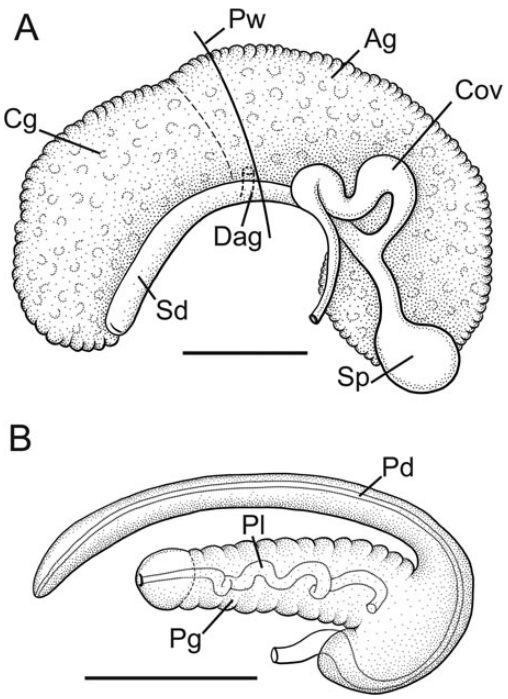

Figure 6. Reproductive anatomy of the 'masked duskysnail' (USNM 1258915). **A.** Female glandular oviduct and associated structures (viewed from left side). **B.** Penis, dorsal surface. Abbreviations: Ag, albumen gland; Cg, capsule gland; Cov, coiled oviduct; Dag, duct to the albumen gland; Pd, penial duct; Pg, penial gland; Pl, penial lobe; Pw; posterior wall of pallial cavity; Sd, spermathecal duct; Sp, sperm pouch. Scale bar = 250 μm.

those of *L. pupoides* (Burch & Tottenham, 1980: fig. 305), which is distributed in northern Atlantic Coastal drainages, but are slightly larger (maximum shell height about 3.5 mm). They are nearly identical to specimens of *L. walkeri* from the Great Lakes region that we have examined (Fig. 7C). The supposedly distinctive 'mask' of pigment on the neck and proximal section of the snout of the masked duskysnail (Fig. 8A, B) is shared by *L. retromargo* (Thompson, 1968: fig. 27H) and *L. pupoides* (Fig. 8C); the pigmentation of the other species of *Lyogyrus* has not been described or illustrated. The penis of the masked duskysnail

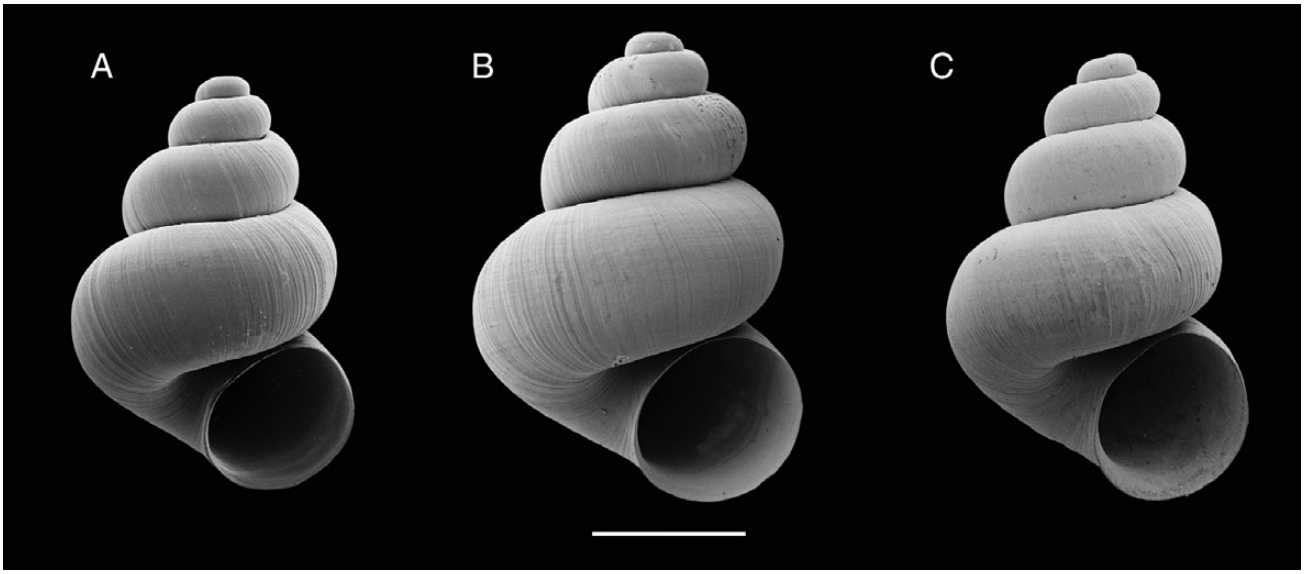

Figure 7. Scanning electron micrographs of shells of the 'masked duskysnail' (**A, B**) and *Lyogyrus walkeri* (**C**). **A.** USNM 1258915, Fish Lake, Washington. **B.** USNM 1262996, Upsata Lake, Montana. **C.** USNM 622780, inlet of Waubasacan Lake, northeast of Bedford, Michigan. Scale bar = 1.0 mm.

Figure 8. Photographs of the Masked duskysnail (**A, B**) and *Lyogyrus pupoides* (**C**) showing closely similar headfoot pigmentation. **A.** USNM 1258915. **B.** USNM 1262996. **C.** USNM 1296760, Pond along Mill Brook, Scarborough, Maine. Scale bars = 1.0 mm.

(Fig. 6B) closely resembles those of the four congeners that have been illustrated previously (*L. walkeri*, Berry, 1943: fig 2; *L. granum*, *L. pupoides*, *L. retromargo*, Thompson, 1968: fig. 37A–C). Further morphological comparisons are precluded by the paucity of data for eastern American *Lyogyrus*.

DISCUSSION

Our results show that the putative species of western American *Amnicola* is not substantially differentiated morphologically or genetically and does not form a separate, evolutionarily independent lineage relative to eastern American *A. limosa*. Based on these findings we conclude that the 'Washington duskysnail' is not a distinct species and that all of the western *Amnicola* populations are *A. limosa*. This finding extends the range of *A. limosa* from western Montana westward almost to the Pacific margin (Fig. 1). Although the western *Amnicola* populations do not merit recognition as distinct species, our data suggest that they have been genetically isolated from eastern congeners for at least 1 myr based on COI molecular clocks derived for other hydrobioid gastropods (Wilke, 2003; Hershler & Liu, 2008). Thus it may be appropriate to treat the western *Amnicola* populations as a separate management unit for conservation purposes. Our genetic data also suggest that the taxonomy of eastern American *Amnicola* needs to be revisited (e.g. to investigate the distinction between *A. dalli* and *A. limosa*); however, this is beyond the scope of the current paper.

As mentioned above, the scant morphological description of the masked duskysnail in the grey literature is not sufficient for generic assignment. Herein we have provided congruent genetic and morphological evidence that this snail belongs to *Lyogyrus*, which was previously thought to be restricted to eastern North America. *Lyogyrus* is a poorly known genus that has been little studied taxonomically during the past 50 years (but see Thompson, 1968; Thompson & Hershler, 1991). Most of the nine (Thompson & Hershler, 1991; Turgeon et al., 1998; Hershler, 1999) currently recognized congeners are known only from shells and species limits within the genus have not been reassessed since Thompson's (1968: 163) brief synopsis. Although our study is constrained by this paucity of data, we have not found clear morphological evidence supporting recognition of the masked duskysnail as a new species. There are no previously published DNA sequences for *Lyogyrus* and thus we cannot adequately evaluate the possible genetic distinctiveness of the masked duskysnail, although our finding that this snail differs from specimens of *L. pupoides* by 3.9% COI sequence divergence (together with the difference in shell size mentioned above) suggests that these are probably different species. We recommend that the western populations be treated as '*Lyogyrus* sp.' pending further study of previously described congeners, particularly *L. walkeri*, which closely resembles '*Lyogyrus* sp.' morphologically (as noted above) and is also the most geographically proximate congener (ranging westward to Lake Winnipeg, southern Manitoba; Clarke, 1973).

Our results also provide the first molecular phylogenetic evidence of the relationships of *Lyogyrus*, which had been treated as a subgenus of *Amnicola* during much of the last century (e.g. Thompson, 1968; Burch & Tottenham, 1980) before being elevated to its current taxonomic status (Thompson & Hershler, 1991). Our molecular phylogenetic analyses (Fig. 2) clearly indicate that *Lyogyrus* is not closely related to *Amnicola*, but instead forms a clade with *Marstoniopsis*, a Palaearctic genus, and *Taylorconcha*, which is

distributed in the northwestern United States. This grouping is also supported by the unique female reproductive anatomy of these genera, which are the only members of the amnicolid clade having a single sperm pouch (*Marstoniopsis*, Szarowska, 2007: fig. 198; *T. serpenticola*, Hershler *et al*., 1994: fig. 11C).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *Journal of Molluscan Studies* online.

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