Population structure and mantle display polymorphisms in the wavy-rayed lampmussel, *Lampsilis fasciola* (Bivalvia: Unionidae)

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Abstract: Genotypes from 10 polymorphic DNA microsatellite loci were used to make assessments of population structure, measurements of gene flow, and attempts to genetically segregate polymorphic host fish-attracting mantle displays for the wavy-rayed lampmussel, *Lampsilis fasciola* Rafinesque, 1820 — an endangered species in Canada. Specimens were collected from seven localities — six in the Great Lakes drainages of Ontario, Canada, and one from the Little Tennessee River in North Carolina, USA. Four distinct and sympatric mantle display morphologies were observed on female *L. fasciola*. Displays could not be distinguished genetically using analysis of molecular variance and genotypic assignment tests. The diversity of mantle displays was correlated with the overall genetic diversity observed among populations of *L. fasciola*. In managing populations of *L. fasciola* for propagation, augmentation, and translocation, polymorphic lures should be represented in proportion to what is observed in wild populations. Through moderately high F_{ST} values and high assignment to population in genotype assignment tests, genetic structure was evident among the river drainages. Within-drainage gene flow was very high, and sampling localities within the Ontario drainages displayed panmixia. Efforts in artificial propagation and possible translocations to reintroduce or augment populations should be made to maintain the substantial levels of genetic variation while maintaining distinctiveness.

Résumé : Les génotypes de 10 locus microsatellites polymorphes d'ADN ont servi à évaluer la structure de population, mesurer le flux génique et tenter de discriminer génétiquement les déploiements polymorphes du manteau qui servent à attirer les poissons hôtes chez la lampsile fasciolée, *Lampsilis fasciola* Rafinesque, 1820 — une espèce en péril au Canada. Les spécimens proviennent de sept localités — six des bassins versants des Grands Lacs en Ontario, Canada, et une de Little Tennessee River en Caroline du Nord, É.-U. Quatre morphologies distinctes et sympatriques de déploiement du manteau s'observent chez les femelles de *L. fasciola*. Il n'est pas possible de distinguer ces déploiements sur une base génétique à l'aide de l'analyse de la variance moléculaire et des tests d'attribution génotypique. La diversité des déploiements du manteau est en corrélation avec la diversité génétique globale observée chez les populations de *L. fasciola*. Dans la gestion des populations de *L. fasciola* pour fins de propagation, de croissance et de translocation, les leurres polymorphes devraient être représentés dans des proportions semblables à celles qu'on observe dans les populations sauvages. Il existe une structure génétique évidente au sein des bassins versants des rivières d'après les valeurs moyennement élevées de F_{ST} et le taux élevé de succès des assignations à la population dans les tests d'attribution génotypique. Le flux génique à l'intérieur des différents bassins versants est très élevé et il existe une panmixie aux points d'échantillonnage situés dans les bassins versants ontariens. Les efforts de propagation artificielle et les translocations possibles pour réintroduire ou consolider les populations devraient viser à maintenir des niveaux significatifs de variation génétique, tout en préservant leur caractère distinctif.

[Traduit par la Rédaction]

Introduction

Nearly all freshwater mussels (also known as naiads or unionids) are obligate parasites on fish during the larval stage. In response to this unique life history, unionids belonging to the tribe Lampsilini (Unionidae: Ambleminae) have evolved a fascinating diversity of host-attraction strategies (Zanatta and Murphy 2006*b*) to facilitate the infestation of a host fish with the mussel's glochidia larvae. The mantle flap display is best known and most remarkable in species of *Lampsilis* Rafinesque, 1820 (Kraemer 1970). Female mussels' flaps are remarkably good mimics of small fish (darters and minnows). They have false eyespots, fins, pigmentation, such as spots, and a false lateral line. Movement and twitches of the mantle facilitate the deception (Haag et al. 1995). Mantle flaps and other modes of host attraction have been shown to elicit attacks from potential hosts (Haag and Warren 1999).

The wavy-rayed lampmussel, *Lampsilis fasciola* Rafinesque, 1820 is a widespread unionid found throughout much of the Ohio and Tennessee river drainages and lower Great

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Lakes drainage (Metcalfe-Smith et al. 2000*a*). Although populations are considered to be secure in most jurisdictions of United States, it is an endangered species in Canada (COSEWIC 2006). In Canada, it is found in several Ontario watersheds of the lower Great Lakes. Reproducing populations occur in the Grand, Thames, and Maitland rivers, and smaller populations are found in the Ausable River and Lake St. Clair (Morris 2006). Morris (2006) reported that the determination of the genetic distinctiveness of populations of *L. fasciola* in Ontario was an important knowledge gap for species recovery. In addition to possible genetic differences among river drainages, female *L. fasciola* have a diversity of mantle displays. This diversity could indicate the existence of cryptic species.

Mimicry in parasitic organisms is used to fool potential hosts. Variation in mimicry can delineate host races and species. For example, in the cuckoo (*Cuculus canorus* L., 1758), mimicry has lead to the delineation of discrete host races (Gibbs et al. 2000; Avilés and Møller 2004). However, not all parasitic organisms form distinct host races (e.g., Fanelli et al. 2005). In the freshwater mussel genera *Cyprogenia* and *Epioblasma*, polymorphic conglutinates (packages of glochidia) that mimic worms and mantle displays are diagnostic for species (Jones et al. 2006; Serb and Barnhart 2007). It is possible that the polymorphic mantle flap displays in *L. fasciola* are used to attract different hosts. If true, then each lure type could represent a distinct species or host race.

Microsatellite loci have been developed and characterized for unionids (Eackles and King 2002; Geist et al. 2003; Jones et al. 2004; Shaw et al. 2006; Zanatta and Murphy 2006*a*). These molecular markers have revealed significant population structure in the European pearl mussel, *Margaritifera margaritifera* (L., 1758). Many populations both within and among river drainages had unique alleles and high pairwise F_{ST} values and genetic distances (Nei 1972; Geist and Kuehn 2005). Similarly, populations of *Lampilis cariosa* (Say, 1817) along the Atlantic coast of Maine showed significant population structure (Kelly and Rhymer 2005). Genetic variation among unionid populations in rivers draining into salt water would be expected to be high because there would be much less opportunity for the transport of glochidia by host fish between populations. This relationship of riverine distance to genetic distance has been confirmed in the central basin of North America using the critically endangered *Epioblasma torulosa rangiana* (I. Lea, 1838) (Zanatta and Murphy 2007*a*). Microsatellite DNA markers have also been used to assist in distinguishing among several closely related species of critically endangered *Epioblasma* in the Tennessee and Cumberland river drainages (Jones et al. 2006) and in diagnosing management units and distinct taxonomic units in the imperiled snuffbox mussel, *Epioblasma triquetra* (Rafinesque, 1820) (Zanatta and Murphy 2007*b*).

In combination with knowledge of life history, molecular data are critical in planning for the perpetuation of imperiled freshwater mussels. Ecology, captive care, and propagation have been emphasized in the planning for the recovery of endangered freshwater mussels (National Native Mussel Conservation Committee 1998). Animals should be relocated into areas that contain similar genetic profiles to augment populations (Villella et al. 1998). Data on the genetic characteristics of mussel populations are needed to make informed decisions regarding the numbers, localities, and logistical concerns of potential relocations or population augmentation through artificial propagation. Herein, we use multilocus microsatellite genotypes to assess population structure and gene flow in *L. fasciola* and to determine if the sympatric polymorphic mantle displays segregate genetically.

Methods

Sample localities, mantle display observations, and tissue collection

In total, 127 specimens of *L. fasciola* were collected from seven localities: from the USA, 35 from the Little Tennessee River near Needmore, Swain Co., North Carolina (35°19′23.67″N, 83°31′21.92″W); from Ontario, Canada, 16 from the Grand River near Waterloo (43°29′39.61″N, 80°28′15.17″W); 21 from the Grand River near Kitchener (43°24′13.58″N, 80°25′58.17″W); 18 from the North Thames River, near St. Mary's (43°12′31.04″N, 81°12′26.10″W); 16 from the Middle Thames River, near Thamesford (43°2′49.56″N, 80°59′37.41″W); 9 from the South Maitland River, near Summerhill (43°41′4.56″N, 81°32′27.64″W); and 12 from the Middle Maitland River near Wingham (43°51′35.92″N, 81°19′9.87″W) (Fig. 1).

In situ photographs of mantle displays were taken using an underwater digital camera (Olympus™). Descriptions of the mantle displays were made using the terminology of Kraemer (1970).

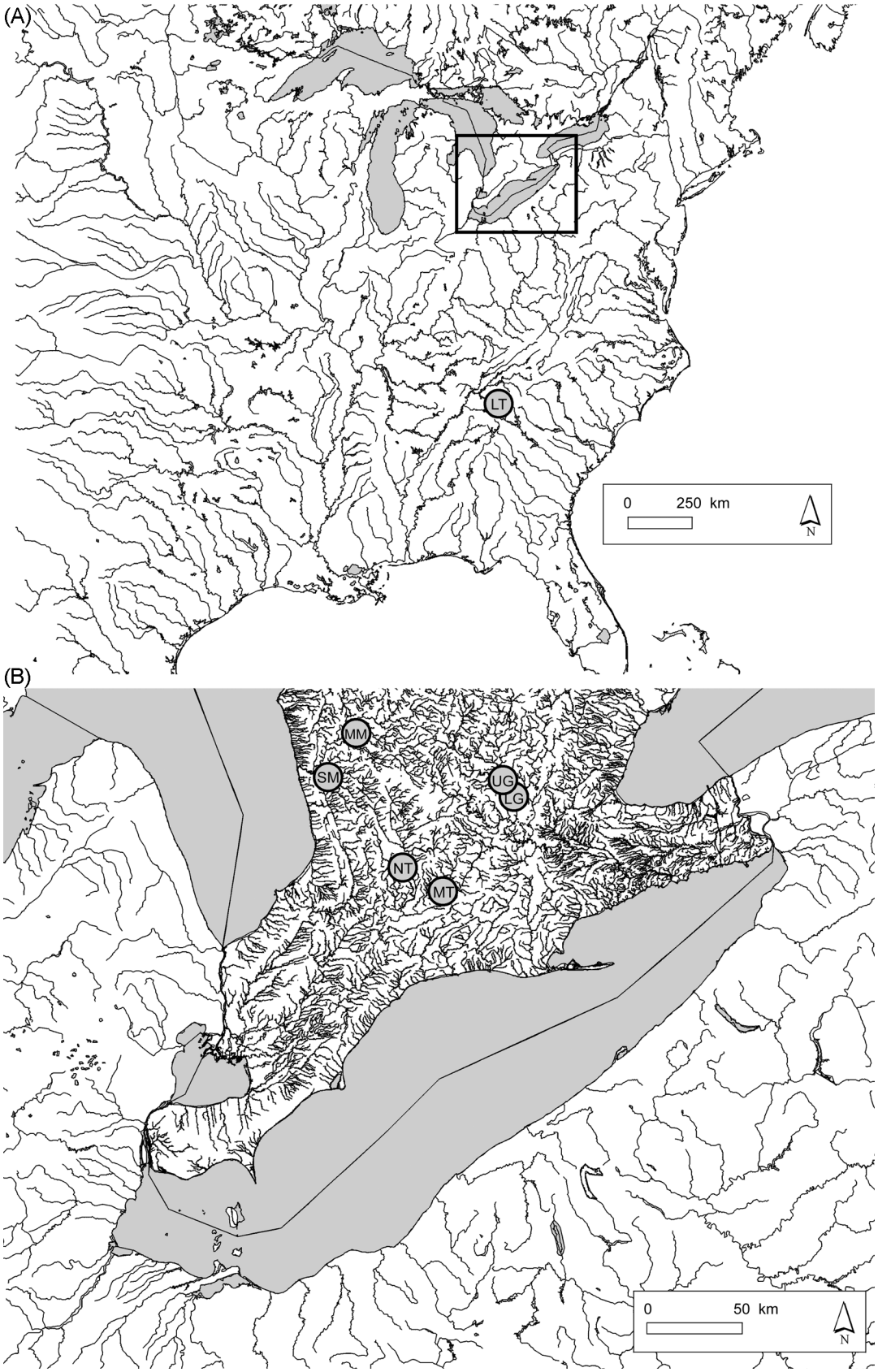
Mantle tissue (~30 mg) was nondestructively excised following protocols by Berg et al. (1995). Tissues were placed in cryovials, preserved in 95% ethanol or frozen on dry ice, and subsequently stored at –80 °C.

DNA extraction and genetic analyses

Total genomic DNA was extracted from ~15 mg of frozen preserved mantle tissue samples by standard phenol extraction (Hillis et al. 1996). A suite of 10 microsatellite loci were used (GenBank accession Numbers: AY650389–AY650398, AF512384–AF512398, and DQ396404). These loci were developed for other unionid species: *Epioblasma capsaeformis* (I. Lea, 1834) (Jones et al. 2004), *Lampsilis abrupta* (Say, 1831) (Eackles and King 2002), and *E. torulosa rangiana* (Zanatta and Murphy 2006*a*).

The characterization of these loci followed methods slightly modified from Zanatta and Murphy (2006*a*). Forward PCR primers were ordered with an M13 tail on the 5′ end following the protocol of Schuelke (2000). PCR reactions were done in a 25 μL solution containing 1.0 μL of genomic DNA, 8 pmol of each the forward primer with M13 tail, reverse primer, and a 6-FAM or VIC-labeled M13 primer, 0.3 μmol/L dNTP, 1× PCR buffer (2.0 mmol/L Tris–HCl, 10 mmol/L KCl, 0.01 mmol/L EDTA, and 0.1 mmol/L DTT), 2.0 μmol/L $MgCl_2$, and 1.5 U *Taq* (1 U ≈ 16.67 nkat) (Promega™ or Fisher™). Each PCR run (94 °C for 2 min; 35 cycles of 94 °C for 40 s, 58 °C for 40 s, 72 °C for 1 min; a final extension step of 72 °C for 1 min and a hold at 4 °C — Eppendorf Mastercycler 534X thermocycler) included a negative control. Double-stranded

Fig. 1. The distribution of populations where tissue collections were made for *Lampsilis fasciola*. Sample site localities: (A) in the USA, Little Tennessee River (LT) near Needmore, North Carolina; (B) in Ontario, Grand River (UG) near Waterloo; Grand River (LG) near Kitchener; North Thames River (NT) near St. Mary's; Middle Thames River (MT) near Thamesford; South Maitland River (SM) near Summerhill; Middle Maitland River (MM) near Wingham.



PCR products were visualized with a 1 kb+ ladder to estimate fragment length in a 1.0% agarose gel stained with ethidium bromide. Nonoverlapping amplified microsatellite loci were multiplexed and genotyped with a LIZ size standard (dye set DS-33) using an Applied Biosystems (ABI) 3100 automated sequencer and scored using ABI's GeneMapper® software.

Statistical analyses

Genetic diversity in each sample (for the microsatellite DNA data set) was summarized as allelic richness (A), measured as the mean number of alleles per locus after correcting for sample size, and expected heterozygosity (H_E). Allelic richness was calculated in FSTAT v. 2.9.3.2 (Goudet 1995). These data were standardized for sample size using rarefaction (Petit et al. 1998). Detection of deviations from Hardy–Weinberg equilibrium and randomization tests for linkage disequilibria were conducted using GENEPOP v. 3.4 (Raymond and Rousset 1995).

The microsatellite data set was tested for genotyping errors due to stuttering, short allele dominance, and null alleles using a Monte Carlo simulation of expected allele size differences using MICRO-CHECKER (Van Oosterhout et al. 2004). Allele size-difference frequencies were determined to deviate from expectations if they fell outside the Bonferroni-corrected 95% confidence interval generated by the simulation. Predicted frequencies of null alleles were calculated according to the method developed by Brookfield (1996).

A hierarchical analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was used to estimate the partitioning of genetic variance within and among populations. An AMOVA was carried out in Arlequin v. 2.0 (Schneider et al. 2000) using data pooled across loci. F_{ST} (Weir and Cockerham 1984) was calculated for all pairs of populations in FSTAT v. 2.9.3.2 (Goudet 1995). Probability values for F statistics were calculated to test the null hypothesis of panmictic populations by permuting genotypes among populations to calculate the probability of obtaining equal or greater F_{ST} by chance distribution of genotypes. Sequential Bonferroni correction (Rice 1989) was used to correct P values for multiple comparisons.

A second AMOVA was done to estimate the partitioning of genetic variation between the observed mantle display types. Only female specimens were used in this analysis. The AMOVA was implemented in Arlequin v. 2.0 (Schneider et al. 2000) using data pooled across loci. F_{ST} (Weir and Cockerham 1984) was calculated between groups of individuals sharing mantle display types. Corresponding to the F_{ST} values, gene flow was roughly estimated using N_m (number of migrants per generation), calculated using $N_m = (1/F_{ST} - 1)/4$.

Genetic distances between populations were estimated using Nei D_A genetic distance (Nei et al. 1983) as implemented in DISPAN (Ota 1993). The resulting distance matrix was used to construct a neighbour-joining (NJ) network in MEGA version 3.1 (Kumar et al. 2004). Bootstrapping was performed by first generating 1000 distance matrices, which were then used to generate 1000 NJ trees in DISPAN (Ota 1993).

Assignment tests were used to estimate the probability of each individual originating from a given population (Paetkau et al. 1995; Rannala and Mountain 1997). GENECLASS2 (Piry et al. 2004) was used to implement the assignment tests (Rannala and Mountain 1997), using the "as is" option. This technique estimated the posterior probabilities of actual allele frequencies given the observed allele frequencies from that population; it then estimated the probability for each individual of belonging to any of the populations. For each population, χ^2 was calculated to test whether the level of assignment was greater than could be expected by chance.

A second set of assignment tests were used to estimate the probability of each individual originating from a given mantle display type (Paetkau et al. 1995; Rannala and Mountain 1997). As with the AMOVA, only females were used. In this test, the expectation was that individuals should assign to the correct display type 95% of the time.

A test to detect recent population bottlenecks using infinite allele, stepwise, and two-phased mutation models was implemented in BOTTLENECK (Cornuet and Luikart 1996).

Isolation-by-distance was measured by comparing Nei D_A to geographic distance (measured in river kilometres) for pairs of populations. Geographic distances between populations were measured in ArcView GIS v. 3.2 (Environmental Systems Research Institute 2001). The distance between the Great Lakes drainage and the Ohio and Mississippi river drainages was estimated by measuring modern land distance between the Maumee and Wabash rivers. These rivers were connected at the end of the last Pleistocene glaciation (Calkin and Feenstra 1985) and were the hypothesized vector for the postglacial reinvasion of the Lake Erie drainage for freshwater mussel species originating from the Mississippi Basin (Graf 2002). The statistical significance of the correlation between geographic and genetic distance matrices were tested using a Mantel test in GENEPOP v. 3.4 (Raymond and Rousset 1995).

Results

Mantle displays

Mantle displays occurred on all female *L. fasciola* collected. Specimens of *L. fasciola* averaged 65 mm in length, with the mantle display extending ~3–4 cm along the ventral margin. Four distinct mantle displays were observed in the field: the most common had darter-like variants, with midlateral spots, dorsal spots, simple appendages, and a distinct eyespot (Fig. 2C); an orange display, lacking appendages, and an eyespot was observed at most localities (Fig. 2A); less common was a hellgrammite-like (*Corydalis* sp.) display that was dark, with a generally patternless dorsum contrasting with lighter sides — the dark pigment extended lobe-like into lighter area, it lacked an eyespot, and it had simple appendages (Fig. 2B); and another group of variable, but more or less similar fish- or crayfish-like displays, that were termed "flamboyant attractors", with gaudy colors and patterns, some compound (branched) appendages, and a poorly defined eyespot (Fig. 2D). Tissue collections and mantle display observations in North Carolina were made in April–July, and the populations from Ontario were sampled in mid-July.

These mantle displays were not distributed equally among populations (Table 1). Greater diversity was observed in the Little Tennessee River's population than in Ontario, where

Fig. 2. Typical examples of mantle display diversity in *Lampsilis fasciola*: (A) orange, no appendages, no eyespot; (B) hellgrammite-like, dark, generally patternless dorsum contrasting with lighter sides (sublateral), dark pigment extends lobe-like into lighter area, lacks eyespot, simple appendages; (C) darter-like variants, midlateral spots, often with dorsal spots, simple appendages, distinct eyespot; (D) other variable fish-like or crayfish-like display, "flamboyant attractor", gaudy colors and patterns, some compound (branched) appendages, eyespot present but not well-defined (photo credits: S.J. Fraley, North Carolina Wildlife Resources Commission).

the darter-like morphology was dominant. The flamboyant attractor type displays (Fig. 2D) were not observed in any of the populations from Ontario. Within the population in the Little Tennessee River, the darter-like morphs showed greater variety of pigment patterns and colors than those in Ontario. The sex ratio bias towards female mussels (Table 1) could be attributed to the higher visibility of the mantle displays present in the female mussels. A significant correlation (r = 0.776, P = 0.040, Fig. 3) was found between the allelic richness (A) of populations (Table 2) and the number of mantle display types observed, with the higher genetic diversity being found with larger numbers of mantle display types.

Genetic analyses

All 10 microsatellite loci amplified for *L. fasciola* were highly polymorphic, with a total of 154 alleles observed (5–25 alleles/locus, mean = 15.4). Allele size ranges of the loci used overlapped with those observed for *E. capsaeformis* (Jones et al. 2004), *Lampsilis abrupta* (Eackles and King 2002), and *E. torulosa rangiana* (Zanatta and Murphy 2006*a*). Genetic diversity, as measured by allelic richness (A), varied somewhat by population (1.63–1.87 alleles/locus), with the lowest allelic richness occurring in the South Maitland River and the highest in the Little Tennessee River (Table 2). Similarly, mean expected heterozygosities ranged from 0.63 to 0.87 (Table 2).

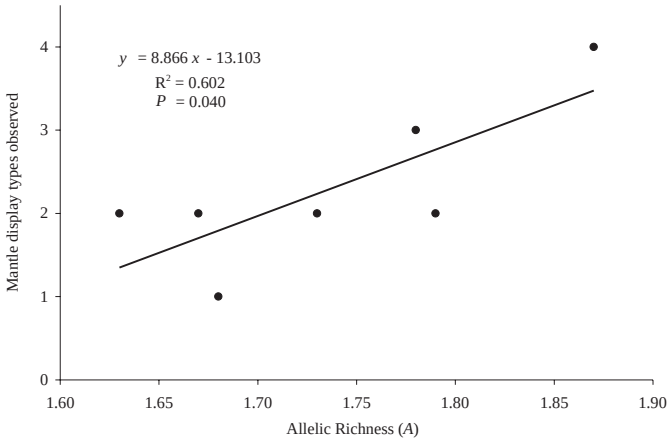
Significant deviations from Hardy–Weinberg expectations occurred at 8 out of 70 locus–population combinations after a Bonferroni correction (Table 2). These deviations indicated the possible presence of null alleles or other locus-specific genotyping errors. Analysis of the microsatellite data set showed no evidence for genotyping errors due to stuttering of large-allele dropout, suggesting the presence of nonamplifying alleles as a probable source of genotyping error. Estimated null frequencies varied by population and locus, but ranged as high as 0.247 for one locus–population combination (*LabC24*), with a mean frequency of 0.059. Randomization

Table 1. Sex ratios and diversity of mantle displays collected from each population of *Lampsilis fasciola*.

		Sex		Mantle display morphology			
River drainage	Population*	Male	Female	Orange lure (Fig. 2A)	Helgrammite-like (Fig. 2B)	Darter-like (Fig. 2C)	Other fish-like forms (Fig. 2D)
Grand	LG	4	17	2	3	12	—
	UG	1	15	1	—	14	—
Maitland	MM	6	6	2	—	4	—
	SM	4	5	—	—	5	—
Thames	MT	—	16	3	—	13	—
	NT	2	16	3	—	13	—
Little Tennessee	LT	—	35	6	6	17	6

*LG, Lower Grand; UG, Upper Grand; MM, Middle Maitland; SM, South Maitland; MT, Middle Thames; NT, North Thames; LT, Little Tennessee.

Fig. 3. Regression of allelic richness (*A*) versus number of mantle displays observed in populations of *Lampsilis fasciola*. *P* value calculated using *t* test of the Pearson correlation.



tests for linkage disequilibrium by locus and population did not indicate any significant linkage disequilibria (Bonferroni-corrected) for any of the dilocus combinations in *L. fasciola*.

Significant population structuring was found for *L. fasciola*. Of the total variation, 2.5% was due to differences among populations ($P < 0.001$). Based on F_{ST}, significant differences were observed among most pairs of populations after correcting for multiple comparisons (Table 3); however, the differences were not significant within drainages.

The levels of gene flow, inferred from N_m (number of migrants between populations or generations), between populations were all moderate to high, ranging from 1.172 to 11.32 (Table 3). Values for $N_m < 1$ indicate low levels of gene flow (0 = no gene flow); increasing values reflect progressively higher levels of gene flow.

Sample sizes of mantle display types were sufficient to compare the orange lure type (Fig. 2A) with all other display types (Fig. 2). No significant structure occurred between these two groups in the AMOVA ($P = 0.085$). Of the total variation, only 0.75% was due to differences between the observed mantle display groups. The pairwise F_{ST} of 0.0075 between the two groups was not significant (AMOVA, $P = 0.113$).

Using pairwise Nei D_A genetic distances (Nei et al. 1983), the branching pattern formed in the NJ network (Fig. 4) closely resembles the branching pattern of the rivers of origin among the sample populations (Fig. 1).

We evaluated the level of assignment to drainage and to population using the criteria described by Rannala and Mountain (1997). Based on cross-validation, on average, individuals of *L. fasciola* were assigned to their own population nearly 95% of the time and to their own drainages with 100% accuracy (Table 4). Chi-square tests revealed that assignments to population and to drainage were greater than would be expected by chance for all populations ($P < 0.01$, 1 degree of freedom (df)). All of the misclassified individuals were assigned to the next-nearest population geographically, suggesting that misclassified individuals could be the result of recent gene flow from nearby populations.

In addition to the assignment tests, we examined the level of assignment to mantle display type. Sufficient numbers of the other mantle display morphologies allowed the formation of two groups: female *L. fasciola* with orange lure vs. other lures. Using cross-validation, individuals with the orange display (Fig. 2A) were assigned to the correct group only 35% of the time and were significantly less than the 95% correct assignment expected ($P < 0.001$, Table 5), whereas the other lure types were assigned to the correct group 86% of the time, not significantly different from the 95% correct assignment expectation ($P > 0.05$, Table 5).

Evidence for recent bottlenecks were found in the Middle Thames and North Thames river populations of *L. fasciola* under a stepwise mutation model and a Wilcoxon's sign-rank test (Luikart and Cornuet 1998; $P = 0.012$ and 0.007, respectively). However, no evidence of bottlenecking was found under an infinite allele or two-phased mutation models, under Wilcoxon's sign-rank tests ($P > 0.05$).

A Mantel test revealed a significant correlation between geographic and genetic distance for *L. fasciola* ($r = 0.829$, $P = 0.011$, Fig. 5).

Discussion

Genetic population structure

The microsatellite analyses showed significant genetic population structure and indicated that the sampled populations of *L. fasciola* were not panmictic. Moderate gene flow appears to have recently occurred between all of the sampled populations. Gene flow was most evident between the Ontario drainages. However, this may be a result of the limited number of individuals sampled per locality in the presence of a relatively large number of alleles (up to 25 alleles/locus). This result may be related to the movement of the host fish for *L. fasciola*, smallmouth bass (*Micropterus*

Table 2. Locus name, allele size range, and GenBank accession Number, with number of alleles (private alleles in parentheses) and observed (H_O) and expected (H_E) heterozygosities for *Lampsilis fasciola* by locus and population.

		Populations							
		Grand Drainage		Maitland Drainage		Thames Drainage		Little Tennessee Drainage	
Locus, allele size range, and GenBank accession No.		LG	UG	MM	SM	MT	NT	LT	All *L. fasciola*
Etr114	No. of alleles	10 (0)	10 (2)	4 (0)	3 (0)	11 (0)	6 (1)	15 (9)	25
130–195 bp	H_E	0.86	0.88	0.63	0.58	0.87	0.73	0.90	0.88
DQ396404	H_O	0.94*	0.86*	0.50	0.44	1.00	0.67	0.73	0.77*
	n	19	14	12	6	14	15	15	98
Ecap1	No. of alleles	12 (0)	11 (0)	5 (0)	5 (0)	9 (1)	14 (3)	8 (0)	23
168–218 bp	H_E	0.86	0.89	0.89	0.87	0.83	0.90	1.00	0.90
AY650389	H_O	1.00*	0.60*	1.00	1.00	0.93	1.00	1.00	0.91*
	n	19	15	4	5	14	16	4	77
Ecap5	No. of alleles	11 (2)	12 (1)	6 (0)	4 (0)	7 (0)	11 (2)	10 (5)	25
226–291 bp	H_E	0.85	0.82	0.78	0.80	0.84	0.85	0.90	0.87
AY650393	H_O	0.82	0.80	0.78	1.00	1.00	0.94	0.89	0.88
	n	17	15	9	6	13	16	9	85
Ecap10	No. of alleles	6 (1)	5 (0)	2 (0)	1 (0)	2 (0)	3 (0)	8 (4)	10
140–158 bp	H_E	0.69	0.71	0.10	Only 1 allele	0.08	0.39	0.84	0.56
AY650398	H_O	0.36	0.69	0.10	—	0.08	0.35	0.60	0.35*
	n	14	13	10	1	12	17	10	77
LabC2	No. of alleles	4 (0)	3 (0)	2 (0)	2 (0)	4 (0)	4 (0)	4 (1)	5
150–168 bp	H_E	0.69	0.60	0.53	0.36	0.52	0.64	0.77	0.66
AF512384	H_O	0.50*	0.44	0.86	0.43	0.64	0.50	0.83	0.57*
	n	18	9	7	7	14	16	6	77
LabC23	No. of alleles	6 (0)	4 (1)	5 (0)	4 (0)	7 (1)	5 (1)	12 (4)	14
196–234 bp	H_E	0.77	0.71	0.70	0.64	0.90	0.72	0.92	0.81
AF512385	H_O	0.80	0.75	1.00	0.75	1.00	0.82	1.00	0.86*
	n	20	16	11	8	7	17	16	95
LabC24	No. of alleles	4 (0)	4 (0)	3 (0)	4 (0)	5 (0)	5 (0)	4 (0)	5
152–167 bp	H_E	0.74	0.68	0.63	0.76	0.67	0.61	0.61	0.74
AF512386	H_O	0.21*	0.20*	0.36	0.29	0.20*	0.35	0.57	0.31*
	n	19	15	11	7	15	17	14	98
LabD111	No. of alleles	7 (1)	9 (0)	2 (0)	5 (0)	7 (0)	7 (0)	10 (1)	15
246–305 bp	H_E	0.77	0.77	1.00	0.77	0.69	0.70	0.97	0.78
AF512395	H_O	0.87	0.69	1.00	0.86	0.80	0.69	0.83	0.78
	n	15	13	1	7	15	16	6	73
LabD206	No. of alleles	7 (1)	7 (0)	4 (0)	4 (0)	6 (0)	8 (1)	3 (2)	11
219–259 bp	H_E	0.78	0.83	0.64	0.76	0.47	0.83	0.83	0.84
AF512397	H_O	0.71	0.85	0.75	0.63	0.55	0.77	1.00	0.72
	n	14	13	8	8	11	13	2	69
LabD213	No. of alleles	8 (0)	10 (0)	7 (0)	4 (0)	6 (0)	13 (3)	14 (3)	21
185–279 bp	H_E	0.84	0.87	0.89	0.80	0.86	0.92	0.93	0.90
AF512398	H_O	0.82	0.63	0.50	0.67	0.67	0.89	0.79	0.74*
	n	11	16	8	3	6	18	14	76
Mean H_E		0.79	0.78	0.68	0.63	0.67	0.73	0.87	
Allelic richness		1.79	1.78	1.68	1.63	1.67	1.73	1.87	

*Indicates locus–population combinations with H_O significantly different from H_E, after a sequential Bonferroni correction (Rice 1989), experiment-wide $\alpha = 0.05$.

dolomieu Lacepède, 1802; Zale and Neves 1982; McNichols and Mackie 2002, 2003; McNichols et al. 2004) and the fairly continuous nature of the mussel population within these reaches (Morris 2006). Smallmouth bass are moderately vagile (VanArnum et al. 2004) within a river reach and appear to be moving *L. fasciola* glochidia between the localities sampled within drainages.

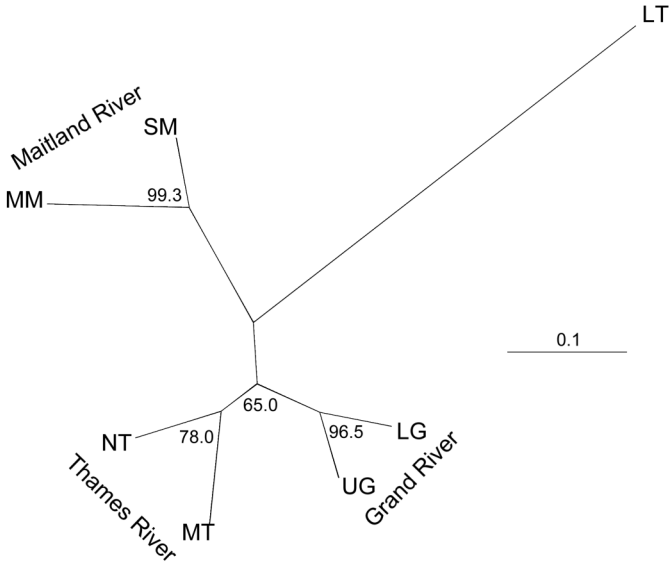
Populations of *L. fasciola* in the Great Lakes drainage are now effectively isolated because zebra and quagga mussels (*Dreissena* spp.) largely eradicated unionid populations from the Great Lakes (Schloesser and Nalepa 1994; Zanatta et al. 2002; Schloesser et al. 2006). Although nothing is known of the geneflow or population structure of *L. fasciola* through the Great Lakes prior to the zebra mussel invasion, this former habitat of *L. faciola* now forms an effective genetic barrier between the remaining riverine populations in the Great Lakes drainage. The pattern of genetic distance in *L. fasciola* closely paralleled the branching patterns of the Grand, Thames and Maitland rivers in the Great Lakes. This study provided further evidence that unionid mussels may be ideal organisms for the study of isolation by distance as a significant correlation between genetic distance

Table 3. Pair-wise F_{ST} (Weir and Cockerham, 1984) values (below diagonal) and number of migrants N_m (above diagonal) for all populations of *Lampsilis fasciola*.

	Grand Drainage		Maitland Drainage		Thames Drainage		Little Tennessee Drainage
Population	LG	UG	MM	SM	MT	NT	LT
LG	—	∞	1.842	2.111	3.022	5.745	2.453
UG	–0.0013	—	1.172	2.931	4.151	9.328	2.301
MM	0.1195*	0.1758*	—	∞	1.905	2.354	1.172
SM	0.1059*	0.0786*	–0.0090	—	3.316	3.432	1.296
MT	0.0764*	0.0568*	0.1160*	0.0701*	—	11.32	1.210
NT	0.0417*	0.0261*	0.0960*	0.0679*	0.0216	—	1.587
LT	0.1019*	0.0980*	0.1758*	0.1617*	0.1712*	0.1361*	—

*Indicates significant pairwise F_{ST} after adjusting for multiple comparisons via a sequential Bonferroni correction (Rice 1989), experiment-wide $\alpha = 0.05$.

Fig. 4. An unrooted neighbour-joining network based on Nei D_A (Nei et al. 1983) genetic distance for seven populations of *Lampsilis fasciola*. Numbers indicate nodes with bootstrap support of more than 50% for 1000 replications.



and riverine distance was found in *L. fasciola*. Unionid mussels may be ideal organisms for a stepping stone model because they are found in fragmented, patchy populations (mussel beds) along interconnected freshwater river systems. Thus, their difficulty-of-dispersion is a function of riverine linear geography.

Genetic diversity

Within-population genetic diversity (allelic richness and expected heterozygosities) was estimated to investigate the possibility of loss of variation. Allelic richness was highest in the unglaciated, Little Tennessee River's population. The populations in the formerly glaciated Great Lakes drainages in Ontario had somewhat lower allelic richness, possibly related to a founder effect of relatively few individuals.

Both of the sampling localities in the Thames River showed limited evidence (significance under two of four models) of a recent genetic bottleneck. This could be evidence of decline of *L. faciola* in the Thames River drainage to very small numbers, followed by a recovery of the populations. Although historical data are not available in the Thames River, the mussel populations in the Grand River have shown evidence of recovery in recent decades (Metcalfe-Smith et al. 2000*b*). Like the recovery of unionids in the Grand River, recovery of *L. fasciola* after a genetic bottleneck in the Thames River could be attributed to improvements in water quality in recent decades.

Deviations from Hardy–Weinberg equilibrium are not unusual. Heterozygote deficiencies in allozyme and microsatellite loci have been reported as causes for Hardy–Weinberg disequilibrium in other freshwater mussels (Nagel et al. 1996; Johnson et al. 1998; Jones et al. 2006; Zanatta and Murphy 2007*a*, 2007*b*) and other bivalves (Zouros and Foltz 1984; Raymond et al. 1997). A likely source of disequilibrium in this study is the limited sample sizes in the presence of high allelic diversity. Analyses indicated that excess of homozygotes at several loci may also have resulted from nonamplifying alleles, possibly as a consequence of motif anomalies or mutations in the flanking region. Nonamplifying alleles may be quite common in bivalves, even in species for which the microsatellite primers were designed (McGoldrick et al. 2000). The low level (5.9%) of estimated null-allele frequency likely makes this factor a nonissue for the microsatellite data used in this study. A recent study showed that mean null-allele frequencies as high as 20% did not significantly change the results in parentage analyses using a simulated data set (Dakin and Avise 2004).

Mantle display polymorphisms

Limited sample sizes limited our ability to fully test the existence of cryptic species in *L. fasciola* as an explanation for the polymorphic mantle displays. Additional sampling of the rare lure types may be warranted to fully test this hypothesis. However, the available samples and the results largely reject the existence of cryptic species.

Graf (1997) proposed a theoretical mechanism for sympatric speciation in unionids involving a switch in the species of host fish used. Although not necessarily supported by the results of the analysis of microsatellite DNA loci, the sympatrically occurring polymorphic mantle displays observed in *L. fasciola* may represent a mechanism during early stages of sympatric speciation (sensu Maynard Smith 1966). Few definitive examples of sympatric speciation in mussels are known (Graf 1997). Smallmouth and largemouth bass (*Micropterus dolomieu* and *Micropterus salmoides* (Lacepède, 1802), respectively) have been shown to be the best

Table 4. Results of maximum likelihood assignment tests (Cornuet et al. 1999) by population and by drainage for *Lampsilis fasciola*.

	Grand Drainage		Maitland Drainage		Thames Drainage		Little Tennessee Drainage
	LG	UG	MM	SM	MT	NT	LT
Population							
Lower Grand (LG)	**19**	1	—	—	—	—	—
Upper Grand (UG)	2	**15**	—	—	—	—	—
Middle Maitland (MM)	—	—	**10**	—	—	—	—
South Maitand (SM)	—	—	2	**9**	—	—	—
Middle Thames (MT)	—	—	—	—	**15**	—	—
North Thames (NT)	—	—	—	—	1	**18**	—
Little Tennessee (LT)	—	—	—	—	—	—	**17**
Observed correctly classified	19	15	10	9	15	18	17
Expected correctly classified	3	2.29	1.71	1.29	2.29	2.57	2.42
χ^2 (1 df)	26.84	21.30	14.44	13.52	21.30	26.99	25.47
Correctly classified (%)	**90.4***	**93.8***	**83.3***	**100.0***	**93.8***	**100.0***	**100.0***
Drainage							
Grand River	**21**	**16**	—	—	—	—	—
Maitland River	—	—	**12**	**9**	—	—	—
Thames River	—	—	—	—	**16**	**18**	—
Little Tennessee River	—	—	—	—	—	—	**17**
Observed correctly classified	21	16	12	9	16	18	17
Expected correctly classified	5.3	4.0	3.0	2.3	4.0	4.5	4.3
χ^2 (1 df)	25.2	19.2	14.4	10.8	19.2	21.6	20.4
Correctly classified (%)	**100.0***	**100.0***	**100.0***	**100.0***	**100.0***	**100.0***	**100.0***

Note: χ^2 values are for the test of whether individuals assign more frequently to their own population than would be expected by chance if there were no differences among populations (1 df). Values in bold indicate the number of individuals that correctly assigned to their population of origin.

*Significant at $\alpha = 0.05$.

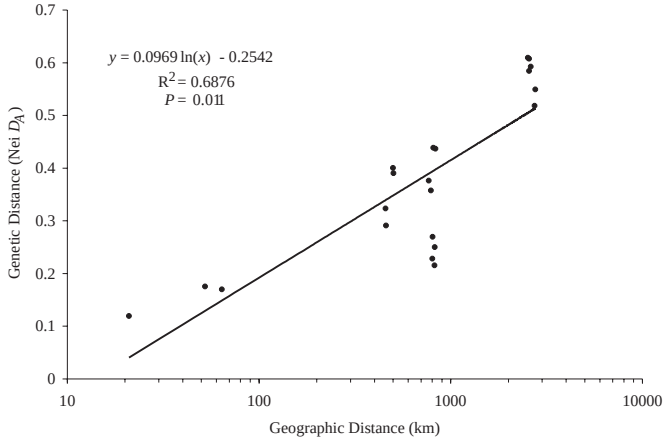
Table 5. Results of maximum likelihood assignment tests (Cornuet et al. 1999) by mantle display morphology for *Lampsilis fasciola*.

	Mantle display type	
	Orange lure	Other lures
Orange lure (Fig. 2A)	**6**	10
Other lures (Figs. 2B, 2C, 2D)	11	**64**
Observed correctly classified	6	64
Expected correctly classified	15	70
χ^2 (1 df)	86.4	12.94
Correctly classified (%)	**35.3***	**86.5**

Note: χ^2 values are for the test of whether individuals assign more frequently to their own lure type with a 95% expectation of accuracy (1 df). Values in bold indicate the number of individuals which correctly assigned to their own population.

*Represents significant difference from expected value at $\alpha = 0.05$.

Fig. 5. Regression of geographic (river) distance (km) versus genetic distance (Nei D_A) for *Lampsilis fasciola*. *P* value calculated using a Mantel test.



hosts for *L. fasciola* (Zale and Neves 1982; McNichols and Mackie 2003). However, the presence of multiple mantle display morphologies in *L. fasciola* could be a precursor to host switching and sympatric speciation as described by Graf (1997) for unionids.

Two recent studies may conform to the model of sympatric speciation for unionids proposed by Graf (1997), a switch in host fish likely leading to speciation as evidenced by divergences in mtDNA and (or) nuclear loci and host attraction-related behaviour and morphologies. Strongly diverged maternal (mtDNA) lineages occur sympatrically across the range of the western fanshell, *Cyprogenia aberti*

(Conrad, 1850) (Serb 2006). These lineages correspond closely with polymorphic conglutinates observed in *C. aberti* (Serb and Barnhart 2007). Closely related *Epioblasma florentina walkeri* (Wilson and H.W. Clark 1914) and *Epioblasma capsaeformis* occur sympatrically throughout the Tennessee River drainage. A switch in hosts from the greenside darter (*Etheostoma blennoides* Rafinesque, 1819) to the fantail darter (*Etheostoma flabellare* Rafinesque, 1819), or vice-versa, could have resulted in speciation, followed by divergence in molecular (mtDNA and nuclear DNA loci) and mantle pad morphology (Jones et al. 2006) in these species.

Because the molecular markers used were unable to identify potential cryptic species divided along the use of the polymorphic mantle displays in *L. fasciola*, reasons for the maintenance of this polymorphism are questioned. A hypothesis for the maintenance of polymorphic mantle displays in *L. fasciola* is similar to the hypotheses proposed by Serb and Barnhart (2007) in the polymorphic conglutinates of *Cyprogenia*. Host fish may develop an aversion to one type of mantle display after an initial encounter because of discomfort associated with glochidia attachment. The polymorphic mantle display morphologies in *L. fasciola* might have a selective advantage because potential host fish would be less likely to have previous experience with that display type, thereby maintaining the polymorphism in the species. A study of differences in the timing of polymorphic displays could indicate cryptic species, whereas lack of specific timing would support the hypothesis for maintaining the polymorphism. Further experimentation could determine whether one mantle display type elicits more attacks by hosts than others or if attacks are elicited more frequently by different display types under various conditions that exist during the time of display in their native habitat (e.g., water clarity, temperature, and light levels). No major difference in host attack frequency would indicate no selection for any particular lure type and selection for the maintenance of the polymorphisms.

The maintenance of polymorphic mantle displays may be somewhat parallel to the Red Queen hypothesis (Van Valen 1973). This hypothesis states that coevolutionary cycles are formed between hosts and parasites because of frequency-dependent selection. As such, if a given lure type is favoured by a host fish, it will increase in frequency until it is so common that there will be selection against that lure type in the host fish, and the fish will start to avoid it. This will increase the relative fitness of other lure types, and so on. This mechanism could maintain lure type polymorphisms by preventing any type from becoming too frequent.

Acquired host immunity could also help drive the maintenance of mantle display polymorphisms. Host immunity studies have shown that host fish recently infected with glochidia develop a significant immune response to further infestation for a period of time (Watters and O'Dee 1996; Dodd et al. 2006). The mantle display polymorphisms in *L. fasciola* could be an adaptation in response to this host immunity in that individuals with different morphologies are genetically different enough that the immune response is lessened, thus making more hosts available to more mussels for infestation. However, this hypothesis may not be viable. A study using largemouth bass showed that fish developed a significant immunity to glochidia from a variety of unionid species after infestation by the glochidia of a single species (Dodd et al. 2005).

The genetic link to the polymorphic mantle displays may be sex-linked. Because microsatellite alleles are inherited from each parent, they may not be suitable for finding genetic differences linked to a single sex. Findings in a gastropod suggest that a mammalian-like "X-linked" mode of sex determination may exist in molluscs (Avise et al. 2004). However, sex determination is, as yet, unknown for the Unionidae; hence, sex-specific markers have yet to be developed. Adding the problem of doubly uniparental inheritance of the mitochondrial genome observed in the Unionidae (Hoeh et al. 2002) makes the usually maternally-inherited mitochondrial DNA markers suspect as well. This is a critically important area of research for this group of animals, especially considering the conservation and recovery of many species.

Conservation implications

Although the polymorphic mantle displays in *L. fasciola* were found to be genetically indistinguishable using a suite of microsatellite loci, their diversity was correlated with genetic diversity. This absence of some display morphologies in Ontario could be a reflection of the low number of specimens collected from some localities. Further molecular analysis may be required to determine how these polymorphisms occur. In managing populations of *L. fasciola* for propagation, augmentation, and translocation, we recommend that polymorphic lures be represented in approximate proportions to what is observed in wild populations.

Moderate to high gene flow appeared to have been recently occurring between all of the sampling localities. Within-drainage gene flow was highest, and sampling localities within the Ontario drainages displayed panmixia. The relatively recent construction of impoundments on the Grand and Thames rivers and the introduction of dreissenid mussels have further isolated the remaining populations in Canada. Many of the intervening riverine and lacustrine habitats are now inhospitable to *L. fasciola*. Likewise, populations in the upper Little Tennessee River system are isolated by impoundments. Although not detectable today, this will ultimately lead to ever-increasing genetic divergence and isolation due to drift.

The importance of maintaining genetic diversity is well recognized. Although many of the populations of *L. fasciola* in Canada have experienced decline, it is still relatively healthy in several drainages. Because Canadian populations of *L. fasciola* are in the same geographic area facing similar threats to their status, they do not merit listing by the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) as separate designatable units (COSEWIC 2005; Green 2005). However, based on moderate F_{ST} values and nearly no misclassification between drainages in the assignment test, we recommend that populations in each of the drainages sampled in this study be treated as separate management units (sensu Moritz 1994). Efforts in artificial propagation and possible translocations to reintroduce or augment populations should be made to maintain the substantial levels of genetic variation while maintaining distinctiveness. If the population from a particular region has been extirpated or is too small to propagate, collections of brood

stock from the nearest viable population, in riverine distance and known genetic profile, is recommended.

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