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Microsatellite loci for dreissenid mussels (Mollusca: Bivalvia: Dreissenidae) and relatives: markers for assessing exotic and native populations

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Abstract

We developed and tested 14 new polymorphic microsatellite loci for dreissenid mussels, including the two species that have invaded many freshwater habitats in Eurasia and North America, where they cause serious industrial fouling damage and ecological alterations. These new loci will aid our understanding of their genetic patterns in invasive populations as well as throughout their native Ponto-Caspian distributions. Eight new loci for the zebra mussel *Dreissena polymorpha polymorpha* and six for the quagga mussel *D. rostriformis bugensis* were compared with new results from six previously published loci to generate a robust molecular toolkit for dreissenid mussels and their relatives. Taxa tested include *D. p. polymorpha*, *D. r. bugensis*, *D. r. grimmi*, *D. presbensis*, the 'living fossil' *Congeria kusceri*, and the dark false mussel *Mytilopsis leucophaeata* (the latter also is invasive). Overall, most of the 24 zebra mussel (*N* = 583) and 13 quagga mussel (*N* = 269) population samples conformed to Hardy–Weinberg equilibrium expectations for the new loci following sequential Bonferroni correction. The 11 loci (eight new, three previously published) evaluated for *D. p. polymorpha* averaged 35.1 alleles and 0.72 mean observed heterozygosity per locus, and 25.3 and 0.75 for the nine loci (six new, three previously published) developed for *D. r. bugensis*. All but three of these loci successfully amplified the other species of *Dreissena*, and all but one also amplified *Congeria* and *Mytilopsis*. All species and populations tested were significantly divergent using the microsatellite data, with neighbour-joining trees reflecting their evolutionary relationships; our results reveal broad utility for resolving their biogeographic, evolutionary, population and ecological patterns.

Keywords: Bivalvia, *Dreissena*, Dreissenidae, exotic species, invasive species, microsatellites, PCR primers, quagga mussel, zebra mussel

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Ponto-Caspian dreissenid mussels (Mollusca: Bivalvia: Dreissenidae) are invasive in a variety of freshwater North American and Eurasian ecosystems, where they cause significant ecosystem shifts and economic damage (Strayer 2009). The zebra mussel *Dreissena polymorpha polymorpha* and the quagga mussel *D. rostriformis bugensis* have spread across the two continents via canals, shipping and ballast. The taxonomic identities and systematic relationships of the dreissenids, including all of the taxa used here, are revised and clarified by Stepien *et al.* (2012). High-resolution nuclear DNA markers allow assessment of the comparative population genetic structures of these species, providing tools for analysing their introduction pathways and predicting likelihood of future spread, as well as for resolving their respective biogeographic histories (see baseline work for these taxa and their relatives by Stepien *et al.* 2001, 2003, 2005 using earlier techniques). The present investigation developed and tested 14 new unique polymorphic dinucleotide, trinucleotide and tetranucleotide nuclear DNA microsatellite loci to provide improved resolution for *D. p. polymorpha* (*N* = 583) and *D. r. bugensis* (*N* = 269), and evaluated the results of cross-species amplifications for each species.

We conducted new in-depth analyses for two populations of *D. p. polymorpha* from Gibraltar Island, Lake Erie, OH, USA (*N* = 24 sampled by us in 2004, with its invasion discovered there in 1998), and from San Justo Lake, CA, USA (*N* = 24 collected in 2008, when its invasion was reported). Our evaluations of *D. r. bugensis* concentrated on two introduced populations from Gibraltar

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Island, Lake Erie, OH, USA (N = 25 collected in 2007, with its invasion reported there in 1998), and Lake Mead, NV, USA (N = 24 collected in 2007, when its invasion was reported). We compared these population-level data with broadscale patterns across their North America and Eurasia ranges using 583 *D. p. polymorpha* (24 samples from 21 sites) and 269 *D. r. bugensis* (13 samples from 12 sites), analysed by us in Brown & Stepien (2010). We then conducted cross-species amplifications of *D. p. polymorpha* and *D. r. bugensis* for the microsatellite loci developed for the other species, using the four test populations.

We evaluated amplification of these loci for four additional related taxa: *D. r. grimmi* from the Caspian Sea, *D. presbensis* (formerly '*D. stankovicki*') from Lake Ohrid in Macedonia, the dark false mussel *Mytilopsis leucophaeata* from the southern Mississippi River at Baton Rouge, LA, USA, and the rare 'living fossil' *Congeria kusceri* from karst caverns in Metkovic, Croatia (see Stepien *et al.* 2001 for the latter's evolutionary relationships). We also tested previously published microsatellite loci developed for dreissenids by Wilson *et al.* (1999), Naish & Boulding (2001) and Astanei *et al.* (2005) for all six taxa, to evaluate their utility in a larger taxonomic arena and to compare results with those from our new loci.

To develop microsatellite primers, we employed an enrichment protocol described by Glenn & Schable (2005). Genomic DNA (gDNA) was extracted from a single zebra mussel and a single quagga mussel from Gibraltar Island, Lake Erie. DNA was digested with either *RsaI* and *XmnI* or *BstUI* and *XmnI* for enrichments (New England Biolabs, Ipswich, MA, USA). Following restriction digest, SuperSNX24 linkers (ordered from Integrated DNA Technologies, Coralville, IA, USA; sequences in Glenn & Schable 2005) that serve as priming sites for PCR were ligated onto the ends of gDNA fragments. Two different sets of biotinylated probes [trinucleotides: $(ACT)_{12}$, $(AAT)_{12}$, $(AAG)_8$, $(ATC)_8$ and $(AAC)_6$; tetranucleotides: $(AAAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(ACAT)_8$ and $(AGAT)_8$] were hybridized to gDNA, and the resultant probe-gDNA complex was added to streptavidin-coated magnetic beads (Dynabeads® M-280; Invitrogen, Carlsbad, CA, USA). This mixture was washed twice with 2×SSC, 0.1% SDS and four times with 1×SSC, 0.1% SDS at 53 °C. For the final two washes, the mixture was incubated for 1 min in a 53 °C water bath. After the last wash, enriched fragments were removed from the biotinylated probe by denaturing at 95 °C and precipitated with 3 M sodium acetate and 95% ethanol. Enriched fragments then were used as a template in a 'recovery' PCR, and PCR fragments were cloned using the TOPO-TA Cloning® kit (Invitrogen) following the manufacturer's protocol. Fragments inserted into vectors then were amplified using universal M13 primers, and PCR products were cleaned using MultiScreen-PCR Filter Plates following the manufacturer's protocol (Millipore, Billerica, MA, USA). DNA sequencing was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with sequencing reactions run on an ABI 3730 DNA Analyzer. Primers for microsatellites were developed from flanking sequences using Primer3 (http://primer3.sourceforge.net/). One hundred and forty-three clones were sequenced from the *D. p. polymorpha* library and 88 clones were sequenced from the *D. r. bugensis* library. Of these, 48 clones of *D. p. polymorpha* and 23 clones of *D. r. bugensis* had microsatellite repeat regions that contained five or more repeats; these were used for further testing.

The microsatellite PCR amplification mixture contained 0.6 units *Taq*, 50 μM nucleotides, 1× PCR buffer (50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris-HCl), 0.5 μM of each primer (Table 1) and approximately 30 ng of template in 10 μL. Amplification encompassed a 2-min initial denaturation at 94 °C; this was followed by 35 cycles of 30 s at 94 °C, 1 min annealing at a primer-specific temperature (Table 1), and 30 s at 72 °C, with a final 5 min extension at 72 °C on an MJR DYAD thermalcycler (Bio-Rad Laboratories, Hercules, CA, USA). Amplification products were diluted 1:50, of which 1 μL was added to 13 μL of formamide and ABI Gene Scan 500 size standard and loaded onto a 96-well plate for analysis on an ABI 3130 XL Genetic Analyzer using ABI GENEMAPPER v4.0. Output profiles were checked to confirm allelic size variants, and representative alleles were sequenced to verify that length polymorphisms were because of variation in copy number of single repeat motifs. The mitochondrial cytochrome *b* gene additionally was sequenced to confirm species identity following Brown & Stepien (2010).

Of the 48 new loci tested for zebra mussels, we selected eight loci for use in the present study that amplified reliably, generally were in Hardy–Weinberg equilibrium (HWE) following sequential Bonferroni correction (Holm 1979) across the more extensive population analyses presented in Brown & Stepien (2010), did not show overall widespread evidence of null alleles, and did not exhibit linkage disequilibrium (see Table 1). Similarly, of the 23 loci tested for quagga mussels, we selected six new loci that met those criteria (Table 1). These loci are the ones we present and include here.

We additionally re-evaluated microsatellite loci developed by other researchers, including three for *D. p. polymorpha* from Naish & Boulding (2001: *Dpol*A6, B6 and C5), two from Astanei *et al.* (2005: *D pol*B8-9) and six for *D. r. bugensis* by Wilson *et al.* (1999: *Dbug*1-6). In our experiments, two locus primer sets did not amplify reliably (*Dpol*B6 and C5), and thus were not used. The

Table 1 Characteristics of microsatellite loci isolated from the A. zebra mussel *Dreissena polymorpha polymorpha* and B. quagga mussel *D. rostriformis bugensis*. We reanalyse and summarize results for (a) 24 zebra mussel and (b) 13 quagga mussel population samples from Brown & Stepien (2010), with novel concentration here on population-level analyses of two invasive zebra mussel samples from the San Justo, CA reservoir (2008) and Gibraltar Island, Lake Erie, OH (2004), and two invasive quagga mussel samples from Lake Mead, NV (2007) and Gibraltar Island, Lake Erie, OH (2007). Locus, GenBank accession numbers, primer sequence (for the new loci), repeat motif, annealing temperature (T_A), range of repeat sizes (size, bp), range of number of repeats, number of alleles (N_A), and average observed and expected heterozygosity are indicated. Asterisks denote samples that did not conform to Hardy–Weinberg equilibrium expectations after sequential Bonferroni correction, which were attributed by analyses with MICRO-CHECKER to the possible influence of null alleles

a)

Locus	GenBank accession number	Sequence/Source	Repeat motif	T_A (°C)	Size (bp)	Across all populations from Brown & Stepien (2010) (N = 583, 24 samples, 21 sites) Native and Introduced		San Justo Reservoir, CA (N = 24) Introduced		Lake Erie, OH (N = 24, 2004) Introduced	
						N_A	*Ho*	N_A	H_O/H_E	N_A	H_O/H_E
*Dpo*04	GU213457	F: CTTCAAACGTAACGCACTCG R: TGCACAATTCAATGCAAAAAC	$(AAC)_7AC(AAC)_4$	50	225–366	13	0.62	4	0.63/0.57	3	0.25/0.65*
*Dpo*101	GU213458	F: AGACTGTGCTTCAGGGATCG R: GATGCATACCTCGACCTCGT	$(TGA)_{14}$	52	186–339	35	0.81	11	0.92/0.83	8	0.63/0.84*
*Dpo*171	GU213459	F: TAATCGCATTCCGGCGTAG R: GAAATGGATTGAAACGAAAGAAA	$(AC)_{15}$	50	125–271	41	0.85	10	0.50/0.80*	9	0.46/0.87*
*Dpo*173	GU213460	F: CTCACCATGCGAATGTCCTA R: CGCTTTTATCTGGAGCTCTG	$(TAGA)_7AAGA(TAGA)_{27}$	52	96–288	38	0.71	10	0.83/0.66	9	0.54/0.80*
*Dpo*221	GU213461	F: GACTTTATTTGTAATGAGGATGTTGG R: TGGGTTGGTGTCAATGTTTC	$(TGA)_{25}$	52	66–195	34	0.78	5	0.92/0.70	10	0.88/0.72
*Dpo*260	GU213462	F: TTGTTGGATTCGGTGGAATA R: CCATAGATCCGTTTGCGAGT	$(TAGA)_{35}$	50	112–292	35	0.72	10	0.67/0.76	4	0.83/0.72
*Dpo*272	GU213463	F: TGGTCACCTTGCTTCTGGAT R: CGTGTGTTAGTCAGTTTAATG	$(AACT)_8$	50	142–286	13	0.38	4	0.71/0.64	5	0.58/0.76
*Dpo*281	GU213464	F: AAGCCGGTCTGTTGAGTAGG R: AGCTTGACGATTAGCCAGGA	$(TG)_{53}$	52	124–280	44	0.62	6	0.67/0.63	5	0.50/0.79*
*Dpol*A6	AF317427	Naish & Boulding (2001)	–	58	280–397	29	0.83	12	0.83/0.85	10	0.50/0.68*
*Dpol*B8	AF317428	Astanei *et al.* (2005)	–	54	122–380	58	0.70	11	0.58/0.85	7	0.50/0.79*
*Dpol*B9	AF317429	Astanei *et al.* (2005)	–	58	195–402	46	0.87	10	0.58/0.83	8	0.38/0.87*
			Total (mean)			386 (35.1)	(0.72)	93 (8.5)	(0.70/0.74)	78 (7.1)	(0.55/0.78)

Table 1 Continued

b)

Locus	GenBank accession number	Sequence/Source	Repeat motif	T_A (°C)	Size (bp)	Across all populations from Brown & Stepien (2010) (N = 269, 13 samples, 12 sites) Native and Introduced		Lake Mead, NV (N = 24) Introduced		Lake Erie, OH (N = 24) Introduced	
						N_A	*Ho*	N_A	H_O/H_E	N_A	H_O/H_E
*Dbu*74	GU213465	F: ATAAACGCCGGTGCATTAAA R: TGCATGTTTATTGCTGAAAACT	$(AG)_9GG(AG)_3GG(AG)_{31}ATTC(AG)_5AC(AG)_5$	50	153–297	34	0.77	2	0.29/0.51	16	0.40/0.94*
*Dbu*75	GU213466	F: CCACACTGCCAAAATGTAGC R: TCCAGCAAAATATCGAGCAA	$(TGGA)_{13}$	50	152–212	13	0.75	6	0.46/0.60	7	0.68/0.79
*Dbu*92	GU213467	F: TGCCCGCTTGATTTCAATAC R: TCCAGAAAGAAATAATTAAGTTTAAGG	$(TATC)_{33}$	50	170–298	13	0.54	2	0.54/0.51	2	0.40/0.37
*Dbu*93	GU213468	F: TTGTCCTCGTGGGCTTAAAT R: ACTGACTGGCGATTGCTCTT	$(TGTC)_{21}$	52	94–178	19	0.59	5	0.46/0.56	8	0.40/0.81*
*Dbu*110	GU213469	F: GGGGCAACTGGACTCATTTA R: CAATGTGTCGAAACCCTTAGTG	$(TG)_{11}$	52	151–199	17	0.76	7	0.54/0.84	6	0.56/0.78
*Dbu*141	GU213470	F: CATTAATGGGTGGGTTTTGG R: TTGACGTGAACAGAGGGAAT	$(TTAG)_8(TAAG)(TTAG)_{29}$	50	234–378	25	0.85	9	0.54/0.88*	12	0.50/0.86*
*Dbug*1	AF095846	Wilson *et al.* (1999)	–	56	121–325	36	0.79	12	0.38/0.92*	10	0.44/0.88*
*Dbug*4	AF095849	Wilson *et al.* (1999)	–	56	158–353	31	0.76*	6	0.17/0.75*	8	0.12/0.76*
*Dbug*5	AF095850	Wilson *et al.* (1999)	–	62	214–388	40	0.91*	7	0.25/0.77*	21	0.40/0.94*
			Total (mean)			228 (25.3)	(0.75)	56 (6.2)	(0.40/0.70)	90 (10.0)	(0.57/0.79)

unreliability of these loci probably resulted from incorrect sequences published by Naish & Boulding (2001), which just recently were corrected (Naish & Boulding 2011). We included *Dpol*A6 from Naish & Boulding (2001) and *Dpol* B8-9 from Astanei *et al.* (2005) in the present analyses. Of the six microsatellite loci developed by Wilson *et al.* (1999) for *D. r. bugensis*, three did not amplify reliably for us (*Dbug*2–3 and 6) and thus were not used here. Our evaluations included the three loci that amplified well (*Dbug*1 and 4–5). We utilized these previously published loci and our new loci in cross-species amplifications using our four test population samples of *D. p. polymorpha* and *D. r. bugensis*, as well as the four related taxa: *D. r. grimmi*, *D. presbensis*, *C. kusceri* and *M. leucophaeata*.

Our data are deposited at Dryad and may be accessed via http://dx.doi.org/10.5061/dryad.8621 or doi:10.5061/dryad.8621. Locus characteristics used in this investigation are summarized in Table 1, including number of alleles, observed and expected heterozygosities, and tests for deviations from HWE and linkage disequilibria determined using GENEPOP v4.0 (Rousset 2008). All loci were found to be unlinked and there were few overall deviations of population samples from HWE following sequential Bonferroni correction (Holm 1979) across the zebra and quagga mussel samples from Eur-

Table 2 Cross species amplification of a) zebra mussel loci in quagga mussels (Lake Mead, NV and Lake Erie, OH) and b) quagga mussel loci in zebra mussels (San Justo Reservoir, CA and Lake Erie, OH). Populations and loci tested were those reported in Table 1. Range of repeat sizes (size, bp), number of alleles (N_A), average observed/expected heterozygosity, and number of individuals that successfully amplified per sample ($N_{amp.}$) are indicated. Asterisks denote samples that did not conform to Hardy–Weinberg equilibrium expectations after sequential Bonferroni correction (Holm 1979), which were attributed by analysis with MICRO-CHECKER to the likely influence of null alleles. – Indicates that the locus did not amplify for any of the samples that we tested

a)

		Lake Mead, NV (N = 24) Introduced			Lake Erie, OH (N = 24) Introduced		
Locus	Size (bp)	N amp.	N_A	H_O/H_E	$N_{amp.}$	N_A	H_O/H_E
*Dpo*04	207–273	0	–	–/–	14	8	0.36/0.85*
*Dpo*101	183–315	5	3	0.00/0.80	14	10	0.14/0.93*
*Dpo*171	151–251	1	1	0.00/0.00	12	7	0.08/0.82*
*Dpo*173	136–288	8	2	0.00/0.00	13	6	0.15/0.82*
*Dpo*221	63–225	22	9	0.55/0.80	18	15	0.61/0.90
*Dpo*260	88–260	10	3	0.10/0.51	7	6	0.57/0.65
*Dpo*272	–	–	–	–/–	–	–	–/–
*Dpo*281	126–224	16	11	0.25/0.92*	20	20	0.50/0.94*
*Dpol*A6	220–301	1	1	0.00/0.00	24	2	0.00/0.00
*Dpol*B8	215–263	24	7	0.71/0.64	24	13	0.79/0.68
*Dpol*B9	–	–	–	–/–	–	–	–/–
	Total (mean)	87 (10.8)	37 (4.6)	(0.28/0.38)	146 (16.2)	87 (9.6)	(0.44/0.66)

b)

		San Justo Reservoir, CA (N = 24) Introduced			Lake Erie, OH (N = 24) Introduced		
Locus	Size (bp)	$N_{amp.}$	N_A	H_O/H_E	$N_{amp.}$	N_A	H_O/H_E
*Dbu*74	122–157	7	7	0.14/0.95*	14	12	0.29/0.93*
*Dbu*75	152–208	20	2	0.00/0.10	17	8	0.47/0.89*
*Dbu*92	174–262	19	2	0.05/0.05	17	4	0.35/0.59
*Dbu*93	94–274	3	1	0.00/0.00	5	6	0.60/0.95
*Dbu*110	–	–	–	–/–	–	–	–/–
*Dbu*141	226–306	3	3	0.67/0.83	20	13	0.80/0.92
*Dbug*1	121–293	21	7	0.43/0.82*	21	13	0.38/0.91*
*Dbug*4	164–332	10	4	0.00/0.73*	17	11	0.18/0.92*
*Dbug*5	208–352	22	14	0.59/0.85	23	21	0.70/0.95*
	Total (mean)	105 (13.1)	40 (5)	(0.24/0.54)	134 (16.8)	88 (11.0)	(0.47/0.88)

asia and North America used in our expanded study (Brown & Stepien 2010) for the new loci (Tables 1 and 2). We tested for the presence of null alleles with MICRO-CHECKER v2.23 (http://www.microchecker.hull.ac.uk; Van Oosterhout *et al.* 2004, 2006) and detected few indications of these with the new loci across our broadscale population analyses (Brown & Stepien 2010). In the case of the loci developed by other researchers, those by Wilson *et al.* (1999) for *D. r. bugensis* were out of HWE for some population samples, reflecting heterozygote deficiency and possible null alleles (Tables 1 and 2). A tendency for heterozygote deficiency was observed by both us and Wilson *et al.* (1999), as well as in a later study using those loci in Therriault *et al.* (2005), who did not find evidence for null alleles at most loci. In their analyses with *D. p. polymorpha*, Naish & Boulding (2001) discerned heterozygote deficiency at locus *Dpol*A6 as well as at other loci they tested, whereas Astanei *et al.* (2005) found some significant departures from HWE, which were alleviated when they corrected for null alleles. Heterozygote deficiencies have been described for many bivalves, as originally discerned with allozyme loci (see Zouros & Foltz 1984). Wilson *et al.* (1999) discussed a biological explanation for heterozygote deficiency of dreissenid mussels, which they attributed to their planktonic dispersal and possible allele-frequency differences among cohorts leading to a Wahlund effect. Alternatively, they raised the less likely possibility of HWE deviation because of selective pressures from differential mortality of larvae. These possibilities merit further investigation.

We here identified 386 alleles for the 11 loci for zebra mussels, with a mean of 35.1 alleles per locus and a mean observed heterozygosity (H_o) of 0.72 (Table 1a). The number of alleles observed for our new loci ranged from 13 (*Dpo*04 & 272) to 44 (*Dpo*281), with H_o ranging from 0.38 (*Dpo*272) to 0.85 (*Dpo*171). Our values for the loci from Astanei *et al.* (2005) ranged higher, to 58 alleles (*Dpol*B8) and H_o = 0.87 (*Dpol*B9). For locus *Dpol*A6, we observed 29 alleles and H_o = 0.83 from 583 *D. p. polymorpha* individuals across North America and Eurasia, whereas Naish & Boulding (2001) found 20 alleles and H_o = 0.66 from 648 Great Lakes individuals; these differences appear to reflect our wider versus their narrower geo-

Table 3 Cross amplification of a) zebra mussel loci and b) quagga mussel loci for four additional related taxa

	D. presbensis (*N* = 1)		*D. r. grimmi* (*N* = 4)		*Congeria kusceri* (*N* = 3)		*Mytilopsis leucophaeata* (*N* = 4)	
Locus	Size (bp)	N_A	Size (bp)	N_A	Size (bp)	N_A	Size (bp)	N_A
a)								
*Dpo*04	261–342	2	246–363	2	228–249	3	225–297	4
*Dpo*101	–	–	195–288	4	204–210	2	195–312	3
*Dpo*171	185	1	143	1	143	1	141–281	4
*Dpo*173	–	–	136–240	4	120–192	3	148–284	3
*Dpo*221	–	–	75–141	6	60–195	2	159	1
*Dpo*260	296	1	116–248	2	120–140	3	132–280	2
*Dpo*272	–	–	–	–	–	–	–	–
*Dpo*281	–	–	142–306	2	140–194	4	152–238	5
*Dpol*A6	–	–	322–373	2	226	1	–	–
*Dpol*B8	266–290	2	–	–	251–353	3	293	1
*Dpol*B9	243–258	2	324–390	2	219–318	3	198–201	2
	Total (mean)	8 (1.6)		25 (2.7)		25 (2.5)		25 (2.7)
b)								
*Dbu*74	153	1	199–229	4	155–231	3	143–259	3
*Dbu*75	172–296	2	156–216	4	172	1	160–280	4
*Dbu*92	258–294	2	174–258	3	166–310	4	174–262	3
*Dbu*93	94	1	94–114	3	94–118	2	94	1
*Dbu*110	–	–	161–171	2	163	1	161–167	3
*Dbu*141	258	1	274–306	5	222–262	3	298–302	2
*Dbug*1	133	1	133–161	2	229	1	129–261	2
*Dbug*4	–	–	194	1	227	1	185–260	2
*Dbug*5	229–265	2	274–283	4	229–238	2	205–292	5
	Total (mean)	10 (1.4)		28 (3.1)		18 (2)		25 (2.8)

Ranges of repeat sizes (size, bp) and numbers of alleles (N_A) are given. – = Indicates that the locus did not amplify for any of the samples that we tested

graphic sampling scales. Our results for loci *Dpol*B8 and *Dpol*B9 similarly had higher values of H_o = 0.70 and 0.87, respectively, than were found from a narrower sampling range and 310 individuals (H_o = 0.52 and 0.66, respectively) by Astanei *et al*. (2005).

We identified 228 alleles for the nine loci developed for quagga mussels, with a mean of 25.3 alleles per loci, and 0.75 mean observed heterozygosity (Table 1b). The number of alleles identified for our new loci ranged from 13 (*Dbu*75 & 92) to 34 (*Dbu*74), with observed heterozygosity ranging from 0.54 (*Dbu*92) to 0.85 (*Dbu*141). Our values with the Wilson *et al*. (1999) loci used here ranged higher, to 40 alleles and 0.91 observed heterozygosity (*Dbug*5).

Quagga mussels were cross-amplified using primers developed for the zebra mussel loci (Table 2a) and zebra mussels for the quagga mussel loci (Table 2b). Of 11 zebra mussel loci tested with quagga mussels, two failed to amplify *(Dpol*B9, *Dpo*272), one was monomorphic (*Dpol*A6) and the remaining eight were polymorphic in at least one of the two population samples (Table 2a). A cross species amplification by Naish & Boulding (2001) for their locus *Dpol*A6 failed; in contrast, ours amplified but had low diversity (two alleles). Nine quagga mussel loci were tested for zebra mussels; *Dbu*110 failed to amplify, and the remaining eight loci were polymorphic (Table 2b). We also tested amplification of all loci for four additional taxa: *D. r. grimmi*, *D. presbensis*, *C. kusceri* and *M. leucophaeata*; results indicate broad utility of these loci, but limited sample size (*N* = 1–4) in the present study precluded their further analysis at the population level (Table 3).

The relationships among the six taxa and four representative populations of *D. p. polymorpha* and *D. r. bugensis* were evaluated using Cavalli-Sforza chord distances (Cavalli-Sforza & Edwards 1967) and neighbour-joining trees (Saitou & Nei 1987) constructed in PHYLIP v.3.68, with 1000 bootstrap pseudoreplicates (Felsenstein 1989). We analysed the data separately for the microsatellite loci designed for zebra mussels and for quagga mussels, resulting in the trees shown in Fig. 1a (zebra mussel loci) and Fig. 1b (quagga mussel loci). The relationships supported by two trees were similar to each other and to previously published phylogenies of dreissenid mussels and their relatives (Stepien *et al*. 2002, 2003, 2005), with the loci discriminating among the tested species and populations. The trees based on the zebra mussel and quagga mussel loci appeared similar, except that the latter placed *C. kusceri* within *Dreissena*, reflecting apparent homplasy (Fig. 1b). This general congruence suggests that these loci are useful in discriminating among the various dreissenid taxa. Notably, the tree based on the zebra mussel loci (Fig. 1a) closely resembled the topology of dreissenid taxa phylogenies derived from new analyses of DNA

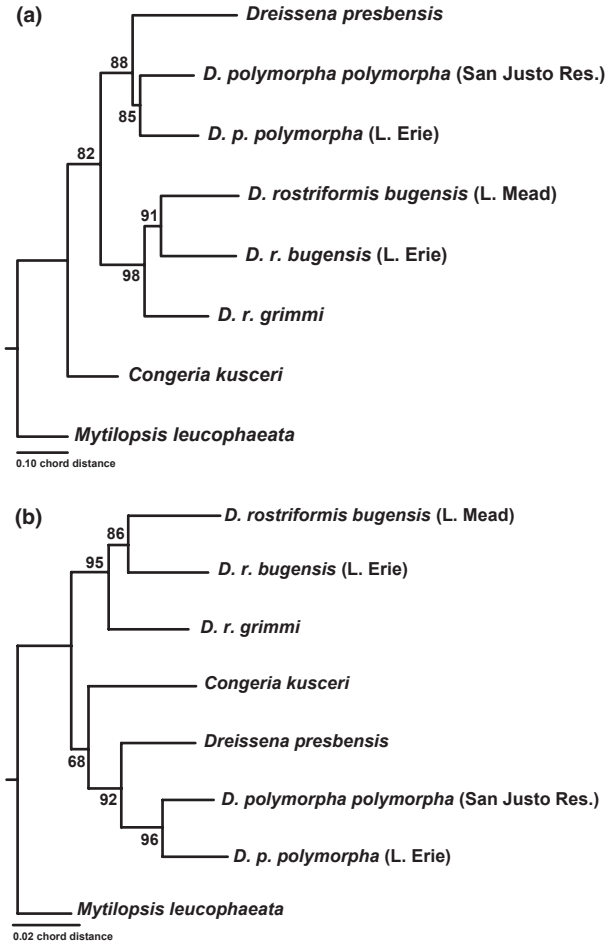

Fig. 1 Neighbour-joining tree showing relationships among dreissenid populations and related taxa using our microsatellite data, Cavalli-Sforza chord distances (Cavalli-Sforza & Edwards1967) and PHYLIP v3.68 (Felsenstein 1989). Bootstrap percentage values are from 1000 pseudoreplicates and branch lengths are proportional to genetic divergence. a) is based on loci developed for *D. p. polymorpha*, and b) is based on loci developed for *D. r. bugensis*. The two sets of loci developed for each species were run separately to test their independent utility to resolve species relationships among the taxa.

sequence data from four separate genes (mitochondrial DNA COI, cytochrome *b*, and 16S RNA, and nuclear 28S RNA) (Stepien *et al*. 2012).

These microsatellite markers will aid future assessment of genetic diversity, population structure, and biogeography across native and introduced dreissenid mussel distributions (see Brown & Stepien 2010). They add to a few loci previously developed for zebra (five; Naish & Boulding 2001; Astanei *et al*. 2005) and quagga mussels (six; Wilson *et al*. 1999). Given the results showing cross-species amplification, it is likely that these loci may serve as the basis for work in related taxa. Our study thus provides the starting point for a standardized micro-

satellite toolkit to aid future population genetic investigations of invasive dreissenid mussels and their relatives.

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