

# Amplified fragment length polymorphism (AFLP) analysis of the genetic structure of the zebra mussel, *Dreissena polymorpha*, in the Mississippi River

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## SUMMARY

1. We predicted that zebra mussel, *Dreissena polymorpha* (Pallas), genetic structure in the Mississippi River would follow a model of invasive species genetics, which predicts low genetic structure among populations of recently established species. This prediction was upheld in our previous genetic study using allozymes, however, one locus yielded anomalous results.
2. We employed amplified fragment length polymorphism (AFLP) analysis as a neutral marker to assess the amount of genetic structure within and among populations, and as a test of expected population structure from both invasion genetic theory, and the results from our previous study.
3. There was greater spatial differentiation, as measured by  $F_{st}$ , observed using AFLP's than for allozymes ( $P < 0.001$ ). There was no evidence that AFLP variation conformed to an isolation by distance model, and genetic relationships of populations, as measured by AFLP markers, were not similar to those detected in our allozyme survey.
4. The lack of concordance between these two genetic marker systems probably reflects their differential responses to drift, migration, and selection occurring during this rapid invasion. Strong population structure is counter to predictions that populations of invasive species will not be differentiated, as with observations based on allozyme markers. Therefore, newly established species may require genetic surveys using multiple marker systems to evaluate population structure.

*Keywords:* amplified fragment length polymorphism, genetics, invasive species, zebra mussel

## Introduction

The founding of multiple populations from a genetically homogenous source should result in genetically similar populations (Hutchison & Templeton, 1999). Following colonisation, populations may diverge because of genetic drift when gene flow is restricted. Divergence between populations is expected to increase with their degree of spatial separation, and

population structure may eventually approach isolation by distance (Wright, 1942). However, evolutionary forces such as habitat fragmentation and extinction/colonisation events may result in population structure that lack a geographic pattern (McCaughey, 1993). Recent colonisation events like the invasion of North America by zebra mussels (*Dreissena polymorpha*, Pallas) in the late 1980's, provide the opportunity to study the formation of population genetic structure when there has been insufficient time to reach a drift/migration equilibrium.

Zebra mussel genetics in North America has been studied previously and results are fairly consistent.

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Zebra mussel populations have high intrapopulation genetic variation (Hebert, Muncaster & Mackie, 1989) and little genetic divergence (Marsden, Spidle & May, 1995; Lewis, Feder & Lamberti, 2000). However, these studies are based on allozyme markers, which may not be well suited for the study of invasion genetics because they often lack sufficient polymorphism to detect genetic differentiation on a recent time scale (Holland, 2000). Another problem with allozyme allele frequencies is they may be influenced by selection. In zebra mussel populations significant allele frequency gradients have occurred for subsets of loci over relatively small geographic distances (Fetisov *et al.*, 1991; Marsden *et al.*, 1995; Lewis *et al.*, 2000; Elderkin, Klerks & Theriot, 2001). Allele frequency gradients have also occurred in response to variation in thermal regimes (Fetisov *et al.*, 1991). If these gradients are because of selection it is likely that they would obscure the effects of gene flow and founder events in the genetic structure of invasive species.

We found a significant north-south gradient in allele frequencies of leucine aminopeptidase (Lap) locus in the Mississippi River (Elderkin *et al.*, 2001) while five other loci exhibited no gradient or spatial structure. We hypothesised that selection on Lap is maintaining the gradient in allele frequencies, while the lack of differentiation at the other loci correspond to the prediction of minimal genetic differentiation among populations of invading species (Elderkin *et al.*, 2001). Previous studies on invertebrates have found that allozyme allele frequencies are suspected to be influenced by selection (Watt, 1977; Hilbish & Koehn, 1985; Karl & Avise, 1992; Schmidt & Rand, 2001). Typically, anomalous allozyme results are better understood by assessing the population structure with other molecular markers that are unlikely to be under selection (Karl & Avise, 1992; Davies, Villablanca & Roderick, 1999; Schmidt & Rand, 1999).

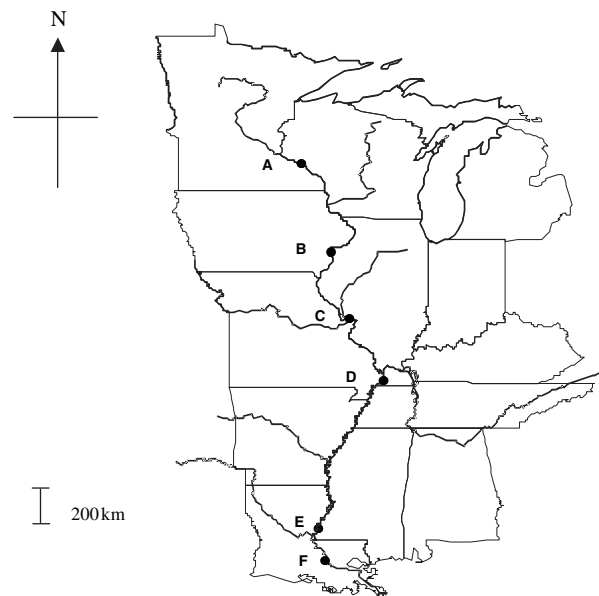
The results from our previous allozyme study suggested that another molecular marker was necessary to resolve zebra mussel population genetics in the Mississippi River. Based on previous work on the genetics of invasive species and the patterns we observed in allozymes, we expected that a second neutral marker would find high genetic variation, no heterogeneity of intrapopulation variation, low population structure, and no evidence of isolation by distance. Furthermore, we predicted that any genetic

structure detected by another set of neutral markers would not be similar to that observed in Lap, which we thought to be under selection. We tested these hypotheses using amplified fragment length polymorphism (AFLP) profiles to assess population structure in zebra mussels. This marker system allows for examinations of a large number of neutral polymorphic loci and thus should be better suited to the genetic analysis of recent invasions than allozymes which typically have low levels of polymorphism.

## Methods

### Collection

Individuals were collected from six sites along the Mississippi River. We sampled locations approximately 200–300 river miles apart along the latitudinal gradient (Fig. 1). The collection sites used for this study were Minnesota, Illinois I, Illinois II, Kentucky, Mississippi, and Louisiana (details see Table 1 and Elderkin *et al.*, 2001). We collected mussels from the sides of floating moorings such as docks, or from permanently docked barges. Live mussels were shipped back to Lafayette, Louisiana where they were maintained in 10 gal. aquaria until they were processed.



**Fig. 1** Sampling locations of zebra mussels, *Dreissena polymorpha*, in the Mississippi River. The sampling locations are from north to south: (A) Minnesota, (B) Illinois I, (C) Illinois II, (D) Kentucky, (E) Mississippi and (F) Louisiana.

**Table 1** Sampling location: location column is the U.S. state where populations were collected and these population names are referenced throughout the text. For a more specific location, the city column (the nearest city to each collection location), and the latitude and longitude are listed for each collection site. We also include number of individuals sampled ( $n$ ) from each location, a calculation of expected heterozygosity ( $H$ )  $\pm$  SD, and percent polymorphic loci (% $P$ ).  $H$  and % $P$  based on data from amplified fragment length polymorphism (AFLP) analysis. The % $P$  is split into calculations for each primer set, B, blue fluorescing; G, green fluorescing.

Location	City	$n$	Latitude ( $^{\circ}$ N)	Longitude ( $^{\circ}$ W)	$H$ (SD)	% $P$	% $P$ (B)	% $P$ (G)
Minnesota	Lake Pepin	29	44.4650	92.2923	0.11 (0.14)	49	50	42
Illinois I	Moline	25	41.4851	90.4887	0.09 (0.13)	35	40	30
Illinois II	Alton	24	38.9039	90.1540	0.09 (0.13)	35	32	39
Kentucky	Columbus	32	37.7595	89.1018	0.09 (0.14)	45	40	44
Mississippi	Natchez	13	31.5478	91.3885	0.07 (0.13)	36	26	37
Louisiana	Baton Rouge	23	30.4489	91.1260	0.11 (0.13)	46	41	52

### DNA extraction

Soft mussel tissue was removed and stored separately at  $-80^{\circ}\text{C}$  in a microcentrifuge tube along with a few drops of storage buffer (Hebert & Beaton, 1993). We removed an average of 100-mg of soft tissue from each of approximately 30 individuals from each sampling location (only 13 were available for the Mississippi site). This tissue was blotted, weighed, and total DNA was isolated using a mini-beadbeater, mechanical disruption method (FastDNA kit; Krackeler Scientific Inc., Albany, NY, U.S.A.), at  $5\text{ m s}^{-1}$  for 2 s. DNA extracts were purified using a GeneClean spin silica glass spin filter kit (Krackeler Scientific Inc.).

### Amplified fragment length polymorphism procedure

We performed AFLP analyses (Vos *et al.*, 1995) following the procedures in PE Biosystems AFLP plant mapping kit (Applied Biosystems, Foster City, CA, U.S.A.). Minor modifications included: (a) omission of bovine serum albumin because of inhibition of subsequent polymerase chain reaction (PCR) amplifications, (b) a 1 : 10 dilution of restriction/ligation reactions with sterile water prior to preselective amplification, (c) a 1 : 2 dilution of preselective amplification reactions with sterile water prior to selective amplification, and (d) a precipitation of selectively amplified DNA prior to gel loading to lower signal to noise ratio and maximise peak detection. We used a target amount of initial DNA, which was standardised across all individuals; 550 ng of DNA was used per individual in each reaction. Two combinations of AFLP selective primer sets were used; MseI-CAA and

(5-carboxyfluorescein – FAM) EcoRI-ACT (Blue fluorescing), and MseI-CAA and (6-carboxy-4',5'-dichloro-2',7'-dimethoxy-fluorescein – JOE) EcoRI-ACG (Green fluorescing). We used this combination to maximise the number of bands produced and to permit multiplexing through use of different dyes in a single electrophoresis lane.

### Data collection and scoring

Selective amplification products were sized with an internal base pair standard, ROX dye-labelled Genescan 500 (Applied Biosystems). AFLP products were analysed in 5% Long Ranger Hydrolink (Cambrex Bioscience, Rockland, ME, U.S.A.) denaturing polyacrylamide gel using an automated DNA sequencer (ABI 377, Applied Biosystems). Gels and running conditions were performed using standard protocols (Applied Biosystems).

The AFLP patterns were captured and peaks were assigned base-pair sizes using GeneScan analysis software (version 3.1, Applied Biosystems). Genotyper software (version 2.1, Applied Biosystems) was used to assemble a data matrix consisting of the calculated sizes for all fragments from each individual mussel. Peak intensity was used to determine the presence/absence of a fragment in the generated electropherogram and only peaks above 20-rfu were selected. The peak data were converted to binary data by scoring for presence or absence of a fragment. Three replicate AFLP reactions per individual were used to eliminate variation in the analysis of individuals and populations because of anomalous PCR products, gel artefacts, or artefacts of detection. Only PCR fragments that were highly replicable were used

(approximately 66% of the total number of fragments were highly replicable); this process identified 412 AFLP loci for analysis.

#### Data analysis

We assessed within-population genetic diversity using average unbiased expected heterozygosity ( $H$ ) and the estimated proportions of polymorphic loci (% $P$ ). Estimates of  $H$ , based on allele frequencies, were calculated using the methods of Lynch & Milligan (1994). We tested for homogeneity of  $H$  among populations using a Kruskal–Wallis test. We estimated % $P$  where an allele was polymorphic if a fragment was present at an individual locus in less than 95% of the total number of individuals in the sample (Travis, Maschinski & Keim, 1996).

We used two methods to calculate the levels of among-population genetic diversity. First, we used the program Tools for Population Genetic Analysis (TFPGA) (Miller, 1997) to calculate theta ( $\theta$ ) (Weir & Cockerham, 1984) an unbiased estimator of Wright's (1965)  $F_{st}$ . We calculated 95% confidence intervals around our estimates of  $\theta$  by bootstrapping with 1000 replicates. To assess interpopulation genetic structure without assuming Hardy–Weinberg equilibrium, we conducted analysis of molecular variance (AMOVA) using the program Arlequin 2.0 (Schneider *et al.*, 2000) which provides an  $F_{st}$  analogue ( $\phi_{st}$ ) and an estimate of the proportion of variance among populations (Excoffier, Smouse & Quattro, 1992). We tested for differences in estimates of  $\theta$  from previous allozyme analyses of the same populations (Elderkin *et al.*, 2001) using the Lewontin–Krakauer test (Lewontin & Krakauer, 1973) modified by Pogson, Mesa & Boutilier (1995) and Barker *et al.* (1997).

We also calculated genetic distances (Nei, 1978) for each pairwise combination of populations. For a visual representation of the genetic distances among populations, we used a cluster analysis of all samples based on the unweighted pair group method with arithmetic averages (UPGMA). This method is simply used to generate a dendrogram of how similar our populations are to one another, not to infer an evolutionary clock among populations. Also, we included data from our previous work using allozymes (Elderkin *et al.*, 2001) as a comparison of the genetic distance using different molecular markers. We did not bootstrap the data, as

bootstrapping fragment data like AFLP's (i.e. data in 1 and 0's) yields ambiguous results, because the fragments do not evolve independently and eliminating one fragment could create two (J. Felsenstein, unpublished data).

We used a Mantel test for an association between genetic distance (Nei, 1978) as measured by AFLP markers and geographic distance. A positive association would suggest migration rates between populations decrease with increasing distance as expected under an isolation by distance model of gene flow (Wright, 1942). We also tested for an association of genetic distance estimates calculated using AFLP markers and allozymes. All Mantel tests were implemented using TFPGA.

## Results

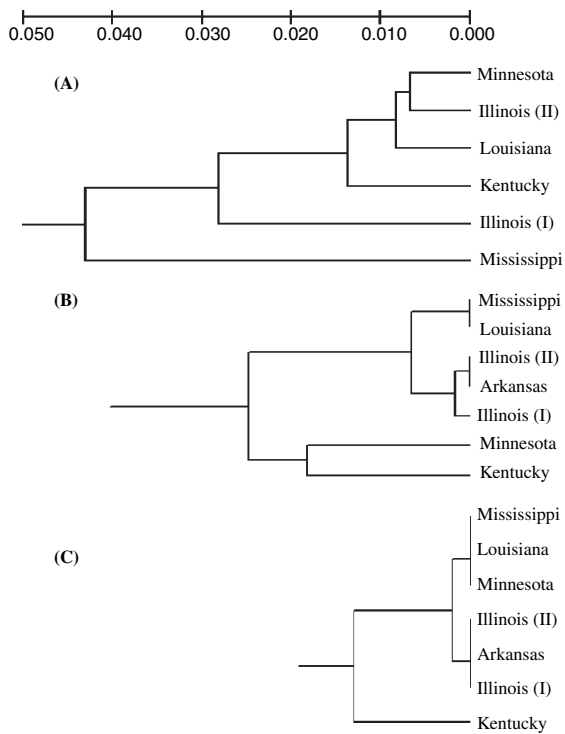
### Genetic variation

Using AFLP analysis we found significant heterogeneity among population estimates of  $H$  ( $P = 0.001$ ), however, analysis of the allozyme data did not reveal any heterogeneity of  $H$  ( $P = 0.995$ ). The frequencies of polymorphic loci were similar within populations, with the highest at both Louisiana and Minnesota and lowest at Mississippi (Table 1). The majority of loci were polymorphic in more than one population and there were only 20 loci out of 412 that were polymorphic in only a single population.

### Population differentiation

Mississippi River populations of *D. polymorpha* exhibited significant interpopulation genetic differentiation ( $\theta = 0.16$ , 95% CI = 0.127–0.187). AMOVA also revealed significant among population variation ( $\phi_{st} = 0.17$ ; 17% among population variation, 83% within populations;  $P < 0.001$ , 1023 nonparametric permutations). Population differentiation estimates calculated for allozymes ( $\theta = 0.002$ ) were significantly lower than estimates from AFLP analysis.

The calculated pair-wise genetic distances among populations was relatively high when calculated using AFLP's (Fig. 2A). The UPGMA cluster analysis shows there are different genetic distances among populations depending on the marker used (Fig. 2A,B). Also, the effect is further exaggerated with the exclusion of the Lap locus (Fig. 2B,C).



**Fig. 2** Cluster analysis of zebra mussel populations using unweighted pair group method using arithmetic means (UPGMA). Top bar represents Nei's (1978) genetic distance measures for (A) amplified fragment length polymorphism (AFLP), (B) six allozyme loci, (C) five allozyme loci, excluding leucine aminopeptidase (Lap).

The Mantel test revealed no significant association between genetic distance and geographic distance between sampling locations ( $P = 0.55$ ). We also compared genetic distance matrices based on allozyme analysis to the genetic distance matrix from AFLP analysis. There was no significant association between genetic distances obtained using allozymes and AFLP ( $P = 0.90$ ).

## Discussion

The AFLP analysis reveals that zebra mussels in the Mississippi River exhibit population structure at large geographic scales but do not show a correlation between genetic structure and geographic distance. These results are similar to recent studies of zebra mussels (Stepien, Taylor & Dabrowska, 2002) and their sister taxa (Wilson, Naish & Boulding, 1999), but differ from previous studies using allozymes (Marsden *et al.*, 1995; Lewis *et al.*, 2000) including our own (Elderkin *et al.*, 2001) which found little variation at

five allozyme loci. However, even among studies using highly variable markers, our results using AFLP loci have found relatively high estimates of population differentiation (Wilson *et al.*, 1999; Muller, 2001; Stepien *et al.*, 2002).

The extinction and colonisation pattern observed within other rivers may explain the population structure. Organisms with planktonic larvae have a difficult time colonising lotic systems (Horvath *et al.*, 1996). In large river systems, mussel populations are spatially heterogeneous, theoretically colonising only the most suitable habitat, or where planktonic larvae reach settlement stage, or a combination of both. Also, established populations may suffer from poor recruitment, which would increase the probability of local extinction. Because of these two factors, researchers have noticed that suitable habitats for zebra mussels are sometimes vacant and only a percentage of suitable areas are colonised at any one time (Stoeckel *et al.*, 1997). This colonisation dynamic could be the cause of founder events, which would increase genetic structure (McCauley, 1993).

Jump dispersal from boats (as opposed to dispersal by the river current) is considered a vector for zebra mussel's introduction to novel habitats (Schneider, Ellis & Cummings, 1997; Pollux *et al.*, 2003). In the Mississippi River, boat mediated transport could also explain why we observe the significant differences among populations while the variation has no geographical pattern. In 1992, zebra mussels were observed throughout much of the northern Mississippi River, and colonised the southern Mississippi River in 1993 (<http://www.usgs.gov>). This rapid spread of mussels, as well as their appearance far upstream of possible colonisation from other river drainages, makes it unlikely that gene flow is occurring primarily from downstream drift. A unidirectional model of gene flow based on dispersal of drifting juveniles would have resulted in spatially proximate populations sharing greater genetic similarity, something we observed in neither allozymes nor AFLPs.

Other potential explanations for population structure have been identified in recent studies. Multiple, genetically distinct, European source populations have been identified to have colonised the U.S. (Stepien *et al.*, 2002). This scenario could contribute to population genetic structure if individuals of different sources were not randomly distributed in

the Mississippi River. Also, Lewis *et al.* (2000) suggested asynchronous spawning with no larval mixture as a probable explanation, which could cause genetic drift among cohorts resulting in increased population structure among populations and low genetic variation.

Using an initial human mediated colonisation, Muller (2001) explains two genetic outcomes resulting from recent colonisation of river ecosystems. The first (colonisation from a single source), results in a low genetic structure among and low variation within populations. The second (colonisation from multiple sources), results in structure among populations with no isolation by distance and unusually high variation within populations. In most cases, zebra mussels studies using allozymes showed only the high population variation of the second prediction (Marsden *et al.*, 1995; Lewis *et al.*, 2000) with no population structure. However, in our study and other recent studies using genetic markers other than allozymes (Wilson *et al.*, 1999; Stepien *et al.*, 2002), populations had high population structure and no isolation by distance, thus supporting the second prediction.

Analysis of the AFLP data resulted in a much higher intrapopulation genetic differentiation than was found from the allozyme data (AFLP  $\theta = 0.17$  versus Allozyme  $\theta = 0.002$ ). Although a significant amount of interpopulation genetic differentiation was also exhibited by Lap in the prior study, it exhibited a significant isolation by distance structure that is lacking from the AFLP data.

The results based on AFLP's do not confirm the hypothesis that the genetic structure of zebra mussels is one of high gene flow and no structure, as would be expected based on most of the allozyme loci examined by Elderkin *et al.* (2001). The AFLP results also do not support a isolation by distance model of more limited gene flow in a linear environment which could have explained allele frequency variation of Lap. In addition, AFLP's did little to shed light on the hypothesis that Lap is under directional selection in Mississippi River. Instead, these results indicate that separate stochastic and non-stochastic forces may affect allozyme and AFLP loci differently within the Mississippi River.

Insight gained in this study shows that any model of genetics of invasive species, or population estimates of gene flow among populations should be based on more than one genetic marker. Variation observed in AFLP loci is different than that detected

for allozymes suggesting that non-stochastic forces are maintaining allele frequencies at the allozyme loci among populations. Our results support commonly held hypotheses related to the genetics of invasive species only when we used a highly variable genetic marker. Additional analysis of different marker systems and a more complete sampling of source populations are needed to better understand the forces influencing the genetic variation of zebra mussels in North America.

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