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Extensive allozyme monomorphism in a threatened species of freshwater mussel, *Margaritifera hembeli* Conrad (Bivalvia: Margaritiferidae)

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Abstract

A threatened but under-studied component of freshwater biodiversity in North America is the native freshwater mussels (Bivalvia: Unionoidea: Margaritiferidae and Unionidae). Genetic data suggest that these mussel species generally exhibit levels of variability similar to other invertebrates. We surveyed allozyme variation in the Louisiana Pearlshell, *Margaritifera hembeli* (Margaritiferidae), a threatened freshwater mussel. Five examined populations are monomorphic for 25 allozyme loci, the first report of a native freshwater mussel species with extensive allozyme monomorphism. Low genetic diversity appears to be characteristic of margaritiferids, as an ANOVA indicated that mussels of the family Margaritiferidae have significantly lower levels of heterozygosity than the mussels of the family Unionidae. Margaritiferids have strong habitat preferences and modification of habitat leads to rapid loss of populations. Although bottlenecks are known to cause low genetic variability, margaritiferids may exhibit meta-population structure with extinction/re-colonization dynamics leading to low genetic variability. Margaritiferids generally exhibit a patchy distribution with a colonization rate that is approximately twice that of extinction. Tests of the metapopulation hypothesis will require additional allozyme population genetic data as well as hypervariable microsatellite loci.

Introduction

Freshwater biota are underrepresented in conservation research (Clark and May 2002; Stein et al. 2002) despite the observation that freshwater biodiversity is in crisis (Abell 2002). A large component of this freshwater biodiversity in North America is the unionoidean bivalves (native freshwater mussels; Bivalvia: Unionoidea: Margaritiferidae and Unionidae), the most endangered fauna in North America with 69% of species listed as extinct, imperiled or vulnerable (Stein et al. 2002). Unionoidean diversity is greatest in the United States with approximately 300 recognized species, primarily located in the Mississippi River basin and the southeast (Williams et al. 1992).

Research on the biology of freshwater mussels is a key component of conservation efforts (Neves 1992); however, unionoidean's unique life histories complicate conservation efforts. Many unionoideans are slow growing, long lived species with low recruitment rates (Bauer 1987, 1992; Payne and Miller 2000). Recruitment is limited by a parasitic stage requiring a specific piscine or amphibian host; thus, despite extremely high fecundities, only a small percentage of larvae survive past metamorphosis to the juvenile stage (Coker et al. 1921; Payne and Miller 2000). The need for conservation efforts has been heightened by the introduction of the zebra mussel (*Dreissena polymorpha*), as bio-fouling by this exotic has particularly impacted freshwater mussels (Neves 1992; Williams et al. 1992; Ricciardi et al. 1998).

An understanding of species and population level genetic structure and diversity is essential in conservation management (Avise 1996), particularly in unionoideans where such studies are lacking for most species (Lydeard and Roe 1998). Allozyme diversity

has been sampled in several species of North American Unionoidea (Davis and Fuller 1981; Davis et al. 1981; Kat 1983a, b, c; Davis 1984; Stiven and Alderman 1992; Mulvey et al. 1997; Berg et al. 1998; Johnson et al. 1998; Mulvey et al. 1998; Kandl et al. 2001) with the range and average levels of genetic polymorphism and heterozygosity similar to most invertebrates (Nevo et al. 1983). In general, margaritiferids show levels of polymorphism at the lower end of the range for unionoideans. Average polymorphism and heterozygosity were low (P = 0.259, H = 0.014) among 11 populations of European *Margaritifera margaritifera* (Machordom et al. 2003), consistent with a previously sampled N. American population (Davis et al. 1981). Although sample sizes were small and levels of polymorphism were correlated with sample size, polymorphism and heterozygosity in the population with the greatest sample size (P = 0.074, H = 0.018, N = 21.4) were still at the lower range for these bivalves. A single population of *Margaritifera auricularia* has also been sampled and, as with *M. margaritifera*, both polymorphism and heterozygosity were low (P = 0.222, H = 0.010, N = 33.4).

Margaritiferids have a maritime distribution, usually located within 250 km of open sea, and commonly use anadromous fishes as parasitic hosts. *Margaritifera hembeli* is limited in geographic distribution to small 2nd and 3rd order streams in the Red River drainage in north-central Louisiana, U.S.A. (Shively and Vermillion 1998; Shively 1999; Johnson and Brown 2000). This species is listed as threatened under the U.S. endangered species act and as critically endangered by the IUCN. Populations are patchily distributed into large "beds" in shallow areas with stable substrata of sand and gravel; streambed between populations is often inhabited sparsely or not at all (Johnson and Brown 2000). Johnson and Brown (1998) examined populations at four stream sites and concluded that dispersal of *M. hembeli* among drainages is limited, resulting in life history variation between populations. We examined variation at 25 allozyme loci for five populations of *M. hembeli*, including three examined by Johnson and Brown (1998). Additionally, we used a nested analysis of variance (ANOVA) to test for differences in heterozygosity at the family, genus and species levels for the freshwater Unionoidea.

Methods

Allozyme analysis

Individuals were non-destructively sampled by immersing the whole animal in 0.005M serotonin solution until foot extension and excising a small section (*ca* 0.25 cm^2) of mantle tissue (Berg et al. 1995); the tissue was immediately frozen in liquid nitrogen and the individual was then returned to the streambed. Samples were collected from thirty individuals each at Beaver Creek, Moccasin Creek, Cress Creek (Grant Parish, LA) and Loving Creek (Rapides Parish, LA); only ten individuals could be located at Gray's Creek (Grant Parish, LA). Descriptions of sample sites are available in Johnson and Brown (2000), Shively and Vermillion (1998) and Shively (1999).

Tissue samples were homogenized in 100 μl of double-deionized H_2O and centrifuged for 10 min. at 20,000 X G and 4 °C. Starch gel electrophoretic techniques and allozyme stain recipes followed those of Murphy et al. (1996) and Shaw and Prasad (1970). Loci were scored immediately following staining. Twenty-five loci were examined with 4–5 loci examined per gel. Enzyme loci, buffer systems and the number of individuals scored per locus are given in Table 1.

Heterozygosity analysis

Population heterozygosity estimates were obtained from published studies; if heterozygosities were not available they were calculated from published allele frequencies. Species names were synonymized according to Williams et al. (1992); analyses were run with the Margaritiferidae defined according to Williams et al. (1992, single genus *Margaritifera*) and alternatively according to Smith (2001, two genera). Three hierarchical levels were defined for the nested ANOVA analysis; species, genus and family, with populations being treated as individual observations. The specific model nested species within genus nested within family. Species and genus levels were treated as random effects. Taxa were divided into two families, the Unionidae and Margaritiferidae; these two families are sister clades (Hoeh et al. 1998, 2001) and as such the family level was treated as a fixed effect. Population heterozygosity estimates were arc-sine square root transformed prior to analysis and weighted according to the number of individuals

Table 1. Enzyme, E.C. number, number of loci and number of individuals sampled for *M. hembeli*

Enzyme	E.C. No.	No. loci	No. ind.
Aspartate Aminotransferase[B]	2.6.1.1	1	130
Arginine Kinase[C]	2.7.3.3	1	10
Cytosol Aminopeptidase[D]	3.4.11.1	1	130
Creatine Kinase[B]	2.7.3.2	1	130
Esterase[B]	3.1.1.–	3	130
Glyceraldehyde-3-Phosphate Dehydrogenase[B]	1.2.1.12	1	10
Glucose-6-Phosphate Isomerase[D]	5.3.1.9	1	130
Guanylate Kinase[C]	2.7.4.8	1	130
Hexokinase[E]	2.7.1.1	1	10
l-Iditol Dehydrogenase[C]	1.1.1.14	1	130
Isocitrate Dehydrogenase[D]	1.1.1.42	1	10
Malate Dehydrogenase[C]	1.1.1.37	2	130
Malate Dehydrogenase (NADP+)[C]	1.1.1.40	1	130
D-Octopine Dehydrogenase[F]	1.5.1.11	1	130
Peptidase[F]	3.4.–.–	3	130
Phosphogluconate Dehydrogenase[E]	1.1.1.44	1	130
Phosphoglucomutase[E]	5.4.2.2	2	130
Superoxide Dismutase[E]	1.15.1.1	2	130

Buffer systems: A-amino citrate pH 6.9; B-tris citrate pH 8.0; C-lithium hydroxide pH 8.1; D-tris-EDTA-borate pH 8.0; E-tris-EDTA-borate pH 8.7.

sampled. Least squares mean estimates of heterozygosity were also calculated for each treatment at each of the three levels. All analyses were carried out using Proc GLM of the software package SAS (SAS Institute Inc. 1989).

Results

Allozyme analysis

All five populations of *M. hembeli* are fixed for the same allele at the twenty-five allozyme loci examined (Table 1). Five enzyme systems indicated multiple loci with two of these staining for three different loci. Only ten individuals were genotyped for four loci due to limited tissue sampling.

Heterozygosity analysis

In all, 283 heterozygosity estimates were analyzed, including those from this study; these 283 estimates are distributed among 60 species in 19 genera. Estimates from Kat (1982) were excluded because the author preferentially selected the most heterozygous loci for genotyping. Estimates from Kandl et al. (2001) were excluded because of the low sample sizes. The proportion of estimates in each family was unbalanced, with *ca*. 91% of observations from the family Unionidae. At the generic level, *Unio* has the highest proportion of observations (28.98%) with *Anodonta* (21.20%), *Elliptio* (14.49%) and *Margaritifer*a (8.48%) being the only other genera with more than 5% representation. Results were similar regardless of the taxonomic distinction used for the margaritiferids. The dataset is available from the authors upon request.

The model was highly significant, indicating substantial partitioning of variance among the levels (Table 2). The species effect was the most significant, despite an F-value that is one-fourth of the family effect; this is likely due to the large number of degrees of freedom at this level. The effect of genus was not significant and had the lowest F-value, but it is important to note that the generic level variance is estimated from the unionids because of the limited number of margaritiferid genera. In contrast, the family effect, which is the highest level, was significant and produced the greatest F-value.

Least squares mean heterozygosity estimates were also calculated for each treatment at all levels. At the family level, margaritiferids had a ten-fold lower heterozygosity estimate than the unionids (Table 3), consistent with the significant ANOVA analysis. Generic level heterozygosities varied from a low of 0.0054 for *Margaritifera* to a high of 0.158 for *Quadrula* (Table 3). At the species level, *M. hembeli* is the only species with a heterozygosity estimate of 0, excluding the value for *Microcondylaea bonellii*, which is based on a sample of 12 individuals. Species level heterozygosities range from 0 to 0.294; all three species of *Margaritifera* are at the low end of this range, comprising three of the six lowest heterozygosity estimates.

Discussion

Species viability

Relatively low genetic variability has been shown to reduce the fitness of populations through expression of genetic load (Vrijenhoek 1994; Vrijenhoek 1996; Pamilo and Palsson 1998; Crnokrak and Roff 1999; DeRose and Roff 1999; Hedrick and Kalinowski 2000;

Table 2. Results of ANOVA analysis of arc-sine transformed heterozygosities

Source	D.F.[A]	Type III sums of squares	Mean square	F value	*p*
Family	1	4.58	4.58	8.29	0.0111
Error[B]	15.85	8.79	0.55		
Genus (Family)	17	9.05	0.53	1.58	0.0969
Error[C]	64.62	21.81	0.34		
Species (Family∗Genus)	42	18.24	0.43	2.44	<0.0001
Error[D]	222	39.44	0.18		

[A]degrees of freedom.
[B]1.078 × MS(Genus(Family)) – 0.0227 × MS(Species(Family∗Genus)) – 0.0549 × MS(Error).
[C]0.623 × MS(Species(Family∗Genus)) + 0.377 × MS(Error).
[D]MS(Error).

Hansson and Westerberg 2002; Keller and Waller 2002; Reed and Frankham 2003), a phenomenon that has a strong environmental component (Bijlsma et al. 1999; Haag et al. 2002). *Margaritifera hembeli* is the dominant mussel in these small nutrient-limited headwater streams (Shively and Vermillion 1998; Shively 1999); whether this is due to competitive exclusion by *M. hembeli* or a lack of suitable habitat for other mussel fauna is unclear. Margaritiferids show strong habitat preferences and changes in substrate can cause a shift from margaritiferids to other unionid species (Vannote and Minshall 1982). Given the strong habitat preference of these mussels (Johnson and Brown 2000) and the relative success despite low genetic variation, the modification of mussel habitat by anthropogenic activities (Shively and Vermillion 1998; Shively 1999) is of great concern.

Causes of low genetic variation

Low or no genetic variability has been reported in a number of vertebrates (Bonnell and Selander 1974; O'Brien et al. 1983; Merola 1994); however, it appears to be less common in invertebrates (Nevo et al. 1983). The absence of detectable allozyme variation in several populations of *M. hembeli* is in contrast to other North American unionoideans. Previous studies have detected variation in unionoideans, with levels of polymorphism and heterozygosity ranging from 5.6–60% and 0.010–0.281 respectively.

Allozyme variation has been observed in nearly all populations of congeneric margaritiferids sampled. Levels of polymorphism and heterozygosity in populations of margaritiferids are low (3.7–14% and 0.003–0.037 respectively)(Davis 1984; Machordom et al. 2003), although low polymorphism may be a factor of sample size (Machordom et al. 2003). The observation of lower heterozygosity in the Margaritiferidae relative to the Unionidae is supported by ANOVA analysis of heterozygosities. Thus, we conclude that margaritiferid genetic diversity is generally lower than unionid genetic diversity. We hypothesize that there are biological characteristics unique to the Margaritiferidae that are responsible for the reduced genetic diversity.

Demographic events, such as species bottlenecks, are commonly hypothesized as causes of reduced genetic variation (Merola 1994; O'Brien 1994; Hedrick 1996). The divergence of the margaritiferids likely occurred during the Cretaceous period (Smith 2001); this is supported by uncorrected cytochrome *c* oxidase subunit I nucleotide divergence between these taxa (12–14%) (Machordom et al. 2003, Curole unpublished data). Thus, adequate time has elapsed for recovery from bottleneck events that may have occurred in the lineage leading to these species. Current population sizes do not reflect a recent bottleneck for *M. hembeli* (approximately 35,000 individuals), *M. margaritifera* (between 100,000 and 1 million mussels in Scotland alone, Hastie et al. 2000) or *M. auricularia* (2,000 individuals, Araujo and Ramos 2000). A large reduction in species sizes may have occurred during a historical climate change event; however, it is difficult to reconcile bottleneck species sizes with the observation that margaritiferid mussel beds (1–3 m^2) may have densities of over 300 individuals per square meter (Vannote and Minshall 1982; Johnson and Brown 1998; Hastie et al. 2000).

Meta-population structure, with cells undergoing frequent extinction, can also lead to low levels of

Table 3. Least squares mean estimates of heterozygosities for all taxa analyzed

Taxon	No. pop. (No. ind.)	Mean H
Margaritiferidae		0.0054
Margaritifera		0.0054
M. auricularia	1 (33.40)	0.0100[J]
M. hembeli	5 (26.00)	0.0000
M. margaritifera	18 (7.9)	0.0143[C,D,J]
Unionidae		0.0575
Alasmidonta undulata	2 (17.5)	0.0126[I]
Amblema		0.0921
A. elliotti	2 (2.4)	0.0554[K]
A. neisleri	1 (7.8)	0.1420[K]
A. plicata	8 (10.8)	0.0879[F,K]
Anodonta		0.0613
A. anatina	26 (16.7)	0.0438[L,M]
A. cataracta	2 (20)	0.0700[G]
A. cygnea	7 (19.1)	0.0095[L,M]
A. fragilis	3 (20)	0.0465[G]
A. gibbosa	1 (20)	0.1470[G]
A. grandis	1 (20)	0.1920[E]
A. implicata	3 (20)	0.1298[G]
A. species I	6 (13.2)	0.0095[L,M]
A. species II	10 (7.6)	0.0053[L,M]
A. woodiana	1 (31.7)	0.1010[M]
Elliptio		0.1053
E. arctata	1 (35)	0.1120[E]
E. buckleyi	1 (28)	0.1050[E]
E. cistelliformis	1 (25)	0.0800[E]
E. complanata	20 (22.4)	0.0985[E,I,N]
E. congaraea	1 (22)	0.1870[E]
E. crassidens	3 (25.3)	0.1700[E]
E. dilatata	1 (23)	0.1060[E]
E. fisheriana	1 (15)	0.0850[E]
E. foliculata	1 (13)	0.1000[E]
E. icterina	1 (17)	0.1880[E]
E. lanceolata	1 (17)	0.0210[E]
E. mcmichaeli	2 (22.5)	0.1619[E]
E. producta	1 (16)	0.0490[E]
E. shepardiana	1 (15)	0.0770[E]
E. sp.	4 (21.8)	0.1219[E]
E. waccamawensis	1 (30)	0.0990[E]
Elliptoideus sloatianus	1 (21)	0.0820[E]
Fusconia		0.0936
F. flava	1 (16)	0.1070[E]
F. succissa	1 (25)	0.0810[E]
Lampsilis		0.1008
L. cariosa	3 (12.00)	0.2938[N]
L. fullerkati	1 (31.91)	0.1690[N]
L. radiata	10 (19.20)	0.0560[H,N]
L. siliquoidea	1 (20)	0.1130[E]
L. species	1 (20)	0.0390[H]
L. splendida	1 (20)	0.0590[H]
L. teres	1 (25)	0.0560[E]
Leptodea ochracea	5 (26.8)	0.0533[H,N]
Megalonias		0.0376
M. boykiniana	3 (12.7)	0.0212[E,K]
M. nervosa	7 (2.5)	0.0584[K]
Microcondylaea bonellii	1 (12.0)	0.0000[L]
Plectomerus dombeyanus	3 (23.9)	0.0479[F]
Potomida littoralis	6 (19.3)	0.0739[J,L]
Pseudanodonta complanata	2 (7.5)	0.0040[M]
Quadrula		0.1580
Q. pustulosa	3 (15.3)	0.1387[F]
Q. quadrula	12 (39.1)	0.1738[B,E,F]
Quincuncina infuctata	1 (25)	0.0830[E]
Unio		0.0612
U. crassus	8 (20.7)	0.0673[L]
U. elongatulus	9 (49)	0.0639[A]
U. pictorum	57 (30.3)	0.0831[A,L]
U. tumidus	8 (32.5)	0.0354[L]
Uniomerus		0.1050
U. carolinianus	1 (5)	0.0860[E]
U. declivis	1 (3)	0.1190[E]
U. excultus	1 (18)	0.1110[E]
Utterbackia imbecilis	1 (1.9)	0.0270[K]

A: Badino et al. (1991); B: Berg et al. (1998); C: Chesney et al. (1993); D: Davis and Fuller (1981); E: Davis (1984); F: Johnson et al. (1998); G: Kat (1983a); H: Kat (1983b); I: Kat and Davis (1984); J: Machordom et al. (2003); K: Mulvey et al. (1997); L: Nagel and Badino (2001); M: Nagel et al. (1996); N: Stiven and Alderman (1992); O: this study.

genetic variability (Hedrick 1996; Hedrick and Gilpin 1997; Pannell and Charlesworth 2000). *Margaritifera hembeli*, *M. margaritifera*, *M. auricularia* and *M. falcata* exhibit a highly contagious distribution (range of Morisita's index = 13–74) strongly correlated with microhabitat factors, consistent with a metapopulation structure (Vannote and Minshall 1982; Araujo and Ramos 2000; Hastie et al. 2000; Johnson and Brown 2000). In contrast, surveys of several unionids indicate that microhabitat factors are not strongly correlated with mussel presence and distributions are less contagious (range of Morisita's index = 0.7–8.8) (Kesler and Bailey 1993; Strayer and Ralley

1993; Hornbach et al. 1996; Haag and Warren 1998). Thus, margaritiferids, in contrast to unionids, are highly dependent on abiotic microhabitat factors and exhibit a highly contagious distribution with high local densities as a result of this dependence. Strong substrate dependence and high local densities may also result in higher extinction rates for beds (see below). In addition, allozyme analysis of *M. margaritifera* populations supports a meta-population structure (F_{ST} = 0.320) (Machordom et al. 2003).

The extinction and colonization dynamics of these mussels are poorly understood. Extirpated beds of *M. hembeli* have been observed where the streamflow has shifted and mussels have failed to move (Bolden and Brown 2002). Siltation of beds and reduced stream flow also lead to the loss of populations (WWF 1990). At the other end of this spectrum, spates have also been shown to strip beds of resident individuals for both *M. margaritifera* and *M. falcata* populations (Vannote and Minshall 1982; Hastie et al. 2001). These events likely occur on a 50–100 year scale, suggesting that a small proportion (5–10%) of beds are extirpated every couple to few generations (Vannote and Minshall 1982; Hastie et al. 2001).

At a minimum the colonization rate is twice that of the extinction rate. Within 30 months after denudation of two *M. margaritifera* beds, approximately 10% of the bed had been colonized (Young and Williams 1983). Assuming a constant rate of colonization, the beds would return to original population sizes in 25 years, which is near the generation time of 20 years for these mussels (Bauer 1987). Hermaphroditism has also been shown to occur in *M. margaritifera*, *M. auricularia* and *M. falcata* (but not *M. hembeli*) (Heard 1970; van der Schalie 1970; Bauer 1987; Hansten et al. 1997) and could contribute to any bottleneck effect occurring during colonization, although there is no genetic evidence of inbreeding in *M. margaritifera* or *M. auricularia* (Machordom et al. 2003). The availability of genetic fingerprinting and population assignment algorithms could allow a finer discrimination of the mode of colonization (migrant pool vs. propagule pool, see Pannell and Charlesworth 2000) and any bottleneck effects that may occur.

We hypothesize that the low levels of heterozygosity in the Margaritiferidae are a result of the underlying population dynamics due to meta-population structure of species in this family. In contrast to a historical bottleneck, the meta-population model predicts fewer rare alleles than neutrality expectations at hypervariable loci and disequilibrium between linked loci (Hedrick 1996). Identification of hypervariable genetic loci is the next step in evaluating the hypothesis of meta-population structure for this family of bivalves.

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