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A genetic comparison of two species of clam shrimp in the genus *Eulimnadia*: An electrophoretic approach

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Abstract

Herein we report the first genetic comparison among species in the genus *Eulimnadia*. Multilocus genotypic patterns (using six allozyme loci) were compared for a total of 2277 clam shrimp from nine populations from Arizona and New Mexico. Seven of these populations were morphologically typed as *Eulimnadia texana* Packard and two were typed as *Eulimnadia diversa* Mattox. All populations were hermaphrodite-biased, and highly inbred (inbreeding coefficients ranging from 0.33 to 0.98). Genetic distances showed the two species to be within the range described for other arthropods. One of the two *E. diversa* populations appeared to be a hybrid between *E. texana* and *E. diversa*, showing electrophoretic patterns similar to both species, although morphologically, they were typed as *E. diversa*. A phenogram (generated using coancestry distances and a neighbor joining algorithm) placed this hybrid population half-way between these two species, and a breakdown of individuals within this hybrid population (based on allozyme scores) indicated individuals very similar to the second *E. diversa* population, and two groups of apparent hybrid individuals. Therefore, the distinction between these two species is questionable due to their apparent hybridization in this area of Arizona. Genetic population structuring was noted among the seven *E. texana* pools. Estimated migration rates were less than one migrant per generation. Even in the geographically close pools in New Mexico, which were separated by only hundreds of meters, significant sub-structuring was noted, and estimates of migration rate were less than two migrants per generation.

Introduction

Although branchiopod crustaceans are ubiquitous, outside of the cladocerans, they are understudied. In particular, genetic studies, either at the population or at higher levels, are limited to a handful of papers (Sassaman, 1989; Sassaman & Weeks, 1993; Tinti & Scanabissi, 1996; Hanner & Fugate, 1997; Sassaman et al., 1997; Weeks & Zucker, 1999; Weeks et al., 1999, 2000; Remigio & Hebert, 2000; Spears & Abele, 2000). For example, taxonomic relationships among three of the families of clam shrimp (Spinicaudata) have only recently been genetically examined (Spears & Abele, 2000). But, to date no intergeneric or interspecific genetic comparisons have been reported among these organisms. Hence, taxonomic relationships, heretofore based solely on morphological characters, have yet to be genetically tested in these clam shrimp.

Shrimp in the genus *Eulimnadia* are known to show great morphological variation in taxonomically important factors, such as growth lines on the carapace and rostrum shape (Mattox, 1959; Sissom, 1971; Vidrine et al., 1987; Belk, 1989; Weeks et al., 1997). This variation makes species identifications within the genus *Eulimnadia* problematic (Sissom, 1971; Belk, 1989). In 1989, Belk collapsed seven species of *Eulimnadia* (*E. alienata*, *E. francesae*, *E. inflecta*, *E. thompsoni*, *E. ventricosa*, *E. oryzae*, and *E. diversa*) into a single, morphologically variable species, *E. diversa*, stating that these 'seven nominal species are only different-sized populations of a single species' (Belk, 1989: 117). Belk (1989) concluded there are seven *Eulimnadia* species in North America (*E.*

agassizii, *E. antlei*, *E. antillarum*, *E. astraova*, *E. cylindrova*, *E. diversa*, and *E. texana*) and that egg shell morphology is the most reliable character for species identification. However, there has yet to be a genetic comparison among any of the seven *Eulimnadia* species, nor any test for reproductive isolation (e.g., hybridization experiments) among these species. Thus, we are left with no genetic corroboration of the morphological distinctions among *Eulimnadia* species.

Herein we report the first interspecific genetic comparisons in the Spinicaudata. Comparisons of two of the seven *Eulimnadia* species, *E. texana* and *E. diversa*, were made. We used six electrophoretic loci to compare seven populations of *E. texana* with two populations of *E. diversa*. We show that genetic differentiation between these two species is consistent with sister species, but that hybridization between these species is occurring in one of these nine populations. Additionally, these data allow the first evaluation of genetic population structure within the Spinicaudata.

Methods and materials

Rearing protocol and data collection

Soil containing clam shrimp cysts was collected from six sites in New Mexico (AMT1, JD1, JT3, JT4, LTER, and SWP5), located on the USDA Jornada Experimental Range within Dona Ana Co. (south-central New Mexico), and three sites in Arizona (BAP, WAL, and YCOM; the latter two being 'Portal 1' and 'Portal 2' in (Sassaman, 1989) near Portal in Cochise Co., near the base of the Chiricahua Mountains. These samples were then transported back to the laboratory in Akron. Sub-samples of soil (250 ml) from all populations were hydrated using filtered tap water. Hydrations were in 37-l aquaria housed in an environmentally controlled room under continuous light (Durotest sunlight-simulating fluorescent bulbs), at 25–27 °C, and continuous aeration (see Sassaman & Weeks, 1993; Weeks et al., 1997). All aquaria were supplemented with 10 ml of baker's yeast solution (1 g dried yeast per 100 ml water) per day. After reaching sexual maturity in these aquaria (at approximately 5–7 days), shrimp were sexed and then frozen for electrophoresis.

Electrophoretic assays were conducted using cellulose acetate gel electrophoresis (Richardson et al., 1986). All shrimp were scored for six polymorphic loci: Fum (fumarate hydratase, EC 4.2.1.2), Idh-1, Idh-2 (isocitrate dehydrogenase, EC 1.1.1.42), Mpi (mannose-phosphate isomerase, EC 5.3.1.8), Pgm, (phosphoglucomutase, EC 5.4.2.2), and Pgi (Glucosephosphate isomerase, EC 5.1.3.09). All gels were run using 'Buffer C' from Richardson et al., (1986), which produces the clearest bands for these enzymes in this species (Weeks et al., 1999). Allele designations were labeled by increasing anodal mobility (ranging from 'a' to 'e'). All alleles were compared across populations to provide uniform scoring designations.

Table 1. Percent males, location information, sample size, inbreeding coefficient (f), and average alleles per polymorphic locus (A_p) for the nine sampled *Eulimnadia* populations (Pop)

Pop	% Males	Species[1]	Location[2]	N	F	A_p
AMT1	28.1[a]	Et	NM	69	0.521	2.3
JD1	17.8[a]	Et	NM	196	0.285	2.0
JT3	17.9	Et	NM	20	0.552	2.4
JT4	20.9[a]	Et	NM	699	0.341	2.4
LTER	24.2[a]	Et	NM	82	0.423	2.8
SWP5	20.3[a]	Et	NM	287	0.370	2.2
WAL	24.2[a]	Et	AZ	725	0.334	2.7
BAP	8.8	Ed	AZ	61	0.903	2.0
YCOM	0.6	Ed	AZ	138	0.976	2.4

[1] Et – *Eulimnadia texana*; Ed – *Eulimnadia diversa*; species identification via Denton Belk.
[2] AZ – Portal, Arizona; NM – Las Cruces, New Mexico.
[a] From Weeks & Zucker (1999).

Electrophoretic data analyses

Genetic distances and allozyme diversity statistics were calculated for the individuals and populations using the computer software package, Genetic Data Analysis, vers. 1.0 (Lewis & Zaykin, 2001). These measures included Wright's (1951) F-statistics, Nei's (1978) estimates of genetic distance, and Reynolds et al. (1983) coancestry distance (see also Weir, 1996). The latter distance measure was used to further generate a phenogram for the populations using the neighbor joining method (Saitou & Nei, 1987).

Genetic population structure was assessed using Wright's F-statistics (Wright, 1951), as modified by Weir (1996). The F-statistics and their bootstrapped 95% confidence intervals were generated using the program Genetic Data Analysis (Lewis & Zaykin, 2001).

Results

Species identification was based on egg shell morphology (Belk, 1989) and was corroborated by identification by Denton Belk (pers. com.). These morphological identifications suggested seven populations of *E. texana* (AMT1, JD1, JT3, JT4, LTER, SWP5, and WAL) and two populations of *E. diversa* (BAP and YCOM) (Table 1). Six of the nine populations were collected in New Mexico, and three were from Arizona (Table 1). All populations were hermaphrodite-biased, inbred, and had few alleles per polymorphic locus (Table 1).

Electrophoretic comparisons among the populations showed that one of the two*E. diversa* populations (BAP) was clearly genetically distinct from all of the seven *E. texana* populations (Table 2). The BAP shrimp were fixed for alleles at IDH-2 and PGI that were not found at any frequency in these other seven populations. Differentiation at the remaining five loci was not as dramatic (Table 2). Most of the differences among populations were primarily due to differences in gene frequencies rather than to a plethora of private alleles. Overall, only four private alleles were found out of 25 identified: Idh-1 d in YCOM, Idh-1 c in WAL, Fum a in LTER, and Pgi b also in LTER (Table 2).

Genetic distances are shown in Table 3. Nei's (1978) genetic distance is given for comparisons with other crustaceans, but is not an appropriate measure for highly inbred populations (Brown, 1979). In species where inbreeding is common, Nei's genetic distance calculations can be inflated because of the increased likelihood of fixed differences among populations due to the increased levels of homozygosity associated with close inbreeding (Brown, 1979). Coancestry distances are more reliable for recently diverged populations that differ primarily due to genetic drift and inbreeding (Weir, 1996). Coancestry distances show the seven *E. texana* populations to be similar to one another (mean $\pm$ S.E. = 0.194 ± 0.021), which was approximately half as much as the distance between the two *E. diversa* populations (0.358, Table 3). Average distances between the two *Eulimnadia* species was 0.815 ± 0.084.

The neighbor-joining phenogram reflected these patterns (Fig. 1). BAP was the most distinct of the nine populations, with YCOM approximately halfway between BAP and the remaining seven populations. The populations also clustered geographically. The three Arizona populations (WAL, YCOM, and BAP) all clustered together. Among the New Mexico populations, SWP5, JT4, and LTER are all geographically close, JT3 and JD1 are similarly close to one another, and AMT1 is the geographically most remote of the six New Mexico populations (Fig. 2).

An unsuspected result was that though YCOM did cluster with BAP, it was rather intermediate between BAP and the *E. texana* populations. When reviewing the genetic data (Table 2), it was clear that part of this intermediate distance relationship was due to mixtures of both *E. diversa* and *E. texana* electrophoretic patterns at Pgi and Idh-2 (Table 2). Therefore, it was

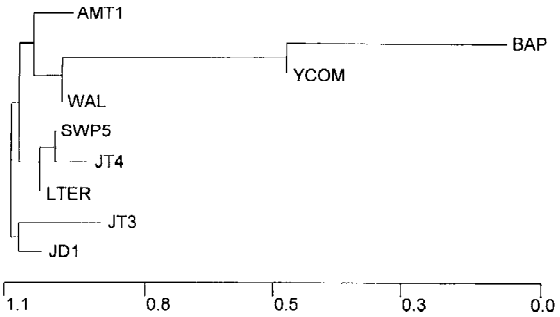
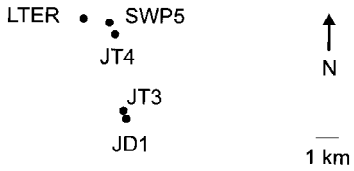


Figure 1. Neighbor-joining (Saitou & Nei, 1987) phenogram based on Reynolds et al. (1983) coancestry distance (Table 3). Population codes are given in Table 1.



• AMT1

Figure 2. Geographic distances among the six *Eulimnadia texana* populations in New Mexico. The one additional *E. texana* population (WAL) was ~230 km west of the New Mexico populations in southeastern Arizona.

Table 2. Allele frequencies in all *Eulimnadia* populations

Locus	AMT1	JD1	JT3	JT4	LTER	SWP5	WAL	YCOM	BAP
Pgm									
A			0.550	0.913		0.939		0.027	0.289
B	1.000	1.000	0.450	0.087	0.994	0.061	0.670	0.434	0.711
C					0.006		0.330	0.540	
Idh-1									
A			0.105				0.007		
B	0.939	0.789	0.711	0.936	0.980	0.931	0.970	0.842	1.000
C							0.023		
D								0.158	
E	0.061	0.211	0.184	0.064	0.020	0.069			
Idh-2									
A	0.007			0.016		0.030	0.001		
B	0.993	1.000	0.975	0.968	0.969	0.970	0.788	0.538	
C			0.025	0.016	0.031		0.211		
D								0.462	1.000
Fum									
A					0.015				
B	0.430	0.841	0.895	0.569	0.732	0.724	0.660	0.004	
C	0.570	0.159	0.105	0.431	0.254	0.276	0.340	0.996	1.000
Mpi									
A				0.002			0.401		
B			0.088		0.173	0.228	0.159		
C	0.262	0.394	0.382	0.869	0.545	0.614	0.225	0.8070.500	
D	0.397	0.606	0.529	0.129	0.237	0.158	0.215	0.1930.500	
E	0.341				0.045				
Pgi									
A								0.448	1.000
B					0.052				
C	0.992	1.000	1.000	1.000	0.931	0.996	0.989	0.455	
D						0.004	0.011	0.097	
E	0.008				0.017				

possible that YCOM received an intermediate distance score because it was actually a mixture of both species that we did not recognize. We thus used alleles at Idh-2 and Pgi as diagnostic characters between these species to see if we could genetically identify two separate populations within YCOM. YCOM shrimp were reclassified into *E. diversa* (Idh-2 = d/d and Pgi = a/a; YCOM1) and *E. texana* (Idh-2 = b/b and Pgi = c/c; YCOM2). However, all YCOM shrimp could not be categorized into these two, separate groups (which would have indicated a mixture of reproductively isolated species): 20% of the population was of 'mixed' genotypes, indicative of hybridization between *E. texana* and *E. diversa* (Table 2). Comparing electrophoretic patterns across all loci clearly revealed that YCOM was not merely a mixture of two reproductively isolated species (Table 4).

Construction of a phenogram for all nine populations (with YCOM split among the three sub-types; Table 4) confirmed the hybrid nature of YCOM. In

Table 3. Nei's (1978) (above diagonal) and coancestry (Reynolds et al., 1983; Weir, 1996) (below diagonal) genetic distances among the nine populations of *Eulimnadia*

Population	AMT1	JT3	SWP5	BAP	WAL	YCOM	JD1	LTER	JT4
AMT1		0.145	0.056	0.653	0.086	0.321	0.058	0.042	0.063
JT3	0.350		0.096	0.968	0.129	0.551	0.064	0.094	0.134
SWP5	0.183	0.270		0.778	0.062	0.347	0.039	0.002	0.017
BAP	1.045	1.184	1.104		0.809	0.210	0.844	0.758	0.692
WAL	0.171	0.214	0.137	0.820		0.356	0.090	0.062	0.090
YCOM	0.481	0.563	0.554	0.358	0.434		0.476	0.372	0.254
JD1	0.208	0.217	0.144	1.220	0.194	0.662		0.027	0.069
LTER	0.136	0.246	0.008	1.115	0.130	0.518	0.103		0.024
JT4	0.243	0.434	0.072	1.205	0.238	0.502	0.270	0.097	

Table 4. YCOM separated into sub-groups. YCOM1 had the electrophoretic patterns of *Eulimnadia diversa*, YCOM2 had the pattern of *E. texana*, and YCOM3 had patterns of both (see Table 2). Sample sizes were: YCOM1, 51; YCOM2, 52; YCOM3, 26

Locus	YCOM1	YCOM2	YCOM3
Pgm			
A	0.051	0.025	
B	0.769		0.375
C	0.179	0.975	0.625
Idh-1			
B	1.000	0.133	0.760
D		0.867	0.240
Idh-2			
B		1.000	0.712
D	1.000		0.288
Fum			
B	1.000	1.000	1.000
Mpi			
C	0.854	0.833	0.769
D	0.146	0.167	0.231
Pgi			
A	1.000		0.269
B			
C		1.000	0.231
D			0.500

(Note: individuals that were not positively scored for both PGI and IDH-2 were not included in this table).

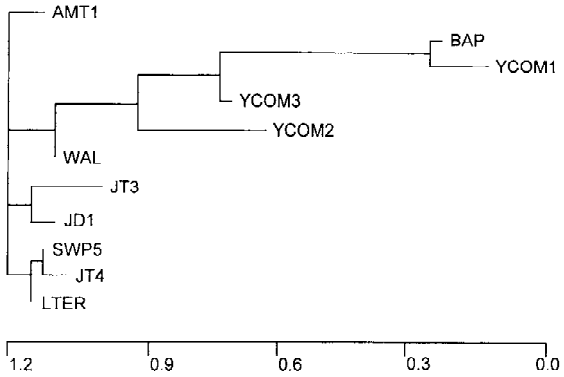


Figure 3. Neighbor-joining (Saitou & Nei, 1987) phenogram based on Reynolds et al. (1983) coancestry distance (Table 5) with the YCOM population divided into three subpopulations as described in the text. Population codes are given in Table 1.

this analysis BAP grouped closely with YCOM1, with YCOM3 placed nearly half-way between *E. texana* and *E. diversa* (Fig. 3). The '*E. texana*' sub-type (YCOM2) was placed rather far from the nearest *E. texana* population (WAL; Table 5; Fig. 3). The remaining populations retained their geographically structured clustering (Fig. 2), similar to that portrayed in Figure 1.

Population sub-structure was analyzed among the seven *E. texana* populations using Wright's (1951) *F*-statistics. All three *F*-statistics were positive (F_{IS} = 0.366; F_{ST} = 0.284; F_{IT} = 0.546), suggesting both inbreeding within populations, and population subdivision. In fact, estimates of both of these factors were found to be significantly different from zero (95% CI: F_{IS} = 0.212 – 0.536; F_{ST} = 0.158 – 0.524; see Lewis & Zaykin, 2001, for bootstrap estimates of 95% CI values). The estimated gene flow rate among populations,

using $(1 - F_{ST}) / 4F_{ST}$ (Wright, 1931), was therefore 0.63 migrants per generation (Nm). Considering only the six, geographically close New Mexico populations (Fig. 2), all three F-statistics were still found to be positive, but differences among populations were less apparent (F_{IS} = 0.347; F_{ST} = 0.158; F_{IT} = 0.450). Nonetheless, both estimates of inbreeding within populations and population subdivision were also found to be significantly different from zero (95% CI: F_{IS} = 0.212 – 0.508; F_{ST} = 0.065 – 0.252). This estimated population subdivision was robust to exclusion of the one furthest removed population (AMT1; see Fig. 2): the F_{ST} estimate with this population excluded was 0.144 (95% CI: 0.063 – 0.228). Among these six populations, gene flow was estimated at 1.33 migrants per generation (including all six populations).

Discussion

Two clear conclusions emerged from the genetic comparison between *E. texana* and *E. diversa*. The first is that the two *Eulimnadia* species were genetically distinct from one another at the six allozyme loci examined. The two *E. diversa* populations (BAP and YCOM1) were both distantly related to *E. texana* (average D_{Nei} = 0.78) [Note: we are reporting Nei's (1978) genetic distances herein because only these distances were found in previous comparisons among arthropod species. However, we used the more recent Reynolds et al. (1983) distances to construct the phenograms.] This distance is typical for sister species within the same genera in insects (Nei, 1976), and substantially smaller than between differing conchostracan genera (D_{Nei} = 2.48 – 2.60) (Tinti & Scanabissi, 1996). One complicating factor for comparing distances in these taxa is the level of inbreeding exhibited by both clam shrimp species. In the current comparison, differences among species are confounded by the level of inbreeding: the two *E. diversa* populations are also the two most highly inbred populations. Thus, it is impossible to determine how much of the genetic distance between the two species is due to fixed differences among species versus a fixation of alleles due to extreme inbreeding. A more extensive genetic survey among several populations from each species will allow such a distinction.

The second conclusion was that one of the two *E. diversa* populations (YCOM) was clearly a mixture of *E. diversa* and a hybrid swarm of *E. diversa*/*E. texana* individuals. The description of *E. diversa* as a separate species from *E. texana* is based exclusively on morphological differences, primarily in the morphology of the cysts (Belk, 1989). No experiment has examined the level of reproductive isolation between these species. Genetic data collected from YCOM suggests that hybridization is occurring between *E. diversa* and *E. texana* in this population. Both the '*E. texana*' genotype (YCOM2) and the 'hybrid' genotypes (YCOM3) were found to be intermediate to *E. diversa* and *E. texana* (Fig. 3). Since the YCOM1 shrimp clustered tightly with BAP, and both YCOM2 and YCOM3 were intermediate to *E. diversa* and *E. texana*, it appears that hybridization is progressing from *E. diversa* to *E. texana*, but not vice versa. This could occur in one of two ways: (a) only matings between *E. diversa* males and *E. texana* hermaphrodites are viable (due to behavioral or genetic reasons), or (b) YCOM is (or was) a mixture of *E. texana* hermaphrodites and *E. diversa* males and hermaphrodites. The second possibility seems more likely. BAP (which is geographically close to YCOM: 630 m between ponds) has a low level of males, whereas in YCOM very few males (0.6%) have been found. It is therefore likely that propagules from BAP have colonized YCOM, and that hybridization has been through *E. diversa* males mating with *E. texana* hermaphrodites. Since males are either now completely absent, or at least very rare in YCOM, it is reasonable to suggest that such hybridization was incomplete (i.e., *E. diversa* hermaphrodites from BAP could continue to self, thus preserving their *E. diversa* genotypes), and thus 'pure' *E. diversa* individuals were still found in YCOM. Current experiments are under way to attempt to hybridize these two species in the laboratory.

The apparent hybridization between *E. diversa* and *E. texana* sheds some doubt on the true distinction between these two species. Although the genetic distances are within the range of other crustaceans (Hedgecock et al., 1982) and are somewhat larger than among subspecies of arthropods (Nei, 1976), these measures may be inflated by the large amount of inbreeding typical of these two species. Since these species appear to be inter-fertile (at least in this section of Arizona), it is possible that the egg shell morphological variants are primarily marking geographic 'races' or sub-species rather than truly, evolutionarily-distinct species. Again, a wider genetic survey combined with hybridization experiments will allow us to determine whether these two species are truly evolutionarily distinct.

Table 5. Coancestry distances (Reynolds et al., 1983; Weir, 1996) among the nine populations of *Eulimnadia*, with YCOM split into the three sub-groups as in Table 4

Population	AMT1	JT3	SWP5	BAP	WAL	YCOM1	YCOM2	YCOM3	JD1	LTER
JT3	0.350									
SWP5	0.183	0.270								
BAP	1.045	1.184	1.104							
WAL	0.171	0.214	0.137	0.820						
YCOM1	1.245	1.448	1.170	0.189	0.848					
YCOM2	0.703	0.814	0.704	1.325	0.440	1.387				
YCOM3	0.604	0.591	0.695	0.594	0.486	0.658	0.328			
JD1	0.208	0.217	0.144	1.220	0.194	1.380	0.929	0.833		
LTER	0.136	0.246	0.008	1.115	0.130	1.267	0.781	0.632	0.103	
JT4	0.243	0.434	0.072	1.205	0.238	1.175	0.638	0.664	0.270	0.097

Lastly, these genetic data allow a first glimpse into population subdivision within *E. texana*. Wright's *F*-statistics clearly show significant levels of population sub-division, with migration rates of less than one migrant per generation across all populations surveyed. Interestingly, even with populations that were only several hundreds of meters apart, we found low migration rates (< 2 migrants per generation) and significant population sub-division. Much of this is likely due to low levels of cyst transfer among pools coupled with the high rates of genetic drift found in highly selfing populations.

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