Evolution, 50(2), 1996, pp. 952–957

PATERNAL MITOCHONDRIAL DNA DIFFERENTIATION FAR EXCEEDS MATERNAL MITOCHONDRIAL DNA AND ALLOZYME DIFFERENTIATION IN THE FRESHWATER MUSSEL, *ANODONTA GRANDIS GRANDIS*

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Key words.—Allozymes, Anodonta, doubly uniparental inheritance, mtDNA.

Received June 24, 1994. Accepted April 28, 1995.

Comparisons of population structure revealed by organellar genomes and protein polymorphisms have yielded strikingly discordant patterns in oysters (Buroker 1983; Reeb and Avise 1990; Karl and Avise 1992), horseshoe crabs (Selander et al. 1970; Saunders et al. 1986), the copepod *Tigriopus californicus* (Burton and Lee 1994), the California closed-cone pines (Millar et al. 1988; Hong et al. 1993; Strauss et al. 1993), and perhaps also in lodgepole pine (Wheeler and Guries 1982; Dong and Wagner 1993, 1994). For example, allelic frequencies at protein polymorphisms in the American oyster, *Crassostrea virginica,* are relatively homogeneous from Maine to Texas (Buroker 1983), suggesting extensive gene flow mediated by long distance dispersal of pelagic larvae. However, nuclear and mtDNA polymorphisms reveal the contrasting pattern of a sharp genetic discontinuity in northeastern Florida, suggesting only limited and local dispersal. Furthermore, the relationship between heterozygosity and components of fitness may differ between protein markers and DNA markers (Pogson and Zouros 1994). In the sea scallop, *Placopecten magellanicus,* growth rate is positively correlated with allozyme heterozygosity, but not with heterozygosity at DNA markers (Pogson and Zouros 1994). These and other studies (review in Mitton 1994) clearly indicate that various types of gene markers can give discordant results when they are used to estimate population structure, gene flow, and selection intensities.

Plants contain two organellar genomes, one chloroplast genome and one mitochondrial genome, and most animals carry a single mitochondrial genome (Avise 1994). In animals, the inheritance of mtDNA is generally maternal, although a low level of paternal transmission does occur in mouse (Gyllensten et al. 1991) and *Drosophila* hybrids (Satta et al. 1988; Kondo et al. 1992). Recently, Skibinski et al. (1994) and Zouros et al. (1994) described a unique pattern of mtDNA inheritance in the blue mussel, *Mytilus edulis,* which they referred to as "doubly uniparental inheritance." In this mode of inheritance, the transmission of mitochondrial types depends upon the sex of the offspring. Males receive approximately five of the M-type (male) mitochondria from their father (Longo and Dornfield 1967) and approximately 10^4 of the F-type (female) mitochondria from their mother (Billett 1979), but the M-type mitochondria replicate faster and become codominant as the animals develop and grow. In females, the F type mitochondria remain predominant and if the M type mitochondria exist in females, they cannot be detected by the usual detection methods. Males preferentially package the M-type mitochondria into sperm while females package only the F-type mitochondria in the egg. The maternally inherited F-type mtDNA and the paternally inherited M-type mtDNA do not recombine, allowing these mitochondrial forms to diverge.

Here we report the results of a study of genetic variation of the freshwater mussel, *Anodonta grandis grandis* (Bivalvia: Unionidae), which has revealed gender-specific mtDNA. The differentiation of paternal mtDNA far exceeds the differentiation of maternal mtDNA and allozymes.

The Life Cycle of Anodonta grandis grandis

A typical bivalve usually discharges gametes into the water and externally fertilized eggs develop into free-swimming trochophore, then veliger larvae (Barnes 1968). After dispersal, the larvae settle out of the water column and transform into sedentary adults. However, the life cycles of species in the family Unionidae are atypical for bivalves. They display an indirect but specialized development. Eggs are released into the water tubes of the suprabranchial cavity and are fertilized there by sperm brought in by the ventilating water currents. The zygotes remain in the water tubes through early development, and are brooded in a marsuipum of modified gill lamella, where they develop through the veliger stage. However, the veliger, which is called a glochidium in the family Unionidae, has become a highly modified parasitic larva. In *Anodonta,* the glochidium is enclosed by two valves, each bearing a hook. The glochidia leave the gills through the suprabranchial cavity and exhalant siphon and become attached to the fins and other surfaces of fish. The glochidia undergo metamorphosis and eventually fall to the bottom and burrow in the mud, where they complete development and gradually assume the adult habit (Barnes 1968).

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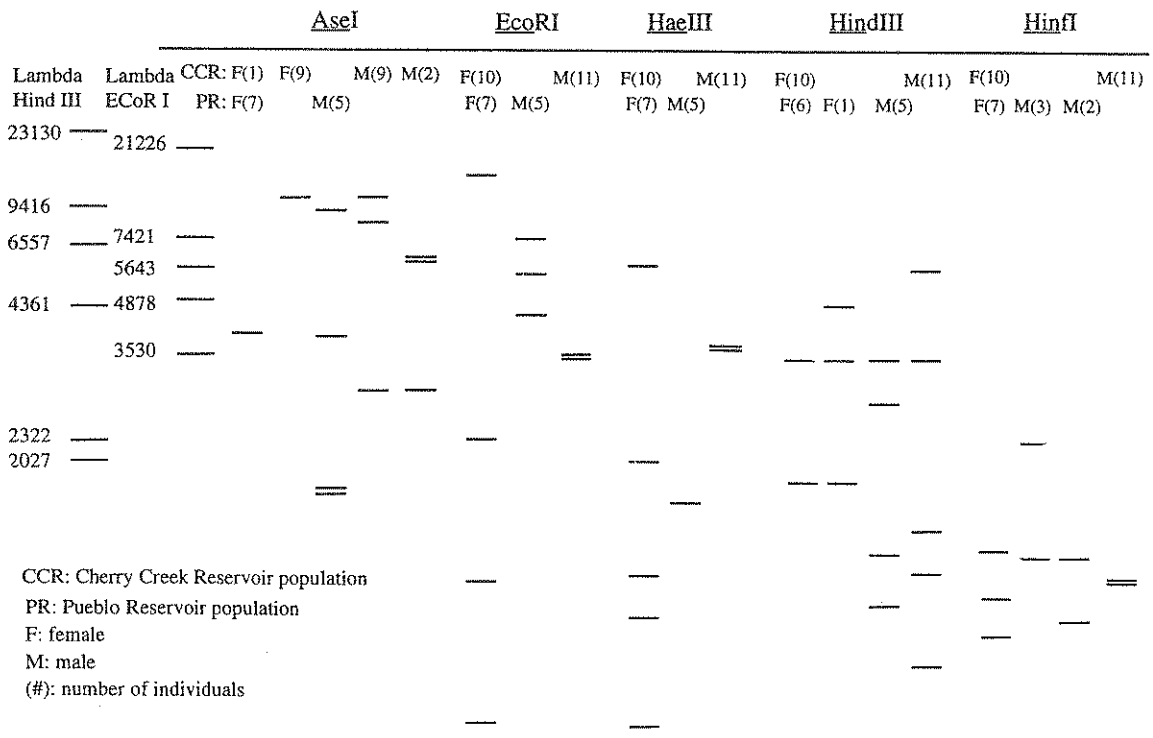

FIG. 1. Diagrammatic representation of all the *Ase*I, *Eco*RI, *Hae*III, *Hin*dIII, and *Hin*fI fragment patterns observed in 33 samples of the giant *floater (Anodonta grandis grandis* Say) collected in Cherry Creek Reservoir and Pueblo Reservoir.

MATERIALS AND METHODS

Specimens

Anodonta grandis grandis is commonly referred to as the giant floater, for it reaches a length of 23 cm, and it floats if air becomes trapped between the valves.

Anodonta were collected from two drainages in Colorado: the Arkansas River drainage; Pueblo County, Pueblo Reservoir (T20S, R66W, sections 33/36) in October 1993 by S. J. Herrmann, 12 specimens (7 females, 5 males); and the South Platte River drainage; Adams County, Cherry Creek Reservoir (T5S, R67W, sections 1/12), in November 1993 by S.-K. Wu and H.-P. Liu, 21 specimens (10 females, 11 males). We assessed gender based on the presence or absence of the marsupial gill. Mussels with a marsupium are female, and mussels lacking a marsupium are male. In our samples, all individuals with a marsupium also carried glochidia.

Mitochondrial DNA Restriction Fragment Length Polymorphisms

For each individual, total DNA was extracted from gonadal tissue with a modified CTAB procedure (Rowan and Powers 1992). In a preliminary study, total DNA was digested with 15 restriction enzymes, *Ase*I, *Bam*HI, *Bcl*I, *Bgl*I, *Bsi*HKAI, *Eco*RI, *Hae*III, *Hha*I, *Hin*dIII, *Hin*fI, *Kpn*I, *Msp*I, *Pst*I, *Sac*I, and *Xho*I. Five of these, *Ase*I, *Eco*RI, *Hae*III, *Hin*dIII, and *Hin*fI, were chosen to digest all samples collected from Pueblo Reservoir and Cherry Creek Reservoir. DNA fragments were separated on a 1% agarose gel, and transferred to a nylon membrane (Magnagraph nylon membrane, Micron Separations Inc.; Southern 1975). After the transfer, the DNA was bound permanently to the membrane by placing it between sheets of 3MM blotting paper and baking it at 80°C for 30 min. Mitochondrial DNA fragments were detected by hybridizing with

TABLE 1. Enzyme systems examined and their electrophoretic requirements.

Enzyme	Locus	Buffer system	Condition	Staining reference
Aminopeptidase-leucyl-alanine (AP-ala)	1	Lithium Hydroxide[1]	40mA, 7h	Harris and Hopkinson 1976
Aminopeptidase-leucyl-tyrosine (AP-tyrosine)	2	Lithium Hydroxide	40mA, 7h	Harris and Hopkinson 1976
Glutamate Oxaloacetate Transaminase (GOT)	1	Lithium Hydroxide	40mA, 7h	Shaw and Prasad 1970
Isocitrate Dehydrogenase (IDH)	2	Tris-Citrate[2]	45mA, 4h	Shaw and Prasad 1970
Malate Dehydrogenase (MDH)	2	Tris-Citrate	45mA, 4h	Shaw and Prasad 1970
Mannose Phosphate Isomerase (MPI)	1	Lithium Hydroxide	40mA, 7h	Passteur et al. 1987
Phosphoglucomutase (PGM)	2	Tris-Citrate	45mA, 4h	Pasteur et al. 1987
Phosphoglucose Isomerase (PGI)	1	Tris-Citrate	45mA, 4h	Shaw and Prasad 1970

[1] Electrode buffer: stock solution A; Gel buffer: 1:9 mixture of stock solution A & B
stock solution A: 0.03 M lithium hydroxide, 0.19 M boric acid, pH 8.1
stock solution B: 0.05 M Tris, 0.008 M citric acid, pH 8.4.
[2] Electrode buffer: 0.223 M Tris, 0.086 M citric acid, pH 6.3; Gel buffer: 0.080 M Tris, 0.003 M citric acid, pH 6.7.

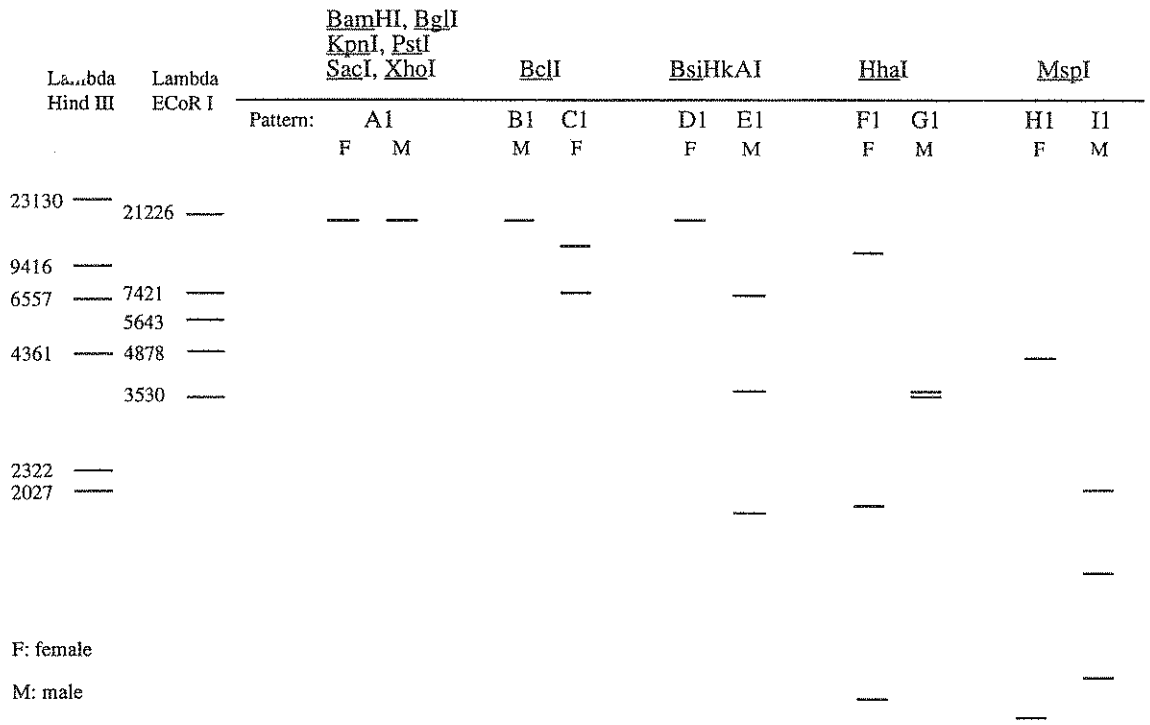

FIG. 2. Diagrammatic representation of all the *Bam*HI, *Bgl*I, *Kpn*I, *Pst*I, *Sac*I, *Xho*I, *Bsi*HkAI, *Bcl*I, *Hha*I, and *Msp*I fragment patterns observed in the preliminary studies in samples of the giant floater (*Anodonta grandis grandis* Say) collected in Cherry Creek Reservoir.

mtDNA probes (30 ng/ml) in hybridization buffer (5X SSC, 0.1% lauroylsarcosine, 0.02% sodium laurylsulfate, 1% vial 11 in the Genius Nonradioactive DNA Labeling and Detection kit; Boehringer Mannheim) at 50C. Because we expected low sequence similarity between target DNA and probe DNA, washing was carried out at a much lower stringency condition (2 × 5 min in 2X SSC at room temperature and 2 × 15 min in 0.5X SSC at room temperature) than used by Fisher and Skibinski (1990).

The entire mtDNA of *Mytilus edulis* (around 17 kb long, supplied by D. O. F. Skibinski) cloned into phage Lambda EMBL3 and maintained in *Escherichia coli* P2392 (Skibinski and Edwards 1987) was used as a probe. DNA template was labeled with digoxigenin-dUTP (random primed method) by using the Genius Nonradioactive DNA Labeling and Detection kit supplied by Boehringer Mannheim and prepared according to their standard protocol. Because templates of 100–10,000 bp label efficiently and produce probes with maximal sensitivity, the mtDNA of *M. edulis* was double digested with *Bam*HI and *Eco*RI to obtain two approximately 2 kb and two approximately 7 kb fragments (Hoffmann et al. 1992) which were purified from low-melting agarose gel before labeling.

To verify that the RFLPs revealed by hybridization were mtDNA, mitochondria were purified from gonadal tissue of one male by the method of differential centrifugation (Kwast and Hand 1993), and mtDNA was extracted, digested with *Eco*RI, run on an agarose gel, and observed first with ethidium bromide and then by Southern hybridization. The bands detected by hybridization using total DNA as described above were identical to the bands from the mitochondrial extraction visualized either by ethidium bromide or by hybridization.

When restriction enzymes other than *Eco*RI were used to cut the DNA, the probe hybridized to one or more fragments, but the sum of the fragments was less than 18 kb (Fig. 1). This is most easily explained by incomplete hybridization between the *Mytilus* probe and the *Anodonta* mtDNA. Both species are bivalves, but they have been separated for at least 200–250 million yr (Purchon 1968), so incomplete hybridization is not unexpected.

Nei (1987) and Nei and Miller (1990) presented statistical methods to estimate the degree of genetic divergence of DNA within and among populations by using either restriction-site or restriction-fragment data. Generally, restriction-site analysis provides more information about the evolution of mtDNA. It is possible to convert restriction-fragment to restriction-site data, when two sequences are closely related. For example, from the disappearance of two fragments and the appearance of a new fragment whose molecular weight equals the sum of the lost fragments, one cleavage site loss can be inferred. However, *A. g. grandis* has highly differentiated fragment patterns in each population, and due to incomplete hybridization, it is difficult to be sure how many substitutions are involved. Therefore, restriction-fragment data were used to estimate sequence divergence within and between populations of *A. g. grandis*.

Isozyme Electrophoresis

For each individual, 0.1–0.2 g of gonadal tissue were cut into pieces with scissors, mixed with 0.5 ml buffer (0.01M sodium phosphate, 0.001M EDTA, 0.001M mercaptoethanol, at pH 7.0), sonicated for 5–10 sec, and centrifuged in a clin-

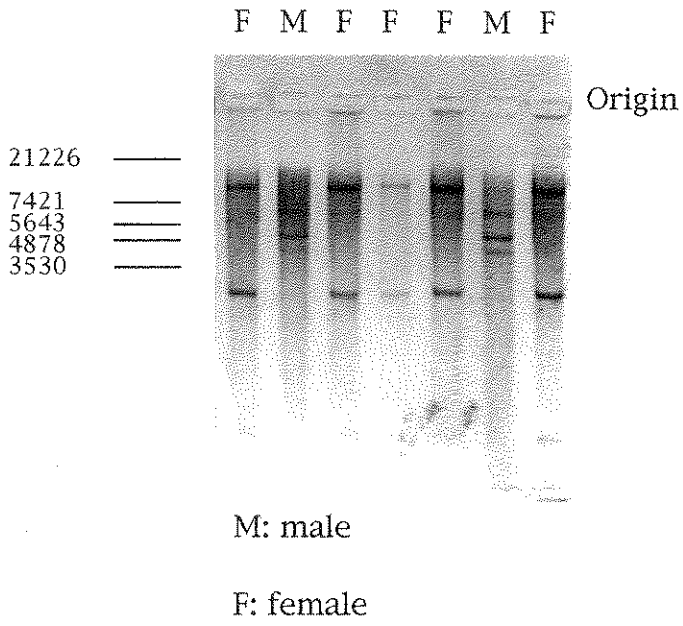

FIG. 3. *Eco*RI digests of mtDNA extracted from samples of the giant floater (*Anodonta grandis grandis* Say) collected from Pueblo Reservoir.

ical Eppendorf centrifuge at 4°C for 5 min. The supernatant was adsorbed onto a piece of Whatman #1 filter paper and inserted into a starch gel. The enzyme systems examined and their biochemical staining conditions are listed in Table 1. Multilocus systems were numbered based on the mobility of their gene products from anode to cathode. All protein patterns were consistent with those expected from autosomal loci with codominant alleles. The allelic terminology utilized relative differences in electrophoretic mobility of homologous gene products with the most common allele in the Cherry Creek Reservoir population designated as 100 and the value of the origin being zero (Utter et al. 1987).

Statistical Procedures

To estimate levels of genetic differentiation between populations, the unbiased genetic distance coefficients (D) of Nei (1978) were calculated from allelic frequencies of the enzyme polymorphisms with the Biosys-1 computer program (Swofford and Selander 1981). For restriction fragment data, the average number of nucleotide substitutions within and between populations was estimated by using the method of Nei (1987) with the RESTSITE computer program (Miller 1991).

RESULTS

Mitochondrial DNA Restriction Fragment Length Polymorphisms

Among 15 restriction enzymes used in the preliminary survey, six (*Bam*HI, *Bgl*I, *Kpn*I, *Pst*I, *Sac*I, and *Xho*I) recognized only one restriction site in the mtDNA of *A. g. grandis*. These enzymes reduced the circular mtDNA molecule to a single, large band shown (Fig. 1, pattern A1). In a comparison with fragments of lambda DNA produced by digestion with EcoRI or HindIII, the size of the mtDNA genome of *A. g. grandis* is estimated to be 18 kb.

All of the enzymes except, *Bam*HI, *Bgl*I, *Kpn*I, *Pst*I, *Sac*I, and *Xho*I, revealed distinctly different mtDNA RFLPs between males and females (Figs. 1–3). Females all share similar mtDNA haplotypes, regardless of whether they were collected from Cherry Creek Reservoir or Pueblo Reservoir. The mtDNA haplotypes of females are shared between localities, but the mtDNA haplotypes of males differ between localities.

Within each of these populations, the mtDNA sequence difference between males and females is 6.8% (Fig. 4). The mtDNA sequences of females between these localities are virtually identical: the divergence is estimated to be 0.4% (Fig. 4). However, the sequence difference among males between these localities is 11.5% (Fig. 4).

Isozyme Electrophoresis

The electrophoretic examination of eight enzymes allowed the resolution of the gene products of 12 presumptive loci (Table 1). Of these 12 loci, three (PGM-1, PGM-2, IDH-1) were polymorphic (Table 2). There were no differences between the sexes, but the allelic frequencies at all three polymorphic loci were significantly different between populations. At all three polymorphic loci, allelic frequencies were almost fixed for the common allele in the Cherry Creek population, but were more intermediate (0.375–0.583) in the Pueblo population. Genotypic proportions were consistent with Hardy-Weinberg expectations for all loci in all population samples. The genetic distances between population, based on all 12 loci, was D = 0.055.

DISCUSSION

The mtDNA haplotypes of males differ from the mtDNA haplotypes of females; the average sequence divergence be-

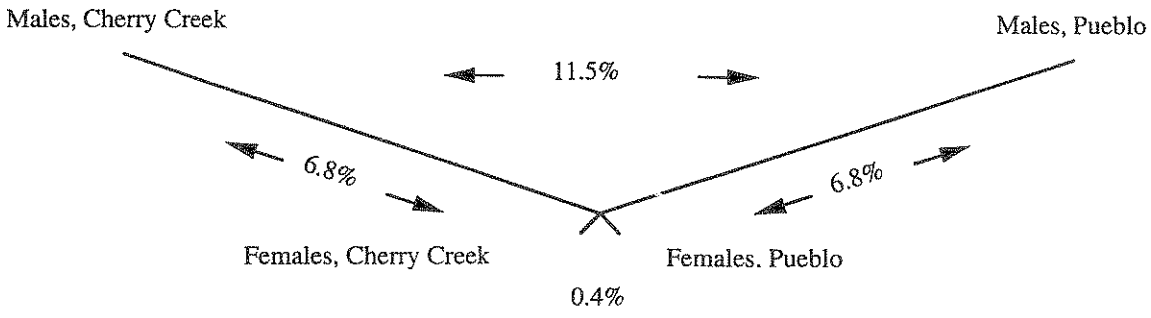

FIG. 4. Mitochondrial DNA sequence divergence between sexes and localities.

TABLE 2. Allele frequencies at three polymorphic loci in the populations of *Anodonta grandis grandis* from Pueblo Reservoir and Cherry Creek Reservoir.

Locus	Allele	Pueblo Reservoir (n = 12)	Cherry Creek Reservoir (n = 21)
IDH-1	100	0.500	0.976
	80	0.500	0.024
PGM-1	107	0.417	0.048
	100	0.583	0.952
PGM-2	100	0.375	0.905
	80	0.625	0.095

tween male and female mtDNA is 6.8% (Fig. 4). Separate maternal and paternal transmission and the lack of recombination between these systems has allowed mutations to accumulate, producing highly differentiated mtDNAs. The M- and F-types of mtDNA differ by 10–20% in the blue mussel, *Mytilus edulis* (Fisher and Skibinski 1990; Hoeh et al. 1991).

Allozyme polymorphisms, maternal mtDNA and paternal mtDNA reveal disparate degrees of genetic differentiation among populations of the giant floater. The differentiation revealed by allozymes and maternal mtDNA is slight, whereas the differentiation revealed by paternal mtDNA is great. The discrepancy in the magnitudes of differentiation can not be explained by differences in gene flow between males and females. One hypothesis to explain the disparate degrees of mtDNA differentiation focuses upon the modes of inheritance and the genetic environments within males and females. In the blue mussel, males carry both types of mtDNA in their somatic tissues, but females usually have only the F-type mtDNA. Thus, the F-type mtDNA must operate in isolation in females, but the M-type mtDNA works in the presence of the F-type mtDNA in males (Skibinski et al. 1994; Zouros et al. 1994). If this is also the case for *A. g. grandis,* selective constraints might be less restrictive for the M-type mtDNA, allowing faster evolution and greater differentiation. This hypothesis is consistent with high estimates of evolutionary rates in chloroplast DNA (cpDNA) from nonphotosynthetic species (Wolfe et al. 1992).

Although heteroplasmy for the M and F mitochondria is commonly found in the somatic tissues of male blue mussels (Fisher and Skibinski 1990; Hoeh et al. 1991; Zouros et al. 1992), we found no evidence of heteroplasmy for the M and F mtDNAs in our samples. However, heavy infestations of water mites in the mantle and gill tissues forced us to use only gonadal tissue for extraction of DNA. Because males preferentially package the M-type mitochondria into sperm, the exclusive use of gonadal tissue precluded detection of heteroplasmic males (Hurst and Hoekstra 1994; Skibinski et al. 1994; Zouros et al. 1994).

ACKNOWLEDGMENTS

We thank D. O. F. Skibinski, University of Wales, for kindly providing the mitochondrial DNA probe; A. Klein, University of New Hampshire, for technical advice; and S. Herrmann, University of Southern Colorado, for collecting specimens from the Pueblo Reservoir. We also thank R. Grosberg, two anonymous reviewers, B. Farrell, B. Latta, Y. Linhart, D. Olmstead, and T. Ranker for reading earlier versions of this manuscript and providing valuable suggestions. This work was supported by grants to H.-P.L. from the Department of Environmental, Population, and Organismic Biology, University of Colorado at Boulder, the University of Colorado Museum, the Western Society of Malacologists, the Southwest Shell Club, the Santa Barbara Shell Club and the San Diego Shell Club. This manuscript was prepared in the University of Georgias Savannah River Ecology Laboratory and supported by Contract DE-AC09-76SR00-819 between the U.S. Department of Energy and the University of Georgia Savannah River Ecology Laboratory.

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