Characteristics and Distribution of Large Tandem Duplications in Brook Stickleback (*Culaea inconstans*) Mitochondrial DNA

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ABSTRACT

Most animal mitochondrial DNAs (mtDNAs) range in size from 15 to 18 kb, but increased sizes up to ~40 kb are occasionally found. We investigated large size variation in mtDNA of the brook stickleback fish, *Culaea inconstans*, and characterized four large (2.7–5.8 kb) tandem duplications. Duplications differ in size, frequency of occurrence, and degree of associated heteroplasmy, but each includes the control region and one or more adjacent genes. Duplications are correlated with two mtDNA lineages sampled from 31 populations. L₁ duplications (3.2–4.8 kb) were present in all lineage I individuals ($n = 121$, 19 populations); 53 fish were heteroplasmic due to variation in the copy number of a tandemly repeated 270-bp sequence within the duplicated region. In contrast, duplications L₂, L₃, and L₄ (2.7–5.8 kb) occurred in only 117 of 174 lineage II fish, in eight of 14 populations. Nine fish with L₃ or L₄ duplications were heteroplasmic, possessing some mtDNAs that lacked duplications (normal-length mtDNAs). Heteroplasmy in L₂ was associated with a small variable region near the ND5 gene. Phylogenetic analysis of restriction sites in *Culaea* mtDNAs and haplotype-defining sequence differences present in both copies argue for multiple independent events that gave rise to three of the four duplications.

UNLIKE mitochondrial genomes in plants, protists, and fungi, most metazoan mitochondrial DNAs (mtDNAs) are small, genetically compact, and relatively uniform in size (~15–18 kb) (BORST and GRIVELL 1981; ATTARDI 1985; BROWN 1985). Although gene arrangements are variable among metazoans, a particular arrangement appears to be relatively conserved within major metazoan groups (e.g., vertebrates) and gene content is generally the same in all animals (reviewed by GRAY 1989; MORITZ *et al.* 1987; WOLSTENHOLME 1992; BOORE and BROWN 1994).

Intraspecific mtDNA size variation occurs but is usually small (<1 kb) and is usually confined to noncoding portions of the mtDNA, such as the control region in vertebrates (reviewed by BROWN 1985; HARRISON 1989; MORITZ 1991a). Larger size variation also occurs, but much less frequently in metazoan mtDNAs. Tandem duplications of the control region and immediately adjacent structural genes have been reported in newts (WALLIS 1987), lizards (MORITZ and BROWN 1986, 1987; MORITZ 1991a), and fish (GACH and REIMCHEN 1989; RICHARDSON and GOLD 1991), often in polyploid or parthenogenetic species (MORITZ and BROWN 1987; MORITZ 1991b). We report here the widespread occurrence of large tandem duplications within a diploid fish, *Culaea inconstans* (Gasterosteidae). We have characterized the structure, genetic organization, and geographic and phylogenetic distribution of the duplica-

tions in hopes of inferring their mechanisms of origin and of assessing any evolutionary changes that have occurred.

MATERIALS AND METHODS

Population sampling: We analyzed 295 fish from 31 localities representing 22 drainages. Minnow traps and seines were used to capture 275 individuals from 20 drainages between 1986–1992 (Table 1). Samples from Minnesota and Wisconsin ($n = 10$) were obtained from bait dealers whose stock was locally collected (Wisconsin) or when the identity of the trapper was known (Minnesota) and the collection site could be confidently inferred. Alberta fish ($n = 10$) were second generation crosses of local populations.

Preparation and analysis of mtDNA: Mitochondrial DNA was prepared from a combination of fresh heart, liver, spleen, muscle and gonadal tissues by differential and equilibrium cesium chloride gradient centrifugation (BROWN 1980; DOWLING *et al.* 1990). Single and double restriction enzyme digests were performed according to suppliers' directions (Bethesda Research Labs, Boehringer-Mannheim, and New England Biolabs), except that all incubations were at 37°. Digests were end-labeled at room temperature or at 37° with ³²P-dNTPs using the large "Klenow" fragment of *Escherichia coli* DNA polymerase (BROWN 1980). Component DNA fragments were resolved by overnight electrophoresis on 0.8–1.2% agarose and 3.5–5% polyacrylamide vertical gels using 1× TBE buffer (DOWLING *et al.* 1990) and visualized by autoradiography (BROWN 1980). Size standards consisting of lambda phage DNA digested with *Hind*III and phiX 174 RF DNA digested with *Hae*III were included in one or two lanes of each gel.

Mitochondrial genome size was estimated by averaging sums of fragment lengths produced by single restriction endonuclease digests. All samples were digested with *Eco*RI and *Hind*III to assay mtDNA lineage and size. A subset of samples from each size class was digested with additional enzymes to

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TABLE 1
Sampling localities

Designation	Drainage	Locality
Alberta	Hudson Bay	Edmonton, AL, Canada
Quebec	St. Lawrence River	Gatineau Park, QU, Canada
Iowa	Mississippi River	Dickinson Co., IA
Michigan-1	Lake Huron	Alpena Co., MI
Michigan-2	Lake Michigan	Emmet Co. (Pellston)
Michigan-3	Lake Michigan	Emmet Co. (Mackinaw City)
Michigan-4	Lake Superior	Chippewa Co.
Michigan-5	Lake Superior	Luce Co.
Michigan-6	Lake Michigan	Alger Co.
Michigan-7	Lake Superior	Marquette Co.
Michigan-8	Lake Superior	Gogebic Co.
Michigan-9	Lake Michigan	Iron Co. (Paint River)
Michigan-10	Lake Erie	Washtenaw Co. (Saline River)
a		Fosdick Rd.
b		Grass Rd.
c		Textile/Zeeb Rds.
d		Willis Rd.
Michigan-11	Lake Erie	Washtenaw Co. (Huron River)
a		Jackson/Dancer Rds.
b		Cobb Rd.
c		Kaiser Rd.
d		George Reserve
Michigan-12	Lake St. Clair	Macomb Co.
Michigan-13	Lake Erie	Oakland Co.
Michigan-14	Lake Huron	Iosco Co.
Michigan-15	Lake Michigan	Roscommon Co.
Michigan-16	Lake Michigan	Kalkaska Co.
Minnesota	Mississippi River	St. Paul, MN
Minnesota	Mississippi River	Big Fork
New Mexico	Canadian River	Merrick Lake, NM
Ohio	Lake Erie	Medina Co., OH
Pennsylvania	Lake Erie	Crawford Co., PA
Wisconsin	Mississippi River	Minong, WI

further characterize size variation. Error of the size estimates was calculated to be $\leq 6\%$ based on repeated measurement of size standards.

Transfer-hybridization: To determine whether the sequences responsible for length variation contained structural genes, probes containing portions of known gene sequences were hybridized to *Culaea* mtDNA immobilized on nylon membranes (GeneScreen Plus, Dupont), following procedures described by HOEH *et al.* (1991). After digestion and gel electrophoresis, DNA was transferred to nylon membranes and hybridized with random-primed, ^{32}P -labeled mtDNA sequences in 0.36 M NaCl, 0.02 M sodium phosphate, 0.02 M EDTA, 7% SDS, 0.5% nonfat powdered milk. Membranes were washed four times in $2\times$ SSC (0.3 M NaCl, 0.03 M sodium citrate pH 7.0), 1% SDS at 65° for 5–10 min, followed by two washes with $0.1\times$ SSC, 0.1% SDS at 65° for 45 min, then exposed to X-ray film for 1–7 days at -70° . Before hybridization with the next probe, filters were stripped of the previous probe with successive washes of boiling $0.1\times$ SSC, 0.1% SDS.

Gene sequences used to probe the immobilized mtDNA

FIGURE 1.—Comparison of small and large mtDNAs illustrating fragment patterns of enzymes with zero (I), one (II), and two (III) recognition sites within the duplicated region. *Hind*III (I), *Eco*RI (II), and *Bsp*HI (III) digests of small (lanes 1, 3 and 5) and large (L_4 ; lanes 2, 4, and 6) mtDNAs. The duplication is seen in lane 2 as an increase in the largest band from 8.6 to 11.3 kb, in lane 4 as a novel 2.7-kb band, and in lane 6 as a stoichiometric doubling of the 1.9-kb fragment and a novel 0.8-kb band. M, *Hind*III-digested lambda DNA size standard (in descending order, 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.56 kb).

included whole cloned mtDNA from threespine stickleback fish, *Gasterosteus aculeatus*, a cloned 1.4-kb fragment of gorilla mtDNA containing the 12S rRNA, valine tRNA and part of the 16S rRNA genes (HIXSON and BROWN 1986), and PCR-amplified portions of the 12S rRNA (450 bp), 16S rRNA (600 bp), and cytochrome *b* (307 bp) genes from *Culaea*. Amplification conditions were generally as described by INNIS and GELFAND (1990), using 5 μl of purified mtDNA template and 1.5 units of Taq DNA polymerase per sample. Amplifications were performed for 30 cycles at 94° (1 min), 50° (2 min), and 72° (3 min). Primers used to amplify gene sequences for cytochrome *b* are given in KOCHER *et al.* (1989), for 12S rRNA in PALUMBI *et al.* (1991), and for 16S rRNA in GACH (1993).

Restriction mapping of size variation: mtDNA restriction sites were mapped using single and double digests of the following 17 enzymes: *Acl* (A), *Bam*HI (B), *Bcl*I (C), *Nde*I (D), *Eco*RI (E), *Bsp*HI (F), *Apa*LI (G), *Hind*III (H), *Bgl*II (L), *Sma*I (M), *Nco*I (N), *Pvu*II (P), *Ssa*II (S), *Bst*II (T), *Mlu*I (U), *Xho*I (X) and *Nhe*I (Y). An approximate alignment of the restriction map with the standard vertebrate gene order map

FIGURE 2.—Transfer-hybridization experiments showing hybridization of whole mtDNA and 12S rRNA gene sequences to mtDNA fragments. *Hind*III (H), *Eco*RI (E) and *Sst*II (S) digests of small (lanes 2, 4, and 6) and large (L_2 ; lanes 1, 3, and 5) mtDNAs. M, *Hind*III-digested lambda DNA. Duplication-associated fragments are indicated by arrows. (A) Hybridization of total *G. aculeatus* mtDNA to *Culaea* mtDNA digests. (B) Hybridization of PCR-amplified 12S rRNA gene sequences from *Culaea* to the same filter. Note hybridization of probe to the conserved 1.7-kb *Sst*II (*Sac*II) fragment.

(BROWN 1985; JOHANSEN *et al.* 1990) was determined using the two highly conserved *Sst*II (= *Sac*II) sites present in the ribosomal genes (CARR *et al.* 1987), along with the loci of hybridization of 12S rRNA, 16S rRNA, and cytochrome *b* gene sequences to *Culaea* mtDNA.

Localizing restriction sites on large mtDNAs was difficult because restriction fragments associated with size variation could not always be positively identified in the absence of standard-length mtDNAs. This was overcome by mapping mtDNA from individual fish with multiple duplication size classes (heteroplasmic individuals), or by combining mtDNA samples with different duplication size classes to create heteroplasmic samples. Both approaches permitted stoichiometric distinction of duplication-associated fragments, which were always fainter than other bands from the same digest and varied in length as predicted by duplication size.

Densitometry: The intensity of bands resulting from digestion of mtDNAs with *Nhe*I was quantified by densitometry to test for a correlation between the number of small fragments and the sizes of L_1 . Exposed autoradiographic films were scanned with an LKB Ultrosan XL Laser Densitometer. Band intensity was estimated from the area under corresponding densitometric peaks, determined by integration methods using the LKB 2400 Gelscan software package.

Quantification of divergence: Sequence divergence was estimated from the proportion of restriction sites shared be-

tween mtDNAs using Equations 21 and 28 of NEI and TAJIMA (1983).

RESULTS

Physical characteristics of *Culaea* mtDNA: Of 295 mtDNAs examined, 57 were 17.4 kb long, while 238 were larger (range 20–24 kb). To exclude the possibility that this observation resulted from incomplete enzymatic digestion, aliquots of the larger sized samples were digested with a suite of enzymes. Estimated mtDNA size for a given sample was always the same (within the expected 6% measurement error), indicating that incomplete digestion had not produced artificially elevated size estimates. When digests of large and standard-length mtDNAs are compared (Figure 1), the fragment profiles are identical except that large mtDNAs display either (1) a single fragment larger than the comparable small fragment (I, Figure 1), (2) an additional fragment, equal in length to the difference between the large and small fragments in (1) (II, Figure 1), or (3) a band of doubled intensity and a novel fragment, the summed size of which equals the novel fragment size observed in (2) (III, Figure 1). Taken together, these observations are consistent with the presence of a single, tandemly repeated sequence. The three patterns (I, II and III, above) are produced by enzymes recognizing zero, one, or two sites, respectively, within a tandemly duplicated sequence (MORITZ and BROWN 1987).

Some individuals in two localities displayed further modifications of the above fragment profiles: (1) fragments normally 3–4 kb larger than those in small mtDNAs were larger by ~7 kb, (2) novel bands were twice as dense as in other samples, and (3) comigrating (double) bands increased in intensity by 50% as measured by densitometric analysis. These observations suggested the presence of a third tandemly replicated copy. All fragments observed in digests of large *Culaea* mtDNAs, including novel fragments produced by enzymes cleaving within the additional sequences, hybridized with whole *G. aculeatus* mtDNA (Figure 2), indicating that the added sequences were due to duplication of standard mtDNA sequences and not to incorporation of exogenous DNA (Figure 2). *G. aculeatus* mtDNA is 16.6 kb long and lacks the additional sequences observed in *Culaea* (GACH and REIMCHEN 1989; O'REILLY *et al.* 1993).

Physical characteristics of duplications: Restriction mapping revealed four distinct tandem duplications, designated L_1 – L_4 (Table 2, Figure 3). The sizes of the duplicated regions ranged from 2.75 kb (L_4) to 5.6 kb (L_2). Larger mtDNAs contained longer duplicated sequences, except in L_1 , for which the endpoints were identical, regardless of mtDNA size.

No variation in the size of L_3 or L_4 was seen within individuals or populations, but incremental variation of ~200 bp was observed with L_2 , which encompasses a region of minor length variation in mtDNA lineage II,

TABLE 2
Restriction endonuclease characterizations of duplicated sequences

	Size variant			
	L ₁	L ₂	L ₃	L ₄
Size (kb)	3.2–5.1	5.5–5.8	3.8	2.7
Frequency	121/121	37/37	41/51	38/49
Heteroplasmy	53/121	7/37	1/41	8/38
<i>AclI</i>	0.25, 0.60, 2.7–3.9 ^a	0.86, 4.6–4.9	3.8	2.7
<i>ApaI</i>	3.2–5.1	5.5–5.8 ^b	1.27, 2.5	1.27, 1.46
<i>BamHI</i>	I	I	I	I
<i>BclI</i>	I	5.5–5.8	3.8	I
<i>BglII</i>	I	I	I	I
<i>BspHI</i>	I	5.5–5.8 ^{a,b}	1.9, 1.8 ^a	1.9, 0.8 ^a
<i>BstEII</i>	I	5.5–5.8 ^a	3.8 ^a	2.7 ^a
<i>EcoRI</i>	3.2–5.1	5.5–5.8	3.8	2.7
<i>HindIII</i>	3.2–5.1 ^a	5.5–5.8	I	I
<i>MluI</i>	No cuts	5.5–5.8 ^a	I ^a	I ^a
<i>NcoI</i>	I	I	I	I
<i>NdeI</i>	I	5.5–5.8 ^a	3.8 ^a	2.7 ^a
<i>NheI</i>	(3.1), 3.4, ~0.27 ^a	1.5, 2.5, 1.5–1.8 ^a	3.8	2.7
<i>PvuII</i>	3.2–5.1	I	I	I
<i>SmaI</i>	3.2–5.1 ^a	5.5–5.8	3.8	I
<i>SstII</i>	3.2–5.1	1.7, 3.8–4.1	1.7, 2.1	2.7
<i>XhoI</i>	I	5.5–5.8 ^b	I	I

The absence of novel, duplication-associated fragments is indicated by I (e.g., see *BamHI*); the presence of novel and doubled (in italics) fragments in kb. Frequency is the proportion of the samples of a given size variant over all populations in which that size variant was observed. Heteroplasmy is the proportion of the samples with multiple duplication sizes (L₁) or with both large and normal-length genomes (L₂, L₃, L₄).

^a Site gain.

^b Site loss.

presumably in the vicinity of ND5 and ND6. Variation in the size of L₂ occurred in this region of the mtDNA, resulting in size differences within and among individuals and populations.

L₁ differed in size among populations, among individuals, and within individuals in discrete increments of ~270 bp (Figure 4), suggesting the presence of a variable number of small, tandemly repeated sequences within one or both copies of the duplication. The possibility that additional tandem repeats cause the incremental variation was investigated by further restriction mapping. Variation was observed in the novel fragments produced by all enzymes except *NheI* (Figure 4), which produced a more complex pattern. All samples digested with *NheI* display a novel 3.4-kb fragment that is cleaved only by those enzymes with recognition sites in the duplicated region, e.g., *EcoRI* and *PvuII*, suggesting that the fragment is associated with the duplication. Additionally, stoichiometric variation occurs in an ~270-bp *NheI* fragment, and individuals with larger duplications have more copies of this 270-bp fragment (Figure 4, Table 3). Slight length variation among duplications is mirrored by length variation in this *NheI* fragment, from 240 to 280 bp among individuals. These observations and the L₁ restriction map (Figure 5, Table 4) are consistent with a model of size variation in which L₁ in-

cludes a region with a tandemly repeated, ~270-bp DNA sequence containing an internal *NheI* site (Figure 6). Larger L₁'s have more copies of this repeated sequence, in some of which small additions and/or deletions have also occurred.

The presence of an additional 3.15-kb *NheI* fragment in two samples (Figure 4, *NheI* lanes 1 and 8) is puzzling. This fragment was also cleaved only by digestion with enzymes with recognition sites within L₁, suggesting that it is associated with the duplication. The sum of *NheI* fragment sizes from these two digests is 24 kb, which is 3 kb larger than other enzymatic digests of the same samples. Because the 3.4- and 3.15-kb fragments differ in size by an amount equal to one copy of the small repeated segment, the fragments may be derived from one another by gain and loss of one copy of the small repeated sequence or an *NheI* site within an external copy of the small repeated sequence. We can presently offer no convincing explanation for the apparent stoichiometric equality of the two fragments, although nonstoichiometric labeling may be a cause (BROWN 1980).

Gene content and location: Gene content of the duplicated regions was examined by a combination of restriction mapping and transfer-hybridization experiments. Probes containing cytochrome *b* gene sequences

FIGURE 3.—Diagrammatic alignment of duplicated regions with the linearized cleavage map of *C. inconstans* mtDNA and with the canonical vertebrate gene order map. --- indicate possible extensions of duplications past known restriction sites. Only restriction sites common to all individuals sampled are shown. Size variation in lineage II mtDNAs responsible for size variation in L_2 is indicated as a sawtooth line in the region of ND6.

hybridized to novel fragments in individuals with L_1 and L_2 . Probes with 12S rRNA gene sequences hybridized to novel fragments in individuals with L_2 , L_3 and L_4 . Finally, probes with 16S rRNA sequences hybridized to novel fragments in all individuals with L_2 and L_3 . All probes also hybridized to some fragments present in all individuals, including those with standard-length genomes. Restriction mapping indicates that all of the duplications span the control region and some portion of the 12S rRNA gene. In addition, L_1 and L_2 contain several other genes, including ND5, ND6, and cytochrome *b*.

Geographic distribution: The four duplications have differing geographic distributions (Figure 7). L_1 is ubiquitous throughout the northern range of *Culaea*, with isolated occurrences in two drainages in southern Michigan. L_2 was obtained only from two adjacent drainages south of Lake Erie. L_3 and L_4 are each confined to single drainages in southern Michigan. Duplications are fully correlated with two major mtDNA lineages (Table 5) that differ in sequence by an estimated $6.4 \pm 1.26\%$ (GACH 1996); L_1 occurs only in lineage I individuals and L_2 , L_3 , and L_4 only in lineage II individuals.

Heteroplasmy: The frequency of individuals with two or more size classes of mtDNA (size heteroplasmy) varied among duplications and lineages (Table 5). In lineage II, heteroplasmy was infrequently associated with L_2 , L_3 , and L_4 . Nine of 117 fish had normal-length mtDNAs coexisting with L_3 or L_4 . Heteroplasmy associated with L_2 was, in contrast, due to the internal length variation discussed above; no fish with L_2 also contained normal-length mtDNA. In lineage I, all individuals had mtDNAs with L_1 , along with a much higher frequency of size heteroplasmy (Table 3). Each of the 53 hetero-

plasmic samples contained two or more duplication size classes, presumably produced by variation in the copy number of the 270-bp repeat discussed above. Size heteroplasmy has been observed in mtDNAs from a wide array of organisms (reviewed in WOLSTENHOLME 1992) and has usually been attributed to variable copy number of small, tandemly-repeated sequences located within or directly adjacent to the control region. If the inferred alignment of the *Culaea* restriction map with the standard vertebrate genetic map is correct, the tandemly repeated sequence responsible for variation in L_1 is noteworthy in occurring outside the control region (ÁRNASON and RAND 1992).

DISCUSSION

Duplications of the size and nature of those encountered in *Culaea* are infrequent among animal mtDNAs. This brings to seven the number of studies documenting large tandem duplications in animal mtDNA (see MORITZ 1991a; RICHARDSON and GOLD 1991; GJETVAJ *et al.* 1992).

Duplication formation: The duplications in *Culaea*, like those described in other vertebrate mtDNAs, contain the control region and immediately adjacent genes, and reinforce the inference that this region is especially vulnerable to such events (*e.g.*, MORITZ and BROWN 1987; STANTON *et al.* 1994). Sequences essential to the initiation and regulation of mtDNA replication are found in the control region. During mtDNA replication, the control region and immediately adjacent sequences remain single-stranded for a lengthy period of time (reviewed by CLAYTON 1992). Perturbation of the

FIGURE 4.—Variation in size and stoichiometry of the L_1 duplication in eight individuals. (A) 1.2% agarose gel; *HindIII* and *NheI* digests of the same eight samples; lane 9, *HindIII*-digested lambda DNA size standard. Arrows indicate duplication-associated fragments in both digests. The novel 3.2-kb fragment in *HindIII* lane 7 corresponds to the absence of two smaller fragments and is due to a site loss in this individual. Substoichiometric bands in *HindIII* lanes 2, 4, 5, and 8, and the size variation among samples in the *HindIII* digests are notably absent in *NheI* digests. All duplication-associated fragments were determined by restriction mapping. (B) Portion of the corresponding 5% polyacrylamide gel showing the smallest (240–270 bp) *NheI* fragment. Size variation occurs among individuals, and stoichiometric variation is coincident with duplication size variation visible in A (see especially *NheI* lane 8).

replication process could give rise to tandemly duplicated sequences, through replication slippage and mispairing between original and nascent DNA strands (LEVINSON and GUTMAN 1987). Replication slippage may also be the mechanism responsible for change in copy number of the internal repeated sequence in L_1 and would be consistent with the frequent length heteroplasmy that we and others have observed (CASANE *et al.* 1994).

Secondary structure is a second possible factor that may be involved in generating mtDNA duplications. MORITZ and BROWN (1987) noted that many lizard (*Cnemidophorus*) mtDNA duplications terminated near tRNA genes (within resolution limits of restriction site analysis) and hypothesized that secondary structures formed by adventitious folding of single-stranded tRNA genes during mtDNA replication might promote duplication. Patterns of endpoint location in another lizard (*Heteronotia*) and in *Culaea* also support this hypothesis. Duplications in *Heteronotia* display substantial association of endpoints with tRNA genes (MORITZ 1991a), and six of 10 *Heteronotia* duplications terminate within a 0.3-kb region of the ND1-ND2 gene boundary, which encompasses three tRNA genes. In *Culaea*, as many as five of the eight duplication endpoints may coincide with tRNA genes. While some endpoints may be shared through common ancestry [*e.g.*, L_3 , L_6 and L_8 in *Heteronotia* (MORITZ 1991a); L_3 and L_4 in *Culaea* (see below)], the action of some common factor in determining endpoint formation must be considered. The association of all duplication endpoints with stem-and-loop structures in *Cnemidophorus* (STANTON *et al.* 1994) and the frequent association of duplication endpoints with tRNA genes (which contain such structures) found in this and other studies strongly suggests that stem-and-loop structures are important in the duplication process.

The possible role of hybrid parthenogenesis in the formation of duplications has been considered in lizards. MORITZ and BROWN (1987) found that duplication frequency in *Cnemidophorus* was weakly correlated with triploidy, but not with hybridity. Duplications in hybrid triploid *Heteronotia* were equally frequent in animals possessing one or two haploid sets of nuclear chromosomes from the mtDNA donor species (C. MORITZ, personal communication). *Culaea* is neither parthenogenetic nor polyploid (CHEN and REISMAN 1970), although the independent occurrence of three large tandemly repeated sequences in two Michigan populations subject to faunal introductions (J. MERNA and R. BAILEY, personal communication) reprises hybridity as a possible causative factor of length variation in this species. Interactions between mtDNA and nuclear gene products are a fundamental aspect of mtDNA replication and transcription (CLAYTON 1982, 1984; CHANG and CLAYTON 1987) and a hybrid system may increase the error rate of some processes and lead to duplication. Regardless of how the original duplications arose, the presence of three repeat copies only in putative hybrids suggests that nucleocytoplasmic disruptions may be involved in length variation.

The high frequency of duplications in *Culaea* indicates conditions that predispose this fish to mitochondrial size variation. Hypotheses for this phenomenon encompass cellular, physiological and ecological mechanisms. Unique physiological or biochemical properties may predispose *Culaea* to mtDNA duplication forma-

TABLE 3

Expected and observed measurements of 270-bp *NheI* fragment intensity

Sample	Density ratio			
	Trial		Average	Expected
	1	2		
1	1.26	1.25	1.26	1.28
2	0.73	0.84	0.78	1.00
3	2.64	2.37	2.51	2.36
4	2.38	1.67	2.03	2.02
5	2.33	1.89	2.11	2.00
6	2.09	2.31	2.20	2.12
7	7.78	9.66	8.72	8.29

Density ratio = relative intensity (absorbance units $\times$ height) of 270-bp fragment band when compared to a 1.2-kb fragment band in the same lane of a 5.0% polyacrylamide gel. Expected values were calculated as $\sum p_i/n$ where p_i = relative frequency of duplication size class i , and n = predicted copy number of the 270-bp fragment based on observed duplication size. Copy number was predicted by sizing the variable fragment observed in all Type II digests (3.4 kb = 1 copy, 3.6 kb = 2, 3.9 = 3, etc.). An additional 0.5 copies were included in sample 7 to account for the presence of a novel 3.15-kb *NheI* fragment present at 50% intensity. Samples correspond to lanes 2–8 in Figure 4. Spearman's rank correlation coefficient $r_s = 0.964$ ($P < 0.01$) for expected and observed copy number.

tion. Duplications may have arisen due to secondary contact between the two lineages indicated by mtDNA; perhaps they are the legacy of an ancient hybridization event that continues to disrupt processes of mtDNA replication. Alternatively, there may be a selective advantage in possessing multiple copies of genes associated with cellular respiration (for example, our observations suggest that *Culaea* appears to be physiologically tolerant of very low oxygen levels). Whatever the reason, our limited sampling of related taxa indicates a much lower frequency of duplication events in other members of the Gasterosteidae.

Duplication age and origin: Animal mtDNA duplications are considered to be evolutionarily ephemeral and to have had recent, multiple origins in both *Heteronotia* and *Cnemidophorus* (MORITZ and BROWN 1987; MORITZ 1991a,b). Two separate lines of evidence, one based on sequence modification within the duplication and the other on phylogenetic analysis, address similar questions of age and multiple origins in *Culaea* duplications. Sequence divergence in either or both copies of the duplicated region can indicate the age of a duplication event. Mutations will accrue independently in each copy, resulting in sequence differences if there is no mechanism for copy correction. Although small tandem repeats may be subject to concerted evolution (e.g., RAND 1994), large duplications appear to evolve independently (MORITZ and BROWN 1986; CANTATORE *et al.* 1987; MORITZ 1991a). Only Alberta samples (L_1)

FIGURE 5.—Cleavage map and model proposed to explain size variation in duplication L_1 . A 3.6-kb duplication is shown; letters and subscripts refer to Table 4. □ indicates 270-bp repeated sequences; ■ denotes the junction of the two duplicate sequences. Although three repeats are modelled, when Y_3 is lost, as is postulated for the majority of stickleback mtDNAs, digestion with *NheI* (Y) produces only a single 270-bp fragment.

displayed copy-specific site differences, suggesting that all four duplications are of recent origin.

All *Culaea* mtDNA duplications are similar in their general location and gene content. However, as noted above, duplications in other taxa are also similar in these respects, which does not provide evidence of common origin. Instead, independent origins of L_1 (in lineage I) and L_2 – L_4 (lineage II) are strongly suggested by the presence of lineage-defining sequence differences in both copies of the repeat. For example, *Acd*, *HindIII*, and *SmaI* sites that characterize lineage I in cytochrome *b* and the control region are also present in L_1 . No site variation in duplicated sequences was detected among haplotypes with L_1 , indicating a single origin for this duplication. Eight restriction site differences distinguish populations with L_2 from those with L_3 and L_4 (GACH 1996). Four of these differences are within a duplicated region (Table 2) and are found in both copies of the repeated sequence, indicating separate origins of L_2 and ($L_3 + L_4$) variants. No restriction site variation was found between populations with L_3 and L_4 , suggesting that these duplications may be derived from a single origin (the apparent sequence divergence between L_3 and L_4 results from L_3 's greater length and the incorporation of an additional *SstII* recognition site). The polarity of evolution can be inferred from the number of mutation events required to produce L_3 from L_4 , and vice versa. If mtDNA is viewed as a string of letters, $xyzabcde$, where $a = 2.7$ kb and $b = 1.1$ kb, then $L_3 = xyzabcde$ and $L_4 = xyzaabcde$. Derivation of L_4 from L_3 requires only the deletion of b . The opposite derivation, of L_3 from L_4 , requires not only the duplication of b ($xyzaabbcde$) but its transposition to the junction between the duplicate copies ($xyzababcde$). Thus it is much more plausible from a parsimony perspective that the direction of evolution in this mtDNA lineage is shortened from L_3 to L_4 .

The pattern of duplication formation can also be considered within an evolutionary framework at the population level. When phylogenetic methods are used to generate hypotheses of population relationships, additional traits of interest can be superimposed on the

TABLE 4

Expected and observed fragment lengths generated by restriction endonuclease digestion of L_1 with *NheI* and additional restriction enzymes

Enzyme	Fragment	Fragment length (kb)	
		Predicted	Observed
<i>EcoRI</i>	E_1-Y_1	0.6	0.6
	E_2-Y_4	0.875	0.9
	Y_3-E_2	2.3	2.2
	Y_2-E_2	2.5	2.45
<i>SstII</i>	S_1-Y_1	0.5	0.57
	S_2-Y_4	0.7	0.84
	Y_3-S_2	2.4	No data
	Y_2-S_2	2.7	2.45
<i>HindIII</i>	H_1-Y_1	2.2	2.17
	H_2-Y_4	2.4	2.38
	Y_3-H_2	0.8	0.77
	Y_2-H_2	1.1	1.02
<i>PvuII</i>	P_1-Y_1	2.6	2.5
	P_2-Y_4	2.75	2.8
	Y_3-P_2	0.35	0.27
	Y_2-P_2	0.625	0.595
<i>ApaLI</i>	G_1-Y_1	1.15	1.15
	G_2-G_3	1.25	1.27
	G_3-Y_4	0.09	0.09
	Y_3-G_2	1.75	No data
	Y_2-G_2	2.0	1.85

Data pertain to the model of L_1 size variation. Abbreviations as in Figure 5.

resulting cladogram(s) to determine the number of times each trait was generated (FUNK and BROOKS 1990). Plotting duplication classes onto a network of *Culaea* populations grouped as haplotypes (Figure 8) reveals two patterns. The most parsimonious occurrence of L_1 is at the base of lineage I, since L_1 is present in all lineage I haplotypes and nowhere else. The remaining duplications occur in only two haplotypes within lineage II, and two competing hypotheses address their origin. Either an ancestral duplication appeared early in lineage II, followed by derivation and loss in some populations, or duplications arose independently in the Erie and Mich-C* haplotypes. The first (single-origin) hypothesis requires three steps, one gain and two losses, without considering the divergence of L_2 , L_3 , and L_4 . The second (multiple-origin) hypothesis requires only two steps and is preferable under a strict parsimony model. The multiple-origin hypothesis is also consistent with the restriction site modifications discussed above, in which haplotype-defining differences are present in both copies of the duplicated sequence. The possible origin of L_3 and L_4 from a single ancestral duplication is suggested by their presence in the same mitochondrial haplotype. Thus, L_1 likely arose as a single event, while the L_2 - L_4 group has probably been generated by at least two.

The present range of *Culaea* is thought to result from

FIGURE 6.—Expected restriction fragment patterns under the proposed model of L_1 size variation. Linearized mtDNAs with cleavage sites are depicted on the left, corresponding fragment patterns after restriction endonuclease digestion and electrophoresis are depicted on the right. (A) Enzymes with a single recognition site in the duplicated region produce a fragment pattern with incremental size differences among mtDNAs (arrow) containing additional copies of a small internal repeated sequence. The resultant fragment pattern is similar to *HindIII* digests in Figure 4. (B) Enzymes with a recognition site in the internal repeated sequence produce uniform fragment sizes, but with increasing density of the band containing a small fragment (arrow) corresponding to increasing numbers of the repeat, similar to *NheI* lanes in Figure 4.

habitat colonization and range expansion from several postulated refugia, following a period of extensive glaciation that ended 17,000–8000 years ago (BAILEY and SMITH 1981). The limited phylogenetic and geographic

FIGURE 7.—Geographic distribution of mtDNA duplications. L_1 is represented by Δ and $\blacktriangle$, L_2 by $\blacksquare$, L_3 and L_4 by $\bullet$. $\circ$ indicate populations with normal-length mtDNAs.

TABLE 5

Sample size, mtDNA lineage and duplication parameters for *Culaea inconstans* populations. Population designations as in Table 1

Locality	Sample size	Lineage	Class	Frequency	Duplication parameters	
					Heteroplasmy	
					Size ^a	Presence ^b
Alberta	10	I	L ₁	10	1	0
Quebec	9	I	L ₁	9	0	0
Iowa	4	I	L ₁	4	0	0
Minnesota	4	I	L ₁	4	2	0
Wisconsin	6	I	L ₁	6	4	0
Michigan						
1	13	I	L ₁	13	6	0
2	3	I	L ₁	3	2	0
3	5	I	L ₁	5	4	0
4	13	I	L ₁	13	10	0
5	18	I	L ₁	18	8	0
6	5	I	L ₁	5	1	0
7	6	I	L ₁	6	0	0
8	6	I	L ₁	6	4	0
9	1	I	L ₁	1	0	0
10						
a	49	II	L ₄	38	0	8
a ^c	2	I	L ₁	2	2	0
b	2	I	L ₁	2	0	0
c	1	II	L ₄	1	0	0
d	1	II	L ₄	1	0	0
d	6	I	L ₁	6	3	0
e	6	I	L ₁	6	5	0
11						
a	20	II	L ₃	20	0	0
b	17	II	L ₃	17	0	0
c	13	II	L ₃	3	0	1
d ^c	6	I	L ₁	6	1	0
12	7	II	—	—	—	—
13	7	II	—	—	—	—
14	4	II	—	—	—	—
15	6	II	—	—	—	—
16	6	II	—	—	—	—
Ohio	18	II	L ₂	18	0	0
Pennsylvania	19	II	L ₂	19	7	0
New Mexico	6	II	—	—	—	—

^a Number of individuals with more than one size class of duplication.

^b Number of individuals with duplications which also had normal-length mtDNA.

^c Triplication in one or more individuals.

distributions of L₂, L₃, and L₄ strongly suggest that they have arisen since that time. The widespread distribution of L₁ indicates that it originated before postglacial colonization or that recent and potentially rapid colonization of postglacial habitat has occurred by populations carrying L₁.

Evolutionary considerations: The striking disparities in duplication frequency and incidence of heteroplasmy between L₁ and L₂–L₄ suggest that they are governed differently by evolutionary forces. L₂–L₄ behave similarly to most duplications described in other taxa in displaying little heteroplasmy, variable endpoints, and in occurring at a low frequency. Of pre-

viously described large length variants, only those in *Cnemidophorus cozumela* (MORITZ and BROWN 1987) and a subgroup of *Heteronotia* distinguished by extensive mtDNA sequence divergence (MORITZ 1991a) compared in frequency with L₁, with large genomes in every animal. Differences in ecology or in nucleotide sequence between *Culaea* mtDNA lineages I and II would seem unlikely to cause different frequency and distribution patterns of mtDNA duplications. One possibility is that the L₁ and L₂–L₄ frequency and distribution differences are due to endpoint-associated structures that confer different levels of stability on the duplicated region.

FIGURE 8.—Phylogenetic hypothesis of population relationships and evolution of length variants within *Culaea*. A cladogram of haplotype relationships was constructed under maximum parsimony (SWOFFORD 1990) using the presence/absence of 94 mtDNA restriction sites as characters (GACH 1996). Two related gasterosteid fishes, *G. aculeatus* and *Pungitius pungitius*, were included as outgroups. Population designations follow Table 5 and below: Iowa (Iowa, Mich 10a–d); Erie (Ohio, Pennsylvania); Mich-A (Mich 16); Mich-B (Mich 12, 15); Mich-C* (Mich 10a, d); Mich-C (Mich 10a, 13, 14); Mich-N (Michigan 1–9, Wisconsin); *Gasterosteus*, *G. aculeatus*; *Pungitius*, *P. pungitius*.

The few instances of size heteroplasmy occurring in L_2 , L_3 and L_4 are confined to three populations. An unfixed duplication experiences frequency changes through drift (unequal reproduction of molecules) and mutation (generation or loss of duplications). Inheritance studies in stickleback (GACH 1993), cow (HAUSWIRTH and LAIPIS 1985), yeast (BIRKY 1991), and rabbit (CASANE *et al.* 1994) indicate that intracellular drift can be quite strong, especially during oogenesis and development. Mutation rate, if high, might also account for high duplication frequencies. One *Culaea* population, Mich-10a, offers strong evidence for mutation as a factor shaping the distribution of mtDNA duplications. In this population, L_4 and a novel *Hind*III restriction site occur in variable frequency within individuals. The *Hind*III site exists in both large and normal-length genomes, with and without L_4 (Figure 9). Intermolecular recombination is not thought to occur among metazoan mtDNAs (MORITZ *et al.* 1987; GRAY 1989), although the data addressing this question are neither direct nor conclusive. Therefore, either the restriction site or the duplication have incurred multiple mutation events (*e.g.*, KOEHLER *et al.* 1991).

Unlike *Cnemidophorus*, *Heteronotia*, and *Triturus*, no internal deletions were observed in *Culaea* mtDNA duplications. Deletion events in *Cnemidophorus* and *Heteronotia* point to the likely ephemerality of this type of length variation (MORITZ and BROWN 1987; MORITZ 1991a; ZEVEKING *et al.* 1991), supporting the suggestion of a selective advantage of small size (WALLACE 1982; SEDEROFF 1984; ATTARDI 1985; CLARK-WALKER 1985) through the faster replication of small molecules. (SOLIGNAC *et al.* 1984; RAND and HARRISON 1986). L_1 is a possible exception, given its high frequency and lack

FIGURE 9.—*Hind*III digest of Michigan-10a individuals displaying a novel *Hind*III site in both large and small mtDNAs. Lane 1, L_4 mtDNA; lane 2, L_4 mtDNA with novel site; lane 3, small mtDNA; lane 4, small mtDNA with novel site; lane 5, heteroplasmic individual with all four genomes. M, *Hind*III-digested lambda DNA size standard.

of secondary modifications. Inheritance data for L_1 and L_4 (GACH 1993) strongly suggest that *Culaea* mtDNA is not necessarily subject to selection for small size *per se*. Duplication of mtDNA segments has been proposed as a possible mechanism for evolutionary gene rearrangement (MORITZ and BROWN 1986, 1987; CANTATORE *et al.* 1987; PÄÄBO *et al.* 1991). Among vertebrates, birds deviate from the standard gene order by a single transposition (DESJARDINS and MORAIS 1990, 1991) and marsupials by rearrangement of the L-strand replication origin and surrounding five tRNAs (PÄÄBO *et al.* 1991), though no large duplications have been reported in any avian or marsupial species. Duplications or duplicated regions may evolve into pseudogenes (MORITZ and BROWN 1986, 1987; CANTATORE *et al.* 1987; ZEVEKING *et al.* 1991), become inverted, and alter their position in the molecule. No rearrangements were observed in *Culaea*, either by transfer-hybridization or by comparison with other stickleback genomes (GACH 1996).

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