J Mol Evol (1991) 33:152–155

Journal of Molecular Evolution

© Springer-Verlag New York Inc. 1991

Cloning and Characterization of *Daphnia* Mitochondrial DNA

D.J. Stanton,[1]* T.J. Crease,[1] and P.D.N. Hebert[2]

[1] Department of Biological Sciences, University of Windsor, Windsor, Ontario N9B 3P4, Canada
[2] Department of Zoology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Summary. The mitochondrial genome of *Daphnia pulex* (Crustacea, Cladocera) was cloned as a single fragment into the plasmid vector pUC12. The genome size, estimated from restriction endonuclease fragment lengths, is 15,400 ± 200 base pairs. The GC content, estimated from thermal denaturation studies, is 42%. The positions of 39 cleavage sites were mapped for 14 restriction enzymes. The distribution of these sites within the genome is random ($P = 0.44$). Heterologous hybridizations with *Drosophila sylvestris* mitochondrial DNA (mtDNA) probes indicate that gene orders within *Daphnia* and *Drosophila* mtDNAs are similar.

Key words: Crustacea — Cladocera — Restriction mapping — GC content — Genome size — Gene order

Introduction

Restriction fragment pattern (RFP) analysis of mitochondrial DNA (mtDNA) has become an extremely useful tool in population genetics. However, studies of mtDNA variability in natural populations of crustaceans have not been forthcoming. This is regrettable, given the ecological and economic importance of many of these species, as well as the confused state of crustacean phylogenetics and taxonomy (Schram 1983). Consequently, molecular cloning of *Daphnia pulex* (Crustacea, Cladocera) mtDNA was undertaken. Such a clone can be used as a hybridization probe for large scale population surveys and for the investigation of mitochondrial genome evolution within the Crustacea.

**Present address:* Department of Biology, University of Michigan, Ann Arbor, MI 48109, USA
Offprint requests to: D.J. Stanton

Materials and Methods

Mitochondrial DNA Extraction

A single parthenogenetic clone of *D. pulex* from Amana, Iowa, was reared in mass quantities. Mitochondrial DNA was extracted from approximately 100 g of *Daphnia* per extraction following procedures outlined in Lansman et al. (1981). Animals were washed, resuspended in grinding buffer, and homogenized on ice with a motor-driven Teflon pestle and glass sleeve. Cellular debris was pelleted by low-speed centrifugation. Intact mitochondria were recovered by centrifugation and purified on discontinuous sucrose gradients (Lansman et al. 1981). Mitochondria were lysed with SDS and phenol extracted. Mitochondrial DNA was ethanol precipitated and purified by CsCl/propidium iodide ultracentrifugation (Wright et al. 1983).

Cloning

Approximately 0.5 μg of purified pUC12 DNA (Vieira and Messing 1982) and 1.0 μg of purified mtDNA were cleaved with Bam HI according to manufacturer's specifications (BRL). Ligation was carried out using T4 ligase in a 50-μl reaction volume according to manufacturer's specifications (BRL). Twenty microliters of the ligation mixture were added to 200 μl of competent *Escherichia coli* (JM103) cells, prepared according to the procedure of Hanahan (1983). Transformation and screening of transformed cells was performed according to procedures outlined in Maniatis et al. (1982).

Plasmid Extraction

In order to confirm the identity of recombinant inserts, miniplasmid preparations were made from selected colonies according to the procedure of Klein et al. (1980). Confirmed recombinants were stored as glycerol stocks at −70°C. Large-scale plasmid preparations were performed according to the alkaline lysis procedure (Maniatis et al. 1982) to provide mtDNA for characterization. Restriction fragment patterns for the cloned mtDNA were compared to those obtained from purified *Daphnia* mtDNA in order to confirm that the entire genome had been cloned.

Characterization

Physical Mapping. Mitochondrial DNA (10 ng per digest) was digested according to manufacturer's specifications (BRL) with the restriction endonucleases listed in Table 1. Fragments thus generated were radioactively end-labeled, electrophoresed on agarose and acrylamide gels, and analyzed using the procedure of Wright et al. (1983). Lambda DNA cut with Hind III was used

Table 1. Number of restriction sites and genome size estimates

Enzymes			No. of sites		GC content				Size
No.	Name	Sequence	Observed	Expected	0	2	4	6	(kb)
1	Apa I	GGGCCC	4	1.3				4	15.4
2	Bam HI	GGATCC	1	2.5			1		15.4
3	Bgl II	AGATCT	8	4.8		8			15.4
4	BstE II	GGTNACC	2	2.5			2		15.4
5	Eco RI	GAATTC	5	4.8		5			15.4
6	Eco RV	GATATC	3	4.8		3			15.4
7	Pst I	CTGCAG	4	2.5			4		15.3
8	Pvu II	CAGCTG	1	2.5			1		15.5
9	Sca I	AGTTCA	4	4.8		4			15.4
10	Sma I	CCCGGG	1	1.3				1	15.6
11	Sst I	GAGCTC	2	2.5			2		15.4
12	Sst II	CCGCGG	1	1.3				1	15.2
13	Xba I	TCTAGA	2	4.8		2			15.4
14	Xho I	CTCGAG	1	2.5			1		15.5
15	Dra I	AAATTT	10	9.2	10				
	Sum		39	42.9	10	22	11	6	215.6
	Mean		2.79	3.07	10	4.4	1.8	2.0	15.4

The expected number of cut sites was calculated according to Lansman et al. (1981). The calculations assume a molecule 15.4 kb in length with a GC content of 42% and a random distribution of nucleotides. GC content refers to the number of G:C pairs in the recognition sequence of each enzyme. Size refers to the cumulative size of all fragments observed for each restriction endonuclease. The Dra I cut sites were not mapped and are excluded from all subsequent analyses

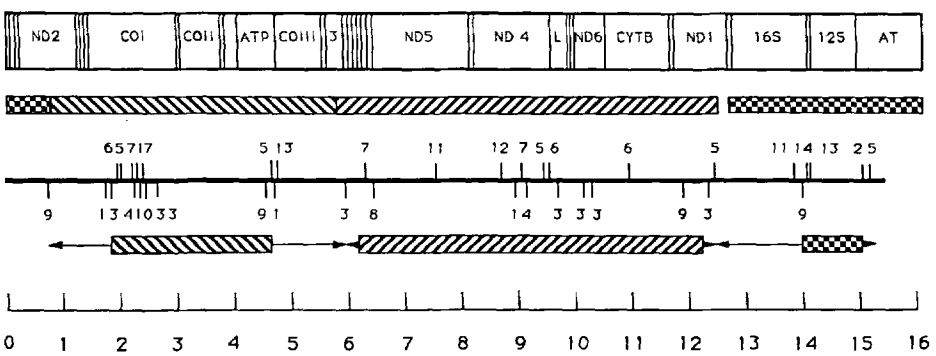

Fig. 1. Restriction map and hybridization. Genetic map drawn to scale from *Drosophila yakuba* (Clary and Wolstenholme 1985). Positions of tRNAs are shown as open boxes. Shaded areas represent *Drosophila sylvestris* probes (DeSalle et al. 1987). Restriction map is of *Daphnia pulex* cloned mtDNA. Number designations for restriction endonucleases are given in Table 1. Cut site positions are generally ±50 bp given the resolution of the mapping technique. *Daphnia* fragments to which probes hybridized are shown by shading. Arrows show that the probe hybridized to the fragment but the exact endpoint within the fragment is unknown as there are no restriction sites within the fragment. Scale shown is in kilobases.

as a molecular weight standard. Sizing of fragments was accomplished using the DNAGEL program (Kieser 1984) modified according to Grewe and Hebert (1987).

GC Content. The T_m value was measured using a Beckman DU-8 spectrophotometer with attached T_m module. Five micrograms of mtDNA were suspended in 300 μl of 1 × standard saline citrate (SSC: NaCl, 0.15 M; citric acid, 0.015 M, pH 7.0) and degassed. Absorbance values were automatically recorded at 260 nm as the temperature was raised from 25°C to 98°C at a rate of 0.5°C per min. Calf thymus DNA and salmon sperm DNA were used as reference standards, having known GC contents of 42% and 43%, respectively (Marmur and Doty 1962). The GC content was determined from the T_m value according to the conversion factor of Marmur and Doty (1962).

Genetic Mapping. Daphnia pulex mtDNA was cleaved with restriction endonucleases, electrophoresed in agarose, and transferred to nitrocellulose filters according to the procedure of Southern (1975). Three subclones of the *Drosophila sylvestris* mtDNA genome (DeSalle et al. 1987) were used as probes (Fig. 1). *Drosophila* mtDNA was nick-translated to high specific activity using the procedure of Rigby et al. (1977). Hybridization was carried out according to procedures outlined in Maniatis et al. (1982). Low stringency washes were used (SDS 0.1%, SSC 0.5×, 37°C, 60 min).

Results

Cloning

The entire *D. pulex* mitochondrial genome was successfully cloned as a single fragment in the pUC12 vector. However, yields of this recombinant plasmid were low, and glycerol stocks were unstable.

Table 2. The expected number of restriction sites

	GC content						
	0	2	4	6	χ^2	P	Significance
Observed	10.0	4.4	1.8	2.0	$\chi^2_{(0.05,3)} = 7.8$		
Expected							
GC = 34%	19.9	5.3	1.4	0.4	12.4	<0.01	**
GC = 42%	9.2	4.8	2.5	1.3	0.7	>0.80	NS
GC = 50%	3.7	3.7	3.7	3.7	12.6	<0.01	**

P is the probability that the observed deviation from the expected ratio can be attributed to chance. The level of significance: NS = not significant; ** = significant at the 0.01 level. The mean of the observed number of sites for each class was taken from Table 1. The expected number of sites for each class was calculated as in Table 1, using the GC contents shown

For this reason, clones were constructed using Pst I sites and the methods outlined above. Three subclones were obtained that represent all but 200 base pairs of the entire mitochondrial genome. These subclones are substantially more stable than the Bam HI clone.

Characterization

Physical Mapping. The size of the *D. pulex* mitochondrial genome, determined as the consensus length of all restriction fragment patterns (Table 1), was found to be 15.4 ± 0.2 kb. Cleavage site positions were determined by the double digest procedure (Fig. 1). A total of 39 sites were mapped for 14 enzymes with an average of 2.79 cleavage sites per enzyme (Table 1). The cleavage sites were analyzed to determine if they were randomly distributed (Watson 1961), using the computer program of Adams and Rothman (1982). When cleavage sites were pooled, they were found to be randomly distributed throughout the genome ($P = 0.44$).

GC Content. The T_m value was 87°C, which corresponds to an estimated GC content of 42%. The average number of cleavage sites observed was in close agreement with the number expected based on this GC content (Table 1). The ratio of the number of cleavage sites observed for enzymes with differing proportions of GC base pairs in their recognition sequences was compared to the expected ratio by χ^2 analysis. There was close agreement between the observed ratio and the ratio expected for a GC content of 42%. However, the ratio was significantly different from that expected for a GC content of 34% or 50% (Table 2).

Genetic Mapping. Drosophila sylvestris mtDNA probes were found to hybridize to various fragments of *Daphnia* mtDNA (Fig. 1). The exact boundaries of the coding regions are unknown, due to lack of resolution in the physical map and the large number of coding regions within each probe. However, the inferred gene positions are consistent with the gene order observed in *Drosophila yakuba* (Clary and Wolstenholme 1985), but are inconsistent with that observed in humans (Anderson et al. 1981).

Discussion

Genome Size

Animal mtDNA displays extreme size economy in general (Borst and Grivell 1981). Even given this economy, the size of the *D. pulex* mitochondrial genome is near the lower limit of the range reported for animals. It is unlikely that this is due to the elimination of coding regions, as all animal mtDNAs, except those in nematodes (Wolstenholme et al. 1987), contain the same coding regions. However, in different species of *Drosophila* the size of the AT-rich region is seen to covary with total genome size (Fauron and Wolstenholme 1976, 1980a,b). The small size of the *Daphnia* mitochondrial genome might, therefore, be due to a reduction in size of an analogous region.

GC Content

The GC content reported here is high for invertebrates, which generally display lower GC contents (range 21–43%) than vertebrates (range 37–50%) (Brown 1983). The *Daphnia* value (42%) is dramatically different from that reported for decapod crustaceans (29%) (Skinner and Kerr 1971), but similar to that of the more closely related *Artemia salina* (38%) (Schmitt et al. 1974). Increased GC content would of course be expected as a consequence of a reduction in the size of the AT-rich region.

Genetic Mapping

The inferred gene arrangement in *Daphnia* mtDNA is consistent with the gene order observed in *Dro-*

sophila (Clary and Wolstenholme 1985) and the partial gene order determined for *Artemia* (Batuecas et al. 1988). Sequence analysis of these genomes is currently underway in order to determine if differences in gene order exist. Such data, in coordination with other gene order determinations, could be important in addressing the issue of arthropod monophyly (Schram 1983). The cloned DNA has already been used as a hybridization probe in large-scale population surveys of *D. pulex* (Crease et al. 1989). The clone has also been successfully employed as a heterologous probe for many other species within the genus, as well as other crustaceans as distantly related as decapods (Stanton 1988). Such data could be very useful in the determination of phylogenetic relationships among the Crustacea.

Acknowledgments. The authors acknowledge the help and technical assistance of the following: Barb Zielinski, Yousef Ozeer, Peter Grewe, Neil Billington, Lou Densmore, D.T.N. Pillay, Guy DeLanversian, and Tom Dowling. We also thank W. Brown for advice and support, as well as generously providing access to equipment. J. Adams provided access to computer programs and invaluable technical assistance. Thanks also to those who read earlier drafts of the manuscript including: Neil Billington, Margaret Beaton, Wes Brown, and Tom Dowling. We acknowledge the support of an NSERC grant to P.D.N.H. and an NIH grant to A.R. Templeton.

References

Adams J, Rothman ED (1982) Estimation of the phylogenetic relationships from DNA restriction patterns and selection of endonuclease cleavage sites. Proc Natl Acad Sci USA 79: 3560–3564

Anderson S, Bankier AT, Barrell BG, deBruijn MHL, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJH, Staden R, Young IG (1981) Sequence and organization of the human mitochondrial genome. Nature 290:457–465

Batuecas B, Garesse R, Calleja M, Valverde JR, Roberto M (1988) Genome organization of *Artemia* mitochondrial DNA. Nucleic Acids Res 16:6515–6529

Borst P, Grivell LA (1981) Small is beautiful—portrait of a mitochondrial genome. Nature 290:443–444

Brown WM (1983) Evolution of animal mitochondrial DNA. In: Nei M, Koehn RK (eds) Evolution of genes and proteins. Sinauer, Sunderland, MA, pp 62–88

Brown WM, George M, Wilson AC (1979) Rapid evolution of animal mitochondrial DNA. Proc Natl Acad Sci USA 76: 1967–1971

Clary DO, Wolstenholme, DR (1985) The mitochondrial DNA molecule of *Drosophila yakuba*: nucleotide sequence, gene organization, and genetic code. J Mol Evol 22:252–271

Crease TJ, Stanton DJ, Hebert PDN (1989) Polyphyletic origins of asexuality in *Daphnia pulex*. II. Mitochondrial DNA variation. Evolution 43:1016–1026

DeSalle R, Freedman T, Prager EM, Wilson AC (1987) Tempo and mode of sequence evolution in mitochondrial DNA of Hawaiian *Drosophila*. J Mol Evol 26:157–164

Fauron CMR, Wolstenholme DR (1976) Structural heterogeneity of mitochondrial DNA molecules within the genus *Drosophila*. Proc Natl Acad Sci USA 73:3623–3627

Fauron CMR, Wolstenholme DR (1980a) Extensive diversity among *Drosophila* species with respect to nucleotide sequences within the adenine + thymine-rich region of mitochondrial DNA molecules. Nucleic Acids Res 8:2439–2452

Fauron CMR, Wolstenholme DR (1980b) Intraspecific diversity of nucleotide sequences within the adenine + thymine-rich region of mitochondrial DNA molecules of *Drosophila mauritiana, Drosophila melanogaster,* and *Drosophila simulans*. Nucleic Acids Res 8:5391–5410

Grewe PM, Hebert PDN (1987) Mitochondrial DNA diversity among stocks of the lake trout *Salvelinus nemaycush*. Great Lakes Fishery Commission Research Completion Report. Windsor, Ontario, pp 1–59

Hanahan D (1983) Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol 166:557–580

Harrison RG, Rand DM, Wheeler WC (1987) Mitochondrial DNA variation in field crickets across a narrow hybrid zone. Mol Biol Evol 4:144–158

Kieser T (1984) DNAGEL: a computer program for determining DNA fragments sizes using a small computer equipped with a graphics tablet. Nucleic Acids Res 12:679–688

Klein RD, Selsing E, Wells RD (1980) A rapid microscale technique for isolation of recombinant plasmid DNA suitable for restriction enzyme analysis. Plasmid 3:88–91

Lansman RA, Shade RO, Shapira JF, Avise JC (1981) The use of restriction endonucleases to measure mitochondrial DNA sequence relatedness in natural populations. III. Techniques and potential applications. J Mol Evol 17:214–226

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Marmur J, Doty P (1962) Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J Mol Biol 5:109–118

Rigby PWJ, Dieckmann M, Rhodes C, Berg P (1977) Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J Mol Biol 113:237–251

Schmitt H, Grossfeld H, Beckmann JD, Littauer UZ (1974) The biogenesis of mitochondria. Academic Press, New York

Schram FR (1983) Crustacean phylogeny. Balkema, Rotterdam, pp 1–22

Skinner DM, Kerr MS (1971) Characterization of mitochondrial and nuclear satellite deoxyribonucleic acids of five species of Crustacea. Biochemistry 10:1864–1872

Southern E (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98: 152–160

Stanton DJ (1988) The evolution of asexuality in *Daphnia pulex*: implications of mitochondrial DNA analysis. PhD thesis, University of Windsor

Vieira J, Messing J (1982) The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19:259–268

Watson GS (1961) Goodness-of-fit tests on a circle. Biometrika 48:109–114

Wolstenholme DR, MacFarlane JL, Okimoto R, Clary DO, Wahleithner JA (1987) Bizarre tRNAs inferred from DNA sequences of mitochondrial genomes of nematode worms. Proc Natl Acad Sci USA 84:1324–1328

Wright JW, Spolsky C, Brown WM (1983) The origin of the parthenogenetic lizard *Cnemidophorus laredoensis* inferred from mitochondrial DNA analysis. Herpetologia 39:410–416

Received June 15, 1988/Revised and accepted February 28, 1991