

Sequence Analysis of Mitochondrial DNA Variation in *Daphnia*

Abstract

Estimates of mitochondrial DNA sequence variability were determined for two closely related species of *Daphnia*. Sequence analysis from two genes, NADH dehydrogenase subunits 4 and 5, revealed a pattern of nucleotide substitution that has a 10 to 1 transition bias. This pattern is consistent with that from other taxa, suggesting the existence of a universal transition bias. Estimates of nucleotide diversity within species were similar to those obtained from other species. The estimated level of amino acid diversity between *Daphnia* and *Drosophila* was approximately as predicted from molecular clock analyses. Estimates of mitochondrial DNA variability among populations were similar for sequence data and restriction site data, suggesting that restriction endonuclease analyses provide accurate estimates of population-level genetic variability.

Introduction

Advances in sequencing technologies over the past decade (Sanger *et al.* 1977) have provided evolutionary biologists with a remarkable tool for assaying genetic variability. Since its inception, DNA sequencing has been used as a molecular approach for inferring phylogenetic relationships among species. However, only recently has DNA sequencing been used to examine genetic variation at or below the species level (see Hillis *et al.* 1991 for a review). One molecule that has received considerable attention is animal mitochondrial DNA (mtDNA).

Animal mtDNA is a relatively small (~14-16 kilobases), covalently closed circular molecule that is maternally inherited without recombination. These properties, along with its supposedly high rate of evolution (Brown *et al.* 1979), have led to its widespread use in evolutionary studies. Brown *et al.* (1982) proposed that transitions (C<->A, T<->C) are strongly favored over transversions (G<->C, G<->T, A<->T, A<->C) in mitochondrial evolution, despite the neutral prediction which suggests that there should be twice as many transversions as transitions based upon their probability of occurrence (eight ways for transversions to occur, four ways for transitions). Brown *et al.* (1982) showed that in closely related primates, 92% of the nucleotide substitutions are transitions. However, in comparison to more distantly related taxa, this transition bias disappears. Apparently, this transition bias is the result of multiple substitutions replacing transitions as transversions (Brown *et al.* 1982; De Salle *et al.* 1987).

The strong transition bias between closely related taxa is also supported with data from rodents (Brown

and Simpson 1982; Kocher *et al.* 1989), birds (Edwards and Wilson 1990), fish (Thomas and Beckenbach 1989), sea urchins (Giorgi *et al.* 1991), nematodes (Thomas and Wilson 1991), and planarians (Bessho *et al.* 1992). However, data from *Drosophila* (Clary and Wolstenholme 1987) suggest that this transition bias may not occur all taxonomic groups. In fact, in *Drosophila* there is a slight bias towards transversions that is very close to the theoretical expectation (but see De Salle *et al.* 1987). The lack of a transition bias in *Drosophila* is most likely the result of the comparison of *D. yakuba* with *D. melanogaster*, which are not very closely related. In fact, when more closely related species of *Drosophila* are compared, the bias becomes more evident (Beckenbach *et al.*, 1993). Since the majority of the data supporting the existence of a bias have come from vertebrates (mammals in particular) or *Drosophila*, the hypothesis of a widespread transition bias needs to be confirmed with data from other protostomate invertebrates.

Mitochondrial DNA (mtDNA) is known to be variable both within and between species (Moritz *et al.* 1987). The amount of mtDNA variability within species tends to be high. For example, Wayne *et al.* (1991) recently reported up to 8% nucleotide divergence within a single population of black-backed jackals. Between-population estimates of divergence can sometimes approach ten percent (Avice *et al.* 1987). These observations are in agreement with predictions from molecular clock analysis. Calibration of mtDNA molecular clocks for protein-coding genes predicts significant levels of divergence at zero divergence time (i.e., within species), suggesting the potential for high levels of site-specific heterozygosity (Lynch and Jarrell,

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1993). However, some species with large dispersal capabilities or those that have suffered recent bottlenecks may show low levels of diversity (Avisé *et al.* 1987).

Daphnia are protostomate, freshwater microcrustaceans that inhabit temporary ponds and permanent lakes over much of Europe and North America. Restriction-site studies (Crease *et al.* 1990), reveal that *Daphnia* exhibit significant amounts of variability for mtDNA both within and between populations. In this manuscript, I report on the pattern of nucleotide substitution within and between two species of *Daphnia* and provide estimates of locus-specific nucleotide diversity for portions of the NADH dehydrogenase subunits 4 and 5 (ND4, and ND5) genes. Data on mtDNA sequence variability in *Daphnia* provide valuable insights about the pattern of nucleotide substitution and also allow estimates of variability obtained from sequencing and restriction-site analysis to be compared. Jarrell (submitted) has shown that estimates of variability from restriction endonuclease analyses tend to be lower than estimates of variability measured from sequence data. Finally, these data for within-species levels of locus-specific heterozygosity can be used to test the prediction that significant levels of baseline heterozygosity should be present for certain loci and should be positively correlated with the rate of evolution of these genes.

Materials and Methods

Field Sampling and Collection Localities

Samples were taken from seven *Daphnia pulex* populations (FC19, FC23, 17, TSP, PA, BUS, and AMZ) and four *Daphnia pulicaria* populations (ODL, I.CU, KLA, and HOS); (see Table 1 for a complete description of the populations). One individual was isolated from each of the eleven samples, placed in conditions that favor asexual parthenogenesis (Lynch 1983), and allowed to multiply until enough individuals were present to provide an adequate amount of tissue for DNA manipulation. The accumulated individuals were placed into 1.5 ml eppendorf tubes and kept at -80° Celsius prior to DNA isolation. One randomly chosen clonal isolate per population was used for further study.

DNA Extraction and Cloning

Following the procedure of Crease *et al.* (1989), approximately 100 μ g of *Daphnia* tissue (wet

TABLE 1. Sampling Localities of *Daphnia* Populations.

Population	Location	Habitat
<i>D. pulex</i>		
PA	Warren Co., IN	Temporary
TSP	Vermilion Co., IL	Temporary ¹
BUS	Champaign Co., IL	Temporary
AMZ	Lane Co., OR	Temporary
FC19	Essex Co., Ontario, Canada	Temporary
FC23	Essex Co., Ontario, Canada	Temporary
17	Essex Co., Ontario, Canada	Temporary
<i>D. pulicaria</i>		
ODL	Klamath Co., OR	Permanent
I.CU	Deschutes Co., OR	Permanent
HOS	Deschutes Co., OR	Permanent
KLA	Klamath Co., OR	Permanent

¹Although the pond was permanent, there was an annual extinction of the *Daphnia* population each fall followed by repopulation in the spring.

weight, whole animals) was ground in 400 μ l of a solution containing 0.1 M NaCl, 30 mM Tris (pH 8.0), 10 mM EDTA, 10 mM β -mercaptoethanol, and 0.5% Triton X-100, and subjected to two rounds of phenol extraction followed by two rounds of chloroform:IAA (24:1) extraction. DNA was precipitated by the addition of 0.1 volumes 2M NaCl and 2.5 volumes 100% ethanol, and then resuspended in doubly distilled water.

A genomic DNA library was constructed for each of the eleven individuals by ligation of \sim 0.5 μ g of *Bam*III digested genomic DNA with 1 μ g of pre-digested lambda phage (Promega Packagene system). *E. coli* MM294 were infected with the recombinant phage and plated onto LB plates. Recombinant clones were screened for the presence of mtDNA inserts by plaque hybridization techniques (Maniatis *et al.* 1982) using radiolabelled *Daphnia* mtDNA (Stanton *et al.* 1991).

Previous analyses of *Daphnia* mtDNA had established that part of the ND4 gene and part of the ND5 gene were at opposite ends of a 1.5 kb *Apa*I-*Sst*I fragment (Figure 1; Crease *et al.* 1990). The desired subclones were produced by ligation of these fragments into pBS vector. One μ g of each mtDNA recombinant was digested with *Apa*I plus *Sst*I. 0.2 μ g of pBS vector was digested with the same complement of enzymes to produce compatible ends, and treated with phosphatase to prevent self-ligation. In both cases, the enzymes (restriction enzymes and phosphatase) were removed by extraction with phenol. After ethanol

Daphnia pulex

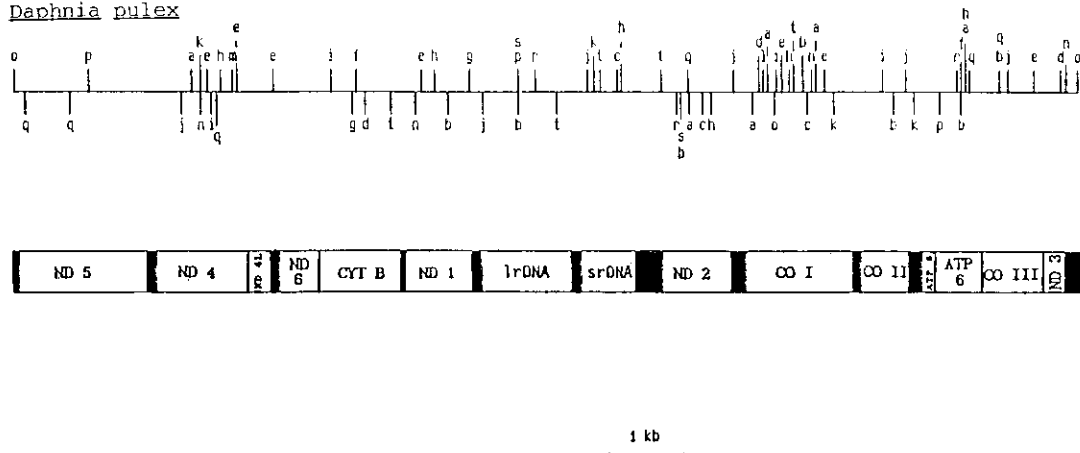


Figure 1. Restriction map of *Daphnia* mitochondrial DNA (modified from Crease *et al.* 1990). The letter codes are as follows: a= ApaI; b= AvaI; c= BamHI; d= BclI; e= BglIII; f= BstEII; g= ClaI; h= EcoRI; i= EcoRV; j= HindIII; k= HpaI; l= MluI; m= NcoI; n= PstI; o= PvuII; p= SstI; q= StuI; r= XbaI; s= XhoI; t= XmnI. Conserved sites within species are shown above the line.

precipitation, the fragments were resuspended in 10 ul of water and combined to make a total of 20 ul. The ligation was carried out by adding 10 ul of ligation mix [20 ul 10X ligation buffer (600 mM Tris, pH 7.5, 70 mM MgCl₂, 600 mM PEG, 10 mM DTT, 1 mM ATP) and 1 unit of T4 ligase]. This mixture was incubated overnight at 16o C. Ten ul of this ligation mixture were added to 100 ul of competent *E. coli* (XL-1 Blue), prepared by the method of Hanahan (1983). Following transformation, 10 ul of the transformation mixture were plated on "blue" Luria-Bertani (LB) agar plates containing 0.5% IPTG, 40 ug/ml X-gal, and 100 ug/ml Ampicillin. The plates were incubated overnight at 37° C, and white colonies were picked according to Maniatis *et al.* (1982). Plasmids were screened for the appropriate inserts by isolation from transformed bacterial cells using the "mini-prep" procedure of Maniatis *et al.* (1982) and subsequent electrophoresis on 0.8% agarose gels.

DNA Sequencing

To prepare DNA of sequencing quality, 10 ml cultures of the desired clones were grown overnight in LB media and plasmid purified by the use of Promega Magic Mini-preps. Three micrograms of purified double-stranded DNA were alkaline-denatured using 0.2M NaOH at room temperature for 10 minutes. The mixture was neutralized by

the addition of 0.5 volumes 0.9M sodium acetate and the DNA was precipitated by the addition of three–four volumes of 100% ethanol. This pellet was washed with 70% ethanol and resuspended in 7 ul of doubly distilled water. The DNA was sequenced using [³⁵SdATP] and T7 DNA polymerase with the Sequenase kit (United States Biochemical). All reactions were carried out according to manufacturer's directions. The M13-universal and M13 reverse primers were used in sequencing the ND4 and ND5 genes. All sequencing reactions were electrophoresed on 8% polyacrylamide gels, which were subsequently dried and exposed to autoradiographic film for 12-24 hours.

Sequence Analysis

The GCG group of programs for the VAX were used to align and translate sequence data. Consensus sequences were generated by determining the most frequent nucleotide for each site. The *Drosophila* genetic code (Clary and Wolstenholme 1985) was used to translate nucleotide sequences into amino acid sequences. Nucleotide diversity (average number of nucleotide differences per site) was estimated by equation 10.4 of Nei (1987) using 170 bp sequences for each gene that were present for all 11 individuals (one per population).

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      1 GCCCAATTTTCTAATCCTATCTTATTTTGATTAATACTGTTGGCGTTTCT 50
          ||| | | | | | | | | | | | | | | | | | | | | | | | | |
9034 TTTATGTTTAATTATGATTTATTATATTTTTGTTTATTATGTGCTTTTTT 8985

      51 AGTCAAGCTTCCCGTATATTTTGGACATTTGTGACTTCCTAAGGCCGATG 100
          ||| | | | | | | | | | | | | | | | | | | | | | | | | |
8984 AGTGAATAACCAATATTTTGTAGTACATTTATGATTACCTAAAGCTCATG 8935

     101 TTGAGGCCCCAGTTGCAGGGTCTATAATTCTAGCGGGGTTTTATTAAAG 150
          |||| | | | | | | | | | | | | | | | | | | | | | | | | | |
8934 TTGAAGCTCCTGTATCTGGTTCTATAATTTTAGCTGGTATTATATTAAAA 8885

     151 TTAGGGGGGTACGGAATAGTTCGAGTTGTCCCTTACCGTCGAGTTAAGTC 200
          |||| | | | | | | | | | | | | | | | | | | | | | | | | | |
8884 TTAGGAGGTTATGGTTTATTACGAGTAATTAATTTTTTACAATTAATAAAA 8835

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Figure 2. Alignment of *Daphnia* NADH dehydrogenase subunit 4 gene with *Drosophila yakuba*. *Daphnia* sequence is on top.

Results

Comparison of Nucleotide Sequences

The consensus sequences for ND4 and ND5 from the eleven individuals are aligned with *Drosophila yakuba* sequences in Figures 2 and 3 respectively. Overall, there were two mitochondrial types for ND4 and five different types for ND5 (Table 2). Summed over both loci, six different mitochondrial types are evident, indicating a high degree of sequence polymorphism. The sample frequency for all but one of these types is 2/11. The other type had a frequency of 1/11. There were two polymorphic nucleotide sites in ND4 (positions 33 and 40) and five in ND5 (positions 84, 89, 105, 123, and 210). The estimated nucleotide diversity (average number of nucleotide differences per site) was 0.22% for ND4 and 0.69% for ND5. The combined nucleotide diversity is estimated to be 0.51 %.

Both ND4 and ND5 showed divergence at the amino acid level. There were four amino acid substitutions among the eleven sequences at two different sites (position 30 in ND4 and position 89 in ND5). These two changes were tryptophan → cysteine in ND4 and isoleucine → threonine in ND5. The estimate of amino acid diversity (combined over both species for each locus) is 0.2%, about the same as the nucleotide diversity for ND4, but substantially less than the nucleotide divergence for ND5.

Contrary to the result from restriction endonuclease analyses (Crease *et al.* 1990), indi-

TABLE 2. Variable nucleotide sites for ND4 and ND5.

Individual	ND4 Position		ND5 Position				
	33	40	84	89	105	123	210
PA	A	C	T	C	C	C	T
TSP	A	C	T	C	C	C	T
BU	A	C	C	T	T	C	C
AM	A	C	C	T	T	C	C
FC19	C	T	T	T	C	C	T
FC23	C	T	T	T	C	C	T
17	A	C	T	T	T	C	C
ODL	A	C	C	T	T	T	C
LCU	A	C	C	T	T	T	C
HOS	A	C	T	T	C	C	T
KIA	A	C	T	T	C	C	T

viduals from the two *Daphnia* species do not form distinctive clades, as there are no fixed differences between the two species. The mtDNA genotypes between species are no more distant to each other than are genotypes within species.

Pattern of Substitution

Patterns of nucleotide substitution (Table 3) show that most of the substitutions among the eleven individuals were synonymous (i.e., did not result in an amino acid substitution) and occurred mainly at third base positions in codons. Combining all loci, there were eighteen synonymous substitutions

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1 GAATCCTCTAGGATGGGATGGGTT.GGGCTAATTTCTTACGGTCTAGTAA 49
  | | | | | | | | | | | | | | | | | | | | | | | | | | | |
7818 CATTCTATTAGGGTGAGATGGATTAGGACTTGTTCCTTATTGTTTAGTAA 7770

50 TTTATTATCCTTCTAAAAAGTCAAGTAGCGCGGGTATAATCACAGTCTTA 99
  | | | | | | | | | | | | | | | | | | | | | | | | | | | |
7769 TTTATTTTCAAATATCAAATCTTACAATGCTGGAATATTAAGTGCATTA 7720

100 TCTAACCGGGTGGGGGATGTGTGCATCTTACTCTCAATCGCTTGATTTCGT 149
  | | | | | | | | | | | | | | | | | | | | | | | | | | | |
7719 TCTAATCGAATTGGAGATGTAGCTCTTCTTCTTGCTATTGCTTGAATATT 7670

150 A...TCAGTAGGAGATTTTAATTTTAGAATTTGGGCCCTTTATAATAAT 195
  | | | | | | | | | | | | | | | | | | | | | | | | | | | |
7669 AAATTATGGTAGATGAAATTATATTTTATTATTAGAAAGTTATACAA.AAT 7621

196 GTAGAGTTTAGAAATGGGTTCTCTTCTTGTATTACTGGCCGCTATAAC 245
  | | | | | | | | | | | | | | | | | | | | | | | | | | | |
7620 GAATTTTCAATATTAATAATTGGTAGTTAGTTATATTAGCTGCTATAAC 7571

246 TAAAAGAC 252
  | | | | | |
7570 TAAAAGAG 7563

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Figure 3. Alignment of *Daphnia* NADH dehydrogenase subunit 5 gene with *Drosophila yakuba*. *Daphnia* sequence is on top.

TABLE 3. Patterns of Nucleotide Substitution in *Daphnia*. Data are summed over all individuals.

Gene	bp	syn	non-syn	tsit	tvr
ND4	170	2	2	2	2
ND5	169	16	2	18	0
TOTAL	339	18	4	20	2

NOTE: bp = base pairs, syn = synonymous, non-syn = non-synonymous, tsit = transitions, tvr = transversions, ND4 = NADH sub-unit 4; ND5 = NADH sub-unit 5

and four non-synonymous substitutions distributed among the eleven individuals. There exists a strong transition bias in *Daphnia* mtDNA evolution measured both within and between species. The transition:transversion ratio for the two loci combined was 10:1.

Discussion

Estimates of diversity from restriction endonuclease (RE) data for *D. pulex* average about 0.006 (Crease

et al. 1990). Estimates of nucleotide diversity obtained from sequence data generally agree with those obtained from RE studies, but average slightly lower. Per locus estimates are very similar, but the total estimate is low due to little variability in ND4.

Sequence analysis of *Daphnia* mtDNA genes does not elucidate phylogenetic relationships among populations and species that are suggested for these populations at both the isozyme electrophoresis level (data not shown) and the mtDNA genome restriction level (Crease *et al.* 1990). A larger survey of sequence variability within the species would most likely reveal the patterns of phylogenetic structure that are evident in the larger restriction-site survey.

Amino-acid substitutions observed in this analysis occurred in both genes. In fact, estimates of amino-acid diversity for the ND4 gene are nearly as large as the estimates for nucleotide diversity. It has been suggested that the amino-acid replacement rate should approach the nucleotide substitution rate in genes that are less functionally constrained (Nei 1987). This has been the case

for other mtDNA genes from other organisms (*Drosophila*, Clary and Wolsteholme 1985; *Apis*, Pumo *et al.* 1992). The estimated degree of intraspecific variability at the amino acid level is shown to be on the same order as was predicted previously by molecular clock analyses (Lynch and Jarrell, 1993), despite the effects of recent bottlenecks (e.g., Pleistocene glaciations) on *Daphnia* populations.

When the equations 13 and 14 of Lynch and Jarrell (1993) are applied to estimated levels of amino acid divergence between *Daphnia* and *Drosophila*, estimates of the date of divergence of these two species are obtained (Table 4). The estimates for the two genes give similar estimated dates of divergence. When averaged over both loci, the data suggest that the *Daphnia-Drosophila* split may have occurred about 320 million years ago. Fossil *Daphnia*-like animals are known from the Permian (280 million years ago; Smirnov 1970), so this may be a relatively accurate estimate.

TABLE 4. Amino Acid Divergence, Δ , and Estimated Time to Common Ancestor (MY) with 95% confidence limits between *Daphnia* and *Drosophila*.

	ND1	ND5
Δ	0.283	0.370
MY +/- CL	300 +/- 109	340 +/- 138

If all nucleotide substitutions are equally likely to occur, then transversions should outnumber transitions by two to one over evolutionary time. This is clearly not the pattern observed. Data from a variety of taxa and for a variety of genes indicate that there is in fact a transition bias. Data from *Daphnia* mitochondrial DNA confirm this bias as well. For *Daphnia*, there were nearly ten transitions for each transversion. Although this is less than the bias that is observed for many vertebrates, it is still substantially different from the neutral prediction. Thus, it is possible that this transition bias is a fairly widespread phenomenon.

The cause of the bias is unknown. One hypothesis is that variations away from the neutral expectation are caused by activation during DNA replication of mutator genes that favor transitions (Jukes 1987). Such mutator genes are known to exist in bacterial (Cox and Yanofsky 1967) and viral (Speyer 1965) systems, but their existence in eucaryotic systems is hypothetical.

DeSalle *et al.* (1987) suggested that the cause of the bias is due to inefficient repair systems of mitochondria. There is evidence for lower levels of repair for mitochondria (Clayton 1984, 1991), and bacterial mutants deficient in repair show mutational spectra that are very similar to that of mtDNA (Schaaper and Dunn 1987). However, recent research with γ -DNA polymerase, the only known eucaryotic DNA polymerase to be found in mitochondria, suggests that it may possess 3' to 5' exonuclease activity and proof-reading capabilities (Kunkel and Musbaugh 1989). Moreover, certain types of repair enzymes have been identified in mitochondrial fractions (Tomkinson *et al.* 1990), although their use still remains questionable.

In light of the genetic code, transversions should not outnumber transitions by a factor of two. In protein-coding DNA, the majority of synonymous changes within two-fold and six-fold degenerate codon groups are transitions. Among two-codon groups, transversions in the third position of the codon result in an amino acid change, whereas a transition at the third position specifies the same amino acid. The same pattern is observed in the first position of the triplet code in six codon groups. Thus, it seems likely that transitions are selectively favorable to transversions and should occur more frequently during the replicative process. The observations that transitions are the most likely mispair during replication (Topol and Fresco 1976) and that the bias is fairly widespread among different taxa and loci, support the contention that selection has shaped the replicative machinery to be more tolerant of transitional changes during replication. Thus, it is unnecessary to invoke any special properties of mitochondria to explain a transition bias.

Expanding on the rationale of Jukes (1987), the neutral prediction of nucleotide substitution should not be for a transversion bias if the majority of substitutions are likely to be synonymous (as is the case for mtDNA which is under strong functional constraint (Jukes and Bhushan 1986)). Using the human mtDNA genetic code, 62 of the potential 126 synonymous substitutions are transitions, suggesting a neutral ratio of nearly one transition to each transversion (due to the difference in genetic code, there exists a slight expected transversion bias, 1.25:1 for nuclear DNA). Deviations away from this neutral prediction would most likely reflect the amino acid composition and codon usage bias of proteins.

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Literature Cited

- Avise, J. C., J. Arnold, R. M. Ball, E. Bermingham, T. Lamb, J. F. Neigel, C. A. Reeb, and N. Saunders. 1987. Intraspecific phylogeography: The mitochondrial DNA bridge between population genetics and systematics. *Ann. Rev. Ecol. Syst.* 18:489-522.
- Beckenbach, A. T., Y. W. Wei, and H. Liu. 1993. Relationships in the *Drosophila obscura* species group, inferred from mitochondrial cytochrome oxidase II sequences. *Mol. Biol. Evol.* 10:619-634.
- Bessho, Y., T. Ohama, and S. Osawa. 1992. Planarian mitochondria I. Heterogeneity of cytochrome c oxidase subunit I gene sequences in the freshwater planarian, *Dugesia japonica*. *J. Mol. Evol.* 34:324-330.
- Brown, G. G. and M. V. Simpson. 1982. Novel features of animal mtDNA evolution as shown by sequences of two rat cytochrome oxidase subunit II genes. *Proc. Natl. Acad. Sci. USA* 79:3246-3250.
- Brown, W. M., M. George, Jr., and A. C. Wilson. 1979. Rapid evolution of animal mitochondrial DNA. *Proc. Natl. Acad. Sci. USA* 76:1967-1971.
- Brown, W. M., E. M. Prager, A. Wang, and A. C. Wilson. 1982. Mitochondrial DNA sequences in primates: tempo and mode of evolution. *J. Mol. Evol.* 18:225-239.
- Clary, D. O. and D. R. Wolstenholme. 1985. The mitochondrial DNA molecule of *Drosophila yakuba*: nucleotide sequence, gene organization, and genetic code. *J. Mol. Evol.* 22: 252-271.
- _____. 1987. *Drosophila* mitochondrial DNA: conserved sequences in the A+T-rich region and supporting evidence for a secondary structure model of the small ribosomal RNA. *J. Mol. Evol.* 25:116-125.
- Clayton, D. A. 1984. Transcription of the mammalian mitochondrial genome. *Ann. Rev. Biochem.* 53:573-594.
- _____. 1991. Replication and transcription of vertebrate mitochondrial DNA. *Ann. Rev. Cell Biol.* 7:453-478.
- Cox, E. C. and C. Yanofsky. 1967. Altered base ratios in the DNA of an *Escherichia coli* mutator strain. *Proc. Natl. Acad. Sci. USA* 58:1895-1902.
- Crease, T. J., M. Lynch, and K. Spitze. 1990. Hierarchical analysis of population genetic variation in mitochondrial and nuclear genes of *Daphnia pulex*. *Mol. Biol. Evol.* 7:144-158.
- Crease, T. J., D. J. Stanton, and P. D. N. Hebert. 1989. Polyphyletic origins of asexuality in *Daphnia pulex*. II. Mitochondrial DNA variation. *Evolution* 43: 1016-1026.
- De Salle, R., T. Freedman, E. M. Prager, and A. Wilson. 1987. Tempo and mode of sequence evolution in mitochondrial DNA of Hawaiian *Drosophila*. *J. Mol. Evol.* 26:157-164.
- Edwards, S. V. and A. C. Wilson. 1990. Phylogenetically informative length polymorphisms and sequence variability in mitochondrial DNA of Australian song-birds (*Pomatostomus*). *Genetics* 126:695-711.
- Giorgi, C., F. DeLuca, and e. Saccone. 1991. Mitochondrial DNA in the sea urchin *Arbacia lixula*: nucleotide sequence differences between two polymorphic molecules indicate asymmetry of mutations. *Gene* 103:249-252.
- Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166: 557-580.
- Hillis, D. M., A. Larson, S. K. Davis, and E. A. Zimmer. 1991. Nucleic acids III: sequencing. In: *Molecular Systematics*, D. M. Hillis and C. Moritz (eds). Sinauer Press, MA.
- Jukes, T. H. 1987. Transitions, transversions, and the molecular evolutionary clock. *J. Mol. Evol.* 26:87-98.
- Jukes, T. H. and V. Bhushan. 1986. Silent nucleotide substitutions and G+C content of some mitochondrial and bacterial genes. *J. Mol. Evol.* 24:39-44.
- Kocher, T. D., W. K. Thomas, A. Meyer, S. V. Edwards, S. Paabo, F. X. Villablanca, and A. C. Wilson. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proc. Natl. Acad. Sci. USA* 86: 6196-6200.
- Kunkel, T. A. and D. W. Musbaugh. 1989. Exonucleolytic proofreading by a mammalian DNA polymerase γ . *Biochemistry* 28:988-995.
- Lynch, M. 1983. Ecological genetics of *Daphnia pulex*. *Evolution* 37: 358-374.
- Lynch, M. and P. E. Jarrell. 1993. A method for calibrating molecular clocks and its application to animal mitochondrial DNA. *Genetics* 135: 1197-1208.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Publications, Cold Spring Harbor.
- Moritz, C., T. E. Dowling, and W. M. Brown. 1987. Evolution of animal mitochondrial DNA: relevance for population biology and systematics. *Ann. Rev. Ecol. Syst.* 18:269-292.
- Nei, M. 1987. *Molecular Evolutionary Genetics*. Columbia University Press, MA.
- Pumo, D. E., C. J. Phillips, M. Barcia, and C. Millan. 1992. Three patterns of mitochondrial DNA nucleotide divergence in the meadow vole, *Microtus pennsylvanicus*. *J. Mol. Evol.* 34:163-174.
- Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463-5467.
- Schaaper, R. M. and R. L. Dunn. 1987. Spectra of spontaneous mutations in *Escherichia coli* strains defective in mis-match correction: the nature of *in vivo* DNA replication errors. *Proc. Natl. Acad. Sci. USA* 84: 6220-6224.
- Smirnov, N. N. 1970. Cladocera (Crustacea) iz permskikh otlozheniy Vostochnogo Kazakhstana. *Paleontol. Zh.* 3: 95-100.
- Speyer, J. 1965. Mutagenic DNA polymerase. *Biochem. Biophys. Res. Comm.* 21:6-10.
- Stanton, D., T. J. Crease, and P. D. N. Hebert. 1991. Cloning and characterization of *Daphnia* mitochondrial DNA. *J. Mol. Evol.* 33:152-155.

- Thomas, W. K. and A. T. Beckenbach. 1989. Variation in Salmonid mitochondrial DNA: evolutionary constraints and mechanisms of substitution. *J. Mol. Evol.* 29:233-245.
- Thomas, W. K. and A. C. Wilson. 1991. Mode and tempo of molecular evolution in the nematode *Caenorhabditis*: cytochrome oxidase II and calmodulin sequences. *Genetics* 128:269-279.
- Tomkinson, A. E., R. T. Bonk, J. Kim, N. Bartfield, and S. Linn. 1990. Mammalian mitochondrial endonuclease activities for ultraviolet-irradiated DNA. *Nucleic Acids Res.* 18:929-935.
- Topol, M. D. and J. R. Fresco. 1976. Complementary base pairing and the origin of substitution mutations. *Nature* 263:285-289.
- Wayne, R. K., A. Meyer, N. Lehman, B. Van Valkenburgh, P. W. Kat, T. K. Fuller, D. Girman, and S. J. O'Brien. 1991. Large sequence divergence among mitochondrial DNA genotypes within populations of eastern black-backed jackals. *Proc. Natl. Acad. Sci. USA* 87: 1772-1776.

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