

THE USE OF MITOCHONDRIAL DNA TO STUDY RELATEDNESS IN PELAGIC SPECIES

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SUMMARY

The Southwest Fisheries Center is initiating a study which will apply a new technique for measuring genetic variability to the old problem of defining the "unit stock" for population analyses and management needs. Traditionally, unit stocks have been identified by particular morphological, ecological or, more recently, electrophoretic characters. However, not all populations of exploited species can be characterized by any of the above means due to a lack of phenotypic variability. We have applied this new technique for investigating relatedness to skipjack tuna from the Atlantic and Pacific Oceans. Endo-restriction nuclease analysis of mitochondrial DNA, which has been used successfully to demonstrate population structure in mammals, indicated a surprisingly high degree of genetic similarity between skipjack tuna from the two oceans. These results suggest continued genetic contact between the two populations since the uplift of the Panama land ridge 3.1 million years ago.

RESUME

Le "Southwest Fisheries Center" a entrepris une étude visant à appliquer une nouvelle technique de mesure de la variabilité génétique à la sempiternelle question de comment définir l'unité de stock" pour les besoins des analyses de population et de la gestion. Les unités de stocks ont traditionnellement été

identifiées au moyen de caractéristiques propres morphologiques, écologiques ou, plus récemment, électrophorétiques. Les populations d'espèces exploitées ne peuvent cependant pas toutes être identifiées par l'un ou l'autre de ces moyens, du fait d'un manque de variabilité phénotypique. Nous avons appliqué cette nouvelle technique de recherche de la relation au listao des océans Atlantique et Pacifique. L'analyse de la nucléase endo-restrictive de l'ADN mitochondrial, utilisée avec succès pour illustrer la structure des populations chez les mammifères, signalait pour les listaos un degré étonnamment élevé de similitude génétique entre les deux océans. Ces résultats suggèrent des contacts génétiques suivis entre les deux populations depuis le soulèvement de la chaussée panaméenne il y a 3,1 millions d'années.

RESUMEN

El Southwest Fisheries Center está dando comienzo a un nuevo estudio que aplicará técnicas recientes encaminadas a medir la variabilidad genética del viejo problema de definir una "unidad de stock", para análisis sobre población y ordenación. Tradicionalmente, las unidades de stock han sido identificadas por alguna característica propia de tipo morfológico, ecológico o, más recientemente, electroforético. Sin embargo, no todas las poblaciones de especies explotadas pueden

incluirse en las características mencionadas, debido a falta de variabilidad fenotípica. Se aplicó esta nueva técnica para investigar la relación entre los listados del Atlántico y Pacífico. Los análisis de nucleasá endorestrictiva de DNA mitocondrial, que ha sido utilizada con éxito para demostrar la estructura de la población de mamíferos, señaló un sorprendentemente alto nivel de similitud genética entre los listados de ambos océanos. Estos resultados sugieren que ha habido contactos genéticos ininterrumpidos entre las dos poblaciones desde la elevación de la cordillera de Panamá, hace 3,1 millones de años.

Introduction

Critical to the realistic application of population dynamics models in fisheries biology is an understanding of what constitutes the unit stock. All too often the definition of a stock is based upon what is operationally or politically convenient and has little biological reality. Ideally, before undertaking assessment studies, the fisheries scientist should carefully map the self-contained and self-perpetuating genetic units (Gulland 1971). But for pelagic populations which lack obvious geographic isolation and are experimentally difficult to separate into subspecies, such an idealized course of action is usually not followed.

In the past, attempts to identify subspecies of pelagic organisms have used morphological, ecological or, more recently, electrophoretic characters (for skipjack tuna, see Argue 1981). These studies have proven more difficult than population analyses of terrestrial or freshwater species for a variety of reasons: (1) Pelagic species like tunas and marine mammals generally exhibit low phenotypic within taxa variability (Sharp in Selander 1976). Thus electrophoretic studies of protein variation in pelagic species require huge sample sizes (Argue 1981, Fujino 1969, Fujino 1970, Fujino and Kang 1968, and Fujino et al. 1981). And (2) pelagic stocks often intermingle in complex and overlapping migratory patterns and isolation of the breeding units is likely to occur only during reproductive periods (Iles and Sinclair 1982). These observations, as well as the widespread temporal and spatial dispersion of most pelagic populations, demand complex and expensive sampling. Clearly, new and more powerful techniques of population analysis must be designed to facilitate the task of stock segregation.

The Southwest Fisheries Center is initiating a study which will apply a new technique for measuring genetic variability to the problem of defining the unit stock for pelagic species. The technique requires smaller sample sizes because of its greater resolution of the underlying genetic differentiation which must accompany stock segregation. The technique, which can quantitatively define the relatedness between any two individuals or groups, involves the analysis of the DNA of the mitochondria (mtDNA). Fragments, created by the action of restriction endonucleases on the mtDNA, are electrophoretically separated and compared between individuals in order to

calculate the frequency of nucleotide (base pair) differences. The greater the differences, the more distant the relationship. Although the restriction endonuclease approach does not necessarily detect all of the variation in the mtDNA genome, it is much simpler and more practical than completely sequencing the mtDNA molecule (Nei and Tajima 1981).

In this report, we briefly describe the mtDNA molecule, its genetics, isolation and purification, and its application to the study of population structures. Our goal is not a manual of techniques but rather to provide a sense of the degree of complexity and utility of the analysis. We also report on the results of a preliminary investigation of skipjack tuna collected in the Pacific and Atlantic Oceans. The report concludes with a description of our future plans for the mtDNA technique.

mtDNA genetics

The mtDNA molecule is a covalently closed-circular duplex DNA. The genome is functionally conservative, consisting of the same genes in all multicellular organisms. The mtDNA molecule consists of 15,000 to 18,000 nucleotide base pairs which encode rRNA's, tRNA's, and mRNA's specifying hydrophobic membrane-associated polypeptides (Avisé et al. 1979a, Brown 1981, and Gray 1982).

The mitochondrial genome has properties which make it ideal for population or evolutionary studies. Purification is relatively easy. Sufficiently large amounts can be obtained from even small animals to allow its characterization by a number of different procedures. It is small in size and can be completely sequenced if desired. Most importantly, rates of evolution of mtDNA are 5 to 10 times faster than nuclear DNA (Brown et al. 1979, 1982, Ferris et al. 1981a and b, and Gray 1982). Consequently, mtDNA analysis results in a magnified view of genetic differences among closely-related species (Ferris et al. 1981a and b) and within disjunct populations of the same species (Avisé et al. 1979a and Ferris et al. 1982). Rates of sequence divergence for individuals or groups are calibrated by examining organisms whose evolutionary history is known from the geological record. For instance, Avisé et al. (1979a) estimate that evolutionary rates of mtDNA in

the genus Peromyscus are from 1.5×10^{-6} to 7.5×10^{-8} substitutions per nucleotide per year. Later in this report, we make more use of these observations when we compare the relatedness of skipjack tuna from the Atlantic and Pacific Oceans and contrast their variability to that found in mammal populations.

Analogous but opposite to male-surname heredity, the mtDNA genome is maternally inherited (Awise et al. 1979a and b, Brown et al. 1981, Giles et al. 1980, and Lansman et al. 1981). Although both the ova and the sperm contain mitochondria, during fertilization mitochondria from the sperm are not incorporated into the zygote. Thus, lack of sexual recombination between generations simplifies tracing of lineages and each mtDNA genotype provides relatively complete and unambiguous data about the female line to which it belongs (Lansman et al. 1981). As a result, the individual organism is the operational taxonomic unit.

The mtDNA analytical technique

Analysis of relatedness utilizing the mtDNA technique (Figure 1) requires (1) collections of material, (2) isolation of mitochondria, (3) purification of mtDNA, (4) digestion of mtDNA with restriction endonucleases, (5) determination of the size of the resultant mtDNA fragments, and (6) calculation of sequence divergence.

Maximum yields of mtDNA are obtained from freshly killed specimens (Lansman et al. 1981). Analyzing mtDNA from frozen or degraded samples is complicated because of contamination by nuclear DNA. Since the cell contains 200-500 times more nuclear DNA than mtDNA, severe contamination requires that a more involved visualization procedure be followed (Ferris et al. 1981 and Lansman et al. 1981). For large animals, careful freezing of sample tissues, such as liver, heart, or ovaries with no subsequent defrosting results in adequate samples (George 1982). Sufficiently large sample size (10's of grams) compensates for the loss of harvest efficiency from frozen samples. Our current laboratory work is designed to determine the optimal compromise between sampling convenience and sample adequacy. Long-term (months/years) storage methods are also being investigated.

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In the laboratory, mitochondria are separated from the other cellular constituents by slow speed centrifugation and are then lysed to release the mtDNA. The next step, the separation of the mtDNA from the other mitochondrial constituents (protein, RNA's, and glycogen) and the remaining nuclear DNA, is the rate limiting step of the procedure. mtDNA is purified by ultracentrifugation ($160,000 \times g$) in a cesium chloride gradient for a minimum of 36 hours. However, modern equipment, now available, may reduce this time to 8 hours.

However, if nuclear DNA contamination is severe, an alternate process for isolating and visualizing the mtDNA must be followed. The "Southern method" (Ferris et al. 1981a and Lansman et al. 1981) is operationally more complex, but it does not require the long periods of ultracentrifugation. The alternate method is based upon DNA-DNA hybridization techniques and can detect minute amounts of mtDNA in crude tissue extracts of all cellular DNA.

Over 200 restriction enzymes are now available from commercial sources. These enzymes recognize a specific sequence of 4 - 6 nucleotides and cut the mtDNA strand within the sequence (Figure 2). The size of the resultant fragments are determined by electrophoresis on agarose gels; each restriction enzyme employed on a given sample will result in a specific restriction phenotype (pattern). The sizes of each fragment in numbers of base pairs will sum to approximately 16,000, the number of base pairs of the original molecule. The differences in the gel banding patterns between individuals reflect differences in the mtDNA nucleotide sequence. Pair-wise comparisons between individuals for each restriction enzyme used allows determination of the number of fragments shared per total number of fragments.

A simple index of relative genetic similarity between two individuals can be calculated as the proportion of fragments shared in their digestion profiles,

$$F = 2 N_{xy} / (N_x + N_y)$$

where N_{xy} is the number of fragments shared by the two organisms and N_x and N_y are the number of fragments in the digestion profiles of sample x and y , respectively. If one assumes that all fragment changes are the result of single substitutions of single base pairs, the frequencies and distribution of cleavage sequences in the DNA are similar to those expected in random sequences of DNA with the same proportion of base pairs, and that all digestion fragments are observed, then the number of base substitutions per nucleotide (p) which separate a given pair of organisms can be calculated (Awise et al. 1979a and b, Lansman et al. 1981, and Nei and Tajima 1981),

$$p = 1 - [-F/2 + ((F^2 + 8F)/2)^{0.5}] (1/n)$$

where F is the proportion of fragments shared and n is the number of base pairs recognized per cleavage site (Lansman et al. 1981).

Restriction analyses of mtDNA sequence have been undertaken on a great variety of mammals (humans and great apes--Brown 1980 and Denaro et al. 1981, Ferris et al. 1981a and b, and Giles et al. 1980. pocket gopher--Awise et al. 1979b. mice--Awise et al. 1979a. rats--Brown et al. 1981, Castora et al. 1980, and Ferris et al. 1982) and have recently been initiated on lower vertebrates. The number of base substitutions per base pair (p) range from 0 to 0.25 depending on the evolutionary distance between organisms compared (Figure 3). The p values show a clear geographic pattern. For instance, gophers collected in the same geographic area exhibited p 's near zero while comparisons of gophers from geographically disjunct populations resulted in a mean $p = 0.03$ between any two individuals (Awise et al. 1979b). Typically conspecific organisms exhibit p values of less than 0.03 and individuals of closely related species show mean p values between 0.08 - 0.25 base substitutions (Lansman et al. 1981).

Knowledge of mtDNA mutation rates allows the development of a time scale of divergence among individuals and populations. Base substitution rates for mammals are about 0.02 base substitutions per lineage per million years (Brown et al. 1982). For instance, since the mean sequence heterogeneity between any two randomly selected humans is 0.0018-0.0036, the human species appears to be

young and appears to have originated from a single mating pair that existed from 90,000 to 180,000 years ago (Brown 1980). Analysis of fragment patterns support the notion that the major racial groups have developed within the last 10,000 to 50,000 years (Denaro et al. 1981 and Ferris et al. 1981b) and that Asia is genetically central to the radiations which gave rise to the racial groups (Denaro et al. 1981).

mtDNA diversity in Atlantic and Pacific skipjack tuna

This preliminary study of skipjack tuna mtDNA presents a first view of mtDNA variation within natural populations of fishes. The objectives were (1) to demonstrate that tuna mtDNA could be purified with the procedures outlined above (or visualized with the Southern technique) and 2) to determine from restriction site variation, the amount of sequence divergence between tunas from the Atlantic and Pacific Oceans.

Work by Argue (1981), Fujino (1969), Fujino and Kang (1968), and Fujino et al. (1981) suggest that skipjack tuna from the two oceans are closely related; they failed to find loci at which fixed electrophoretic differences occurred although they did demonstrate some slight allelic frequency differences. Subspecies of mammals and fishes studied generally show some fixed allelic differences or major allelic frequency differences at several loci (Awise 1974), and would be expected to show even more mtDNA diversity.

Liver and heart were obtained from 6 skipjack tuna caught off Hawaii and 6 from the Santos Basin off Brazil. For these fish, the Southern method was used. For a pooled sample of 3 additional tunas from Hawaii, collected at a different time from the other 6, and one sample from Puerto Rico, the conventional mtDNA purification procedure, outlined above, was followed.

In summary, 14 fragments were scored in the 12 tunas by the Southern method. Five different restriction enzymes were employed (Table 1). None showed fixed differences between the Atlantic and Pacific specimens. In the 12 tunas, one SacI variant was present in Hawaiian fish. From the purification procedure, a total of 25 fragments were scored in the Hawaiian specimens, and 26 in the Puerto Rican one. In this comparison, 3 fragments

were different, which can be attributed to a single PstI mutation in the Puerto Rican fish. The Hawaiian PstI pattern is the same as that in the Brazilian tunas. Thus the Puerto Rican skipjack tuna was a variant. Finally, a variant was found in one of the Hawaiian tunas for AvaII. All variants appeared to differ by the loss or gain of 1 restriction site. Table 1 summarizes the fragment patterns, w is the "wild" or common type and var is the "variant".

The mean genome size of skipjack tuna mtDNA, estimated from the sums of fragments, is 16,900 nucleotide pairs (bp's). This value is very close to that reported by Berg and Ferris (MS) for salmonid mtDNA and 400 no larger than human mtDNA (Brown 1981).

The actual number of base pairs examined in the 5 restriction enzyme analysis by the Southern method can be precisely determined. AvaII recognizes a 5 base pair sequence and produces 3 fragments, thus 15 base pairs were examined. Following the same reasoning for the rest of the enzymes, a total of 73 bp's were examined with the Southern method and an additional 96 were examined with the purification procedure, for a total of 169 base pairs. It must be emphasized that the number of sites examined so far is smaller than the current mammalian studies, and more variation is expected to be found with the use of more enzymes. However, some interesting conclusions may be drawn.

How variable are tunas compared to mammals? The class of enzymes examined here, mostly recognizing 6 base pair sequences, do not reveal as much variation as 4 base cutters (Ferris et al. 1981b) but were chosen because they are technically easier to handle. Thus all of the estimates of divergence are minimum estimates. Table 2 compares the number of mtDNA morphs found in skipjack tuna with those of chimpanzee (Pan troglodytes) which exhibit typical mammal variation (Ferris et al. 1981b). For the enzymes used in this study, the chimps exhibited 12 morphs for 10 animals tested, whereas for the skipjack tuna only 8 morphs were discovered in 16 animals tested. It is interesting to note that humans exhibit 1/5 the variability of chimps, yet a recent study which examined over 150 fragments per individual demonstrated a number of fragments which were racially predominant (Johnson et al. MS). Thus, even though the skipjack tuna did not exhibit as much variability as some mammals, the level of mtDNA variability detected appears to be sufficiently high to

detect subpopulation divisions if they indeed existed.

Most notable is the striking lack of differentiation between Atlantic and Pacific skipjack tuna. Although a more exhaustive search will likely turn up more variants, and perhaps a fixed one, the data here suggest that the two forms share essentially the same mtDNA gene pool. There is essentially 0% sequence divergence ($p = 0$). Even if a fixed site difference were found in the present experiment, the divergence would be $1/169$ or $p = 0.006$ which is less than the p value seen between any mammalian allopatric populations. The genetic similarity of skipjack tuna from the Atlantic and Pacific Oceans is in direct contrast to the high degree of genetic differentiation which has been demonstrated electrophoretically between closely related pairs of New World tropical marine shorefishes of the Atlantic and Pacific which were separated by the uplift of the Panama land bridge (Vawter et al. 1980). The mtDNA similarity of Atlantic and Pacific skipjack tuna parallels the observation that the dolphin (Coryphaena hippurus), a strictly pelagic fish, electrophoretically demonstrates no significant interocean variability (Rosenblatt and Waples, personal communication).

This lack of differentiation between Atlantic and Pacific skipjack tuna is interesting from the standpoint of their isolation. If the uplift of the Isthmus of Panama 3.1 MY ago (Keigwin 1978, 1982) indeed isolated the Atlantic and Pacific skipjack tunas and if mtDNA evolves at about 2% sequence divergence per million years, we would expect a divergence of $p=0.06$ (Figure 3). Clearly, our data indicate that the Atlantic and Pacific skipjack tunas have not been isolated that long. Fishery catches of skipjack tuna around the tip of Africa indicate that no barrier exists for migration between the Indian and Atlantic Oceans (see Fisheries Agency of Japan, Annual reports of effort and catch by the Japanese longline fishery, and Silas and Pillai 1982). The boundary of the skipjack tuna's range is considered to be the 20-degree surface isotherm (Sund et al. 1981). Sufficiently warm water (>20 degrees) does move around the Cape of Good Hope (Davies 1962). The southward flowing Angulhis Current and the northward flowing Canary Current provide a route for movement from the Indian to the Atlantic Ocean. Furthermore, temperatures have been cooling from an interglacial maximum about 750 years ago (Emiliani 1955).

Thus the results of this study suggest that the skipjack tuna from the Atlantic and Pacific Oceans are not isolated by the Isthmus of Panama. However, there is the alternate hypothesis that the skipjack tuna are subspecies by other criteria than mtDNA and that their mtDNA is not diverging rapidly. Since in all other organisms studied to date (Borst and Grivell 1981) the mtDNA molecule is evolving very rapidly, this alternative hypothesis is less likely. Furthermore, isolation of several million years should have resulted in some fixed allelic differences between Atlantic and Pacific tunas. None have been demonstrated (Fujino 1969, Fujino 1970, Fujino and Kang 1968, and Fujino et al. 1981).

Future work at the SWFC

The SWFC is committed to evaluating the mtDNA technique. Initial plans are to develop optimal sample handling procedures so that shipboard collecting is facilitated and mtDNA harvest are enhanced. We will attempt to develop a cyroprotectant and to optimize the homogenization buffers for fish rather than using those developed for mammalian systems.

We will extend the skipjack tuna work with more samples from a variety of sites, and will initiate an albacore and an anchovy mtDNA subspecies study. Currently, the SWFC has a great variety of marine mammal specimens in frozen storage. We intend to use the Southern method for a preliminary study of the adequacy of these samples.

Summary

In summary, we wish to stress that the mtDNA technique has a degree of genetic resolution which before now has not been focused on fishery stock problems or population investigations of highly migratory animals. In this study we have investigated the genetic material itself, not gene products, the genotype rather than the phenotype.

In this pilot study of mtDNA variability in skipjack tuna from the Atlantic and Pacific Oceans, no fixed base pair differences were demonstrated

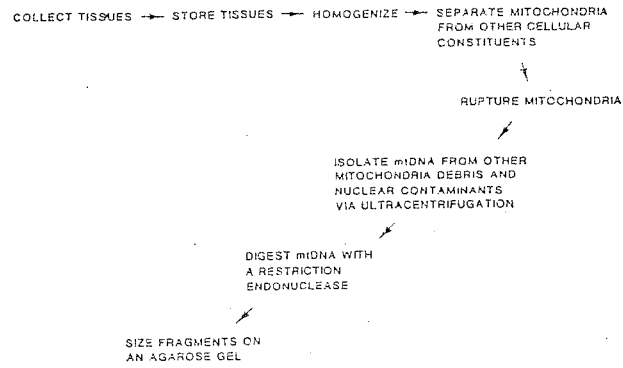
between fish from the two oceans. These results, although preliminary, suggest current or very recent genetic contact between skipjack tuna caught off Hawaii and those caught off Brazil. Perhaps it is time to consider the null hypothesis of genetic studies. That is, the failure of previous techniques to establish significant levels of differentiation in skipjack tuna may not be due to the use of insufficiently variable characters but rather that such differentiation does not, indeed, exist.

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PURIFICATION OF mtDNA



VISUALIZATION OF mtDNA FRAGMENTS

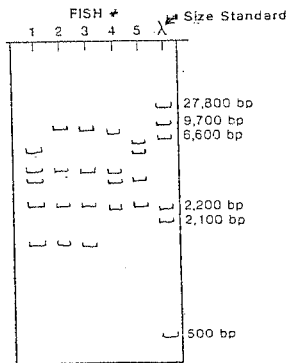


Figure 1. Flow chart of the procedures in a typical restriction endonuclease analysis of mtDNA. The size standard is produced from a restriction endonuclease digest of lambda phage DNA; the size of the fragments produced by specific restriction enzymes are available from the enzyme manufacturer.

RESTRICTION ENDONUCLEASE

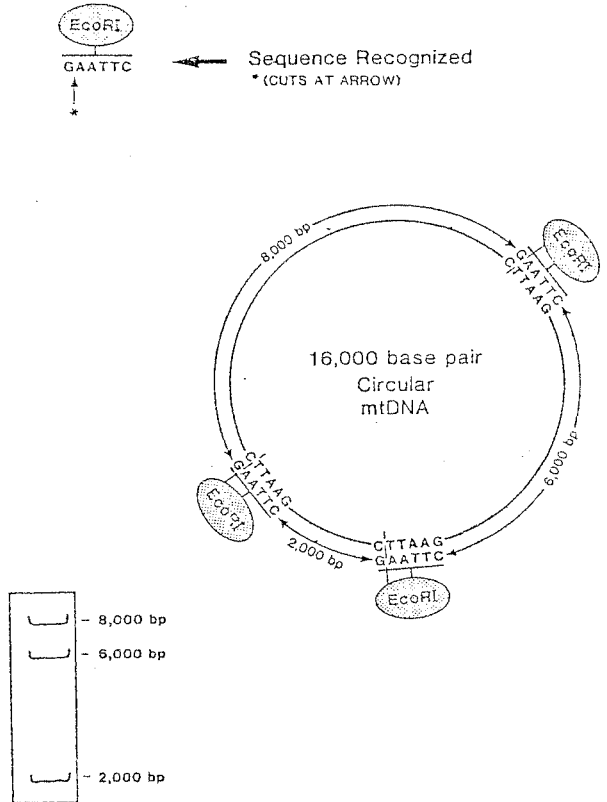


Figure 2. Operation of a restriction endonuclease on a hypothetical mtDNA molecule.

mtDNA RATE OF EVOLUTION

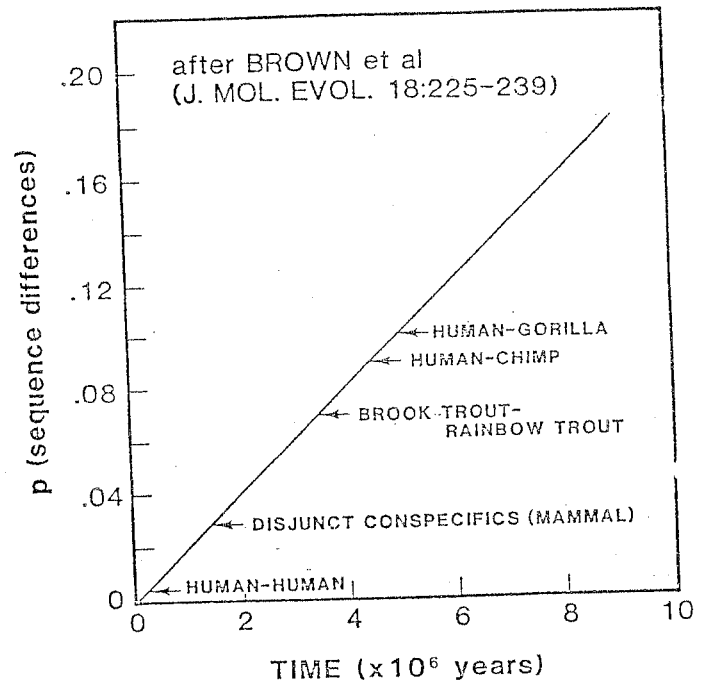


Figure 3. mtDNA rate of evolution. The p value is the number of base pair substitutions per nucleotide base pairs in the mtDNA genome. Skipjack tuna mtDNA genome has approximately 16,900 nucleotide base pairs.