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PCR Analysis of the Small Indian Mongoose Mitochondrial DNA

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Abstract

A non-coding region of the mtDNA, the D - loop region, was analyzed from small Indian mongooses (Herpestes auropunctatus) inhabiting three islands in the Caribbean. The PCR procedure was subjected to various manipulations including time, temperature, and MgCl2 concentrations. Two sets of primers with known homology to human, rat, and mouse mtDNA were used. A total of 11 multiple band patterns were observed. The multiple band pattern is most likely a result of mispriming to nuclear DNA contamination of the mtDNA enriched preparation.

Introduction

The unifying theme in biology is evolution or as one of the great evolutionary biologists, Theodosius Dobzhansky, has written, "Nothing in biology makes sense except in the light of evolution", (Dobzhansky, 1973). In its simplest form, evolution implies that all existing organisms are modified descendants of one or a few simple ancestors that arose long ago (Smith, 1989). For centuries man has labored to reconstruct the paths that evolution has taken to arrive at the diversity of organisms alive today. In doing so, two basic approaches have been employed. Classical evolutionists examine fossil records, as well as morphological and physiological characters, to determine evolutionary relationships (patterns) of organisms.

The other approach to the study of evolution involves delineating the processes that lead to observed patterns. These processes can be broadly classified into two categories: deterministic and stochastic. Deterministic processes are those that shape the genetic structure of a population through such predictable mechanisms as selection or migration. Stochastic processes include mutation and genetic drift. Genetic drift is the loss of genetic variation through the random loss of individual organisms and is a very important mechanism in populations with small numbers.

Before the mid-1960s, evolutionary mechanisms were merely speculative (Nei, 1987). Early evolutionists believed that mutations were responsible for generating variation and that natural selection was the driving force for evolution. They also argued that the evolutionary change occurred gradually over time (Nei, 1987). Therefore, experimental verification of proposed theories was difficult to obtain due to a researcher's short lifetime. That is, researchers were unable to observe tangible genetic changes of populations except under special conditions. Thus, the pre-1960's evolutionists relied on extensive mathematical studies to support theories designed to explain changes in genetic structure.

By the mid-1960s, however, the methods of evolutionary study were drastically changing. Molecular techniques had revealed that DNA was the chemical substance of genes and, furthermore, stored all developmental information. Theoretically, the evolution of organisms could now be studied by analyzing the nucleotide sequences of DNA. Molecular techniques permitted studies of evolution without the constraints of species boundarys and allowed genes within and between species to be analyzed quantitatively (Nei, 1987). During the past 30 years many molecular techniques have been adapted to the study of evolution.

Initially, direct sequencing of nucleotides proved to be difficult and expensive. However, researchers were able to study evolutionary changes of genes by analyzing amino acid sequences of proteins. Proteins, of course, are direct products of genes and the sequences of proteins are determined by the nucleotide sequences of DNA. Amino acid sequencing elucidates the exact number of amino acid differences, which amino acids are involved and their location. Amino acid sequencing, then, reveals the nucleotide substitutions in the gene itself.

Although amino acid sequencing does provide an abundance of information, the technique is also time consuming and expensive (Nei, 1987). Thus, this technique is impractical for studies of genetic structures in natural populations of organisms.

Analysis of protein variation through electrophoretic techniques was adapted as a simpler approach to the study of genetic variation. This method allows charged molecules to migrate across a voltage potential applied to a supporting matrix such as a gel. Each protein's migration pattern depends upon the overall charge of the molecule, overall size, and to some extent its 3-dimensional shape. This technique is much faster and less expensive than amino acid sequencing, and provides good estimates of genetic variation in populations (Mettler et al, 1988).

While protein electrophoretic studies have contributed substantially to the study of evolution, there are some disadvantages to the method. Electrophoretic analysis of proteins can not reveal the number of amino acid differences, which amino acids

are substituted, or the location of the substitutions (Mettler et al, 1988). This method may also underestimate the actual amount of genetic variation. Some mutations are merely substitutions of one charged amino acid for another of the same charge. In this case, the overall charge and possibly the structure of the polypeptide would remain the same. Of course, this type of mutation would be undetectable by protein electrophoresis. Thus, the method fails to separate polypeptides that are truly different.

Recently, polymerase chain reaction (PCR) and restriction enzyme techniques have been introduced into the study of molecular evolution. Restriction enzymes recognize specific nucleotide sequences and cut the DNA at or near those sites. The result is fragments of DNA that can be electrophoretically separated. The restriction fragment length polymorphism (RFLP) patterns generated can be compared within and between species to estimate genetic variation. However, the number of fragments generated tends to be very large and the result will be a gel that is very difficult to interpret (Mettler et al, 1988). Hence, this technique is better suited for use with DNAs that are small such as mitochondrial DNA (mtDNA).

PCR is a method that permits the amplification of defined regions of the DNA molecule. By selecting primers that flank the region of interest, a specific DNA sequence can be amplified. The new copies as well as the original then serve as templates for further amplification (Weaver and Hedrick, 1989). In this way, the amount of DNA grows exponentially, resulting in millions of copies of a specific DNA sequence in a matter of a few hours. As with most molecular techniques, PCR has its disadvantages. For instance, good DNA templates must be used to avoid amplification of non-target sequences. The conditions under which the reaction is run must be tightly controlled as well to avoid amplification of unwanted regions and correct primers are required. Overall, PCR techniques can be difficult to perfect.

The focus of this research has been to study the evolutionary processes, specifically the founder effect, at work in introduced insular populations of mongoose through use of PCR amplification of the D-loop region of mtDNA. The founder effect has been an important and controversial force in evolutionary studies since it was originally conceived by Mayr in 1942. Founder events occur when a relatively small number of individuals are removed from their original population and are moved into another environment to establish a new population. Mayr (1942) argued that founder populations most likely do not contain all the genetic variation of the original population. Furthermore, the founder population will suffer even more loss of genetic variation due to genetic drift. These pre- and post-founder event processes add up to the process known as founder effect.

Crow and Kimura (1964) offered a mathematical analysis that suggested the founder effect (loss of genetic variation) is not as dramatic as suggested by Mayr. According to their analysis, 75 % of genetic variation will be maintained through a founder event of two. Additionally, Nei et al (1975) expanded the analyses of founder effect to include the impact of population growth rates upon genetic structure in post-founder populations. They concluded that retention of high levels of genetic heterozygosity, as estimated through protein electrophoresis, was likely when post-founder populations exhibited rapid population growth rates. Both studies indicated that overall levels of genetic variation would be minimally affected by founder effects, but that a loss of rare alleles would be expected.

The small Indian mongoose (Herpestes auropunctatus) is a species that has undergone founder events. The populations inhabiting the islands in the Caribbean Sea and Pacific Ocean provide an opportunity to examine effects founder events may have had on the genetic structure of these insular populations. In many cases, the date of introduction of the animals onto the islands and the founder population size is well documented. For instance, the first founder event occurred in 1872 when nine mongooses (imported from India) were introduced onto Jamaica. Between 1882 and 1884 two to four pairs of mongooses were introduced onto the island of St. Croix
(Espeut, 1882). Because these animals originated from the founder population on Jamaica, they experienced yet another founder event.

Several scientists have been intrigued by the unique circumstances surrounding the colonization of these islands and have attempted to determine the genetic structure of some of the mongoose populations. A protein electrophoretic analysis of 39 loci of populations inhabiting five islands (St. Croix, Jamaica, Puerto Rico, Hawaii, and Oahu) was conducted to assay current levels of genetic variation. The study revealed no significant differences in heterozygosity levels between these insular populations (Hoagland, 1988). Their analysis appears to support the observation that Nei et al's (1975) contention that a rapid population growth rate will maintain heterozygosity within founder populations. Hoagland's study failed to detect any influence of small introductions on subsequent genetic structure. However, as mentioned above, protein electrophoresis generally underestimates levels of genetic variation. Therefore, it is possible that differences in levels of genetic variation exist, but may only be detected by more sensitive methods such as PCR.

The mtDNA molecule is a logical choice for analysis of genetic variation. The molecule has a simple sequence organization, no introns, and recombination is absent. Additionally, mtDNA is maternally inherited.

Thus, the effective population size is smaller than that for nuclear DNA (Harrison, 1989). Finally, while the coding regions of mtDNA are highly conserved, the D-loop region is subject to mutations. Consequently, the D-loop region is a sensitive diagnostic tool that can detect genetic variation.

Materials and Methods

Frozen liver from previously collected tissue samples of mongooses captured on three islands (St. Croix, U.S.V.I., Jamaica, and Hawaii) was used as a source of DNA. An enriched mtDNA preparation was isolated from ten samples from each island according to procedures developed in the laboratory of C. W. Kilpatrick (University of Vermont). Verification of mtDNA was achieved through agarose gel electrophoresis and ethidium bromide staining (Sambrook et al., 1989). DNA samples were resuspended in ddH2O and stored at -20 OC until amplification by polymerase chain reaction. Peromyscus maniculatus DNA was also isolated and analyzed as a control using the same procedures.

DNA amplification was carried out using an EricComp Thermocyler (EricComp, CA), and reagents for all reactions were obtained from a GeneAmp Kit (Promega, Madison, WI). Two sets of primers, with known homology to sequences flanking the D-Loop region of human, rat, and mouse mtDNA were obtained from Oligos Etc. (Gulleford, Ct.). Cycle variations included time, temperature, number of cycles (either one or two), and cycle repetition. Times ranged from 0.5 - 3.0 min in step one and from 0.5 - 2.0 min in steps two and three. Temperatures ranged from 42 - 500 C in step two and from 68 - 72 0 C in step three.

Results

The mtDNA enriched preparation of mongooses and P. maniculatus appeared as a smear on the agarose gel following ethidium bromide staining. In most cases, a bright, large band could be seen in the 17 kb region.

PCR amplification of the mtDNA enriched preparation resulted in multiple band patterns. A total of 11 different multiple band patterns were obtained, four from P. maniculatus and seven from mongoose DNA (Table 1). The number of bands per pattern ranged from one to four. However, the average number observed was three. Band size ranged from 100 to 1400 (+/- 50 bp). Bands amplified in the presumptive D-loop region (approximately 1400 bp for mongooses and 1000 bp for P. maniculatus) were large and stained intensely. Smaller bands were generally smaller and stained less intensely.

Discussion

The above results are not what I had expected. I had hoped to see one large band in the 1400 bp (+ 50 bp) region for mongooses and one band approximately 1000 bp for P. maniculatus and not multiple band patterns. Still, I was able to achieve amplification of the presumptive D-loop region in some of the band patterns. The extraneous bands are most likely a result of promiscuous amplification. This type of amplification occurs when the primers anneal not only to the target sequences flanking the D-loop region but also to homologous sequences found elsewhere on the DNA strand. The remaining band patterns (those without D-loop amplification) can be explained as a result of degradation of the mtDNA.

Table 1. Representative patterns obtained from PCR amplification of mtDNA enriched preparations of mongoose and Peromyscus maniculatus DNA. Lanes A, C, J, and K represent P. maniculatus PCR amplified DNA and lanes B, D, E, F, G, H, and I represent PCR amplified mongoose DNA. bp designates number of base pairs +/- 50.

bp	A	B	C	D	E	F	G	H	I	J	K
1400						—					
1000	—		—							—	—
750	—			—						—	—
500				—			—			—	
400					—			—	—		—
300		—	—	—	—	—	—				
150			—		—	—					—
100						—					

Multiple band patterns can often be eliminated through modification of the reaction conditions. Often, primers that are annealing to other regions of template DNA do so because the annealing temperature is set

too low. The low temperature allows the primers to non-specifically anneal to the DNA strand. Raising the temperature 2 - 50 C creates a more stringent condition in which the primers should anneal to their complementary sequences. Often, the annealing step is extended in addition to (or instead of) lowering the annealing temperatures. The extension of time better allows the primers to find their complementary sequences. Furthermore, the Taq polymerase will extend the primer sequence which locks the primer to its complement on the template. Finally, MgCl2 concentrations may also be manipulated. Too little MgCl2 in the buffer will result in no PCR product and too much MgCl2 will result in multiple band patterns (Sambrook et al, 1989). Each of the above outlined solutions were attempted in a systematic manner. However, each procedure resulted in multiple band patterns.

Primers, of course, are another possible cause for the multiple band patterns. Normally, primers are the last solution considered due to the high cost of replacing each pair of primers. Primers may be any sequence, but relatively equal numbers of each nucleotide without internal repeats or self-homology is ideal (Sambrook et al, 1989). Internal repeats or self-homology often result in mispriming or primer dimers and, ultimately, random base insertion during the extension step. One set of primers (XT) were tested for possible dimer activity: a PCR reaction was run with only the primers and no template DNA. No amplification was observed. The sequences of the primers were then sent to Dr. Rod Sobieski at Emporia State University. Dr. Sobieski was able to check the XT primers against 172,358 sequences through the National Center for Biotechnology Information at NIH which accesses Genbank and the European Molecular Biology Library through Internet. The XT primers were found to be homologous to human, rat, and mouse mtDNA as expected. However, the primers were also found to be homologous to three human nuclear DNA sequences.

Another pair of primers (Ld) were selected. The Ld primers were run under the same reaction conditions as the XT primers. Unfortunately, the Ld primers generated the same multiple band patterns as the XT primers. Again, the primer sequences were sent to Rod Sobieski for sequence comparisons through Internet. The Ld primers were found to be homologous only to human and rat mtDNA. Because no matter which procedure I attempted resulted in multiple band patterns, both pairs of primers and the template were sent to Dr. Sobieski's lab. Dr. Sobieski ran the same reaction conditions and produced the same results.

As mentioned above, no matter which procedure was attempted, the same multiple band patterns were obtained. PCR requires a relatively pure template. Any contamination present in the reaction solution will be amplified. Thus, amplification of non D-loop regions, specifically nuclear regions, was most likely occurring. Cesium chloride (CsCl) density gradients provide a more accurate means of separating mtDNA from nuclear DNA than the protocol employed during this project (Sambrook et al, 1989). Cesium chloride is a heavy salt which forms a gradient when spun in the ultracentrifuge. The high density particles of CsCl fall to the bottom of the tube while the lower density particles remain near the top of the solution. DNA, when added to the solution, will seek an equilibrium position at the point in the tube where its density exactly matches that of the CsCl. Thus, the two types of DNA, nuclear DNA and mtDNA, will form two separate bands in the tube. Another option which could be considered in the future is Zimmerman et al's (1988) technique. In this approach, nuclear DNA is destroyed by treating the preparation with DNase before the cells are ruptured. A sucrose gradient is then used to extract the mtDNA. In view of the laboratory facilities available, the latter technique is the most feasible.

The PCR technique has been lauded as one of the most important discoveries in molecular genetics. The DNA polymerase that drives the PCR reaction was even voted molecule of the year by Science in 1989. Yet, those that attempt the technique know that it is not as simple as it seems and certainly not as easy as some scientific papers would have you believe. Rather, this technique reinforces the idea that science is, indeed, an art form. PCR is an extremely sensitive procedure that requires a certain finesse to be successful. Still, the PCR technique is an exciting procedure that offers a wide range of applications. Furthermore, the technique's ability to go beyond traditional boundaries and the freedom it extends to the researcher are some reasons PCR was chosen for this project. PCR will be an instrumental tool which will help to settle evolutionary controversies such as the one involving the importance of founder effect. Continued PCR analysis of the mtDNA D-loop region will undoubtedly contribute significant data that may find that founder has affected genetic variation in introduced insular populations of small Indian mongooses.

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