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USING RANDOM SEQUENCE PRIMERS IN THE POLYMERASE CHAIN REACTION TO IDENTIFY GENDER-SPECIFIC GENETIC MARKERS IN HOUSE WRENS

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ABSTRACT

In order to fully understand the biology of asexually reproducing organism, it is essential that one is able to distinguish the males from the females. In determining the gender of monomorphic birds, standard techniques including visual identification, surgery, and karyotyping are impossible or impractical for large-scale studies. A reliable gender identification method that uses genetic markers identified within the DNA would be an asset to the researcher because it would require only a minimal blood sample which could be collected in the field without harming the bird and stored easily for long periods of time. Griffiths and Tiwari (1993) described such a technique based on the generation of RAPD markers (Random Amplified Polymorphic DNA). The use of RAPDs involves the amplification of genomic DNA in the polymerase chain reaction (PCR) using primers of arbitrary oligonucleotide sequence to generate a range of DNA fragments that can be separated by agarose gel electrophoresis. This study employs this method to generate a reliable sex probe for the house wren (Troglodytes aedon), using RAPDs to isolate female-specific markers from random locations on the W sex chromosome. Results indicate that after extensive manipulation of the Griffiths and Tiwari protocol, consistant PCR amplification of house wren DNA was achieved. However, further research is necessary to find a primer that will yeild W specific fragments in large samples of wrens. If successful, the sex probe will be used in future studies of house wren reproductive strategy. Specifically, gender identification information of house wren nestlings will be used to investigate the maternal condition hypothesis.

INTRODUCTION

Visual gender identification in birds can be difficult or impossible in sexually monomorphic species. Sex identification is further complicated in juvenile birds due to the absence of secondary sex characteristics. Surgical gender verification is reliable but not practical because it is invasive and may place the individual at great risk (Longmire *et al.* 1993). Furthermore, surgeries are time-consuming and can only be performed by experienced field technicians, making large-scale gender identification an impossible task. Karyotype analysis has been used in birds to locate the W sex chromosome found only in heterogametic females; however, such studies have met with limited success due to difficulties in obtaining avian chromosome spreads and due to indistinguishable sex chromosomes (Prus and Schmutz 1987),

A reliable gender identification method that uses genetic markers identified within the DNA would be an asset to the researcher because it would require only a minimal blood sample that could be collected in the field without harming the bird, and then stored easily for long periods of time (Arctander 1988). The difficulty in developing such a molecular sex probe lies in finding sex chromosome-linked markers in the genome. In mammals, the discovery of the SRY gene (Sex Determining Region - Y chromosome) has virtually eliminated this difficulty (Foster *et al.* 1992). In birds, however, no such widely conserved sex-specific gene has been described and gender markers must be isolated from DNA sequences that are not common to all avian genera (Tone *et al.* 1984).

Molecular sex probes have been successfully developed for some species of birds. These probes have made use of either Restriction Fragment

Length Polymorphism (RFLP) analysis, which hybridizes W-specific DNA fragments (Griffiths and Holland 1990), or W-specific minisatellite and microsatellite DNA analysis (Longmire *et al.* 1993, Rabenold *et al.* in press). These techniques are slow, labor intensive, and require large amounts of DNA, and thus are not any more practical for field studies than are non-molecular methods (Sabo *et al.* in press). The generation of genetic markers by Random Amplified Polymorphic DNA (RAPDs) as described in Williams (*et al.* 1990) represents an alternate method for the identification of W-specific markers and is favored due to its economic and temporal feasibility. The use of RAPDs as a sex probe does not depend on the existence of preknown target nucleotide sequences, as is the case in the use of microsatellite and minisatellite DNA analyses, nor on the design of specific target primers as in the use of RFLPs (Williams *et al.* 1990, Welsh and McClelland 1990). Rather, this technique involves the amplification of genomic DNA fragments by using random primers in a polymerase chain reaction (PCR) (Mullis *et al.* 1986, Mullis and Faloona 1987, White *et al.* 1989). PCR is a technique for *in vitro* replication of specific DNA sequences by simultaneous primer extension on complimentary strands of denatured DNA. *Taq* DNA polymerase carries out the synthesis in the 5' to 3' direction on each single stranded template after the DNA has been denatured by heating. The strands are then reannealed when the reaction is cooled. PCR is a cyclic amplification reaction that will produce over a million copies (nanogram amounts) of DNA after only twenty cycles of heating and cooling. RAPD analysis uses PCR and oligonucleotide primers of arbitrary sequence to generate a range of DNA fragments, presumably from numerous locations within the genome. When a primer is able to generate identifiable bands from random locations on the W chromosome, it can be employed in sexing birds.

Griffiths and Tiwari (1993) described the use of RAPDs to generate reliable gender identification markers for the great tit (*Parus major*), the jackdaw (*Corvus monedula*), and the zebra finch (*Tenopygia guttata*). They used a single primer to produce fragments specific to females, and then seperated the bands by agarose gel electrophoresis. This paper describes the use of a variation of Griffiths and Tiwari's method to generate a reliable sex probe for house wrens (*Troglodytes aedon*). The house wren is a sexually monomorphic species that is known to be a valuable study organism because of their willingness to breed in nestboxes. The development of a molecular sex probe will enable researchers to investigate questions concerning adaptive offspring sex ratios and related aspects of reproductive strategy.

METHODS

DNA Collection

The DNA used to develop the sex probe was extracted from blood samples drawn from adult house wrens in the summers of 1991 and 1993. These samples were collected from captured wrens breeding in nestboxes located about 30 km northeast of Bloomington, IL (see Harper *et al.* 1992). Adult wrens were visually sexed in the hand at the time of collection by the presence of brood patch only in females, and by the males' much larger cloacal protuberance. Approximately 50-200 ul of blood was collected from each adult in microcapillary tubes from the brachial vein where it crosses the elbow. Blood samples collected in 1991 were stored in phosphate-buffered solution (PBS) preservative while blood used from the 1993 field season was preserved in Longmire's Solution (P. Parker, personal communication). DNA samples from both seasons were used to control for the possibility of pipetting errors

associated with the increased viscosity of the samples stored in Longmire's Solution.

Polymerase Chain Reaction

Genomic DNA was prepared from samples from several males and females using standard phenol extraction methods (Sambrook *et al.* 1989), and was amplified by low stringency polymerase chain reaction (PCR) (Lewin 1990). Amplifications were carried out with a single, ten-base pair primer (a 10-mer) of arbitrary sequence, which produces a range of fragments from the genome wherever the primer binds at opposing sites within 2-3 kb of each other (Welsh and McClelland 1990).

Initially we followed the PCR protocol outlined in Griffiths and Tiwari (1993), but this was modified in response to inconsistencies in amplification products (see Results). Original PCR amplifications were carried out in 10 ul total volume containing 20-100 ng of genomic DNA, 200 uM of each dNTP, 5-10 pmol of primer, 0.33 units of *Taq* polymerase, 50 mM of KCl, 10 mM of Tris-HCl, 1.5 mM of $MgCl_2$, and 0.1% Triton X-100. Primers were purchased from Operon as a kit containing twenty random sequence 10mers. Primers arrived freeze-dried and were diluted in sterile TE to the correct concentrations as they were used. Solutions of $MgCl_2$, *Taq* polymerase, and each dNTP were purchased from Promega. A reaction buffer solution, also purchased from Promega, contained the correct concentrations of KCl, Tris-HCl, and Triton X-100. All reactions were prepared in 0.6 ml microcentrifuge tubes. The thermal profile for PCR was 38 cycles of 1 minute at 37 °C, 3 minutes at 72 °C with a ramp rate of 6 seconds/degree between annealing and extension steps, and 1 minute at 94 °C.

Extreme caution was exercised to insure that sterile conditions were maintained at the laboratory bench. Any contamination from previously amplified products or from any other DNA source (including, but not limited to hair roots or sloughed-off skin cells from the PCR operator) could act as a target template for the reaction resulting in amplification of the wrong source (a false-positive signal) (McPherson *et al.* 1991). Microcentrifuge tubes, distilled water, and pipette tips were all autoclaved and kept specifically for PCR, and were stored apart from equipment used for any other preparations. Disposable gloves were worn at all times and changed frequently. Sterile paper bench covering was changed at the onset of all preparations. Negative reagent controls containing water instead of template DNA were included with each reaction to control for contamination during preparation by highlighting any false-positives.

Gel Electrophoresis

Fragments produced by PCR were separated by size in 1.4% agarose gel electrophoresis (Williams *et al.* 1990). Gels were loaded with 20 ul total volume per well consisting of 10ul ddH_2O, 6 ul urea tracking dye, and 4 ul of PCR product. Separation was achieved at 100 volts for 2-3 hours. Bands resulting from the amplification were then visualized by staining the gel with ethidium bromide and photographing it under ultraviolet light. Gels containing ambiguous banding or excessive background staining were destained in water prior to photographing.

RESULTS AND DISCUSSION

Because of the extreme sensitivity of PCR, successful DNA amplification depends a great deal on the conditions of the reaction and the concentration of the reagents. When beginning PCR on an organism for the first time, inordinate amounts of time are typically spent solely in obtaining the parameters which will produce the desired results. Such has been the case in our quest for the house wren sex probe. The results of one set of trials were always the basis for the modifications implemented the following week.

The first amplifications followed the Griffiths and Tiwari protocol without modification. Genomic DNA from two males and from two females was used in two concentrations. Stock concentrations of the extracted samples were calculated to be between 244.2 and 607.6 ug/ml by spectrophotometry. Dilutions of 10:1 gave concentrations within the range specified by the protocol. Additionally, samples of 100:1 dilutions were included to see if less DNA would lead to less non-specific primer binding (McPherson *et al.* 1991). The results (Fig. 1) indicate that some amplification did occur, but the degree of inconsistency prevented any conclusions as to the optimal DNA concentration or as to the value of this primer as a sex probe. Subsequent trials with this and other primers yielded similarly inconsistent banding such that the reaction conditions required modification. No false-positives occurred in the negative controls suggesting that the bands produced were from the wren template, and that no contamination had occurred.

In response to the sporadic amplification of the initial trials, DNA from blood samples collected in 1991 was tested for its ability to generate more consistent banding. The DNA used in the first reactions was originally

collected into Longmire's Solution in 1993. The extracted DNA from these samples was very viscous and the level of accuracy in pipetting such small quantities for each reaction was in question. The 1991 samples that were collected into PBS buffer were less viscous. For these reactions the 1993 DNA source was retested along with the 1991 source to control for both the viscosity of the template and for the possibility that the 1993 samples were simply a poor source of DNA. If the 1991 samples proved to amplify with any degree of consistency, additional tests would have to be performed to determine whether the problem with the 1993 was indeed its viscosity, or if it simply was a poor template. The results of these side-by-side PCR attempts are shown in Figure 2. Neither the 1991 samples nor the formerly tested 1993 samples produced any consistent amplification, which indicated that the source of DNA was not the sole problem with our reaction conditions and that the other reagents would have to be explored for their role in obtaining successful amplification. For all subsequent protocol manipulations (except for those involving EDTA), the 1993 DNA samples were again used because results could be compared to the few bands it was able to produce in trials following the original Griffiths and Tiwari protocol. Only one reagent was manipulated in any one trial so that any consistency in banding could be attributed to the effects of its manipulation.

Inquiries to other researchers familiar with PCR troubleshooting suggested that different concentrations of *Taq* polymerase be tested. The reactions were run with three new *Taq* concentrations; 0.70 units/RXT (double the amount in previous reactions), 1.0 units/RXT, and 3.5 units/RXT (both of the latter concentrations were chosen because of ease of calculations). The results of these reactions are shown in the gel photograph of Figure 3. As was the case with the DNA manipulation, no bands were produced in any of the

reactions. To determine if the polymerase itself had become defective, possibly due to repeated thawing and refreezing or due to a defective stock from the supplier, new *Taq* polymerase was ordered and tested with similar lack of results (Fig. 4).

Intensive literature review revealed that PCR may be inhibited by the presence of a high concentration of EDTA. In PCR, $MgCl_2$ is a source of Mg^{2+} ions required by the *Taq* polymerase for synthesis of primer extensions. The presence of EDTA inhibits amplification by decreasing the nuclease activity by binding free metal ions (McPherson 1991). EDTA was present in the sterile TE that was used as a primer dilution and in the final step of the DNA extraction process. To test if either of these EDTA sources were inhibiting amplification, new dilutions of primer in sterile water were used. In conjunction with the new primer preparation, two independent experiments were also conducted; the first to determine whether or not the dialysis at the end of the phenol extraction of DNA into its buffer solution was inhibiting the reaction, and the second to determine if increased $MgCl_2$ concentration would increase the degree of amplification. In the first experiment we did not change the $MgCl_2$ concentration from that used in previous reactions, but we used DNA collected in 1990 that was extracted into sterile water instead of the buffer solution. The second experiment involved the use of the 1993 DNA samples (in buffer solution containing EDTA) in conjunction with four new concentrations of $MgCl_2$ (2.0mM, 3.0 mM, 4.0mM, and 5.0mM). The reactions performed with DNA stored in water did not succeed in producing bands, but those with increased $MgCl_2$ concentrations and 1993 DNA did produce bands (Fig. 5). These results indicated that the EDTA present in the 1993 DNA samples was not an inhibitor to any degree that could not be compensated for with an increased concentration of $MgCl_2$. All subsequent

reactions were carried out using that $MgCl_2$ concentration that yielded the best results (4.0mM).

Although the degree of amplification produced by the new $MgCl_2$ concentration was marked improvement over that found in previous trials, consistency was still lacking even in reactions with identical reagent composition. This is demonstrated in the results of an experiment in which the same reagents were used, including the same DNA sample, in the preparation of four reactions for each bird. The purpose of these repeated reactions was to investigate the possibility that inconsistency was due simply to errors in pipetting accuracy. It was speculated that working with such extremely small volumes of each reagent made it impossible to adhere exactly to the concentrations specified in the protocol. My suspicions were confirmed by the observance of amplification in only some of the reactions (Fig. 6). Thus, the inconsistent amplification was due to procedural problems associated with our method of preparation, rather than due to a problem of reagent manipulation.

A new technique of reaction preparation was employed that would decrease the chances of pipetting imprecise amounts of each reagent. When performing multiple reactions with a single primer, the only reagent that is different for each reaction is the DNA source. In this experiment one large bulk reaction was mixed with all reagents except for the DNA. From this large "cocktail" of reagents, individual reactions were aliquoted to respective microcentrifuge tubes and the DNA for each reaction was added to the tube. This allowed for pipetting larger volumes and would insure that proper mixing of reagents had occured; thus the only problem encountered would be with the pipetting of DNA. The presence of amplification product in every sample (Fig. 7) indicated that pipetting error was indeed the cause of the inconsistent results.

The achievement of consistent PCR amplification paves the way for the isolation of the molecular sex probe. The future development of the project will involve two major forms of experimentation: First will be the surveying of numerous primers in hopes of finding one that will generate bands only in female wrens. The second phase of experimentation will be to use said primers in PCR with DNA samples from numerous adult birds of known sex. This will insure researchers of the universal ability of the probe to accurately determine the gender of house wrens.

FUTURE APPLICATIONS

The availability of a reliable and efficient means of determining the sex of the offspring produced in a population or within discrete breeding units (i.e. family units) represents a great potential for application in studies concerning adaptive offspring sex ratios. In populations of sexually reproducing vertebrates it has been proposed that sex ratios of offspring will be maintained at unity; in addition to the random segregation of sex chromosomes predicted by Mendelian assortment, Fisher (1958) hypothesized that observed equal sex ratios should result from stabilizing selection due to the increased reproductive success of the rarer sex. However, numerous recent studies have indicated that selection at the level of the individual may result in parents increasing their reproductive fitness by producing skewed sex ratios. This may be in response to environmental conditions, or in response to their own or their mate's physical fitness (Trivers and Willard 1973, Meyers 1979, Weatherhead 1983, Gowaty and Lennartz 1985, Austad and Sunquist 1986). Parents can increase their fitness by producing more offspring or by producing highly competitive

offspring, both of which result in more of the parent's genes being passed on to subsequent generations.

The maternal condition hypothesis (Trivers and Willard 1973) is most often cited to describe a parent's facultative manipulation of its offspring sex ratio around the mean population sex ratio. This hypothesis states that a female in good condition should produce more offspring of that sex facing the greatest variance in reproductive success. Males usually exhibit greater variance in reproductive success because their investment in the subsequent generation is much more variable due to the fact that some males may successfully mate many times, while others may not mate at all. Because a healthy female should produce strong competitors, she would benefit if she produces more of the sex that has the greatest opportunity to maximize its investment in the next generation (usually males). In contrast, a female parent in poor condition should produce more offspring of that sex that is less variant in its probable success.

Possible mechanisms for the adjustment of sex ratios include facultative adjustment prior to conception (e.g. non-random segregation of sex chromosomes), and manipulation after conception via sex-biased offspring mortality (Griffiths 1992). Fundamental to the ability to test the maternal condition hypothesis and its proposed mechanisms in birds is the large-scale, accurate determination of offspring gender. The success of the development of a molecular sex probe will determine the extent to which a study population of house wrens can be investigated with respect to these and similar questions.

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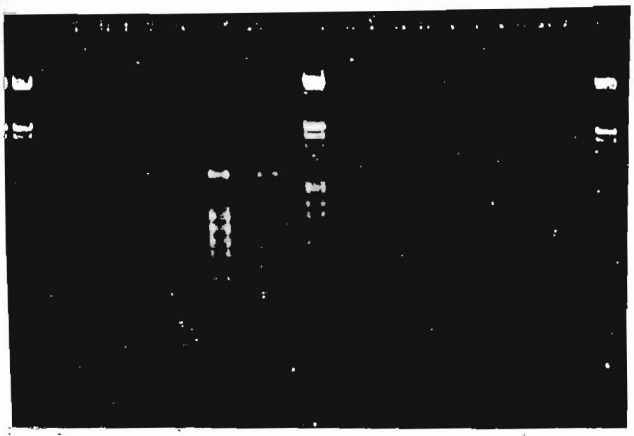

Figure 1. Agarose gel showing PCR amplification of only two samples (lanes 9, 11) using unmodified protocol from Griffiths and Tiwari. Lanes 1,13,25 show Ecor1-HindIII nucleic acid marker.

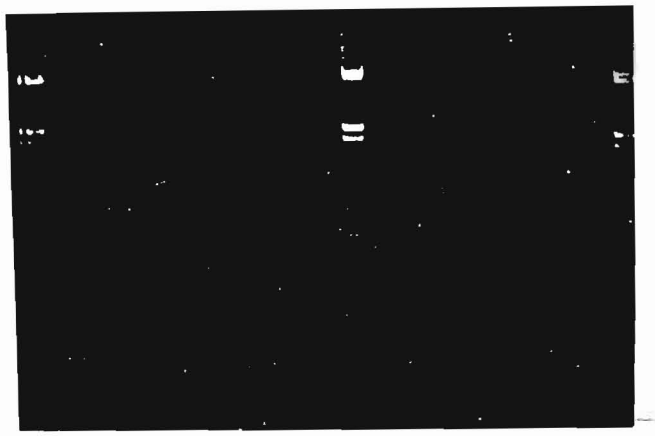

Figure 2. Comparison of PCR using DNA Stored in different buffers. Lanes 2-14 contain samples in PBS, Lanes 16-24 contain samples in Longmire's, and lanes 1, 15, and 25 contain marker.

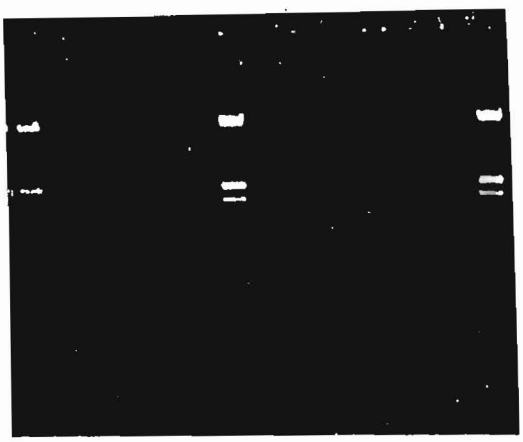

Figure 3. Testing whether increased concentrations of Taq polymerase affects amplification. Lanes 2-7 are female wren DNA, lanes 9-16 are males, each with three different amounts of Taq. Lanes 1,8,and 17 contain marker.

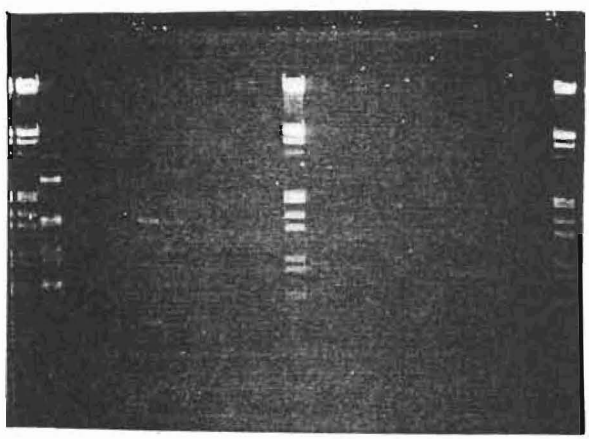

Figure 4. PCR using two stocks of Taq polymerase to determine if the original stock had become defective. Lanes 2-4 contain old stock, lanes 5-22 contain reactions with new Taq, and lanes 1,12, and 23 contain marker.

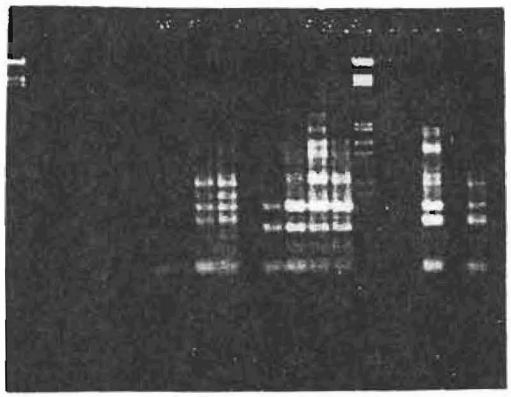

Figure 5. Two PCR experiments to determine the effect of EDTA. Lanes 2-5 contain DNA extracted without EDTA, lanes 6-24 contain samples with four concentrations of $MgCl_2$, lanes 1,16 contain marker.

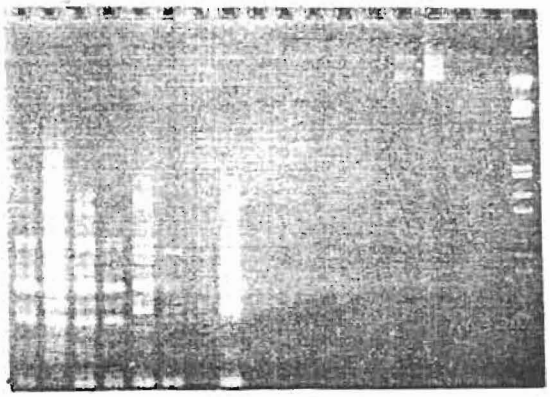

Figure 6. Testing for procedural error with multiple repeat reactions. Lanes 1-8 contain reactions with two individuals repeated four times each, lanes 11-17are the same reactions but in 50 ul total volume instead of in 10 ul, marker is in lane 18.

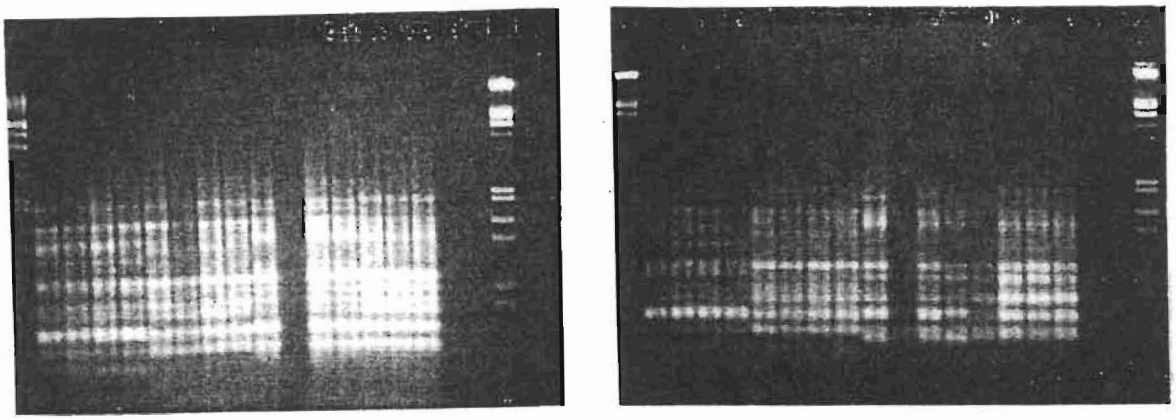

Figure 7. Two sets of reactions showing successful amplification in two different total volumes, each using the "cocktail" method of reagent pooling in preparation. The photo left shows reactions in 10 ul: Lanes 2-18 are samples of four individuals repeated four times each, marker in lanes 1, 19. The photo right shows the same reaction but prepared in 25 ul total volume.