Optimizing PCR Amplification of Sry Gene for Sex Identification in Ctenomys sociabilis

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Abstract

Two different primer sets were used in PCR amplification of the Sry gene and the Zfy/Zfx gene as a method of sex identification in Ctenomys sociabilis, the colonial tuco-tuco. Varying PCR conditions and reaction set-ups were tested to optimize the protocol for determining the sex of this species. Optimum annealing temperature for the Sry-related sequence was shown to be ~51°C and a PCR touchdown was necessary to eliminate amplification of nonspecific products. The Zfy/Zfx-related gene sequence had an optimum annealing temperature of ~57°C, but the primer set was specific enough to anneal consistently at 51°C. Optimum DNA concentration results need further study, but it was found that the total DNA needed for PCR ranges from 20-40ng per 25uL reaction. While the amplification of the Sry region was successful, the variability of the yield indicates that designing a more specific primer set for tuco-tucos would be the next logical course of action.

Introduction

The colonial tuco-tuco, Ctenomys sociabilis, is a subterranean rodent native to the Neuquen Province, Argentina. These rodents are social, meaning that they live in groups of multiple related adult females and at most one male. The sex ratio of adults is highly female biased in the burrow. In order to preserve this female to male ratio in the burrow, males need to leave the burrow they were born in at a higher rate than females. Long-term field studies of C. sociabilis have demonstrated that males disperse more frequently than females from their native burrow (Lacey et al. 2004). Consequently, it is hypothesized that predators eat males more
routinely than females due to the inherent riskiness of dispersing. This sex-biased pattern of dispersal is important for group living and could provide evidence for a species living socially. The major premise of males dispersing at a higher frequency than females and the assumption that these males are more likely to be preyed upon make the foundation for which this experiment rests on.

According to previous studies, *C. sociabilis* underwent a genetic bottleneck 3,000 to 5,000 years ago (Lacey 2001). There is debate as to whether the species was social before the bottleneck or if the reduction of genetic diversity led the species to become social. A way to indirectly examine if colonial tuco-tucos were social before the bottleneck would be to see if, prior to this event, the frequency of dispersal by males exceeded that of females. Assuming that animals that leave their natal burrow are more likely to be killed by predators, we can study the bones of tuco-tucos killed more than 3,000 years ago to determine if dispersal was male biased at this point in time. Using the information gleaned from this protocol I have optimized, another scientist may test the hypothesis that the historic samples collected before the genetic bottleneck will be male biased. This future data should yield important clues as to what the social system was before the dramatic reduction in population size and genetic diversity.

Argentinian biologists have provided the lab with historic samples of bones and teeth of *Ctenomys sociabilis* collected from caves. Deposition of these bones is the result of owl predation. Due to the nature of owl pellet deposition, bones collected from the caves provide a continuous temporal sequence of material dating from around 2,000 to 10,000 years ago. From these specimens, DNA can be extracted, amplified via PCR, and examined for an X and Y chromosome marker to determine the sex of the individuals represented in the historical material.
Specifically, sex will be determined by assessing whether a sample contains 2 PCR bands (X and Y chromosomes = male) or only a single PCR band (X chromosome only = female).

My project consisted of using modern day *C. sociabilis* tissue samples of known sex in order to optimize PCR conditions for genetic sexing of this species. Once these best conditions for PCR reactions have been determined, another scientist can apply the same PCR protocol to the older, more degraded genetic samples in a future study.

**Materials and Methods**

*Subject Samples*

Ten liver tissue samples were provided by Professor Lacey from her modern tuco-tuco subjects in the lab. Modern samples are used in this experiment for two main reasons. One, they are of known sex. Two, because they are modern, the DNA has not undergone the degradation and deamination associated with historic samples. The liver samples were taken from 5 males and 5 females and assigned a letter code so that knowing the sex of the sample did not bias the analysis of gels.

♀ 144D = A  ♂ 575 = B  ♂ 657 = C  ♂ 61B = D  ♀ 41F = E  ♂ 500 = G  ♀ 123 = F  ♀ 64C = H  ♂ 223 = I  ♀ C7B = J

After the gels were run and examined for the desired bands, the assigned code was matched to the identification number of the sample to compare the sex of the sample and the sex indicated by the gel. The original tissue samples were then sub-sampled, meaning that I took roughly half of the sample and transferred them to different tubes to avoid contamination of the original samples and to apply the code names. They were suspended in EDTA DMSO buffer.
DNA Extraction

DNA for PCR reaction was extracted using a Qiagen DNeasy Blood and Tissue Kit. I primarily followed “Protocol: Purification of Total DNA from Animal Tissues (Spin-Column Protocol)” with some modifications. 180uL of ATL buffer was added to a 1.5uL microcentrifuge tube. Approximately 25mg of tissue was removed from the liver sample, placed into the tube, and cut into small pieces with forceps to increase surface area for enzyme activity. 20uL of proteinase K was added, the whole solution was mixed by vortexing, and incubated in the thermomixer for 1-3 hours (55°C), or overnight. The function of these last steps was to degrade the tissues and allow for the isolation of the DNA.

Once the samples were removed from the thermomixer, they were vortexed for 15 seconds. 200ul of AL buffer and 200ul of 96-100% ethanol was added to each of the samples and then they were vortexed again for 15 seconds. The entire solution of each sample was individually transferred into the DNeasy mini spin column placed in a 2ml collection tube to collect the flow through from centrifuging. I Centrifuged at 8000rpm for 1 min and discard flow through and collection tube. The spin column was placed into a new collection tube, 500uL of AW1 buffer was added to the spin column, and centrifuged at 8000rpm for 1 minute. The collection tube and flow through were again discarded. The spin column was placed in a new collection tube, 500uL of AW2 buffer was added, and it was centrifuged at 14,000rpm for 5 minutes. It was important in this step to make sure that there was no droplet underneath the spin column because any remaining ethanol would interfere with future reactions. Lastly, the spin columns were placed in a new 1.5mL microcentrifuge tube and 100uL of warmed AE buffer was
added to the spin column. The samples were centrifuged at 8000rpm for 1 minute and the remaining flow through was the purified, extracted DNA.

Creating DNA dilutions

In order to create a more simplistic and uniform protocol, the previously extracted DNA samples were functionally a stock solution. DNA dilutions were made adjusting for concentration so that a uniform volume of each sample could be added during PCR preparation. Because the extractions yield varying amounts of DNA, the concentrations were measured using a Nanodrop. The Nanodrop was cleaned with 1.5uL of dH2O and then blanked with 1.5uL of AE buffer because that is what the DNA is suspended in. 1.5-2uL of each DNA sample was added and the measurement of the concentration was recorded in ng/uL. The goal of dilutions was to make a solution that gives 20ng of DNA in 5uL for a 25uL PCR reaction. Using the formula $V_1C_1 = V_2C_2$, a dilution of all 10 samples was made.

PCR Amplification

Two sets of primers were added to each reaction tube to amplify a region of the X chromosome and a region of the Y chromosome. The logic behind this co-amplification was to have the band correlated to the X chromosome function as a positive control for a successful polymerase chain reaction (PCR). This positive control was provided by amplifying a 447/445bp region of the Zfy-Zfx genes with primers taken from Aasen and Medrano (1990). The critical band necessary for sex determination was provided by amplifying a 157bp region of the Sry gene, a gene only found on the Y chromosome and therefore only possessed by males. The SRYA-3 and SRYA-5 primers were taken from Bryja and Konecny (2003).
The amplifications were performed under conditions that varied in each individual trial of the experiment. In general, the conditions for each 25uL reaction tube were as follows unless otherwise specified in the trial: 13.84uL water, 2.5 uL Roche buffer, 1uL SRYA-3 primer, 1uL SRYA-5 primer, 0.5uL P1 Primer (for X chromosome), 0.5uL P2 Primer, 0.5uL 10mM dNTPs, 0.16uL 5U/uL Roche Taq, and 20ng (5uL) DNA. The cycling conditions were, unless otherwise specified, 1 cycle of denaturation at 93°C (5 min) followed by 30 cycles of denaturation at 93°C (1 min), annealing at 50°C (1 min), and elongation at 72°C (1 min). There was a final elongation period at 73°C (5 min) and a final hold at 8°C (∞). The PCR amplifications were performed in a PCR Thermocycler (Bio-Rad).

*Gel Electrophoresis*

2.5g of general purpose agarose was combined with 100mL TBE buffer to create a relatively higher density gel of 2.5% agarose. 3uL of PCR products and 2uL of green loading dye were mixed together on parafilm and added together into the wells for each individual sample. 5uL of a 100bp ladder was added so that once the gel was run, the length of the sample fragments could be compared to fragments of known size. All of the gels were run at 100V for 45 minutes, then soaked in an ethidium bromide bath for 20 minutes. The ethidium bromide binds to the DNA and allows the bands in the gel to be visualized with UV light.

*Results and Discussion*

There was amplification of the Sry-related gene in males, but the amount of amplified DNA varied significantly and was often low in yield. Based on the results of PCR amplifications in female subjects, it can be concluded that no Sry-related gene was amplified. The co-amplification of the Zfy/Zfx genes resulted in fairly consistent amplification yield and serves
as an excellent positive control. Therefore, the following experimental adjustments made in each trial aimed to optimize the yield of the Sry-related band.

**Removing Nonspecific Products Through Change of Annealing Temperature**

The optimal annealing temperature is where the primers in a PCR form the maximal number of hydrogen bonds with the template DNA to create a maximally stable association. At both suboptimal and superoptimal annealing temperatures, non-specific products may be extended and the yield of desired product is reduced (Rychlik et al. 1990). In the following three separate trials, all factors were held constant while the annealing temperature was the tested variable. I started with an annealing temperature of 48°C in the first trial, moved to 50°C in the second trial, then attempted a custom touchdown in the last trial. An annealing temperature of 50°C showed less nonspecific products than 48°C, and the touchdown resulted in only the desired products.

Touchdowns are a PCR method that increase specificity of the primers at higher temperatures and increase efficiency by lowering the annealing temperature towards the end. If the primer binds to the Sry gene during the high temperature phases, subsequent rounds of PCR will amplify this gene of interest instead of amplifying nonspecific sequences (Don et al. 1991). The cycling conditions of the touchdown were denaturation at 93°C (5 min), 6 cycles of 93°C (1 min), 60°C decreasing to 59,57,55,53,52 each cycle (1 min), 72°C (1 min), followed by 25 cycles of conditions described in the methods section as well as the final extension and hold. The reduction in amplification of nonspecific products can be seen by the gels from these trials in Figure 1.
Figure 1: the gels decrease the amount of nonspecific products from left to right. The center gel highlights the desired fragment sizes, in base pairs. The third gel shows that only the desired bands were amplified, but the reaction in the fourth well failed and therefore has no product. This kind of failed reaction is the reason I used the Zfy-Zfix as a positive control. If there were no positive control, this male subject would have been mistaken for female.

Varying DNA Concentration to Improve Product Yield

The amount of DNA used in a PCR is a common variable to adjust due to its large effect on the outcome of the reaction. Using too high of a concentration of DNA can lead to packed DNA in the reaction vessel and can lead to poor synthesis due to inhibited diffusion of Taq polymerase. Too low total DNA increases the chance of contamination and degradation by enzymes (Cankar et al. 2006). In the following five trials, all factors were held constant, with an annealing temperature of 50°C and the tested variable being the amount of total DNA added. Because I used the dilutions described in the Methods section, the concentration is constant, therefore the total DNA in the reaction was adjusted by adding a variable amount of the dilutions and making up for that volume difference with the addition or removal of water. DNA volumes in the trials were 2.5uL (10ng), 5uL, 7.5uL, 10uL, and 12.5uL.

The results of these gels are inconclusive due to the variability of product yield. In trials that were repeated multiple times under the same PCR conditions and same DNA concentration, the product yield was wide-ranging. This variable success of the Sry-related gene amplification could be attributed to the low affinity the primer has for the tuco-tuco gene sequence. Any small disturbance in the reaction from variables such as human error could result in the complete absence of a product. Further trials are needed to optimize the DNA concentration, but the total DNA needed for each 25uL reaction has been determined to be in the range of 20-40ng.
References


