Posters

IDENTIFICATION OF SEX SPECIFIC DNA REGIONS IN THE SNAKE GENOME USING A SUBTRACTIVE HYBRIDIZATION TECHNIQUE

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SUMMARY

Karyotypic studies have shown that a ZZ/ZW sex chromosome system is used by snakes, which chromosomally resembles the ZZ/ZW system used by birds. However genetic studies have shown that *SOX 3*; the sex determining gene in mammals and *DMRT1*; which is the hypothesised sex determining gene in birds, are both located on autosomal chromosomes in the snake. Therefore it has been suggested that the snake sex chromosome system is unique and has evolved independently of the bird's system. This paper describes a subtractive hybridization method, using physical separation of biotinylated 'driver' DNA. The novel application of this technique was its use in identifying sex specific DNA regions within the genome of the Australian python; *Morelia spilota imbricate*. Female DNA enrichment was achieved using this technique and resulted in the identification of two non-sex specific repeating elements. The conclusion from this work is the identification of female specific DNA in snakes requires further subtractive hybridization enrichment and a more efficient screening procedure.

INTRODUCTION

Through karyotyping it has been shown that all species of snake use a common sex chromosome system, which consists of a single pair of ZZ (male) or ZW (female) sex chromosomes (Ezaz *et al.* 2006). Examination of the snake ZZ/ZW system has revealed that it is karyotypically similar to the ZZ/ZW sex chromosome system found in birds (Ezaz *et al* 2006). Therefore a theory that both bird and snake sex chromosomes have evolved from a common ancestor was conceived (Ezaz *et al.* 2006). However recent genetic research has revealed that *SOX 3*; the primary sex determining gene in mammals and *DMRT1*; which is hypothesised to be the sex determining gene in birds, are both located on autosomal chromosomes in the snake (Matsubara *et al.* 2006). It has now therefore been suggested that the snake sex chromosome system is unique and has evolved independently of the avian system despite its similarities (Matsubara *et al.* 2006). Our current research involves using a subtractive hybridization method, for the novel application of identifying sex specific DNA regions within the genome of the Australian carpet-python; *Morelia spilota imbricate*. By identifying sex specific regions we hope to assist in the study of snake sex chromosomes and the evolution of the snake's sex determination mechanism, which to date is still largely a mystery.

MATERIALS AND METHODS

Subtractive hybridization preparation. Male and female genomic DNA was isolated from ethanol preserved blood samples using white cell lysis buffer (10mM Tris.Hci, pH 7.6; 10mM EDTA, pH 8.0; 50mM NaCl; 200mg/ml Proteinase K (Promega); 0.1% SDS) followed by sequential phenol/chloroform extractions and isopropranol precipitation (Mathew 1985; Taggart *et al.* 1991). Male and female DNA was then double digested with 2.5 units *AluI* and *XmnI* and ligated with 50 units of T4 DNA Ligase to specifically designed double-stranded linkers RHZ (male linker) and RHW (female linker) respectively. This was performed as a single cycled reaction, to increase ligation efficiency, under the following thermal profile, 26× (16˚C for 15min, 37˚C for 30min); and heat inactivated at 65˚C for 20min.

Subtractive hybridization. A unique feature of the male linker RHZ, was biotin bound to the 5 prime end of the forward strand. This allowed the RHZ linker and ligated male DNA fragments to be almost irreversibly bound to streptavidin coated magnetic beads (Dynabeads®, Invitrogen), following manufactures instructions. Then female linker ligated DNA (2.5µg) was heat denatured and added to the magnetic beads coated with male DNA at a molar ratio of 2:1 male to female. The beads in the hybridization mixture were removed from the solution, after incubation with constant rotation at 55˚C for 1 hour, using a preheated magnetic particle concentrator (55˚C). The removed male DNA bound beads at this point contained female DNA fragments which have hybridized to the complementary male DNA and were discarded. The supernatant, enriched for unique female-specific DNA was used for repeated subtractive hybridization enrichments. This was achieved by replacing the female linker ligated DNA in the process described above with the enriched product from the previous subtractive hybridization. For subsequent enrichments fresh male DNA attached beads were used. The subtractive hybridization process was performed a total of 3 times at a stringency of 0.8×SSC (1.5M NaCl; 0.15M sodium citrate).

Screening. The sample enriched for female specific DNA was amplified with a RHW-F primer. The PCR was performed under the following conditions; volume equivalent to 15ng of template DNA before the subtractive hybridization cycles, 1.5mM $MgCl_2$, 200µM dNTPs, 0.6mM RHW-F primer, 1 unit Bioline DNA Polymerase, 1×PCR buffer (Promega) as recommended by the manufacturer and water to a final volume of 50µl. The PCR cycling conditions were: 1× (95˚C for 10min); 30× (94˚C for 45s, 55˚C for 45s, and 72˚C for 1.5 min); 1× (72˚C for 1hour extra extension for TA cloning); and held at 14˚C. Purified PCR product (3µl) was cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen). The TOPO cloning reaction (2µl) was used to electroporate TOP10 *E.coli* (20µl) (Invitrogen). The subsequent plating out of the transformed E.coli resulted in the isolation of 102 plasmids with potential sex specific DNA inserts. A dot blot analysis was performed to identify any sex specific DNA inserts. Only 51 of the 102 isolated identified were screened due to time constraints. Radioactive DNA probes of each plasmid isolate were prepared by incorporating ^{32}P label dCTP into a PCR reaction using M13 primers. Then each radioactive probe was hybridized to 2 male and 2 female genomic DNA samples. Subsequent DNA sequencing of selected plasmids followed.

RESULTS AND DISCUSSION

Subtractive hybridization preparation. An advantageous modification to the subtractive hybridization method was the combining of the genomic DNA digestion and linker ligation reactions together in a single cycled reaction. This cycling method was more efficient and less time and labor intensive then separating the digestion and ligation reactions. Firstly, de-phosphorylation of the genomic DNA to prevent concatamerization is not required in this method and secondly the introduction of XmnI prevents linker dimer formation, thereby increasing the efficiency of the linker ligation.

Subtractive hybridization. Using a molar ratio of 2:1, male to female DNA, three consecutive subtractive hybridization cycles were performed. At the end of each cycle a small aliquot of elution was kept for analysis, which can be seen in figure 1. When comparing the initial female linker ligated starting material (figure 1, lane 2), to the three sequential subtractive hybridization cycles (figure 1, lanes 3-5) a reduction in intensity of the DNA products is observed. This indicates a reduction in the concentration of female DNA, which decreases with each subsequent enrichment cycle. There is also a constant reduction in the observed fragment size range across the

three subtractive hybridization cycles.

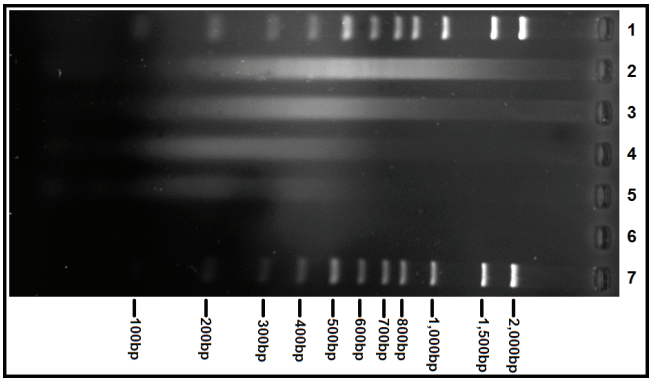

Figure 1. PCR of three consecutive subtractive hybridization cycles

Figure 1 shows the amplified product of a PCR using a RHW-F primer and the DNA from a RHW linker ligation (lane 2), elute from subtractive hybridization cycle 1 (lane 3), elute from subtractive hybridization cycle 2 (lane 4), elute from subtractive hybridization cycle 3 (lane 5) and a RHW negative control (lane 6). Lanes 1 and 7 are a molecular size marker (100kb ladder from Axygen).

Screening. Six of the 51 isolates screened for sex specific DNA gave a positive hybridization result, when analyzed using snake genomic DNA dot blots. This indicates an efficiency of 11.76% of the screening procedure, which was considerably lower than expected. Each blot had a probe positive control, which was found in virtually all instances to be very strong, indicating that the probe incorporated the radioactive tag. Subsequent investigations into this lowered efficiency revealed that the genomic DNA used for the membrane blots was of low quality and highly fragmented. Initial the DNA extractions were tested and concluded to be of highest quality; however, the DNA had degraded over time, due to its improper storage in purified water not a suitable buffer as required. Therefore, it is highly likely that the reason for the apparent lack of hybridization, in the negative blots was due to the use of degraded genomic DNA. It would be appropriate to repeat these experiments using pristine DNA, as it may reveal potential sex specific target sequences, however due to time limitations this was not possible.

Three clones, which did react with the genomic DNA on the dot blots, albeit at a lower signal strength, were DNA sequenced. DNA sequence analysis of the 731bp sequence from isolate 3E showed that it formed part of a probable repeating element. The repeating element is most likely a SINE or a LINE and was identified by BLAST analysis to match with a number of non-coding regions in a variety of unrelated organisms. This element is not unique to snakes and from the dot blot analysis was not identified to be sex specific. Sequence analysis of the isolate 5H resulted in the identification of another repeating element 140bp in length. This region was identified by BLAST analysis to occur in the intronic region of genes from two snake species and in a microsatellite region from a third snake species. In *Trimeresurus flavoviridis,* the sequence was found to have a high degree of similarity to sequences from two different intronic regions within the phospholipase A2 inhibitor gene. Furthermore, its coupling to a microsatellite sequence from a species of snake would indicate that it is most likely a SINE. Coupled consecutive SINE and microsatellite sequences have been reported to occur in other species. For instance 24% of dG-dT and dC-dA microsatellites found in the porcine genome are associated with the PRE-1 SINE and 5.6% are associated with Artiodactyl Repetitive Element 1 and 2 Porcine SINE (Alexander *et al*

1995). Furthermore, in cattle, horses and whales similar SINE elements have been found to be associated with microsatellites (Band and Ron 1996; Gallagher *et al.* 1999; Buchanan *et al. 2*001). Therefore, in conclusion it appears that these two elements are most likely of the SINE family. However, it also poses the question as to the efficiency of the capture process to remove these repetitive elements.

CONCLUSIONS

The aim of this experiment was to use a modified subtractive hybridization method to isolate and identify sex specific DNA regions from the genome of the Australian carpet python; *Morelia spilota imbricate*. Subtractive hybridization through the physical separation of biotinylated 'driver' DNA is not a novel technique and has been used previously to isolate tissue specific genes and in the recovery of full-length open reading frames (Rodriguez and Chader 1992; Meyer *et al.* 2007). However, the use of the subtractive hybridization technique, described in this paper, to isolate sex specific DNA regions of a complex genome is a novel application. This subtractive hybridization technique was significantly modified in order to increase its efficiency of capture and to assist in monitoring the progress of the experiment.

One of the major downfalls of the technique was the temperamental nature of the subtractive hybridization process and the difficulty in monitoring it. We chose to design sex specific linker and use PCR as a means to investigate and track the hybridization efficiency of the process. However, PCR amplify DNA fragments bound to the streptavidin coated magnetic beads proved to be difficult and resulted in us trying to investigate varying intensities of smeared DNA. It has been suggested that the process required more controls, in the form of a male on male and a female on female subtractive hybridization procedure. In order to identify how much DNA was being removed from each cycle. This would have increased the cost of the project and due to the reduced sensitivity of the PCR we believe no further conclusions would have been made by adding these controls. Ultimately a more sensitive process was needed to track the removal of the female DNA. A possible solution would be to label the female DNA with a fluorescent or radioactive molecule, so it could be tracked through the system more accurately.

Although the identification of two repeating elements did not satisfy the initial aim of this experiment, the detection of these regions showed that the subtractive hybridization technique was working to capture DNA. Initially it was thought that repeating elements common to both male and female would be removed during the subtractive hybridization process. However we showed that after three subtractive hybridization cycles at 50˚C, repeating elements still persisted. Therefore to eliminate more repeating elements and increase the efficiency of the technique we suggest adding one or two more subtractive hybridization cycles to the procedure and increasing the hybridization temperature by 5-10˚C. Future research will be focused towards re-performing the dot blot screening process, using non-degraded fresh DNA. As we hope snake sex specific DNA regions might still be identified within the 102 isolates.

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