# Higher Returns in Prawn Aquaculture:
# I. Genetics of Sex Determination
# II. Advances in Prawn Cell Culture

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Queensland Government
Department of Primary Industries and Fisheries


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## OBJECTIVES:

1. Establish the genetic mechanism of sex determination in penaeid prawns and provide genetic markers that allow sex to be identified before visible signs are evident.

2. To identify and isolate the gene or genes involved in and ideally responsible for triggering sex determination. Studies of the potential for hormones to induce sex reversal will also be undertaken.

3. Aim to produce sex-reversed prawns as broodstock by introducing sex-determining genes into fertilised eggs to produce transgenic sex-reversed animals.

# NON TECHNICAL SUMMARY:

## OUTCOMES ACHIEVED TO DATE

We established a more detailed understanding of the genetic mechanisms responsible for sex determination in prawns and discovered a high degree of genetic variability between individuals of the same species. Sex determination in penaeid prawns is directed by a single genetic locus or a small number of loci, rather than fully differentiated sex chromosomes. This information influences the methods future researchers will use in manipulating sex ratios of commercially farmed crustacean species. Further, laboratory cell culture techniques developed within this research enable improved methods of molecular visualization of crustacean genes. Our research expands knowledge of the genetic structure of prawn populations, and influences future farming practices and harvesting strategies for wild caught prawns.

The cell culture techniques developed in this research are now used by researchers and their students working on diseases within crustacean species. Experiments designed to test the most effective techniques for preventing bacterial and viral diseases in commercial prawns may be carried out far more quickly growing prawn cells in controlled laboratory circumstances, infecting them with single or multiple disease agents, then trialing potential disease control methods.

Female penaeid prawns grow faster and may have higher feed conversion efficiency than do males. If a technique could be devised to bias the sex ratio of larvae produced so that the proportion of females in each brood outnumbered the males, the commercial grower could achieve dramatically improved production.

To enable control of larval sex ratios, the goal of this grant was to identify the genetic material in prawn chromosomes that triggers development into a male or female prawn. Once it is possible to identify the genes controlling the sex of maturing prawn larvae, those genes can be targeted and manipulated through molecular techniques.

Genetic and chromosomal sex determination is not understood in most crustacean species. Penaeid prawns possess numerous, uniformly small chromosomes so that potential sex chromosomes have never been identified with classical karyotypic microscopic studies. Molecular genetic techniques provide a new tool for increased resolution of sex-determining factors.

We studied two cohorts of genetically inbred prawns to directly reduce the natural genetic variability between individuals and to further accentuate the genetic variability between the sexes. The first experimental group was bred from a match between two wild-caught *Penaeus monodon*. The offspring from this mating were all siblings (an F1 generation). These prawns were grown by Dr. David Hewitt and Mr. Shane Hansford at Bribie Island Aquaculture Research Centre. The second experimental group was bred from another commercial species, *Penaeus japonicus*. Dr. Nigel Preston and Dr. Peter Crocos supplied an F2 generation from pond-reared prawns grown at the CSIRO Marine Laboratories in Cleveland. Creating a genetically inbred F2 generation requires more time because two wild caught *Penaeus japonicus* are mated to obtain F1 offspring. Then two siblings from the F1 experimental group must be raised to maturity and bred so that their offspring possess increased genetic similarity, as an F2 generation.

Our studies revealed an unexpectedly high genetic variability between individuals of the same prawn species. Further, we discovered that prawns use a system of genetic sex determination that is distinct compared to the chromosomal organisation of most well known organisms.

Our results indicate that sex determination in penaeid prawns is directed by a single genetic locus or at most a small number of loci, rather than fully differentiated sex chromosomes. Male/female differences within the genomic sequences of prawn DNA are slight. In order to successfully isolate genetic markers for only one to several loci it was necessary to resort to the most genetically similar, inbred prawns. Laboratory-reared spawners are reliably obtained from *P. japonicus* where as rearing *P. monodon* to spawning condition in captivity is much more difficult. These reproductive characteristics of *P. japonicus* enabled captive breeding to further maximise the genetic similarity of siblings. We also expanded our molecular methods to include the technically complex techniques of subtractive hybridisation. We then successfully located three genetic markers for the male sex and one genetic marker for the female sex in *Penaeus japonicus.*

Our original objectives were forced to change due to the unexpected high degree of genetic variability found between individuals of the same species. To succeed in obtaining the genetic markers of sex we were forced to depend on the F2 generation. These were unavailable in the early stages of the project, due to the complex nature of breeding an F2 generation (two siblings hatched from the same parents, then raised to maturity and bred so that their offspring are genetically similar).

Although the markers were more difficult to obtain than we originally predicted, finding fewer markers after the rigorous search has two advantages: 1) The positions of the genetic markers we isolated are closer to the actual sites of genetic sex determination. 2) Future manipulation of those genes for improved production will be less complex, with fewer genes.

The second part of our research developed fortuitously when we succeeded in growing cells isolated from *Penaeus monodon* under laboratory cell culture conditions. We began this research to obtain prawn chromosome preparations and to have an experimental system in place to explore sex-reversal genetic techniques *in vitro*. In addition to the sex determination studies, cell culture techniques have a broader application to the aquaculture industry. Cell culture methods are used routinely in human and veterinary medicine, but are not well developed for marine organisms. With increases in prawn aquaculture and viral diseases in Queensland, the study of prawn viruses has become a high priority and cell culture techniques have immediate importance.

Cell culture methods have been necessary to understanding viral infectivity and replication in humans and animals. These techniques allow viruses to be harvested and purified more efficiently than is possible from whole organisms. This is especially helpful in prawn research as it allows researchers to isolate pure strains of virus from an infected animal that may contain mixtures of pathogenic organisms.
To understand the specific effects of different viral strains or select for disease resistance in prawns, cell culture techniques are essential.

Our research used the most current biotechnological tools available to study the genetics of sex determination and the complimentary area of prawn cell culture research. Both areas have direct application to the economic development of the prawn industry and cell culture work has immediate relevance to disease control of emerging prawn viruses.

**KEYWORDS: Sex determination, penaeid prawns, *Penaeus monodon, Penaeus japonicus,* cell culture, aquaculture.**

## Acknowledgments

We wish to thank Dr. David Hewitt, Mr. Shane Hansford, Dr. Nigel Preston and Dr. Peter Crocos for kindly supplying the genetically inbred prawns. We could not have accomplished this work without their support and contributions. We wish to also thank our hardworking co-workers, Dr. T. Mahony, Mr. Roger Mitchell, and Mr. Nick Wade. We gratefully acknowledge the financial support of the Fisheries Research and Development Corporation grant 94/070.

# 2. Background

## 2.1 Genetics of Sex Determination

Female penaeid prawns grow faster and may well have higher feed conversion efficiency than do males (Hansford and Hewitt, 1994). If a technique could be devised to bias the sex ratio of larvae produced so that the proportion of females in each brood outnumbered the males, the commercial grower could achieve dramatically improved production. This principle is integral to the commercial success of the Atlantic salmon industry in Tasmania where breeding “males” are produced by reversing the sex of genetic females with hormone treatment. All production stock are therefore produced from matings between genetic females, with the result that all progeny are females.

Far less is known about sex determination in penaeid prawns. Hormonal manipulations of the androgenic gland of the freshwater prawn *Macrobrachium rosenbergii* have had limited success in influencing sex ratios (Malecha *et al*., 1992) and it is not even known if the male or female is genetically dominant (in mammals, such as humans, males have a Y chromosome which is dominant in sex determination). In marine organisms there are many examples where females possess the dominant chromosome(s). In either case, an understanding of the sex determining mechanisms in pawns would enable direct genetic manipulation of the DNA of parental stock such that the larvae produced would be all female and more commercially successful.

Few studies have attempted to document chromosomal sex determination in prawns. Research with the freshwater prawn *Macrobrachium rosenbergii* suggests that females may be heterogamous (ZW) and males homogamous (ZZ), but the sex ratios of offspring in the studies did not completely support the model, implying that the explanation of sex determination is more complex (Sagi and Cohen, 1990; Malecha *et al*., 1992). Cytological chromosome spreads have been prepared for a variety of species: *Penaeus aztecus*, *P. duorarum*, *P. vannamei*, *P. setiferus* (Chow *et al*., 1990), *P. chinensis* and *P. monodon* (Siang, 1988 and Xiang *et al*., 1990 in Lester and Pante, 1992) and for *P. japonicus* (Hayashi and Rujiwara, 1988 in Lester and Pante, 1992) to obtain numerical counts of chromosomes. Unfortunately, these studies did not document details of chromosome morphology or identify potential sex chromosomes. Before it is possible to genetically manipulate sex in *Penaeus* we need to identify specific chromosomes or genetic loci that are consistently distributed with either male or female gametes during meiosis.

With that knowledge it is possible to dissociate genetic sex from physical sex. Observation of naturally occurring variants and experimental manipulation will allow dissection of the processes of sex determination and will lead to the ability to alter the sex ratio.

## 2.2 Advances in Prawn Cell Culture

To obtain quality prawn chromosome preparations and to provide a basis for the sex reversal experiments, we started experimenting with cell culture techniques. We succeeding in growing cells isolated from *Penaeus monodon* under laboratory cell culture conditions. Although our molecular work later demonstrated that prawns are not dependent on differentiated chromosomes we continued with cell culture methods because they have such broad significance for future transgenic studies and prawn disease research. Cell culture methods are used routinely in human and veterinary medicine, but are not well developed for marine organisms. With increases in prawn aquaculture and viral diseases in Queensland, the study of prawn viruses has become a high priority and cell culture techniques have immediate importance.

Cell culture methods have been essential to understanding viral infectivity and replication in humans and animals. These techniques allow viruses to be harvested and purified more efficiently than is possible from whole organisms. This is especially helpful in prawn research as it allows isolation of pure strains of virus from an infected animal that may contain mixtures of pathogenic organisms.

In the outbreak of mid-crop mortality syndrome in Queensland, the research of Dr. Leigh Owens of James Cook University has shown that a mixture of 3 different viruses may be responsible for the mortalities. The different pathogens can be viewed in electron micrographs and identified from the external structure of each virus. To understand the specific effects of different strains or select for disease resistance in prawns, a more detailed understanding of the viral genetic material is required. Further, one viral strain may not be as harmful to a prawn as another but combinations of infection may become detrimental.

The genetic material of viruses can only be studied effectively when a strain is isolated and purified. The best way to accomplish this is to grow and harvest the virus in cell culture. Unlike bacteria, which can be grown in a broth or on solid nutritive medium, viruses require living host cells to replicate.

Cell culture methods grow a sheet of specific cells in a nutrient solution inside sterile flasks. Different cell types can be grown and challenged by various strains of virus, individually and in experimental combinations. Further, the cell-to-cell transmission of virus can be studied as the virus is replicating in the living cells. Molecular analysis can then reveal the DNA and protein interactions that allow a virus to attach to a cell and inject its nucleic acid to take over the normal functions of the cell to produce more virus. These techniques have been well established in human, cattle and insect cells. Cells of marine animals have been more difficult. A small number of laboratories have successfully grown prawn cells in primary culture but viral research has been hampered by an inability to keep cells growing.

# 3. Need

Prawn aquaculture has not met its potential in Australia. In order for commercial growers to be competitive with countries that have sources of less expensive labour, Australian aquaculture businesses need to apply biotechnological advances to their culture practices. Aquaculture facilities are aware of the potential advances that could be made with molecular techniques but have difficulty gaining access to the technology and expertise to make the transition.

Genetic manipulation of sex ratios to create production stocks that are all female fills several needs. In addition to more efficient supply of market size prawns, Australian businesses could market pure female larval stock to growers internationally by maintaining control of the genetically superior parental stock. Further, the ability to reduce grow-out time in commercial aquaculture facilities or to market highly desirable larval stock reduces the risk of production stocks contracting disease.

Recent economic losses from prawn diseases in Queensland highlight the need for additional defences for our aquaculture industry. Cell culture techniques developed throughout this project provide a foundation for future research techniques to combat viruses.

# 4. Original Objectives

1. Establish the genetic mechanism of sex determination in penaeid prawns and provide genetic markers that allow sex to be identified before visible signs are evident.

2. To identify and isolate the gene or genes involved in and ideally responsible for triggering sex determination. Studies of the potential for hormones to induce sex reversal will also be undertaken.

3. Aim to produce sex-reversed prawns as broodstock by introducing sex-determining genes into fertilised eggs to produce transgenic sex-reversed animals.

## 4.1 Modified Objectives

Our original objectives were forced to change due to the unpredicted high genetic variability found between individuals and the discovery that prawns use a system of genetic sex determination that is distinct compared to the chromosomal organisation of most well known organisms. Although the official time period of this grant ended, further tests of the F2 generation continued. Due to the longterm and complex nature of breeding an F2 generation (two siblings hatched from the same parents, then raised to maturity and bred so that their offspring are genetically similar) we obtained these individuals late in the project. They have now contributed the key finding of locating sex specific DNA sequences.

The proposed hormone work was one of the major focuses of collaborations between AIMS and Bribie Island research groups. Both those laboratories were better equipped with seawater facilities and researchers with specific expertise in the biochemistry of the hormones. Research in that area is ongoing and it was not sensible for our group to pursue the same topics.

The transgenic work is one of the major focuses of Dr. Nigel Preston's group (CSIRO Cleveland) in collaboration with researchers at CSIRO in Canberra. They have direct access to prawn embryos through established breeding programs and are conducting experiments injecting reporter genes into embryos. It was most practical for our group to focus on the cell culture work because in addition to its benefits for viral studies the progress of transgenic research will be enhanced because DNA can be introduced to cultured cells for experimentation when live embryos are unavailable or in short supply. Further, activity of reporter genes may be observed more quickly in a monolayer of cells than an entire developing embryo.

Our research used the most current biotechnological tools available to study the genetics of sex determination and the complimentary area of prawn cell culture research. Both areas have direct application to the economic development of the prawn industry and cell culture work has immediate relevance to disease control of emerging prawn viruses.

# 5. Methods

## 5.1 Genetics of Sex Determination: F1 Experimental Methods

### 5.1.1 Random amplification of polymorphic DNA (RAPD)

The larvae from a single mated pair of *Penaeus monodon* were reared at Bribie Island Aquaculture Research Centre by Dr. David Hewitt and Shane Hansford. The full siblings were grown to a carapace length of three centimetres to enable sex identification based on external morphology of the gonopores.

DNA was extracted from 23 individuals of each sex (total of 46 prawns) from the sibling culture using Qiagen tissue columns. The DNA was quantified using a fluorometer and a dilution of 2.5 nanograms DNA per microliter was made for each individual. Bulked samples were mixed using an equal volume of each individual to make a pooled sample for males and a pooled sample for females. These DNA samples were screened for random polymorphisms, individually and bulked by sex, using PCR amplification with RAPD primers.

The RAPD PCR reactions were carried out in 48 well plates with each reaction mix consisting of 12.5 microliters.

Each PCR reaction contained:

| | |
|---|---|
| 1.25 μl | 10x GeneAmp PCR buffer I (Applied Biosystems) |
| 1 μl | Template DNA (2.5 ng/ml) |
| 0.75 μl | 25 mM MgCl |
| 0.5 μl | 10 mM each of dATP, dGTP dCTP and dTTP |
| 0.125 μl | Ampi-Taq DNA polymerase (5 U/ μl) |
| 1 μl | 10 μM RAPD primer (Operon technologies) |

PCR cycling conditions: were:

94$^{o}$C for 60 sec for one cycle

94$^{o}$C for 5 sec
40$^{o}$C for 45 sec
72 $^{o}$ C for 60 sec for forty cycles

72 $^{o}$ C for 600 sec
4 $^{o}$ C Hold for one cycle

Amplification products were analysed by gel electrophoresis in 2% Sea Kem agarose (FMC, Focklad, M.) in 0.5x TBE buffer. Gels were stained with ethidium bromide and viewed and photographed on a UV transilluminator.

A total of 320 primers were screened and each amplification reaction was performed using a single primer.

Statistical linkage analyses was performed using the program Mapmaker 0.3 1993 Whitehead Institute for Biomedical Research.

### 5.1.2 Subtractive hybridisation

Subtractive hybridisations were conducted following the protocol of Lisitsyn *et al.* (1993).

*P. monodon* DNA probes were made by column-purifying the PCR amplified products from the last steps of the subtractive hybridisation protocol (QIAquick PCR Purification Kit). The purified DNA fragments were then digested with *Mbo*I restriction enzyme (Stratagene) and cloned using pUC18 plasmid (Pharmacia cat. no. 27-5260-01). Plasmid clones were purified using RPM columns (Integrated Sciences cat. no. 2070-200).

Probes were labelled using DIG-II-dUTP (Boehringer Mannheim cat. no. 1573 152). The labelling polymerase chain reaction (PCR) reaction mix was: 75.5 μl water, 10μl Boehringer Mannheim PCR buffer, 6.25μl of 25mm $MgCl_2$, 1 μl of 10mm, dNTPs, 1μl of 1mM dUTP, 2μl each of forward and reverse M13 primers or RAPD primers at 20 pm/μl, 1μl of *Taq* polymerase (Boehringer Mannheim) and 1 ng of template DNA. The reaction mix was divided into 15 μl reaction volumes to facilitate the quick transfer of temperature. For cloned templates the amplification protocol was 94$^{o}$C for 3 minutes, then 35 cycles of: 94$^{o}$ C for 5 sec, 62$^{o}$C for 0.45sec, 72$^{o}$C for 1 minute on a Perkin Elmer 9600 machine. For non-cloned templates the cycles were the same except the 62$^{0}$C phase was reduced to 40$^{0}$C.

For the Southern transfer (Maniatis *et al*. 1982), genomic DNA was isolated from male and female individual prawns. These samples were purified with Boehringer Mannheim columns (cat. no. 1796828), digested with *Msp*I (New England Biolabs) and electrophoresed on a 1% agarose gel in TAE buffer (Maniatis *et al*. 1982) for 5 hours at 70V. The gel was transferred to Hybond-N Amersham non-charged membrane overnight using capillary action and the DNA was fixed to the membrane using UV (Maniatis *et al*. 1982). The probe was hybridised to the membrane at 50$^{o}$C using the standard DIG protocol (DIG System User's Guide for Filter Hybridisation, Boehringer Mannheim).

## 5.2 Genetics of Sex Determination: F2 Experimental Methods

The F2 prawns were obtained from Dr. Peter Crocos and Dr. Nigel Preston from CSIRO Cleveland. *Penaeus japonicus* prawns were used due to their lifecycle, as it is easier to obtain inbred lines. RAPD analysis was conducted following the protocol of Bentley and Bassam 1996. This protocol used Stoffel enzyme (Perkin-Elmer-Cetus) in place of *Taq* polymerase and the subsequent banding profiles were run on acrylamide gels and silver stained for more detailed resolution amplification profiles. The thermocycling conditions were 94$^{o}$C for 5 min for 1 cycle, then: 94$^{o}$C for 30 secs, 52$^{o}$C for 1 min, 51$^{o}$C for 1 min, 50$^{o}$C for 1 min, 49$^{o}$C for 1 min, 48$^{o}$C for 1 min, for 35 cycles, then 72$^{o}$C for 5 minutes for 1 cycle. Southern blots were performed as described for the F1 methods except *P. japonicus* probes were labelled without prior cloning.

## 5.3 Advances in Prawn Cell Culture

Prawns were stunned by immersion in seawater that was chilled to a slush in the -70$^0$ C freezer and then submerged for 5 minutes in chilled 75% ethanol. Tissues were dissected from prawns 13-19cm rostrum to telson length. Tissues were dissected in a laminar flow hood and placed into a petridish containing growth medium containing 100 μg/ml streptomycin and 100 U/ml penicillin.

Four cultures were composed of anterior dorsal hematopoietic tissues, including the anterior aorta and adjacent connective tissues and the anterior membranes of the heart. These tissues were dissected for each individual culture. In *P. monodon* we were unable to distinguish discrete patches of hematopoietic tissues dorsal to the foregut, but throughout the decapods, the anterior membranes of the heart and connective tissue dorsal to the aorta are known to be rich in hematopoietic tissue (Martin and Hose, 1991). Tissue samples were combined with the corresponding lymphoid organs of each animal, cut into fragments and dissociated in a ground-glass tissue homogeniser with a clearance of 100μm. Another four individual cultures were composed of ovarian tissue that was cut into fragments and cells were dissociated with the tissue homogeniser as described above.

These eight cultures were established at different times over a period of 11 months. Each culture was monitored weekly for 6 weeks. Every week the cells were scraped from the flask, and the percentage of viable cells determined using trypan blue exclusion and the haemocytometer. The culture was then split in half and fresh medium added.

The primary cultures were seeded at 4 x 10 $^4$ cells /ml, in 5 mls of Sf900 II media (Invitrogen) in Corning 25 cm$^2$ flasks and maintained at 28$^o$C.

Growth rates were calculated assuming exponential cell growth as follows:

$$N_t = N_o e^{rt}$$

where $N_o$=initial density and $N_t$=density at time t

thus,

$$\text{culture growth rate } r = \frac{\ln(N_t/N_o)}{t} \text{ where t is measured in weeks}$$

So $N_o$ was the initial seeded number of 4x10$^4$cells/ml or the cell count just before passage divided by two.

# 6. Results/Discussion

## 6.1 Genetics of Sex Determination

### 6.1.1 Random amplification of polymorphic DNA (RAPD) from F1 prawns

RAPD analyses of male and female prawn bulk DNA samples identified a number of putative markers that appear to be sex linked. To further examine the validity of these markers the RAPD reaction was repeated using DNA from individual prawns. In most cases these putative markers did not appear to be sex-linked. Those that appeared to sex-linked using individual DNA samples were further investigated. These investigations included cloning and sequencing of the marker band. In two cases the marker sequence was utilised to develop sequence characterised amplification reactions (SCAR) that utilised highly specific PCR primers to detect the identified marker. Over 320 oligonucleotides in RAPD reactions to detect sex specific markers, the more detailed explanations for five of these putative markers are illustrated below. To confirm if the amplified products were indeed linked to sex determination in prawns the following experiments were performed:

a. The RAPD PCR was repeated using a large number of individuals.
b. The putative differential amplification product was recovered, cloned and sequenced.
c. The resulting DNA sequence was utilised to develop a sequenced characterised amplification reaction (SCAR).
d. The SCAR was utilised to screen multiple individuals.

Only two putative markers were deemed significant enough for conversion into SCARs. These markers were identified with RAPD primers O2 and S7.

### 6.1.1.1 Characterisation of male specific marker amplified by RAPD Primer O2

Initial screening of bulked DNA from males and females indicated that the O2 primer amplified a specific band in male DNA with an approximate size of 500 base pairs (bp) (Figure 1). On the same gel five other primers also indicated putative sex specific products, D16, D16, N5, N19, O1, O2 and O18, interestingly all of the putative products were amplified in male DNA (Figure 1). To determine if these amplicons were sex specific the RAPD reactions were repeated using template DNA purified from four individual male and female prawns. Only O2 produced similar a similar sex specific amplicon as observed in the bulk DNA amplifications (Figure 2). The amplification profiles of primers N5, N19 and O1 are also shown in Figure 2 but demonstrate, as found in many cases, the apparent sex specific profiles are not reproducible on individual DNA. To further validate the specificity of the O2 amplification profile the RAPD reaction was repeated on twelve male and twelve female DNA samples. As previously demonstrated the 500 bp product was amplified from all of the male DNA samples (Figure 3). Interestingly the product was also amplified from two of the female samples, but was absent from the remaining ten female samples (Figure 3). Despite the amplification of the apparent male product in two female samples it was decided to clone and sequence this product.

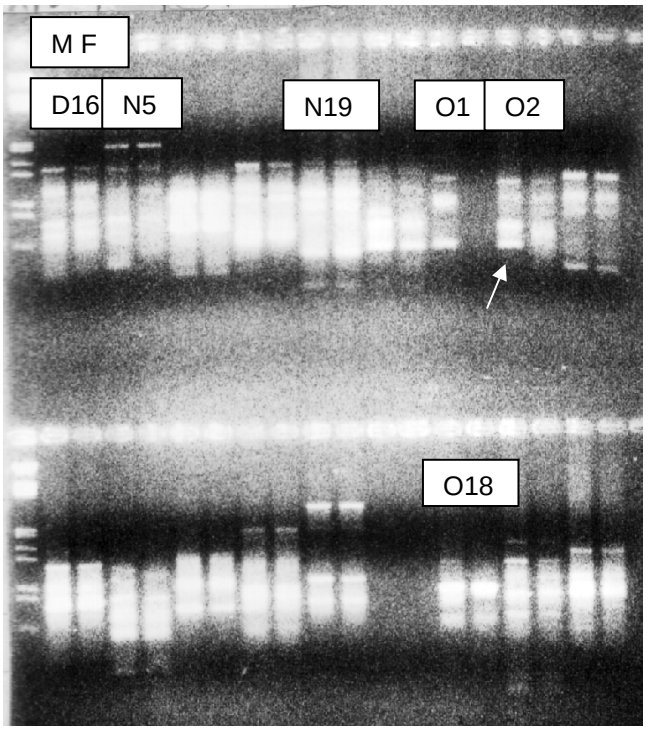


**Figure 1:** Primary product profiles derived from RAPD amplifications using Operon primers from bulk (9 individuals) male and female DNA samples. Samples are loaded in pairs in relation to the RAPD primer and alternate male and female respectively. Of the amplification profiles analysed on this gel D16, N5, N19, O1, O2 and O18 were selected for further analyses. Arrows indicate, where obvious, the putative differential amplification products. Dramatic amplification differences such as those observed for O1 where there was a complete lack of amplification in one sample were not repeatable and most likely due to experimental error. RAPD reactions were performed as described in the Methods section and resolved in 2% Agarose gels.

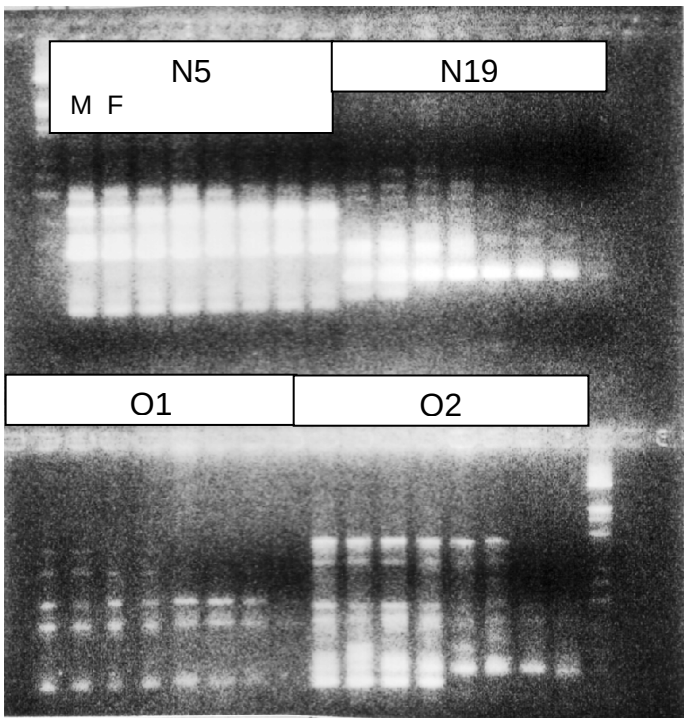


**Figure 2:** Product amplification profile of Operon RAPD primers N5, N19, O1 and O2 on four individual male and female prawns. Samples are loading in sets of eight reactions for each primer. The first four reactions using male template DNA while the remaining four are from female DNA for each primer. In this case only the male specific band from the O2 amplification reactions appeared to be sex specific. Amplifications were performed using 2.5 ng of template DNA for each reaction and 0.5μl of the respective primer.

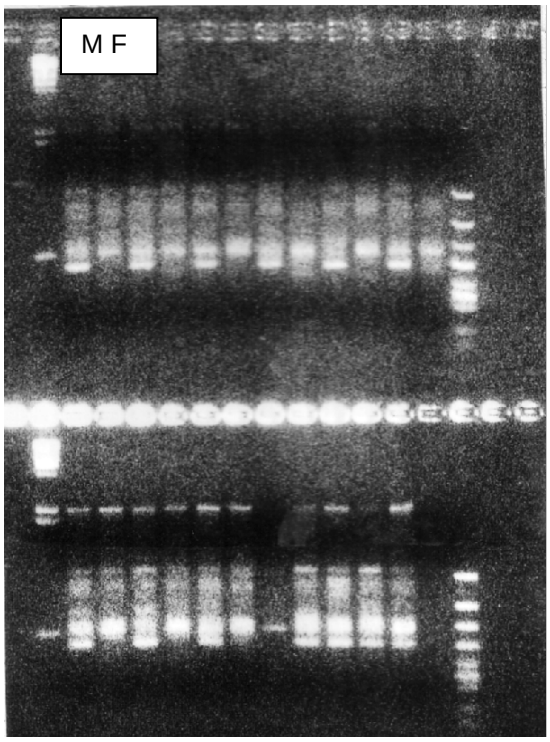


**Figure 3:** Amplification profile of Operon primer O2 on DNA from twelve individual male and female prawns. Samples are loaded alternating male (M) and female (F) DNA templates across the gel.

```
1     ACGTAGCGTC AATGGGGAGG GGGGTAGATT AAACAAACAA ACAAAATATA
                              SCAR primer O2F

51    AAAGAACTAA GGTGTTATGA GAGTGTGTAA ATATACAAAA GAGGAGAGTG

101   GAGTGTGTTT TTTCTAACTG TACTTATAAT CAGTTTACGC TGAAGAGTGT

151   TGGCGAGACA GGAAAGGAAT CTGAACGGAC GGACGTAAAC ACTACGTCAC

201   TGCACATGTA AAGGGGGTCA TCTCGAGACT CTTTTGTAAC CACATGAACG

251   TCTTCTTGTC TTTGTGTCNT CTGCCTAGGA ATCGTGACTC CCYTTGTTGT

301   TTCGCTGGTA AACCTTTTGC MAAACTGTGT GTTATATAGA CAGGGTAAAA

351   GTAAATAAGT ATGGAAAAAA GAAGGAAGTA TTTKATTTAC TGTTGAGTGC

401   TTCGCTGAGA AGGAATATTC CGGTGCATTT CTTGTCGTGG TGTAAAAAAA

451   TCCMAAATTT CGAATGCGTT TTTATTGTGA TGGACGCTAC GT
                       SCAR primer O2R
```

**Figure 4:** Nucleotide sequence of the RAPD product amplified from male prawn DNA using RAPD primer O2. The sequences corresponding to the O2 primer are shown in bold text. The sequences corresponding to the O2 sequence characterised amplification reaction (SCAR) primers are underlined and labelled accordingly.

The nucleotide sequence of the O2 RAPD product is shown in Figure 4. Comparison of this sequence to current nucleotide and protein sequence databases (February 2005) did not find any significant matches with existing sequence data.

To determine if the nucleotide sequence of O2 was specific for the male prawn genome it was decided to develop a sequence characterised amplification reaction (SCAR). This was done by designing PCR primers specific for the O2 sequence which are shown in the relative positions on the nucleotide sequence of the O2 product in Figure 4.

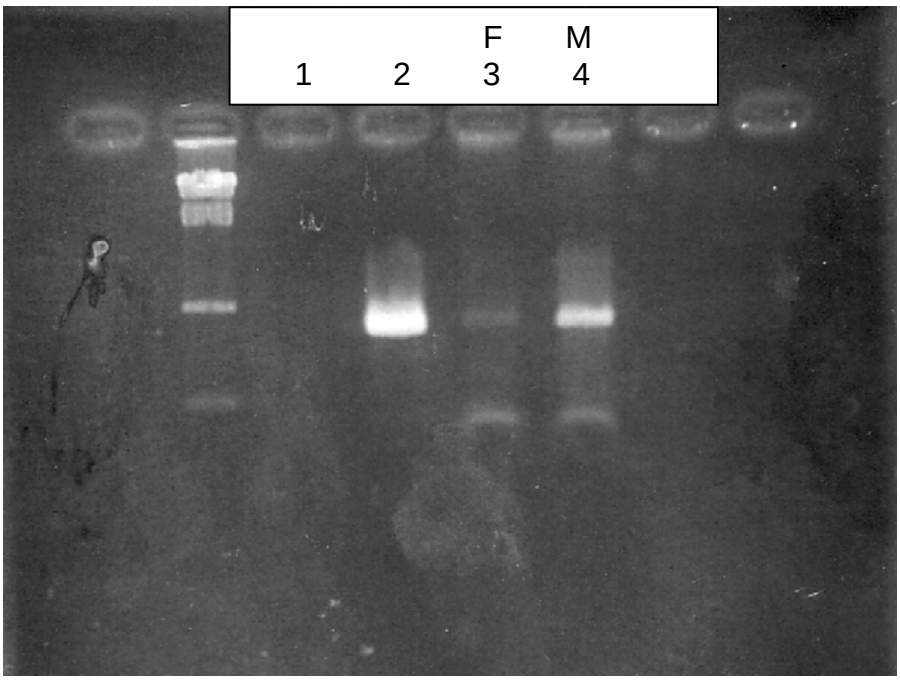


**Figure 5:** Analysis of bulk male and female prawn DNA samples with the O2 SCAR. Lane 1: No DNA control; Lane 2: Amplification control; Lane 3: Bulk Female DNA (5 ng); Lane 4: Bulk Male DNA (5 ng): Amplification conditions: 94$^{o}$C/120s; (94$^{o}$C/5s, 60$^{o}$C/10s, 72$^{o}$C/30s) X35; (72$^{o}$C/600s; 25$^{o}$C/hold).

The oligonucleotide primers O2F and O2R were synthesised designed and synthesised for the O2 SCAR primers. Initial attempts to amplify the O2 product were unsuccessful using DNA from individual prawns. Sequential analysis of the reagents indicated that the O2F primer was of poor quality (data not shown). Resynthesis of this primer did not improve the consistency of the amplification profiles using DNA of individual prawns. However amplifications from bulk male and female DNA samples clearly demonstrated a difference between the sexes (Figure 5). While the initial screening results indicated that the amplicon was specific to males this appears to be an over simplified interpretation of the results.

There are three possible explanations for the amplification of differential amounts of O2 from males and females.

1. There maybe a difference in the copy number which is sex dependent. If this was the case then it is apparent that males have more copies than females. This may explain why when the O2 operon primer is used to amplify from individual animals some females appear to amplify quite strongly. The marker appears to sex specific in the initial RAPD reaction as the other amplicons out compete the amplification of the lower copy number marker in females thus the product appears to be specific to males. However when the reaction is converted to a SCAR where only one specific amplicon is targeted it is amplified from genomes with low copy number (i.e. females). It is should also be noted that the PCR utilised in this case can not be considered to be quantitative PCR and as such all discussion regarding copy number can only be considered to be speculative.

2. It is possible that some of the females used to create the bulk female mix may in fact be males that have been incorrectly identified. Further some prawns in this study were identified that appeared to be hermaphroditic. This identification was only apparent during dissection of the individuals in question, clearly the inclusion of these types of animals would significantly have a significant effect on this type of study.

3. Perhaps the females that appear to contain the O2 marker are due to a cross over event in chromosome replication. If this were the case it would indicate that the O2 marker is not tightly linked to the sex-determining region of the prawn genome.

### 6.1.1.2 Characterisation of male specific marker amplified by RAPD Primer S7

Initial screening of bulked DNA from males and females indicated that the S7 primer amplified a specific band in male DNA with an approximate size of 600 bp (Figure 6). On the same gel four other primers also indicated putative sex specific products, J14, J17, S1, S2 & S7, indicated putative amplification products that were male specific (Figure 6). To determine if these amplicons were sex specific the RAPD reactions were repeated using template DNA purified from individual male and female prawns. Only the S7 produced similar a similar sex specific amplicon as observed in the bulk DNA amplifications (Figure 7A). The amplification profiles of primers J14, J17, S1, and S2, did not produce sex specific profiles on individual DNA.

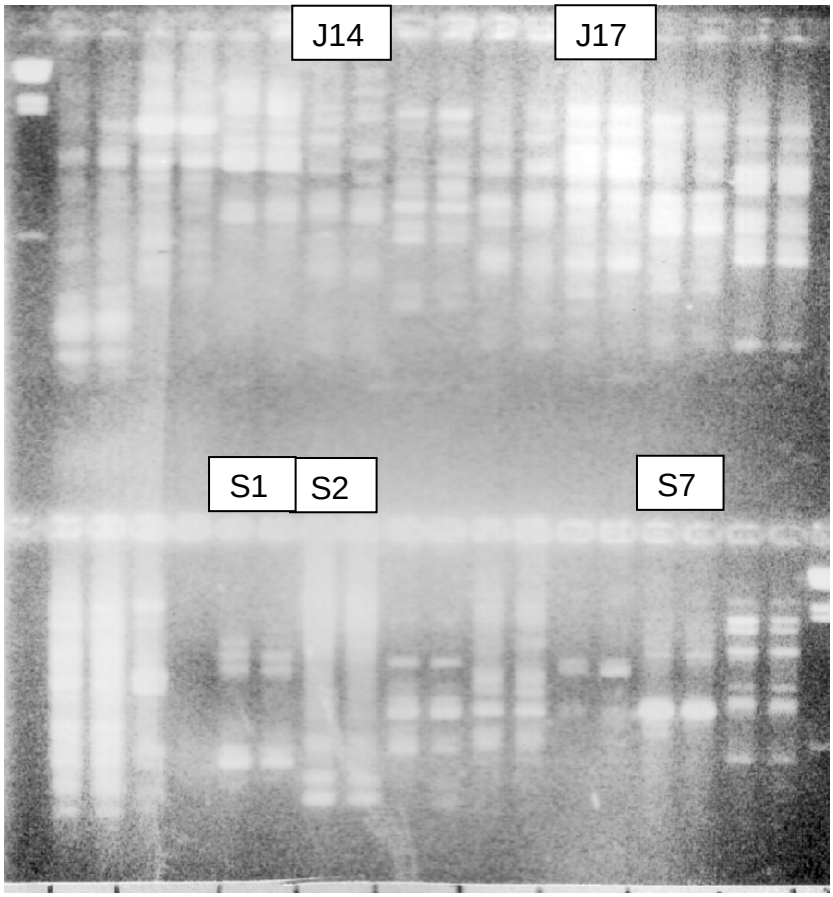


**Figure 6:** Primary product profiles derived from RAPD amplifications using Operon primers from bulk (9 individuals) male and female DNA samples. Samples are loaded in pairs in relation to the RAPD primer and alternate male and female respectively. Of the amplification profiles analysed on this gel J14, J17, S1, S2 and S7 were selected for further analyses. Arrows indicate, where obvious, the putative differential amplification products. Dramatic amplification differences such as those observed for O1 where there was a complete lack of amplification in one sample were not repeatable and most likely due to experimental error. RAPD reactions were performed as described in the Methods section and resolved in 2% agarose gels.

To further validate the sex specificity of the S7 amplification profile the RAPD reaction was repeated on twelve male and twelve female DNA samples. As previously demonstrated the 600 bp product was clearly amplified from the majority male DNA samples (Figure 7A & 7B). The apparent male specific band amplified from S7 was subsequently cloned and sequenced. The nucleotide sequence of the S7 RAPD product is shown in Figure 8. Comparison of this sequence to current databases (February 2005) did not find any significant matches with existing sequence data.

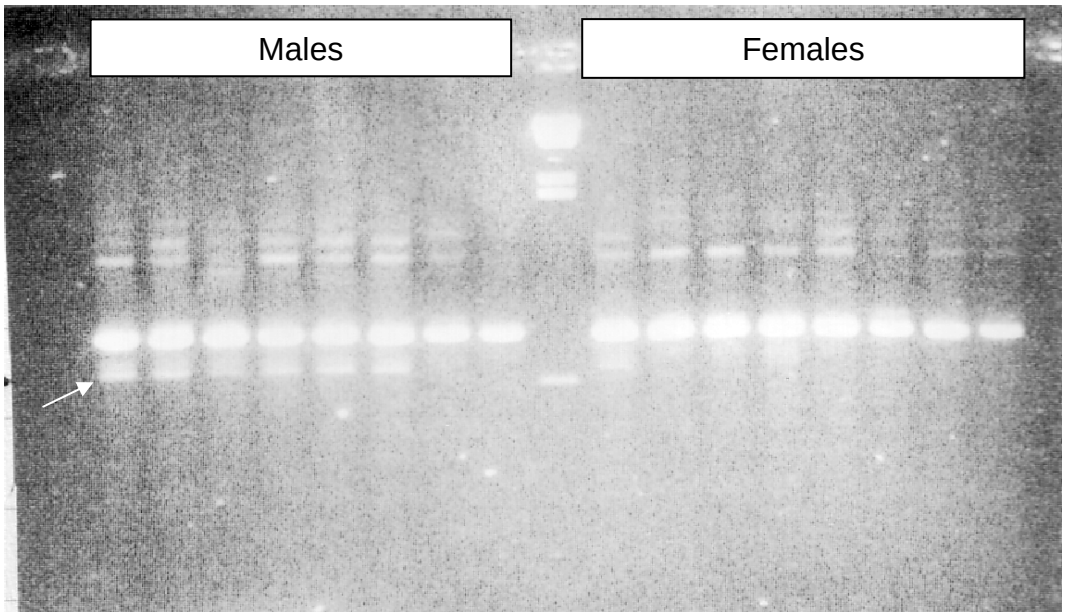


**Figure 7A**

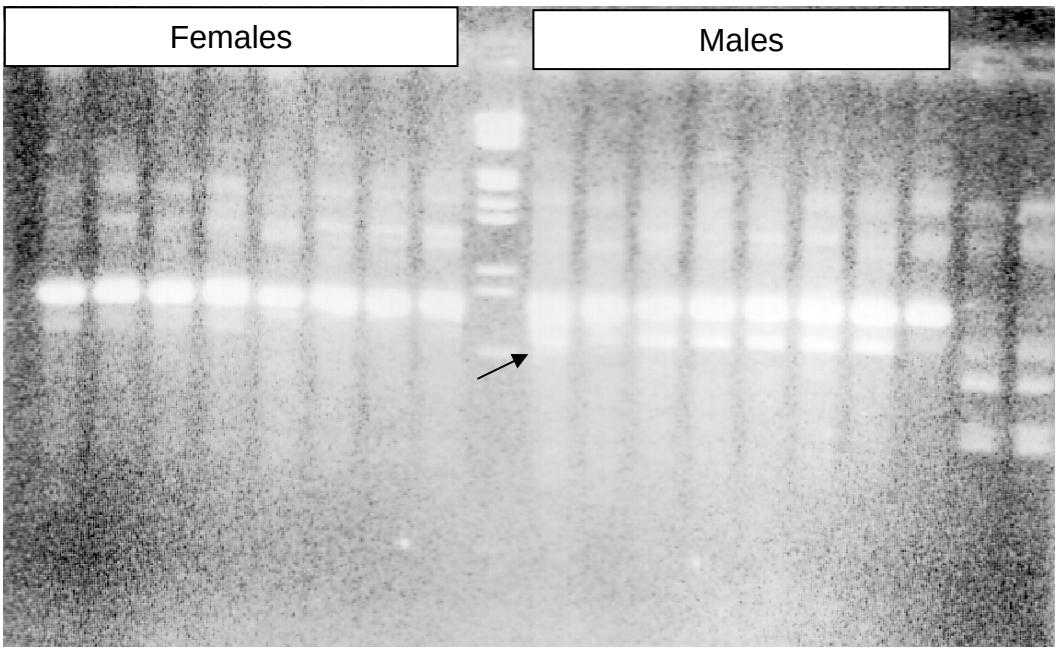


**Figure 7B**

**Figure 7:** RAPD amplification profile of Operon primer S7 on DNA from sixteen individual male and female prawns. Arrow indicates putative male specific amplicon.

```
1      TCCGATGCTG TCAAGACTAC CTACAGTATG AAATAATACA ACGATACAGT

51     TATGAAATAA TGTCTTTTAT AGTGACATGC CAGACATTAT ATCTGGTGAC
                                        SCAR primer S7F

101    AAAAATTTTA AACAAAACCT GAGAGAGTAG ATTATCTCCC ATTTATAACA

151    AAAATATCTA TAGATTATTT CAGATTCTTG GTAATTGTAT TCAACTTGTG

201    GTAAATATGT CCAAATTATT ATGAGATAAC ACATGAAATG GAGCAGTAGA

251    ACATTTATAT ATATAGCTCC ATCATCTTAA AAGTAAATAA AAACCAGTTA

301    TATTGAATAC AACAATAATG GTACTACATA GACTTACACA AAATTCTTAT
                            |   |
                           RsaI

351    TAATAACCCA AATAATCACT TAACCAACAC TCATGGTTAA AGCCAACACT
                                           SCAR primer S7R

401    CCATTGCACA TTATAAAATC CTGTCACTGG CAATACAGAA TATTTAATCT

451    AATTAATTTA GAATTTTTCC CTTCTAAAAT AAGACTGGCA TTACACTAAA

501    GATAGAATTT AAATAAGTGT TGTACTTTAT TTACTTAAAC CCTGTTTTCT

551    TTTTTTTTAT AACCAAGTTT TTAAAATAAT TCACCCACAT CCTACAGCAT

601    CGGA
```

**Figure 8:** Nucleotide sequence of RAPD marker amplified with Operon RAPD primer S7. Sequences corresponding to the RAPD primer S7 are shown in bold at the termini of the sequence. The sequences corresponding to the S7 SCAR primers are underlined and labelled accordingly. Also illustrated is the location and sequence of the restriction endonuclease site *Rsa*I.

To determine if the nucleotide sequence of S7 was specific for the male prawn genome it was also developed in to a sequence characterised amplification reaction (SCAR). The SCAR was developed by designing PCR primers specific for the S7 sequence as illustrated in Figure 8 along with the relative positions on the nucleotide sequence within the S7 product.

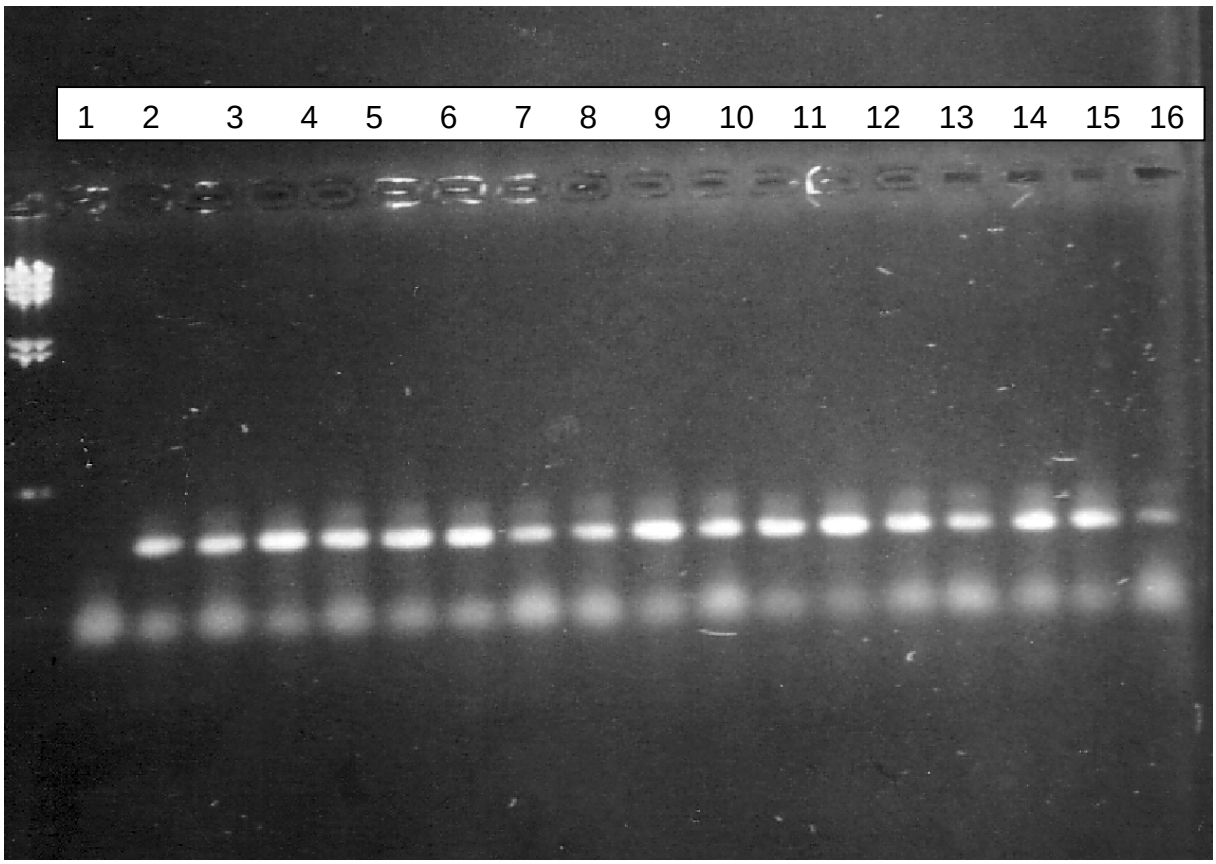


**Figure 9:** Amplification of the putative male specific marker S7 using a SCAR from 50 ng of DNA isolated from individual male and female prawns. Lane 1: No DNA control; Lanes 2 to 8: Individual Male samples; Lane 9: Bulk Male; Lanes 11 to 17 Individual Female samples; Lane 18: Bulk Female. Amplicons were resolved on a 1.5% agarose gel.
Amplification conditions: 94$^{o}$C/120s; (94$^{o}$C/10s, 50$^{o}$C/20s, 72$^{o}$C/60s) X5; (94$^{o}$C/10s, 55$^{o}$C/20s, 72$^{o}$C/60s) X30; (72$^{o}$C/600s; 25$^{o}$C/hold).

Initial attempts to amplify the S7 product from individual male and female DNA preparations are shown in Figure 9. As was the case with the O2 SCAR all samples appeared to contain the sequence corresponding to the S7 SCAR amplicon. Interestingly two of the controls included in this experiment were the bulk (9 individuals of each sex) male and female DNA samples as can be seen in Figure 9 the amplicon for the bulk male sample is much stronger than the corresponding amplicon for the bulk female sample. This is also analogous to the SCAR amplifications for the putative O2 marker. As with the O2 marker there are a number of possible explanations, such as differential copy number between the sexes, incorrect identification of the individuals sex, or that the putative marker is not tightly linked to a sex determining locus in prawns (see description of O2 marker for more descriptive explanation).

One further explanation was also investigated for the S7 marker. The S7 SCAR amplicon (329 bp) is much shorter than the primary RAPD amplicon (606 bp). The reason for this was to facilitate the design of the oligonucleotides. As can be seen from the nucleotide sequence of the S7 RAPD product the nucleotide sequence between the S7R oligonucleotide and the 3' terminal copy of the S7 RAPD primer is made of highly repetitive sequences with long stretches of only one nucleotide, these

types of sequences are not useful for the design of oligonucleotides for specific amplifications as required for the SCAR. There it is possible that the S7 SCAR amplicon is not being amplified from the same region of the genome as the S7 RAPD product. If this were the case it is possible that the amplicons from the female reactions were in fact a different nucleotide sequence compared to the males that coincidently had the approximately the same size. To determine if this was the case it was decided to digest the amplicons of the S7 SCAR with the restriction endonuclease *Rsa*I which cuts both the RAPD and SCAR S7 amplicons as shown in Figure 8.

Following SCAR amplification the resultant products were purified and digested with *Rsa*I. As can be seen in Figure 10 despite differences in the yield of products, which appears to be sex-specific, the *Rsa*I digestion confirms the same DNA sequence is amplified from both males and females, this is also the case with respect to both bulk and individual DNA samples.

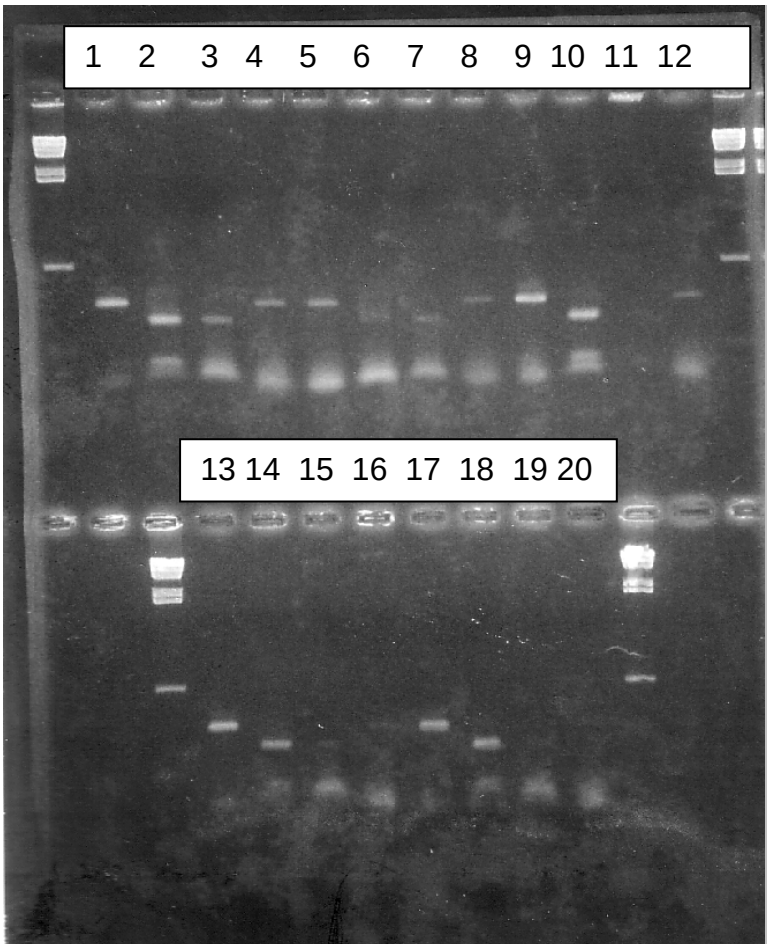


**Figure 10:** Digestion of the S7 SCAR products with *Rsa*I. Following purification amplicons were digested with 10 U of *Rsa*I for 4 hr. Digestion products were then resolved on a 1.5% agarose gel.
Lane 1: Bulk Male; Lane 2: Bulk Male, digested; Lane 3: Bulk Female, digested; Lane 4: Bulk Female; Lane 5: Male; Lane 6: Male, digested; Lane 7: Female; digested; Lane 8: Female; Lane 9: Male; Lane 10: Male, digested; Lane 11: Female; digested; Lane 12: Female; Lane 13: Male; Lane 14: Male, digested; Lane 15: Female; digested; Lane 16: Female; Lane 17: Male; Lane 18: Male, digested; Lane 19: Female; digested; Lane 20: Female.

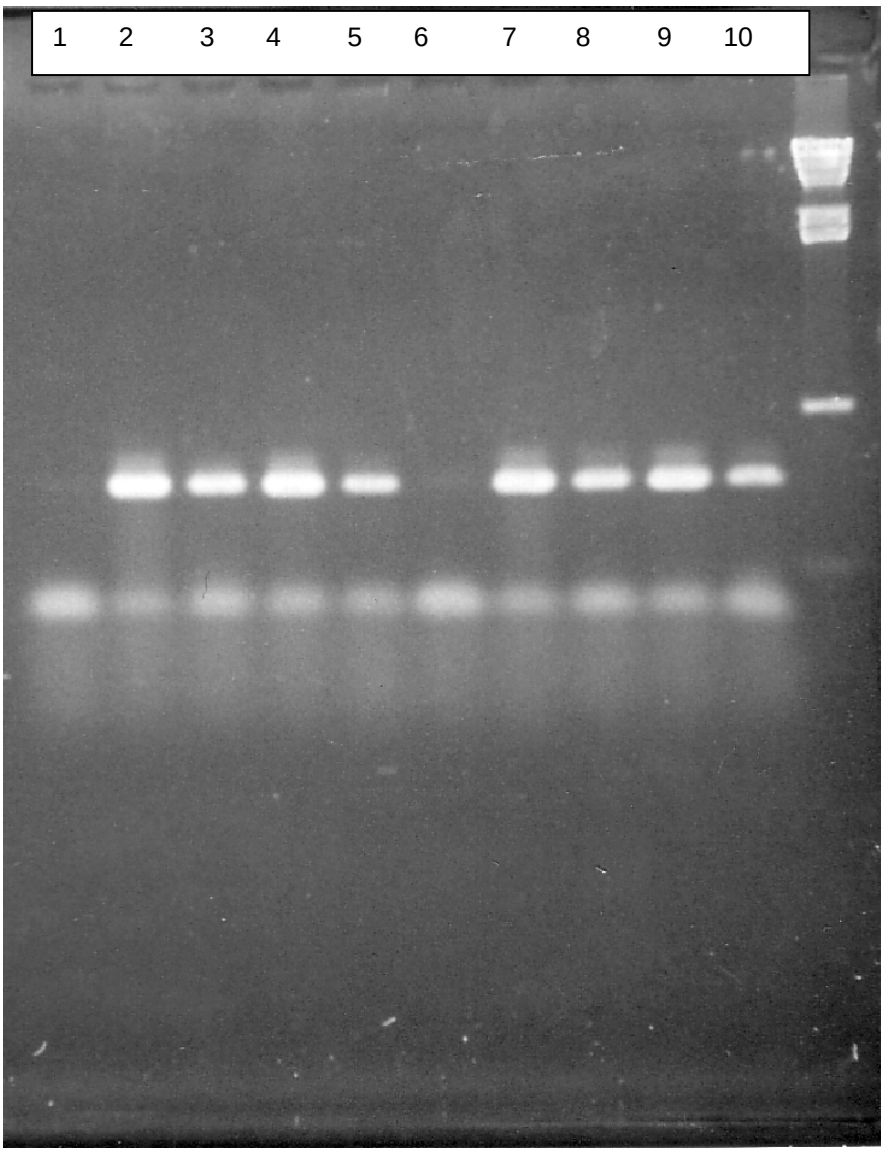


**Figure 11:** Analysis of bulk male and female prawn DNA samples with the S7 SCAR under PCR limiting conditions. PCR amplification was limited to 25 cycles compared to 35 cycles for all other reactions. Lanes 3 and 5 correspond to the 0.5 ng of male and female DNA and the best example of the differential amplification between the bulk samples.
Lane 1 & 6: No DNA control; Lane 2 & 7: Male DNA 5 ng; Lane 3 & 8: Male DNA (0.5 ng); Lane 4 & 9: Bulk Female DNA (5 ng): Lane 5 & 10: Bulk Female DNA (0.5 ng).
Amplification conditions: $94^{o}C/120s$; ($94^{o}C/10s$, $60^{o}C/15s$, $72^{o}C/60s$) X25; ($72^{o}C/600s$; $25^{o}C/hold$).

A further explanation for both the O2 and S7 SCARs failing to provide sex-specificity on all individuals tested is that the RAPD reaction is detecting a nucleotide polymorphism(s) associated with the RAPD primer-binding sites. If this were the case then neither the O2 nor the S7 SCARs would be able to confirm the sex-specificity of the RAPD amplification as the binding sites of both reactions are locating with the primary RAPD amplicon. To investigate this would require isolation and sequencing of the binding regions of the O2 and S7 primer binding regions from the prawn genome as detected in the RAPD reaction. This would be a highly laborious and costly process that was considered beyond the scope of this study

when considered in terms of the likelihood of successfully identifying a sex discriminating marker for prawns.

The apparent differences in amplification between bulked male and female DNA samples were further investigated. As previously discussed the PCR amplification so far described with respect to the S7 marker, both RAPD and SCAR, have been purely qualitative in nature. As a result it was decided to investigate if the differences indicated between the bulk DNA samples could be further validated. In order to reduce the qualitative nature of the S7 SCAR it was decided to reduce the number of amplification cycles from thirty-five to twenty-five and to test effects of varying the concentration of template DNA in the reactions.

Reducing the amount of template DNA added to the S7 SCAR resulted in an apparent drop in the amount of amplicon produced. Figure 11 demonstrates that at a template concentration of 5 ng per reaction there appeared to be more amplicon produced when using male DNA compared to female DNA. Further reducing the template concentration confirmed this apparent relationship. As shown in Figure 12 ten fold reduction in the template concentrations to 0.5 ng and 0.05 ng also indicated that male DNA permits the amplification of more S7 amplicon. Though it is not clearly apparent in Figure 12 a product was produced when limited template DNA to 0.05 ng of male DNA however no product was produced for the corresponding female DNA concentration. Through limiting the reaction length and template concentration the S7 SCAR is semi-quantitative.

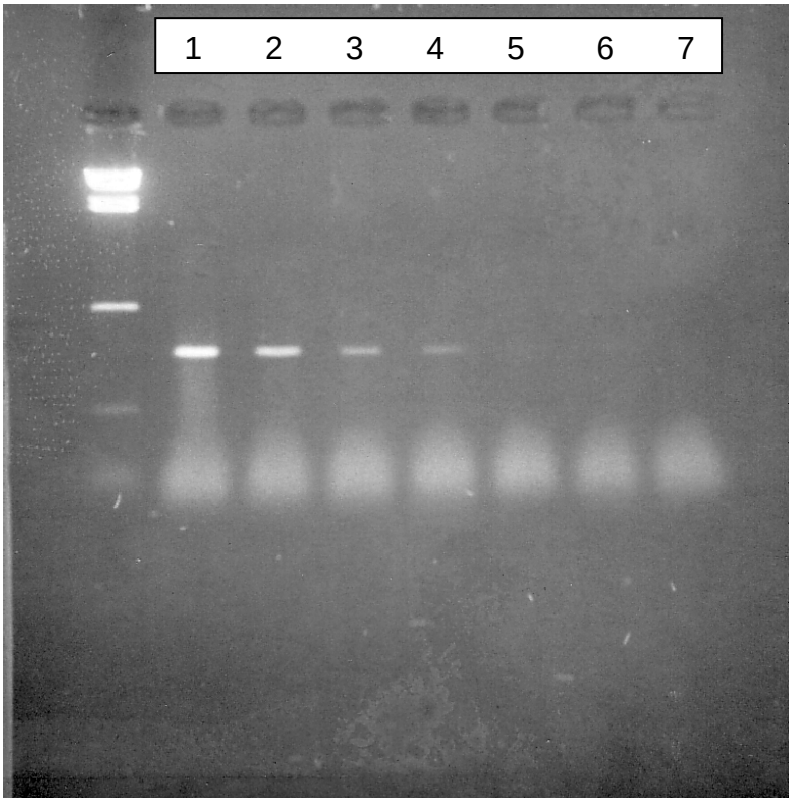


**Figure 12:** Titration of the effect of varying the concentration of template DNA from bulk samples of male and female prawn DNA samples with the S7 SCAR.
Lane 1 No DNA control; Lane 2 Male DNA 5 ng; Lane 3 Female DNA 5 ng; Lane 4 Male DNA 0.5 ng: Lane 5 Female DNA 0.5 ng: Lane 6 Male DNA 0.05 ng Lane 7 Female DNA 0.05 ng. Amplification conditions: 94$^{o}$C/120s; (94$^{o}$C/10s, 65$^{o}$C/15s, 72$^{o}$C/60s) X25; (72$^{o}$C/600s; 25$^{o}$C/hold).

### 6.1.1.3 Characterisation of male specific marker amplified by RAPD Primer P5

Initial screening of bulked DNA from males and females indicated that the P5 primer amplified two specific bands from male DNA with approximate sizes of 1600 bp and 500 bp respectively (data not shown). To determine if these amplicons were sex specific the RAPD reactions were repeated using template DNA purified from individual male and female prawns Figure 13. In addition the P5 amplicons were cloned and sequenced. The sequences of the P5 amplicons are shown in Figure 14. The smaller amplicon was 321 bp in length (Figure 13A). Only 354 bp of the larger amplicon sequence was determined (Figure 13B). Database searches conducted with these sequences did not identify any identity to known sequences. Comparison of the sequences of the two amplicons did not demonstrate any identity.

Based on the previous analysis of the O2 and S7 markers the putative P5 marker was not further investigated using other PCR amplification technologies.

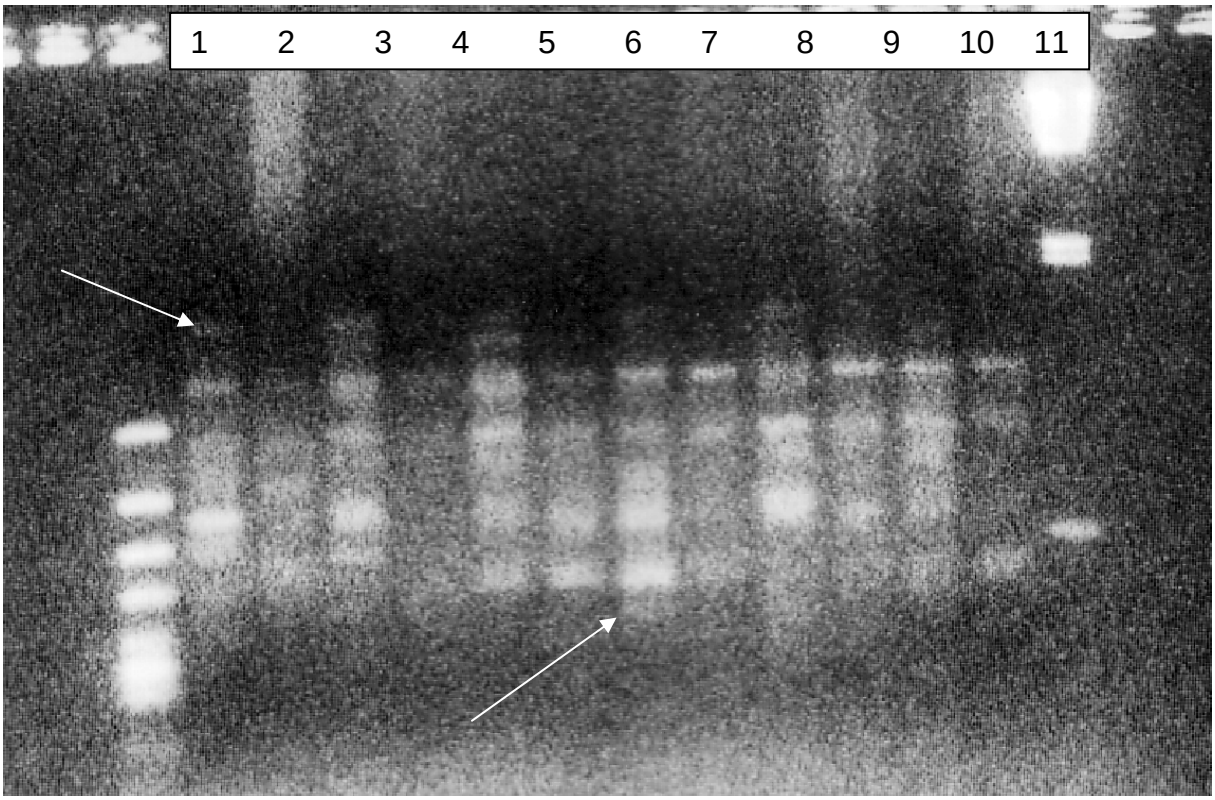


**Figure 13:** RAPD amplification profile of Operon primer P5 on DNA from six individual male and female prawns. Arrow indicates putative male specific amplicon. Lanes 1: 3, 5, 7, 9 & 11; Individual male DNA templates; Lanes 2, 4, 6, 8, 10, 12: Individual female DNA templates

```
  1    CCCCGGTAAC AGCTAAGGCC TCTCCTTATT ATCTCCAACA ATCCGATTTC
 51    GACCGAGTTA TTAAGACCTT AAACGACCTC TTTCAGATCC CTACGACCCA
101    TCAGCAGCTG CTACTGACTT CCACAAGCCG CCATTTTACA GCCTACCGCT
151    CGTTTCTGCG ATGTCTTTCA ACCTATCATG ACCTACCATC TACTGCGACC
201    TATCAGGCCT CCTAACCATT TCTAATGGAT TCTATATATT TCCAATCACA
251    TGAGCTTTTC AATATCTAAT TTCAATTATT TGCTTCCCGA GAAATAATTG
301    GTTTTATGAC AGTTACCGGG G
```

**Figure 14A:** P5 small amplicon

```
  1    CCCCGGTAAC AAGACATGCT AAATCAGAAT CTTATGGAGA TAAGGATGAG
 51    GCTTATCAGT TATCAGATAT CTGTGCCACT AATAGCGTTC CATATTCCTT
101    GTATCGGGGT TATCCTTCAA AACCGGATTC CTGAACTAAA GCCACGTTTT
151    CTCAGCCACT TAACTATTGT AAGTATGAAG TAAGTATCGG TGTTTCTTTA
201    ACAGTTAGGA TGCTTTTTTA TTTGTTTTGT TTTGTTTTTC TTAGAGGGAG
251    TACTGTACAT AACGTCCATA TTGCCTGAAA CATAGTAAGA AGAACAACAC
301    GATTTTGCTC ATGGCCTTCA GTGGAGAATA TTTTGAGGAA TCACATGAGA
351    ATAA
```

**Figure 14B:** P5 large amplicon (incomplete)

**Figure 14:** Nucleotide sequence of RAPD marker amplified with Operon RAPD primer P5. Figure 14A: Nucleotide sequence of smaller P5 amplicon. Figure 14B: Partial sequence of the larger P5 amplicon. Sequences corresponding to the RAPD primer P5 are shown in bold at the termini of the sequence.

### 6.1.1.4 Characterisation of male specific marker amplified by RAPD Primer H9

Initial screening of bulked DNA from males and females indicated that the H9 primer amplified a specific band in male DNA with an approximate size of 600 bp (data not shown). To determine if these amplicons were sex specific the RAPD reactions were repeated using template DNA purified from individual male and female prawns (Figure 15). In addition the H9 amplicon was cloned and sequenced. The sequence of the H9 amplicon is shown in Figure 16. Database searches conducted with this sequence did not identify any sequences with significant identity.

As the amplicon was not present in all individual male samples and based on the previous analysis of the O2 and S7 markers the putative H9 marker was not further investigated using PCR amplification protocols.

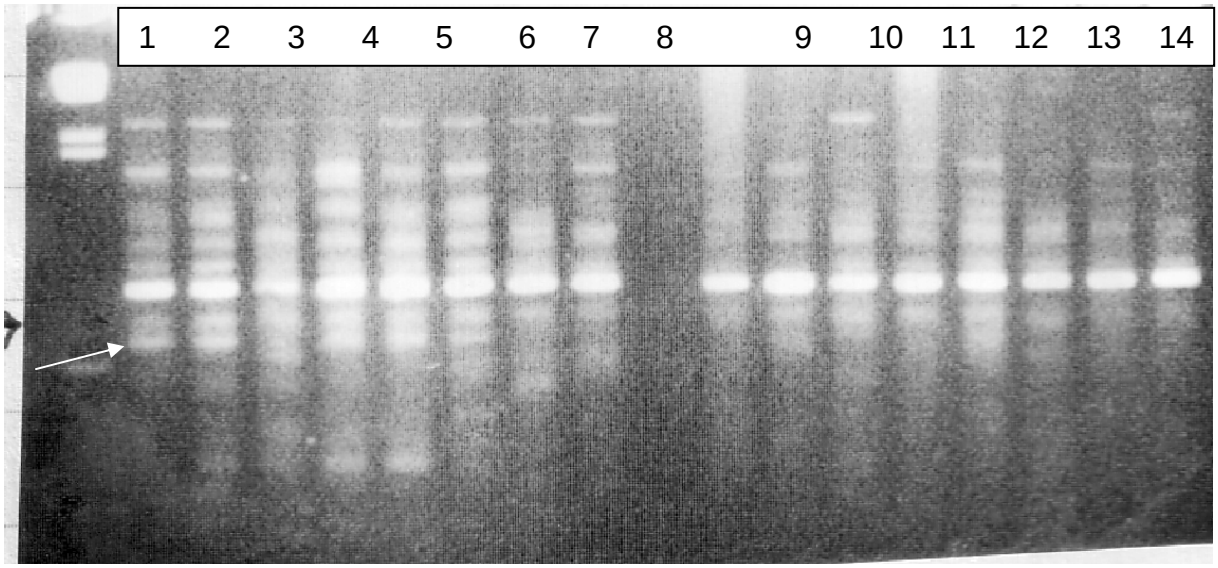


**Figure 15:** RAPD amplification profile of Operon primer H9 on DNA from eight individual male and female prawns. Arrow indicates putative male specific amplicon. Lanes 1 to 8: Individual male DNA templates; Lanes 9 to 16: Individual female DNA templates.

```
  1    TGTAGCTGGG TCAGATATGT GTTTTTCTTC TTCTCGCTTA TTGATACAGA
 51    CTCTGTCGTT ATCAATTCCA TTTATTAATA TATATGCGAT GAGCGTTTTG
101    TTGCTACAAC TTGCATTATT CTCGTAACCG GTTCCACGAT TTGCATAATA
151    GTGTCATTAT CATTTTCATC TTCATCATCA GCTCTGCCAT TGGTAGTATG
201    GATTATCTTT GGAGAAATGT AAGAATACCT CATTAAATCG GTAGTAATCA
251    CTCAAACGGA CACATAAATA AGCGGCTACC ATCATCGTAT TCACCATTAT
301    CATAATCACA TTATCATTTC TATTTCCACC ATTATCATGA CTGGCATACC
351    AGGCTAATAG GTATTCCTAT TCTCATCGCG AACAACAAAA CATTACATTC
401    CCTTCCATTA TTCCTAATAT TGCCATGTTA CTAAAAATAT CCTTGCCATC
451    ATTCCTGATT ACAAGTATAT TAAAAACTAT TCATCTATCC TCACCGTTAT
501    CATCACCATC CTTACCCAAA GCCAGCCAGA CAAACATTCA TTAAAAACTA
551    TCATTTTTAT ACTCATCCTT ATCATCACCA TCCCTACCCA ACCCAGCTAC
601    A
```

**Figure 16**: Nucleotide sequence of RAPD marker amplified with Operon RAPD primer H9. Sequences corresponding to the RAPD primer H9 are shown in bold at the termini of the sequence

### 6.1.1.5 Characterisation of male specific marker amplified by RAPD Primer J10

Initial screening of bulked DNA from males and females indicated that the J10 primer amplified a specific band in male DNA with an approximate size of 400 bp (data not shown). To determine if these amplicons were sex specific the RAPD reactions were repeated using template DNA purified from individual male and female prawns (Figure 17). The putative marker was present in all four male samples tested and absent from all female samples. The J10 amplicon was cloned and sequenced. The sequence of the J10 amplicon is shown in Figure 18. Database searches conducted with this sequence did not identify any sequences with significant identity.

To further investigate the specificity of the marker an additional eight male and eight female DNA samples were tested (Figure 19). It was evident that the J10 amplifications were similar to those previously examined and was also failing to yield consistent sex associated amplification. As the amplicon was not present in all individual male samples and based on the previous analysis of the O2 and S7 markers the putative J10 marker was not further investigated using PCR amplification protocols.

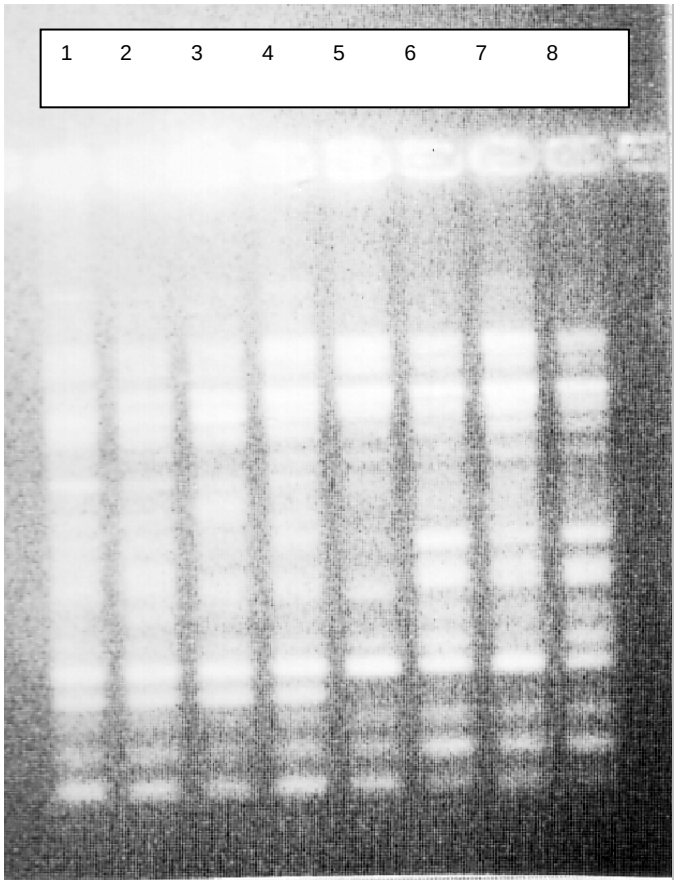


**Figure 17**: RAPD amplification profile of Operon primer J10 on DNA from eight individual male and female prawns. Arrow indicates putative male specific amplicon. Lanes 1 to 4: Individual male DNA templates; Lanes 5 to 8: Individual female DNA templates.

```
  1    AAGCCCGAGG GAGGGAAGAA ATTTACGCTG GCATAATCTA GAATCTATTT
 51    CCTGTGATTT AGGAAGATGC TCTCGCACTG TACTGTAGAC GTATGATTCG
101    GAGTGACTGA GCTCTGTCAC ATTGCTTGTG ACTTCAGAGG AAAGGGGTAC
151    AAACTGTTCA AAGTTCTTGA TGCTTGGGAG CCCAGATAGC CATGCAGCAG
201    TTGCAGCTGC TATACTTTTC TCTAAGTAAT GCTGACTGTC GATACTAATA
251    TTAGTATCTC CATCTTCTTC TTCTTCTTAT TCATCTCCTC CTCTTCCTCC
301    TCCTCCTCCT CCTCCTTCTC TTCCTCCTCC TCCTCCTCAT TATCCCCCAT
351    TTCCCCTATT TTATCCTCGG GCTT
```

**Figure 18:** Nucleotide sequence of RAPD marker amplified with Operon RAPD primer J10. Sequences corresponding to the RAPD primer J10 are shown in bold at the termini of the sequence

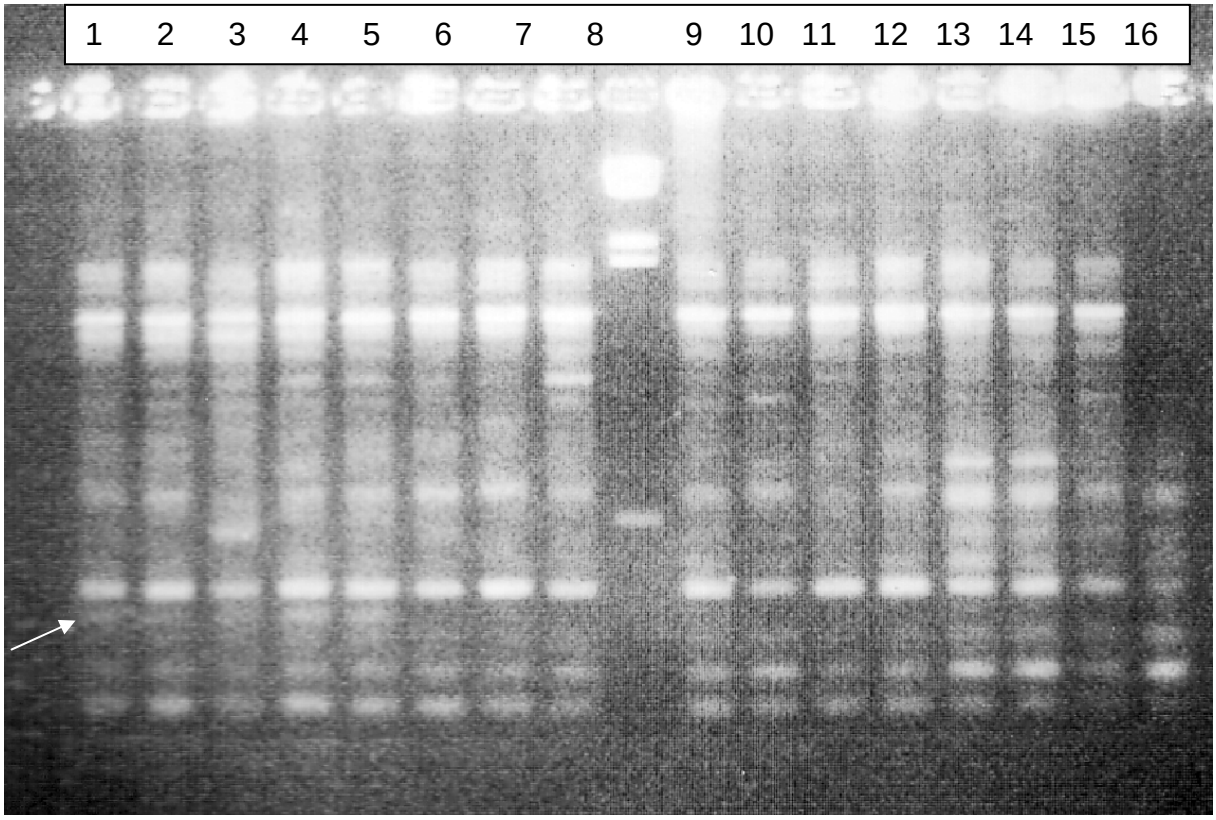


**Figure 19:** RAPD amplification profile of Operon primer J10 on DNA from sixteen individual male and female prawns. Arrow indicates putative male specific amplicon. Lanes 1 to 8: Individual male DNA templates; Lanes 9 to 16: Individual female DNA templates.

### 6.1.1.6 Summary of RAPD analyses of *Penaeus monodon*

Amplification products from bulked samples of prawn DNA yielded numerous differences between male and females. In most cases these putative differences were not maintained when the same primers were used across 18 individuals, 9 of each sex. These results suggest that there is a high degree of individual variability where certain bands that amplify from a few individuals overwhelm other representative RAPD patterns within the bulk. A few individuals may possess specific sequences that match the primers more accurately than other individuals in the bulk so that only a few individuals may yield very strong bands that the majority of individuals in the bulk do not possess. In addition, since RAPD primers are more likely to successfully amplify sequences that are highly repeated, the product bands may also reflect a few individuals who have a higher copy number of sequences that match the specific primer. More closely related siblings and a larger number of individuals included in the bulk reduced this problem. None the less, a combined approach using bulked DNA to screen a larger number of RAPD primers first, then subsequent analysis of the promising primers across individual samples is an efficient way to scan for appropriate markers.

Penaeid prawn species possess diploid chromosome numbers close to 88 chromosomes (Chow *et al*. 1990). Using the RAPD technique the primers produced an average of 8 bands for each primer. Although we tested 320 different primers we located only two markers in *Penaeus monodon* that were statistically linked to sex. These did not remain consistent when further testing compared them across larger numbers of individual DNA preparations. If we were working with a species that possessed differentiated sex chromosomes we would expect a vast number of markers distinguishing the sexes. Thus our results suggest that sex determination in penaeid prawns is directed by a single locus, or a small number of genetic loci.

We identified fourteen candidate sex-linked DNA sequences (Table 1). Thirteen of those potential markers occurred most frequently in the male sex. We analysed those and the one female candidate across a minimum of 18 individuals (9 male and 9 female) through linkage analysis, but only two of the bands analysed were statistically linked to the male locus with LOD scores of 6.2 (band S7) and 4.5 (band O2), Mapmaker program 0.3 1993.

Thus, sex determination in prawns has a genetic basis in that animals of one sex contain DNA sequence that is not present or is at low copy number in the other sex.

Six of the candidate sequences were successfully cloned into plasmid vectors. Alignment analysis of the sequences did not reveal regions of overlap among these sequences indicating that the regions we have cloned are in different positions with respect to the male locus.

The first set of specific primers we designed from the sequence data of our S7 and O2 clones did not distinguish male and female individuals. The results indicate that this particular marker may occur in very low copy number in females so that the preliminary primers we designed were not specific enough to distinguish males and females in the PCR process. The appropriate band amplified in both males and females. In a situation like this the PCR technique may not be to our benefit. The high sensitivity can pick up very low copy numbers in one sex and yield a bold band that might mask real male/female differences. Another important observation was

that the amplification profiles obtained were in some cases dependent on the method of DNA isolation as in some cases different profiles were obtained from the same animals if the DNA was isolated using different methodologies. Thus when randomly screening for genetic polymorphisms it is clearly important to use strictly standardised protocols so that all samples can be accurately compared. This type of approach should aid in the prevention of introducing artefacts that could potentially bias the interpretation of results.

| Marker | Sex linked to | LOD Score | Sequenced |
|---|---|---|---|
| O2 | Male | 4.5 | Yes |
| S7 | Male | 6.7 | Yes |
| J10 | Male | NS | Yes |
| H9 | Male | NS | Yes |
| P5 | Male | NS | Yes |
| D16 | Male | NS | No |
| AB5 | Male | NS | No |
| AB16 | Male | NS | No |
| E8 | Female | NS | No |
| G18 | Male | NS | No |
| G9 | Male | NS | No |
| L6 | Male | NS | No |
| L9 | Male | NS | No |
| L16 | Male | NS | No |

**Table 1:** Summary of the fourteen candidate sex-linked DNA markers identified in *Penaeus monodon*. The Marker name refers to the Operon RAPD primer used to amplify the respective marker bands. LOD scores were calculated for each marker using amplification data from a minimum of 18 individuals (9 male and 9 female) through, but only two of the bands analysed were statistically significant with a score > 3. NS indicates not significant based on the data analysed. The linkage analysis was performed using the Mapmaker program (V0.3 1993).

### 6.1.2 Subtractive Hybridisation of F1 generation prawns

We then tried a more refined and technically difficult approach, Representational Difference Analysis, also called Subtractive Hybridisation (Lisitsyn *et al*. 1993) to identify small differences between the sequences of male and female prawn DNA. This new technique yielded 19 fragments of DNA from the process that enriched for distinct male DNA and 3 fragments from the process that enriched for distinct female DNA. Seven of the male fragments yielded sequence data and ranged in size from 100 to 500 base pairs in length. The sequences of these fragments are clearly distinct from one another. Of the 3 female fragments, one yielded clear sequence data and it is distinct from the male fragments. These sequences are also all distinct from those of the first two probes, S7 and O2.

#### 6.1.2.1 Southern blotting analysis of subtractive hybridisation fragments

We used these probes to screen 63 Southern blots (data not shown). The probes are clearly specific for *Penaeus monodon* prawns, they do not hybridise with *P. japonicus*, but in combination with the restriction enzymes, *Sau*3a, *Eco*RI, *Pst*I, *Msp*I and *Taq*I, they do not distinguish between male and female individuals. It is possible that further study of different restriction enzyme digests could reveal an association, but we chose the alternative approach of experimenting with more closely related individuals, the F2 generation. Our aim was to reduce the between-individual variability and accentuate the differences between males and females.

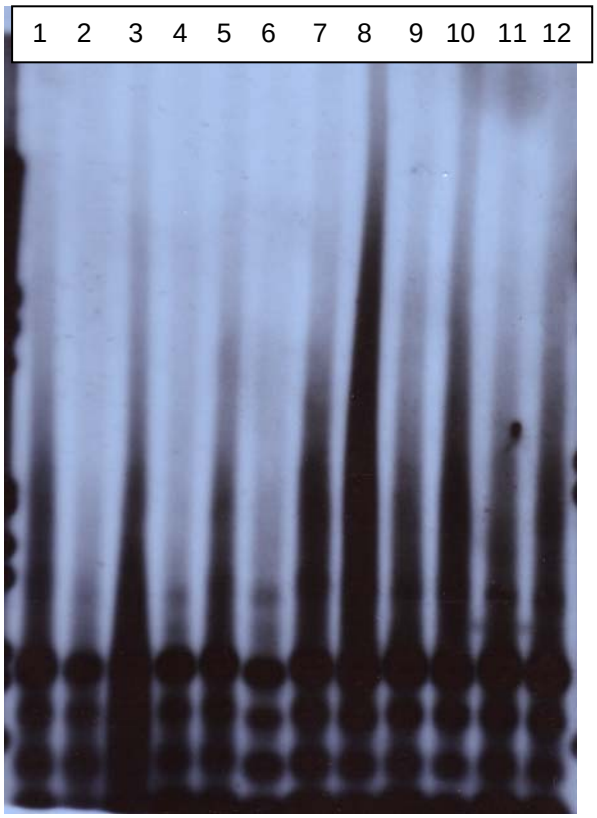


**Figure 20:** Southern blotting analysis of individual male and female prawn genomic DNA samples with T a putative male specific marker. Lanes 1 to 8 Male DNA samples, Lanes 9 to 16 Female DNA samples. All DNA samples were digested with restriction enzyme *Sau*3A.

### 6.1.2.2 Sequence analysis of subtractive hybridisation fragments

Attempts were made to sequence fourteen of the DNA fragments isolated through subtractive hybridisation experiments. To facilitate sequencing fragments were first cloned into a plasmid vector. Clear unambiguous nucleotide sequence was obtained for ten of the fourteen fragments. The resulting nucleotide sequences were then compared to the latest (February 2005) nucleotide and protein sequence databases at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov,) to identify any identity with known sequences. These analyses are summarised in Table 2. Two fragments, 5M and 19F, had identity to known sequences the corresponding Genbank accession numbers are shown in Table 2.

| Fragment Name | Sex-specificity (Tentative) | Sequenced | Database | |
|---|---|---|---|---|
| | | | Nucleotide | Protein |
| 5M | Male | Yes | AY654015 | ND |
| 7M | Male | Yes | Nil | Nil |
| 9M | Male | Yes | Nil | Nil |
| 11M | Male | Yes | Nil | Nil |
| 14M | Male | Yes | Nil | Nil |
| 15M | Male | Yes | Nil | Nil |
| 19F | Female | Yes | AY049200 | ND |
| 24F | Female | No | NA | NA |
| AM | Male | Yes | Nil | Nil |
| HM | Male | No | NA | NA |
| IM | Male | No | NA | NA |
| SM | Male | No | NA | NA |
| TM | Male | No | NA | NA |
| UM | Male | Yes | Nil | Nil |

**Table 2:** Summary of the putative sex-specific fragments isolated using subtractive hybridisation from male and female *Penaeus monodon* prawns.

Fragment 19F was 113 base pairs in length and was isolated from experiments targeting female specific DNA sequences (Figure 21A). Database search with the sequence of Fragment 19F indicated high identity to a microsatellite sequence from *Farfantepenaeus paulensis* (pink shrimp) as shown in Figure 21B. *F. paulensis* is a peneid prawn from the Western Atlantic Ocean and the microsatellite sequence was reported by Maggioni & Rogers (2002). As illustrated in Figure 21B the overall identity of 19F to the microsatellite sequence from *F. paulensis* microsatellite was approximately 80%.

The *F. paulensis* microsatellite sequence is reportedly specific this species of prawn. Thus it is unclear why a reasonable level of homology to a P. monodon sequence would occur. One explanation might be that the region common to both *P. monodon* and *F. paulensis* is common to all prawn species, while the flanking sequences permit differentiation of species. It would be necessary to sequence the DNA flanking Fragment 19F in *P. monodon* to answer this question.

```
  1    GATCATCCCA CAGGCCTCCT CCACCCAGGG TTGTCTTGAA AAGAGACAAC

 51    CTGATGGGTA GGGTCATCCA CAGGGAAATG AGCTAAGTGC ACATGTAGCC

101    TAAGTTGGCG ATC
```

**Figure 21A:**

```
                      1                                                  50
Fpa421             (1) GATCATGGGGTACAGTTGGCAGGACCACGTGTCCATCCGACGACTTCACC

                      51                                                100
Fpa421            (51) AAGAGACTAGCATGGGACCTGTTACTTGCATAATCTGGGACCGCCAACTC
Fragment 19F       (1) -----------------------------------GATCGCCAACTT

                     101                                                150
Fpa421            (101) AGGCTATACGGCCACCGAGCTCGTTTCCCTGTCGATGACTCTGCCCATCA
Fragment 19F       (13) AGGCTACATGTGCACTTAGCTCATTTCCCTGTGGATGACCCTACCCATCA

                     151                                                200
Fpa421            (151) GGTTGTCAATTTTACGCGACAATCGTGCATGGAGGAGACCCGTGGGAAGA
Fragment 19F       (63) GGTTGTCTCTTTT-CAAGACAACCCTGGGTGGAGGAGGCCTGTGGGATGA

                     201                                                250
Fpa421            (201) CCTAGGAAGTCATGGCTTAGGCAGCTCGACCAGACATGTCATGGAGAACT
Fragment 19F      (112) TC--------------------------------------------------

                     251                                                300
Fpa421            (251) AGAGATGGGCCGAGGGCCTGTCTGGCGACTCGCCGTGAGGGACCCTCGTG

                     301                                                350
Fpa421            (301) GCTGGAAGCGAAGGATGGGTGCAACTATGCGTCCCCCGTCGGCGTTAGCT

                     351                                                400
Fpa421            (351) CCTTTGGTGGTGATGATGACACACACACACACACACACACACGCACACAC

                     401                                             447
Fpa421            (401) ACACACATGCTCATACTCACACTCACACTCACTCAGGCAGGTAACCA
```

**Figure 21B:**

**Figure 21:** Sequence analysis of the *Penaeus monodon* fragment 19F isolated from subtractive hybridisation experiments. Figure 21A: Nucleotide sequence of 19F. Bold text indicates sequence with high identity to microsatellite sequence Fpa421 from *Farfantepenaeus paulensis* (pink shrimp, Genbank Accession AY049200). Figure 21B: Alignment of fragment 19F to a microsatellite sequence from *F. paulensis* (Maggioni & Rogers, 2002). Identical nucleotide residues are shaded.

The most interesting database searching result was obtain with the sequence of Fragment 5M, that 110 base pairs in length and was isolated from experiments targeting male specific DNA sequences (Figure 22A). Database search (February 2005) with the sequence of Fragment 5M indicated high identity to a *P. monodon* amplified fragment length polymorphism (AFLP) sequence (clone AFE50M31) that is associated with either sex or growth traits. The sequence is listed on Genbank with the reference: Sraphet, S. & Triwitayakorn, K. (Unpublished) Development of AFLP genetic markers for sex and growth traits of Black Tiger Shrimp (*Penaeus monodon*) Submitted (13 June 2004) to Molecular Biology and Genetics. This study was conducted in Thailand.

To date the reference does not appear in PubMed thus indicating that finalisation of the manuscript to publication has not occurred or it has been rejected for publication. As a result it is not possible to determine is the sequence for AFE50M31 is being reported as a sex-linked marker.

```
  1    GATCAGATGA AATATATACA GATGGTTCGA AATCTGAAAA TGGTGTGGGT

 51    TTTGCAGTAG TGAGCAGAAG GAAGACTGCA TCTGACAGTC TTTCAAAAGC

101    AGCCTCGATC
```

**Figure 22A:**

```
                1                                                  50
AFE50M31    (1) TACAATTCCACCCTTGGACTAATCAGGTCGACCTGGATTTGGTCGGAATT

               51                                                 100
AFE50M31   (51) GGGAAAGGGGAGAGATCCGATCCAGAAGTCAGAGAGATTTTTTCAGCATA

              101                                                 150
AFE50M31  (101) CTTACCAAGGGATCAGATGCAATATATACAGATGGTTCAAAGTCTGAAAA
Fragment 5M (1) ----------GATCAGATGAAATATATACAGATGGTTCGAAATCTGAAAA

              151                                                 200
AFE50M31  (151) TGGTGTGGGCTTTGCAGCAGTGAGCAGAAGGAAGACTGCATCTGCCAGTT
Fragment 5M(41) TGGTGTGGGTTTTGCAGTAGTGAGCAGAAGGAAGACTGCATCTGACAGTC

              201                                                 250
AFE50M31  (201) TTTTTCTCTAGCAGCCTCGATCTTCACTGCAGAGGTACATGCCATCCTTG
Fragment 5M 91) TT--TCAAAAGCAGCCTCGATC--------------------------

              251
AFE50M31  (251) CAGCAG
```

**Figure 22B**

**Figure 22:** Sequence analysis of the *Penaeus monodon* fragment 5M isolated from subtractive hybridisation experiments. Figure 22A: Nucleotide sequence of 5M. Figure 22B: Alignment of fragment 5M to *P. monodon* clone AFE50M31 (Sraphet & Triwitayakorn, Unpublished. Genbank Accession AY654015). Identical nucleotide residues are shaded.

Of the 110 bp of fragment 5M had 89% identity to the sequence of the AFLP clone AFE50M31 which was 256 bp in length (Figure 22B). As illustrated in Figure 22B there are a number of differences between the sequences indicated that there maybe significant polymorphism associated with sex-determination in *P. monodon*. Or given that the sequence for AFE50M31 was determined using *P. monodon* from Thailand the indicated differences maybe due to geographical variation.

Southern blotting of individual male and female DNA samples with the 5M fragment did not reveal any sex specificity (Figure 23). The results of the Southern blot indicated what appeared to primarily non-specific binding to both male and female DNA samples as indicated by the large smears in Figure 23. At the time (May 1996) there were no database matches to the sequence of Fragment 5M as AY654015 was not deposited into Genbank until June 2004. Considering the likelihood of AY654015 being a sex-linked marker further investigation into fragment 5M may have been warranted. However given the number of putative markers being investigated at the time (Tables 1 & 2) a very stringent screening procedure was in place to devise a manageable workload. Further Southern experiments using DNA cut with a variety of restriction enzymes may have yielded clearer results regarding Fragment 5M being a valid sex-specific marker.

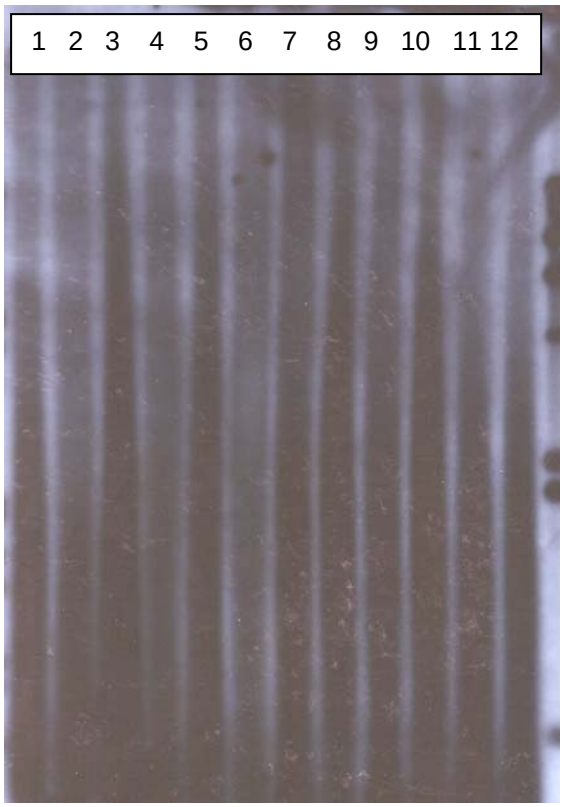


**Figure 23:** Southern blotting analysis of individual male and female prawns with the subtractive hybridisation fragment 5M. This fragment was isolated in experiments targeting the isolation of male specific sequences. All genomic DNA was digested with *Sau*3A prior to electrophoresis and blotting. Lanes 1 to 6: Male DNA samples; Lanes 7 to 12: Female DNA samples.

No significant homologies were identified for the sequenced subtractive hybridisation fragments. The nucleotide sequences of the fragments 7M, 9M, 11M, 14M, 15M, 24F, AM, HM, and UM are shown in Appendix 4.

### 6.1.3 RAPD analysis of DNA from F2 generation prawns

In order to improve the resolution of the RAPD amplification profiles and thus increase the likelihood of identifying a robust sex-linked marker it was decided to analyse the RAPD amplicons using silver stained acrylamide gels rather than agarose gels stained with ethidium bromide. Although this procedure is more time consuming, the combination of acrylamide and silver staining techniques allows superior visualisation of more delicate bands for each RAPD primer. Rather than an average of 8 bands shown from agarose, each primer yielded an average of 32 bands in acrylamide. An example of the relative sensitivities of these two methods of analysis is shown in Figure 24. In spite of the increased resolution of larger numbers of bands, we still could not surmount the problem of the high degree of genetic variability between individuals masking the specific genetic differences between the sexes.

To further improve the likelihood of identifying a sex specific marker DNA from an F2 generation of prawns was used. In this case the individuals utilised in these experiments we used 120 individuals for each male and female pool of DNA. We screened 159 RAPD primers on 35 acrylamide gels. Six putative differential bands were revealed but when these primers were screened across 8 males and 8 females with PCR, the resulting bands did not distinguish between male and female individuals. Figure 25 illustrates typical RAPD amplification profiles from F2 generation prawns using bulked male and female DNA. One of these RAPID primers was also used to screen DNA from individuals of each sex. As was typically obtained in previous analyses the putative markers did not appear to be sex specific (Figure 26).

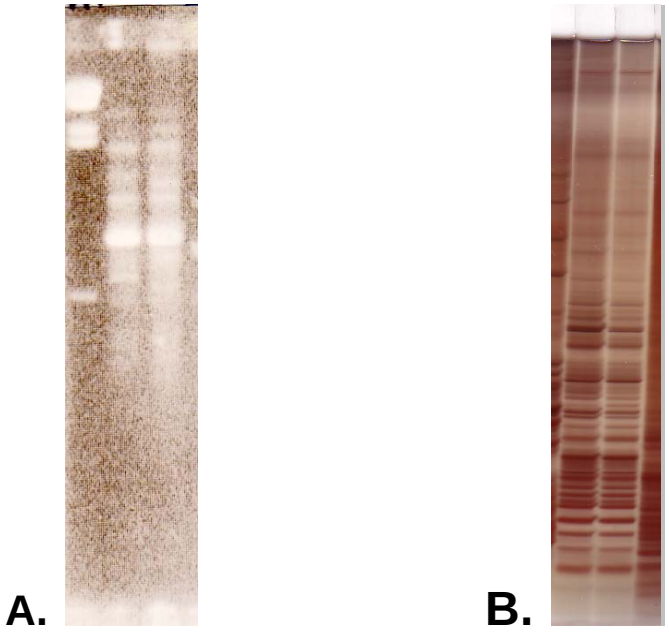


**Figure 24:** Comparison of the amplification profile using Operon RAPD primer H9 sensitivity and complexity. **Figure 24A:** Amplicons resolved in an agarose gel and visualised by ethidium bromide staining. **Figure 24B:** Amplicons resolved in an acrylamide gel and visualised by silver staining.

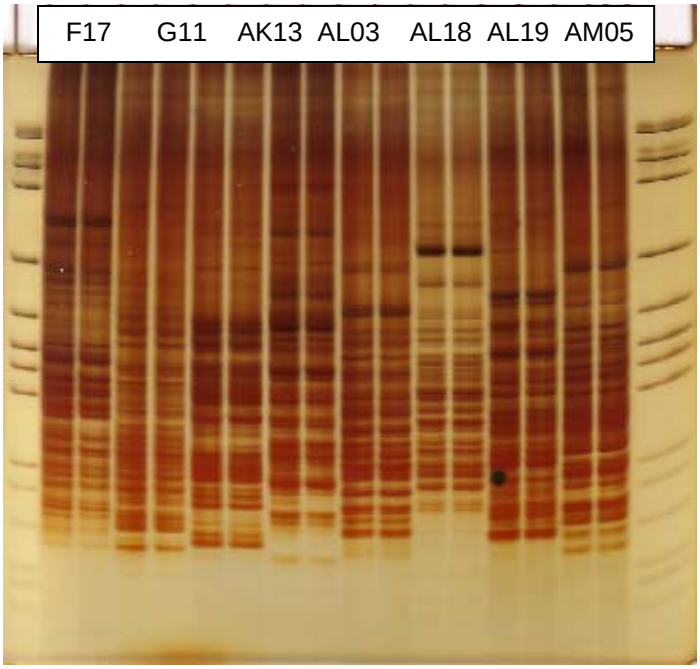


**Figure 25:** Primary product profiles derived from RAPD amplifications using Operon primers from bulk male and female DNA samples. Samples are loaded in pairs in relation to the RAPD primer and alternate male and female respectively. Of the amplification profiles analysed on this gel AM09 was selected for further analysis. RAPD amplicons were resolved on acrylamide gels and visualised by silver staining.

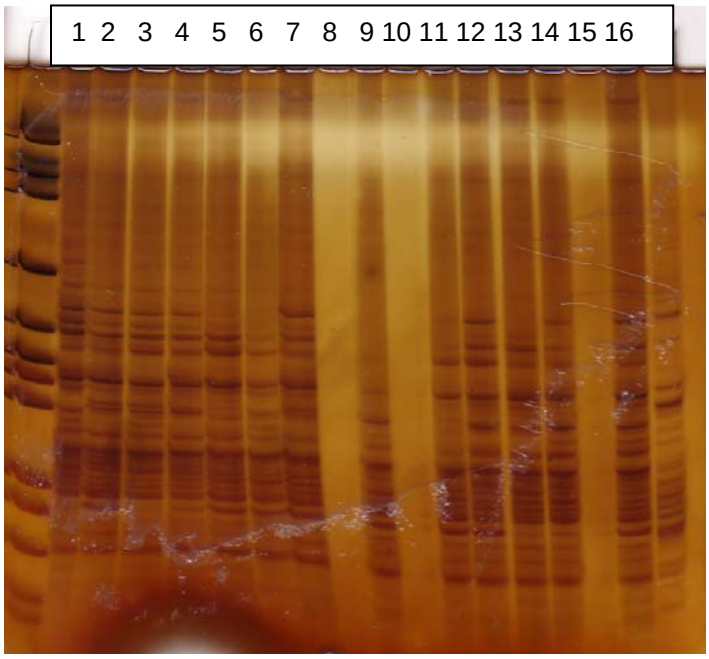


**Figure 26:** Amplification profile of Operon primer AM09 on DNA from eight individual male and female prawns. Samples are loaded alternating male female across the gel. Lanes 1 to 8 Male DNA samples; Lanes 9 to 16 Female DNA samples. Lack of amplification in lanes 8, 10 and 15 are likely due to failure of the reaction and not due to actual genetic differences within the genomes of these individuals. There were no clearly distinguishable sex specific amplicons identified.

### 6.1.4 Subtractive Hybridisation of F2 generation prawns

To gain further genetic resolution, we applied the subtractive hybridisation protocol (Lisitsyn *et al*. 1993) to the F2 *Penaeus japonicus* siblings. We used this technically complex approach for the DNA from 120 male individuals and the DNA from 120 female individuals. These unprecedented, large sample sizes from the closely related F2 individuals succeeded in yielding the sex specific markers we were searching for. We isolated 18 differential bands and have screened nine of them across Southern blots. From these nine bands we have located 3 genetic markers for the male sex and one genetic marker for the female sex. Southern blots provide the most rigorous test for sexual specificity of the primers.

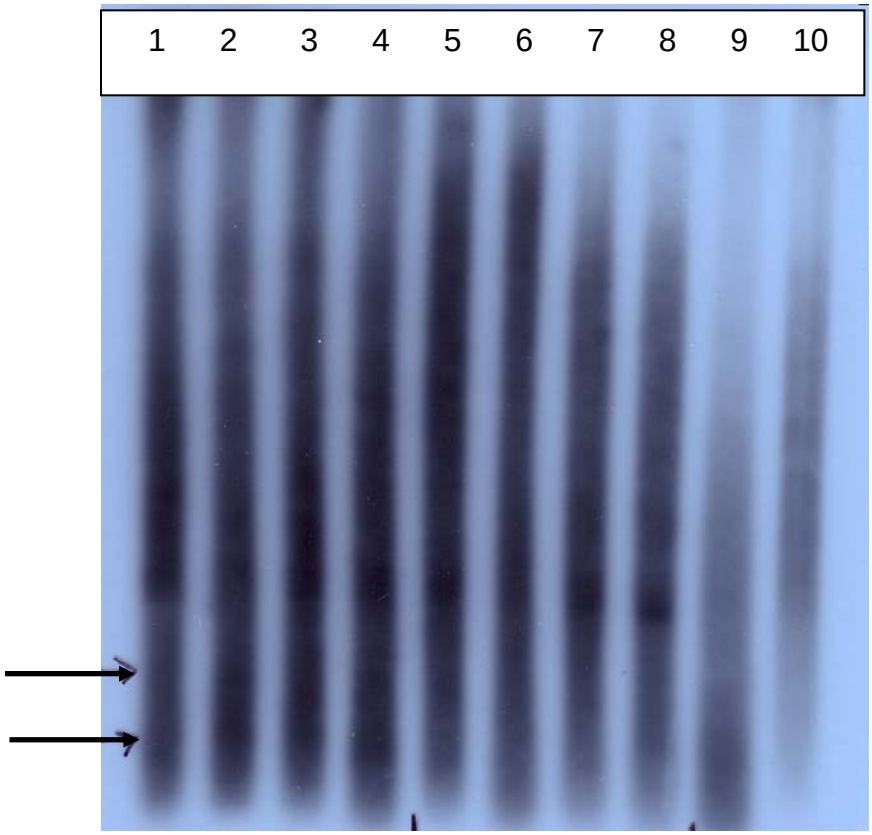


**Figure 27:** Southern blotting analysis of a male specific genetic marker, Probe i12F, identified using subtractive hybridisation on *Penaeus japonicus* F2 generation prawns. Arrows indicate probe hybridising bands that are specific to *P. japonicus* Male DNA. All genomic DNAs were digested with *Sau*3A prior to electrophoresis and blotting. Lane 1 to 4: *P. japonicus* Male DNA; Lane 5 to 8 Female DNA; Lane 9 *P. monodon* Male DNA; Lane 10 *P. monodon* female DNA.

Two of the male specific markers were identified with the subtractive hybridisation fragment, Probe i12F. As can be seen in Figure 27 Probe i12F, hybridised specifically to two bands in DNA from *P. japonicus* males. Interestingly Probe i12F did not appear to hybridise at all with DNA from either male or female *P. monodon* prawns (Figure 27). While it would be necessary to validate this finding across a larger number of samples it does indicate that these markers are not transferable between *Penaeus* species. It would appear reasonable to assume that the gene or genes controlling involved in sex-determination would be similar, if not identical, within the

genus. Thus Probe i12F may not be tightly linked to the sex-determining locus of *P. japonicus*.

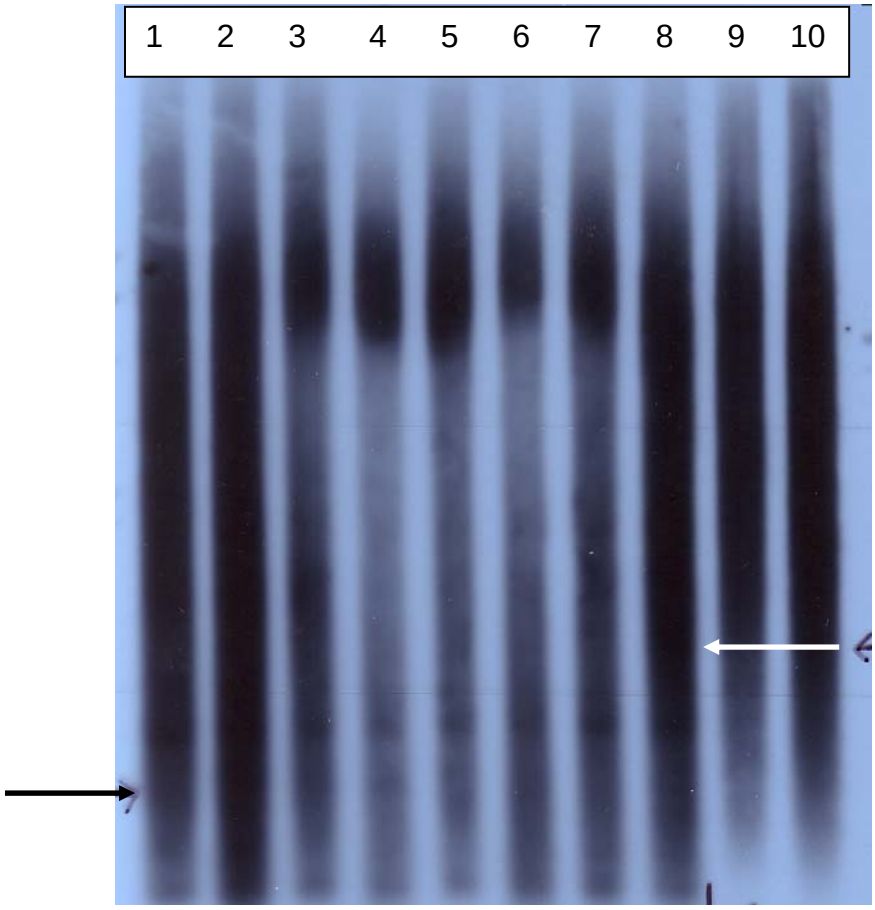


**Figure 28:** Southern blotting analysis the genetic marker, Probe i12FB, identified using subtractive hybridisation on *Penaeus japonicus* F2 generation prawns. Probe i12FB appears to specifically hybridise in a sex specific pattern for each sex: male (black arrow) and female (white arrow),. All genomic DNAs were digested with *Msp*I prior to electrophoresis and blotting. Lane 1 to 4: *P. japonicus* Male DNA; Lane 5 to 8 Female DNA; Lane 9 *P. monodon* Male DNA; Lane 10 *P. monodon* female DNA.

The third male specific marker and the female specific marker were identified with the subtractive hybridisation fragment, Probe i12FN. As can be seen in Figure 28 Probe i12FN, hybridised specifically a *Msp*I digestion product in DNA from *P. japonicus* males. Interestingly Probe i12FN also appears to hybridise to a *Msp*I digestion product in DNA from *P. japonicus* females, though the band is distinctly different to the male product. Unlike Probe i12F, Probe i12FN appear to hybridise to DNA from male and female *P. monodon* prawns (Figure 28). Though in the case of *P. monodon* no specific bands were obvious.

Screening of male and female prawns from both species with genomic DNA digested with multiple restriction enzymes would aid in clarifying the sex-specific nature of both of these probes.

The significance of one marker giving specific and differential identification of each sex requires further investigation to fully explain this finding.

### 6.1.5 Additional Results

We have also screened 353 prawns to search for individuals whose physical sex appears aberrant from normal male and female structures. Dr. David Hewitt located one individual that possessed male morphological features, the gonopores and pentasma of a normal adult male, but the internal dissection yielded mature ovaries. This discovery is the first case of aberrant sexual morphology noted in penaeid prawns and will be studied in detail now that we have confirmed sex-linked probes.

We also prepared slides for *in situ* analysis of chromosome spreads of male and female prawns and started to experiment with PRINS techniques (Gosden and Lawson 1994) for *in situ* hybridisation. When the tissue was dissected directly from prawns the slides were limited by too few dividing cells to observe clear chromosome spreads. This lead us to develop cell culture techniques to try to stimulate and synchronise cell division in sufficient numbers to obtain clear chromosome spreads. Although dividing cells were obtained in these preparations, cell synchronisation and staining difficulties still limited clear chromosome preparations to low numbers.

## 6.2 Advances in prawn Cell Culture

In the hematopoietic/lymphoid tissue cultures, the first week of culture showed several different cell morphologies (Figure 29). Spindle-shaped cells usually started to show after 12 hours but did not occur in every culture. Rounded cells that ranged in size from 10 to 15 μm occurred in all cultures and divided more rapidly. When confluent and the cells were passaged by scraping, only the round cells continued to proliferate. After passaging, the cells continued their growth in suspension but did not form adherent monolayers.

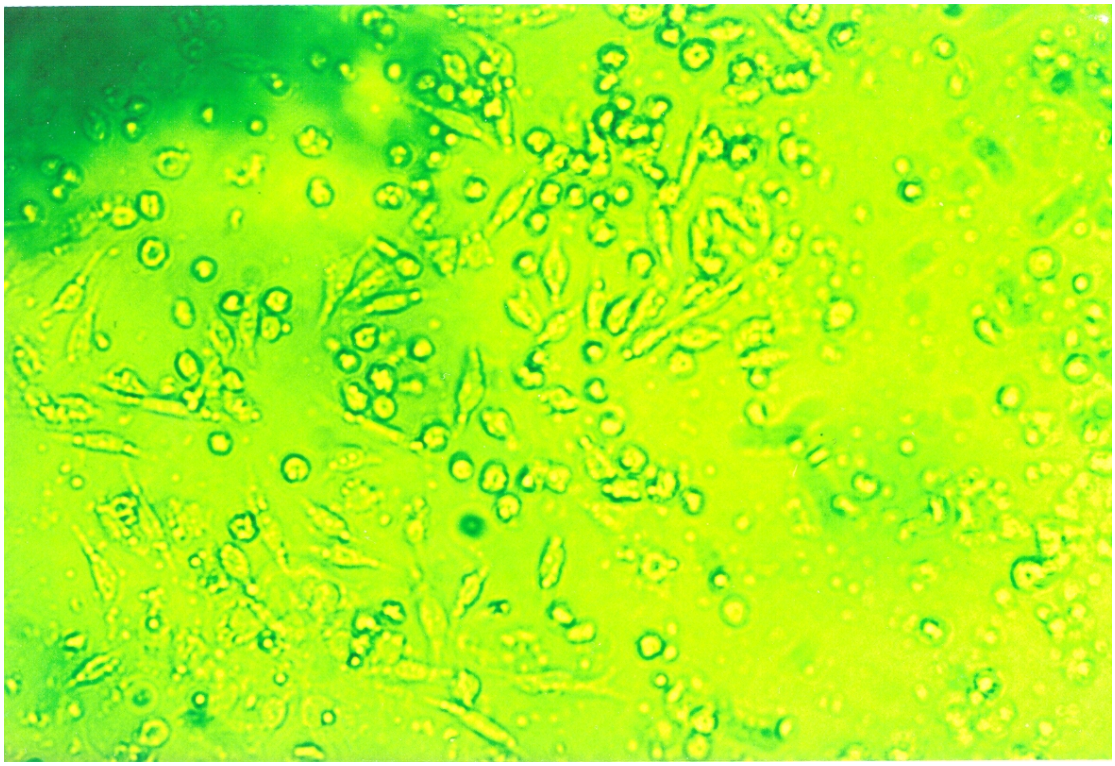

**Figure 29:** Cell morphologies from a 22-hour culture of hematopoietic tissues mixed with the paired lymphoid organs from *Penaeus monodon.* Photo taken at 420 X magnification.

In the four cultures of combined hematopoietic and lymphoid cells, there was considerable variability in growth rates between cultures (Figure 30). In two of the cultures, cell division rates dropped at the second week then increased in the 3rd week. In every culture cell division rates were strongest in the first few weeks and by the fifth and sixth week growth rate declined to low levels. These cultures continued to live for five or more months but the cells divided at very low rates.

Initial attempts to establish cell cultures utilised precondition Sf900 II media from insect cell cultures at a ratio of 1:1 with fresh media. Precondition media was utilised to determine the effects of any growth factors or promotants secreted by the insect cells on the viability of the prawn cells. Later experiments indicated that preconditioning of the media by the insect cells had no effect on the establishment of the prawn cell cultures. As a result all cultures were subsequently established in fresh media.

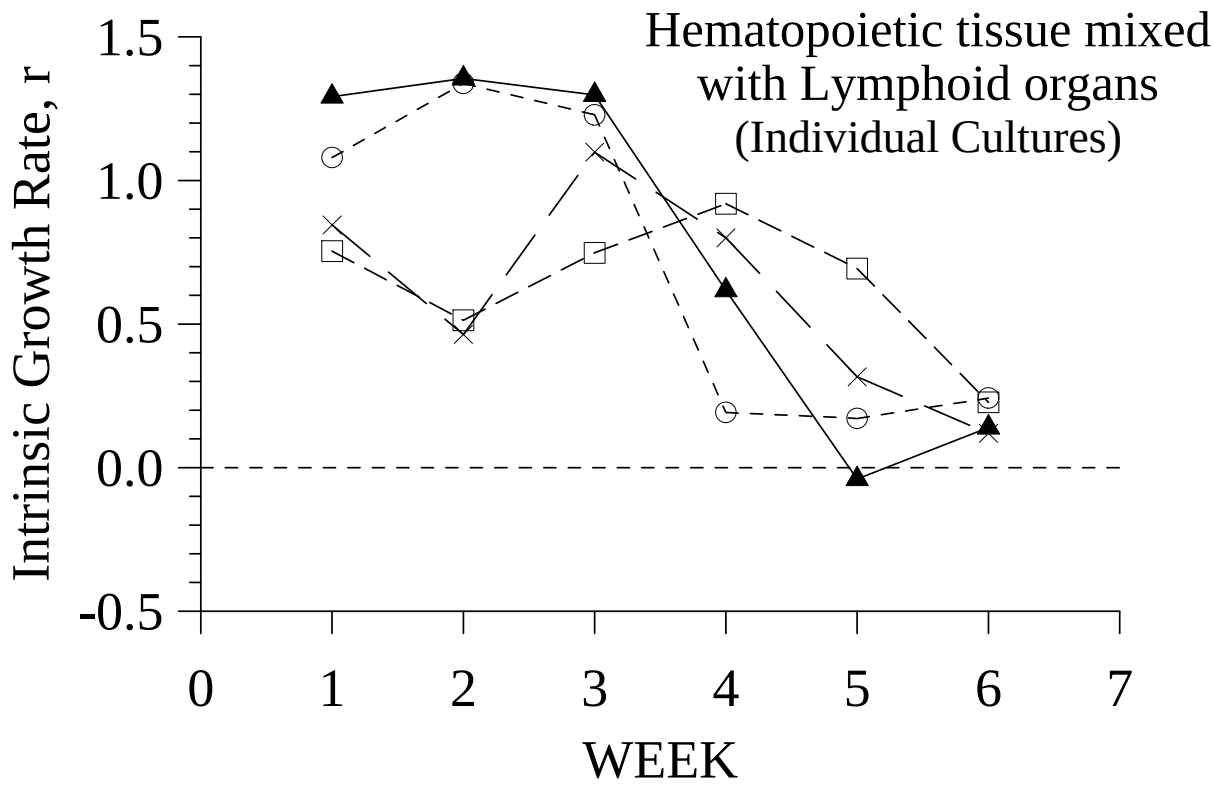


**Figure 30:** Growth rates plotted against time in weeks for 4 individual cultures of hematopoietic tissues mixed with paired lymphoid organs from *Penaeus monodon*.

In the ovarian cultures (Figure 31) the larger oocytes were diluted out with time but the smaller cells, 10 to 15 μm in size proliferated and grew in suspension after passage. Once again there was considerable variability in growth rates between individual cultures. Figure 32 shows the mean and standard error of the cultures combined by cell type. The ovarian cell division rates dropped in the third week, earlier than the hematopoietic/lymphoid cells and then had a slight rise toward the 6th week. These cells continued to survive for 5 months but at a very slow division rate.

Although these cultures all declined in 5-6 weeks, the strong growth rates early in the life of the cultures make them suitable for future testing of genetic modification of sex determination and virological experiments.

The development of viable cell cultures for prawn cells is highly significant. These methods and results were subsequently published in a peer reviewed journal (See Appendix 4).

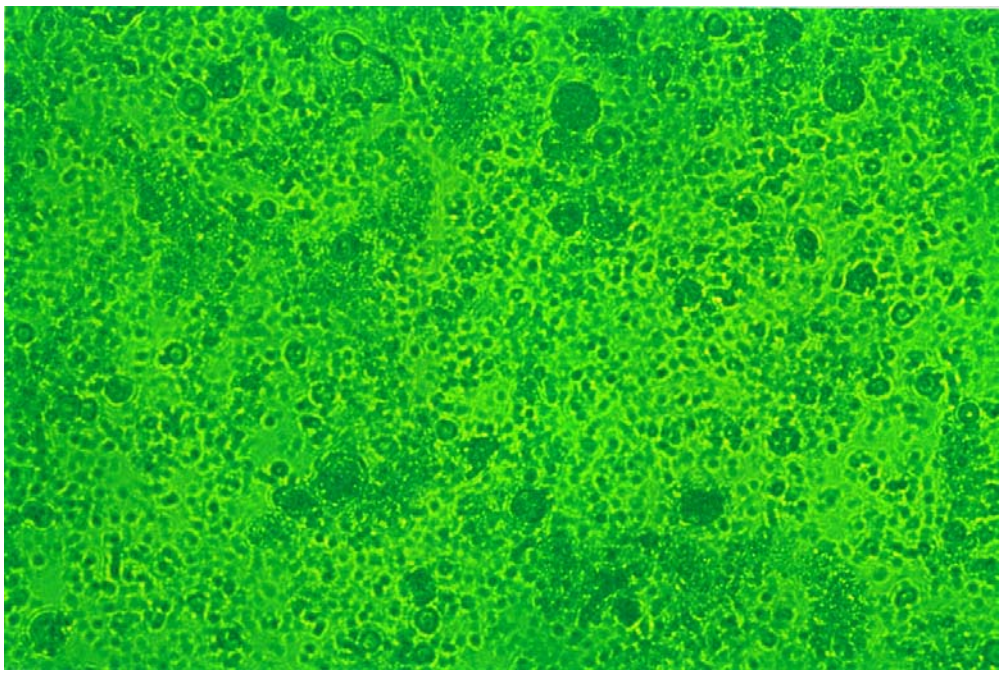

**Figure 30:** Cell morphologies from a 6-day culture of *Penaeus monodon* ovarian cells. The larger cells are oocytes but the smaller cells are the ones that proliferate in culture. Photo taken at 210 X magnification.

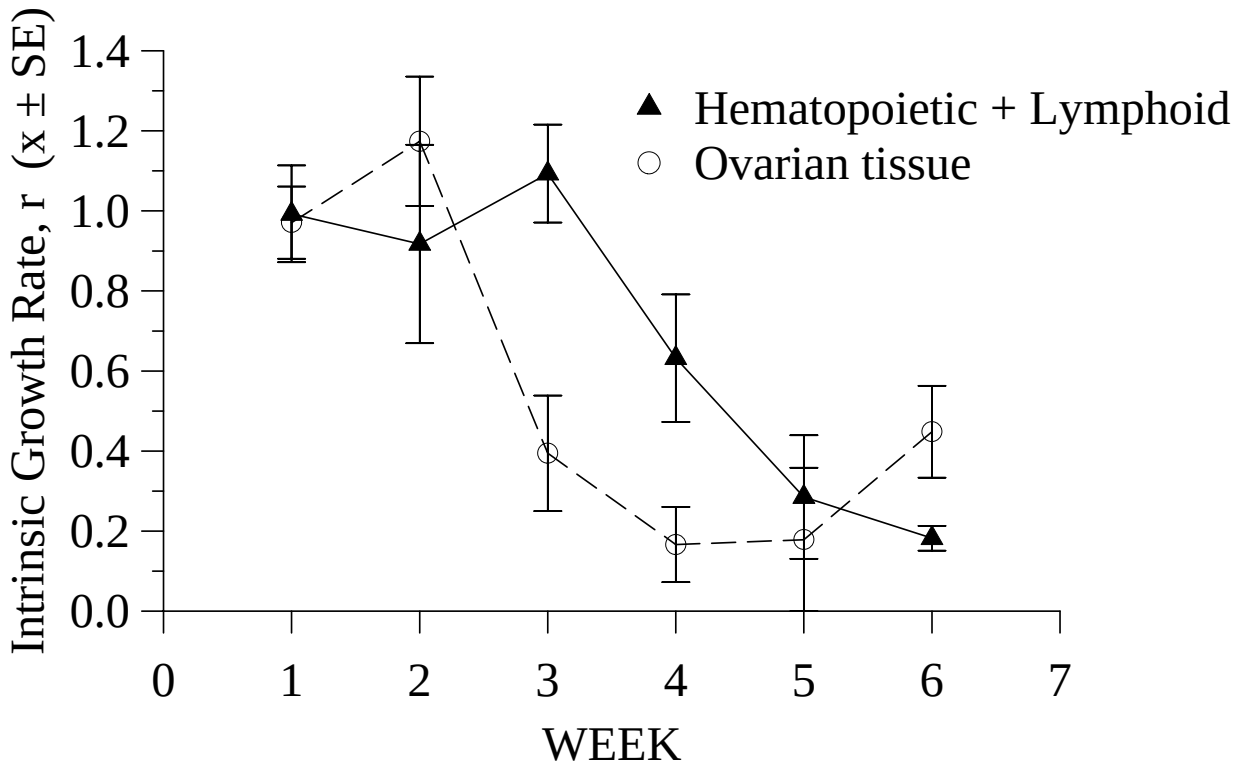


**Figure 31:** Mean and standard error values for growth rates, plotted against time in weeks, with *Penaeus monodon* cultures pooled by cell types.

## 7. Benefits and Adoption

This project benefits penaeid prawn aquaculture business in Australia, through increased knowledge of the genetics of two commercial species, *Penaeus monodon* and *P. japonicus*. Further sequence analysis of the sex specific markers will allow them to be used to target and direct genes responsible for sex determination in larvae. In addition to economic efficiency, control of sex determination could potentially create an international market for genetically superior larval stock.

Female *Penaeus monodon* are 30% larger than males at harvest. This is based on a study by Shane Hansford where males at six months returned a mean weight of 25g (n= 84) compared with a female mean weight of 33g (n=98). That work further suggested that the higher growth rate of females was due in part to higher feed conversion efficiency, but more detailed studies are required to confirm these observations.

When growers are given the technology to ensure that all prawns grown are female they will significantly increase aquaculture production through earlier turnoff with the greatest advantage being savings in feed costs. The total value of *P. monodon* produced by aquaculturists in Queensland in 1992/93 was $10.1m at an average price of $12.24/kg. The industry used 1,723 tonnes of feed at approx. $1,350/tonne, for a total feed cost of approx. $2.33m. Hansford's data indicate that females reach average market size (30g) approx. one month earlier than males, leading to a direct savings of almost $1m on present figures for the Queensland industry alone. This would be enhanced if females do indeed have a higher feed conversion rate.

Many growers prefer to use wild-caught females rather than laboratory raised parents because they believe the larvae produced are more robust. Yet when monocultures of female larvae are routinely available from laboratory-reared parents they are likely to out-perform the mixed-sex larval stock from wild-caught parents. In addition to benefits for the growers, this would benefit Australia's management of natural resources by relieving some of the fishing pressure on spawning females in the wild - 2,700 spawners were caught from the wild in 1992/1993 to supply Queensland hatcheries.

It should be pointed out that technology for manipulating the sex ratio of *Penaeus monodon* and *P. japonicus* will, almost certainly, be transferable to other prawn species and could well be applicable to all decapod crustaceans.

In addition to those benefits mentioned for sex determination, the cell culture findings will aid prawn transgenic research and disease prevention. In 1995 the prawn industry estimated $5 million economic loss from disease. Cell culture techniques developed fortuitously within this project provide a foundation for study of prawn viral replication *in vitro*, the key step to successful means of interrupting the infective cycle.

## 8. Further Development

The genetic markers we isolated for *Penaeus japonicus* need to be thoroughly tested against *P. monodon* to determine if they are specific to *P. japonicus* or can be used across other penaeid species. Since the markers we isolated are adjacent to or overlapping the sex determining genes, we are hopeful that some may be functional in *Penaeus monodon*. The genetic markers we isolated in the present study will continue to be characterised in our laboratory. These regions will be cloned and sequenced to yield further sequence data within the sex determining genes. These sequences are a powerful tool to manipulate sex-determining genes in prawn larvae.

## 9. Planned Outcomes

The knowledge and technologies gained from this project influence the methods future researchers will use to study the genetics and manage the sex ratios of commercially farmed crustacean species. We established a more detailed understanding of the genetic mechanisms responsible for sex determination in prawns and discovered a high degree of genetic variability between individuals of the same species. Sex determination in penaeid prawns is directed by a single genetic locus or a small number of loci, rather than fully differentiated sex chromosomes. We located 3 genetic markers for the male sex and one genetic marker for the female sex. Although the markers were more difficult to obtain than we originally predicted, finding fewer markers after the rigorous search has two advantages: 1) The positions of the genetic markers we isolated are closer to the actual sites of genetic sex determination. 2) Future manipulation of those genes for improved production will be less complex, with fewer genes. Our research expands knowledge of the genetic structure of prawn populations and will influence future farming practices and harvesting strategies for wild caught prawns. Further, laboratory cell culture techniques developed within this research will enable improved methods of molecular visualization of crustacean genes.

The cell culture techniques developed in this research are now used by researchers and their students working on diseases within crustacean species. Experiments designed to test the most effective techniques for preventing bacterial and viral diseases in commercial prawns may be carried out far more quickly growing prawn cells in controlled laboratory circumstances, infecting them with single or multiple disease agents and then trialing potential disease control methods.

## 10. Conclusions

We have successfully isolated 3 genetic markers for the male sex and one genetic marker for the female sex in *Penaeus japonicus*. We accomplished our goal to establish the genetic mechanism of sex determination in penaeid prawns and to isolate genetic markers that will aid in the manipulation of sex ratios for mono-sex production.

Our findings indicate that sex determination in prawns has a genetic basis in that animals of one sex contain DNA sequence that is not present or is at low copy number in the other sex. Sex determination in penaeid prawns is directed by a single genetic locus or a small number of loci, rather than fully differentiated sex chromosomes. Male / female differences within genomic sequences of prawn DNA are slight. In order to successfully isolate genetic markers for only one to several loci it was necessary to resort to the more technically difficult molecular methods of subtractive hybridisation. It was also necessary to minimise the normally high genetic variation of individuals by using large sample sizes of 120 prawns for each sex, obtained from an F2 generation. Although the additional techniques caused delays the markers we obtained are adjacent to or may overlap the sex determining genes. These results are especially desirable because once the markers can be sequenced we will have our first access to manipulating the male and female sex determining factors.

Both sex determination studies and the complimentary area of prawn cell culture have direct application to the economic development of the prawn industry and cell culture work has immediate relevance for testing genetic modification of sex determination and disease control of emerging prawn viruses.

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## Appendix 1: Intellectual Property

We succeeded in isolating 4 DNA fragments of *Penaeus japonicus* that can be used as genetic markers of sex. Three of these markers differentiate male DNA and one of the markers differentiates female DNA.

## Appendix 2: Staff

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## Appendix 3: Nucleotide sequences of subtractive hybridisation fragments

The sequences were isolated from *Peneaus monodon* subtractive hybridisation experiments. No identity was found to current sequence databases (February 2005).

### Fragment 7M (Male associated)

```
  1   GATCAATATC TTGTTTATTG TTAAAGTCTC TACAAATGTA CTCAGTGGCC
 51   CTTACAAGGG ACCGGGTCGA CATTGGAGTC CATGTAATGT TGTCACGTTA
101   AGCACGCCAC CGGTCTGACG GCAACTCGGA ATCTAGGCCC TGTCATCCCA
151   GCCACACACC ACGATGTCGG CGACTGGAGC CACAAACTCT GCCTTTACTC
201   GCCATTGGAA GCCAGGCATG CCAATATCTC CAGCTTGCTG CCACTCCGGA
251   TAGCAGTAGC CCTGCCACGG GCACCCCCCG CGATCCCGAC GCCTCGACAC
301   TCCGAATAAT CAACTGGGAC TCACGTCCTA CTTC
```

### Fragment 9M, (Male associated)

```
  1   GATCTGGAGG CGTCCAACAG GGTTCGCTAT GAACAGATTC TGGAGCGACT

 51   GGTACCTGGG AAAAGGTGCT GGTTGTCTGG ATGTACGGGG AGTCTGGAAG
101   GCAACGCCCG TCCACTACAC TGGGACGGCT TAACACCTTT CCTGACGAAA
151   ATCTCGGTCC GCGATC
```

### Fragment 11M, (Male associated)

```
  1   GATCCTCTCA GCTCCAAAGG GGGGGGGGGG CGCCACCGGC CATCCCTGAG
 51   GGAGAGAAGG AAGGAGGGAG AGAGAGATAA GGGAATTTCA AGGTGCTGAT
101   GAGCCACCTC GGGGGTCCCT GGATTTCGTG GAGATGAGGG AGGGGTAGAG
151   ACCCGGTCAA CACGTTGGTA CGAGTATCCG GCCCGCGCTG TTCTGACTCT
201   TCCATACCAA ATTAGCCCTT CCCTACAAAA AATAATACCA TTTATTTCTC
251   TCGCTTTCCA ATATCAATGA TCCACCACAC AAATTACATC GTCCTCGTTT
301   AGCCCTGAAG AAAAGACTGG CCCGCCGATT ATGCC
```

### Fragment 14M, (Male associated)

```
  1   GATCGACGGT TCCTCAAGGA CAGGGAGAGA GTCCTGCGAG CAGGACACAG
 51   GACGGAAGTC CTGGGAATCG GTGGACCAAT TACTAATACT TCCGCGCTGT
101   CCCATGTGCT CCCTCTCCAG AGGGAGAGGG AGCTCTCCCT TGGCTTTGGG
151   AATGATC
```

## Fragment 15M, (Male associated)

```
  1   GATCGACGGG GAGTGCGGGA GTGAGCGCAC GGGTGAGTGC GCGAGTGAGC
 51   GCACGGGCGA GCGAGTGAGC GCACGGGTGA GCGAGTGAAC GAGTGCCAGC
101   CCGAATGACA GGGAAGGATC
```

## Fragment 24F, (Female associated)

```
  1   AAAATAGTGT TTGGTTATGA TGGTCATAGT TTGTTTTCCT GTGTGAAATT
 51   GCTATCCCTT CATATATCCC ACACACACAT ACGAGCCGGA AGTATAACTG
101   TGTAACGTCT GGGGTGCCTA GTGAGTGAGC TAGCTCACAT TA
```

## Fragment AM, (Male associated)

```
  1   GATCTNGCCC NCCCCATTCN TTGAATTGGC GGGAAAATGT GTTTCTTCCT
 51   TTTAATACAA TTGATATCAA TACTGTTATT ATTGTTATTG ATGTTATGAT
101   TATTACAGTA TCATTAAAAT CTCGTTAACA TCTAAGACAA TGCAACTAGT
151   GGCAAAAGAT C
```

## Fragment HM, (Male associated)

```
  1   GGATCACCCC TCCTAATGAA TGATGCAATC ATAGTCCACC CCAATGTCAG
 51   GTGCCACTGA TGAACCACCT CCCTCATCAC CCCTCCTCCC CTCCACACCC
101   GTCCCCACAA TATTCTTCGT GTTGGAACTT GTACCCGGAT C
```

## Fragment UM, (Male associated)

```
  1   GGATCACCCC TCCTAATGAA TGATGCAATC ATAGTCCACC CCAATGTCAG
 51   GTGCCACTGA TGAACCACCT CCCTCATCAC CCCTCCTCCC CTCCACACCC
101   GTCCCCACAA TATTCTTCGT GTTGGAACTT GTACCCGGAT C
```

## Appendix 4: Scientific publications arising from this work:

West, L., Mahony, T., McCarthy, F., Watanabe, J., Hewitt, D. and Hansford, S. (1999) Primary cell cultures isolated from *Penaeus monodon* prawns. Methods in Cell Science **21**: 219-223. (**Copy attached**)