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ABSTRACT

The advantages and disadvantages of the commonly used techniques for the study of biodiversity and germplasm of organisms are compared. Among all these advanced techniques available, we have established the technique for Random Amplified Polymorphic DNA (RAPD) markers and optimised the conditions for the study of tropical ornamental fish using zebra fish as a model. Optimization was done with the long and short primers which require different conditions. Some of the primers were shown to be very useful as species markers whereas others were selected as useful markers for DNA fingerprinting. After the biodiversity of the organism is established, DNA microinjection technique will be applied to inject foreign DNA into fertilised eggs. Several genes, including the CAT bacterial genes and some other fish genes will be cloned in different plasmid vectors and injected into the embryos. Expression of these transgenic genes at adult stage will be studied.

INTRODUCTION

The study of fish genetics provides information for the inheritance of desirable ornamental traits (eg. colour). It can also facilitate selective breeding in order to obtain specific or new strains and to reduce the deterioration of the fish genetic stock caused by inbreeding. The techniques used in genetic studies include the molecular techniques based on DNA profiling. The principle of individual uniqueness forms the basis for DNA fingerprinting (Kirby, 1992). The study of population genetics is to study the genetic structure of populations and the principles by which the genetic structure changes. Genetic structure refers to the types and frequencies of genes or genotypes present in a population (Nei, 1979).

The data obtained from DNA fingerprinting can be used to analyze the various phenotypic traits at the molecular level. This information is important in agriculture where there is a need to produce a particular selected genotype. Identifying molecular markers linked to these desirable traits in the parents will increase the chance of obtaining progeny with the desirable phenotypic traits and increasing the specificity and reliability of breeding programmes.

The aims of this study are to optimise the newly developed DNA RAPD technique for the genetic study of the tropical ornamental fish *Brachydanio*. The level of genetic variation and the pattern of inheritance of RAPD DNA profiling from F1 population was verified. An attempt will be made to obtain markers to some desirable ornamental phenotypic traits like length of the fins and tails or colour pigmentation. The facility of DNA microinjection has been set up for the study of micro-injecting plasmid DNA containing foreign genes. Several genes, including the CAT bacterial genes and some other fish genes have been cloned in different plasmid vectors. Our long term goal will be the attempts of producing transgenic tropical fish with special ornamental values.

Methods Used For The Study of Biodiversity

Before the birth of molecular biology, classical breeding programmes relied on the selection of superior individuals among the segregating progenies. The selection was based on the physical appearances which may sometimes be influenced by the environmental conditions. This is especially true in tropical ornamental fish whose colour pigmentation can be affected by the food source(Purdom, 1993). These classical phenotype-based selections are usually restricted as there are many characteristics that are controlled by multigenic traits.

Isoenzyme Analysis

Individual proteins could be used as molecular markers as proteins are the direct products of individual genes. Changes in gene sequence can be reflected in the change of protein structure. Protein markers can be monitored for genetic differences in an easily assayed enzyme system. The different molecular forms of enzymes, known as isozymes, change the rate of migration through a gel matrix in response to an electrical field. Therefore, isozyme studies seem to be the answer to the classical breeder's problems which initially has to rely on limited phenotype based experiments. However, the application of isozyme markers are limited by the number of enzymes that can be assayed (Gan *et al.*,1977). There are usually fewer than fifteen good enzyme systems found to be polymorphic in any one species and optimization of each buffer system must be done for each enzyme (Gan *et al.*, 1984). The region of the genome covered via isozyme studies are limited to only functional genes, but not the structural genes. The non-coding regions of the genome and any DNA changes in the wobble positions of the genetic codons that do not lead to amino acid change will not be detected by using isozyme analysis. This limitation results in the under estimation of genetic variation in organisms. Furthermore there are some enzymes which are confined to certain tissue types and are developmentally regulated. (Michelmore and Hulbert, 1987). Therefore , emphasis has been shifted towards other molecular techniques.

Restriction Fragment Length Polymorphisms (RFLP)

RFLP has been used successfully in the identification of a wide variety of DNA markers for gene mapping or the linkage studies among human (Schurr *et al.*,1990), plants and animals (Beckmann, 1991). RFLP utilizes DNA hybridization technology whereby radioactive labelled DNA probes identify differences in the size of specific DNA fragments following digestion by a restriction endonuclease (Beckmann and Soller, 1986).

Variable Number Tandem Repeats (VNTR)

Another technique which is also based on DNA hybridization is termed variable number tandem repeats (VNTR). This technique detects the repeated DNA sequences of different length resulting from variable number of serial repeats of core sequences within the genome (Jeffery *et al.*,1985; Nakamura *et al.*, 1987).

Polymerase Chain Reaction (PCR)

PCR is used to amplify DNA which is then sequenced (Newbury and Lloyd, 1993). This technique reveals variation at a very high level of resolution, differences are measured up to differences at the single base pair level where a single base pair change can be detected through temperature gradient gel electrophoresis (TGGE).

The Limitations

The disadvantages of RFLP and VNTR based techniques are the requirement for large amount of DNA samples. This can be a major problem when samples are scarce and precious. The PCR amplification techniques require information of the gene sequence in order to design the appropriate primers. This can be difficult at times when one is investigating a sample with little or no prior genetic information.

Random Amplified Polymorphic DNA (RAPD)

A PCR based technique using random primers to generate novel polymorphisms have been reported and named as Random Amplified Polymorphic DNA (RAPD) by Williams *et al.* (1990). This technique was also coined as Arbitrarily Primed PCR (AP-PCR) by Welsh and McClelland (1990) and DNA Amplification Fingerprinting (DAF) by Caetano-Anolles *et al.*(1991). DAF utilizes primers of 5 to 8 base pair (bp) long, RAPD uses primers of 10 bp and AP-PCR uses longer primers of more than 18 bp.

The RAPD assay is based on the principal that a single short oligonucleotide of randomly chosen DNA sequence, when mixed with genomic DNA and a thermostable DNA polymerase and then subjected to temperature cycling conditions resembling those of the PCR will prime the amplification of several DNA fragments. The DNA products are then separated on agarose gel electrophoresis and visualised with ethidium bromide staining. The mechanism of RAPD utilizes the fact that at a low annealing temperature, primers can be expected to anneal to many sequences with a variety of mismatch. Some of the mismatches would be within a few hundred base pairs of each other and on the opposite strands. Hence, RAPD detects for the presence of these inverted repeat sequences within the genome which is within the 400 bp amplifiable limit of PCR.

In PCR theory, the number of amplified products depended on the length of primers, size of the target genome and the probability that a given DNA sequence which is complementary to the primer sequence would occur in the genome on the opposite DNA strands, in opposite orientation which is within the limits of PCR amplification (Innis and Gelfand, 1990). Experiments have shown that the number of bands generated is independent of genome complexity over a wide size range (william *et al.*,1990). The final products of the reaction seem to be determined by competition among substrates, rather than the total number of potentially available sites. Primers differing by a single nucleotide can give rise to a whole new profile of amplified bands, as the change in sequence may or may not affect the affinity of the previous primer-template duplex, resulting in the disappearance or appearance of new bands.

Application of RAPD

Since 1991, RAPD has been applied to a wide range of applications, from population studies, construction of genetic linkage maps, DNA fingerprinting to the study of genetic diversity. RAPD has been used in the identification of strain variations in the fungi isolates *Aspergillus fumigatus* (Brown *et al.*,1992) and the *Leptosphaeria maculans*(Goodwin and Lanis, 1991), in the construction of genetic maps in the plant *Arabidopsis thaliana*(Reiter *et al.*, 1992) and the conifer *Apicea glauca* (Tulsieram *et al.*, 1992). RAPD markers have also been used to identify a nematode resistance gene (Lankhorst *et al.*, 1991) and a *Pseudomonas* resistant gene (Martin *et al.*,1991).

Advantages and Disadvantages of RAPD

RAPD has the advantages of being technically simple, quick to perform, requiring only small amounts of DNA (about 1000 times less than RFLP) from a crude DNA preparation and involves no radioactive materials (Waugh and Powell, 1992). RAPD also has another major advantage that it requires little or no knowledge of the biochemistry or molecular biology of the species.

The information derived from RAPD at the nuclear genomic level has the advantage over RFLP genetic diversity studies, as a larger proportion of the genome can be analyzed. In population studies, RAPD analysis can add a new dimension to the available data currently based on organelle genome and isozyme (Tulsieram *et al.*, 1992).

The draw back of RAPD technique is that the DNA polymorphisms revealed segregate as dominant markers. It does not readily differentiate between heterozygotes (one copy) and dominant homozygous (two copies of an amplifiable region in diploid material).

MATERIALS AND METHODS

The primer sequences used is listed in Table 1. RAPD reactions were done in a 10 μl reaction volume overlaid with mineral oil. The 5X reaction mix contained 67mM Tris HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 200 μg/ml gelatine, 4 Mm MgCl2, 200 μM dNTPs. The final concentrations in each RAPD reaction was set at 1X reaction mix, 1μM primer, 50ng DNA template and 0.4 U of Taq polymerase (Biotech International, Australia) and 4 Mm Magnesium. Autoclaved milli-Q water was then added to a final volume of 10μl. The temperature profile for the long primers (> 15 bp) consists of 4 cycles at 94°C for 60 s, 40°C for 60 s and 72°C for 120 s, followed by 50 cycles at 94°C for 60 s, 60°C for 60 s and 72°C for 120 s. An extra 10 min elongation step was done to ensure complete elongation of partially amplified products. The temperature profile for short primers consists of 50 cycles at 94°C for 30 s, 36°C for 60 s and 72°C for 60 s. An extra 10 min elongation step was also done to ensure complete elongation of partially amplified products. The reactions were all performed in a Perkin Elmer DNA thermal cycler.

Amplified products were resolved by electrophoresis at 8v/cm on a 1.8% TBE (0.089M Tris, 0.089M Boric acid and 0.002M EDTA) agarose gel. The gel was stained with Ethidium bromide and the products were visualised and photographed under UV.

RESULTS AND DISCUSSION

Inter and Intra Species Comparison using RAPD

RAPD was used to see if this technique could possibly differentiate between three species of zebrafish(*B. rerio,B. frankie and B. alboliatus)*. Seven of the eleven primers were selected since they gave clear DNA amplified profiles. The selected primers were five long primers ML5, ML6, R1, X2, 58SR and two short primers AB3 and R28. Seven randomly selected fish DNA templates were chosen from each *Brachydanio* species. The intensity of bands were not given different weightage during scoring. The bands generated were sharp and the level of non specific background were at a minimum.

Fig. 1 and Fig. 2 represent the RAPD DNA profiles amplified by using the primers ML5 and R28. Results showed that each primer was able to generate at least one major RAPD band which was unique to the *B. albolineatus* species. The intensity of these unique bands were strong and the bands were consistently present in all the DNA templates of *B. albolineatus*. However, there was no unique band generated by any of the seven primers which could easily differentiate

between the species of *B. rerio* and *B. frankie*. Both species shared the major bands and this result was consistent with all the seven primers.

The RAPD DNA profiles for each primer were analyzed. Table 2 represents the DNA patterns when primer AB3 was used. In general, each primer detects on average 13 loci in the zebrafish genome and at least 2 major RAPD bands. There are on the average 1.5 shared bands per primer between the three species and 4 unique bands per primer for each species.

Tables 3-9 represent the values of index of similarity between the three *Brachydanio* species for the seven primers analyzed. The within species similarity index values for *B. frankie, B. rerio* and *B. albolineatus* respectively are 0.84, 0.84 and 0.92. The value of similarity index if equal to one means complete similarity. The lower the value the more difference they are.

Results showed that there is a close relationship between the species of *B. frankie* and *B. rerio* with similarity index values of 0.71 to 0.90 for all the seven primers analyzed. These values are about the same as the within species values calculated. However, the similarity coefficient indicates that species *B. albolineatus* is least similar to either *B. frankie* or *B. rerio* species with values ranging from 0.2 to 0.4 for the seven primers analyzed.

CONCLUSION

The parameters used for the analysis of RAPD for zebra fish were established. The DNA profiles generated was easy to score, had a low background and the bands are sharp, intense and repeatable. RAPD DNA profile showed that the species *B. frankie* and *B. rerio* were closely related to one another, the index of similarity between these two species was quite similar to the value obtained from within species in all primers assayed. These results suggested that most probably *B. frankie* and *B. rerio* are in fact the same species or subspecies. This hypothesis was further confirmed by the breeding experiments when these two species were mated, fertile offsprings were produced. The third species, *B. albolineatus*, is genetically less related to both the *B. frankie* and *B. rerio*. The inheritance pattern of the RAPD bands in a F1 population was segregating in a 3:1 ratio.

Table 1. Primer sequences

R4	5'TCCTACGCAC	10bp
R28	5'ATGGATCCGC3'	10bp
AB2	5'CCGTGACTCA3'	10bp
AB3	5'GCCGCCACCA3'	10bp
AB6	5'GACATTACTG3'	10bp
LR7	5'TACTACCACCAAGATCT3'	17bp
58SR	5'TCGATGAAGAACGCAGG3'	17bp
R1	5'GTCCATTCAGTCGGTGCT3'	18bp
X2	5'GTTCACGGTGGTCTCCAT3'	18bp
ML6	5'CAGTAGAAGCTGCATAGGGTC3'	21bp
ML5	AAGGAAACTCGGCAAATTATCCTC3'	24bp

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Figure 1

Screening of three *Brachydanio* species with the primer R28. Lanes 1-7 were DNA templates from the *B. frankie* species, lanes 8-14 were DNA templates from the *B. rerio* species, lanes 15-21 were DNA templates from the *B. albolineatus* species. The three species were selected randomly from fish shops and farms in Singapore.

Figure 2

Screening of three *Brachydanio* species with the primer ML5. Lanes 1-7 were DNA templates from the *B. frankie* species, lanes 8-14 were DNA templates from the *B. rerio* species, lanes 15-21 were DNA templates from the *B. albolineatus* species. The three species were select4ed randomly from fish shops and farms in Singapore.