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DNA-level Polymorphisms as Tools in Tilapia Genetics

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Various methods for the detection and analysis of genetic polymorphisms at the DNA-level have been developed. Notable polymorphisms may be found in the mitochondrial DNA and the minisatellites and microsatellites of genomic DNA. Such polymorphisms are exceedingly numerous and may provide a promising basis for a number of scientific as well as practical applications in the genetic studies in Tilapia. An expanded number of genetic markers would increase statistical insights on marker-based population studies which are highly relevant in the assessment and improvement of commercially important stocks of Tilapia. In addition, utilization of these genetic markers could contribute to the rapid elaboration of piscine genomic maps and to the development of markers for health and production-related traits of fish.

Key words: Tilapia genetics, *Oreochromis*, DNA polymorphisms, fisheries management, DNA-markers, microsatellites

Tilapia has become increasingly important in aquaculture in tropical and subtropical countries, such as the Philippines, Taiwan, Israel and Sub-Saharan Africa. It is much appreciated by consumers, being a good and affordable source of protein. Low input and high returns for rearing Tilapia extends the favor to fish farmers as well. There are 20 or more commercially important species, and of these, four are found in the Philippines that contribute significantly to annual fish production in the country. Initially distributed in a neighboring country (Singapore) as an aquarium fish, *Oreochromis mossambicus* was the first to enter the Philippines waters. Because of its unattractive appearance and slow growth rate, the popularity of this species soon declined when *Oreochromis nilotucus* from Israel was introduced. This second species has better physical attributes not only in terms of appearance but in growth as well. *Oreochromis aureus* is a third species, which gained wide acceptance because it produces monosex offspring when crossed with *O. niloticus* (Pullin & Capili 1988). Red Tilapia, a hybrid, is the last of the Philippine species, so called because of its red color in contrast to the grayish brown dark gray color of the other species (Galman et al. 1988).

Problems in fish management, however, still arise in Tilapia production because of its capacity to over breed. At a young age, these fish are able to reproduce, often leading to the overcrowding of fishponds, thus limiting the growth of individual fish. In an effort to address this problem, monosex populations have been produced, generally prepared after three methods. The first of these methods, involved the manual inspection and separation of sexes. A second procedure, which is less labor extensive, involves the use of hormones to induce sex reversal in fry-stage fish. The third method employs the discovery that hybridization of two different species would produce skewed sex ratios, some of which to nearly 100% male offspring. These and other efforts such as the production of superior strains in terms

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of fast growth, tolerance to adverse environmental conditions, resistance to fish diseases, etc. gave roots to a widespread interest in the study of Tilapia genetics (Pullin & Capili 1988).

Stock identification and assessment in Tilapia has been rapidly improved. Morphological description and morphometric analyses were the first tools used to define tilapiine species (Galman & Avtalion 1983, Pante et al. 1988). But these techniques are rather arbitrary, and biochemical means (i.e. electrophoresis of expressed isozymes) soon found a more reliable use in such studies (Macarañas et al. 1986, Galman et al. 1988). Biochemical investigations, however, are still limited in that most of the isozymes are affected by environmental and/or developmental conditions (Galman & Carino 1979). DNA-level investigations were thus developed for fish genetic studies. Such approach provides direct investigations of the genetic make-up of several fish species, thus eliminating the effects of extraneous factors. Furthermore, polymorphisms (variant forms) in the DNA are highly numerous as compared to that of isozymes. This equips DNA-level analysis with a lot of genetic markers from which accurate stock identification and assessment can be deduced. Some of these DNA markers are described in this paper. Each description of the genetic marker ends with a cited paper giving an example of the application of the use of this type of molecular analysis to tilapia research.

Markers at the DNA level

There are two main types of DNA found in the eukaryotic cell, each undertaking a disparate evolution. These are the genomic or nuclear DNA and the mitochondrial DNA (mtDNA); the former being that which is found in the nucleus and is mostly responsible for directing cellular processes, while the latter is exclusively found in the mitochondria and almost independently dictates the functions in this organelle. Markers in the DNA are observable as restriction fragment length polymorphisms (RFLPs). These are variations in the DNA sequence reflected in different lengths of DNA fragments obtained through endonuclease digestion of the DNA molecule. In mtDNA, the RFLP may be analyzed soon after its digestion with restriction enzymes (REs) at specified conditions. For genomic DNA, resultant fragment lengths, however, would be excessively numerous for practical analysis. Instead, portions/subgroups of the genomic DNA are either hybridized or analysed further. One particularly interesting group of genomic DNA is the *satellite* DNA, so called because it forms a "satellite" peak upon absorbance analysis of the DNA. Satellite DNA has no ascertained functions and is simply hypoythesized to take part in binding and stabilizing chromosomal proteins, a structural rather than genetic function. Owing to the lack of function, this DNA derivative is thought to experience low selection pressure thus resulting to high degrees of polymorphism (Turner et al. 1991). One other interesting characteristic is that it is mainly composed of repeated structures present in variable amount (termed as variable number tandem repeats or VNTRs), upon which certain analyses may be based.

There are a number of techniques in molecular biology that may be employed in analyzing DNA-level polymorphism. One of the most basic would be the use of enzymes (REs) isolated from bacteria that cleave the DNA at specific sites . For stock identification and/or assessment using mtDNA, this would usually suffice. Southern blotting, the transfer of electrophoresed DNA to a nitrocellulose film, may be done to produce more permanent records (vs. gels) of the fragment patterns. DNA hybridization may also be done to be able to observe or compare homologous DNA sequences. In this technique, a probe is hybridized to the fragment under a given stringency. Polymerase chain reaction (PCR), the amplification of DNA fragments using primers of known sequences, may be employed for those in which prior knowledge on the DNA length (i.e. the sequence) is available. In the case of Tilapia, wherein neither genomic nor mtDNA has been sequenced, arbitrary primers have been used instead, giving rise to the technique AP-PCR or arbitrarily primed-PCR (Harris et al. 1991). Here there is a random amplification of the DNA and the fragments obtained are sometimes referred to as RAPD or randomly amplified polymorphic DNA. Usually, a combination of molecular biology techniques would yield highly informative data.

Mitochondrial DNA RFLP

For reasons mentioned earlier, DNA-level analyses of genetic make-up proves advantageous against protein analyses. Between mtDNA and genomic DNA, the former would also have certain advantages over the latter, although such advantages are not strictly empirical. To begin with, mtDNA is smaller, making it easier to handle than genomic DNA. Also, it is highly uniform in size, at least among the vertebrates and the invertebrates (i.e. it is 15-18 kb in fish). Due to the maternal inheritance, sequences within a population (most likely originating from a single maternal individual) are highly conserved, so that it is possible to tell the relatedness of the populations of interest (Berg & Ferris 1984). On the other hand, its rapid evolution, which is ten times that of genomic DNA, provides a scale for the quantification of divergence. By plotting the divergence against the degree of divergence, it is possible to pinpoint the divergence between two species from a common ancestry. Using mice mtDNA, the calibration

scale for divergence was found to be 0.02 per 1 million years (Brown et al. 1979).

RFLP analysis of mtDNA may be done through a four-step methodology. The first step involves the isolation and purification of mtDNA using either CsCl- or density gradient centrifugation. The second and third steps following isolation, is the RE digestion and subsequent agarose gel electrophoresis (Alejandrino, 1993). When the fragments are obtained, these are then compared and subjected to statistical tests (Ferris & Berg 1982).

Several investigations on species and subspecies variation of Tilapias have been successfully shown to generate RFLPs. Some enzymes are only able to distinguish between tilapia species but as for *Apa*I, differentiation has been shown down to subspecies level of *O. niloticus* (Seyoum & Kornfield 1992) (Fig. 1). This is a significant finding because of *O. niloticus* economic importance in aquaculture which introduction from different hatcheries and farms all over the world has been uncontrolled and undocumented, as the case in the Philippines where commercial hatcheries and some research institutes have been improving the gene pool of their hatchery stocks with imported stocks from Taiwan, Israel or Africa.

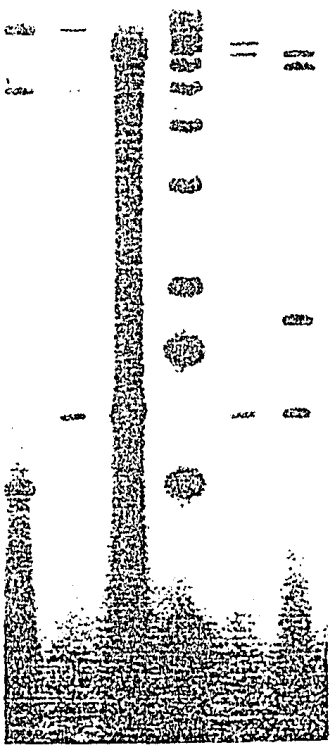

Figure 1. Restriction endonuclease digestion of mtDNA of various *Oreochromis niloticus* (O.n.) subspecies with *Apa*I. Samples (left to right). *O.n. cancellatus, O.n. spilurus spilurus, O.n. vulcani.* 1 kb molecular weight standard, *O.n. sugiltae and O.n. baringoensis* (Source: Seyoum & Kornfield, 1992).

Genomic DNA RFLPs and Satellite DNA

Methods employed in the analysis of genomic DNA include RE digestion, hybridization and amplification via PCR to reveal polymorphic fragments. Several DNA sequences, however, are more practical amplified randomly and thus AP-PCR is used instead to produce RAPDs as was done simultaneously in three different fish species such as *Barbus terazona, Poecilla reticulata* and *O. niloticus* (Fig. 2) (Harris et al. 1991). The PCR technique is highly specific and may generate accurate reflections of polymorphism. However, this technique requires rigidly controlled set-ups that, for instance, a slight sample contamination may cause sizeable errors.

The entire genome is too large and excessively polymorphic that genetic comparisons using RE digestion hardly facilitates RFLP analysis - if at all. However, certain noncoding and repetitive segments of the genome known as *satellite* DNA, thought to be only structurally functional, have now found use in genetic analyses. It has the advantage of ease in analysis in that it is rather small (less than 1 kb) and thus enables even sequence comparisons. Despite the small size, these portions of the genome may contain a high degree of polymorphism. Due to these characteristics, the resolution becomes more defined and detailed studies such as linkage analysis and DNA fingerprinting can be done. Also, amount of DNA required for analysis is rather low thus resulting to a conservation of DNA sample resources, which is crucial in certain instances (e.g. imported germplasm or valuable hatchery genetic resources).

Satellite DNAs have been first observed as extra peaks (- thus the term "satellite") generated upon

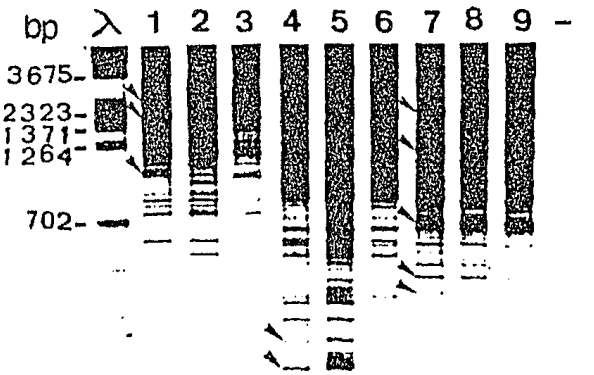

Figure 2. Polymorphic RAPD fingerprints (arrows) generated through AP-PCR in three individuals each of *Barbus tetrazona* (lanes 1-3), *Poecilla reticulata* (lanes 4-6) and *Oreochromis niloticus* (lanes 7-9). Molecular weight marker is *Bst*EII digested lambda phage DNA. The last lane is of negative reaction without template. (Source: Harris et al. 1991).

plotting of DNA content against its buoyant density. Four different satellites are typed according to size; (1) macro-, (2) midi-, (3) micro-; and (4) minisatellites which may be selectively isolated through isopicnic centrifugation, pulse-field electrophoresis, agarose gel electrophoresis, and denaturing gel-electrophoresis, respectively. Of these, the mini-(2-100 bp) and the microsatellites (1-4) are best characterized. Most satellite DNA analysis are thus based on these types.

A general protocol involved in satellite DNA analysis involves basic molecular biology techniques. Initially, isolated genomic DNA is digested with an appropriate RE. Satellite DNA, after gel electrophoresis, is seen as heavily stained bands in the DNA smear. Heavy staining of satellite DNA is due to the presence of this fragment length in large quantities brought about by the multiple restriction sites individually present within a monomer of the repetitive sequence. Polymorphic patterns in the satellite DNA are hypothesized to be generated because of three cases (Fig. 3). In the first case, the recognition sites are isolated outside the repetitive sequences and thus, the satellite is kept intact as a large fragment. Second, the recognition site is found at the repetitive units, but such recognition sites are lost towards the end of the satellite array. Lastly, the lost recognition site may be somewhere within any of the internal tandem

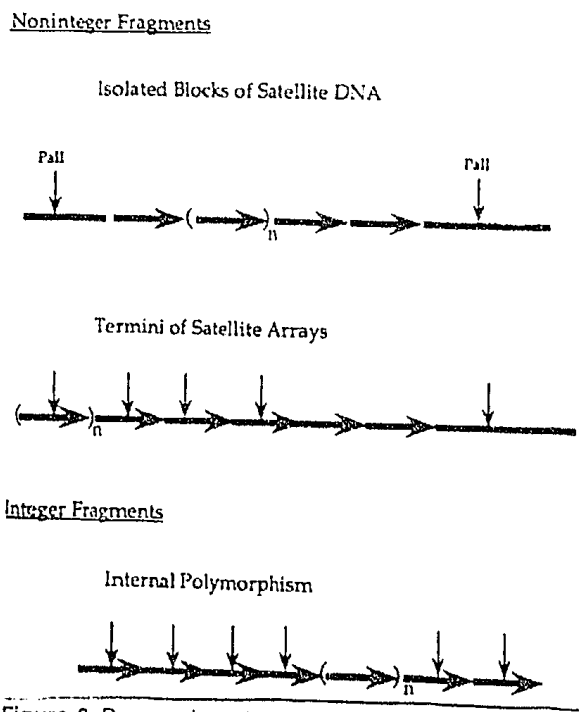

Figure 3. Proposed mechanism for the generation of polymorphic fragments in satellite DNA. Monomer satellite DNAs are represented by horizontal arrows. The vertical arrows represent the proposed *PalI* restriction endonuclease recognition sites (Source: Franck et al. 1992).

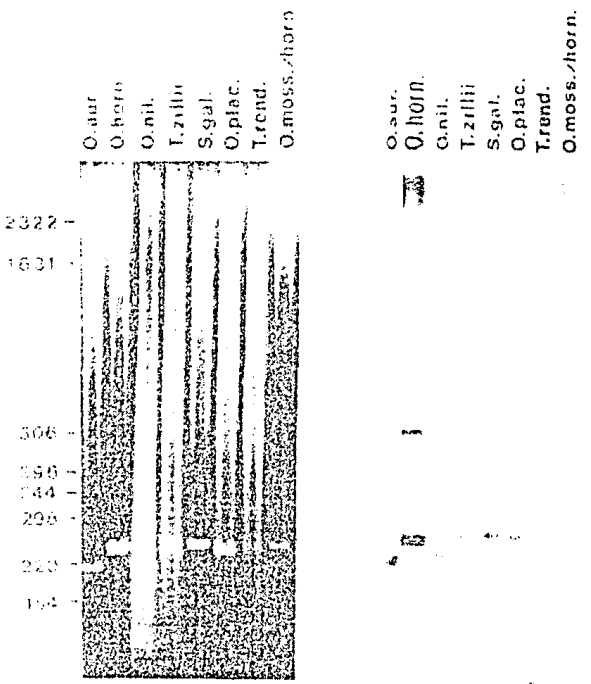

Figure 4. Southern blot and hybridization of a cloned satellite repeat from *O. niloticus* to *Eco*RI digests of various tilapiine DNAs (right panel). Left panel: 2% agarose gel electrophoresis of *Eco*RI digests of genomic DNAs from *O.aureus* (O.aur.), *O. hornorum* (O.horn.), *O.niloticus* (O.nil.), *O. placidus* (O.plac.), *T. zillii* (T. zillii), *T.rendalli* (T.rend.), and the hybrid strain *O. mossambicus x O. hornorum* (O.moss./hom.). Molecular weight markers in base pairs are shown at the left of the figure (Source: Franck et al. 1992).

repeats (Franck et al. 1992). Southern blotting is then done for a more permanent record of the fragment patterns. This type of analysis was used in differentiating different tilapia species (Fig. 4). This showed the utility of microsatellite genetic markers as tools in distinguishing tilapia species particularly those that look morphologically similar, e.g. *O. aureus and O. niloticus or O. mossambicus and O. hornorum*. Furthermore, the generated fragments may be cloned for further analysis, such as PCR and/or sequencing studies, or labelled to be used as a probe in genotyping unknown stocks, mixed or introgressed populations and offspring from parental stocks.

Conclusions

There are several potential and already-utilized applications of the study of DNA-level markers. Among these, genetic branding, phylogenetics, conservation biology, diagnostics and even forensics are relevant to the piscine system. Varietal and parental identification as well as marker-based screening of resource strains

may be greatly facilitated by these studies. Studies on molecular phylogenetics, such as genomic mapping, population and genomic evolution studies, and genetic linkage may be carried out by RFLP analysis or by DNA fingerprinting. In conservation efforts, quantification of introgression is important, as well as accurate delineation between wild stocks from hybrids. Although most valuable in humans disease, diagnosis may also be employed in fishes, particularly if such diseases are gene-related. Identification of loci which is related to disease resistance, immune responsiveness, or cell growth is also part of diagnostics which is valuable in aquaculture. Forensics in game fish sports may also employ DNA-based investigations, as in one particular case where a large bass trout, illegally introduced into the fishing waters, was found to be a native of another locality (Hallerman & Beckman 1988).

The application of DNA-based genetic analysis in tilapia research and stock development and management is still not fully maximized. The limited research available as cited in this paper (Harris et al. 1991, Franck et al. 1992, Seyoum & Kornfield 1992) were carried out separately and independently. They have revealed the potentials of DNA-level polymorphisms as tools for tilapia genetics and management. One of the fundamental concerns of Tilapia genetics is the identification and assessment of available stocks, particularly hybrids from parental species. Steps taken at this particular angle provide insights as to how further studies and management actions may be carried out systematically. Data obtained from this should be able to: (1) provide information regarding the discreteness of stocks; (2) quantify introgression within populations; (3) establish genetic variation or relatedness of different stocks; (4) elucidate evolutionary trends within the Tilapiine genera; and (5) serve as models for studies in other fish systems. Such information is indeed valuable to the overall scientific study of Tilapia and to the management programs necessary for its farming and breeding.

The numerous applications of DNA-level markers which are likely to expand will further the importance of DNA-based analysis in piscine genomic studies. Indeed, DNA-level polymorphism has opened a major highway for tilapia research.

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Figures 1 and 2 were reprinted respectively from Aquaculture 102, Seyoum & Kornfield 1992 and Aquaculture 92, Harris et al. 1991 with permission from Excerpta Medica Inc. Figures 3 and 4 were reprinted from Genome 35, Frank et al. 1992.

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