* CYTOGENETICAL TECHNIQUES IN AQUACULTURE: PRINCIPLES AND APPLICATION

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INTRODUCTION

- Cyto-genetics and Biochemistry have tremendously influenced the agriculture in general and aquaculture sector in particular. However its scientific application in aquaculture sector started later in 1990s.
- The knowledge of fish chromosome and karyotype is a pre requisite to cytogenetic engineering in aquaculture.

FISH CHROMOSOMES:FUNDAMENTAL ASPECTS

- The average size of fish chromosome ranges between 2.5µm (Blarchell,1983). The south American lung fishes *Lepidosiren paradoxa,* the size of the chromosome is very large i. e.15-30µm.
- Amongst the Elasmobranch (sharks, rays and skates) the diploid number of chromosomes varies in between 28-106 but majority of species have chromosome number between 52-100 (Singh *et al*.,1999). However exception lies in three families.
- Among members of Palidonidae and Ascipenseridae the number as well as size of the chromosome is very large due to their polyploidy nature. eg. Asceipencer- tetraploid (2n=118+2); Polydons pathula – octaploid (2n=120).
- The teleost fish show a wide variation in the chromosome number.
- The variation in chromosome number is more pronounced in the order Siluriformes.
- The chromosome number is less variable in the order Perciformes, where most of the species possess 2n=48. Hence majority of fish genetic managers are in the opinion that the 48 acrocentric chromosome constitute the basic diploid karyotype in teleost.

FISH CHROMOSOMES : AT A GLANCE

- The karyotype diversity in fish is arose by polyploidy, Robertsonian fission, fusion and other chromosomal rearrangement like pericentric inversion.
- Chromosomal non disjunction during meiosis produces chromosomal polymorphism within a species. Usually Robertsonian fission and fusion takes place when chromosome number changes without any corresponding changes in the fundamental arm. eg. If two acrocentric chromosomes get fused to form a metacentric chromosome and vice versa without changing in fundamental arm composition. Hence Robertsonian fission and fusion changes the morphology of the chromosome without any alteration in fundamental arm.

Table: showing chromosome number, arm number, DNA content and c value of certain teleost

Sl. No.	Species	2n	NF	relative DNA	C-value	
1.	*Pluronectes* spp.	48	48	0.20	0.7	
1.	*Oreochromis thelolicus*	44	58	0.27	1.0	
2.	*Esox lucius*	50	50	0.39	1.4	
3.	*Carrasius auratus*	104	166	0.40	1.6	
4.	*Cyprinus carpio*	100-104	150-156	0.50	1.8	
5.	*Catla catla*	50	78	-	-	
6.	*Labeo rohita*	50	78	-	-	
7.	*Ctenopharyngodon idella*	48	90	-	-	(Yuital, 1987)
8.	*Hypothalmichthyes molitrix*	48	96	-	-	
9.	*Clarias batrachus*	50	76	-	-	(Pandey & Lotera,1997)
10.	*Clarias gariapinus*	56	88(M)89(F)-		-	(Tengales *et a*l,1992)

Source: A Chrosomal Atlas of Indian Fishes at NBFGR, Lucknow.

FISH CHROMOSOMES : AT A GLANCE

The evaluation of fish karyotype shows three fundamental characteristics-

1. **Polyploidy**

- The polyploidy forms are found naturally in in six different orders of fishes –Ascipenseriformes, Cypriniformes,Beloniformes, Siluriformes, Poeciliformes and Perciformes. Out of which Poecilopsis is a triploid while the Ascipenseriformes is an octaploid species(2n=250+8)(Birstein and vasiliev,1987).
- *Cyprinus carpio* is also a tetraploid species due to its high chromosome number and Cvalue. The most interesting thing about its genome is that although it is polyploid, but many gene loci behaves like a diploid intron of gene expression (Ferris and Whitt,1997). Hence it is suggested that *C carpio* is in the process of depolarization.

2. sex chromosomes

- Almost all types of sex chromosomal mechanism like male heterogamety, female heterogamety and multiple sex chromosomes are observed in fishes. This is the basic reason to describe that the fishes are exhibiting almost all types of sexuality found among the Vertebtrates.
- Male heterogamety (XY-XX) is more prevalent than the the female heterogamety (ZZ-ZW).
- Male heterogamety is found in some deep sea water whjile female heterogamety is more prevalent in several species of cat fishes. (Pandey and Lakra,1997).
- The occurrence of multiple sex chromosomes are reported in in *Chiondraco harmatus*, an Atlantic fish (Caprigion *et al.*,1994).The sex chromosome in rainbow trout is difficult to identify while that of lake trout *Salvelinus namaeush* could be detected by banding technique , in which x chromosomes are having prominent C band while it is reduced in y chromosomes.(Reed and Philip,1995).

3. Chromosomal Ploymorphism

- The karyotypic difference in terms of variation chromosome number is reported in the population of some species. In *Channa puntatus* based on diploid number 32, two chromosomal raises have been identified. How ever the NF value for both the forms are 64. Apart form Robertsonian fusion and fission , the presence of supernumerary chromosome or B chromosome might have lead to the emergence of chromosomal polymorphism in different populations (Valcarcel et al.,1993).

FISH CHROMOSOMES : AT A GLANCE

Methods of Chromosomal preparation in fishes

In fishes chromosomes can be prepared by two resources-

1. *In Vivo*-from the actively binding tissues like kidney, spleen and gills.
2. *In vitro*-from the cultured cells.

- **In vivo method,** 2-3 hours before chromosomal preparation colchicine treatment is required to prevent the polymerization of tubulin that forms microtubules. As a result, cell division is arrested at metaphase stage. The number of metaphase plate obtained depends upon mitotic activities, which is affected by the season, health and age of the individual fish. Prior treatment of mitogen like Phyto-haemaglutanin, Cobalt chloride or Phenyl hydrazine is recommended because they stimulate cell division process and therefore useful in obtaining greater number of metaphasic plates.
- ***In Vitro* Culture method-** A good number of metaphasic plates can be obtained in short term *In vitro* culture of blood cell., abdominal fluid, fin tip or scales epithelium (Fox,1990, Gold *et a*l.,1990). The cultured cells are treated with colchicine two or three hours before the harvest.

The cells obtained from *In vivo* or *In vitro* sources are treated with a hypotonic solution of 0.56% potassium chloride and sodium citrate. As a result, the cell swells in size. The cells are sediment by low speed centrifugation and fixed in methanol acetic acid with two or three changes in between. The chromosomal slides are prepared by air drying method (Harleya and Horney,1985).

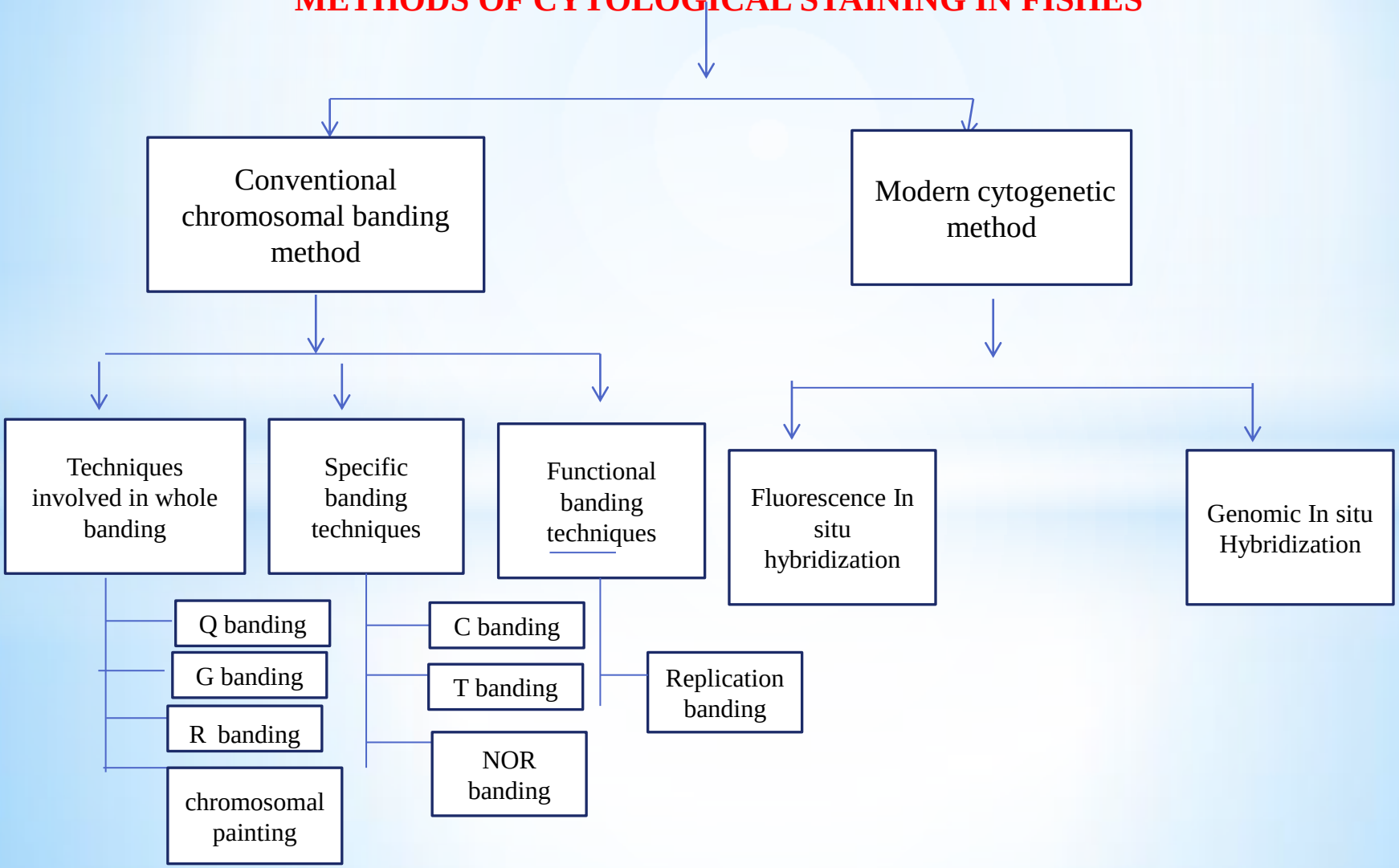
Staining of chromosomes:

- The slides are stained in 4-5% Geimsa solution for the visualization of the chromosomes.
- In many fishes karyotype are symmetrical, since the chromosomes are not much different in size and morphology. e.g. the karyotype in two species of Tilapia consists of 22 pairs of chromosomes.

The development of chromosomal banding techniques provides further advancement in the methodology and structure with enhanced ability. (Summer,1990; Gold *et al*.,1990; Rishi,1989).

METHODS INVOLVED IN CYTOLOGICAL TECHNIQUES IN AQUACULTURE

METHODS OF CYTOLOGICAL STAINING IN FISHES



Conventional Chromosomal Banding Methods

- Conventional cytogenetic testing or routine chromosome analysis is known as Karyotyping. Chromosome bands are produced due to variation in longitudinal structure of the chromatids revealed by various staining technique(Summer,1990).

Techniques involved in whole banding

- Chromosomal banding visualize a certain aspects and region of chromosomes. Each chromosome has a unique banding pattern, which projects the structure of genome and its organization. Each band usually contains from 5-10 Mb. Nearly all banding methods rely on harvesting chromosomes in mitosis by treating cells with tubulin inhibitors (colchicine, Colcemid),which depolarizes mitotic spindle.

Geimsa banding

- It is a cyto -genetic technique to produce a visible karyotype by staining condensed chromosomes. Dark and pale regions can be seen over the chromosomes by treating the slides(chromosome) with diluted trypsinr hot saline citratend staining with Geimsa..

Steps in Geimsa banding

- Metaphase chromosome is treated with trypsin and then stained with Geimsa stain.

* Conventional Chromosomal Banding Methods

- The heterochromatic regions which tend to rich in adenine and Thymine(AT rich) DNA and relatively gene poor, stains more darkly in G banding and other AT specific fluorochromes like Quinacridine , DAPT or Duramycin., while less condensed euchromatin (GC rich) and more transcriptionally active , incorporates less Geimsa stain and apparently these regions appear as light bands in G banding.
- The patterns of bands are numbered in each arm of the chromosome from centromere to the telomere.
- This numbering system allows any band on the chromosomes to be identified and described precisely. These bands are same in appearance on the homologous chromosomes and thus identification becomes easier and more accurate.
- **R banding/serial/transverse banding**
- R banding is a cyto-genetic technique that produces reverse of the G band stains on chromosomes. R banding is obtained by incubating the slides in hot phosphate buffer at 88◦ c, followed by a subsequent treatment of Geimsa dye.
- Resulting chromosomes pattern shows darkly stained dark band in guano- cytosine rich region, while adenine thymine rich regions are more readily denatured by heat.
- This technique is useful for analysing genetic deletion or chromosomal translocation that involves the telomere of the chromosomes.

Limitations of G and R banding in fishes

- The G and R bands are more often not reproducible in fishes because the fish chromosome lack regular distribution of AT and GC rich regions correspond to G and R bands.
- These differences can be revealed further by staining with GC and AT specific fluorochromes, which produce bands in higher vertebrates but stains only GC rich DNA in fish chromosomes.
- These experimental differences clearly suggest that there are some basic differences in chromosomal organizational pattern in fish and higher vertebrates for which the G and R banding are not consistently produced.

* Conventional chromosomal banding Methods

Q banding(Quinacridine banding)

- The era of chromosome banding begin with thr e demonstration of Q banding,which was discovered by Cespersson *et al*.(1968) as most simplest type of banding technique.
- Q banding uses fluorescent staining i.e. by an DNA intercalating agent quinacridine of metaphase chromosome.
- It is thought to reflect the distribution of relatively AT and GC rich classes of DNA throughout metaphase chromosome, while AT rich pairs increases fluorescence(bright band), GC pair suppress it. It helps in detecting either euchromatin or heterochromatin regions.

Chromosome Painting

- Chromosomal painting involves hybridization of each chromosome using a chromosome specific with a unique combination of fluorescent dyes.
- This provides a colourful array of chromosome, each one painted a different colour and this painted chromosome is examined under fluorescent microscope.
- It allows the comparison of entire genome. This method has shown that human chromosome number 6 having hundreds of genes in the major histocompatibility complex have homologous genes in chromosomes number 5 of chimpanzee , chromosome B2 of domestic cat , chromosome 7 of pig and chromosome number 23 of cow. However there in no any authenticated evidence of its use in fishes.

Short comings:

- The incompatible fossil record in many animal groups may pore problems in solving evolutionary or phylogenetic relationship in these species.

Methods of selective banding

C banding (Pardue and Gall,1969)

- C banding is also known as CBG staining and requires mild alkali treatment before Geimsa staining. It is used for banding of constitutive heterochromatin, therefore it is used for identification of centromere

Conventional Chromosomal Banding Methods

It is highly useful for staining of chromosomes regions, which contain repetitive DNA sequence(satellite DNA).These repetitive sequence are often treated adjacent to centromere and distal portion of Y chromosomes. The most significant bands are found on chromosomes 1,9,16 and Y. Among fishes C band can be demonstrated by three different methods-

1. **BSG (Barium hydroxide saline Geimsa technique (SUMMER, 1972)**: It is regarded as standard C banding method. Here slides are hydrolysed by 0.2N HCl, denatured in barium hydroxide solution earlier treated with 2XSSC (Saline sodium citrate) and stained with Geimsa, triton X100 and SDS.

2**. Fluorochromes**: they are also used to detect C banding provided the heterochromatic region is preferably rich in AT or GC sequence. Fluochrome like DAPI or Quinacrine, which bind to AT rich specific regions in the chromosome earlier helped to detect C band in Salmonids and Poecilids.

3. **Treating metaphase chromosome by restriction enzyme** (Lloyad and Thorgaard,1988):

Merits of C banding:

- Information regarding C banding is available in over 100 species of fishes (Khuda Baksh and chakraborti,2000)
- C banding in fishes include not only centromeric region but often used to demonstrate heterochromatin at the telomeric and intercalary region.
- In some cases whole chromosomes are found to be C band positive.

T banding

T banding is slight modification of R banding. It is used for visualization of telomere.

NOR banding

Nucleolar Organiser Region staining or silver staining is an advanced cyto-genetic technique, which requires silver nitrate solution, that stains active ribosomal DNA containing nucleolar organizer region(NOR), where 18S and 28S rRNA are clustered in interphase nuclei. NOR banding can be done by three methods-

1. **Silver staining** (Howell and Black,1980; Gold and Ellison,1983)- Silver stains bind to the non- histone protein of the trascriptionally active NOR region.

2. **Staining with Fluorochromes like Chromomycin A3(CAM) or mithromycin -**bind with GC rich sequence in the chromatin (It binds NOR region irrespective of previous transcriptional activity).

3. **In situ hybridization of the ribosomal DNA probes on the metaphase chromosome.**

Modern Cytogenetic Chromosomal Banding Methods

Molecular cyto genetic testing via Fluorescence *In situ* hybridization (FISH)

In situ hybridization is widely used for mapping directly the metaphase chromosome in which chromosomal preparations on microscopic slides are denatured to convert the double stranded DNA in to single stranded and a probe labelled with radio active isotopes antibodies or even fluorescent dyes containing all of the part of the clonal gene is hybridized to the chromosomal preparation.When the fluorescent dyes is used the hybridized chromosomal preparations can be detected directly under fluorescence microscope and need not to be detected in a gel of Southern blotting.

This technique is now used for homologous chromosome identification and studying the locations of DNA sequence on the chromosome

FISH testing is a method by which an assessment is made for the presence , absence, relative positioning and/or the copy number of specific DNA segment by fluorescent microscopy.

Application in aquaculture:

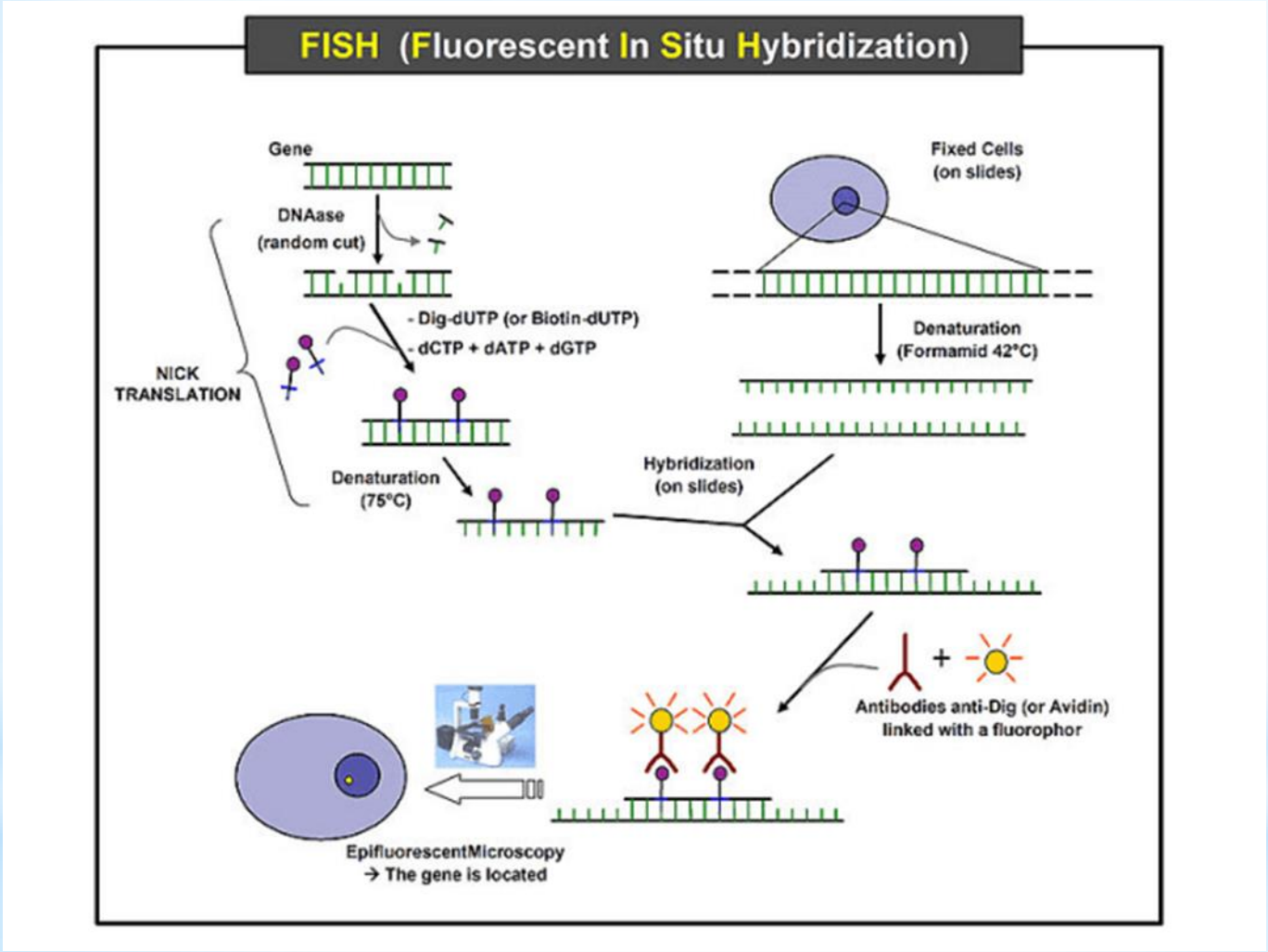
according to Philips and Reed (1996)FSH has tremendous avenues in fish taxonomy and genetic studies of various fish diseases.

It has proven its worth to study structural chromosomal aberration in fishes.

Limitations:

1. Genetic changes that are detected in metaphase chromosome preparation by this type of testing are limited to position and copy number changes primarily deletions and at some instances duplications for the specific chromosomal region for which the employed DNA probes are localized.
2. such studies do not rule out other form of genotypic abnormalities, which may include low level of tissue mosaicism and/ or other form at molecular alterations i.e. single base pair mutations , uni-parental desomics etc.
3.unknown familial genetic polymorphism may result in false positive or negative FISH results, which

*FLUORESCENCE *IN SITU* HYBRIDIZATION



Modern Cytogenetic Chromosomal Banding Methods

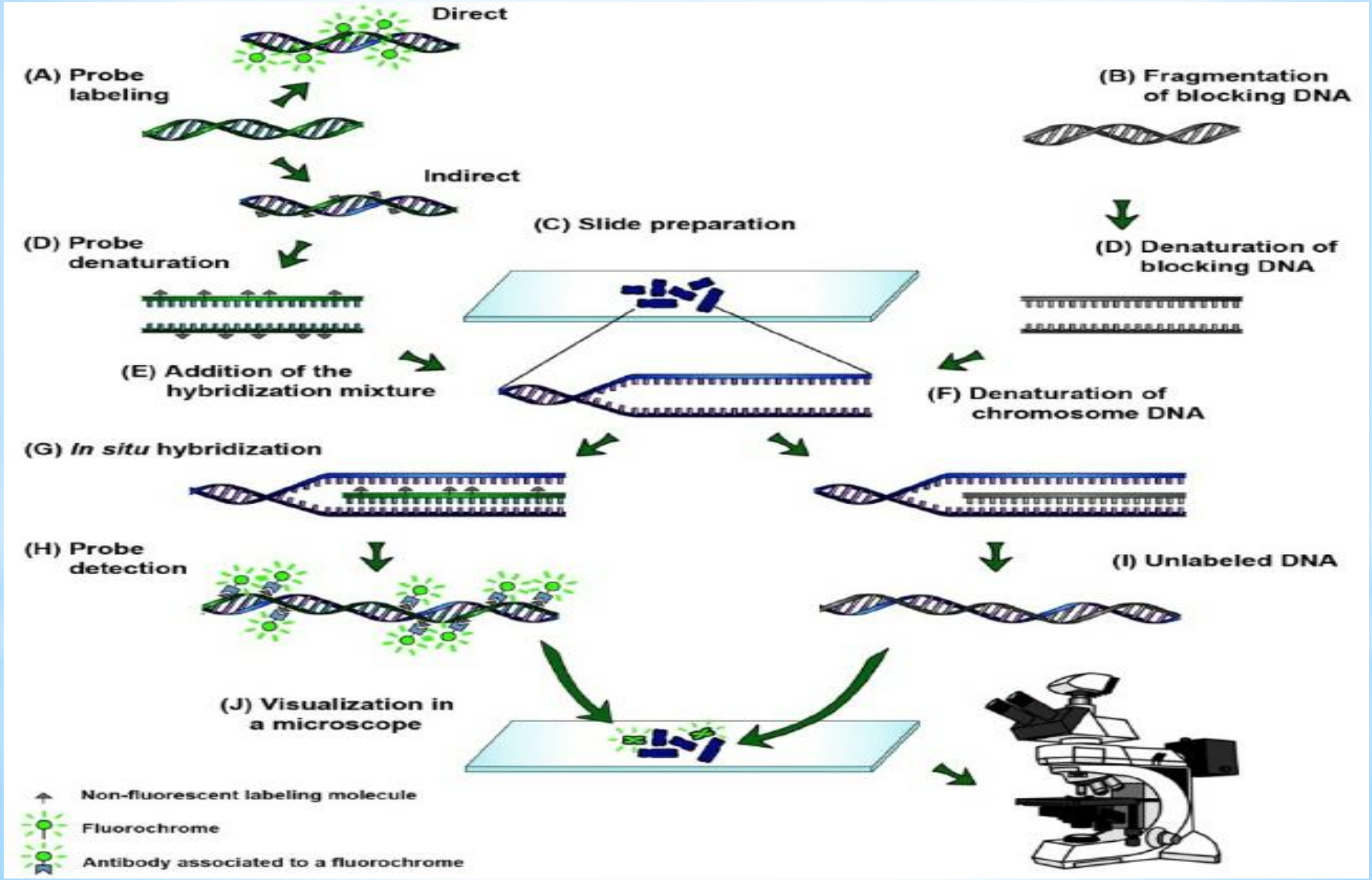
3. Genomic In Situ Hybridization (GISH):

GISH is a cytological technique that allows one to radio label parts of genome within the cells. It was mainly developed for the animal hybrid cell lines (1986) and later used for plants at Plant Breeding Institute, Cambridge(1987),where this techniques got its name.
The GISH is quick, sensitive accurate, informative and a comparative approach rather than absolute one . It shows advancements over FISH technique.

Principle:

The technique is based upon the extraction and subsequently radiolabelling of whole DNA of one organism and to use as a genome probe to target it the genome of other organism. The parts of the genome that are sufficiently similar to the probe hybridize to form a probe-target complex which is now labelled. The remaining non-hybridized parts of genome can be further stained to view them.

* GENOMIC *IN SITU* HYBRIDIZATION



Modern Cytogenetic Chromosomal Banding Methods

Applications of GISH in Aquaculture:

1. The study of fish chromosome started before the beginning of 20th century . According to latest review of Manna (1989), out of 20000 taxonomic species chromosomes of about 1700 species belonging to some 774 genus under 193 families 37 orders of 02 super class Chondrichthyes and Osteichthyes are unevenly known. Among extensively studied families (30-300) of teleost are Characidae, Cyprinidae, Cyprinidontidae, Poecillidae, Channidae, Cichlidae, Gobidae, Salmonidae, . Male heterogamety for XO, XY X1X24 and female heterogamety for ZO , ZW and ZW1 W2 have been reported. some chromosomes polymorphs have been also marked in some species.

2. It is highly useful in- meiotic studies, determination of phylogenetic relationship, determination of the position of translocation break points, comparative genomic studies of malignant and normal cells of individual, unknown genomic identification and to identify the hybridized genome of different species.

2. 3. It gives unique information about the similarity between DNA from related species.

4. It provides data about the physical distribution of sequence which are common or differ between the species being probed and the species used to supply the probed DNA.

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MODERN GENETICAL TECHNIQUES IN AQUACULTURE: AT A GLANCE

1. The concept of molecular genetic approach has long been applied to fisheries. These range from indirect examination of DNA variability using allozyme to direct examination using a variety of molecular markers.

2.The development of molecular genetics whether protein based or DNA based has proven its worth to fishery science (Ward & Grewe,1994,2002).

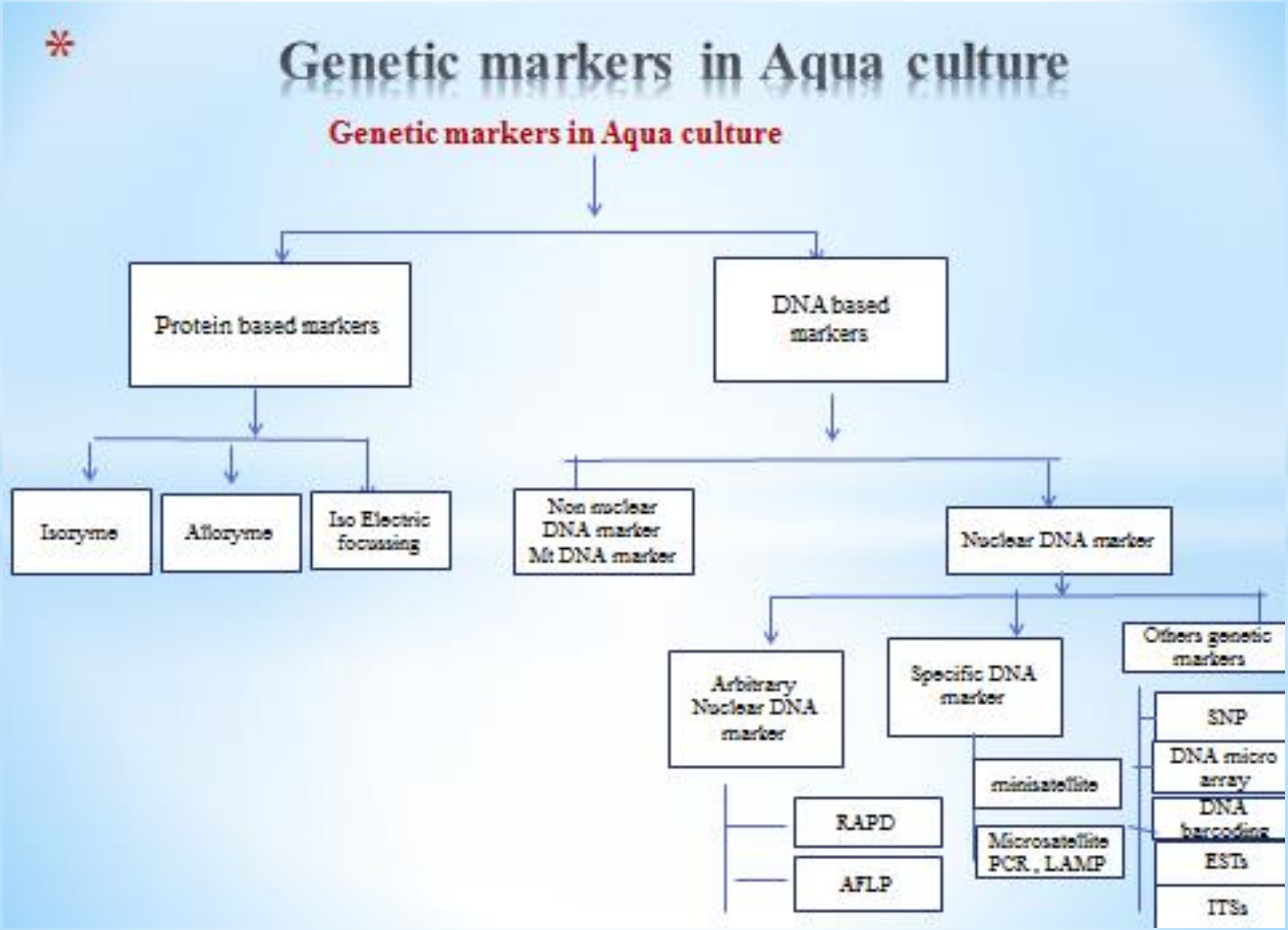
3. Fishery managers are now primarily aimed for resource sustainability and the recovery of depleted populations.

4. Genetic analysis are not only useful to defining stock structure of commercially important fishes rather they are also important in other areas concerned to fishery managers such as conservation and Enhancement programme, the validation of species and the identifications of depleting species.

5. Molecular markers are polymorphic DNA or protein sequences that can be used to identify a chromosomal region.

6. The molecular markers blended with the PCR technology has become the central tool in in manyn areas of fishery research.

MOLECULAR MARKERS : A POTENTIAL TOOL FOR GENETICAL APPROACH IN AQUACULTURE



PROTEIN MARKERS : AT A GLANCE

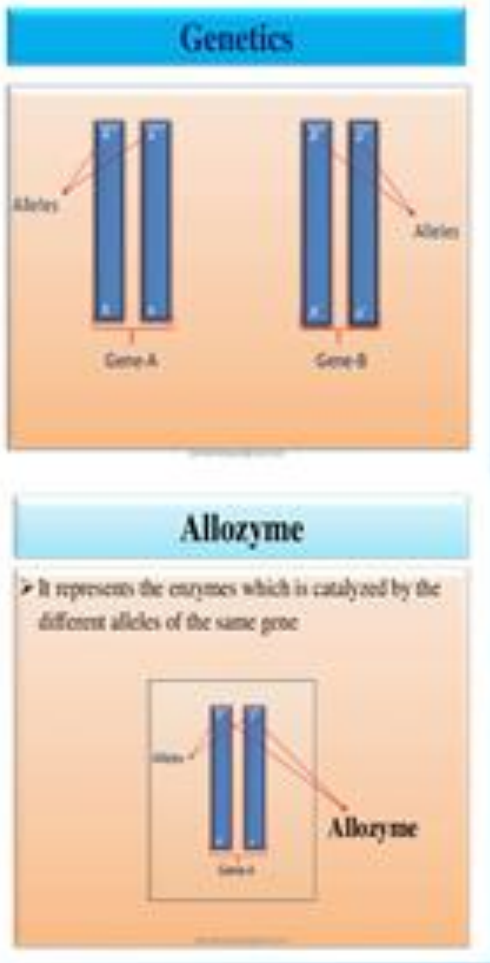
* Molecular markers used in Molecular taxonomy

Protein based markers

- Iso -enzyme s and allozymes were first discovered by R L Hunter and Clement Markert in 1957.
- Both these two variables are now used as interchangeable eg. Lactate dehydrogenase(LDH) chr.-12 and chr-15, malate dehydrogenase, glucose phosphate and glucokinase etc.
- Alloenzymes are common biological enzymes that exhibit high levels of functional evolutionary conservation throughout specific phyla and kingdoms. They are used by phylo-geneticists as molecular markers to gauge evolutionary histories and relationships between different species.
- A Allozyme electrophoresis is a method which can identify genetic variation at the level of enzymes that are directly encoded by DNA protein variants and they will differ slightly in electric charge.
- Allozyme provides us a data of single locus genetic variations which can answer many questions.

Steps in Allozyme analysis

1. Extract allozyme from tissues using standard specific protocol.
2. Then the variation is detected through electrophoresis in an acrylamide or in cellulose acetate gel.
3. Individuals that are homozygous show a single band where as heterozygous individuals show two bands.
4. it is a codominant Mendelian character



* Molecular markers used in Molecular taxonomy

Steps in Allozyme analysis

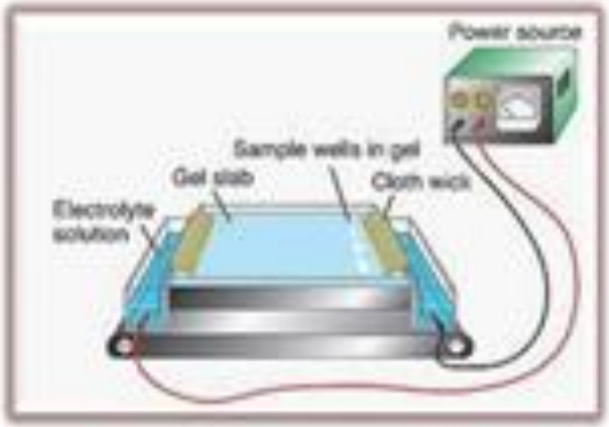
- Extract allozyme from tissues using standard specific protocol.
- Then the variation is detected through electrophoresis in an acrylamide, starch gel or in cellulose acetate gel.
- The protein bands obtained are observed carefully. Individuals that are homozygous show a single band where as heterozygous individuals show two bands. 40-50 individuals can be analyzed per gel.
- It is a co-dominant Mendelian character.

- Molecular markers are versatile tools in various fields other than taxonomy like physiology, embryology, genetic fingerprinting etc.

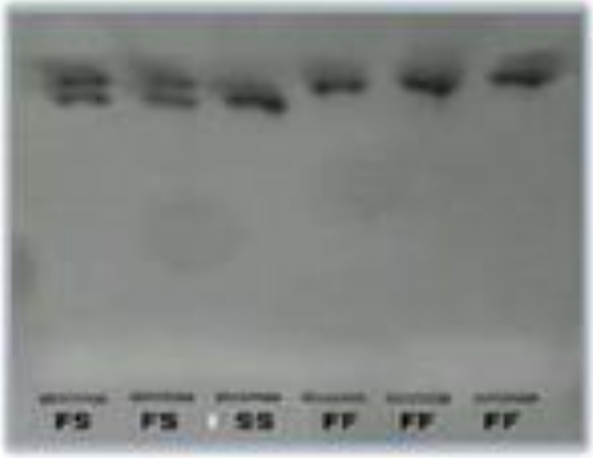
- Molecular phylo-genetics and systematics have been found to be greatly promising in recent years, due to the development of new and diverse method.

- Molecular taxonomic approaches permit an exact and rapid method of distinguishing specimens based on their interspecific variations. These methods allow estimation of the genetic variability of the biota carrying to a super-estimation on the global biodiversity besides the relationships among

Electrophoresis



Protein Pattern after electrophoresis



* Molecular markers used in Molecular taxonomy

Advantages of allozyme

- Due to their simplicity, low cost and requirement of little specialized equipment, it is most widely studied form of molecular evolution.
- It is co dominant type of marker and easily adjustable from species to species.
- The allozyme analysis has general applicability. It has wide spread use in fisheries mainly in Ichthyo- taxonomy, individual identification, establishing the phylogenetic relationship of the different species of fishes, identification and arrangement of the species and stock on the basis of their genetic affinity, identification of genetic diversity within stock and cultured species, study of population structure, genome mapping, study of the genetic behaviour of smolt stage, genetic characterization of the species and stock, to study the genetic drift of the species and stock ,Mixed Stock Fishery Analysis(MSFA), forensic analysis and study of conservation genetics.

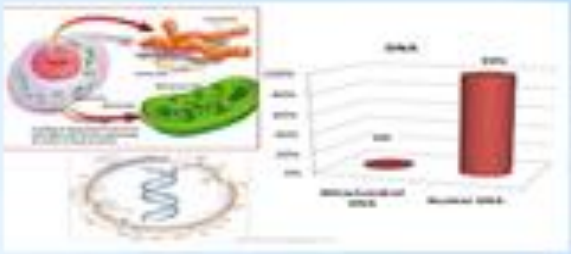
Limitations of Allozyme

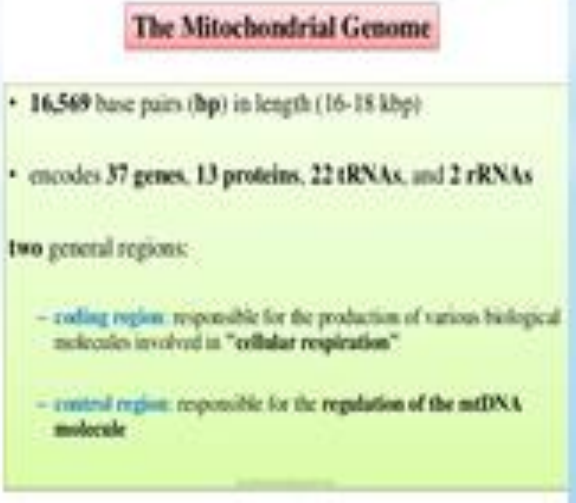
- The technique requires a large amount of tissues and cannot be applied when the organism are very small i.e. larvae form.
- A tissue sampling method is invasive and the fish needs to be sacrificed and the tissues needs to be stored cyto-genetically. A point mutation in nucleotide sequence may not necessarily results in change in amino acid and hence could not be detected by protein electrophoresis.
- presence of limited number of allozyme loci precludes their use in large scale genome mapping.
- Heterozygote deficiencies due to null alleles.

* Molecular markers used in Molecular taxonomy

Mitochondrial DNA marker

- Mitochondrial DNA is non nuclear, remain present within mitochondria.
- Mt DNA is maternally inherited with haploid genome
- The entire genome undergoes transcription as one single unit. They are not subjected to any recombination and hence they are homologous marker .
- They are selectively neutral, occurring in multiple copies in each cell.
- Mt DNA is physically separated from the rest of the cells DNA and so it is relatively easier to isolate from any tissues or blood samples.
- Due to maternal inheritance of Mt. DNA, the effective population size is smaller than nuclear DNA and so Mt DNA variation is more sensitive to population bottle neck and hybridization.
- The difference in the nucleotide sequence of the DNA molecule in mitochondria can be determined directly or indirectly by several methods like-RFLP.
- The newly emerged sequencing technologies have enabled direct sequencing of Mt DNA and several sets of universal primer have been developed from conserved sequence region. Slow evolved gene regions are constantly being used for interspecies comparison while fast evolving gene region are used for population comparison eg. D-loops
- The only non coding region of Mt DNA is D-loop region which is fast evolving gene region and hence mostly used for population comparison. Besides the Cyt b and ND-1 and ND-5/6 gene regions are also being used.
- Mit cytochrome c oxidase I gene(CO I gene) has been identified as universal barcode for species level identification due to its conserved nature across a wide range of taxa.





Application of Mitochondrial DNA Marker in molecular taxonomy

Molecular markers also show significant promise for aquaculture applications

(i) In comparison of hatchery and wild stocks;

(ii) Genetic identification and discrimination of hatchery stocks;

(iii)Monitoring inbreeding or other changes in the genetic variation;

(iv) Assignment of progeny to parents through genetic tags;

(v) Identification of quantitative trait loci (QTL) and use of these markers in selection programmes;

(vi)Assessment of successful implementation of genetic manipulations such as polyploidy and gynogenesis.

Source: (Magoulas, 1998 ; Davis and Hetzel, 2000, Fjalestad et al., 2003, Sahasingha et al., 2003)

DNA Barcodes

- DNA Barcodes are segments of approximately 600 b p of Mit. CO I gene, which is a fast , efficient and inexpensive technique helpful in cataloguing the biodiversity.
- With the increased resolution and maximum information obtained Mt DNA analysis is highly productive in molecular phylo-genetics.

Functions of mtDNA in Ichthyotaxonomy

- Individual identification
- Mixed Stock Fishery Analysis(MSFA)
- To identify the phylogenetic relationship b/w the different species of fishes
- To identify and arrangement of the species and stock on the basis of their genetic affinity
- To identify the genetic diversity with in the stock & cultured species

Uses for mtDNA in Forensics

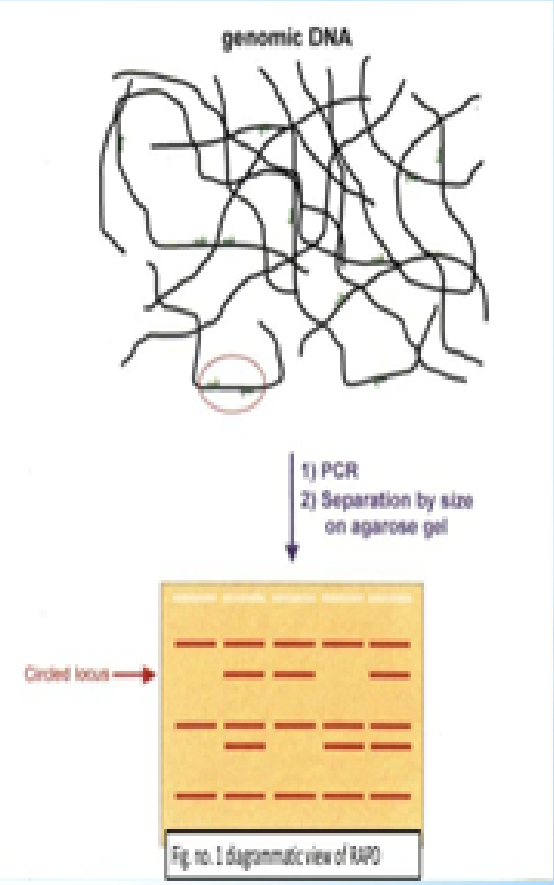
- mtDNA will be used when "biological evidence may be degraded [i.e. charred remains] or in small quantity"
- Cases in which evidence consists only of:
  - hairs
  - bones
  - Teeth
- Missing Persons Cases (use of skeletal remains)
- Establishing Individuals as suspects (hair evidence)

* Arbitrary Nuclear DNA marker in Molecular taxonomy

- Any genetic trait that can be identified with confidence and relative case and can be followed in a mapping population is known as genetic marker.
- Genetic marker is a specific location on a chromosome that is defined by a naked eye polymorphism as differences in electrophoretic mobility of specific protein or as difference in specific DNA sequence. Arbitrary nuclear DNA marker are used when a sequence of DNA of unknown function is targeted.
- The widely used method for amplifying unknown regions are-

Random Amplified Polymorphic DNA (RAPD)

- Random amplified Polymorphic DNA(RAPD) markers are DNA fragments from PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence.
- RAPD analysis is a PCR based molecular marker technil que. Single short oligonucleotide primer is arbitrarily selected to amplify a set of DNA segments distributed randomly through out the genome (anonymous loci).
- RAPD uses random primer to generate multiple PCR products resulting in a fingerprint for a particular species. It is very fast, cheap and show very high amount of polymorphism and the marker does not require the prior



Applications , advantages and disadvantages of RAPD

Applications OF RAPD

- Molecular genetic marker have been developed into powerful tools to analyse genetic relationship and genetic diversity.
- As an extension to the variety of existing technique using polymorphic DNA markers, the RAPD may be used in molecular ecology to determine taxonomic identity, assess kinship relationship, analyse mixed genome samples and create specific probes.

Advantages of RAPD

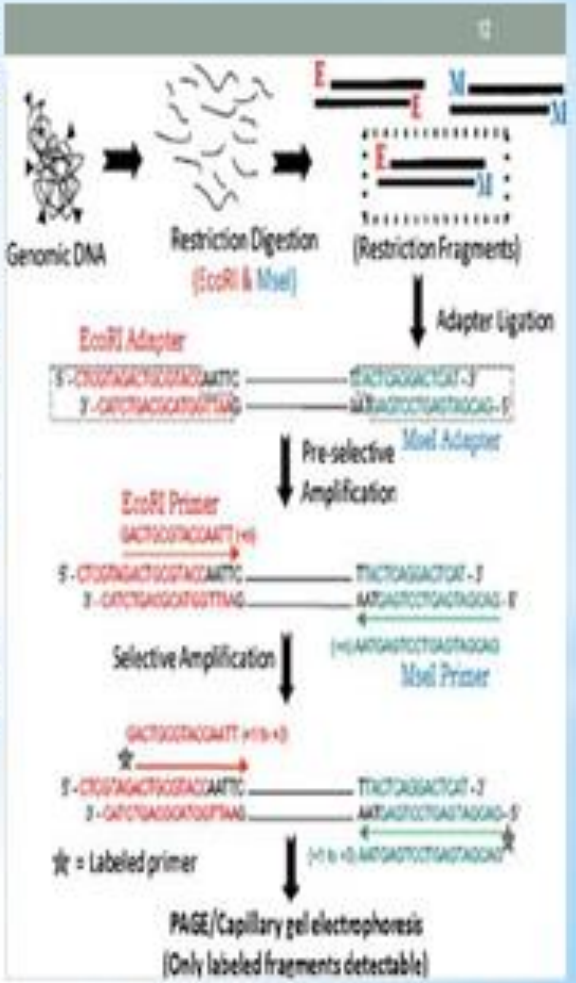
- It simultaneously targets multiple gene loci and is therefore more useful for discriminating closely related or cryptic species.
- DNA fingerprint is generated in a single reaction
- Data may be used for phylogenetic reconstruction in some instances.
- It is suitable for work on anonymous genome.
- It is quick and efficient screening for DNA sequence based polymorphism at many loci and low expanses technique. It does not involve any radio active assays.

Disadvantages of RAPD

- The major drawback of RAPD is the reproducibility and repeatability. At the same time a large number of products generated
- RAPD is a dominant marker and homozygous and heterozygous state can not be differentiated.
- It cannot be used on mixed samples.
- It is only useful as diagnostic, if RAPD fingerprint of unknown specimen has already been resolved for comparison.
- Co dominant RAPD markers observed as different sized DNA segments amplified from the same locus are detected only rarely.

Amplified Fragment Length Polymorphism (AFLP) DNA Markers

- Amplified fragment length polymorphisms (AFLPs) provide an effective means of genotyping, particularly when little is known about the genome or genetics of an organism. It involves ligation of adaptors to digested DNA followed by PCR amplification using primer, that are primarily adaptor and partially gene specific.
- They may combine the benefit of both RAPD and RFLP. The total genomic DNA is digested using two restriction enzymes.
- Restriction enzymes cut the DNA and double stranded nucleotide adaptors are ligated to the ends of the fragments to serve as primer biding site for PCR amplification.
- Fragments are then amplified using PCR and the presence or absence of their varying lengths can then be visualized on polyacrylamide gel or capillary-based platform. Thus genetic polymorphism is studied.



* Applications , advantages and Disadvantages of AFLP

Applications

- The AFLP has the capability to detect various polymorphism in different regions simultaneously.
- AFLP has been widely used for identification of genetic variations in strains or closely related species of plants , fungi animals and bacteria.
- The AFLP technique has been used extensively in criminal and paternity test , also to determine slight variation within the population and linkage studies to generate maps for quantitative Trait Locus(QTL) analysis.

Advantages

- It simultaneously target multiple gene loci and therefore useful for discriminating closely related or cryptic species.
- It is very sensitive and more robust than RAPD.
- Data may be used for phylogenetic reconstruction in some instances.
- The whole genome analysis is possible without any knowledge of known genome sequence by changing the selective nucleotide.

Disadvantages

- It is a complex procedure and requires manipulations in addition to PCR.
- It cannot be used on mixed samples.
- It is only useful as diagnostic if AFLP fragment of the unknown specimen has already been resolved for comparison.
- AFLP is very sensitive for detecting genetic polymorphisms but requires relatively large amounts of high-quality DNA and has difficulty with mixture analysis. Thus, AFLP is not an ideal candidate for genotyping forensic rich samples but has been used for population genetics of plants.

Restriction Fragment Length Polymorphism (RFLP) DNA Markers

The University of Edinburgh

What is RFLP ?

- Restriction Fragment Length Polymorphism is a variation in the length of a DNA fragment produced by a specific restriction enzyme acting on a DNA from different individuals that usually results from a genetic mutation.
- If two organisms differ in the distance between site of cleavage of a particular restriction endonuclease, the length of the fragments produced will be different when the DNA is digested with a restriction enzyme.
- RFLP analysis is the detection of the change in the length of the restriction fragments.

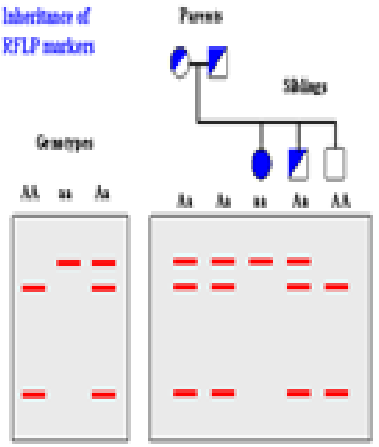
The University of Edinburgh

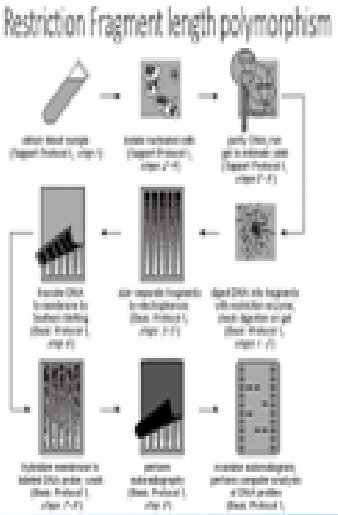
Continuous

- A restriction enzyme cuts the DNA molecule at every occurrence of a particular sequence, called restriction site.
- For Example, HindIII enzyme cuts at AAGCTT.
- If we apply a restriction enzyme on DNA, it is cut every occurrence of the restriction site into a million restriction fragments each a few thousands nucleotides long.

Continuous

- Any mutation or polymorphism of a single nucleotide may destroy (AAGCTT for HindIII) and change the length of the fragment.
- The term polymorphism refers to the slight differences between individuals, in base pair sequences of genes
  or
- A polymorphism is a clinically harmless DNA variation that does not affect the phenotype.
- So RFLP analysis is a technique which is used to detect the change in the length of the restriction fragments.





In molecular biology, restriction fragment length polymorphism (RFLP) is a technique that exploits variations in homologous DNA sequences, known as polymorphisms, in order to distinguish individuals, populations, or species or to pinpoint the locations of genes within a population

Applications, Advantage and Disadvantages of RFLP

Applications

- RFLP can be used for paternity cases or criminal cases to determine the source of a DNA sample (i. e. It has forensic applications).
- RFLP can be used to detect mutations.

Advantages

- It is simple and accurate process and does not require sequence information.

Disadvantages

- It is an expensive process.
- It is very slow, labour intensive and time consuming process.
- It requires a very large amount of DNA
- It is difficult to automate.

Specific DNA markers in Molecular taxonomy

- There are two main classes of repetitive and highly polymorphic DNA- Mini satellite DNA (VNTRs) and Microsatellite DNA(STRs).

Mini-satellite DNA or Variable number of tandem repeats (VNTR)

- Variable number of tandem repeats are a sequence of DNA i.e. repeated tens or hundreds to thousands of time in nuclear genome.
- They repeat in tandem, vary in number in different loci and differently in individuals.
- Minisatellite DNA referring to genetic loci with repeats of length 9-65 bp, are not restricted to Nuclear DNA, rather they are also present in MtDNA or cpDNA .
- VNTR have been thought to arisen due to slippage during DNA replication or as a consequences of unequal crossing over. They may be of two types- Multi-locus mini-satellite and single locus minisatellite.

Multi locus minisatellite

- They are composed of tandem repeats of 9-65bp and have a total length ranging from 0.1to7 kb

Disadvantages

1. Multilocus minisatellite is are generally used in percentage analysis.

They are less useful for population genetics analysis unless we use large sample size.

2. The complexity of mutation process undergone by mini satellite loci is also a limitations.

Single locus minisatellite

Advantages

1. Single locus minisatellite have been very useful and successful detecting in genetic variation within and between population.

2. It has also been used in fisheries for forensic, parentage, genetic identity, estimating mating success and conforming gynogenesis.

Disadvantages

1. Single locus minisatellite analysis requires reasonable quantities of high quality DNA.

Specific DNA markers in Molecular taxonomy : Microsatellite(STRs)

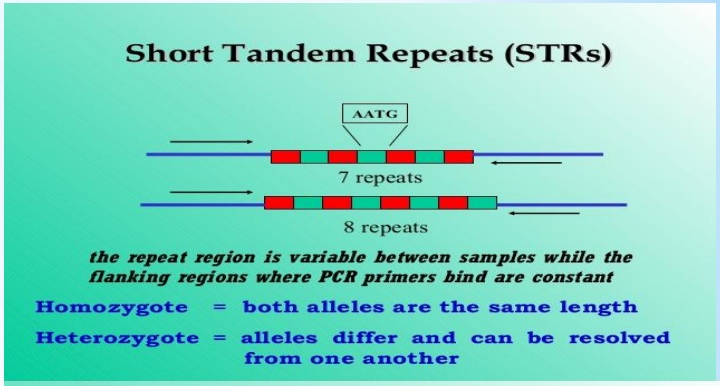
1. **Microsatellite** is a simple DNA sequence which is repeated several times across various points in the DNA of an organism.

2. They are also known as short tandem repeats (STRs), consisting of tracts of repeats of 2-8 bp (1-6) long .They are highly variable and these loci can be used as markers.

3. Microsatellite occurs once in every 10kbp, while minisatellites occur in every1500kbp in fishes. So they are more useful in genome mapping and population genetics studies.
4. They are highly variable, noncoding and selectively neutral.

Principle

Microsatellite analysis involves DNA extraction, amplification of microsatellite nuclei using specific primer in a PCR machine (thermocycler) and examination of band under SDS PAGE .The basic assumption while using microsatellite loci is that the predicted amount of sequence divergence between units of interest is directly related to the length of time since separation.
Cross amplification with primers developed in closely related species is also possible which minmizes the cost associated with detecting microsatellite sequence in different species.
The recent introduction of automated genotyping machine has lead to the use of a large number of samples and loci, which has increased precision and speed with micro satellite analysis.



Microsatellites – Types

Based on repeat pattern

1. Perfect – CACACACACACACACACACA
2. Imperfect – CACACACACA CACACACA CACACACA
3. Compound – CACACACACACA CATACATACATA CATACATACATA
4. Complex – CACACACACACACACA AATAATAATAATAATAATAAT

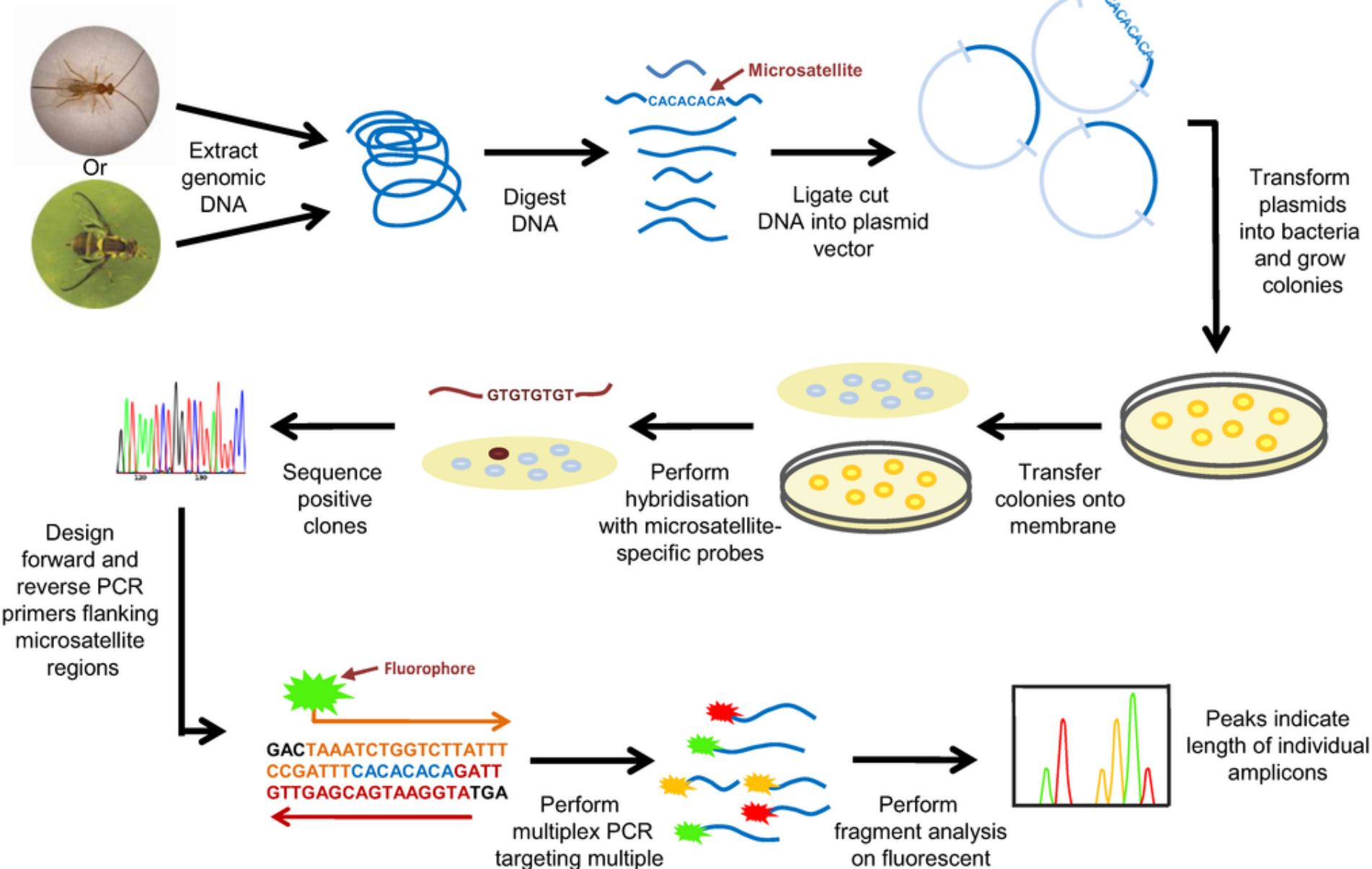
Based on number of base pairs

1) Mono (e.g. CCCCCCCC or AAAAAA)
2) Di (e.g. CACACACACA)
3) Tri (e.g. CCA CCA CCA CCA)
4) Tetra (e.g. GATA GATA GATA GATA GATA GATA GATA)

Minisatellites: - (9 – 65 base pairs repeated from 2 to several hundred times)

CGCCATTGTAGCCAATCCGGGTGCGATTGCAT CGCCATTGT AGCCAATCCGGGTGCGATTGCAT CGCCATTGTAGCCAATCCGGG TGCGATTGCAT CGCCATTGTAGCCAATCCGGGTGCGATTGCAT CGCCATTGTAGCCAATCCGGGTGCGATTGCAT

Development and Use of Microsatellite Marker for Species Identification



Specific DNA markers in Molecular taxonomy :Microsatellite(STRs)

Advantages

1. Micro satellite analysis simultaneously targets multiple gene loci and therefore more useful for discriminating closely related or cryptic species.
2. When fluorescent primers are used fragment analysis is readily automated .
3. Assays can be multiplexed during PCR and detection(fragment analysis) phase.
4. Some microsatellite assays can be applied across a number of different species.

Microsatellite analysis have been extensively used in fisheries and aquaculture for phylo-genetics and phylo-geographical studies, population genetics, structure, biodiversity, conservation, stocking impact and hybridization. Besides, it is extensively used for forensic identification, genome mapping, determination of kinship and behavioural pattern.

Disadvantages

1. Assay development is tissue consuming initially and cannot be used in mixed samples.
2. The presence of null alleles and shutter bands are major constraints of microsatellite markers.
3. Null allele occurs when mutation occur at primary binding sites of microsatellite locus it reduces the accuracy in terms of relatedness analysis and assignment test.
4. Shutter bands occur when a ladder of bands differing in 1-2 bp is seen and these occur due to slipped strand impairing PCR or incomplete denaturation of amplification products.

Specific DNA markers in Molecular taxonomy :Polymerase chain reaction(PCR)

- **Polymerase chain reaction (PCR)** is a method used widely in molecular biology to make millions to billions of copies of a specific DNA sample rapidly, allowing scientists to take a very small sample of DNA and amplify it to a large enough amount to study in detail.
- PCR was invented in 1983 by the American biochemist Kary Mullis. It is fundamental to much of genetic testing including analysis of ancient samples of DNA and identification of infectious agents. Using PCR, copies of very small amounts of DNA sequences are exponentially amplified in a series or cycles of temperature changes.
- Applications of the technique include DNA cloning for sequencing, gene cloning and manipulation, gene mutagenesis**; construction of DNA-based phylogenies**, or functional analysis of genes; diagnosis and monitoring of hereditary diseases; amplification of ancient DNA;] analysis of genetic fingerprints for DNA profiling (for example, in forensic science and parentage testing); and detection of pathogens in nucleic acid tests for the diagnosis of infectious diseases.
- A basic PCR set-up requires several components and reagents, including:a DNA template that contains the DNA target region to amplify, a DNA polymerase(heat-resistant Taq polymerase),two DNA primers that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strands of the DNA target ,deoxynucleoside triphosphates, or dNTPs (sometimes called "deoxynucleotide triphosphates"; buffer solution providing a suitable chemical environment for optimum activity and stability of the DNA polymerase and bivalent cations, typically magnesium (Mg) or manganese (Mn) ions; Mg2+

* Specific DNA markers in Molecular taxonomy :Polymerase chain reaction(PCR)

PCR consists of a series of 20–40 repeated temperature changes, called thermal cycles, with each cycle commonly consisting of two or three discrete temperature steps. The individual steps common to most PCR methods are as follows:

Initialization: This step is only required for DNA polymerases that require heat activation by hot-start PCR. It consists of heating the reaction chamber to a temperature of 94–96 °C (201–205 °F), or 98 °C (208 °F) if extremely thermostable polymerases are used, which is then held for 1–10 minutes.

Denaturation: This step is the first regular cycling event and consists of heating the reaction chamber to 94–98 °C (201–208 °F) for 20–30 seconds. This causes DNA melting, or denaturation, of the double-stranded DNA template by breaking the hydrogen bonds between complementary bases, yielding two single-stranded DNA molecules.

Annealing: In the next step, the reaction temperature is lowered to 50–65 °C (122–149 °F) for 20–40 seconds, allowing annealing of the primers to each of the single-stranded DNA templates. Two different primers are typically included in the reaction mixture: one for each of the two single-stranded complements containing the target region. The primers are single-stranded sequences themselves, but are much shorter than the length of the target region, complementing only very short sequences at the 3' end of each strand.

The processes of denaturation, annealing and elongation constitute a single cycle. Multiple cycles are required to amplify the DNA target to millions of copies. The formula used to calculate the number of DNA copies formed after a given number of cycles is 2n, where n is the number of cycles. Thus, a reaction set for 30 cycles results in 230, or 1,073,741,824, copies of the original double-stranded DNA target region.

Final elongation: This single step is optional, but is performed at a temperature of 70–74 °C (158–165 °F) (the temperature range required for optimal activity of most polymerases used in PCR) for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully elongated.

Final hold: The final step cools the reaction chamber to 4–15 °C (39–59 °F) for an indefinite time, and may be employed for short-term storage of the PCR products.

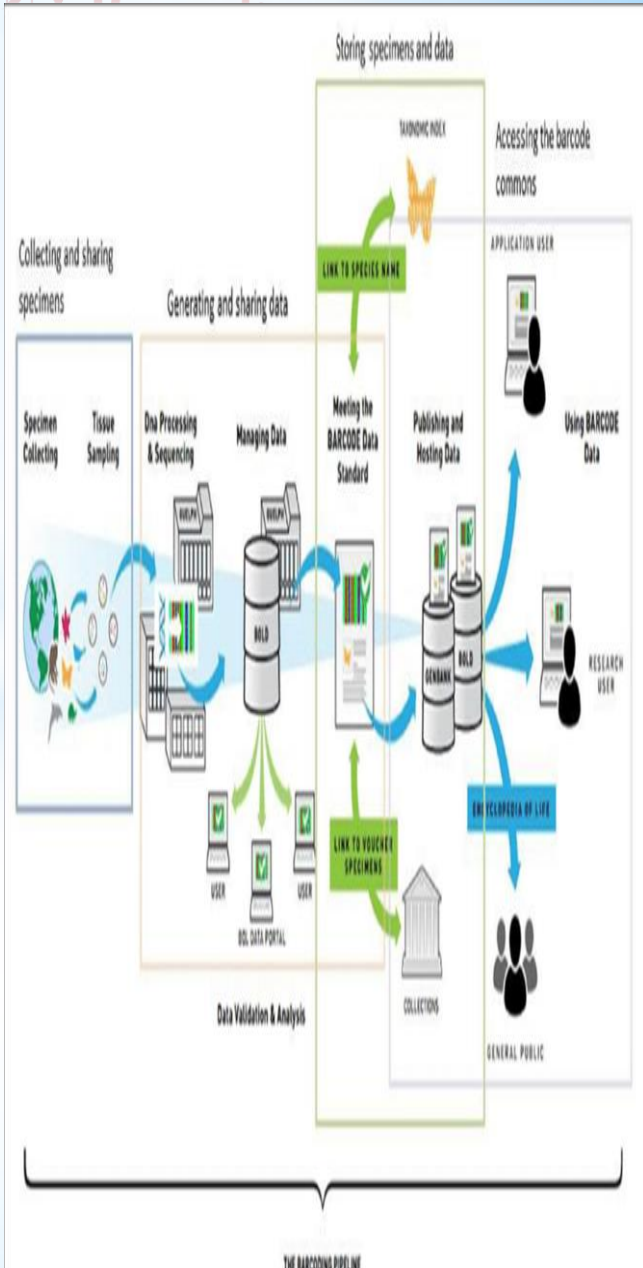
Other Useful DNA Markers in Molecular taxonomy :DNA Micro array

- **DNA Micro array/ DNA microchips** A DNA microarray (also commonly known as DNA chip or biochip) is a collection of microscopic DNA spots attached to a solid surface. Scientists use DNA microarrays to measure the expression levels of large numbers of genes simultaneously or to genotype multiple regions of a genome. A dDNA microarray is a test that allows for the comparisons of thousands of genes at a time.
- The principle behind microarrays is that complementary sequences will bind to each other. The unknown DNA molecules are cut into fragments by restriction endonucleases; fluorescent markers are attached to these DNA fragments. These are then allowed to react with probes of the DNA chip.
- In this technique cDNA or RNA (molecules of known sequences or probe, reporters, oligos, or antisense RNA) covalently attached on the solid surface. Each DNA spot contains pico moles (10−12 moles) of a specific DNA sequence. The experimental DNA or RNA (unknown sequences or test, target, or sample DNA or RNA) are tagged with fluorescent dye and poured over the probe area for hybridization. Probe-target hybridization is usually detected and quantified by techniques like autoradiography, laser scanning, fluorescence, and enzyme detection devices. Such techniques can be used to read the chip surface and hybridization pattern.
- Each DNA spot contains many thousands of copies of a specific DNA sequence, known as probes. These usually correspond to a short section of a gene – generally at the 3′ end. Each microarray includes one or a few probe sets for each interrogated gene. These are used to hybridize a cDNA sample (the target) under high-stringency conditions. Probe–target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labeled targets to determine the relative abundance of transcripts in the target sample. Data on about 700 000 sample hybridizations performed on DNA microarrays are accessible through the databases Gene Expression Omnibus (GEO) at NCBI, and Array Express at EBI.
- They have diverse applications in gene expression analysis, DNA sequencing, characterization of

Specific DNA markers in Molecular taxonomy : DNA Barcoding

DNA barcoding is a method of identifying organisms based on a short, standardized fragment of genomic DNA and has been developed for use by taxonomists, ecologists, conservation biologists, regulatory agencies, and others.

- DNA barcoding involves the use of a single gene to identify a given species through the comparison of nucleotide sequences in the DNA to that of the same gene in other species. This is in marked contrast to the multiple STR approach used for identifying individuals within a species.
- A bar code (often seen as a single word, barcode) is the small image of lines (bars) and spaces that is affixed to retail store items, identification cards, and postal mail to identify a particular product number, person, or location. ... A barcode reader is used to read the code.
- A region of the mitochondrial gene COI (cytochrome c oxidase subunit I) is used for barcoding animals. COI is involved in the electron transport phase of respiration. Thus, many genes used for barcoding are involved in the key reactions of life: storing energy in carbohydrates and releasing it to form ATP.
- DNA barcodes can aid conservation and research by assisting field workers in identifying species, by helping taxonomists determine species groups needing more detailed analysis, and by facilitating



Specific DNA markers in Molecular taxonomy : DNA Barcoding

Advantages

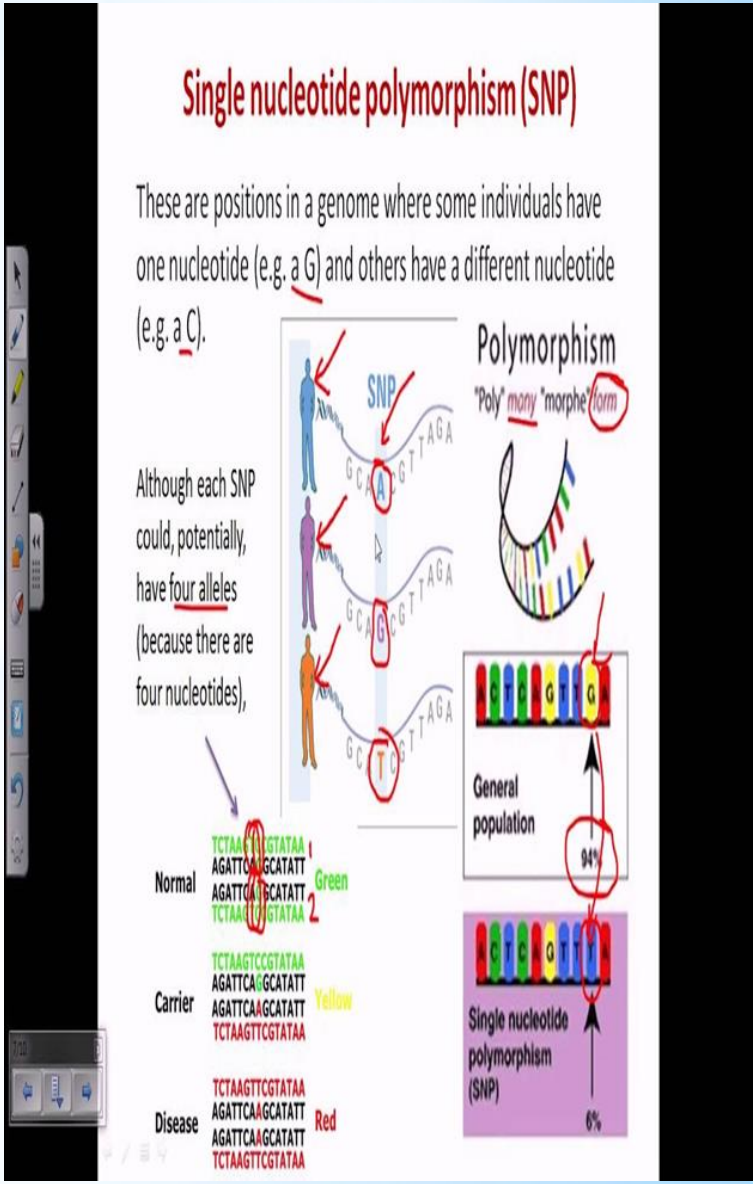
- It is widely used in Arthropod identification.
- Genes primers are available for COI barcode region. COI is widely used for distinguishing closely related and less closely related taxa.
- Alternate markers can be sequenced ,if COI barcode is not differentiating.
- It may be highly useful for taxonomic analysis.

Disadvantages

- It requires a large data base of sequences for comparison.
- Prior knowledge of barcoding region is required when applied diagnostically.
- Individual sequences may not provide sufficient discrimination, when studying cryptic species complexes.
- CO I and other mitochondrial genes are maternally inherited which may result in decreased barcode diversity and thereby slow phylogenetic inferences.

Specific DNA markers in Molecular taxonomy : Single Nucleotide Polymorphism (SNP)

- **A single-nucleotide polymorphism** (**SNP**, pronounced **snip**) is a DNA sequence variation occurring when a single nucleotide adenine (A), thymine (T), cytosine (C), or guanine (G]) in the genome (or other shared sequence) differs between members of a species or paired chromosomes in anindividual.
- A single-nucleotide polymorphism is a substitution of a single nucleotide that occurs at a specific position in the genome, where each variation is present at a level of 0.5% from person to person in thepopulation.
- SNP arise due to single nucleotide substitution (transition /trans-version) or single nucleotide (insertion or deletion).
- Here, point mutation gives rise to different alleles with a particular base at a particular mutational position.
- SNPs are the most abundant polymorphism in the genome (coding and noncoding region) of any organism.
- The SNP variables can be detected using PCR, DNA microarray and Fluorescent technology.
- They are considered as next generation markers in fisheries.



Specific DNA markers in Molecular taxonomy : Expressed Sequence Tags (ESTs)

Expressed sequence tags (ESTs) are relatively short DNA sequences (usually 200–300 nucleotides) generally generated from the 3′ ends of cDNA clones from which PCR primers can be derived and used to detect the presence of the specific coding sequence in genomic DNA.

The identification of ESTs has proceeded rapidly, with approximately 74.2 million ESTs now available in public databases (e.g. GenBank 1 January 2013, all species).
ESTs may be used to identify gene transcripts and analyse their expression analysis, and are instrumental in gene discovery and in gene-sequence determination and linkage mapping.

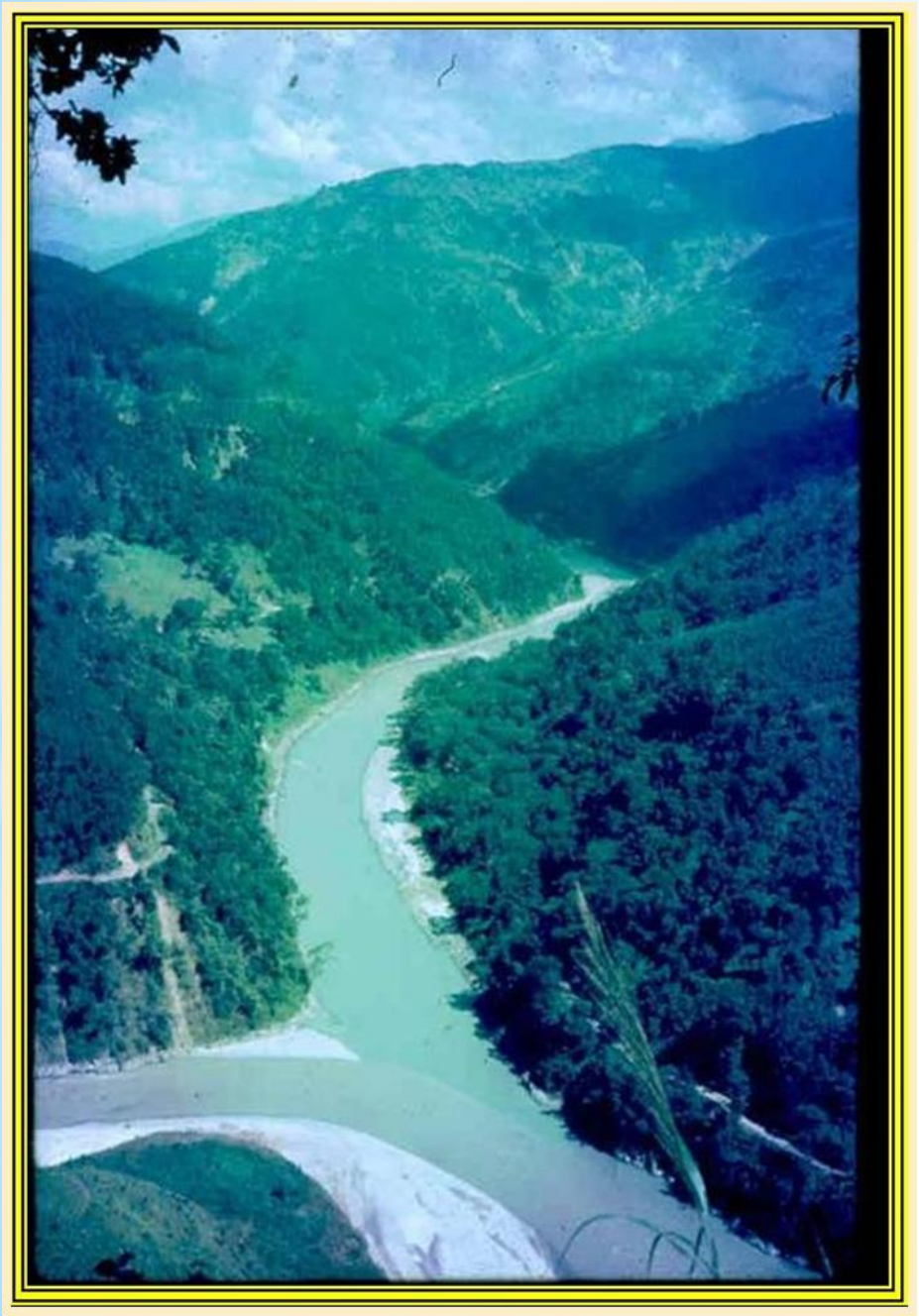
An EST results from one-shot sequencing of a cloned cDNA. The cDNAs used for EST generation are typically individual clones from a cDNA library. The resulting sequence is a relatively low-quality fragment whose length is limited by current technology to approximately 500 to 800 nucleotides.
Because these clones consist of DNA that is complementary to mRNA, the ESTs represent portions of expressed genes. They may be represented in databases as either cDNA/mRNA sequence or as the reverse complement of the mRNA, the template strand.

One can map ESTs to specific chromosome locations using physical mapping techniques, such as radiation hybrid mapping, Happy mapping, or FISH. Alternatively, if the genome of the organism that originated the EST has been sequenced, one can align the EST sequence to that genome using a computer.

The current understanding of the human set of genes (as of 2006) includes the existence of thousands of genes based solely on EST evidence. In this respect, ESTs have become a tool to refine the predicted transcripts for those genes, which leads to the prediction of their protein products and ultimately of their function. Moreover, the situation in which those ESTs are obtained (tissue, organ, disease state - e.g. cancer) gives information on the conditions in which the corresponding gene is acting. ESTs contain enough information to permit the design of precise probes for DNA microarrays that then can be used to determine gene expression profiles.It is comperatively a fast and reliable method to analyse gene expression in particular tissue type under specific physiological condition differentially expresse3d genes could be identified using

Conclusions

1. The cyto- genetical approach in fisheries science can be best elucidated in three basic areas-
   1.Genetic selection and hybridization,
   2. Sex control and sex renewal and
   3. Genetic engineering.
2. The use of molecular techniques in fisheries has increased drastically over the past several years. The RAPD, RFLP AFLP and PCR based technologies have completely revolutionized the genotyping of infectious aquatic organism and several aquatic species.
3. These are useful techniques in establishing pedigree and establishing paternity.
4. It has immense applications in Ichthyo-taxonomy.
5. Various stock identification and characterization is possible using this technique as a tool.
6. These techniques make the opportunity to analyse interspecific gene flow and hybrid speciation. It opens a vast avenue for aquatic health management.
7. Hybridization between species can be well studied by genetic analysis (Seribner et al,2000).
8. Achievements have been made in producing disease resistant varieties and other variance with higher growth rate, more flesh content , gaining length and width in intraspecific hybridization programme. Some of the successful hybrids produced are bass hybrids, Hybrids of Salmonids, Stugeron hybrid, Hybrid of cat fishes etc.
9. The genetic engineering has been used extensively in fishes to induce polyploidy and prevented chromosomal inheritance (Androgenesis and Gynogenesis) in both cultured freshwater and marine water fishes.
10. Transgenesis have been successfully applied to the fish.



THANK YOU