

Fatchiyah



Objectives

- Briefly describe VNTR, STR and RFLP
- Describe the principle, including the target, for: Southern, Western, Northern and Southwestern blots.
- Compare and contrast the 4 blotting methods.
- Given a target state the best method for detection.
- State 2 clinical applications for detecting VNTRs, STRs and RFLPs.
- Given the background information and results of a blot, interpret the results.



- A wide variety of techniques exist in the molecular laboratory.
- Knowledge of the targets and appropriate techniques to detect them are an integral part of molecular methods in the clinical laboratory.
- The following slides provide a review of targets and techniques used to detect the targets.



- VNTR variable number tandem repeats
 - Location in a genome where a short nucleotide is organized as a tandem repeat
 - These can be found on many chromosomes and often show variations in length
 - Each variant acts as an inherited allele allowing used for identification
 - Useful in genetics, biology research, forensics and DNA fingerprinting



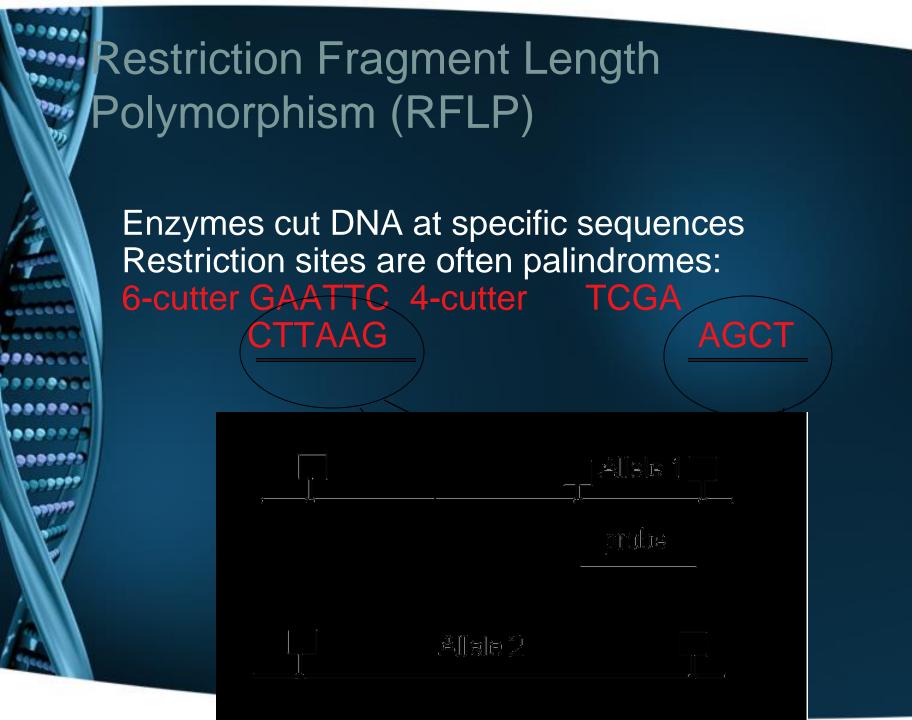
- STR short tandem repeat in DNA
 - Occurs when a pattern of TWO or more nucleotides are repeated and the repeated sequences are adjacent to each other.
 - Pattern can range in length from 2 to 10 bp
 - Typically in non-coding intron region
 - Count how many repeats of a specific STR at a given locus can create unique genetic profile
 - Currently over 10,000 published STR sequences in human genome
 - Prevalent method for determining genetic profiles in forensic cases.

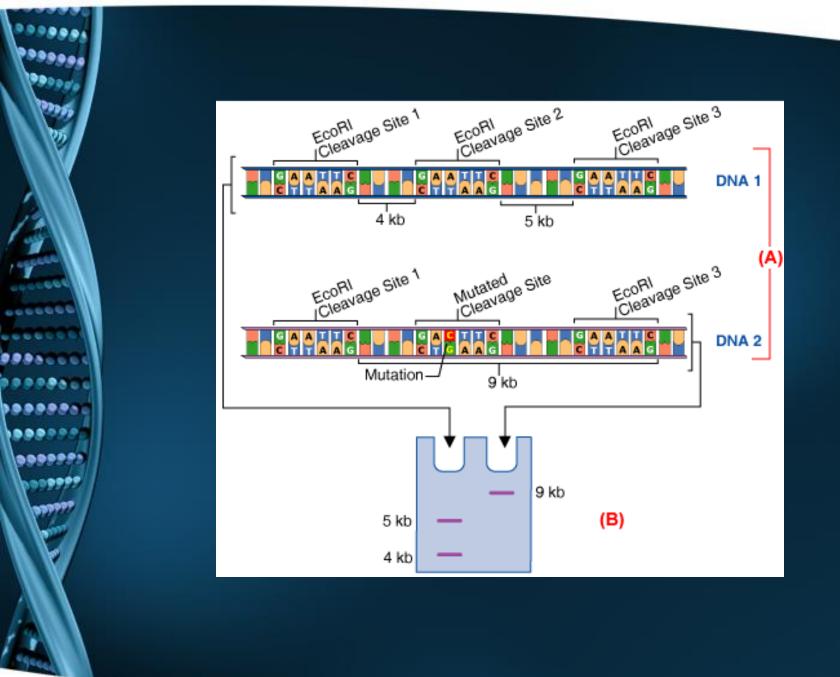


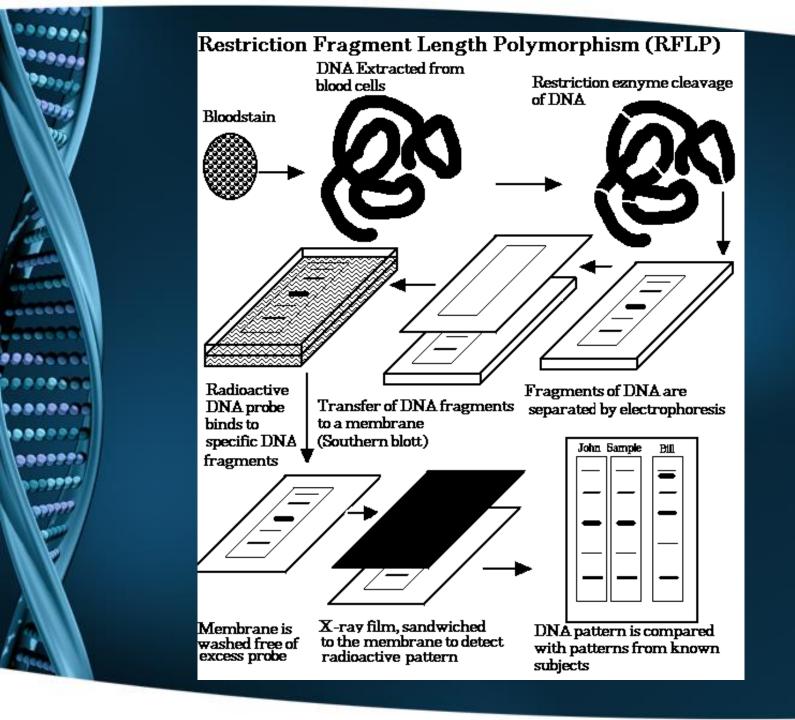
- STR
 - Analysis is performed by extracting nuclear DNA from cells of interest.
- DNA is amplified using PCR.
- Tested by gel electrophoresis or capillary electrophoresis.



- RFLP restriction fragment length polymorphism
 - Variation in the DNA sequence of a genome detected by breaking DNA into pieces with restriction enzymes.
 - Analyze fragment by gel electrophoresis
 - Important tool in genome mapping, localization of genetic disease genes, determination of risk for a disease, genetic fingerprinting and paternity testing







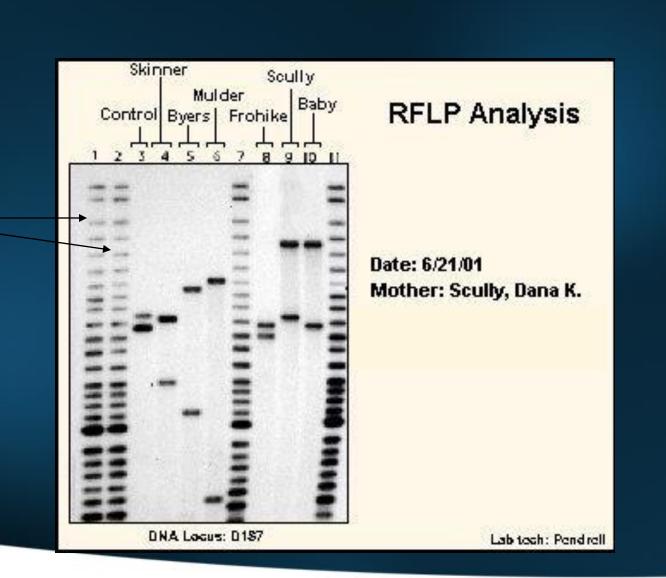


Can be used for species or population identification

Human mt DNA has 2 EcoR1 restriction sites
Honey bee mt DNA has 5 restriction sites

Q: How many bands would you see on a gel after digesting human and honey bee mtDNA with the EcoR1 restriction enzyme? Hint: mtDNA is circular in both humans and honey bees.

Can be used for analysis of relatedness





Using RFLP polymorphism to study population genetic structure and evolution

Advantages: variants are co-dominant; measures variation at the level of DNA sequence, not protein sequence.

Disadvantages: labor intensive; requires relatively large amounts of DNA



RFLP - Applications

- Agriculture direct method for selecting desirable genes such as disease resistance
- Forensics
- Genetic mapping
 - Determine disease status of an individual, ie, Huntington's chorea
 - Cystic fibrosis
 - Sickle cell anemia
- Genetic counseling very important when discussing results with patients or parents who use this technology to have children who are free of genetic disease.



Blotting

- All techniques use electrophoresis to separate.
- Difference in techniques lies in the target
- Four applications
 - Western
 - Southern
 - Northern
 - Southwestern



Western Blot

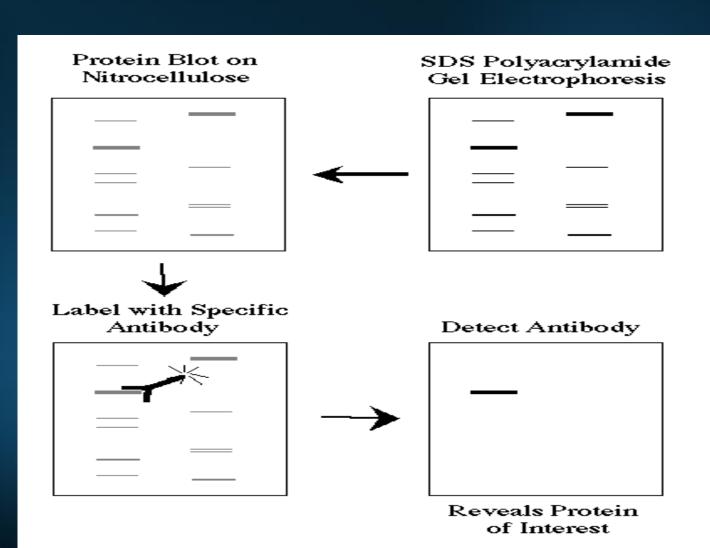
- A technique used to identify and locate proteins based on their ability to bind to specific antibodies.
- Detect protein of interest from a mixture of a great number of proteins.
- Gives information about size of protein in comparison to size marker or ladder.
- Similar in principle to ELISA, but is more specific.



Western Blot

- Procedure
 - Separate proteins by SDS-PAGE
 - Transfer proteins onto membrane
 - Add primary antibody to protein of interest
 - Add secondary antibody, specific for primary antibody, attached to an enzyme
 - Add substrate to visualize bands.
- Question: What is the purpose of the blocking agent? Hint: review SDS-PAGE link at end of presentation.



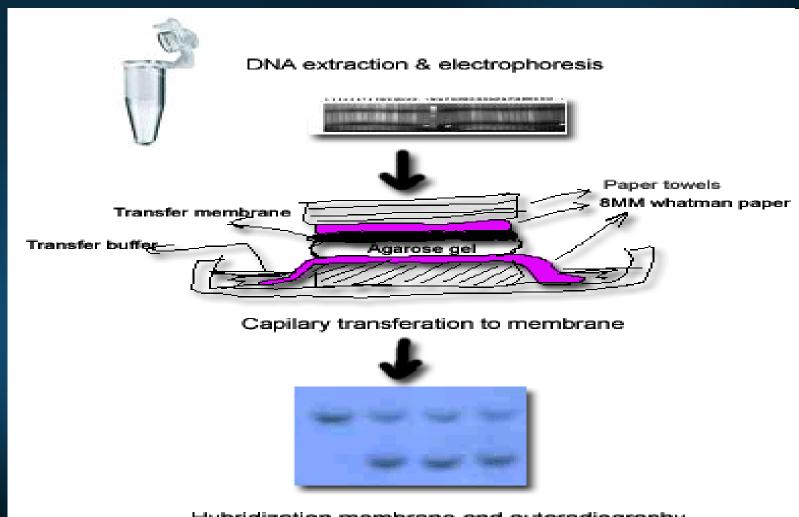




Southern Blot

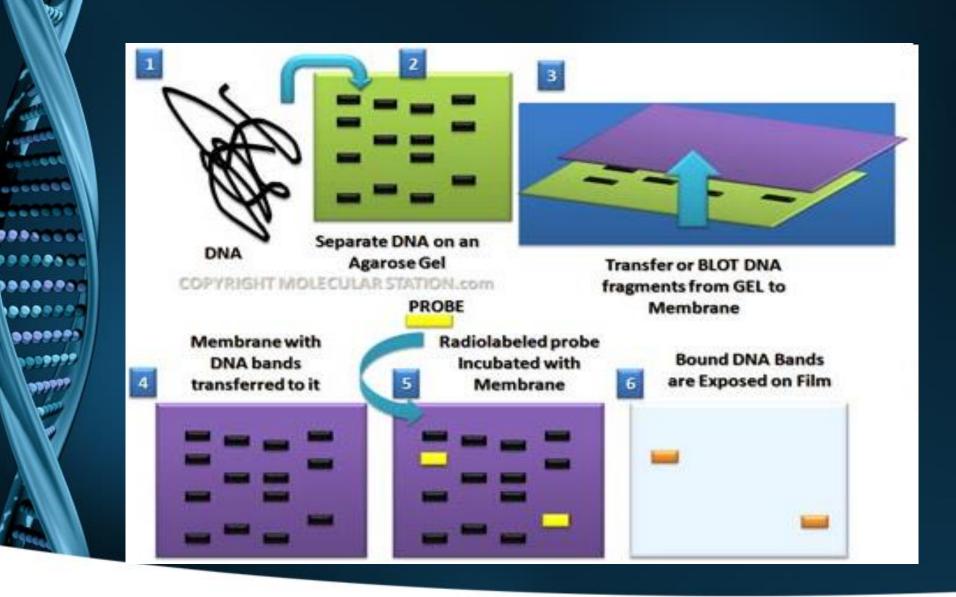
- Southern blot hybridization is one of the most commonly used molecular techniques to detect specific DNA sequences using labeled probes.
- Four steps:
 - DNA extraction
 - Electrophoresis to separate
 - Transfer to membrane
 - Use labeled probes, which will hybridize to specific sequence, to identify sequence of interest

Southern Blot



Hybridization membrane and autoradiography

Southern Blot

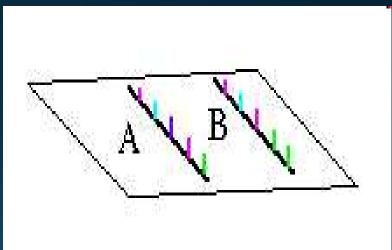


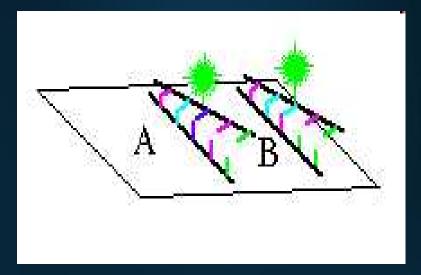


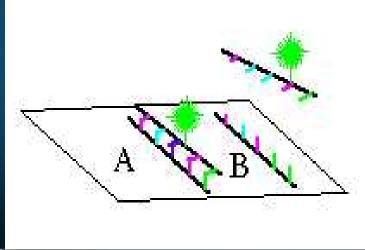
- Used to study gene expression.
- Similar to Western Blot but MAJOR difference is that RNA is analyzed.
- Gels may be run on either agarose or denaturing polyacrylamide, the latter being preferable for smaller RNA fragments.
- Formaldahyde is added to gel and acts as a denaturant to agarose.
- For polyacrylamide, urea is the denaturant.
- Not used much for diagnostic, mainly used in research.

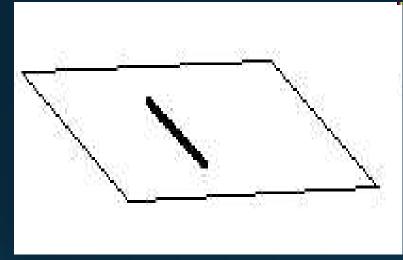


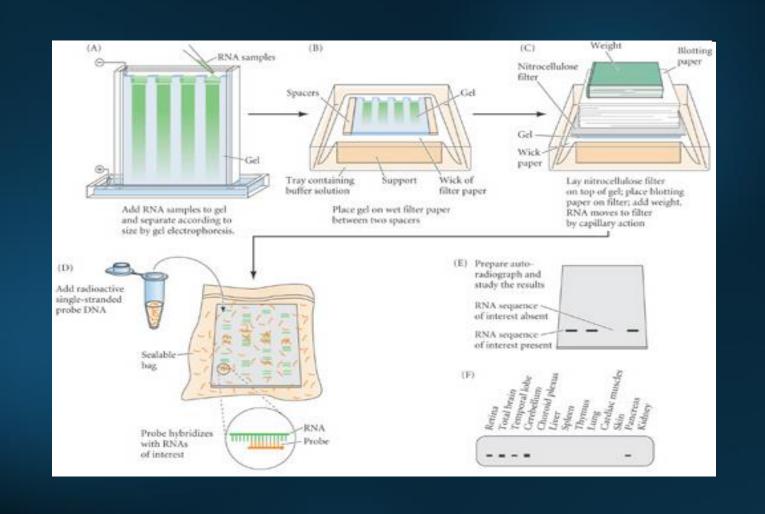
- Three types of RNA: tRNA, rRNA and mRNA
- Northern blot isolates and hybridizes mRNA
- Procedure
 - mRNA extracted from cells and purified
 - Separate with electrophoresis
 - Transfer onto membrane
 - Use labelled probes to identify mRNA of interest













Southwestern Blot

- Combines features of Southern and Western blotting techniques.
- For rapid characterization of both DNA binding proteins and their specific sites on genomic DNA.
- Involves identifying and characterizing DNAbinding proteins (proteins that bind to DNA) by their ability to bind to a specific oligonucleotide probes.
- Identification of protein factors that bind to genes to turn them on or off is therefore important in investigating gene functions.
- Primary use is for research, not clinical applications.



Southwestern Blot

- Procedure
 - Separate proteins using SDS-PAGE
 - Renatured by removing SDS in presence of urea
 - Transfer to membrane
 - Genomic DNA of interest is digested by restriction enzymes, labeled and added to separated proteins.

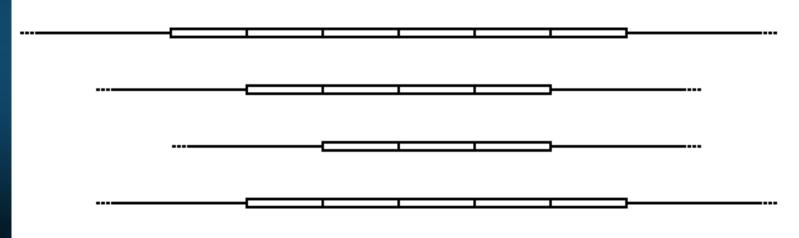
Comparison of Blotting Methods

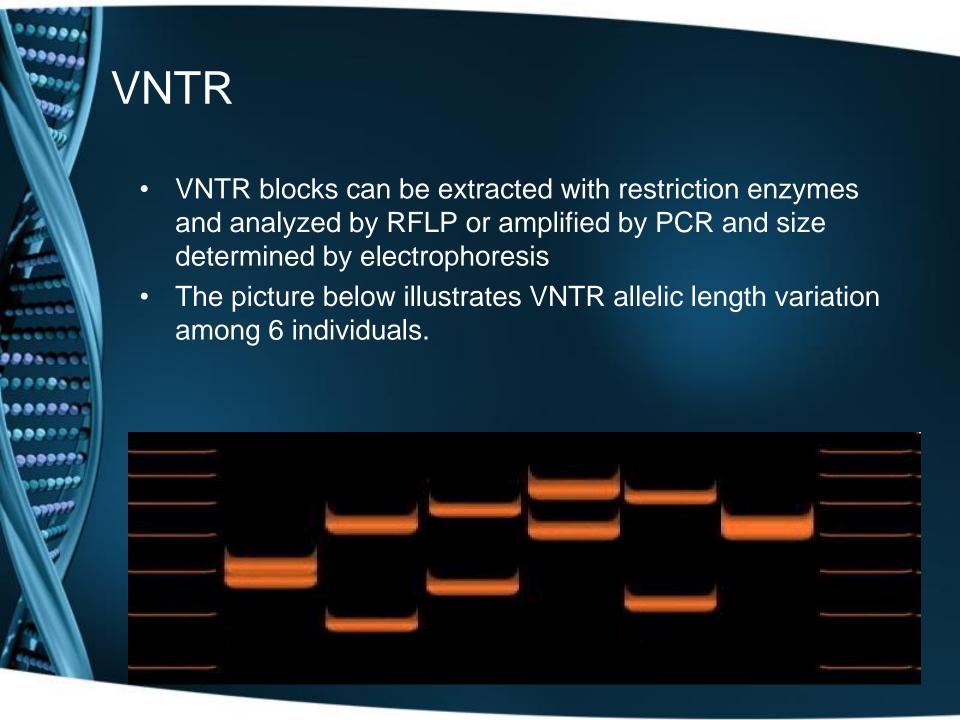
	Southern	Northern	Western	Southwestern
What is separated	DNA cut with restriction enzymes	Denatured RNA	Protein denatured with SDS	Characterizes DNA binding proteins
Probe	Radioactive gene X DNA	Radioactive gene X DNA	Antibody against protein X, labeled with enzyme or radioactivity	Labelled DNA probes
What do you learn	Restriction map of gene X chromosome	How much gene X mRNA is present. How long is gene X mRNA	How much protein X is present. How large is protein X.	Identify expression of specific DNA binding proteins.



VNTR

- Rectangle blocks represent repeated DNA sequences at a particular VNTR location
- Repeats are tandem clustered together and oriented in same direction
- Repeats can be removed or added leading to alleles with different numbers of repeats.







VNTR

- Important source of RFLP genetic markers used in linkage analysis (mapping) of genomes.
- Has become essential to forensic crime investigation.
- May use PCR.
- Size determined by gel electrophoresis and Southern blotting to produce pattern of bands unique to each individual.
- The likelihood of 2 unrelated individuals having same allelic pattern extremely improbable.
- VNTR also being used to study genetic diversity and breeding patterns in animals.



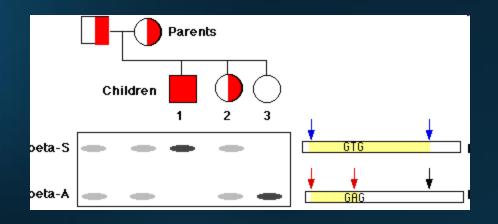
VNTR – Clinical Applications

- Microbiology
 - VNTR typing as the next gold standard in the molecular epidemiology of tuberculosis
 - Genotyping for early diagnosis of M. tuberculosis super-infection or mixed infection.
 - Visit http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi
 for list of organisms
- DNA fingerprinting



STR

- The following diagram illustrates how VNTR analysis can be used to diagnose sickle cell anemia in a family.
- Parents are carriers (heterozygotes)
- Refer to http://tinyurl.com/c9xxr2 for the case study.





STR – Applications

- Forensics
 - The Federal Bureau of Investigation (FBI) has chosen 13 specific STR loci to serve as the standard for CODIS.
 - Mass disasters
 - Paternity testing
 - Military DNA "dog tag"
 - Convicted felon DNA databases
- Bone marrow transplant follow up
 - Important for establishing graft rejection and disease relapse
 - The ratio of allele peak heights between donor and recipient good indication of success



PCR based methods: don't need much DNA

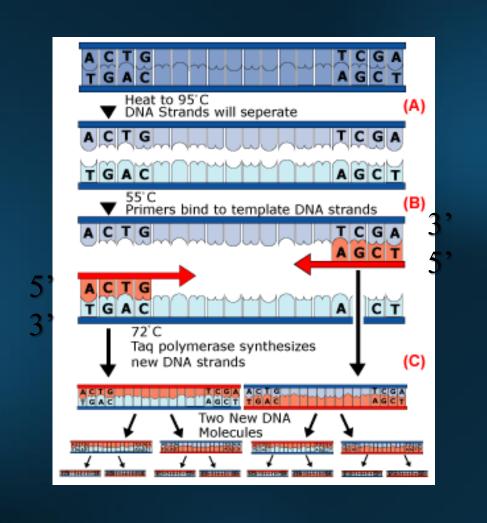
RAPD: randomly amplified polymorphic DNA

AFLP: amplified fragment length polymorphism

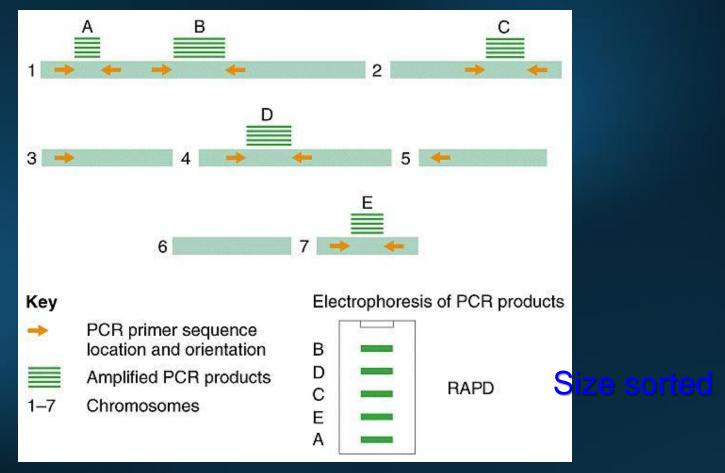
VNTR: variable number tandem repeats; including

microsatellites

PCR: polymerase chain reaction



RAPD: randomly amplified polymorphic DNA

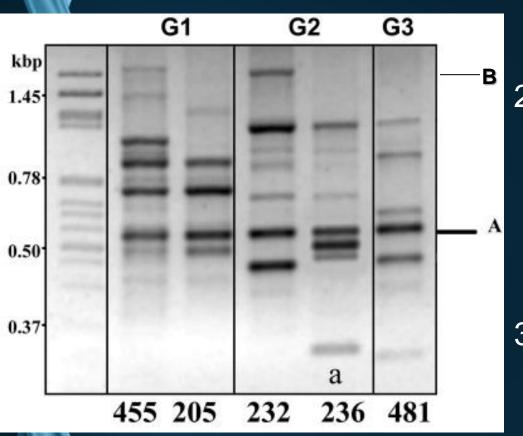




Advantages: fast, relatively inexpensive, highly variable.

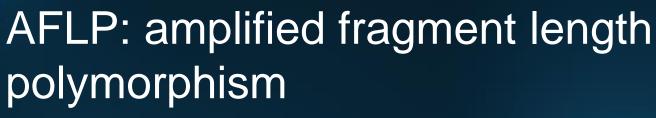
Disadvantages: markers are dominant. Presence of a band could mean the individual is either heterozygous or homozygous for the sequence--can't tell which. Data analysis more complicated.

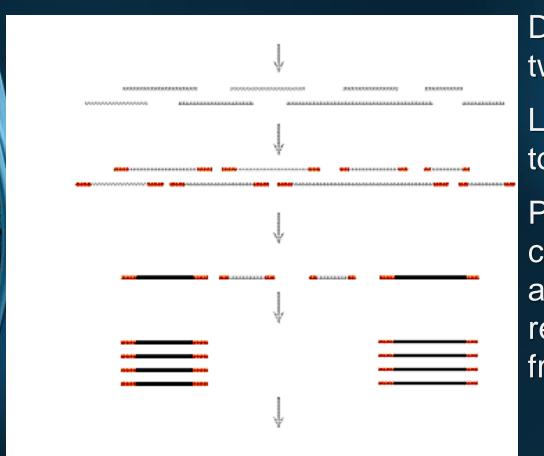
RAPD Analysis



Questions:

- 1. Is the locus represented by band "B" polymorphic?
 Band A?
- 2. Is individual 232 a homozygote or heterozygote for alleles represented by band "B"? What about individual 236?
- 3. Does band "B" represent a longer or shorter DNA fragment than band "A".

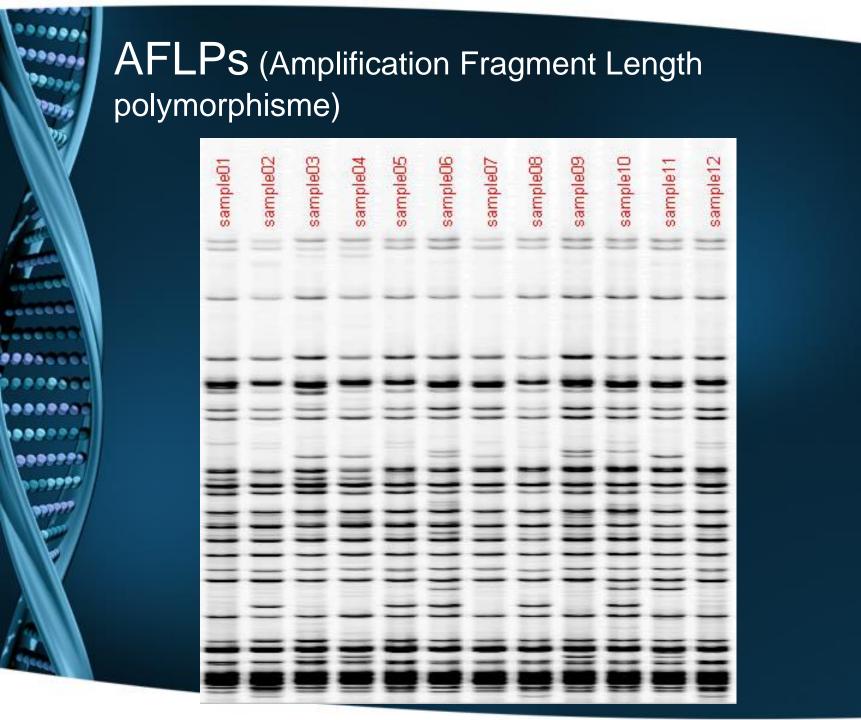




Digestion of DNA with two enzymes

Ligation of adapters to fragment ends

Primers
complementary to
adapters and to 3'
region of some of the
fragments





AFLPs

Advantages: fast, relatively inexpensive, highly variable.

Disadvantages: markers are dominant. Presence of a band could mean the individual is either heterozygous or homozygous for the sequence-can't tell which.



RAPDs and AFLPs

Good for distinguishing between populations

Often used for trait mapping studies because they

are variable between the populations that are

crossed

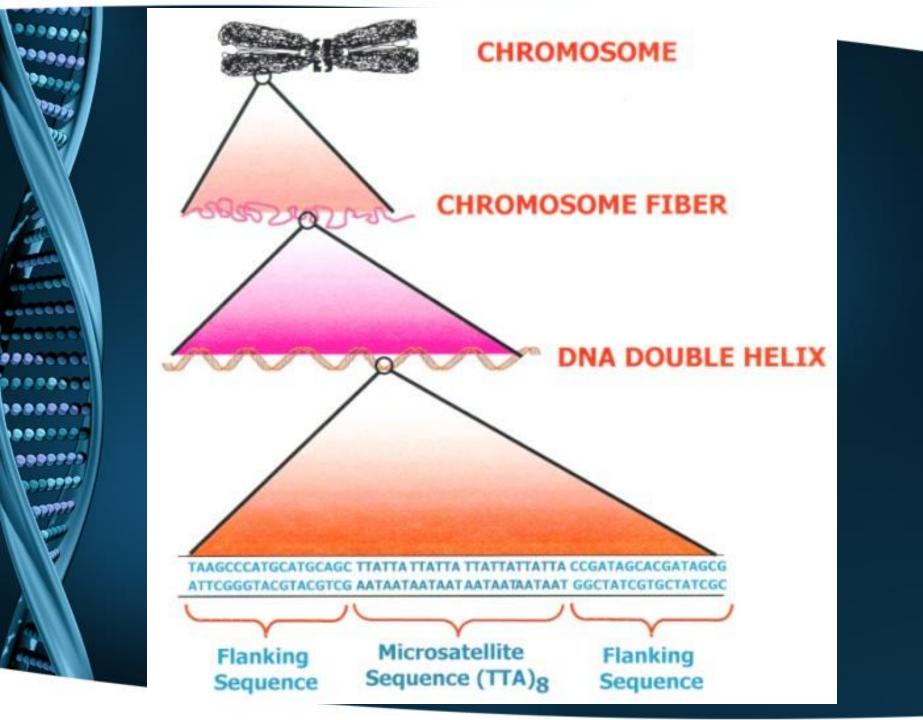


VNTR: variable number tandem repeats

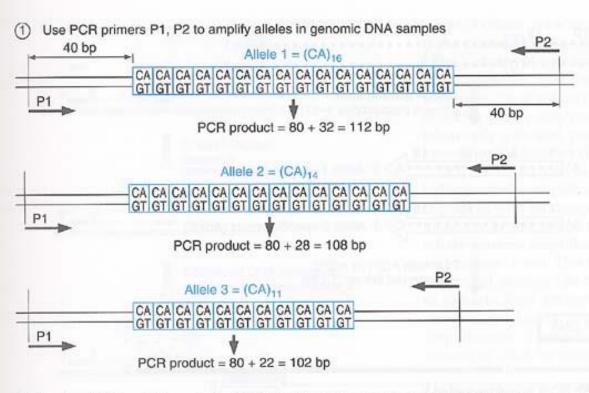
Non-coding regions
Several to many copies of the same sequence
Large amount of variation among individuals in the number of copies



- Not a tiny orbiting space craft
- Most useful VNTRs
- 2, 3, or 4 base-pair repeats
- A few to 100 tandem copies
- Highly variable
- Many different microsatellite loci (1000s) in any species



Design primers to flanking regions

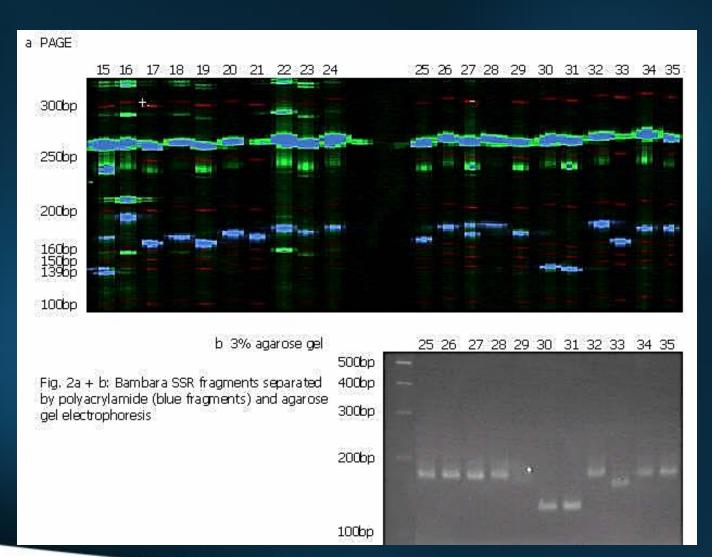


Alleles
1 = 112 {
2 = 108 {
3 = 102 {

- (2) Denature PCR products and size-fractionate by polyacrylamide gel electrophoresis
- 3 Autoradiography

Figure 6.7: PCR can be used to type short tandem repeat polymorphisms (STRPs).

Microsatellite Gels



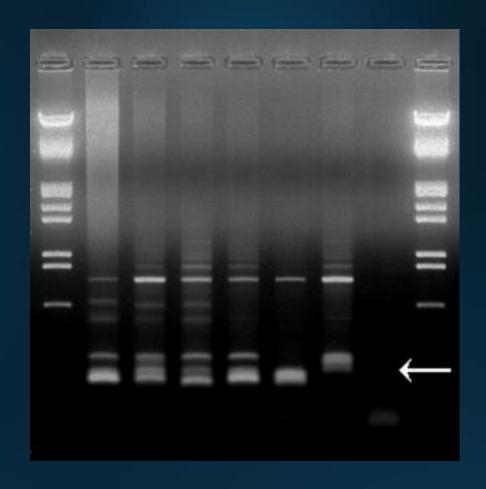


Advantages: highly variable, fast evolving, codomininant

Relatively expensive and time consuming to develop

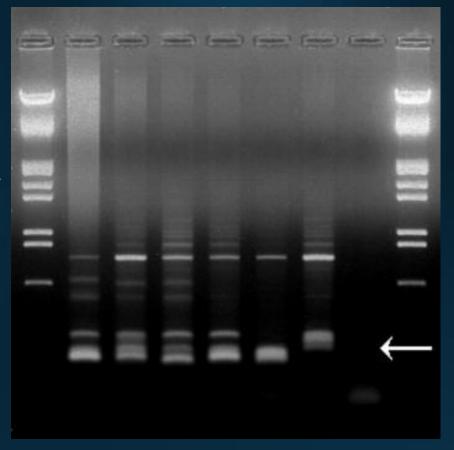
Used for withinpopulation studies;
not as much for
between-population
studies b/c they
evolve too fast

Paternity analysis and
other studies of
kinship

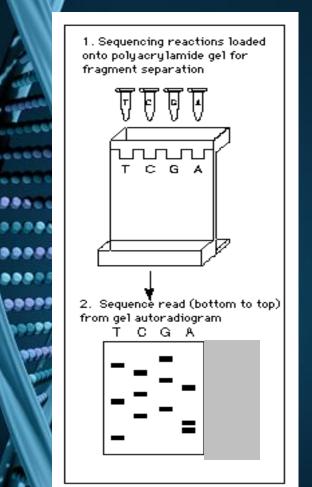


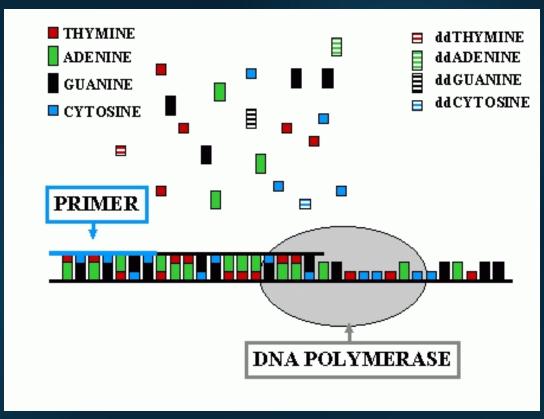
Questions:

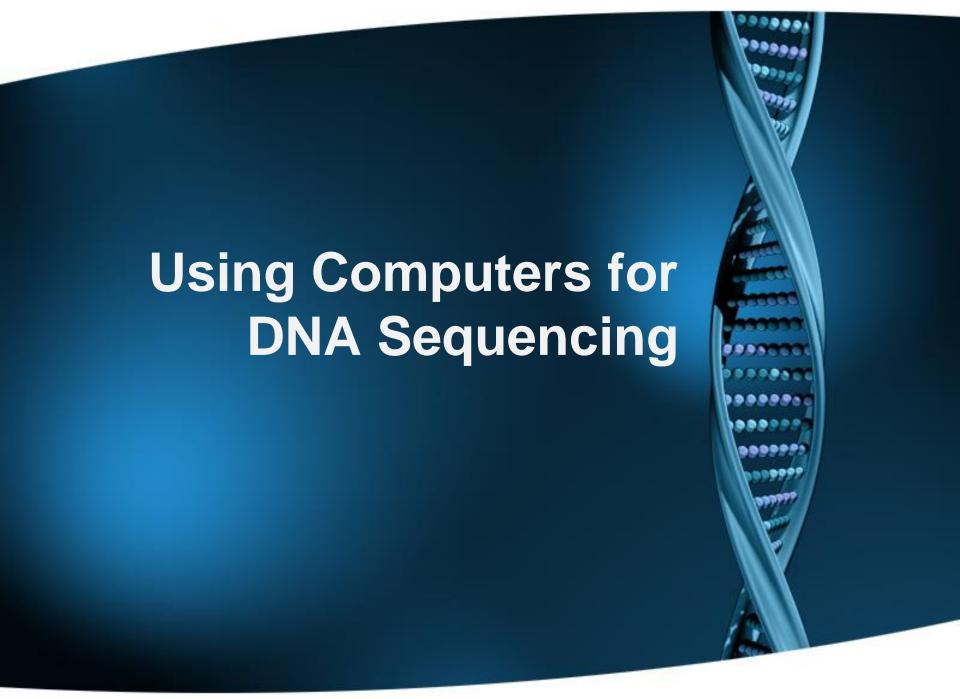
- 1. Is the locus represented by the bands at the arrow polymorphic?
- 2. If it is polymorphic, how many individuals are heterozygous?
- 3. How many individuals are homozygous for the "short" allele?



Sequencing







Sequencing

Often used for phylogenetics (especially sequences of mitochondrial genes).

Iso used for studies of molecular evolution (e.g., compare rates of synonymous vs. non-synonymous substitution)



The Human Genome Project

The Human genome is now officially sequenced. That was a big job, how did they do it?

Is there anything that a knowledge of bioinformatics tells us that we should watch out for in the human genome sequence?



The Biological Basis of DNA Sequencing Technology

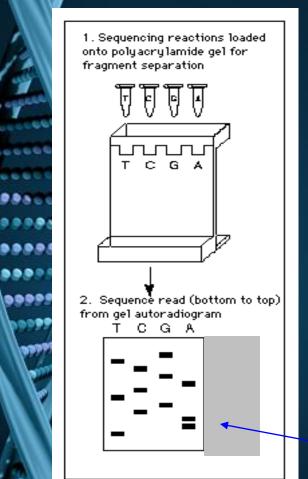
Virtually all DNA sequencing, (both automated and manual) relies on the Sanger method

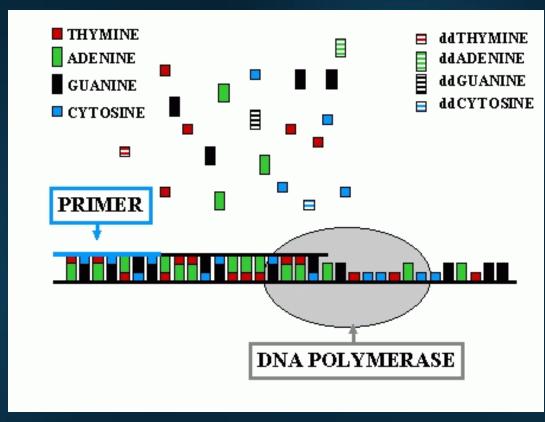
- DNA replication with dideoxy chain termination
- separation of the resulting molecules by polyacrylamide gel electrophoresis.

The DNA fragment to be sequenced must first be cloned into a vector (plasmid or lambda).

Then the cloned DNA must be copied in a test tube (in vitro) by a DNA polymerase enzyme to obtain a sufficient quantity to be sequenced.

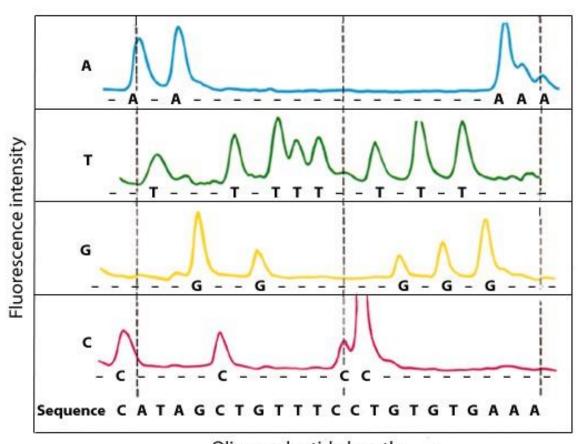
Sequencing





Q: What's the DNA sequence?

fuorescent dyes coupled to each reaction



Oligonucleotide length

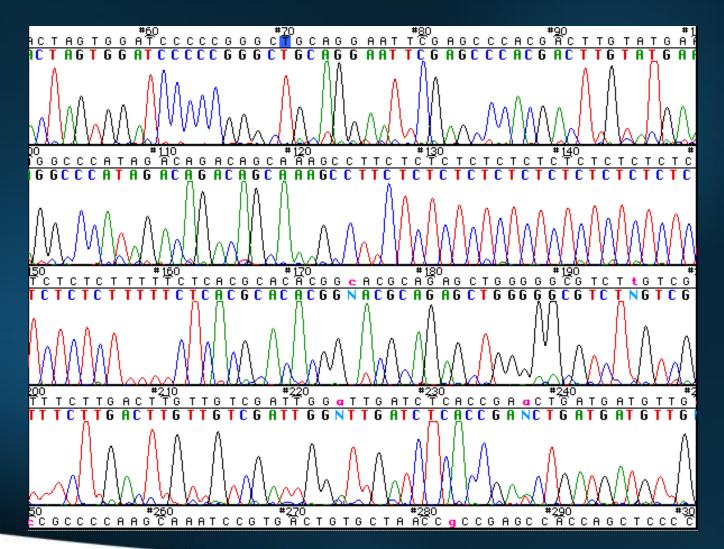


Fluorescent dye coupled to reaction allows visualization of di-deoxy termination events by means of a laser that detects the colored product.

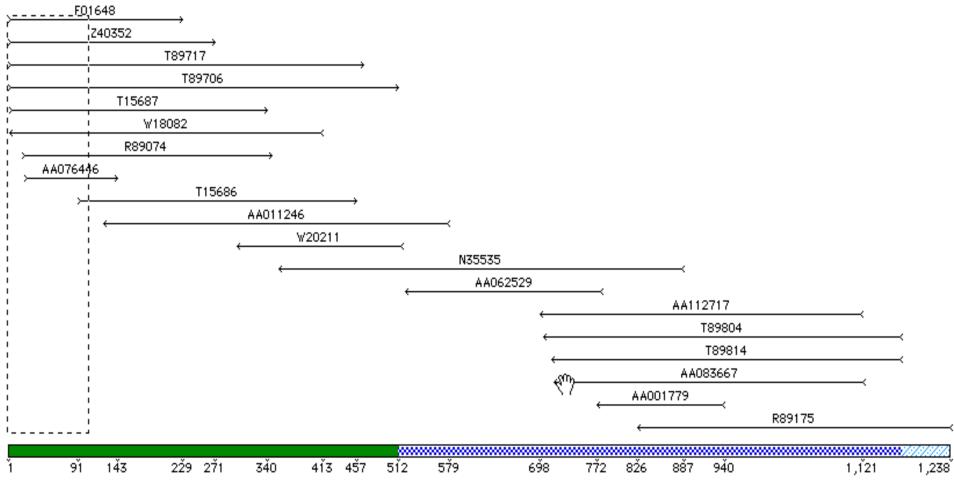
This shows four different reactions as done with the old manual sequencing.

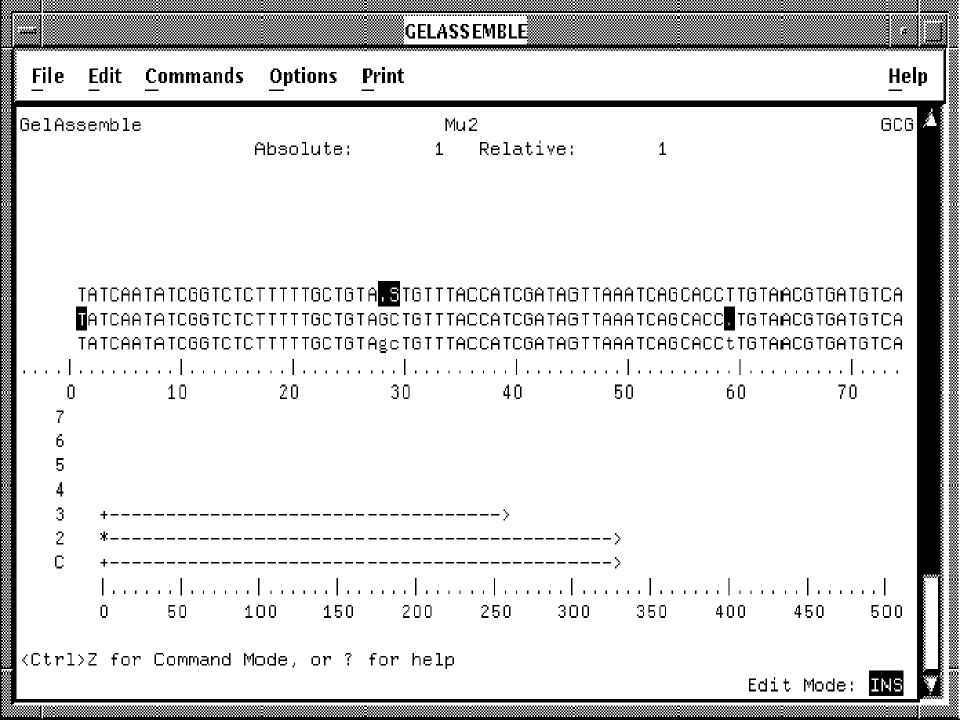
Sample DNA Sequence

from ABI sequencer

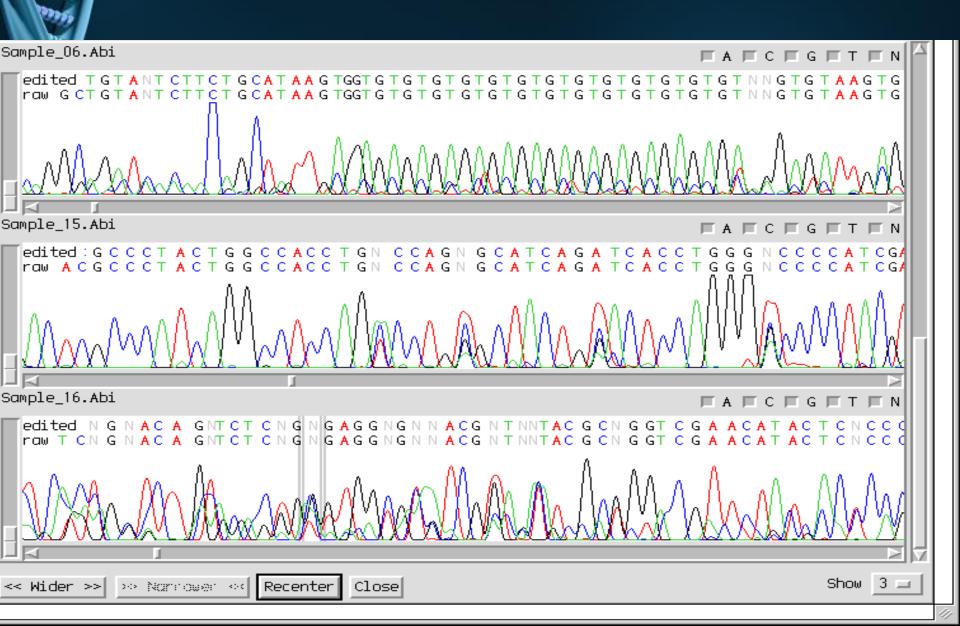


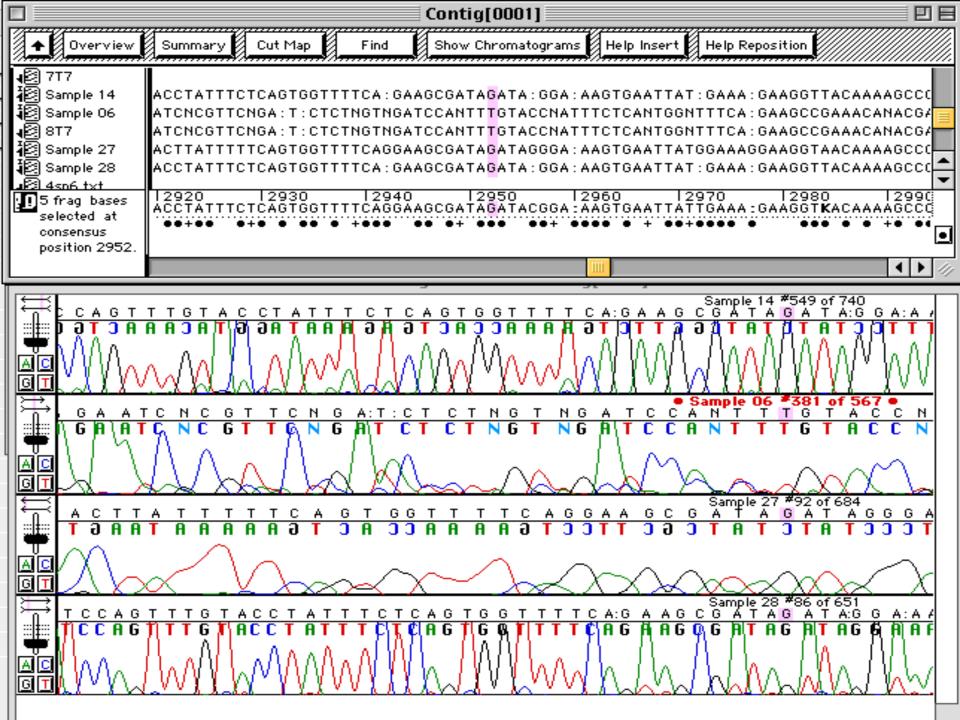


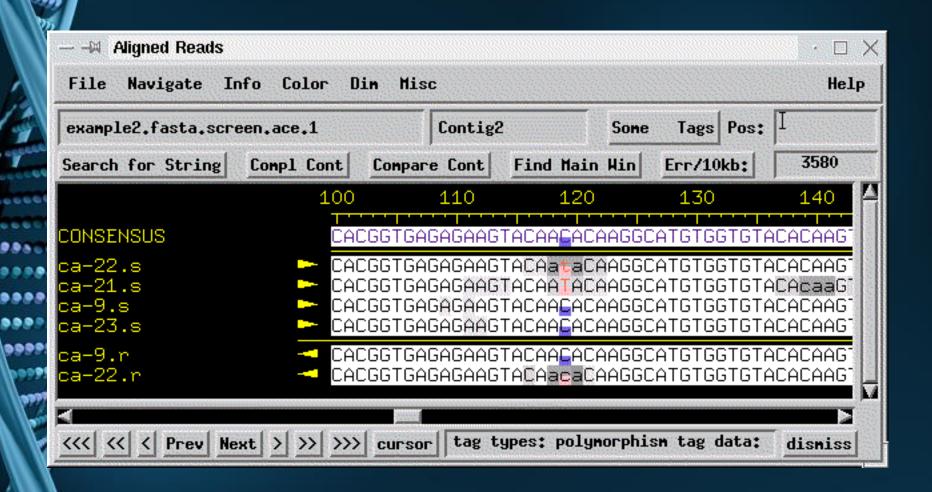


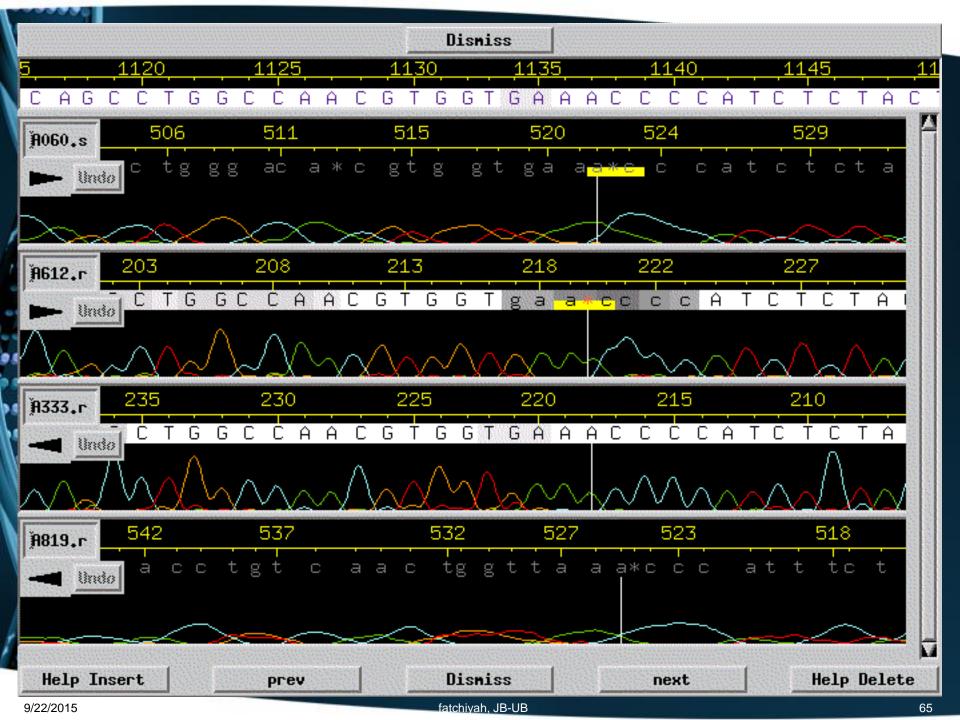


SeqLab has a Chromatogram viewer

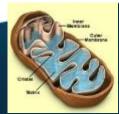




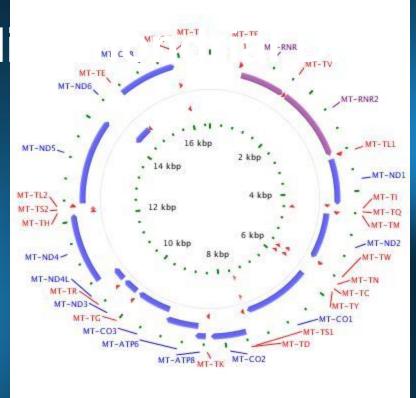








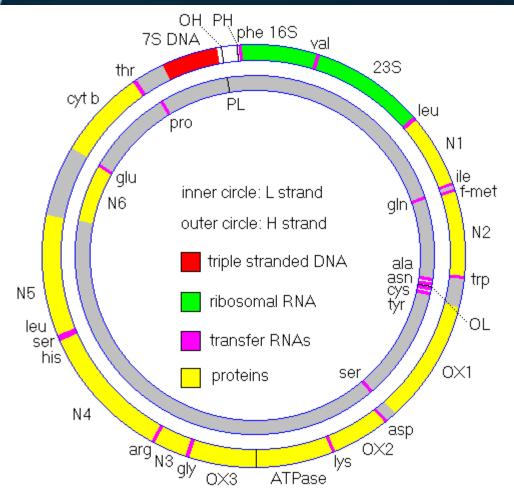
M







The human mitochondrial genome is only 10.0kb among the smallest in the animal kingdom. The circular chromosome was completely sequenced by Sanger's team in 1981 and the map is shown below:



Mitochondrial genes are among the estimated 20,000 to 25,000 total genes in the human genom. numans, mitochondrial DNA spans about 16,500 DNA building blocks (base pairs), representing a small fraction of the total DNA in cells.

What is mitochondrial DNA?

- Mitochondria are structures within cells that convert the energy from food into a form that cells can use. This genetic material is known as mitochondrial DNA or mtDNA.
- Mitochondrial DNA contains 37 genes, all of which are essential for normal mitochondrial function.
- Thirteen of these genes provide instructions for making enzymes involved in oxidative phosphorylation.
 - Oxidative phosphorylation is a process that uses oxygen and simple sugars to create adenosine triphosphate (ATP), the cell's main energy source.
 - The remaining genes provide instructions for making molecules called transfer RNA (tRNA) and ribosomal RNA (rRNA), which are chemical cousins of DNA. These types of RNA help assemble protein building blocks (amino acids) into functioning proteins.



Activities

- Review tutorial on Western Blot http://tinyurl.com/awaut3
- Review VNTR animation http://tinyurl.com/dawjau
- Review DNA fingerprinting http://tinyurl.com/co2yml
- Review DNA forensics Problem set 1 http://tinyurl.com/clsglg
- Review DNA forensics Problem set 2 http://tinyurl.com/b4tftq
- Review the RFLP applications http://tinyurl.com/djddcc



Vocabulary and Acronyms to review

- Intron
- PCR
- Restriction enzyme
- SDS-PAGE review http://tinyurl.com/5c4wrx



References

- Molecular Technologies http://tinyurl.com/b56l43
- Molecular Searching Techniques http://tinyurl.com/bfbeal
- DNA Initiative http://www.dna.gov/basics/analysis/str
- DNA Diagnostics Center http://www.forensicdnacenter.com/dna-str.html
- RFLP <u>http://tinyurl.com/djddcc</u>
- Brief History of Forensic DNA Typing http://tinyurl.com/dmbgvl