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Molecular Markers Used in Gene analysis.

ENZYMES IN GENETIC ENGINEERING

RESTRICTION NUCLEASES

EXO & ENDO NUCLEASES

Types of markers:

1- Non-DNA markers

Protein and isozyme markers.

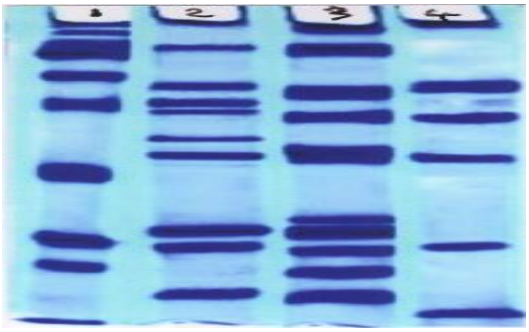
Advantages:

- ✓ **Require relatively simple equipment.**
- ✓ **A robust complement to the morphological**
- ✓ **Assessment of variation**

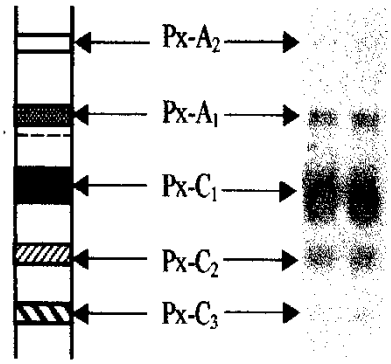
Disadvantages:

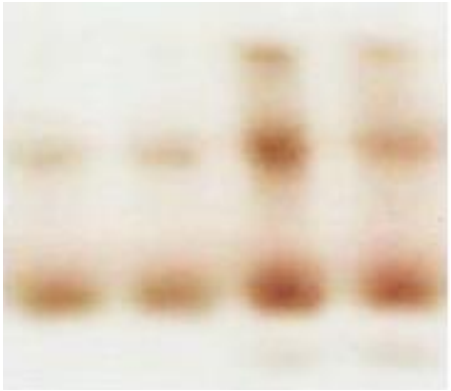
- ✓ **Subject to environmental influences**
- ✓ **Limited in number**

Protein markers



Isozyme markers





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2- DNA based markers:

✓ **Markers based on the differences in the DNA profiles of individuals.**

What is a DNA marker?

- A way of determining a position along a chromosome.
- Markers may identify one specific nt or may be cloned segments of DNA or simply amplified via PCR.
- Single copy markers occur in regions of unique sequence.
- Multiple copy markers contain sequences that are repeated in the genome and thus have multiple positions.
- Genetic markers detect a polymorphism (difference) in the DNA between two individuals.
- Polymorphic markers can be scored to determine the ancestral origin of a portion of DNA; may be co-dominant (distinguish heterozygous from homozygous genotypes) or dominant/recessive (+/-)

- **Factors to consider when choosing markers:**

- Abundance
- Level of polymorphism
- Locus specificity
- Codominance / dominance
- It should be:
- Reproducible
- Evenly and frequently distributed
- Easy, fast and cheap to detect.
- Quantity of DNA required for analysis.
- (RFLPs = 5-10µg, PCR =5-100ng per reaction)

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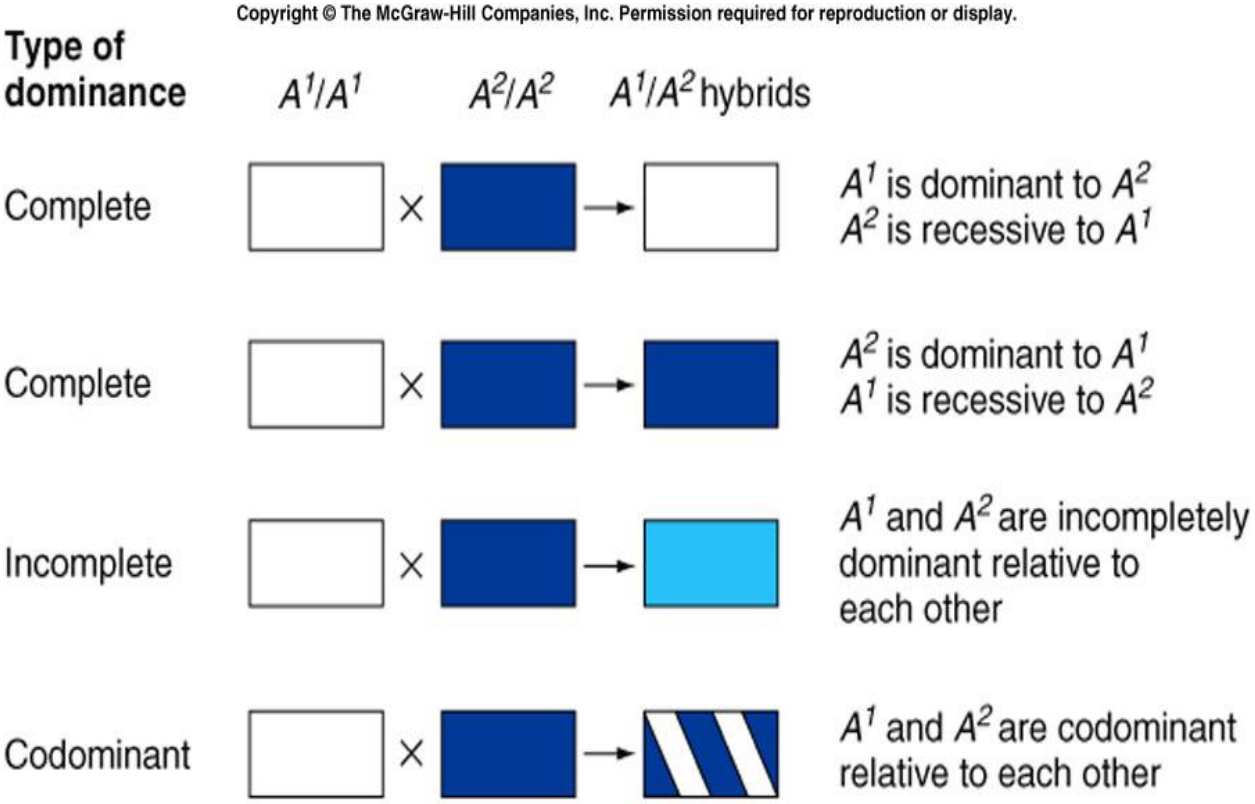
Interactions Between Alleles



Molecular techniques grouped into:

- Non-PCR based approaches*:* Restriction fragment length polymorphism (RFLP).
- PCR based techniques: Random Amplified Polymorphic DNA (RAPD), microsatellites or Simple Sequence repeat (SSR), Amplified Fragment Length Polymorphism (AFLP), *etc.*
- Targeted PCR: Sequence tagged sites (STS), sequence characterized amplified region (SCAR), *etc.*

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RESTRICTION NUCLEASES

Introduction:

A **restriction enzyme** is a nuclease enzyme that cleaves DNA sequence at random or specific recognition sites known as restriction sites. In bacteria, restriction enzymes form a combined system (restriction + modification system) with modification enzymes that methylate the bacterial DNA. Methylation of bacterial DNA at the recognition sequence typically protects the own DNA of the bacteria from being cleaved by restriction enzyme.

There are two different kinds of restriction enzymes:

(1) **Exonucleases** catalyses hydrolysis of terminal nucleotides from the end of DNA or RNA molecule either 5'to 3' direction or 3' to 5' direction. Example: exonuclease I, exonuclease II etc.

(2) **Endonucleases** can recognize specific base sequence (restriction site) within DNA or RNA molecule and cleave internal phosphodiester bonds within a DNA molecule. Example: EcoRI, Hind III, BamHI etc.

History:

In 1970 the first restriction endonuclease enzyme Hind II was isolated. For the subsequent discovery and characterization of numerous restriction endonucleases, in 1978 Daniel Nathans, Werner Arber, and Hamilton O. Smith were awarded the Nobel Prize for Physiology or Medicine. Since then, restriction enzymes have been used as an essential tool in recombinant DNA technology.

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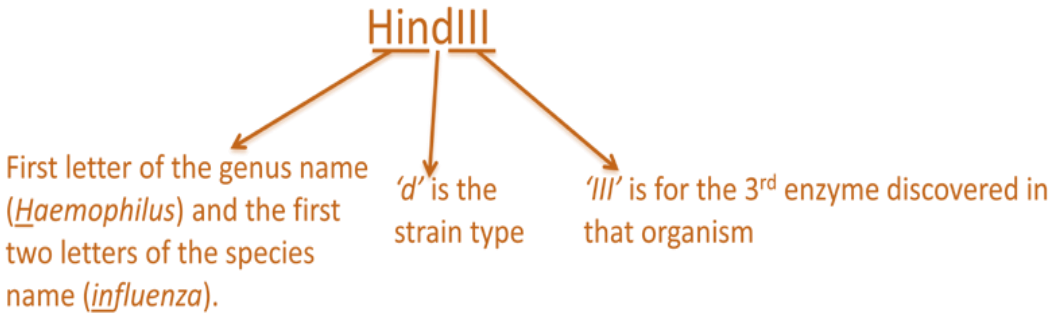
Restriction Endonuclease Nomenclature:

Restriction endonucleases are named according to the organism in which they were discovered, using a system of letters and numbers. For example, *HindIII* (pronounced *"hindee-three"*) was discovered in *Haemophilus influenza* (strain d). The Roman numerals are used to identify specific enzymes from bacteria that contain multiple restriction enzymes indicating the order in which restriction enzymes were discovered in a particular strain.



Classification of Restriction Endonucleases:

There are three major classes of restriction endonucleases based on the types of sequences recognized, the nature of the cut made in the DNA, and the enzyme structure:

- Type I restriction enzymes.
- Type II restriction enzymes.
- Type III restriction enzymes.

Type I restriction enzymes:

- These enzymes have both restriction and modification activities. Restriction depends upon the methylation status of the target DNA.
- Cleavage occurs approximately 1000 bp away from the recognition site.
- The recognition site is asymmetrical and is composed of two specific portions in which one portion contains 3–4 nucleotides while another

portion contain 4–5 nucleotides and both the parts are separated by a non-specific spacer of about 6–8 nucleotides.

- They require S-adenosylmethionine (SAM), ATP, and magnesium ions (Mg2+) for activity.
- These enzymes are composed of mainly three subunits, a specificity subunit that determines the DNA recognition site, a restriction subunit, and a modification subunit.

Type II restriction enzymes:

- Restriction and modification are mediated by separate enzymes, so it is possible to cleave DNA in the absence of modification. Although the two enzymes recognize the same target sequence, they can be purified separately from each other.
- Cleavage of nucleotide sequence occurs at the restriction site.
- These enzymes are used to recognize rotationally symmetrical sequences which is often referred to as palindromic sequence.
- These palindromic binding site may either be interrupted (e.g. BstEII recognizes the sequence 5´-GGTNACC-3´, where N can be any nucleotide) or continuous (e.g. KpnI recognizes the sequence 5´-GGTACC-3´).
- They require only Mg^{2+} as a cofactor and ATP is not needed for their activity.
- Type II endonucleases are widely used for mapping and reconstructing DNA *in vitro* because they recognize specific sites and cleave just at these sites.

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The steps involved in DNA binding and cleavage by a type II restriction endonuclease:

- These enzymes have nonspecific contact with DNA and initially bind to DNA as dimmers.
- The target site is then located by a combination of linear diffusion or "sliding" of the enzyme along the DNA over short distances and hopping/jumping over longer distances.
- Once the target restriction site is located, the recognition process (coupling) triggers large conformational changes of the enzyme and the DNA, which leads to activation of the catalytic center.
- Catalysis results in hydrolysis of phosphodiester bond and product release.

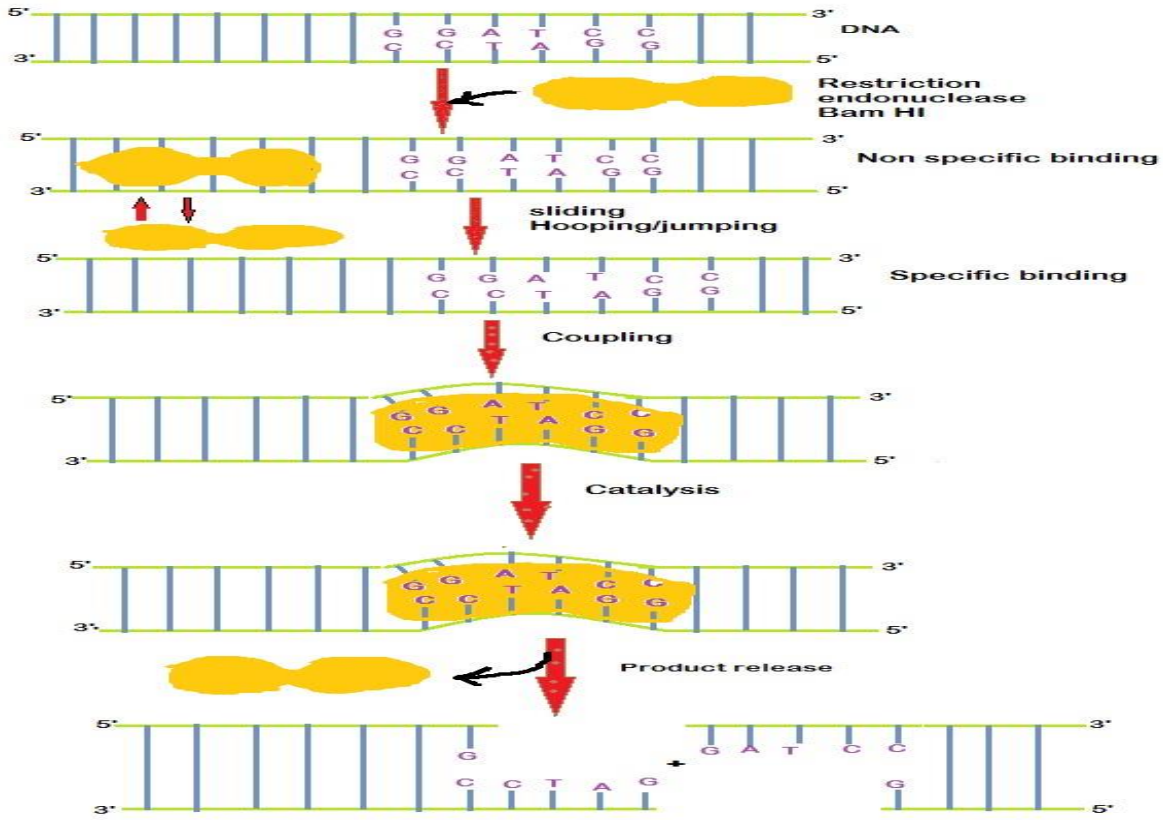

Fig: Structures of free, nonspecific, and specific DNA-bound forms of BamHI.

The two dimers are shown in brown, the DNA backbone is in green and the bases in gray. *BamH*I becomes progressively more closed around the DNA as it goes from the nonspecific to specific DNA binding mode.

Type III restriction enzymes:

- These enzymes recognize and methylate the same DNA sequence but cleave 24–26 bp away.
- They have two different subunits, in which one subunit (M) is responsible for recognition and modification of DNA sequence and other subunit (R) has nuclease action.
- Mg^{+2} ions, ATP are needed for DNA cleavage and process of cleavage is stimulated by SAM.
- Cleave only one strand. Two recognition sites in opposite orientation are necessary to break the DNA duplex.

Property	Type I RE	Type II RE	Type III RE
Abundance	Less common than Type II	Most common	Rare
Recognition site	Cut both strands at a non-specific location > 1000 bp away from recognition site	Cut both strands at a specific, usually palindromic recognition site (4-8 bp)	Cleavage of one strand, only 24-26 bp downstream of the 3′ recognitions Site.
Restriction and modification	Single multifunctional enzyme	Separate nuclease and methylase	Separate enzymes sharing a common subunit
Nuclease subunit structure	Heterotrimer	Homodimer	Heterodimer
Cofactors	ATP, Mg2+, SAM	Mg2+	Mg2+ (SAM)
DNA cleavage requirements	Two recognition sites in any orientation	Single recognition site	Two recognitions sites in a head-to-head orientation
Enzymatic turnover	No	Yes	Yes
DNA translocation	Yes	No	No
Site of methylation	At recognition site	At recognition site	At recognition site

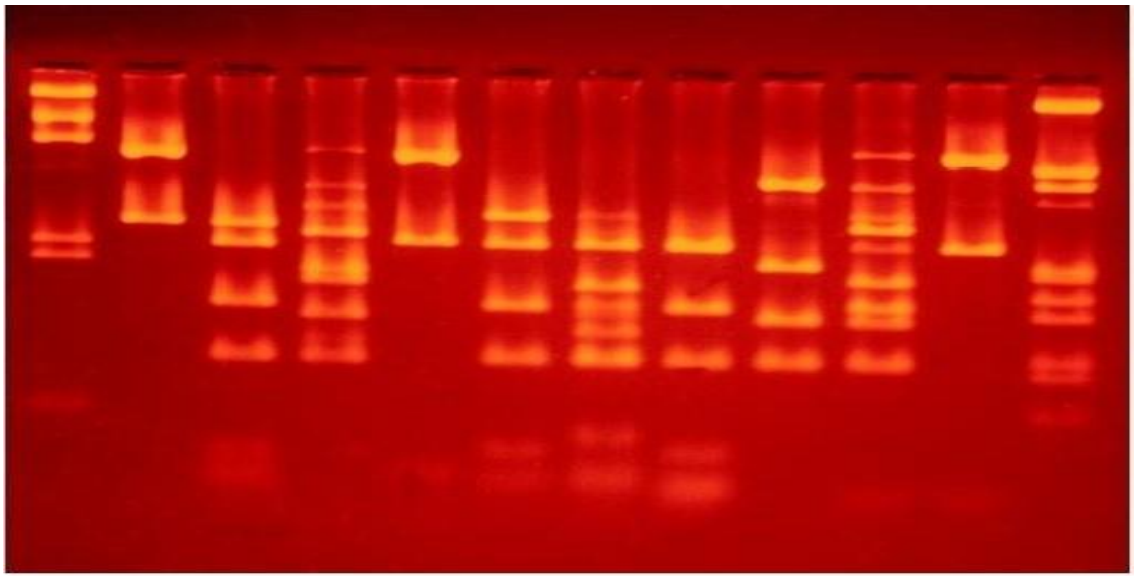

Fig : Cleaving a DNA sequence by a restriction enzyme creates a specific pattern .

Cleaving a single piece of DNA with multiple restriction enzymes creates a "DNA fingerprint." The pattern of fragments can be compared to similar DNA from another source treated with the same enzymes, to determine if the two are identical or different.

Cleavage Patterns of Some Common Restriction Endonucleases:

The recognition and cleavage sites and cleavage patterns of *HindIII*, *SmaI*, *EcoRI*, and *BamHI* are shown. Cleavage by an endonuclease creates DNA sequence with either a sticky end or blunt end. The blunt ended fragments can be joined to any other DNA fragment with blunt ends using linkers/adapters, making these enzymes useful for certain types of DNA cloning experiments.

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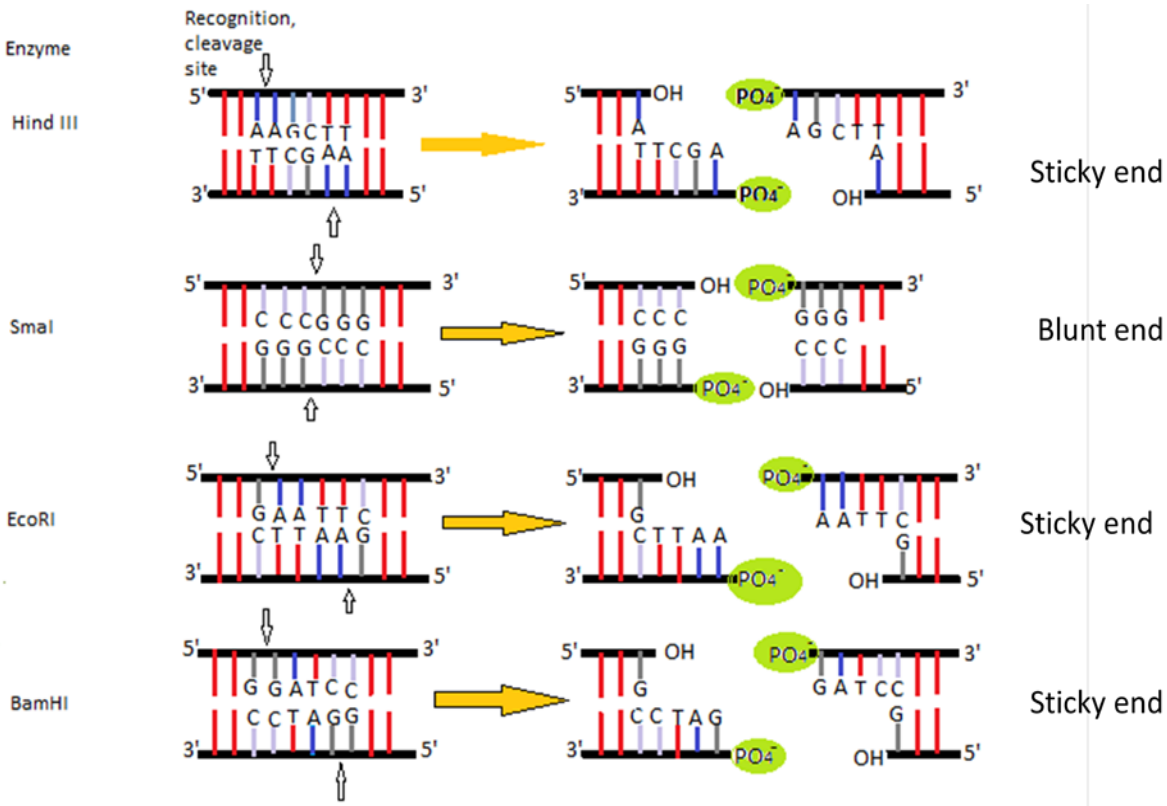

Fig: Cleavage patterns of *HindIII, SmaI, EcoRI* and *BamHI*

Applications:

In various applications related to genetic engineering DNA is cleaved by using these restriction enzymes.

- They are used in the process of insertion of genes into plasmid vectors during gene cloning and protein expression experiments.
- Restriction enzymes can also be used to distinguish gene alleles by specifically recognizing single base changes in DNA known as single nucleotide polymorphisms (SNPs). This is only possible if a mutation alters the restriction site present in the allele.
- Restriction enzymes are used for Restriction Fragment Length Polymorphism (RFLP) analysis for identifying individuals or strains of a particular species.