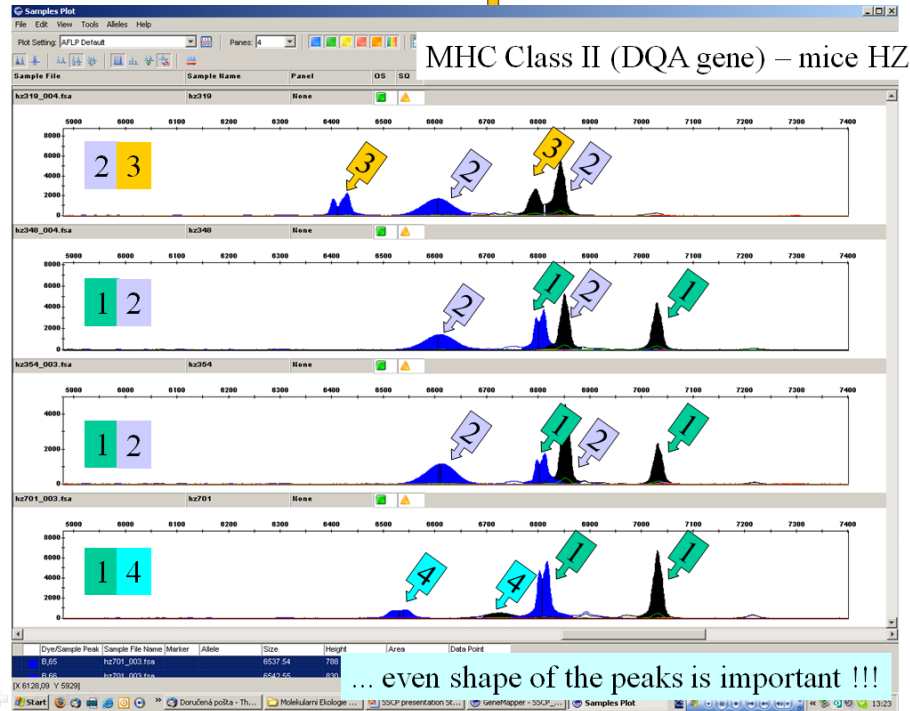
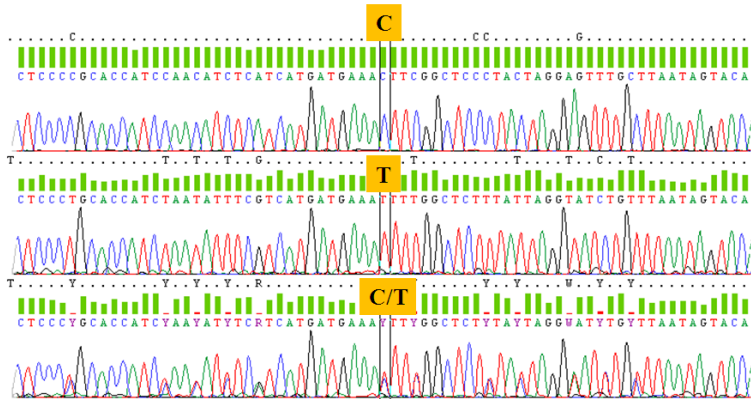


SNPs genotyping - sekvenování? Je drahé a nejasné u heterozygotů

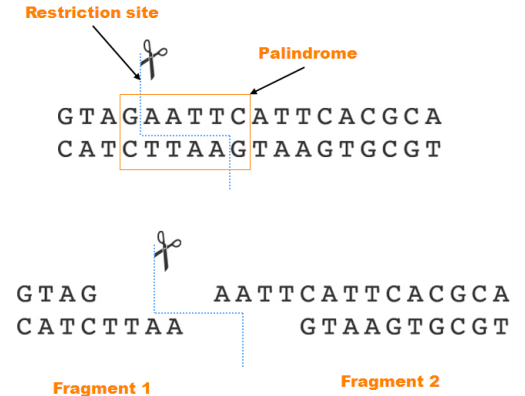


SNP genotyping - old standards

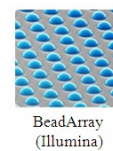
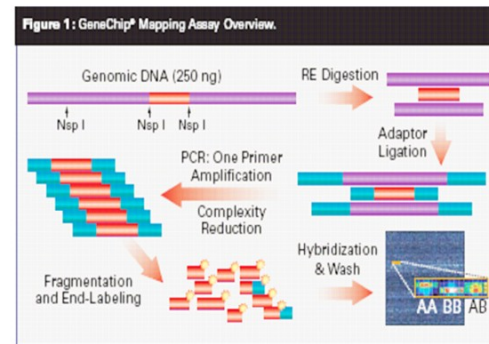
PCR-RFLP
(restriction fragments length polymorphism)

Enzyme Site Recognition

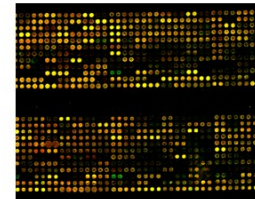
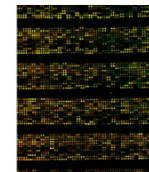
- Each enzyme digests (cuts) DNA at a specific sequence = restriction site
- Enzymes recognize 4- or 6- base pair, palindromic sequences (eg GAATTC)



Detekce: Affymetrix, Illumina



BeadArray (Illumina)



10 – 500 tisíc SNP znaků najednou – „chip technology“

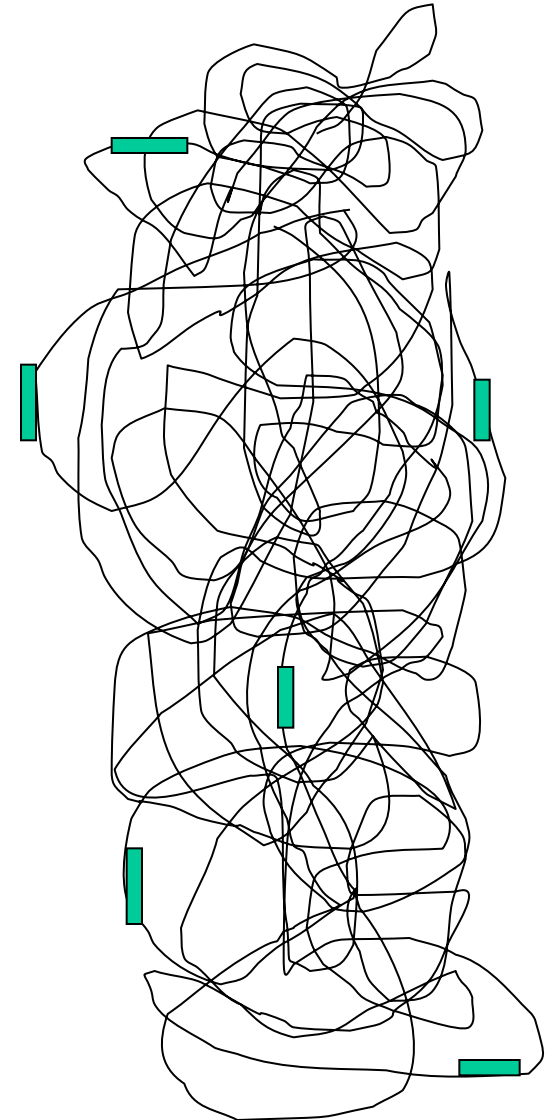
Typy genetických markerů

	Single locus	Codominant	PCR assay	Overall variability
Nuclear multilocus				
Nuclear single locus				
Alozymy	Yes	Yes	No	Low-medium
Mikrosatelite	Yes	Yes	Yes	High
SINE (LINE)	Yes	Yes	Yes	Low
SNPs (sekvence)	Yes	Yes	Yes	Low-high

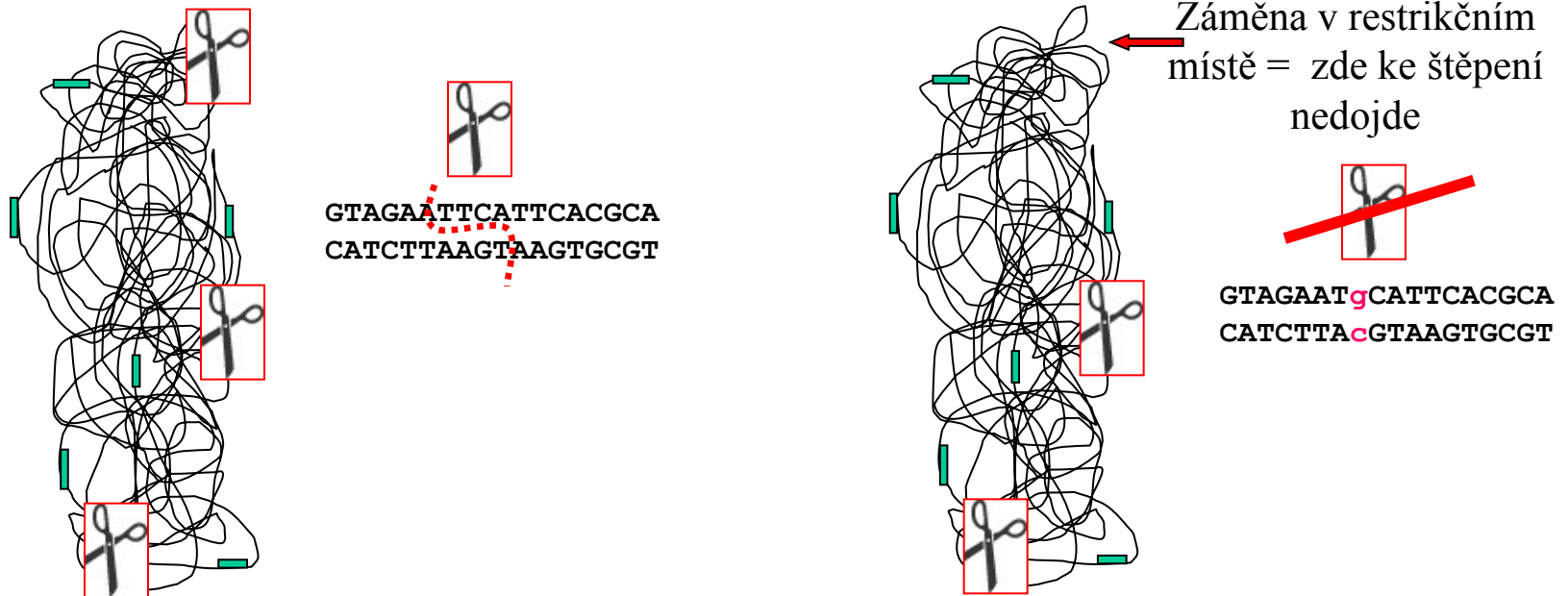
Multi-locus genetic markers

- Mnoho znaků náhodně rozmístěných v genomu - celogenomový scan
- *minisatellite DNA fingerprinting*
- *RAPD* (randomly amplified polymorphic DNA)
- *AFLP* (amplified fragment length polymorphism)
- presence vs. absence **restrikčního místa** (AFLP) či **místa pro dosednutí primerů** (RAPD) = **dominantní znaky** (neodliší heterozygota - proužek na gelu buď je nebo není)
- není nutno znát předem genom studovaného druhu (tj. primery)

Př.: chromozóm 1



Každý jedinec má jedinečný genom



1. Ztráta nebo nabytí restričního místa

Enzyme Site Recognition

- Each enzyme digests (cuts) DNA at a specific sequence = restriction site
- Enzymes recognize 4- or 6- base pair, palindromic sequences (eg GAATTC)

Restriction site

Palindrome

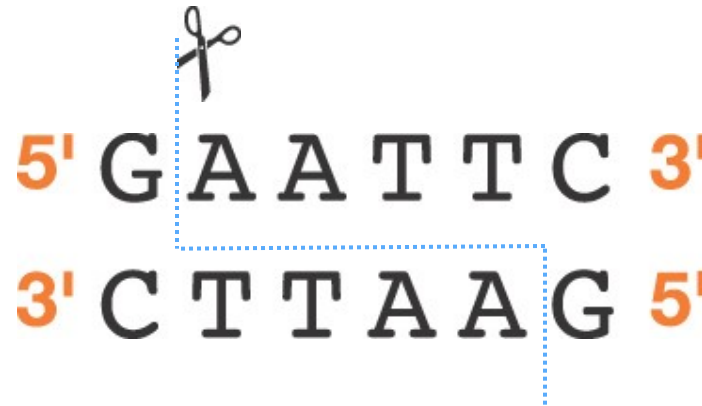
G T A G G A A T T C A T T C A C G C A
C A T C T T A A G T A A G T G C G T

G T A G A A T T C A T T C A C G C A
C A T C T T A A G T A A G T G C G T

Fragment 1

Fragment 2

Common Restriction Enzymes



EcoRI

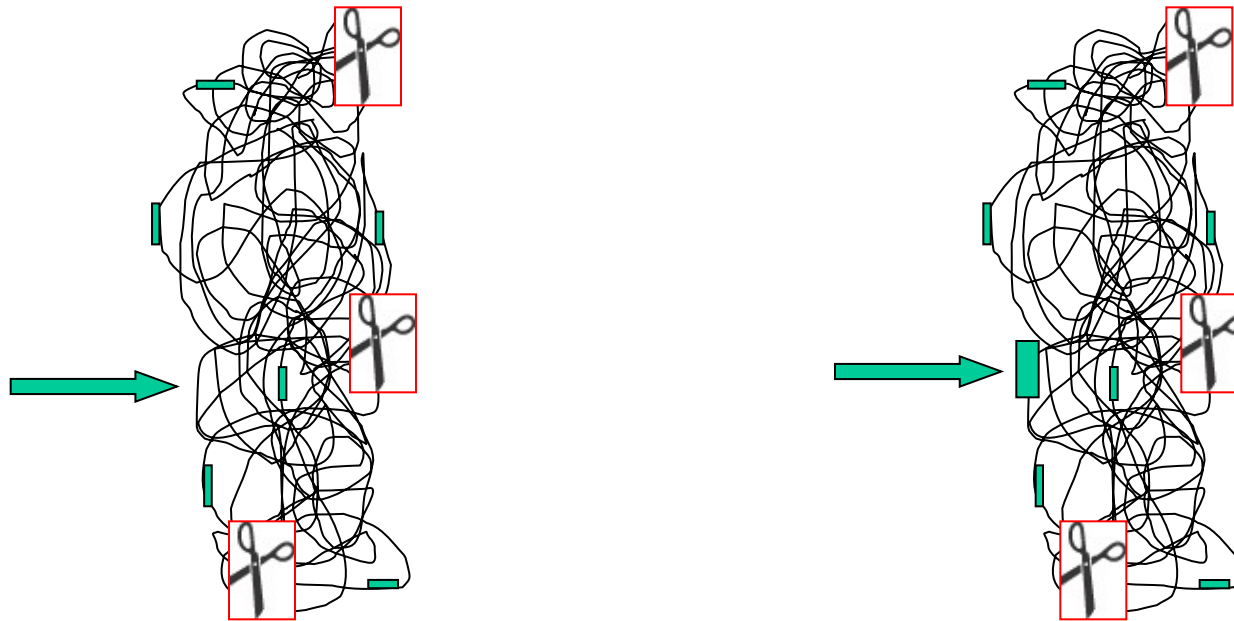
- *Escherichia coli*
- 5 prime overhang



PstI

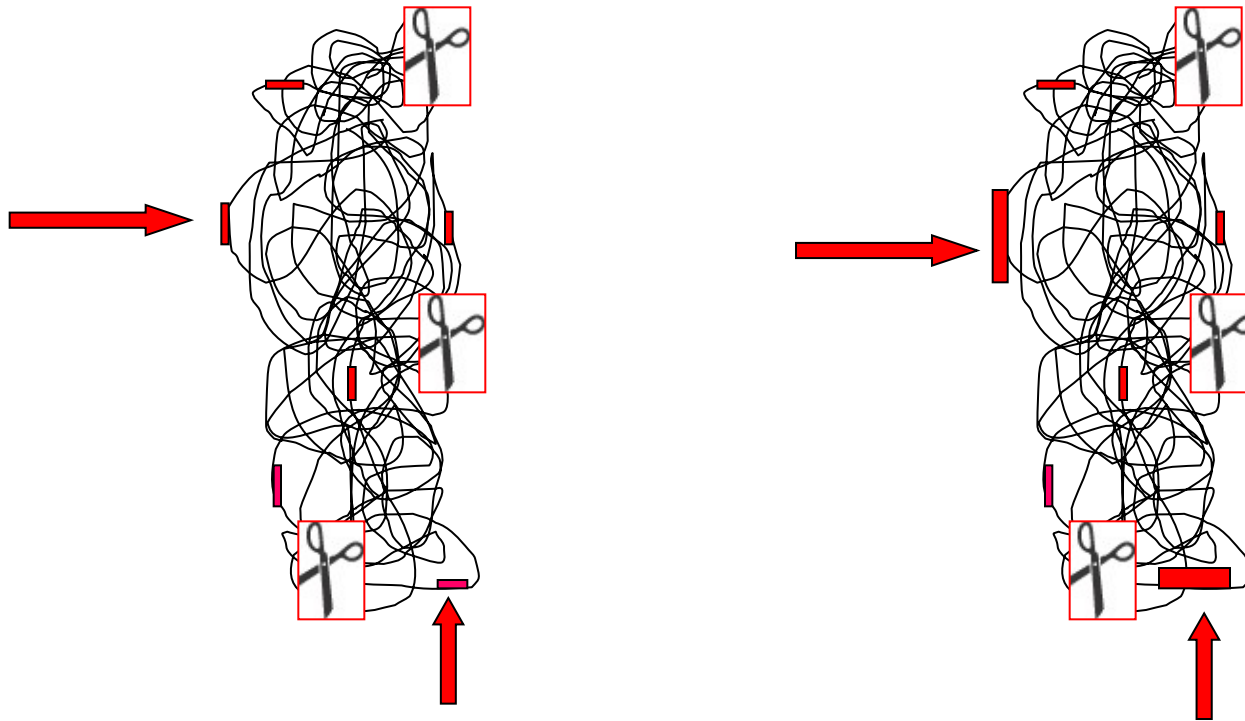
- *Providencia stuartii*
- 3 prime overhang

Každý jedinec má jedinečný genom



2. Ztráta nebo nabytí SINE (např. **Alu** sekvence) nebo LINE

Každý jedinec má jedinečný genom



3. Vysoká mutační rychlost **minisatelitů a mikrosatelitů** -
rozdíly v počtu repeticí, tj. v délce daného úseku

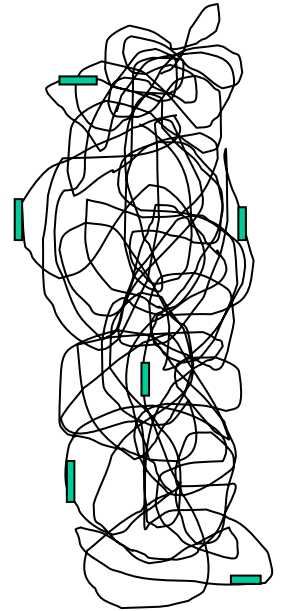
Repetitivní DNA

DNA	Typical sequence length (bp)	Location
Satellites ($>10^6$ repeats/genome)	5-100	Tandem arrays, scattered throughout the genome
Minisatellites ($>10^3$ loci/genome)	20-300	Tandem arrays up to 5 kb in length, scattered throughout the genome
Microsatellites ($>10^4$ loci/genome)	1-6	Tandem arrays up to a few 100 bp in length, scattered throughout the genome
Telomeres	4-8	Tandem arrays up to 1kb in length, at the ends of each chromosome
SINEs ($>10^5$ /genome)	50-500 (100-300)	Interspersed throughout the genome
LINEs ($>10^3$ /genome)	1-5 k	Interspersed throughout the genome

(Minisatellite) DNA fingerprinting

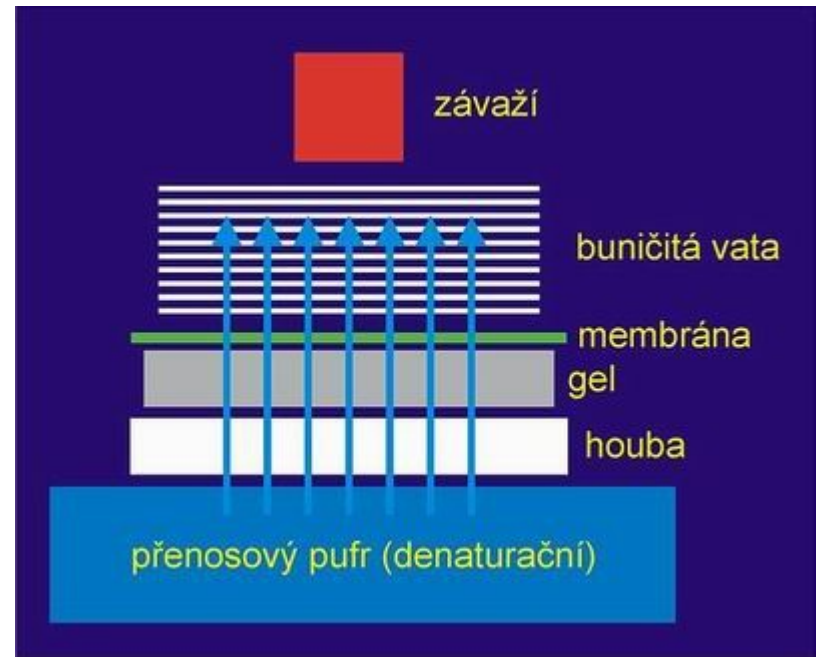
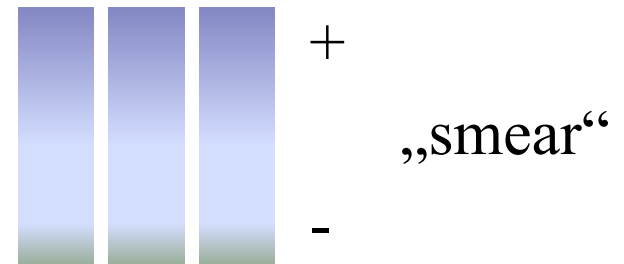
(Jeffreys et al. 1985)

- první celogenomový screening
- restriční štěpení kompletní DNA – sekvenčně specifické **restriční endonukleázy**



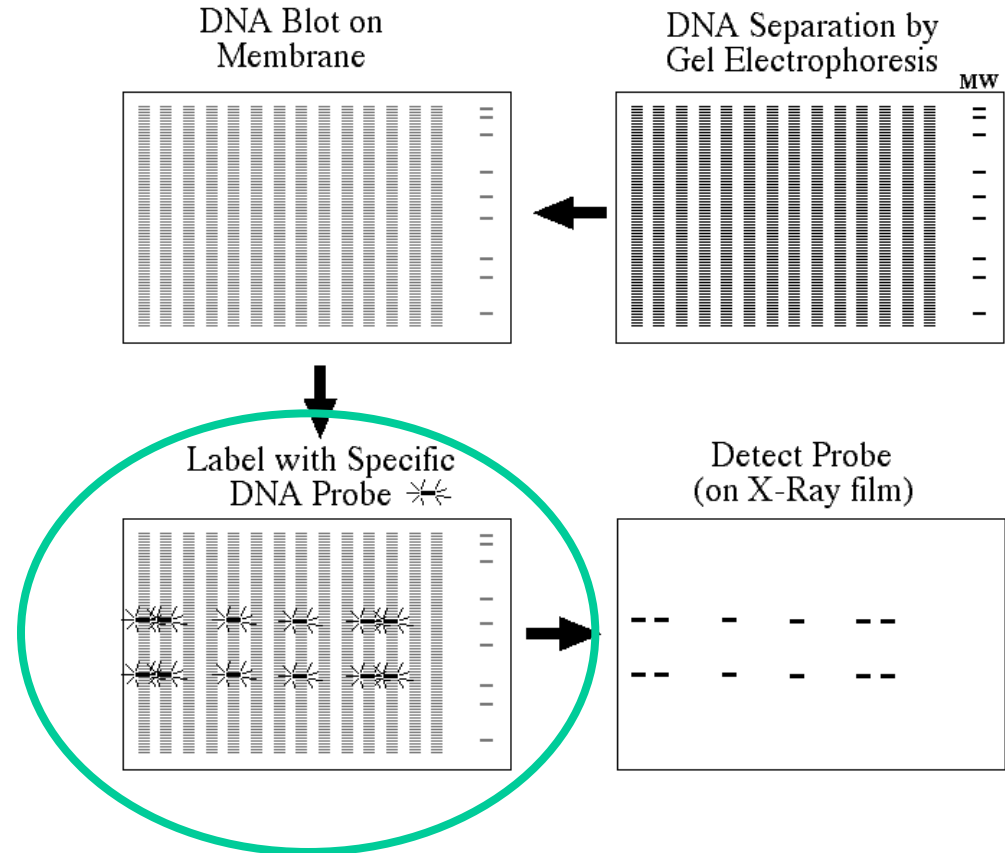
Minisatellite DNA fingerprinting

- elektroforéza rozštěpené DNA
- Southern blotting – přenesení DNA na membránu



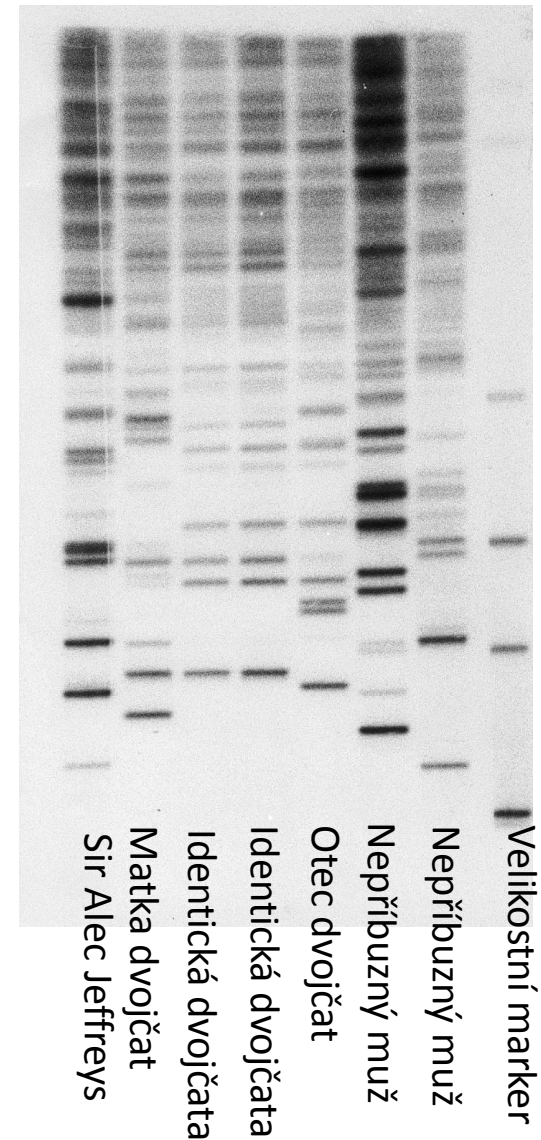
Minisatellite DNA fingerprinting

- elektroforéza
- Southern blotting – přenesení DNA na membránu
- hybridizace se značenou sondou (nejčastěji radioaktivní značení), tj. specifickou sekvencí odpovídající danému minisatelitu (popř. SINE)



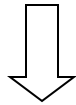
Minisatellite DNA fingerprinting

- elektroforéza
- Southern blotting – přenesení DNA na membránu
- hybridizace se značenou sondou, tj. specifickou sekvencí odpovídající danému minisatelitu
- zásadní objevy např. EPC u ptáků
- v posledních 10-15 letech – přesun k PCR-based metodám



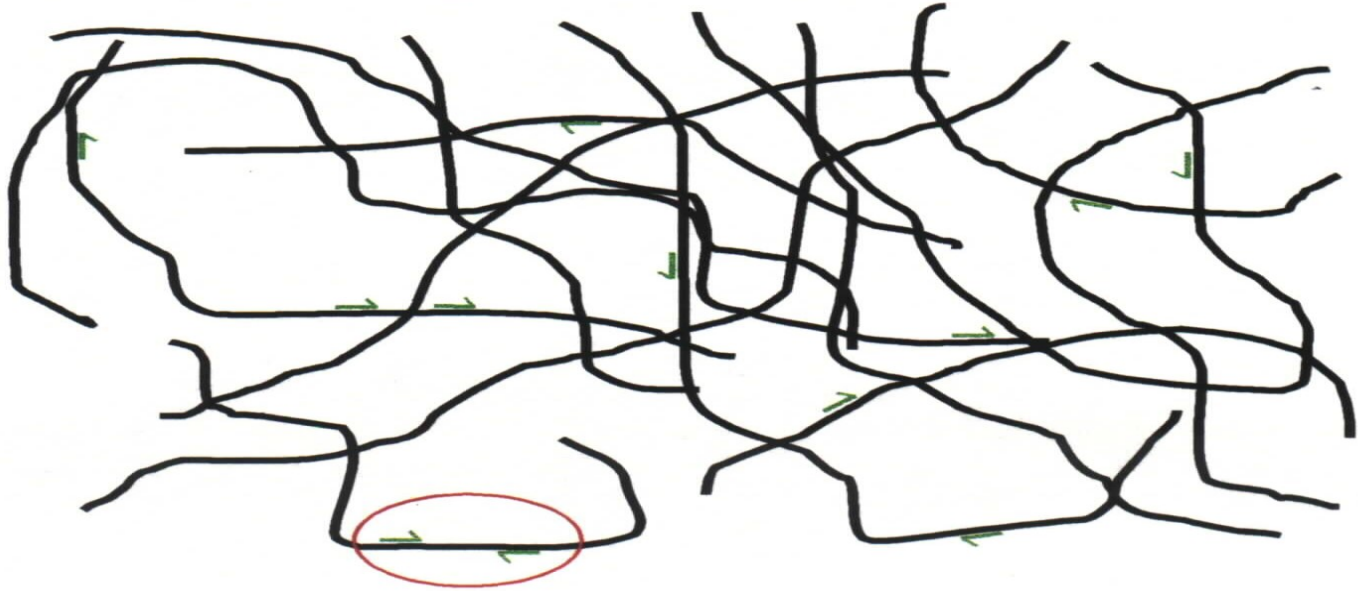
RAPD (randomly amplified polymorphic DNA)

Krátké náhodné oligonukleotidy
(~ 10 bp) jako primery

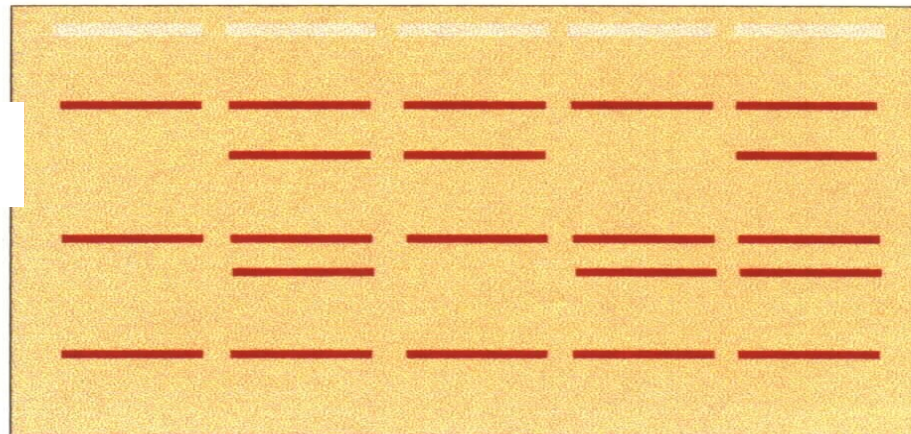


PCR za málo specifických podmínek

genomic DNA



- 1) PCR
2) Separation by size
on agarose gel

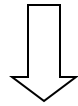


Variabilní DNA detekovaná metodou RAPD je důsledkem ztráty RAPD lokusů v důsledku:

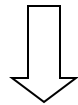
- a) Změna sekvence v místě nasedání primeru
- b) Delece místa nasedání primeru
- c) Velká inzerce mezi dvěma místy nasedání primeru

RAPD - review

Krátké náhodné oligonukleotidy
(~ 10 bp) jako primery

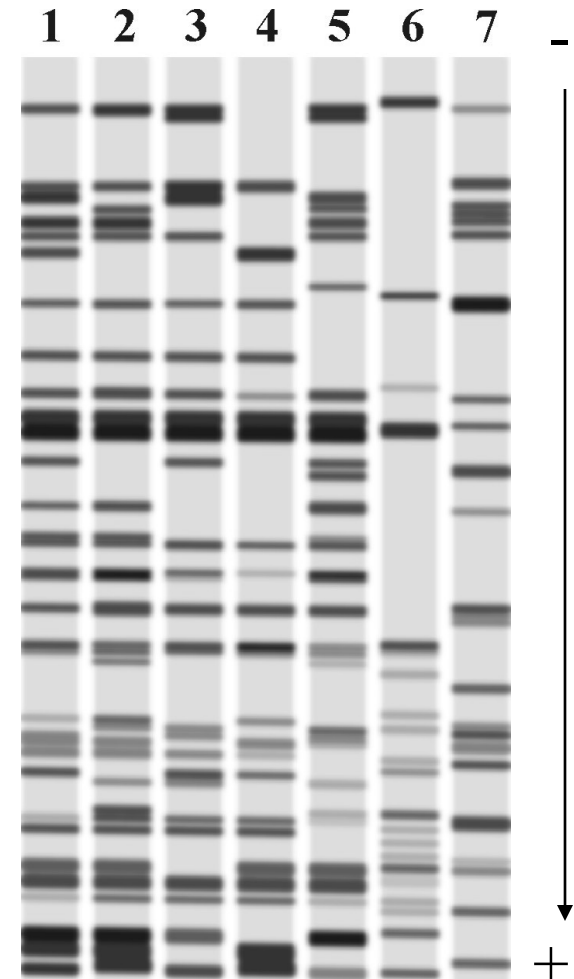


PCR za málo specifických podmínek



Detekce PCR produktů elektroforézou

**Nízká opakovatelnost v důsledku
mnoha faktorů ovlivňujících PCR –
dnes již není akceptována jako
metoda např. pro studium
populační struktury**



AFLP (amplified fragments length polymorphism)

- levná, jednoduchá, rychlá a spolehlivá metoda na generování stovek informativních genetických markerů
- současný screening mnoha různých DNA oblastí distribuovaných náhodně v genomu
- lépe reprodukovatelná než RAPD – obsahuje krok se specifickou PCR
- „genome scan“ – hledání asociací s fenotypovými znaky

Princip AFLP metody („generating AFLP markers“)

(a) AFLP template preparation

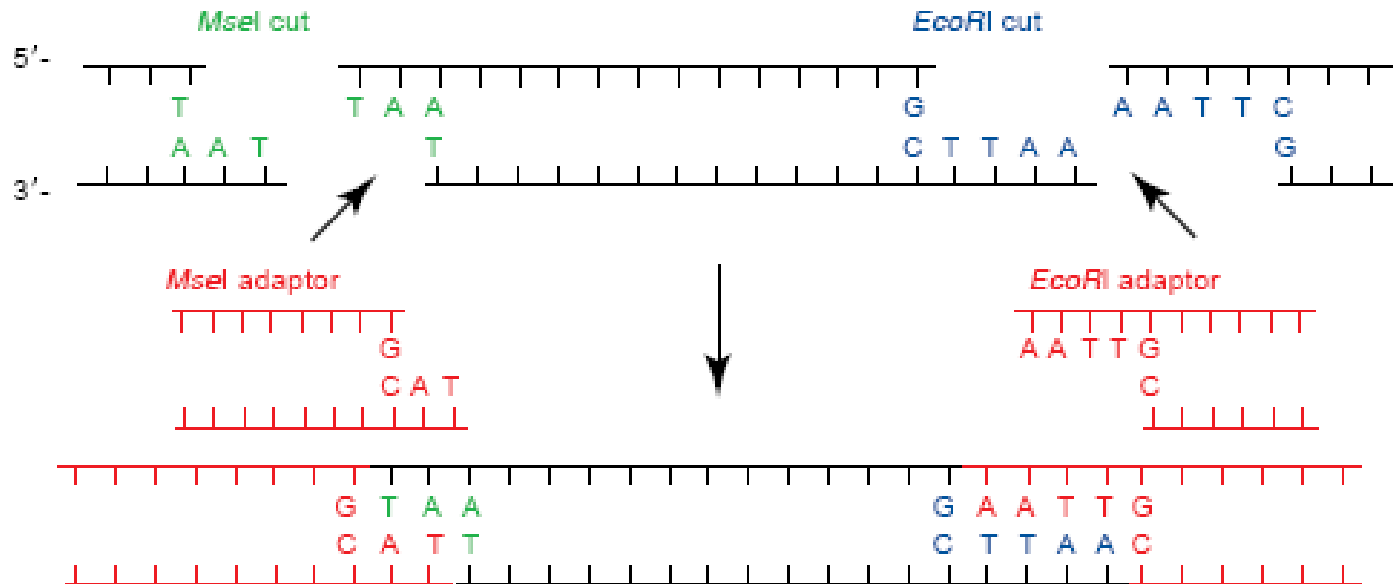
Whole genomic DNA



Restriction enzymes
(*MseI* and *EcoRI*)
and
DNA ligase

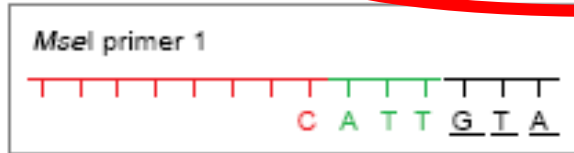


(b) Restriction and ligation

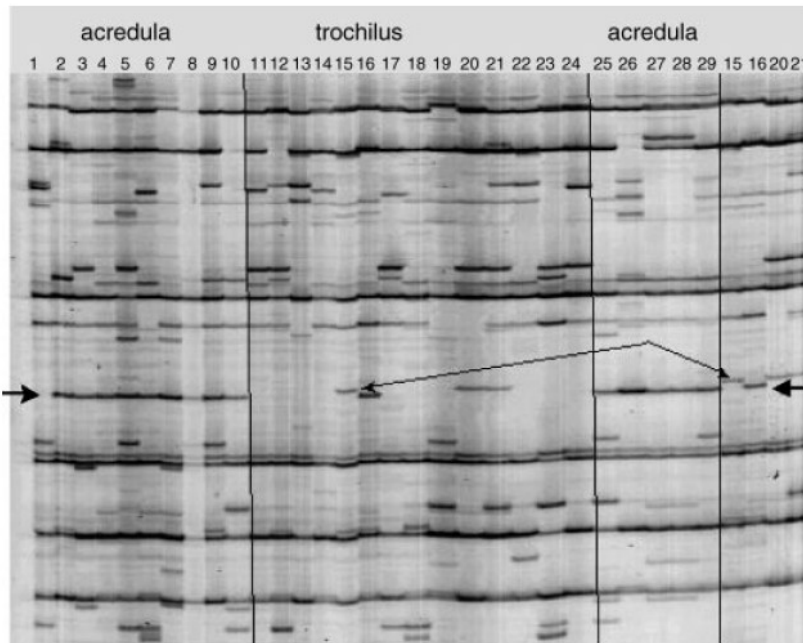
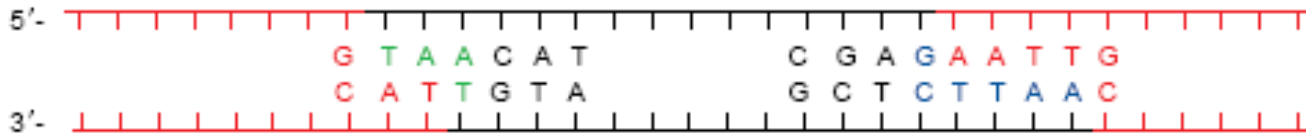


Generating AFLP markers

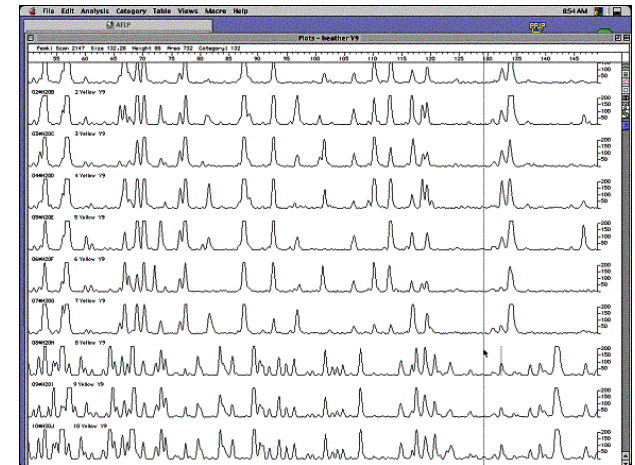
(c) Selective amplification (one of many primer combinations shown)



PCR with primers on adaptors



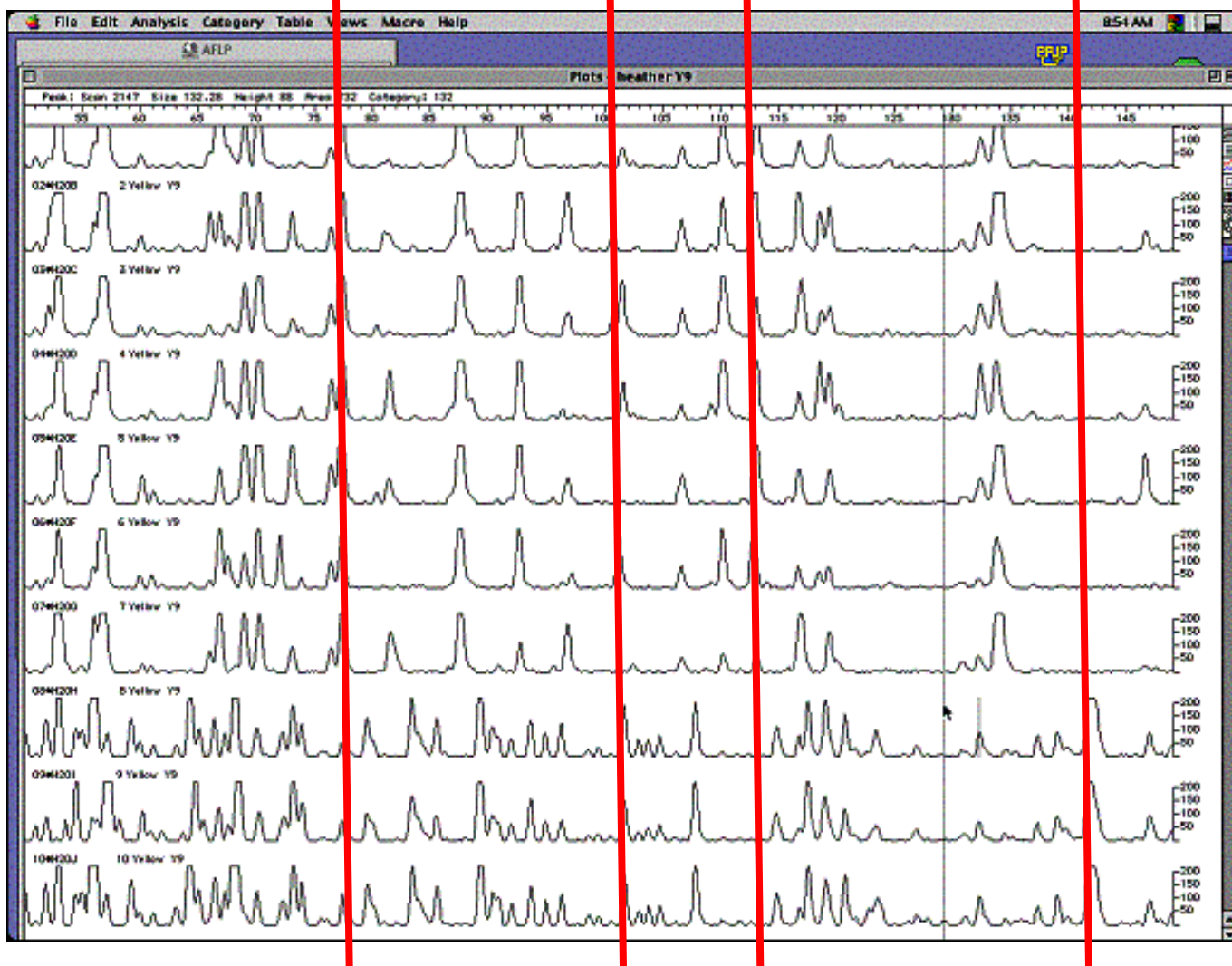
multi-locus genotype



„capillary version“

Ex.:
Combination
MseI + EcoRI

Automatizované čtení elektroforetogramu podle
zadaných kritérií (např. pozice a minimální výška píku)



Budoucnost genetických metod v ekologickém výzkumu

1. Nové postupy při sekvenování DNA („genomics“)

Molecular Ecology Resources (2008) 8, 3–17

doi: 10.1111 /j.1471-8286.2007.02019.x

TECHNICAL REVIEW

**Sequencing breakthroughs for genomic ecology and
evolutionary biology**

MATTHEW E. HUDSON

Department of Crop Sciences, University of Illinois, Urbana, 334 NSRC, 1101 W. Peabody Blvd., IL 61801, USA

4-kapilární sekvenátor

=

96 x 500 bp/12 hodin

=

cca 100 000 bp/den

Next-generation sequencing

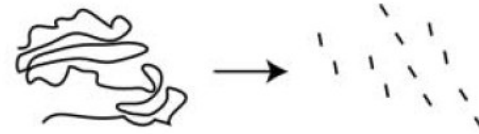
=

cca 1 000 000 000 bp/den

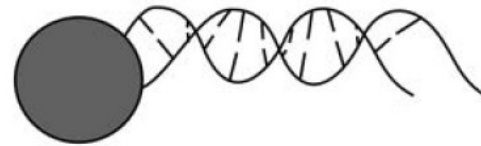
electrophoresis

„Next generation sequencing“

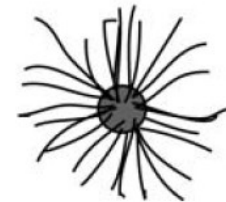
1) Randomly fragment many molecules of target DNA



2) Immobilize individual DNA molecules on solid support



3) Amplify DNA in clonal 'polymerase colony'



„polonies“
(polymerase colonies)

4) Sequence DNA by adding liquid reagents to immobilized DNA colonies



5) Interrogate sequence incorporation *in situ* after each cycle using fluorescence scanning or chemiluminescence



... commercially available since August 2007

454 pyrosequencing

- emulzní techniky amplifikace pikolitrové objemy
- simultánní sekvenování na destičce z optických vláken detekce pyrofosfátů uvolňovaných při inkorporaci bází
- První generace GS20 → 200 000 reakcí najednou (zhruba 20 milionů bp) dnes FLX → 400 000 reakcí najednou eukaryotní genom za týden!!!
- Délka jednotlivých sekvencí 100 - 400



Molecular Ecology (2008) 17, 1629–1635

NEWS AND VIEWS

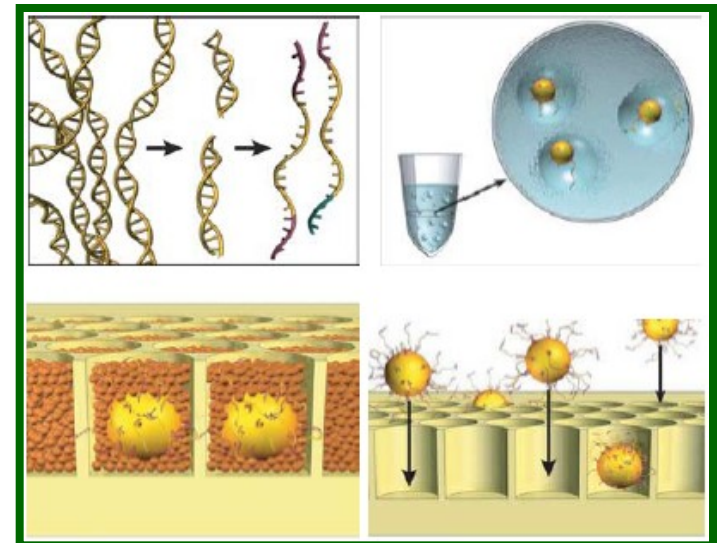
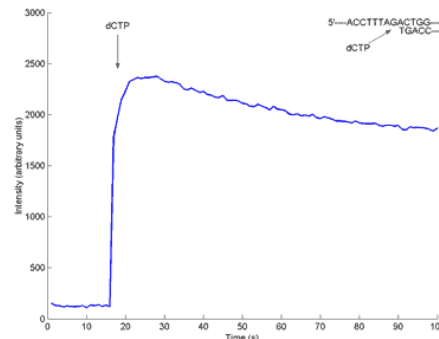
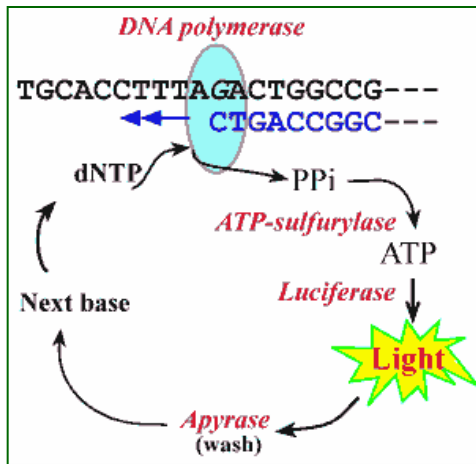
PERSPECTIVE

Sequencing goes 454 and takes large-scale genomics into the wild

HANS ELLEGREN

Department of Evolutionary Biology, Uppsala University, Norbyvägen 18D, SE-75236 Uppsala, Sweden

1 600 000 well plate



Pracovní postup



1

DNA Library Preparation

1. DNA Fragmentation (Nebulization)
2. DNA Fragment Size Selection
3. DNA Sample Quality Assessment (Nebulized or *LMW* DNA Sample)
4. Fragment End Polishing
5. Adaptor Ligation
6. Small Fragment Removal
7. Library Immobilization
8. Fill-In Reaction
9. Single-Stranded DNA Library Isolation
10. DNA Library Quality Assessment and Quantitation

Time: 11 - 72 h

General Laboratory 1



2

Emulsion-Based Clonal Amplification (emPCR)

1. Preparation of the Live and Mock Amplification Mixes
2. DNA Library Capture
3. Emulsification
4. Amplification
5. Bead Recovery
6. DNA Library Bead Enrichment
7. Sequencing Primer Annealing

Time: 11 - 13 h

Controlled Room

Amplicon Room



3

Sequencing / Genome Sequencer FLX Operation

1. The Pre-Wash
2. PicoTiterPlate Device Preparation
3. The Sequencing Run

Time: 11.5 h

General Laboratory 2



4

Data Processing and Analysis

1. Data Processing
 - a) Image Processing
 - b) Signal Processing
2. Data Analysis
 - a) Assembly
 - b) Mapping
 - c) Amplicon Variant Analysis

Time: variable



1. Příprava jednořetězcové DNA knihovny (ssDNA library preparation)

1 DNA Fragmentation (Nebulization):



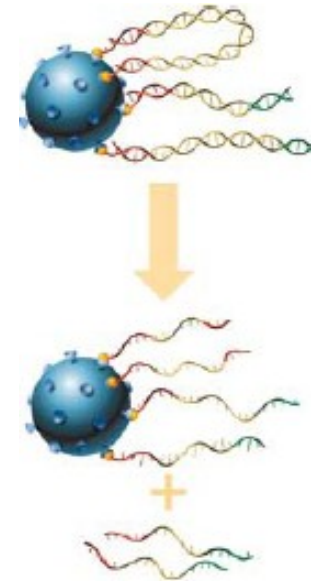
5 Adaptor Ligation:



7 Library Immobilization:



9 ssDNA Library Isolation:



Adaptor A + Adaptor B

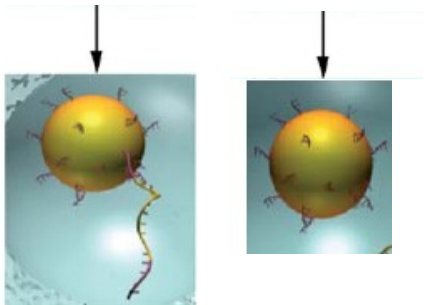
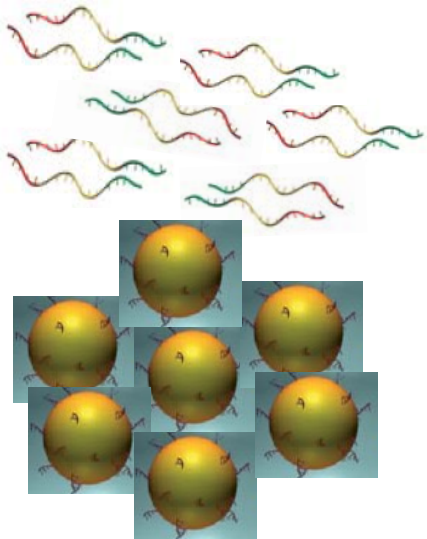
-Slouží jako vazebné místo primerů pro následnou PCR amplifikaci a sekvenování

-Slouží k uchycení na kuličky (na adaptor B je připojen **biotin**)

2. Namnožení každé jednotlivé molekuly pomocí emulzní PCR (emPCR)

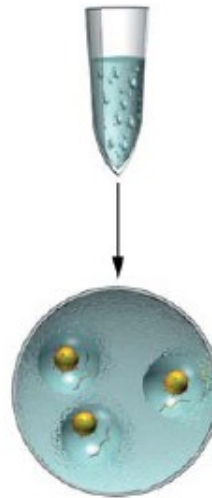
1 DNA Library Capture:

- poměry nastavit tak aby
1 kulička \leq 1 molekula DNA

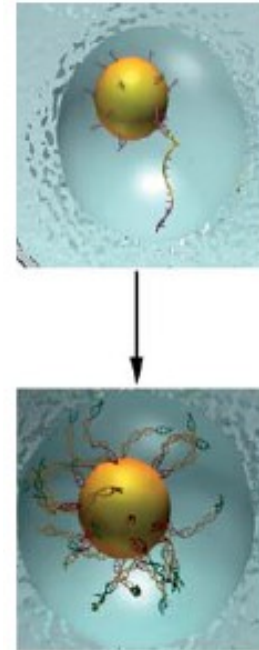


2 Preparation of the Amplific. Mixes

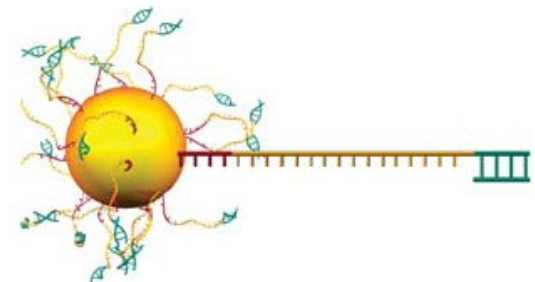
3 Emulsification:



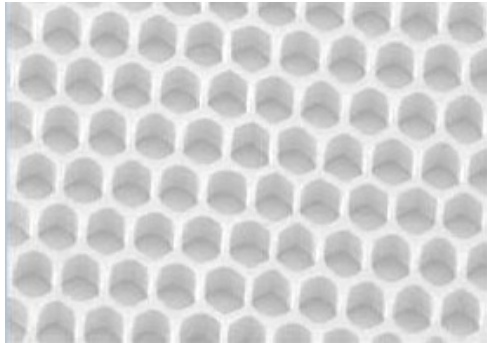
4 emPCR Amplification:



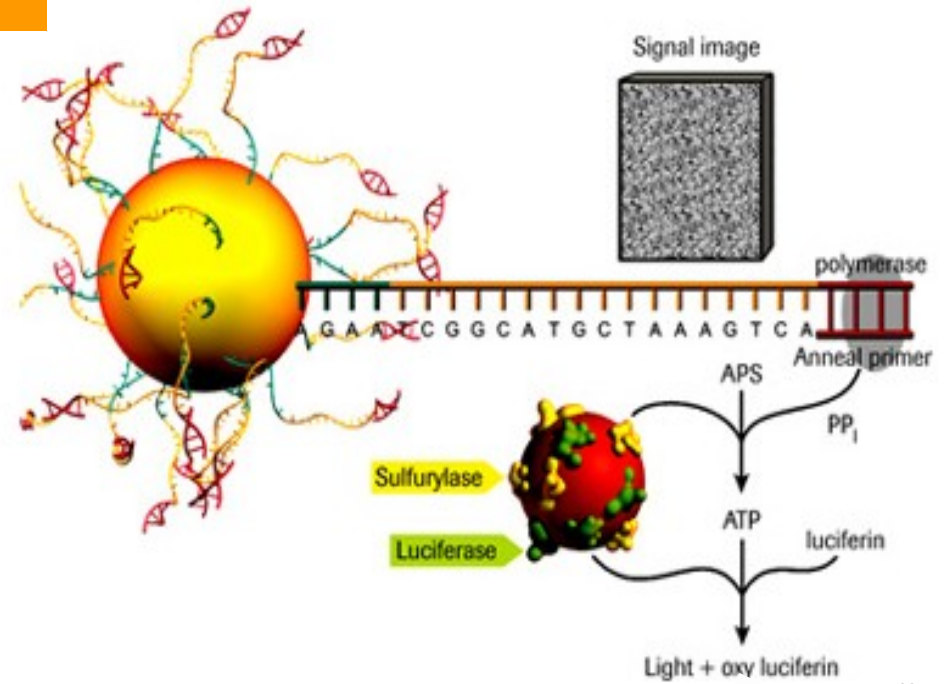
7 Sequencing Primer Annealing:



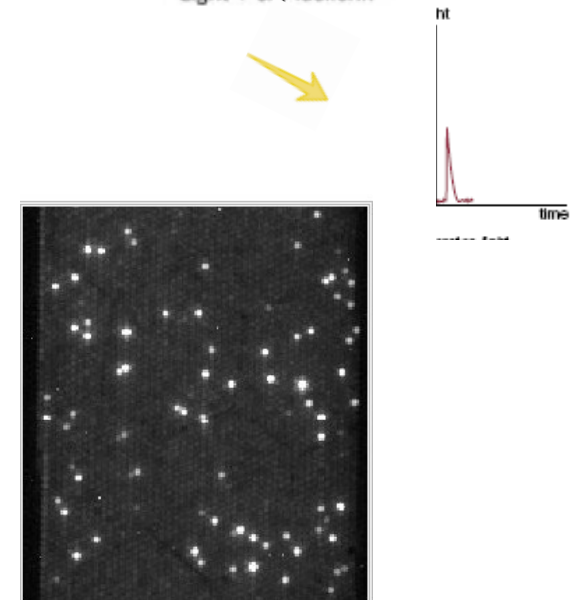
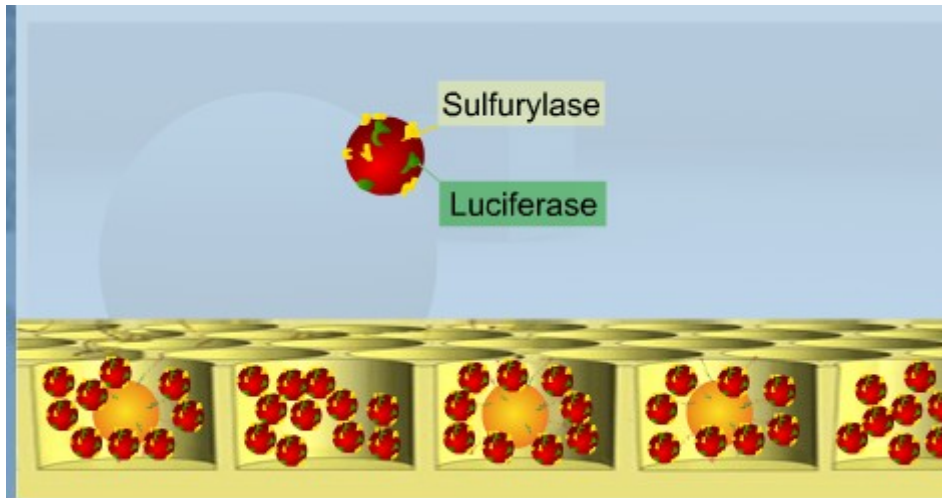
3. Pyrosekvenovani



pikotitrační destička



Na jedné destičce 400 000 až 1milión jamek



3. Pyrosekvenovani - detekce signálu

- postupně se přidávají nukleotidy v definovaném pořadí: např. TACG TACG TACG
- po přidání každého nukleotidu a detekci signálu se nukleotid odmyje a přidá se další odmyje

DNA sekvence: **C T C C G**

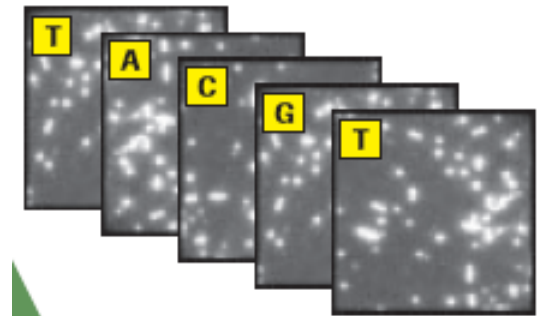
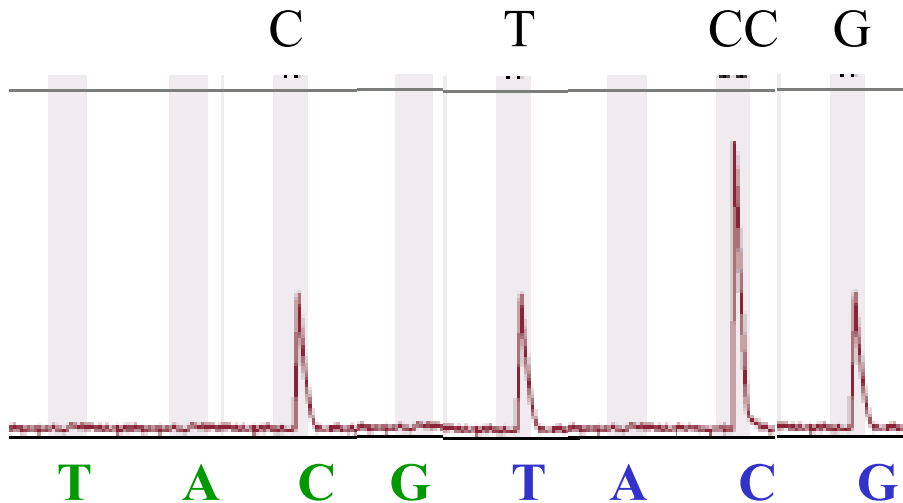


Image Files:
12-15 gigabytes
per run

Problém!!!! Homopolymery např. AAAAAAAAAA

High-throughput - paralelní sekvenování

1 běh (run) = 1 destička:

- 400 000 / 1milión jamek (reads)
- v každé 240 / 400 bp (read length)
- 7.5 / 10 hod

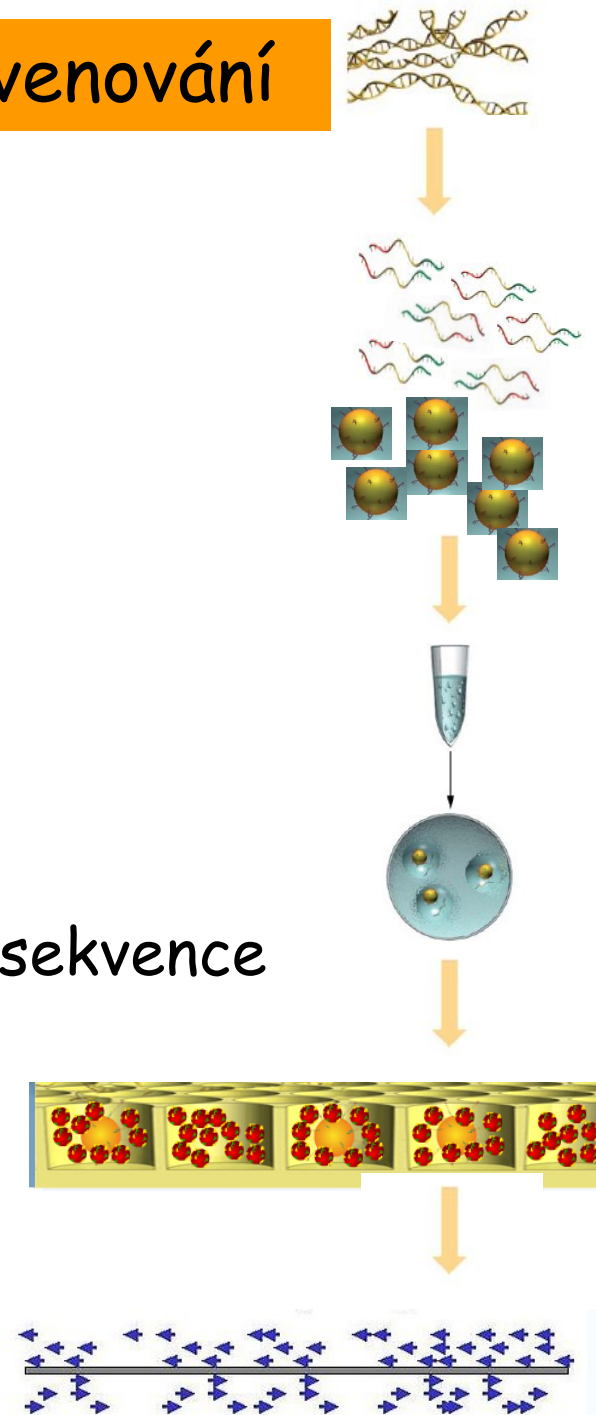
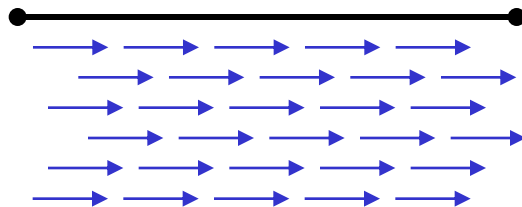
→ 100 Mb / 400 Mb na jednu destičku

→ cena??? 150-350 000 Kč ????

!!! Samozřejmě nestačí mít každou bázi osekvenovanou 1x !!!

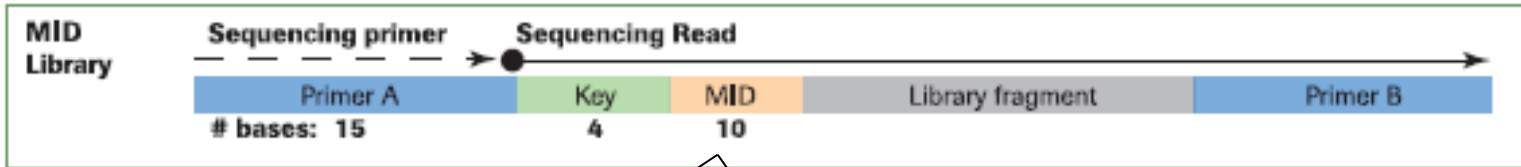
- Pospojování (**reads assembly**) do souvislé sekvence

- Nepřesnosti - pokrytí (**coverage**)

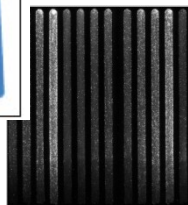
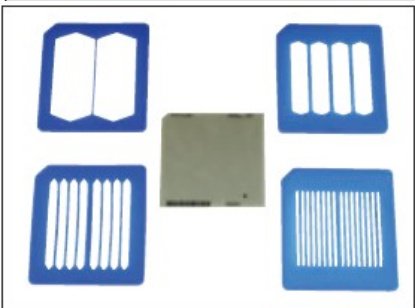
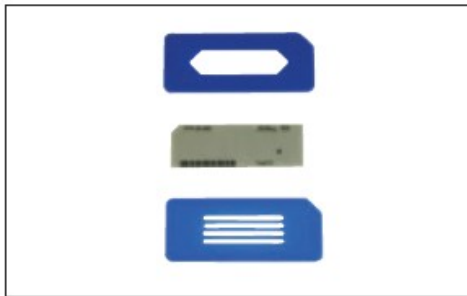


Kapacita destičky **400 Mb**:

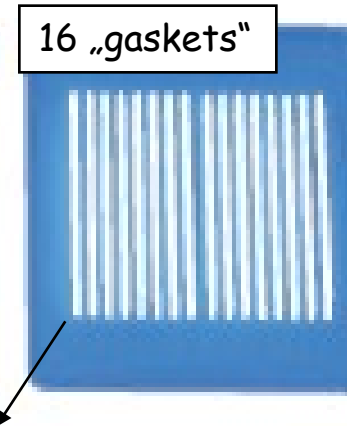
Mus:	2700 Mb	→ 7 run 1x coverage
Caenorhabditis:	100 Mb	→ 1 run 4x coverage
E. coli:	5 Mb	→ 1 run 80x coverage
mitoch. Mus:	0.016 Mb	→ 1 run 25000x coverage
HIV:	0.01 Mb	→ 1 run 40000x coverage



- k dispozici 12 odlišných MID



1. CCCCCCCCCC
2. GGGGGGGGGG
- ...
12. CCCCAAAG



$$\begin{array}{r} 12 \text{ MID} \\ \times \\ 16 \text{ gaskets} \\ = \\ \text{max. 192 vzorků} \end{array}$$

V každém max. 12 vzorků
(každý označen svým MID)

Využití

1. Celogenomové sekvenování de novo
2. Celogenomové resekvenování
3. Sekvenování amplikonů (PCR produktů)
+ to samé i s RNA (resp. cDNA)



1. Celogenomové sekvenování de novo

Problém: **KRÁTKÝ READ LENGTH**

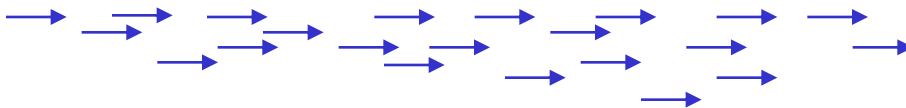
- **400bp** 454 FLX Roche
- **35-75bp** Solexa, Solid
- vs **800-1000bp** Sanger



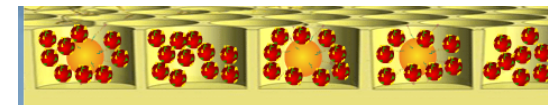
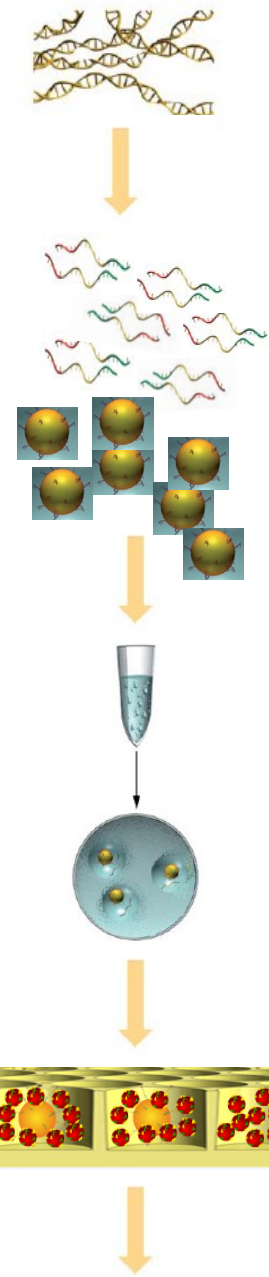
→ Uspořádání (assembly) už není problém z hlediska výpočetní kapacity

!!!! **REPETITIVNÍ OBLASTI** delší než read length !!!!

GTAAAAAAAAAAAAAAAAAAAAAAC



Zvláště komplexní eukaryotické genomy - úseky souvislých oblastí přerušovaných mezerami



2. Celogenomové resekvenování

- podobné problémy jako u de novo, ale méně (větší strukturální přestavby..)

KOMPARATIVNÍ GENOMIKA

- viry, prokaryota, malá eukaryota
- mitochondrie/plastidy/plasmidy

ANCIENT (mt) DNA

- různé směsné, degradované vzorky, např. fosilie

Cell

A Complete Neandertal Mitochondrial Genome Sequence Determined by High-Throughput Sequencing

Richard E. Green,^{1,*} Anna-Sapfo Malaspinas,² Johannes Krause,¹ Adrian W. Briggs,¹ Philip L.F. Johnson,³ Caroline Uhler,⁴ Matthias Meyer,¹ Jeffrey M. Good,¹ Tomislav Maricic,¹ Udo Stenzel,¹ Kay Prüfer,¹ Michael Siebauer,¹ Hernán A. Burbano,¹ Michael Ronan,⁵ Jonathan M. Rothberg,⁶ Michael Egholm,⁵ Pavao Rudan,⁷ Dejana Brajković,⁸ Željko Kučan,⁷ Ivan Gušić,⁷ Märten Wikström,⁹ Liisa Laakkonen,¹⁰ Janet Kelso,¹ Montgomery Slatkin,² and Svante Pääbo¹

3. Sekvenování ampliconů (PCR produktů)

SMĚSNÉ VZORKY

1. Metagenomika/metatranskriptomika

- Celé společenstvo půdních, vodních mikroorganismů, střevní mikroflóra
- PCR genu 16S (18S) rRNA
- lze i kvantifikovat

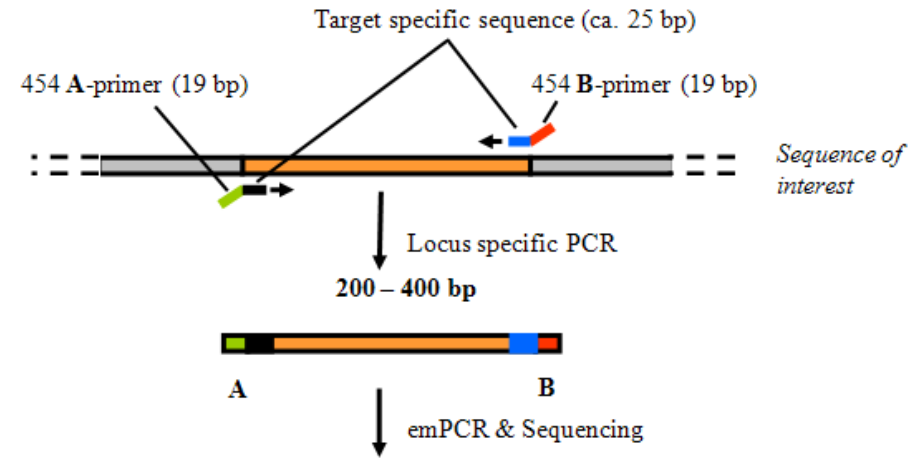
2. Složení potravy, trusu ???

4. Studie u kandidátních genů

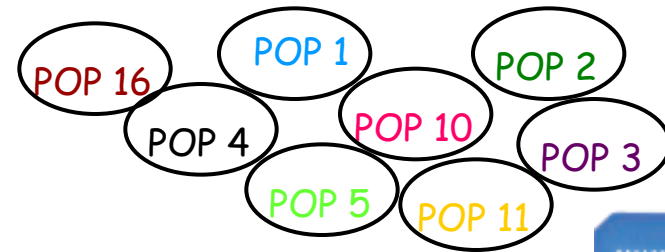
20x
NEMOCNÉ MYŠI

20x
ZDRAVÉ MYŠI

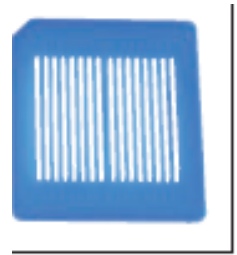
1. PCR např. imunitního genu/genů
2. Sekvenování
3. Které varianty jsou asociovány s chorobou??



3. Populační genetika

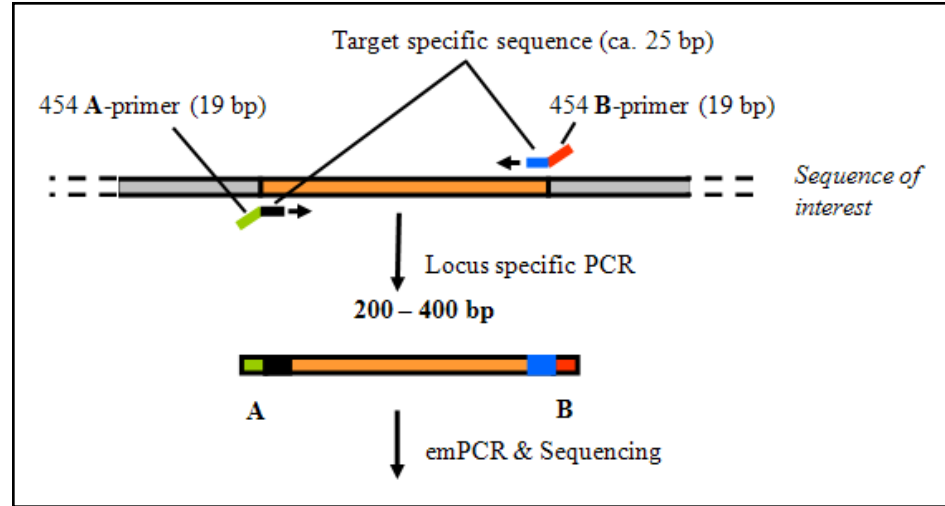
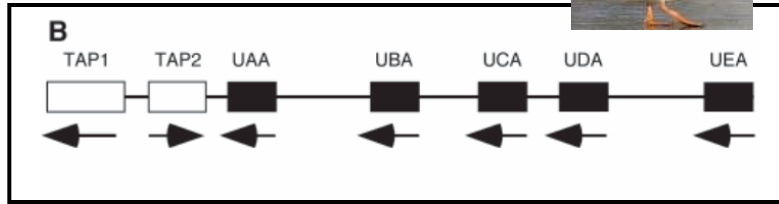


1. PCR genu/genů
2. Sekvenování
3. Zjištění sekvencí variant a frekvencí variant v každé populaci (záleží na pokrytí)



3. Sekvenování amplikonů (PCR produktů)

5. Genové duplikace

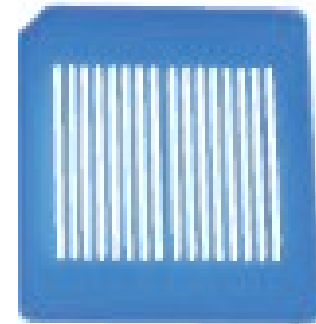


A-adaptor MID Target specific

Označí jedince

Amplifikuje všechny kopie MHC genů

Potřeba k emPCR, sekvenování..

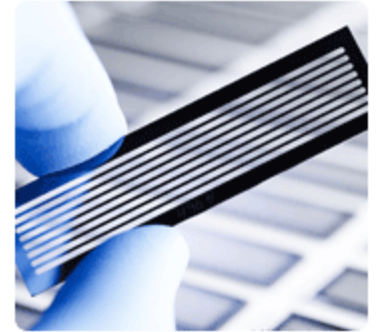


192 jedinců

Solexa/Illumina 1G SBS technology

(SBS = sequencing by synthesis)

- 1 Gb (šestinásobek genomu *Drosophily*)
- Výrazně levnější
- Sekvence délky 35 bp
- Fluorescence, reversibilní terminátory
- Spíš pro resequencing


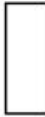




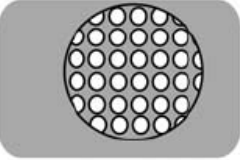
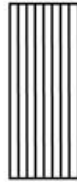
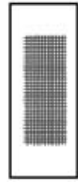
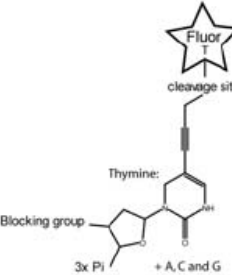


SOLiD

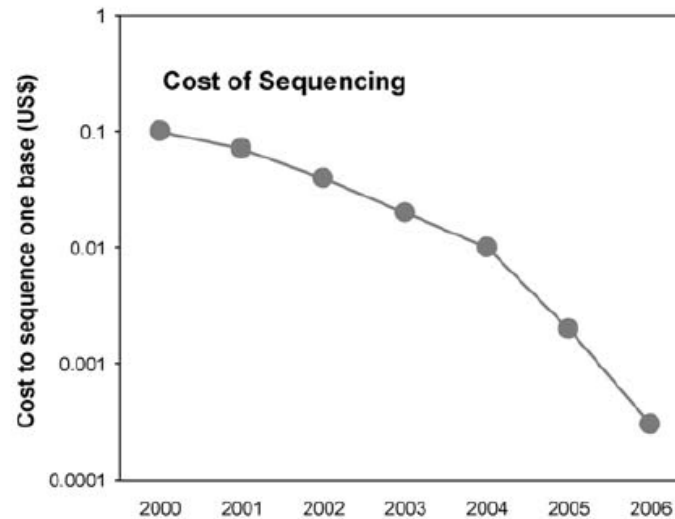
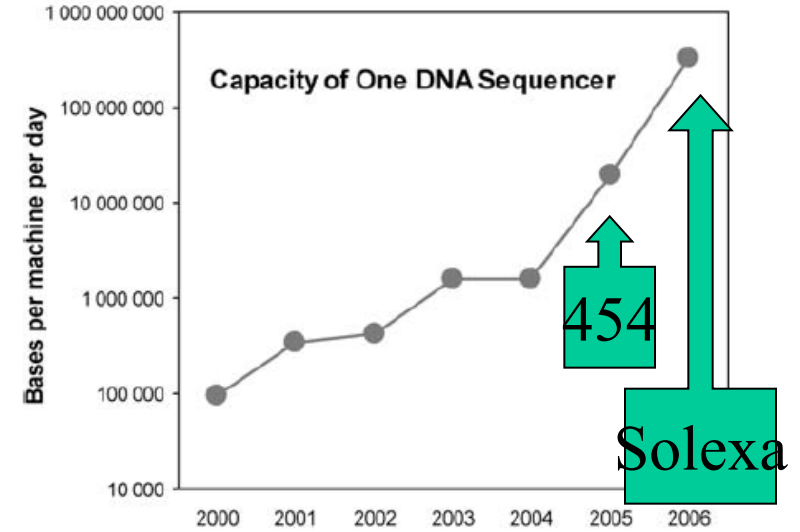
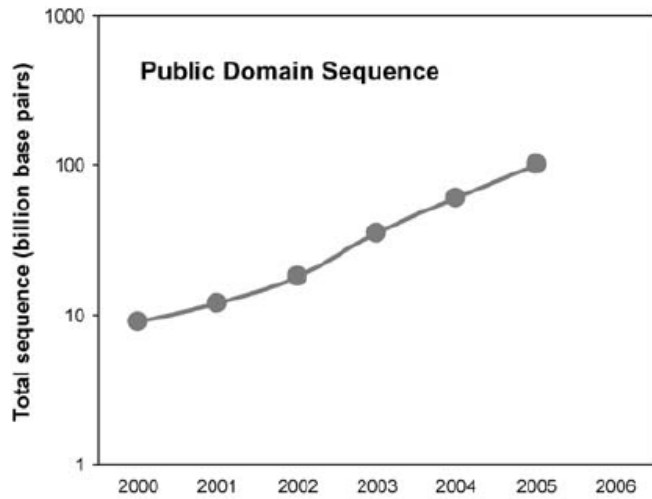
(sequencing by Oligonucleotide Ligation and



... a další (každého půlroku nová technologie - bouřlivý rozvoj !!!)

	454 pyrosequencing	Solexa SBS sequencing	Agencourt / ABI SOLiD polony sequencing
All methods ligate single, randomly sheared DNA molecules to support			
DNA support	 25–36 µm bead	surface of flow cell 	 ~1 µm bead
Amplification	 emulsion-phase PCR	 <i>in situ</i> PCR on solid surface	 emulsion-phase PCR
Sequencing surface	 1 600 000 well plate one bead per well	 8-channel flow cell clusters of DNA randomly located	 Single slide imaged in panels beads random
Sequencing chemistry	<p>Nucleotide incorporation</p> <p>↓</p> <p>PPi ADP + Sulfurylase</p> <p> ↓ ↓</p> <p> ATP ↓</p> <p>Luciferin Luciferase → light</p> <p>pyrosequencing</p>	<p>Fluor T</p> <p>↓</p> <p>cleavage site</p> <p>Thymine: </p> <p>Blocking group 3x Pi + A, C and G</p> <p>reversible-terminator sequencing by synthesis</p>	<p>Fluor G</p> <p>Fluor C</p> <p>Fluor A</p> <p>Fluor T</p> <p>nnnnGzzz</p> <p>nnnnCzzz</p> <p>nnnnAzzz</p> <p>nnnnTzzz</p> <p>Ligation of sequence- specific labeled oligos</p>
Sequence detection	Chemiluminescence (one channel)	Fluorescence (four channel)	Fluorescence (four channel)
Read length and number	100–400 bp > 2 x 10 ⁵ reads	35 bp ~ 4 x 10 ⁷ reads	25 bp (paired) > 10 ⁷ reads

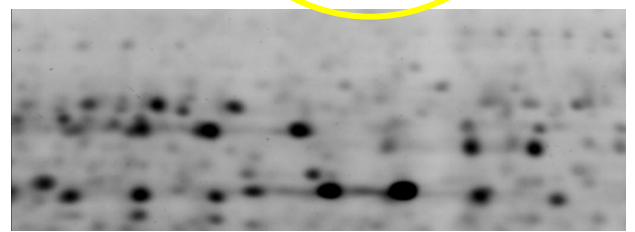
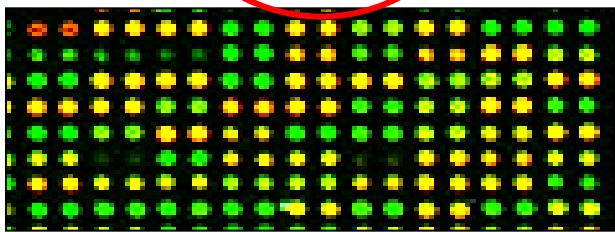
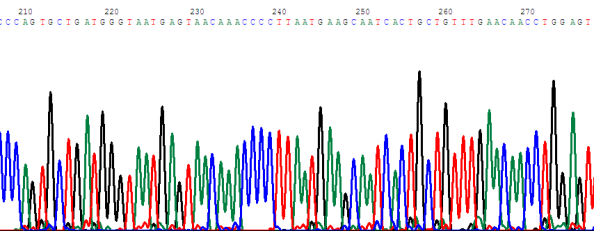
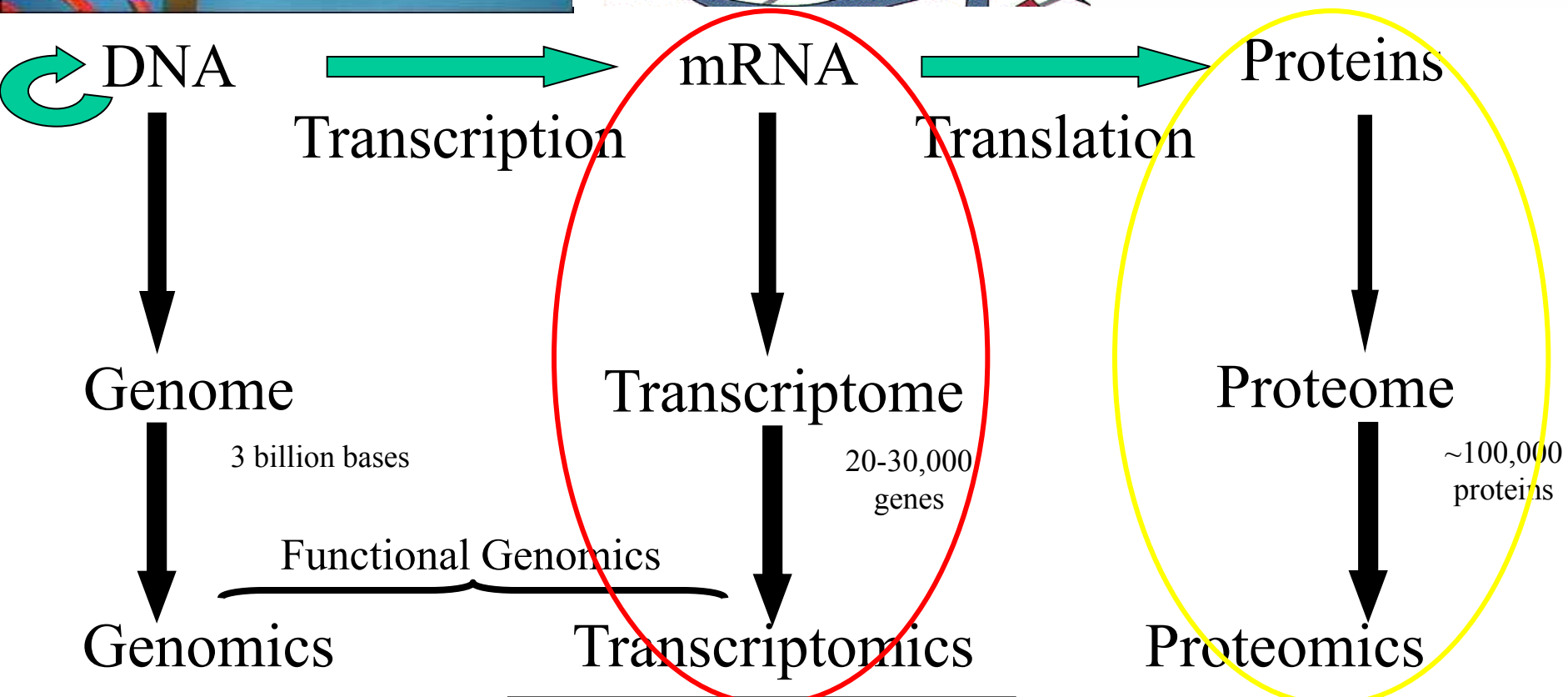
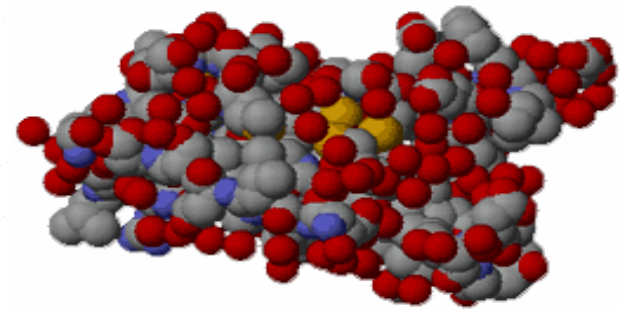
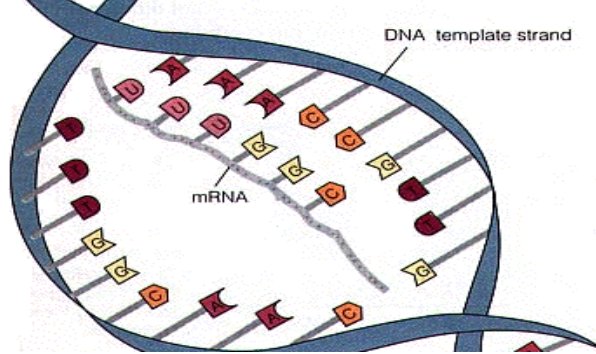
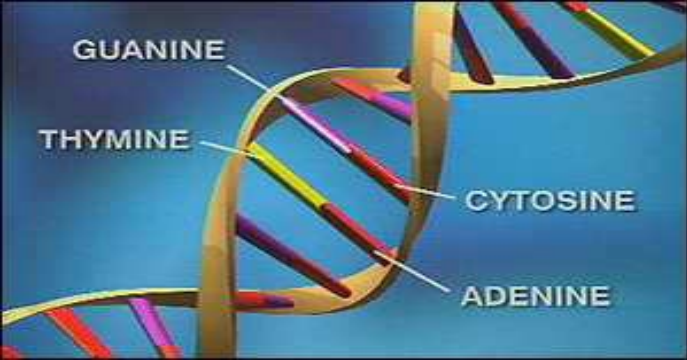
„genomics era“



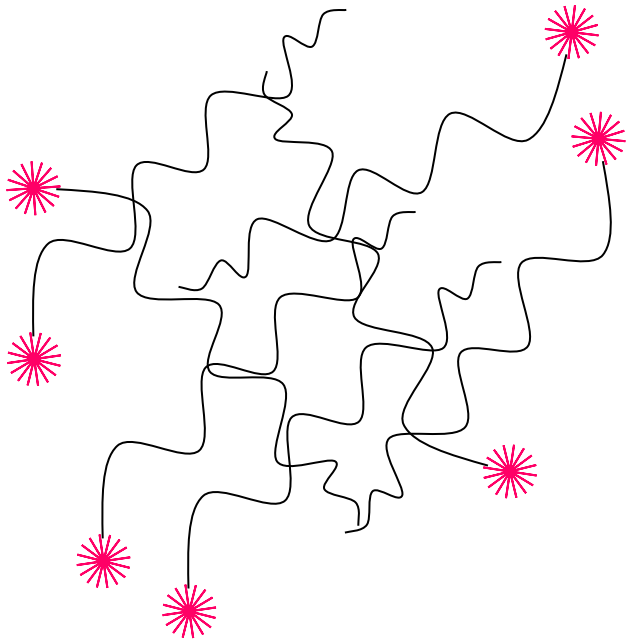
Budoucnost genetických metod v ekologickém výzkumu

2. Analysis of expression by microarrays („transcriptomics“)

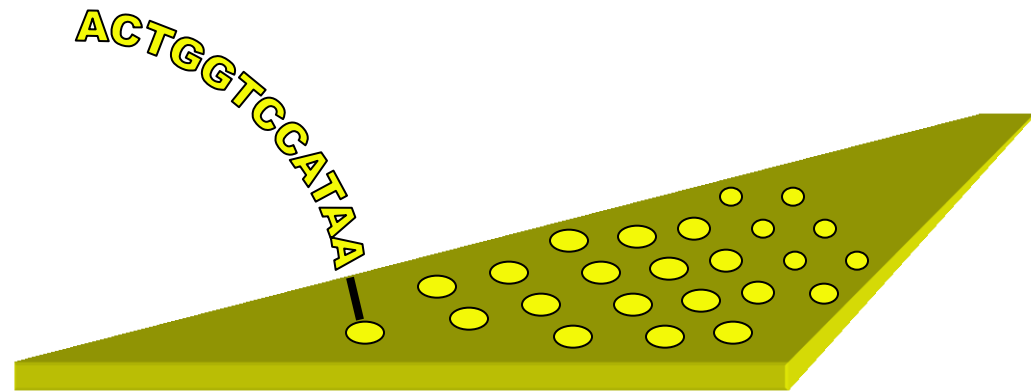
Ranz JM, Machado CA: Uncovering evolutionary patterns of gene expression using microarrays. TREE, 21(1): 29-37



Microarray analysis of transcriptome (~ specific DNA hybridization)



Target (i.e. mix of transcripts in a form of cDNA)

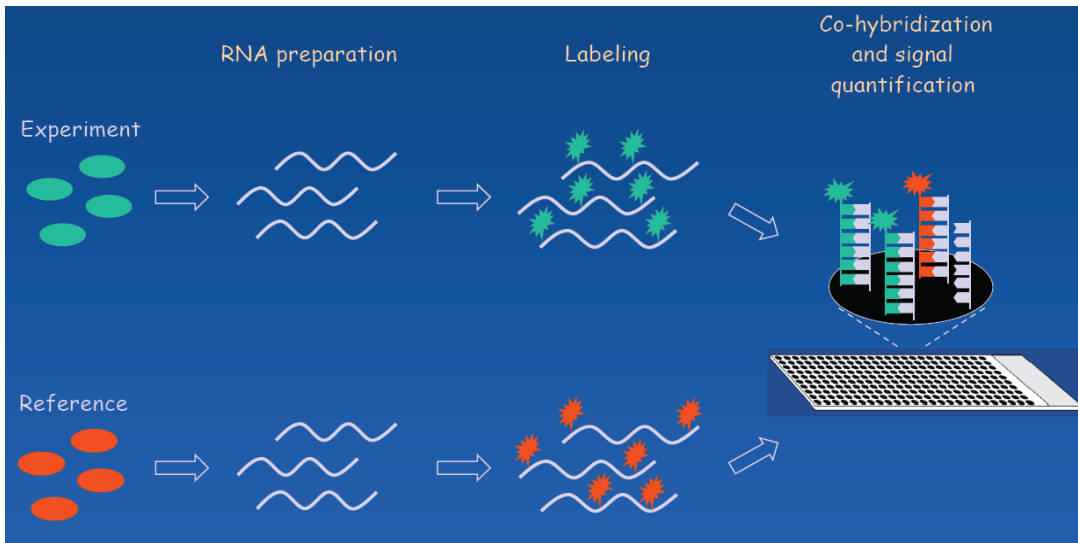


Probe (i.e. synthesized oligonucleotides complementary to particular genes)

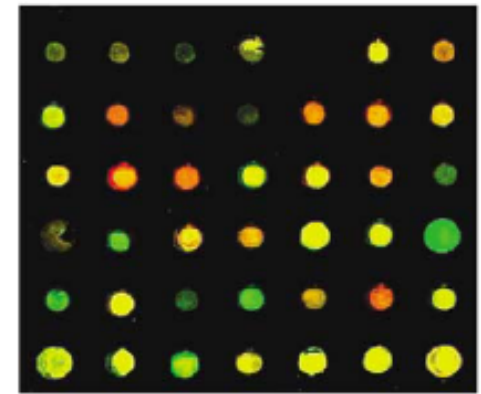
How to get a transcription profile

- vždy srovnání kontroly a „treatment“

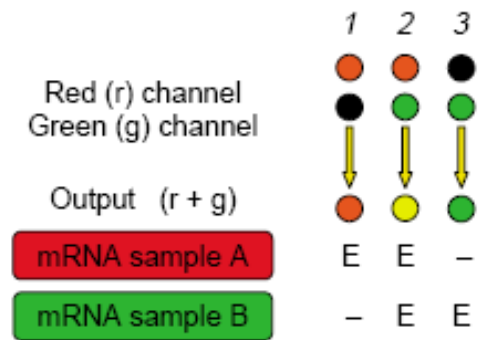
(a)



(b)



(c)



TRENDS in Ecology & Evolution

Case study: Joop Ouborg et al.

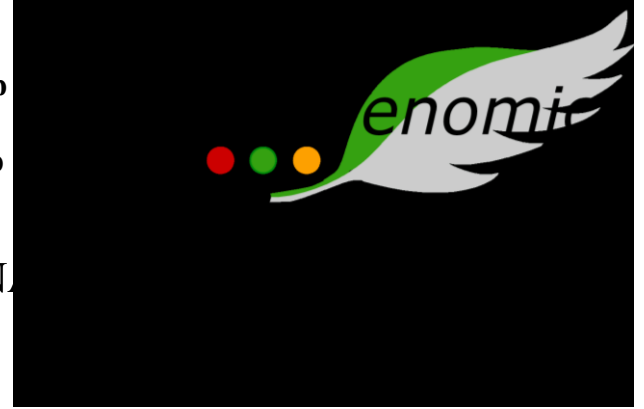
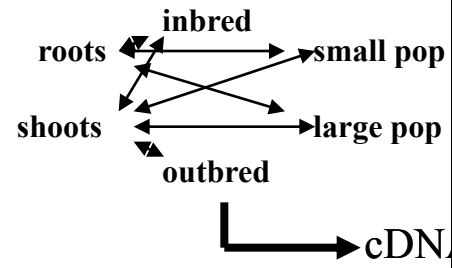
Transcriptional profiling of inbreeding depression and genetic erosion in *Scabiosa columbaria*: the balance between genetic drift and selection in the genetic erosion process.





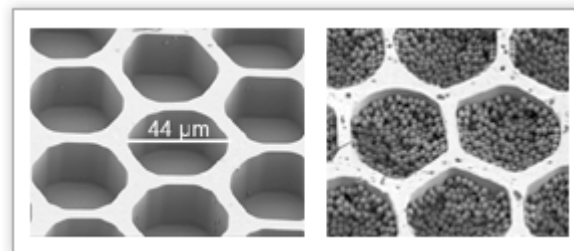
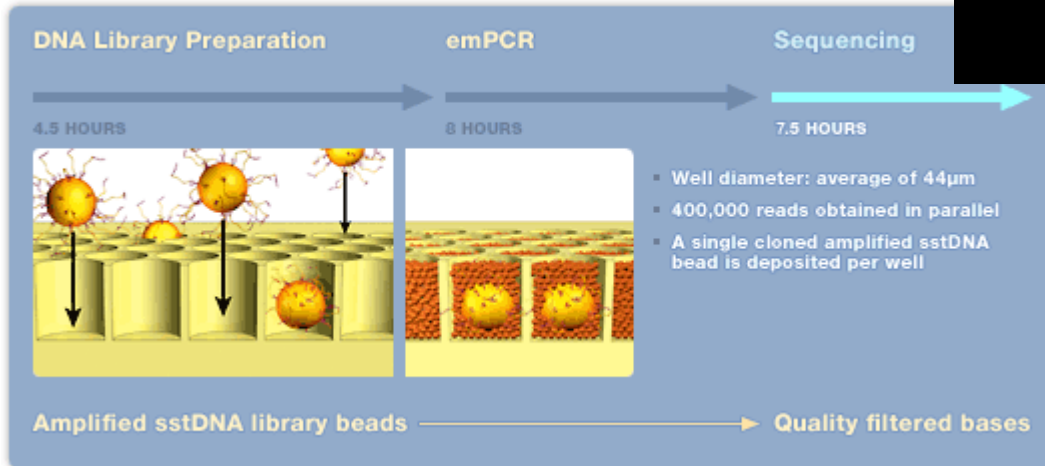
Example:

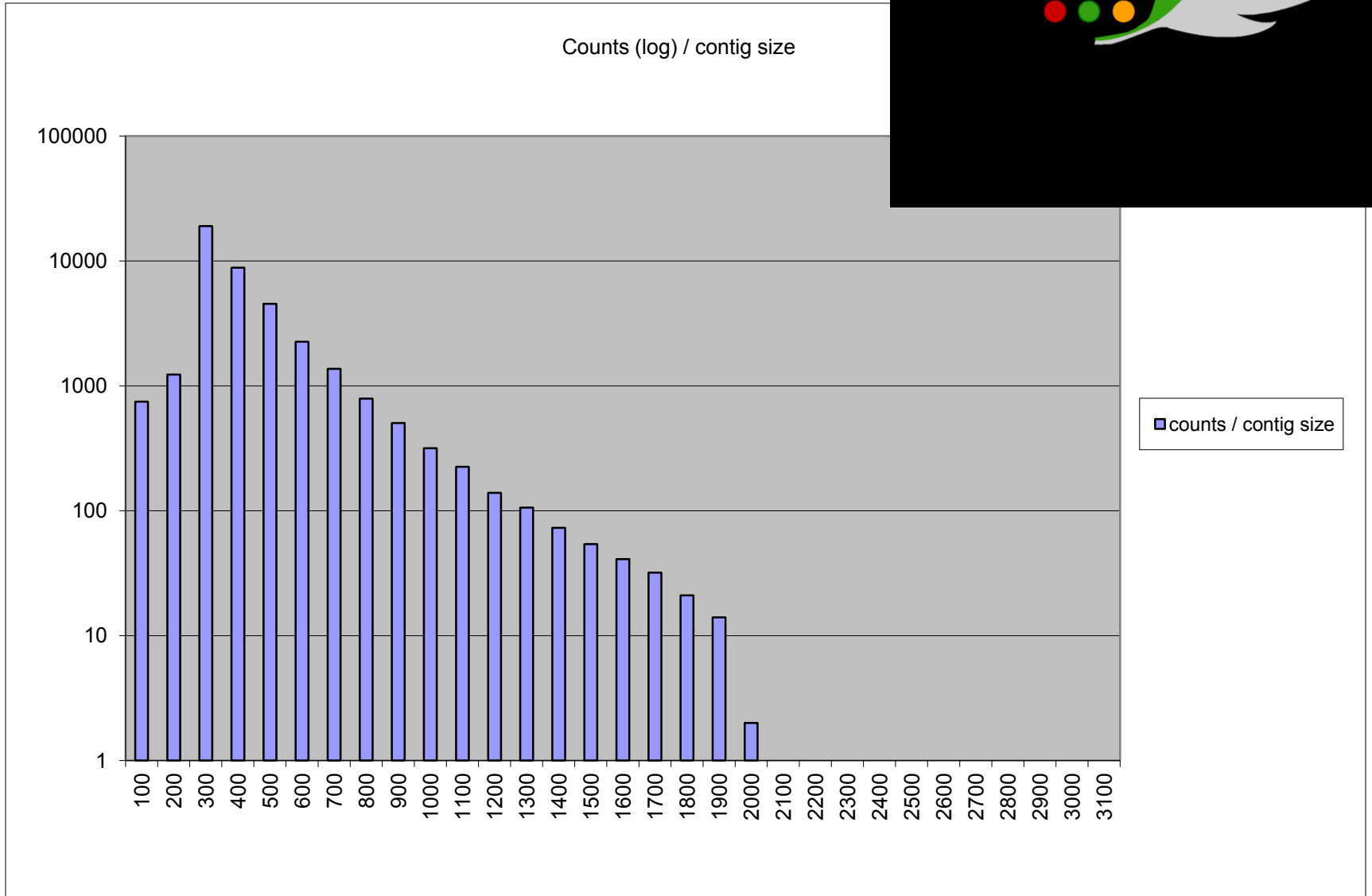
Scabiosa columbaria



cDNA library preparation – 454 sequencing

FIGURE 9





Total number of reads: **528557** Number of contigs: **40302**



In the next phase:

Annotation of these 40.000⁺ ESTs („e
sequence tags“)

Automated programs available, like **BLAST2GO** (<http://www.blast2go.de/>):

just feed a file with the ESTs into the program, and turn it on.....

1 week later you will have the results, being:

- Homology with known sequences
- Known function

The sequences may also be searched for:

EST-associated SSR markers: MISA (<http://pgrc.ipk-gatersleben.de/misa/>)

SNP markers: SNP-mining software like PolyBayes

(<http://genome.wustl.edu/tools/software/polybayes.cgi>)

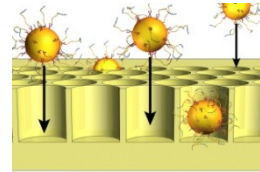
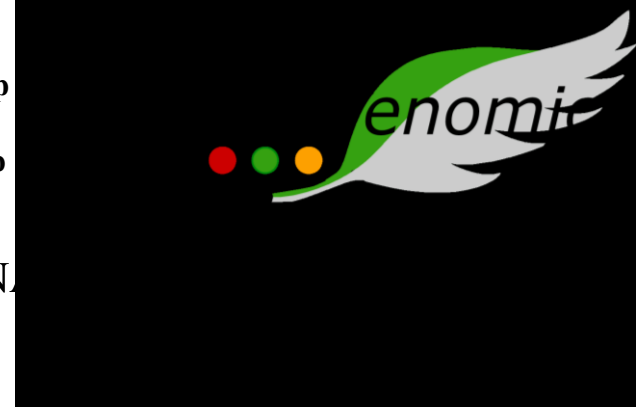
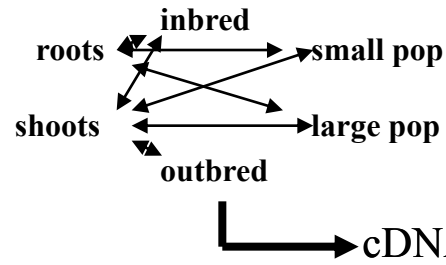
Again by using search software, freeware

ALMOST HALF OF GENES (ESTs) ARE UNKNOWN !!!



Example:

Scabiosa columbaria

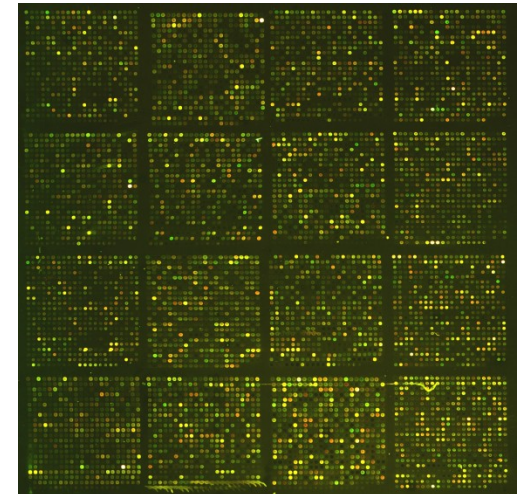
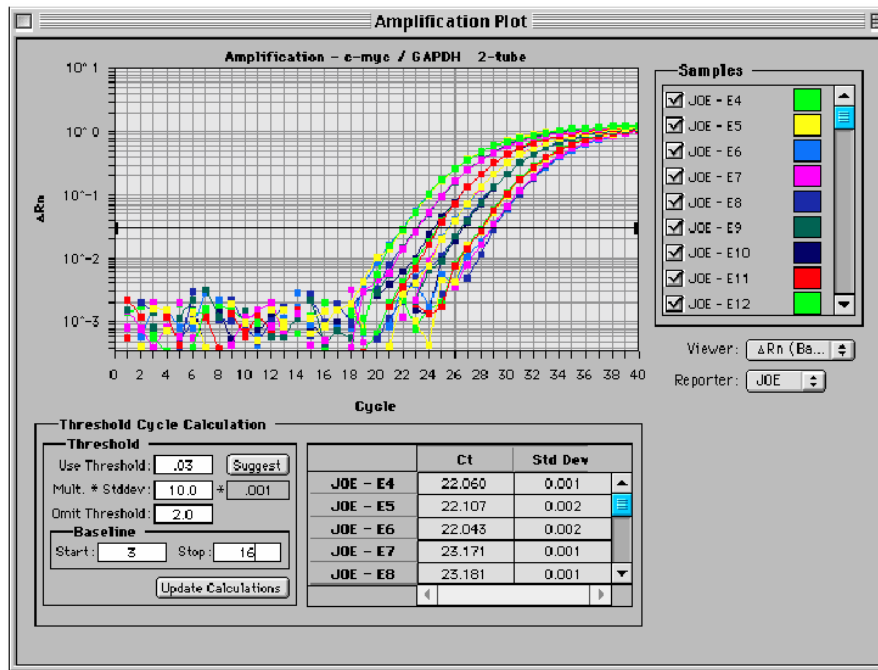


530.000 sequences in one run, leading to ~ 40.000 ESTs

Two methods of detecting transcrip

1. Design of quantitative RealTime-PCR method
EST sequences

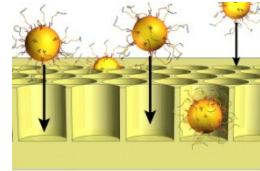
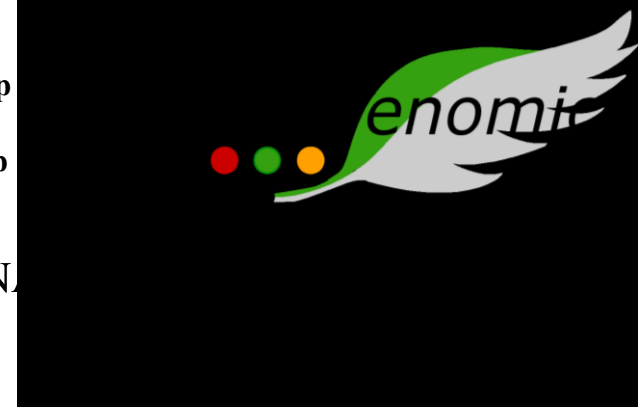
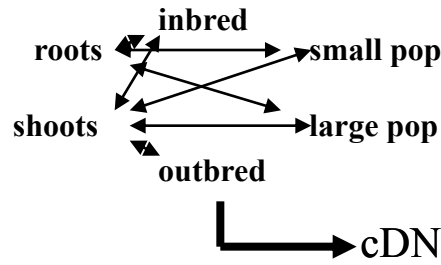
2. Design of a *Scabiosa* specific microarray



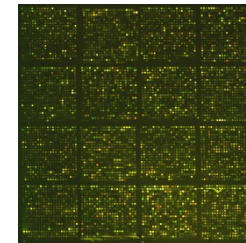


Example:

Scabiosa columbaria



530.000 sequences in one run, leading to ~ 40.000 ESTs



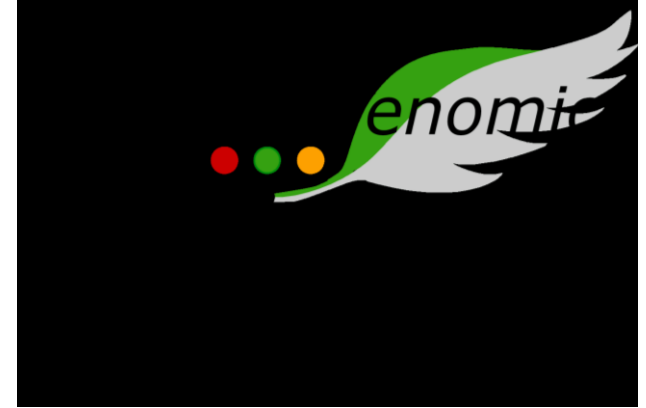
↓
15k – 30k
60-mer
microarrays

Experiment: transcriptional profiling of inbreeding depression



Expected pay-off:

- Ecogenomic approach to conservation genetics: effects of genetic erosion on functional genetic variation
- How does genetic erosion affect evolutionary potential?
- What is the **balance between genetic drift and natural selection** in effects of habitat fragmentation?
- Are there general **inbreeding depression genes**, or is inbreeding depression a random phenomenon?
- **Which genes are involved in inbreeding depression in different life history stages**, and can this explain the non-correlation of IBD between these stages?
- What are the **footprints of selection** in the genomes of individuals from small and large populations?
- What is the **selective value of variation in gene expression**?



Costs/requirements (2008/2009):

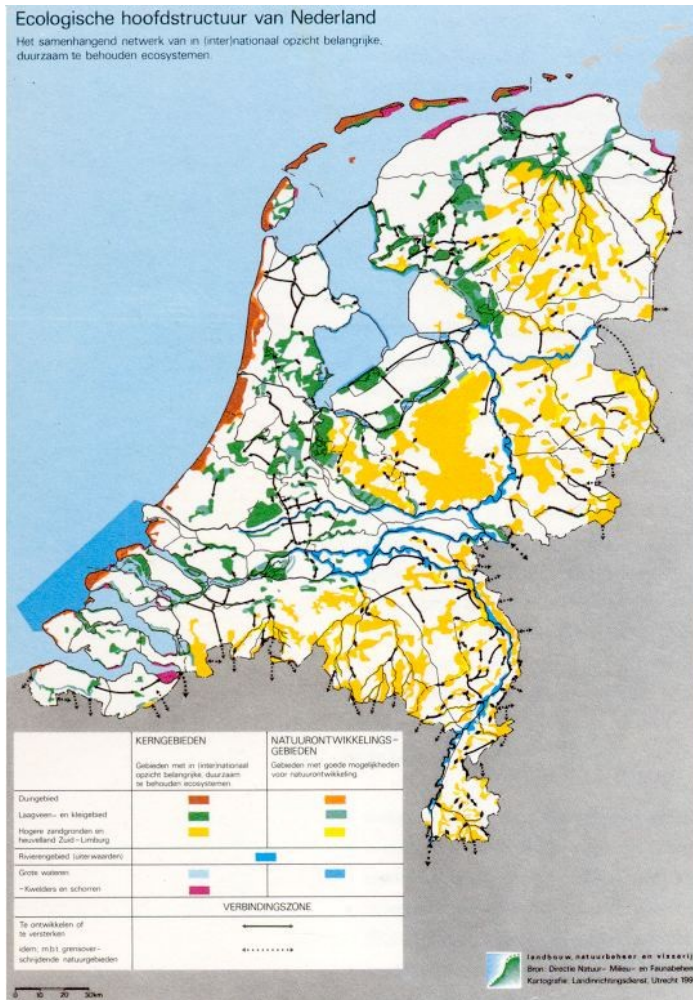
Costs are diminishing continuously

454 FLX-cDNA sequencing : 1 month, 15.000 €
(used to be
200.000 € with Sanger technology)

microarray production: 100 € per array
microarray screening: 150 € per array

cheaper options (like SOLEXA technology) are
becoming available, at much lower costs

Relative costs of conservation genomics:



Projected costs (but this is almost certain a severe underestimation):

20 billion Euro

That is:

20.000.000.000 Euro

That is equivalent to
40.000.000 microarray
runs.....

We live in exciting times !!!