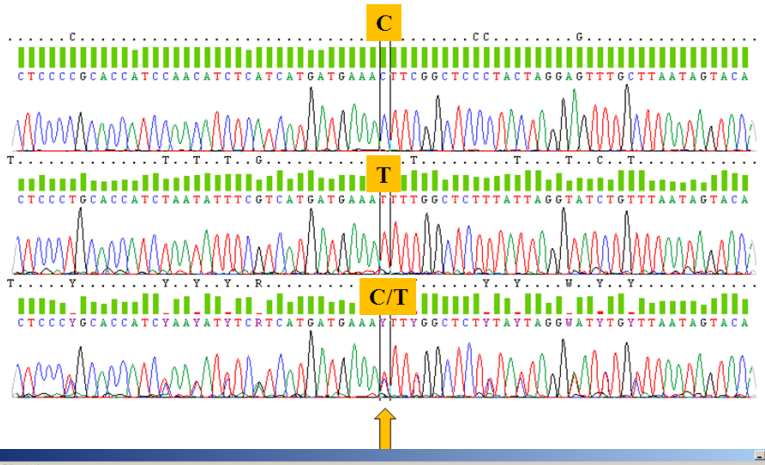
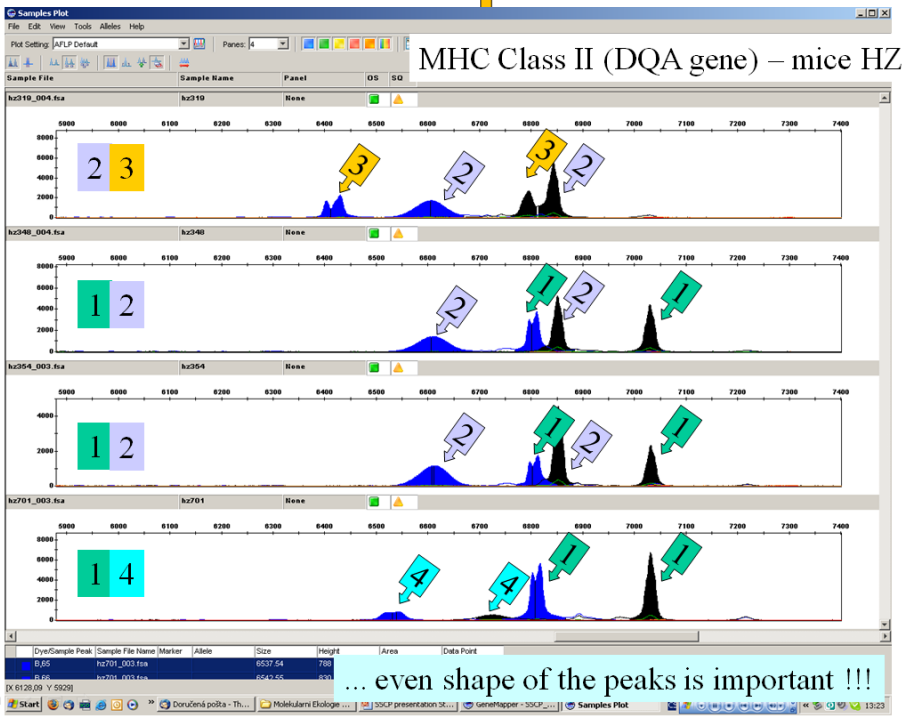
SNPs genotyping - sekvenování?
Je drahé a nejasné u heterozygotů



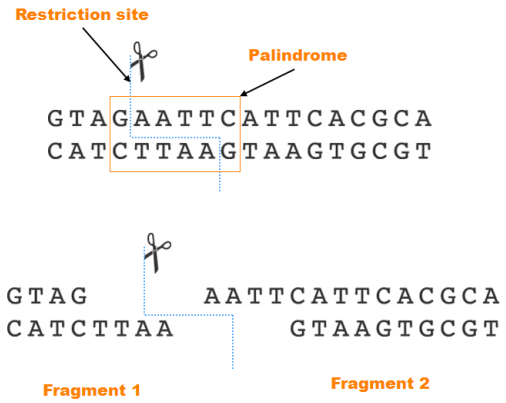


SNP genotyping - old standards

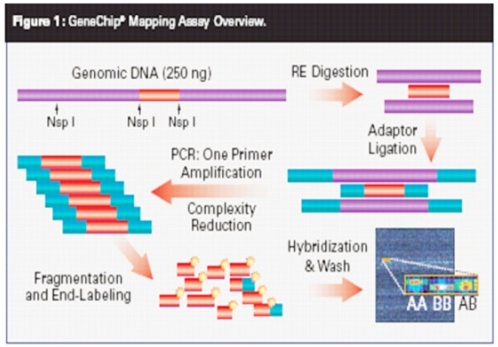
PCR-RFLP
(restriction fragments length polymorphism)

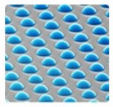
Enzyme Site Recognition

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

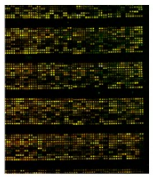


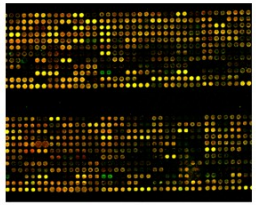
Detekce: Affymetrix, Illumina





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
Mikrosatelity	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***

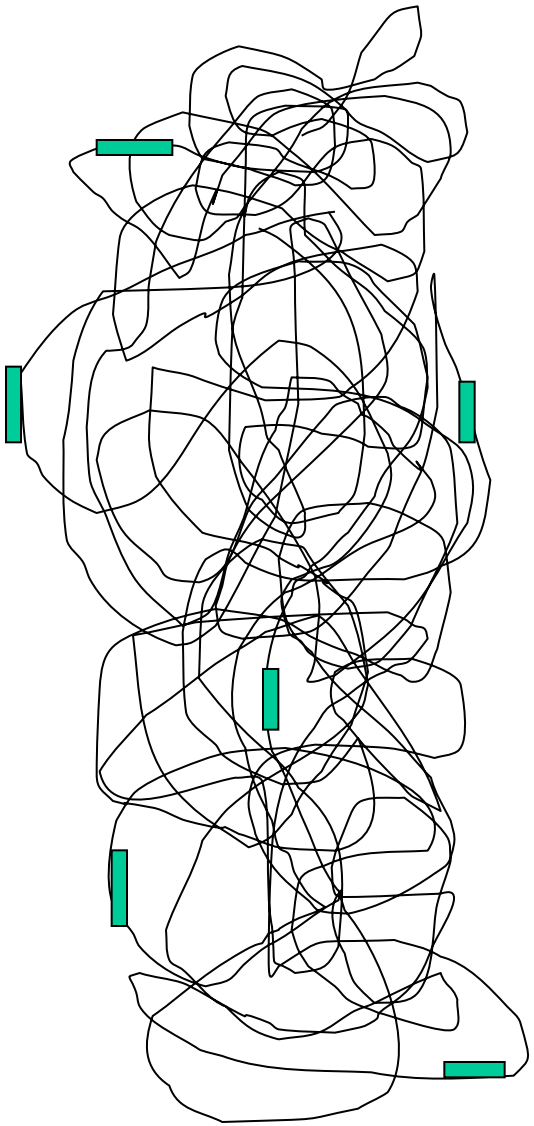
➢ ***RAPD*** (randomly amplified polymorphic DNA)

➢ ***AFLP*** (amplified fragment length polymorphism)

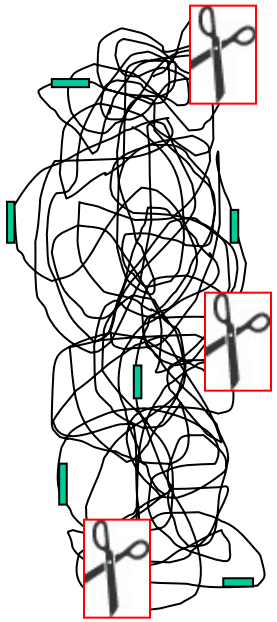
- presence vs. absence **restrikčního místa** (AFLP) **či místa pro dosednutí primerů** (RAPD) = **dominantní znaky** (neodliší heterozygota – proužek na gelu buď je nebo není)

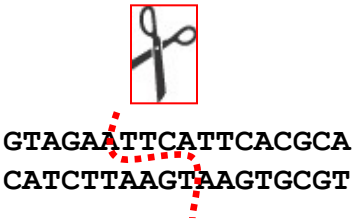
- není nutno znát předem genom studovaného druhu (tj. primery)

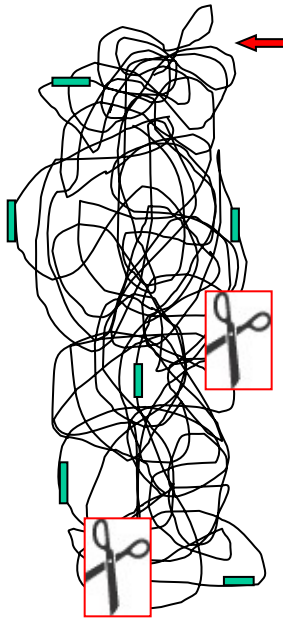
Př.: chromozóm 1



Každý jedinec má jedinečný genom







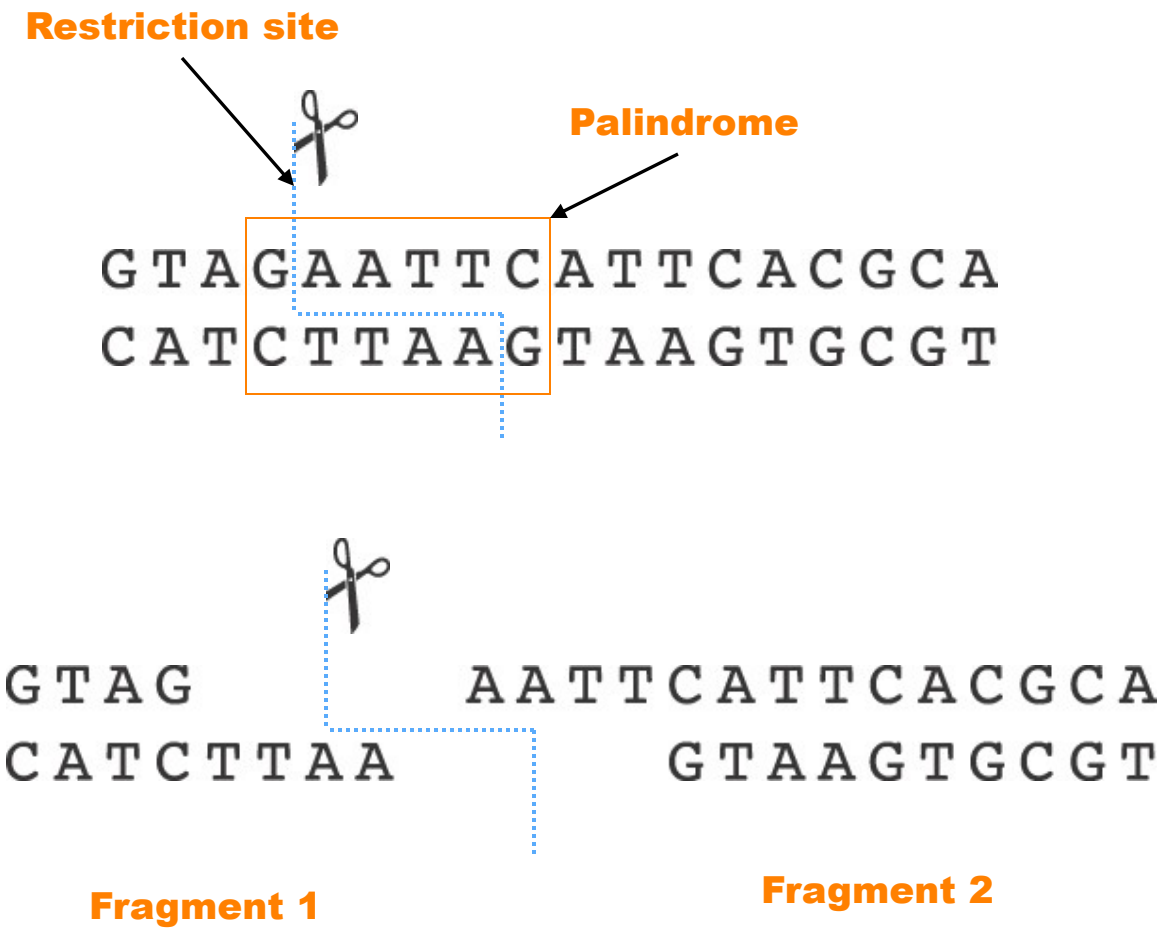
Záměna v restrikčním místě = zde ke štěpení nedojde

GTAGAATgCATTCACGCA
CATCTTAcGTAAGTGCGT

1. Ztráta nebo nabytí restrikčního 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)**

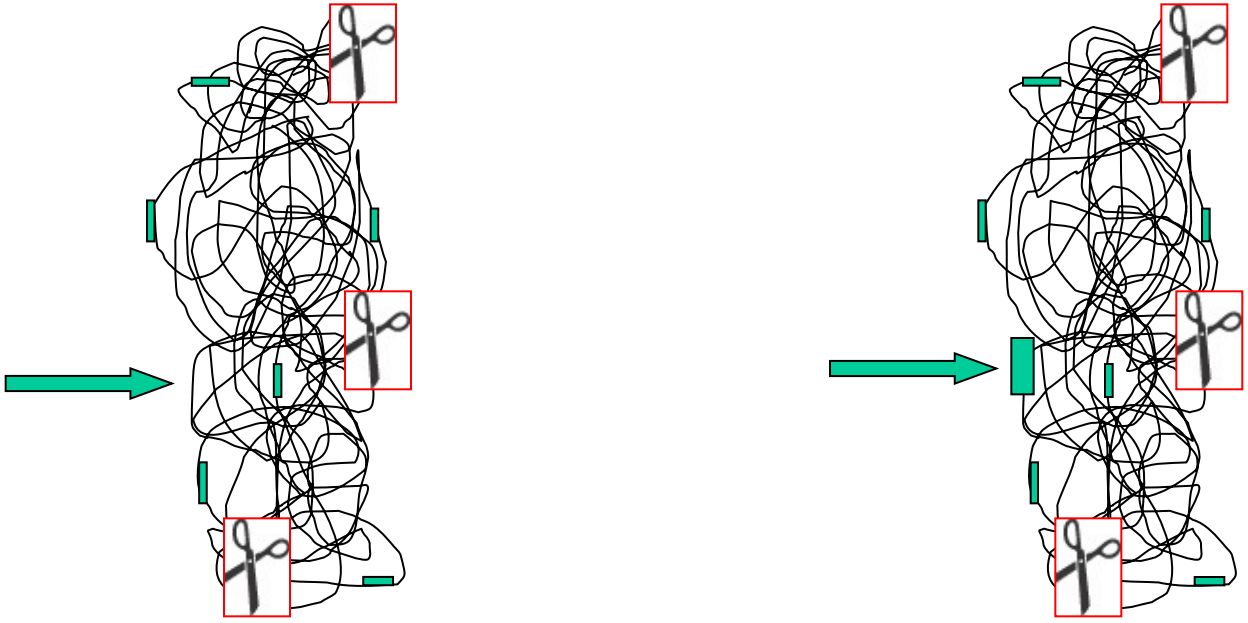


Common Restriction Enzymes

EcoRI
- *Eschericha coli*
- 5 prime overhang

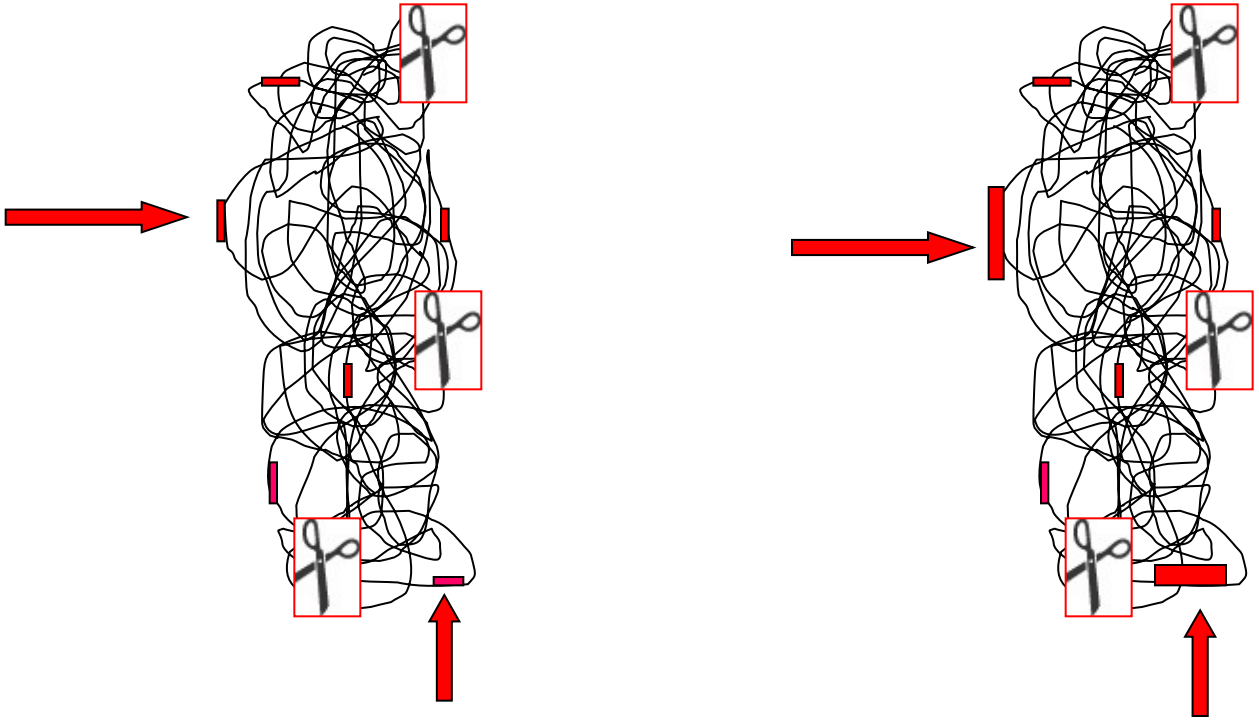
PstI
- *Providencia stuartii*
- 3 prime overhang

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 legth, 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

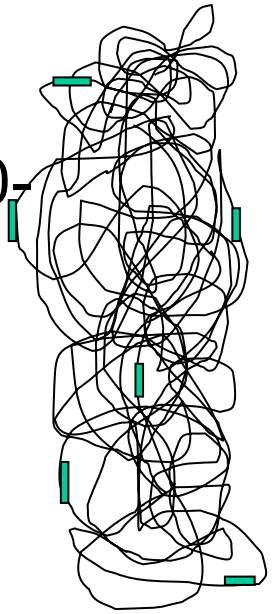
(Minisatellite) DNA fingerprinting
(Jeffreys et al. 1985)

- první celogenomový screening

- náhodně rozmístěné repetice (např. SINE, LINE)

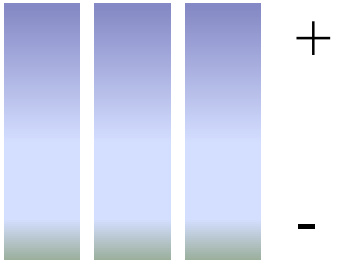
- různě dlouhé minisatelity (různá délka repetic, 10-15 bp „core sequences“)

- restrikční štěpení kompletní DNA – sekvenčně specifické restrikční endonukleázy

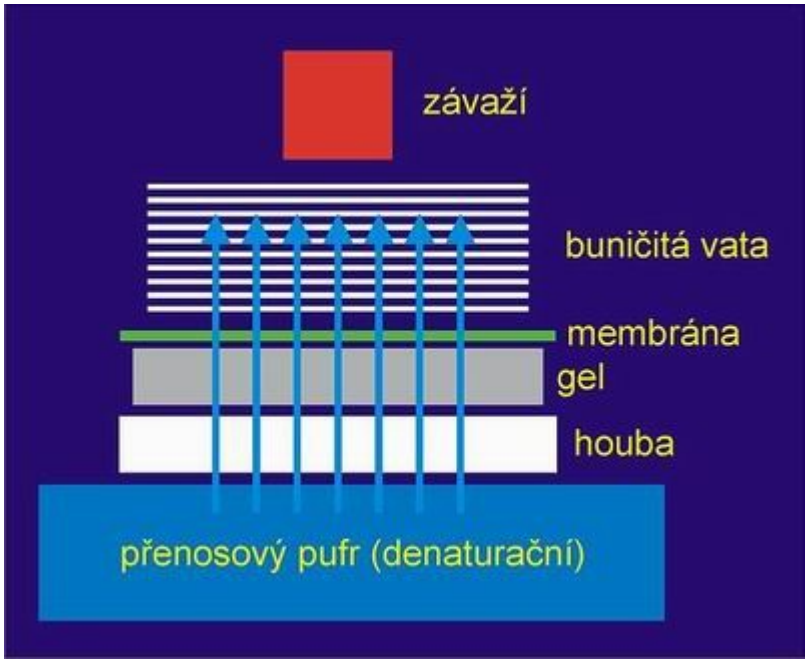


Minisatellite DNA fingerprinting

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

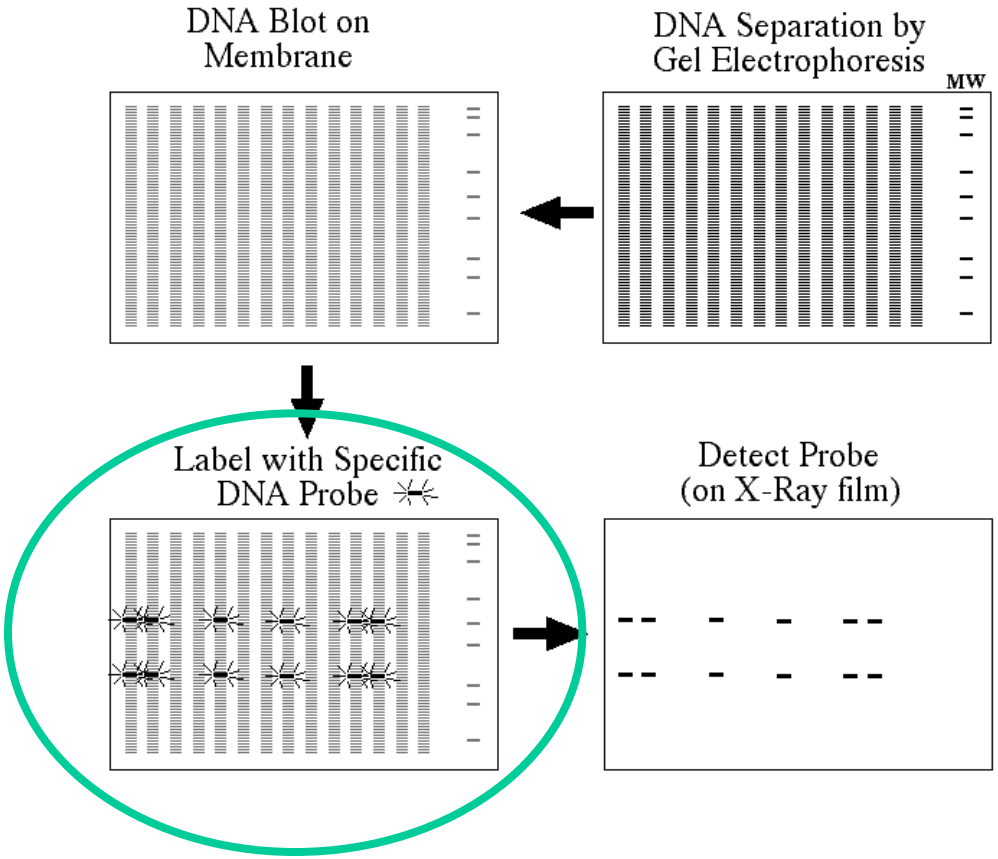


„smear“



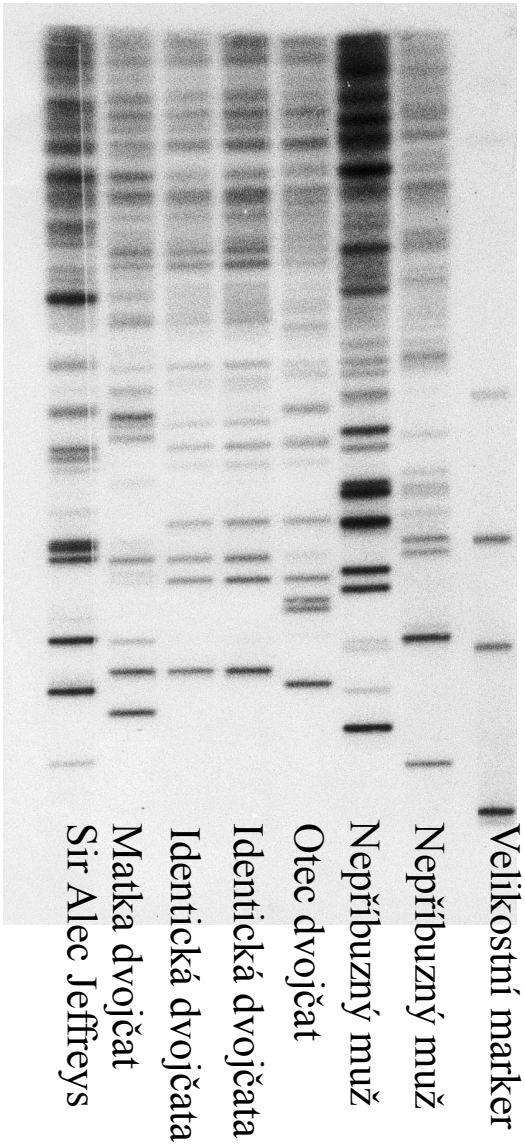
Minisatellite DNA fingerprinting

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



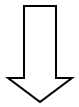
Minisatellite DNA fingerprinting

- 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

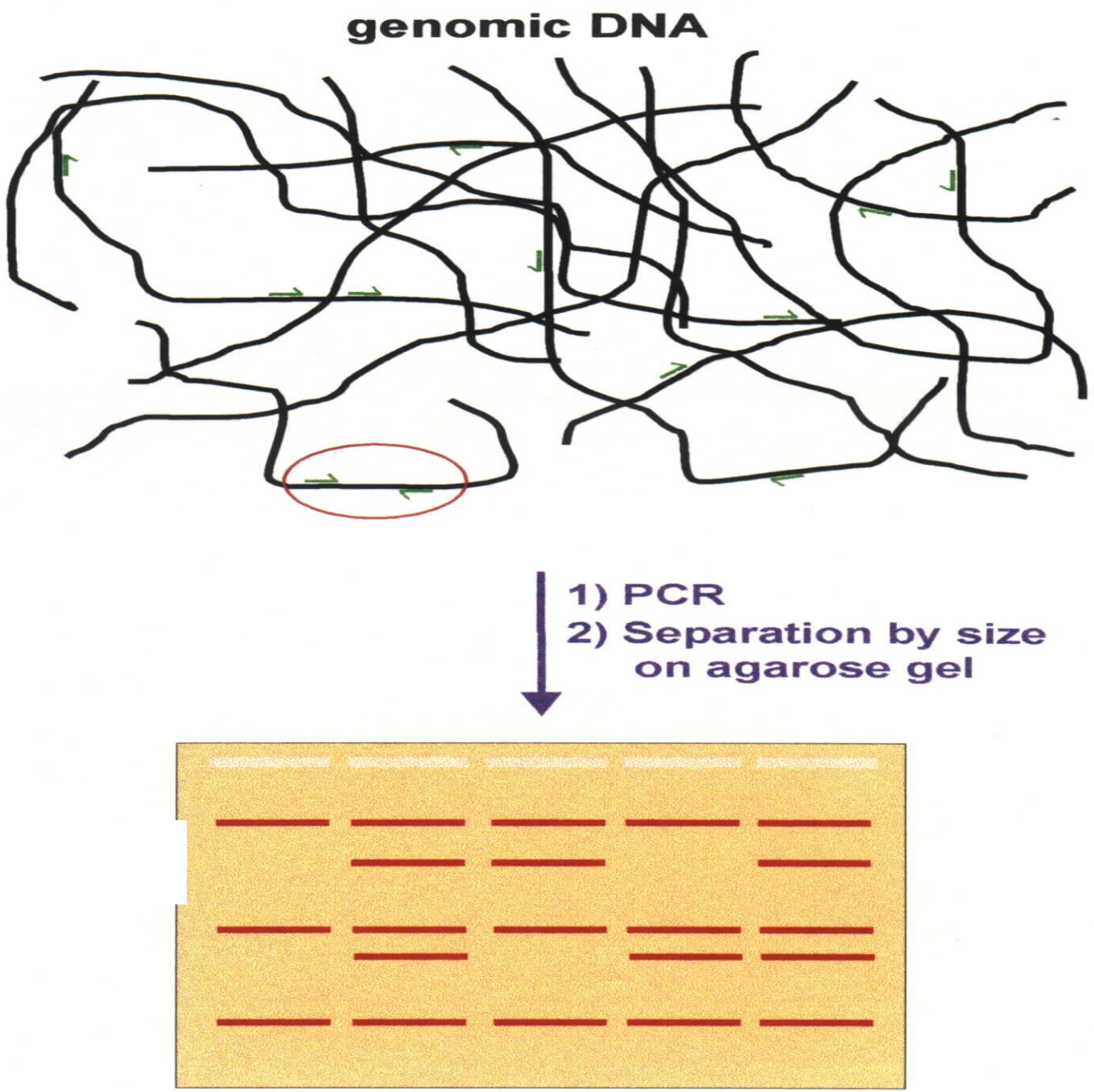


RAPD (randomly amplified polymorphic DNA)

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

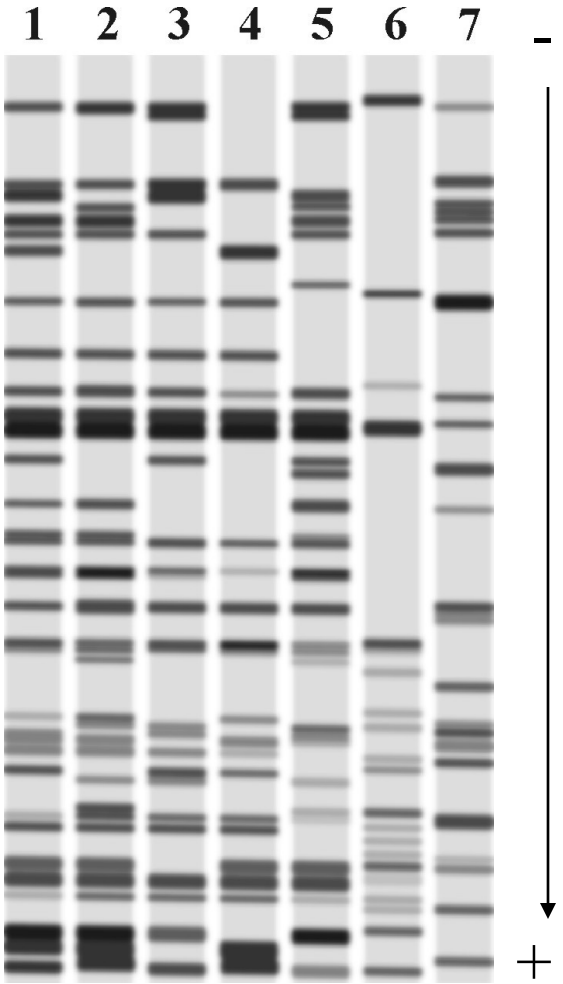
⇩

PCR za málo specifických podmínek

⇩

Detekce PCR produktů elektroforézou

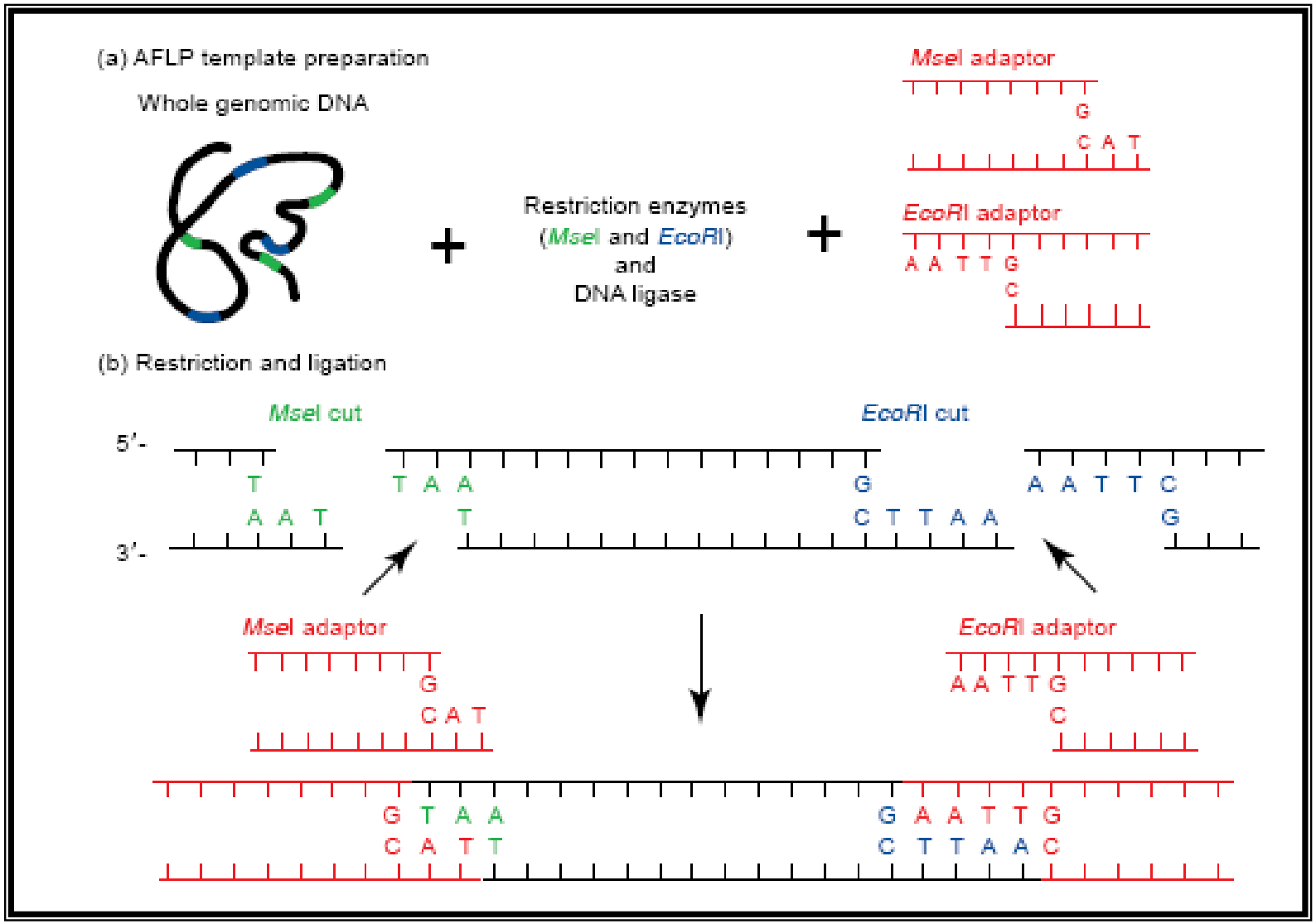
Nízká opakovatelnost v důsledku mnoha faktorů ovlivňujících PCR – dnes již není akceptována jako metoda např. pro studium populační struktury



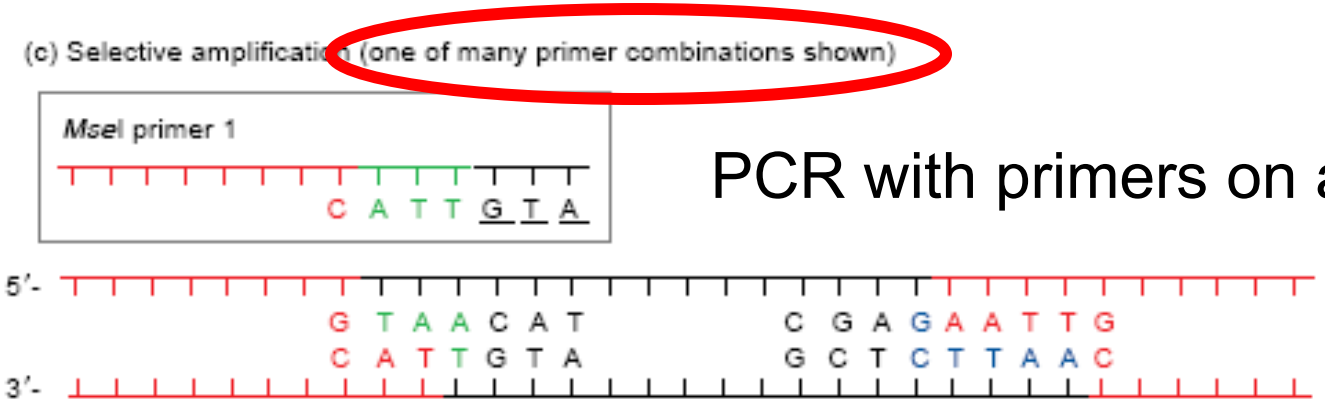
AFLP (amplified fragments length polymorphism)

- 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

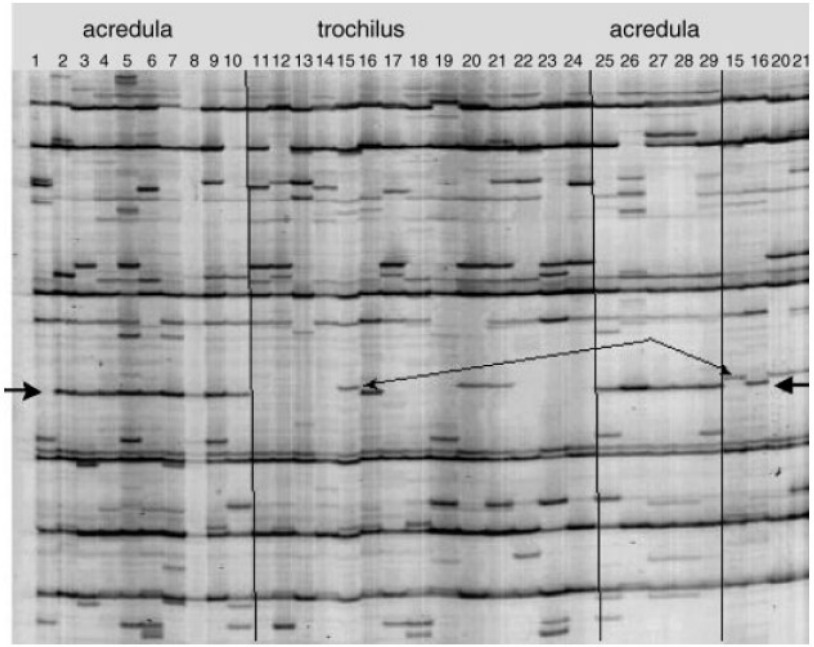
Princip AFLP metody („generating AFLP markers")



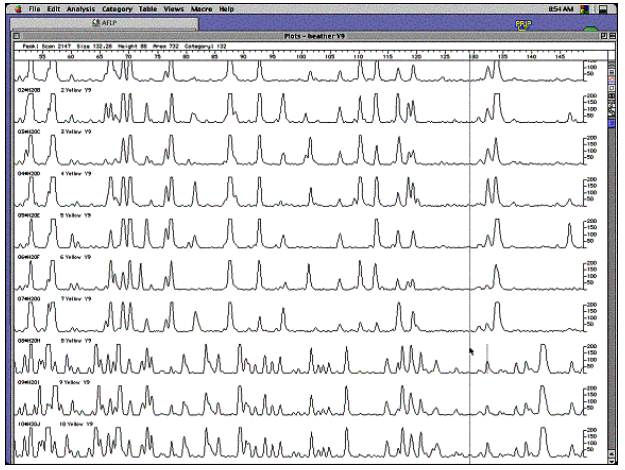
Generating AFLP markers



PCR with primers on adaptors



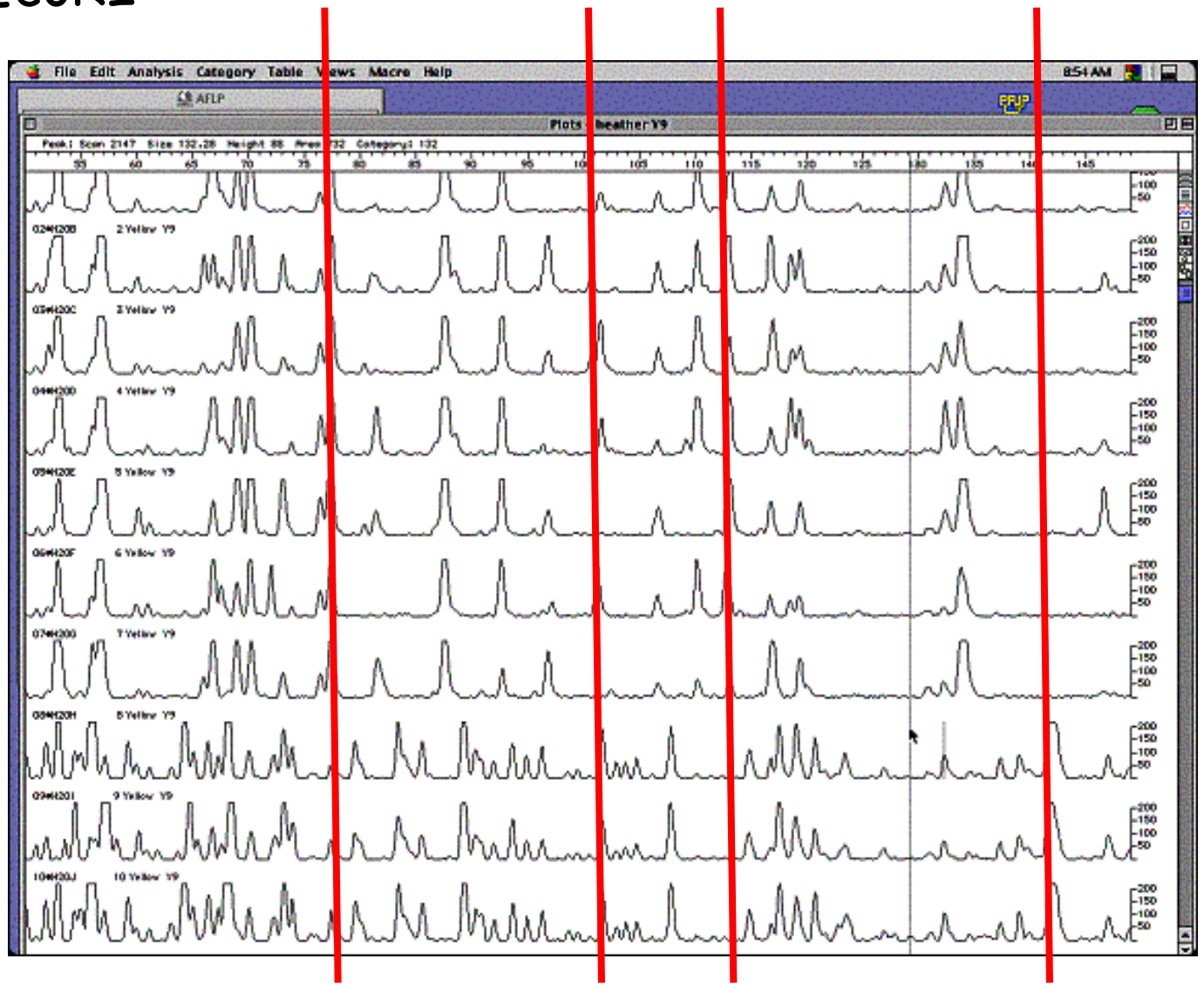
multi-locus genotype



„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

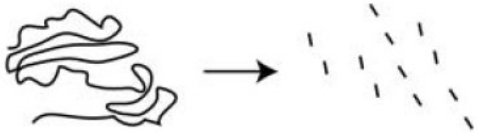
=

cca 1 000 000 000 bp/den

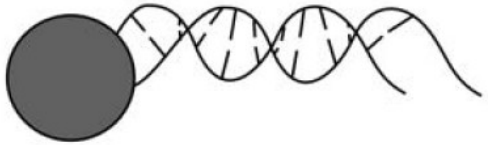
electrophoresis

„Next generation sequencing"

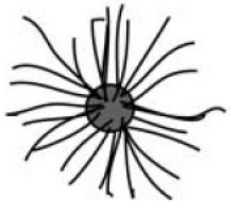
1) Randomly fragment many molecules of target DNA



2) Immoblize individual DNA molecules on solid support



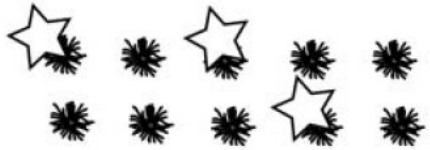
3) Amplify DNA in clonal 'polymerase colony'



„polonies"
(polymerase colonies)

4) Sequence DNA by adding liquid reagents to immoblized 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 bazí
- 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

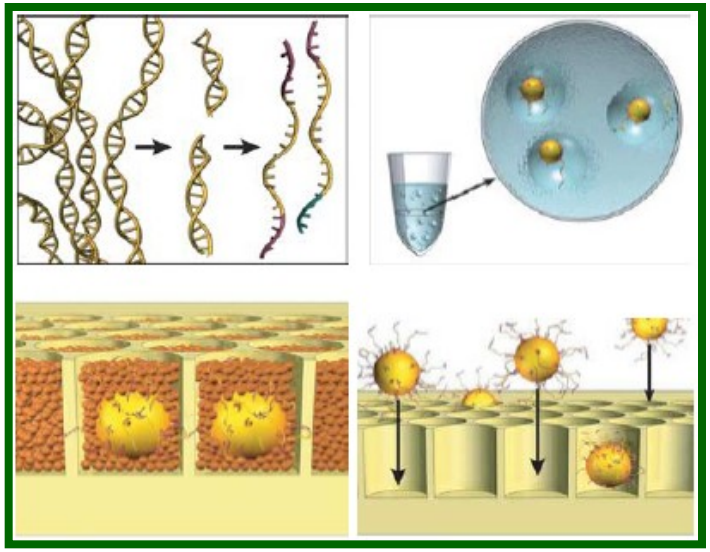
NEWS AND VIEWS

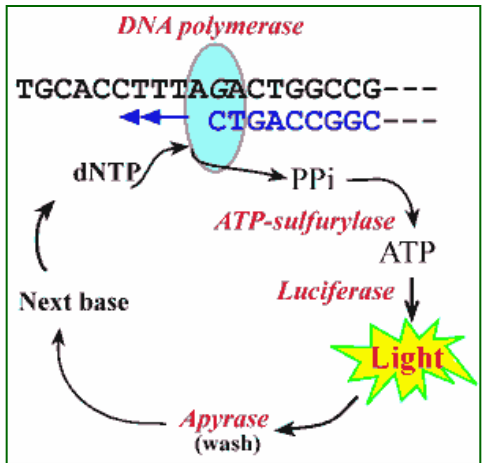
PERSPECTIVE

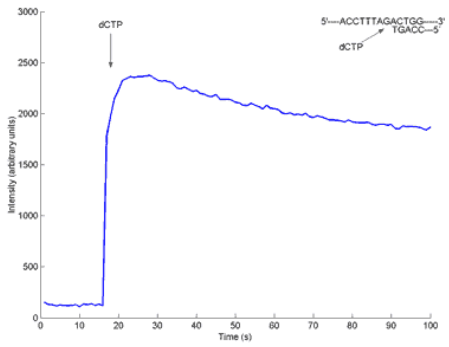
Sequencing goes 454 and takes large-scale genomics into the wild

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

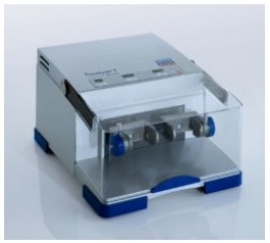
1 600 000 well plate







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

Controlled Room

4. Amplification
5. Bead Recovery
6. DNA Library Bead Enrichment
7. Sequencing Primer Annealing

Time: 11 - 13 h

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

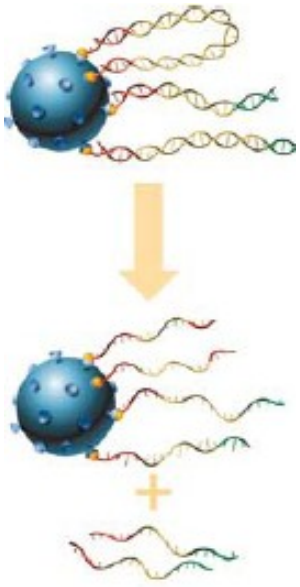
1. Příprava jednořetězcové DNA knihovny (ssDNA library preparation)

1 DNA Fragmentation (Nebulization):

5 Adaptor Ligation:

7 Library Immobilization:

9 ssDNA Library Isolation:



Adaptor A + **Adaptor B**

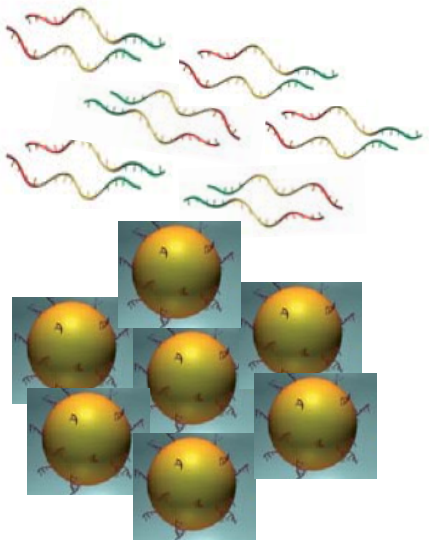
-Slouží jako vazebné místo primerů pro následnou PCR amplifikaci a sekvenování

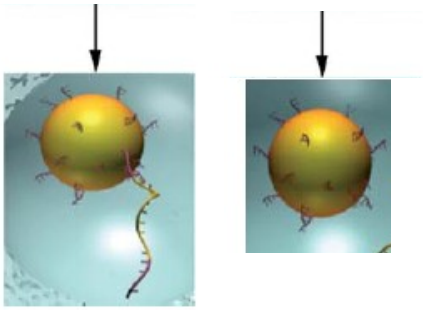
-Slouží k uchycení na kuličky (na adaptor B je připojen biotin)

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

1 DNA Library Capture:

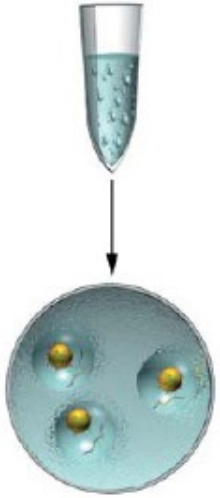
- poměry nastavit tak aby 1kulička ≤ 1 molekula DNA



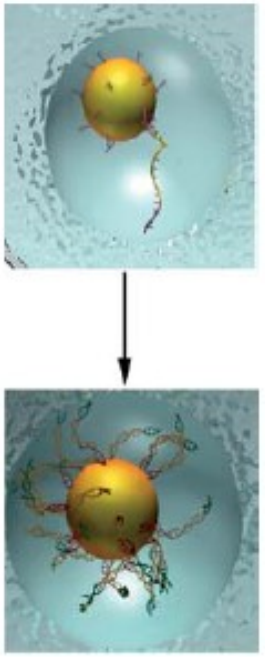


2 Preparation of the Amplific. Mixes

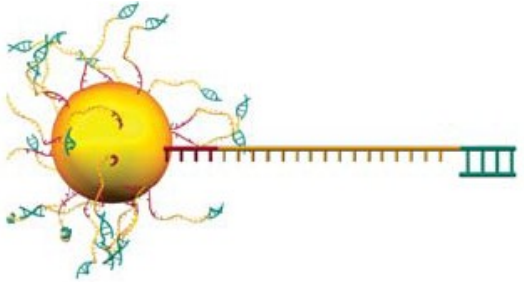
3 Emulsification:



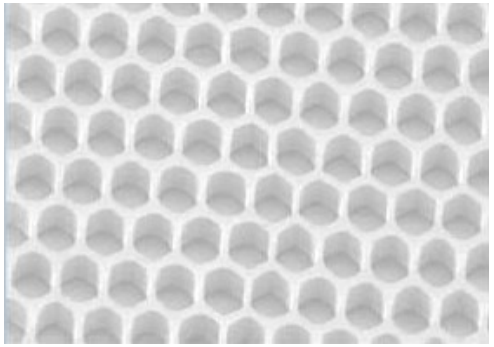
4 emPCR Amplification:



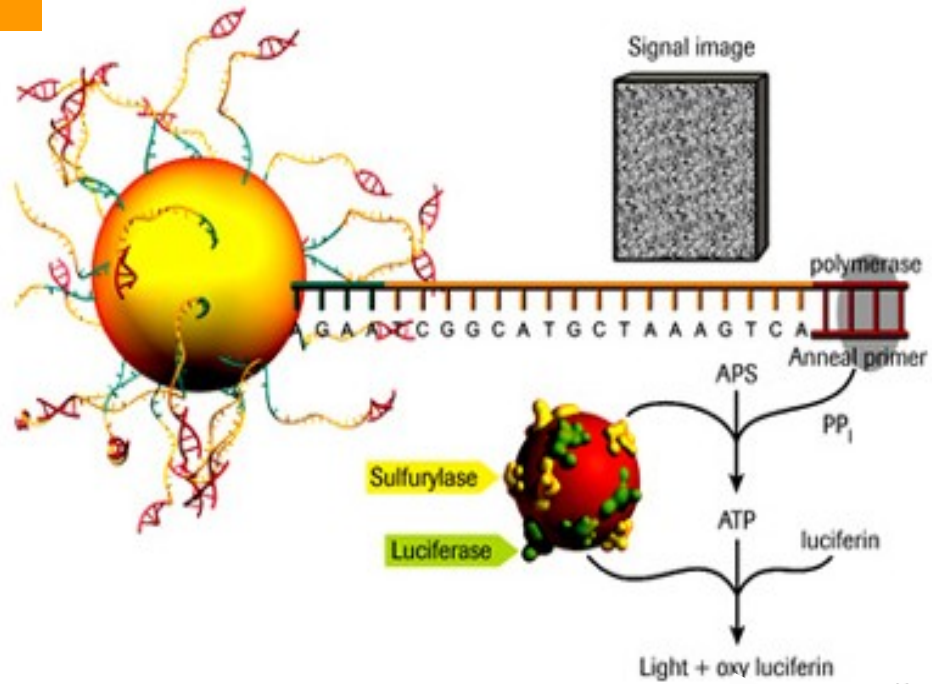
7 Sequencing Primer Annealing:



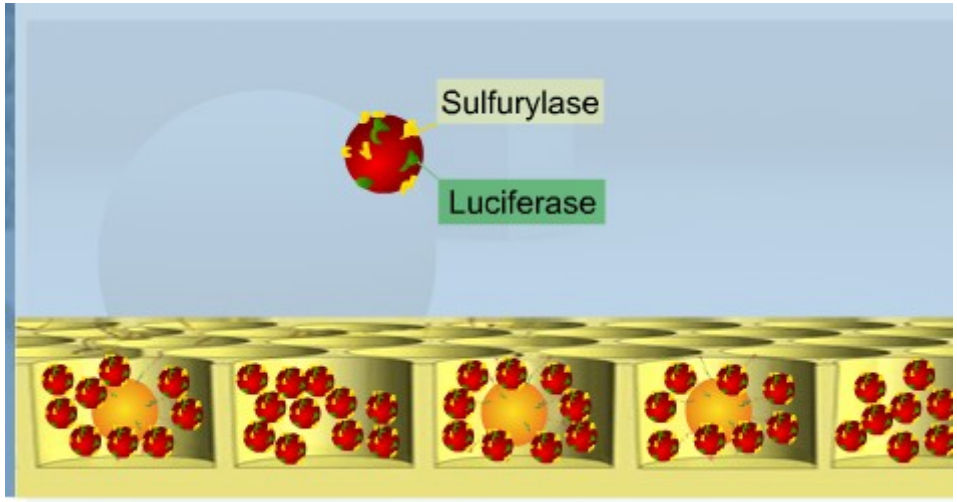
3. Pyrosekvenovani

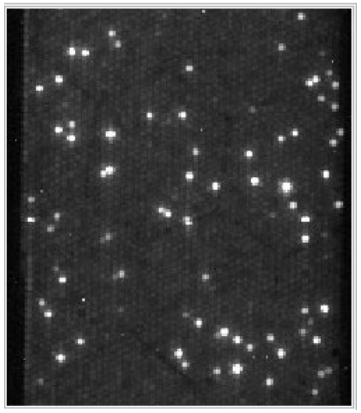


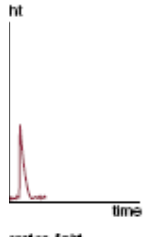
pikotitrační destička



Na jedné desticče 400 000 až 1milión jamek



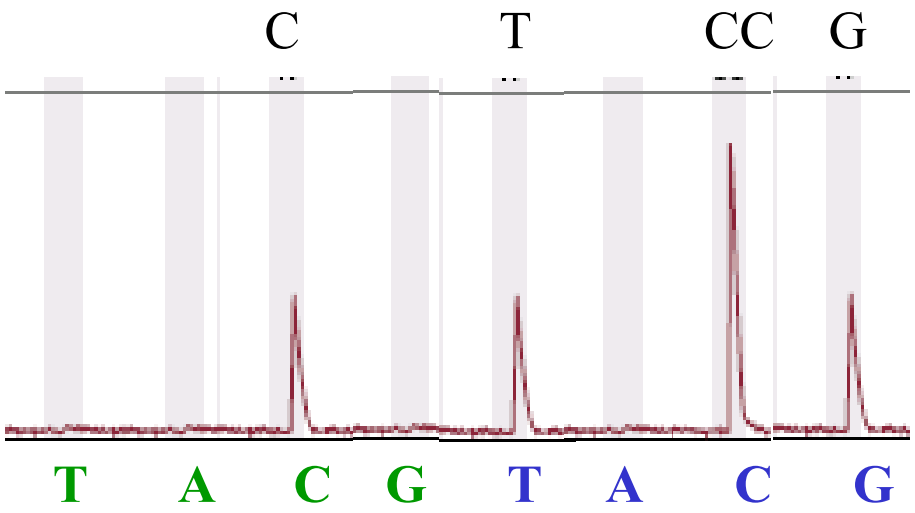




3. Pyrosekvenovani – detekce signálu

- 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**



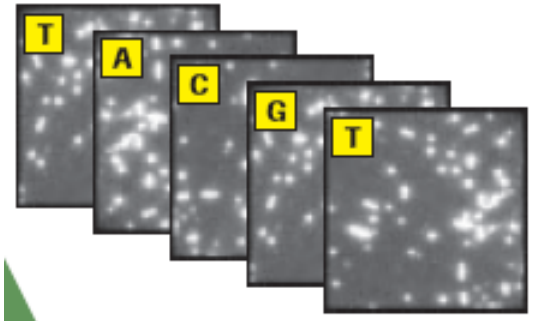
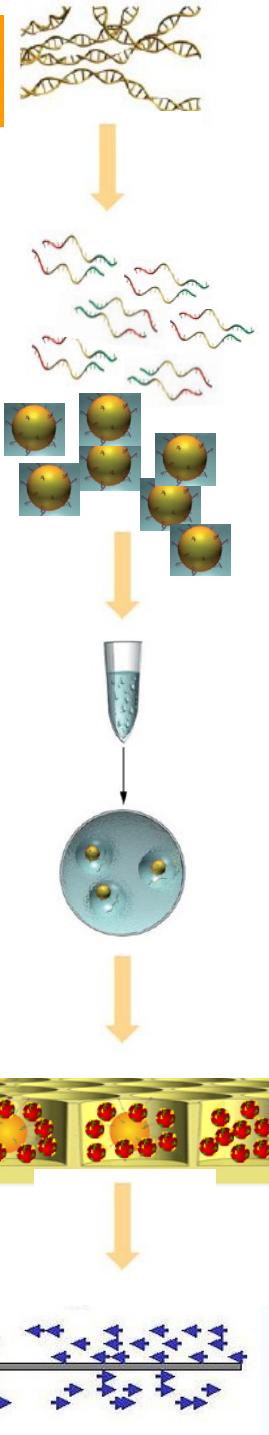


Image Files:
12-15 gigabytes
per run

Problém!!!! Homoplymery např. AAAAAAAAAA

High-throughput – paralelní sekvenování



1 běh (run) = 1 destička:

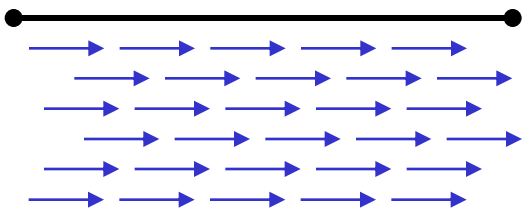
- 400 000/ 1milión jamek (reads)
- v každé 240 / 400 bp (read length)
- 7.5 / 10 hod

→ 100 Mb / 400 Mb na jednu destičku

→ cena??? 150-350 000 Kč ????

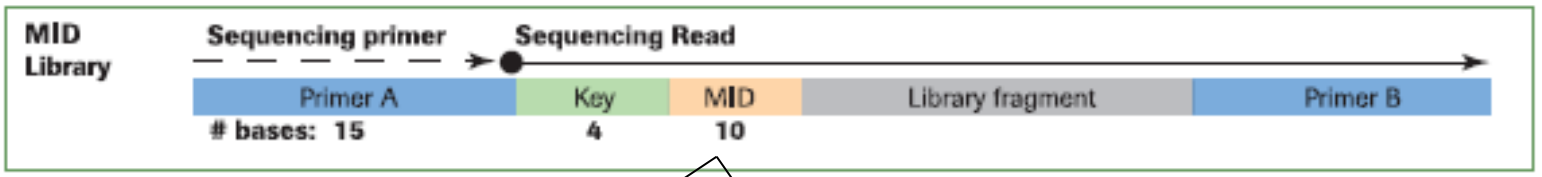
!!! Samozřejmě nestačí mít každou bázi osekvenovanou 1x !!!

- Pospojování (reads assembly) do souvislé sekvence
- Nepřesnosti – pokrytí (coverage)

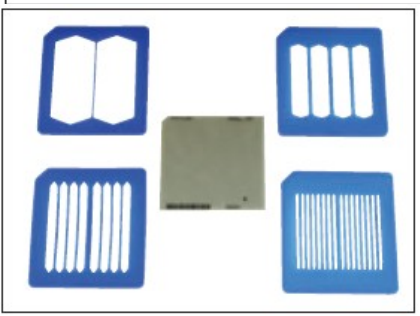


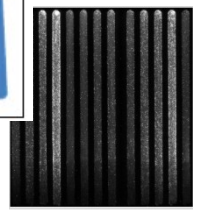
Kapacita destičky 400 Mb:

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

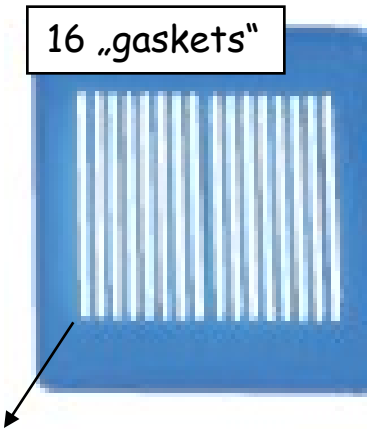


- k dispozici 12 odlišných MID





1. *CCCCCCCCCC*
2. *GGGGGGGGG*
.
.
.
12. *CCCCCAAAG*



12 MID
X
16 gaskets
=
max. 192 vzorků

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

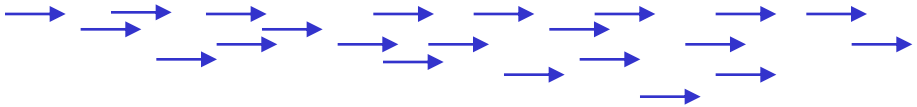
Problém: **KRÁTKÝ READ LENGTH**

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

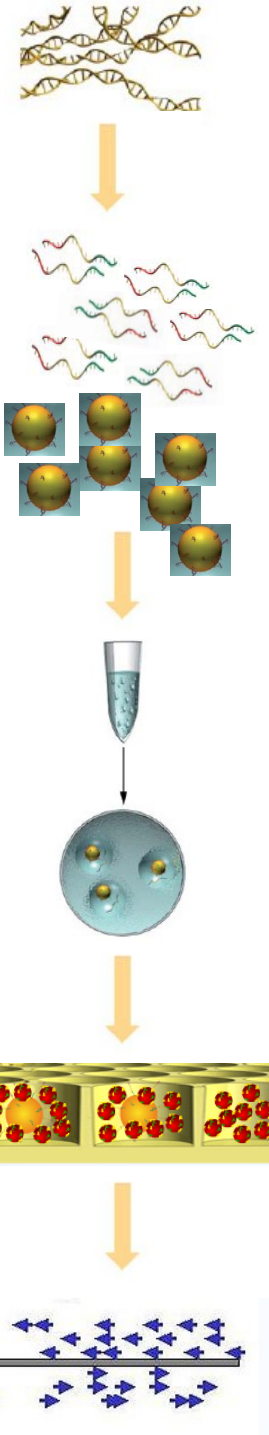
→ Uspořádání (assembly) už není problém z hlediska výpočetní kapacity

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

GTAAAAAAAAAAAAAAAAAAAAAC



Zvláště komplexní eukaryotické genomy – úseky souvislých oblastí přerušených mezerami



1. Celogenomové sekvenování de novo

- získání kompletní uspořádané sekvence celých velkých eukaryotních genomů pomocí next-generation sequencing de novo je problém (ale to je nakonec i u Sangera)
- viry, prokaryota, malá eukaryota, mitochondrie/plastidy/plasmidy

× Ale čo

Genetic Detection and Characterization of Lujo Virus, a New Hemorrhagic Fever–Associated Arenavirus from Southern Africa

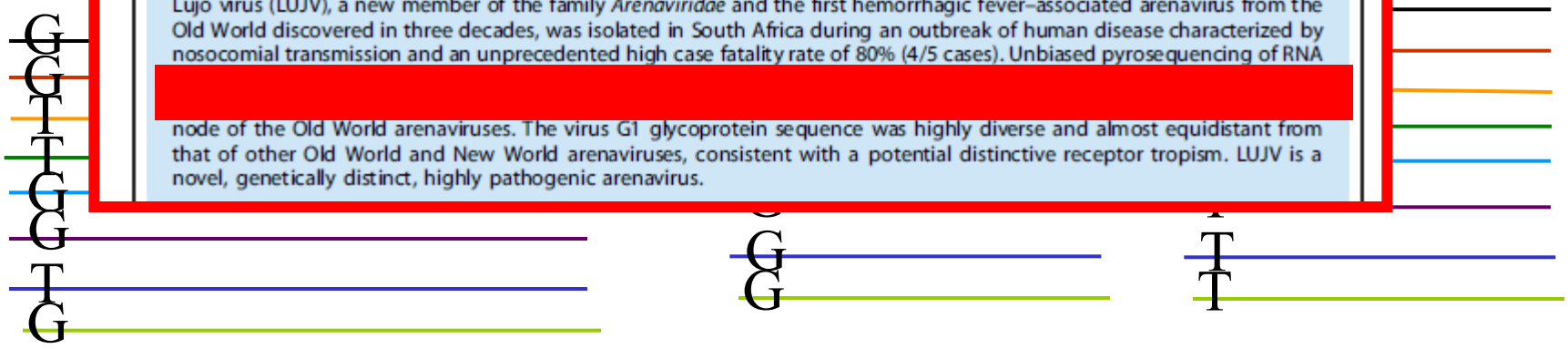
Thomas Briese[1☯*], Janusz T. Paweska[2☯], Laura K. McMullan[3], Stephen K. Hutchison[4], Craig Street[1], Gustavo Palacios[1], Marina L. Khristova[5], Jacqueline Weyer[2], Robert Swanepoel[2], Michael Egholm[4], Stuart T. Nichol[3], W. Ian Lipkin[1*]

1 Center for Infection and Immunity, Mailman School of Public Health, Columbia University, New York, New York, United States of America, 2 Special Pathogens Unit, National Institute for Communicable Diseases of the National Health Laboratory Service, Sandringham, South Africa, 3 Special Pathogens Branch, Division of Viral and Rickettsial Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, United States of America, 4 454 Life Sciences, Branford, Connecticut, United States of America, 5 Biotechnology Core Facility Branch, Centers for Disease Control and Prevention, Atlanta, Georgia, United States of America

Abstract

Lujo virus (LUJV), a new member of the family *Arenaviridae* and the first hemorrhagic fever–associated arenavirus from the Old World discovered in three decades, was isolated in South Africa during an outbreak of human disease characterized by nosocomial transmission and an unprecedented high case fatality rate of 80% (4/5 cases). Unbiased pyrosequencing of RNA

node of the Old World arenaviruses. The virus G1 glycoprotein sequence was highly diverse and almost equidistant from that of other Old World and New World arenaviruses, consistent with a potential distinctive receptor tropism. LUJV is a novel, genetically distinct, highly pathogenic arenavirus.



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

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

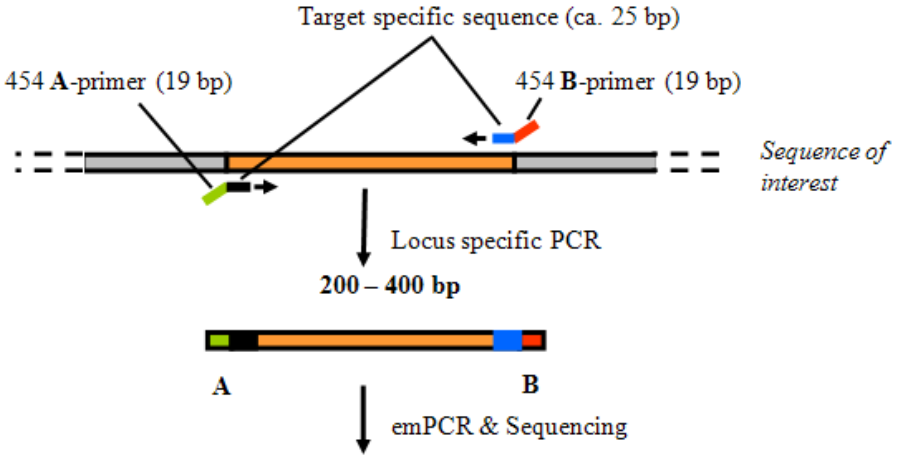
Richard E. Green,[1,*] Anna-Sapfo Malaspinas,[2] Johannes Krause,[1] Adrian W. Briggs,[1] Philip L.F. Johnson,[3] Caroline Uhler,[4] Matthias Meyer,[1] Jeffrey M. Good,[1] Tomislav Maricic,[1] Udo Stenzel,[1] Kay Prüfer,[1] Michael Siebauer,[1] Hernán A. Burbano,[1] Michael Ronan,[5] Jonathan M. Rothberg,[6]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

3.Sekvenování amplikonů (PCR produktů)

SMĚSNÉ VZORKY

1. Metagenomika/metatranskriptomika

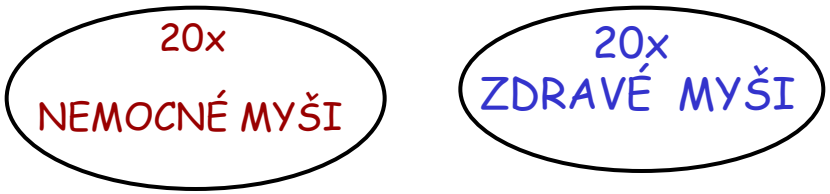
- Celé společenstvo půdních, vodních mikroorganismů, střevní mikroflóra
- PCR genu 16S (18S) rRNA
- lze i kvantifikovat

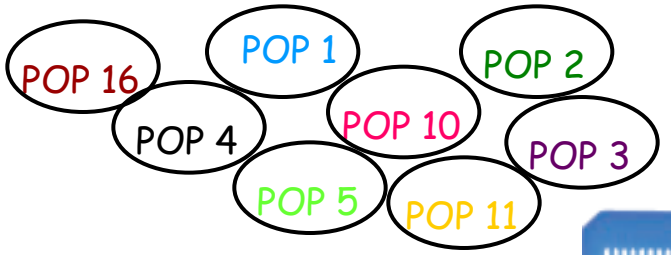


2. Složení potravy, trusu ???

3. Populační genetika

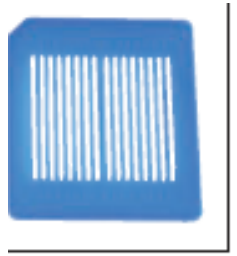
4. Studie u kandidátních genů





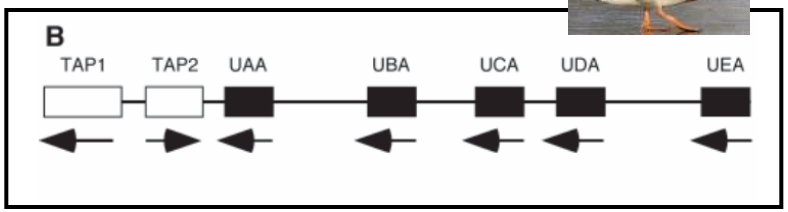
1. PCR např. **imunitního genu/genů**
2. Sekvenování
3. Které varianty jsou asociovány s chorobou??

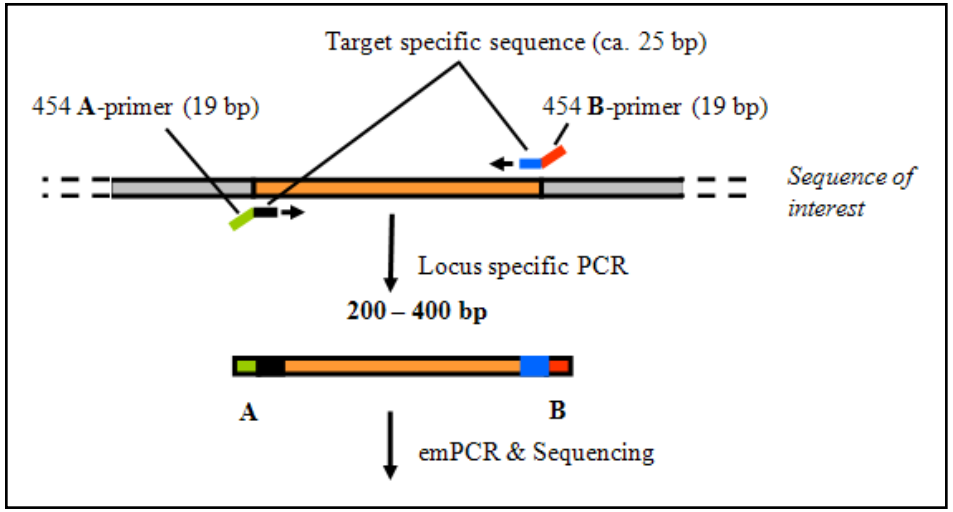
1. **PCR genu/genů**
2. Sekvenování
3. Zjištění sekvencí variant a frekvencí variant v každé populaci (záleží na pokrytí)



3.Sekvenování amplikonů (PCR produktů)

5. Genové duplikace



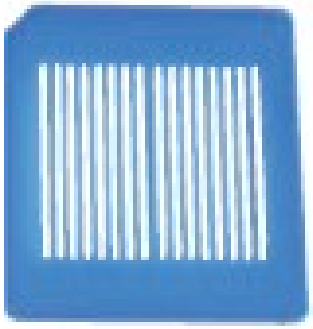


A-adaptor MID Target specific

Označí jedince

Amplifikuje všechny kopie MHC genů

Potřeba k emPCR, sekvenování..

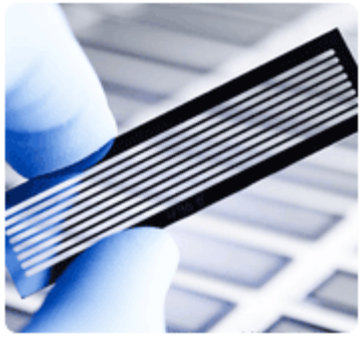


192 jedinců

Solexa/Illumina 1G SBS technology

(SBS = sequencing by synthesis)

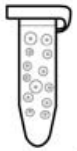
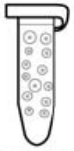
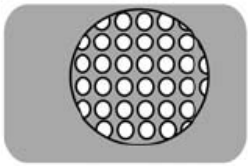
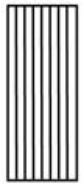
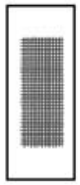
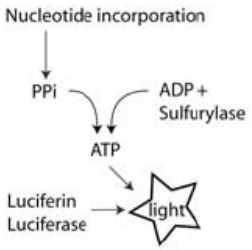
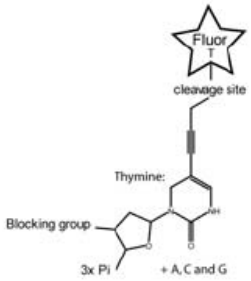
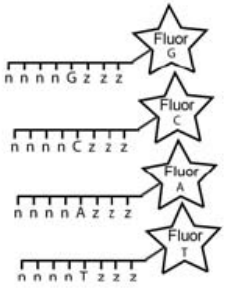
- 1 Gb (šestinásobek genomu *Drosophily*)
- Výrazně levnější
- Sekvence délky 35 bp
- Flourescence, reversibilní terminátory
- Spíš pro resequencing



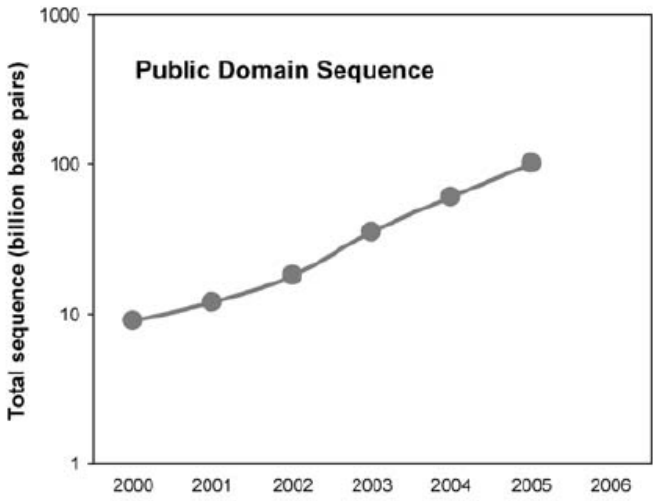
SOLiD

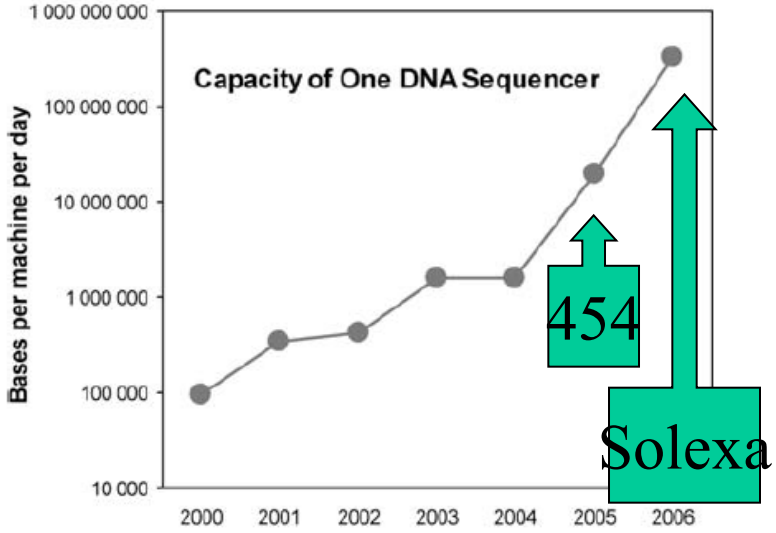
(sequencing by Oligonucleotide Ligation and

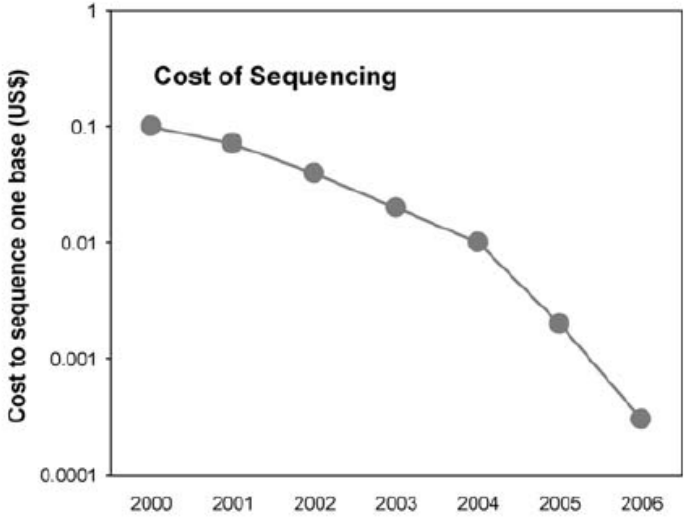
… a další (každého půlroku nová technologie – bouřlivý rozvoj !!!)

	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	 *in situ* 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	 pyrosequencing	 reversible-terminator sequencing by synthesis	 Ligation of sequence-specific labeled oligos
Sequence detection	Chemiluminescence (one channel)	Fluorescence (four channel)	Fluorescence (four channel)
Read length and number	100–400 bp $> 2 \times 10^5$ reads	35 bp $\sim 4 \times 10^7$ reads	25 bp (paired) $> 10^7$ reads

„genomics era“



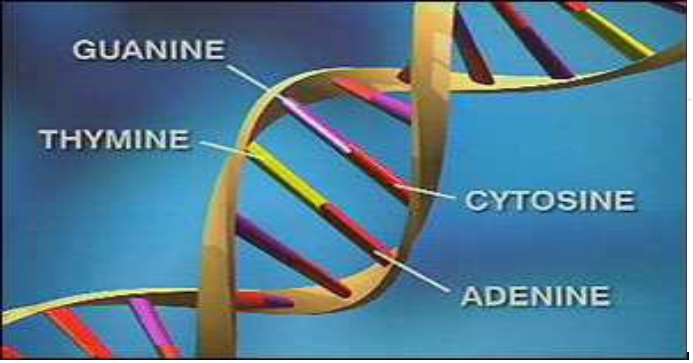


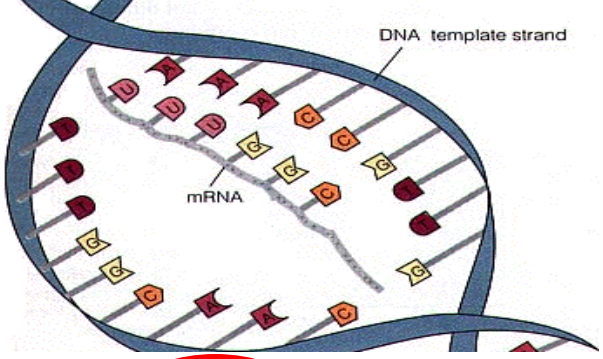


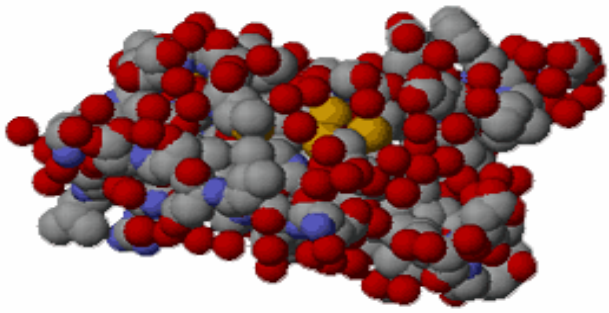
Budoucnost genetických metod
v ekologickém výzkumu

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

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

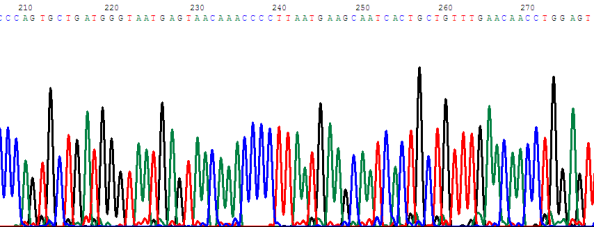


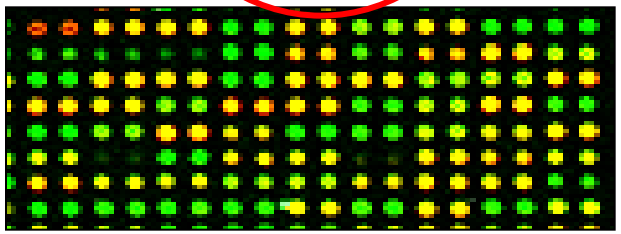


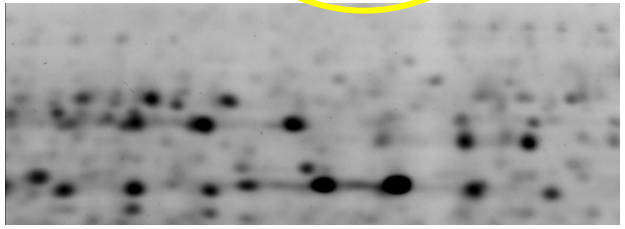


DNA → Transcription → mRNA → Translation → Proteins

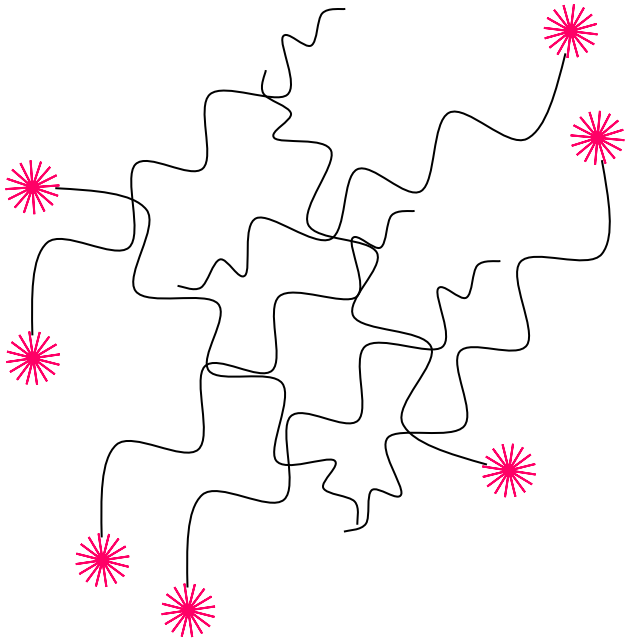
Genome		Transcriptome	Proteome
3 billion bases		20-30,000 genes	~100,000 proteins
Genomics	Functional Genomics	Transcriptomics	Proteomics

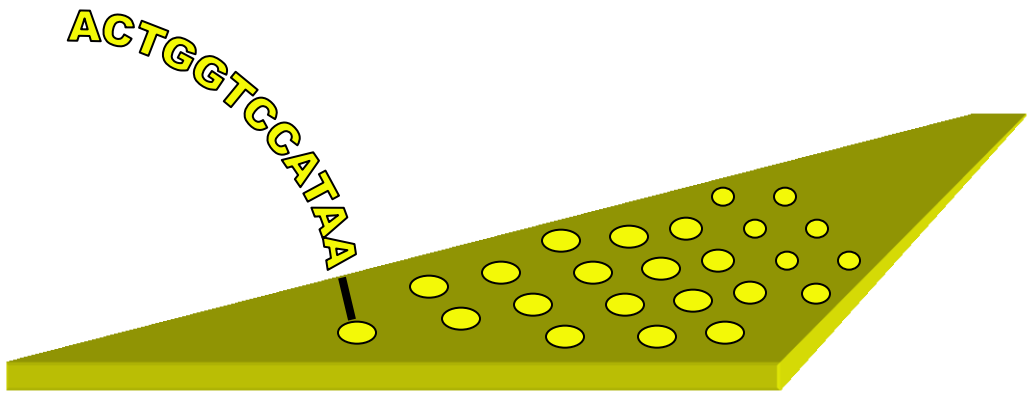






Microarray analysis of transcriptome
(~ specific DNA hybridization)





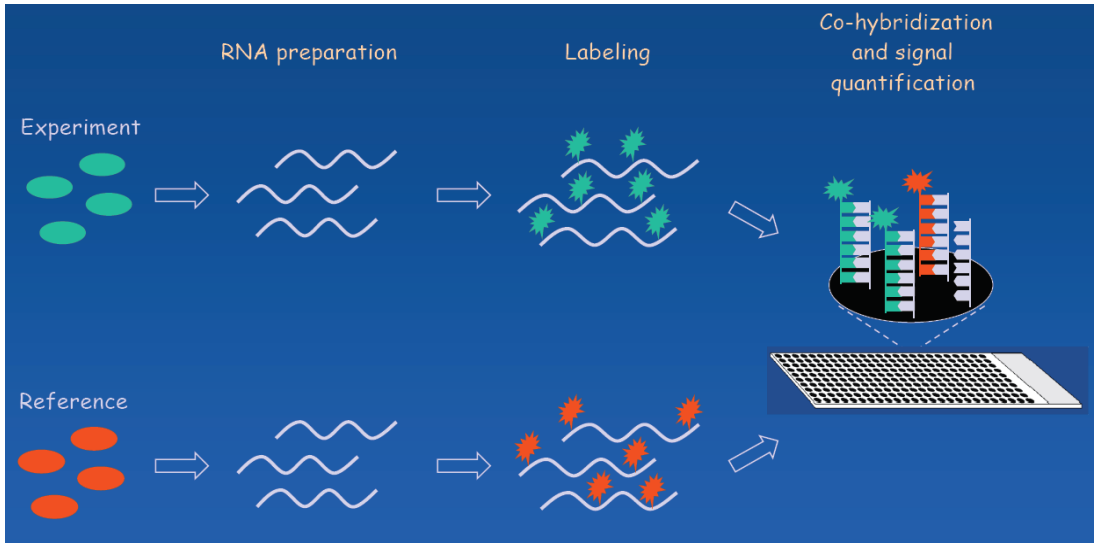
Target **(i.e. mix of transcripts in a form of cDNA)**

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

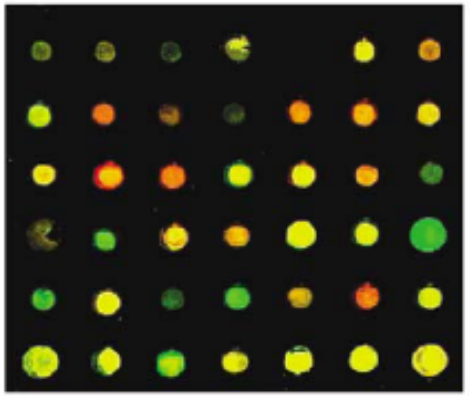
How to get a transcription profile

- vždy srovnání kontroly a „treatement“

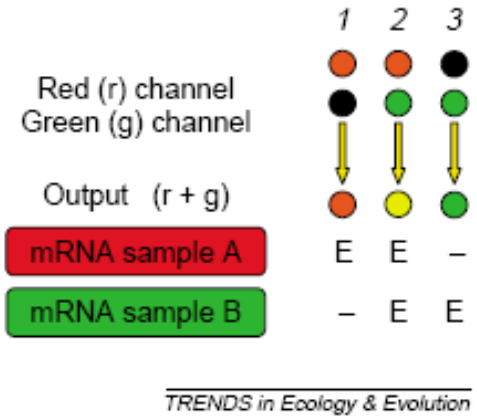
(a)



(b)



(c)



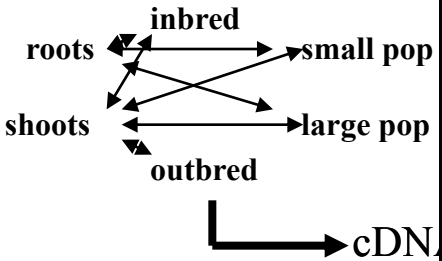
Analysis of expression l

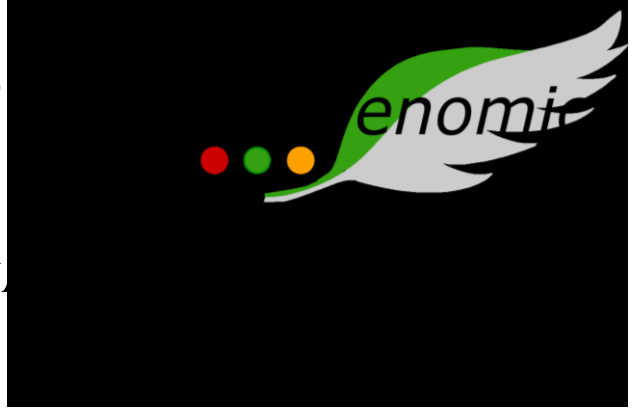
Case study: Joop Ouborg et al.

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

Example:

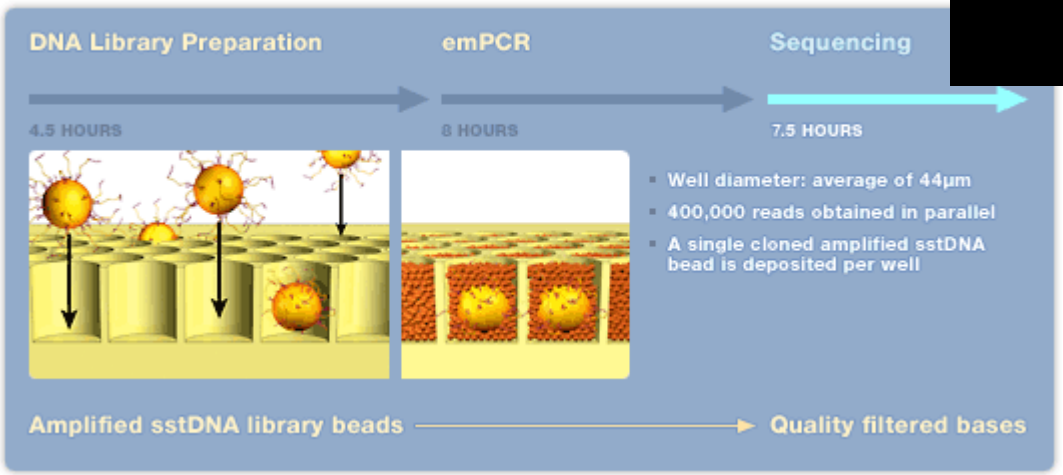
Scabiosa columbaria

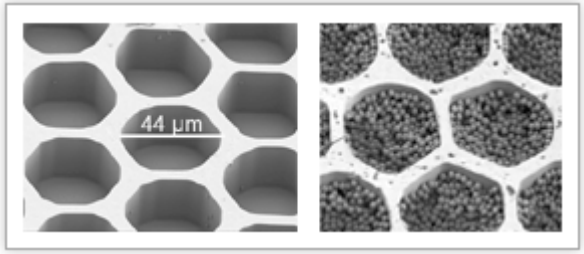


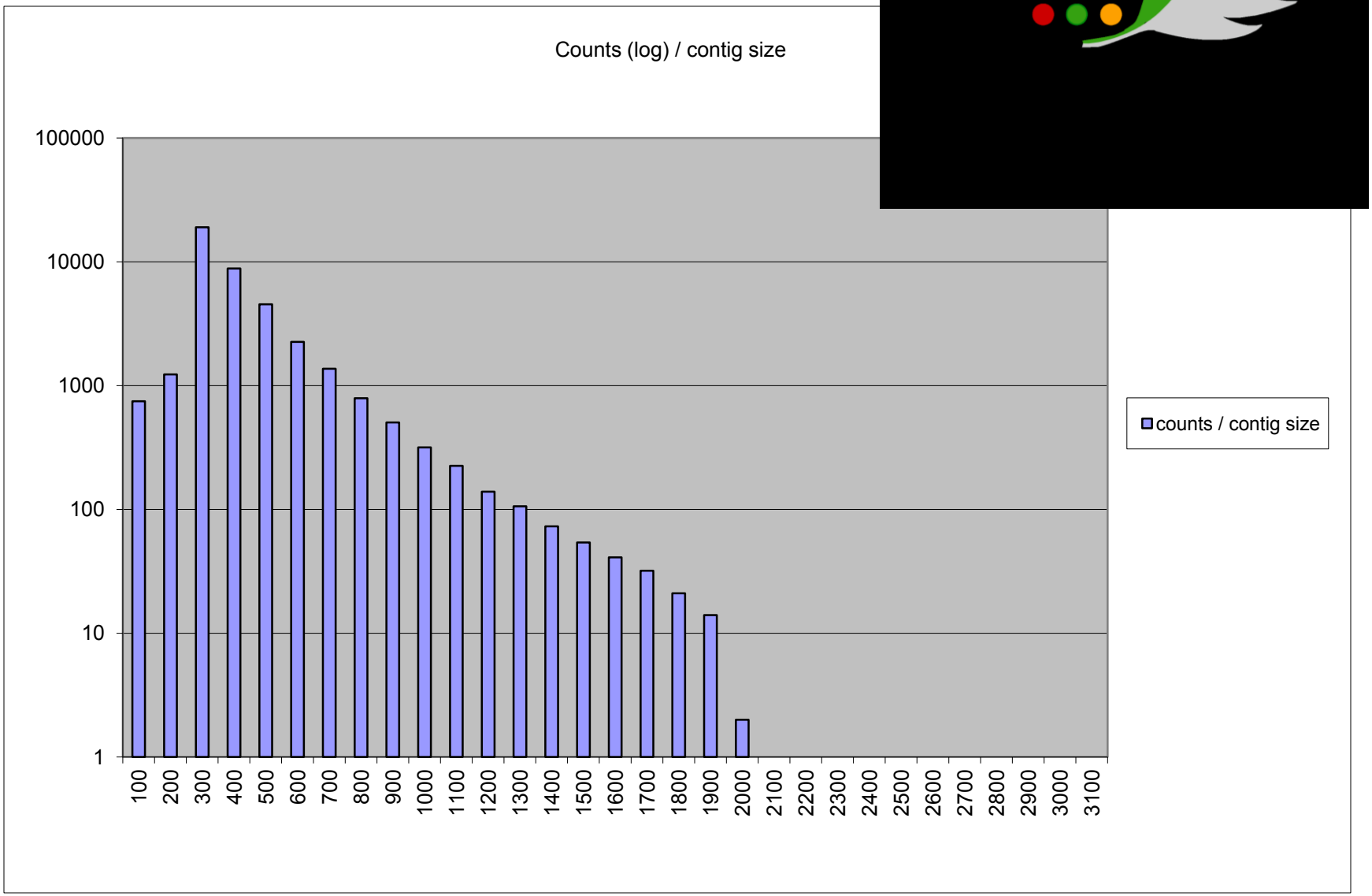


cDNA library preparation – 454 sequencing

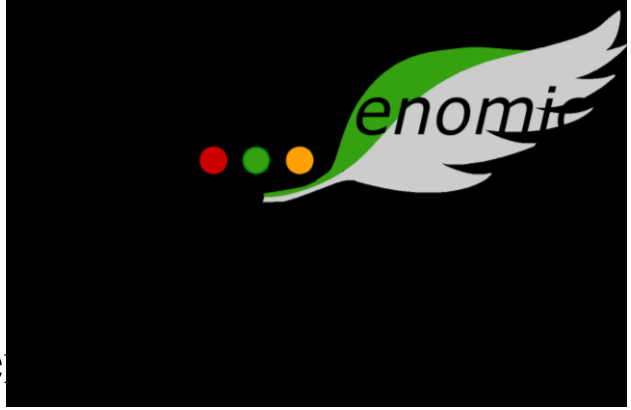
FIGURE 9







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/)

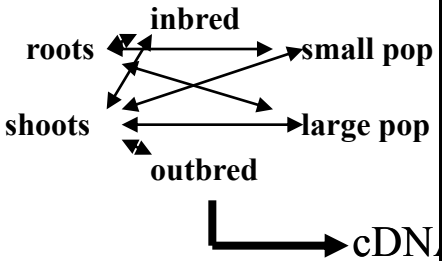
SNP markers: SNP-mining software like PolyBayes
(http://genome.wustl.edu/tools/software/polybayes.cgi)

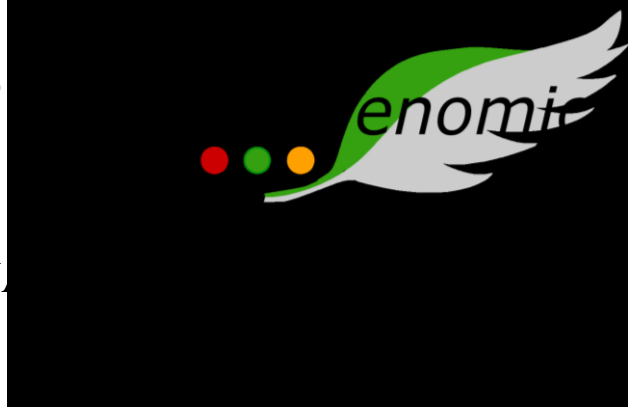
Again by using search software, freeware

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

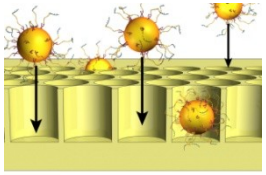
Example:

Scabiosa columbaria





454 LIFE SCIENCES



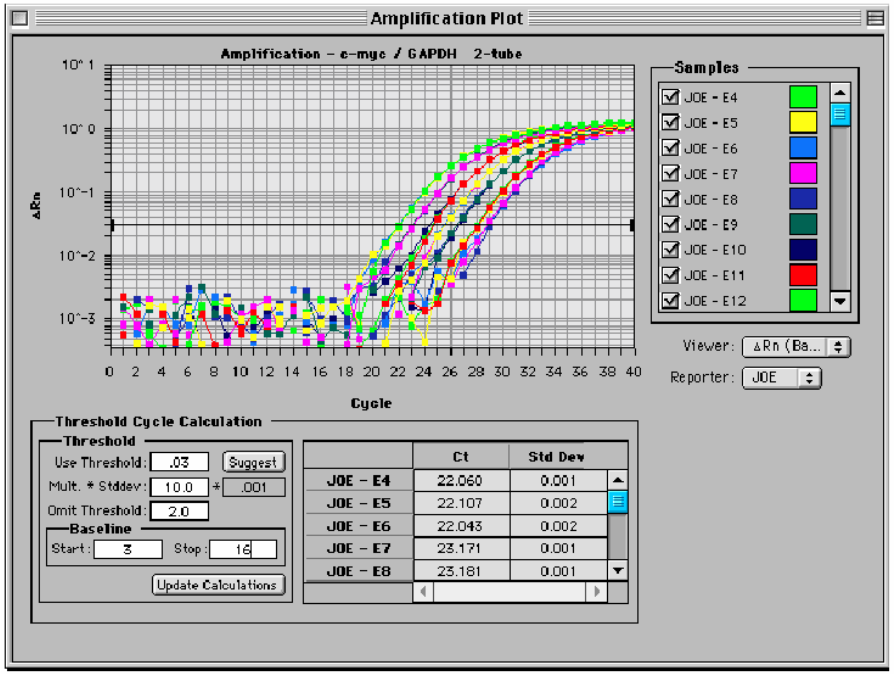
NCBI

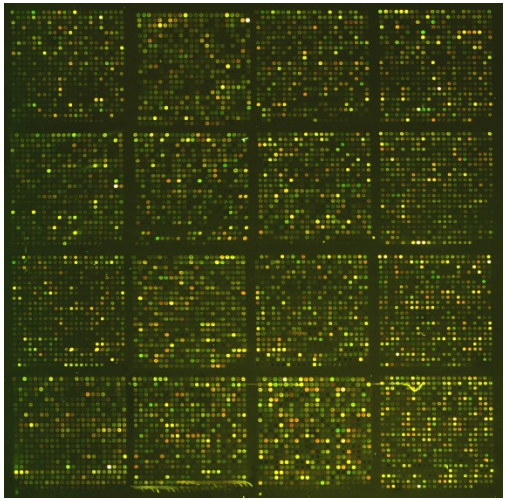
530.000 sequences in one run, leading to ~ 40.000 ESTs

Two methods of detecting transcri

1. Design of quantitative RealTime-PCR met
EST sequences

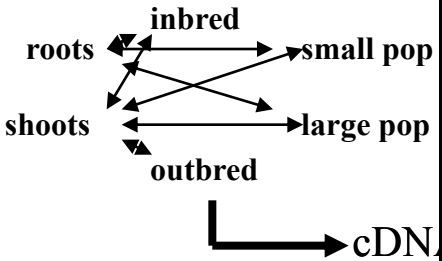
2. Design of a *Scabiosa* specific microarray



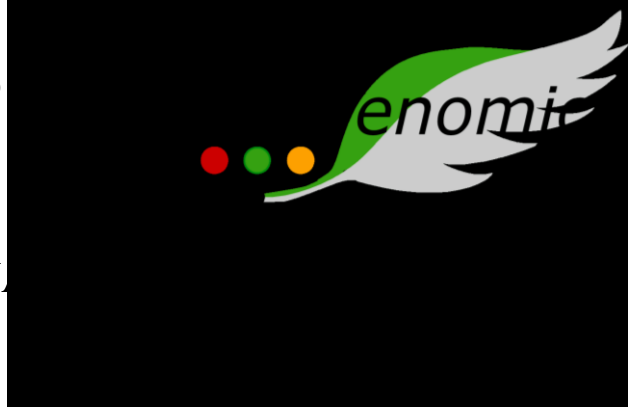


Example:

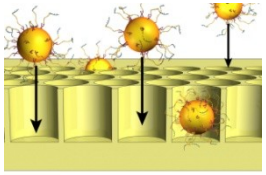
Scabiosa columbaria



cDN



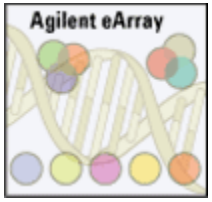
454 LIFE SCIENCES

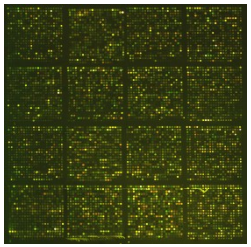


NCBI

530.000 sequences in one run, leading to ~ 40.000 ESTs

Agilent Technologies



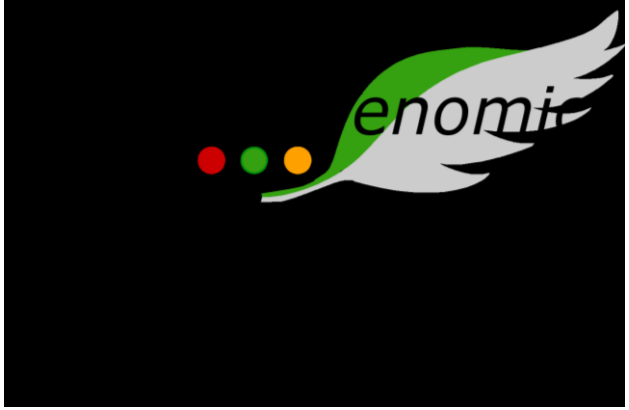


15k – 30k 60-mer microarrays

Experiment: transcriptional profiling of inbreeding depression

Expected pay-off:

- Ecogenomic approach to conservation genet
  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):

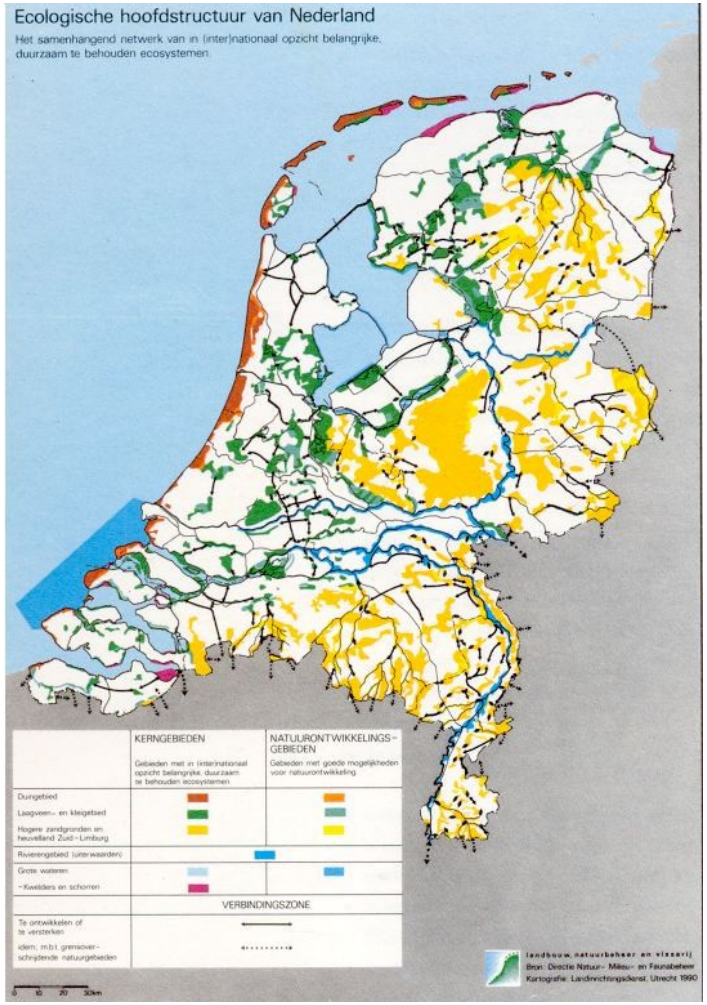
Costs are diminishing continuously

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 !!!