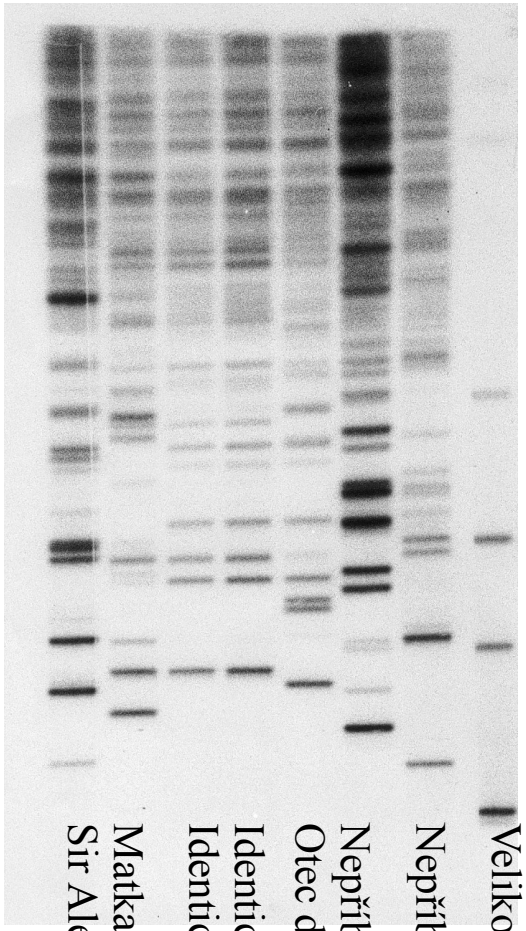
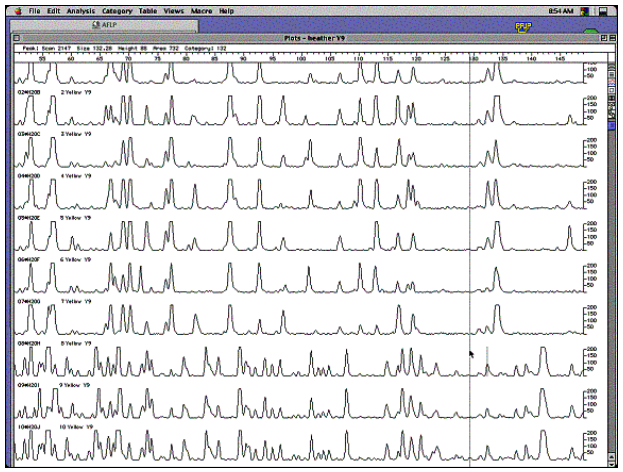
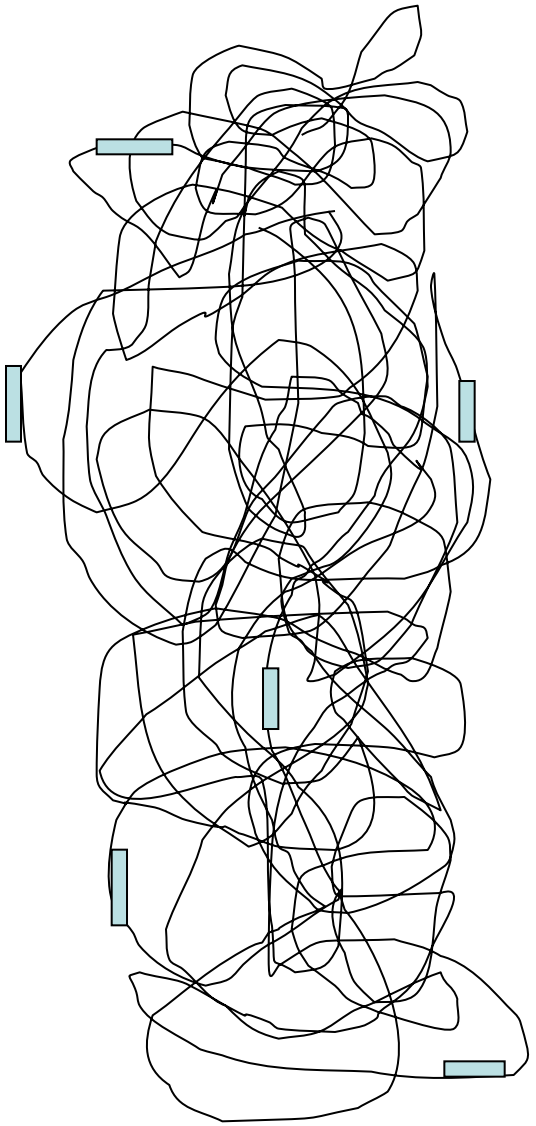


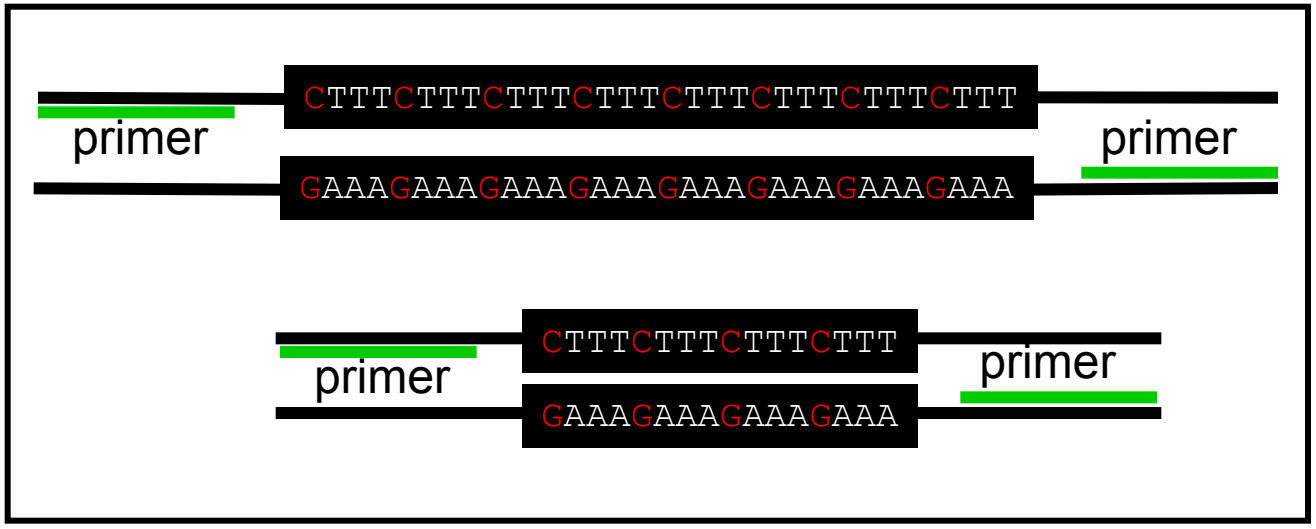
„DNA fingerprinting“

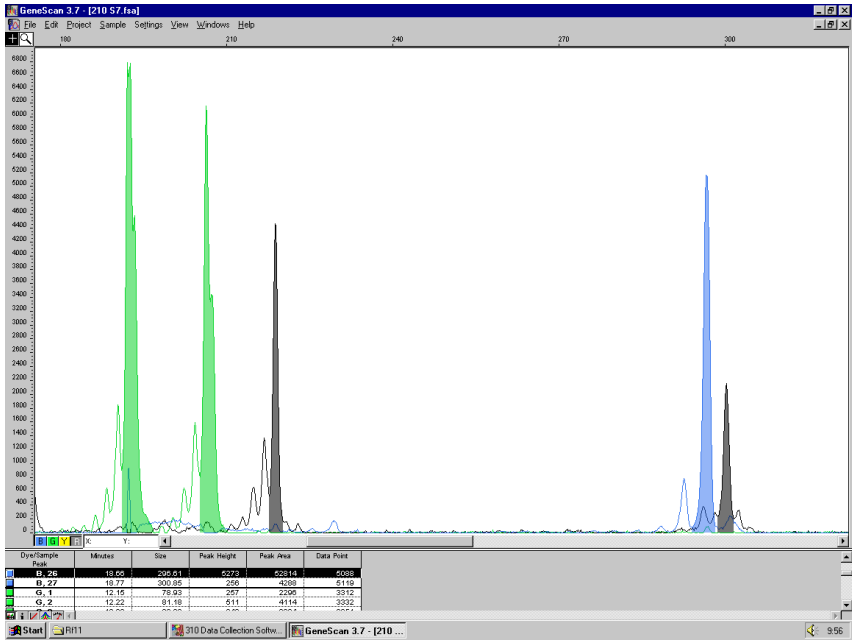


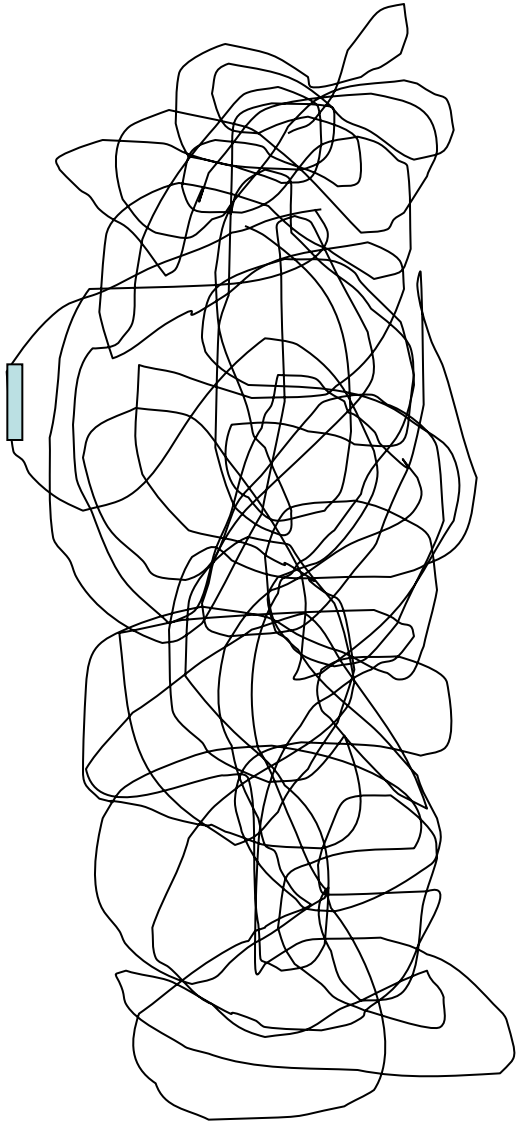
AFLP



Multilocus DNA markery







Mikrosatelity

Single-locus DNA markery

SINE, LINE, etc.

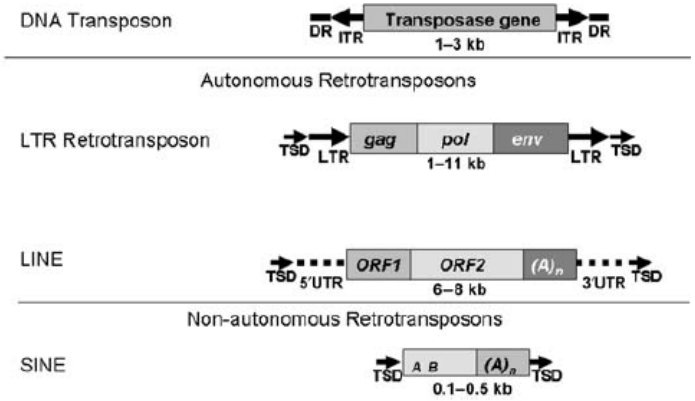
(Shedlock et al. 2004, TREE; Ray et al. 2007, MolEcol)

- **Transposable elements**
- Vytváří kopie (většinou)
- Kopie integrovány na nová místa v genomu
- Obvykle nejsou specificky odstraňovány
- Molekulární fosílie – neexistují homoplasie !!!
- Nesmírně početné
- Člověk – víc jak polovina genomu (ost. druhy – 40-90%)

Objev DNA transpozonů u kukuřice: Barbara McClintock

Typy transposabilních elementů

- **Kódující své proteiny, autonomní,** 1-10 kb
  - **DNA transposony** (cut-and-paste)
  - transposasa
  - **Retrotransposony** (copy-and-paste)
  - **LINE**
    1-2 proteiny, kopie přes RNA
  - **LTR retrotransposony**
    5-6 proteinů, také přes RNA

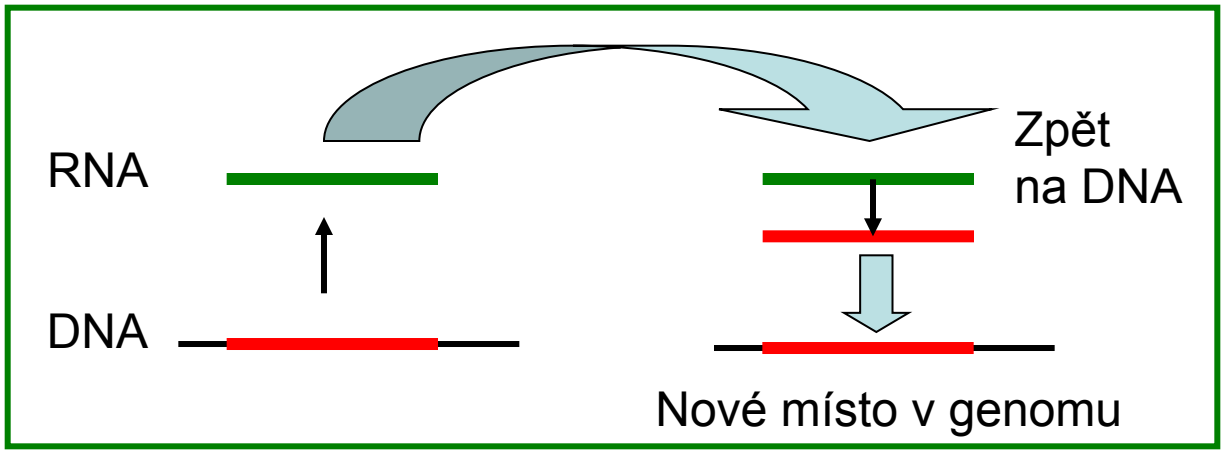


- **Nekódují proteiny, neautonomní,** 100-1000 bp
  paraziti předešlých, např. **SINE** (člověk *Alu* – více než 1 milion kopií) – nejčastěji používané v populačních a fylogenetických studiích

LINE – mechanismus transpozice

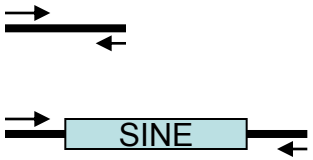
- Kopie přes RNA
- Reversní transkriptáza
- Mašinerii využívají **SINE** (jsou to „paraziti“),

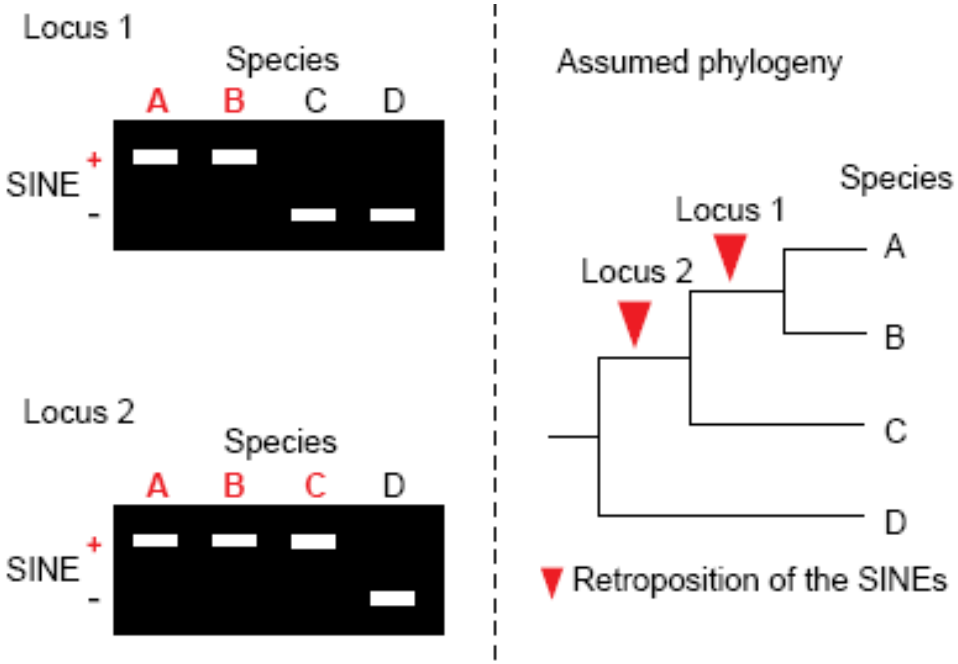
  Alu (SINE) a *L1* (LINE) se stejně rychle množí



- **LTR retrotransposony – opět přes RNA, složitější proces**

Velmi nízké riziko homoplázií → SINE = ideální fylogenetické markery

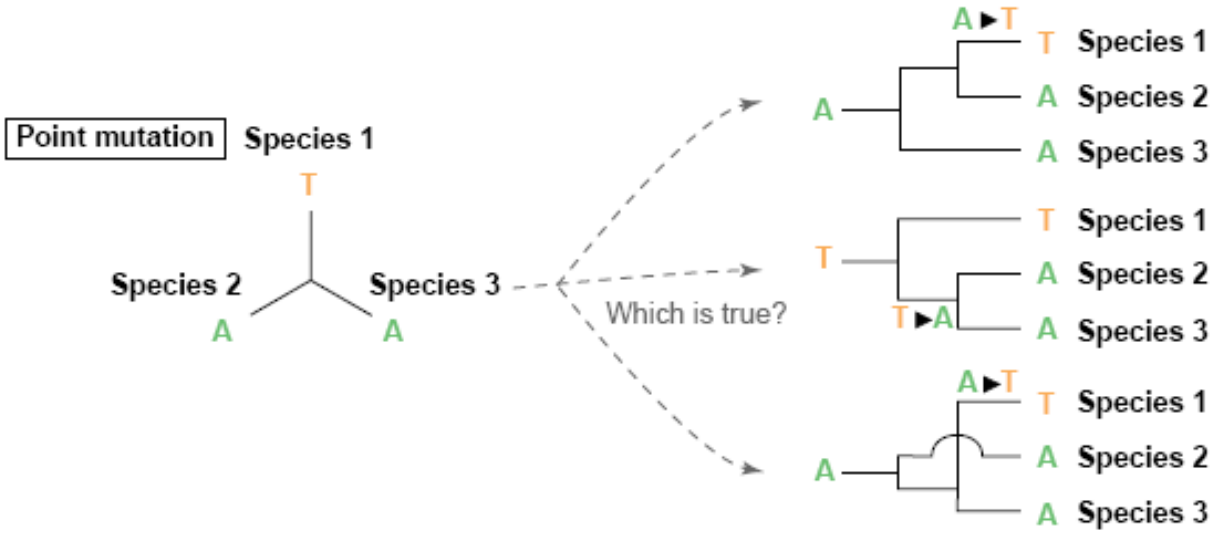




„single-locus marker“

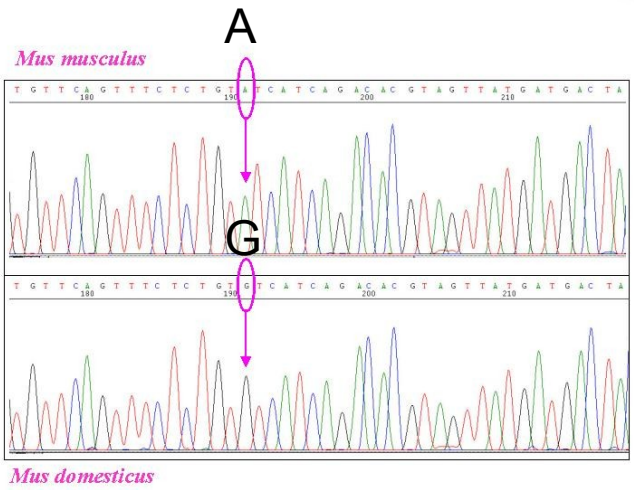
- PCR amplifikace daného úseku a elektroforéza

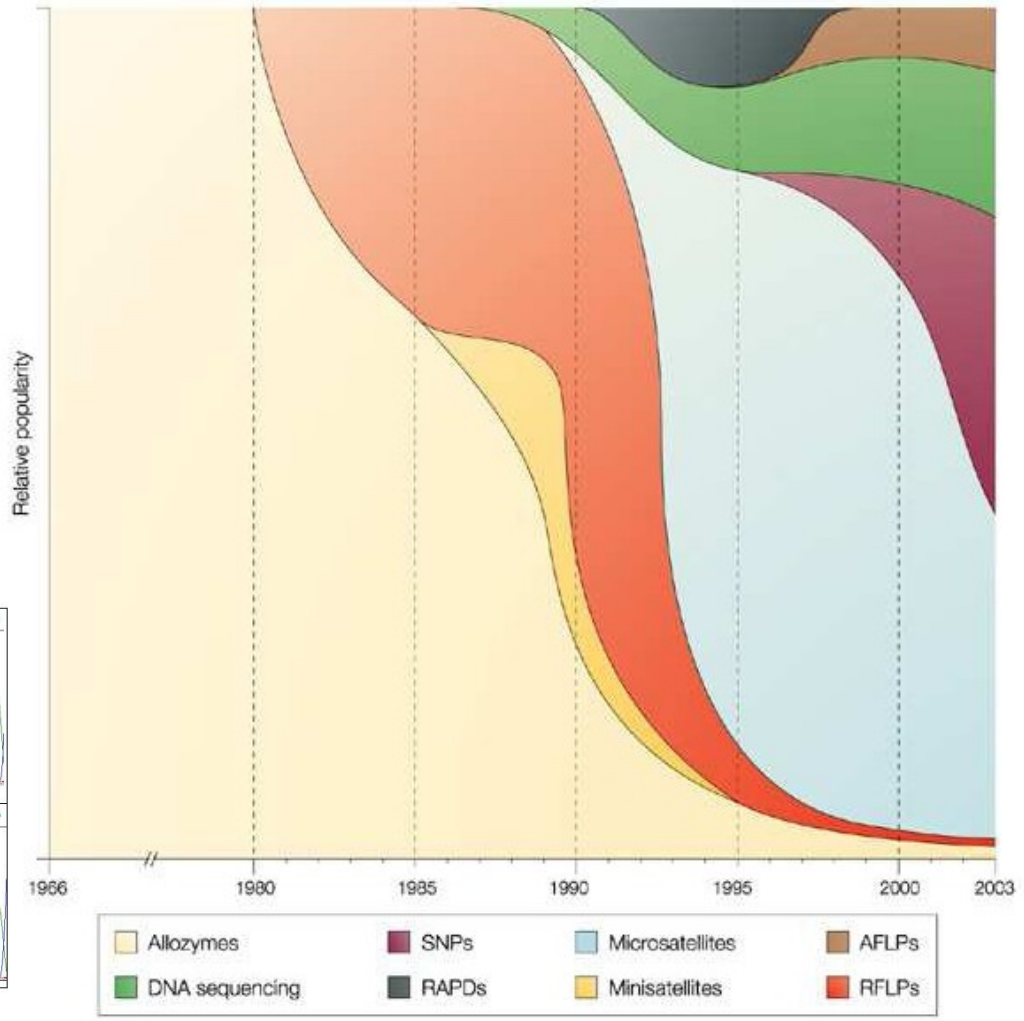
Neexistují zpětné mutace = výhoda oproti sekvenačním datům



Příklad aplikace: kytovci vs. sudokopytníci (hroch je bratr velryby)

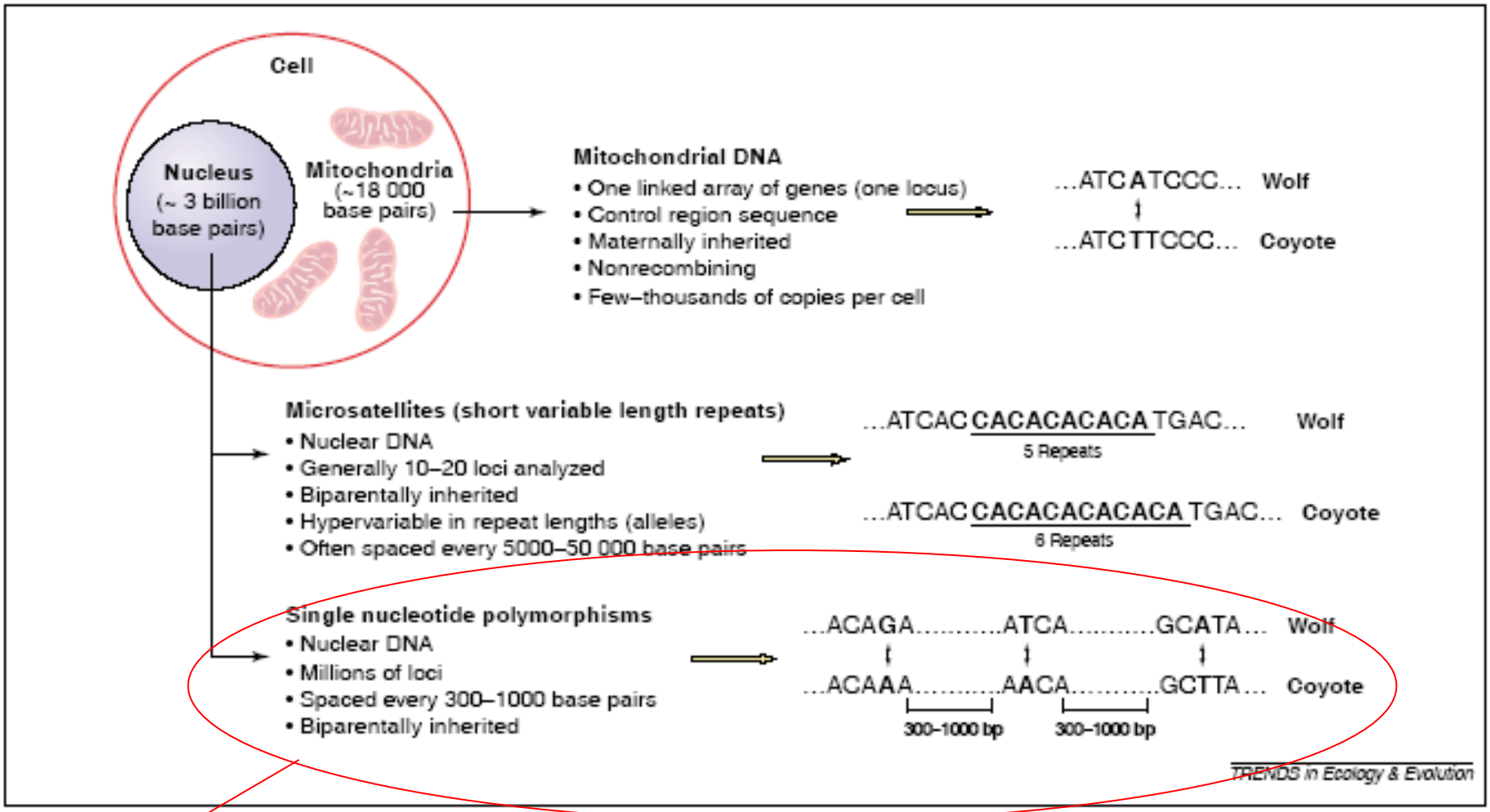
SNP Fashion on the rise





Nature Reviews | Genetics

Single nucleotide polymorphisms (SNPs)

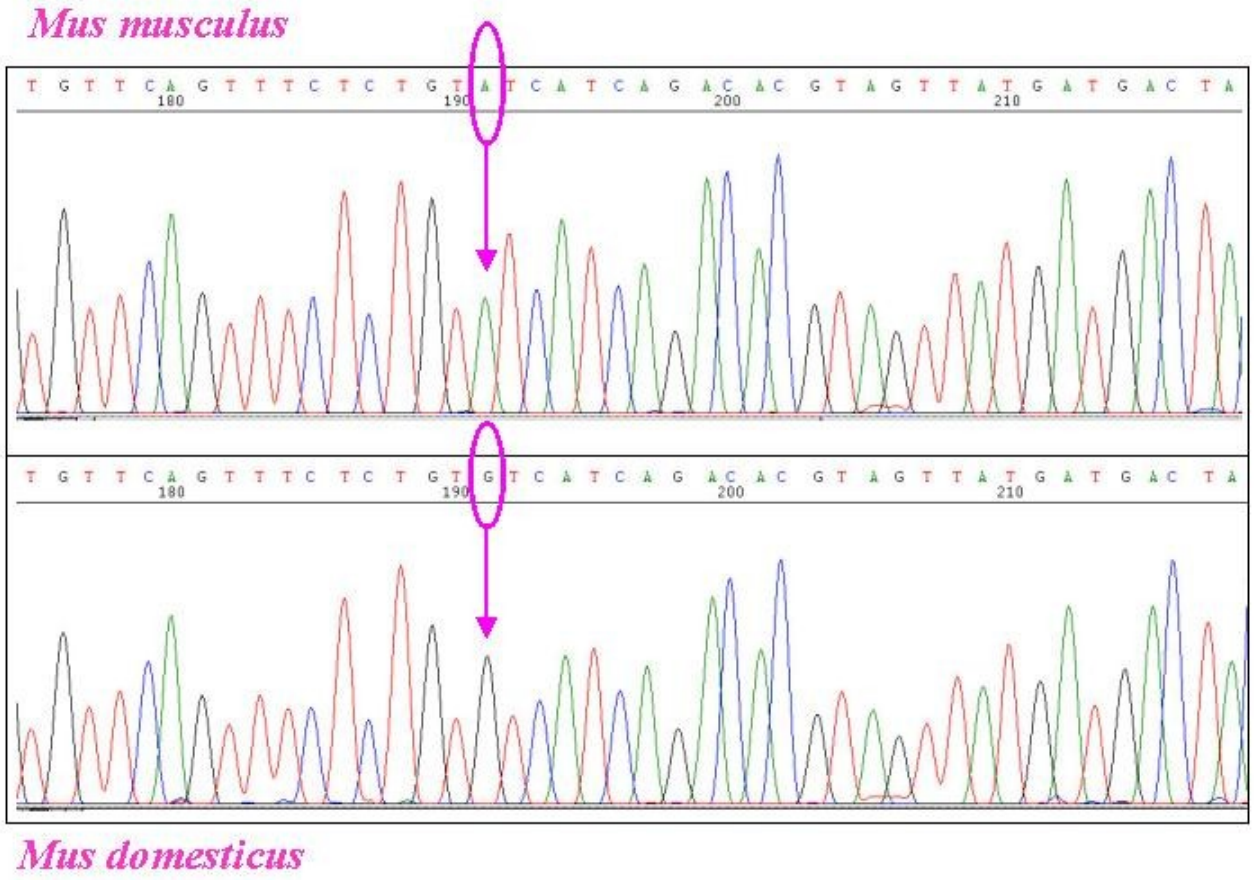


SNPs : nuclear genome (consensus)

Kolik SNPs se vyskytuje u člověka?

- mutační rychlost je ~2.5 x 10^{-8} mutací / místo / gen
- ~150 mutací/diploidní genom/generace
- 6.3 milliard lidí na světě = 945,000,000,000 mutací v současném světě
- 3 miliardy nukleotidů = každý nukleotid je zmutovaný 315 krát

Příklad informativního SNP znaku



transice

A ↔ G

transition: Pu→Pu or Py→Py

transversion: Pu→Py or Py→Pu

Využití SNPs znaků

- identifikace druhu (nebo genetické skupiny) - studium hybridizace
- fylogeografie
- populační genetika (genetická variabilita, identifikace jedinců a vztahů mezi nimi, populační velikost a její změny atd.)

Výhody

- početné a rozšířené v genomu (v kódujících i nekódujících oblastech) – milióny lokusů
- 1 SNP cca každých 300-1000 bp
- Mendelovská dědičnost (vs. mtDNA)
- evoluce je dobře popsatelná jednoduchým mutačním modelem (vs. microsatellites)
- jsou analyzovány kratší fragmenty DNA – neinvazivní genetika

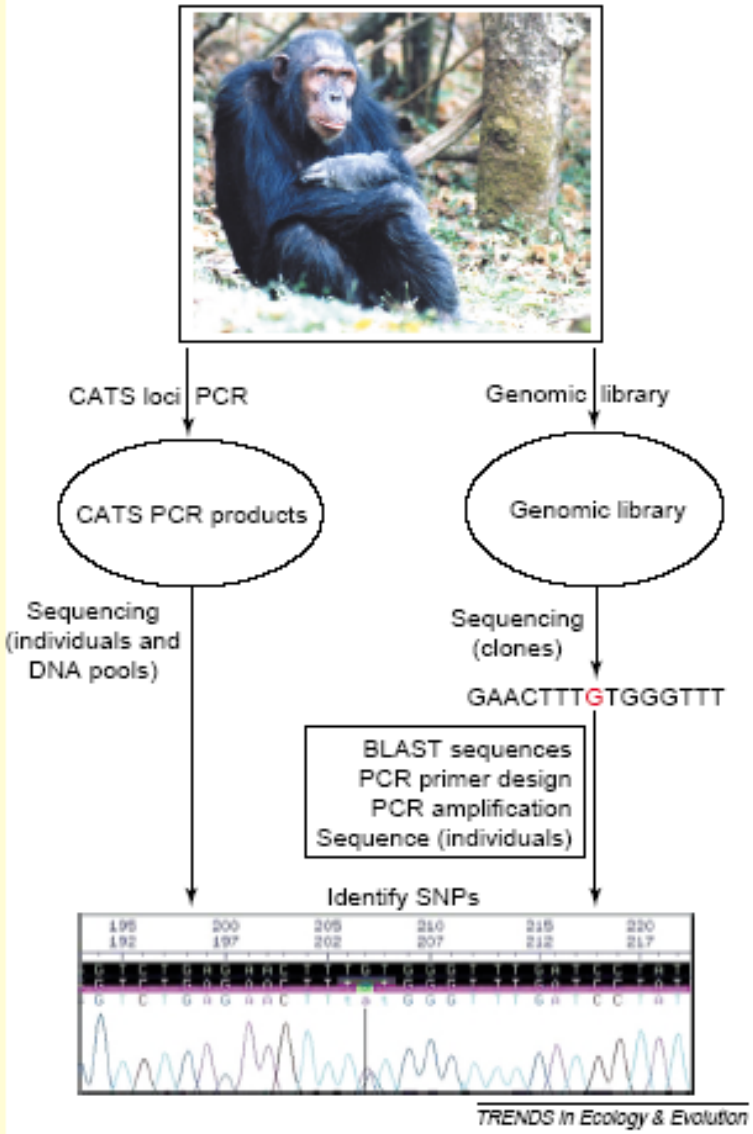
Nevýhody

- „ascertainment bias“ – výběr znaků se provádí na základě jen malého počtu jedinců a nemusí být reprezentativní
- nízká variabilita na lokus (většinou jen 2 alely)
- pro populační genetiku je vyžadován větší počet lokusů (4-10 krát více než u mikrosatelitů)

Metody analýzy

1. Nalezení lokusů („ascertainment“)
2. Genotypizace

Nalezení SNPs

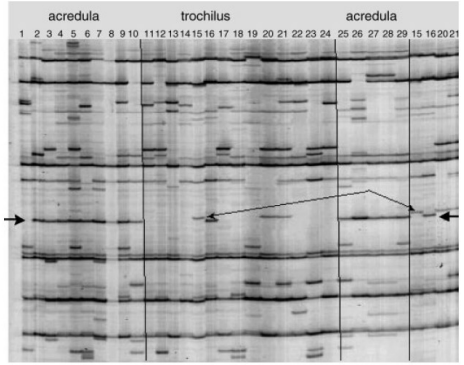


CATS loci = comparative anchor tagged site loci (= cross amplification)

Genomic library = genome restriction + cloning

AFLP = alternative to the genomic library construction (provide PCR fragments, can be transformed to informative SNP)

Next-generation sequencing – analýza více jedinců a hledání polymorfismů



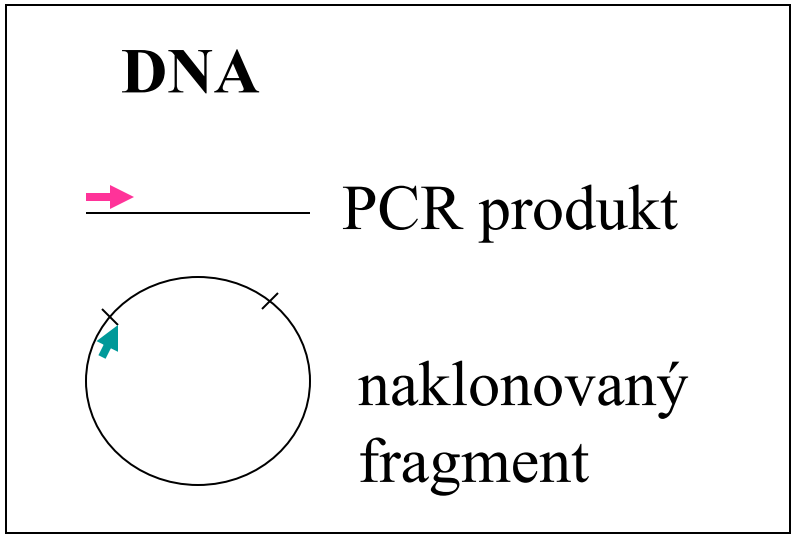
Sekvenování

Sekvencování DNA

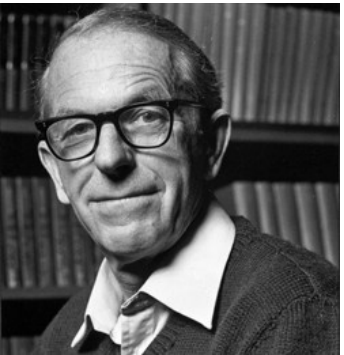
- Maxam-Gilbertova (chemická) metoda:
  bázově-specifická chem.
  modifikace a štěpení
  fragmentů DNA

- Sangerova (enzymatická) metoda:
  terminace replikace
  pomocí ddNTP

Sequencing

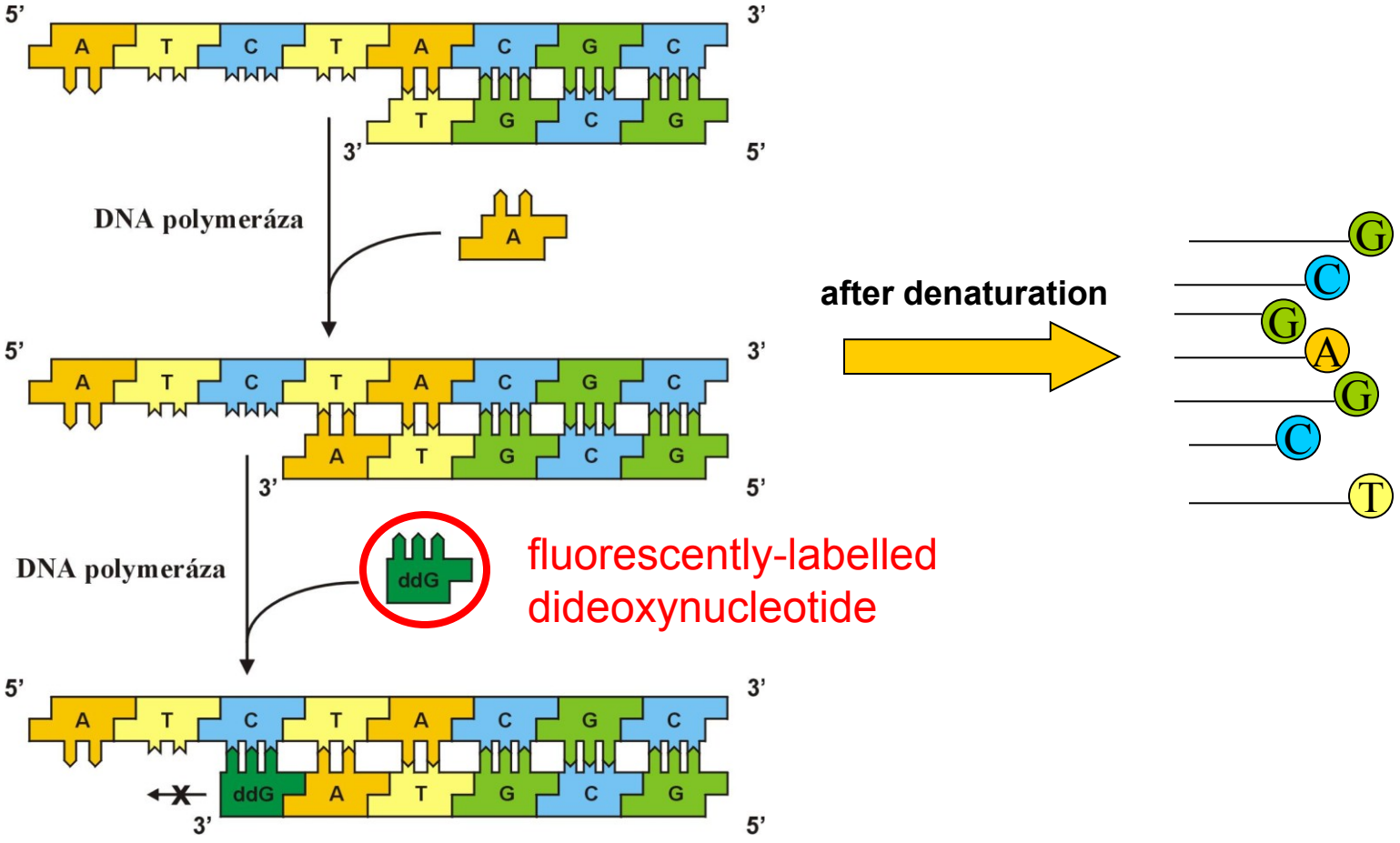


sekvenační reakce se značenými dideoxynukleotidy and jedním specifickým nebo universálním primerem

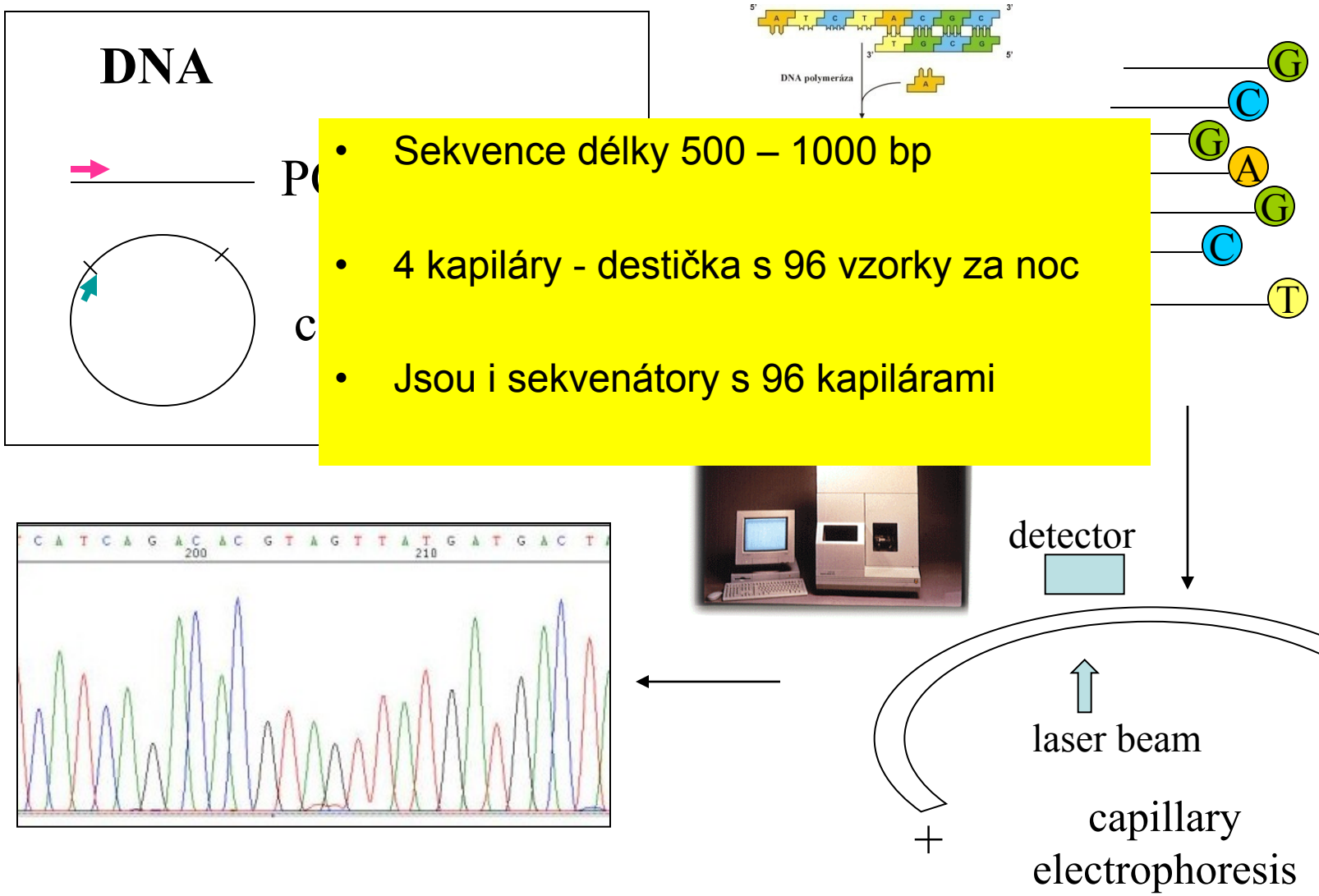


Sangerova dideoxy metoda

Sekvencování DNA

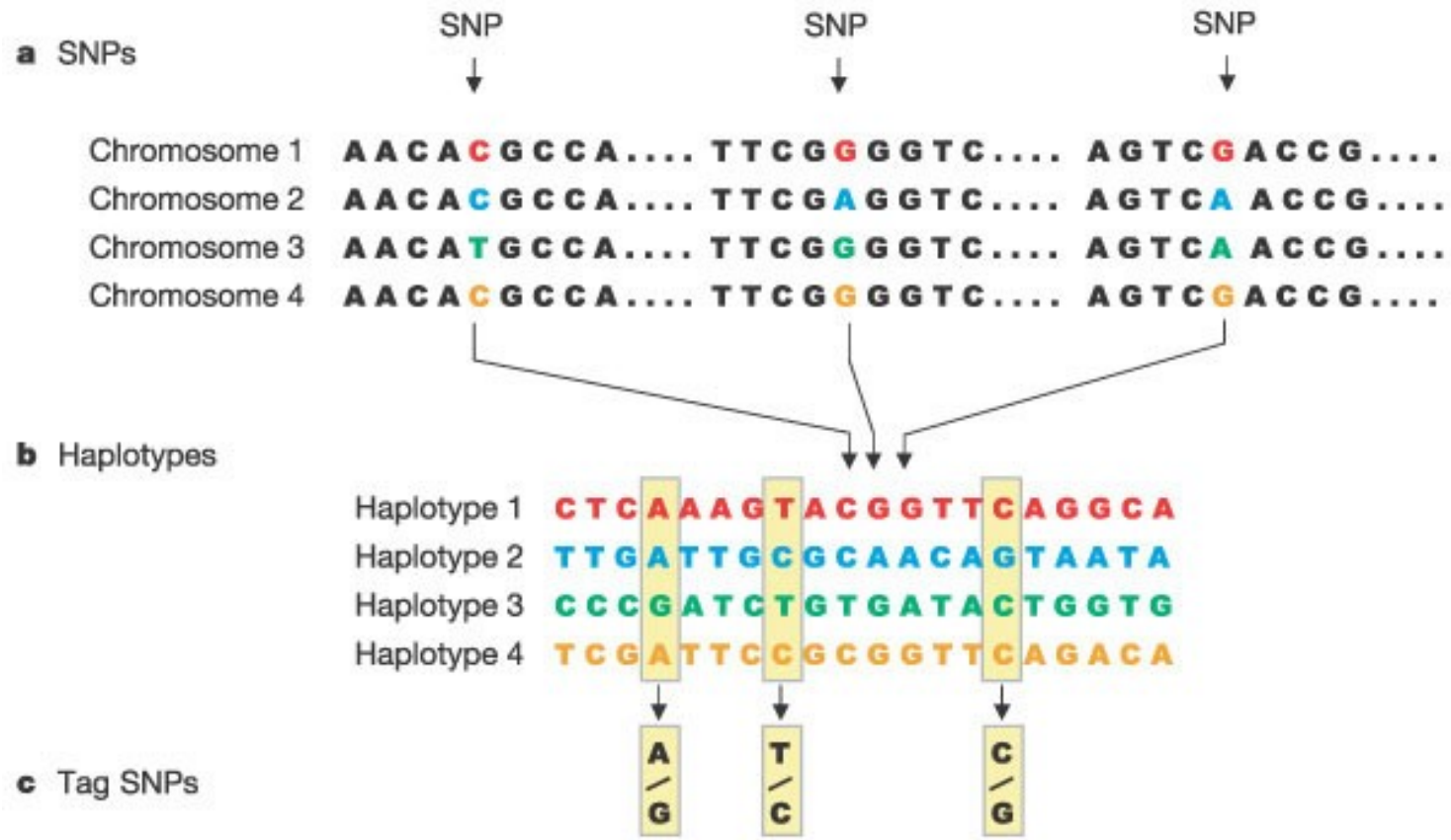


Sequencing



- Sekvence délky 500 – 1000 bp
- 4 kapiláry - destička s 96 vzorky za noc
- Jsou i sekvenátory s 96 kapilárami

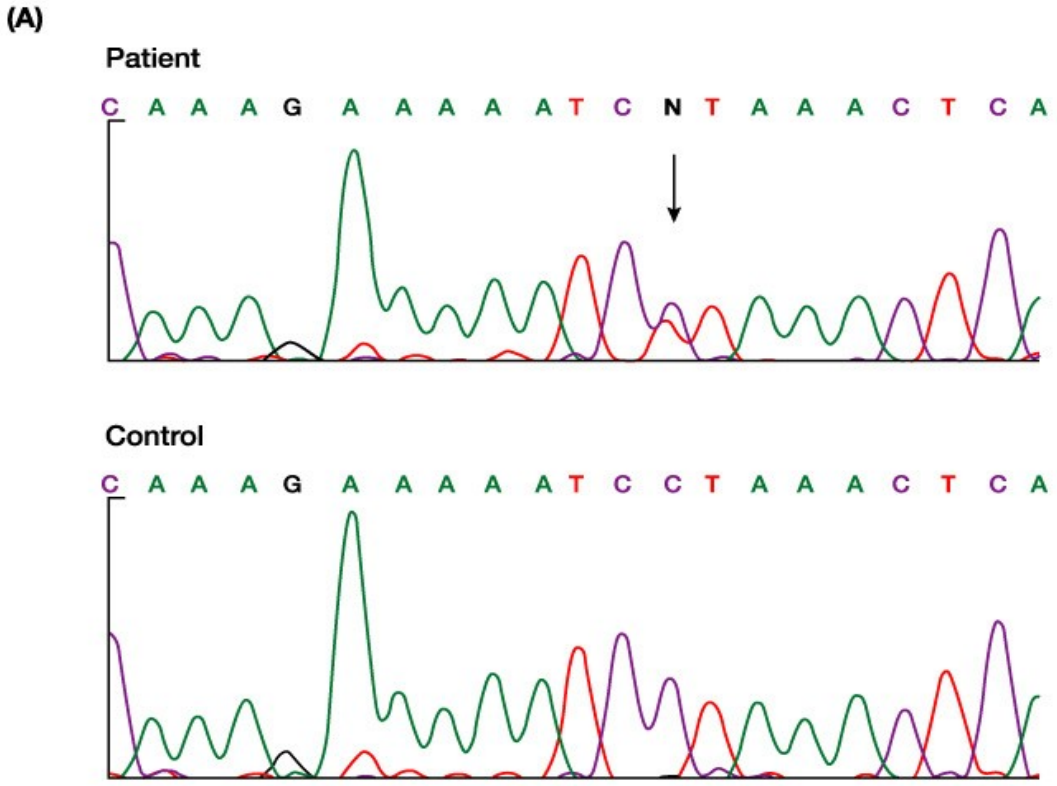
Identifikace různých genotypů u různých jedinců (= homologních chromozómů, tj. variabilita alel)



SNPs genotyping

= zjištění genotypu daného jedince

SNPs genotyping – sekvenování?
Je drahé a nejasné u heterozygotů

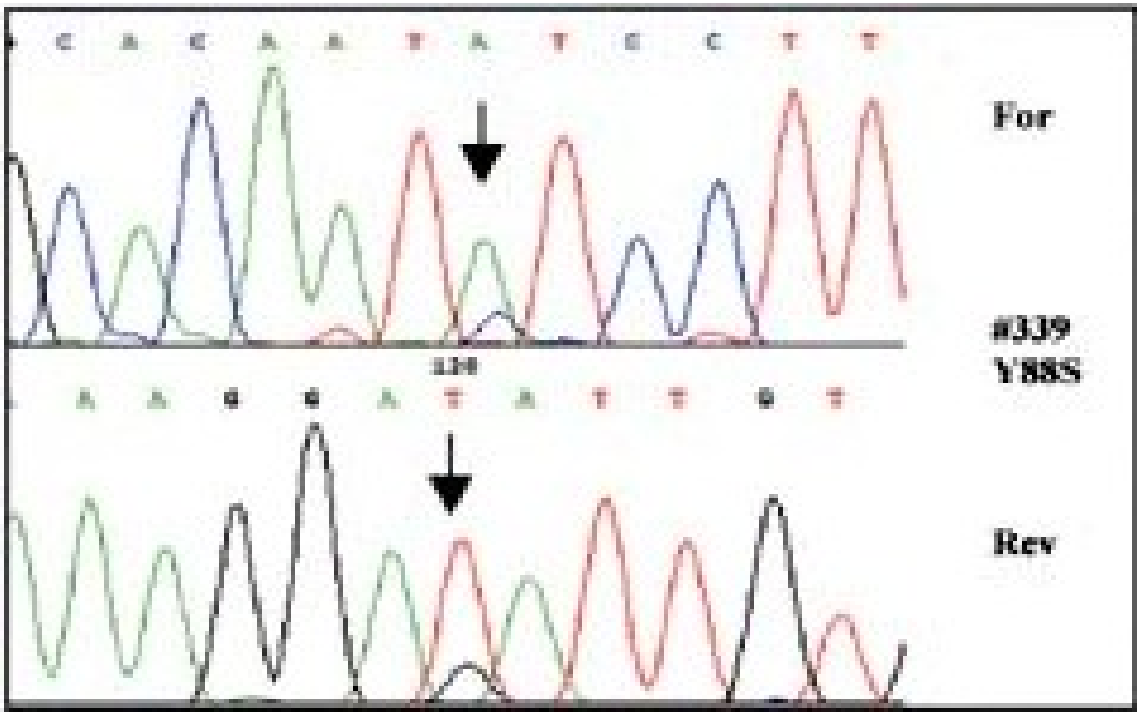


Heterozygote?

Homozygote

Double peak shows heterozygous mutation

Heterozygotes?



Bi-directional sequencing – are you really sure?

SNPs genotyping – klonování a následné sekvenování?
- separation of two (or more in duplicated genes) alleles

each clone contain the only allele

!!! cloning – 1000 Kč
!!! sequencing 1 clone – 150 Kč

ligation, transformation

Ex.: heterozygote = two diff. alleles

SNP genotyping - old standards

PCR-RFLP

(restriction fragments length polymorphism)

Allele A

CCGATCAATGCGGCAA

GGCTAGTTACGCCGTT

cutting by restriction endonuclease

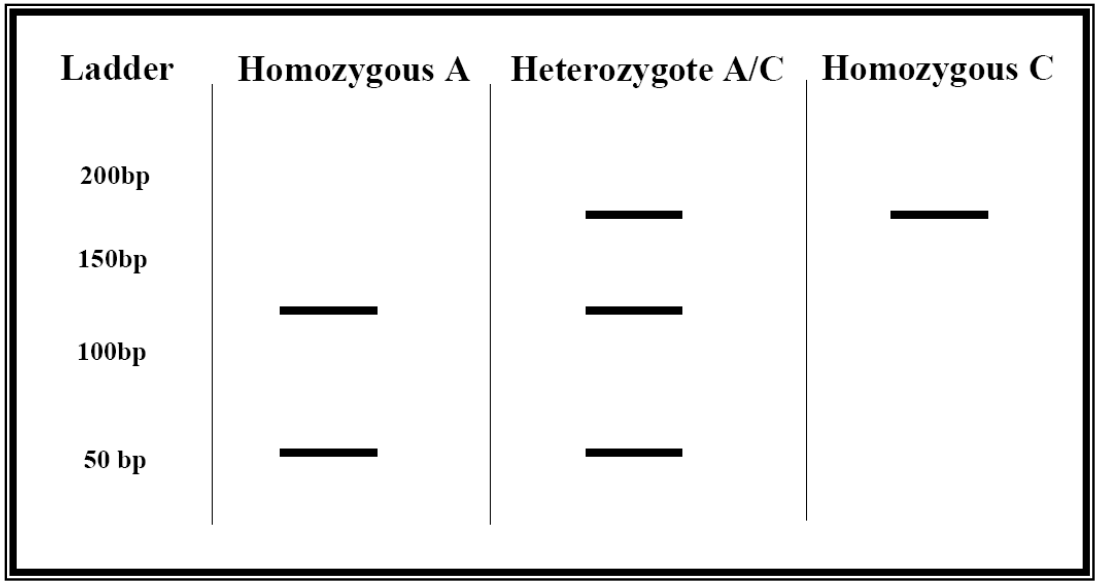
Allele C

CCGATCACTGCGGCAA

GGCTAGTGACGCCGTT

no cut

- neumožní nalézt novou variantu daného SNP (odliší pouze 2 formy daného znaku: +/-)



SNPs genotyping – old standards
Methods of mutation detection

(comparison of specimen's pattern with pattern of known allelles)

- Thermal gradient gel electrophoresis **(TGGE)**
- Denaturing gradient gel electrophoresis **(DGGE)**
- Single-strand conformation polymorphism **(SSCP)**

- = special electrophoresis methods based on differences in mobility of different DNA sequences

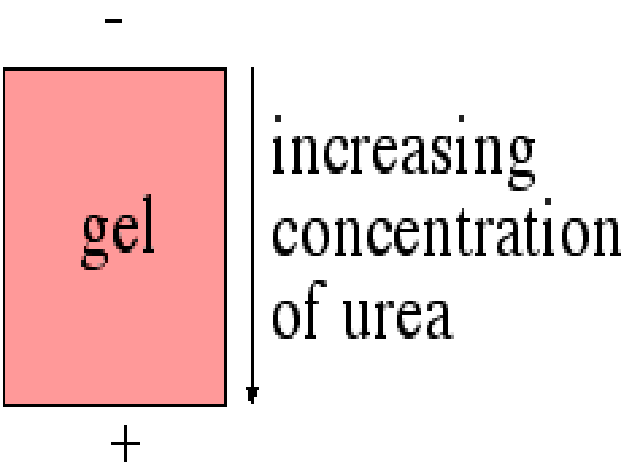
- detekce geneticky podmíněných chorob, např. cystická fibróza

Denaturing gradient gel electrophoresis (DGGE) (TGGE – podobné, ale gradient teploty)

The small (200-700 bp) genomic fragments are run on a low to high denaturant GRADIENT acrylamide gel

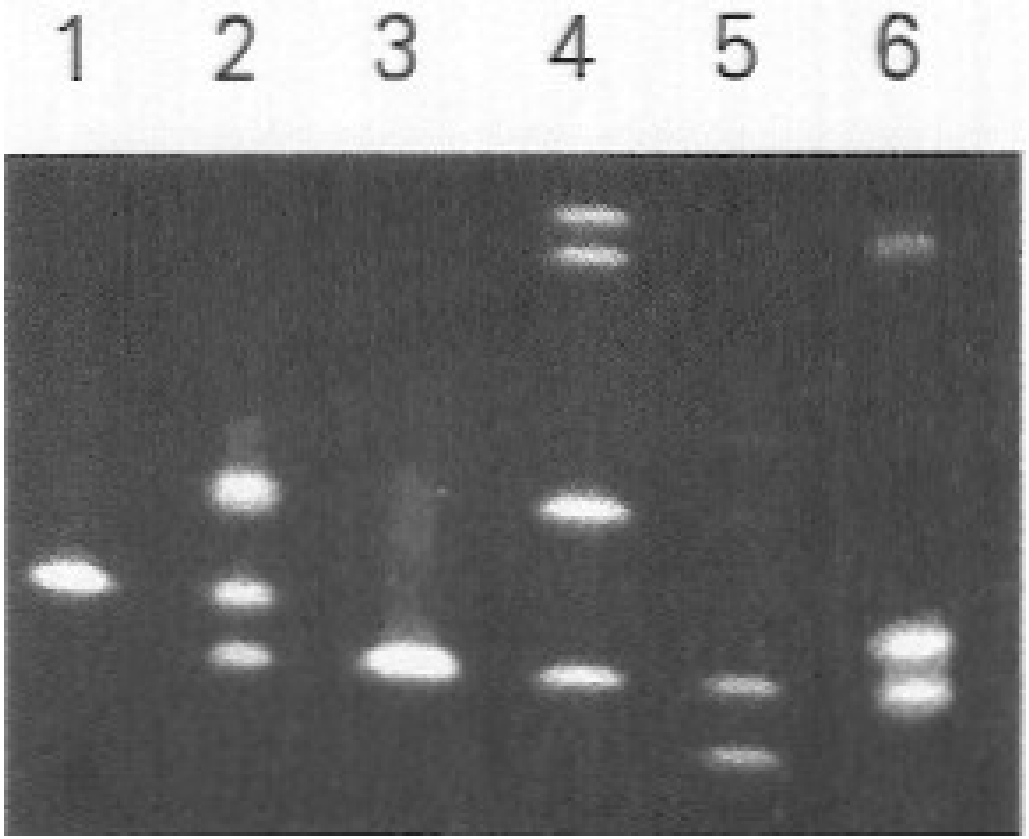
Each fragments move according to molecular weight, but as they progress into more denaturing conditions, each (depending on its sequence composition) reaches

A POINT where the DNA BEGINS TO MELT



They retard, and we will see shift in mobility

We will see different shifts in mobility for differing products



1- normal homozygote
3- homozygous mutations will yield one band on a different position
2, 4, 5, 6 – heterozygous mutations will yield 4 bands (2 homozygous and 2 heterozygous)

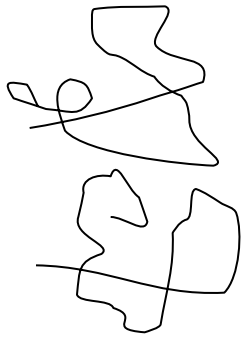
NOT ALL BANDS ARE SEEN !!!!!

www.leveninc.com/cftr_ex.gif

Single strand conformation polymorphism (SSCP)

Allele 1

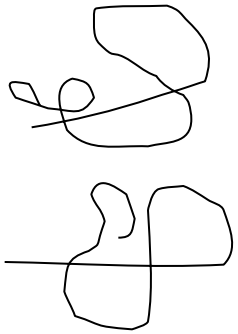
...CGCTTCAGG ...
...GCGAAGTCC...



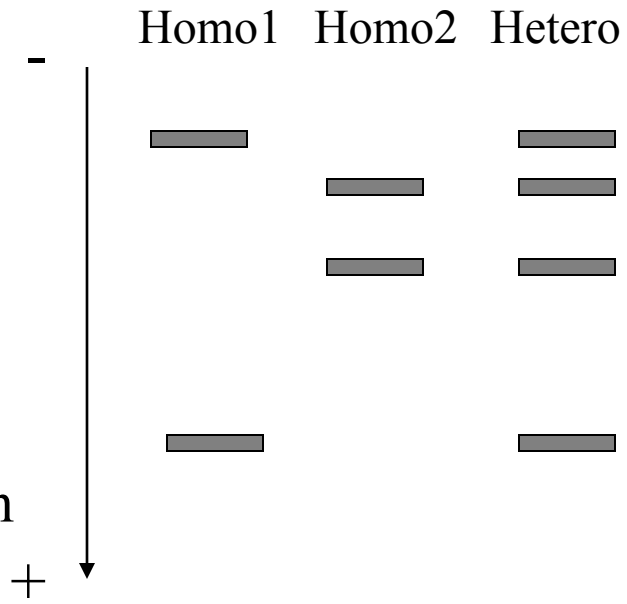
heating - denaturation
snap-cooling → partial renaturation

Allele 2

...CGCTTAAGG ...
...GCGAATTCC...



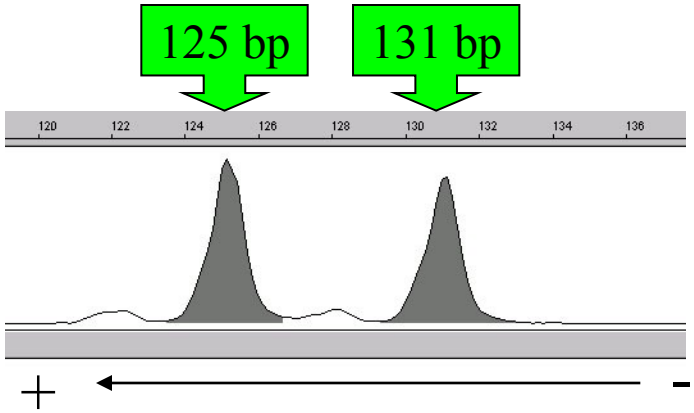
sequence-specific
ssDNA conformations



!!! non-denaturing PAGE

radioisotopes
silver-staining
fluorescent dyes (SYBR gold)

Použití automatických sekvenátorů
(denaturing polymer POP7 – ssDNA, e.g. microsatellites)



Well controlled electrophoresis parameters, high sensitivity

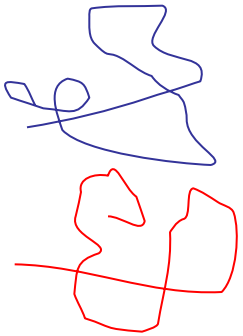
Použití automatických sekvenátorů

Why not non-denaturing electrophoresis?

CAP (conformation analysis polymer) – Applied Biosystems

Allele 1

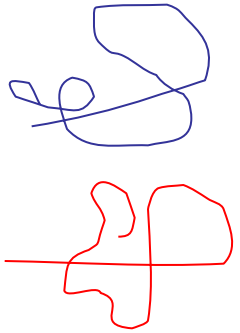
FAM... **C**GCTT**C**AGG ...
... GCGAA**G**TC**C** ***...HEX***



- well controlled electrophoresis

Allele 2

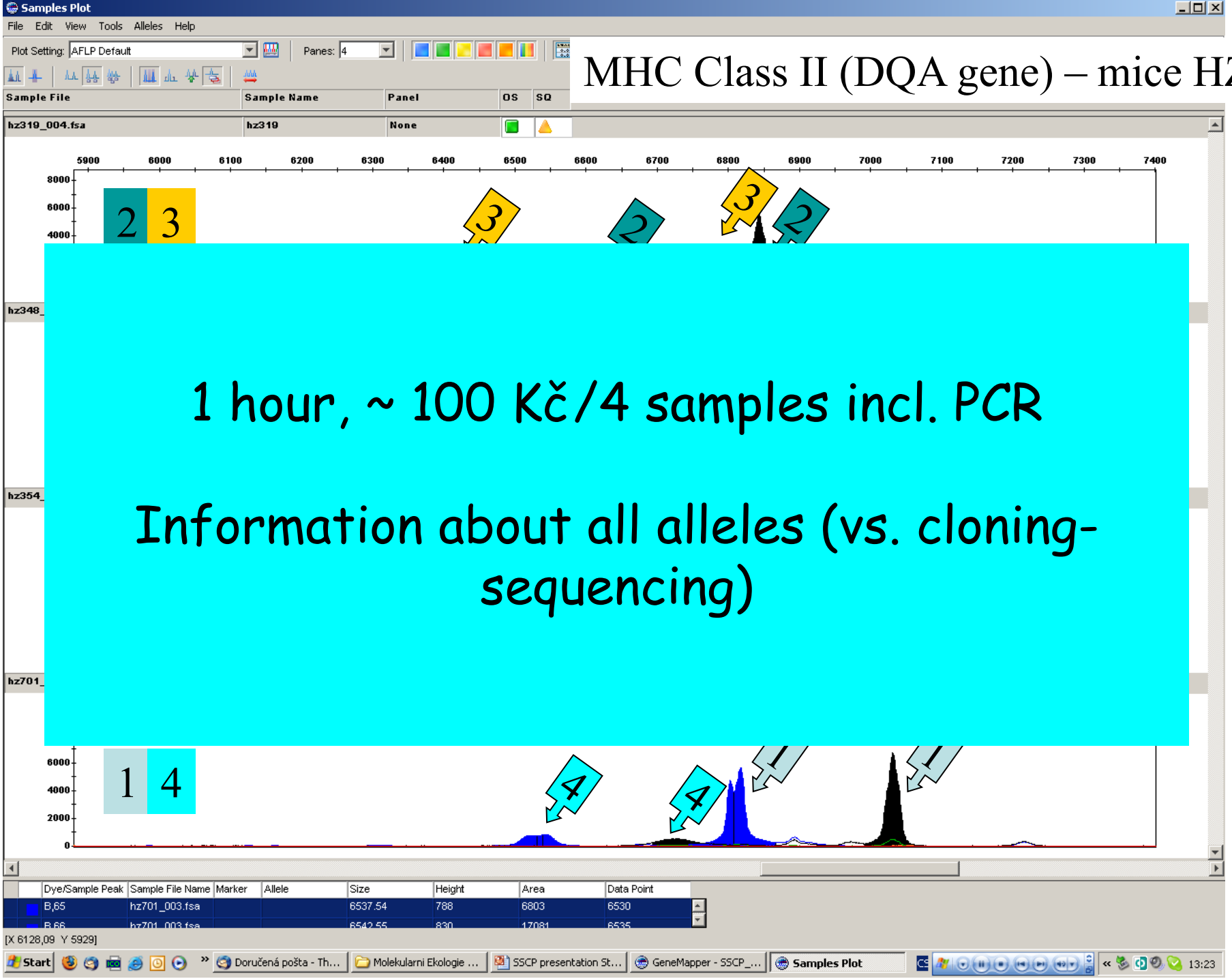
FAM... **C**GCTT**A**AGG ...
... GCGAA**T**TC**C** ***...HEX***



- two fluorescent labels

- high sensitivity

MHC Class II (DQA gene) – mice HZ

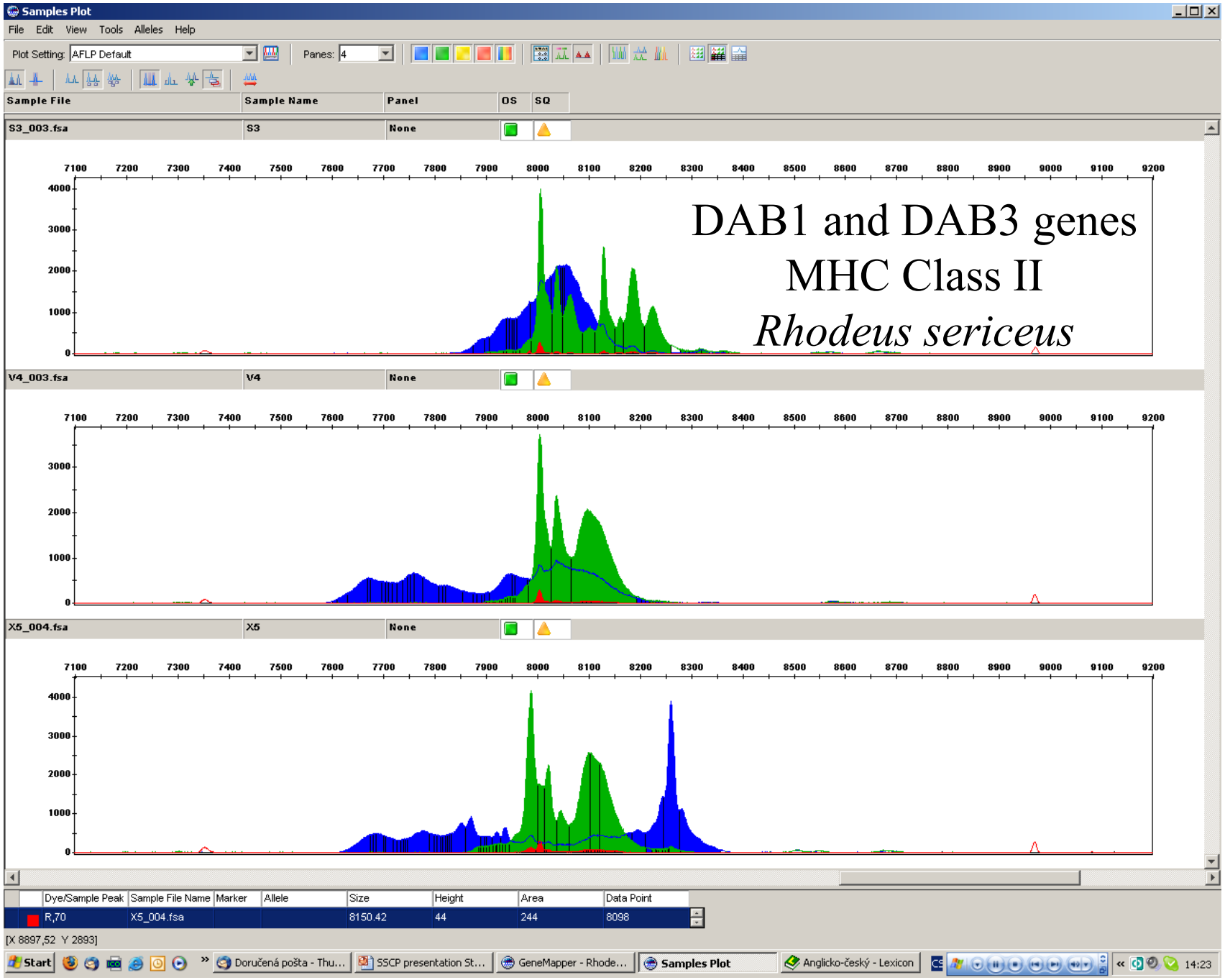


1 hour, ~ 100 Kč/4 samples incl. PCR

Information about all alleles (vs. cloning-sequencing)

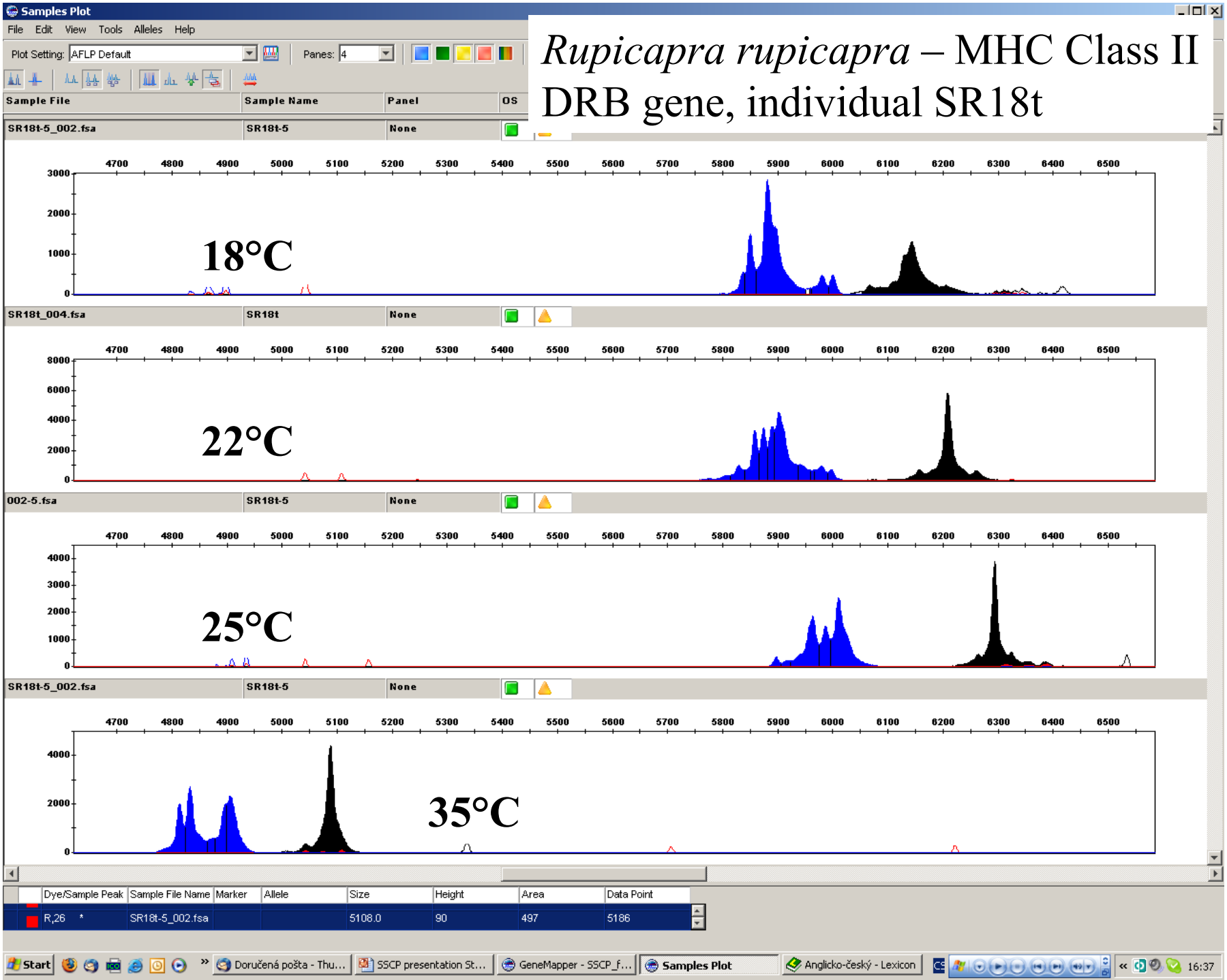
Advantages of CE-SSCP

- high throughput (when using 4, 16, or 96 - capillary sequencer) – time and money saving
- no need of gel preparation and autoradiography
- distinction of two DNA strains by two colour-labeling (usually FAM and HEX)
- potential of multiplexing – not yet used !!!


Samples Plot
File Edit View Tools Alleles Help
Plot Setting: AFLP Default
Panes: 4
Sample File
Sample Name
Panel
OS
SQ
S3_003.fsa
S3
None
V4_003.fsa
V4
None
X5_004.fsa
X5
None
DAB1 and DAB3 genes
MHC Class II
Rhodeus sericeus
Dye/Sample Peak
Sample File Name
Marker
Allele
Size
Height
Area
Data Point
R,70
X5_004.fsa
8150.42
44
244
8098
[X 8897,52 Y 2893]
Start
Doručená pošta - Thu...
SSCP presentation St...
GeneMapper - Rhode...
Samples Plot
Anglicko-český - Lexicon
14:23

Disadvantages

- need for electrophoresis optimisation (running temperature, sieving matrix, dilution of samples)
- „complex" patterns in some sequences
- alleles with the same pattern may rarely occur
- it is necessary to test several run temperatures



Rupicapra rupicapra – MHC Class II DRB gene, individual SR18t

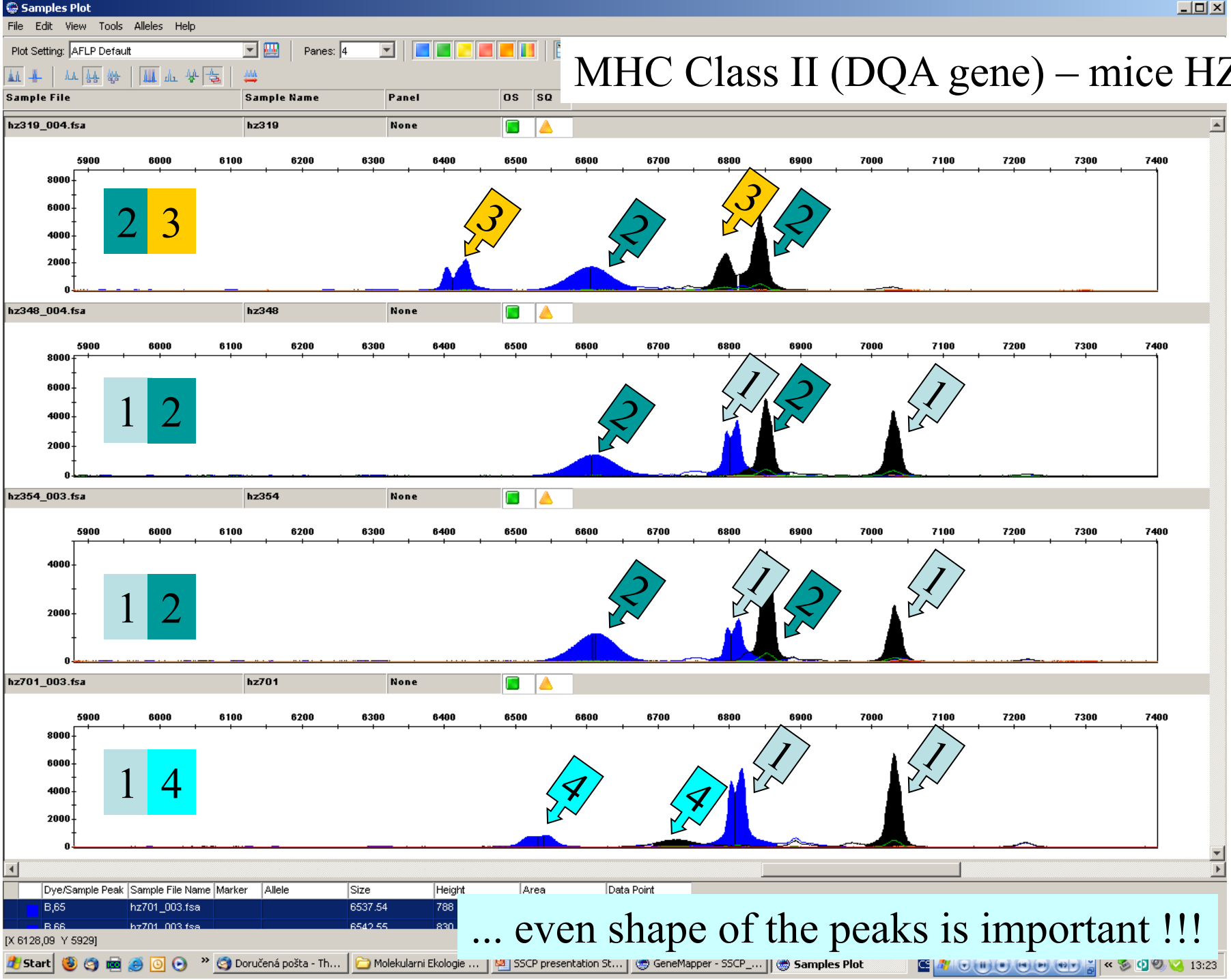
Data analysis

- GeneMapper (Applied Biosystems)
- different „Size Standard" for each temperature
- alignement of more samples

Applications

1) Genotyping of codominant markers (e.g. single copy MHC genes)

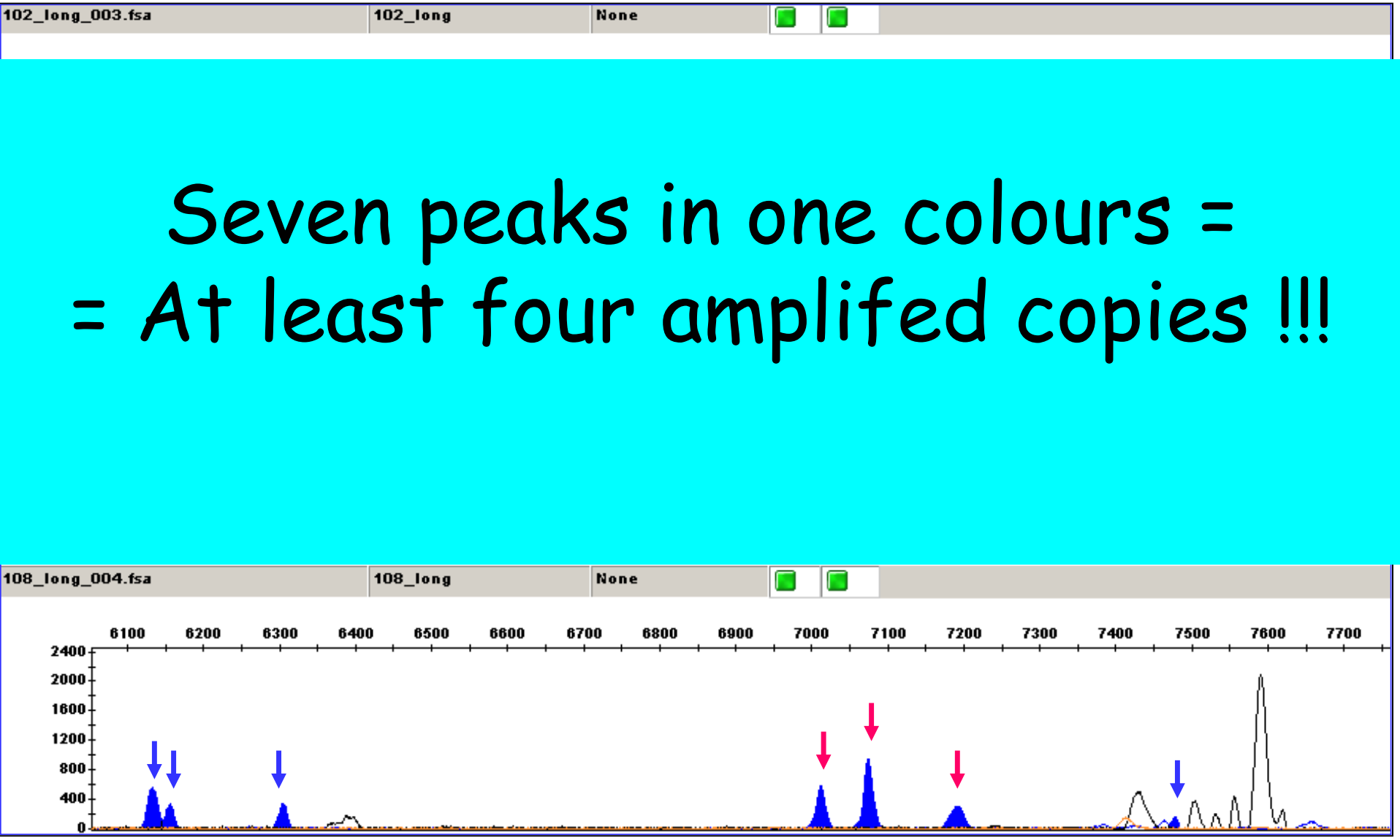
MHC Class II (DQA gene) – mice HZ



... even shape of the peaks is important !!!

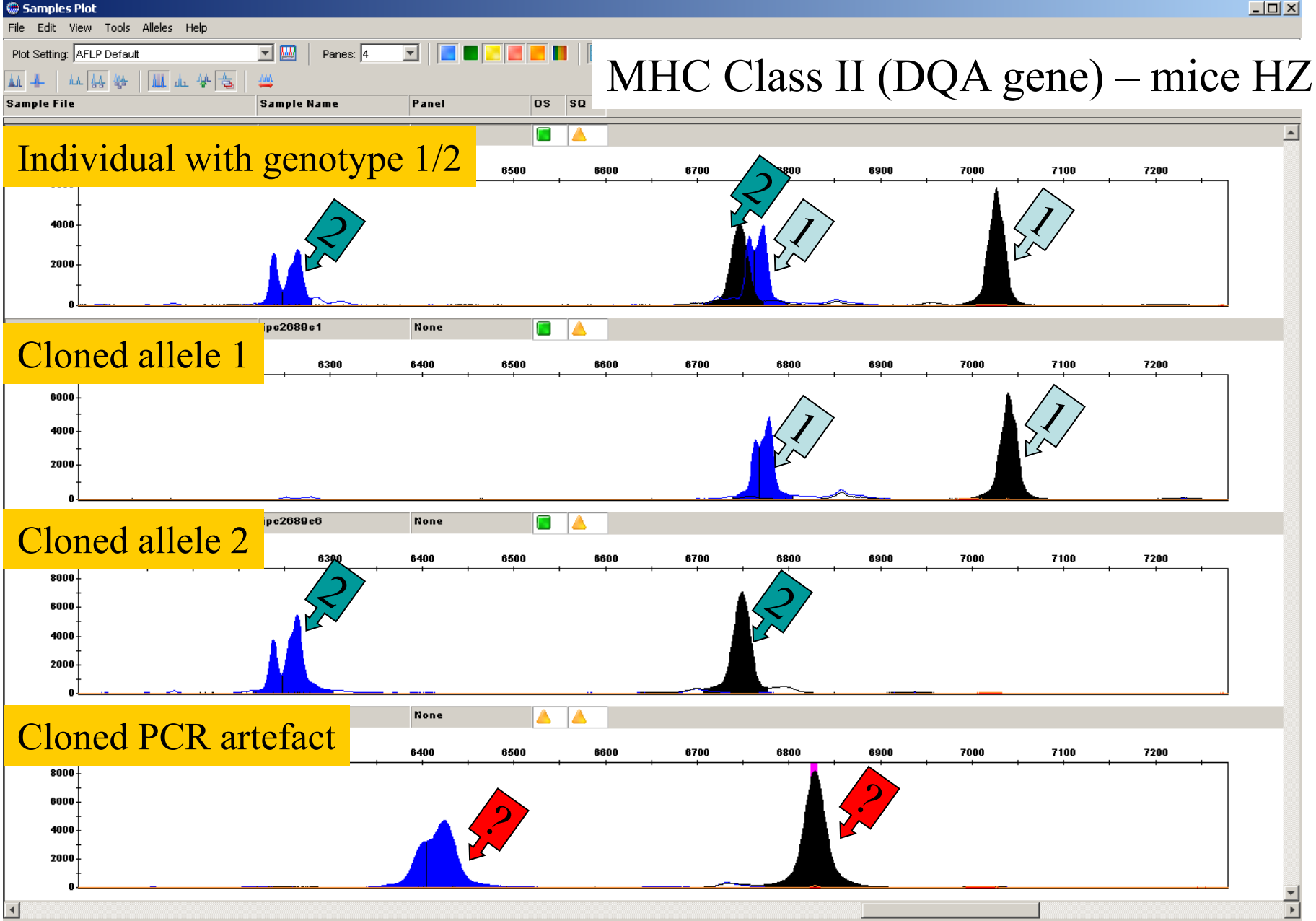
Applications

1) Genotyping of codominant markers (e.g. single copy MHC genes)
2) Identification of number of genes (e.g. duplicated MHC genes)



SSCP of three individuals: - different alleles - same alleles

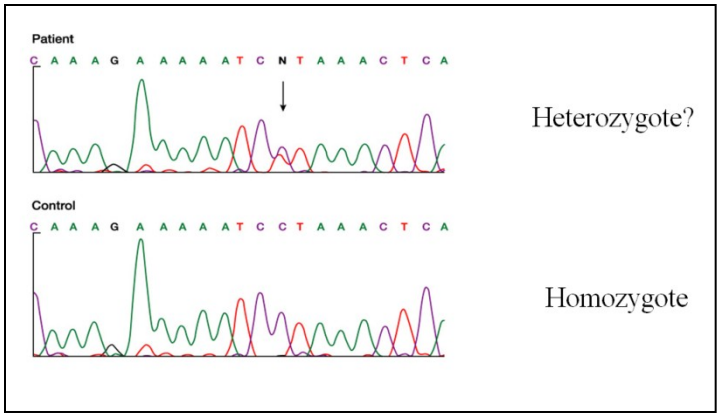
Carpodacus erythrinus – MHC Class I (Promerová et al. 2007)



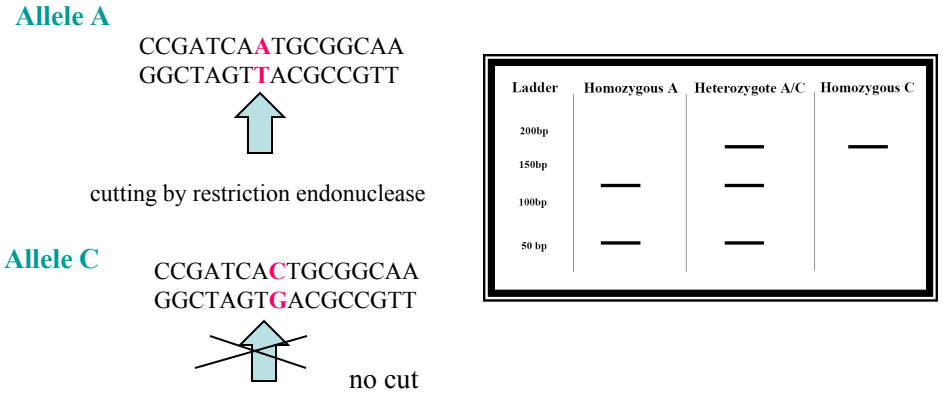
Detection of PCR artefacts during cloning of heterozygotes

SNP genotyping – old classics

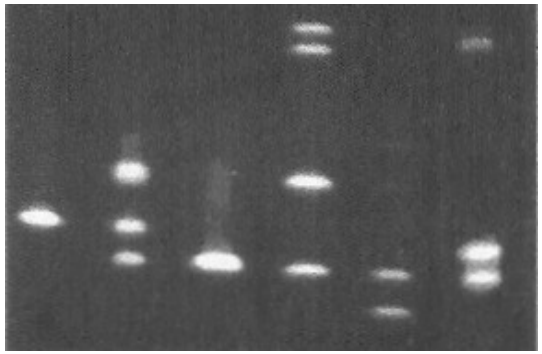
Sekvenování



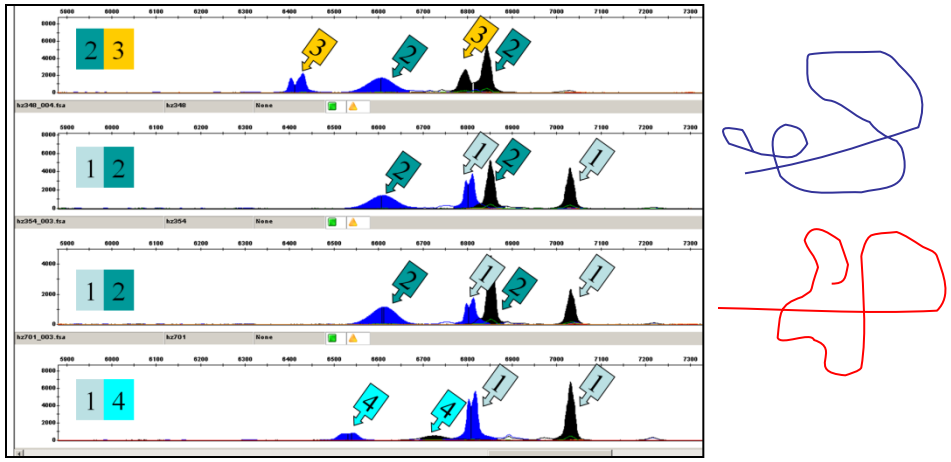
PCR-RFLP (restrikční štěpení)



DGGE, TGGE – gradientové elektroforézy



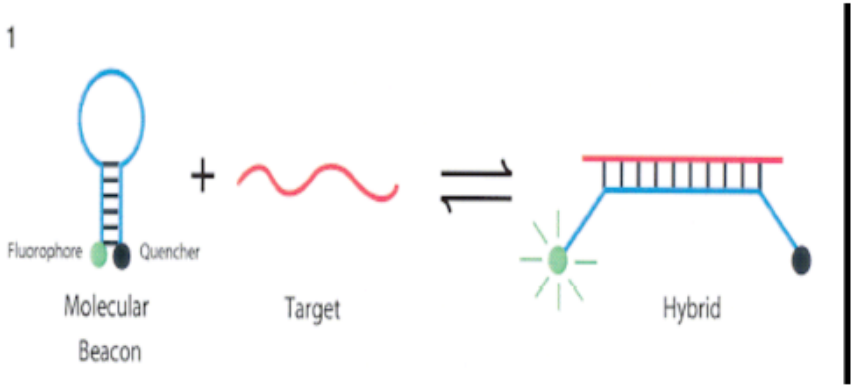
SSCP

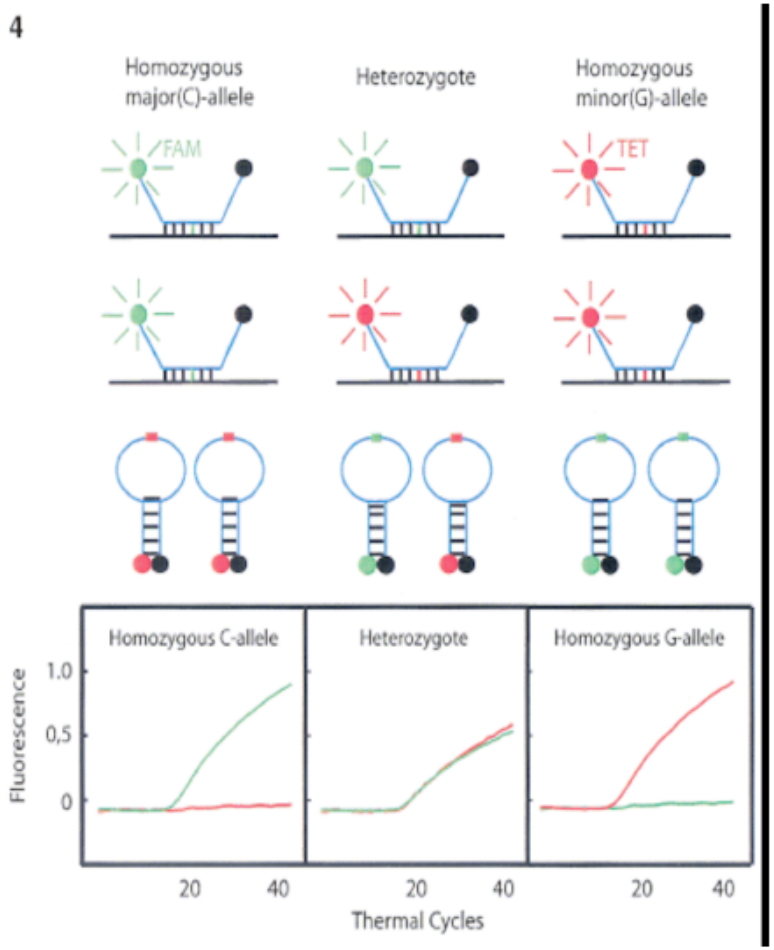


SNP genotyping – new methods

1. real-time PCR se specifickými sondami (TaqMan, molecular beacon)
2. ASPE: allele-specific primer extension
3. SBE: single base extension
4. SNP microarrays (GeneChip method)

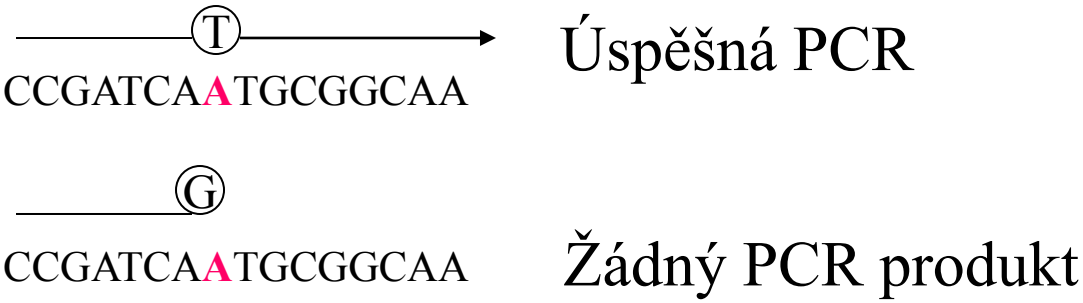
Real-time PCR se specifickou sondou





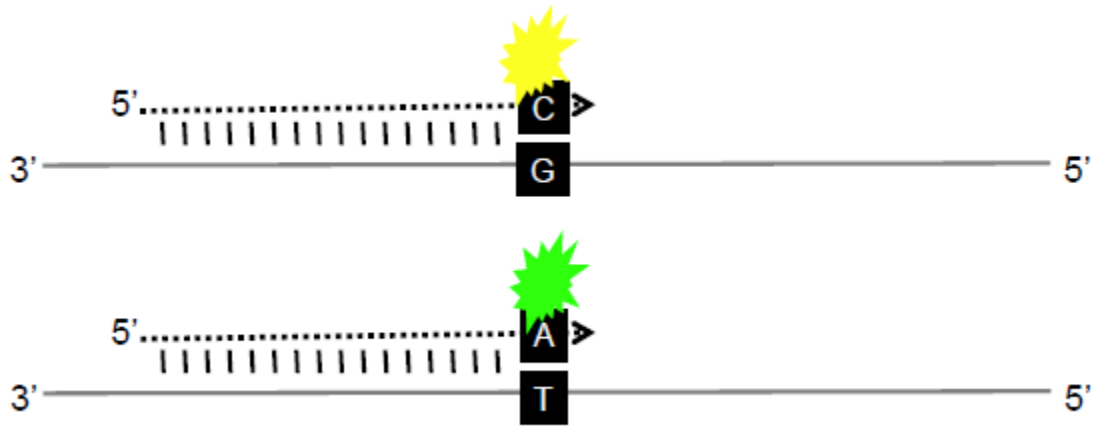
1) TaqMan sondy
2) Molecular Beacons („maják“)

ASPE: allele-specific primer extension



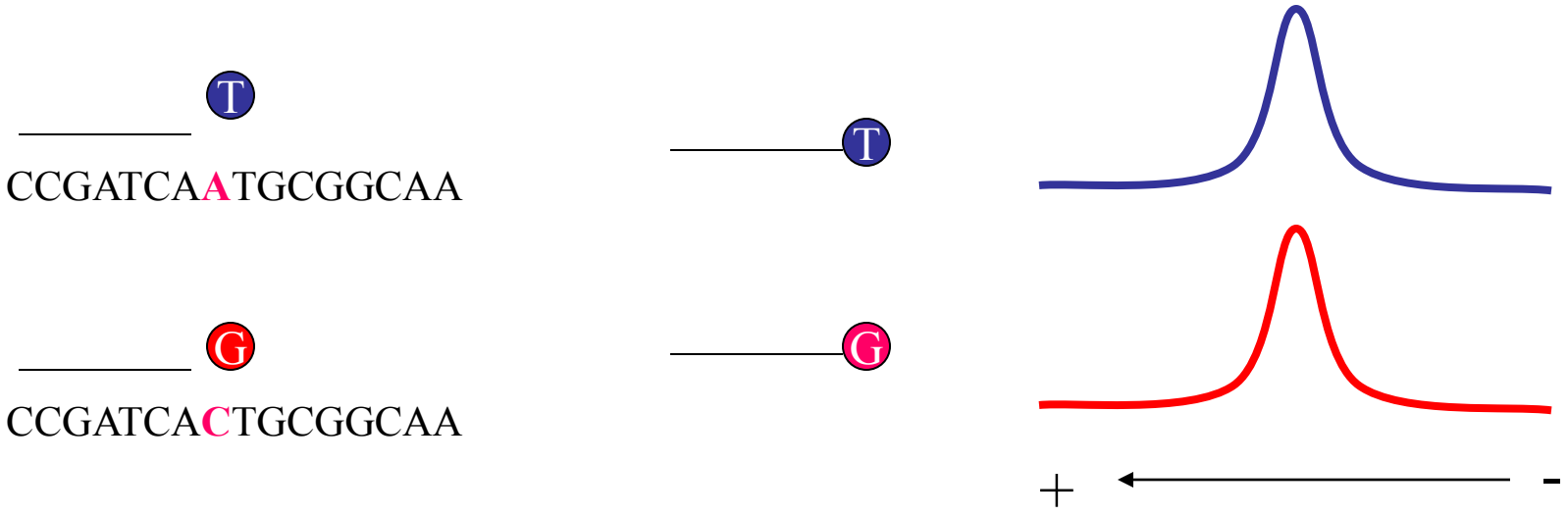
- dvě PCR se specifickými primery
- 3’ terminální nukleotid na primerech je komplementární k SNP nukleotidu
- alelově-specifická amplifikace je umožněna vysoce specifickou polymerázou

ASPE: allele-specific primer extension (automatizovaná verze)



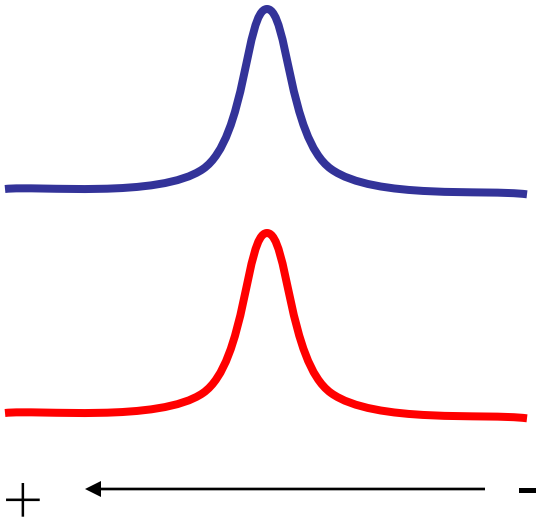
- existují zoptimalizované multiplexy pro modelové druhy (např. člověk 1536 SNPs)
- fluorescenční detekce (Illumina)

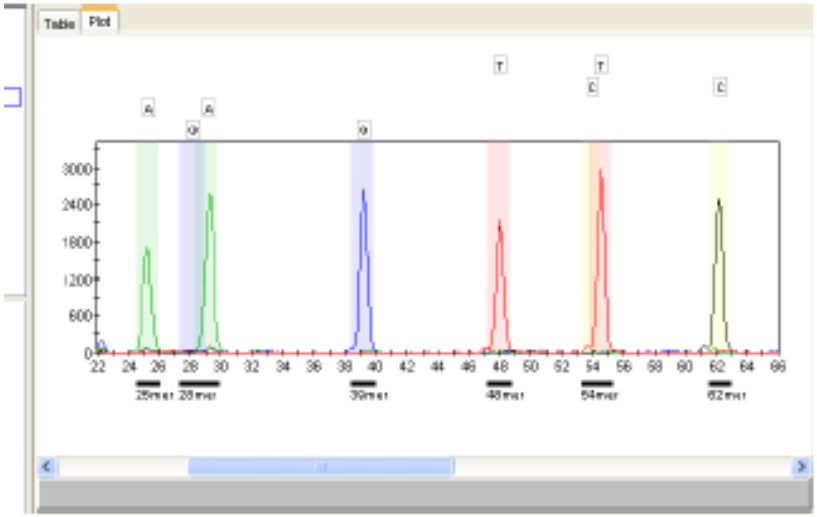
SBE: single base extension



- pouze jeden dideoxynukleotid je přidán k primeru
- detekce různými metodami

Detection or SBE products





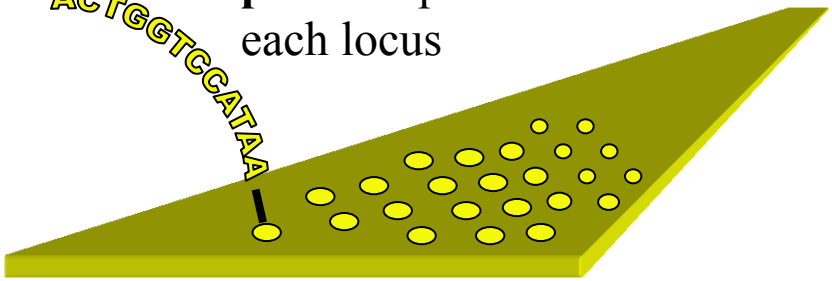
„multiplex version“ – různě dlouhé primery, aby bylo možné odlišit různé lokusy

electrophoresis in a capillary
SNaPShot Multiplex Kit (Applied Biosystems)

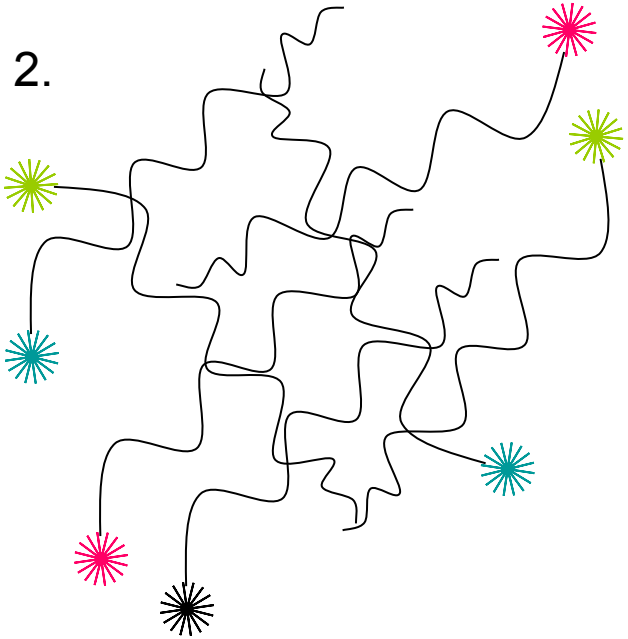
Microarray detection of SBE products

1. **tag** – specific for each locus

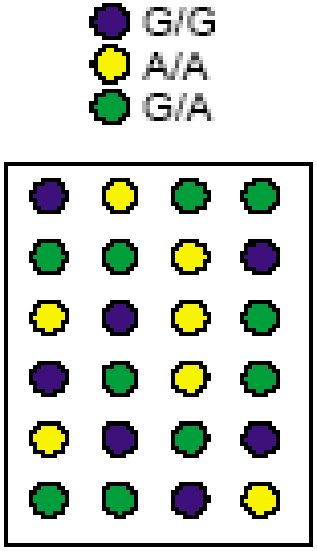
3. **tag-complementary probe -** specific for each locus



2.



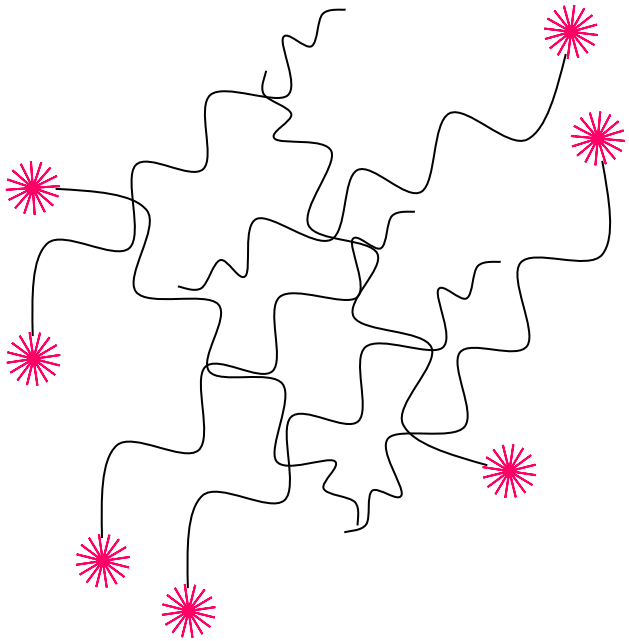
4.



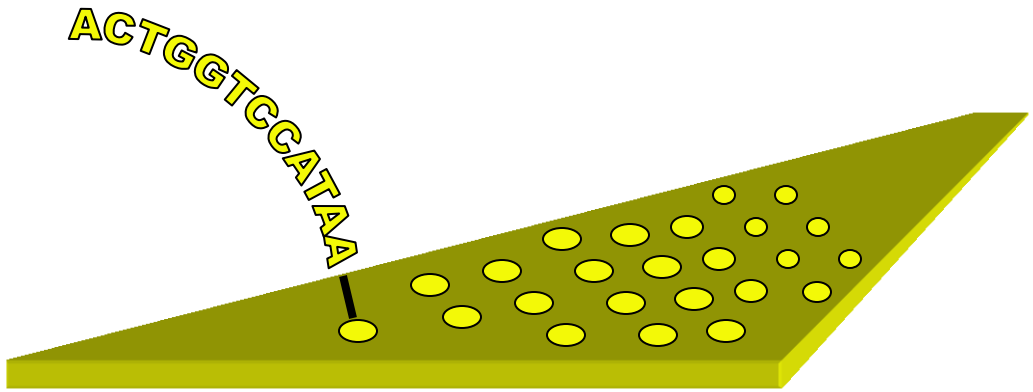
Small-scale “in house” SNP genotyping

multicolor detection (using of 5’ oligonucleotide tags on SBE primers)

Microarray analysis of SNPs
(whole genome approach – „chip technology“)

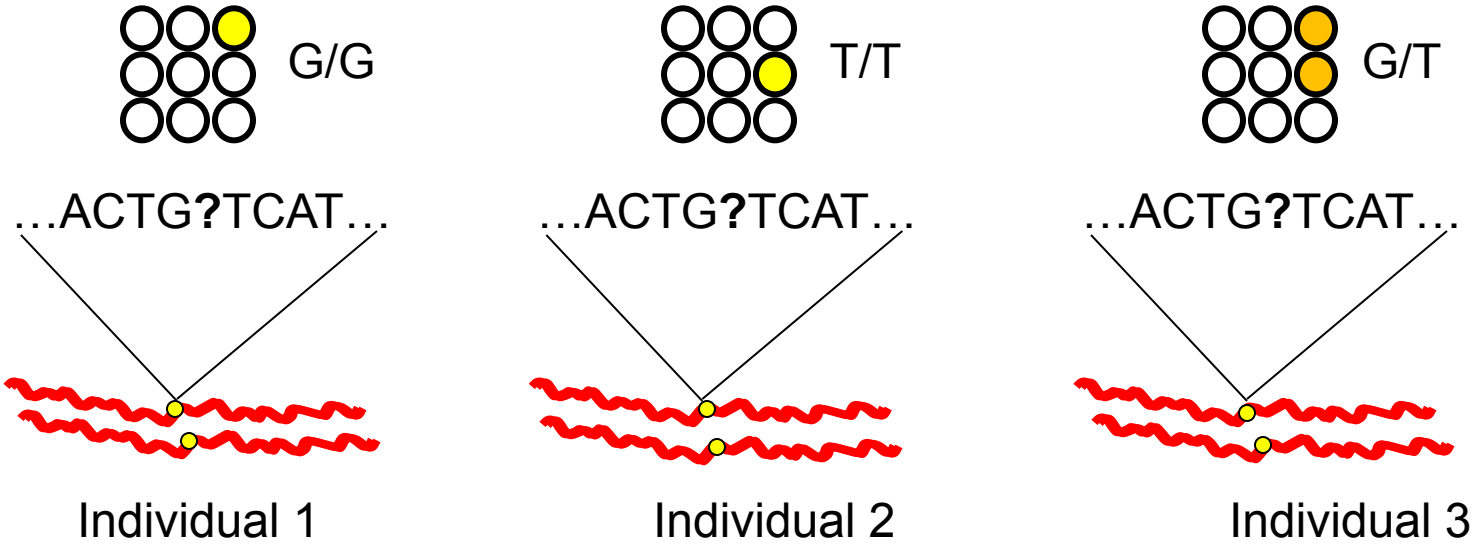


Target

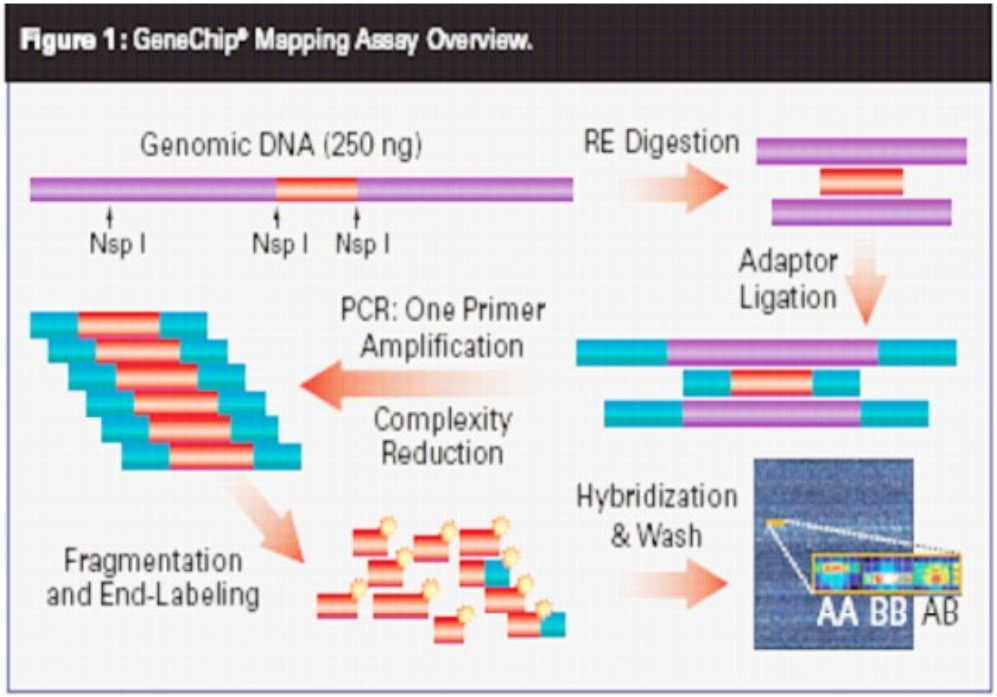


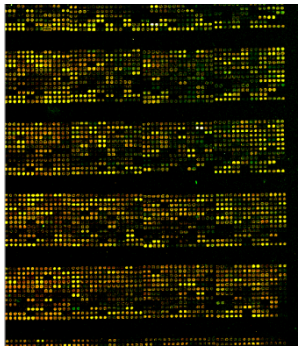
Probe

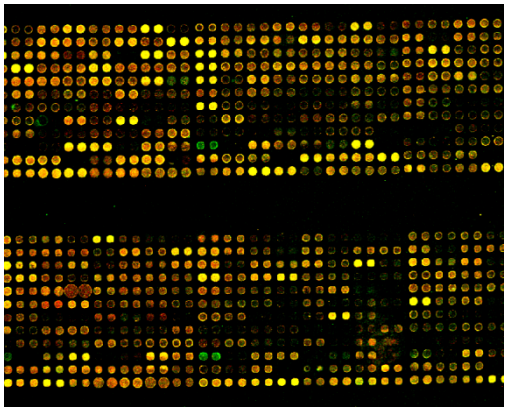
Microarray SNP Genotyping



Detekce: Affymetrix, Illumina aj.







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

Fees - Whole Genome Genotyping									
Platform	SNP multiplex	# samples per array	# genotypes	array $	reagent $	core fee $	Project price per sample	Project price per genotype	volume discount bins
Affymetrix 10K	10,000	1	10,000	185	50	255	**$490.00**	$0.0490	
Affymetrix 50K	50,000	1	50,000	210	50	255	**$515.00**	$0.0103	
Affymetrix 100K (50K x2)	100,000	1	100,000	420	100	510	**$920.00**	$0.0092	
Affymetrix 250K	250,000	1	250,000	470	55	255	**$780.00**	$0.0031	
Affymetrix 500K (250K x2)	500,000	1	500,000	940	110	510	**$1,560.00**	$0.0031	
Affymetrix 500K (250K x2)	500,000	1	500,000	800	110	510	**$1,420.00**	$0.0028	1000-2000 samples
Affymetrix 500K (250K x2)	500,000	1	500,000	700	110	510	**$1,320.00**	$0.0026	2001-5000 samples
Illumina Human-1	109,000	1	109,000	800	na	110	**$910.00**	$0.0083	1-256 samples
Illumina Human-1	109,000	1	109,000	720	na	110	**$830.00**	$0.0076	257-496 samples
Illumina Human-1	109,000	1	109,000	640	na	110	**$750.00**	$0.0069	497-736 samples
Illumina Human-1	109,000	1	109,000	560	na	110	**$670.00**	$0.0061	737-976 samples
Illumina Human-1	109,000	1	109,000	480	na	110	**$590.00**	$0.0054	977+ samles
Illumina HumanHap300	317,000	1	317,000	1100	na	110	**$1,210.00**	$0.0038	1-256 samples
Illumina HumanHap300	317,000	1	317,000	990	na	110	**$1,100.00**	$0.0035	257-496 samples
Illumi									ples
Illumi									ples
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Illumi									ples
Illumina HumanHapS	240,000	1	240,000	700	na	110	**$810.00**	$0.0034	737-976 samples
Illumina HumanHapS	240,000	1	240,000	600	na	110	**$710.00**	$0.0030	977+ samles
Illumina HumanHap550	550,000	1	550,000	1600	na	110	**$1,710.00**	$0.0031	1-256 samples
Illumina HumanHap550	550,000	1	550,000	1440	na	110	**$1,550.00**	$0.0028	257-496 samples
Illumina HumanHap550	550,000	1	550,000	1280	na	110	**$1,390.00**	$0.0025	497-736 samples
Illumina HumanHap550	550,000	1	550,000	1120	na	110	**$1,230.00**	$0.0022	737-976 samples
Illumina HumanHap550	550,000	1	550,000	960	na	110	**$1,070.00**	$0.0019	977+ samles
HumanHap300 + HumanHapS	550,000	1	550,000	1750	na	220	**$1,970.00**	$0.0036	1-256 samples
HumanHap300 + HumanHapS	550,000	1	550,000	1575	na	220	**$1,795.00**	$0.0033	257-496 samples
HumanHap300 + HumanHapS	550,000	1	550,000	1400	na	220	**$1,620.00**	$0.0029	497-736 samples
HumanHap300 + HumanHapS	550,000	1	550,000	1225	na	220	**$1,445.00**	$0.0026	737-976 samples
HumanHap300 + HumanHapS	550,000	1	550,000	1050	na	220	**$1,270.00**	$0.0023	977+ samles

Použití u příbuzných druhů je možné, ale je tam velmi silný „ascertainment bias“