Next-generation sequencing (NGS)

High-throughput sequencing (HTS)

Sanger sequencing

Primer - F - AAGTCAGTCTAA=o

Primer - F - AAGTCAGTCTA=o

Primer - F - AAGTCAGTCT=o

Primer - F - AAGTCAGTC=o

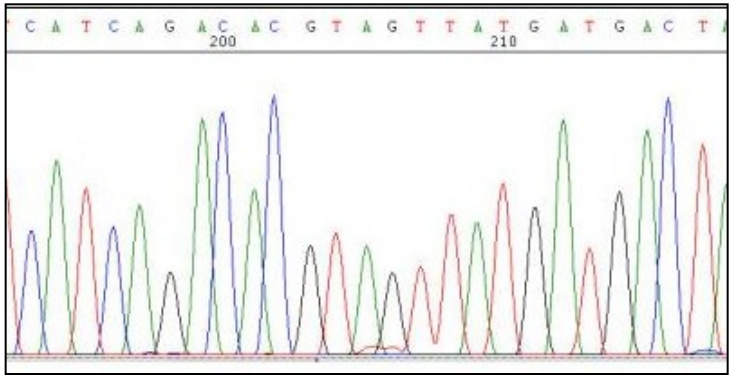
Primer - F - AAGTCAGT=o

Primer - F - AAGTCAG=o

Primer - F - AAGTCA=o

Primer - F - AAGTC=o

-



krátké ---------- dlouhé

(rychlé) ---------- (pomalé)

+

Primer - F AAGTCAGTCTAAATGCGATTGGGA Rev. Primer - R

Rev. Primer - F TTCAGTCAGATTTACGCTAACCCT Primer - R

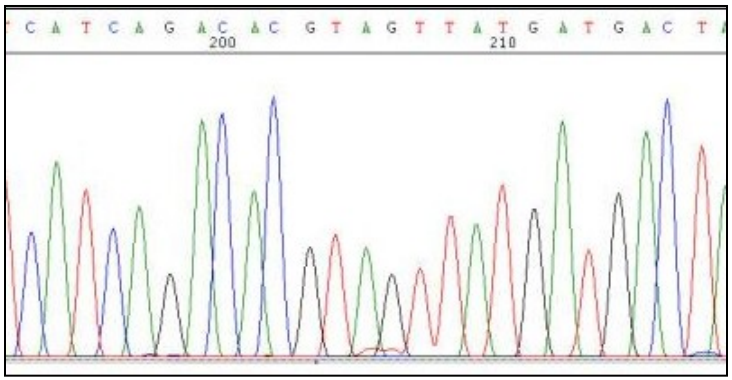
4-kapilární sekvenátor

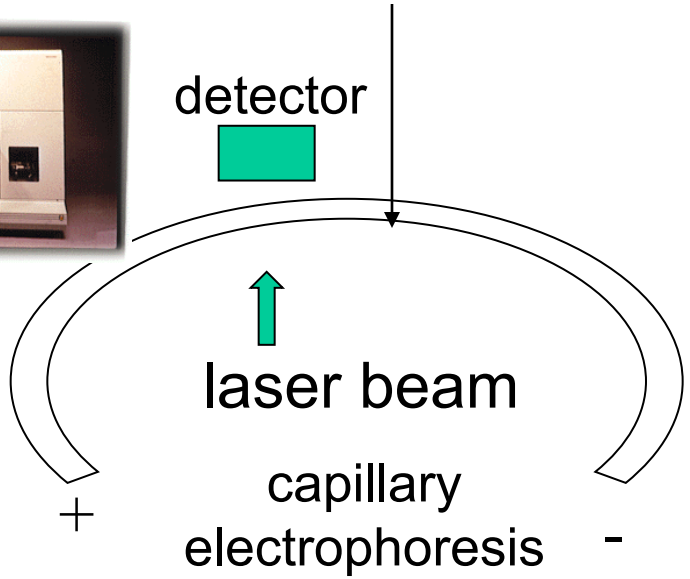
=

96 x 500 bp/12 hodin

=

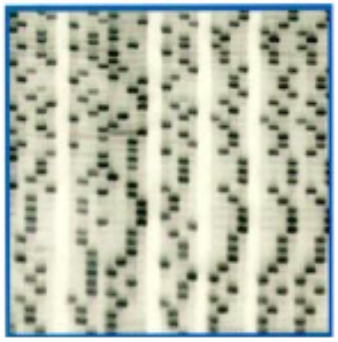
cca 100 000 bp/den





Evoluce Sangerova sekvenování

Pre-1992
"old fashioned way"



S35 ddNTPs
Gels
Manual loading
Manual base calling

1992-1999
ABI 373/377

Fluorescent ddNTPs*
Gels
Manual loading
Automated base calling*

1999
ABI 3700

Fluorescent ddNTPs
Capillaries*
Robotic loading*
Automated base calling
Breaks down frequently

2003
ABI 3730XL

Fluorescent ddNTPs
Capillaries
Robotic loading
Automated base calling
Reliable*

96-kapilární sekvenátor

=

2304 x 500 bp/12 hodin

=

cca 2 400 000 bp/den

HTS (Illumina NovaSeq 6000)

=

cca 6 000 000 000 000 bp/den

electrophoresis

Next-generation sequencing (NGS)

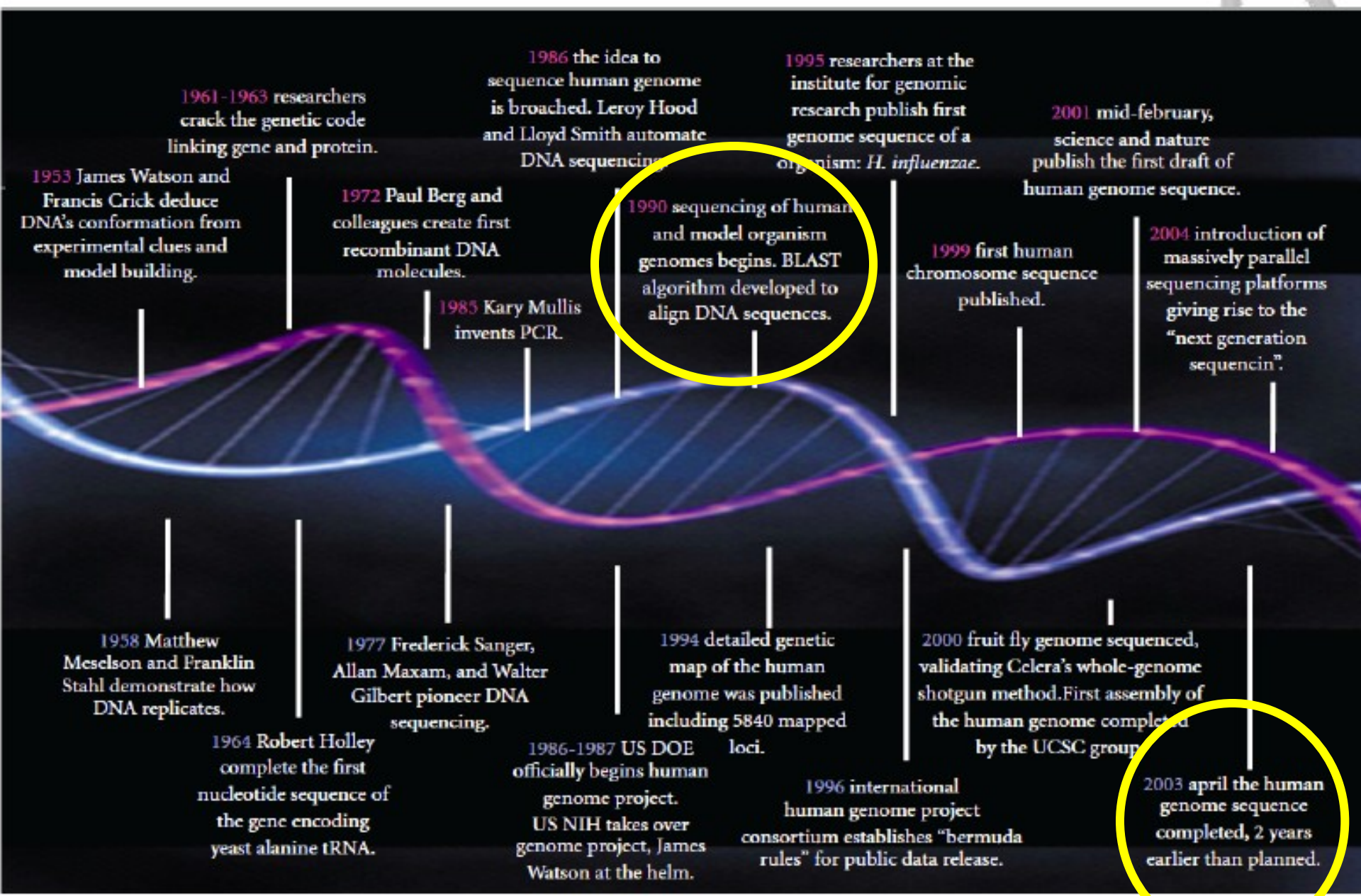
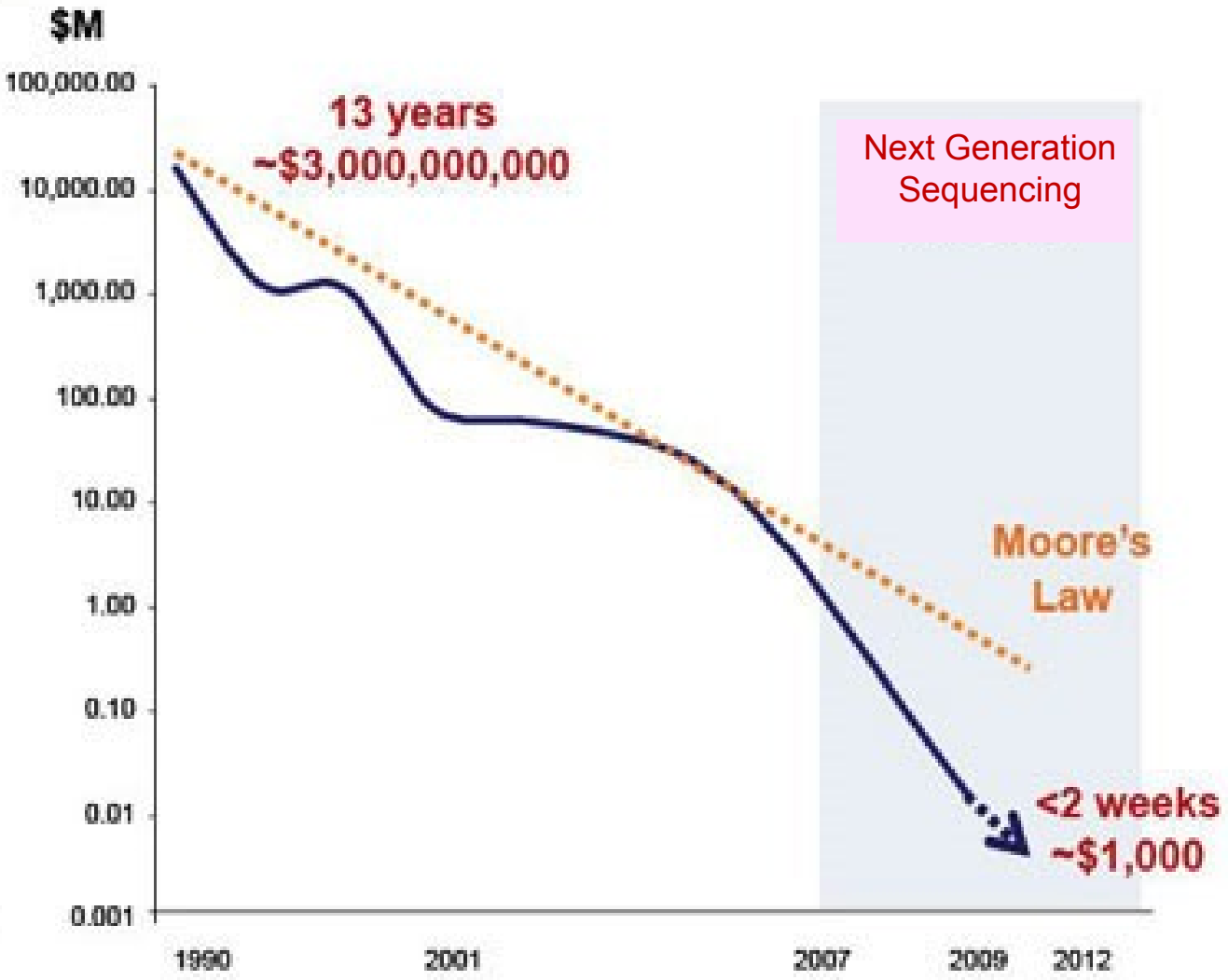


FIGURE 1: Evolution of DNA revolution.

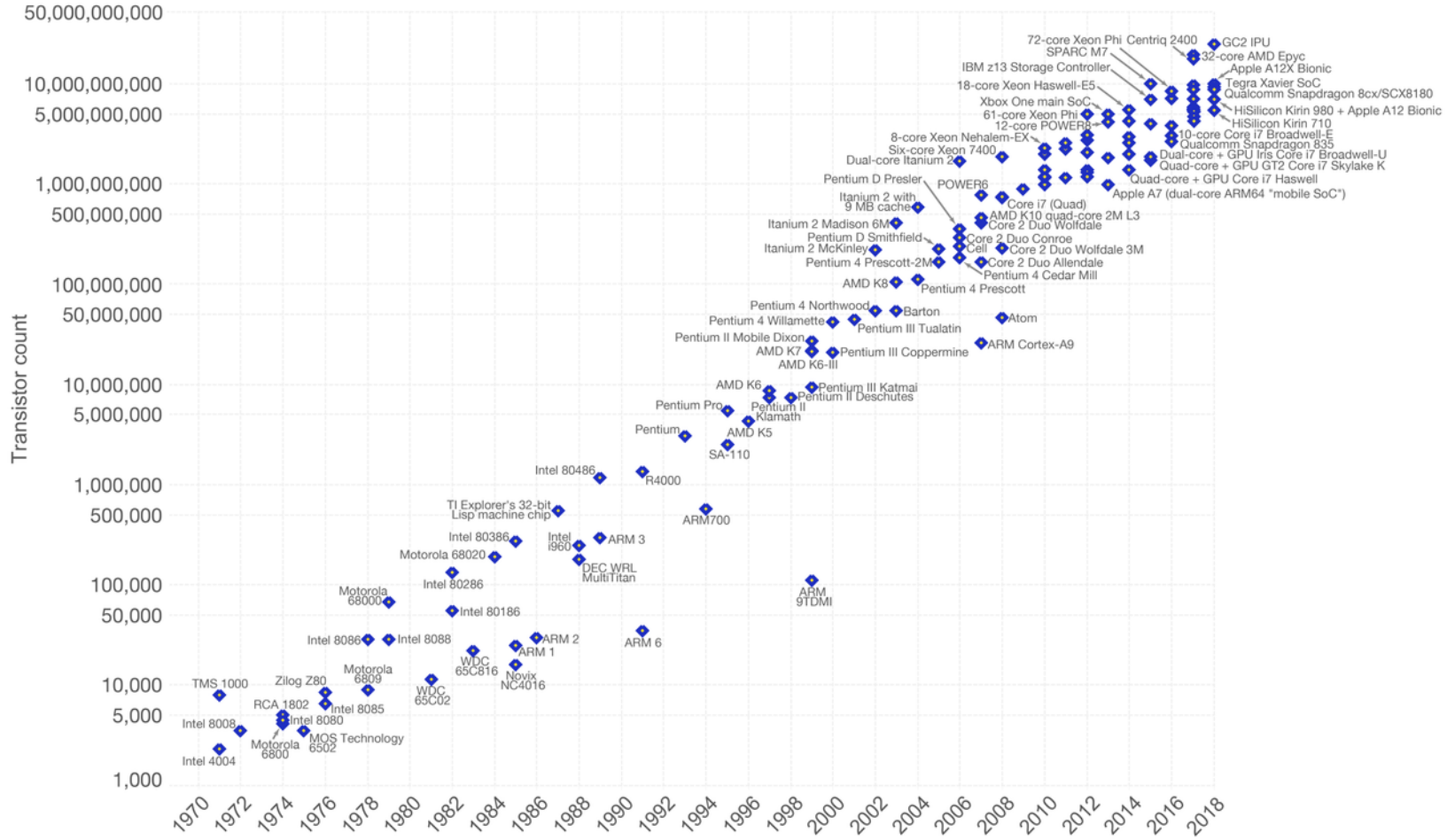
Cost per Human Genome



Moore’s Law – The number of transistors on integrated circuit chips (1971-2018)

Moore's law describes the empirical regularity that the number of transistors on integrated circuits doubles approximately every two years. This advancement is important as other aspects of technological progress – such as processing speed or the price of electronic products – are linked to Moore's law.

Our World in Data



Data source: Wikipedia (https://en.wikipedia.org/wiki/Transistor_count)
The data visualization is available at OurWorldinData.org. There you find more visualizations and research on this topic.

Lidský genom = 3 Gb

cca 5000 lidských genomů/run

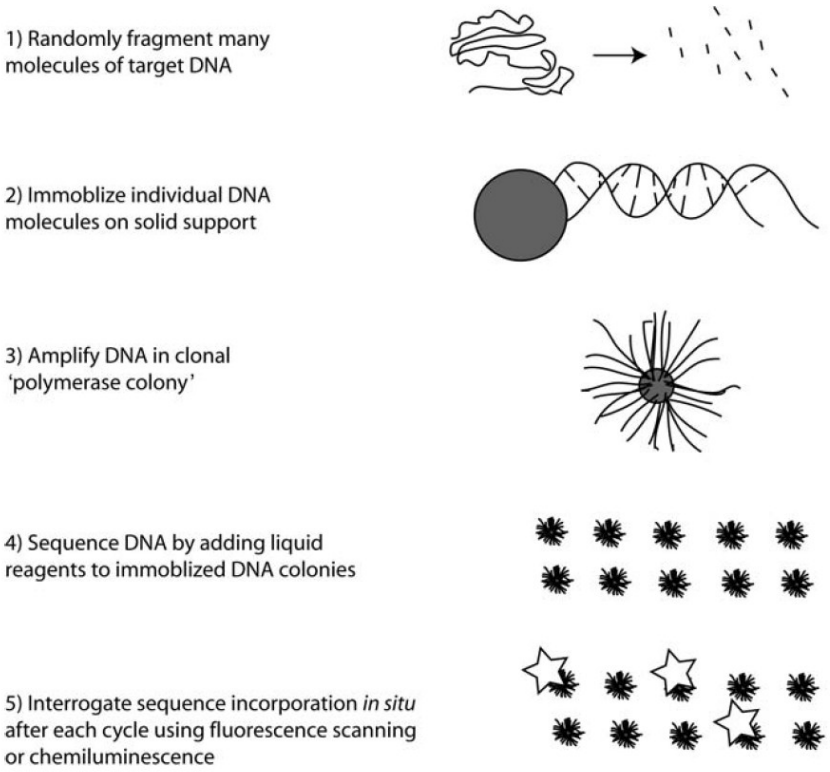
NovaSeq X Series specifications

Output Range	~165 Gb - 16 Tb
Single reads per run	1.6 billion - 52 billion
Read length	2 × 150 bp
Run time	~15 hr - 48 hr

View All NovaSeq X Specs

View AR

Historie „Next generation sequencing“



454 pyrosequencing ... první komerčně dostupná NGS technologie od srpna 2007

2016 – ohlášené stažení z trhu (Roche)

Široké spektrum technologií

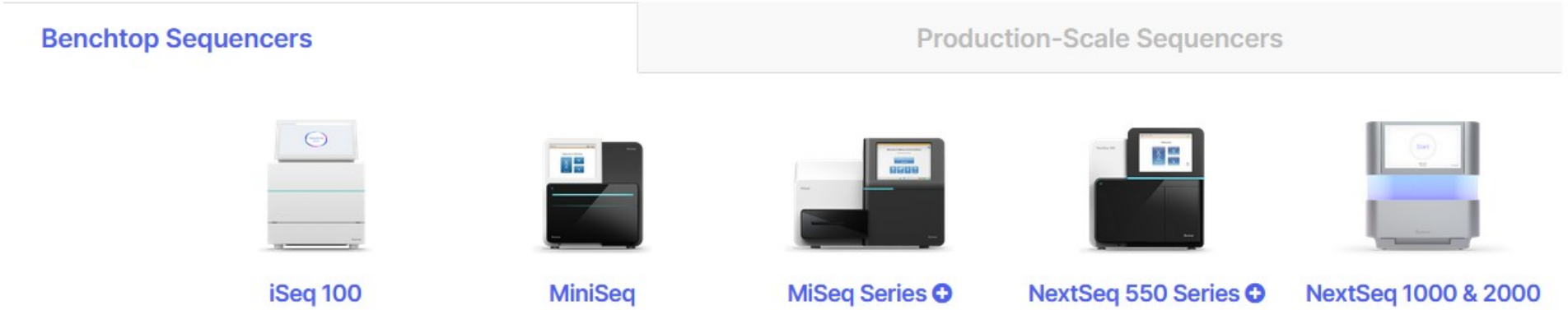
Ale jen některé přežijí

Dnes dostupné NGS platformy

- **Roche 454**
- **Illumina (MiSeq, NextSeq, HiSeq, NovaSeq)**
- ABI SOLiD
- IonTorrent (Life Technologies)
- SMRT (Pacific Biosciences)
- Oxford Nannopore
- …

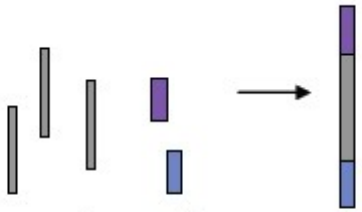
Illumina HiSeq/MiSeq

- v současné době nejrozšířenější typ (cca 70%) na trhu
- v horizontu následujících let její používání spíš poroste
- NextSeq, NovaSeq, etc.



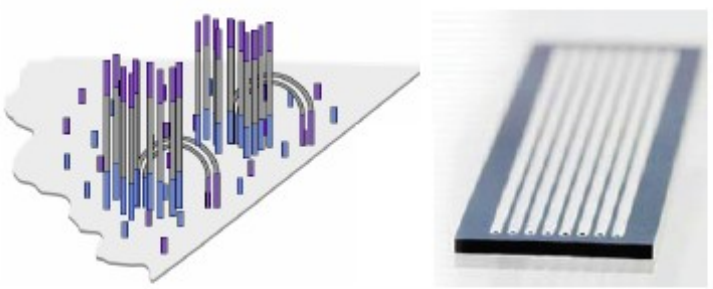
Illumina Sequencing pipeline

1. Sample Prep
(1-5 days)



Ligate adapters

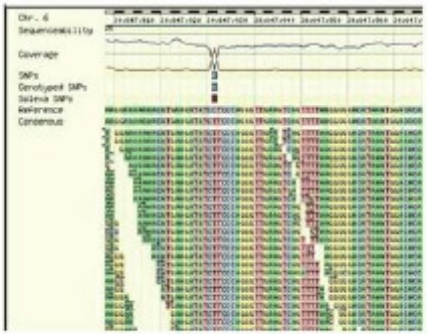
2. Cluster generation on flow cell
(1.5 day)



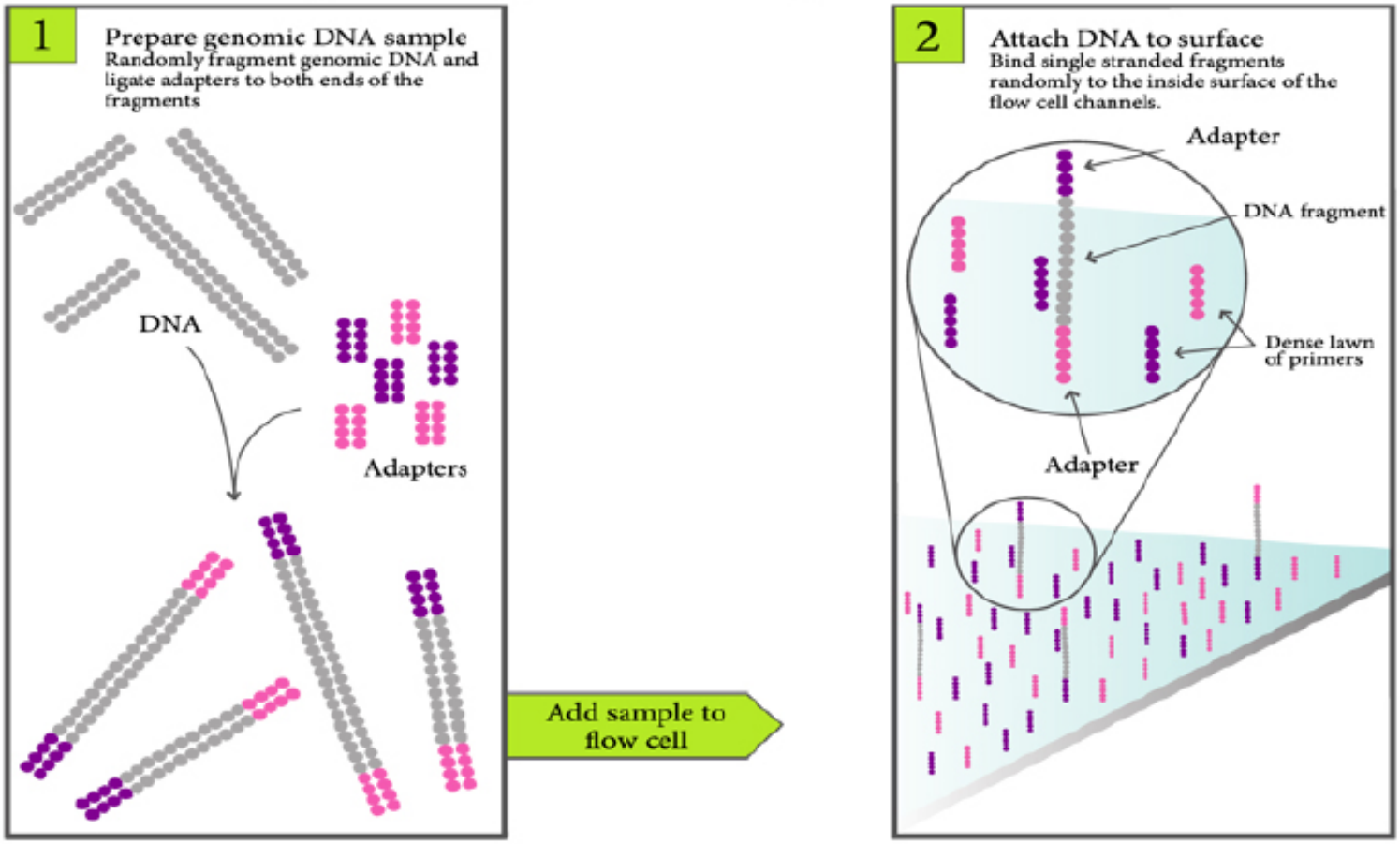
Clonal Single molecular Array

3. Sequencing and imaging
(2-3 days)

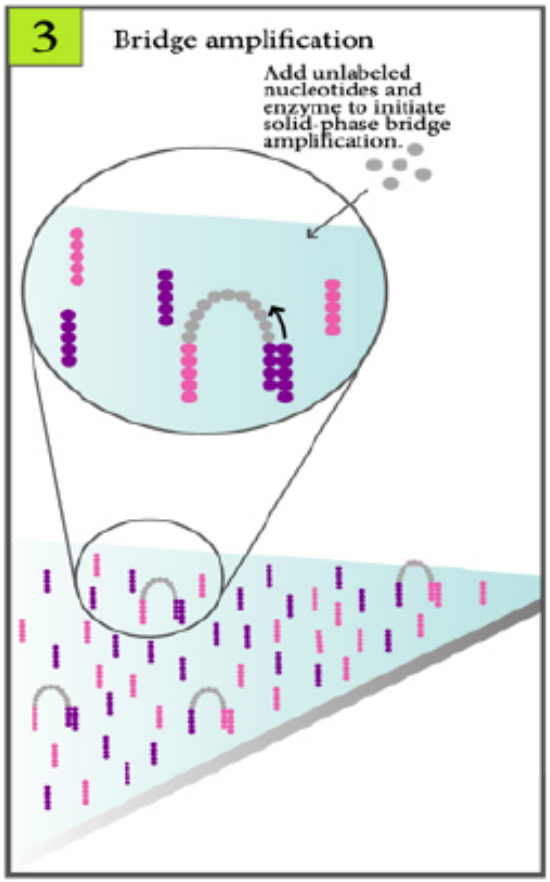
4. Data Analysis
(days-months)

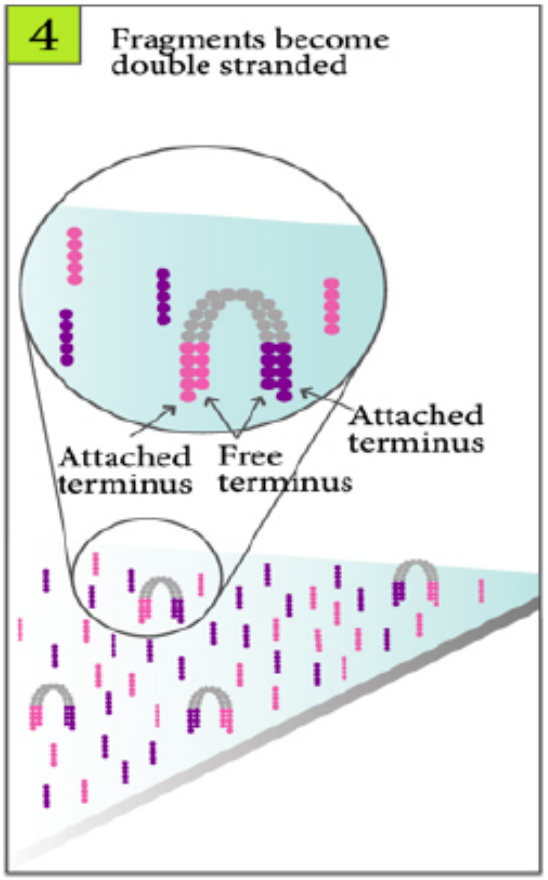


Attach DNA to flow cell

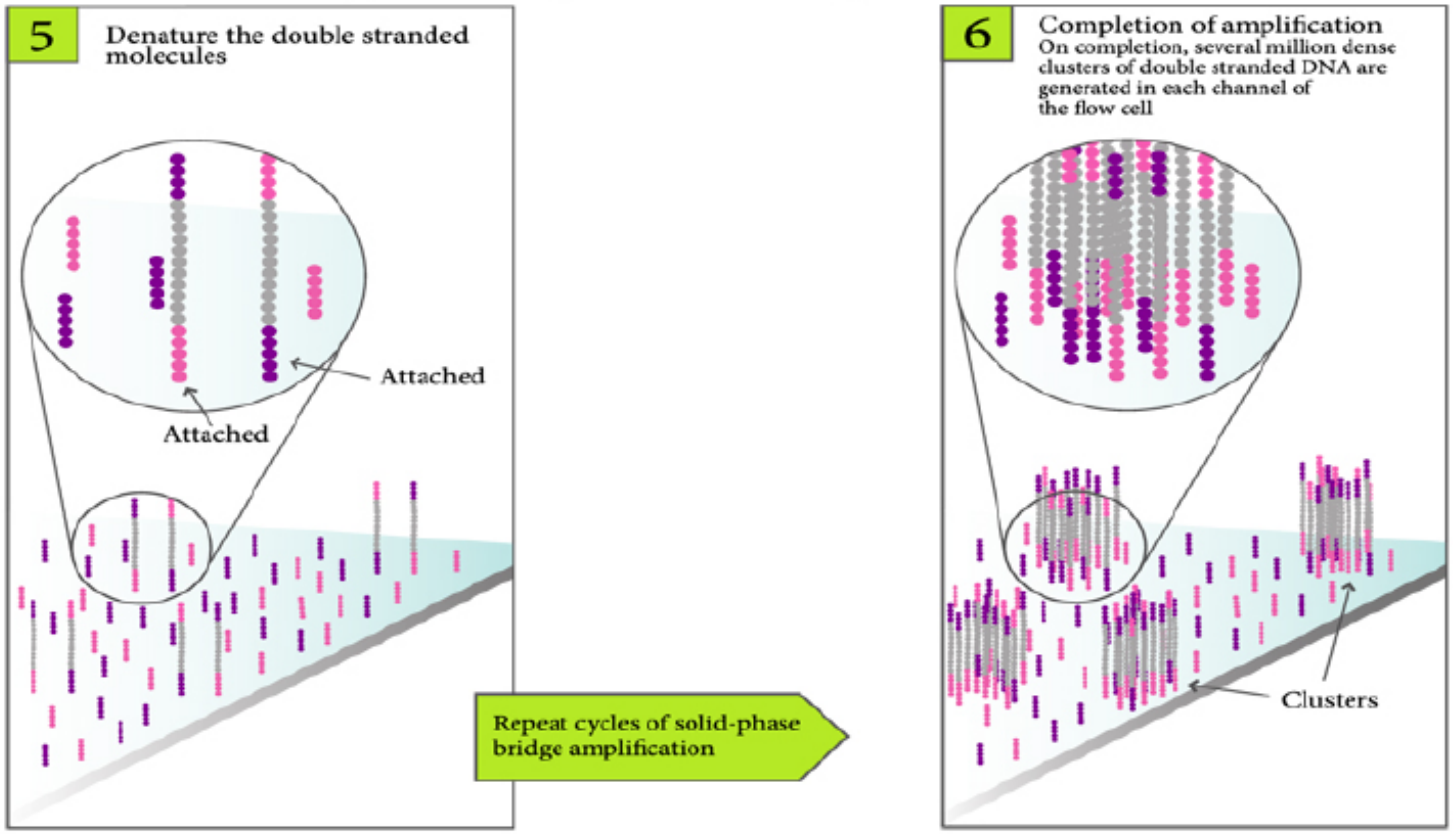


Bridge Amplification



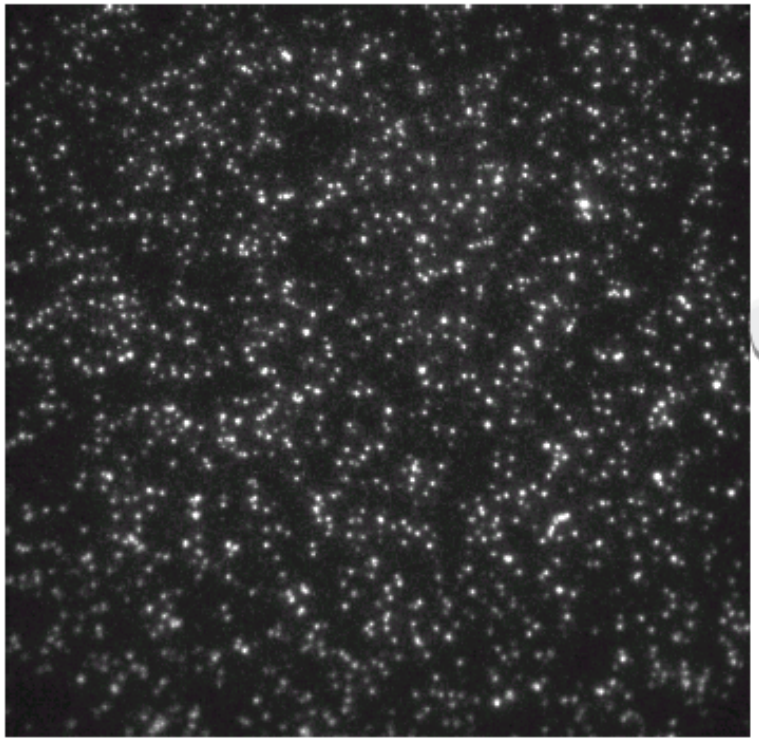


Cluster Generation



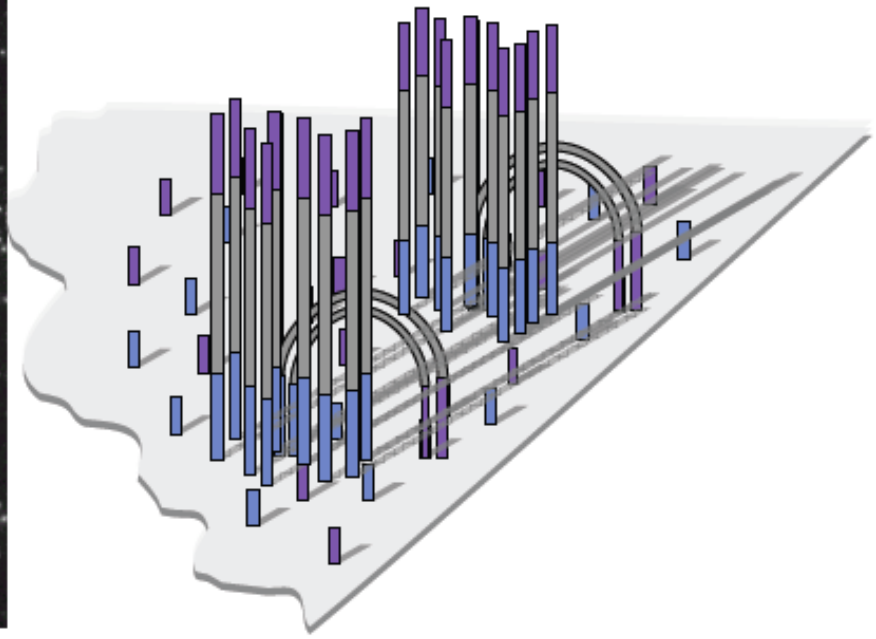
Clonal Single molecular Array

Clonal Single molecule Array



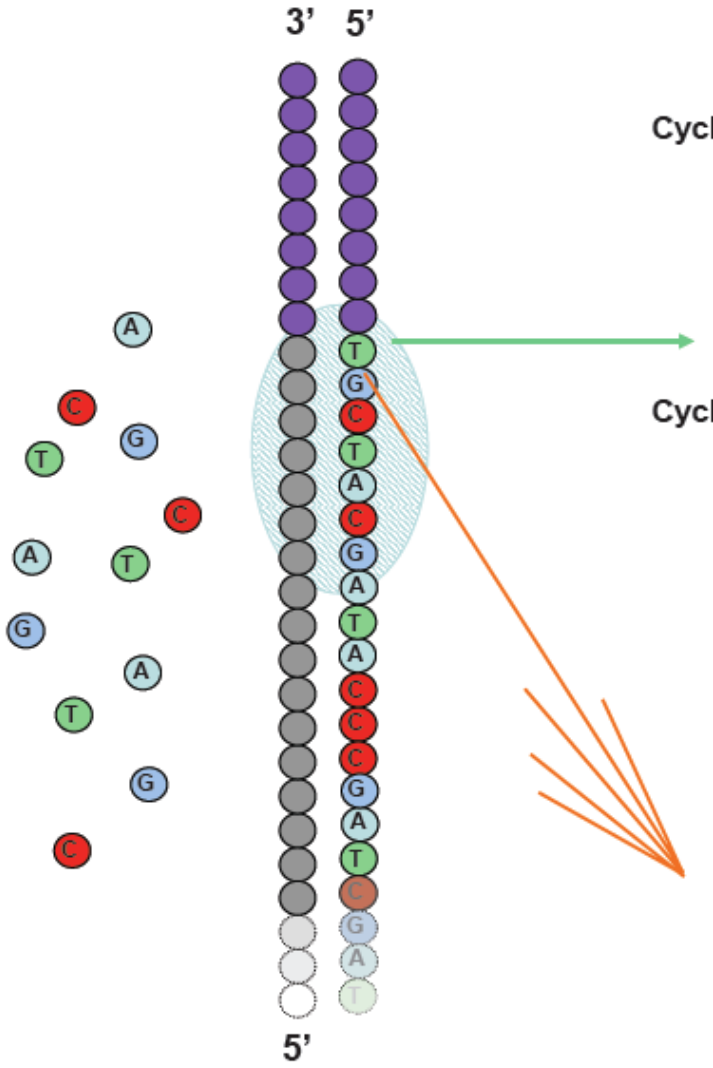
100um

Random array of clusters



~1000 molecules per ~ 1 um cluster
~20-30,000 clusters per tile
~40 M clusters per flowcell

Sequencing By Synthesis (SBS)



Cycle 1: Add sequencing reagents

First base incorporated

Remove unincorporated bases

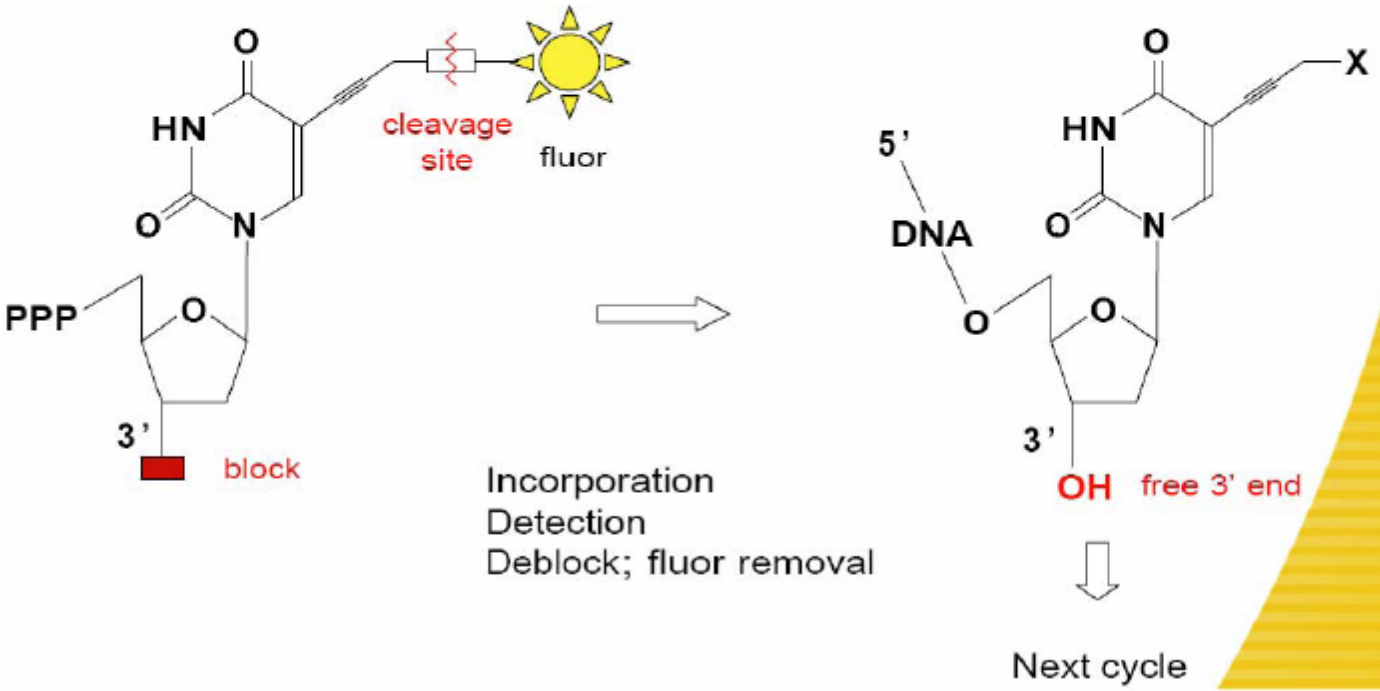
Detect signal

Cycle 2-n: Add sequencing reagents and repeat

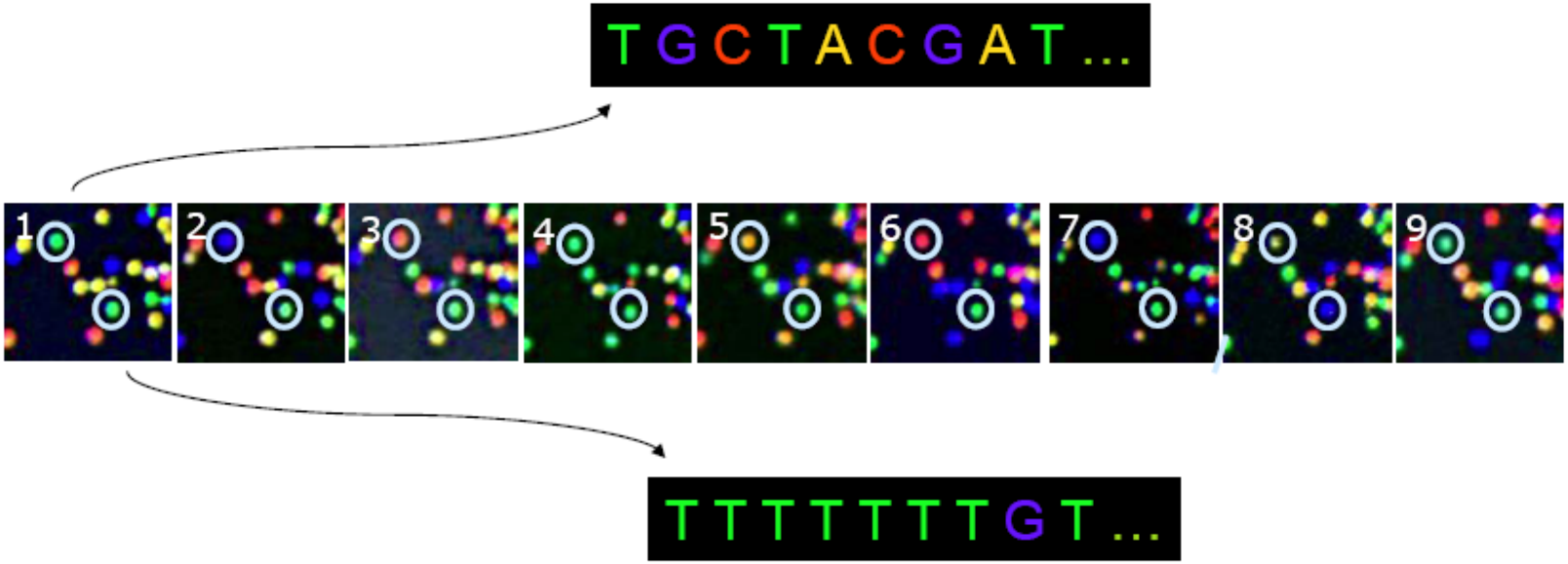
Reversible Terminator Chemistry

Solexa

- All 4 labelled nucleotides in 1 reaction
- Higher accuracy
- No problems with homopolymer repeats

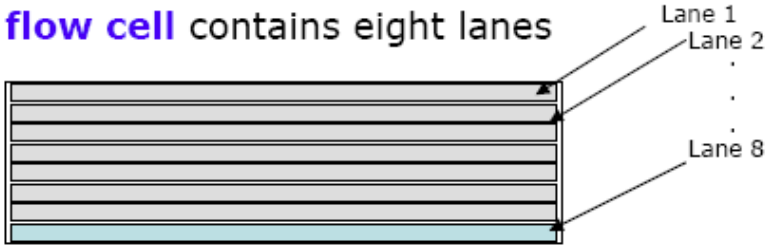


Base Calling From Images

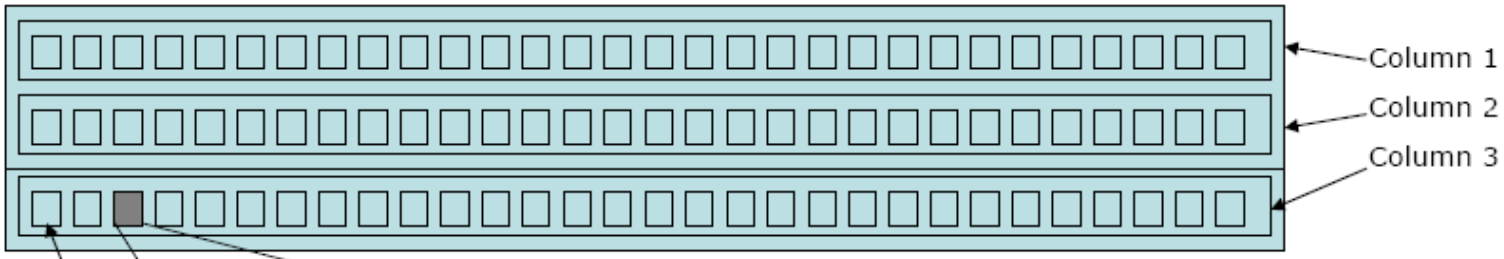


The identity of each base of a cluster is read off from sequential images

A **flow cell** contains eight lanes

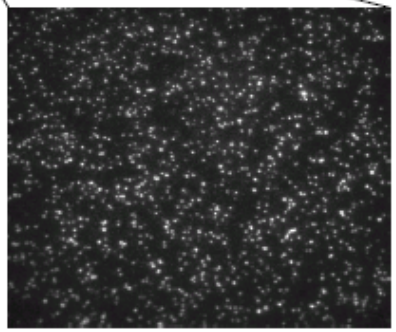


Each **lane/channel** contains **three columns** of tiles



Each **column** contains **100 tiles**

Tile



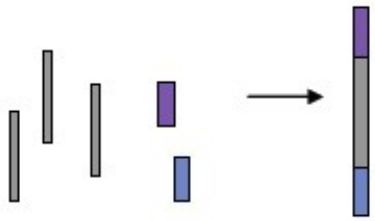
20K-30K Clusters

350 X 350 µm

https://www.youtube.com/watch?annotation_id=annotation_228575861&feature=iv&src_vid=womKfikWlxM&v=fCd6B5HRaZ8

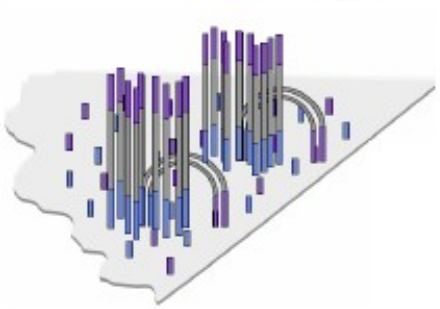
Illumina Sequencing pipeline

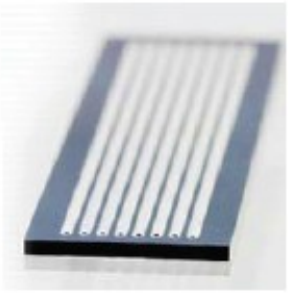
1. Sample Prep
(1-5 days)



Ligate adapters

2. Cluster generation on flow cell
(1.5 day)

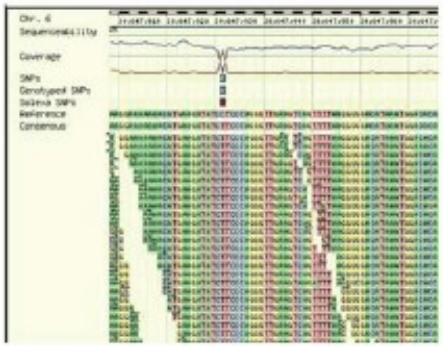




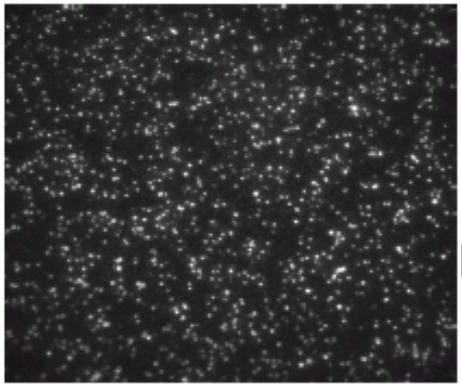
Clonal Single molecular Array

3. Sequencing and imaging
(2-3 days)

4. Data Analysis
(days-months)

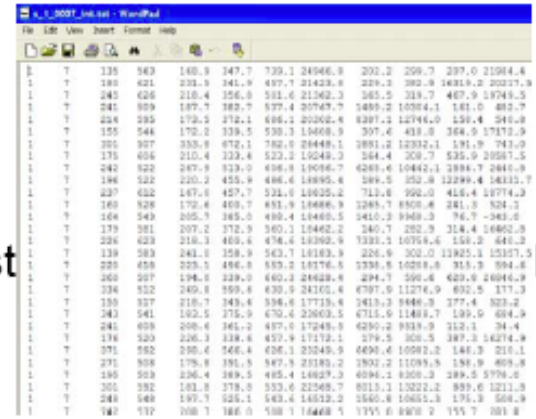


Data Analysis Pipeline



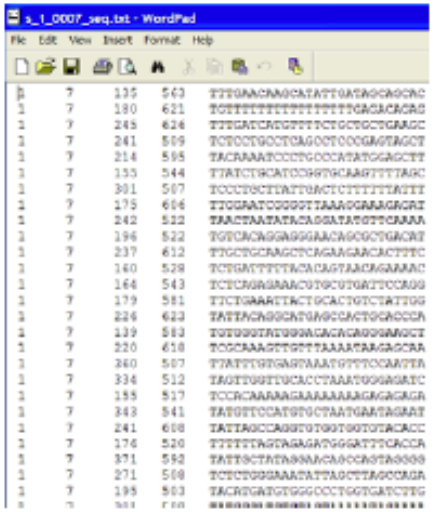
tiff image files
(345,600)

Firecrest



intensity files

Bustard



Sequence files

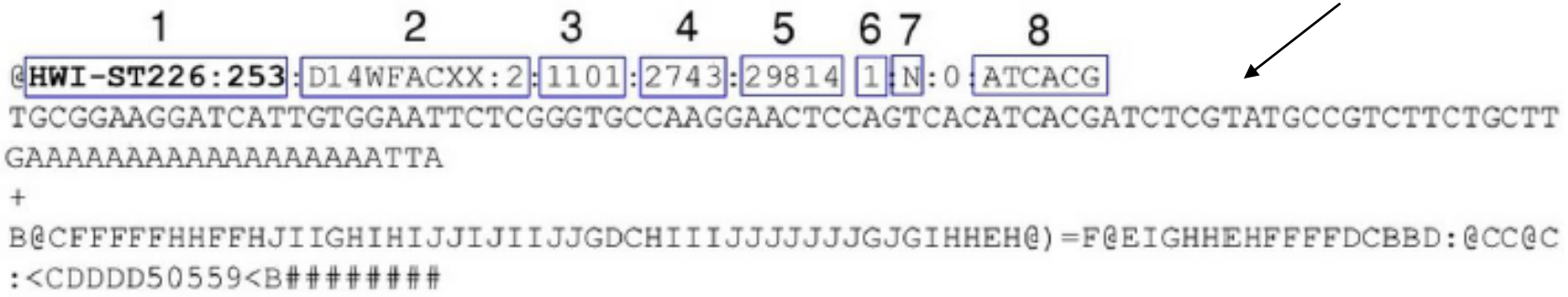
Eland

Alignment to Genome

Additional
Data Analysis

Illumina fastq

= one „read“



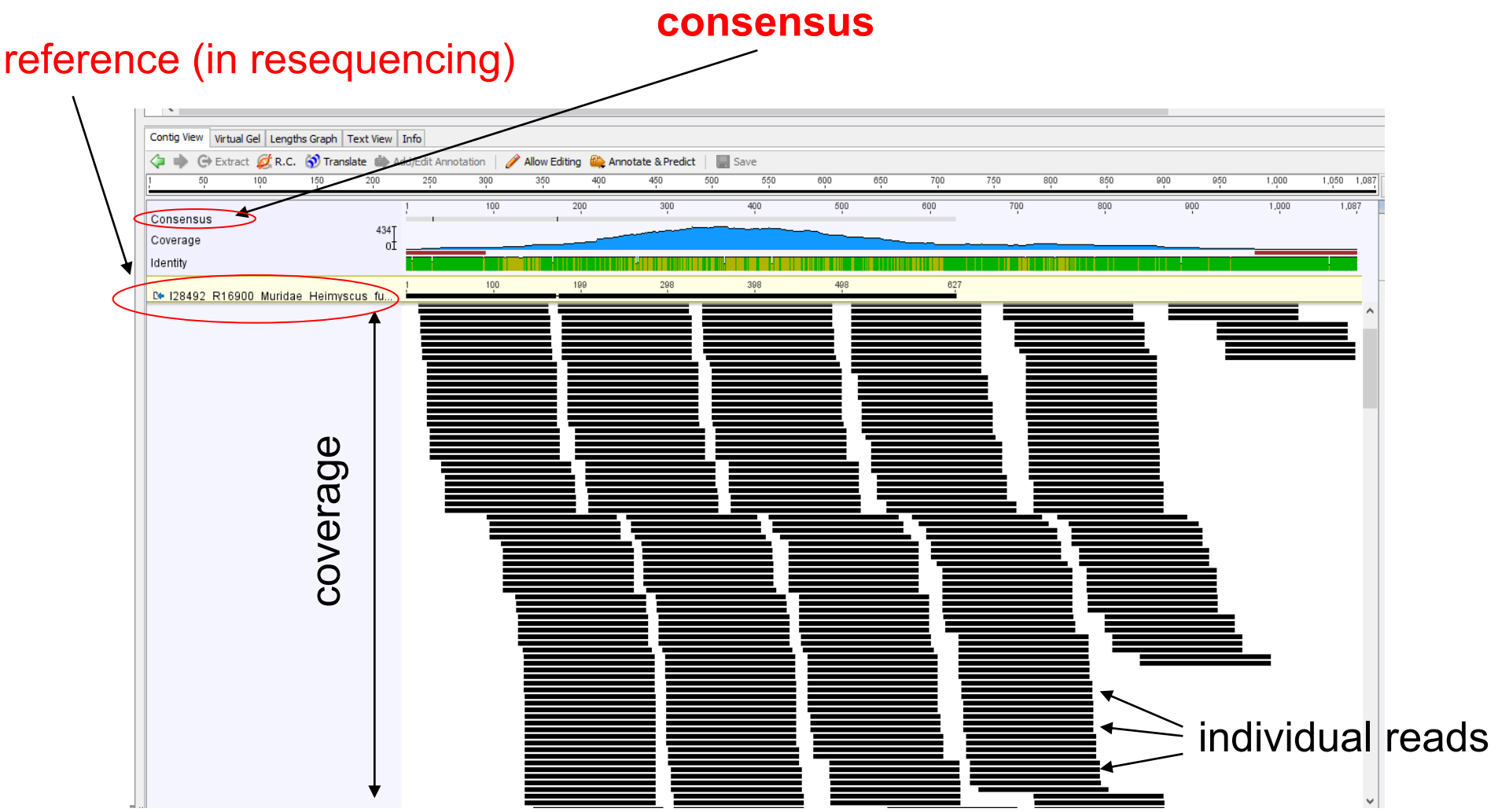
1. unique instrument ID and run ID
2. Flow cell ID and lane
3. tile number within the flow cell lane
4. 'x'-coordinate of the cluster within the tile
5. 'y'-coordinate of the cluster within the tile
6. the member of a pair, /1 or /2 *(paired-end or mate-pair reads only)*
7. N if the read passes filter, Y if read fails filter otherwise
8. Index sequence

All this generates a lot of Data!

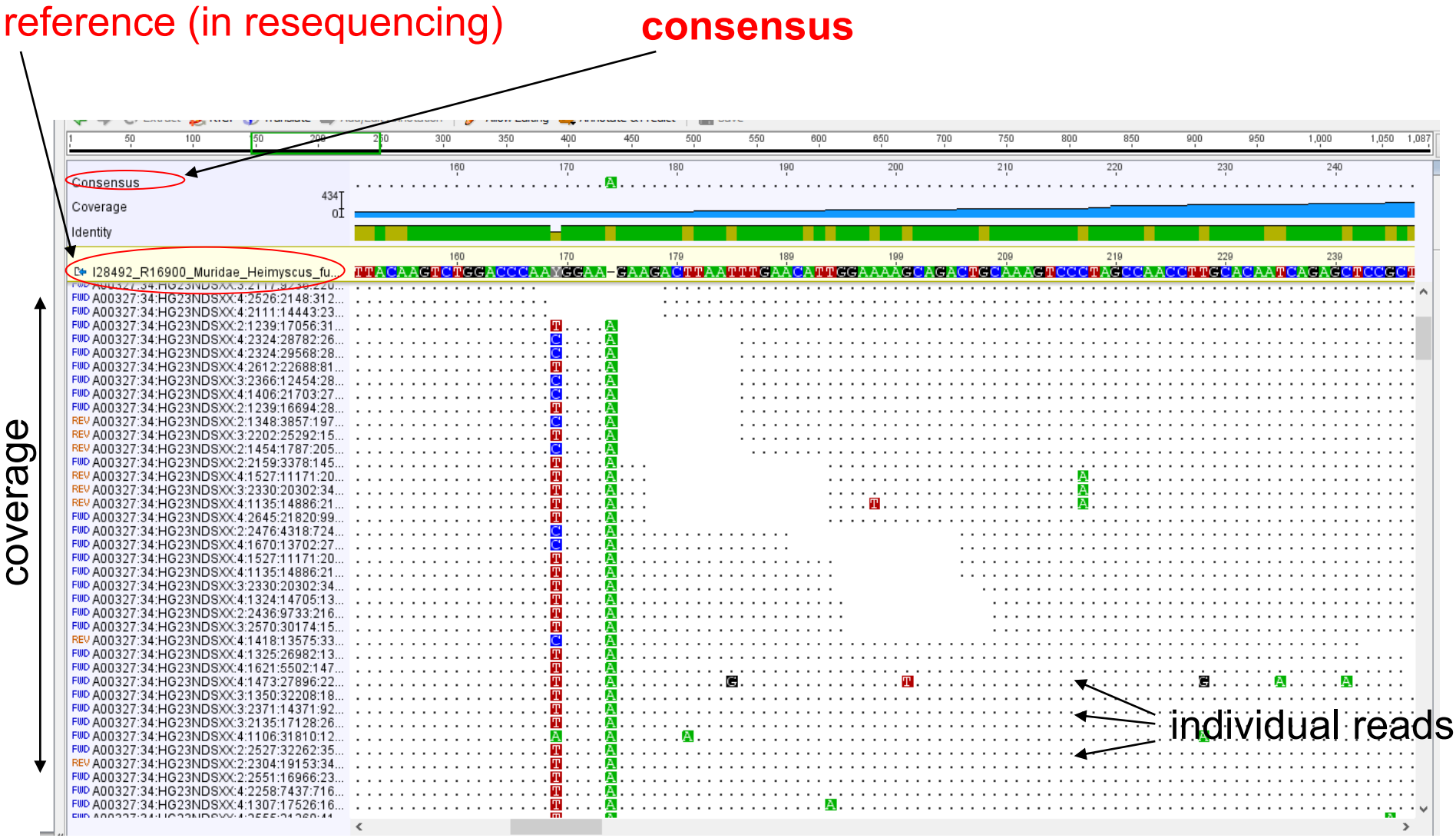
up to 16 TB data/run

- 1 Gig of Space
  - 125,000 pages of text
  - 11 CDs of Music
  - 4000 (1024x768) JPEG images
  - 40,000 pages of PDF
- 1 TB of space
  - 220 Million pages of text
  - 300 hours of video
  - 4,000,000 JPEG images
  - 1,000 copies of the Encyclopedia Britannica
  - 1/10 of the printed Library of Congress

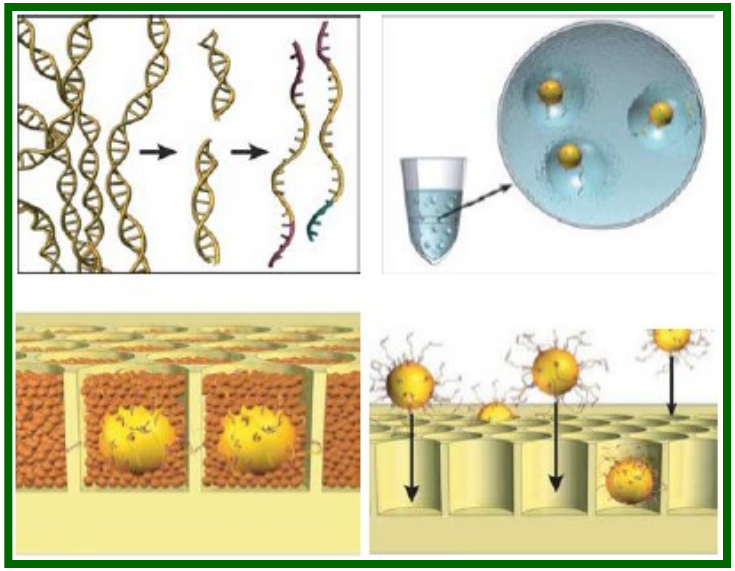
Data analysis in Geneious



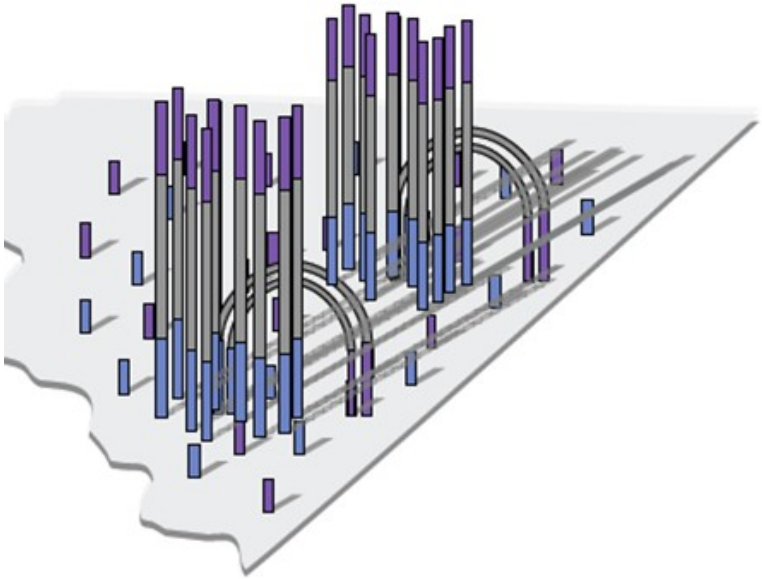
Data analysis in Geneious



Další NGS technologie



454 pyrosequencing
(Roche)



Illumina

Ion Torrent technology

Featured NGS Instruments

Ion GeneStudio S5 System

Scalable targeted NGS to support small and large projects

The Ion GeneStudio S5 system is a scalable, targeted-NGS workhorse with wide application breadth and throughput capability.

Ion Torrent Genexus System

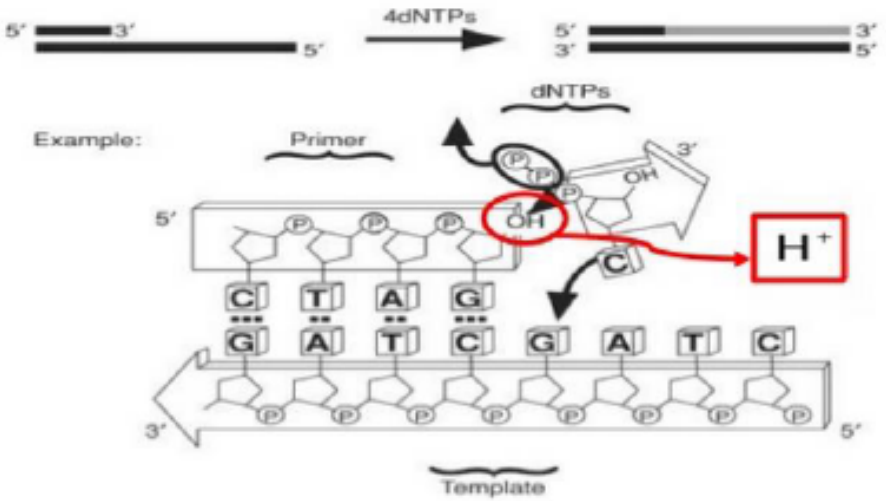
Specimen to report in a single day with a hands-off, automated workflow*

The Genexus System is the first turnkey NGS solution that automates the specimen-to-report workflow and delivers results in a single day with just two user touchpoints.*

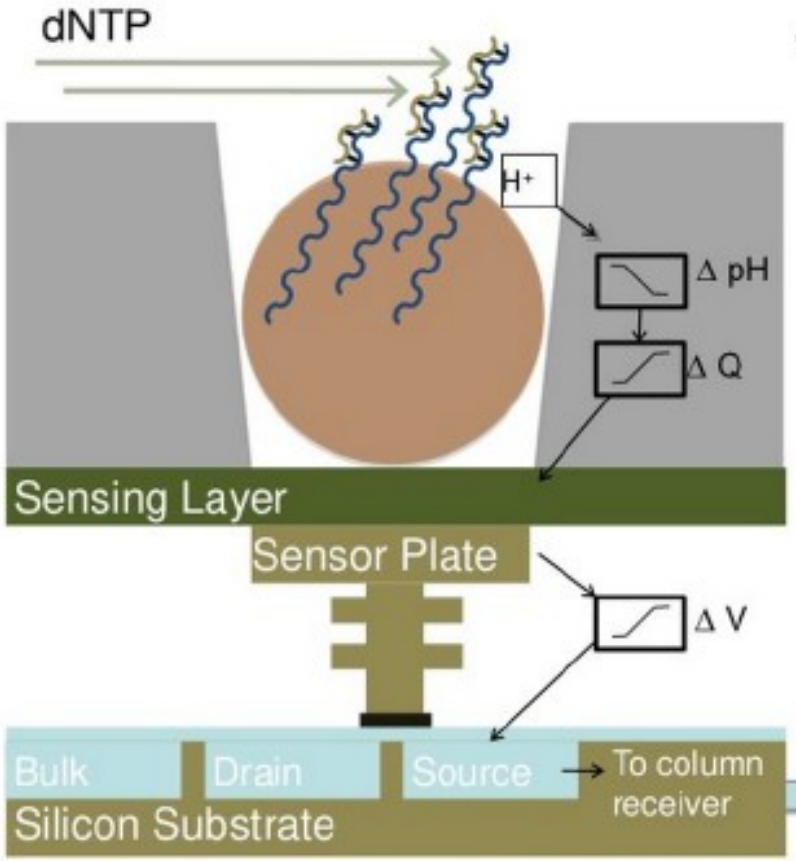
Ion sequencing: ThermoFisher Scientific

Využívá změny pH při syntéze DNA

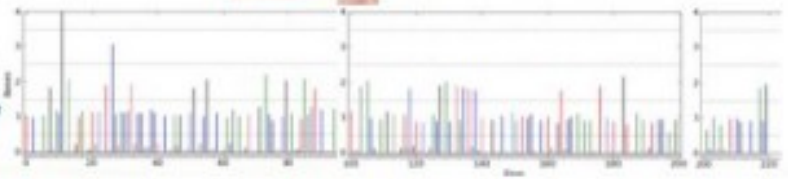
- Ion Semiconductor Sequencing
- Detection of hydrogen ions during the polymerization DNA
- Sequencing occurs in microwells with ion sensors
- No modified nucleotides
- No optics



Ion Torrent



- DNA → Ions → Sequence
  - Nucleotides flow sequentially over Ion semiconductor chip
  - One sensor per well per sequencing reaction
  - Direct detection of natural DNA extension
  - Millions of sequencing reactions per chip
  - Fast cycle time, real time detection



DNBSEQ technology

„DNA Nanoballs (DNB)“ - MGI

BGI Sequencing Services Mass Spec Services Diagnostics & Precision Medicine Resources Company

Unique DNBSEQ™ Sequencing Technology

BGI's Metagenomic Sequencing services are typically executed with proprietary DNBSEQ™ sequencing technology platforms, for great sequencing data at some of the lowest costs in the industry. DNBSEQ™ is a proprietary sequencing technology, first developed by BGI's Complete Genomics subsidiary in Silicon Valley and offers advantages in terms of lower amplification error rates and much lower duplication rates in WGS/WES applications. In addition, studies have shown the index hopping rate in DNBSEQ™ platforms to be much lower when compared to that of other platforms.

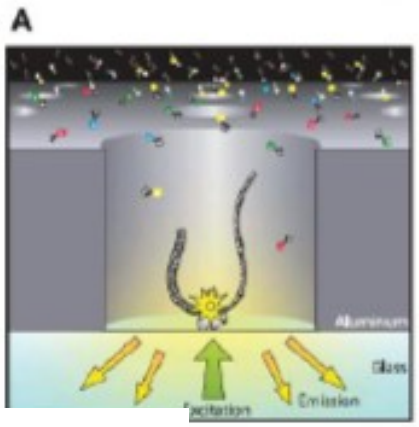
https://www.youtube.com/watch?v=xUVdJN0m38c

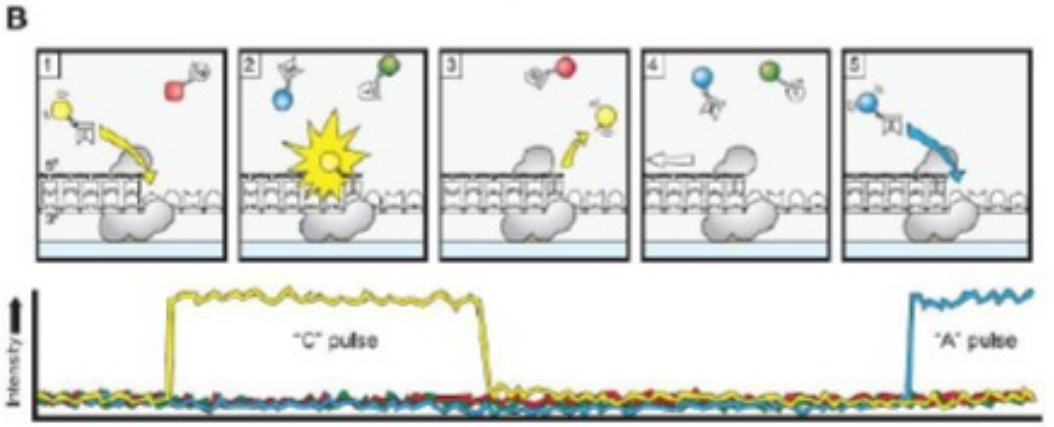
https://en.mgi-tech.com/products/

3rd generation of sequencing (TGS)

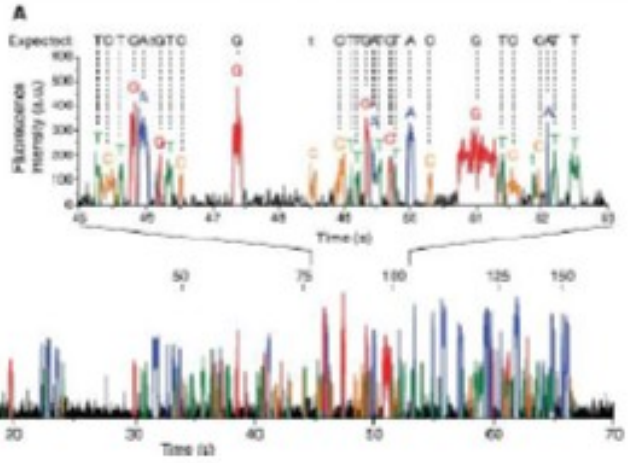
- Dlouhá délka čtení, bez amplifikace
- Přímé čtení oblastí genomu, které je složité analyzovat metodami s krátkými ready
- Rovnoměrné pokrytí genomu – nejsou sensitivní na obsah GC (na rozdíl od platform s krátkými ready)
- (1) PacBio
- (2) Oxford Nanopore

SMRT („single molecule real-time sequencing") – Pacific Biosciences





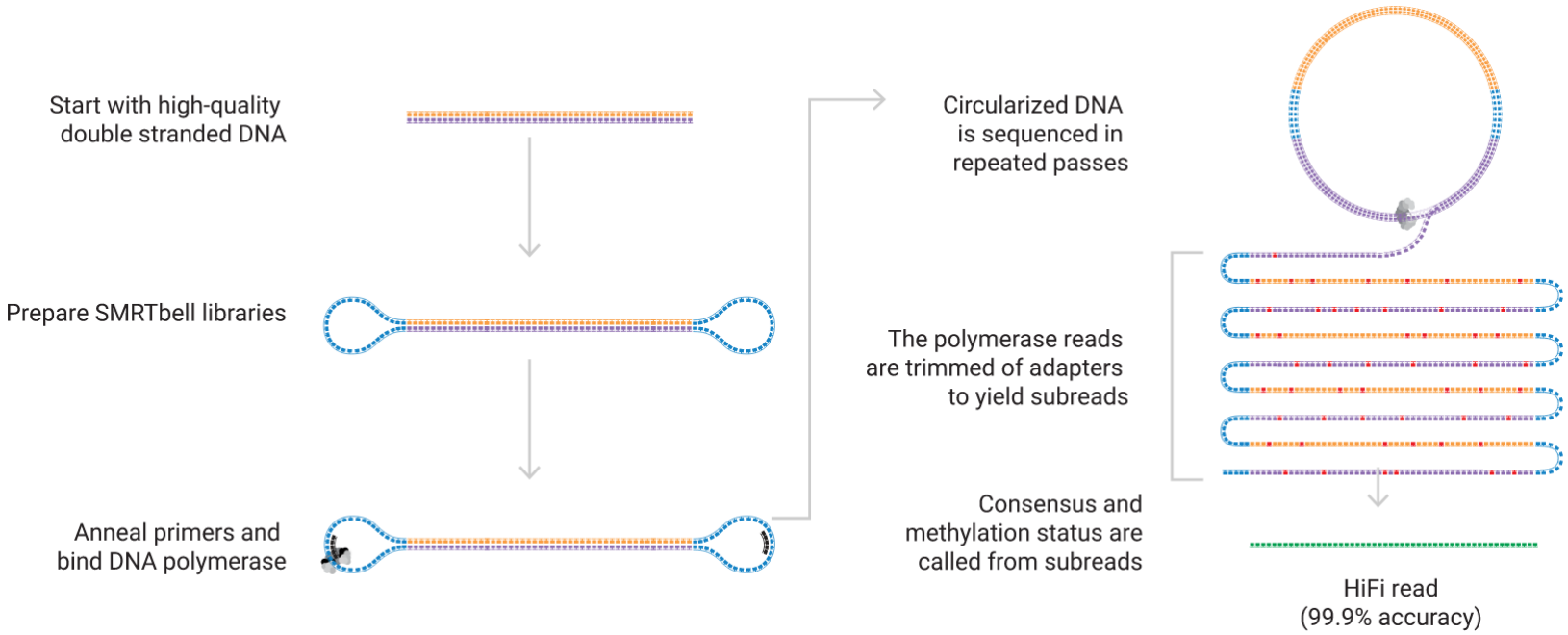
Pacbio RS – raw data



dlouhé čtení (15 kb), hodně chyb

http://www.youtube.com/watch?v=v8p4ph2MAvI

HiFi long-read sequencing



PacBio

dlouhé čtení, velmi přesné

Oxford Nannopore

MinION

512 pores

GridION

5 000 pores

Future Sequencing Technologies

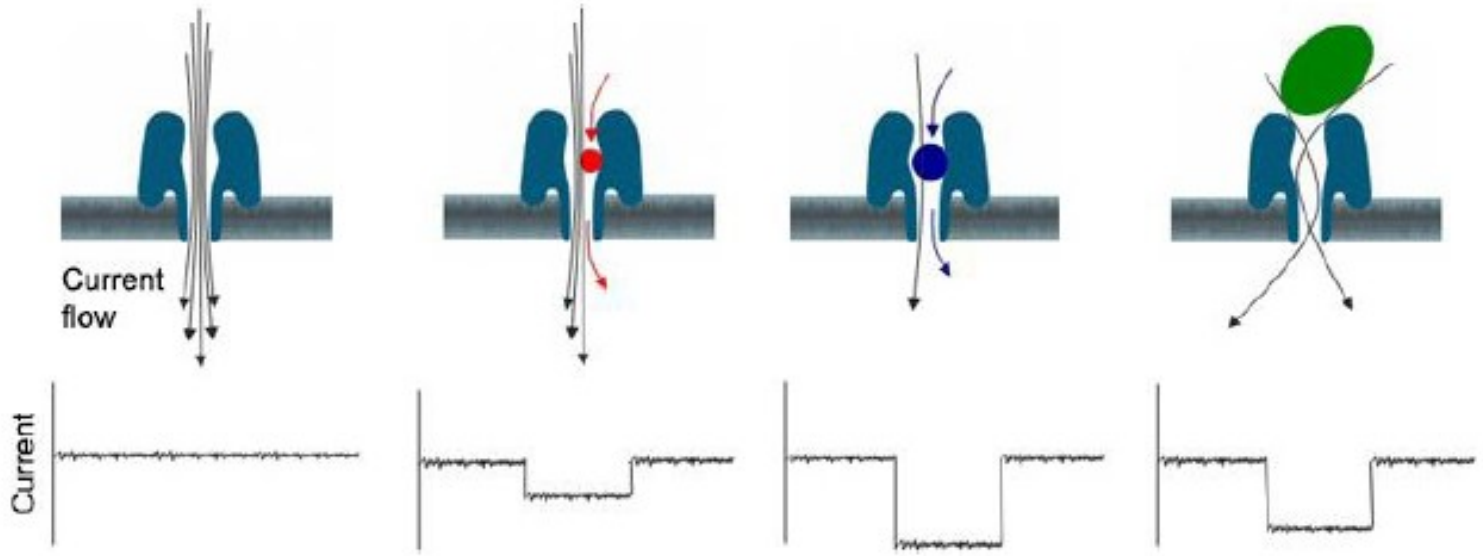
Oxford Nanopore

Nanopore sequencing

up to 50 kb

„Run until sequencing ...“

Princip technologie



https://www.youtube.com/watch?v=CGWZvHIi3i0

Sekvenování přímo v terénu (?)

Ebola outbreak

Quick et al., Nature 2016

Traditional Sequencing vs. Next Generation Sequencing: Data Throughput

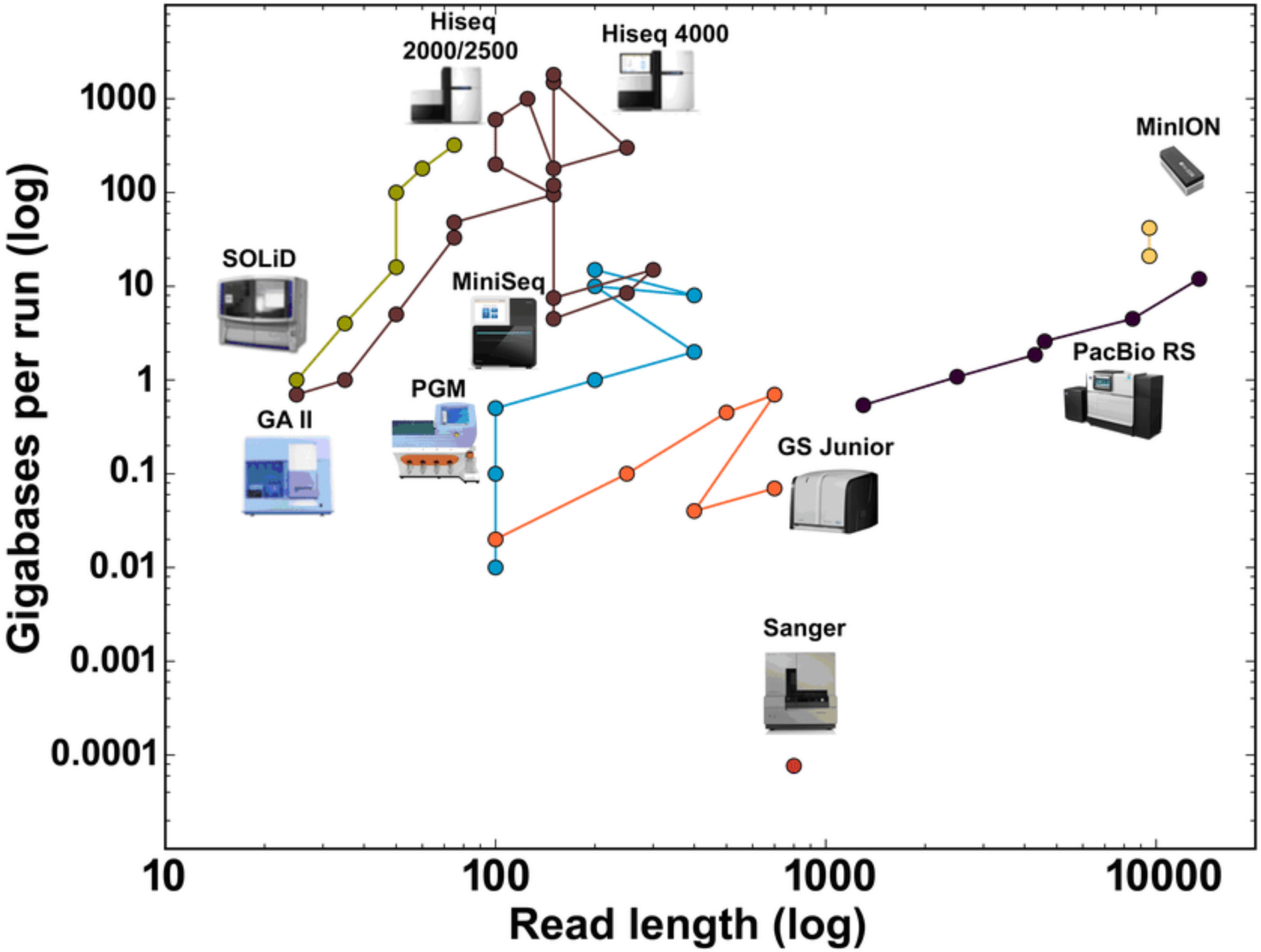
1 x Illumina GAII

Vs.

200+ of 3730xl

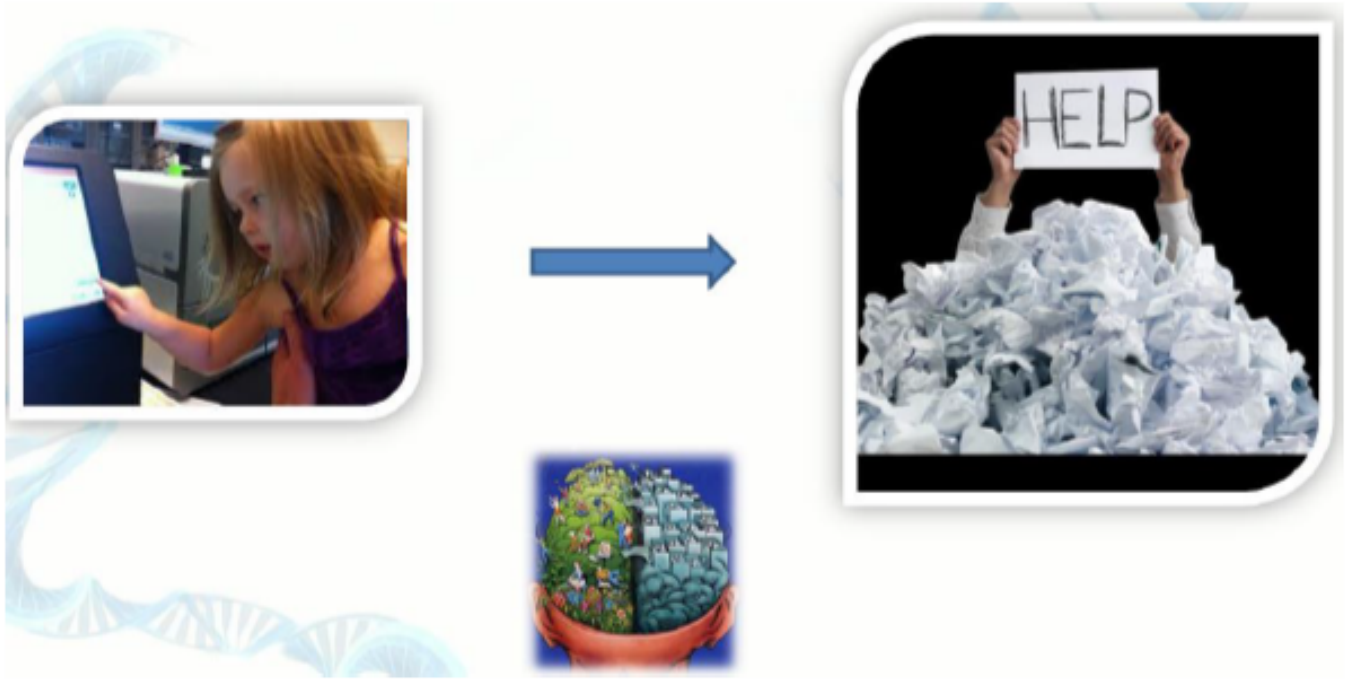
Days vs. Years

The Sequencing Landscape is Changing


Hiseq
2000/2500
Hiseq 4000
MinION
SOLiD
MiniSeq
PacBio RS
PGM
GA II
GS Junior
Sanger
1000
100
10
1
0.1
0.01
0.001
0.0001
Gigabases per run (log)
10
100
1000
10000
Read length (log)

Bioinformatika – největší brzda dalšího rozvoje

Basically, analyzing genomes in interaction with their environment is now feasible and accessible to anyone



Sekvenační strategie

- nutno velmi dobře počítat než se začne sekvenovat
- celkový výtěžek sekvenování = **počet „reads“ * délka „reads“ * coverage**
- zásadně závisí na konkrétním cíli výzkumu a použité technologii

Benchtop Sequencers | Production-Scale Sequencers

iSeq 100

MiniSeq

MiSeq Series

NextSeq 550 Series

NextSeq 1000 & 2000

Popular Applications & Methods	Key Application	Key Application	Key Application	Key Application	Key Application
Large Whole-Genome Sequencing (human, plant, animal)					
Small Whole-Genome Sequencing (microbe, virus)	●	●	●	●	●
Exome & Large Panel Sequencing (enrichment-based)				●	●
Targeted Gene Sequencing (amplicon-based, gene panel)	●	●	●	●	●
Single-Cell Profiling (scRNA-Seq, scDNA-Seq, oligo tagging assays)				●	●
Transcriptome Sequencing (total RNA-Seq, mRNA-Seq, gene expression profiling)				●	●
Targeted Gene Expression Profiling	●	●	●	●	●

Run Time	9.5–19 hrs	4–24 hours	4–55 hours	12–30 hours	11-48 hours
Maximum Output	1.2 Gb	7.5 Gb	15 Gb	120 Gb	330 Gb*
Maximum Reads Per Run	4 million	25 million	25 million †	400 million	1.1 billion*
Maximum Read Length	2 × 150 bp	2 × 150 bp	2 × 300 bp	2 × 150 bp	2 × 150 bp
	Explore iSeq 100	Explore MiniSeq	Compare MiSeq	Compare NextSeq 550	Explore NextSeq 1000 & 2000

NextSeq 550 Series

NextSeq 1000 & 2000

NovaSeq 6000

Popular Applications & Methods	Key Application	Key Application	Key Application
Large Whole-Genome Sequencing (human, plant, animal)			●
Small Whole-Genome Sequencing (microbe, virus)	●	●	●
Exome & Large Panel Sequencing (enrichment-based)	●	●	●
Targeted Gene Sequencing (amplicon-based, gene panel)	●	●	●
Single-Cell Profiling (scRNA-Seq, scDNA-Seq, oligo tagging assays)	●	●	●
Transcriptome Sequencing (total RNA-Seq, mRNA-Seq, gene expression profiling)	●	●	●
Chromatin Analysis (ATAC-Seq, ChIP-Seq)	●	●	●
Methylation Sequencing	●	●	●
Metagenomic Profiling (shotgun metagenomics, metatranscriptomics)	●	●	●
Cell-Free Sequencing & Liquid Biopsy Analysis	●	●	●

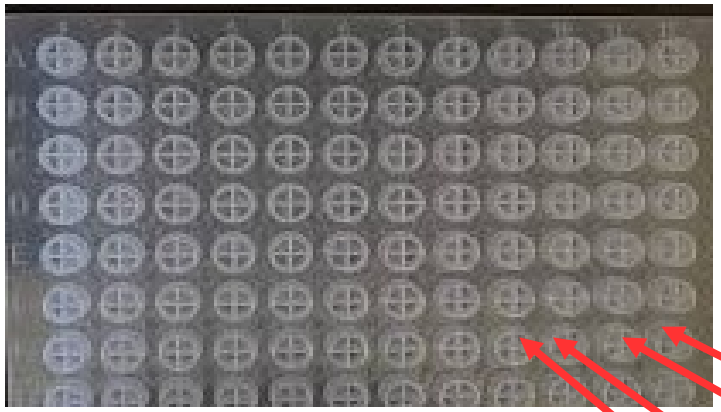
Run Time	12–30 hours	11-48 hours	~13 - 38 hours (dual SP flow cells) ~13–25 hours (dual S1 flow cells) ~16–36 hours (dual S2 flow cells) ~44 hours (dual S4 flow cells)
Maximum Output	120 Gb	360 Gb*	6000 Gb
Maximum Reads Per Run	400 million	1.2 billion*	20 billion
Maximum Read Length	2 × 150 bp	2 × 150 bp	2 × 250**
	Compare NextSeq 550 Request Pricing	Explore NextSeq 1000 & 2000 Request Pricing	Explore NovaSeq 6000 Request Pricing

Sekvenační strategie

…JEDEN VZOREK NA RUN JE MÁLO

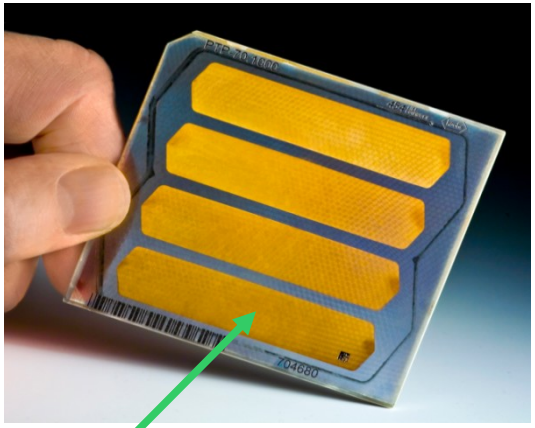
Kapilární sekvenátor

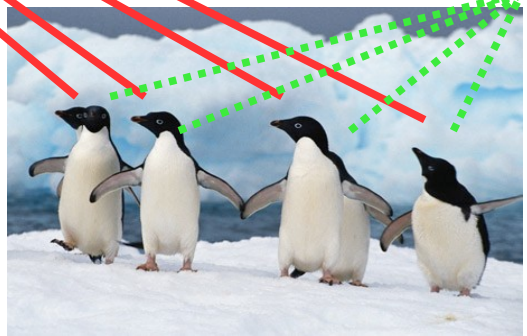
U kapilárních sekvenátorů není problém přiřadit sekvenci k jednotlivým vzorkům na základě pozice na platíčku



Sekvenátor druhé generace

U sekvenátorů druhé generace se najednou sekvenuje pool desítek až stovek vzorků





Sekvenační strategie

…JEDEN VZOREK NA RUN JE MÁLO

Jednotlivé vzorky pro sekvenátory druhé generace se značí tzv. barcody (midy, tagy)

Krátká (obvykle 6-12bp) oligonukleotidová sekvence před primerem (pokud sekvenujeme PCR amplikon) nebo adaptorem (u ostatních genomických knihoven), která je specifická pro daný vzorek (tj. jedince)

Přiřazení identity jednotlivých sekvencí k vzorkům probíhá bioinformaticky

BARCODE PRIMER SEQUENCE

AGCGTAGGTCATTTCGATGCGGTCATGCCTGGATTAAAGCT.....
TTCGTAGGTCATTTCGATGCGGTCATGCCTGGATTAAAGCT.....
TGGGTAGGTCATTTCGATGCGGTCATGCCTGGATTAAAGCT.....
TGCCTAGGTCATTTCGATGCGGTCATGCCTGGATTAAAGCT.....
TGCGCAGGTCATTTCGATGCGGTCATGCCTGGATTAAAGCT.....
TGCGTTGGTCATTTCGATGCGGTCATGCCTGGATTAAAGCT.....

Příklad amplikonů

Sekvenační strategie

AMPLIKONOVÉ SEKVENOVÁNÍ (amplikony kratší než délka readů)

SHOT GUN SEKVENOVÁNÍ

LONG-RANGE PCR + SHOT GUN (amplikony delší než délka readů)

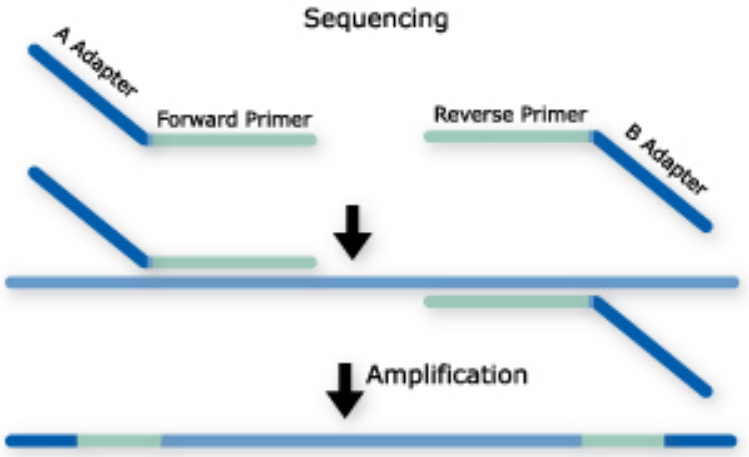
Sekvenační strategie

AMPLIKONOVÉ SEKVENOVÁNÍ

PCR Amplifikace konkrétního úseku daného genomu pomocí specifických primerů (se sekvenačními adaptory)

Následná sekvenace

Taxonomické složení daného vzorku („metabarcoding“), variabilita konkrétních genů apod.



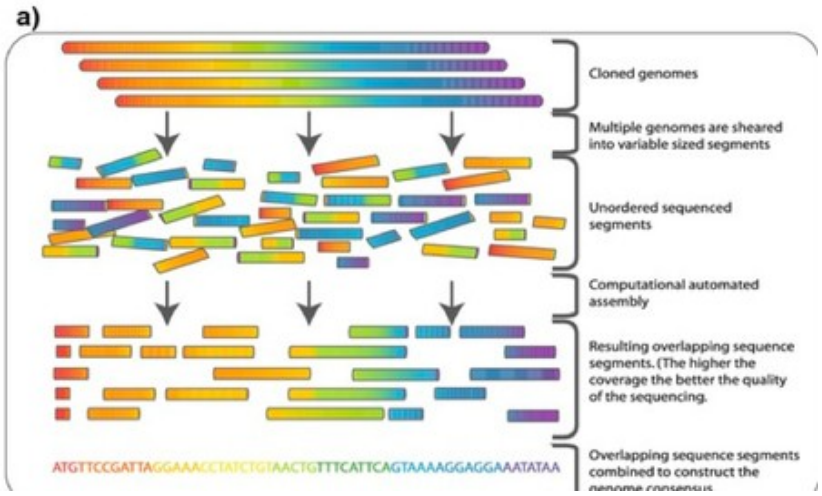
SHOT GUN SEKVENOVÁNÍ

Fragmetace celogenomové DNA (ultrazvukem nebo enzymaticky = „fragmentáza“)

Ligace sekvenačních adaptorů

Následná sekvenace náhodných fragmentů

De novo assembly, resekvenování, transkriptomika, funkční složení daného společenstva



Sekvenační strategie

LONG RANGE PCR + SHOT GUN

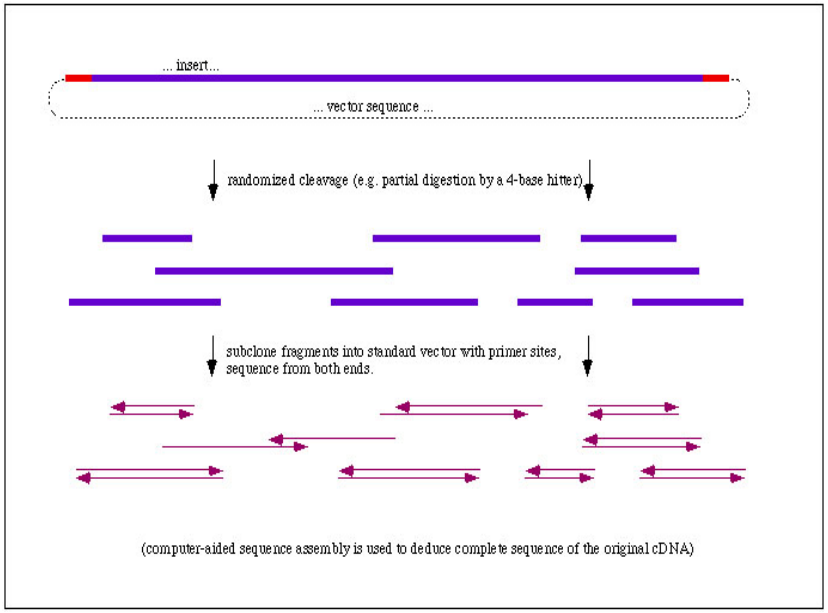
Dlouhé PCR produkty, které nejdou vcelku osekvenovat

Jejich fragmentace

Sekvenování fragmetů

Zpětná rekonstrukce původní sekvence („assembly“)

Použitelné pokud nás zajímá variablita v jednolitém úseku DNA. Např. sekvenace kompletní mitochodrální DNA (3 různé PCR produkty).



Sekvenační strategie

AMPLIKONOVÉ SEKVENOVÁNÍ (amplikony kratší než délka readů)

SHOT GUN SEKVENOVÁNÍ

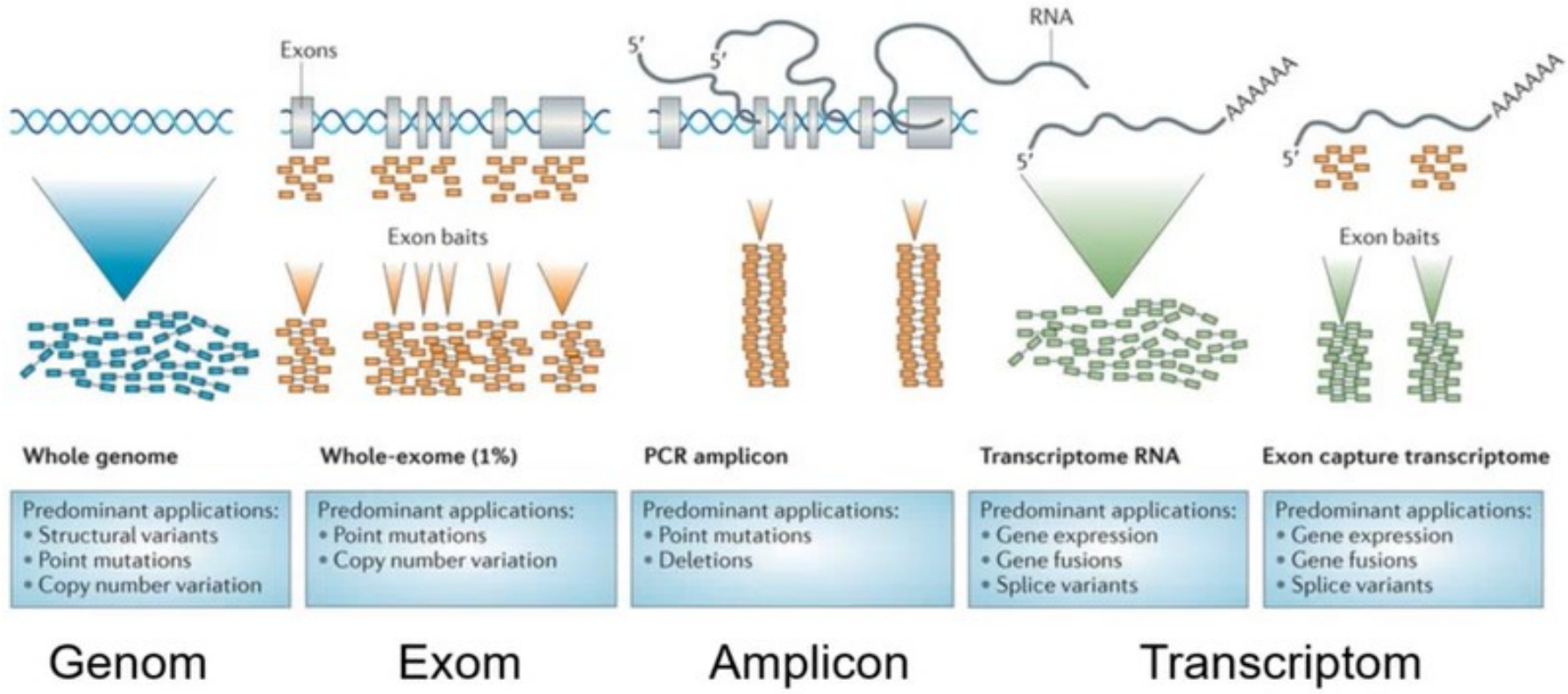
LONG-RANGE PCR + SHOT GUN (amplikony delší než délka readů)

KOMPLETNÍ GENOM (např. virový genom z obohacených vzorků)

REDUKOVANÝ GENOM

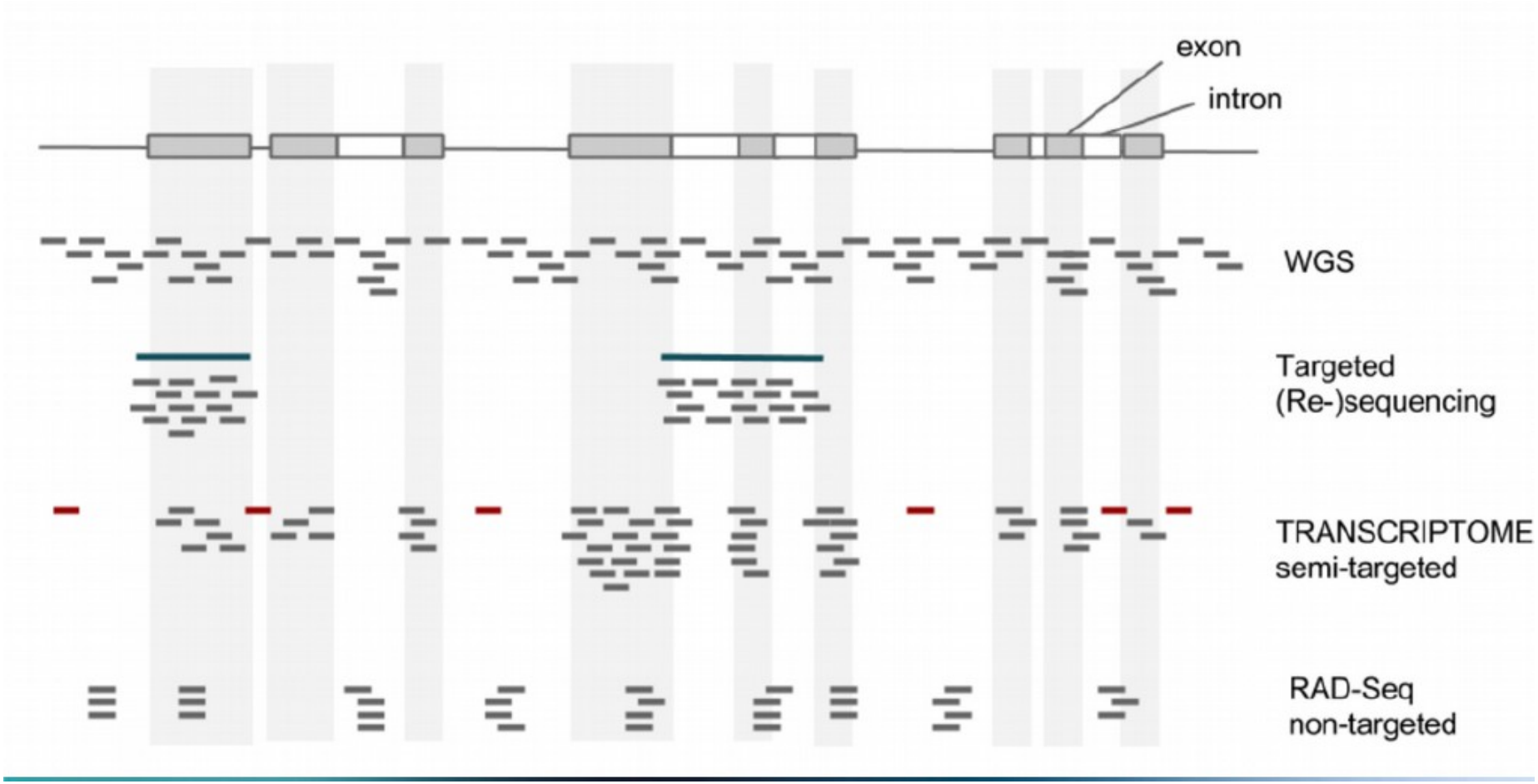
- **PCR** amplicons
- Knihovny obohacené **hybridizací** (development of microsatellite markers, exom, anchored phylogenomics, UCE = ultraconserved elements, etc.)
- Knihovny obohacené o **restrikční fragmenty** (RAD sequencing)
- RNAseq (**transkriptomika** – soubor všech mRNA)

Sekvenační strategie



Simon 2013

Sekvenační strategie



Fuentes-Pardo & Ruzzante 2017

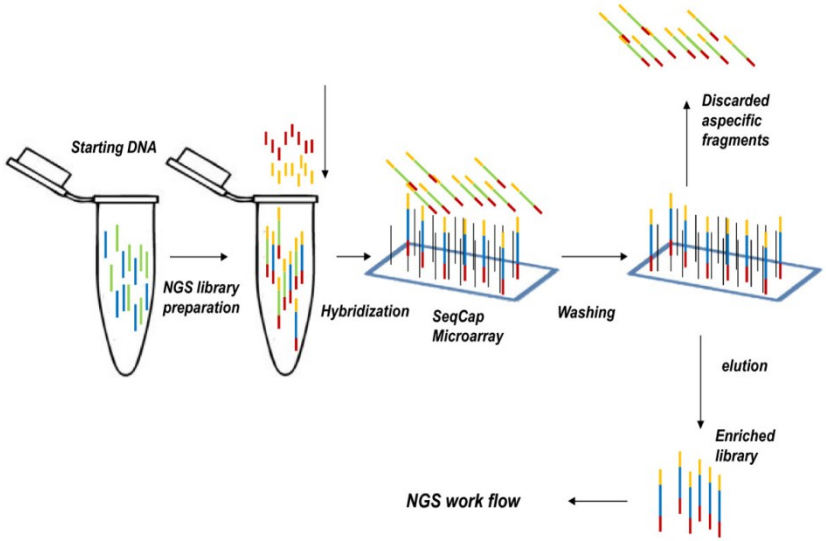
Sekvenační strategie

Obohacené knihovny + shot gun

Separace úseků genomu které nás zajímají na základě jejich hybridizace

Následná sekvenace obohacených knihoven („enrichment by baits“)

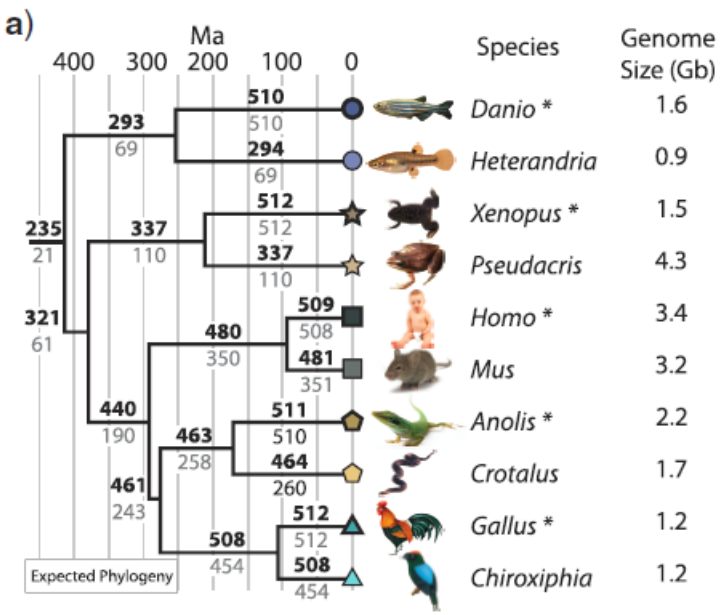
Nové markery (mikrosatelity apod.), kódující oblasti genomu („exom“), „anchored phylogenomics“ apod.

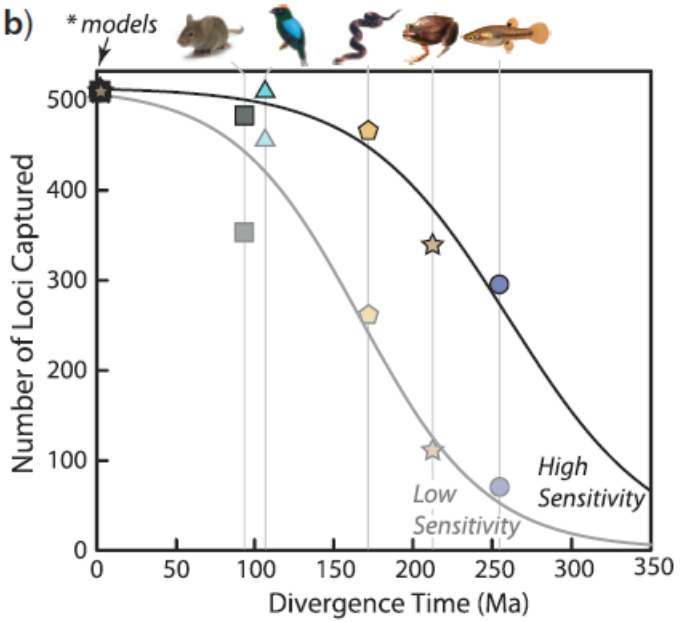


NimbleGen

Anchored phylogenomics

- hundreds of conserved loci
- hybridization enrichement
- u velmi příbuzných taxonů bude málo variability





CENTER FOR ANCHORED PHYLOGENOMICS

ACCELERATING THE RESOLUTION OF LIFE™

Juana, E. Handbook of the Birds of the World Alive (Lynx Edicions, 2015).

LETTER

doi:10.1038/nature15697

A comprehensive phylogeny of birds (Aves) using targeted next-generation DNA sequencing

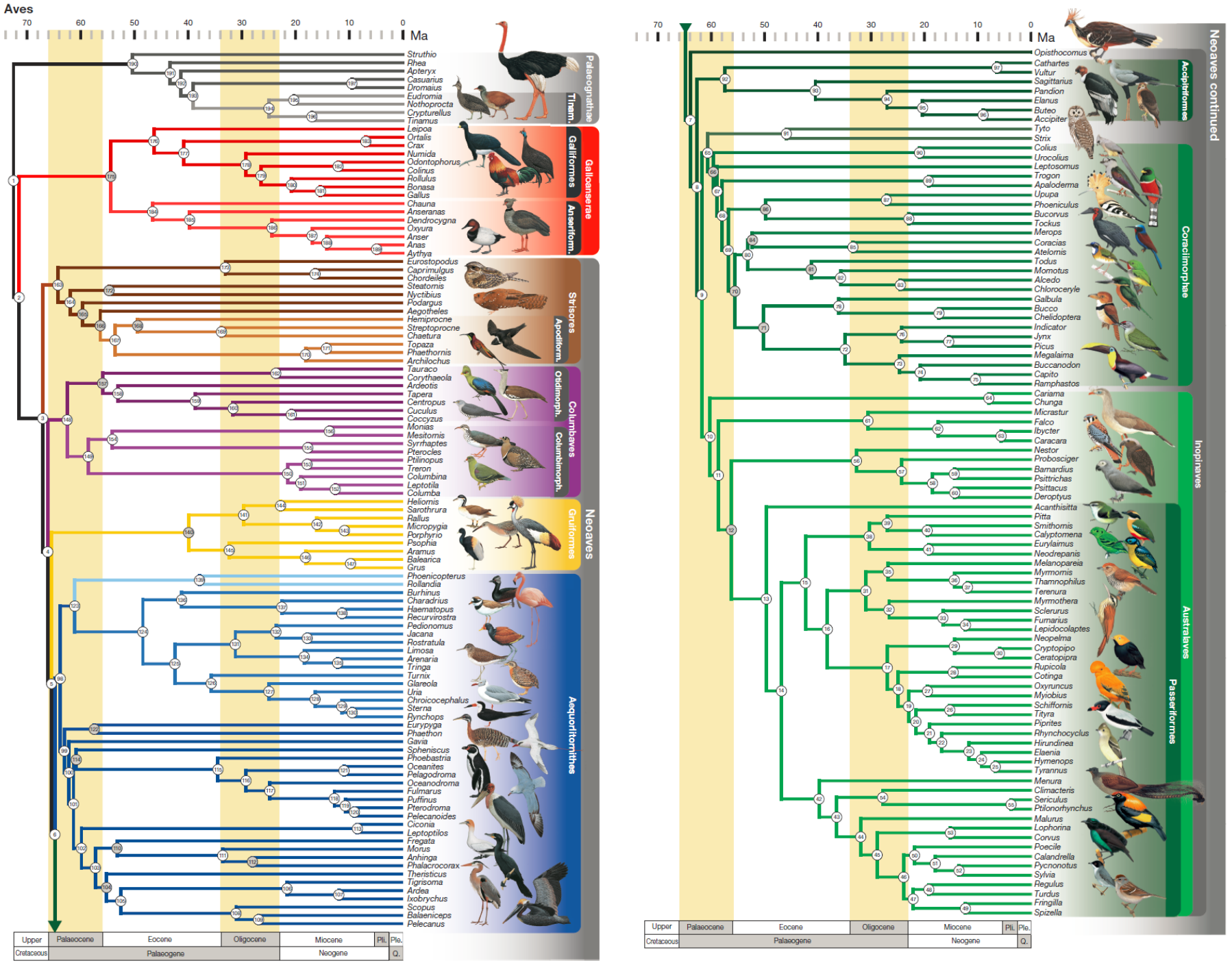
Richard O. Prum[1,2]*, Jacob S. Berv[3]*, Alex Dornburg[1,2,4], Daniel J. Field[2,5], Jeffrey P. Townsend[1,6], Emily Moriarty Lemmon[7] & Alan R. Lemmon[8]

Nature Paper Resolves Bird Tree of Life

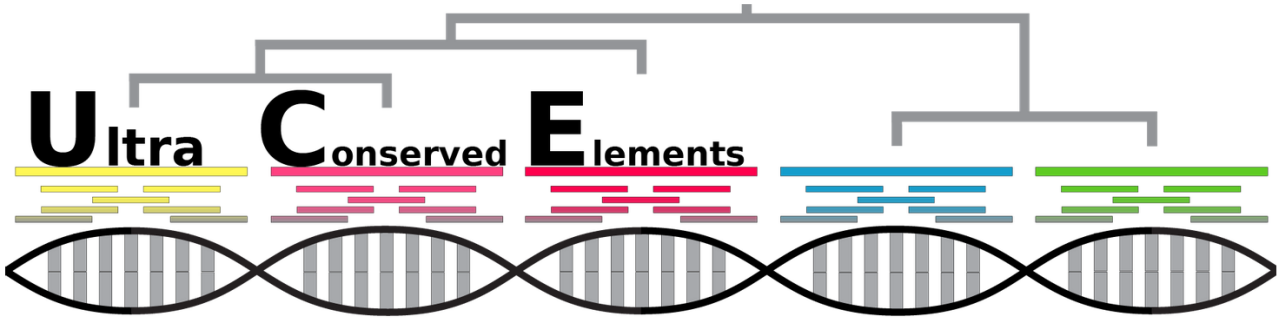
Posted on October 6, 2015 by ameer

October 2015

198 species
259 nuclear loci (ca 1500 bp each)
> 390 000 bp


Aves
70
60
50
40
30
20
10
0
Ma
Struthio
Rhea
Apteryx
Casuarius
Dromaius
Eudromia
Nothoprocta
Crypturellus
Tinamus
Palaeognathae
Tinam.
Leipoa
Ortalis
Crax
Numida
Odontophorus
Colinus
Rollulus
Bonasa
Gallus
Galliformes
Galloanserae
Chauna
Anseranas
Dendrocygna
Oxyura
Anser
Anas
Aythya
Anseriform.
Eurostopodus
Caprimulgus
Chordeiles
Steatornis
Nyctibius
Podargus
Aegotheles
Hemiprocne
Streptoprocne
Chaetura
Topaza
Phaethornis
Archilochus
Apodiform.
Strisores
Tauraco
Coruthaeola
Ardeotis
Tapera
Centropus
Cuculus
Coccyzus
Otidimorph.
Monias
Mesitornis
Syrrhaptes
Pterocles
Ptilinopus
Treron
Columbina
Leptotila
Columba
Columbimorph.
Columbaves
Heliornis
Sarothrura
Rallus
Micropygia
Porphyrio
Psophia
Aramus
Balearica
Grus
Gruiformes
Neoaves
Phoenicopterus
Rollandia
Burhinus
Charadrius
Haematopus
Recurvirostra
Pedionomus
Jacana
Rostratula
Limosa
Arenaria
Tringa
Turnix
Glareola
Uria
Chroicocephalus
Sterna
Rynchops
Eurypyga
Phaethon
Gavia
Spheniscus
Phoebastria
Oceanites
Pelagodroma
Oceanodroma
Fulmarus
Puffinus
Pterodroma
Pelecanoides
Ciconia
Leptoptilos
Fregata
Morus
Anhinga
Phalacrocorax
Theristicus
Tigrisoma
Ardea
Ixobrychus
Scopus
Balaeniceps
Pelecanus
Aequorlitornithes
Upper Cretaceous
Palaeocene
Eocene
Oligocene
Miocene
Plio.
Ple.
Palaeogene
Neogene
Q.
Neoaves continued
Opisthocomus
Cathartes
Vultur
Sagittarius
Pandion
Elanus
Buteo
Accipiter
Accipitriformes
Tyto
Strix
Colius
Urocolius
Leptosomus
Trogon
Apaloderma
Upupa
Phoeniculus
Bucorvus
Tockus
Merops
Coracias
Atelornis
Todus
Momotus
Alcedo
Chloroceryle
Galbula
Bucco
Chelidoptera
Indicator
Jynx
Picus
Megalaima
Buccanodon
Capito
Ramphastos
Coraciimorphae
Cariama
Chunga
Micrastur
Falco
Ibycter
Caracara
Nestor
Probosciger
Barnardius
Psittrichas
Psittacus
Deroptyus
Inopinaves
Acanthisitta
Pitta
Smithornis
Calyptomena
Eurylaimus
Neodrepanis
Melanopareia
Myrmornis
Thamnophilus
Terenura
Myrmothera
Sclerurus
Furnarius
Lepidocolaptes
Neopelma
Cryptopipo
Ceratopipra
Rupicola
Cotinga
Oxyruncus
Myiobius
Schiffornis
Tityra
Piprites
Rhynchocyclus
Hirundinea
Elaenia
Hymenops
Tyrannus
Menura
Climacteris
Sericulus
Ptilonorhynchus
Malurus
Lophorina
Corvus
Poecile
Calandrella
Pycnonotus
Sylvia
Regulus
Turdus
Fringilla
Spizella
Australaves
Passeriformes

https://www.ultraconserved.org/



What are UCEs?

As their name implies, ultraconserved elements (UCEs) are highly conserved regions of organismal genomes shared among evolutionary distant taxa - for instance, birds share many UCEs with humans. UCEs were first described in a wonderful manuscript by Gil Bejerano et al. (2004) from David Haussler's group and subsequently identified in several classes of organisms outside the group of original taxa (Siepel et al. 2005) used to identify these genomic elements. The 27-way vertebrate genome alignment (Miller et al. 2007) identified additional regions of high conservation.

Why are UCEs useful?

We have discovered (see Citations) that we can collect data from UCEs and the DNA adjacent to UCE locations (flanking DNA), and that these data are useful for reconstructing the evolutionary history and population-level relationships of many organisms. Because UCEs are conserved across disparate taxa, UCEs are also universal genetic markers in the sense that the locations (or loci) that we can target in humans are identical, in many cases, to the loci that we can target in ducks or snakes or lizards.

What do UCEs do?

That's an extremely good question, and one to which we do not entirely know the answer (Dermitzakis et al. 2005). UCEs have been associated with gene regulation (Pennachio et al. 2006) and development (Sandelin et al. 2004, Woolfe et al. 2004) and we generally assume that UCEs must be important by the very nature of their near-universal conservation across extremely divergent taxa. However, gene knockouts of UCE loci in mice resulted in viable, fertile offspring (Ahituv et al. 2007), suggesting that their role in the biology of the genome may be cryptic.

Arachnida
14,799 baits for 1,120 UCEs
(Arachnida 1.1Kv1)

Described as part of Faircloth 2017. First use as part of Starret et al. 2017.

Get 1.1Kv1 bait design for Arachnida »

Diptera
31,328 baits for 2,711 UCEs
(Diptera 2.7Kv1)

Described as part of Faircloth 2017. First use has not been published, yet.

Get 2.7Kv1 bait design for Diptera »

Hymenoptera (ver. 1)
2,749 baits for 1,510 UCEs
(Hymenoptera 1.5Kv1)

Described as part of Faircloth et al. 2015. First used as part of Faircloth et al. 2015.

Get 1.5Kv1 bait design for Hymenoptera »

Anthozoa
16,306 baits for 720 UCEs and 1,071 exons
(Anthozoa 1.7Kv1)

Described as part of Quattrini et al. 2017. First use as part of Quattrini et al. 2017.

Get 1.5Kv1 bait design for Hymenoptera »

Coleoptera
13,674 baits for 1,172 UCEs
(Coleoptera 1.1Kv1)

Described as part of Faircloth 2017. First use as part of Baca et al. 2017.

Get 1.1Kv1 bait design for Coleoptera »

Hemiptera
40,207 baits for 2,731 UCEs
(Hemiptera 2.7Kv1)

Described as part of Faircloth 2017. First use has not been published, yet.

Get 2.7Kv1 bait design for Hemiptera »

Hymenoptera (ver. 2)
31,829 baits for 2,590 UCEs
(Hymenoptera 2.5Kv2)

Described as part of Branstetter et al. 2017. First use as part of Branstetter et al. 2017.

Get 2.5Kv2 bait design for Hymenoptera »

Tetrapod probe sets.

Below are several probe designs that we have used to study relationships among amniotes/tetrapods (e.g. Crawford et al. 2012, McCormack et al. 2013). We are constantly evaluating the utility of given probe sets and probe designs, in addition to expanding the number of UCE loci we are targeting. We have several larger bait sets in the works, and we are also working on optimizing probe sets based on their capture success, phylogenetic utility, etc. Please check back for updates.

You can now buy each of these probe sets direct from Arbor Biosciences in the form of a capture kit. Arbor Biosciences has even made **a discounted "pilot" sized kit** available for labs who want to do some test enrichments.

Order enrichment kits from Arbor Biosciences »

2,560 baits for 2,386 UCEs
(Tetrapods-UCE-2.5Kv1)

Described as part of Faircloth et al. 2012. First use as part of Faircloth et al. 2012.

Get 2.5Kv1 bait design for Tetrapods »

5,472 baits for 5,060 UCEs
(Tetrapods-UCE-5Kv1)

Described in Faircloth et al. 2012 and first use as part of Keping et al. 2014.

Get 5Kv1 bait design for Tetrapods »

100 USD/sample

Fish probe sets.

Below are two bait set designs that we have used (1) to understand relationships among the early diverging **teleosts** (Faircloth et al. 2013) and (2) to study the diversification of Acanthomorphs (Alfaro et al. 2018) We are currently working on several other bait set designs, as well as optimizing existing bait sets based on their capture success, phylogenetic utility, etc. Please check back for updates.

You can now buy both probe sets directly from Arbor Biosciences in the form of a capture kit. Arbor Biosciences has even made **a discounted "pilot" sized kit** available for labs who want to do some test enrichments.

Order enrichment kits from Arbor Biosciences »

Actinopterygians
2,001 baits for 500 UCEs
(Actinopterygians 0.5Kv1)

Described as part of Faircloth et al. 2013. First use as part of Faircloth et al. 2013.

Get 0.5Kv1 bait design for Actinopterygians »

Acanthomorphs
2,628 baits for 1,314 UCEs
(Acanthomorphs 1Kv1)

Described as part of Alfaro et al. 2013. First used as part of McGee et al. 2016.

Get 1Kv1 bait design for Acanthomorphs »

Sekvenační strategie

Sekvenování podél restrikčních míst
(Enriched libraries by restriction enzymes)

Fragmetace celogenomové DNA pomocí restrikčních enzymů

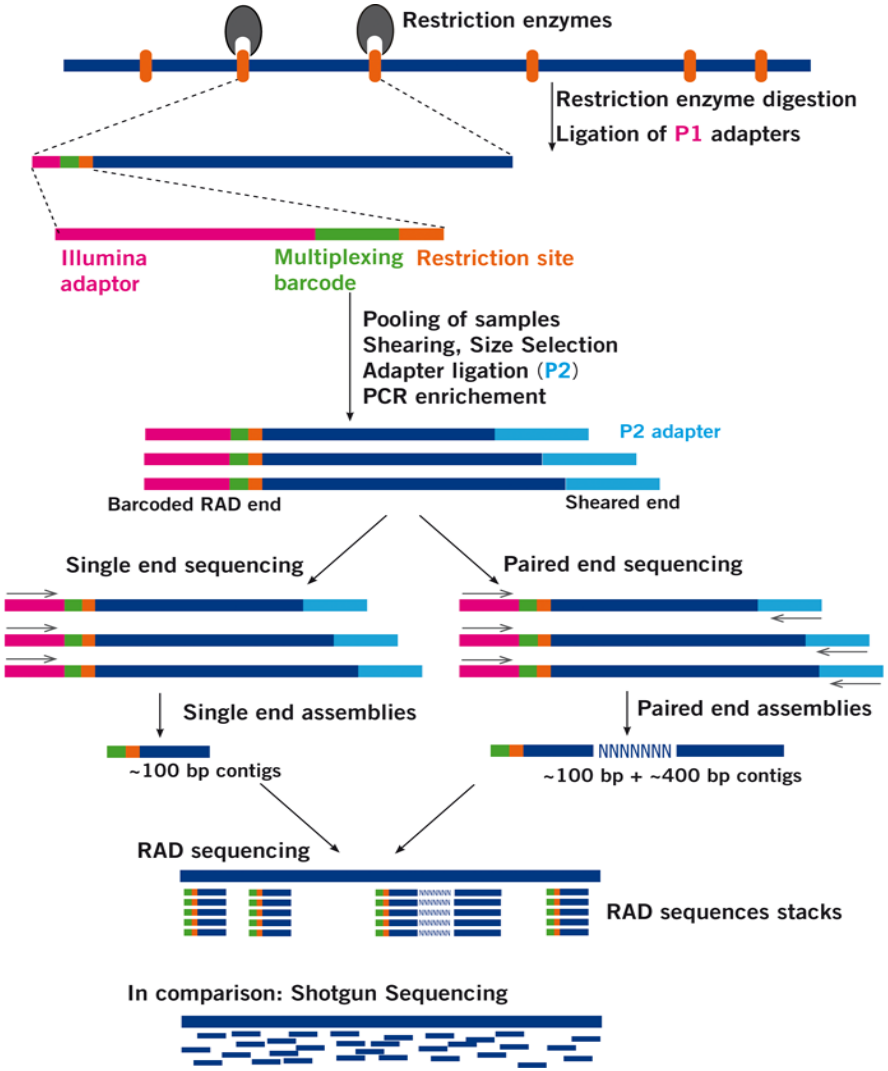
Ligace sekvenačních adaptorů na výsledné fragmenty

Následná sekvenace podél restrikčních míst

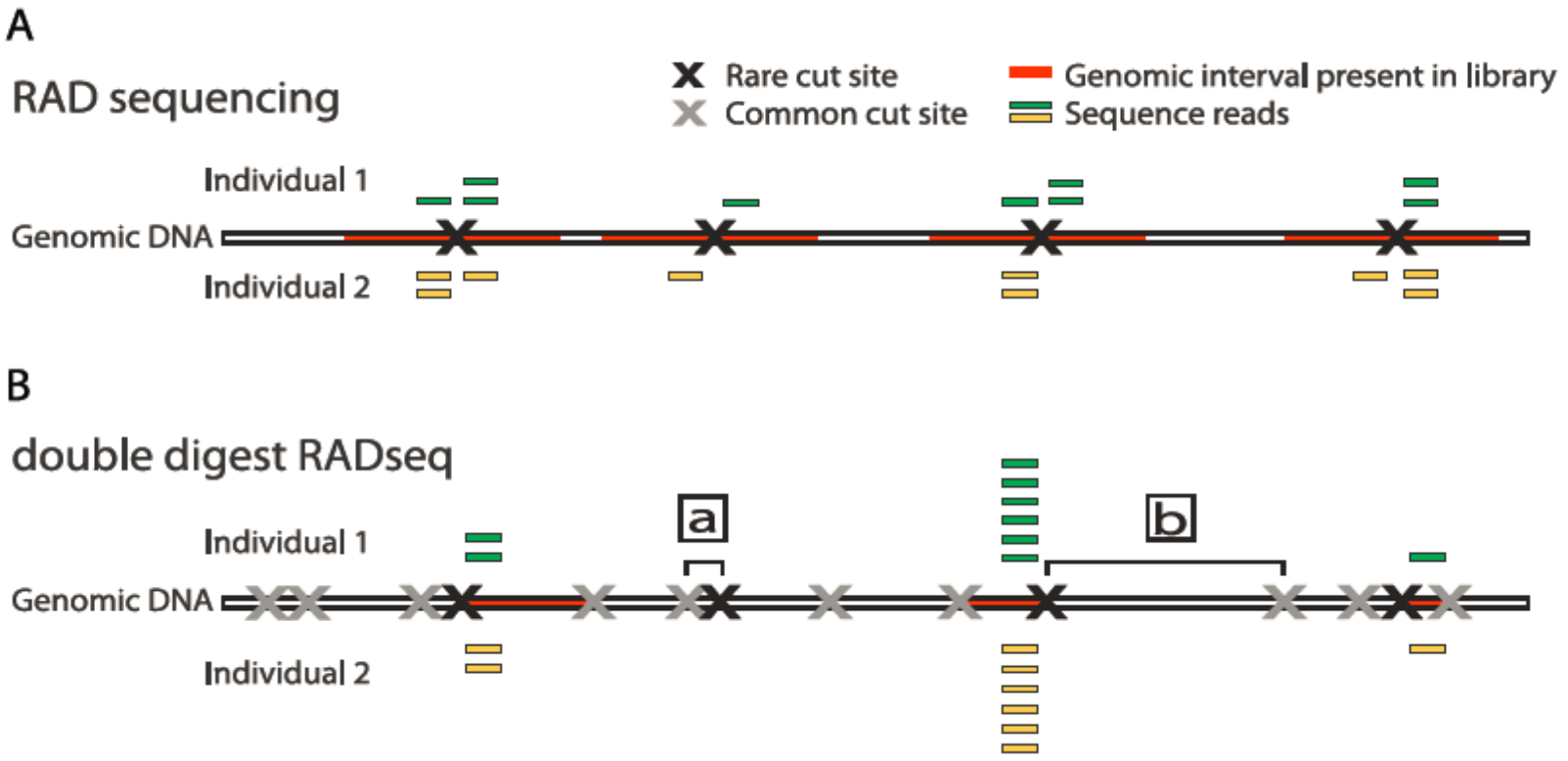
Celogenomové scany genetické variablility

Hledání SNPs, populační genomika (např. RAD-SEQ) apod.

RAD = „**R**estriction sites **A**ssociated **D**na“



RAD vs. ddRAD



Peterson et al PLOS One 2012

Postup ddRAD analýzy

1 – Štěpení

2 – Size-selekce na magnetických kuličkách

3 – Ligace adaptorů

4 – Přečištění na magnetických kuličkách

5 – PCR (namnožení RAD fragmentů, primery s barcody)

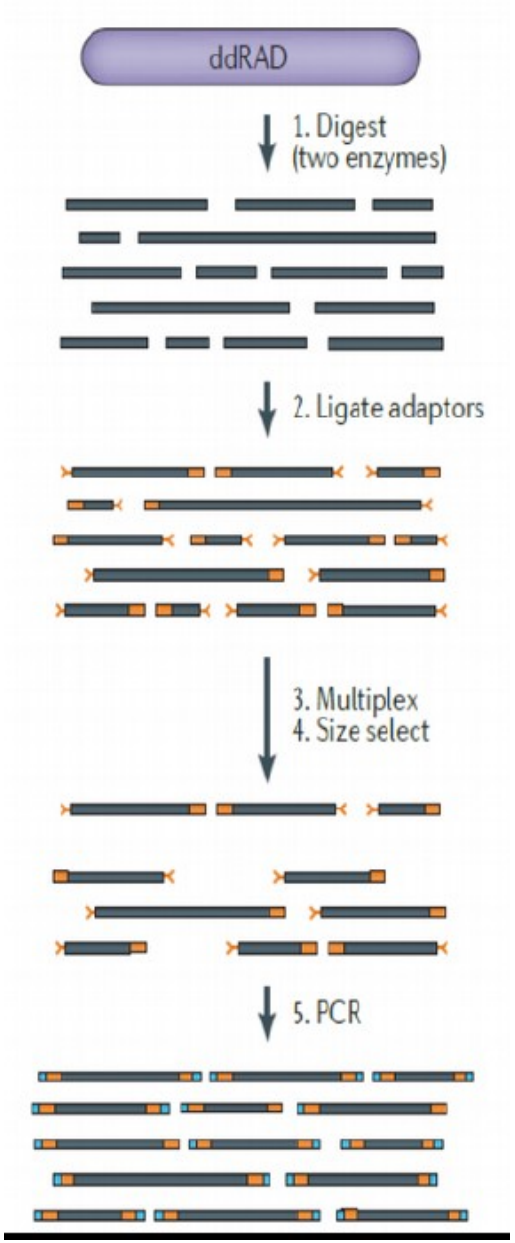
6 – Pooling (ve stejných koncentracích pro různé vzorky = multiplexování)

7 – Size-selekce (Pippin prep) a kontrola na Bioanalyser

8 – qPCR (kvantifikace knihovny)

9 – High-throughput sekvenování

(podle Adapterama)



(podle Peterson 2012)

ddRAD library

o tuto sekvenci nám jde!

DATA ANALYSIS

Complete adapter+insert(EcoRI and MspI) = SEQUENCING LIBRARY :

```
5'-AATGATACGGCGACCACCGAGATCTACACACCGACAAACACTCTTTCCCTACACGACGCTCTTCCGATCTCATCCAAAT          CGAGATCGGAAGAGCACACGTCTGAACTCCAGTCACAGGTCACTATCTCGTATGCCGTCTTCTGCTTG-3'
3'-TTACTATGCCGCTGGTGGCTCTAGATGTGTGGCTGTTTGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGAGTAGGTTTA          GCTCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTGTCCAGTGATAGAGCATACGGCAGAAGACGAAC-5'
                                            <-----                                    -------> <-----                                                  ----->
```

Illumina adapter

sekvenační primery

Illumina adapter

```
ACCGACAA     P5 Index example
TCCAGTGA     P7 Index example
CATCCA       Inline barcode
   (can be preceeded by 1-2 bp to increase complexity)
AATT         RE overhang
CG           RE overhang
----->       R1 sequence
<------      R2 sequence
----->       I1 sequence
<------      I2 sequence
XXXXXX       insert – the RE fragment
```

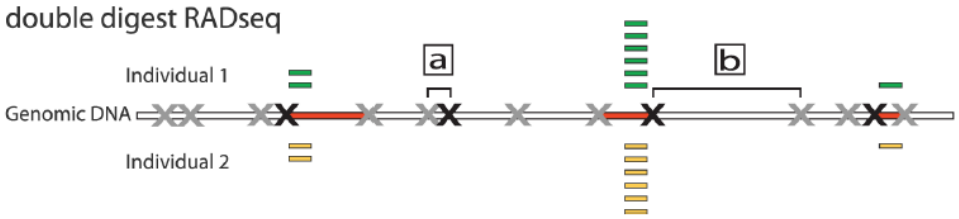
identifikace vzorku (jedince)

Analýza dat z ddRADseq

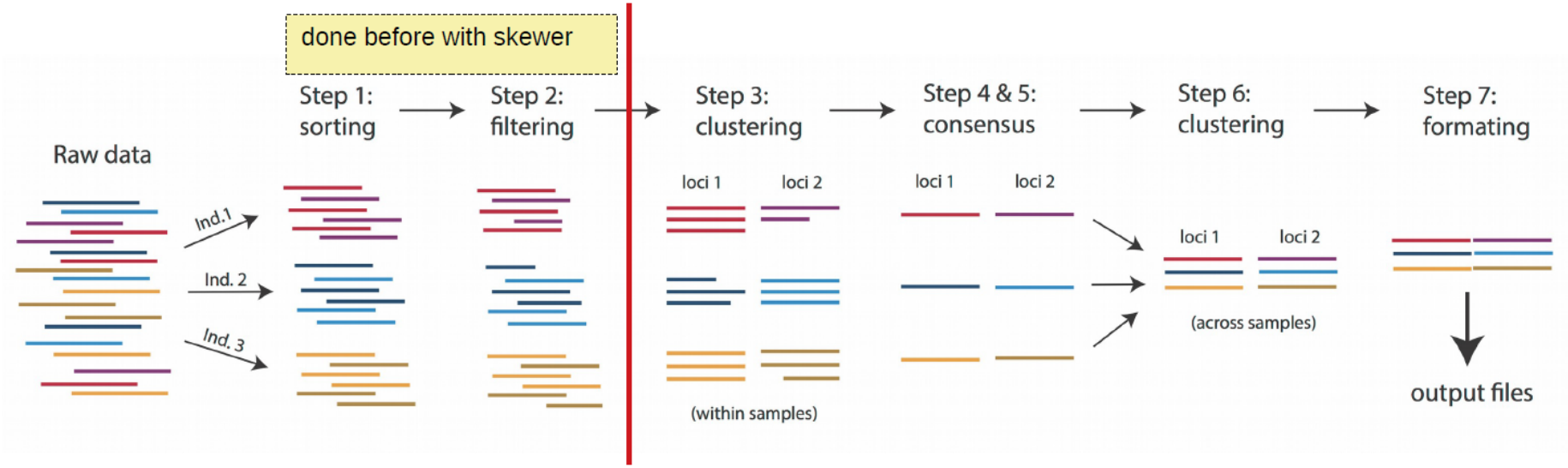
DATA ANALYSIS

B) DEFINE LOCI AND FIND VARIABILITY

SOFTWARE AVAILABLE:
Stacks, dDocent, iPyrad ….



iPyrad denovo assembly workflow (no reference genome)



program Skewer

program iPyrad

- stovky až desítky tisíc lokusů

Aplikace

1. Celogenomové sekvenování de novo

2. Celogenomové resekvenování

3. Sekvenování amplikonů (PCR produktů)

4. Další aplikace – např. hledání klasických DNA markerů (mikrosatelity, SNPs)

1. Celogenomové sekvenování de novo

Problém: **KRÁTKÝ READ LENGTH**

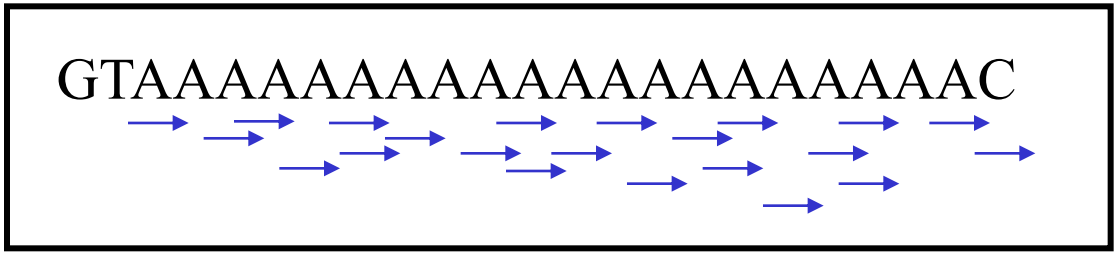
- max 300bp u Illumina, **35-75bp** Solid

  vs **800-1000bp** Sanger

- nové technologie (PacBio, Nannopore) už s tím takový problém nemají -> celé genomy se sekvenují kombinací přístupů s krátkými a dlouhými ready

→ Uspořádání (assembly) ještě stále může být problém z hlediska výpočetní kapacity

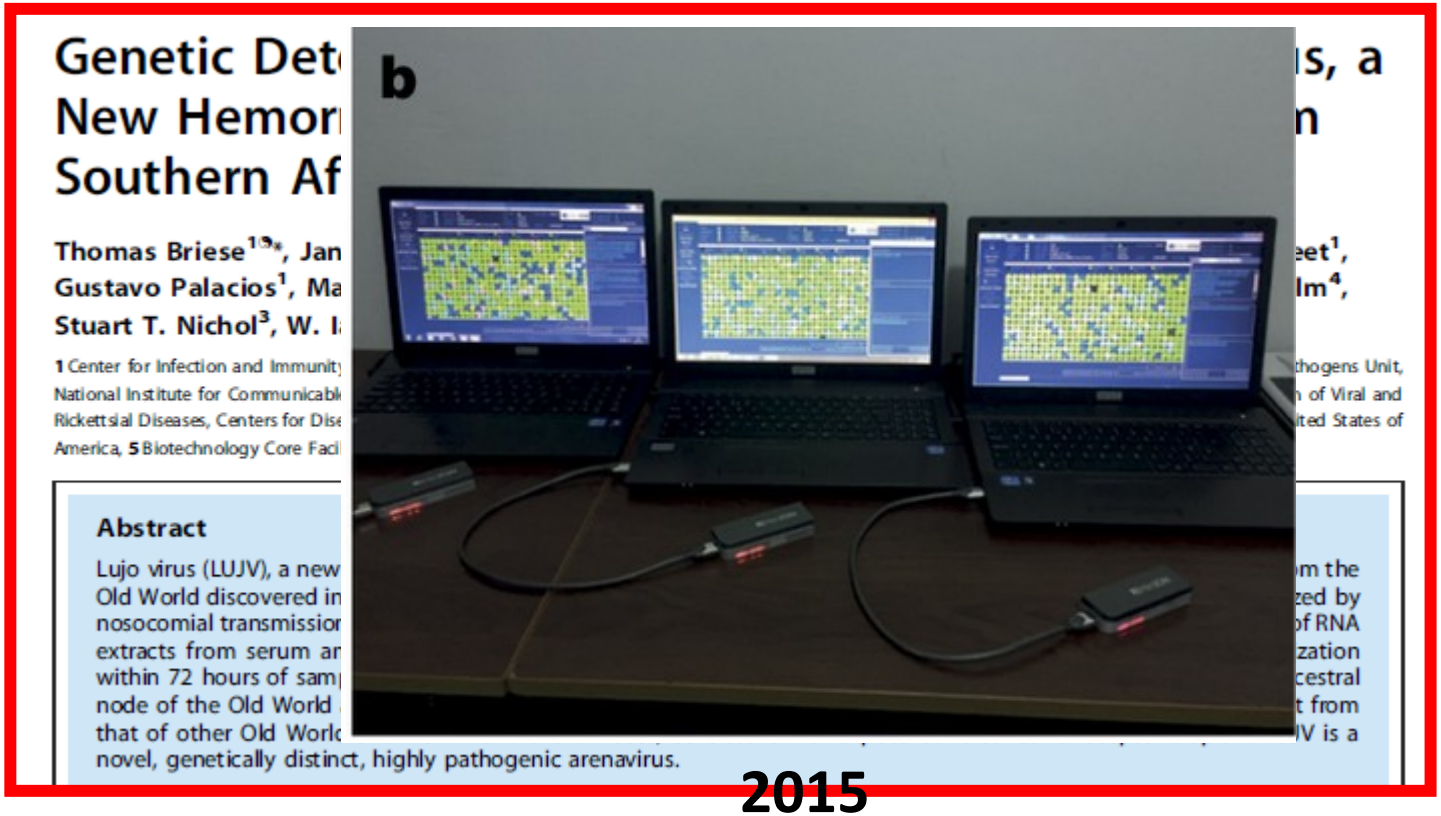
!!!!! REPETITIVNÍ OBLASTI delší než read length !!!!!



Zvláště komplexní eukaryotické genomy – úseky souvislých oblastí přerušených mezerami („contigs")

1. Celogenomové sekvenování de novo

- získání kompletní uspořádané sekvence celých velkých eukaryotních genomů pomocí next-generation sequencing de novo byl donedávna problém (dnes se kombinují dlouhé a krátké ready)
- viry, prokaryota, malá eukaryota, mitochondrie/plastidy/plasmidy – rutinní screening („pathogen hunting“)



2009

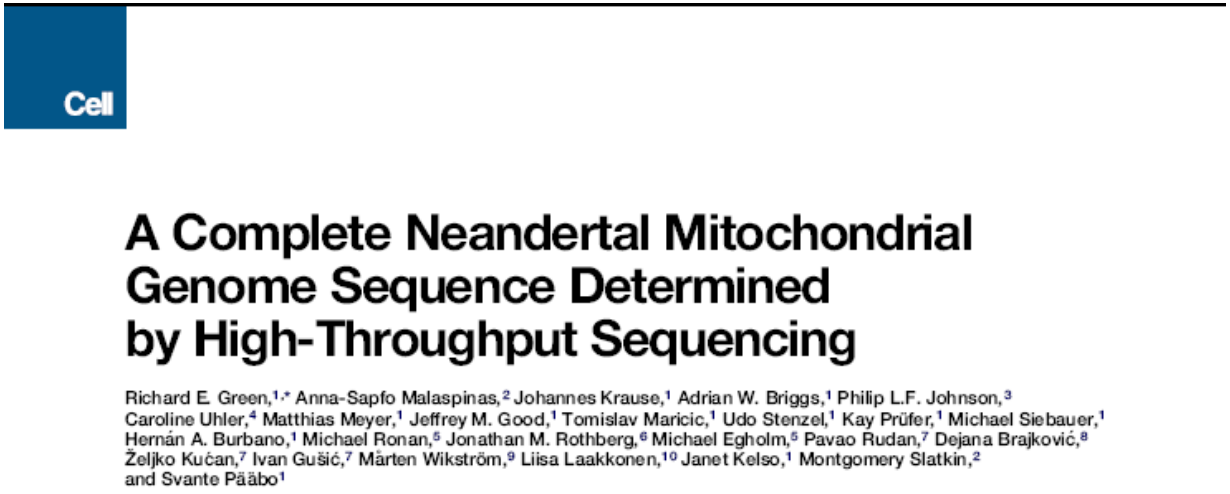
2015

2. Celogenomové resekvenování

- podobné problémy jako u de novo, ale méně

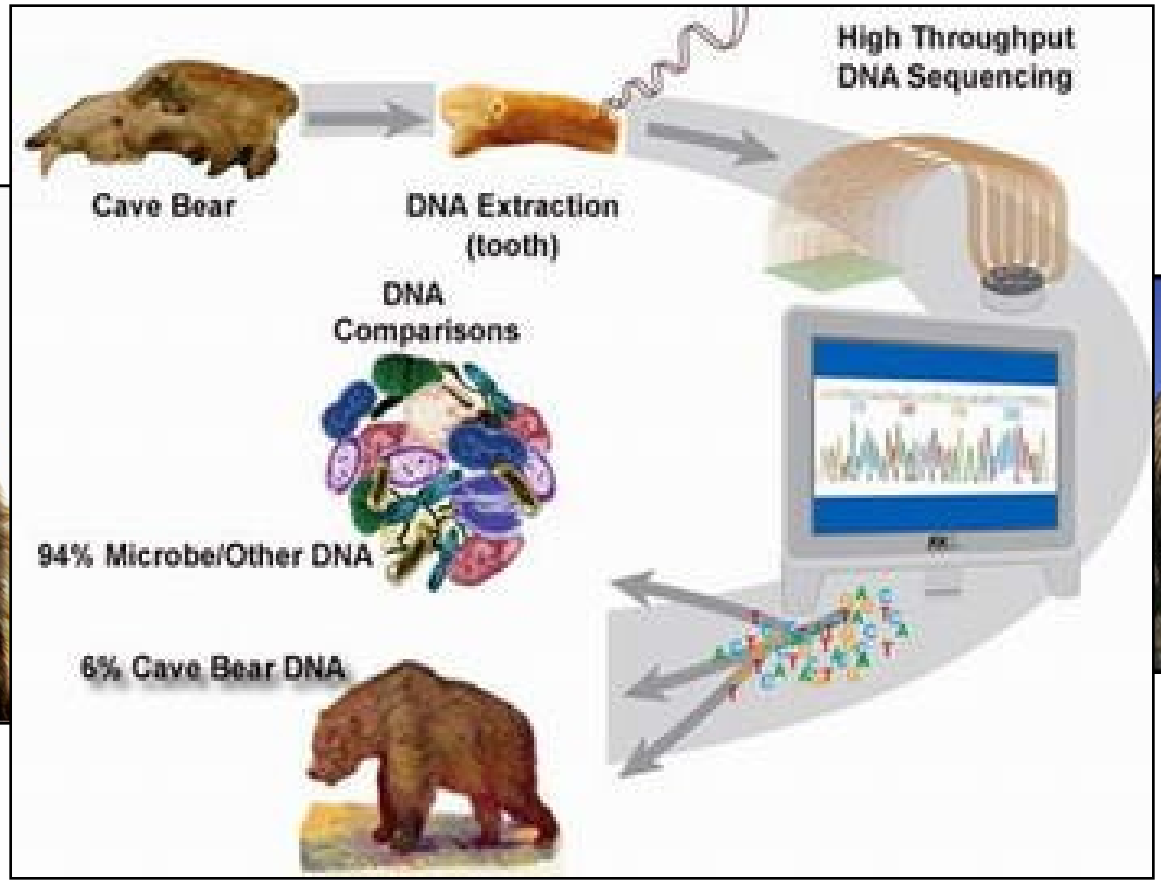
KOMPARATIVNÍ GENOMIKA
- viry, prokaryota, malá eukaryota
- mitochondrie/plastidy/plasmidy

ANCIENT (mt) DNA
- různé směsné, degradované vzorky, např. fosilie



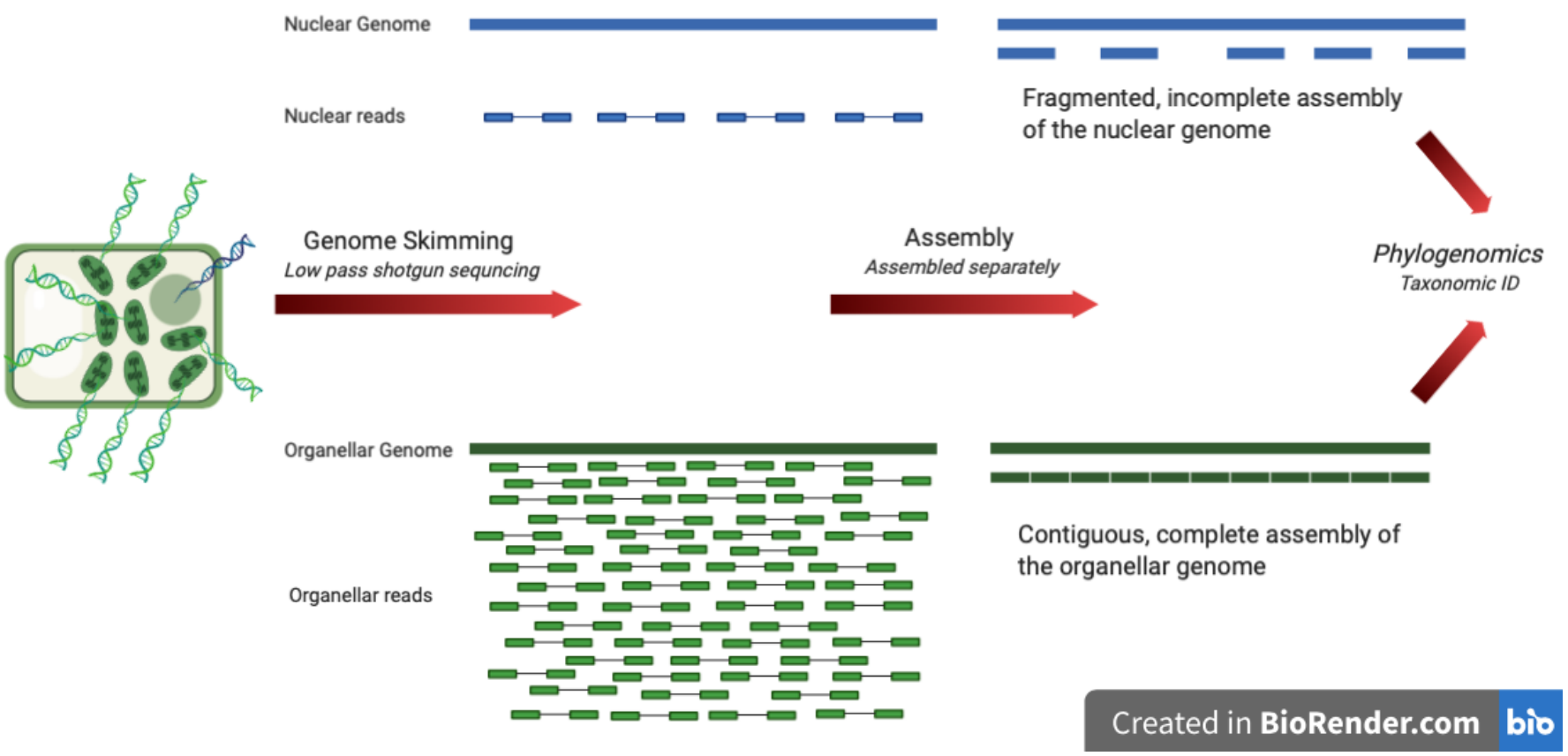
Ancient DNA genomika

- Degradovaná DNA → sekvenování mtDNA
- ale dnes i jaderná DNA ze subfosilního materiálu (jeskynní medvědi, mamuti, neandrtálci ....)



Genome skimming

(low coverage sekvenování kompletní vyizolované DNA)



Analýza muzejního materiálu (např. holotypy)

3.Sekvenování amplikonů (PCR produktů)

SMĚSNÉ VZORKY – **paralelní sekvenování** nahrazuje klonování

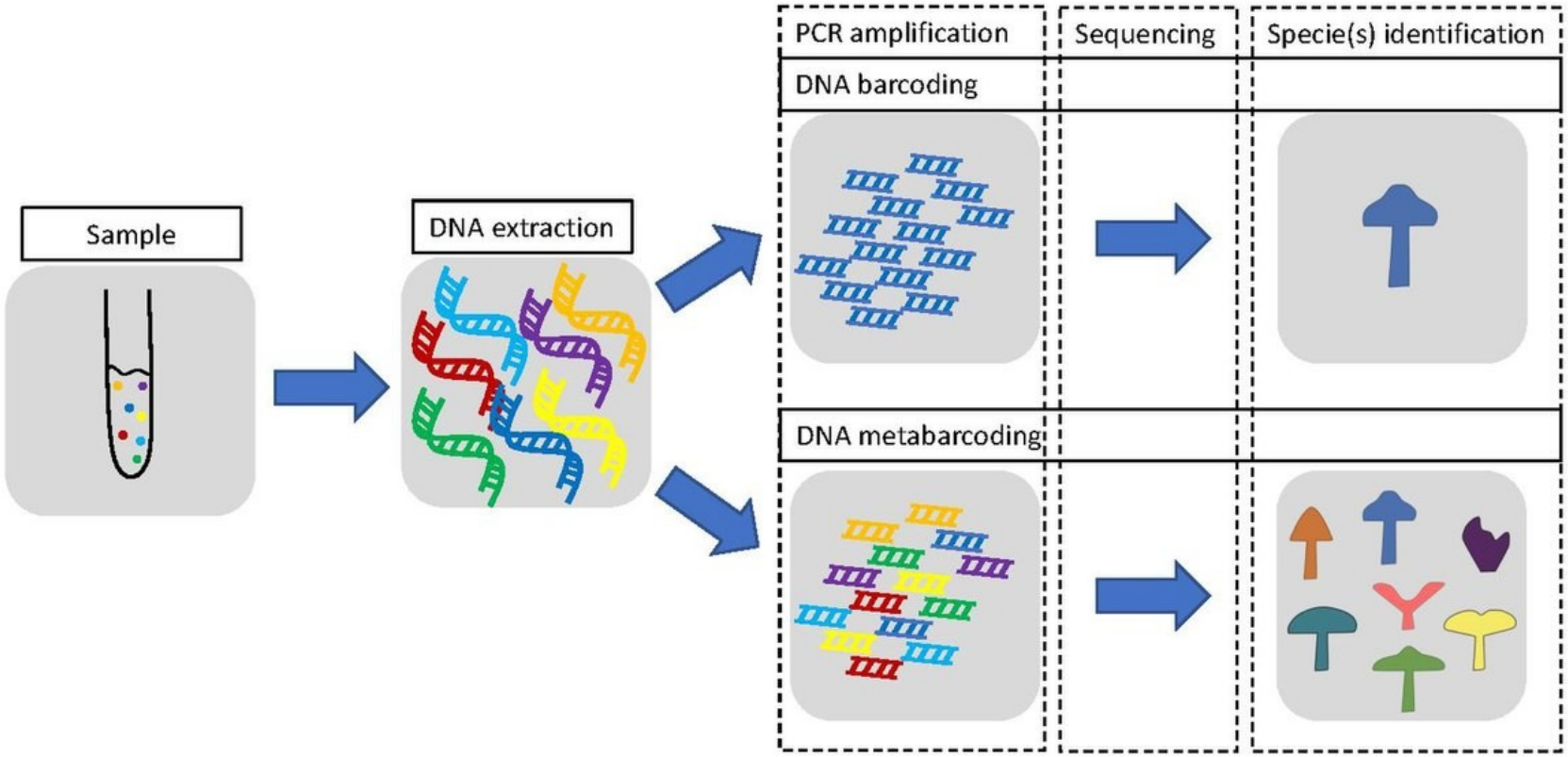
Metagenomika (= hlavně prokaryota)

- Celé společenstvo půdních, vodních mikroorganismů, střevní mikroflóra - **mikrobiom**
- PCR genu 16S rRNA
- lze i kvantifikovat

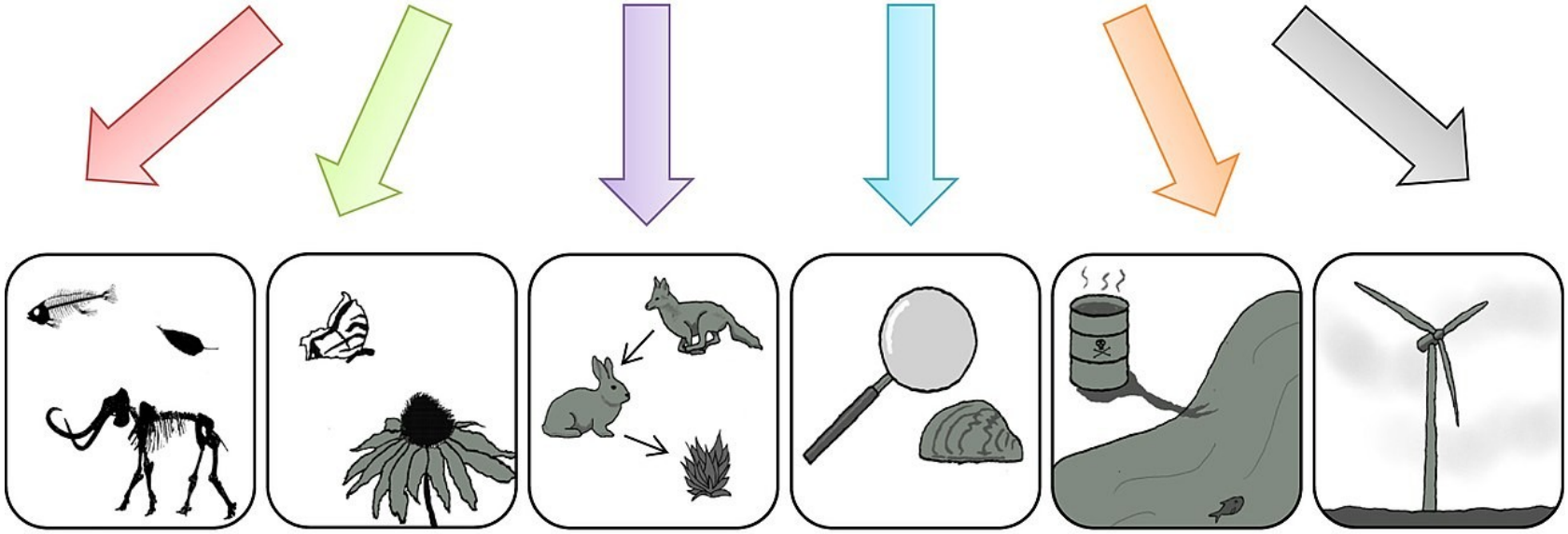
Metabarcoding (= hlavně eukaryota, ale dnes používáno jako obecný termín)

- COI gen, příp. jiný barcodingový marker

Metabarcoding



eDNA Metabarcoding Applications



Ancient Ecosystems	Plant-Pollinator Interactions	Diet Analysis	Invasive Species Detection	Pollution Response	Air Quality Monitoring

eDNA = environmental DNA

Metabarcoding: Taxonomické složení společenstva v environmentální DNA na základě taxonomicky informativního úseku DNA (cyt b, COI, ITS, rRNA...)

- Směsný vzorek enviromentální DNA
- Amplifikace pomoci primerů specifických pro cílovou skupinu, pokrývající taxonomicky informativní úsek (COI, 16s/18s RNA...)
- Paralelni sekvenování
- Filtrování nekvalitních sekvencí
- Klastrování na základě sekvenční podobnosti do OTUs („operational taxonomic units")
- Jejich taxonomické zařazení na základě referenčních databází

Využití: **Analýza druhového složení vzorků kde lze makroskopicky jednotlivé druhy obtížně odlišit**

- Potravní analýza z trusu
- Vzorky půdy
- Mikrobiální společenstva („mikrobiom" – nejen baktérie, ale i houby, prvoci, fágy, ...)
- Permafrost
- Exotická/špatně probádaná společenstva
- Druhově bohatá společenstva („insect traps" v tropech)
- Rutinní analýza velkého množství vzorků

Metabarcoding – příklady využití

Monitoring vzácných, nedávno popsaných druhů savců na základě sekvenování krve pijavic

Výrazně větši úspěšnost prokázání přítomnosti než za použití klasických technik – fotopasti, terénní pozorování apod.

Correspondences

Screening mammal biodiversity using DNA from leeches

Ida Bærholm Schnell[1,2,†],
Philip Francis Thomsen[2,†],
Nicholas Wilkinson[3],
Morten Rasmussen[2],
Lars R.D. Jensen[1], Eske Willerslev[2]
Mads F. Bertelsen[1],
and M. Thomas P. Gilbert[2,*]

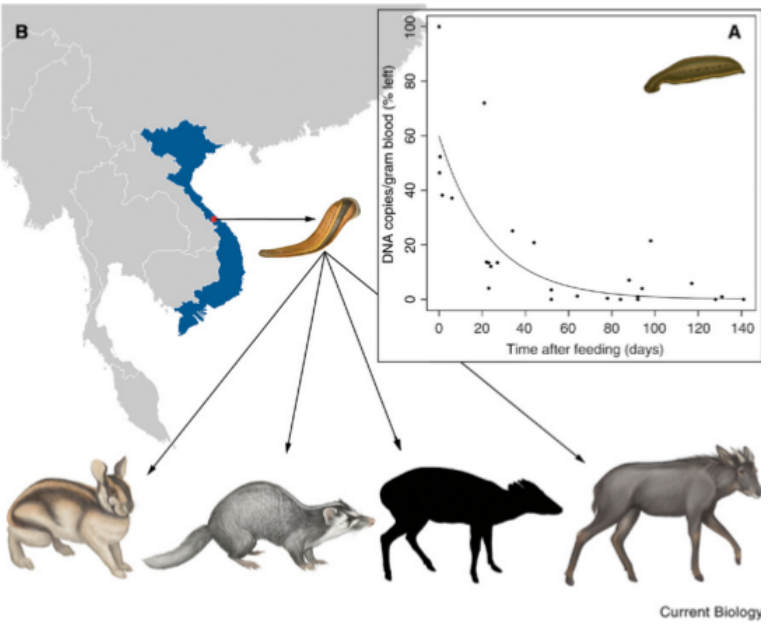
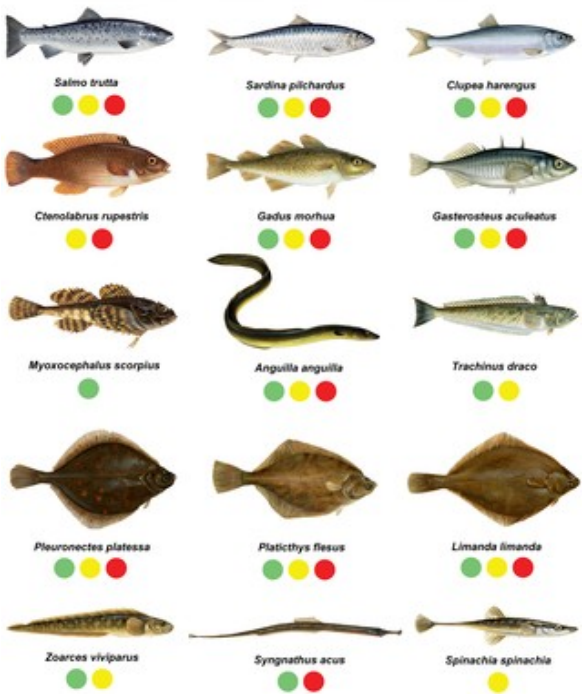
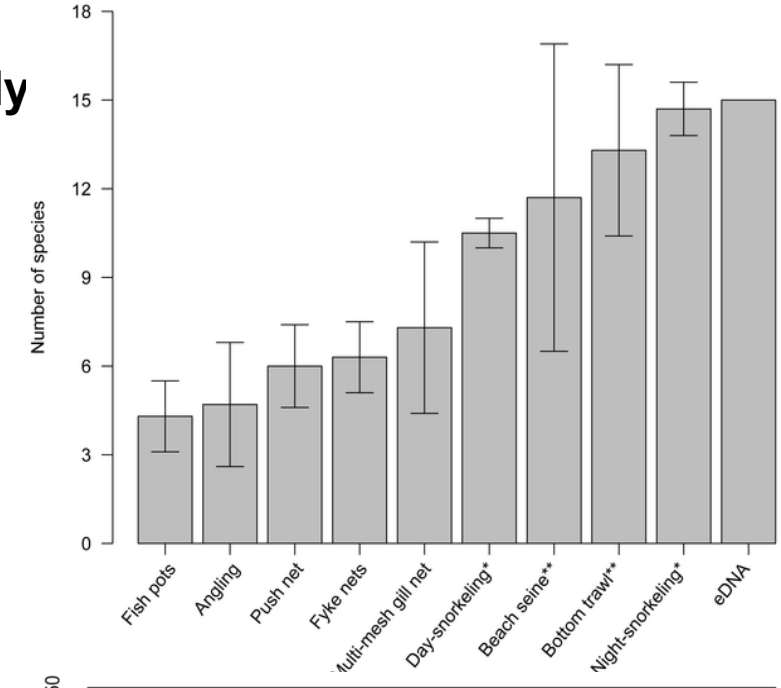


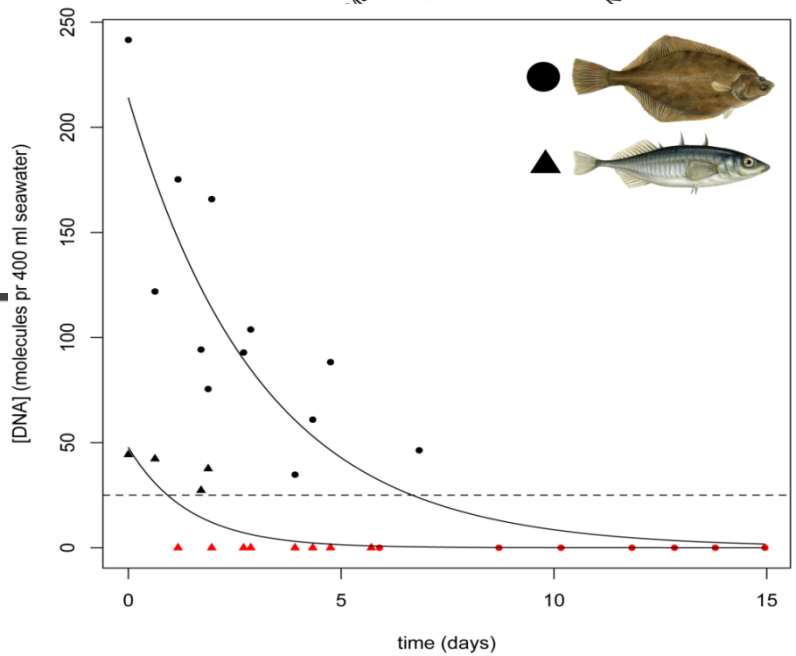
Figure 1. Monitoring mammals with leeches.
(A) Survival of mtDNA in goat blood ingested by *Hirudo medicinalis* over time, relative to freshly drawn sample (100%, ca. 2.4E+09 mtDNA copies/gram blood). Mitochondrial DNA remained detectable in all fed leeches, with a minimum observed level at 1.6E+04 mtDNA/gram blood ingested. The line shows a simple exponential decay model, $p < 0.001$, $R^2 = 0.43$ (Supplemental information). (B) Vietnamese field site location and examples of mammals identified in *Haemadipsa* spp. leeches. From left to right: Annamite striped rabbit, small-toothed ferret-badger, Truong Son munjtac (coat coloration and markings remain unknown), serow. Pictures do not reflect true size proportions. See also Supplemental information.

Metabarcoding – příklady využití

Detekce ryb pomocí izolace eDNA z mořské vody
-taky jedna z nejefektivnějších metod







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PLOS ONE

Detection of a Diverse Marine Fish Fauna Using Environmental DNA from Seawater Samples

Philip Francis Thomsen[1]*, Jos Kielgast[1], Lars Lønsmann Iversen[2], Peter Rask Møller[3], Morten Rasmussen[1], Eske Willerslev[1]*

1 Centre for GeoGenetics, Natural History Museum of Denmark, University of Copenhagen, Øster Voldgade, Copenhagen, Denmark, **2** Freshwater Biology Section, Department of Biology, University of Copenhagen, Helsingørgade, Hillerød, Denmark, **3** Vertebrate Department, Natural History Museum of Denmark, University of Copenhagen, Universitetsparken, Copenhagen, Denmark

Metabarcoding – příklady využití

Analýza potravy

Podíl hospodářských zvířat v potravě irbise je minimální

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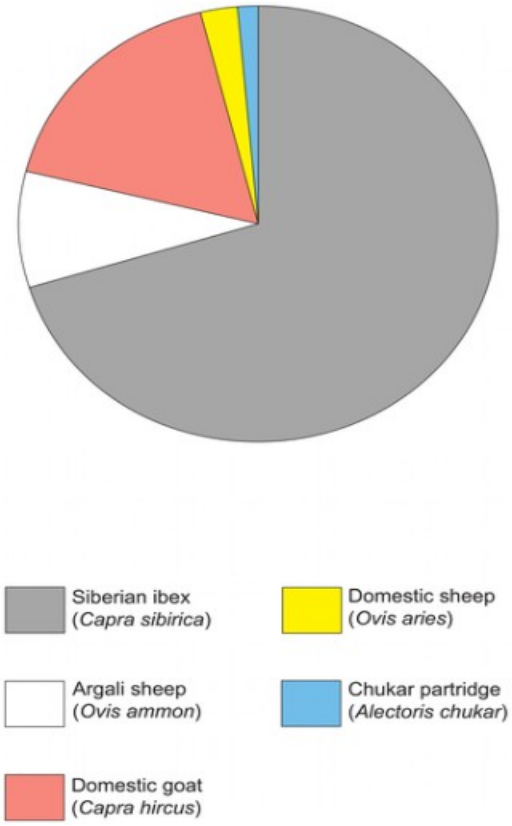
PLoS one

Prey Preference of Snow Leopard (*Panthera uncia*) in South Gobi, Mongolia

Wasim Shehzad[1], Thomas Michael McCarthy[2], Francois Pompanon[1], Lkhagvajav Purevjav[3], Eric Coissac[1], Tiayyba Riaz[1], Pierre Taberlet[1]*

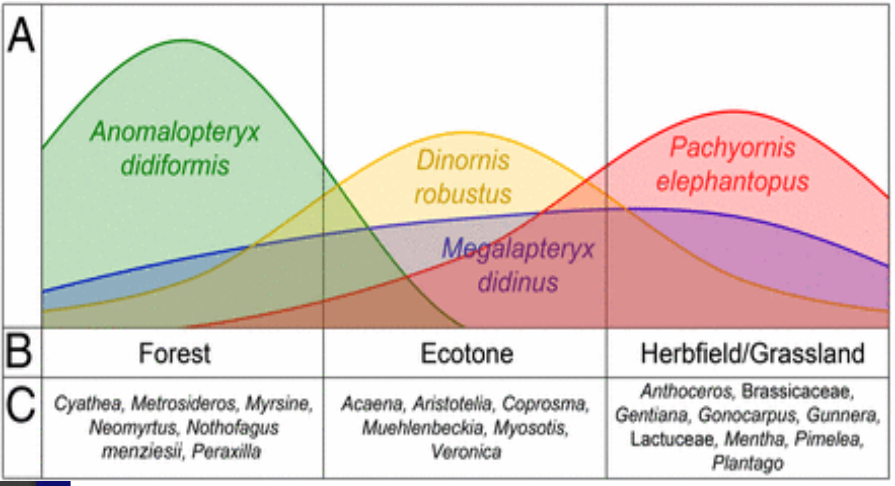
1 Laboratoire d'Ecologie Alpine, Centre National de la Recherche Scientifique, Unité Mixte de Recherche 5553, Université Joseph Fourier, Grenoble, France, **2** Snow Leopard Program, Panthera, New York, New York, United States of America, **3** Snow Leopard Conservation Fund, Ulaanbaatar, Mongolia

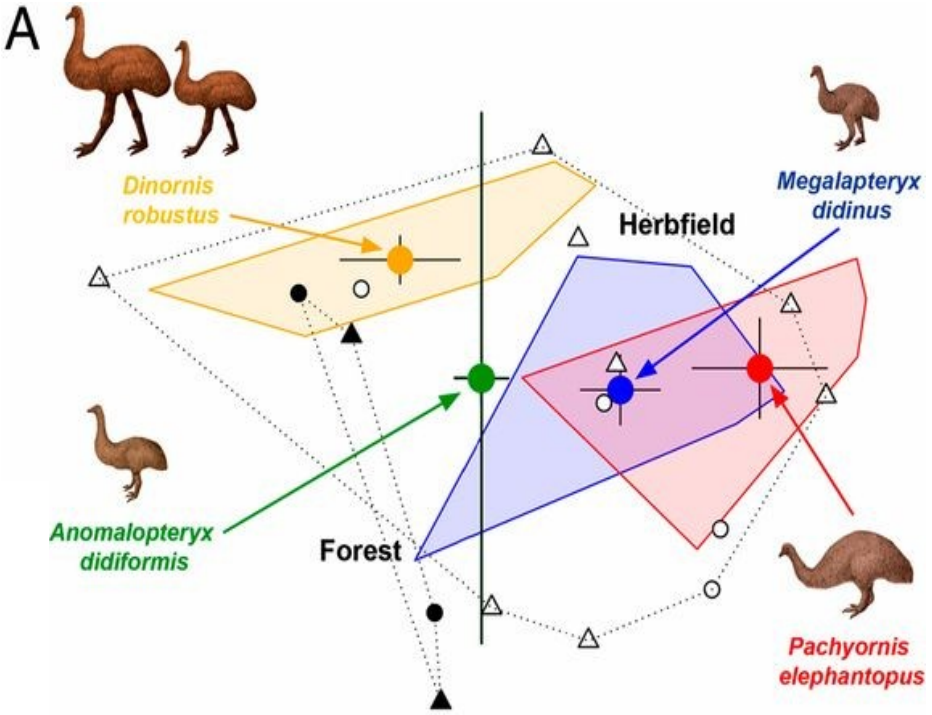


Metabarcoding – příklady využití

Analýza složení společenstva na základě ancient DNA z koprolitů moa (Nový Zéland)

Umožňuje odhadnout typ prostředí které jednotlivé druhy obývaly a separaci ekologických nik





NAS

Resolving lost herbivore community structure using coprolites of four sympatric moa species (Aves: Dinornithiformes)

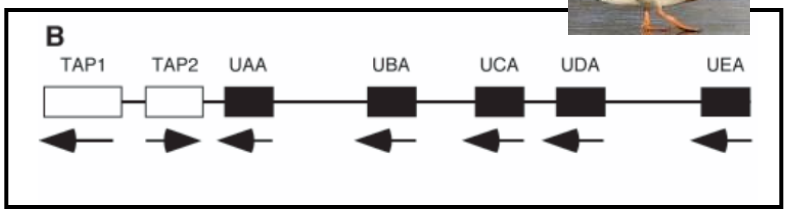
Jamie R. Wood[a,1], Janet M. Wilmshurst[a], Sarah J. Richardson[a], Nicolas J. Rawlence[b,2], Steven J. Wagstaff[a], Trevor H. Worthy[c,3], and Alan Cooper[b]

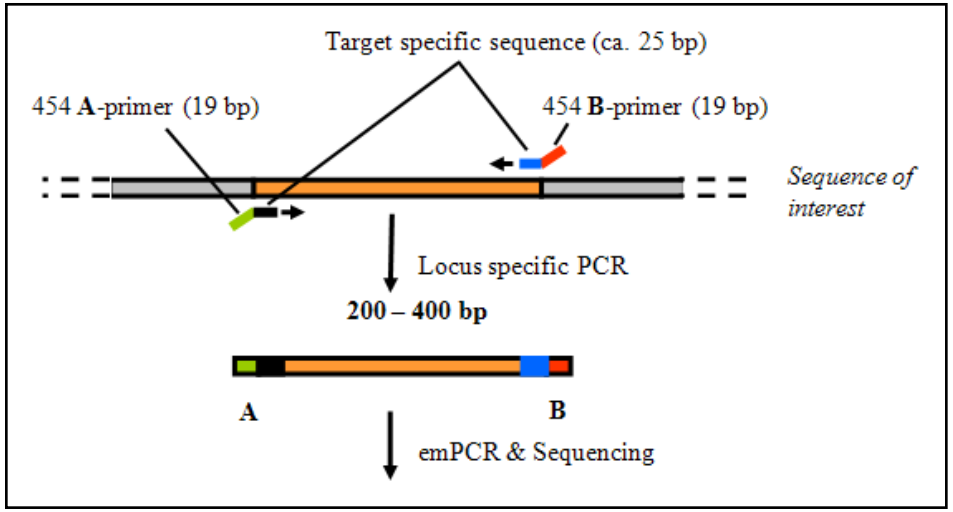
[a]Landcare Research, Lincoln, Canterbury 7640, New Zealand; [b]Australian Centre for Ancient DNA, University of Adelaide, Adelaide, SA 5005, Australia;

3.Sekvenování amplikonů (PCR produktů)

Genové duplikace

(např. MHC geny)





A-adaptor MID Target specific

Označí jedince

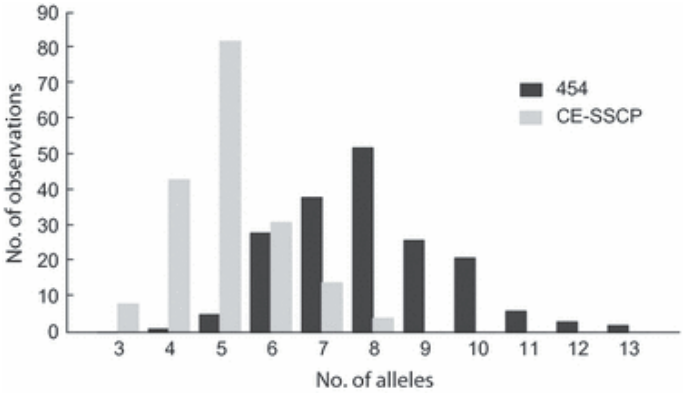
Amplifikuje všechny kopie MHC genů

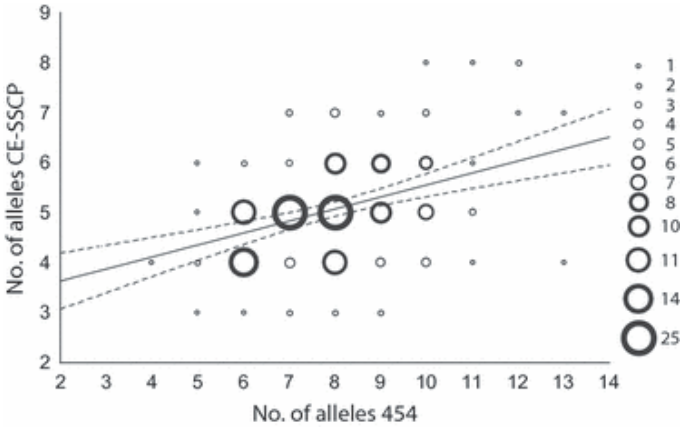
Potřeba k HTS sekvenování

Amplikonové sekvenování

MHC u hýla rudého

- HTS má větší rozlišovací schopnost než SSCP + klonování





MOLECULAR ECOLOGY
RESOURCES
Molecular Ecology Resources (2012) **12**, 285–292
doi: 10.1111/j.1755-0998.2011.03082.x

Evaluation of two approaches to genotyping major histocompatibility complex class I in a passerine—CE-SSCP and 454 pyrosequencing

MARTA PROMEROVÁ,* WIESŁAW BABIK,† JOSEF BRYJA,* TOMÁŠ ALBRECHT,*‡ MICHAŁ STUGLIK† and JACEK RADWAN§

4. Další aplikace – hledání nových genetických markerů

Mikrosatelity

- sekvenování obohacených knihoven

SNPs

- kompletní nebo redukované („enriched") genomické sekvence pro hledání diagnostických SNPs
- např. RAD-sequencing

Hledání nových genetických markerů - mikrosatelity

Obvyklý postup:

-Obohacení („enrichment“) genomické knihovy o mikrosatelitové motivy – sequence capture
-Sekvenování obohacených knihoven
-Detekce mikrosatelitů a navržení vhodných primerů

MOLECULAR ECOLOGY
RESOURCES
Molecular Ecology Resources (2011) 11, 638–644 doi: 10.1111/j.1755-0998.2011.029

High-throughput microsatellite isolation through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries

THIBAUT MALAUSA,* ANDRÉ GILLES,† EMESE MEGLÉCZ,† HÉLÈNE BLANQUART,‡ STÉPHANIE DUTHOY,‡ CAROLINE COSTEDOAT,† VINCENT DUBUT,† NICOLAS PECH,† PHILIPPE CASTAGNONE-SERENO,* CHRISTOPHE DÉLYE,§ NICOLAS FEAU,¶ PASCAL FREY,** PHILIPPE GAUTHIER,†† THOMAS GUILLEMAUD,* LAURENT HAZARD,‡‡ VALÉRIE LE CORRE,§ BRIGITTE LUNG-ESCARMANT,¶ PIERRE-JEAN G. MALÉ,§§ STÉPHANIE FERREIRA‡ and JEAN-FRANÇOIS MARTIN††
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allgenetics

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Experts in Microsatellite Development

Microsatellites (also known as short tandem repeats) are repetitive DNA elements usually found in non-coding regions of the genome. They have h mutation rates, and therefore are frequently highly polymorphic. Variations in the number of repetitions generate different alleles. This makes th appropriate molecular markers for population genetics and molecular ecology projects.

We develop microsatellite markers for your study species

At AllGenetics, we use next-generation sequencing to obtain primer pairs which amplify polymorphic microsatellite loci in your study species. Genor DNA is used to generate genomic libraries. We usually enrich these libraries with 4 to 6 different microsatellite motifs. However, we can customise number of motifs to your needs. We obtain thousands of microsatellite-containing reads by using high-throughput sequencing. Our bionformaticians these reads for primer design. The primers obtained are multiplexed and tested for polymorphism in a number of individuals from different populations

How we work

High quality DNA at a concentration of 100 ng/µL in a minimum volume of 50 µL from a number of individuals is required. Alternatively, we can isol DNA from your samples. These samples should be adequately preserved to ensure DNA integrity. We will deliver tested primer pairs which amp polymorphic loci for your study species. A detailed methodological report and all sequencing reads generated will also be provided.

Our microsatellite development projects are divided into four steps. For your convenience, we can carry out the entire project or only the parts you nee

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32 species validation of a new Illumina paired-end approach for the development of microsatellites

Stacey L. Lance[1*], Cara N. Love[1], Schyler O. Nunziata[1], Jason R. O'Bryhim[1], David E. Scott[1], R. Wesley Flynn[1], Kenneth L. Jones[2]

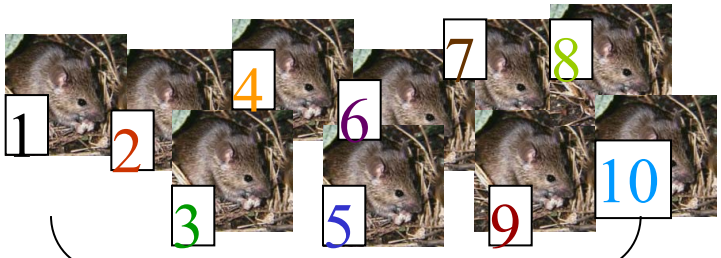
1 Savannah River Ecology Laboratory, University of Georgia, Aiken, South Carolina, United States of America, 2 Department of Biochemistry and Molecular

development service

AllGenetics' microsatellite development service uses high-throughput sequencing to obtain primer pairs which amplify polymorphic microsatellite loci in your study species. The primers obtained are multiplexed and tested for polymorphism in a number of individuals from different populations.

1 › 2 › 3

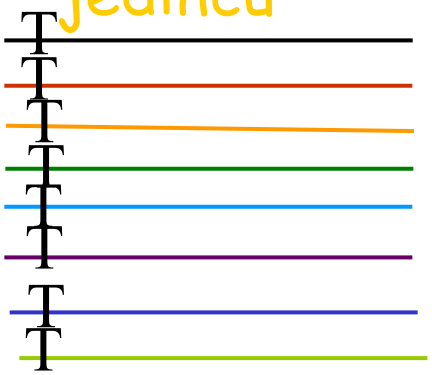
Hledání diagnostických SNP (např. pro studium hybridizace)



smíchat a osekvenovat

10 jedinců

10 jedinců



- hledání zafixovaných polymorfismů - bioinformaticky
- např. pro analýzu v hybridních zónách – identifikace genomických fragmentů, které nepřecházejí hybridní zónu a jsou zodpovědné za udržování druhových hranic (pokud máme referenční genom)

Hledání nových SNPs - RAD-sequencing

Sekvenování podél restrikčních míst

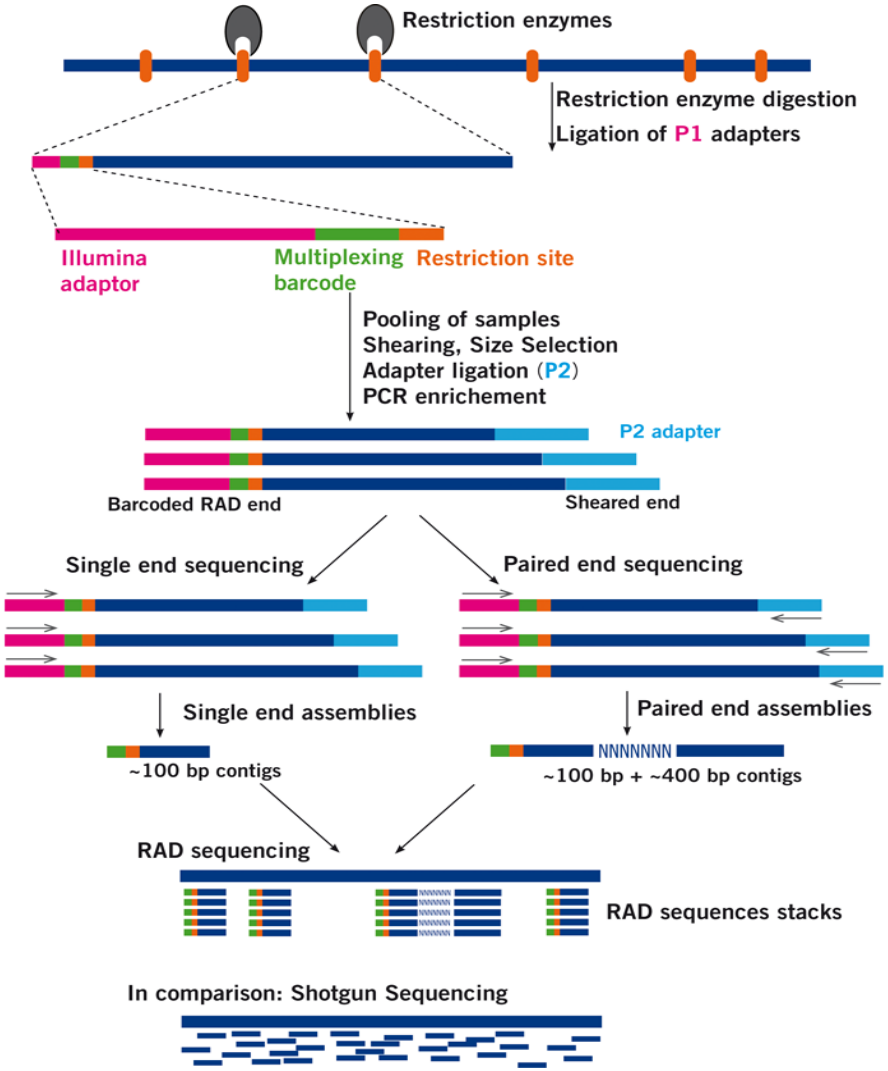
Fragmetace gelogenomové DNA po mocí restrikčních enzymů

Ligace sekvenačních adaptorů na výsledné fragmenty

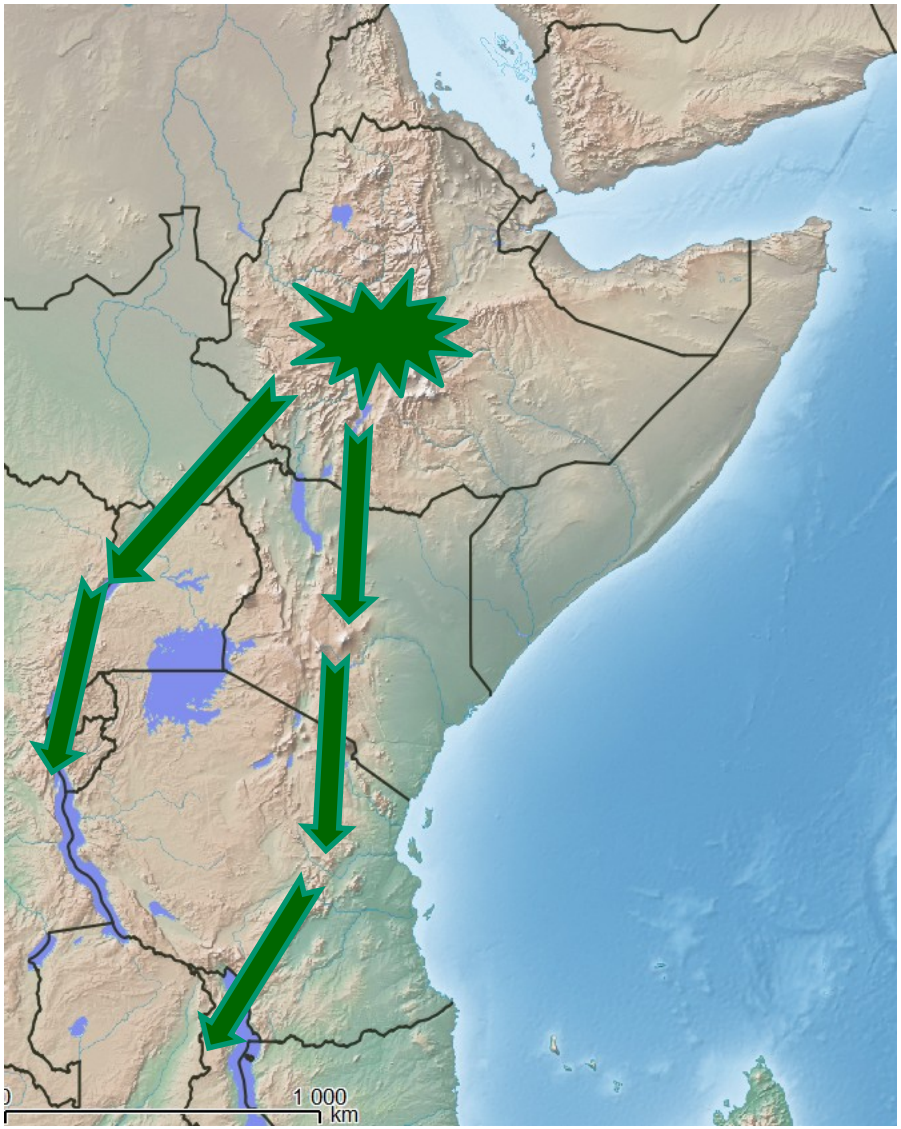
Následná sekvenace podél restrikčních míst

Celogenomové scany genetické variablility

Hledání SNPs, populační genomika (např. RAD-SEQ) apod.



Př.: Fylogenomika hlodavců rodu *Lophuromys*



- ancestral lineage „trapped“ in Ethiopian highlands, where diversified and sourced the colonization of other mountains (**mostly in Pleistocene**)

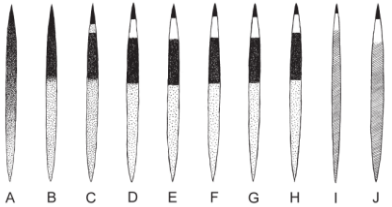
- ***Lophuromys flavopunctatus* complex (9 Ethiopian species)**

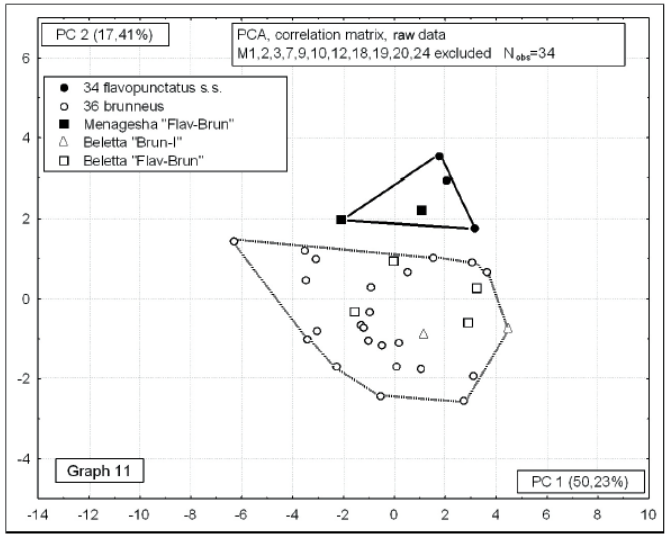
9 endemic species in Ethiopia

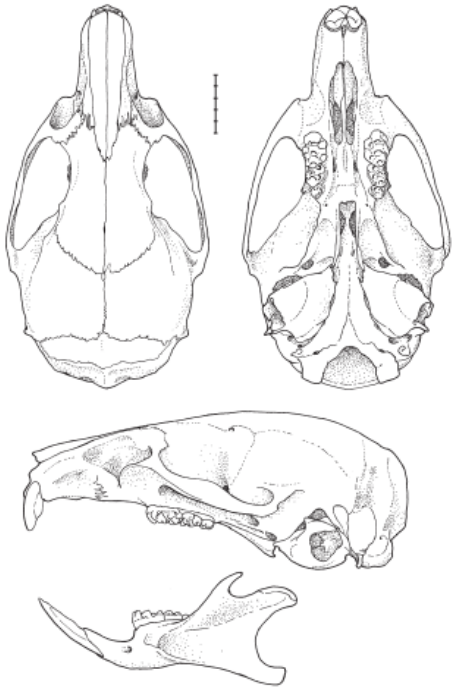
BULLETIN DE L'INSTITUT ROYAL DES SCIENCES NATURELLES DE BELGIQUE — BIOLOGIE, 77: 77-117, 2007
BULLETIN VAN HET KONINKLIJK BELGISCH INSTITUUT VOOR NATUURWETENSCHAPPEN — BIOLOGIE, 77: 77-117, 2007

Morphometric and genetic study of Ethiopian *Lophuromys flavopunctatus* THOMAS, 1888 species complex with description of three new 70-chromosomal species (Muridae, Rodentia)

by Leonid A. LAVRENCHENKO, Walter N. VERHEYEN, Erik VERHEYEN, Jan HULSELMANS & Herwig LEIRS







3.2. Views of skull and mandible of *Lophuromys menageshae* n.sp. (ZMMU S-165969, holotype). Scale bar = 5 mm.

Lophuromys - questions

- Are there really 9 well delimited species?
- Are they easily (genetically) recognizable? (e.g. mtDNA-barcoding)
- What is their distribution and ecological requirements? -> IUCN assessment, etc.

ORIGINAL ARTICLE

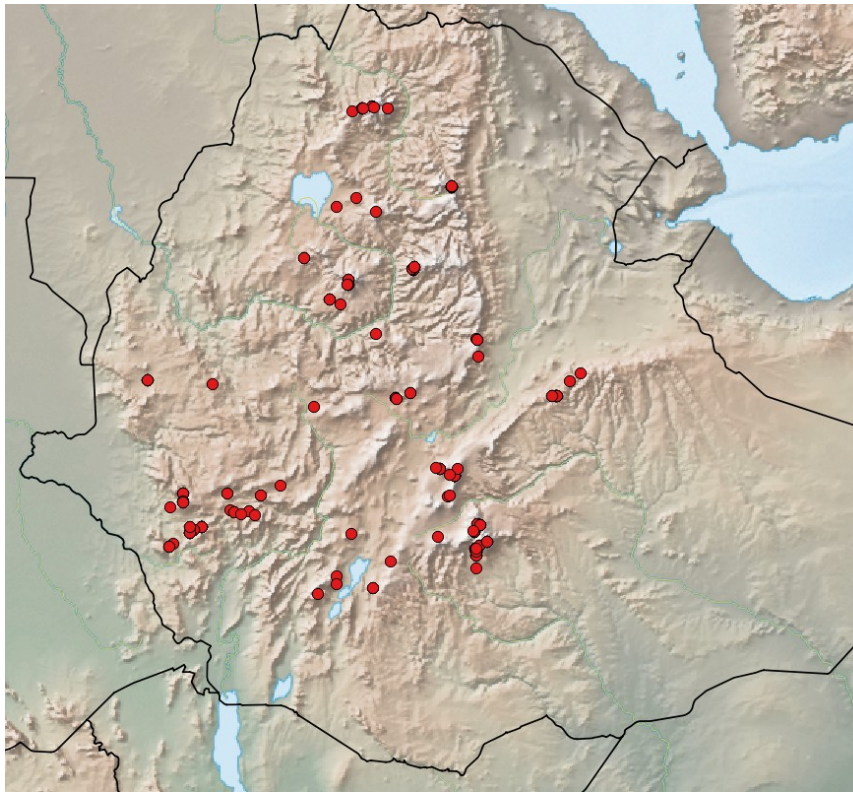
MOLECULAR ECOLOGY WILEY

Complex reticulate evolution of speckled brush-furred rats (*Lophuromys*) in the Ethiopian centre of endemism

Valeria A. Komarova[1] | Danila S. Kostin[1] | Josef Bryja[2,3] | Ondřej Mikula[2] | Anna Bryjová[2] | Dagmar Čížková[2] | Radim Šumbera[4] | Yonas Meheretu[5] | Leonid A. Lavrenchenko[1]

Material and Methods

- cca 500 specimens from all major mountain ranges
- mtDNA marker (CYTB)
- 4 nuclear markers (2 introny + 2 exony)
- **genomic approach – ddRAD sequencing**

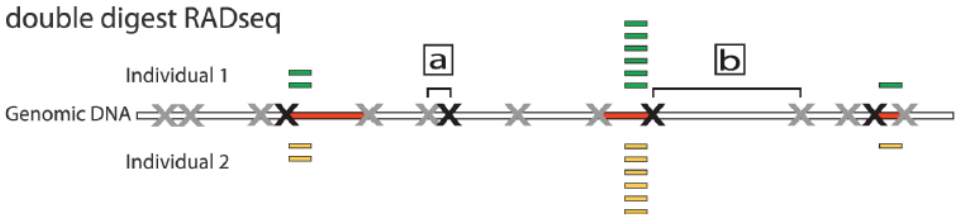


Analýza dat z ddRADseq

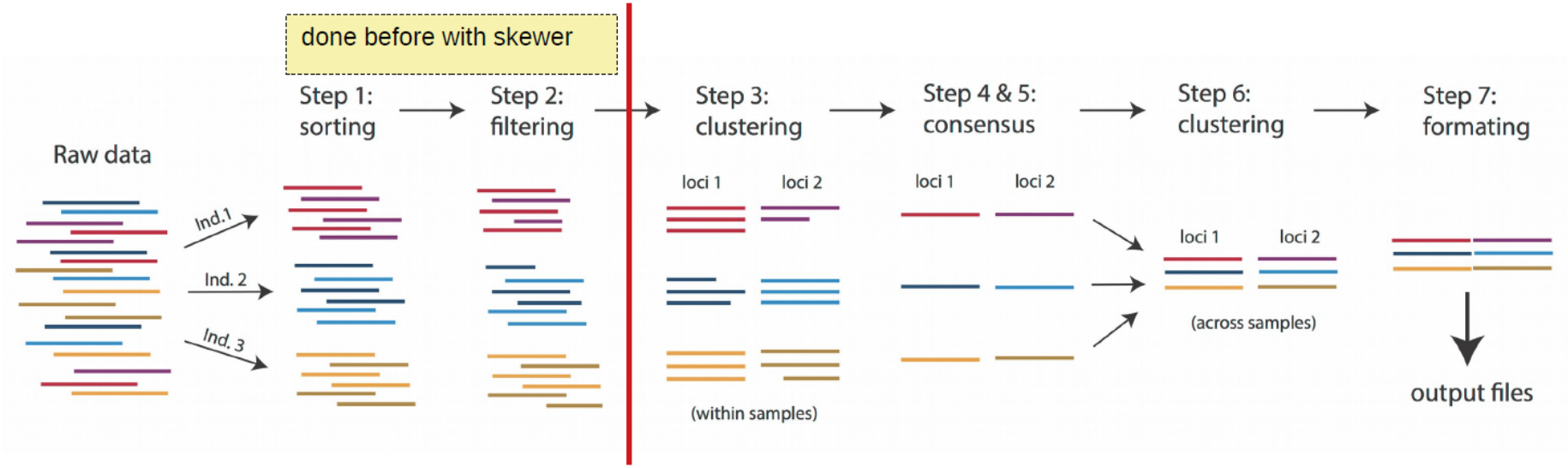
DATA ANALYSIS

B) DEFINE LOCI AND FIND VARIABILITY

SOFTWARE AVAILABLE:
Stacks, dDocent, iPyrad ….



iPyrad denovo assembly workflow (no reference genome)



program Skewer

program iPyrad

- stovky až desítky tisíc lokusů

Retaining well-covered & informative loci

All loci

No. of individuals:	213
No. of loci:	80570
No. of informative loci:	69724
No. of SNPs / PISs per informative locus:	
Min:	1 / 1
25%:	5 / 4
50%:	10 / 9
75%:	20 / 17
Max:	60 / 57
Loci per individual:	
Min:	5178
25%:	9719
50%:	12000
75%:	14607
Max:	23205
Individuals per locus:	
Min:	4
25%:	6
50%:	13
75%:	37
Max:	208
Proportion of missing data:	0.85

HQ loci

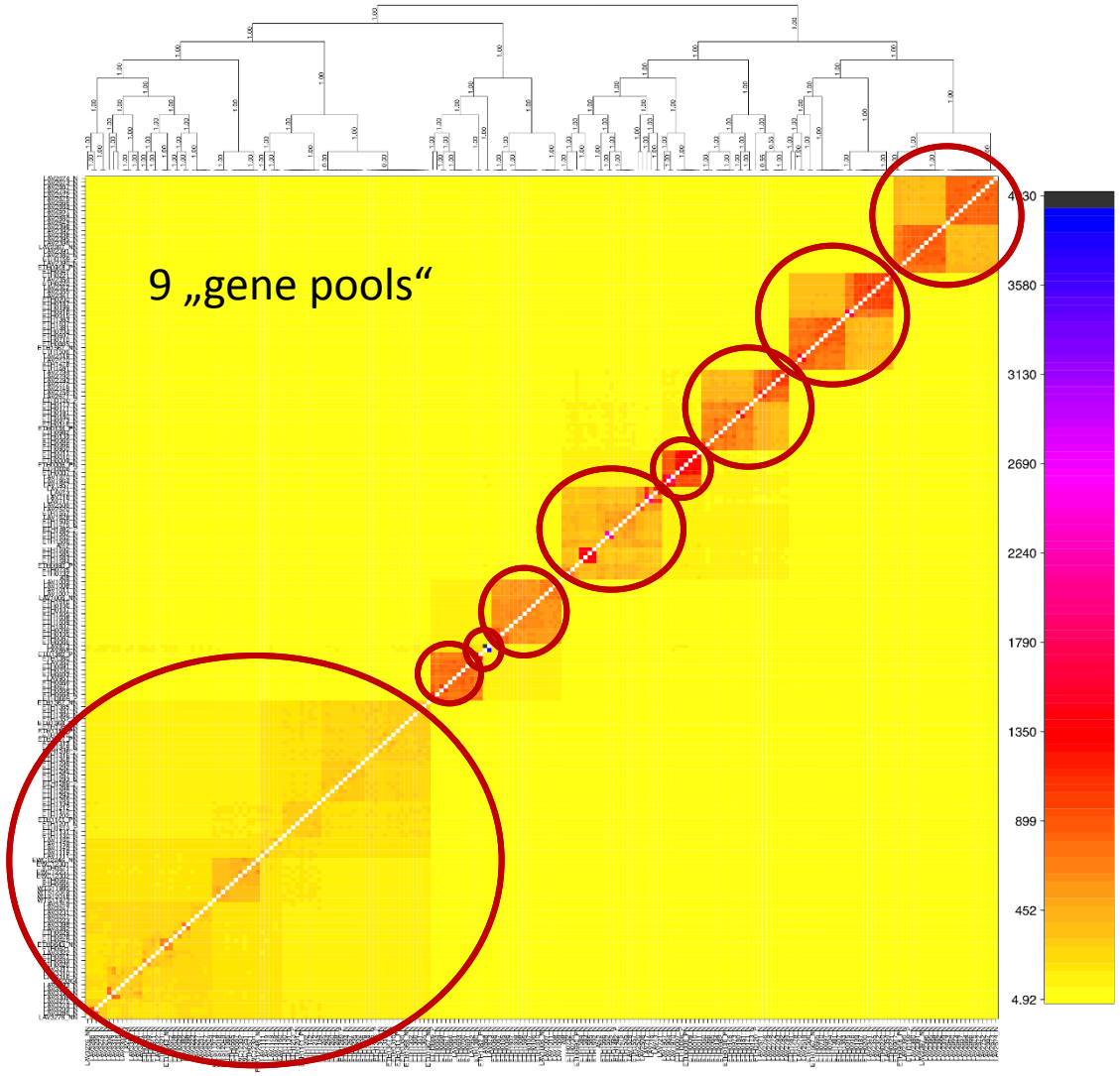
No. of individuals:	213
No. of loci:	15164
No. of informative loci:	15164
No. of SNPs / PISs per informative locus:	
Min:	1 / 1
25%:	17 / 14
50%:	25 / 21
75%:	32 / 28
Max:	57 / 54
Loci per individual:	
Min:	3393
25%:	6912
50%:	8074
75%:	9297
Max:	11912
Individuals per locus:	
Min:	54
25%:	74
50%: ✔	103
75%:	149
Max:	208
Proportion of missing data:	0.47 ✔

80 570 loci → filtering → 15 164 loci

ddRADseq: co-ancestry matrix

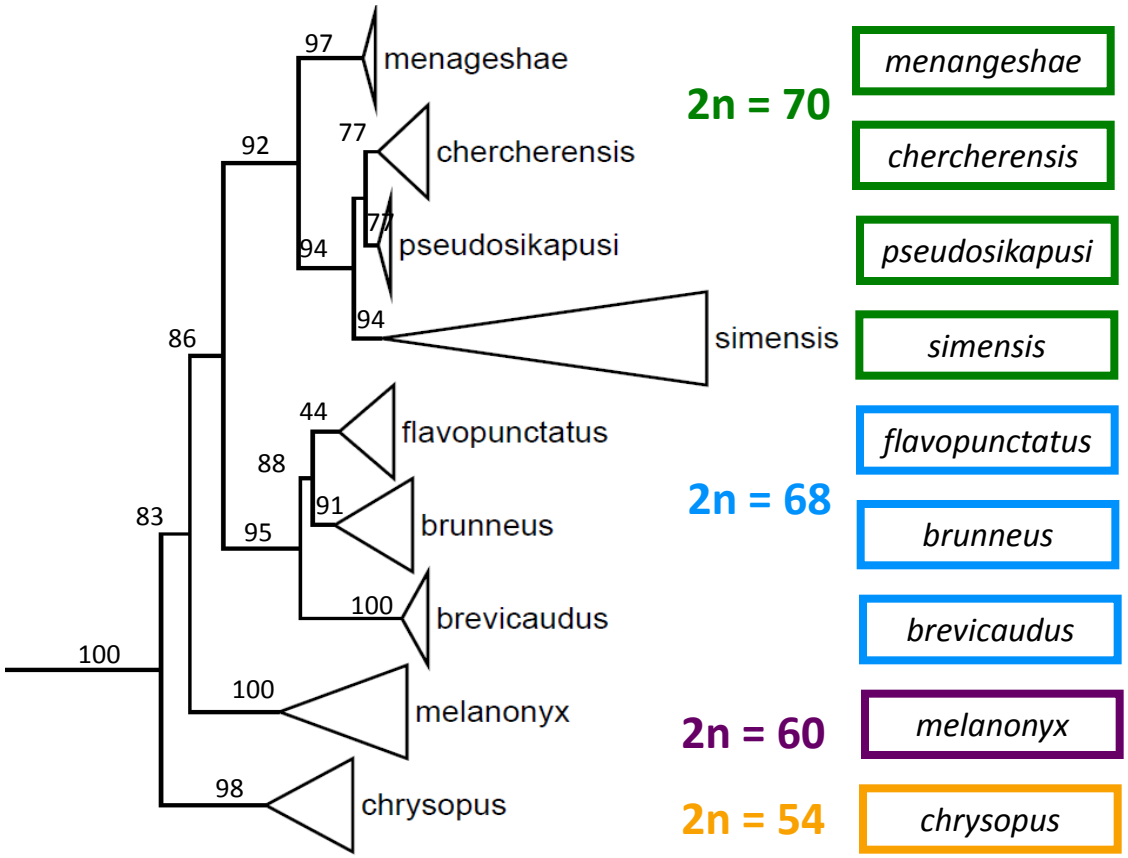
209 individuals

15 623 informative loci



Maximum likelihood analysis of concatenated nuclear dataset

Sanger sequencing



2n = 70

menangeshae

chercherensis

pseudosikapusi

simensis

2n = 68

flavopunctatus

brunneus

brevicaudus

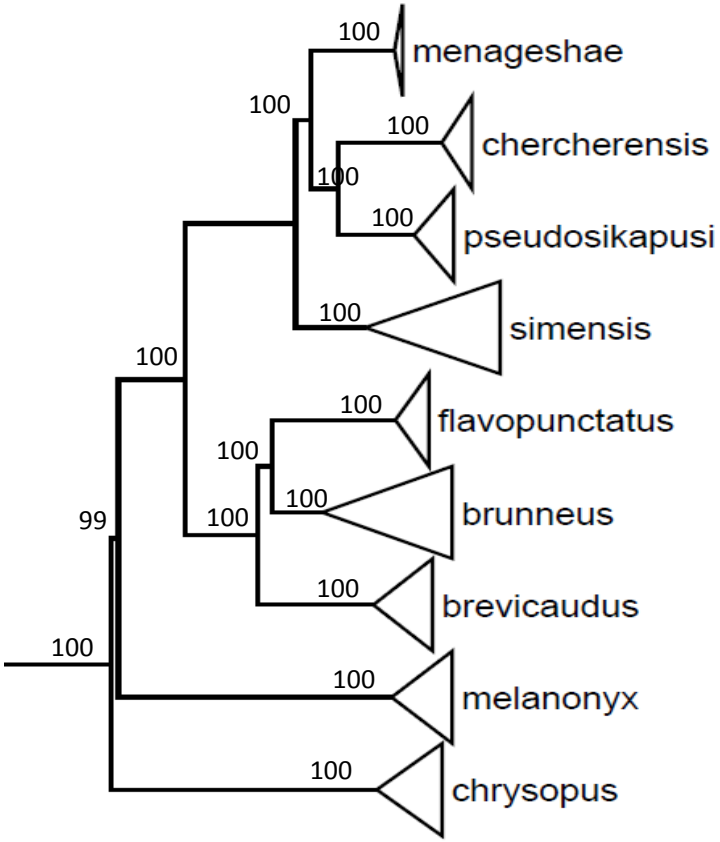
2n = 60

melanonyx

2n = 54

chrysopus

ddRADseq

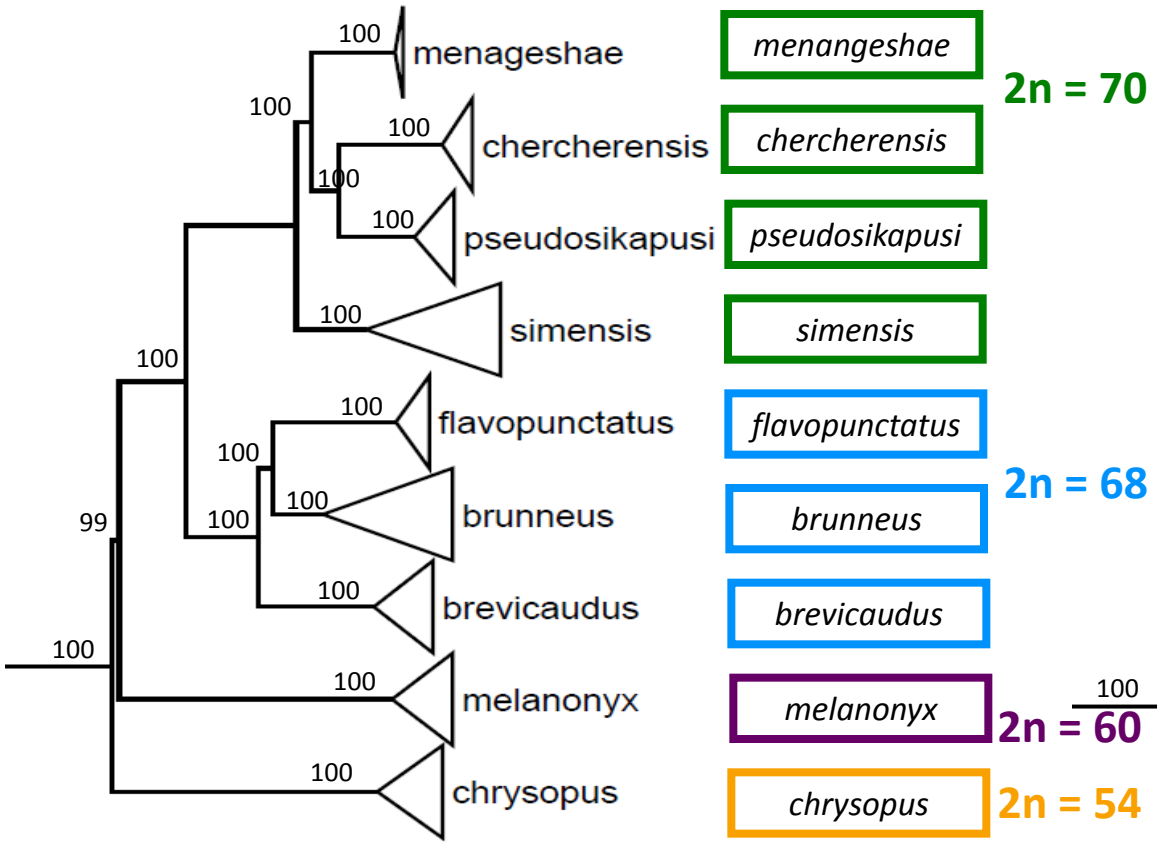


4 nuclear markers (V. Komarova et al.)
(2 604 bp concatenated dataset)

15 623 informative loci

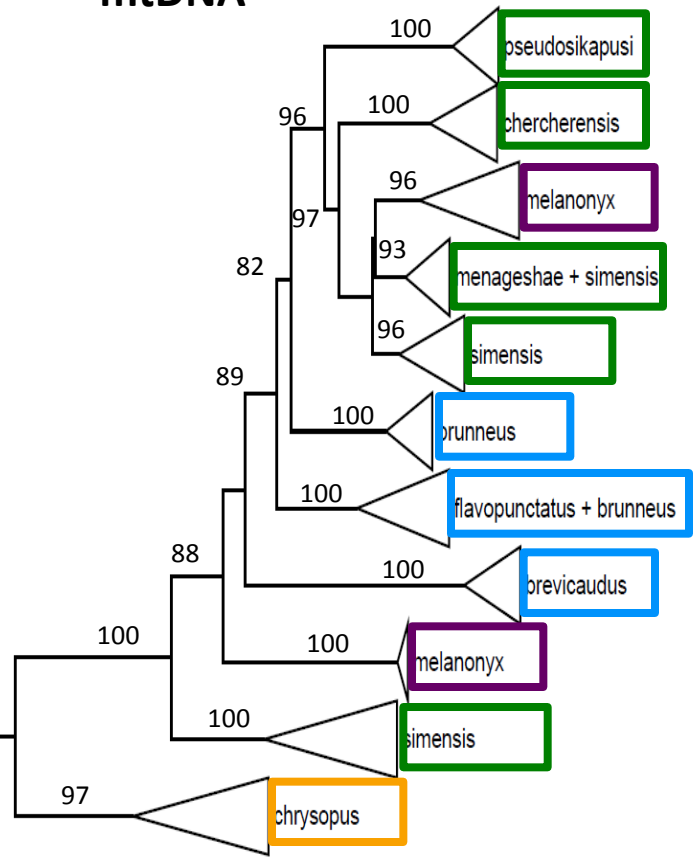
And what about mtDNA?

ddRADseq



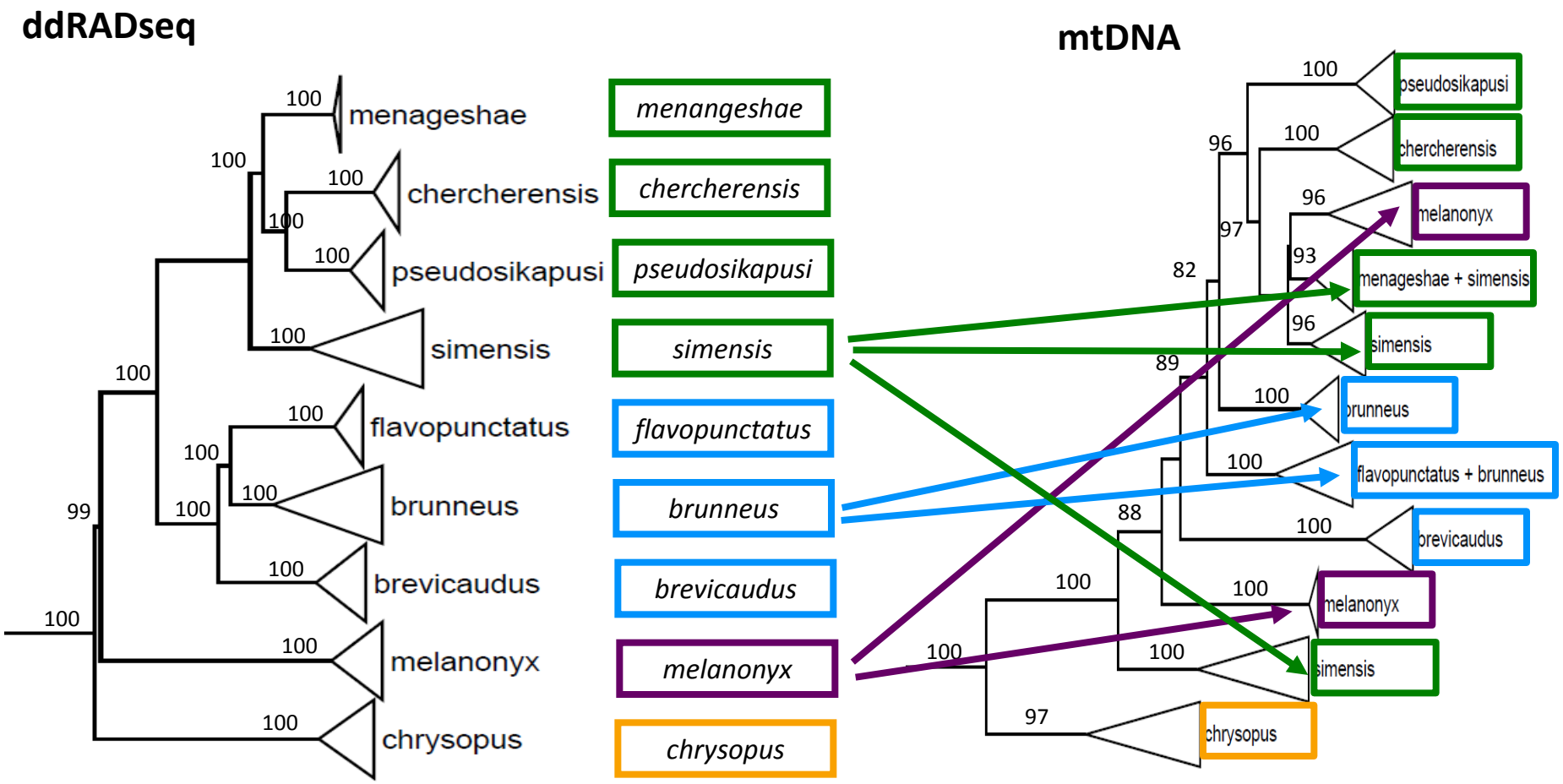
15 623 informative loci

mtDNA



cytochrome *b* (1140 bp)

And what about mtDNA?



15 623 informative loci

cytochrome *b* (1140 bp)

„reticulate evolution" resulting in mtDNA introgression