DNA Profiling e Genetica Forense

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Nelle prossime 5 lezioni vedremo

- Nonhuman forensic genetics
  - DNA barcoding

- Importanza e diffusione di Next Generation Sequencing in human e non human forensic genetics

PLOS | GENETICS

REVIEW

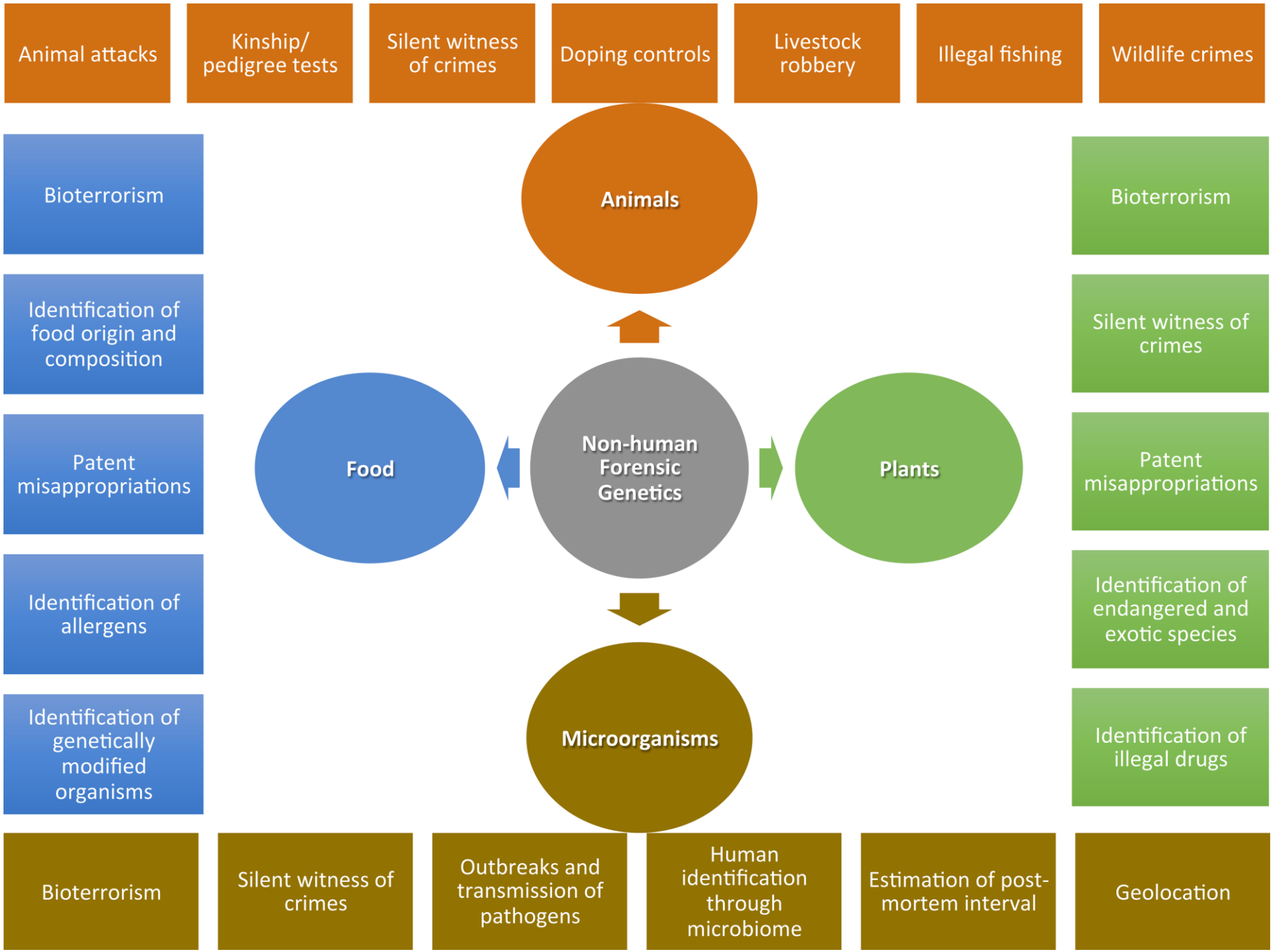
Forensic genetics and genomics: Much more than just a human affair

Miguel Arenas[1,2,3]*, Filipe Pereira[2,4], Manuela Oliveira[2,3,5], Nadia Pinto[2,3,6], Alexandra M. Lopes[2,3], Veronica Gomes[2,3], Angel Carracedo[7,8]*, Antonio Amorim[2,3,5]*

PLOS Genetics | https://doi.org/10.1371/journal.pgen.1006960 September 21, 2017

*Definizione di **Genetica forense** (dal giornale più autorevole nel campo della genetica forense, **Forensic Science International: Genetics)***

"The application of genetics to human and nonhuman material (in the sense of a science with the purpose of studying inherited characteristics for the analysis of inter- and intraspecific variations in populations) for the resolution of legal conflicts"


Animal attacks
Kinship/ pedigree tests
Silent witness of crimes
Doping controls
Livestock robbery
Illegal fishing
Wildlife crimes
Animals
Bioterrorism
Identification of food origin and composition
Patent misappropriations
Identification of allergens
Identification of genetically modified organisms
Food
Non-human Forensic Genetics
Plants
Bioterrorism
Silent witness of crimes
Patent misappropriations
Identification of endangered and exotic species
Identification of illegal drugs
Microorganisms
Bioterrorism
Silent witness of crimes
Outbreaks and transmission of pathogens
Human identification through microbiome
Estimation of post-mortem interval
Geolocation

Broadly speaking, statistical evaluation in NHFG can be required for 3 major scenarios:

1. Individual identification or kinship. It involves cases such as "was a given dog the perpetrator of the attack?" or "is a given foal the offspring of a given highly prized horse?"
2. Species identification. It involves interspecies cases such as "does the label of a processed fish product agree with the species of origin?"
3. Subspecific assignment/identification. It involves cases related with breed, variety, or populations such as "was the attack perpetrated by a dog or by a wolf?"

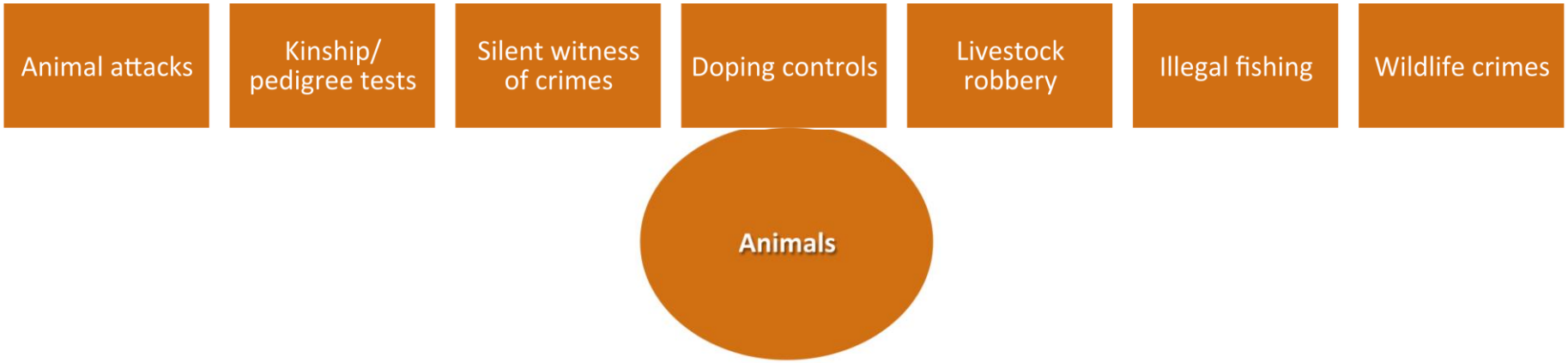
Marcatori:
STR

DNA barcoding
reliable and public databases such as GenBank, EMBL, and Bold

genetic marker depends on the investigated species. For metazoan, the most used markers are regions of the mitochondrial genome (and plastid for Plantae) that can provide accurate distinction between subspecies and autosomal regions of nuclear DNA.

Statistical support

The program STRUCTURE is widely used for the Bayesian assignment of an individual to a population (or subspecies). Again, the statistical evaluation should be performed and reported.



Preferential DNA markers used for individual identification in animals are autosomal **STRs**

STR kits for individual identification and kinship testing in dogs, cats, horses, cattle, bears, deer, badgers (tassi), birds, and koi carps, ***Testudo hermanni***.

Criminal and civil cases: dog or bear attacks, silent witnesses of crimes, identification of samples from sport horses, and in wildlife crime investigations (wildlife forensics), including big cats, mouflons, wild boars, and elephants, among others. Illegal wildlife trade

Forensic zoology often has to deal with degraded samples. In such cases, **mtDNA** may be the only source of genetic information that can be used.

…analisi genetiche sono state eseguite dai tecnici del laboratorio di genetica della conservazione dell'ISPRA. Le metodologie sviluppate, in accordo con quanto previsto nell'ambito del Piano d'Azione per la Conservazione dell'orso Bruno nelle Alpi Centrali (PACOBACE), prevedono l'amplificazione di 14 differenti regioni del genoma (DNA microsatellite) e il sessaggio molecolare di tutti i campioni organici.

In Trentino c'è un orso in meno, hanno ucciso l'orsa KJ2

2017

Trento, catturato l'orso M49: sta bene ed è chiuso nel recinto

2019-2020

Wasser SK, Joseph Clark W, Drori O, Stephen Kisamo E, Mailand C, Mutayoba B, et al. Combating the illegal trade in African elephant ivory with DNA forensics. Conserv Biol. 2008;22(4):1065–71. 10.1111/j.1523-1739.2008.01012.x. pmid:18786100.

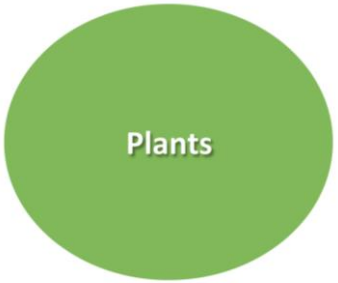
Eichmann C, Berger B, Reinhold M, Lutz M, Parson W. Canine-specific STR typing of saliva traces on dog bite wounds. Int J Legal Med. 2004;118(6):337–42. pmid:15480731.

Frosch C, Dutsov A, Georgiev G, Nowak C. Case report of a fatal bear attack documented by forensic wildlife genetics. Forensic Sci Int Genet. 2011;5(4):342–4. pmid:21315676.

Tsuji A, Ishiko A, Kimura H, Nurimoto M, Kudo K, Ikeda N. Unusual death of a baby: a dog attack and confirmation using human and canine STRs. Int J Legal Med. 2008;122(1):59–62. pmid:17226054.

Plant evidence can provide crucial information for the reconstruction of forensically relevant events or in cases where the crime scene and autopsy reports are not compelling

Forensic botany presents numerous applications such as the identification of the origin of seized illegal drugs



- Bioterrorism
- Silent witness of crimes
- Patent misappropriations
- Identification of endangered and exotic species
- Identification of illegal drugs

.Dufresnes C, Jan C, Bienert F, Goudet J, Fumagalli L. Broad-Scale Genetic Diversity of Cannabis for Forensic Applications. PLoS One. 2017;12(1):e0170522. pmid:28107530.

Cardoso HF, Santos A, Dias R, Garcia C, Pinto M, Sergio C, et al. Establishing a minimum postmortem interval of human remains in an advanced state of skeletonization using the growth rate of bryophytes and plant roots. Int J Legal Med. 2010;124(5):451–6. pmid:19714355.

Review and Future Prospects for DNA Barcoding Methods in Forensic Palynology

Karen L Bell [1], Kevin S Burgess [2], Kazufusa C Okamoto [3], Roman Aranda [4], Berry J Brosi [5]

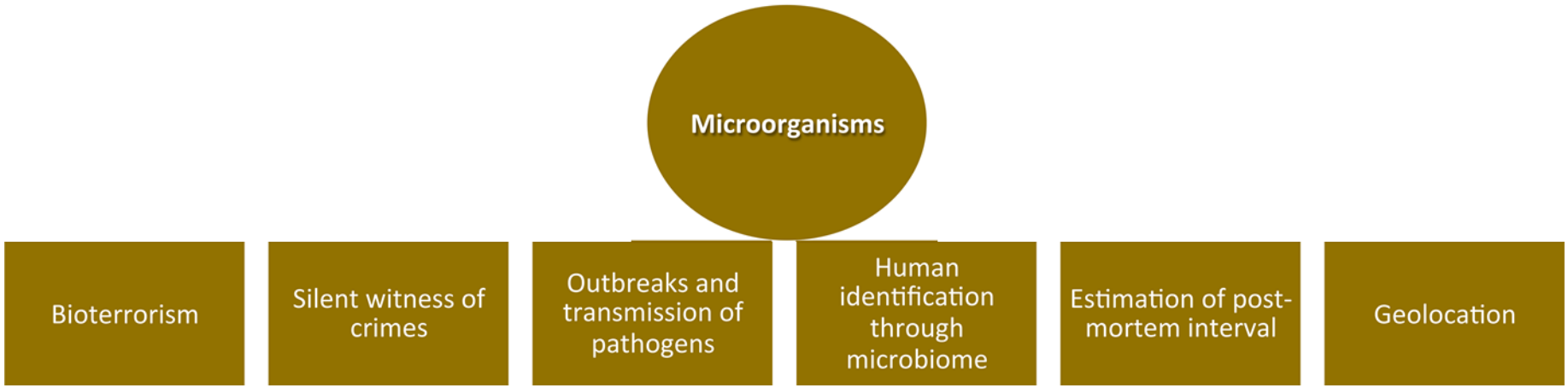
Affiliations + expand

PMID: 26751251 DOI: 10.1016/j.fsigen.2015.12.010

Schmedes SE, Sajantila A, Budowle B. Expansion of Microbial Forensics. J Clin Microbiol. 2016. pmid:26912746.

Budowle B, Schutzer SE, Einseln A, Kelley LC, Walsh AC, Smith JA, et al. Public health. Building microbial forensics as a response to bioterrorism. Science. 2003;301(5641):1852–3. pmid:14512607.

Assiri A, McGeer A, Perl TM, Price CS, Al Rabeeah AA, Cummings DA, et al. Hospital outbreak of Middle East respiratory syndrome coronavirus. N Engl J Med. 2013;369(5):407–16. pmid:23782161; PubMed Central PMCID: PMCPMC4029105.



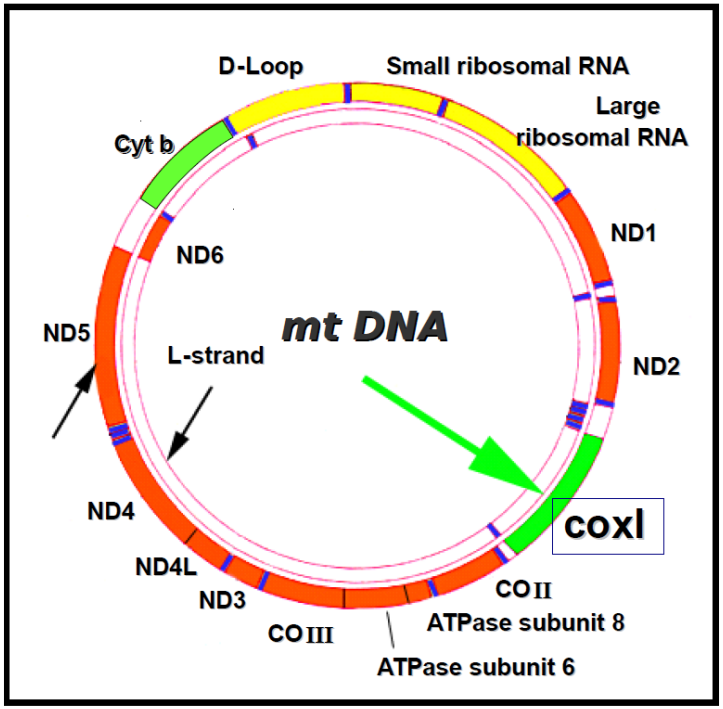
DNA barcoding

DNA BARCODING

A DNA barcode is a short gene sequence taken from standardized portions of the genome, used to identify species.

- **UNIVERSALITY** (standardized, with the same DNA region as far as possible used for a wide range of taxa; e.g. Folmer region; Folmer, 1994).

- **HIGH RESOLUTION** (ability of a certain barcode to differentiate species).



Cosa è il DNA Barcoding

1. Marcatore per l'identificazione di specie e di individui all'interno della stessa specie (**CODICE A BARRE**)

2. Una raccolta di dati (*reference databases*) fruibili anche da non-specialisti
3. Un salto nel passato (possibilità di analizzare campioni museali ed erbari)

Principio generale:

alta variabilità interspecifica

bassa variabilità intraspecifica

DNA BARCODING

- AN INTERNATIONAL NETWORK -

http://www.boldsystems.org

DATABASES

Public Data Portal

A data retrieval interface that allows for searching all 1.3M public records in BOLD using multiple search criteria including, but not limited to, geography, taxonomy, and depository. Search results can be summarized, plotted on high-res maps, and downloaded.

BIN Database

A searchable database of Barcode Index Numbers (BINs), sequence clusters that closely approximate species. This system allows for rapid validation and use of barcode data where taxonomic data are lacking or unverified.

Publications

A collection of barcode publications and publications that have utilized barcode records.

Primer Database

A comprehensive registry of primers used in the generation of barcode sequences. The registry is maintained by users of BOLD.

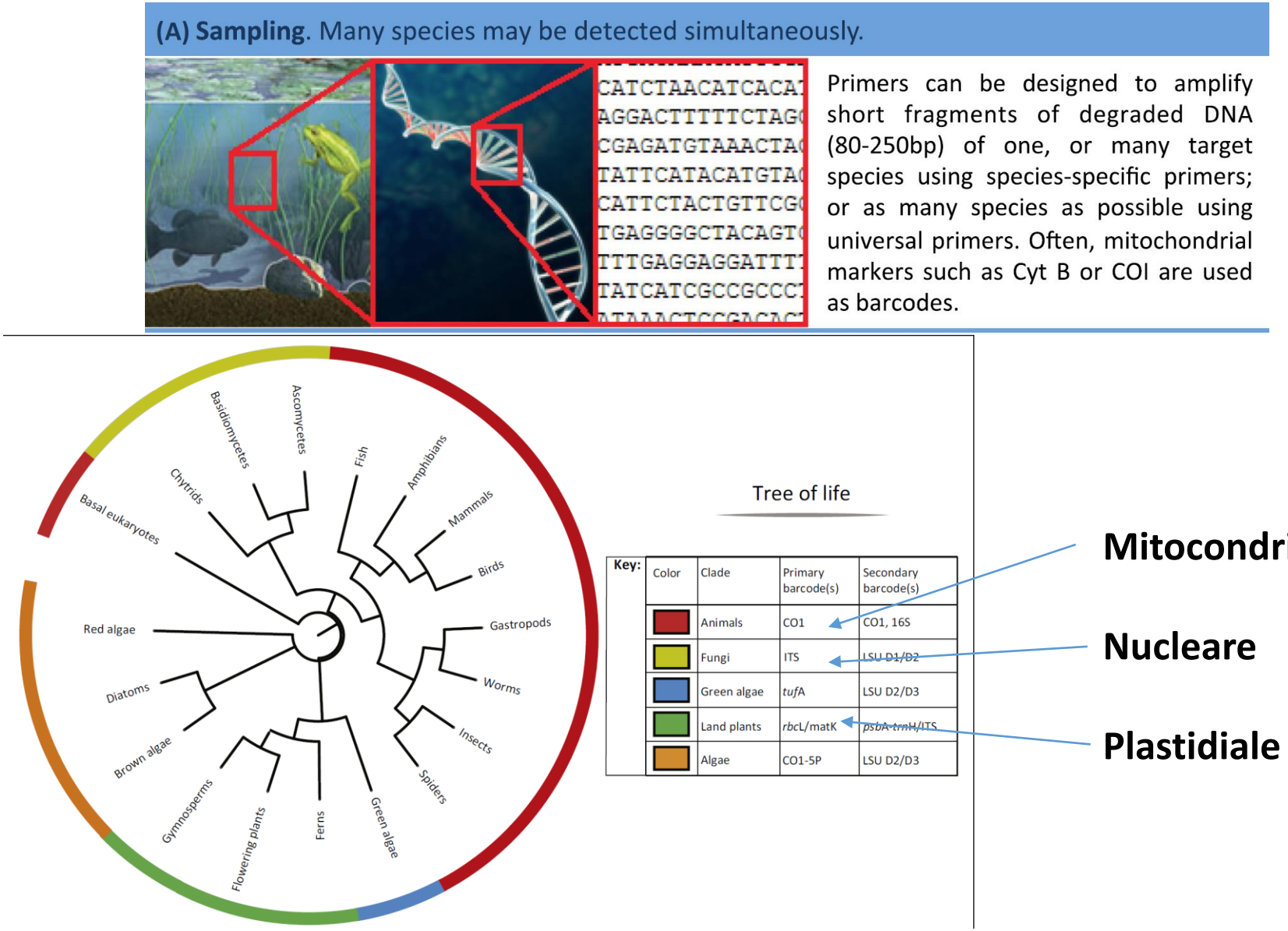


Figure 1. DNA barcodes are becoming a standard tool to discover, describe, and understand biodiversity, specifically to identify species in understudied, microscopic, and highly diverse lineages. DNA barcodes in conjunction with morphological, biochemical, and ecological information are revealing an outstanding diversity of species previously unrecognized through the analysis of morphological variation alone. During the first 8 months of 2014, the Web of Science recorded 310 publications in which DNA barcoding was used in discovering and describing new species, including algae [78], ferns [79], fungi [80], nematodes [81,82], arthropods [83,84], mollusks [85], fish [25], birds [86], and mammals [87]. Early on, it was expected that a single genetic marker would serve as a universal DNA barcode to identify species across the eukaryotic Tree of Life. In practice, different regions of DNA are necessary to provide adequate species identification across lineages. The plastid and nuclear loci indicated here are the most broadly used DNA barcodes for major groups of organisms, including algae [88], land plants [4,89], fungi [5], invertebrates [6], amphibians [7], fish [90], birds [70], and mammals [87,91]. Abbreviations: COI, cytochrome oxidase I; ITS, internal transcribed spacer.

Identificare le specie
IL DNA BARCODING

THE ROYAL SOCIETY

FirstCite® e-publishing

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Biological identifications through DNA barcodes

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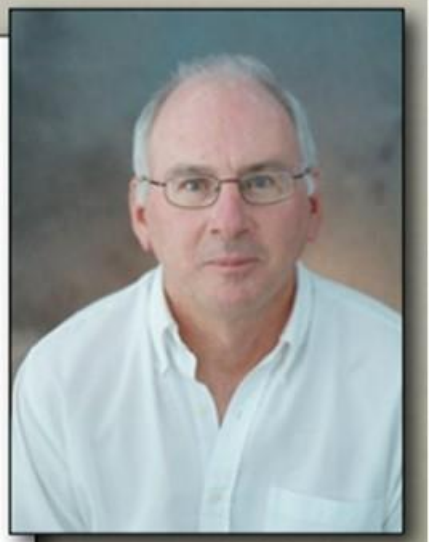
Department of Zoology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Although much biological research depends upon species diagnoses, taxonomic expertise is collapsing. We are convinced that the sole prospect for a sustainable identification capability lies in the construction of systems that employ DNA sequences as taxon 'barcodes'. We establish that the mitochondrial gene cytochrome *c* oxidase I (COI) can serve as the core of a global bioidentification system for animals. First, we demonstrate that COI profiles, derived from the low-density sampling of higher taxonomic categories, ordinarily assign newly analysed taxa to the appropriate phylum or order. Second, we demonstrate that species-level assignments can be obtained by creating comprehensive COI profiles. A model COI profile, based upon the analysis of a single individual from each of 200 closely allied species of lepidopterans, was 100% successful in correctly identifying subsequent specimens. When fully developed, a COI identification system will provide a reliable, cost-effective and accessible solution to the current problem of species identification. Its assembly will also generate important new insights into the diversification of life and the rules of molecular evolution.

Keywords: molecular taxonomy; mitochondrial DNA; animals; insects; sequence diversity; evolution



Paul DN Hebert
Univ. of Guelph
Canada

Da una lezione di M. Casiraghi

Identificare le specie
IL DNA BARCODING

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Biological identificati

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Department of Zoology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

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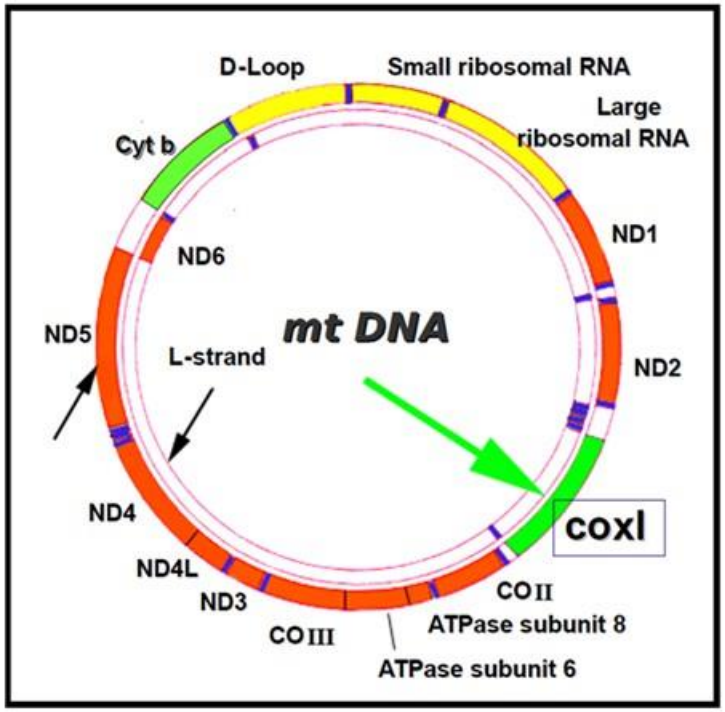
DNA barcoding

Ade esempio, perchè il gene COI?



Regione standard che proviene da un DNA molto abbondante nelle cellule, non ha introni e non ha inserzioni e delezioni.

Primer Database

A comprehensive registry of primers used in the generation of barcode sequences. The registry is maintained by users of BOLD.

Primer Search

SEARCH: Go

IUPAC degenerate base symbols[2]

Description	Symbol	Bases represented					Complementary bases[a]
		№	A	C	G	T	
Adenine	A	1	A				T
Cytosine	C			C			G
Guanine	G				G		C
Thymine	T					T	A
Uracil	U					U	A
Weak	W	2	A			T	W
Strong	S			C	G		S
Amino	M		A	C			K
Keto	K				G	T	M
Purine	R		A		G		Y
Pyrimidine	Y			C		T	R
Not A[b]	B	3		C	G	T	V
Not C[b]	D		A		G	T	H
Not G[b]	H		A	C		T	D
Not T[b]	V		A	C	G		B
Any one base	N	4	A	C	G	T	N
Zero	Z	0					Z

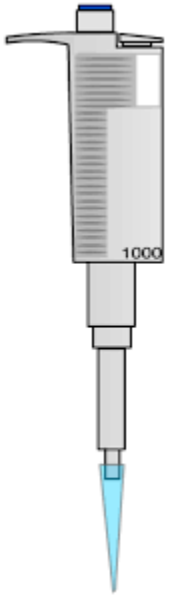
a. ^ I.e., here, read the represented bases in reverse.
b. ^ a b c d Represented by the letter following (excluding U).

SEARCH: shark Go

```
>FISHCOX1R
TARACTTCWGGGTGRCCRAAGAATCA
>S083
TCTACYAACCACAAAGAYATCGGCAC
>S259
ACYAAYCAYAAAGAYATYGGYACC
>R263
TADACTTCIGGRTGICCRAARAATCA
```

Primer degenerati: PCR primer in cui alcune posizioni hanno più di una base possibile.

DNA barcoding: problemi legati al laboratorio



- bisogna disegnare primers che vadano bene per molte OTU

```
>FISHCOX1R
TARACTTCWGGGTGRCCRAAGAATCA
>S083
TCTACYAACCACAAAGAYATCGGCAC
>S259
ACYAAYCAYAAAGAYATYGGYACC
>R263
TADACTTCIGGRTGICCRAARAATCA
```

- bisogna trovare il giusto marcatore (che sia cioè in grado di distinguere tra diverse OTU)
- bisogna amplificare specificamente (non vogliamo contaminazioni da altri marcatori)
- la PCR fa errori, Se il DNA è degradato è difficile amplificare marcatori lunghi > 200 bp

DNA barcoding: problemi con l'analisi dei dati..cosa c'è nel mio campione??

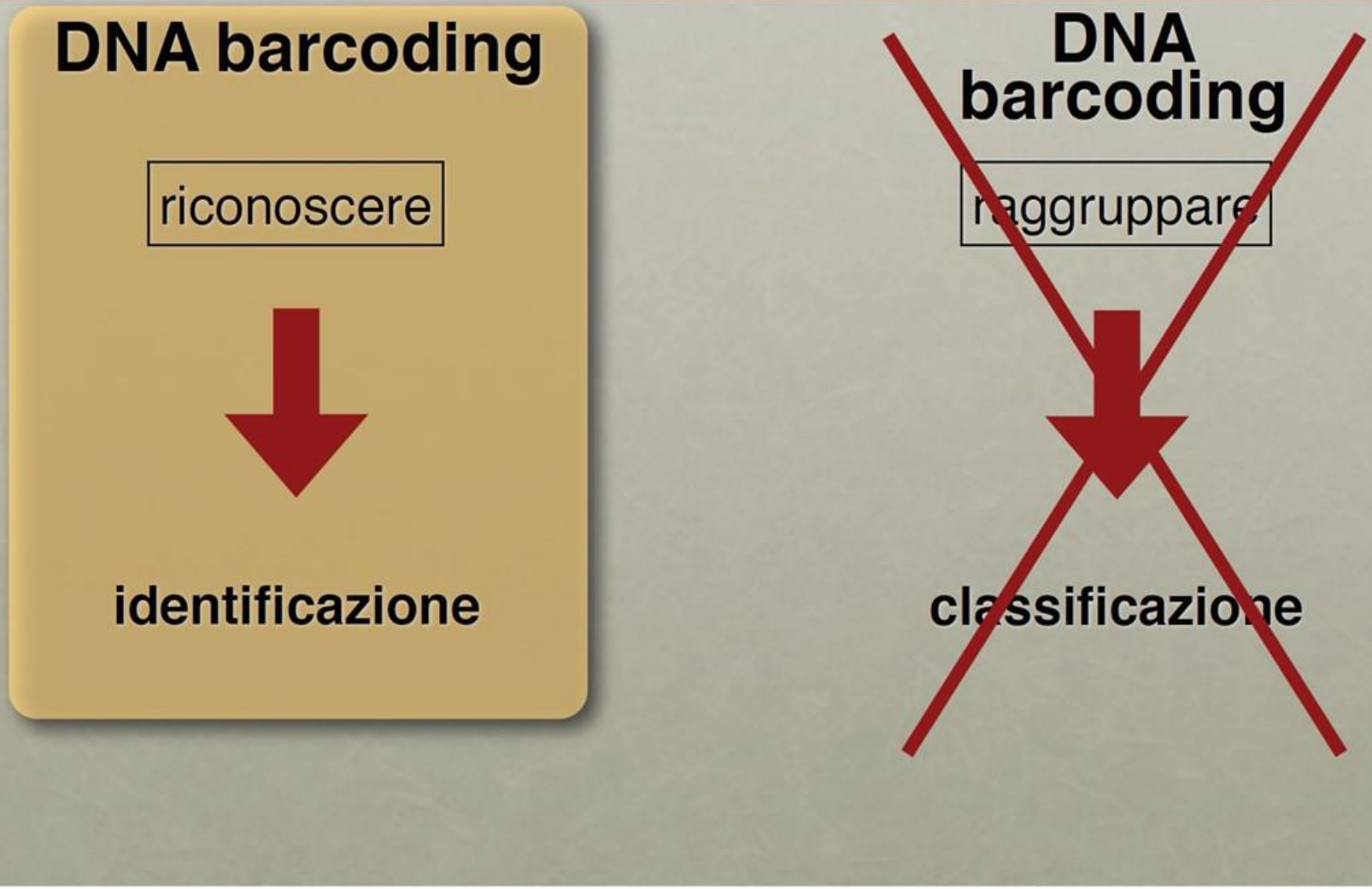
- bisogna avere un database di riferimento che contenga quel marcatore per diversi organismi

- Se si fa una PCR si perde l'informazione dell'abbondanza relativa

DNA barcoding: vantaggi

- Pochi primers per amplificare i frammenti dal maggior numero di diversi organismi possibile
- Pochi metodi di analisi bioinformatica del dato
- pochi marcatori per il maggior numero di specie possibile: mantieni il sistema semplice ed economico!
- il DNA barcoding introduce una standardizzazione negli approcci al modo di collezionare, di trattare i campioni, di analizzare e interpretare i dati
- il DNA barcoding si basa sulla informatizzazione e sul networking; la standardizzazione e la computerizzazione rappresentano uno degli sforzi più grandi nella "democratizzazione" del mondo della ricerca

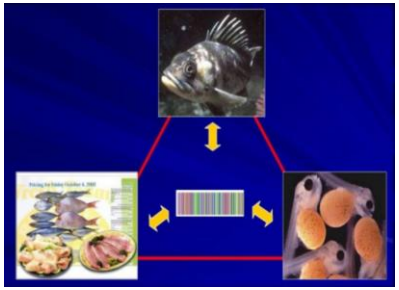
Identificare le specie



DNA barcoding: vantaggi

- campioni difettosi o loro frammenti che non sono adatti alla produzione di preparati morfologici qualitativi

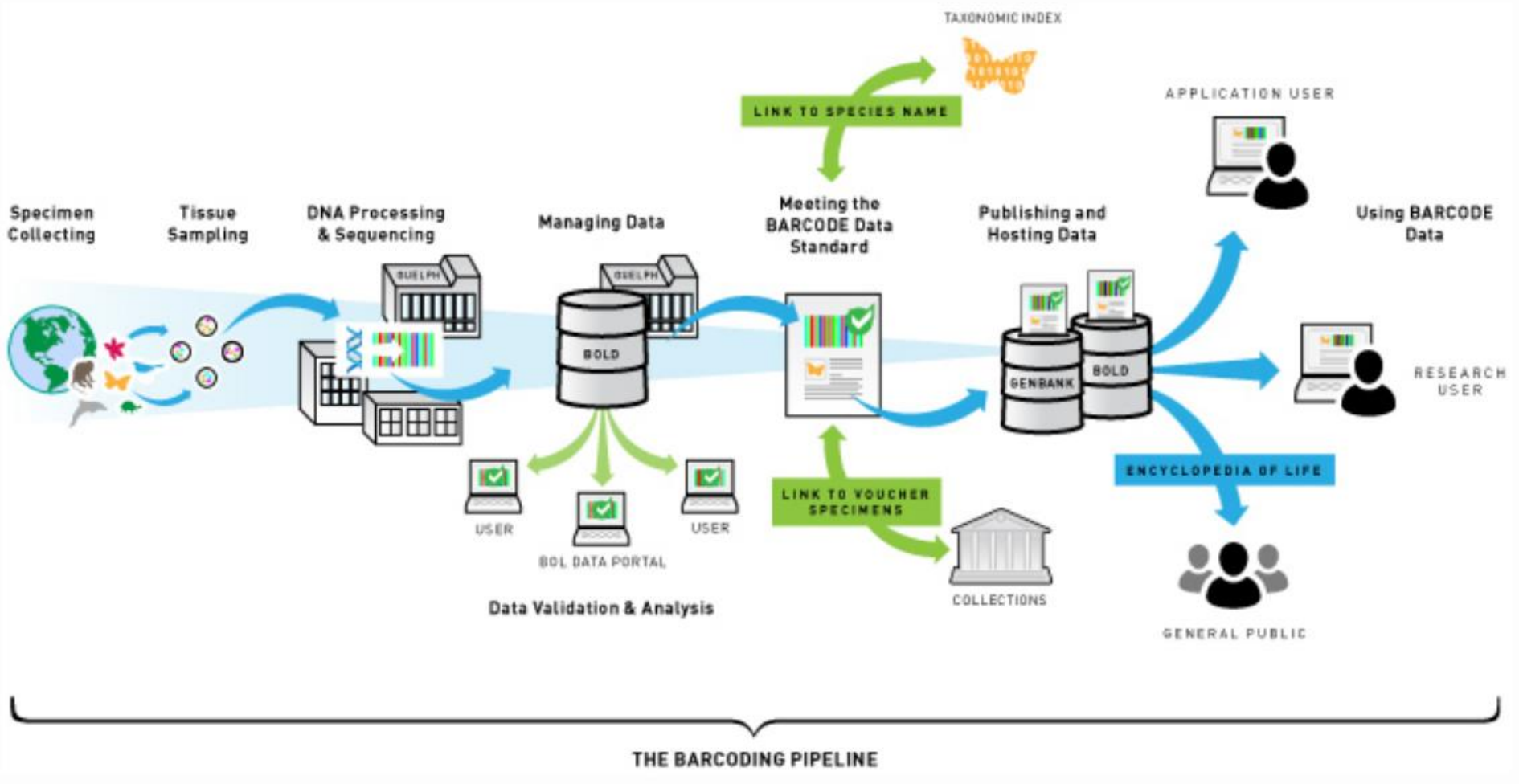
- campioni in qualsiasi fase del ciclo di vita (uova e larve, semi, polline)



- vecchi campioni di raccolta, la certificazione del DNA degli olotipi, senza perdita del campione

- campioni paleontologici (da congelamento eterno, resina di legno, ambra).

Pipeline


TAXONOMIC INDEX
LINK TO SPECIES NAME
APPLICATION USER
Specimen Collecting
Tissue Sampling
DNA Processing & Sequencing
Managing Data
Meeting the BARCODE Data Standard
Publishing and Hosting Data
Using BARCODE Data
BOLD
GENBANK
BOLD
RESEARCH USER
USER
USER
BOL DATA PORTAL
LINK TO VOUCHER SPECIMENS
ENCYCLOPEDIA OF LIFE
COLLECTIONS
GENERAL PUBLIC
Data Validation & Analysis
THE BARCODING PIPELINE

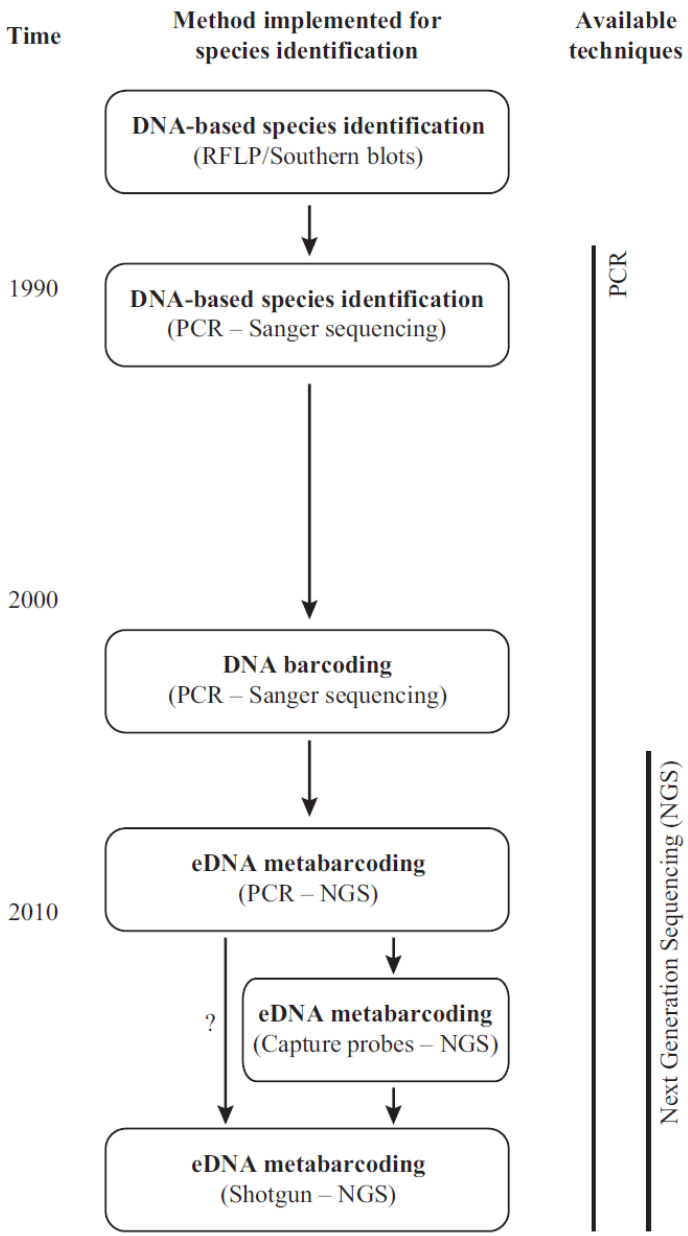
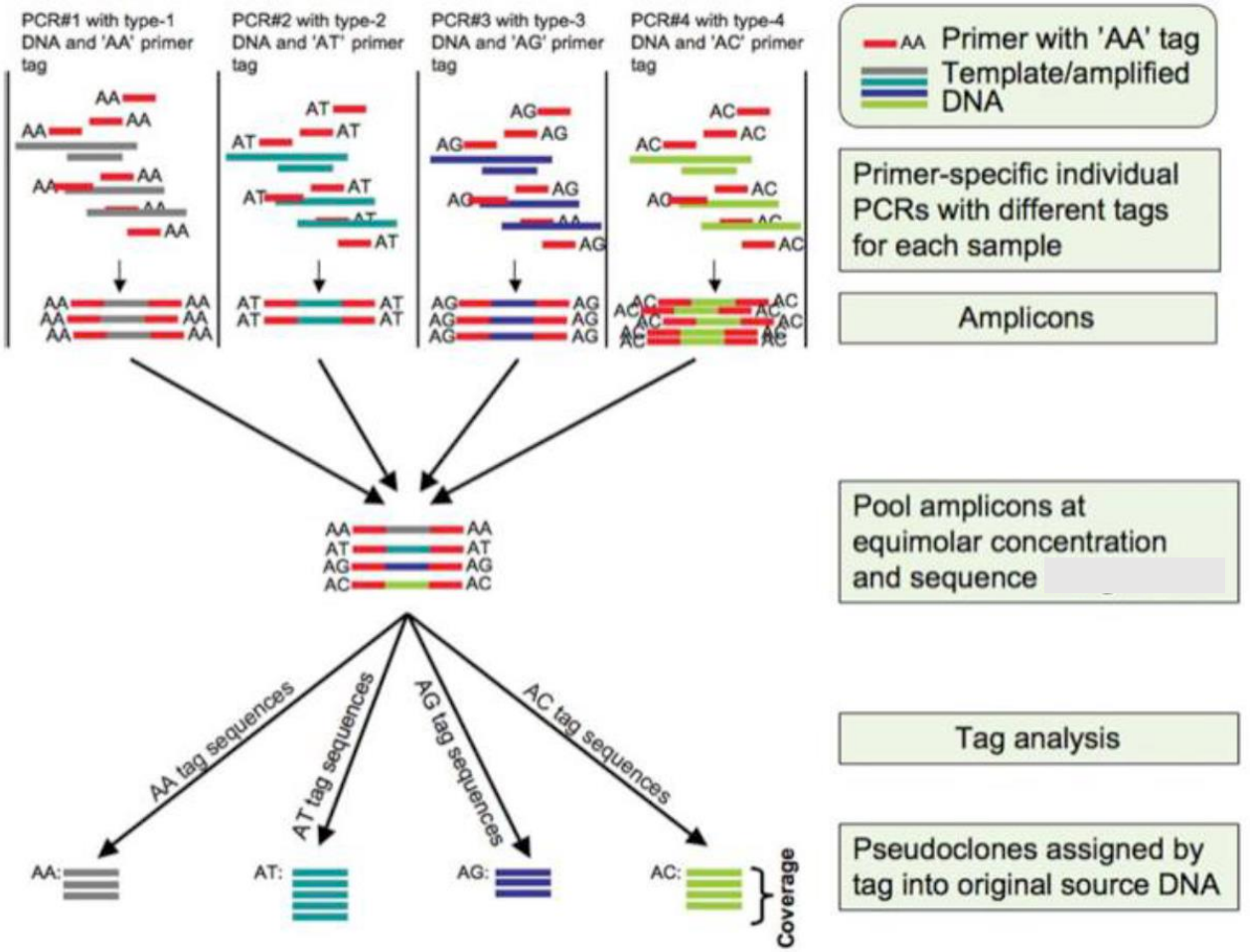
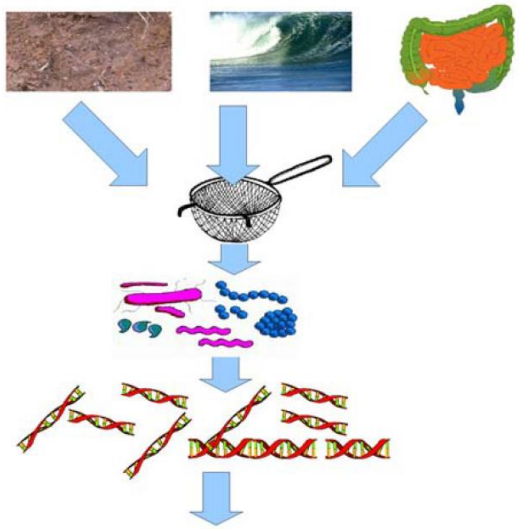


Fig. 1 DNA-based species identification. Past and current approaches, and possible future trends.

Amplificazione di un marcatore per distinguere le OTU e tag per distinguere gli individui



Con i nuovi metodi di sequenziamento e l'aumento di informazioni nelle banche dati si può passare ad uno **shotgun sequencing** (simile ad un sequenziamento genomico, ma di tutto ciò che è stato raccolto nel campione) (vedere il progetto http://extrememicrobiome.org/)

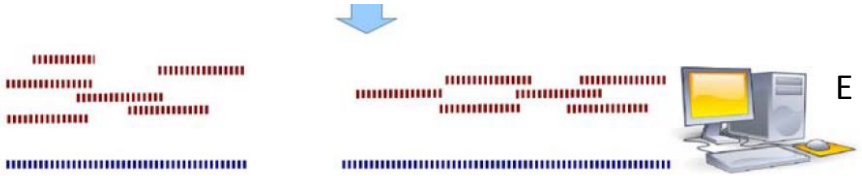


Next-generation sequencing

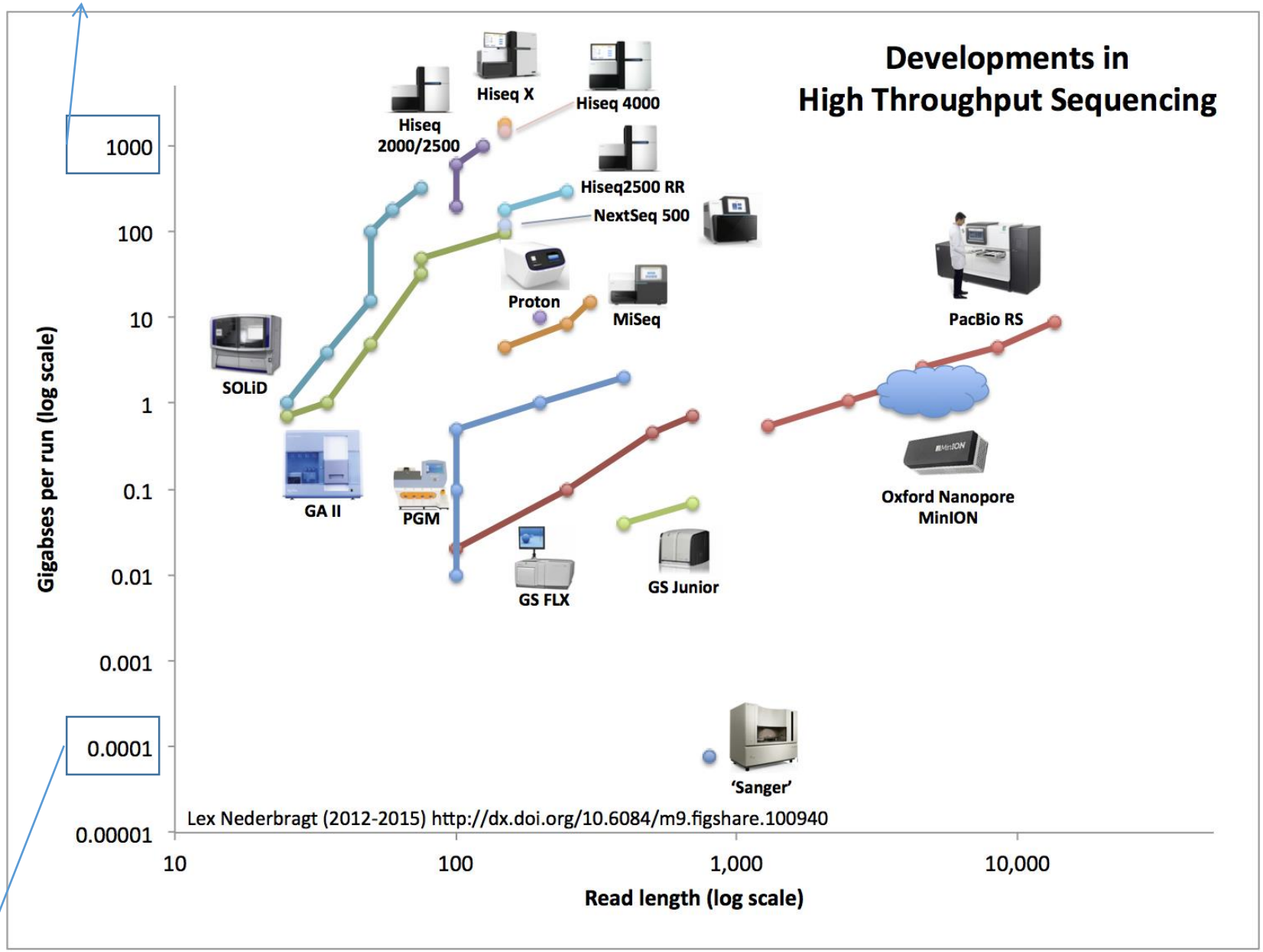
D

http://blog.booleanbiotech.com/nanopore_2016.html

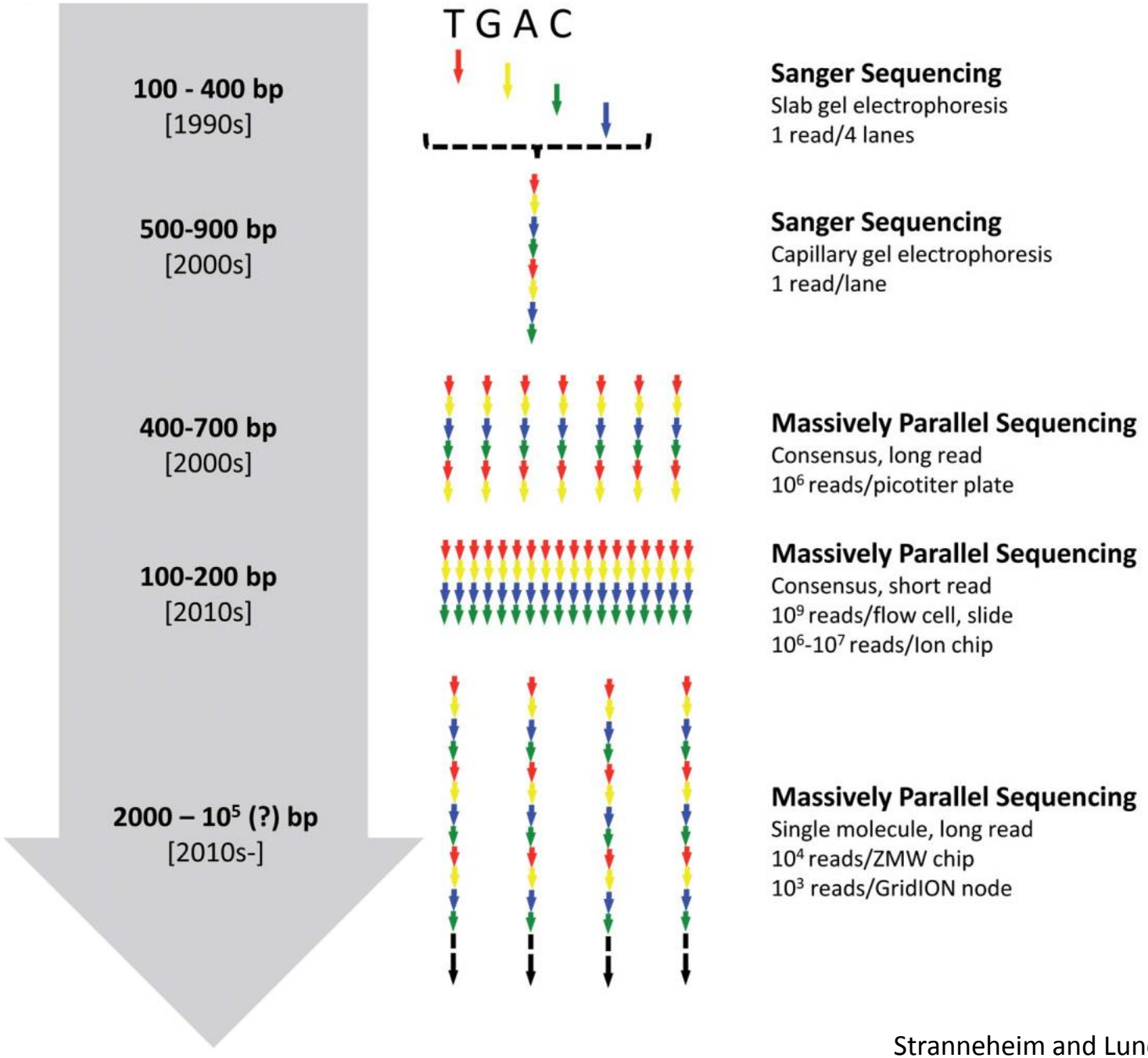
(A) Sampling from habitat
(B) filtering particles, typically by size
(C) DNA extraction and lysis
(D) sequencing
(E) sequence assembly.



Mille miliardi di basi

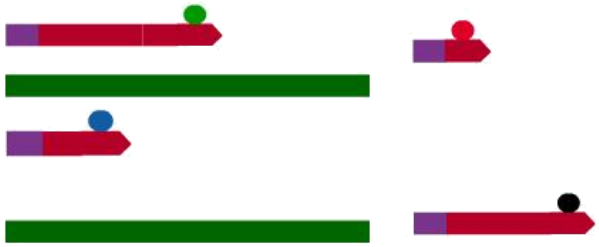


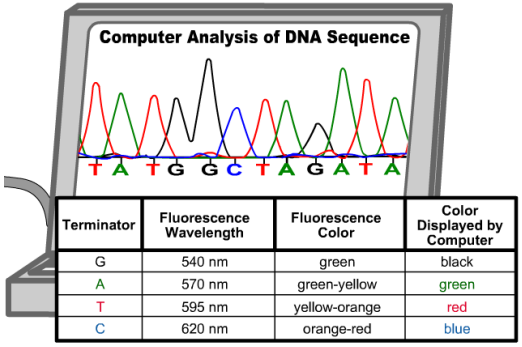
Centomila basi



Stranneheim and Lundeberg 2012

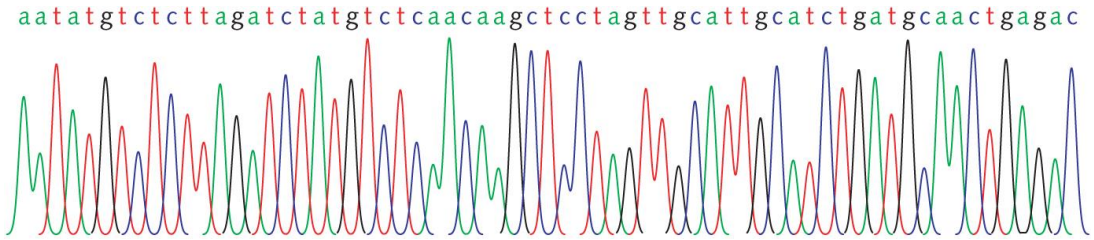
Capillary Electrophoresis ABI 3730, **96**-capillary





Terminator	Fluorescence Wavelength	Fluorescence Color	Color Displayed by Computer
G	540 nm	green	black
A	570 nm	green-yellow	green
T	595 nm	yellow-orange	red
C	620 nm	orange-red	blue

Cromatogramma



Next generation sequencing

File output FASTQ

```
@M02520:79:000000000-ADW7F:1:1101:18569:1519 1:N:0:22

CCTACGGGAGGCTGCAGTGAGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCGACCCCGCGTGTTTGACGACACCCTTTCCC
TTCGTAAACTCCTTTTCTCAGGCCCGTATTTTTTTTCTCTACCTCTCCTTTTCCCTCCCCCTTATTCTTTGCCAGCAGCCCCTGT
CAGTCTGCTGTCGCAAGCTTTTCTCCGTCTTCCTGTCCTTTTTGCTTCTGCTGCCCCCCCCCCTACCTTTTGTTTAACATTCCCTA
CCTCCCCTTCCTCCCCGCCCCTATCCCGCTCTCTCTCCCCTTACT

+

-8ACC=D=5=)8C;B6CFA,@<A8CCEFAF;BB7FE--;@+:+@CEEA,;,+,8@++6+8B+@:@7,,,4,5,,84+:=?A,
9:,,,,+9,,+59C,9,5555,,,,,+86++,:8586++3,,3,383:,,,,,,,,,3,:3++35,,3737,7,,7,,6,,
56*8**8,,,,7,,,,2,,46***1,76,727+***44,++++*5+2+++*++543++,++2**/*1)*1*0*020**0)
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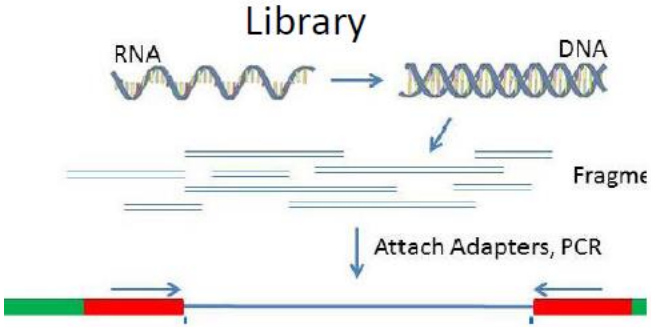
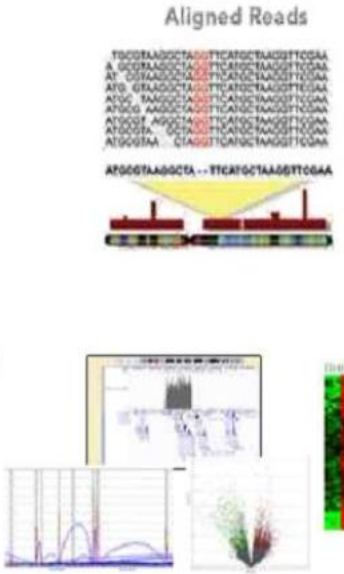
Basic NGS Workflow

Samples preparation



Application specific

Sequencing



Data Analysis

Application Specific

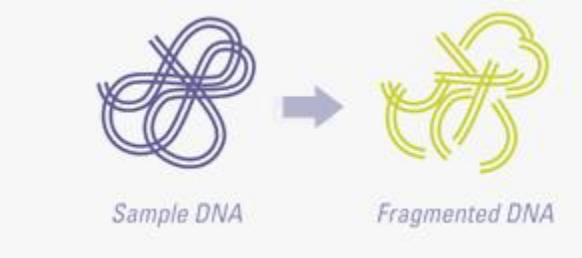
Olson *et al.*

Preparazione della *library*

Il DNA va pre-processato prima di essere sequenziato

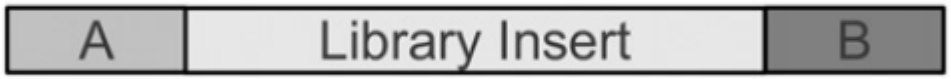
Passaggi:

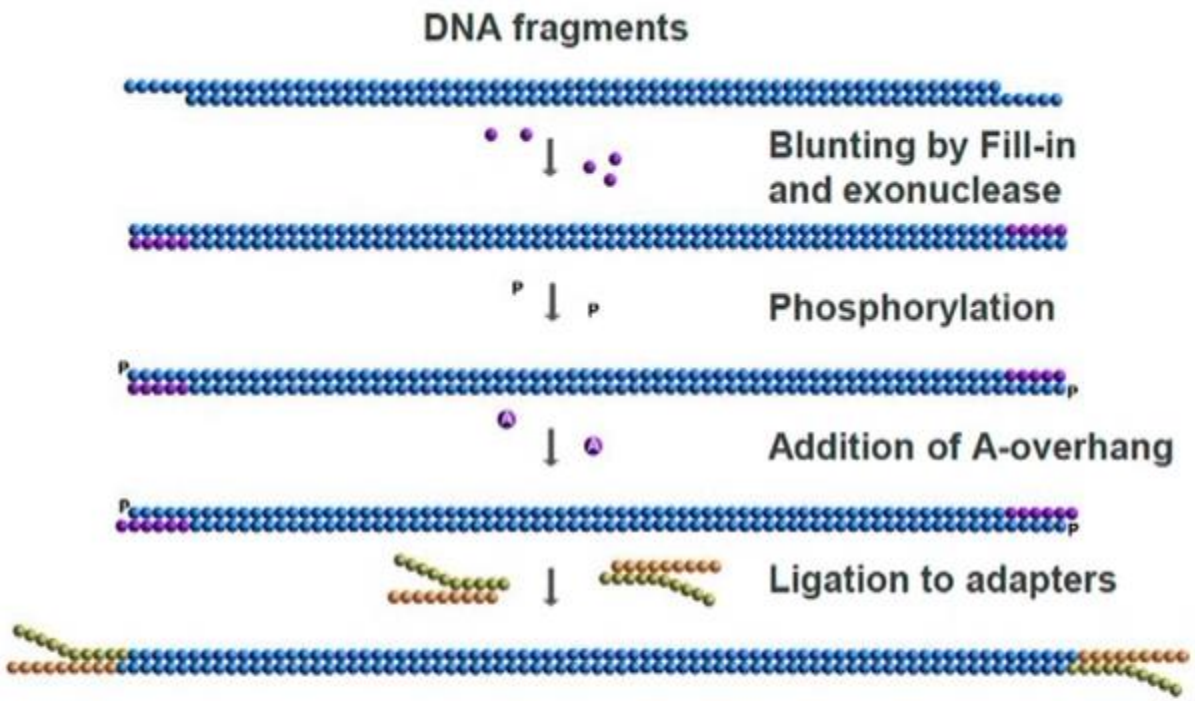
1. Frammentazione (fisica o enzimatica)



2. Le estremità dei frammenti vengono riparate e rese «blunt» o con specifici nucleotidi terminali (es A o T)

3. Alle estremità vengono legati degli adattatori specifici per ogni NGS technology



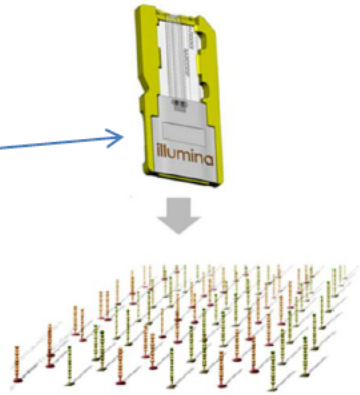


- Shear high molecular weight DNA with sonication
- Enzymatic treatments to blunt ends
- Ligate synthetic DNA adapters (each with a DNA barcode), PCR amplify
- Quantitate library
- Proceed to WGS, or perform exome or specific gene hybrid capture

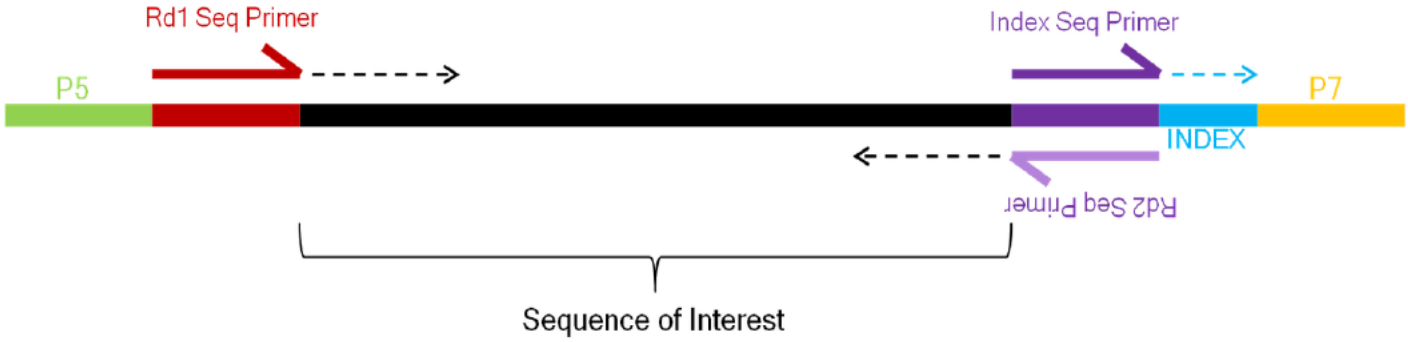
Preparazione della *library:* Illumina

Illumina adaptors

(gli adattatori servono a legare il frammento alla cella dove avviene l'amplificazione e il sequenziamento (P5/P7) e fungono da primer per la reazione di amplificazione prima e di sequenziamento poi)
(Rd1 e Rd2 servono per il paired-end sequencing = sequenziamento a partire da entrambe le estremità di un frammento)



P5/ P7: binding sites to the flow cell
Rd 1 SP: read1 sequencing primer
Rd 2 SP: read2 sequencing primer

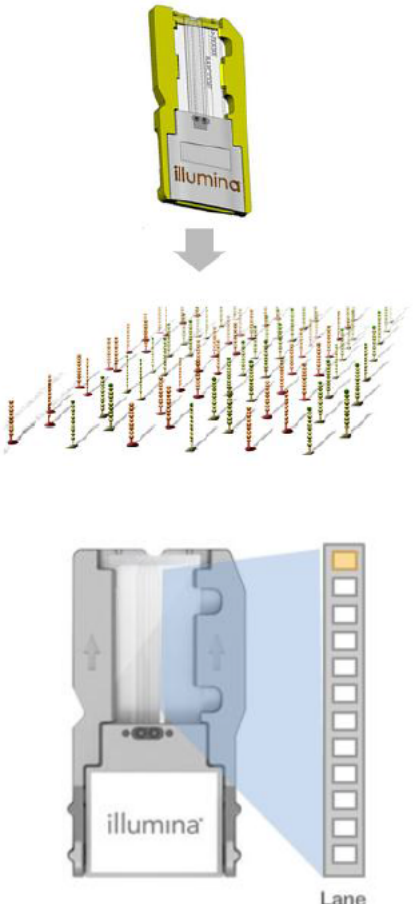


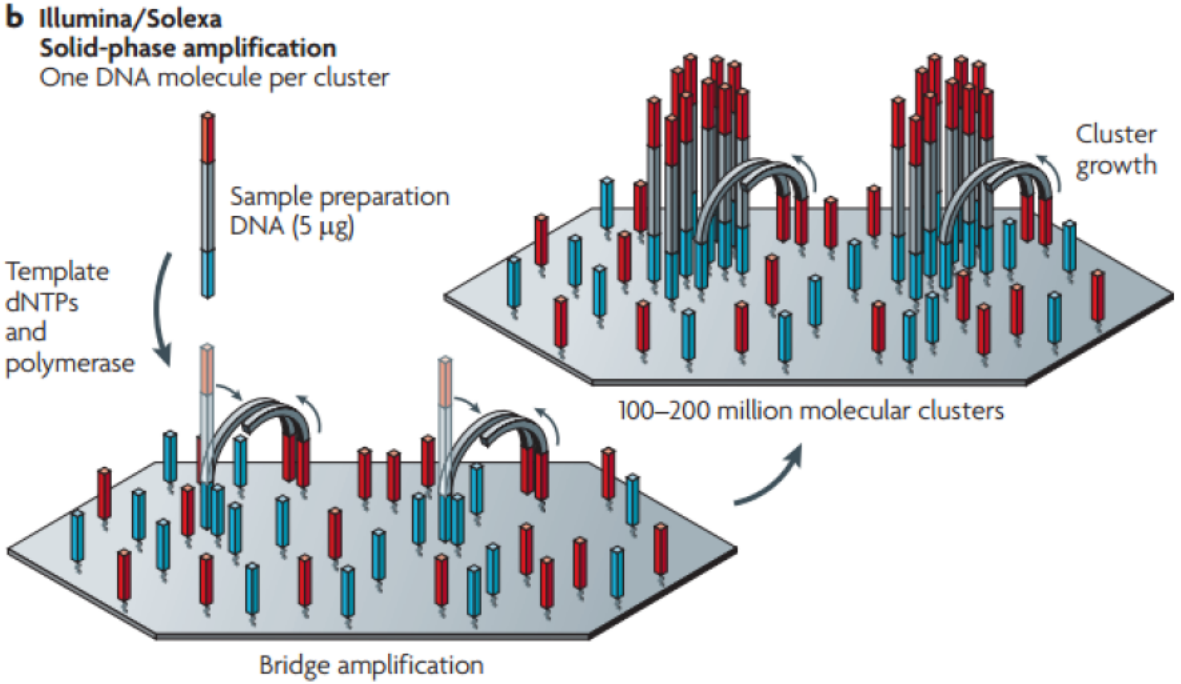
http://nextgen.mgh.harvard.edu/IlluminaChemistry.html

Amplificazione clonale della library
(già nello strumento per NGS!)

Metodologie principali

- Solid-phase cluster generation (Illumina)

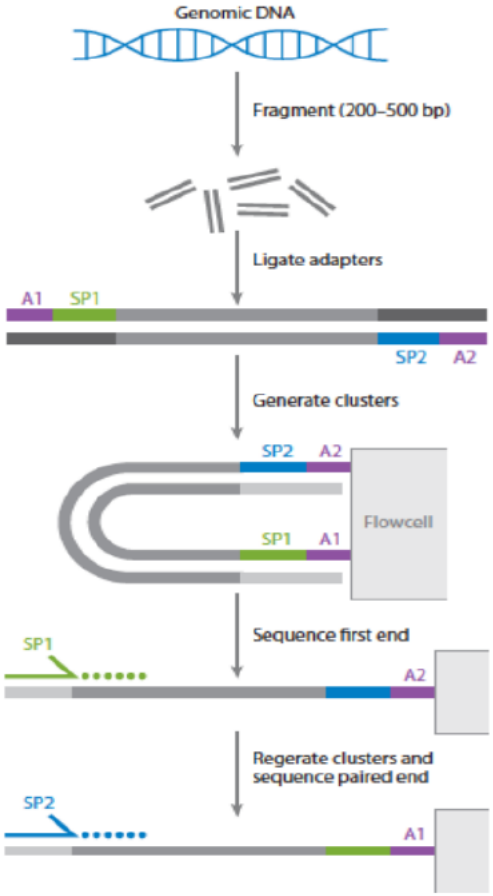




1,000 copies of each fragment are generated by bridge amplification, creating clusters (30-50 million of clusters).

https://www.youtube.com/watch?v=ZlPH9FzUOq8
https://www.youtube.com/watch?v=fCd6B5HRaZ8

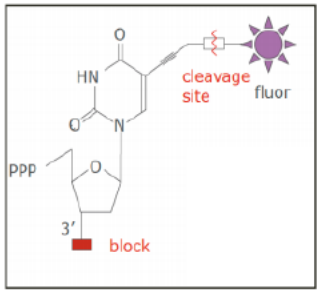
Illumina: summary



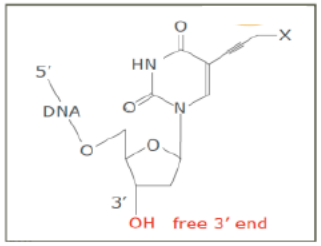
- Shear high molecular weight DNA with sonication
- Polish ends
- Ligate synthetic DNA adapters
- Produce size fractions
- Quantitate
- Amplify library fragments on flow cell surface
- Denature clusters to single-stranded
- Hybridize sequencing primer to linearized ss cluster DNAs
- Proceed to sequencing or hybrid capture

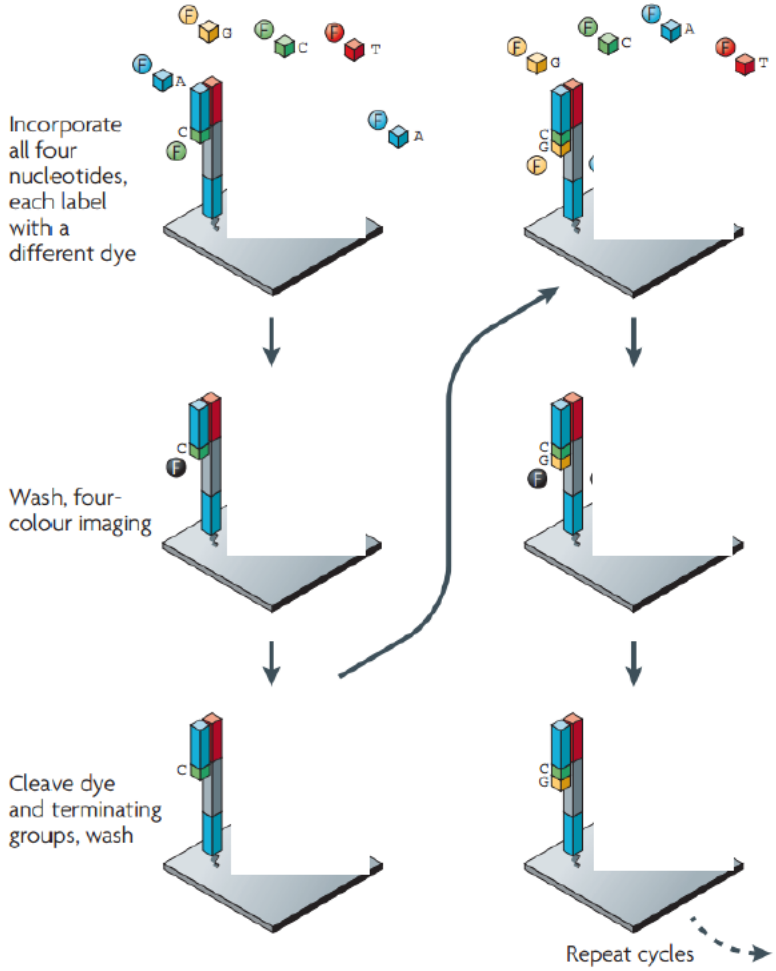
Sequenziamento e rilevazione del segnale

Reversible terminators (Illumina)



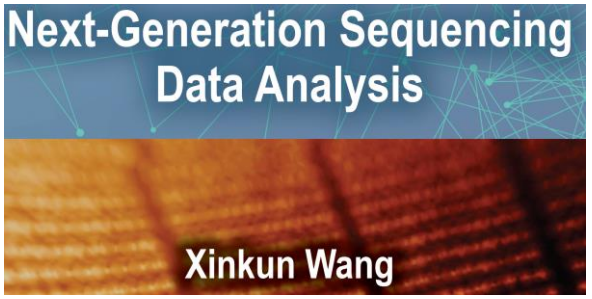
Incorporate
Detect
De-block
Cleave fluor





Mod. from Metzker 2010

Quali campioni e quali dati sono tipici della **metagenomica**?



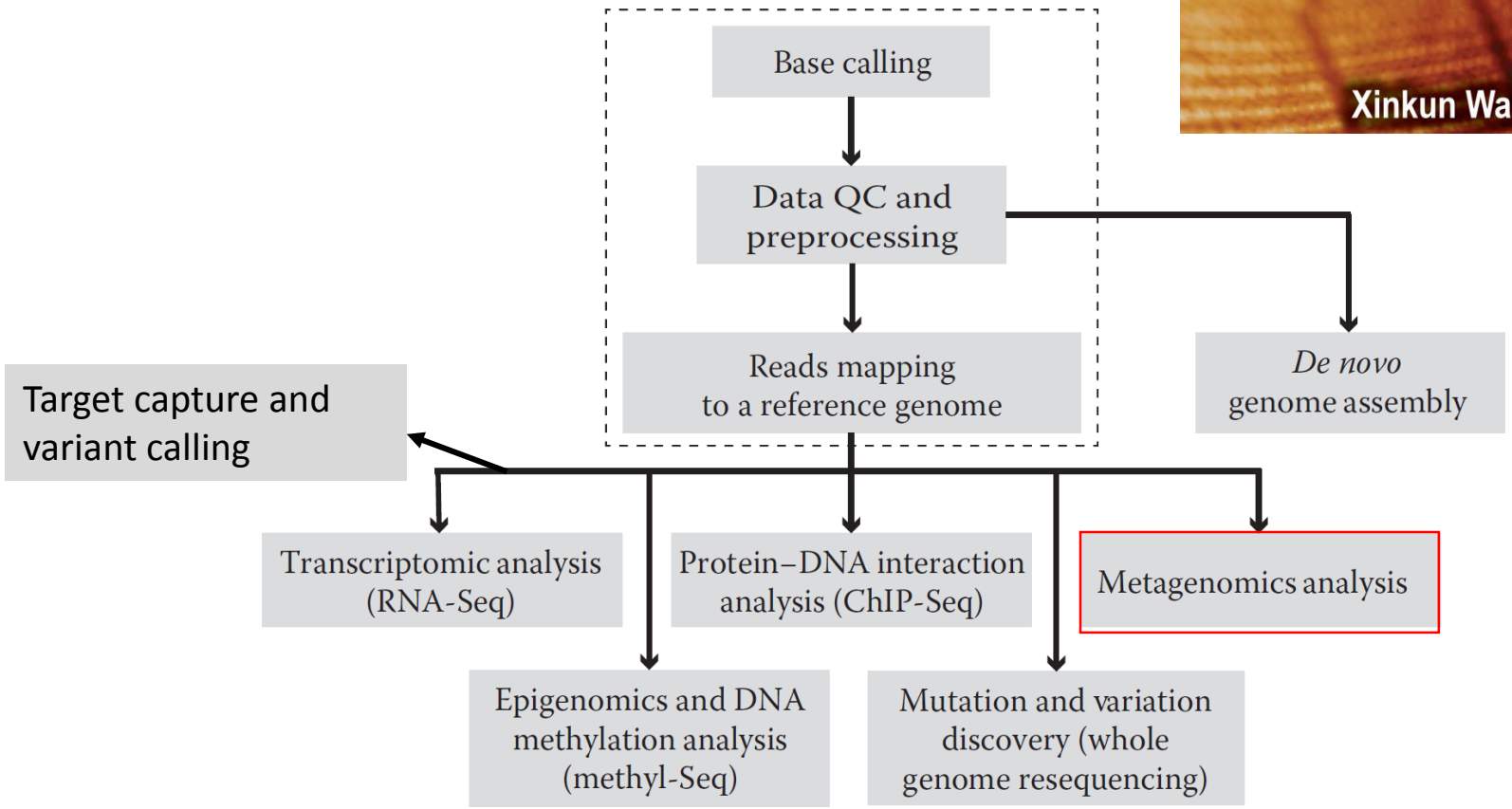
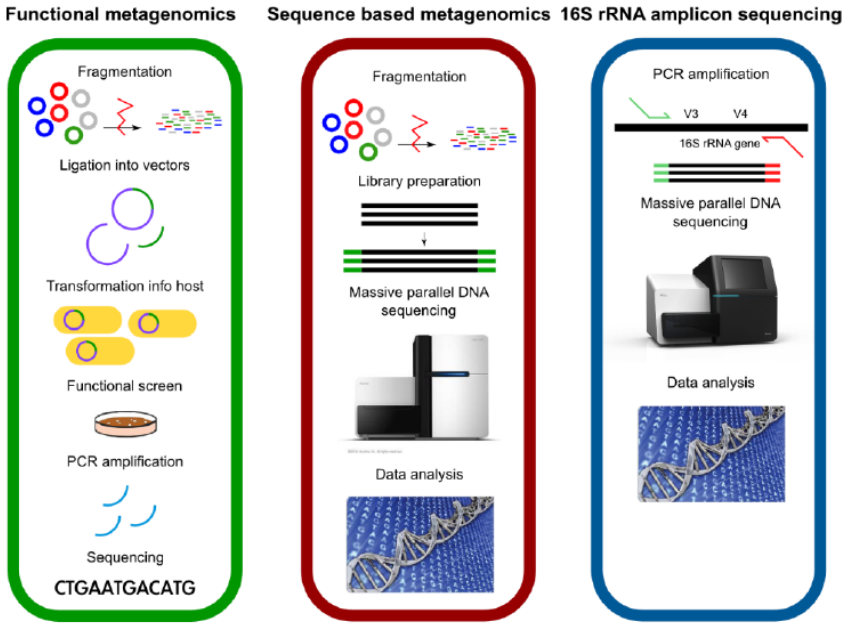


FIGURE 5.1
General overview of NGS data analysis. The steps in the dashed box are common steps conducted in primary and secondary analysis.

Types of metagenomics

Sample collection

DNA extraction

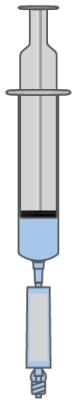


Shot-gun (sequenzio tutto!)

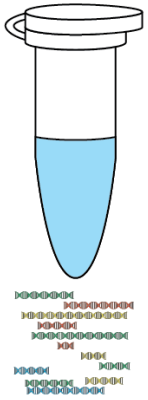
Amplicon-based

Metagenomics

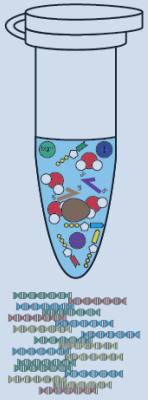
1 Collect an environmental sample



2 DNA extraction from environmental sample



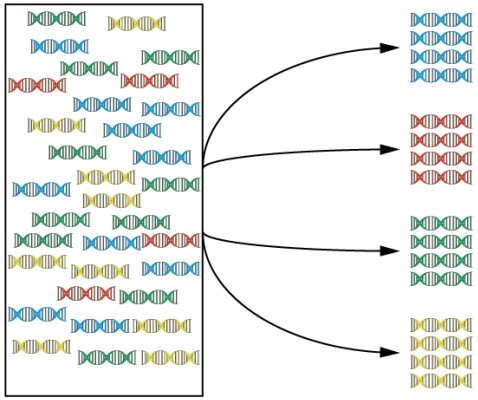
3 Amplify DNA markers



Can be avoided

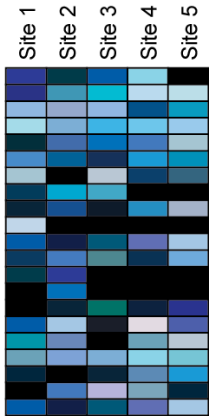
4 High-throughput sequencing

5 Bioinformatic processing



6 Species identification

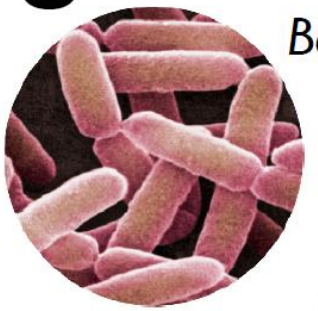
7 Ecological analysis



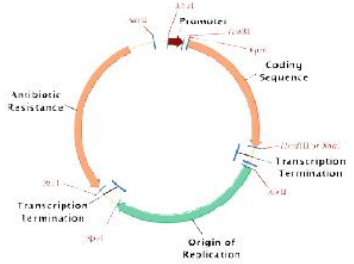
Shotgun metagenomics

- Investigate *all* organisms (and genomic elements)
- Known/unknown
- Culture / Non-culturable (large fraction non-cult.)

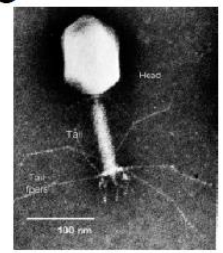
Bacteria



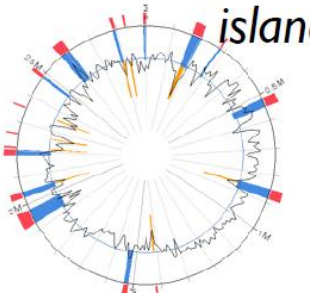
plasmids



phages

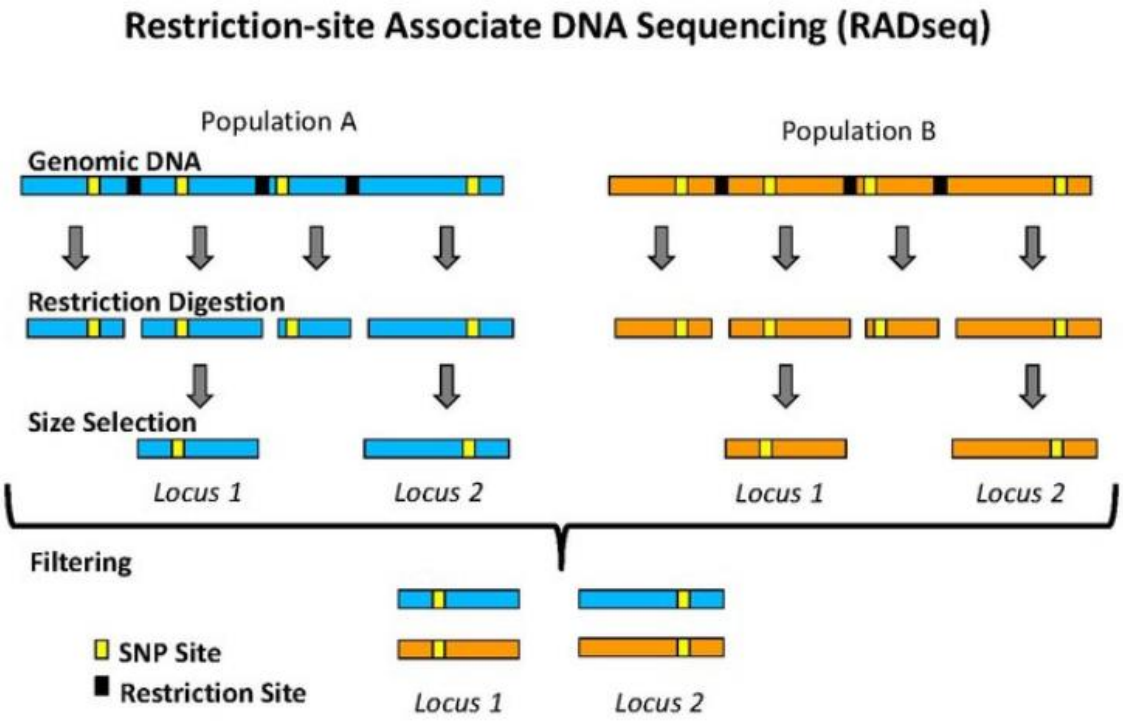


genomic islands



RAD: Restriction site associated DNA markers

Permettono di isolare RAD tags: sequenze di DNA immediatamente fiancheggianti regioni riconosciute da enzimi di restrizione sparse nel genoma. Se contengono siti variabili possono essere usate per genotyping di SNPs (RAD markers).

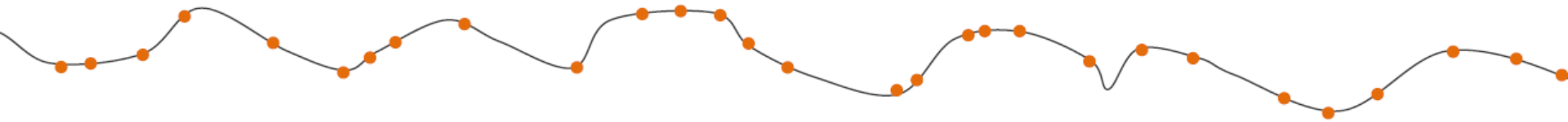


RADseq: a reduced-representation of the genome

By Emiliano Trucchi

RADseq: a reduced-representation of the genome

Whole genomic DNA digestion by restriction enzymes



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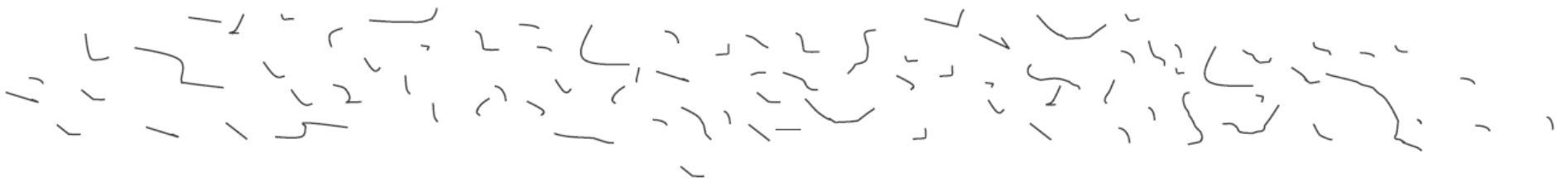


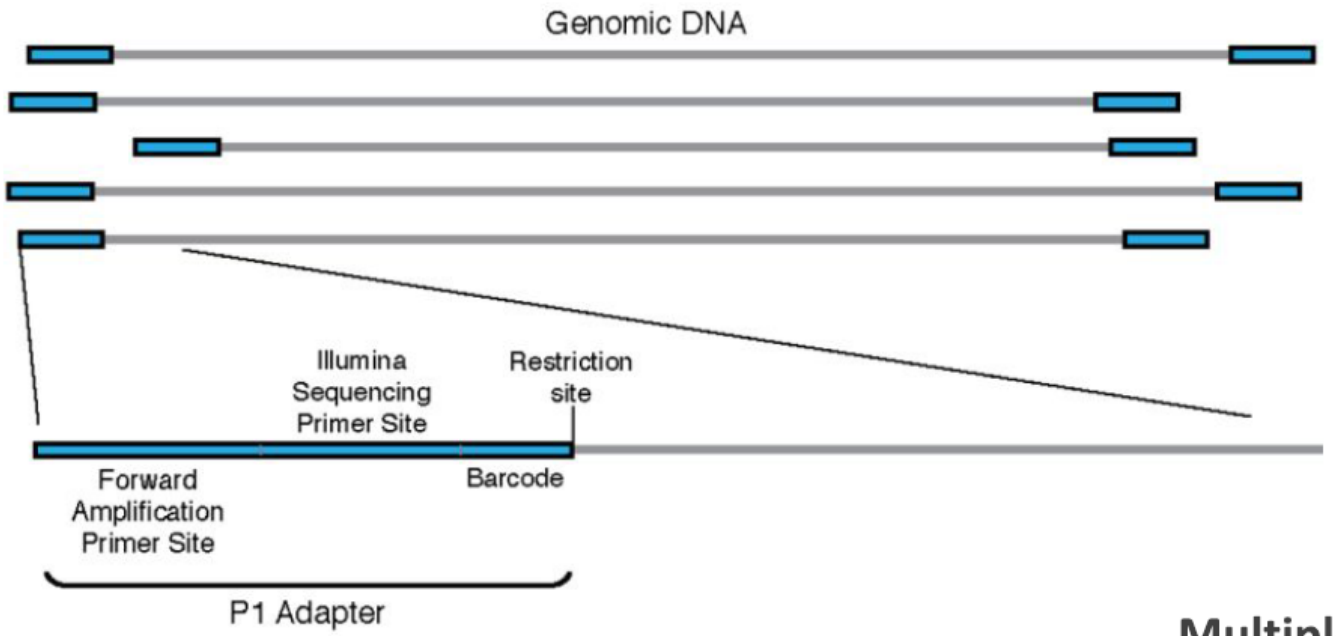
Non-random fragmentation!

By Emiliano Trucchi

RADseq: a reduced-representation of the genome

Whole genomic DNA digestion by restriction enzymes

Ligation of barcoded adapters to restriction site overhangs



Multiplexing!

By Emiliano Trucchi

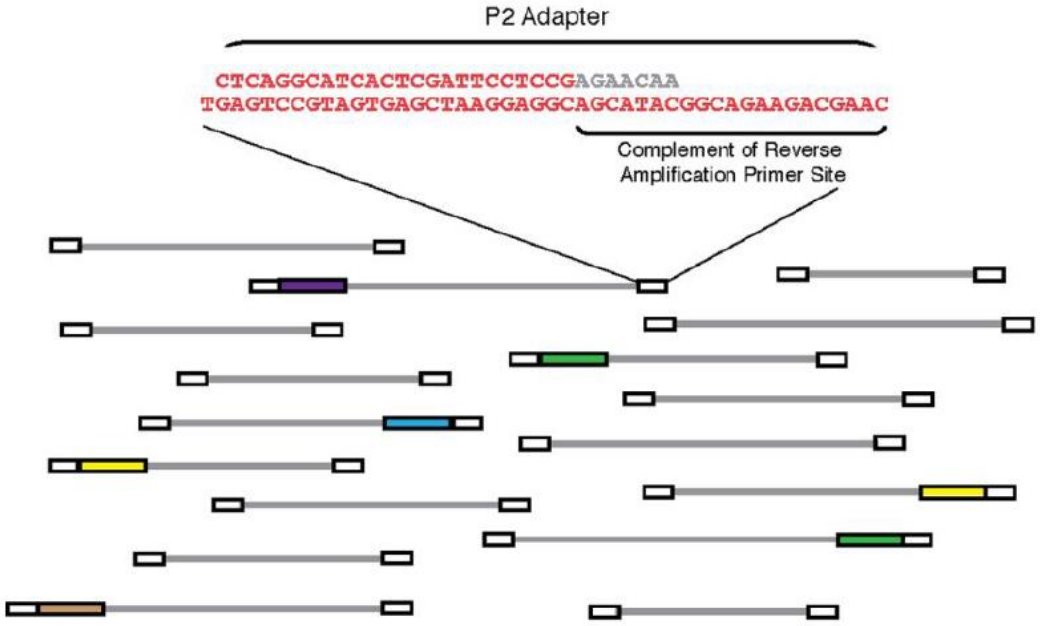
RADseq: a reduced-representation of the genome

Whole genomic DNA digestion by restriction enzymes

Ligation of barcoded adapters to restriction site overhangs

Pooling, Sonication and size selection

Ligation of Y-shaped adapters



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RADseq: a reduced-representation of the genome

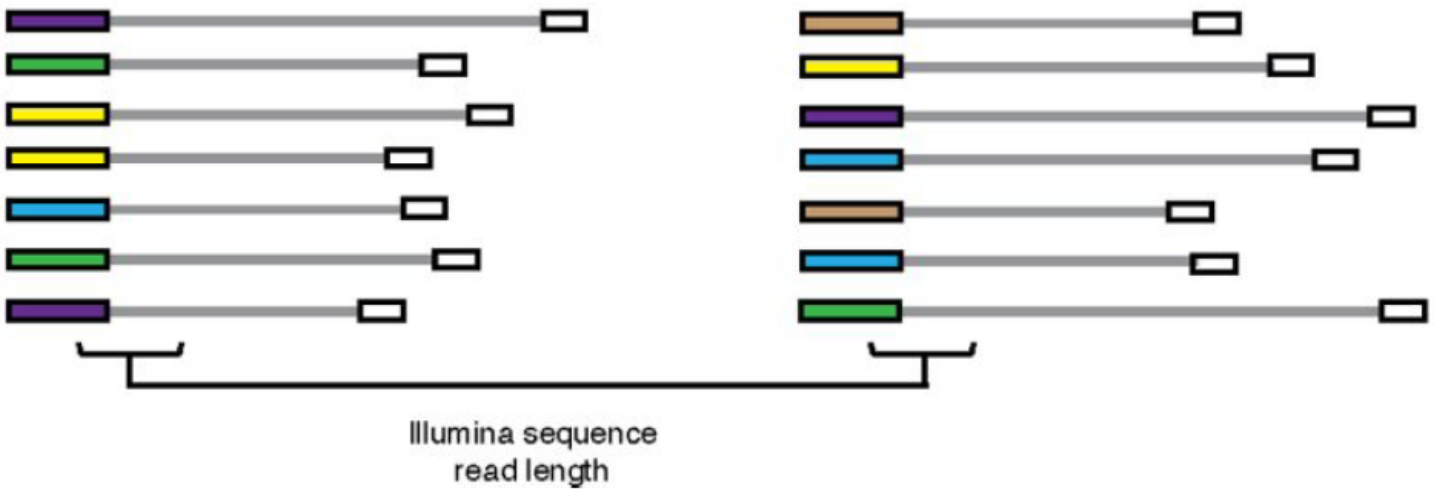
Whole genomic DNA digestion by restriction enzymes

Ligation of barcoded adapters to restriction site overhangs

Pooling, Sonication and size selection

Ligation of Y-shaped adapters

Library enrichment by PCR amplification



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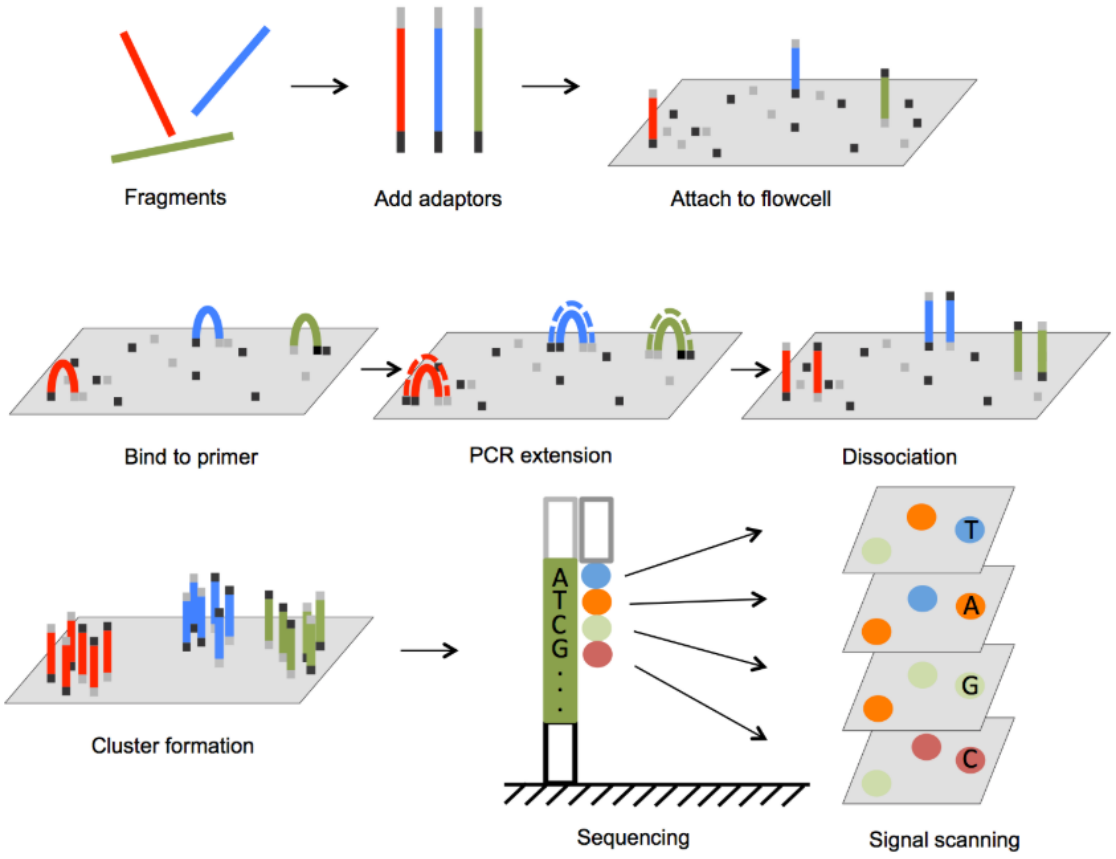
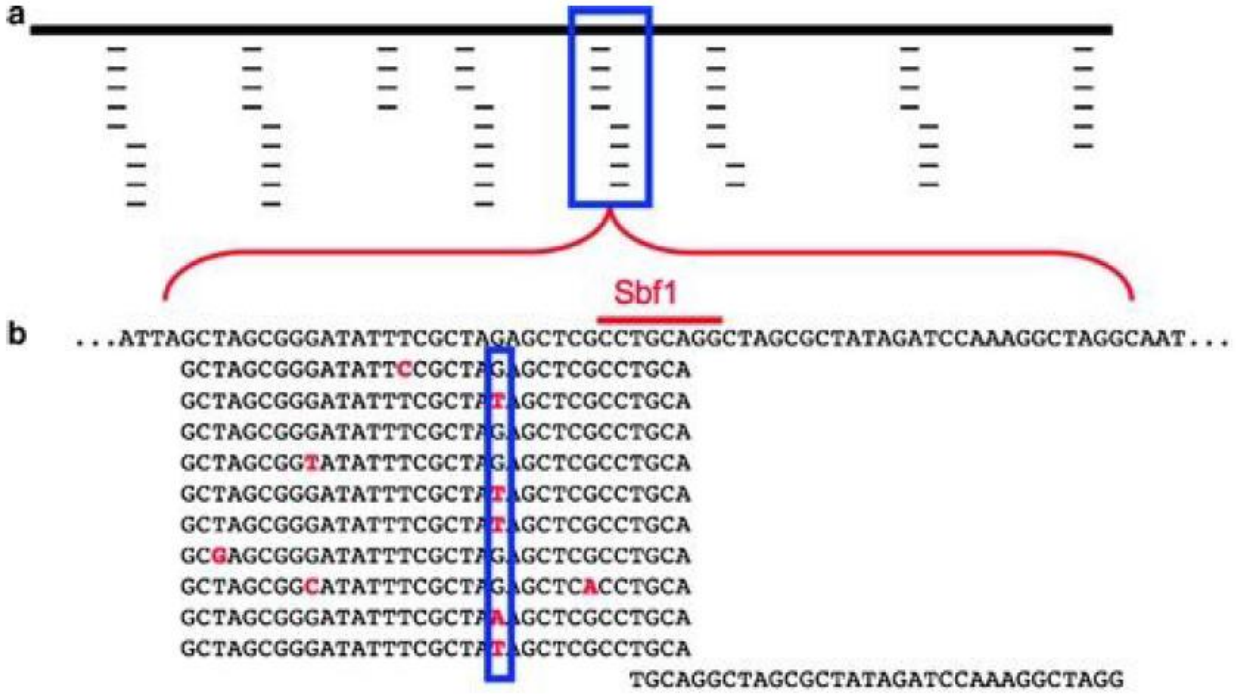
Ligation of Y-shaped adapters

Library enrichment by PCR amplification

Sequencing on ILLUMINA platforms



RADseq: analyzing the data



With or without a reference genome

Stacks, PyRAD, ...

Only with a reference genome

Mapping + GATK, ANGSD, ...