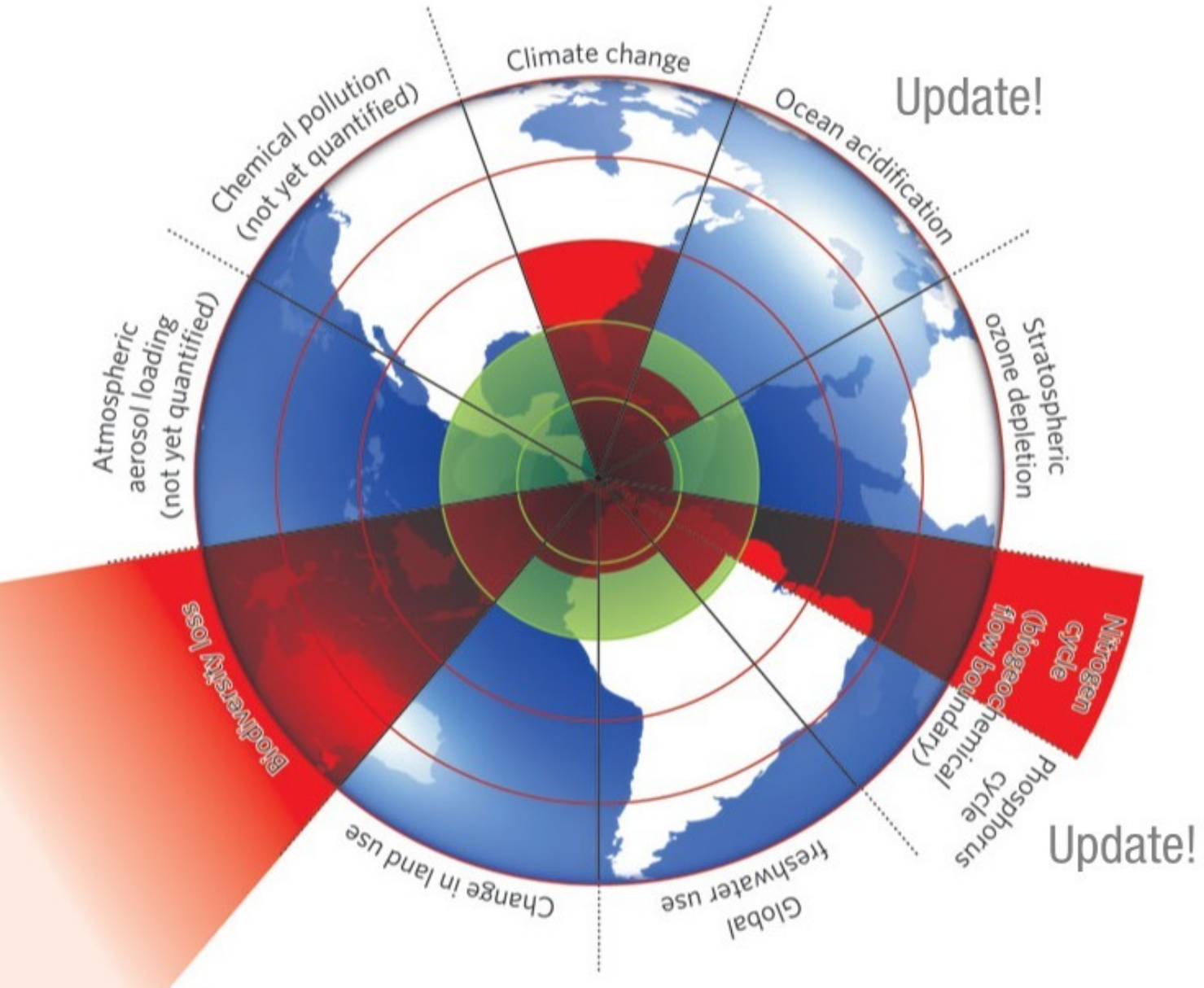
Sequencing technologies & metagenomics

bioinformatics progresses & challenges

Frédéric Mahé
Nov. 17, 2016
Ouagadougou

Planetary boundaries and human development

Rockström et al. (2009) Nature 461 and Steffen et al., (2015) Science 347:6223

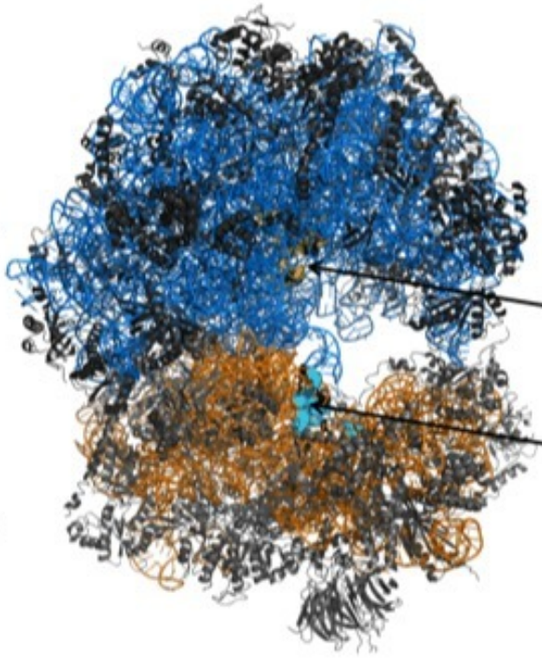


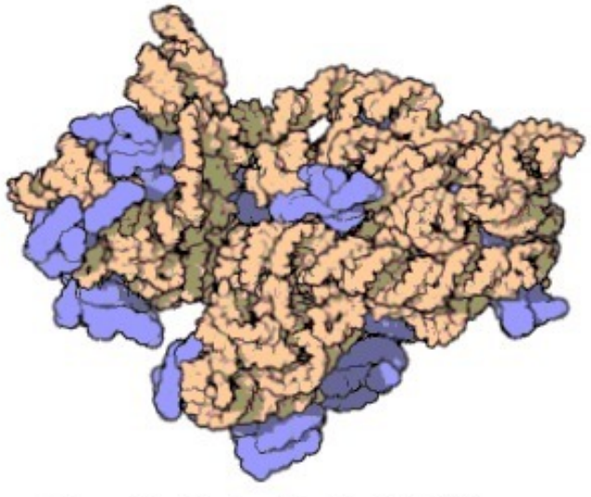
Biodiversity loss (genetic and functional) is a major short-term threat

How can we measure biodiversity?

Small organisms play a major role but are hard to inventory

A universal gene: ribosomal RNA

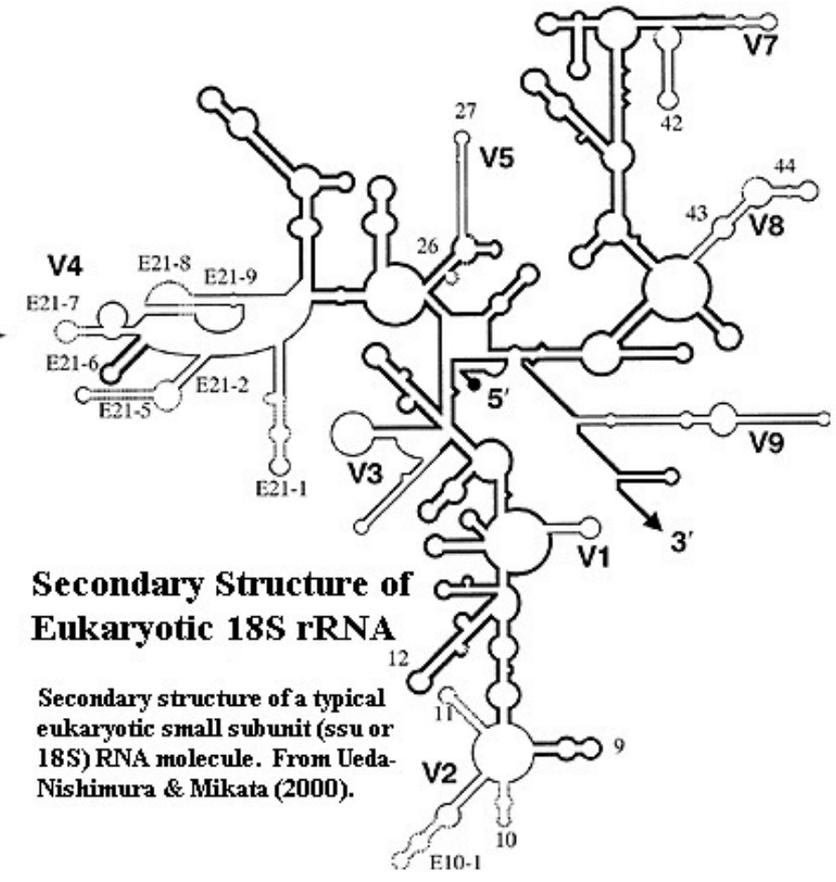




Small Sub-Unit (SSU)

16S | Bacteria
Archaea
Mitochondria
Chloroplasts

18S | Eukaryota



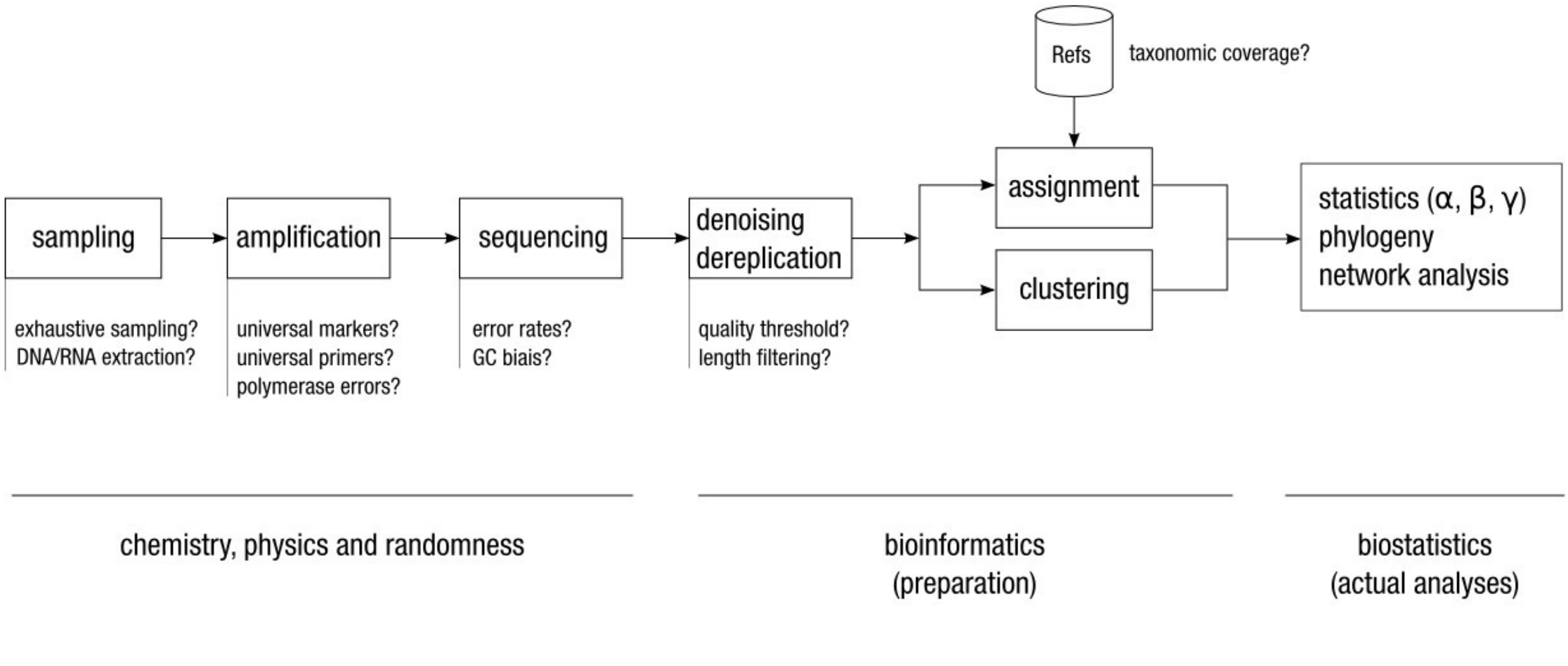
Secondary Structure of Eukaryotic 18S rRNA

Secondary structure of a typical eukaryotic small subunit (ssu or 18S) RNA molecule. From Ueda-Nishimura & Mikata (2000).

other markers can be used (e.g., ITS).
Requirements are: conserved distal regions for primers, variable internal regions, and available sets of reference sequences.

Environmental Metagenomics

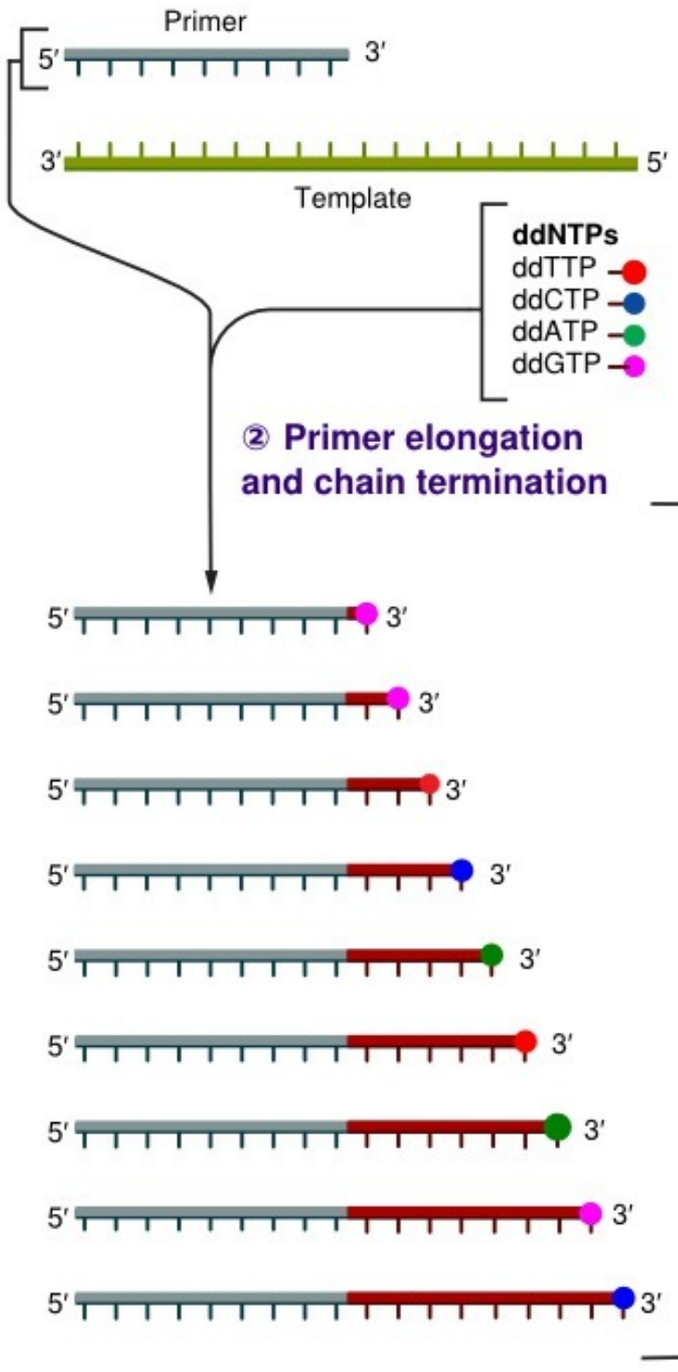
targeted amplification

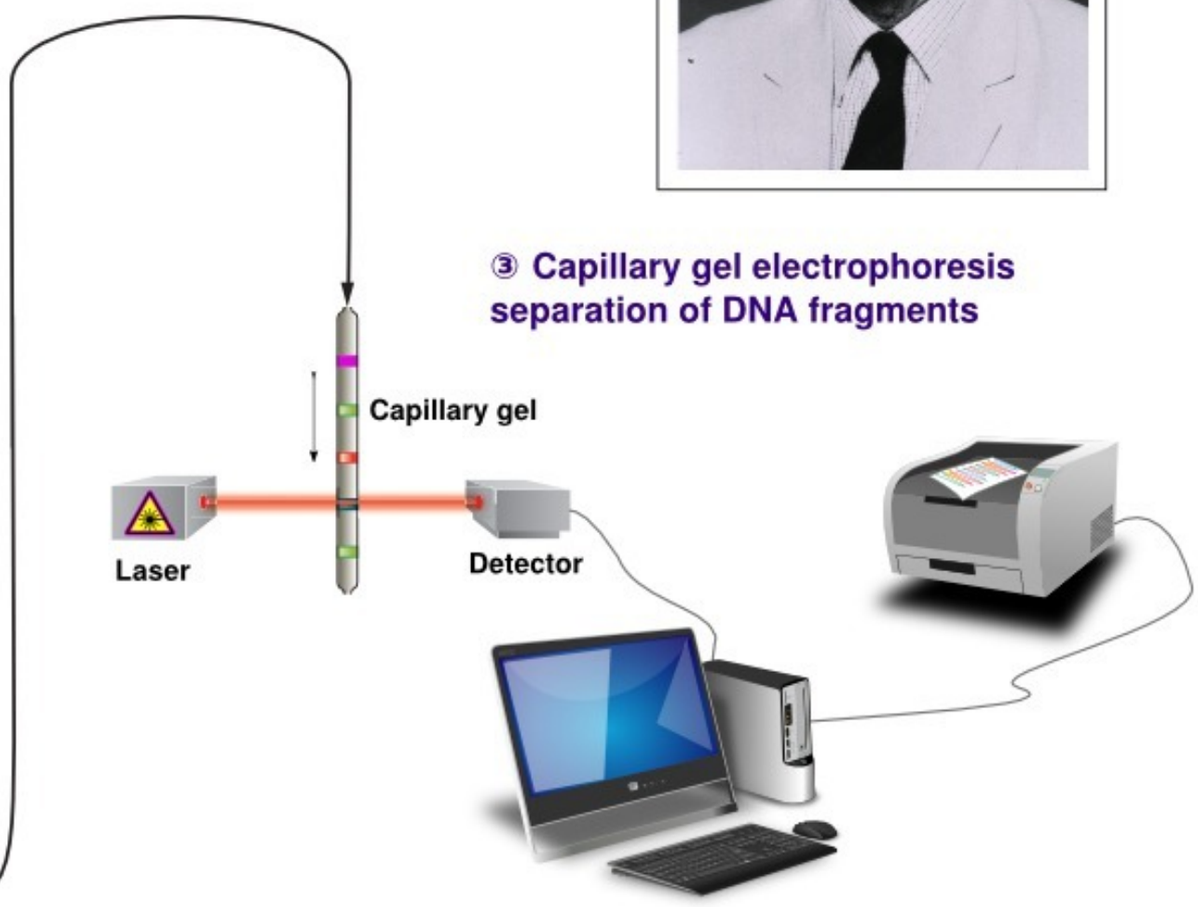


Sanger sequencing technique (1977)

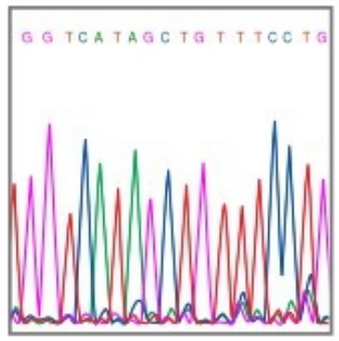
① Reaction mixture

- **Primer and DNA template**
- **ddNTPs with fluorochromes**
- **DNA polymerase**
- **dNTPs (dATP, dCTP, dGTP, and dTTP)**





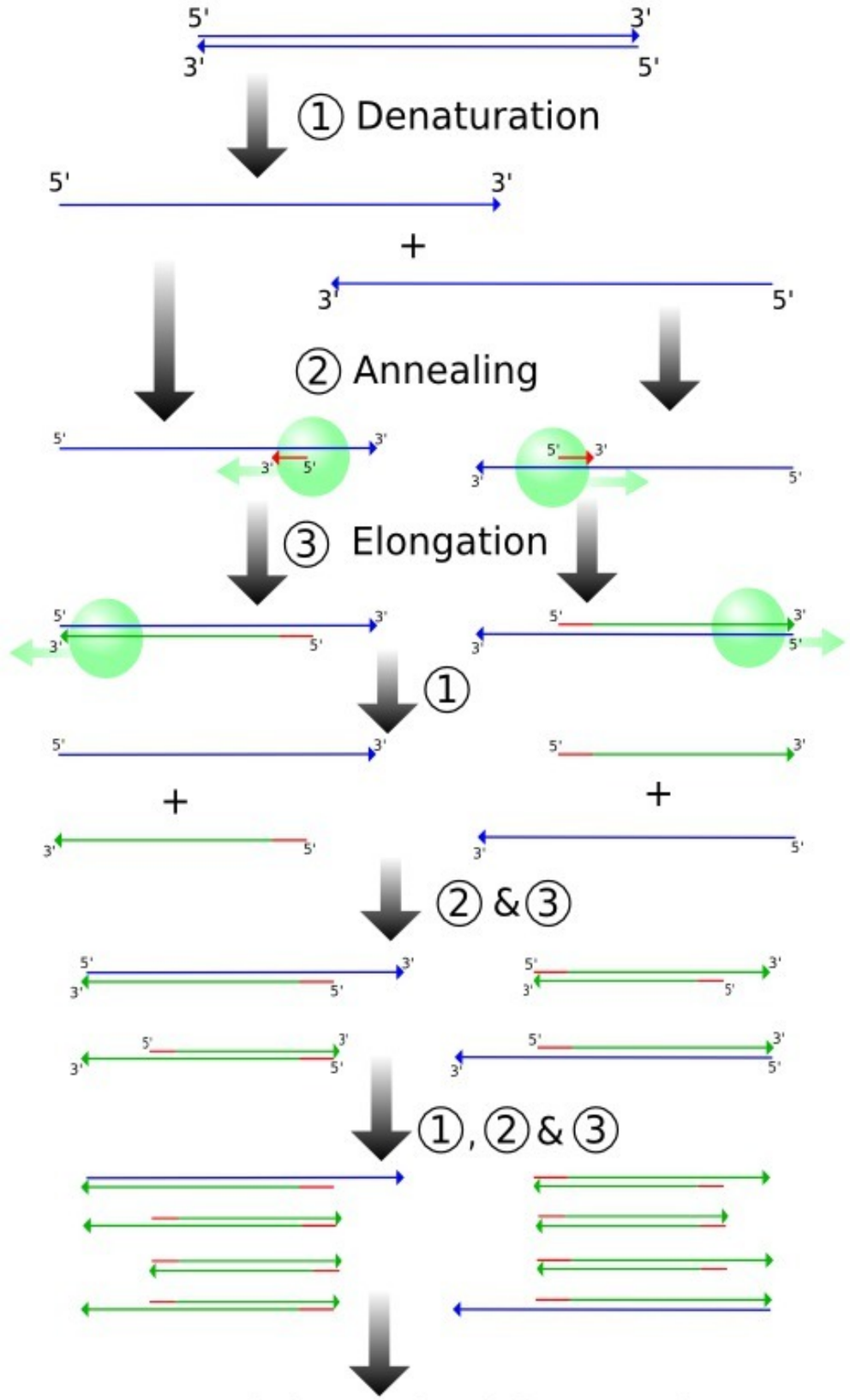
③ Capillary gel electrophoresis separation of DNA fragments



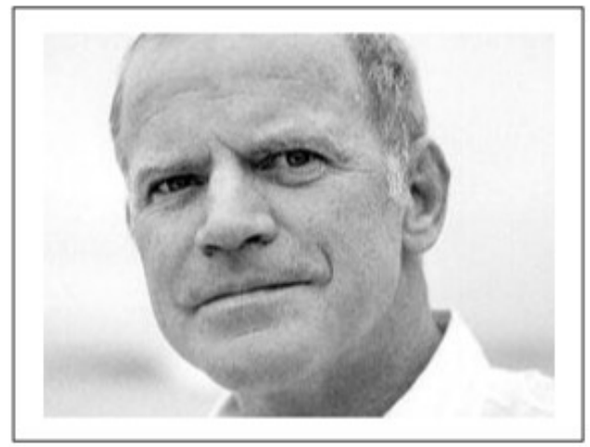
Chromatograph

④ Laser detection of fluorochromes and computational sequence analysis

Polymerase chain reaction (1983)



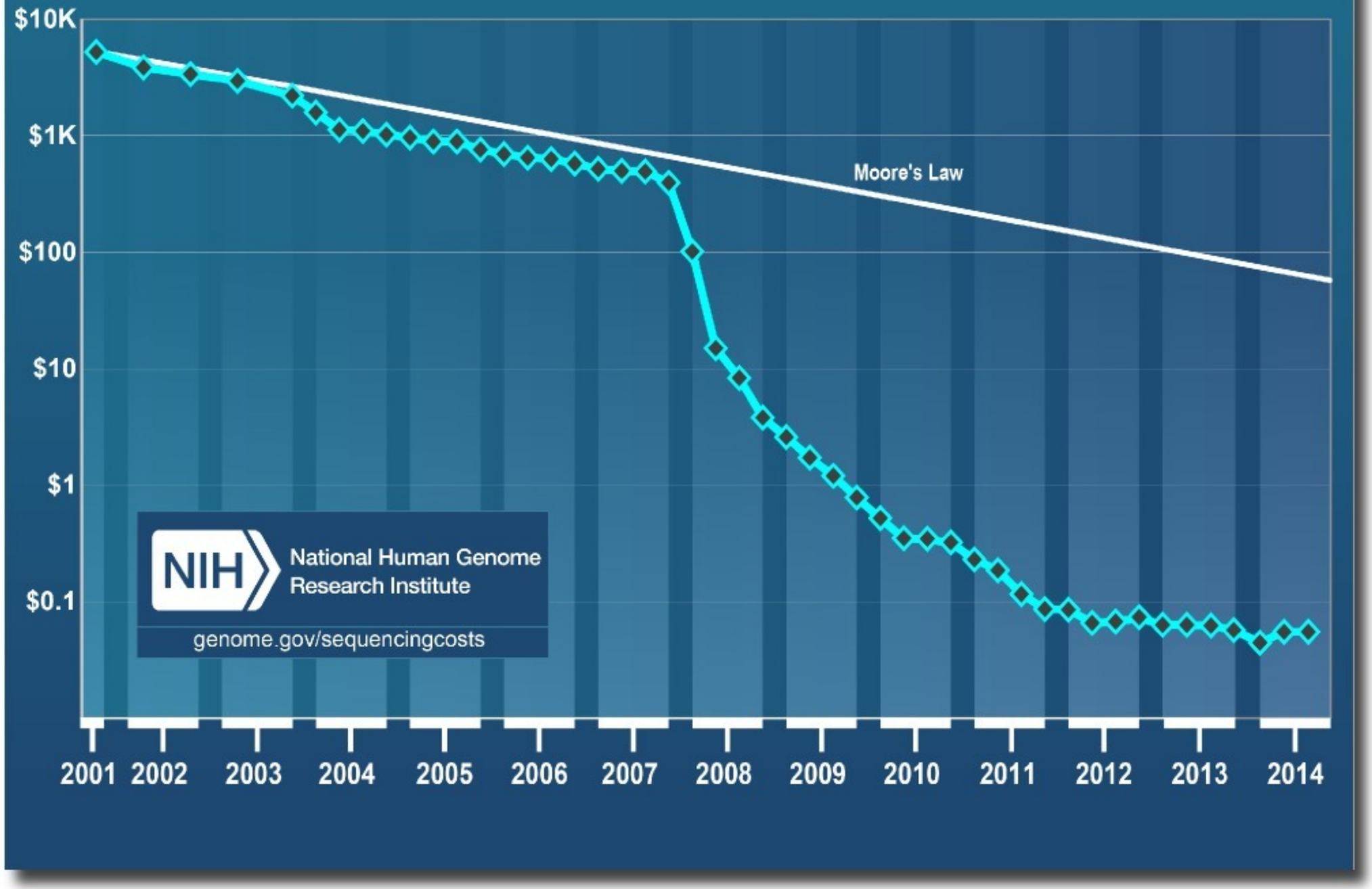
Exponential growth of short product



Kary Mullis (Nobel Prize 1993)

Yikrazuul CC 3.0 By-SA

Cost per Raw Megabase of DNA Sequence



IonTorrent

Ion PGM, Ion Proton

Illumina

HiSeq 3000/4000, MiSeq 3000

allows lots of samples and deep sequencing

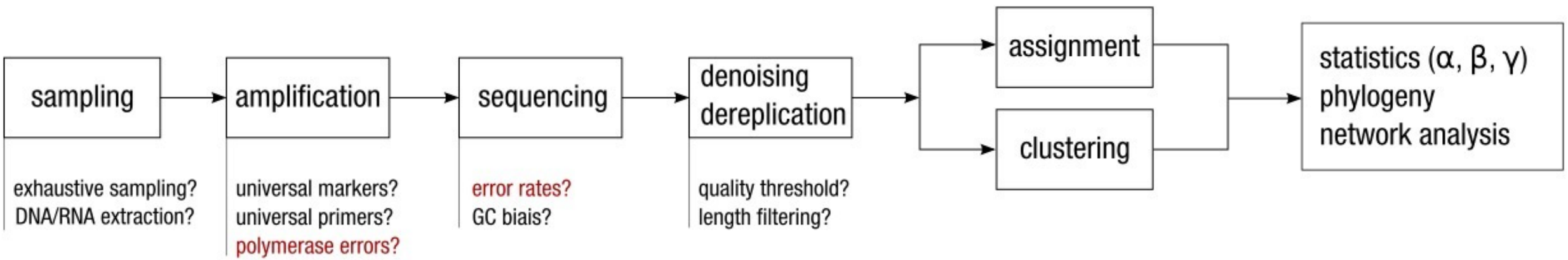
Pacific Biosciences

Oxford Nanopore Technologies

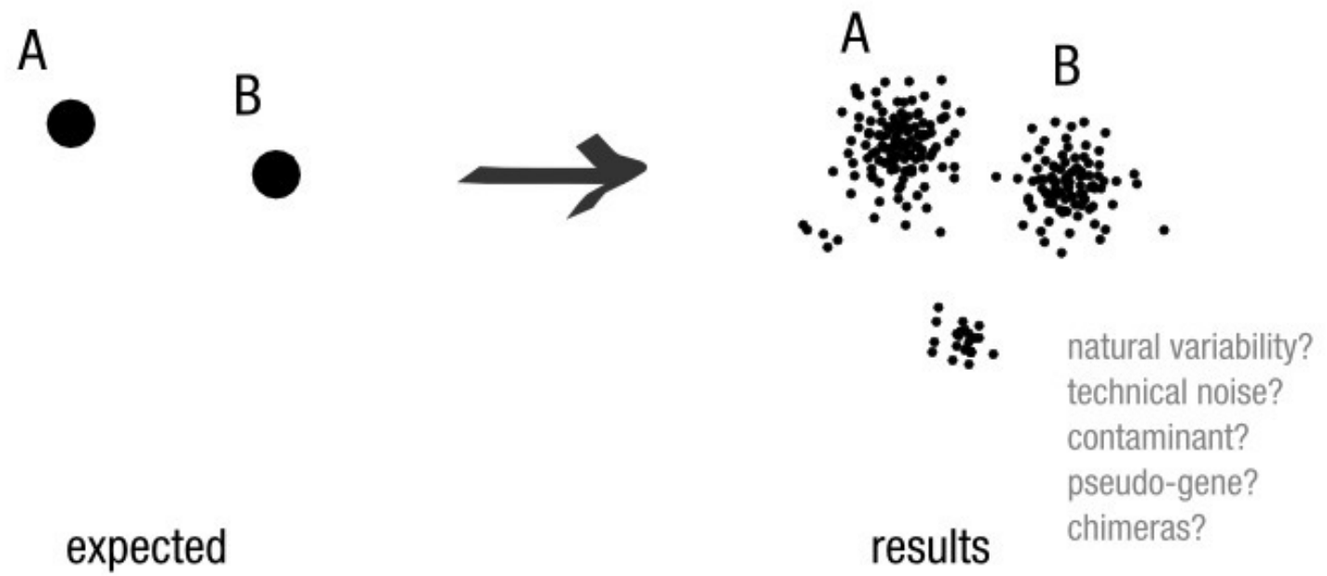
Yes, we need more computers and storage capacity to keep up, but...

Noise is the real challenge

amplification and sequencing are imperfect processes

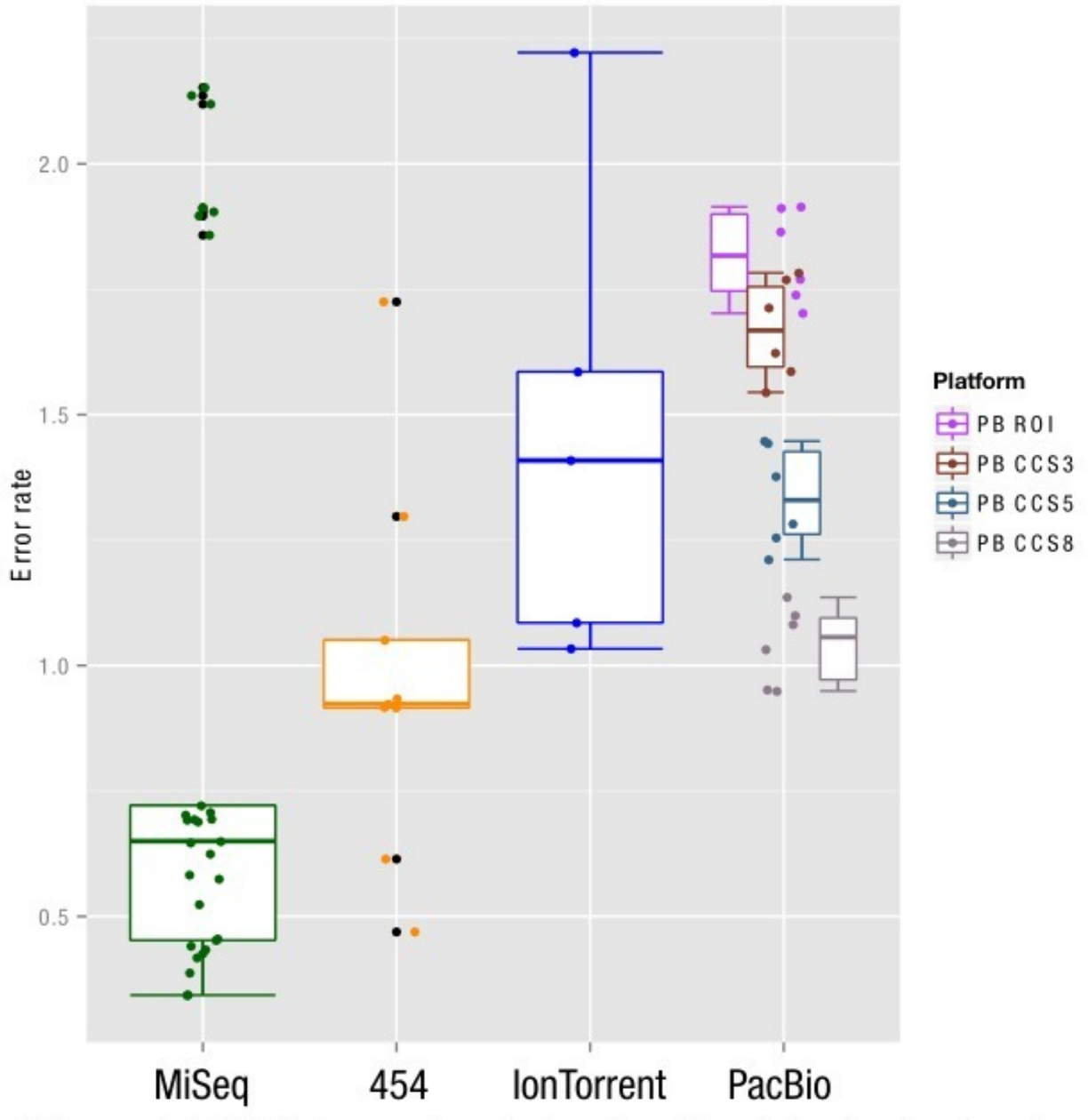


chemistry, physics and randomness

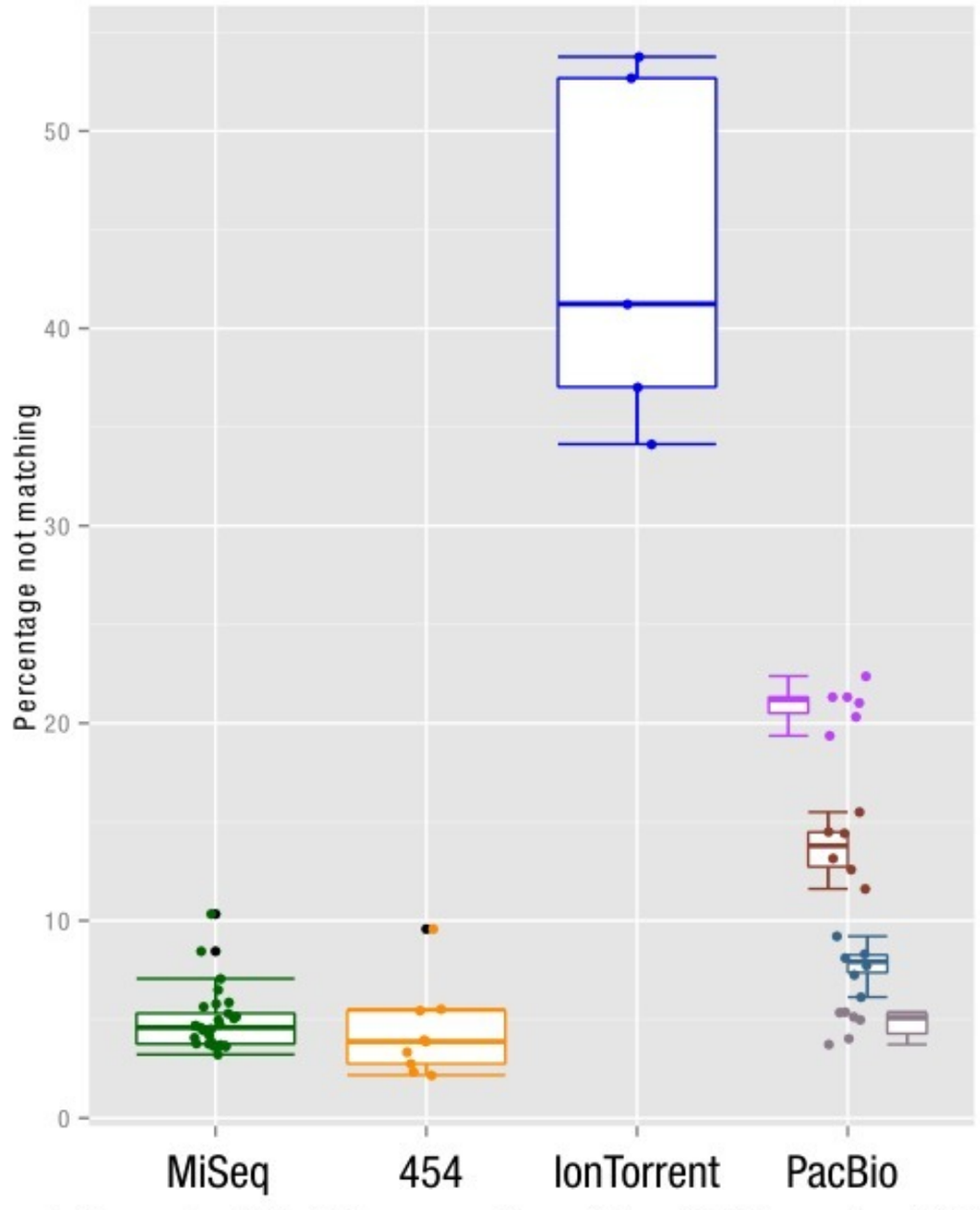


Error rate per sequencing platform

Error rates



Percentage of unassigned reads



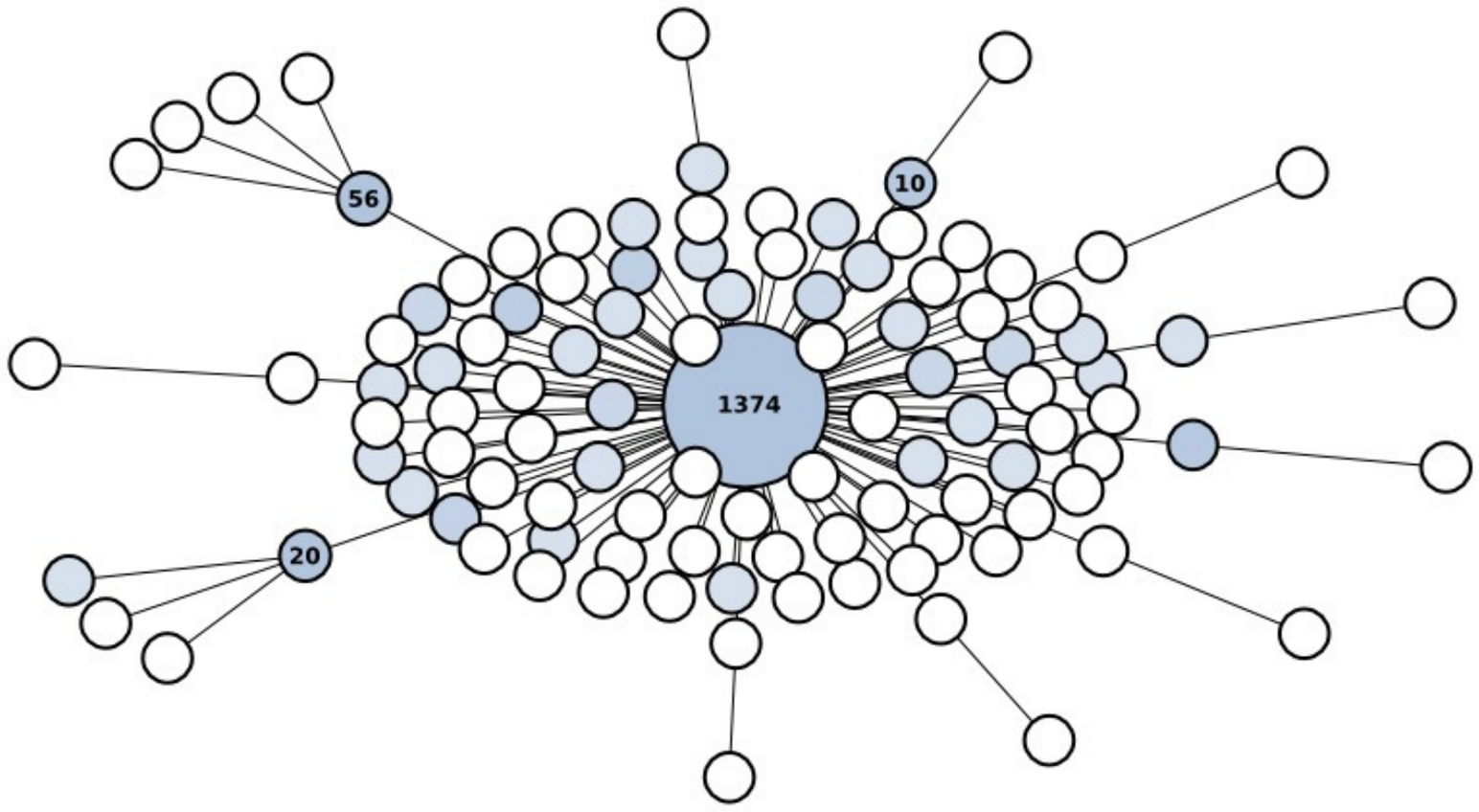
D'Amore et al. (2016) A comprehensive benchmarking study of protocols and sequencing platforms for 16S rRNA community profiling. BMC Genomics, 17:55.

What is the nature of these errors?

2
ACGT
AAGT
AGGT
ATGT
A-GT
AACGT
...

At each position in a sequence:

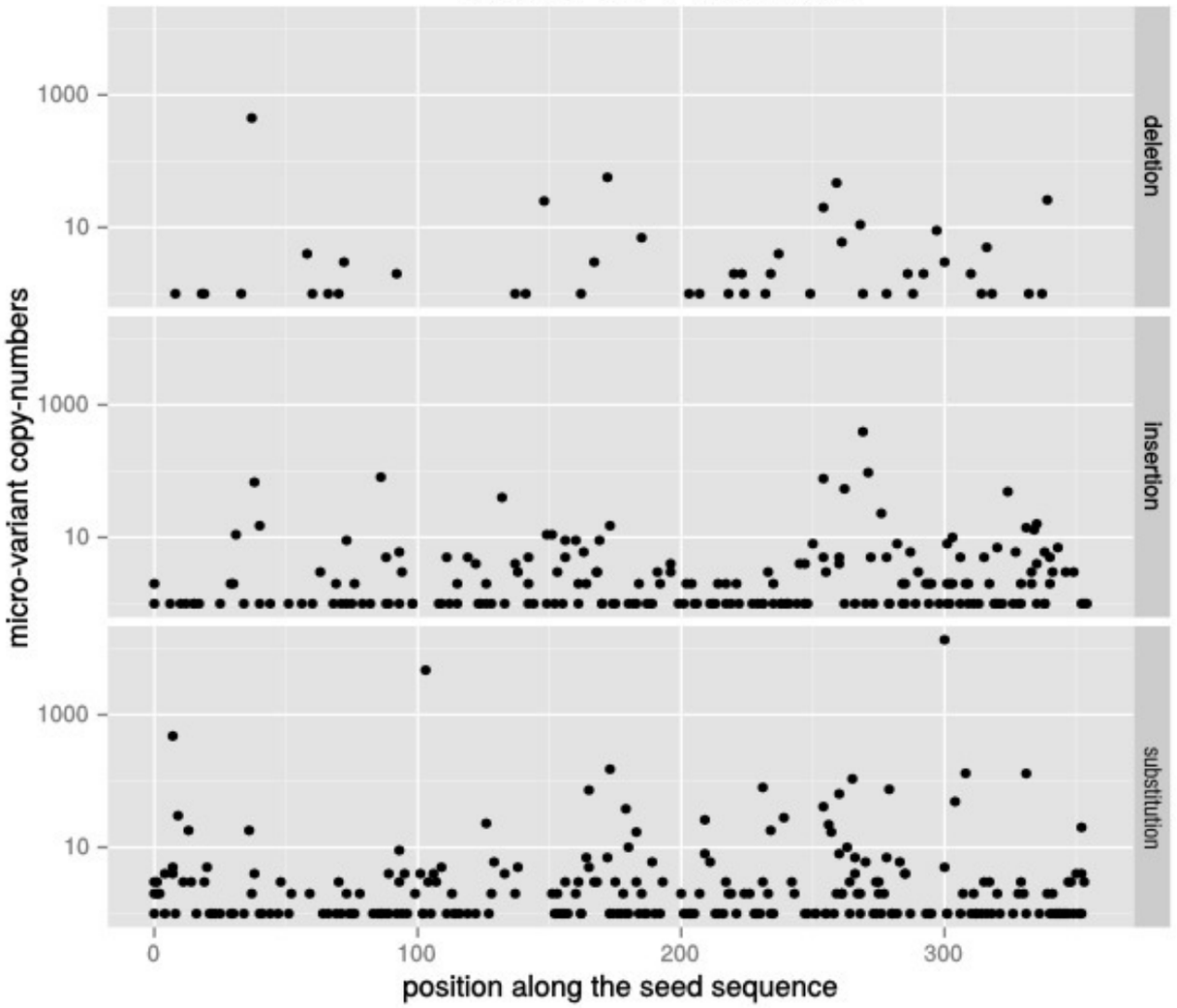
- 3 substitutions (C -> A, G or T)
- 1 deletion,
- 4 insertions

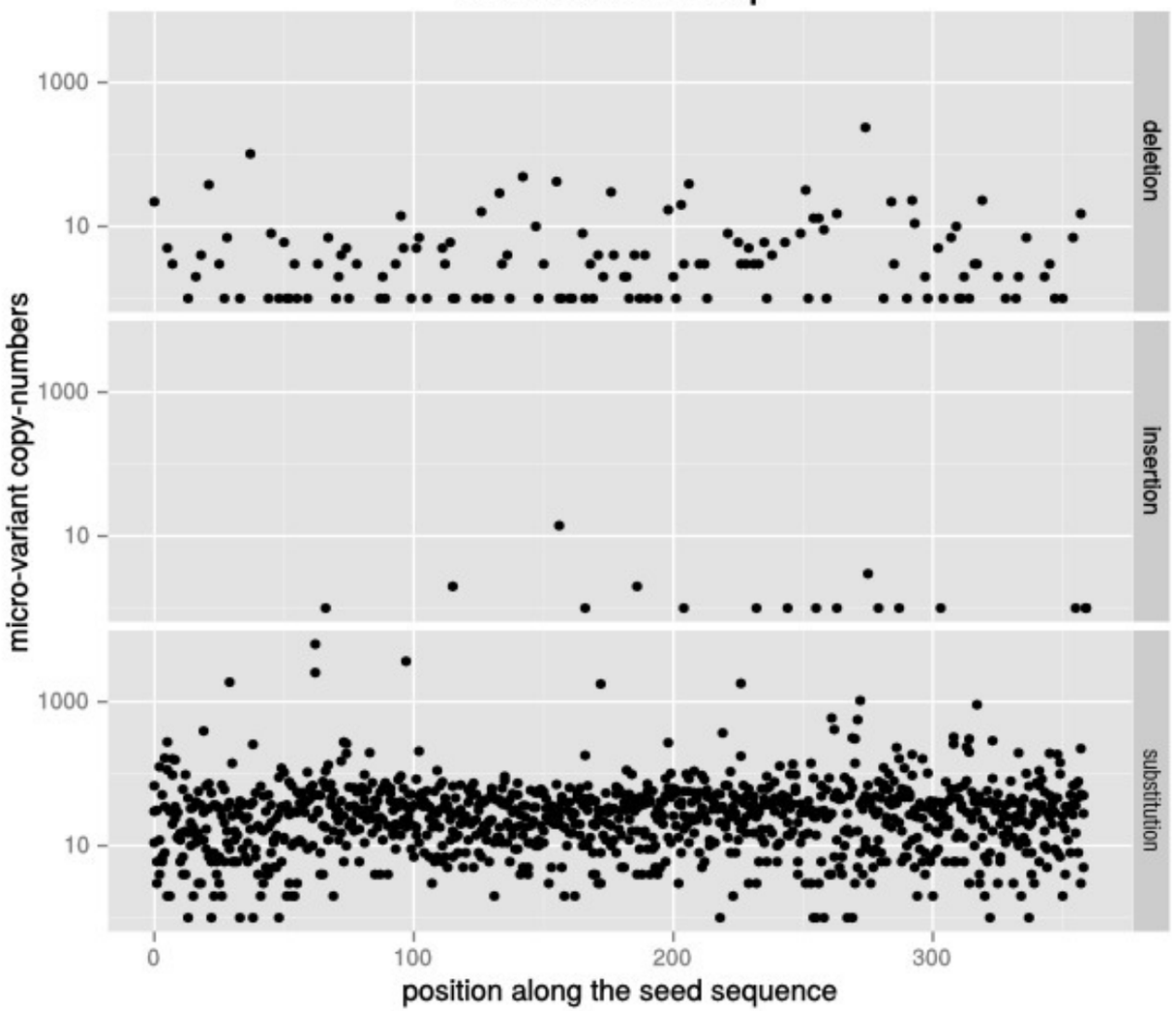


A 380 nucleotide seed (V4), can have up to 2,664 first degree unique micro-variants.

A 130 nucleotide seed (V9), can have up to 914 first degree unique micro-variants.

How are errors distributed?





Roche 454 Titanium (for 100 bp):
- 0.4011 insertions/deletions,
- 0.0543 substitutions.

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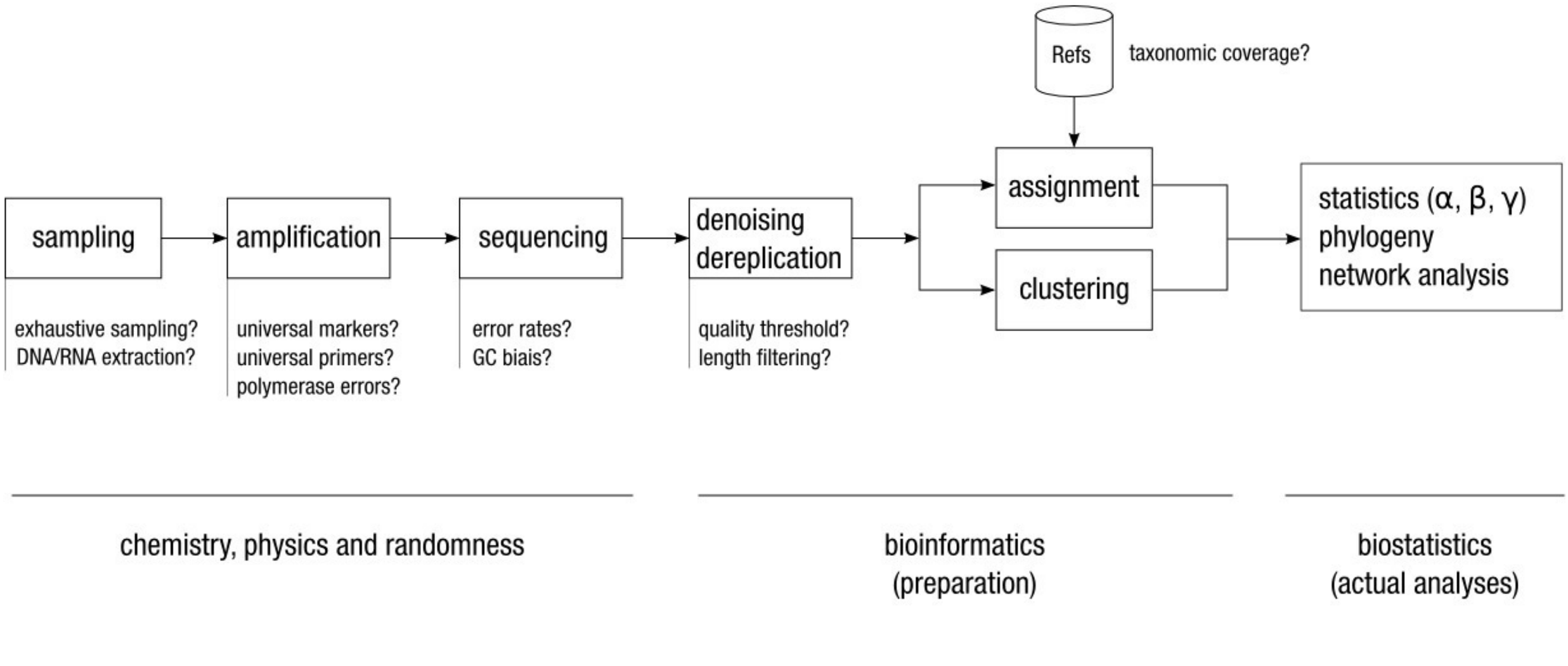
Jünemann *et al*. (2013) Update on 'Performance comparison of benchtop high-throughput sequencing platforms'. Nature Biotechnology 31.

Environmental Metagenomics

targeted amplification



Major metagenomics pipelines

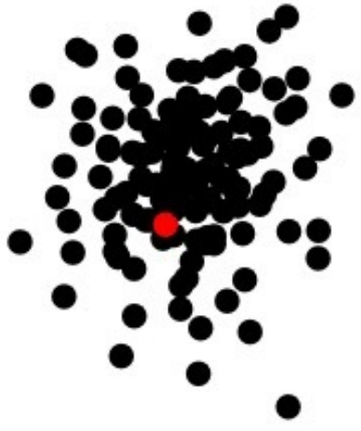
targeted amplification

qiime

Uparse

Mothur (2009)	Qiime (2010)	Uparse (2013)
Patrick Schloss	Gregory Caporaso	Robert Edgar
open-source	open-source	closed-source
single piece	python wrapper	usearch commands
most cited	most used(?)	popular
stats	stats	no stats

How traditional clustering works?



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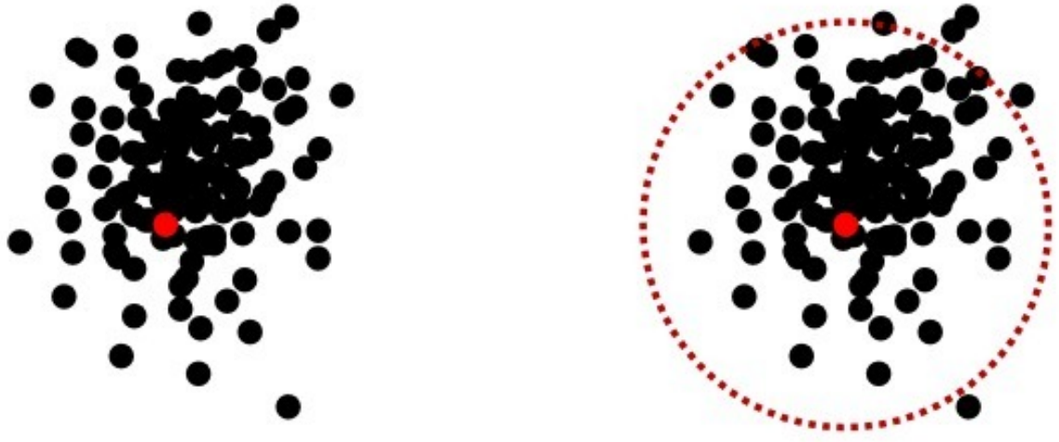

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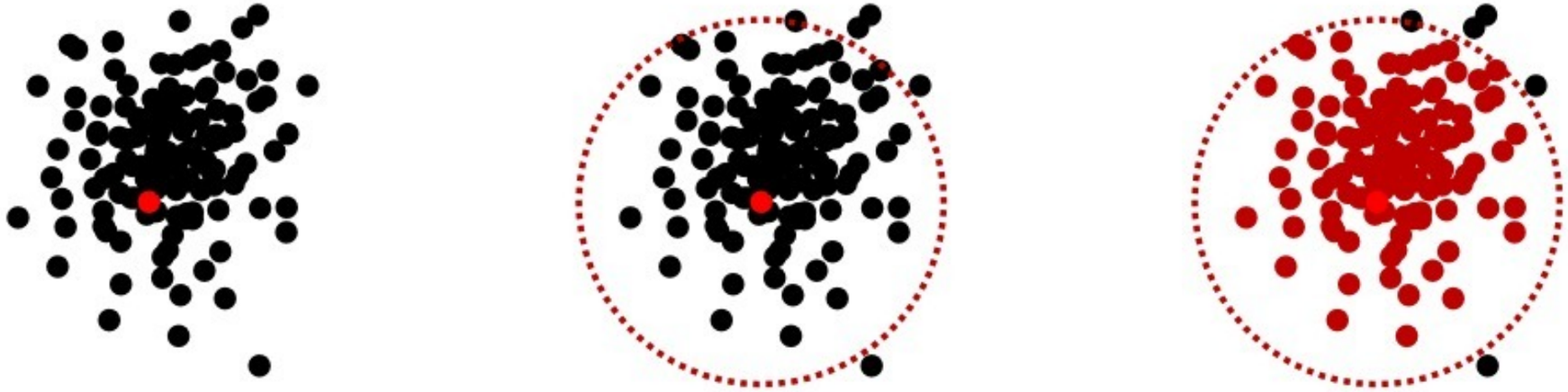

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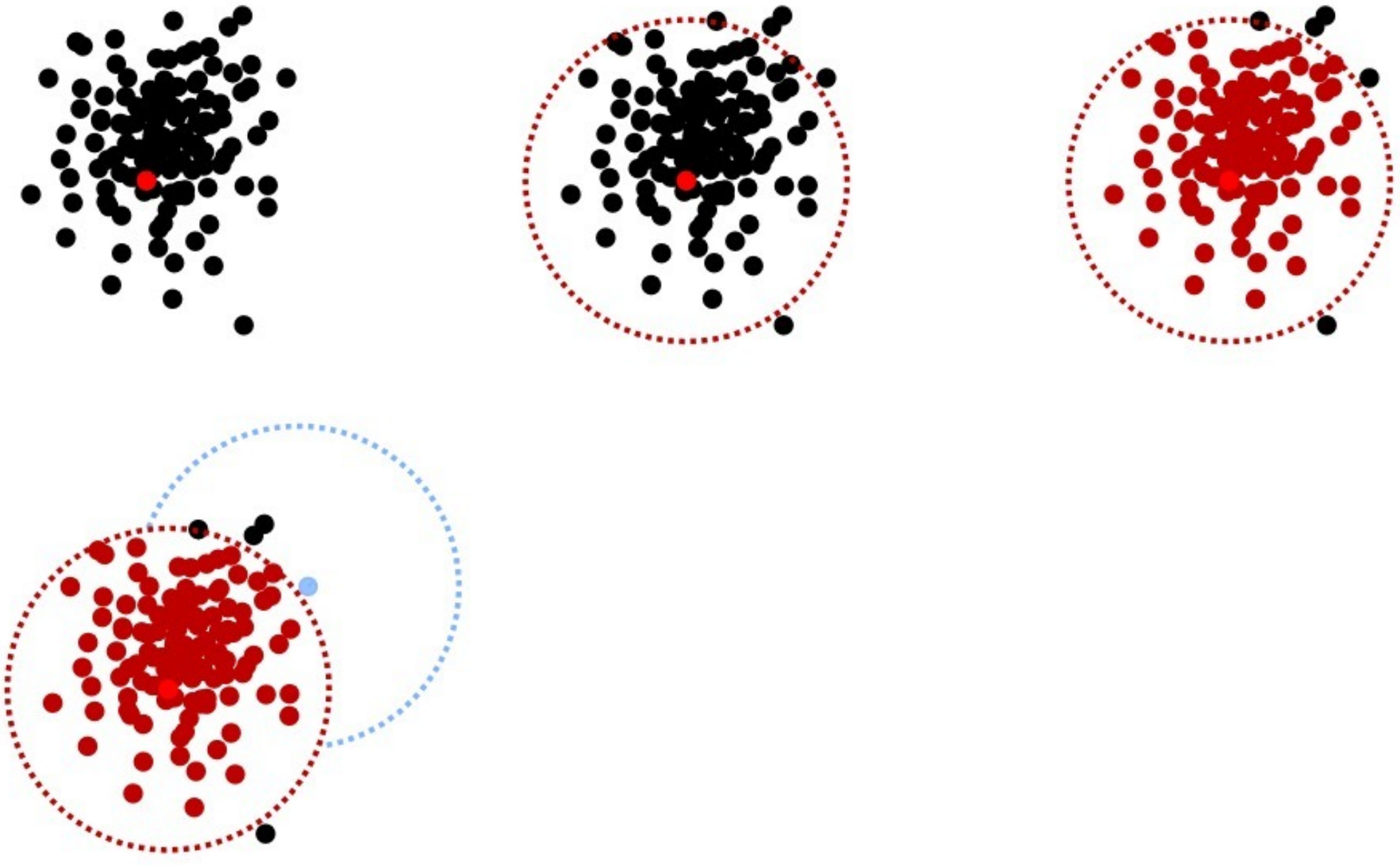

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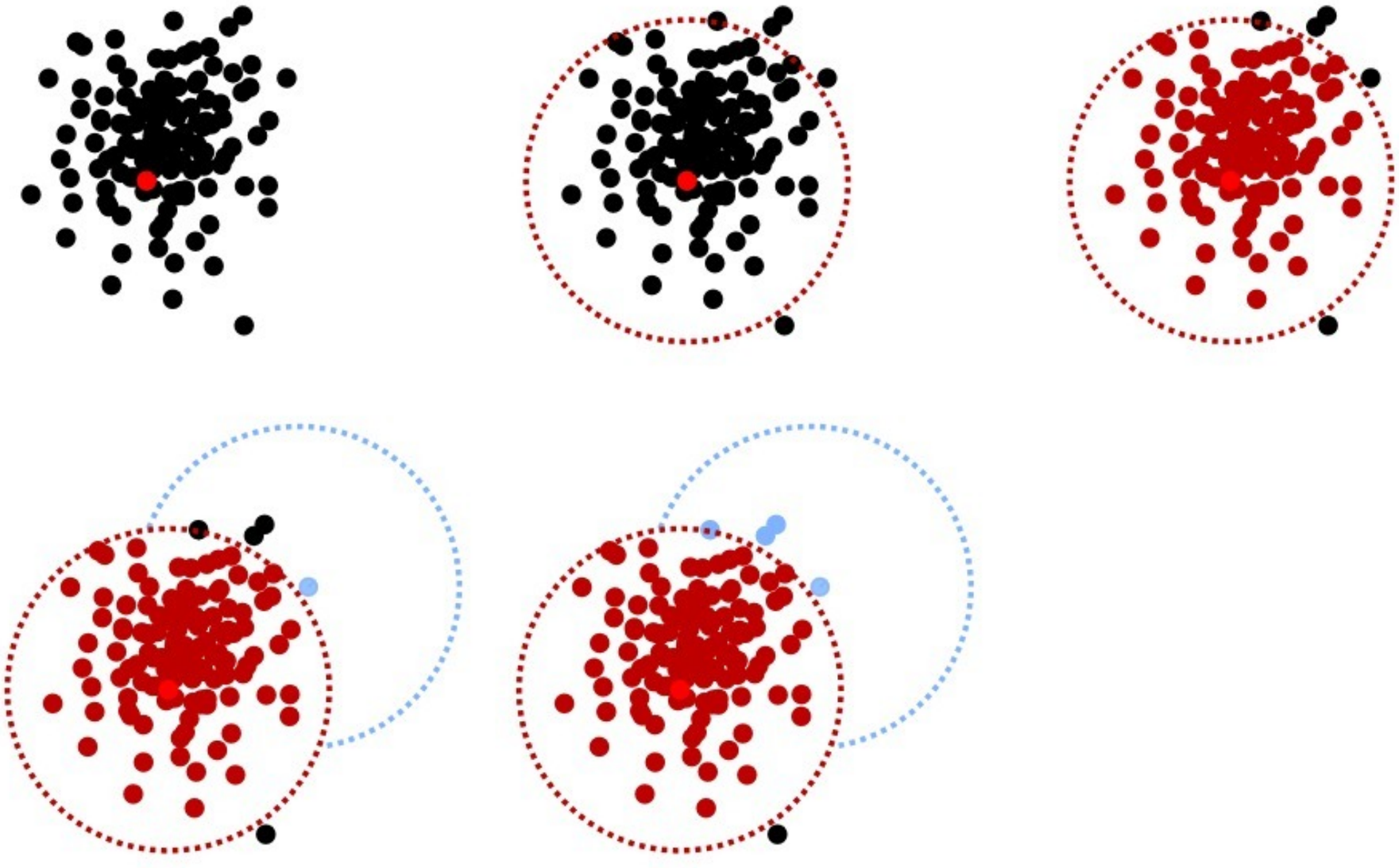

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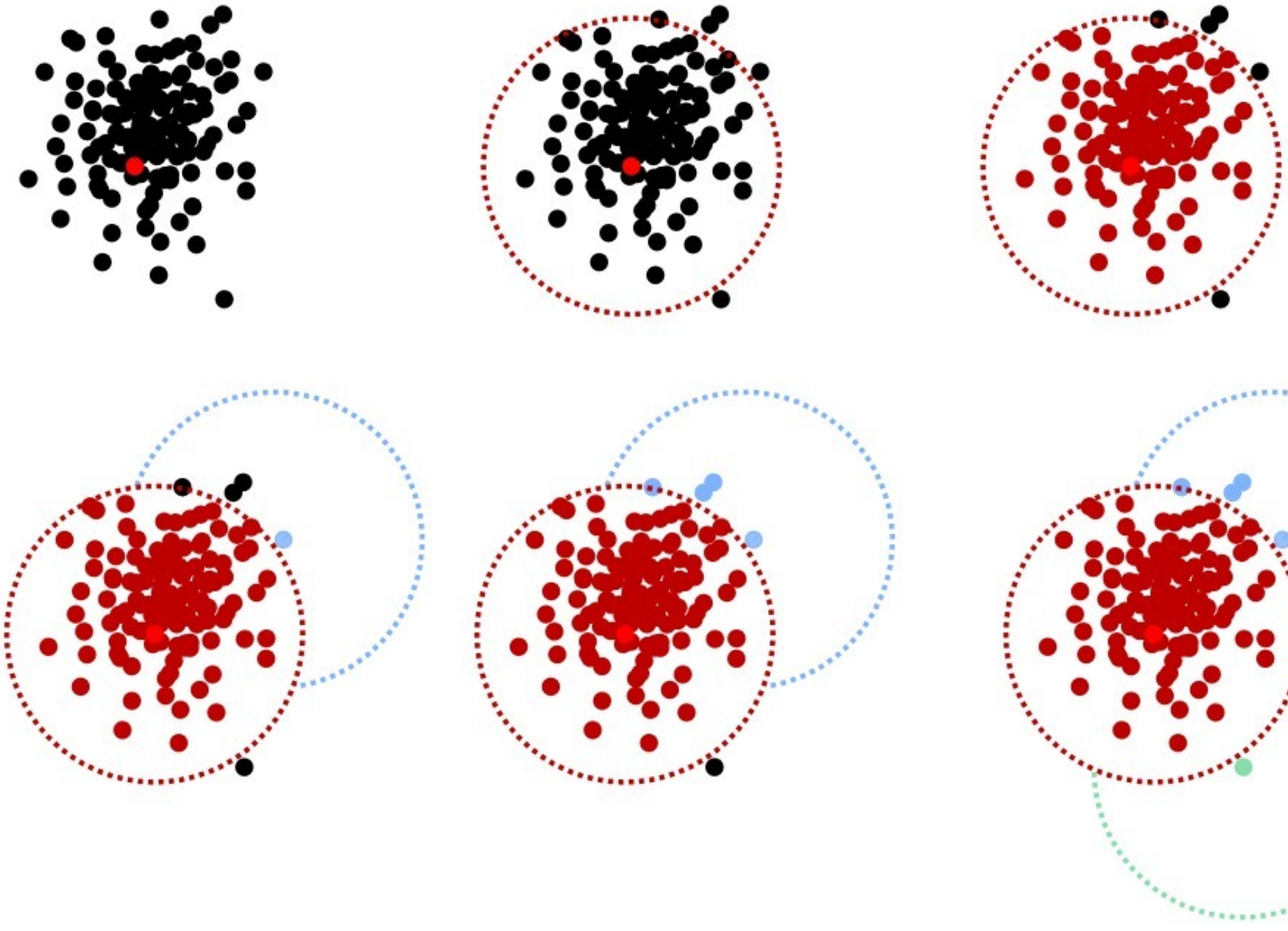
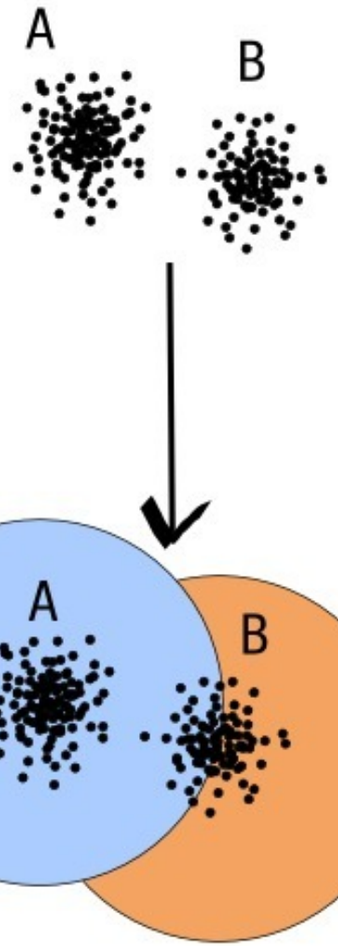
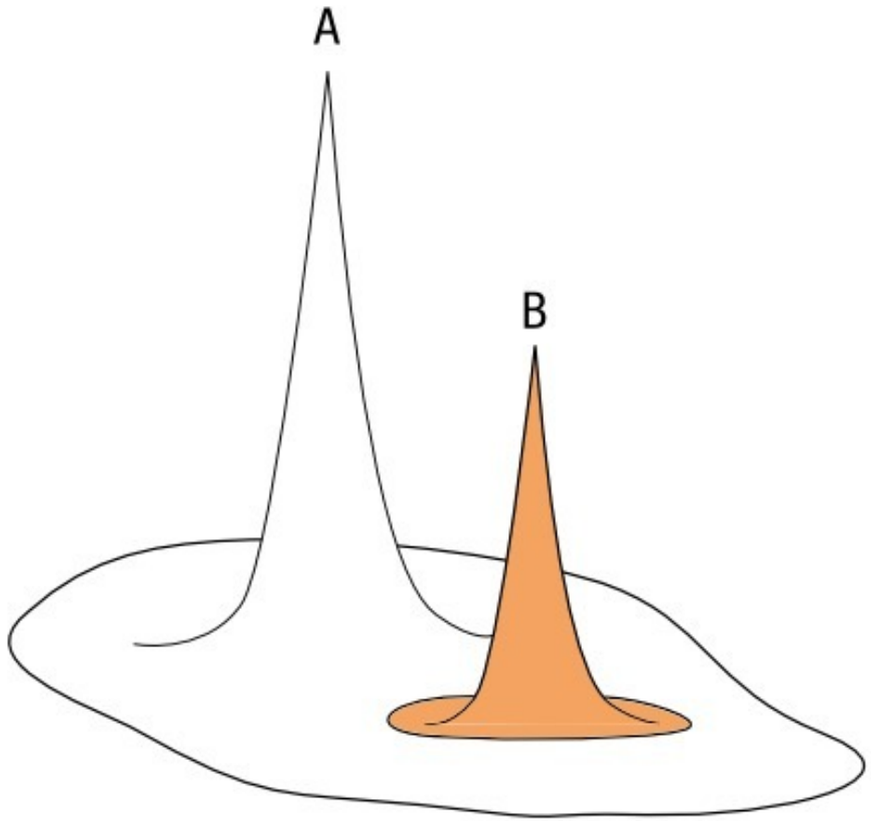


Swarm: fast, exact and high-resolution clustering



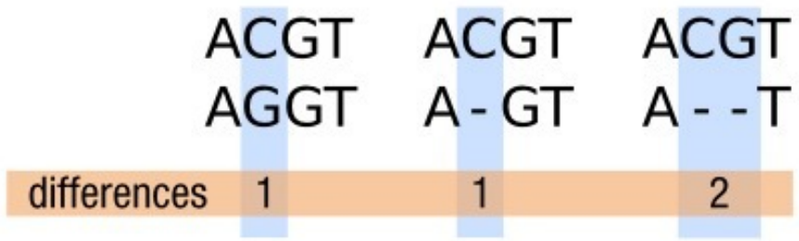
clustering threshold (often 97%) is most of the time unadapted and can mask diversity.



swarm uses abundance values and a new clustering strategy to delineate natural high-quality OTUs.

Swarm clustering method

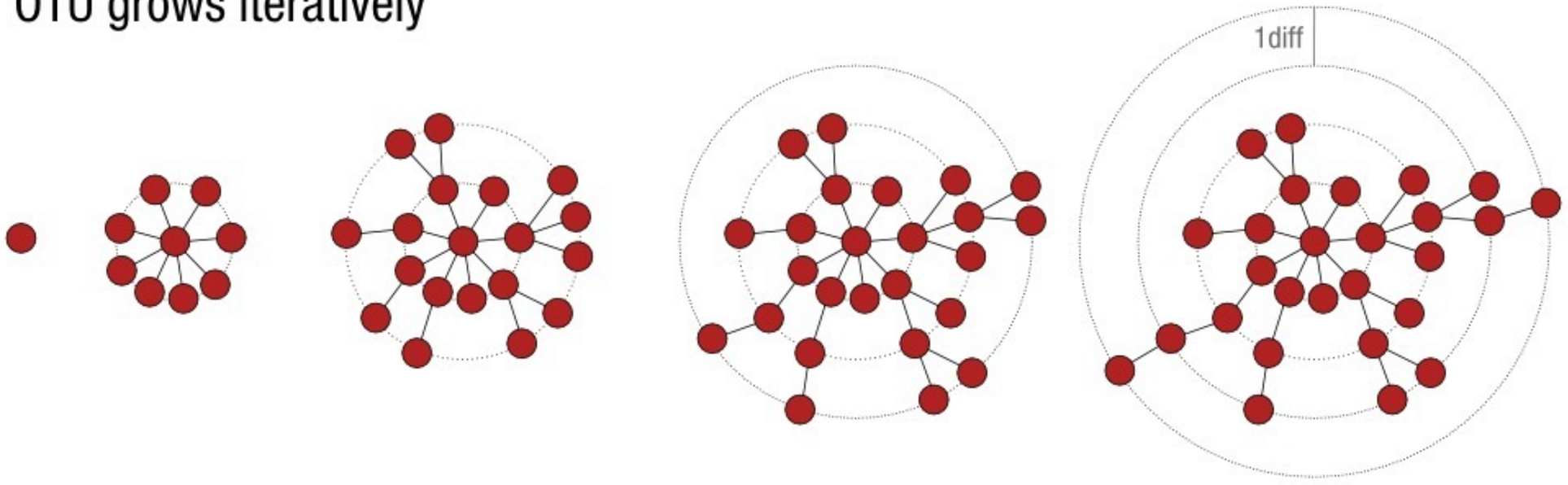
growth phase



Avoid & speed-up comparisons

- composition-based prefiltering
- memoization
- fast Needleman-Wunsch

OTU grows iteratively

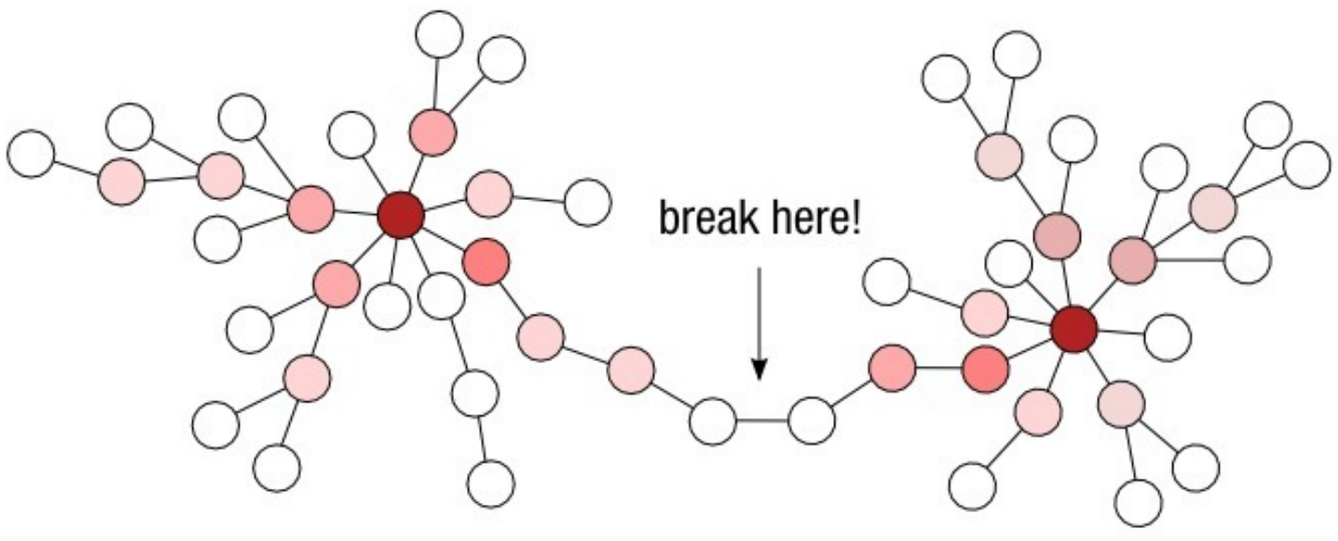


initial seed (randomly picked from amplicon dataset)

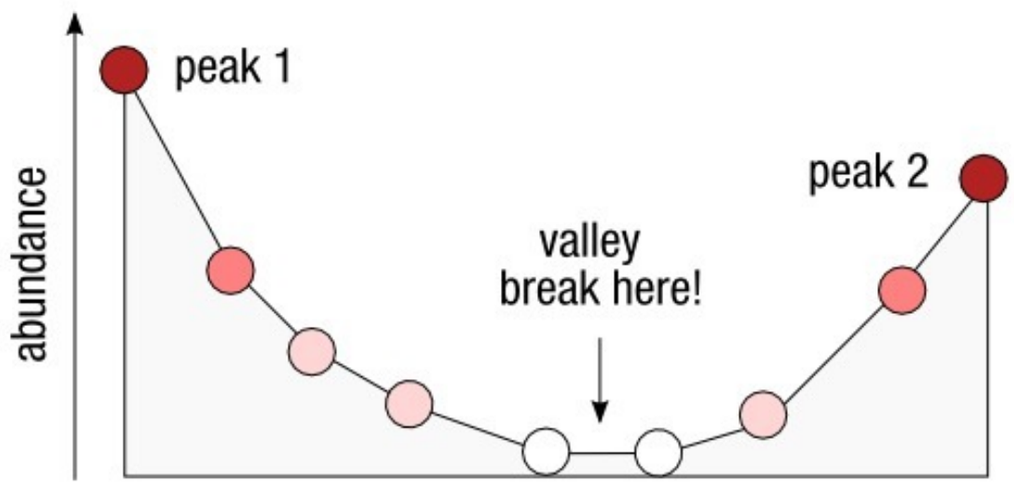
no more closely related amplicons, the process stops

Swarm clustering method

breaking phase

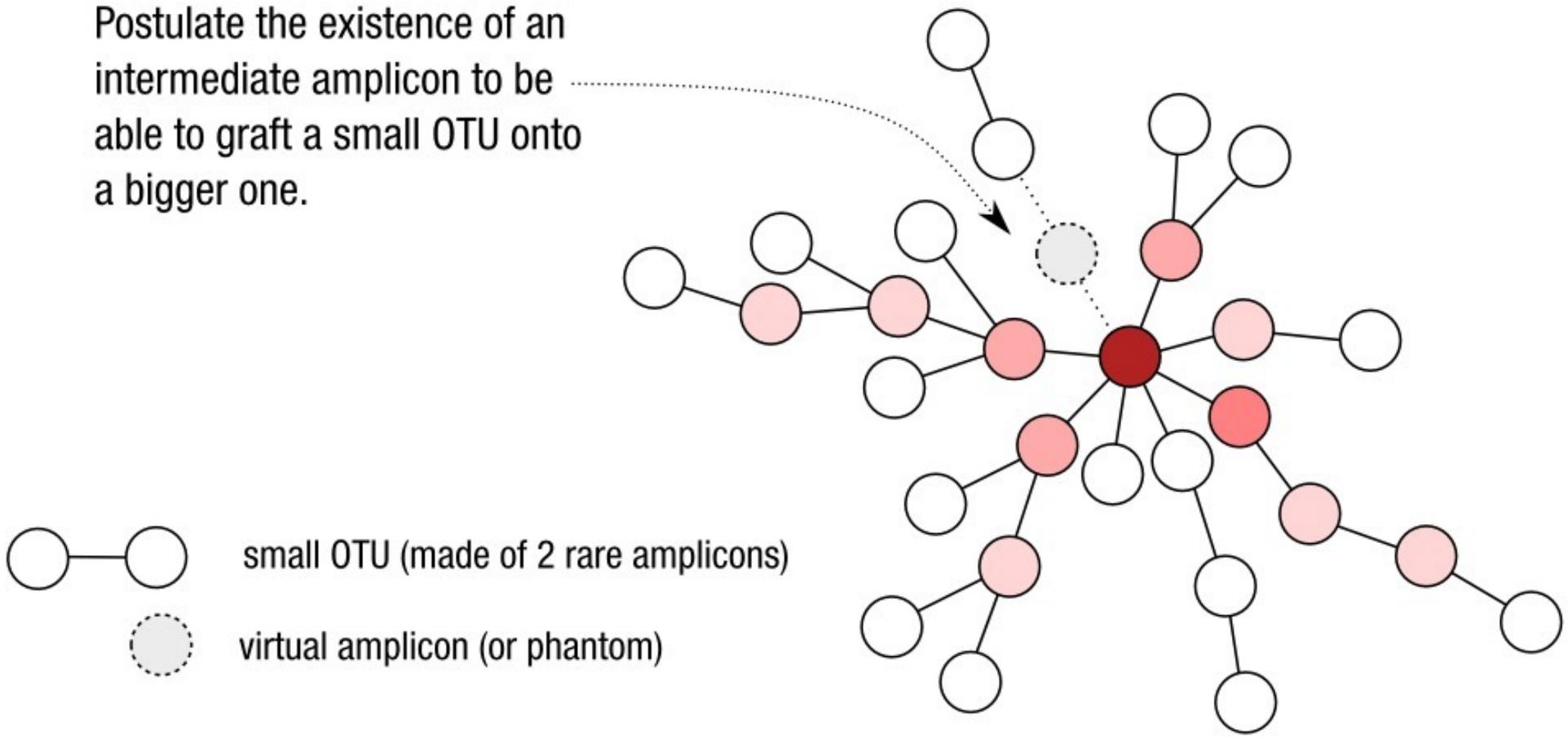


Take into account the abundance of amplicons to produce higher-resolution clusters.

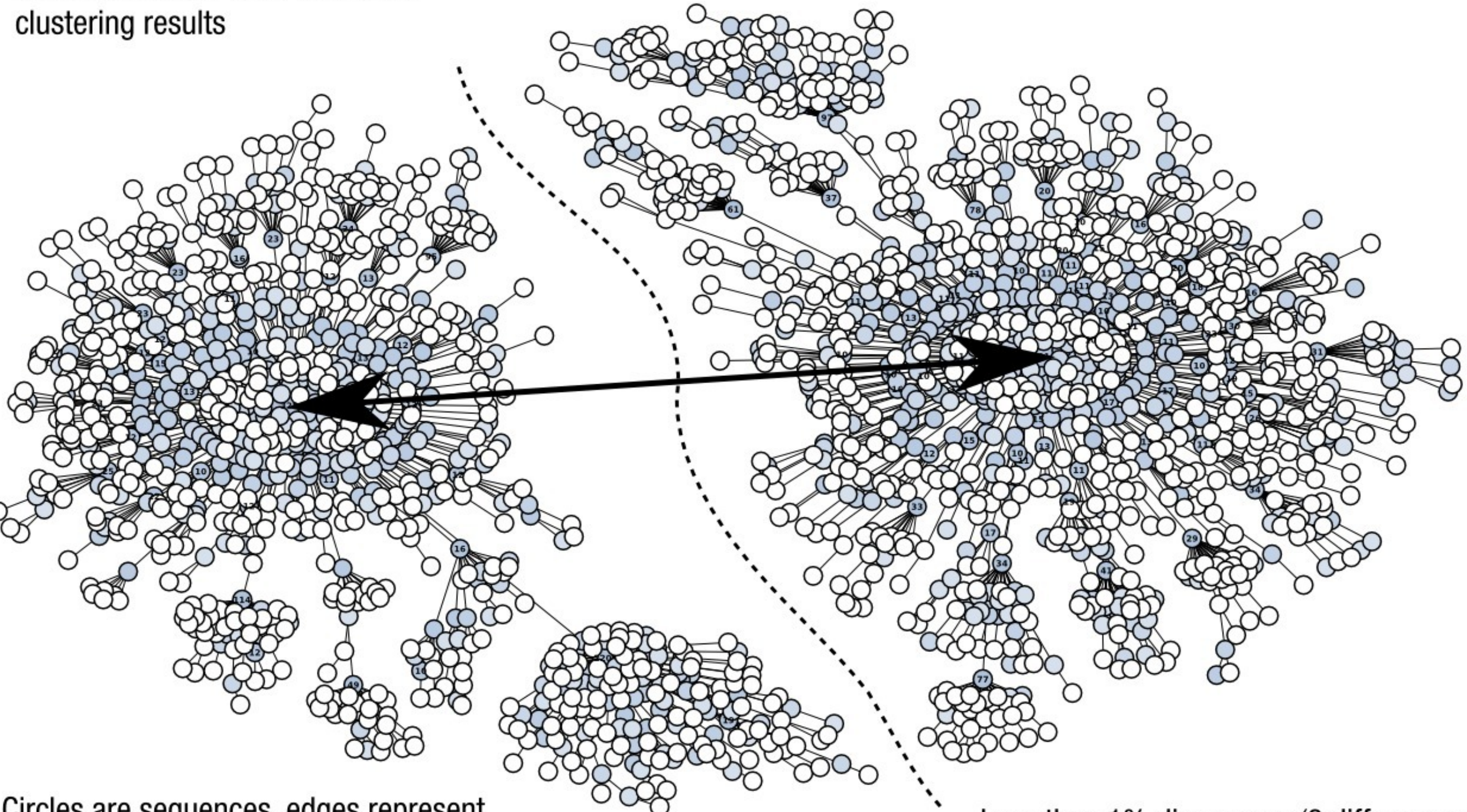


Assuming that original sequences are more abundant than erroneous copies.

Swarm clustering method
grafting phase



Swarm produces high-resolution clustering results



Circles are sequences, edges represent one difference (substitution or indel)

Less than 1% divergence (3 differences) between the two peaks of abundance

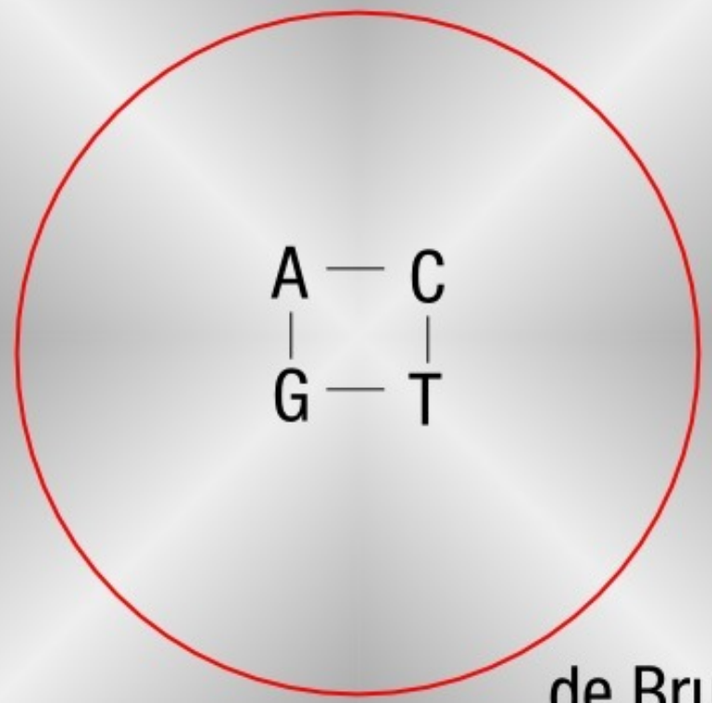
other Hydrozoa genus

new Hydrozoa taxa

Cnidaria (Metazoa)

Lilyopsis rosea

sequence-space



swarm derives from basic observations of the sequence-space

de Bruijn graph in genome assembly

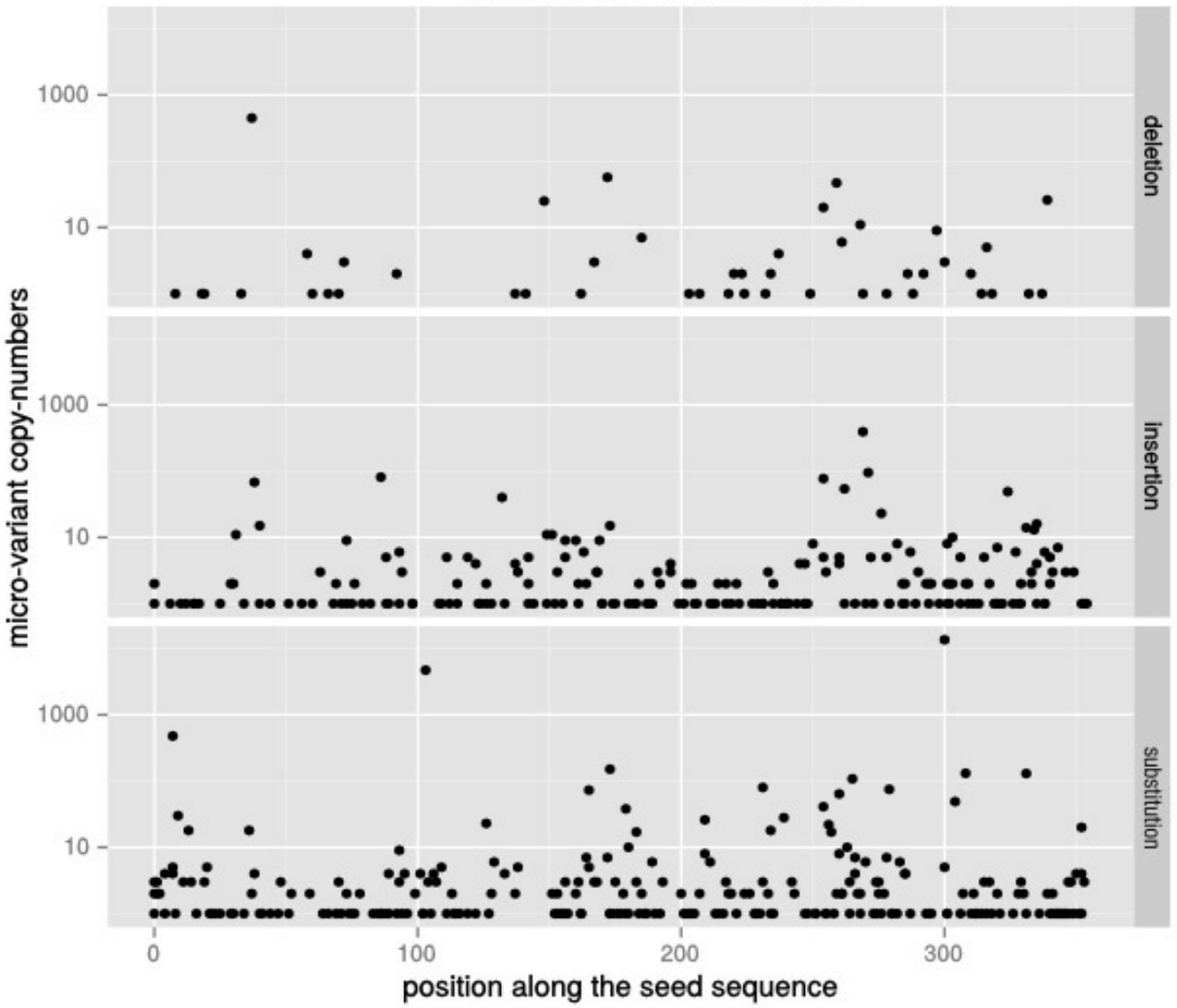
Swarm 2.0 is a highly scalable denoising-clustering method

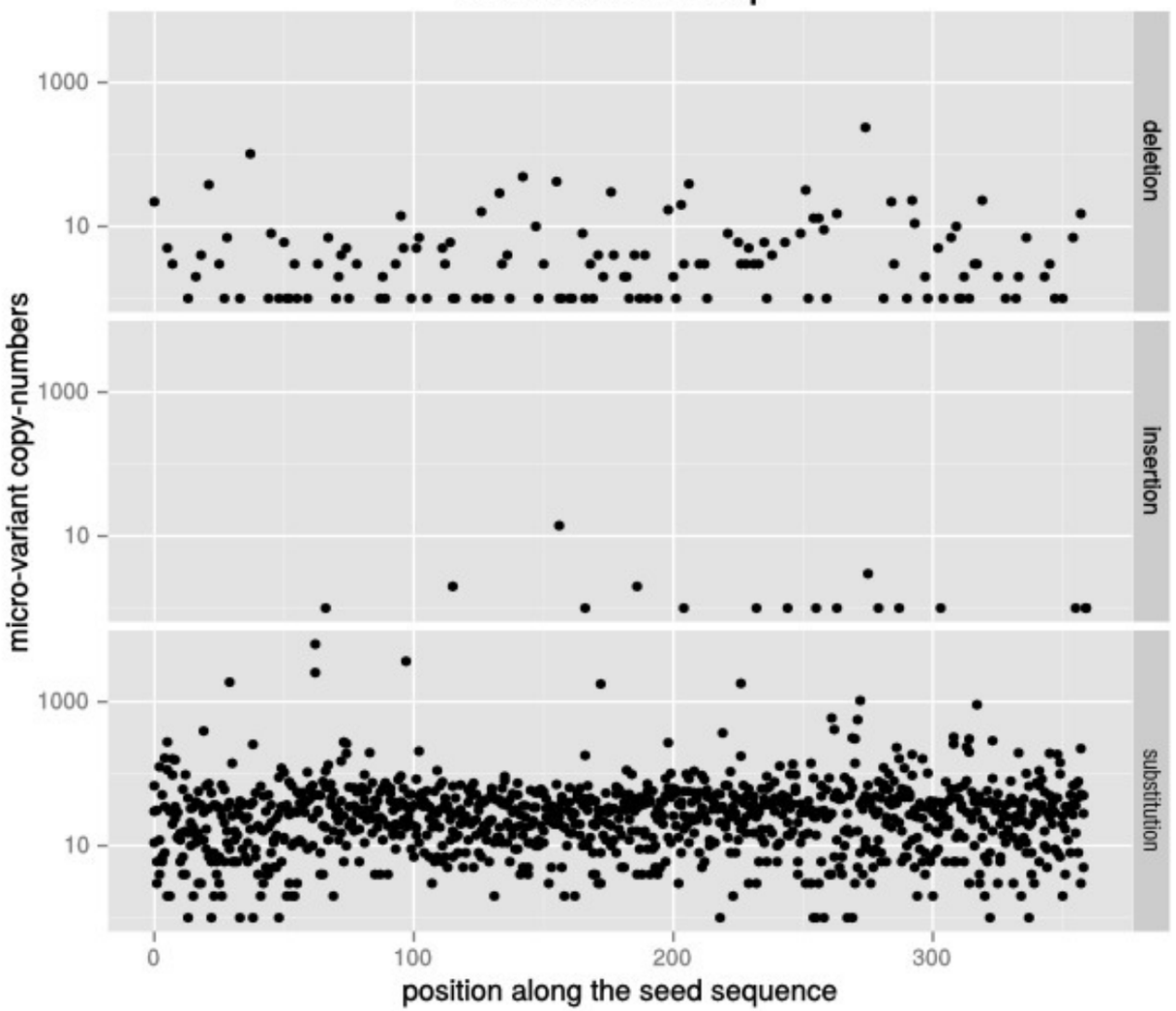
earth microbiome project

28,275 samples
2.3 billion reads

swarm: 5 hours
usearch: >150 days

How are errors distributed?





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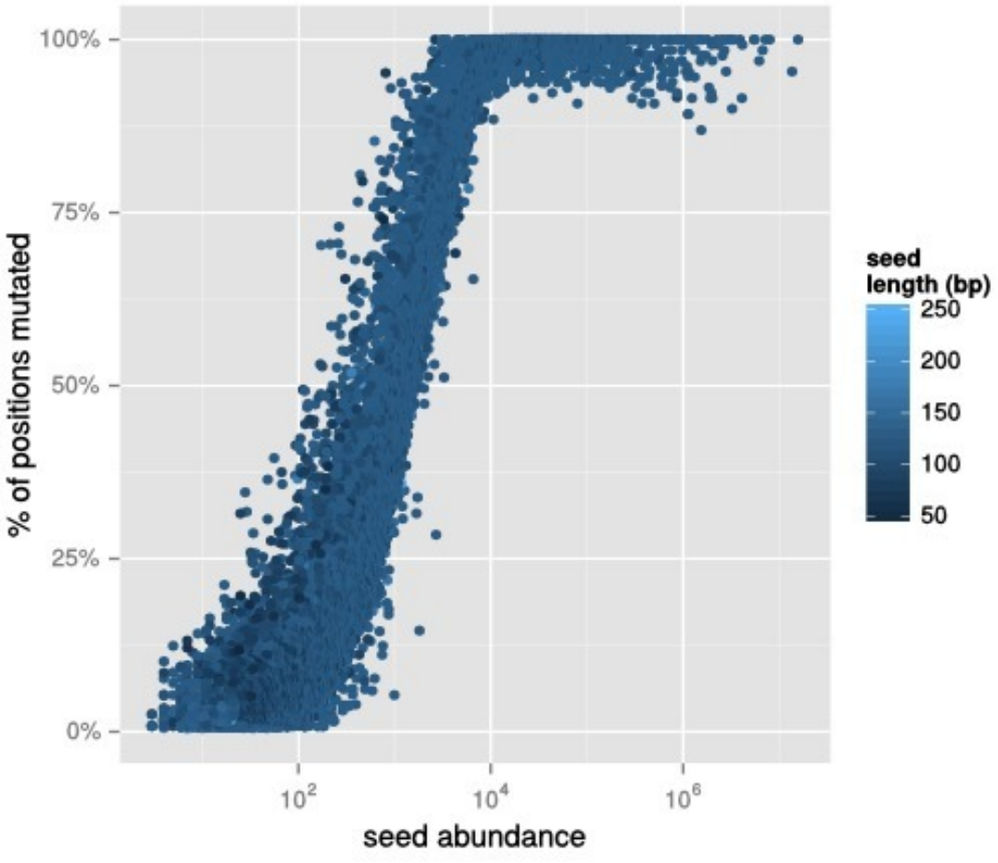
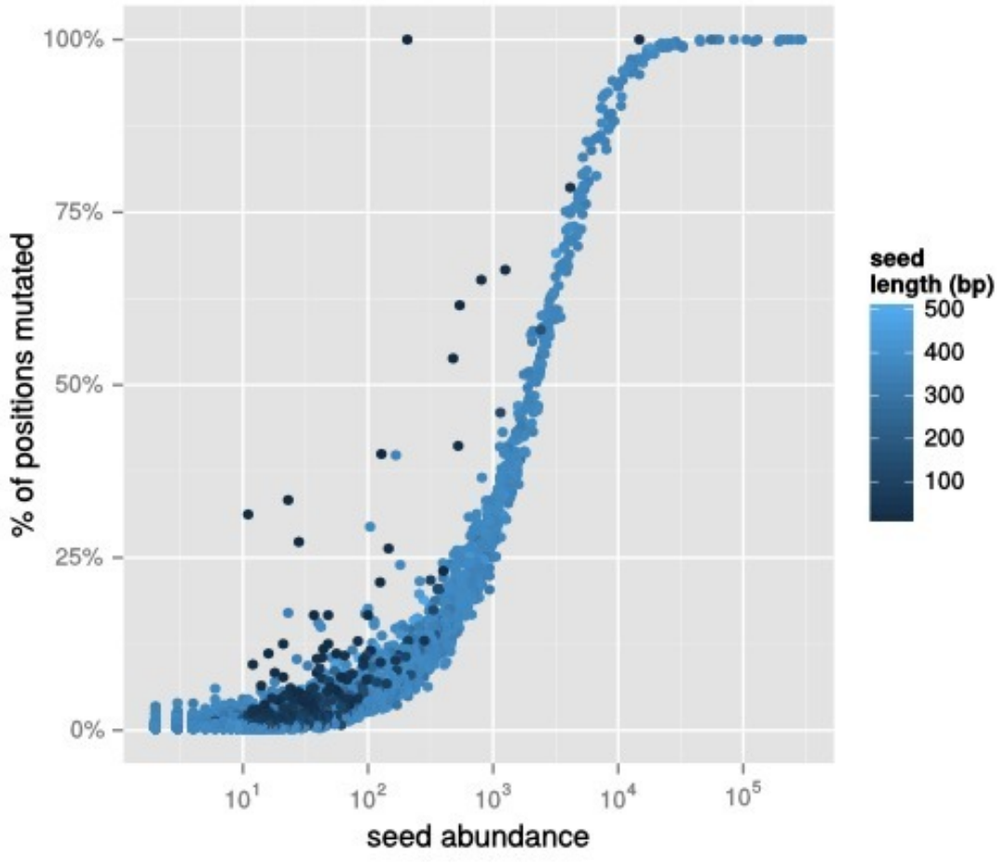
How fast errors accumulate with sequencing depth?



TARA OCEANS
V9

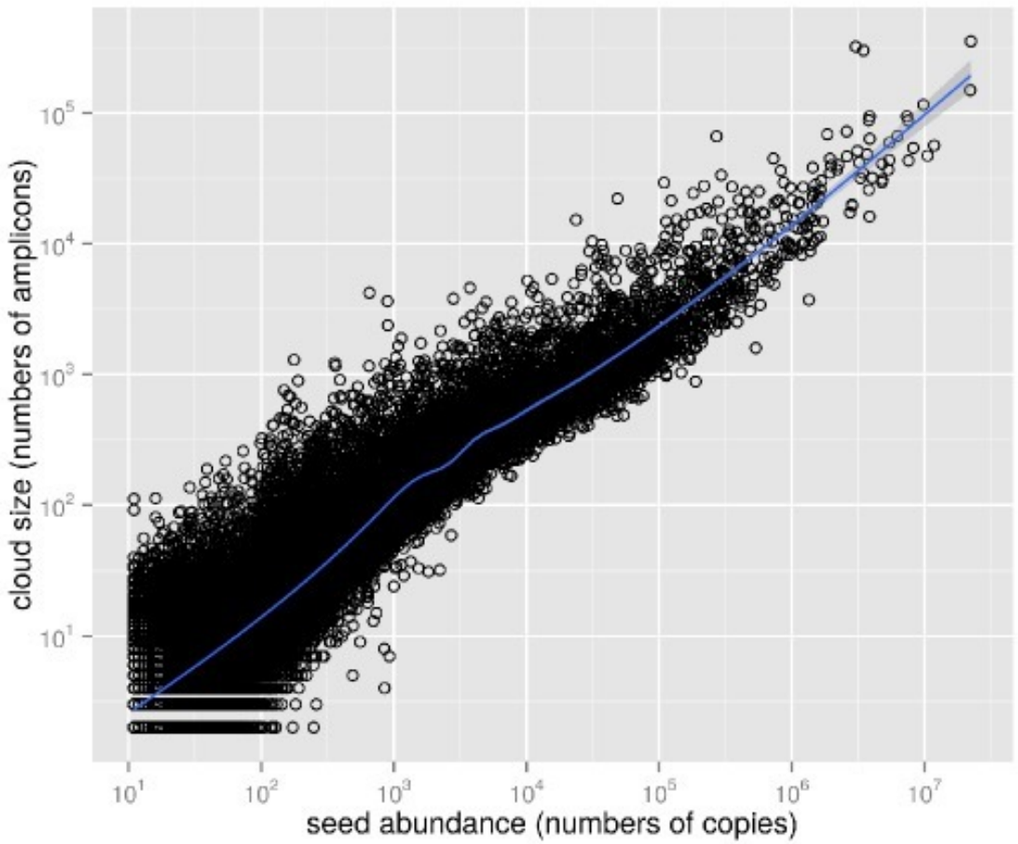
Saturation curve depends on the average seed length and sequencing platform, not on sequencing depth.



Neotropical Forests Soils
V4

Cloud size (and diameter) will vary with the taxa abundance. There is no optimal per-taxa clustering threshold.

Seed versus Cloud



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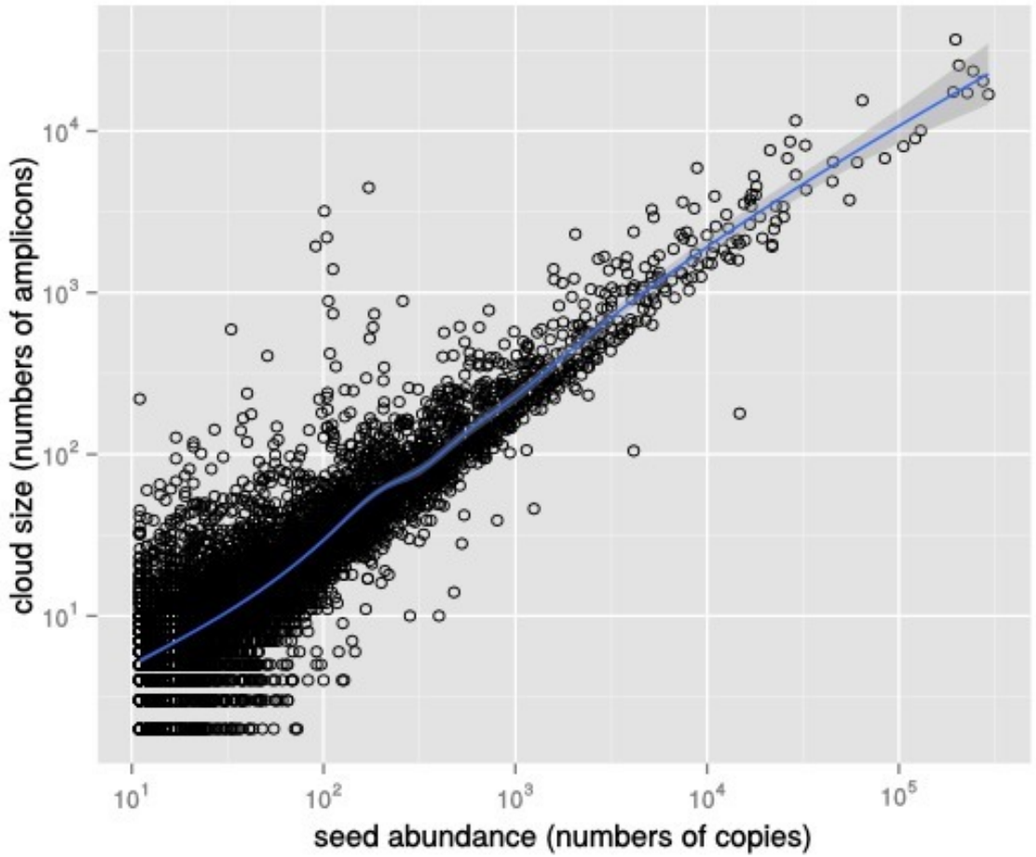
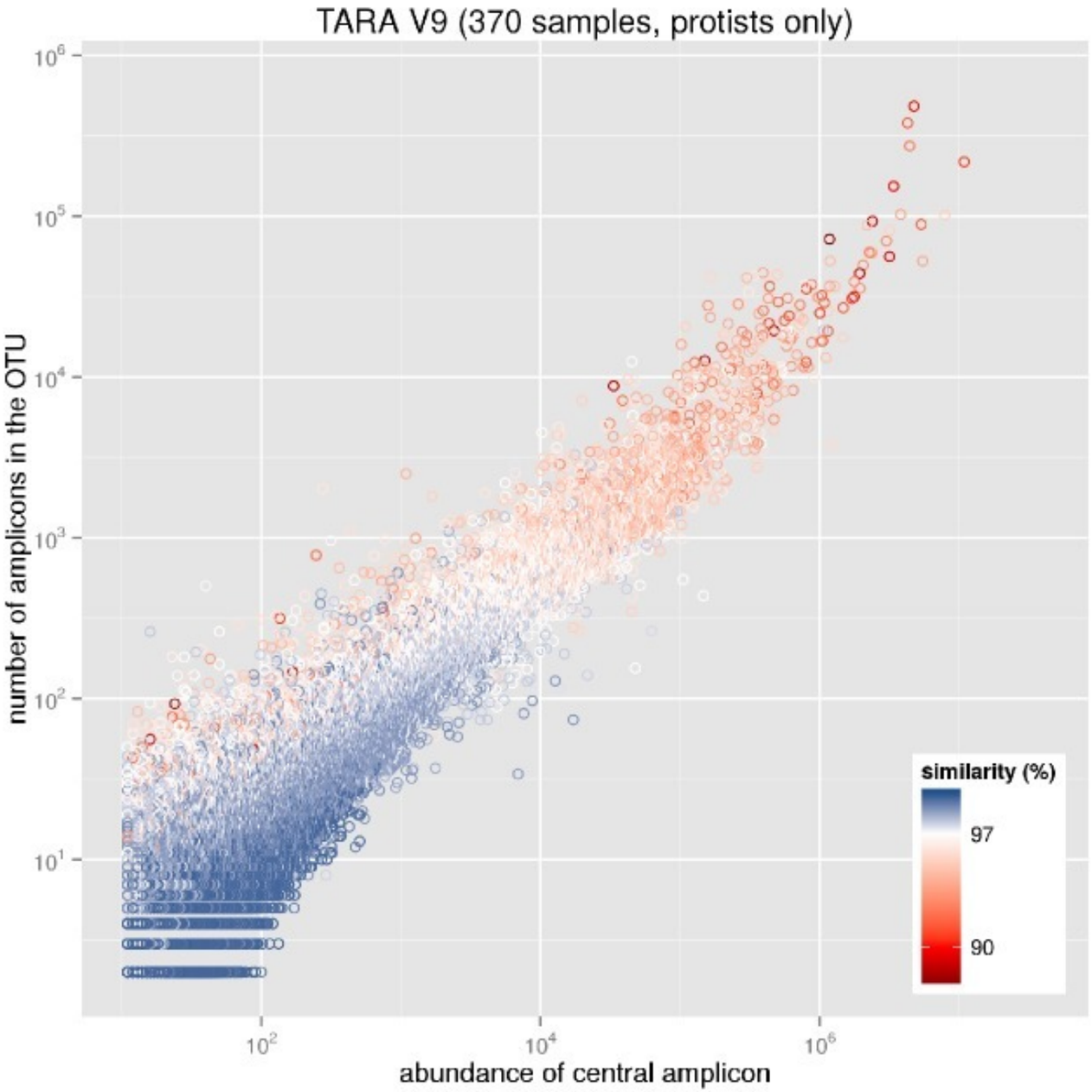
V9

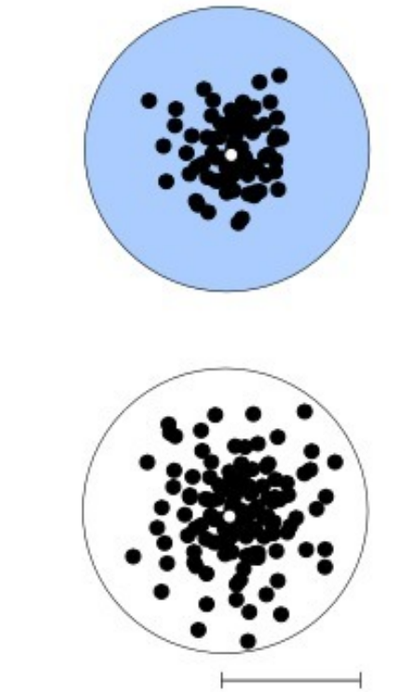


Neotropical Forests Soils
V4

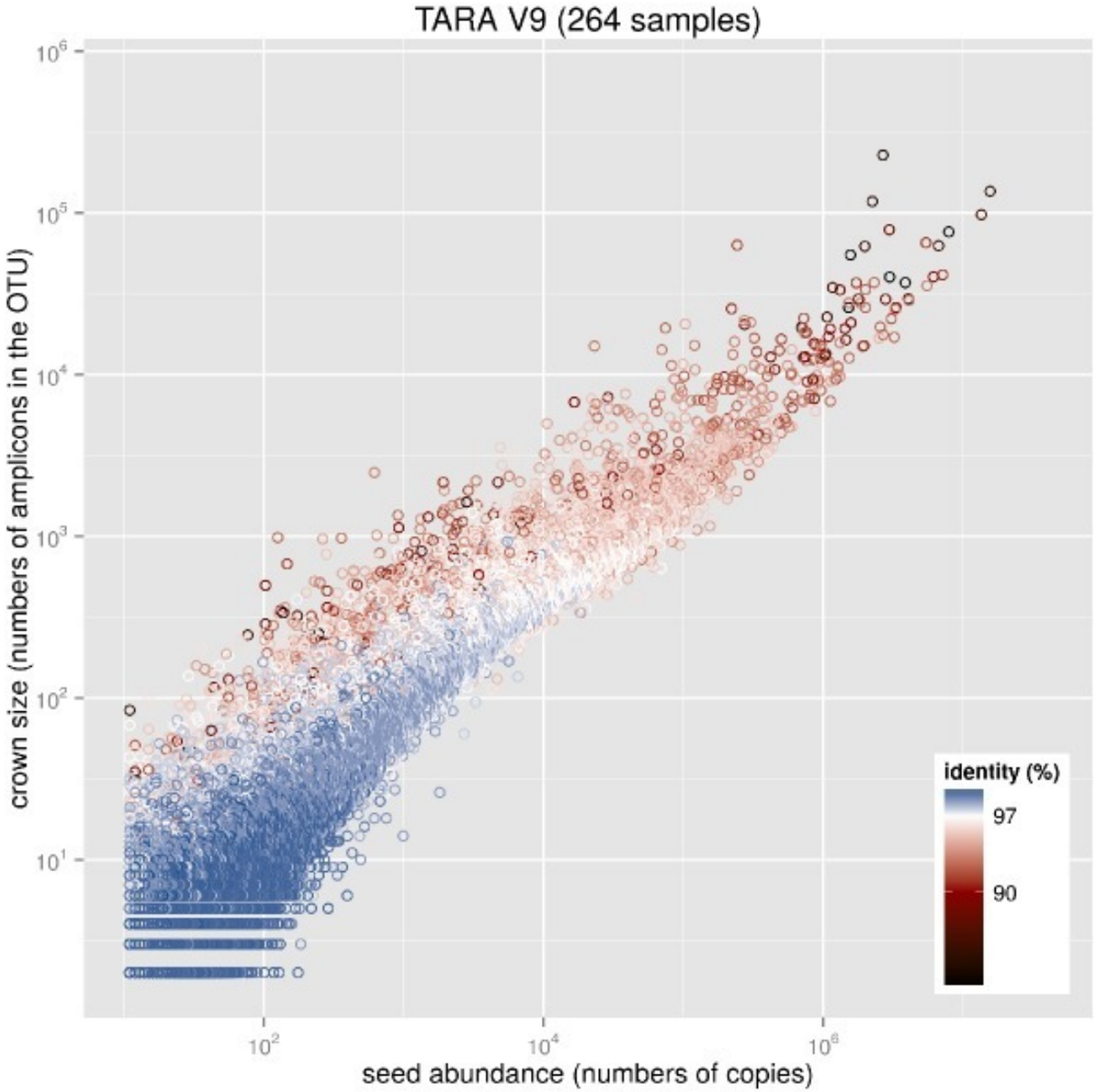
Strong correlation between abundance and the number of micro-variants. Abundant taxa are not known to have more diversified V9 sequences than less abundant taxa. What we see are technical errors.

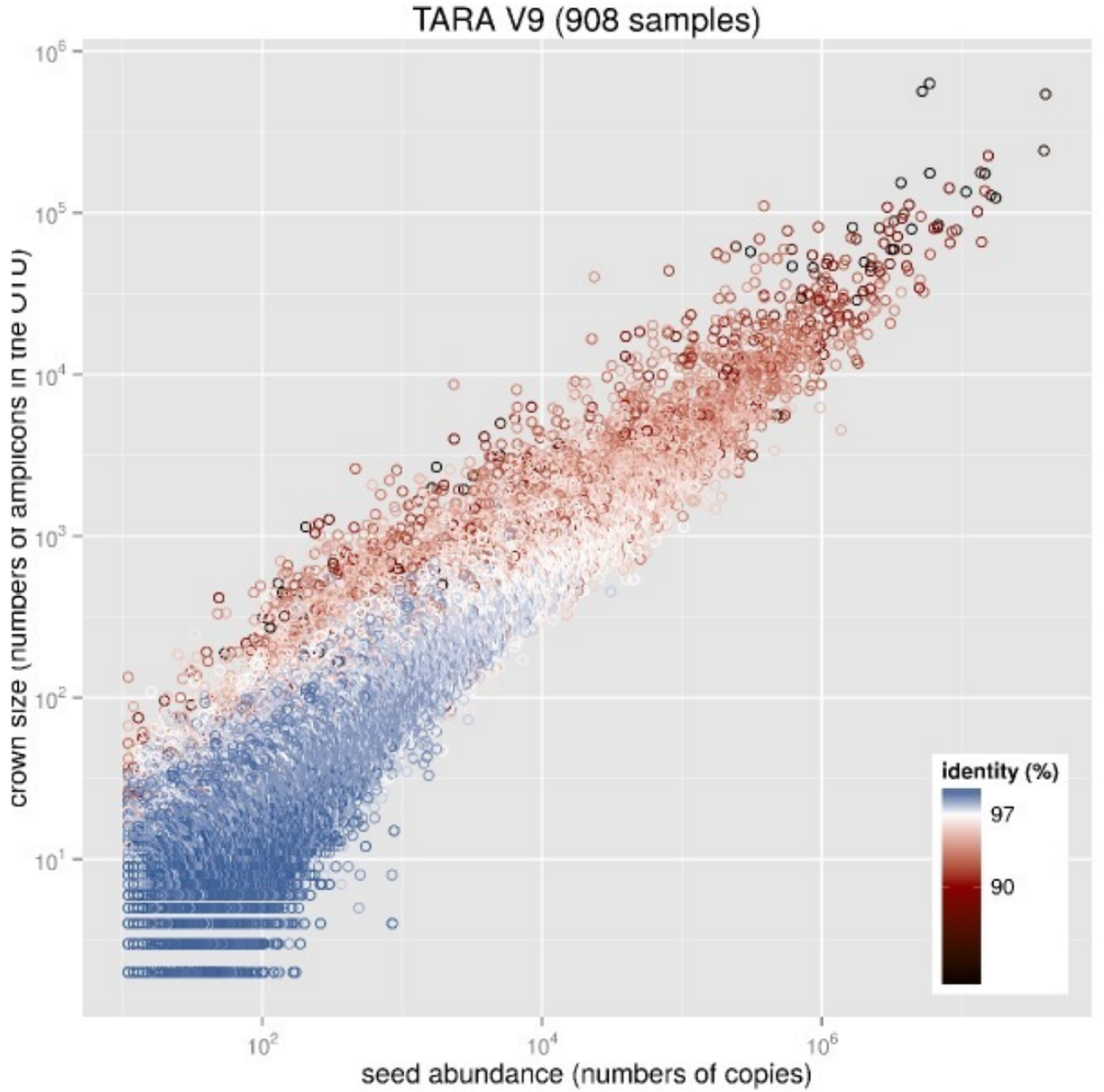
What if we'd used a fix 97%-clustering threshold?





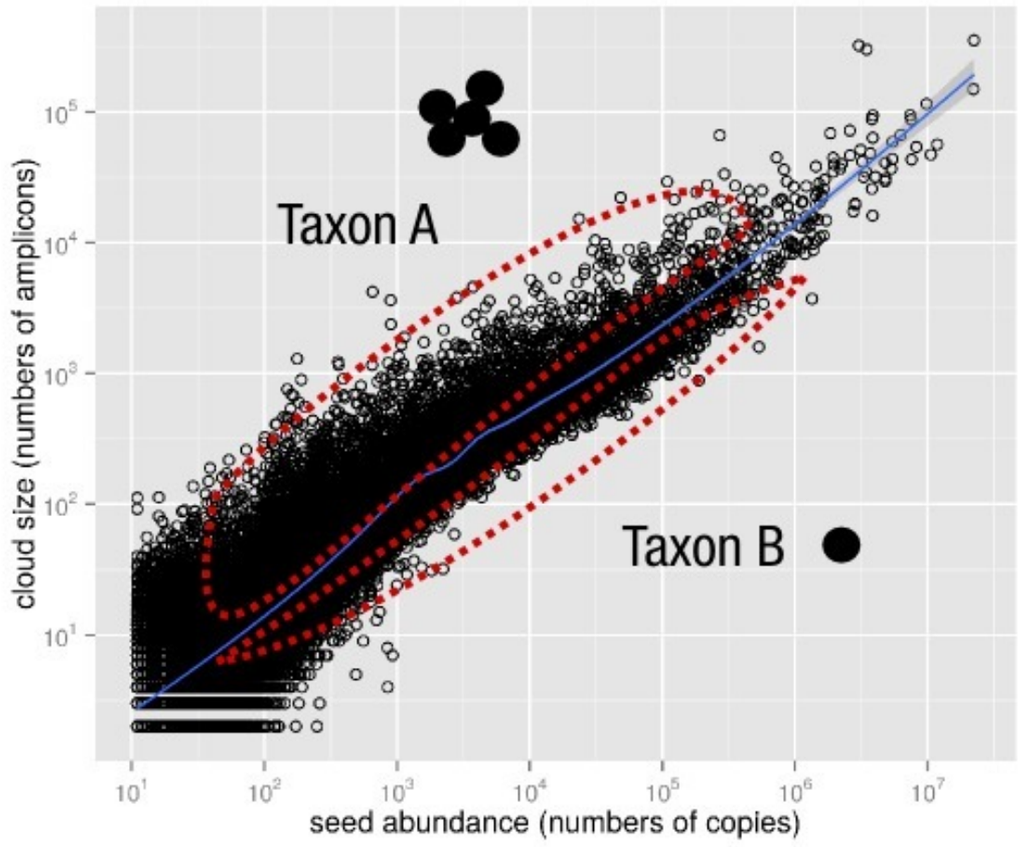
Seed abundance vs cloud vs cluster radius shows 97%-threshold inadequacy





clusters produced with swarm using d = 1

Natural variability?



TARA OCEANS
V9

New algorithms can deal with big data.

Now, how can we reduce noise further?

chimera detection

uchime

UCHIME improves sensitivity and speed of chimera detection

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R.
Bioinformatics. 2011 Aug 15;27(16):2194-200. doi: 10.1093/bioinformatics/btr381


```
A      81 CCTTGGTAGGCCGtTGCCCTGCCAACTAGCTAATCAGACGCgggtCCATCtcaCACCaccggAgtTTTtcTCaCTgTacc 160
Q      81 CCTTGGTAGGCCGCTGCCCTGCCAACTAGCTAATCAGACGCATCCCCATCCATCACCGATAAATCTTTAATCTCTTTCAG 160
B      81 TCTTGGTgGGCCGtTaCCCcGCCAACaAGCTAATCAGACGCATCCCCATCCATCACCGATAAATCTTTAAaCTCTTTCAG 160
Diffs     A       A      p A    A       A                BBBB      BBB     BBBBB BB    BBa B   B BBB
Votes     +       +      0 +    +       +                ++++      +++     +++++ ++    ++! +   + +++
Model     AAAAAAAAAAAAAAAAAAAAAAAAAxxxxxxxxxxxxxxBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBBB
```

Fig. 3. Chimeric alignment showing diffs and votes. This f gure shows a region from an alignment generated by UCHIME. Diffs and votes are annotated. The 'Model' row indicates the three segments of the alignment which are closer to A, the crossover (X) and closer to B, respectively. Diffs are 'A' = diff with Q closer to A in the A segment, 'a' = diff with Q closer to A in the B segment, and similarly for 'B' and 'b'. A 'p' diff indicates that the parents agree but are different from Q. Votes are '+' (yes), '!' (no) and '0' (abstain), indicating whether the corresponding diff supports or contradicts the model.

vsearch: open-source alternative for usearch

clustering, chimera detection, dereplication, searching, sorting, masking and shuffling

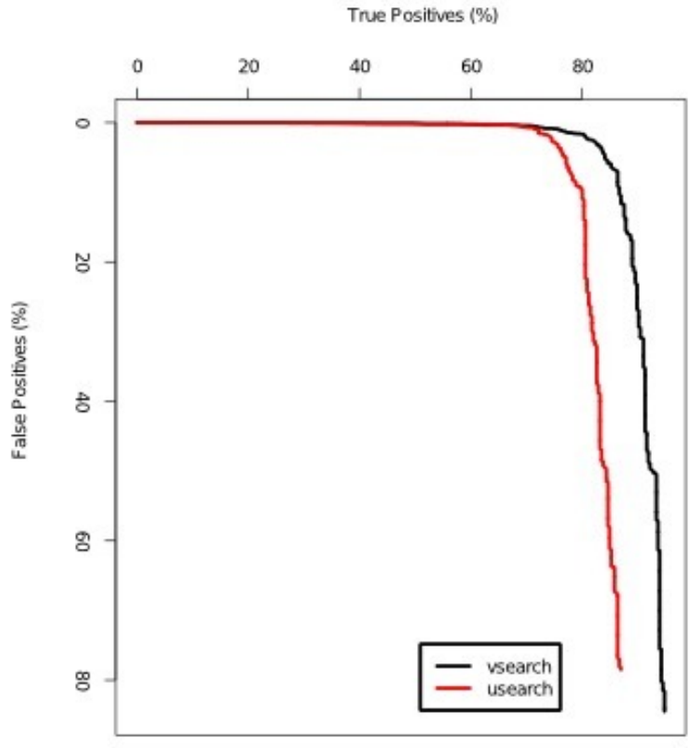
Torbjørn Rognes
Oslo University

usearch (Rob Edgar):
- very important for metagenomics,
- 1,000 citations,
- fundation for QIIME,
- closed-source & costly

vsearch:
- free and open-source,
- fast,
- documented,
- revive the research field

Quantitative Insights Into Microbial Ecology

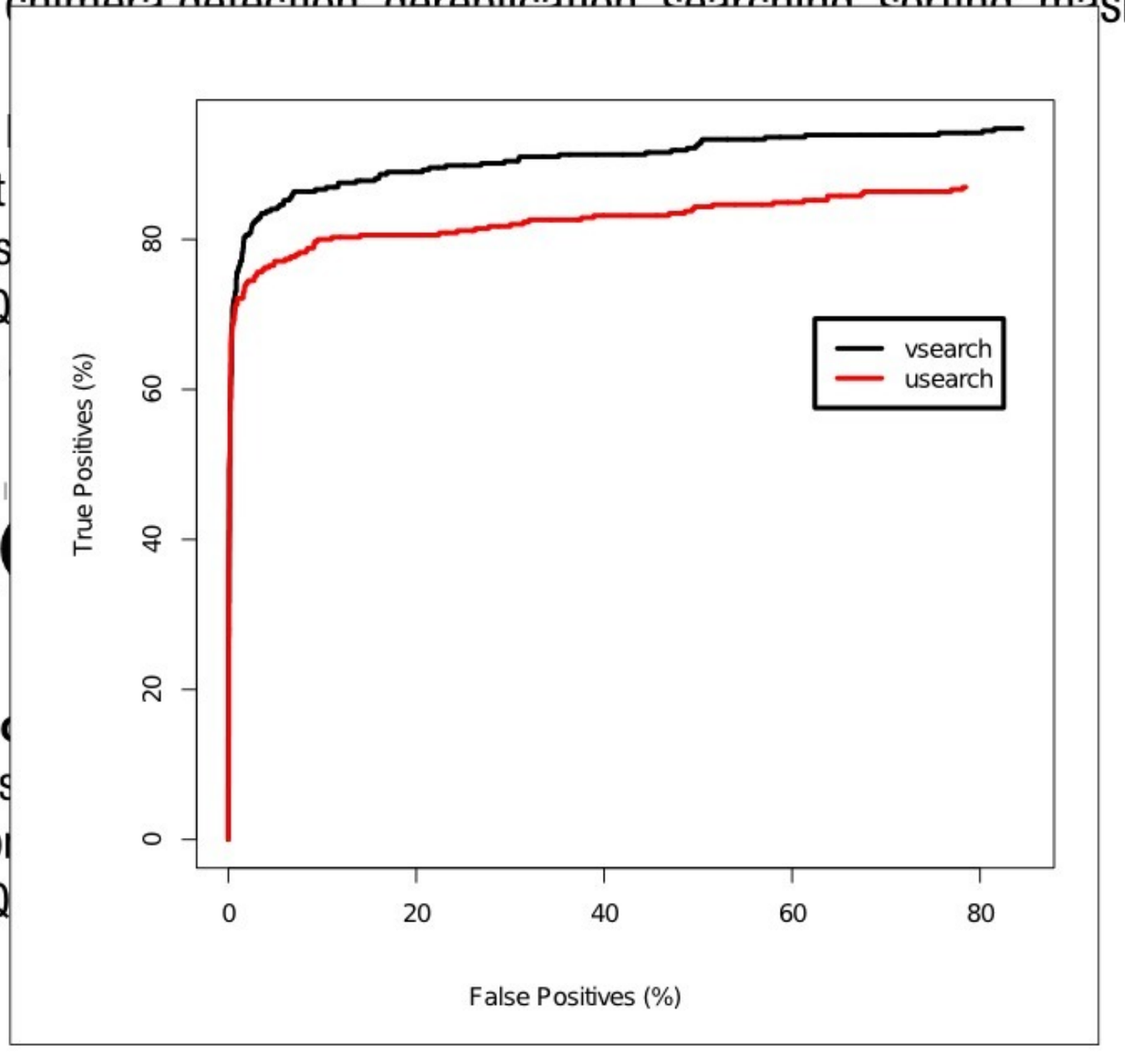
growing success:
- many happy users,
- faster and improved,
- fundation for QIIME 2.0



vsearch: open-source alternative for usearch

clustering, chimera detection, dereplication, searching, sorting, masking and shuffling

usearch (Rob
- very important
- 1,000 citations
- fundation for Q
- closed-source



Quantitative I

growing succ
- many happy us
- faster and impr
- fundation for Q

Torbjørn Rognes
Oslo University

quality filtering

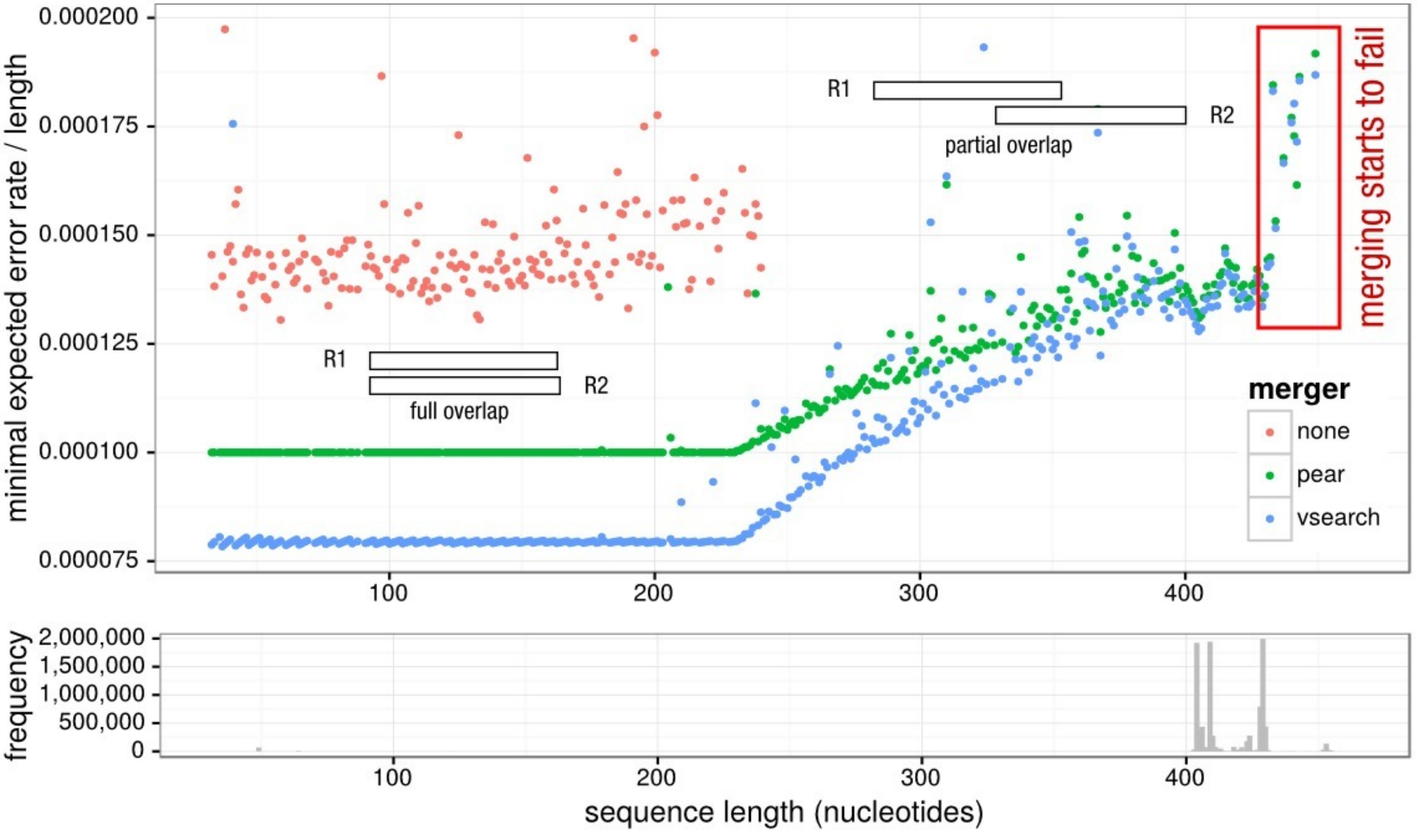
```
@M00185:171:000000000-AJ75U:1
TACGGGAGGCAGCAGTGGGGAATCTTGCGCAATGCGCGAAAGCGTGACGCAGCAACGCCG
+
BCCCC@BBCCCCGGGGGGGGGGHHHHHHGGGGGHFEGGGGGGHGGGGEGGGGGHHHFGGG

@M00185:171:000000000-AJ75U:2
CGGCGTTGCTGCGTCACGCTTTCGCGCATTGCGCAAGATTCCCCACTGCTGCCTCCCGTA
+
HEGCG-BCFGGGGGGGGGFFFFFFGGGGAGAAADFFFFFFFFFFFFFFFFFFFEDA;F
```

expected error rate = Σ Qv

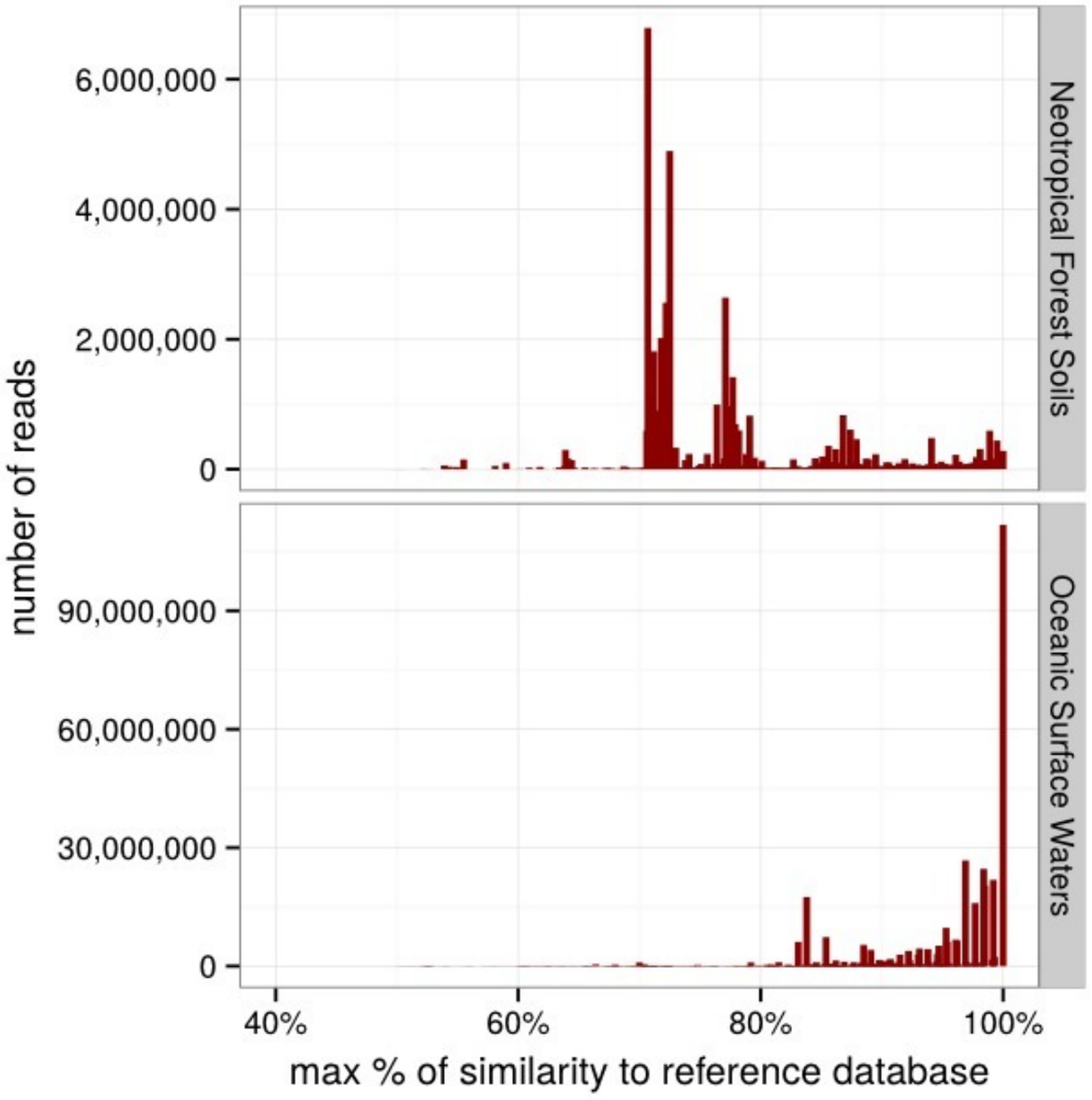
ee = 1.0 (50% chance to have zero error)

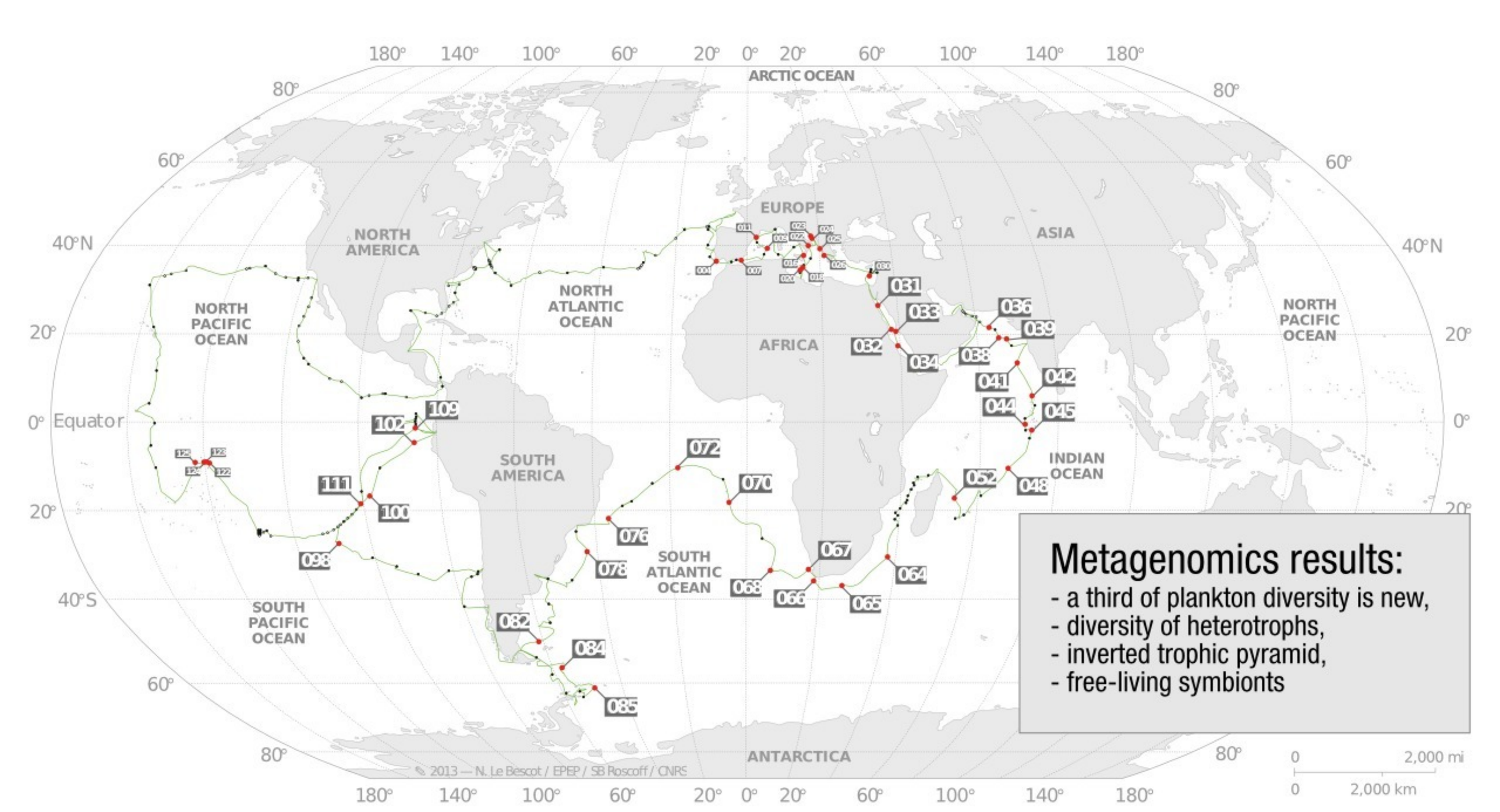
Unique sequences	Σ Q	length
ea51412efcd0b3cc8510436791667c67f186d983	0.0037	48
57e9fa79a7d578ee3fa5dbd4960f8d283b4020b1	0.0042	54
bc42f3dc4a98988d39c23fa5bbf2f3f20c372b66	0.0044	57
8e6a5352d2ba02eb5291ce5a2c4fccc95e5279c6	0.0048	61
282382d34ca8346e1b7968b2084f75249840f819	0.0048	62
092bf0fdd2cf634aba6e3cc5b6ae323db37bbf62	0.0057	73
41873e83fb3666536a7bf0f702cd24805cd11e17	0.0057	73
448b14d7a8657f19f7340da3a31d25652f40b67e	0.0057	73
61c4bb94bf70f1f86e62cad5d7951941a67b8479	0.0057	73
6fc98ad4798e85e9b8b6643786d786701e1eb0d7	0.0057	73
ad967f92c57493caf41f8b86f5bf3397e6bd4a80	0.0057	73
b6b4e3fd88e23a68773ea00a530dcf320826322b	0.0687	73
2a46cc936ef8a263360257420a72921a769440c0	0.0063	80
03dcb427634a4fe6c204a276a9237a3a20c3fe5e	0.0072	92
10dbb1a0a9555c64ad2c0b4f91aec6055175a02b	0.0072	92
121c9a9ac6f8a6fdf1dcf3ca5ecb7c484d8e7fb0	0.0072	92
1222c57cba5c99b5b6013716ec42954f872c27e4	0.0072	92

Lowest expected error observed per sequence length in a 16S V3-V4 MiSeq 2x250 bp dataset



Filtering by taxonomic assignment

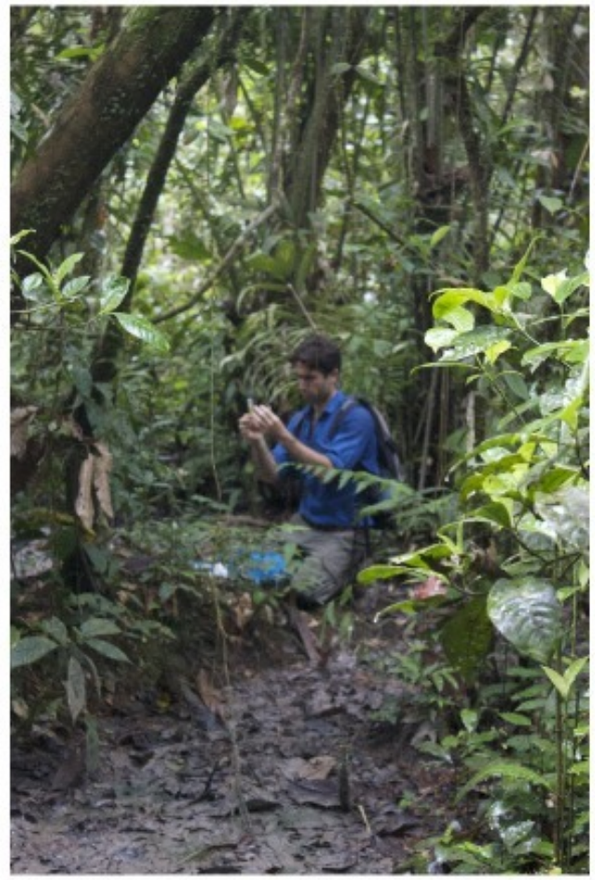




First trip of the TARA OCEANS project

(1.3 billion reads, the 47 sampling stations published so far are in red) de Vargas et al., 2015 Science

Costa Rica
Panama
Ecuador



Neotropical Forests Soil Sampling Project

Early results

- half of unknowns,
- not-so-many fungi,
- dominance of parasits,
- notable endemism,
- hyperdominant taxa

Microbial diversity at the tree line level

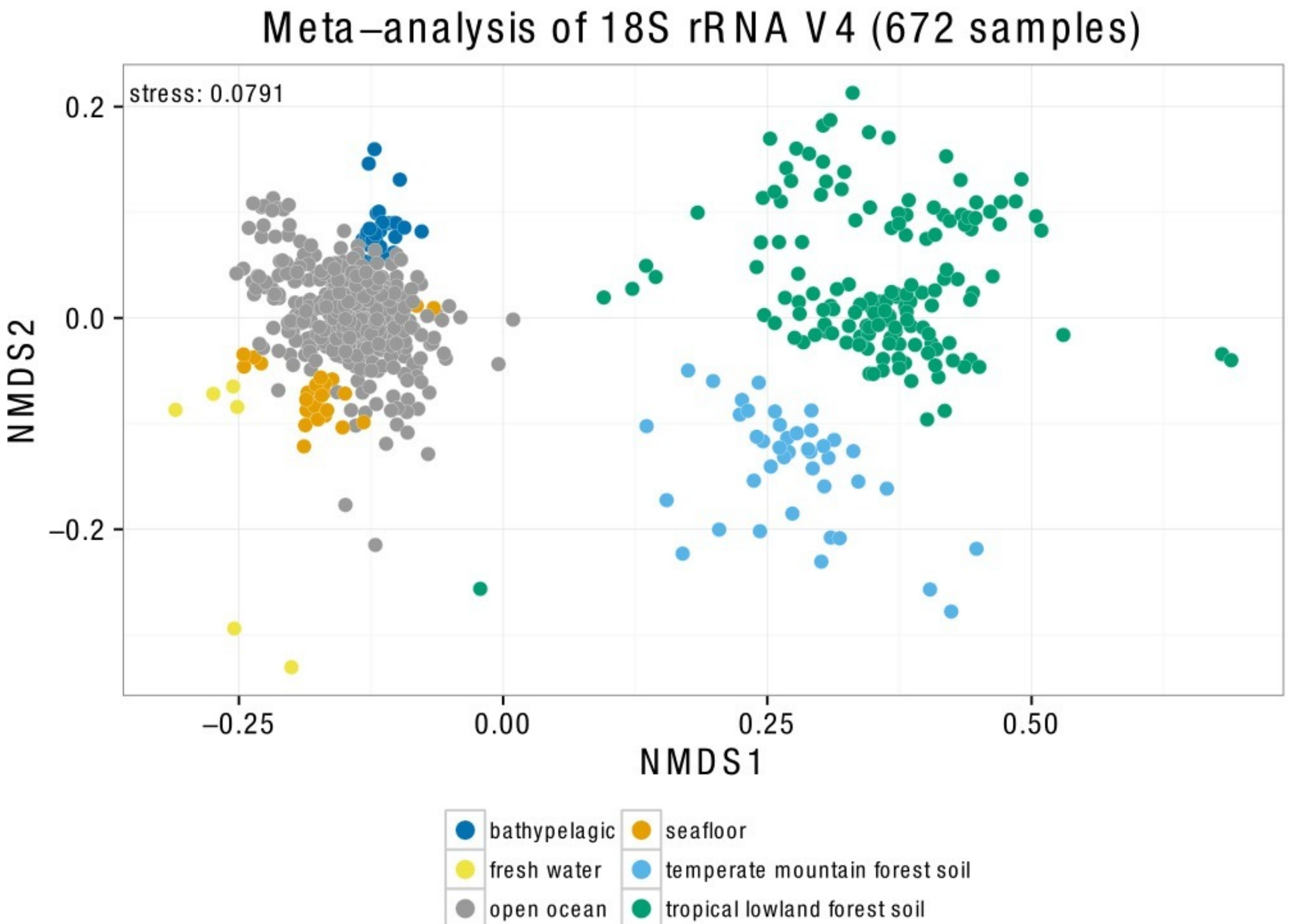
David Wardle & Jordan Mayor, Swedish University of Agricultural Sciences

Sampling

- 7 countries (Australia, Austria, Canada, Chile, Japan, New Zealand, USA),
- 5 transects per country,
- 8 replicates per transect,
- 5 soil samples per replicate,
- soil chemistry

Early results

- few unknowns,
- dominance of fungi,
- weak endemism,
- microbial communities?
- geographical analysis?


Meta–analysis of 18S rRNA V4 (672 samples)
stress: 0.0791
0.2
0.0
−0.2
NMDS2
−0.25
0.00
0.25
0.50
NMDS1
bathypelagic
seafloor
fresh water
temperate mountain forest soil
open ocean
tropical lowland forest soil

What do we need to deal with noise?

faster and more efficient filters (denoising and clustering)
better understanding of PCR/sequencer noise
stronger mathematical background (sequence-space)
robust α, β statistics able to deal with noise
repeated experiments (technical/biological replicates)

Technical noise and robust stats
are our main challenges

Thanks!

https://github.com/torognes/swarm
https://github.com/torognes/vsearch


1 µm