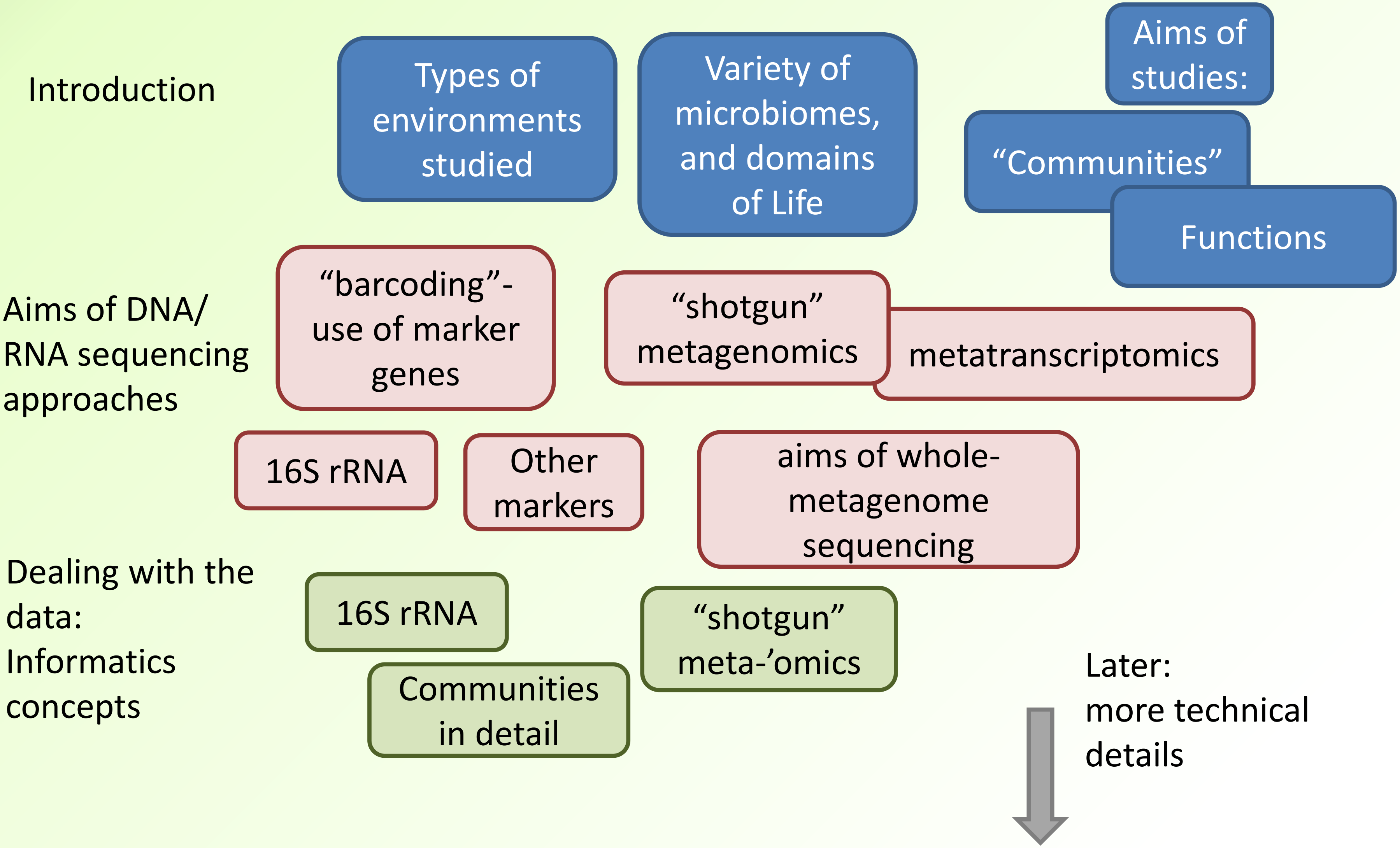
Introducing Microbiome Bioinformatics

Part 4.

Recap: Aims

- **Microbiome analysis**
  - with particular regard to **sequence informatics concepts**
- "Top down" – putting analysis tools and resources in context
- No highly detailed technicalities (yet)
  - No instructions on how to run particular programs
- Why you are using the bioinformatics approaches you use; pros, cons; alternatives

Topics, top-down



Introduction

Types of environments studied

Variety of microbiomes, and domains of Life

Aims of studies:

“Communities”

Functions

Aims of DNA/ RNA sequencing approaches

“barcoding”- use of marker genes

“shotgun” metagenomics

metatranscriptomics

16S rRNA

Other markers

aims of whole-metagenome sequencing

Dealing with the data: Informatics concepts

16S rRNA

“shotgun” meta-’omics

Communities in detail

Later: more technical details

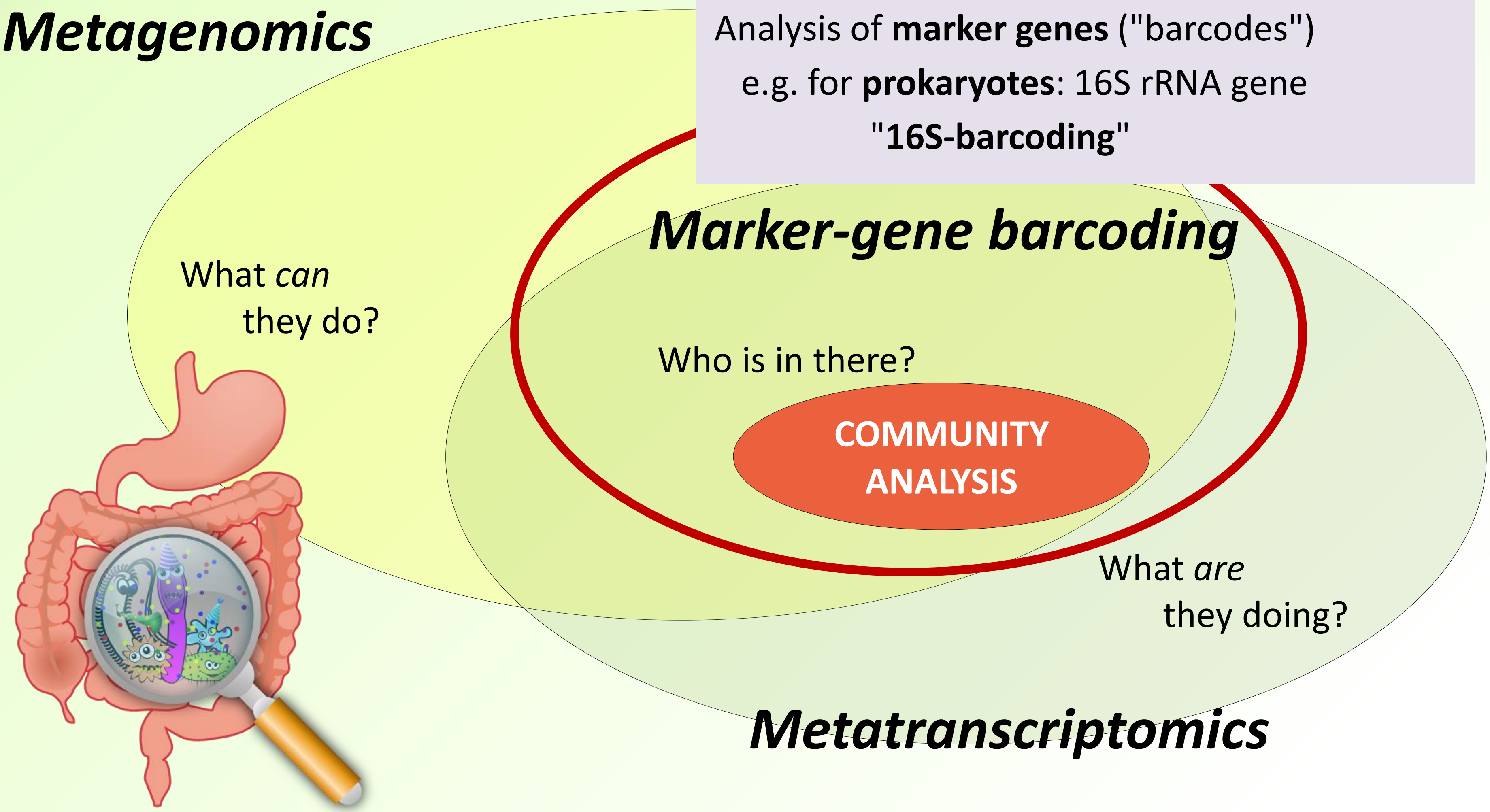
Series of talks

- 3 so far
- Open ended… as long there is demand
- Expected to be every 2 weeks, but all dates will be confirmed in advance
  - *Bite-size bioinformatics mailing list*
- The next few will cover: (*not necessarily in this order…)*
  - 16S analysis for community profiling
  - Classification issues (taxonomies etc)
  - Analysing richness and diversity of those communities
  - Dealing with sequencing and other errors
- Informal and flexible
  - Please interrupt and ask questions
  - Suggestions for topics for further focus

Series of talks

- Part 1: 27/1/2017
  - "Biological and Experimental Stuff that a microbiome bioinformatician needs to know"
  - Overview of marker gene sequencing for community analysis
- Part 2: 10/2/2017
  - Overview of whole-metagenome sequencing
- Part 3: 24/2/2017
  - Focus on metatranscriptomics
- Slideshows
  - http://ghfs1.ifr.ac.uk/ghfs/

- **Who is in there?**
  - In what amounts?



Analysis of **marker genes** ("barcodes")

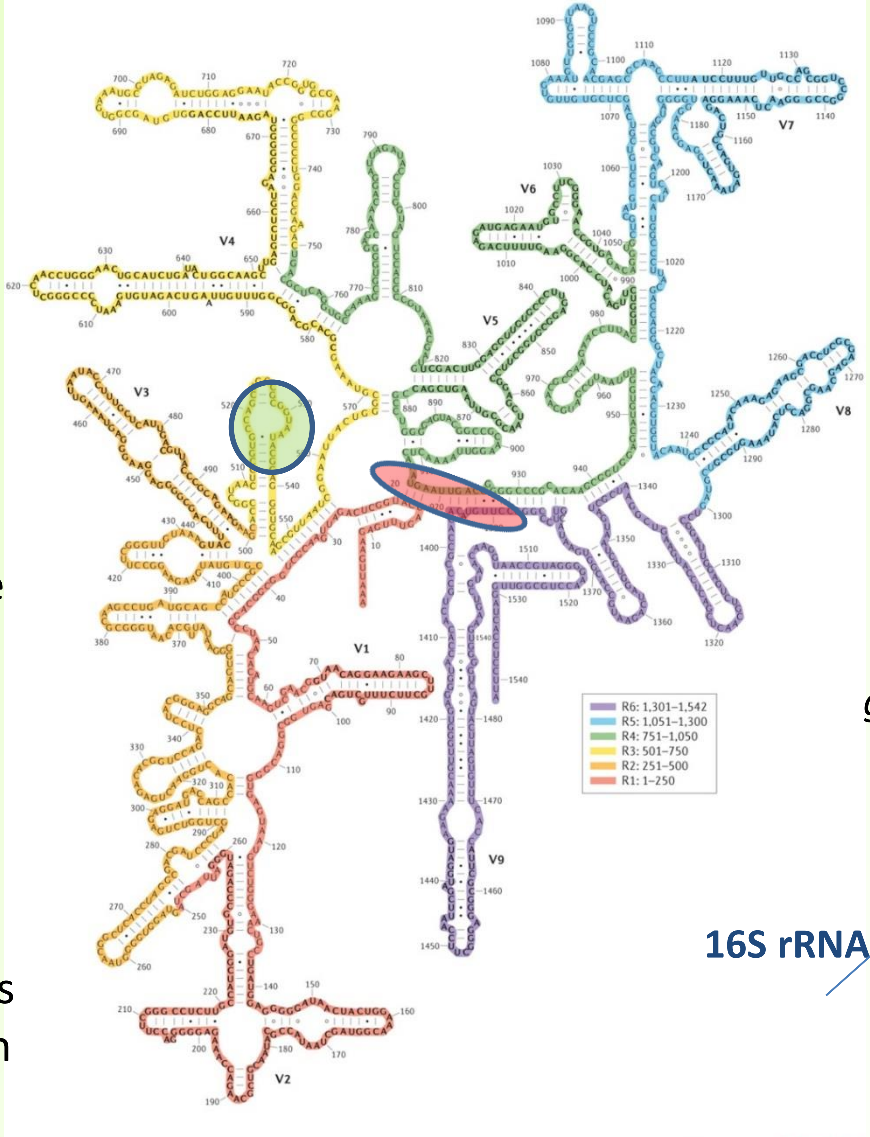
e.g. for **prokaryotes**: 16S rRNA gene

"**16S-barcoding**"

Amplification of a **segment** of the gene which codes for a **variable** region of the 16S rRNA molecule

→Primers

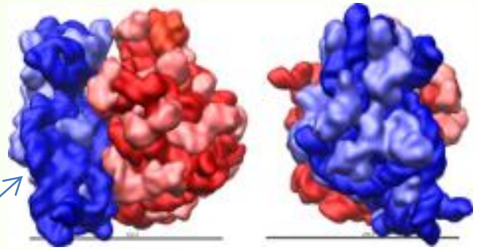
The variable region is chosen to distinguish between taxa



Nature Reviews | Microbiology

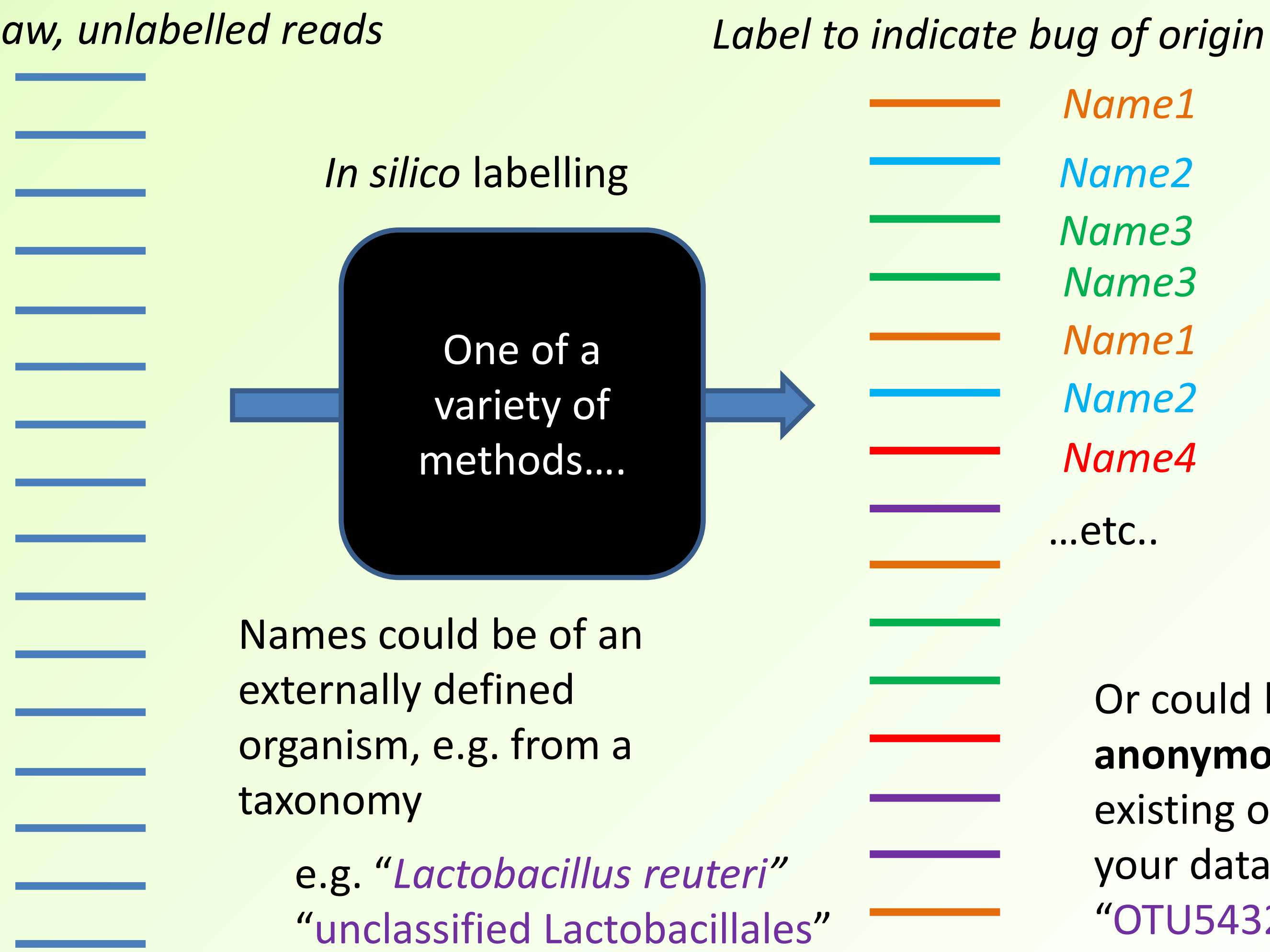
marker gene
(“barcode”)
for ***phylotypes***

gene which codes for…



16S rRNA

Community analysis by marker-gene sequencing

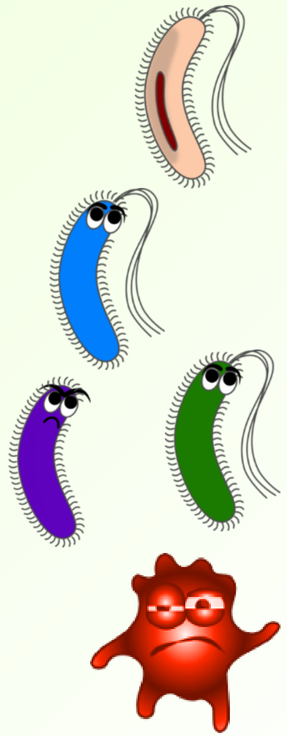


Raw, unlabelled reads

In silico labelling

One of a variety of methods….

Label to indicate bug of origin



Names could be of an externally defined organism, e.g. from a taxonomy

e.g. “*Lactobacillus reuteri*”
“unclassified Lactobacillales”
etc

Or could be **completely anonymous**, a name existing only within your data e.g. “OTU5432”
- Diversity studies still possible

First - some considerations

- Using predefined taxonomies
  - Sequences in a **reference database** have taxonomic annotation
- Using Operational Taxonomic Units (OTUs)
  - **What are OTUs?**
  - **(**for later:
    - **What do they represent?**
    - **Relationship with predefined taxa?)**
- Comparing reads with a database
- Self-comparison (clustering) of a set of reads

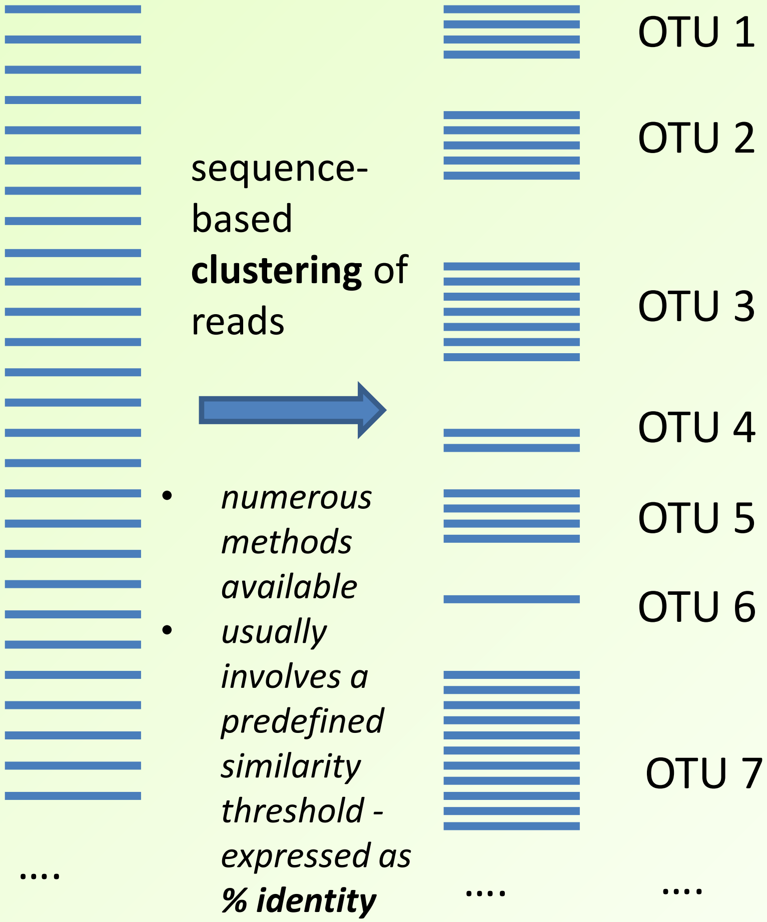
So what about all these methods?

One approach:

Clustering reads into OTUs

collection of reads – must be homologous (e.g., all are amplicons of the same 16S variable region)

Clusters = Operational Taxonomic Units (OTUs)



sequence-based **clustering** of reads

- *numerous methods available*
- *usually involves a predefined similarity threshold - expressed as **% identity***

Number of OTUs = **measure of richness**

This might be all you want to do = end of the analysis.

Or, if you have multiple samples, then you may want to:

(1) Compare **numbers** of OTUs between samples

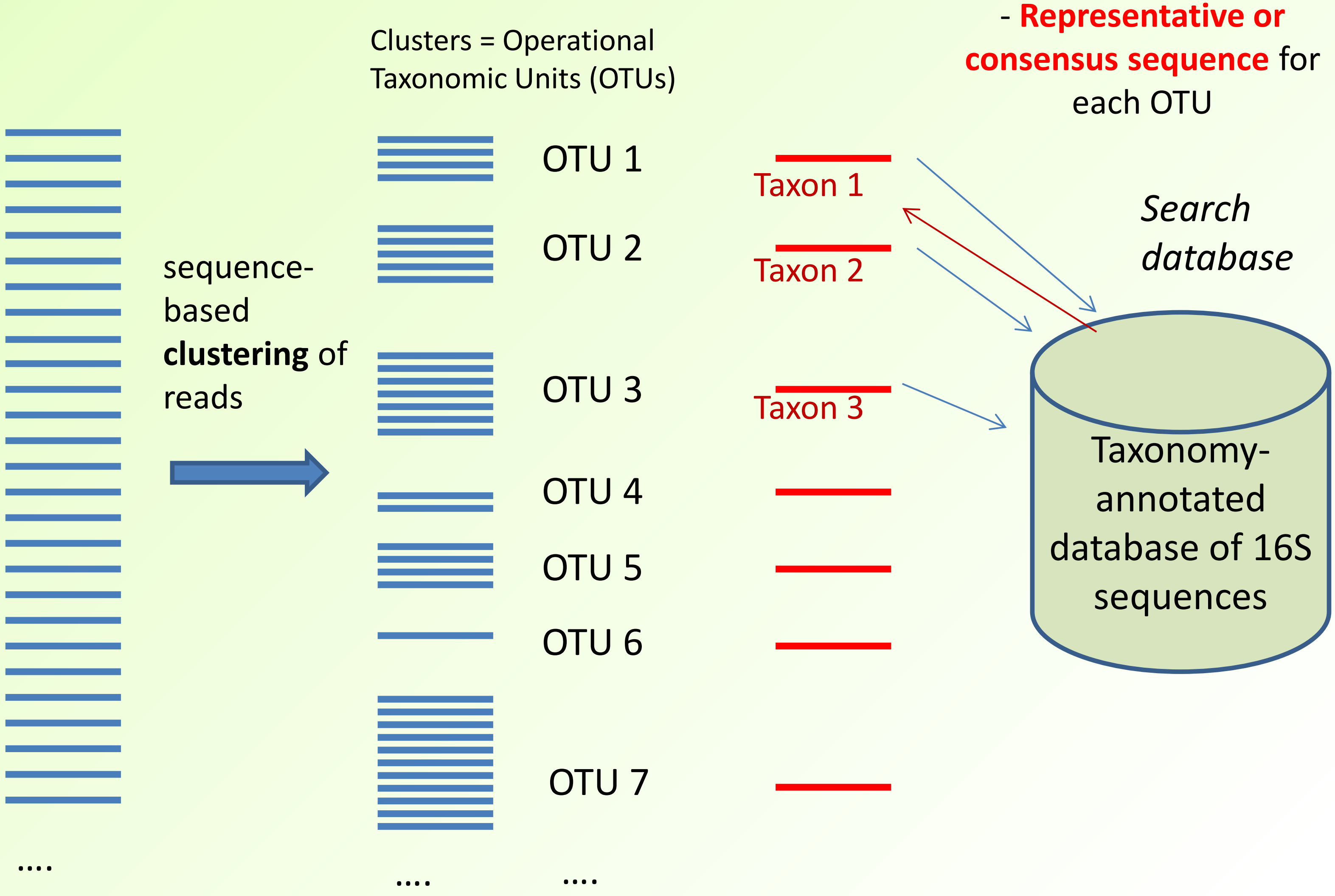
(2) Assess differences in **diversity** between sample

(3) Compare **actual OTUs** (presence/absence, or proportions, between samples)

Distribution of proportions of OTUs = **measure of diversity**

No reference database needed

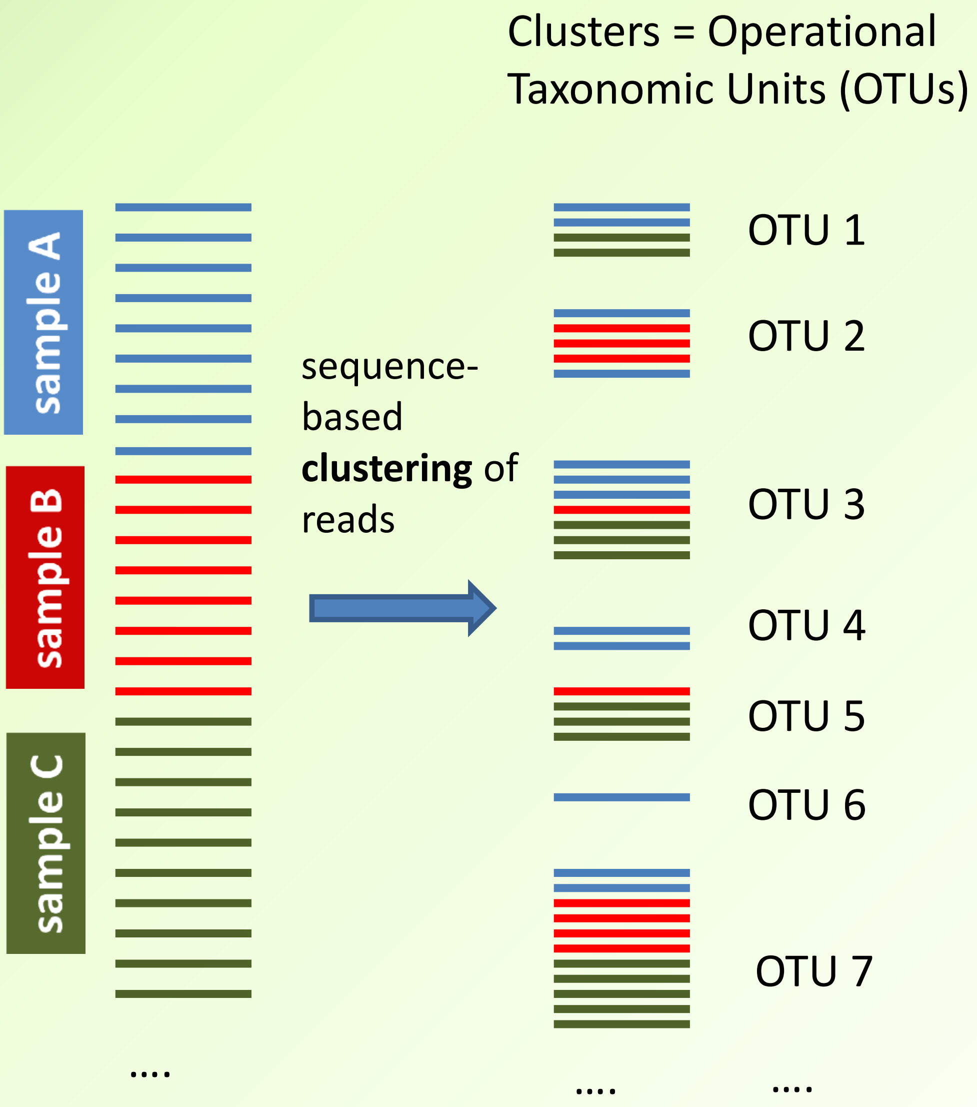
Usually (always?) we will also want to **identify** the OTUs - in terms of a known taxonomic group


Clusters = Operational Taxonomic Units (OTUs)
- **Representative or consensus sequence** for each OTU
sequence-based **clustering** of reads
OTU 1
OTU 2
OTU 3
OTU 4
OTU 5
OTU 6
OTU 7
Taxon 1
Taxon 2
Taxon 3
Search database
Taxonomy-annotated database of 16S sequences
....
....
....

OTU assignment

- "OTU-assignment" is used here to describe the placing of each sequence read into a particular OTU
  - Once that is done, you know **which of your reads are in the same OTUs** as each other
  - **How many different OTUs** there are, etc
- In the general case, that process is distinct from the ***identifying*** of those OTUs
  - "**Identifying**" necessarily means **using some sort of reference**
  - But - assigning reads to OTUs, and identifying the OTUs, **can** be done as part of a single process
- It all depends on which approach is taken

Dealing with multiple samples



Clusters = Operational Taxonomic Units (OTUs)

sequence-based **clustering** of reads

- Ideally, as many sequence reads as possible should be clustered in the same operation
- Which OTU a read is assigned to ***can depend on which other reads are present*** in the clustering exercise
    - May depend on the clustering method used
- So, cluster all your samples’ reads together

So, what ***are*** OTUs?

What do they represent?

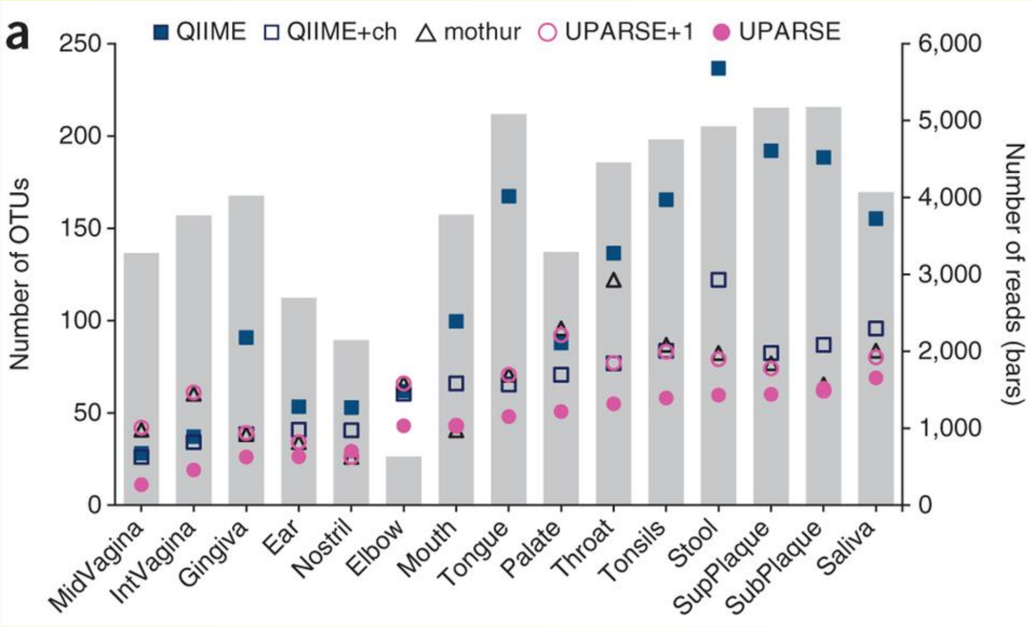
How do they relate to taxa?

…and what’s so special about the number 97?

For now….

- A 97% sequence-identity threshold is often used when clustering reads into OTUs
  - **How** this threshold is used depends a lot on the clustering method
- “Notionally”, the resulting OTUs are roughly equivalent to species…
  - But actually, it’s far from that simple
- Also… 97% identity… of what?

- Different methods (even using the same 97% threshold) can produce **very** different numbers of OTUs



from Edgar (2013) *Nature Methods* **10** (10) 996-8

- But does this actually matter?
  – Discuss….next time

Other methods

Not clustering

Taxon-based

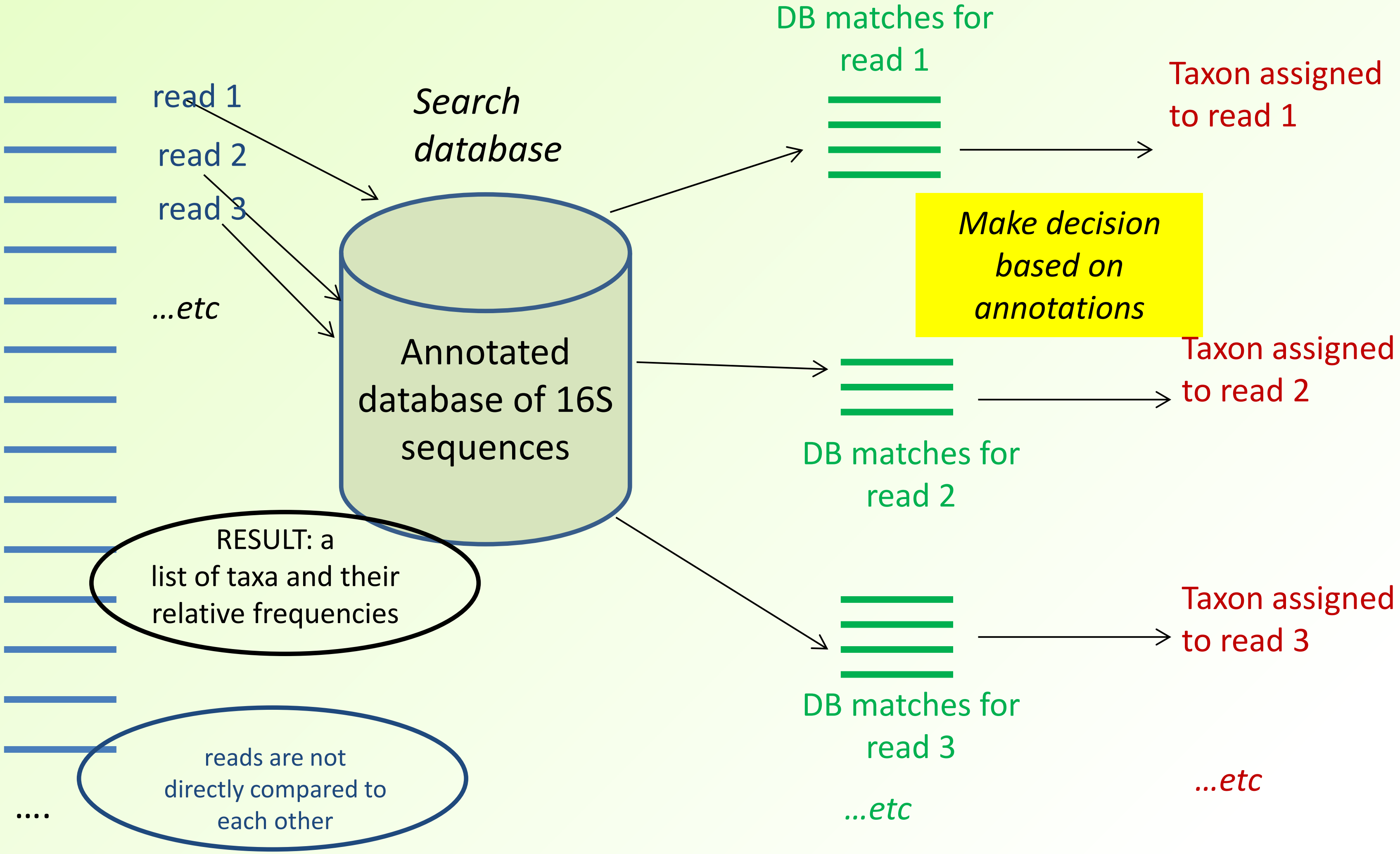
Not OTU-based

Other methods? (not clustering)

- Some methods are a read-by-read approach (“process one read then the next one…”)
  - That is, **each read is processed <u>independently</u> of all the others**
  - This means the process is easy to **<u>parallelise </u>**:
  - many or all reads can be processed at the **same** time
- These methods necessarily involve comparing **<u>each read </u>**with sequences in a reference database
  - There are different approaches
  - In terms of the databases used
  - And **<u>how</u>** the sequence comparison is done

collection of 16S reads

Assigning a taxonomic classification to each **individual** read : ***one example approach***



read 1

read 2

read 3

...etc

Search database

Annotated database of 16S sequences

DB matches for read 1

DB matches for read 2

DB matches for read 3

...etc

Make decision based on annotations

Taxon assigned to read 1

Taxon assigned to read 2

Taxon assigned to read 3

...etc

RESULT: a list of taxa and their relative frequencies

reads are not directly compared to each other

....

Read-by-read methods

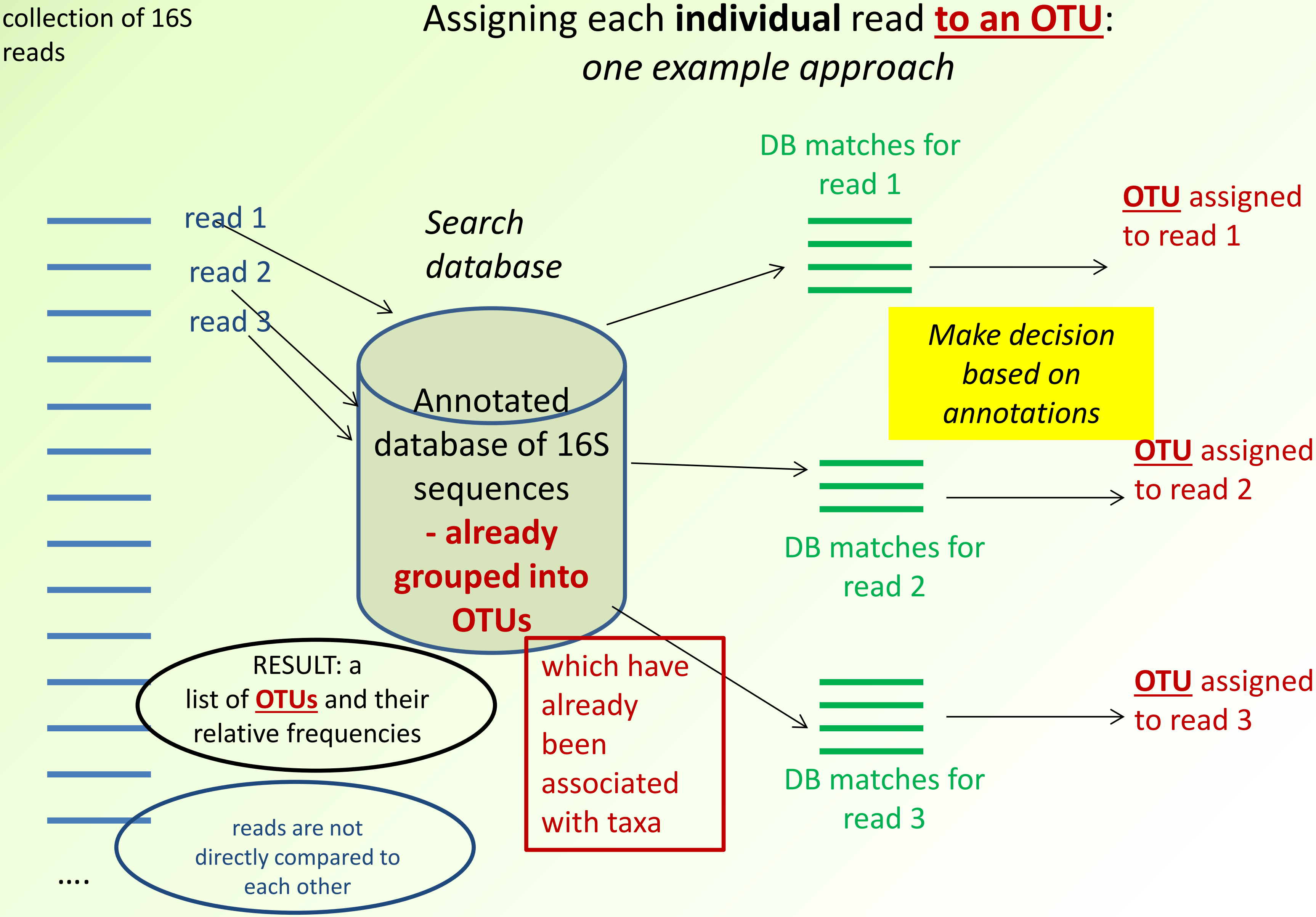
- The reads are each compared to a database, and not to each other
- So this is just as applicable to processing reads from shotgun metagenomics/ metatranscriptomics
  - (but is not the only way of doing this)
- One approach is simply a traditional sequence similarity search (e.g. **BLAST**)
  - With huge numbers of query sequences
  - And using a different database (i.e. not just 16S sequences!)
  - But **making sense of the list of hits can be far from straightforward**
- Can be computationally expensive –
  - if your read set is very large
  - and your reference database is very large too (choose wisely)
  - may actually take up more “wall time” than OTU-clustering, if a fast heuristic clustering method is used
  - unless you have a very large number of processors available
- More about this in future sessions

Back to OTU-assignment….

Other approaches to OTU-assignment

- "**OTU-assignment**" used here to refer to all methods of assigning your reads to OTUs
- Traditionally:
  - OTU-assignment == **OTU-clustering**
  - by whatever clustering method
- Comparison of read sets versus themselves:
  - problematic/impossible for extremely large numbers of reads
  - clustering: difficult to **parallelise** computationally
    - The nature of all-versus-all comparison
  - Some widely-used heuristic methods have been developed
- Has motivated alternative methods of OTU-assignment
  - Using reference databases – of known 16S sequences **already clustered into OTUs** by database curators
  - and the OTUs in the database also **annotated with taxa**

Assigning each **individual** read **to an OTU**: *one example approach*



collection of 16S reads

read 1

read 2

read 3

....

Search database

Annotated database of 16S sequences **- already grouped into OTUs**

which have already been associated with taxa

DB matches for read 1

DB matches for read 2

DB matches for read 3

Make decision based on annotations

OTU assigned to read 1

OTU assigned to read 2

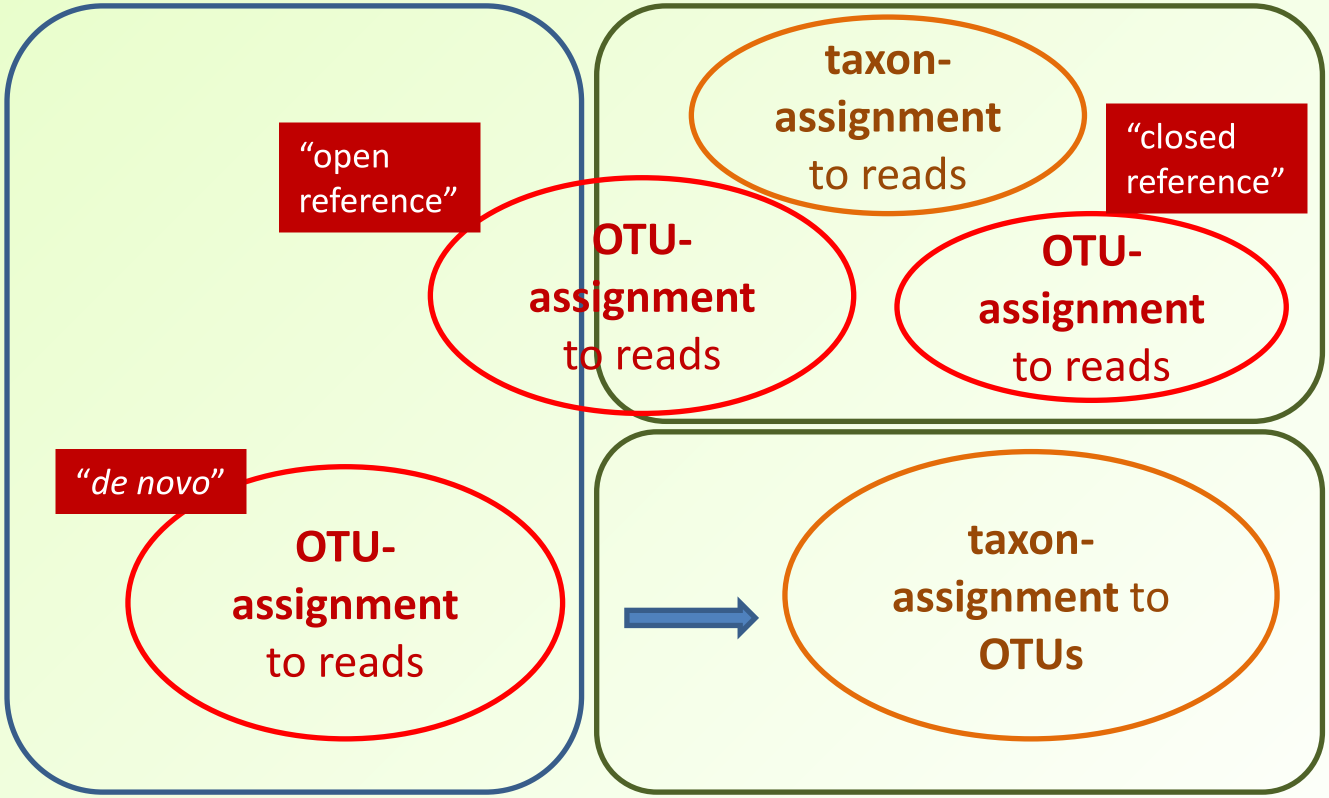
OTU assigned to read 3

RESULT: a list of **OTUs** and their relative frequencies

reads are not directly compared to each other

Clustering : comparing reads with each other (“self-referential”)

Using a reference database



taxon-assignment to reads

“closed reference”

OTU-assignment to reads

“open reference”

OTU-assignment to reads

“*de novo*”

OTU-assignment to reads

taxon-assignment to **OTUs**

processing each **read** independently

processing representative sequences of each OTU

More terminology

- In some contexts, "**OTU-picking**" is used for OTU-assignment
- Potentially confusing: might imply that you are "**picking**" (**selecting**) particular OTUs for your data, from some larger list of OTUs
  - In fact, this is exactly what you ***are*** doing with some methods
  - such as **open- or closed-reference OTU-picking**
- in a different approach, ***de novo* OTU-picking**, there ***is no list of OTUs*** to select from:
  - The list of OTUs is self-generating, from within your data

de novo OTU-assignment

- Clustering reads; no reference database
- Advantages
  - Very systematic; transparent; database-independent
    - You won't get different results if you repeat later, when databases will have changed
    - ***Metrics of richness and diversity will remain unchanged***
    - (but you may well get changes in terms of the later ***identification*** step)
- Disadvantages
  - Speed: very difficult to parallelise
    - Collection of reads must be self-compared
    - Not a problem unless your datasets are huge
  - Does not take advantage of external expert "OTU-curation"

Closed-reference OTU-picking

- Compare each read to reference DB sequences
  - Discard reads which don't match
- Advantages
  - Potentially, speed: can be parallelised
    - Assignment/identification of each read is independent of others
    - Makes use of external curation: use of far larger database collections may enable better OTU-definitions
- Disadvantages
  - Completely dependent on a database
  - ***Even basic metrics of <u>richness</u> and <u>diversity</u> are subject to change,*** *if the reference database changes*
  - If your reads cannot be closely matched to a database sequence, they are ignored
  - If you have novel organisms in your sample, this could be disastrous

Open-reference OTU-picking

- Initially takes the same approach as closed-reference OTU-picking
- But uses a *de-novo* approach for the unidentified reads
- Information on novel organisms will therefore be retained
- Still has the problems of complete database-dependence
- Still has the advantages of access to externally defined OTUs

Whether OTU-clustering or independent read-by-read approaches….

That sounds like a lot of sequence comparison

How are the read sequences compared…. With DB sequences?

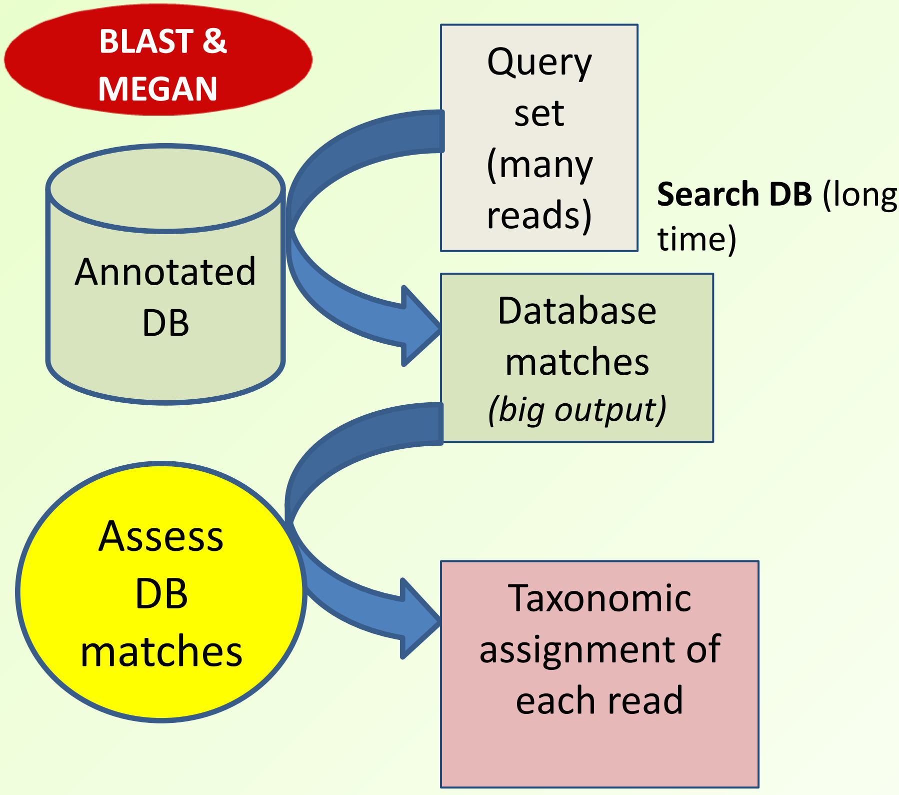
- Some methods use each sequence explicitly – they are **alignment-based methods**
- But:
- Do we need to compare every read sequence with the reference database?
  - This can be time-consuming
  - and produce very large data files
- Are there short-cuts?

Sequence features

- An alternative to using the explicit DNA sequence itself
- For example, **composition** of the sequence
  - E.g. frequencies of particular *k*-mers
- Comparison between sequences' features is much faster than doing a sequence alignment
- May be pre-calculated for sequences in a large reference database
- Calculation for your query set of reads is relatively fast

Direct and indirect comparison with reference database

Direct: two user steps



BLAST & MEGAN

Annotated DB

Query set (many reads)

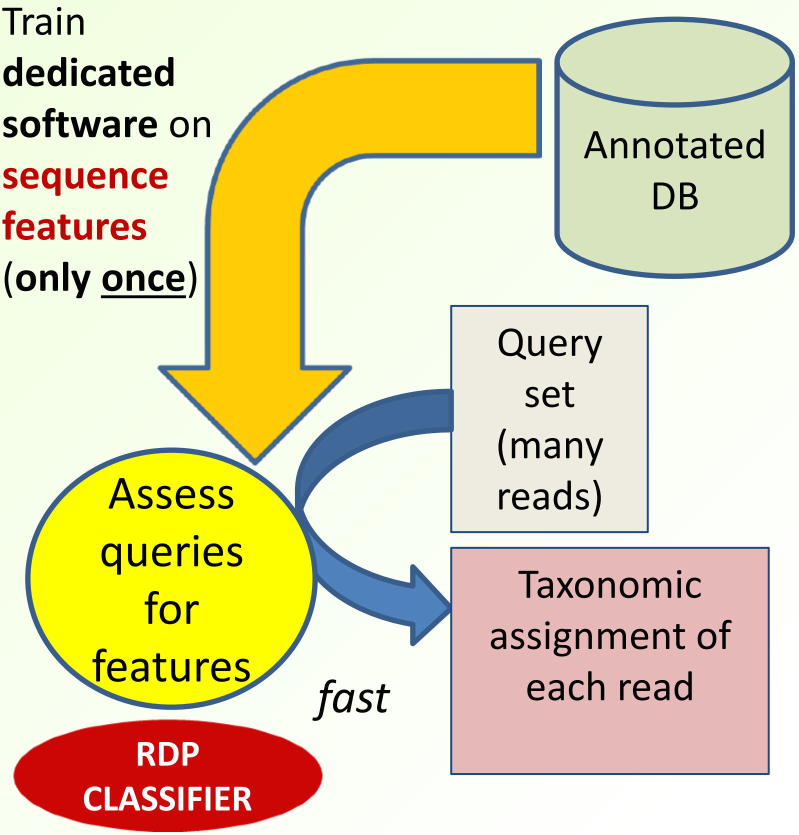
Search DB (long time)

Database matches *(big output)*

Assess DB matches

Taxonomic assignment of each read

Indirect: one user step



Train **dedicated software** on **sequence features** (**only <u>once</u>**)

Annotated DB

Query set (many reads)

Assess queries for features

fast

Taxonomic assignment of each read

RDP CLASSIFIER

How are the read sequences compared…. With each other?

- As in, clustering
- Again, shortcuts are possible
- E.g. pre-screening a large set of reads –
  - using sequence features to filter out pairs of reads of no interest
  - i.e. which cannot possibly have sufficient identity (e.g. 97%)
- Then perform full comparison between pairs of reads which remain