Applications of PCR as a tool for fungal diversity

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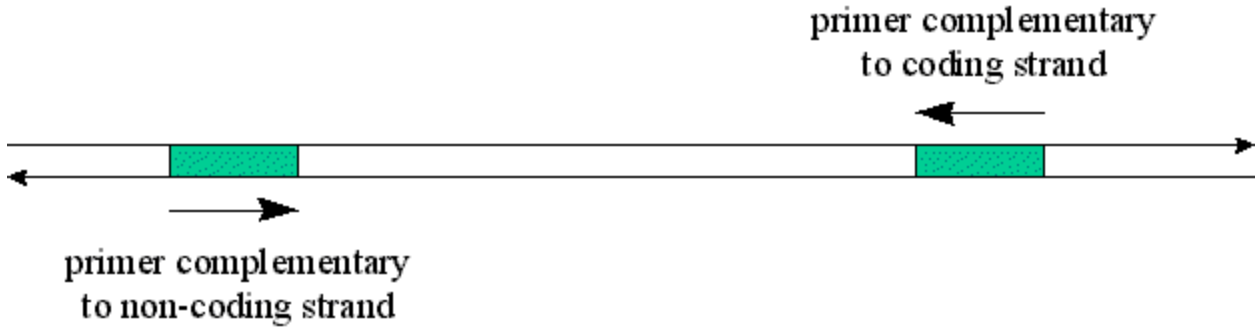
PCR

- Polymerase chain reaction is a culture independent diagnostic tool
- Ribosomal DNA is the most conserved region in the genome with capabilities of phylogenetic divergence

PCR - Polymerase Chain Reaction

- PCR is an *in vitro* technique for the amplification of a region of DNA which lies between two regions of known sequence.
- PCR amplification is achieved by using oligonucleotide primers.
  - These are typically short, single stranded oligonucleotides which are complementary to the outer regions of known sequence.



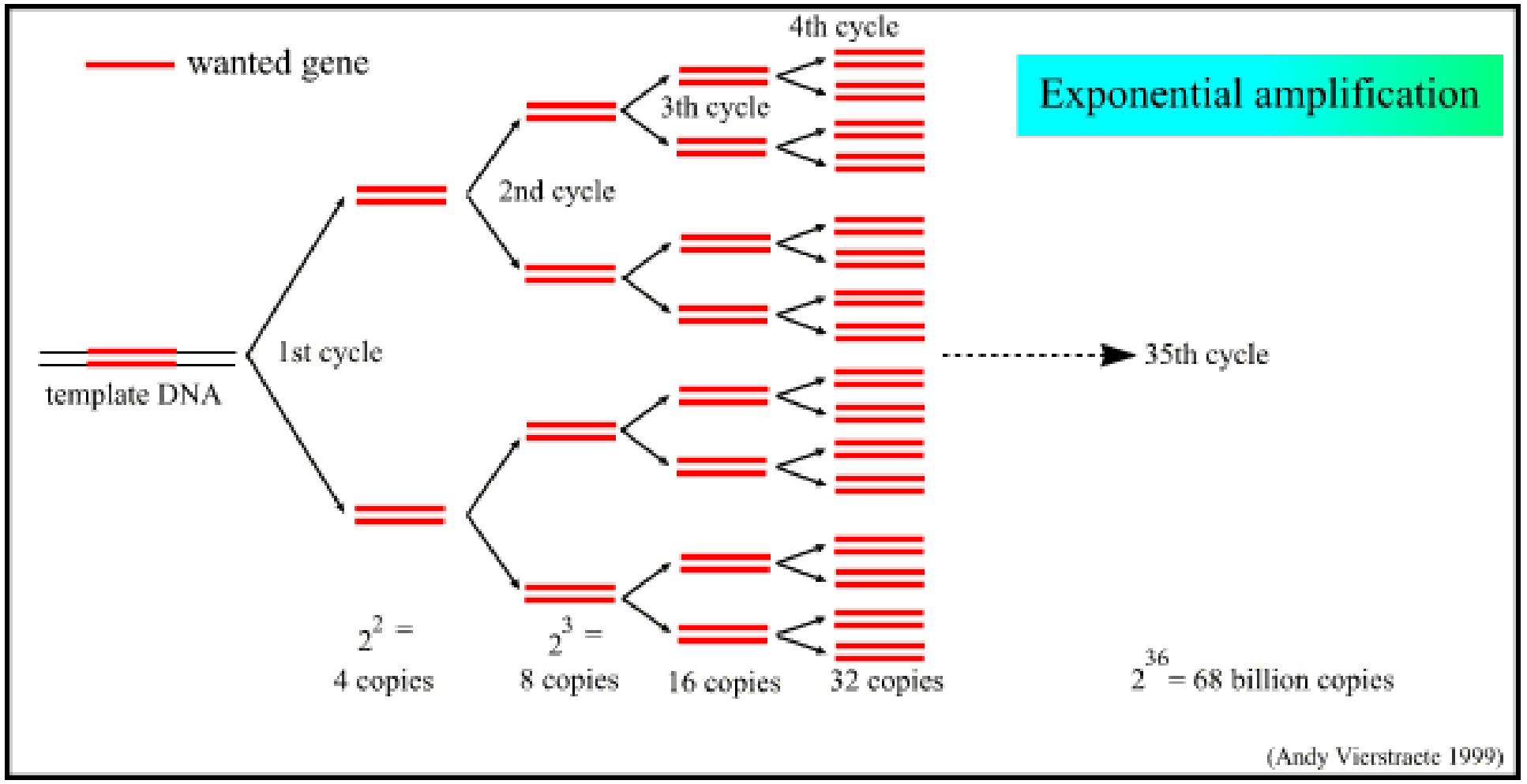
- The oligonucleotides serve as *primers* for DNA polymerase and the denatured strands of the large DNA fragment serves as the *template*.
  - This results in the synthesis of new DNA strands which are complementary to the parent template strands.
  - These new strands have defined 5' ends (the 5' ends of the oligonucleotide primers), whereas the 3' ends are potentially ambiguous in length.

Advantages

- 1. simple
- 2. powerful
  - A. sensitive – sensitivity
  - B. specific – specificity
  - C. reliable – reliability; fidelity
- 3. fast

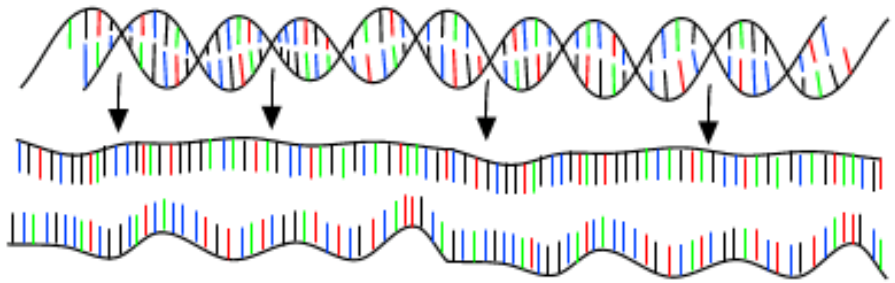
DNA Replication

- Purpose: To duplicate DNA molecule
- Principle:
  - Separation of DNA double-stranded template
  - Primer formation
  - Extension of new DNA strands by a DNA polymerase and deoxyribonucleoside triphosphates (dNTPs)
  - Other proteins involved


wanted gene
4th cycle
Exponential amplification
3th cycle
2nd cycle
1st cycle
template DNA
35th cycle
2^2 =
4 copies
2^3 =
8 copies
16 copies
32 copies
2^{36} = 68 billion copies
(Andy Vierstraete 1999)

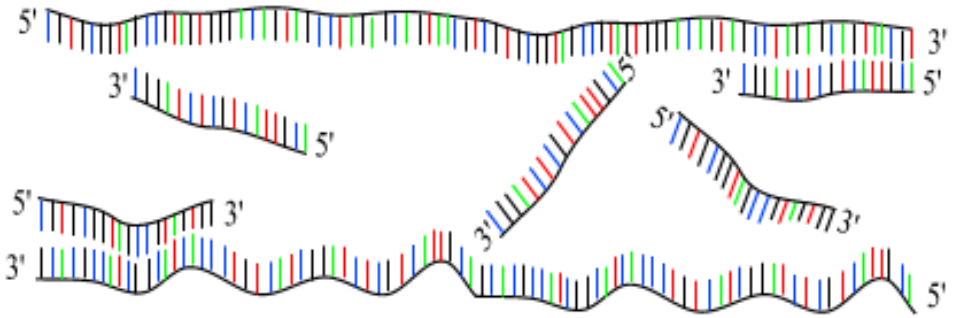
PCR : Polymerase Chain Reaction

30 - 40 cycles of 3 steps :



Step 1 : denaturation

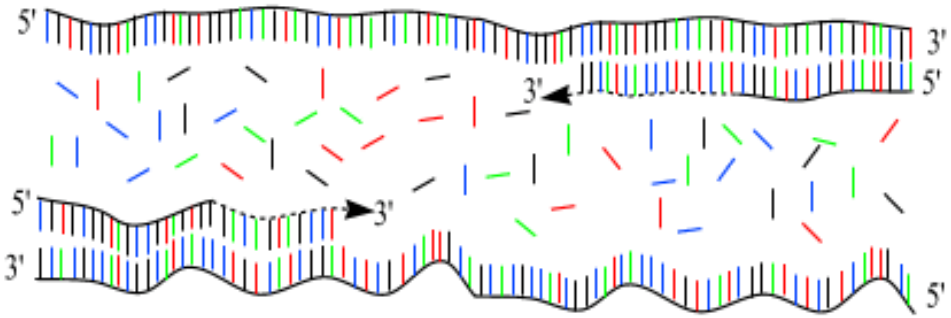
1 minut 94 °C



Step 2 : annealing

45 seconds 54 °C

forward and reverse primers !!!



Step 3 : extension

2 minutes 72 °C

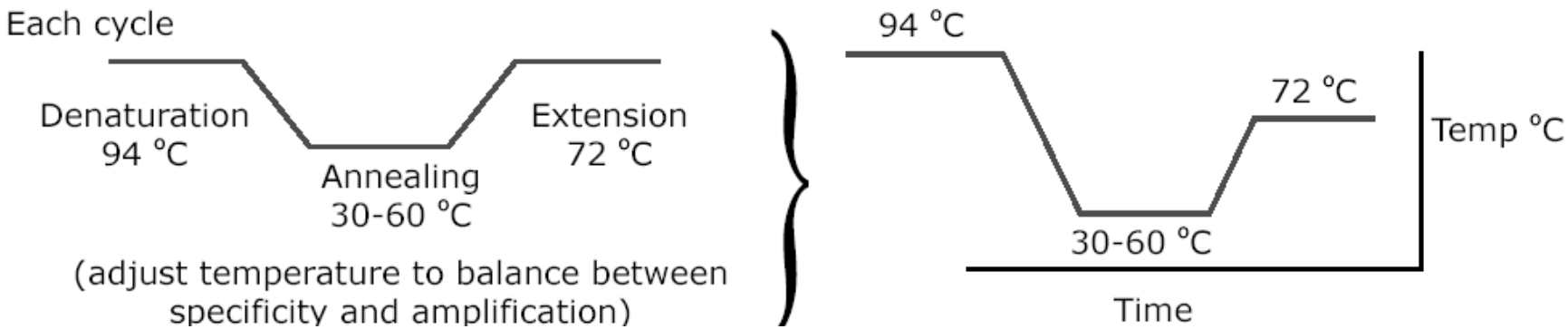
only dNTP's

(Andy Vierstraete 1999)

PCR reaction contains

- Target DNA (example: environmental DNA)
- 2 primers (20-30 nts long)
- Thermostable DNA polymerase
- Nucleotides (dNTPs)
- Mg^{2+} (cofactor for DNA polymerase)

Mix is subjected to temperature cycling



- http://ocw.mit.edu/NR/rdonlyres/Civil-and-Environmental-Engineering/1-89Fall-2004/321BF8FF-75BE-4377-8D74-8EEE753A328C/0/11_02_04.pdf

Primer selection

- **Primer is an oligonucleotide sequence – will target a specific sequence of opposite base pairing (A-T, G-C only) of single-stranded nucleic acids**

- For example, there is a
  - ¼ chance (4-1) of finding an A, G, C or T in any given DNA sequence; there is a
  - 1/16 chance (4-2) of finding any dinucleotide sequence (eg. AG); a
  - 1/256 chance of finding a given 4-base sequence.
- Thus, a **sixteen base sequence will statistically be present only once in every 416 bases (=4 294 967 296, or 4 billion)**: this is about the size of the human or maize genome, and 1000x greater than the genome size of *E. coli*.

Primer Specificity

- Universal – amplifies ALL bacterial DNA for instance
- Group Specific – amplify all denitrifiers for instance
- Specific – amplify just a given sequence

Forward and reverse primers

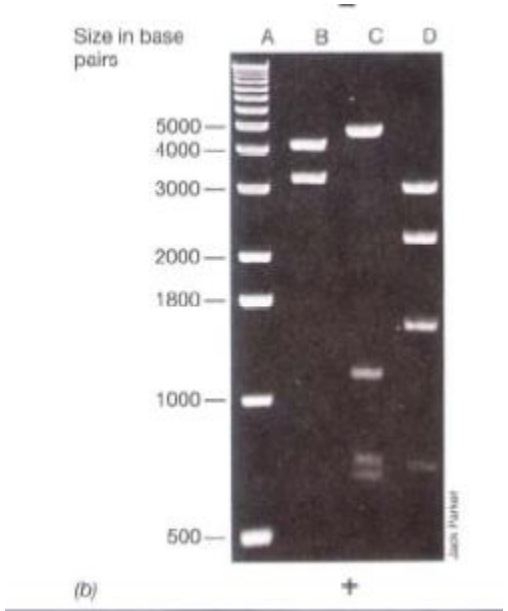
- If you know the sequence targeted for amplification, you know the size which the primers should be anealing across
- If you don’t know the sequence… What do you get?

DNA Polymerase

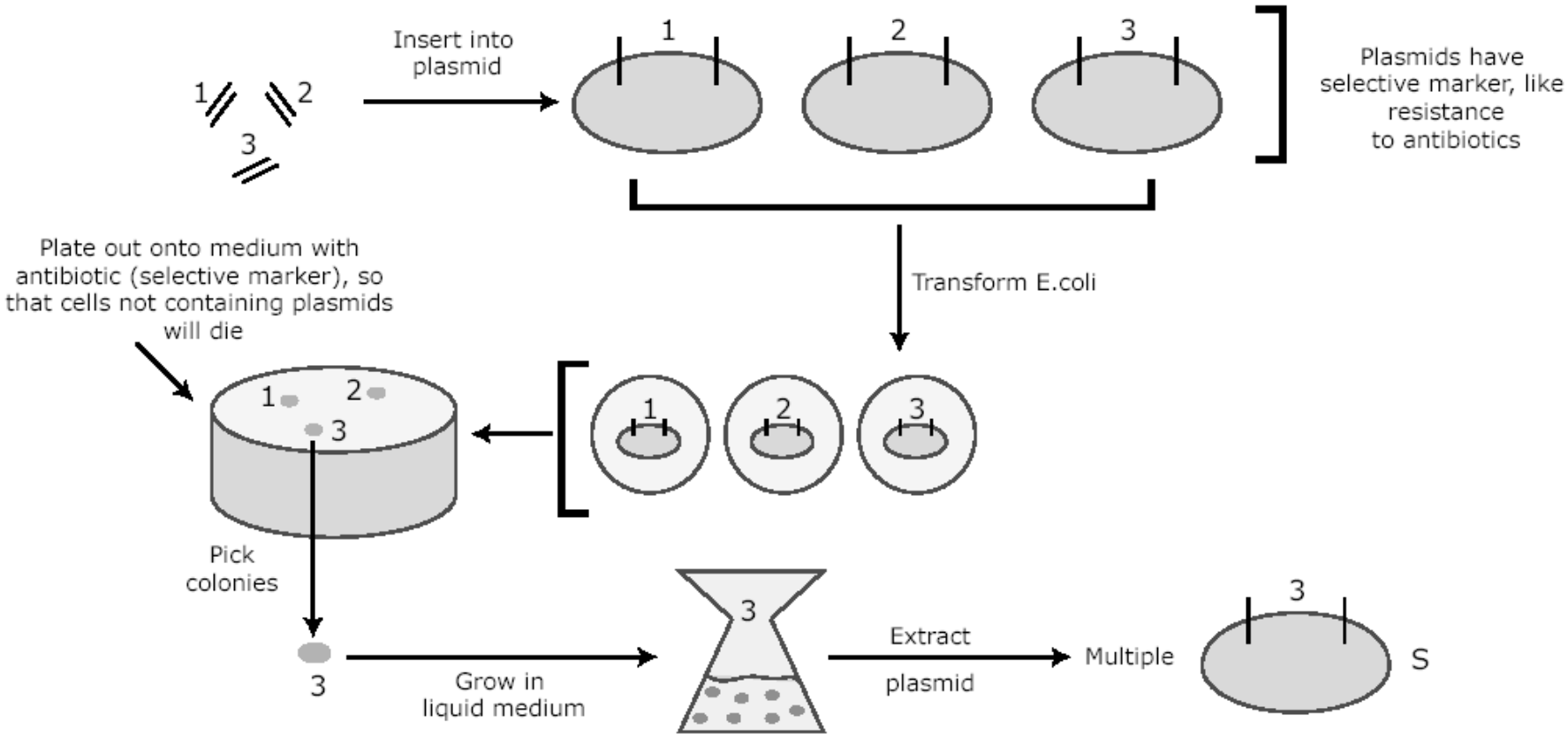
- DNA Polymerase is the enzyme responsible for copying the sequence starting at the primer from the single DNA strand
- Commonly use Taq, an enzyme from the hyperthermophilic organisms *Thermus aquaticus*, isolated first at a thermal spring in Yellowstone National Park
- This enzyme is heat-tolerant → useful both because it is thermally tolerant (survives the melting T of DNA denaturation) which also means the process is more specific, higher temps result in less mismatch – more specific replication

Electrophoresis

- Fragmentation products of differing length are separated – often on an agarose gel bed by electrophoresis, or using a capilarry electrophoretic separation



Clone Library



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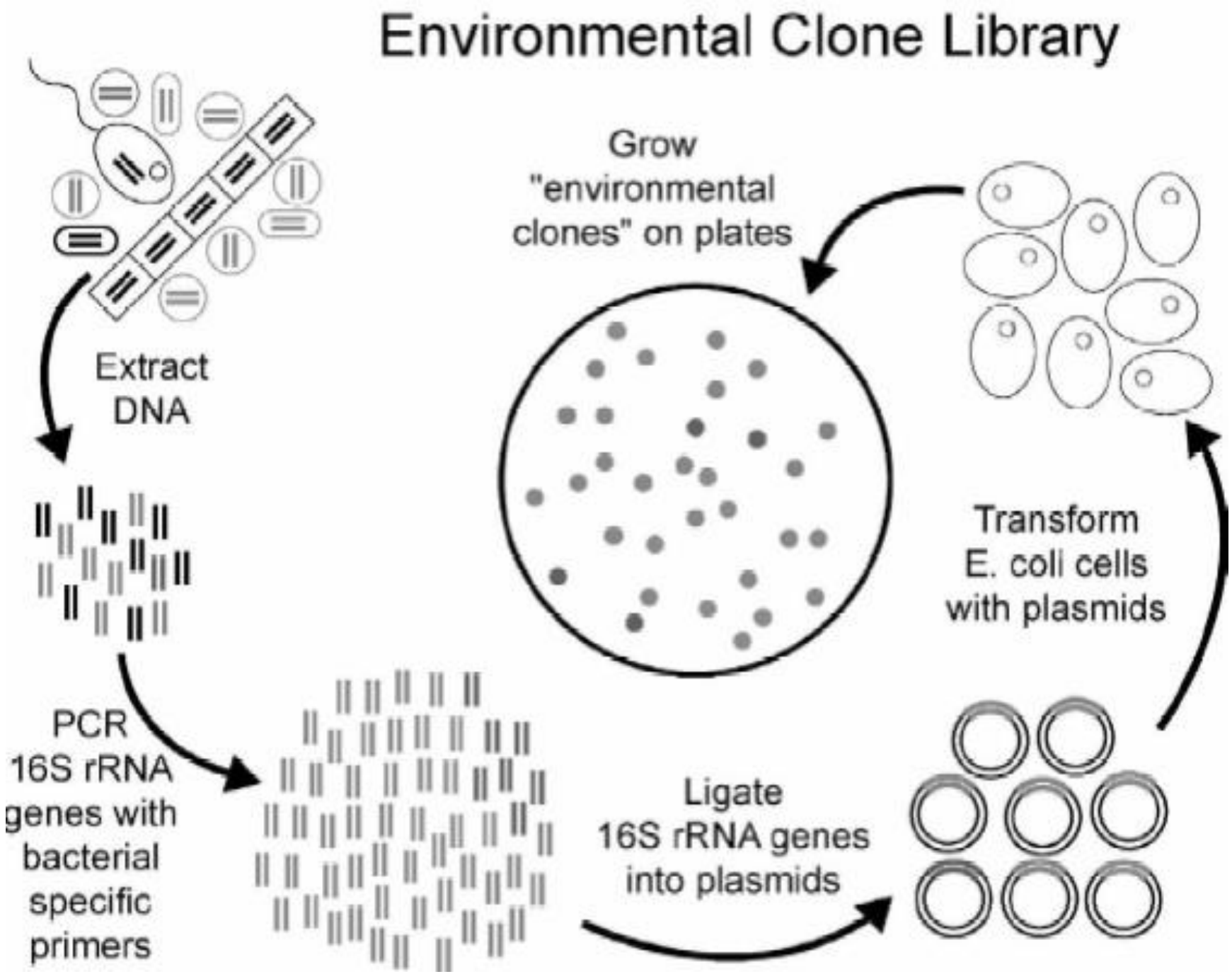
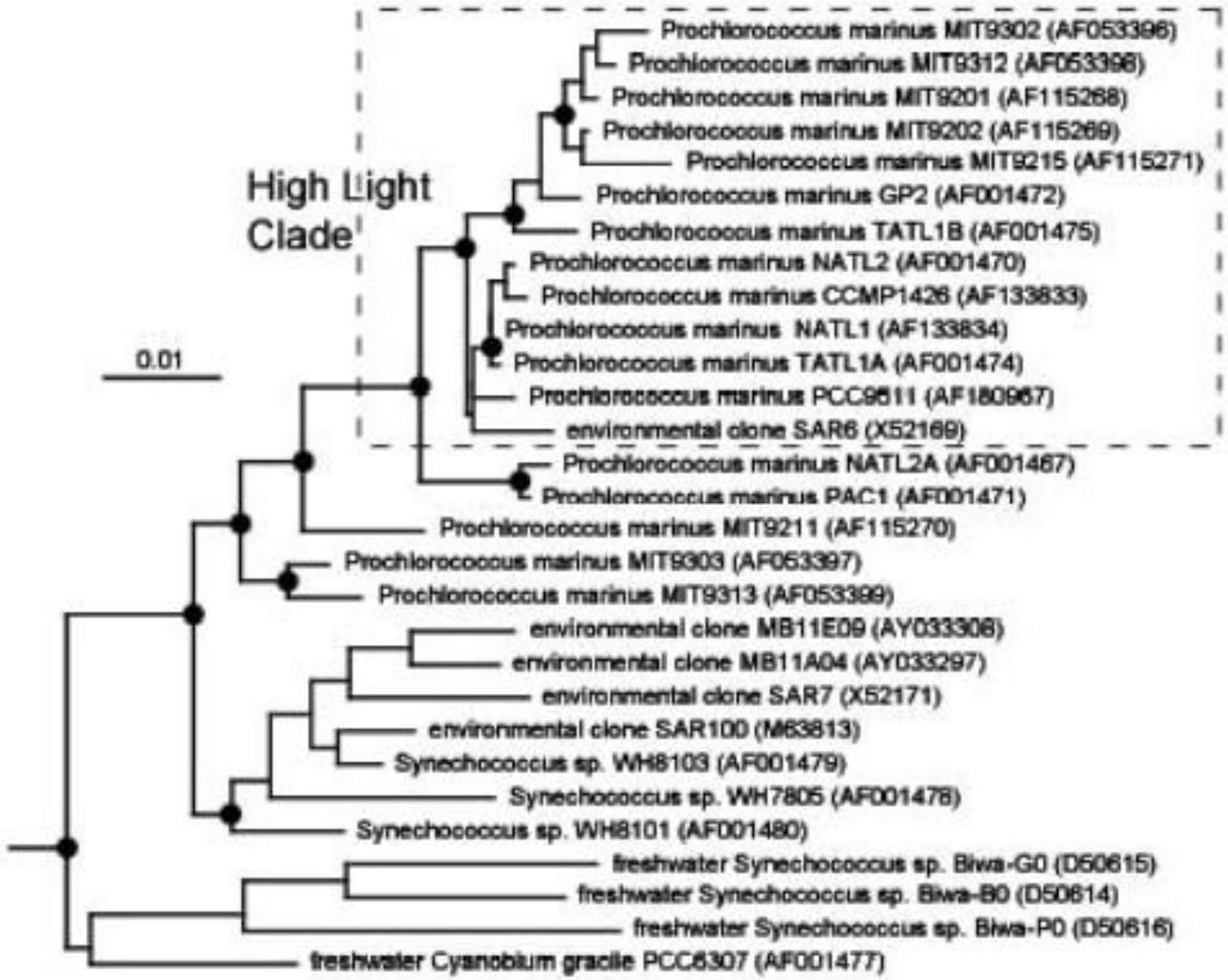


http://www.ifa.hawaii.edu/UHNAI/NAIweb/presentations/astrobiol6.pdf

Link Environmental Gene Clones With Microbes of Known Characteristics



Internal Transcribed Spacer (ITS)

- ✓ ITS refers to a piece of non-functional RNA situated between structural rRNAs.
- ✓ ITS has proven especially useful for elucidating relationships among congeneric species and closely related genera in Asteraceae.
- ✓ ITS region is the most widely sequenced DNA region in fungi.

ITS used for Fungal diversity elucidation

- The whole rRNA gene contains a small subunit (SSU) 18S rRNA, 5.8S rRNA, and a large subunit (LSU) 28S rRNA. Internal transcribed spacer (ITS) region I (ITSI) and ITSII are more variable than the rest of the ribosomal gene subunits and are found between SSU rRNA and 5.8S rRNA and between 5.8SrRNA and LSU rRNA, respectively.

- Besides this, intergenic spacer (IGS) region I (IGSI) and IGSII are found between the end of the LSU and start of the next SSU sequence

CONT'

- **ITS** (Internal Transcribed Spacer) refers to a piece of non-functional RNA situated between structural ribosomal RNAs (rRNA) on a common precursor transcript.

- Read from 5' to 3', this polycistronic rRNA precursor transcript contains the 5' external transcribed sequence (5' ETS), 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA and finally the 3'ETS.

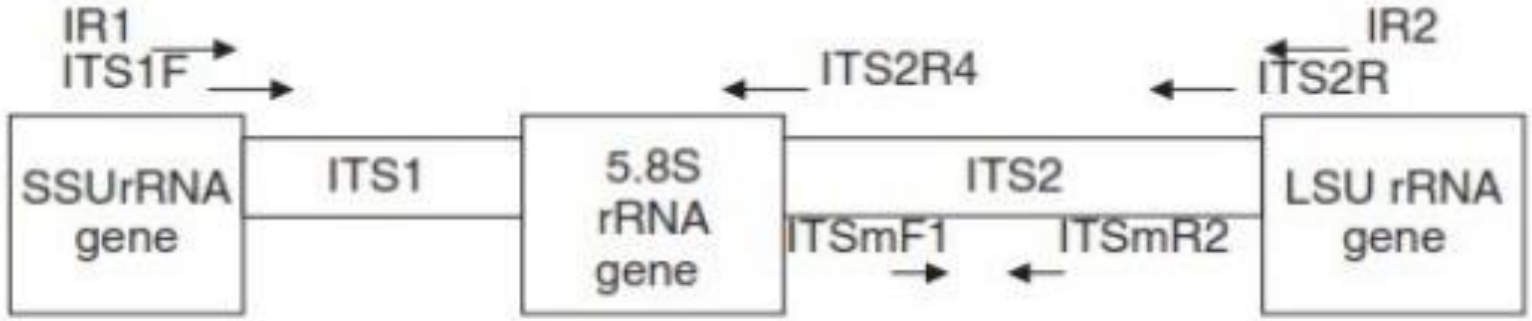
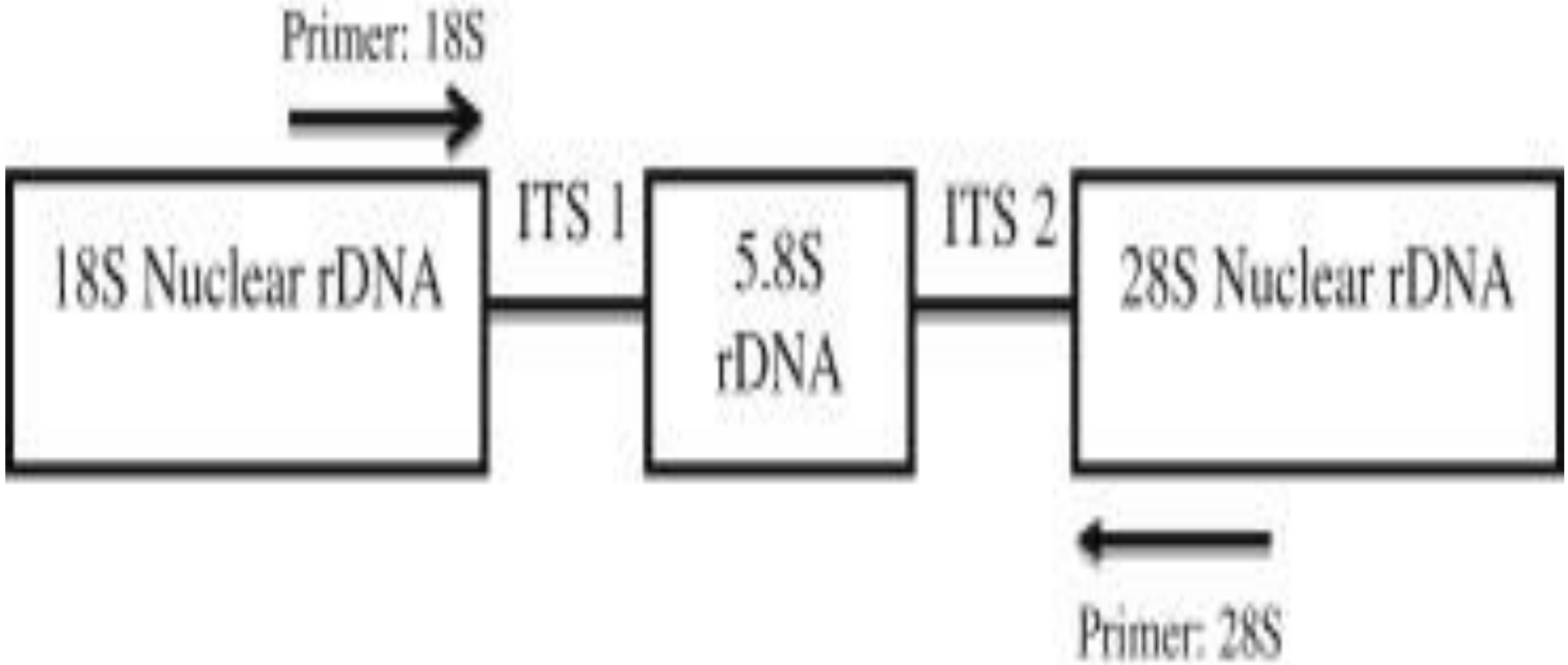
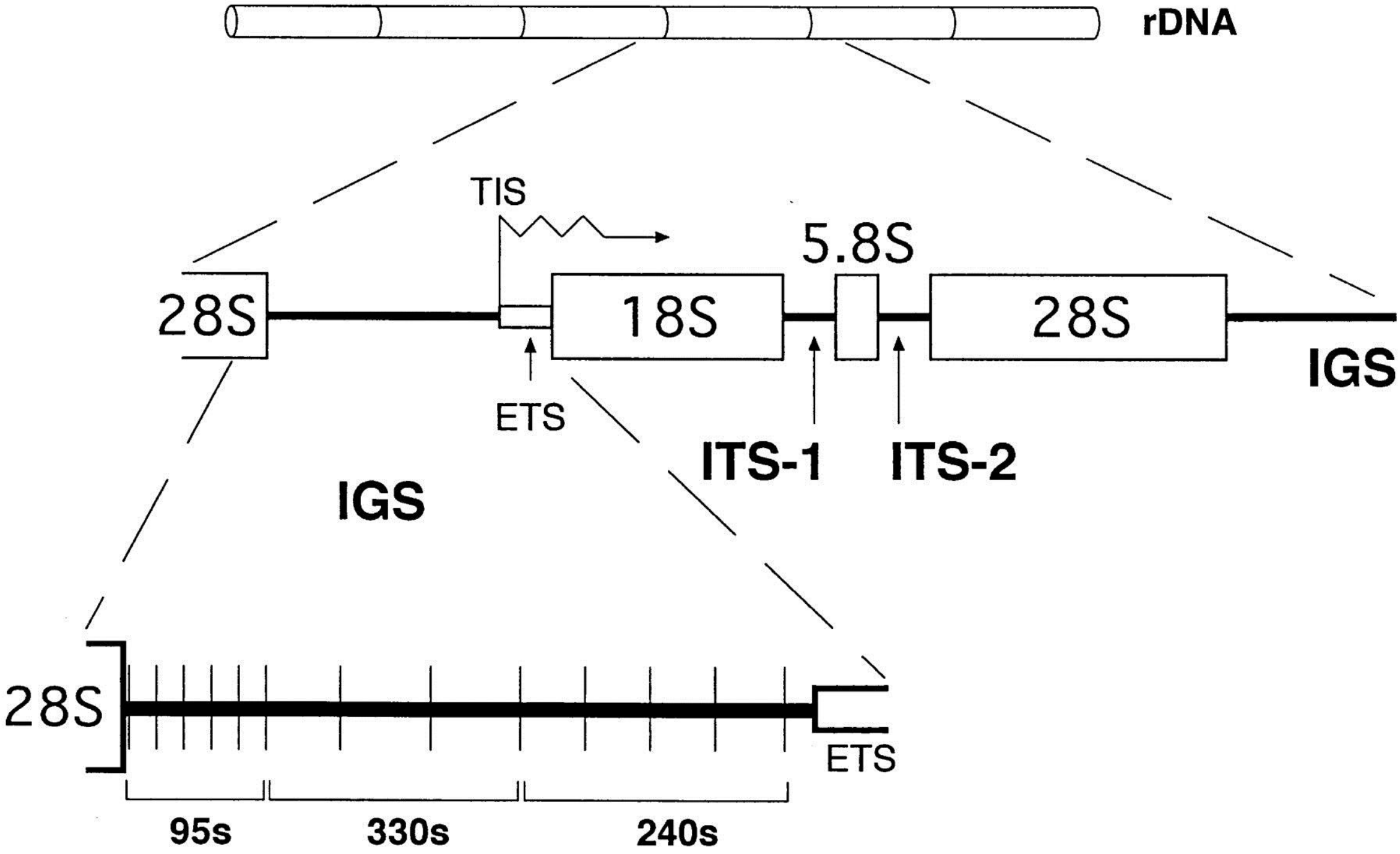


Figure Schematic presentation of the two intergenic spacers (ITS) of the ribosomal (r) RNA operon amplified with primers (= forward; = reverse) specific for *Leishmania* species. SSU = small subunit gene; LSU = Large subunit gene.


Primer: 18S
18S Nuclear rDNA
ITS 1
5.8S
rDNA
ITS 2
28S Nuclear rDNA
Primer: 28S


rDNA
TIS
5.8S
28S
18S
28S
IGS
ETS
ITS-1
ITS-2
IGS
28S
ETS
95s
330s
240s

Sequencing

- sequencing

- Thanks for listening