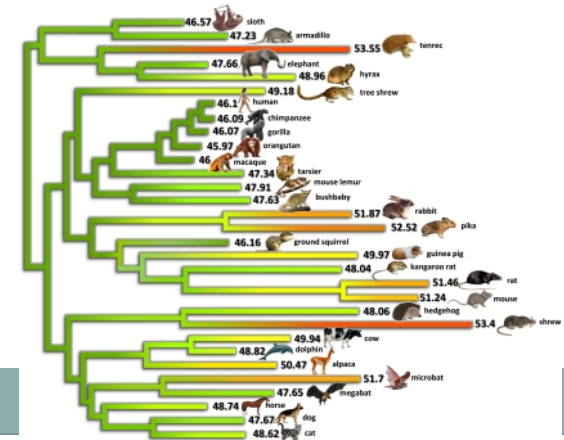
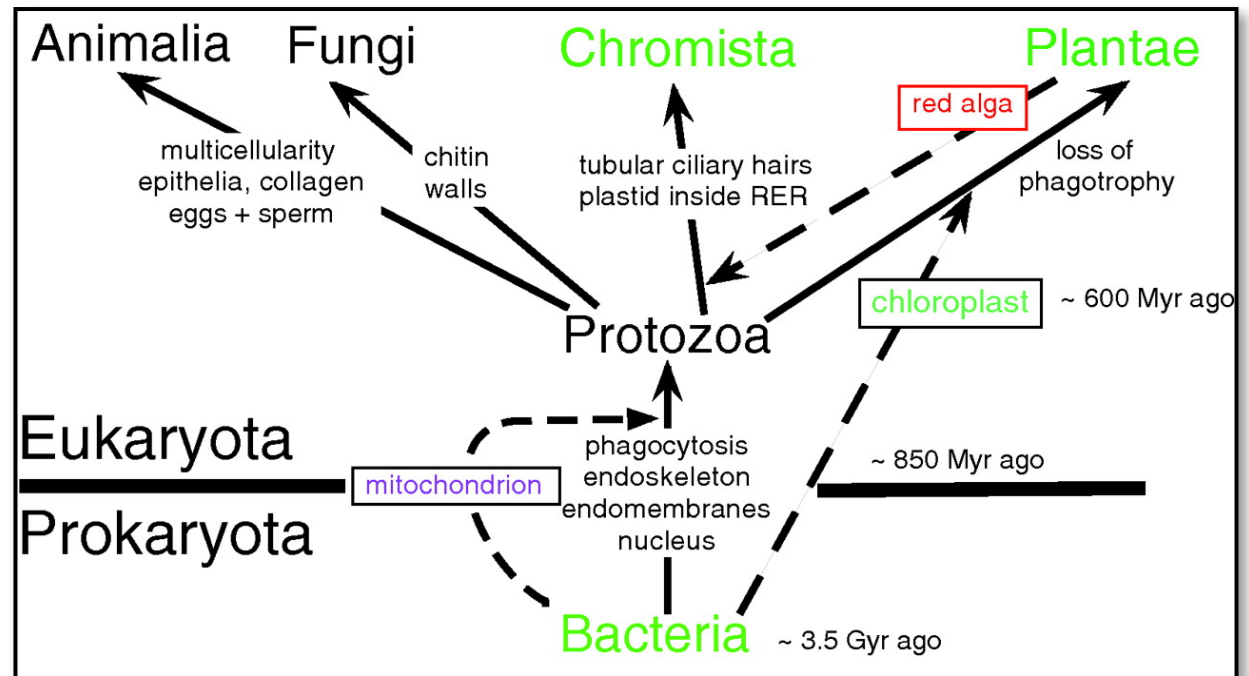


# Phylogenetic Tree

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A phylogenetic tree or evolutionary tree is a branching diagram showing the evolutionary relationships among various biological species based upon similarities and differences in their physical or genetic characteristics.



# The procedure

## 1- DNA extraction

## 2- PCR

Material	Quantity
2x Taq Ready Mix	125 µl
<b>Forward primer:</b> LshCytoD: TTG TAT GCA GAT AAT ATG TGG TGT GTG TTT AGC	10 µl
<b>Reverse primer:</b> LshCytoR: CCA TCT GAA CTC ATA AAA TAA TGT AAAC	10 µl
dd H <sub>2</sub> O	55 µl

### Thermal cycler program:

- 5 min at 95 °C.
- 35 cycles: each composed of
- 30 seconds at 95 °C.
- 30 seconds at 56 °C.
- 1 min at 72 °C.
- A final elongation step at 72 °C for 10 min.

### 3- Gel electrophoresis

### 4- PCR Product Purification

Purification of PCR amplified DNA fragments for sequence analysis

#### **Procedure:**

- 1- Increase the volume of the PCR reaction up to 100  $\mu$ l (add about 85  $\mu$ l of DDW to each PCR tube to be purified).
- 2- Add 5 volumes of binding buffer PB to 1 volume of the PCR sample and mix. For example, add 500  $\mu$ l of Buffer PB to 100  $\mu$ l PCR sample.
- 3- Check that the color of the mixture is yellow. If the color of the mixture is orange or violet, add 10  $\mu$ l of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.
- 4- Place a QIAquick spin column in the 2 ml collection tube provided.
- 5- To bind DNA, apply the sample to the QIAquick column and centrifuge for 30– 60s.

6- Discard flow-through. Place the QIAquick column back into the same tube.

7- To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30– 60s.

8- Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.  
**IMPORTANT:** Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

9- Place QIAquick column in a clean 1.5 ml microcentrifuge tube.  
PCR Product Purification Protocol using Qiagen kit.

10- To elute DNA, add 30 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0– 8.5) to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min.

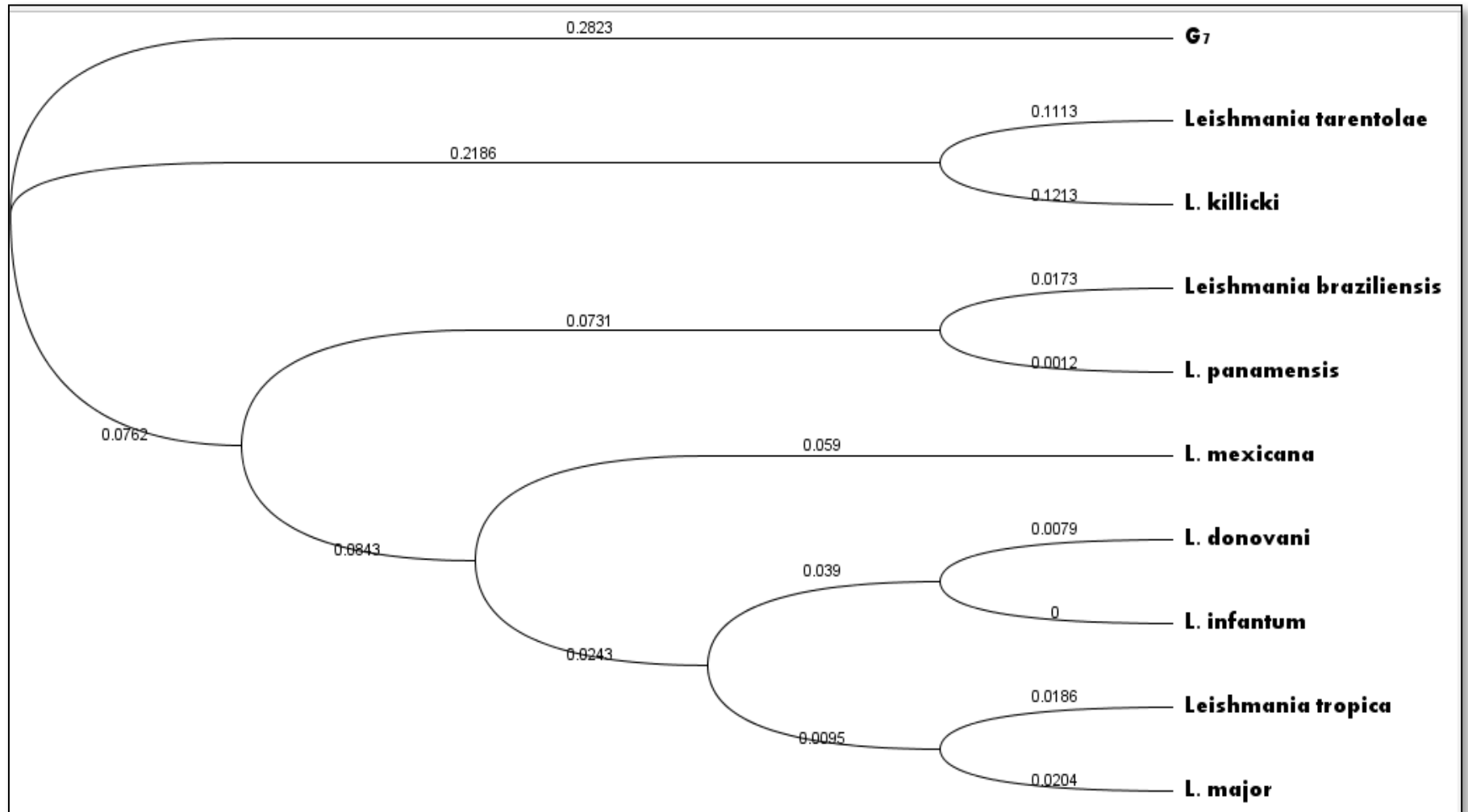
## DNA sequence analysis

- 1- Purify ITS1-PCR and Leishmania cyto-PCR products that need to be identified. PCR cleaning can be carried out using the Qiagene PCR purification protocol. In the current example only purify the positive PCR clinical samples. There is no need to purify the Leishmania reference samples.
- 2- Samples are eluted in low volume (about 30  $\mu$ l) of elution buffer or DDW. Do not dilute as high concentrations of purified DNA give better results.
- 3- For DNA sequencing of PCR products you need to use one of the two oligonucleotide primers in the amplification reaction (if only one strand is to be sequenced). Dilute your primers to 5 pmoles/ $\mu$ l, or according to the instructions of the DNA sequencing service provider.

4- Once the sequence is received it is possible to carry out BLAST (Basic Local Alignment Search Tool) DNA sequence comparison on (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), and generate phylogenetic trees.



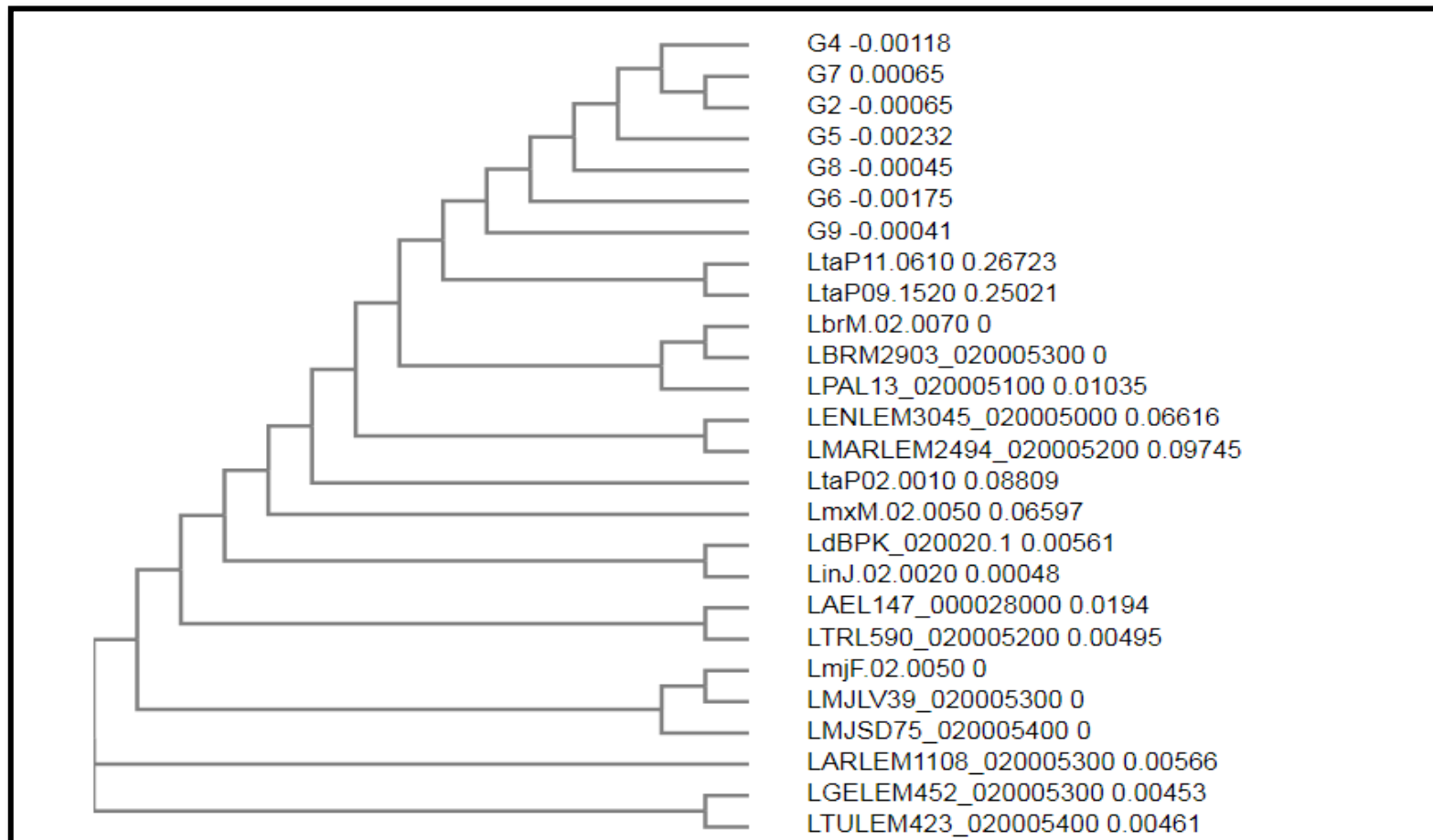
**EBI Bioinformatic Tools** (ie. sequence alignment and phylogenetic tree building).



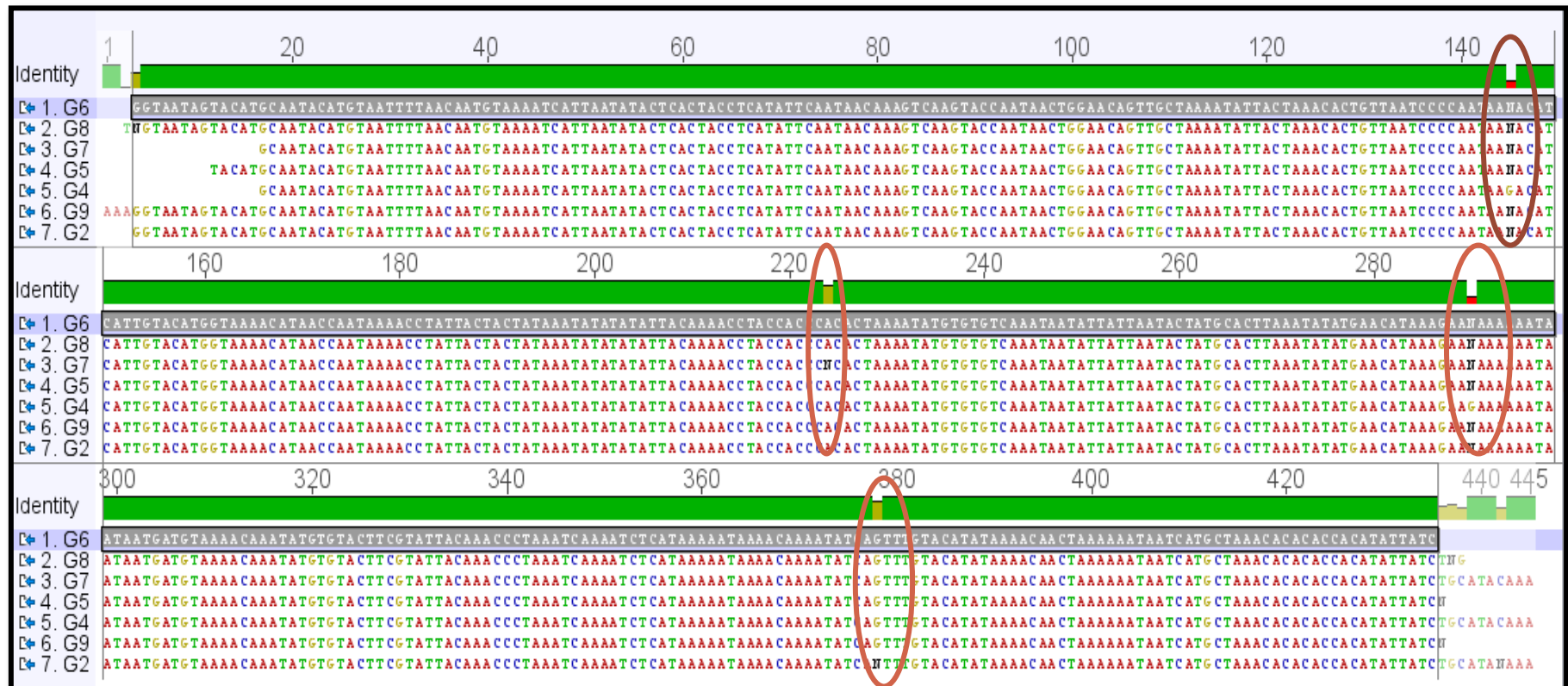
Phylogenetic relationships between group 7 and different members of the genus *Leishmania* based on the nucleotide sequences of the **Cyt b gene**.



Distance Matrix (Substitutions per site)										
	G7	<i>Leishmania tarentolae</i>	<i>Leishmania braziliensis</i>	<i>Leishmania tropica</i>	<i>L. donovani</i>	<i>L. infantum</i>	<i>L. killicki</i>	<i>L. major</i>	<i>L. mexicana</i>	<i>L. panamensis</i>
G7	-	0.59725	0.41722	0.49258	0.57161	0.49258	0.63725	0.49258	0.51986	0.41722
<i>Leishmania tarentolae</i>	0.59725	-	0.52372	0.50603	0.52623	0.52623	0.23262	0.52555	0.60478	0.53934
<i>Leishmania braziliensis</i>	0.41722	0.52372	-	0.22846	0.22433	0.22023	0.66641	0.22599	0.25111	0.018491
<i>Leishmania tropica</i>	0.49258	0.50603	0.22846	-	0.066817	0.060191	0.67346	0.039051	0.11057	0.22640
<i>L. donovani</i>	0.57161	0.52623	0.22433	0.066817	-	0.0061131	0.67346	0.081943	0.11782	0.22228
<i>L. infantum</i>	0.49258	0.52623	0.22023	0.060191	0.0061131	-	0.67346	0.075183	0.11419	0.21818
<i>L. killicki</i>	0.63725	0.23262	0.66641	0.67346	0.67346	0.67346	-	0.38312	0.38312	0.33899
<i>L. major</i>	0.49258	0.52555	0.22599	0.039051	0.081943	0.075183	0.38312	-	0.12331	0.22228
<i>L. mexicana</i>	0.51986	0.60478	0.25111	0.11057	0.11782	0.11419	0.38312	0.12331	-	0.24245
<i>L. panamensis</i>	0.41722	0.53934	0.018491	0.22640	0.22228	0.21818	0.33899	0.22228	0.24245	-



Phylogenetic relationships of various members of the genus *Leishmania* based on the nucleotide sequences of the **Cyt b** gene.



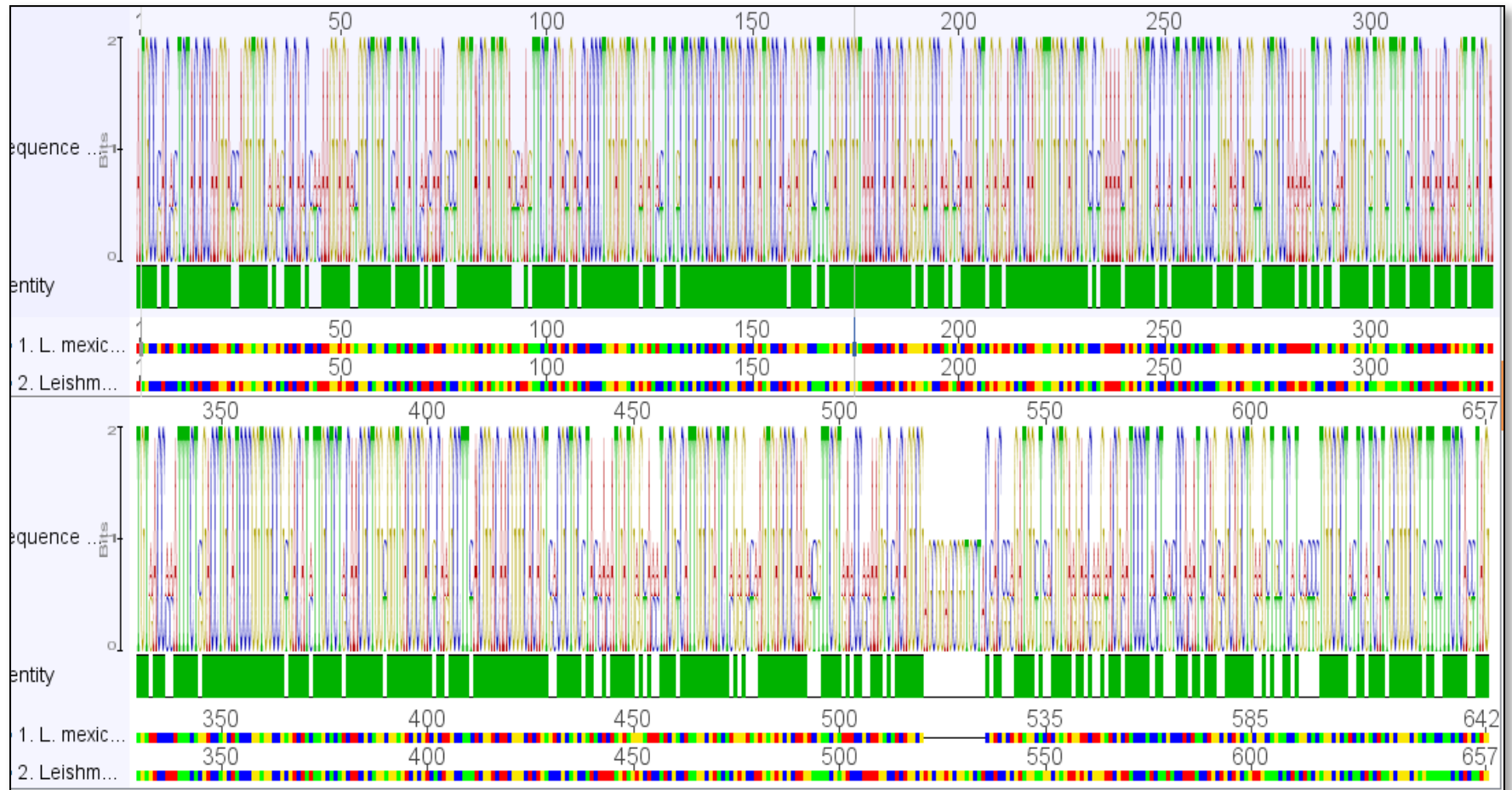
Alignment of the **Cyt b** gene of all tested *Leishmania* groups analysed in this course



Alignment of the **Cyt b gene** of all tested *Leishmania* groups analysed in this course

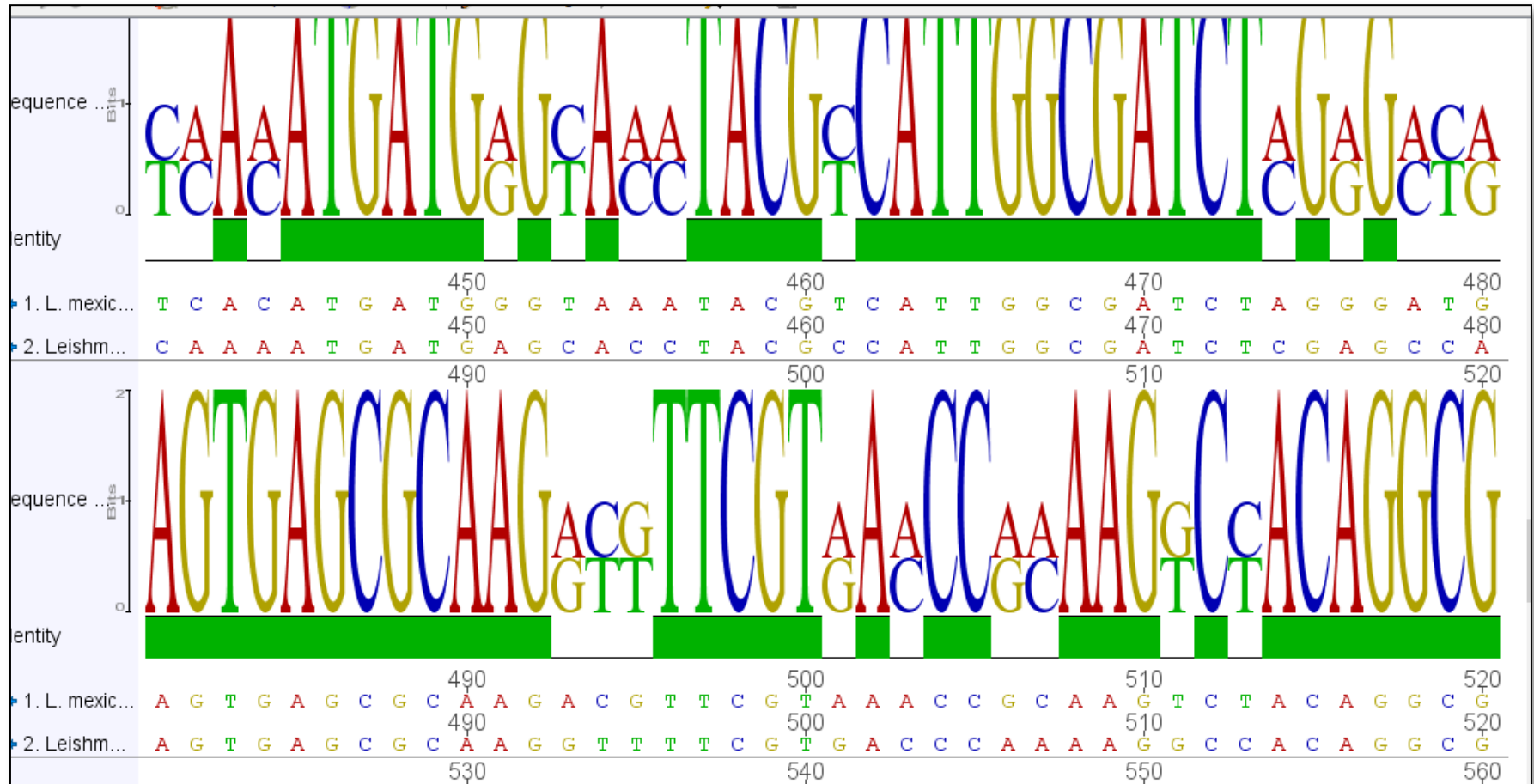
Identity	20 30 40 50 60 70 80 90 100 110 120 130
1. <i>Leishmania braziliensis</i>	TACACCAAGGACGAGGTGGCAGCTCACAAACGTAAGGAGAACGGCTGGCTCATCATCAACAACCTCCGTGTACGATGTGAGCAAGTTCTACGATGATCACCCCTGGAGGTCGAGATCCTCTG
2. <i>Leishmania tropica</i>	TACACCAGGGATCAGGTGGCGGAGCACAAACAGCAAGACGAGTGGCTGGCTCATCATCAATAACGGCGTGTACGATGTGAGCGATTCTACGACGACCACCTGGAGGTCGAGACATTCTT
Identity	150 160 170 180 190 200 210 220 230 240 250 260
1. <i>Leishmania braziliensis</i>	GGCACCGATGCCACAGAGGCTTTCGAGGCGGTAAACCACAGCAGAGGTGCCAAAGCTAGAGGAGCTCAAGGTTGGCGAGCTGTCTGAAAATGAGCGTCGCCACTACATCTCCCTG
2. <i>Leishmania tropica</i>	GGCACCGATGCCACGAGGGCTTCGAGGCGGTAAACCACAGCAGGGGAGCCGTGCGCAAGCTAGAGAAGCTCAAGGTTGGCGAGCTGCCCGAAAACGAGCGTCGCCGCTACATCTCCATG
Identity	280 290 300 310 320 330 340 350 360 370 380 390
1. <i>Leishmania braziliensis</i>	GCCAAAGAAGTCCGCCAACGGTGCTTGGTTTGTATCAATAACAAAGTATACGATGTGACCAAATTTCTCGACCTGCATCCCGGTGGCCGCGACATATTGCTCTGCAACGCTGGTGGTGAC
2. <i>Leishmania tropica</i>	GCGAAAAAGTCGGCTGACGGTGCGTGGTTGTCAATAACAAAGGTGTACGATGTCAACCCGTTTCTGGACCTGCATCCCGGTGGCCGCGACATCTTGTCTTACAACGCCGGCGGCGAC
Identity	410 420 430 440 450 460 470 480 490 500 510 520
1. <i>Leishmania braziliensis</i>	TTTACGGACAACGGGCACAGTCCCGCTGCCTACAAAATGATGAGCACCTACGCCATTGGCGATCTCGAGCCAAGTGAGCGCAAGGTTTTTCGTGACCCAAAAGGCCACAGGCGAGCGGAGGC
2. <i>Leishmania tropica</i>	TTTACGGACAACGAGCACAGCGATACCTGCGTATGAAATGATGGGTAAATACGTCGTTGGCGACGTGGAGCCGAGTGAGTGTAAAGACGCTCGTCAACCGAAAAGGCCACAGGC--GCGAAGC
Identity	548 558 568 578 588 598 608 618 628 638 648 657
1. <i>Leishmania braziliensis</i>	CGCGATGGTTGGCGTGAAGAGCGGGAACGAATCCCTACTTATCCAAATCCAGCAGCAGCTGAAGTTTCTCATCATCGTGGCGCTCTTCATCATTTGCGGGCGTGTCTTCTCAGCTTAG
2. <i>Leishmania tropica</i>	CGCAGATGGTTGCGGTGAAGGACGAGAACCATCCCTCCTTGCCACATTCAAGACCAGCTGAGGCTGCTCATGGCTCTGGCGCTATTGCTCATCGCGGGCGTCTTCTCTCAGCTAG

## *L. Mexicana* vs *L. braziliensis*





# *L. Mexicana* vs *L. braziliensis*



## Restriction Enzyme sites

