SCION
Next generation biomaterials

New diagnostic technologies – what is the New Zealand capability

Tod Ramsfield

7 March 2008

Why?

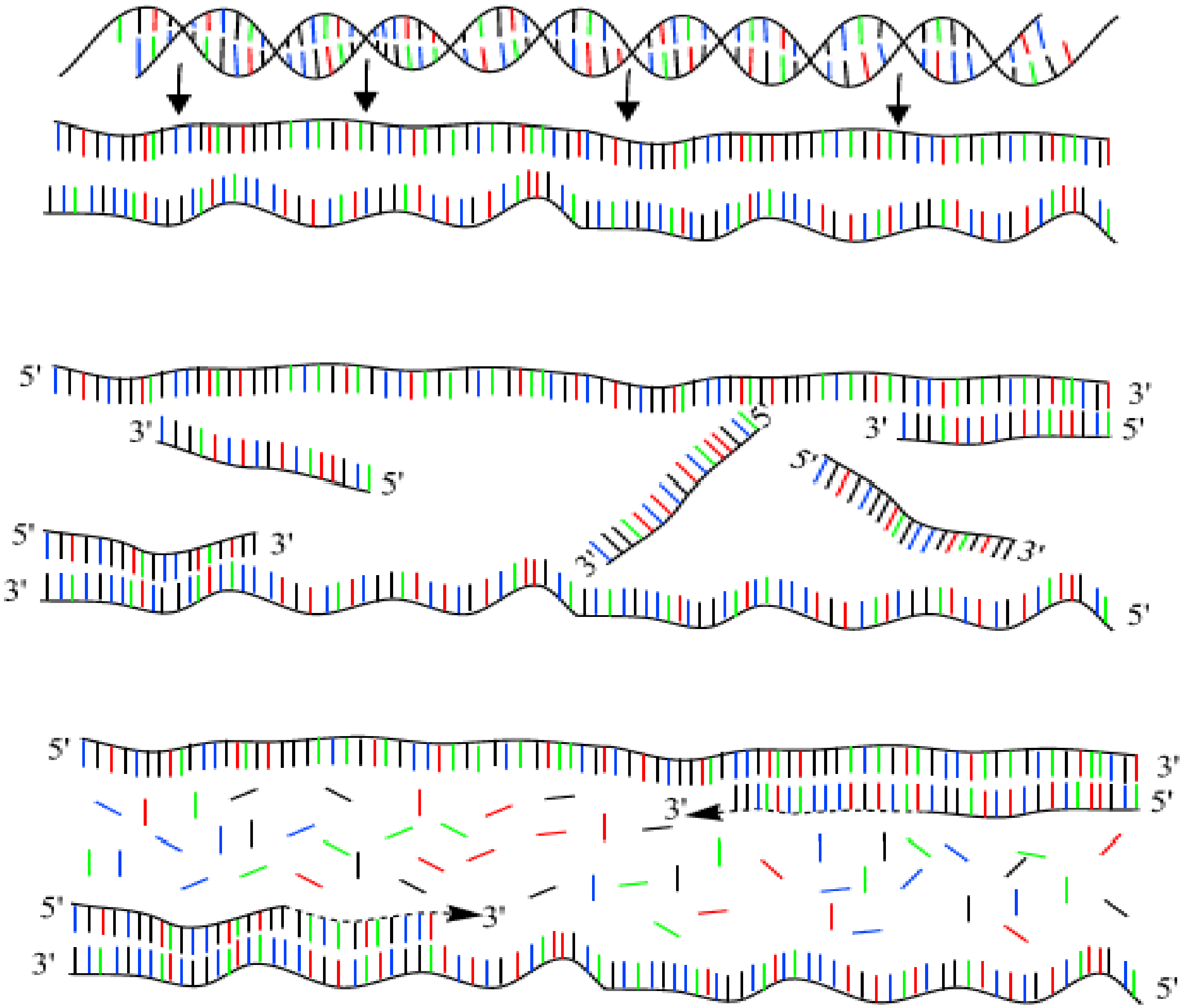
- Time
- Difficult to differentiate species
- Organisms that cannot be cultured
- Sensitivity
- Environmental samples
- Identify new strains

Polymerase chain reaction

- The process whereby DNA is copied exponentially.
- Reaction contains:
    1. Target DNA
    2. Specific PCR primers
    3. Nucleotides (A, G, T, C)
    4. DNA polymerase
- Requires a “thermalcycler”

PCR : Polymerase Chain Reaction

30 - 40 cycles of 3 steps :



Step 1 : denaturation

1 minut 94 °C

Step 2 : annealing

45 seconds 54 °C

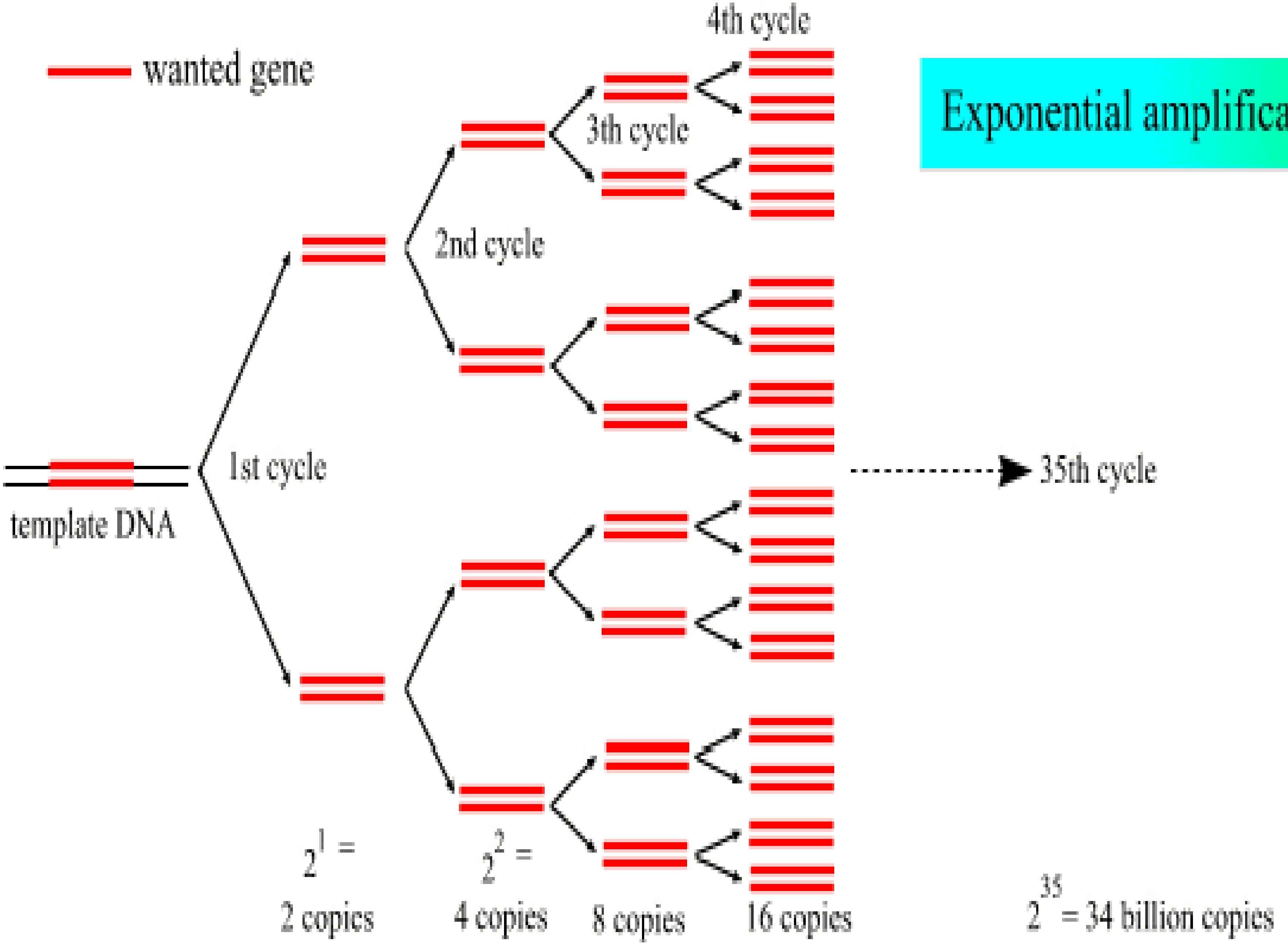
forward and reverse primers !!!

Step 3 : extension

2 minutes 72 °C
only dNTP's

(Andy Vierstraete 1999)

Exponential amplification



wanted gene

template DNA

1st cycle

2nd cycle

3th cycle

4th cycle

35th cycle

$2^1 =$ 2 copies

$2^2 =$ 4 copies

8 copies

16 copies

2^{35} = 34 billion copies

(Andy Vierstraete 2001)

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PCR based IDs

Specific DNA tests for individual organisms:

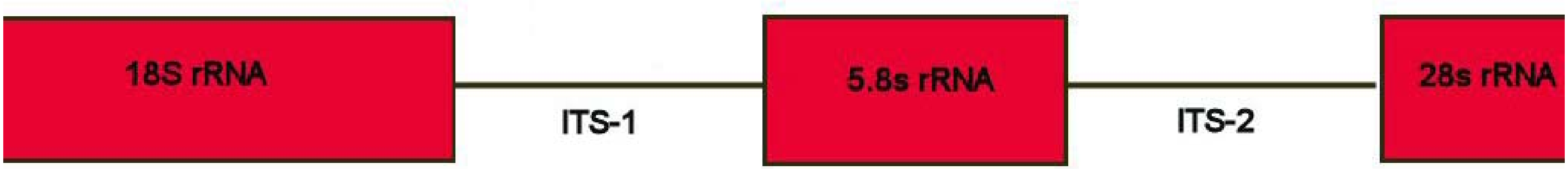
ie. *Endocronartium harknessii*,

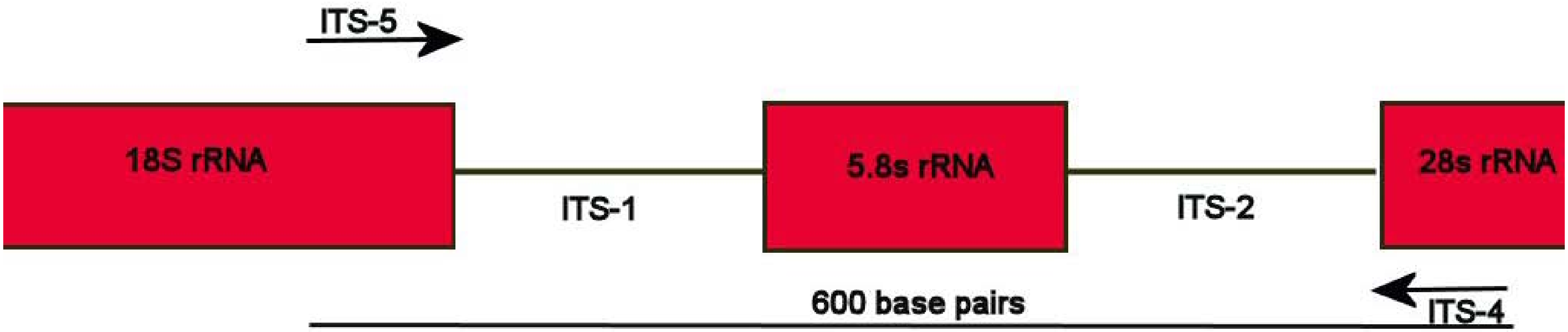
Fusarium circinatum,

Nectria fuckeliana

Often published, ie. *Phytophthora ramorum*

ITS – Fungal ID, ie Nectria





ITS-5

18S rRNA

ITS-1

5.8s rRNA

ITS-2

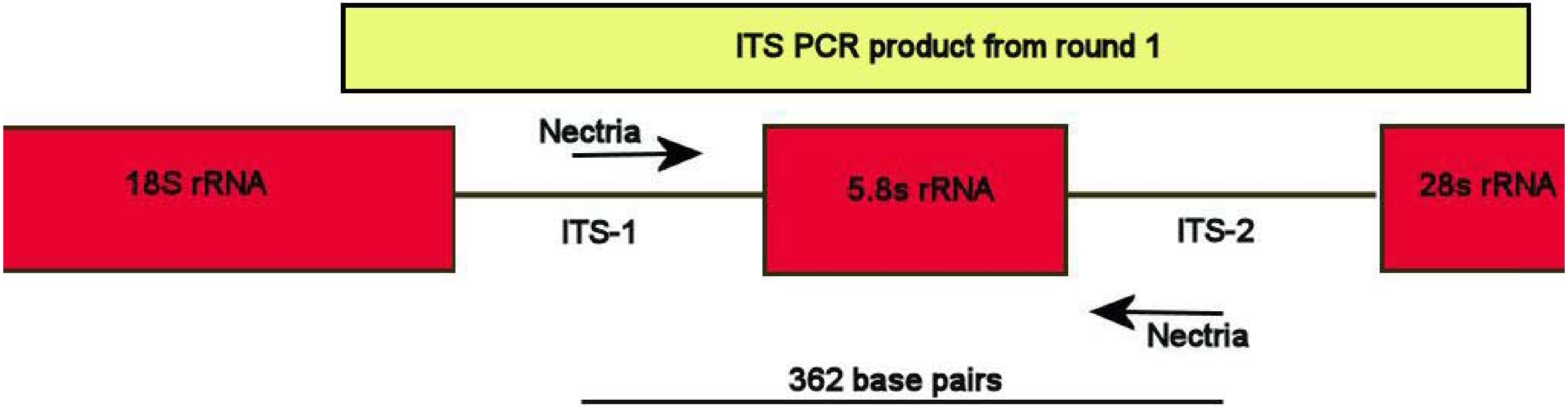
28s rRNA

600 base pairs

ITS-4

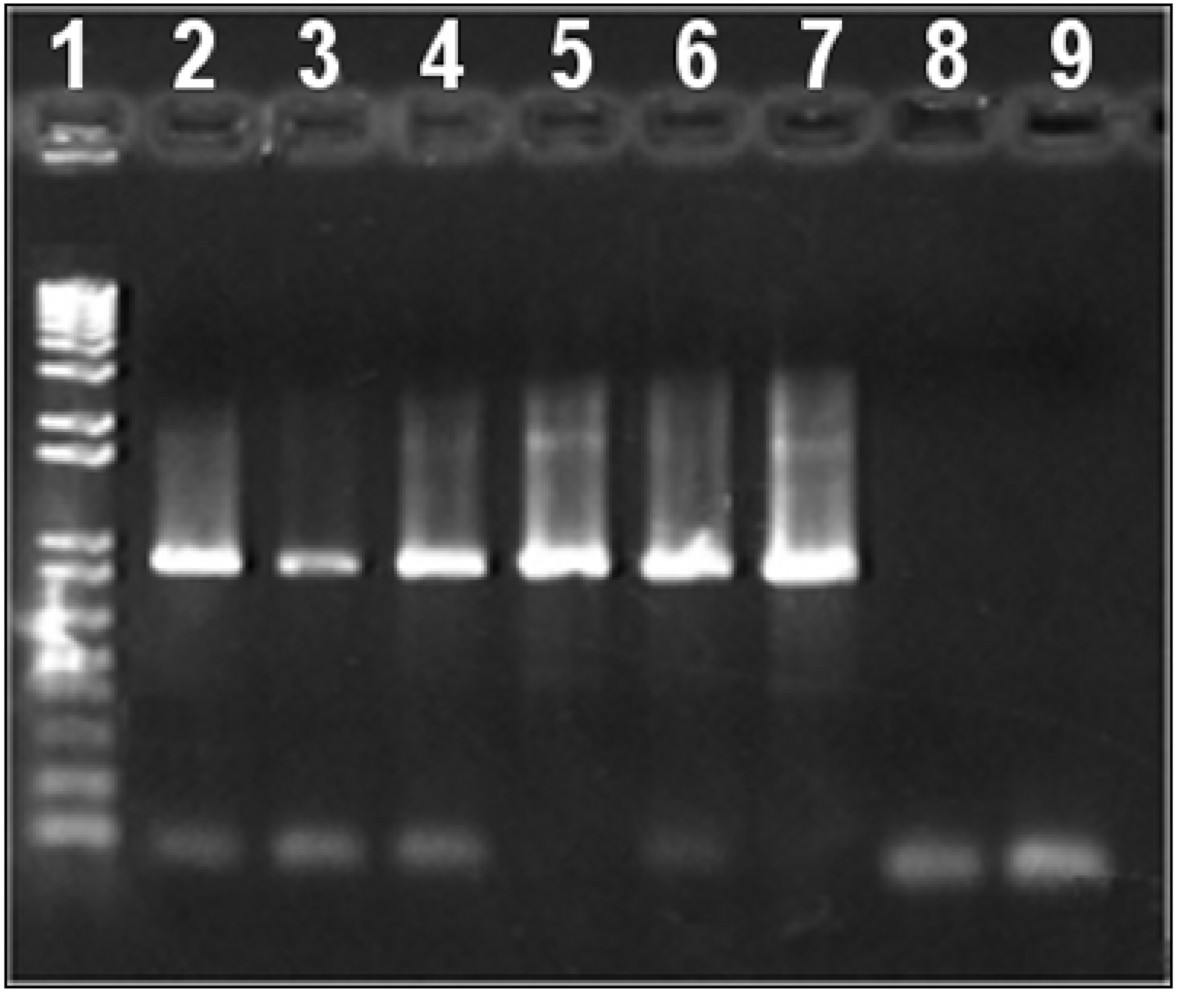
Next generation biomaterials

Nectria nested PCR



Specific PCR Results

Positive result is a band of the expected size.



Potential pitfalls

False negative reactions – intraspecific variability.

False positive reactions – invoke an incursion response.

Both can be extremely costly.

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DNA sequence based IDs

- PCR amplify a variable region using conserved PCR primers.
- Sequence the PCR product.
- BLAST search PCR product sequence in GenBank or other database.

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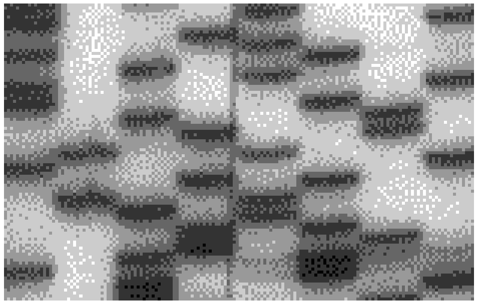
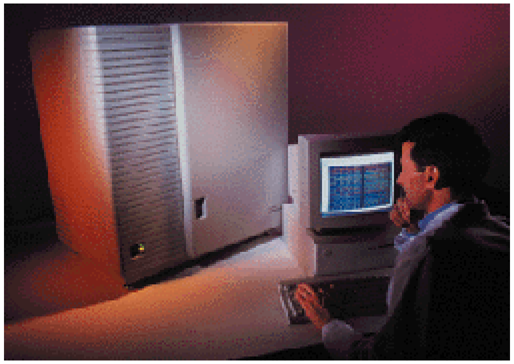
DNA sequencing

Manual sequencing

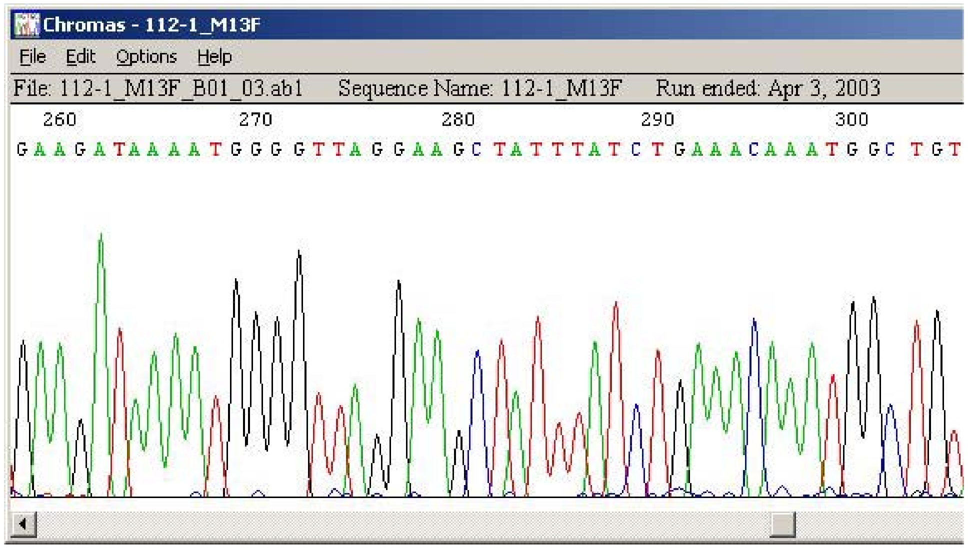


Sanger sequencing, size separation of radioactively labelled DNA

Automated sequencing



Dye termination
Laser reads wavelength



File: 112-1_M13F_B01_03.ab1 Sequence Name: 112-1_M13F Run ended: Apr 3, 2003

```
GAAGATAAAATGGGGTTAGGAAGCTATTTATCTGAAACAAATGGCTGT
```

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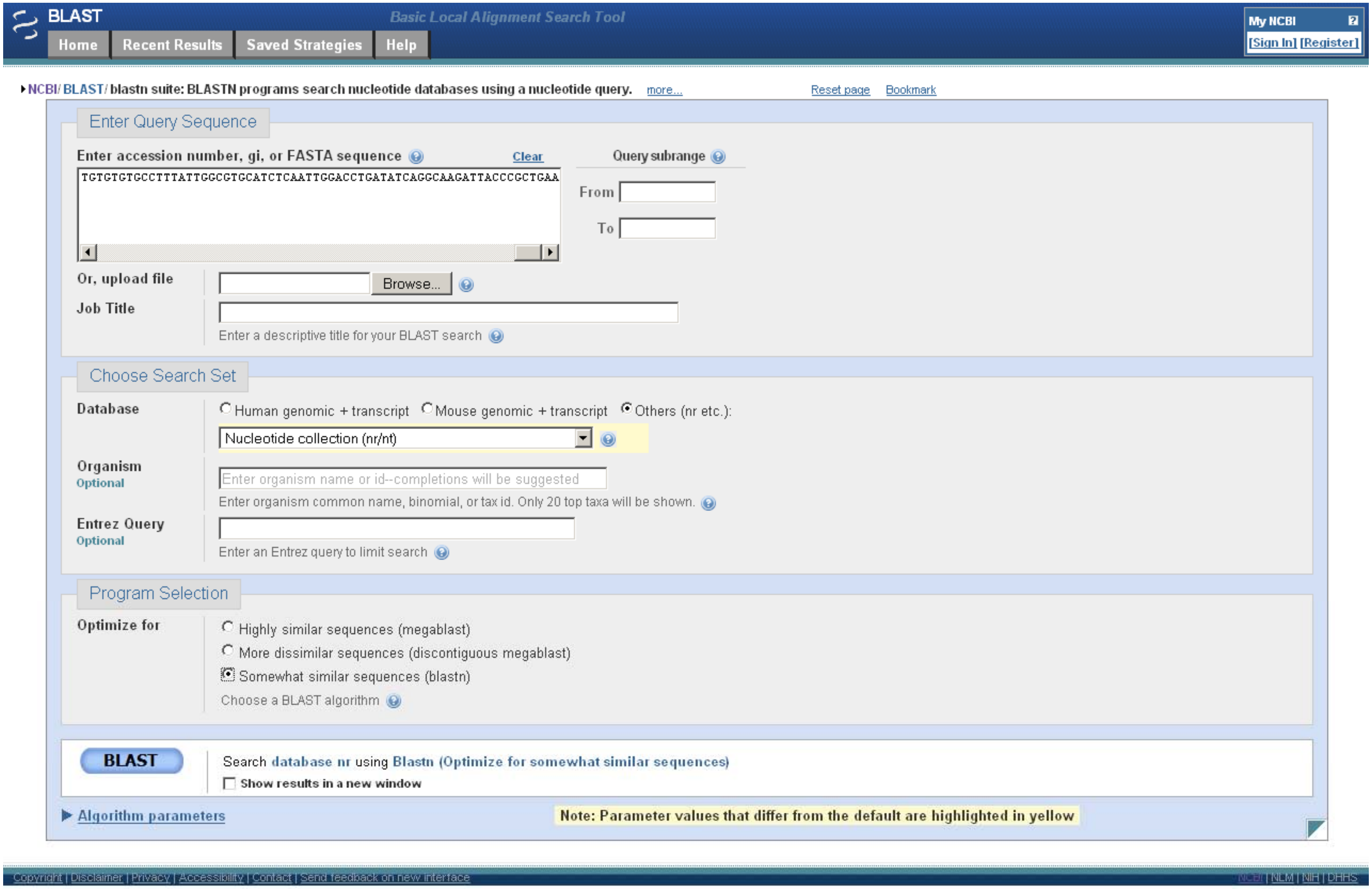
BLAST search



NCBI/ BLAST/ blastn suite: BLASTN programs search nucleotide databases using a nucleotide query. more...

Enter accession number, gi, or FASTA sequence

```
TGTGTGTGCCTTTATTGGCGTGCATCTCAATTGGACCTGATATCAGGCAAGATTACCCGCTGAA
```

Query subrange

From

To

Or, upload file

Job Title

Enter a descriptive title for your BLAST search

Database ○ Human genomic + transcript ○ Mouse genomic + transcript ● Others (nr etc.):

Nucleotide collection (nr/nt)

Organism Optional

Enter organism name or id--completions will be suggested

Enter organism common name, binomial, or tax id. Only 20 top taxa will be shown.

Entrez Query Optional

Enter an Entrez query to limit search

Optimize for

- ○ Highly similar sequences (megablast)
- ○ More dissimilar sequences (discontiguous megablast)
- ● Somewhat similar sequences (blastn)

Choose a BLAST algorithm

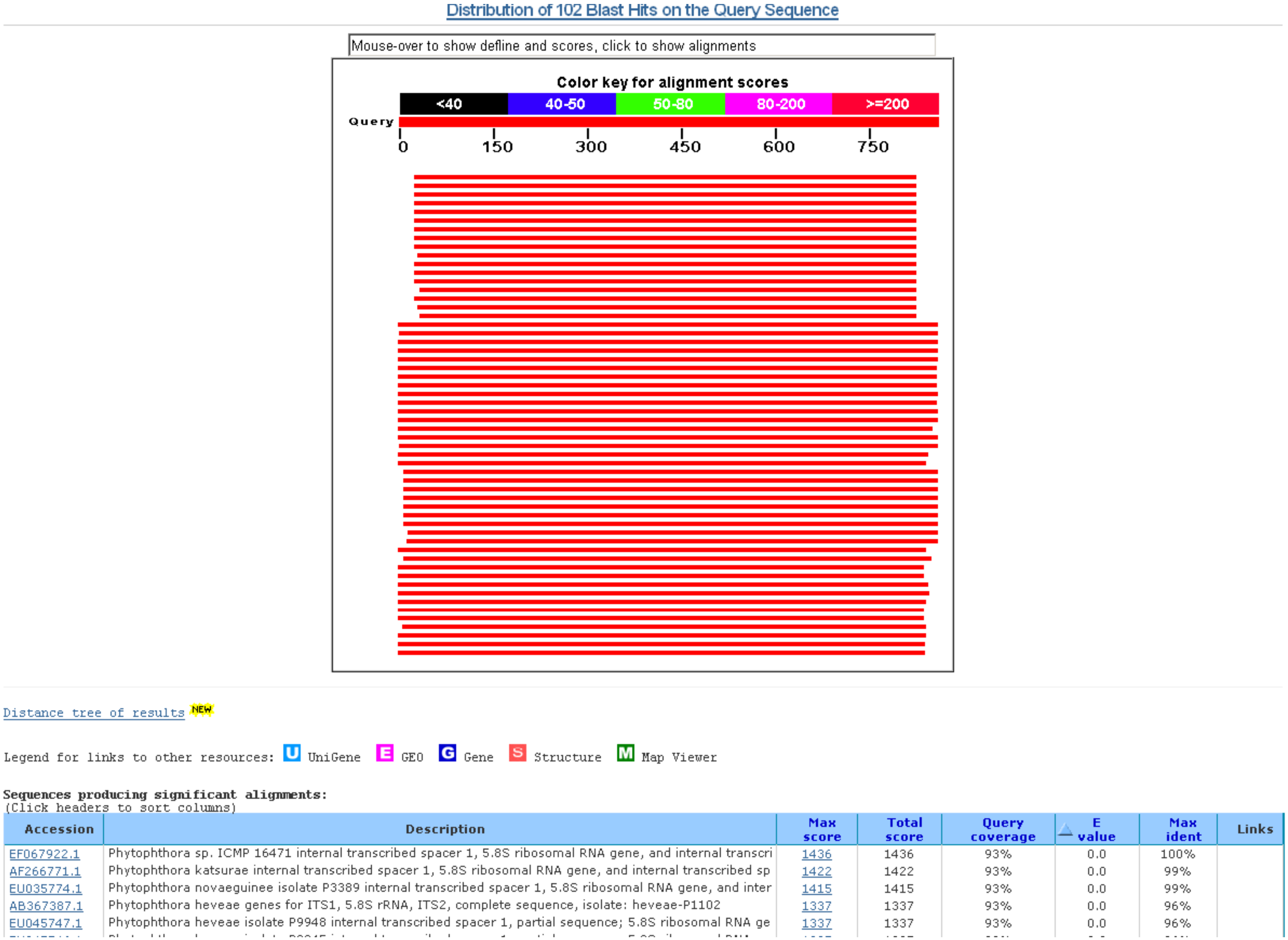
Search database nr using Blastn (Optimize for somewhat similar sequences)

☐ Show results in a new window

Note: Parameter values that differ from the default are highlighted in yellow

Next generation biomaterials

BLAST results



Distribution of 102 Blast Hits on the Query Sequence

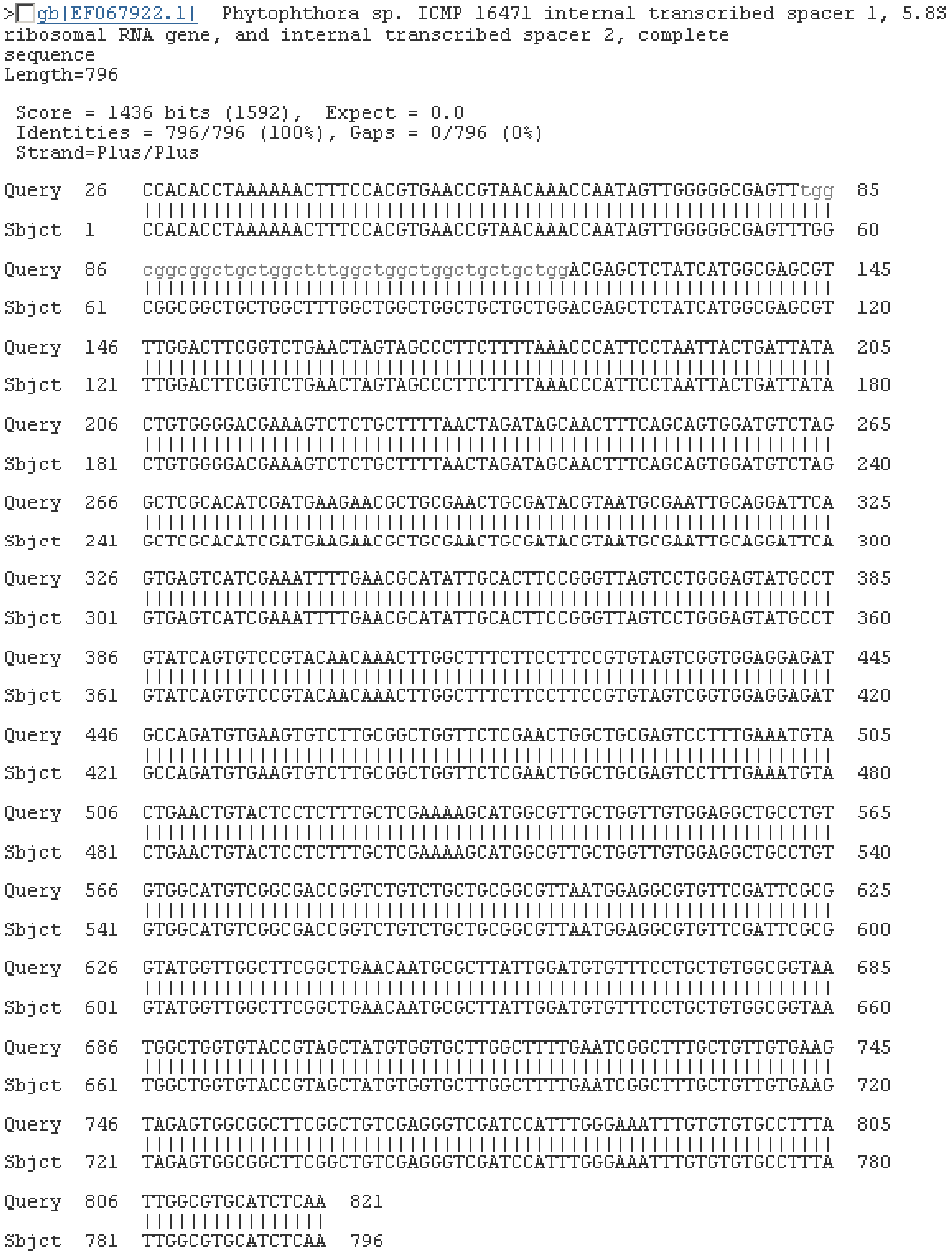
Mouse-over to show defline and scores, click to show alignments

Distance tree of results NEW

Legend for links to other resources: U UniGene E GEO G Gene S Structure M Map Viewer

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	▲ E value	Max ident	Links
EF067922.1	Phytophthora sp. ICMP 16471 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcri	1436	1436	93%	0.0	100%	
AF266771.1	Phytophthora katsurae internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed sp	1422	1422	93%	0.0	99%	
EU035774.1	Phytophthora novaeguinee isolate P3389 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and inter	1415	1415	93%	0.0	99%	
AB367387.1	Phytophthora heveae genes for ITS1, 5.8S rRNA, ITS2, complete sequence, isolate: heveae-P1102	1337	1337	93%	0.0	96%	
EU045747.1	Phytophthora heveae isolate P9948 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA ge	1337	1337	93%	0.0	96%	



```
>☐gb|EF067922.1|  Phytophthora sp. ICMP 16471 internal transcribed spacer 1, 5.8S
ribosomal RNA gene, and internal transcribed spacer 2, complete
sequence
Length=796

 Score = 1436 bits (1592),  Expect = 0.0
 Identities = 796/796 (100%), Gaps = 0/796 (0%)
 Strand=Plus/Plus

Query  26   CCACACCTAAAAAACTTTCCACGTGAACCGTAACAAACCAATAGTTGGGGGCGAGTTtgg  85
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    CCACACCTAAAAAACTTTCCACGTGAACCGTAACAAACCAATAGTTGGGGGCGAGTTTGG  60

Query  86   cggcggctgctggctttggctggctggctgctgctggACGAGCTCTATCATGGCGAGCGT  145
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CGGCGGCTGCTGGCTTTGGCTGGCTGGCTGCTGCTGGACGAGCTCTATCATGGCGAGCGT  120

Query  146  TTGGACTTCGGTCTGAACTAGTAGCCCTTCTTTTAAACCCATTCCTAATTACTGATTATA  205
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TTGGACTTCGGTCTGAACTAGTAGCCCTTCTTTTAAACCCATTCCTAATTACTGATTATA  180

Query  206  CTGTGGGGACGAAAGTCTCTGCTTTTAACTAGATAGCAACTTTCAGCAGTGGATGTCTAG  265
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  CTGTGGGGACGAAAGTCTCTGCTTTTAACTAGATAGCAACTTTCAGCAGTGGATGTCTAG  240

Query  266  GCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCGAATTGCAGGATTCA  325
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  GCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCGAATTGCAGGATTCA  300

Query  326  GTGAGTCATCGAAATTTTGAACGCATATTGCACTTCCGGGTTAGTCCTGGGAGTATGCCT  385
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  GTGAGTCATCGAAATTTTGAACGCATATTGCACTTCCGGGTTAGTCCTGGGAGTATGCCT  360

Query  386  GTATCAGTGTCCGTACAACAAACTTGGCTTTCTTCCTTCCGTGTAGTCGGTGGAGGAGAT  445
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  446  GCCAGATGTGAAGTGTCTTGCGGCTGGTTCTCGAACTGGCTGCGAGTCCTTTGAAATGTA  505
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  GCCAGATGTGAAGTGTCTTGCGGCTGGTTCTCGAACTGGCTGCGAGTCCTTTGAAATGTA  480

Query  506  CTGAACTGTACTCCTCTTTGCTCGAAAAGCATGGCGTTGCTGGTTGTGGAGGCTGCCTGT  565
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  CTGAACTGTACTCCTCTTTGCTCGAAAAGCATGGCGTTGCTGGTTGTGGAGGCTGCCTGT  540

Query  566  GTGGCATGTCGGCGACCGGTCTGTCTGCTGCGGCGTTAATGGAGGCGTGTTCGATTCGCG  625
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  GTGGCATGTCGGCGACCGGTCTGTCTGCTGCGGCGTTAATGGAGGCGTGTTCGATTCGCG  600

Query  626  GTATGGTTGGCTTCGGCTGAACAATGCGCTTATTGGATGTGTTTCCTGCTGTGGCGGTAA  685
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  686  TGGCTGGTGTACCGTAGCTATGTGGTGCTTGGCTTTTGAATCGGCTTTGCTGTTGTGAAG  745
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  746  TAGAGTGGCGGCTTCGGCTGTCGAGGGTCGATCCATTTGGGAAATTTGTGTGTGCCTTTA  805
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721  TAGAGTGGCGGCTTCGGCTGTCGAGGGTCGATCCATTTGGGAAATTTGTGTGTGCCTTTA  780

Query  806  TTGGCGTGCATCTCAA  821
            ||||||||||||||||
Sbjct  781  TTGGCGTGCATCTCAA  796
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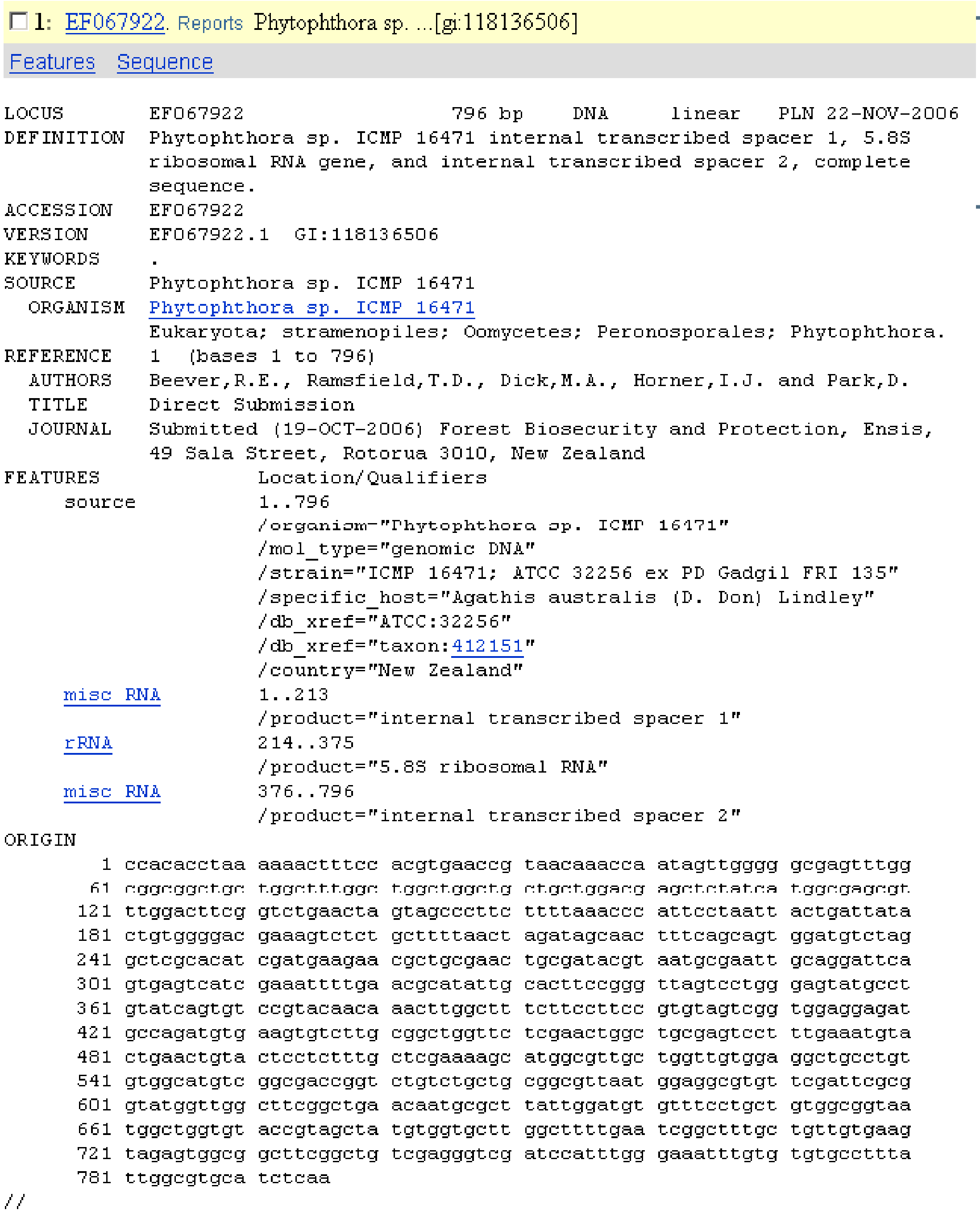

☐ 1: EF067922. Reports Phytophthora sp. ...[gi:118136506]

Features Sequence

```
LOCUS       EF067922                 796 bp    DNA     linear   PLN 22-NOV-2006
DEFINITION  Phytophthora sp. ICMP 16471 internal transcribed spacer 1, 5.8S
            ribosomal RNA gene, and internal transcribed spacer 2, complete
            sequence.
ACCESSION   EF067922
VERSION     EF067922.1  GI:118136506
KEYWORDS    .
SOURCE      Phytophthora sp. ICMP 16471
  ORGANISM  Phytophthora sp. ICMP 16471
            Eukaryota; stramenopiles; Oomycetes; Peronosporales; Phytophthora.
REFERENCE   1  (bases 1 to 796)
  AUTHORS   Beever,R.E., Ramsfield,T.D., Dick,M.A., Horner,I.J. and Park,D.
  TITLE     Direct Submission
  JOURNAL   Submitted (19-OCT-2006) Forest Biosecurity and Protection, Ensis,
            49 Sala Street, Rotorua 3010, New Zealand
FEATURES             Location/Qualifiers
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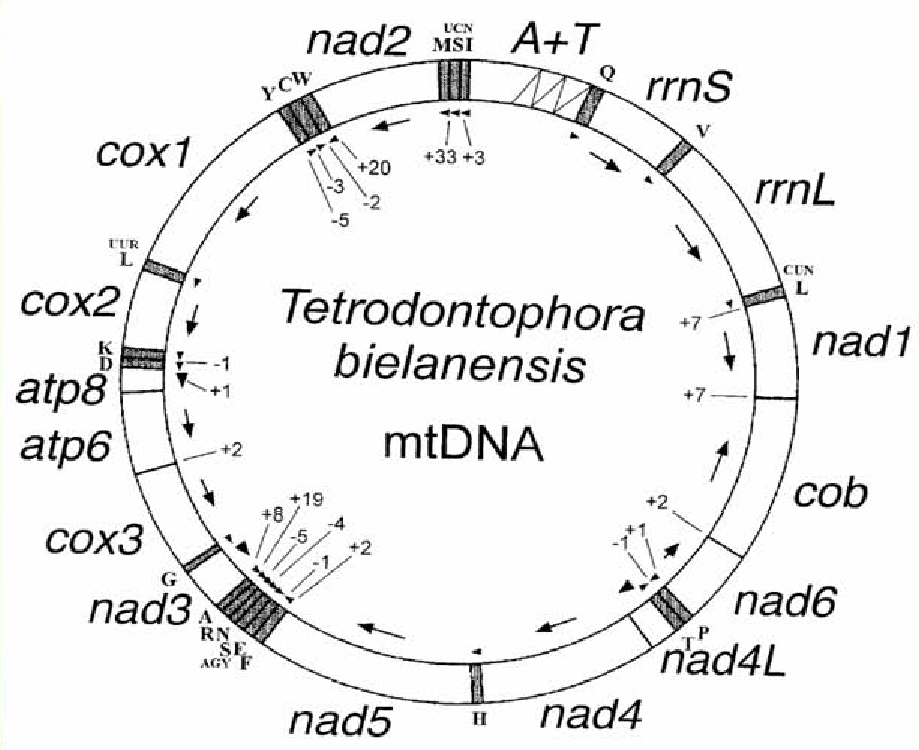
                     /mol_type="genomic DNA"
                     /strain="ICMP 16471; ATCC 32256 ex PD Gadgil FRI 135"
                     /specific_host="Agathis australis (D. Don) Lindley"
                     /db_xref="ATCC:32256"
                     /db_xref="taxon:412151"
                     /country="New Zealand"
     misc_RNA        1..213
                     /product="internal transcribed spacer 1"
     rRNA            214..375
                     /product="5.8S ribosomal RNA"
     misc_RNA        376..796
                     /product="internal transcribed spacer 2"
ORIGIN
        1 ccacacctaa aaaactttcc acgtgaaccg taacaaacca atagttgggg gcgagtttgg
       61 cggcggctgc tggctttggc tggctggctg ctgctggacg agctctatca tggcgagcgt
      121 ttggacttcg gtctgaacta gtagcccttc ttttaaaccc attcctaatt actgattata
      181 ctgtggggac gaaagtctct gcttttaact agatagcaac tttcagcagt ggatgtctag
      241 gctcgcacat cgatgaagaa cgctgcgaac tgcgatacgt aatgcgaatt gcaggattca
      301 gtgagtcatc gaaattttga acgcatattg cacttccggg ttagtcctgg gagtatgcct
      361 gtatcagtgt ccgtacaaca aacttggctt tcttccttcc gtgtagtcgg tggaggagat
      421 gccagatgtg aagtgtcttg cggctggttc tcgaactggc tgcgagtcct ttgaaatgta
      481 ctgaactgta ctcctctttg ctcgaaaagc atggcgttgc tggttgtgga ggctgcctgt
      541 gtggcatgtc ggcgaccggt ctgtctgctg cggcgttaat ggaggcgtgt tcgattcgcg
      601 gtatggttgg cttcggctga acaatgcgct tattggatgt gtttcctgct gtggcggtaa
      661 tggctggtgt accgtagcta tgtggtgctt ggcttttgaa tcggctttgc tgttgtgaag
      721 tagagtggcg gcttcggctg tcgagggtcg atccatttgg gaaatttgtg tgtgccttta
      781 ttggcgtgca tctcaa
//
```

Potential pitfalls

- No DNA sequence from the organism is in GenBank.
- Mis-identification of source organism in GenBank.
- Use other databases, ie. FusID 1.0 or UNITE.

What is a DNA barcode?



The barcode region:

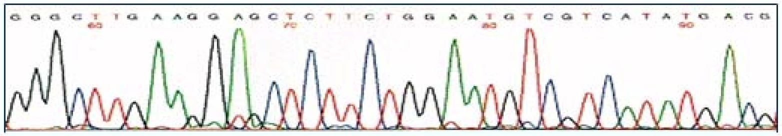
- Cytochrome c oxidase I
- protein-coding
- universal PCR primers
- ubiquitous in life
- relatively rapidly evolving

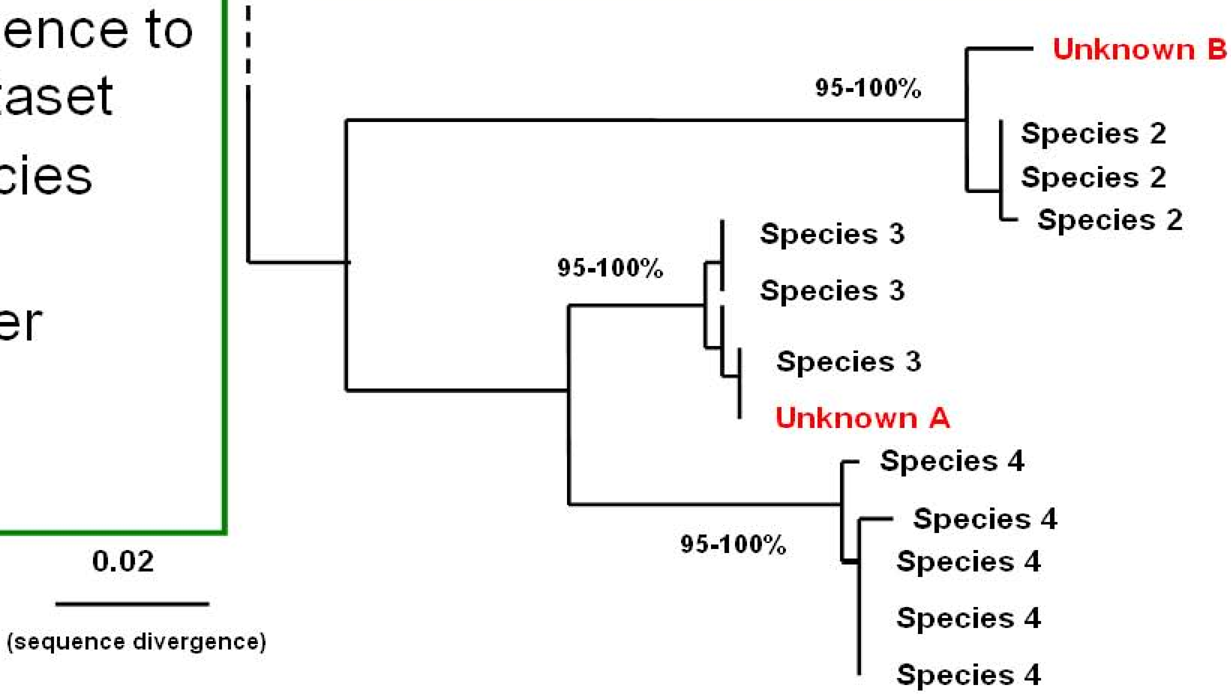
Bio-Protection

Using DNA barcodes for identification

Interrogate the reference dataset

- Append sequence to reference dataset
- Observe species 'clusters'
- Consider other evidence

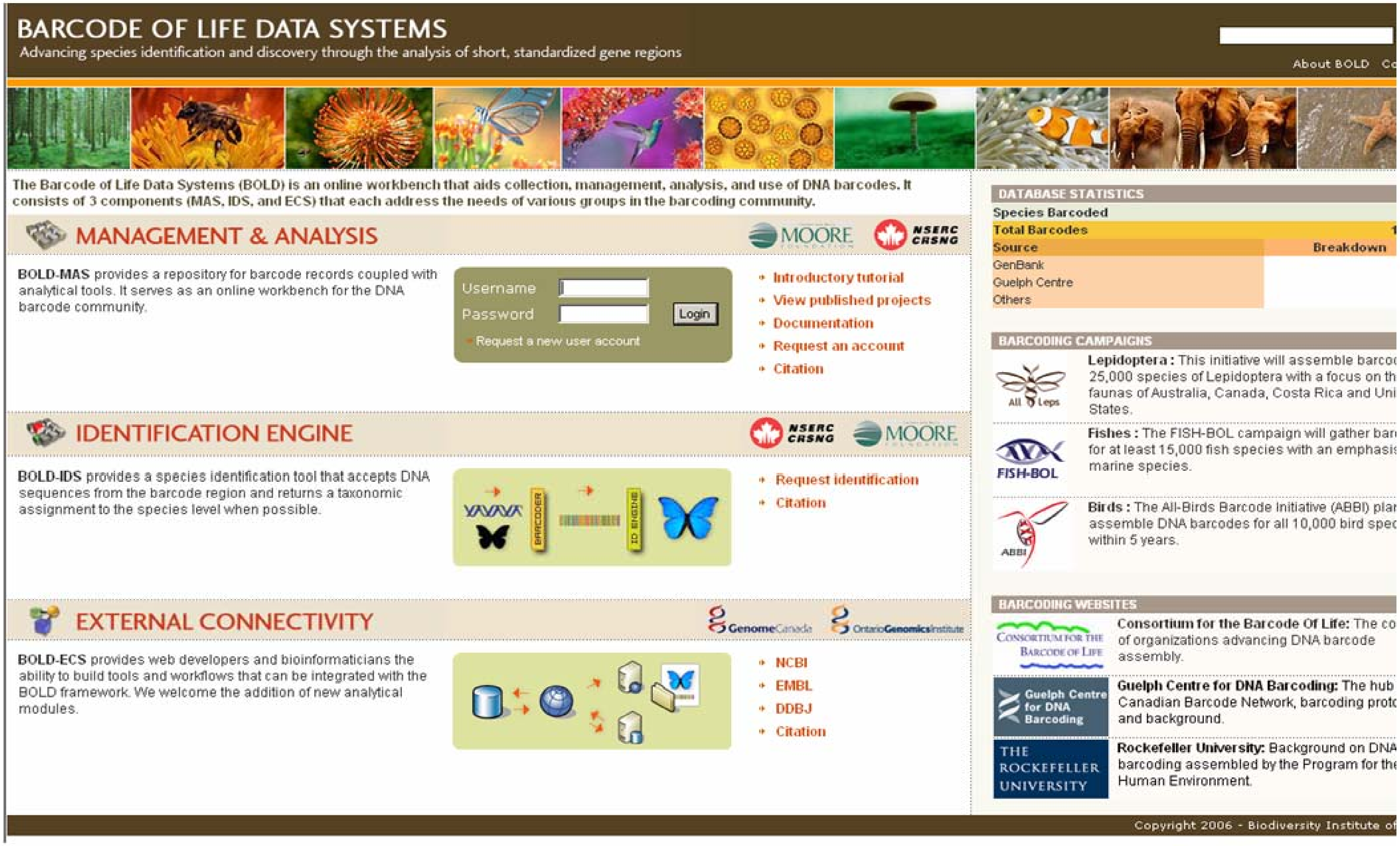




Bio-Protection

BOLD (Barcoding of Life Database)

www.barcodinglife.com



BARCODE OF LIFE DATA SYSTEMS

Advancing species identification and discovery through the analysis of short, standardized gene regions

The Barcode of Life Data Systems (BOLD) is an online workbench that aids collection, management, analysis, and use of DNA barcodes. It consists of 3 components (MAS, IDS, and ECS) that each address the needs of various groups in the barcoding community.

MANAGEMENT & ANALYSIS

BOLD-MAS provides a repository for barcode records coupled with analytical tools. It serves as an online workbench for the DNA barcode community.

- Introductory tutorial
- View published projects
- Documentation
- Request an account
- Citation

IDENTIFICATION ENGINE

BOLD-IDS provides a species identification tool that accepts DNA sequences from the barcode region and returns a taxonomic assignment to the species level when possible.

- Request identification
- Citation

EXTERNAL CONNECTIVITY

BOLD-ECS provides web developers and bioinformaticians the ability to build tools and workflows that can be integrated with the BOLD framework. We welcome the addition of new analytical modules.

- NCBI
- EMBL
- DDBJ
- Citation

DATABASE STATISTICS

Species Barcoded

Total Barcodes 1

Source	Breakdown
GenBank	
Guelph Centre	
Others	

BARCODING CAMPAIGNS

Lepidoptera : This initiative will assemble barcoc 25,000 species of Lepidoptera with a focus on th faunas of Australia, Canada, Costa Rica and Uni States.

Fishes : The FISH-BOL campaign will gather bar for at least 15,000 fish species with an emphasis marine species.

Birds : The All-Birds Barcode Initiative (ABBI) plar assemble DNA barcodes for all 10,000 bird spec within 5 years.

BARCODING WEBSITES

Consortium for the Barcode Of Life: The co of organizations advancing DNA barcode assembly.

Guelph Centre for DNA Barcoding: The hub Canadian Barcode Network, barcoding proto and background.

Rockefeller University: Background on DNA barcoding assembled by the Program for the Human Environment.

Bio-Protection

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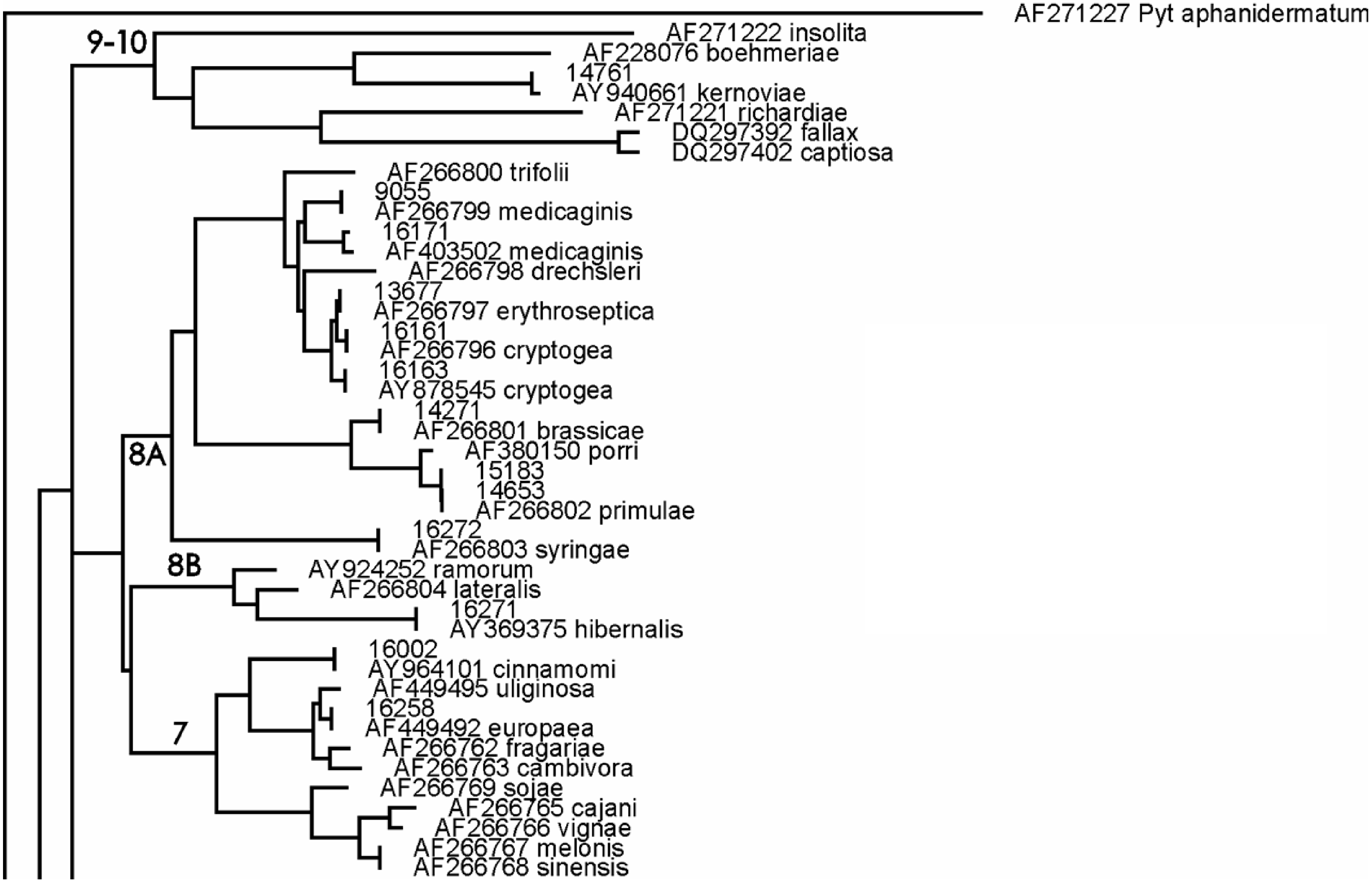
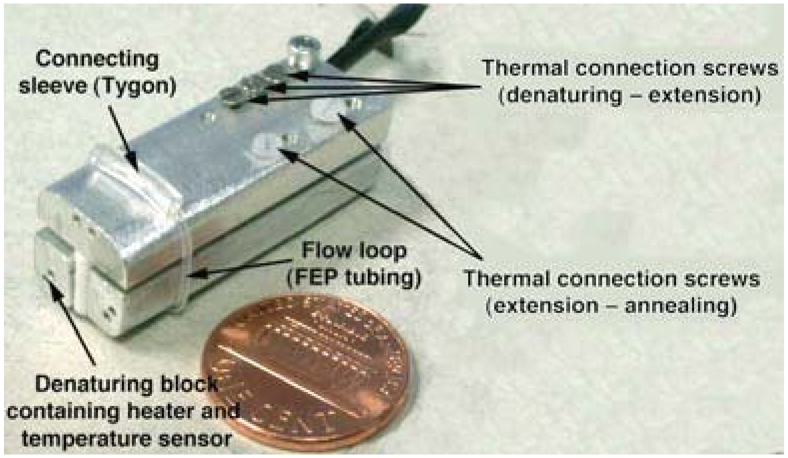
Phytophthora in NZ



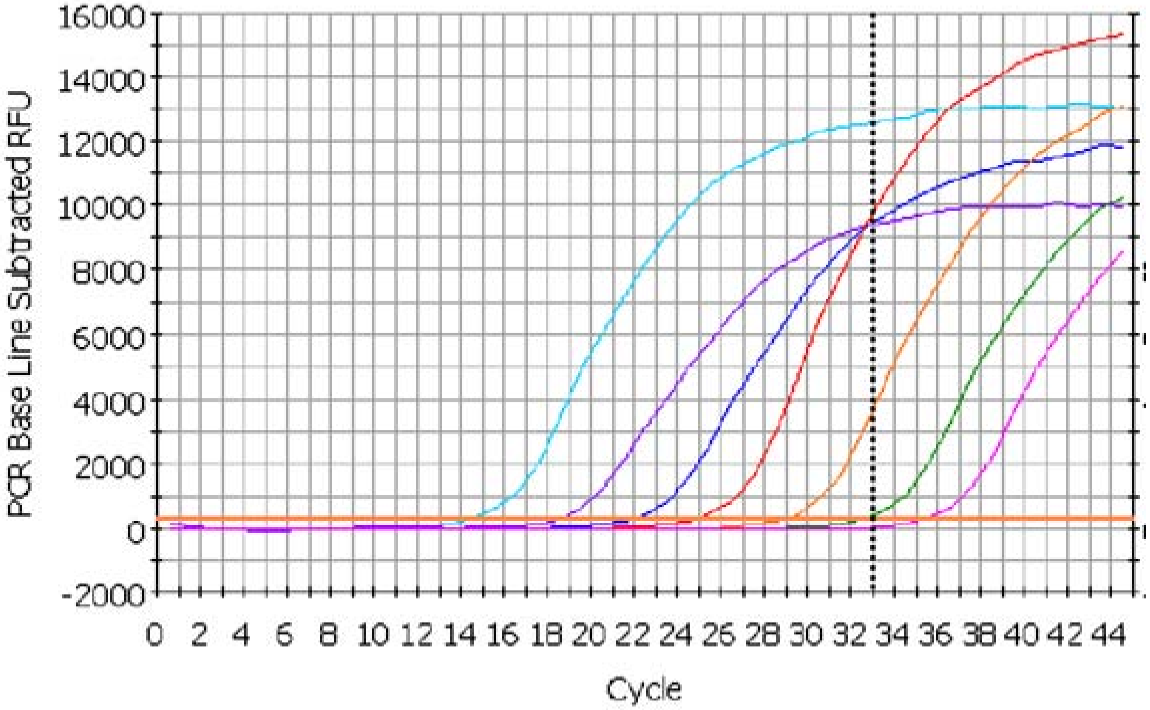
New Technologies

Portable PCR for field-based diagnostics. Crop diseases, animal diseases, human disease bioterrorism.



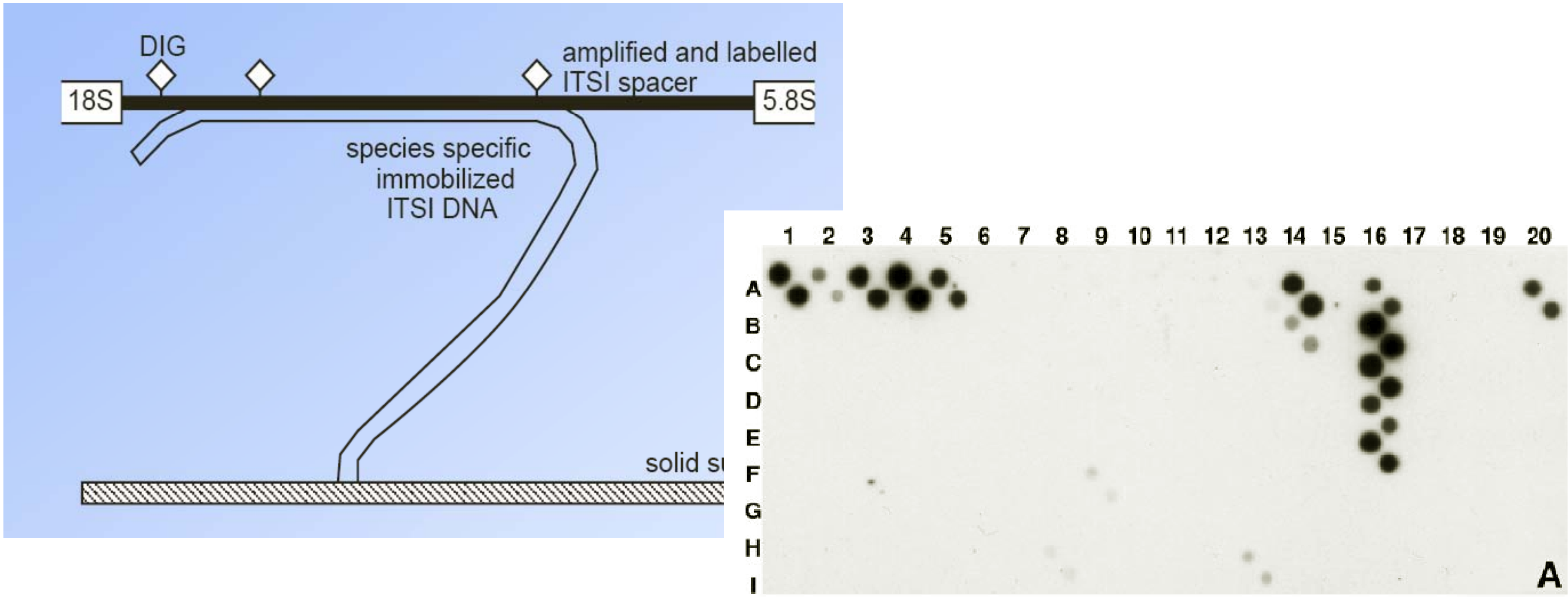
Real time PCR

Monitors amplification progress through fluorescence. Quantitative, rapid.



SERIES OF 10-FOLD DILUTIONS

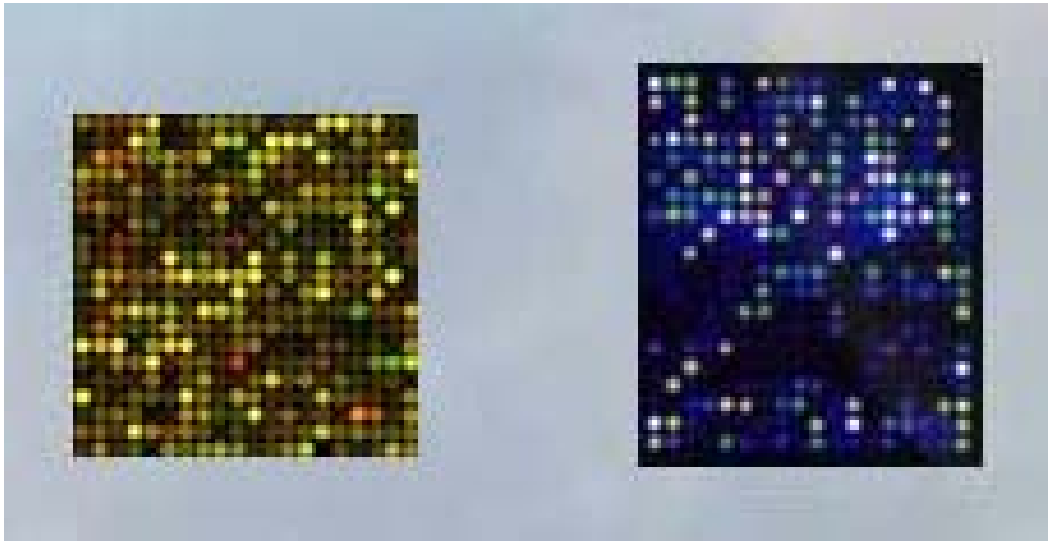
Macroarray



Species specific DNA printed onto array, PCR products hybridized to the membrane, where spots light up, species are identified.
Source: Levesque

Microarray

- 50,000 cDNAs printed onto a 25 x 75 mm slide.
- Build a plant pathogen chip or insect chip.



ELISA

Adgen Phytophthora lateral flow devise.

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New Zealand

- DNA protocols have been developed for specific pathogens that pose a biosecurity threat.
- FRST and MAF Ops funded.
- Tests in use by MAF.
- Lincoln University associated with BAR code of life project.

Conclusions

- Technology is converging – molecular biology and computing.
- DNA sequencing is now very inexpensive.
- Still costs associated with sample preparation.

Conclusions

- PCR based methods offer the ability to detect minute quantities of difficult to identify organisms quickly.
- Problem with databases and arrays – if the organism you are looking for is not present, you will miss it.

Acknowledgements

- Karen Armstrong, Lincoln University
- websites from which photos were obtained:

http://plant.neogeneurope.com/
http://www.microarraystation.com/dna-microarray-protocol/
http://www.biology.usu.edu/labsites/pfrender/Biodiversity%20workshop%202006/Microarray%20Levesque.pdf
https://www.roche-applied-science.com/sis/rtpcr/lightcycler/index.jsp
http://www.rsc.org/chemistryworld/News/2007/May/01050701.asp
http://www.csun.edu/~hcbio027/biotechnology/lec3/sanger.html
http://medgadget.com/archives/2007/09/portable_dna_forensics.html
http://www.smithsdetection.com/eng/1025_3326.php