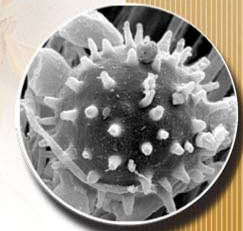




Agriculture and
Agri-Food Canada

Agriculture et
Agroalimentaire Canada



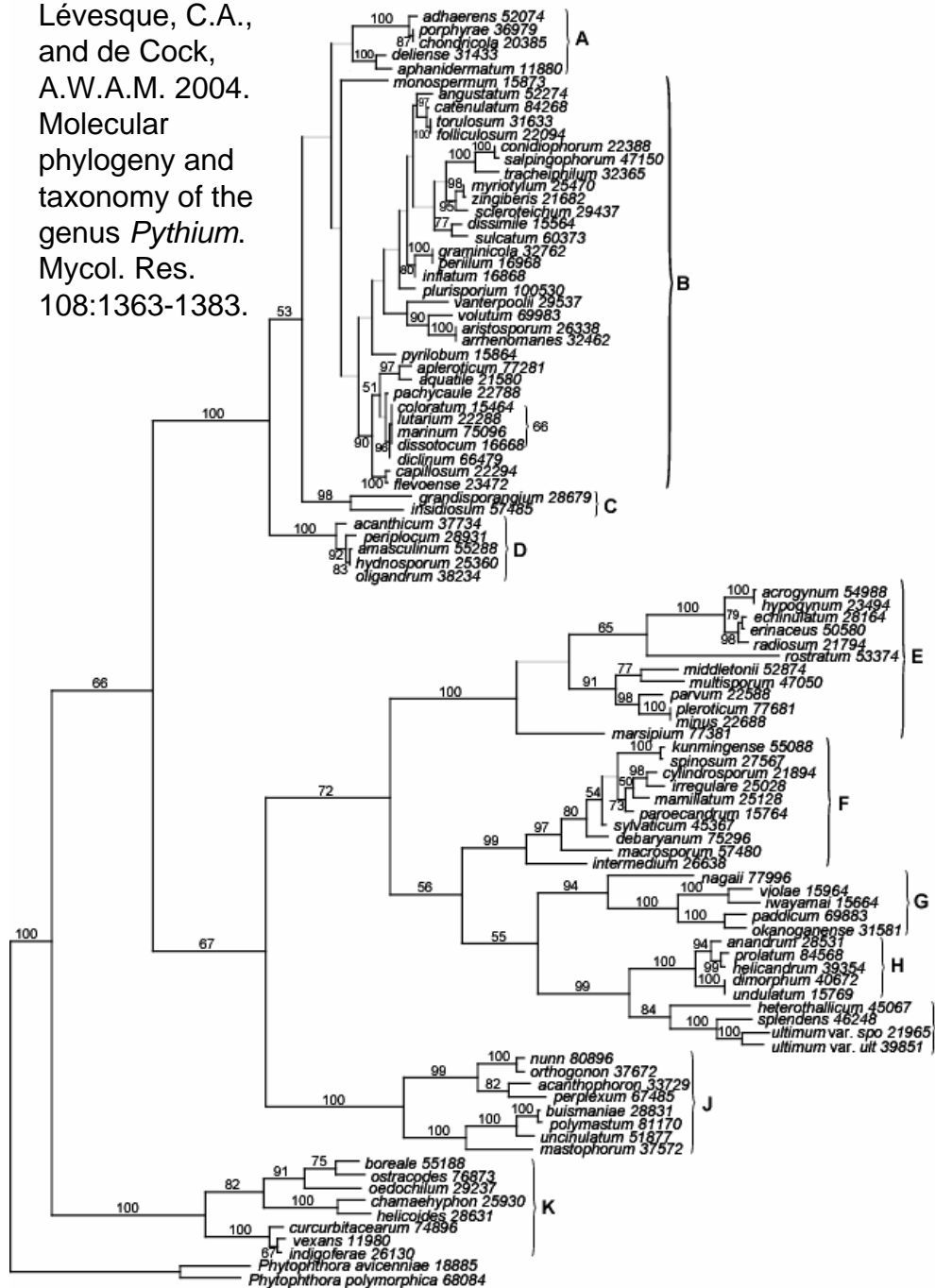
Molecular phylogeny, barcoding and ecology of *Pythium* species.

C.A. Lévesque , G. Robideau, N. Désaulniers, K. Bala, W. Chen, J.T. Tambong
Biodiversity / Mycology, Agriculture and Agri-Food Canada,
Central Experimental Farm, Ottawa, ON
Biology Dept., Carleton University

A.W.A.M. de Cock, Centraalbureau voor Schimmelcultures, Utrecht,
The Netherlands

Canada 

Lévesque, C.A.,
and de Cock,
A.W.A.M. 2004.
Molecular
phylogeny and
taxonomy of the
genus *Pythium*.
Mycol. Res.
108:1363-1383.

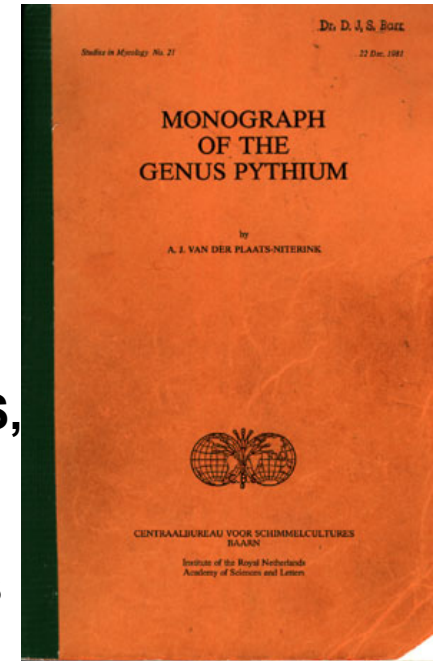


- 10 changes

Evolution of all the species of the genus *Pythium* (ITS and LSU)

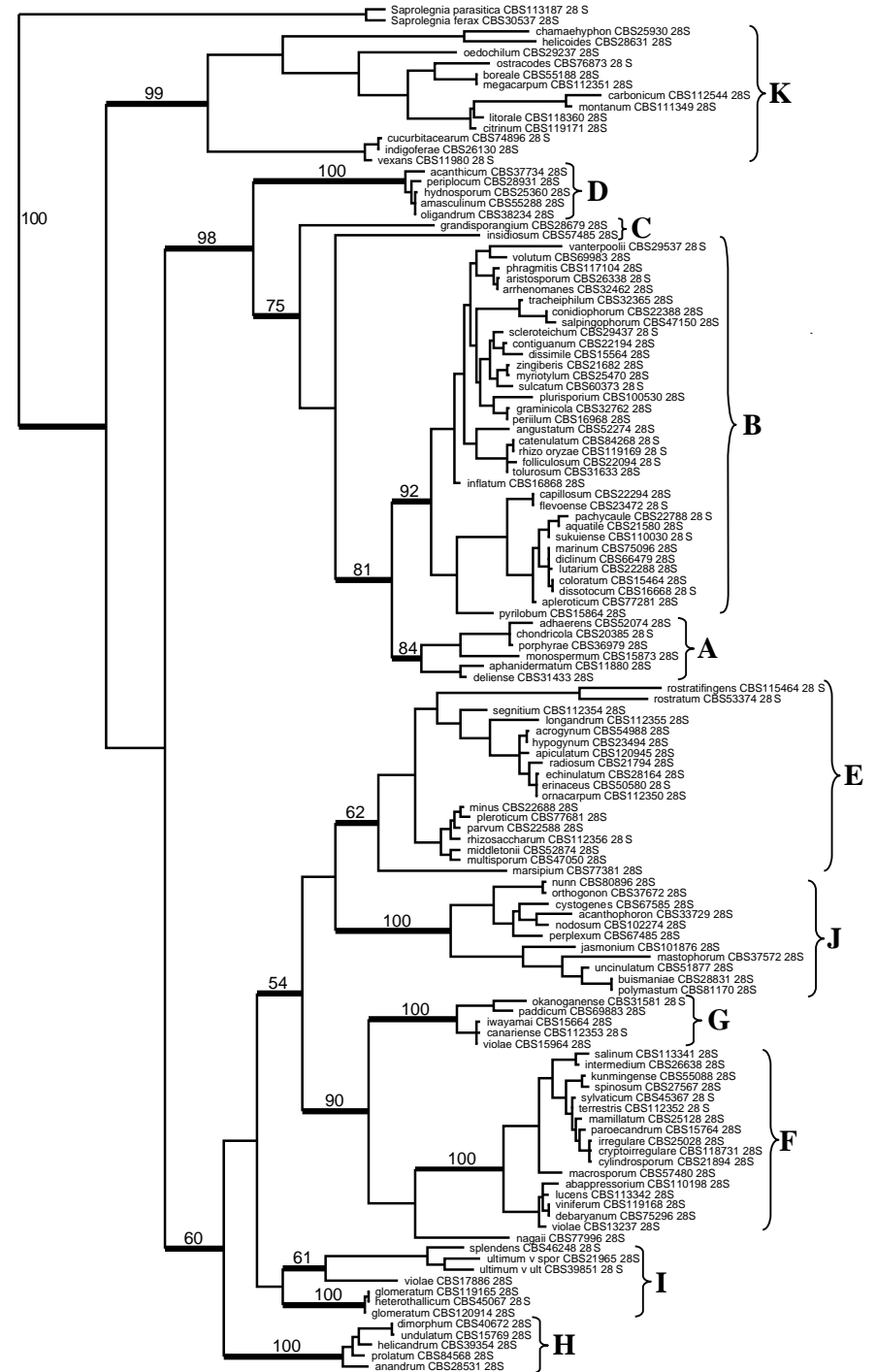


Arthur de Cock, CBS,
Netherlands,
collection of
« ex-type » cultures



LSU Large Subunit rDNA D1 to D3 (127 species)

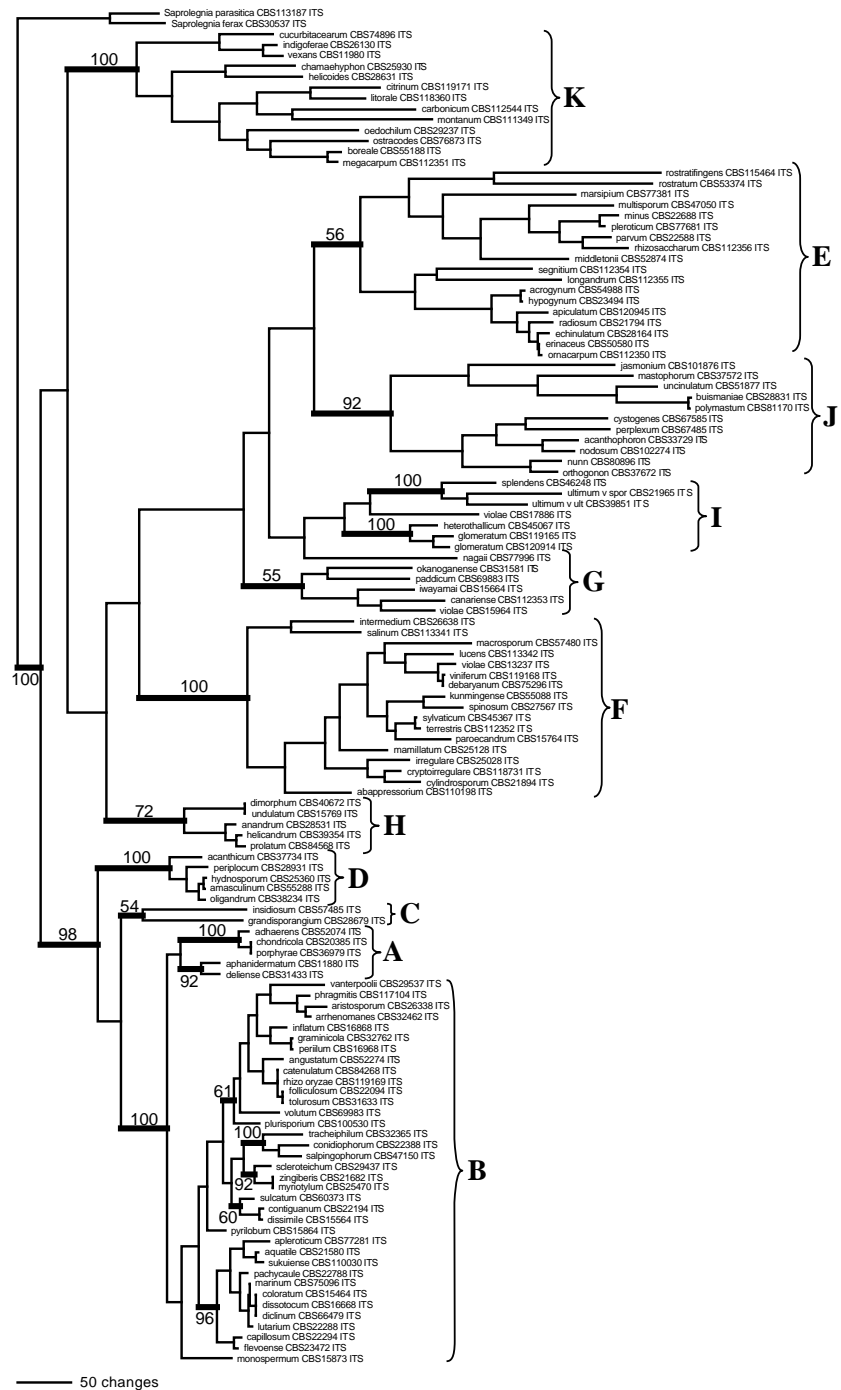
Maximum Parsimony



— 5 changes

ITS Internal Transcribed Spacer rDNA (127 species)

Maximum Parsimony



Global Initiative

Consortium for the Barcode of Life (CBOL)



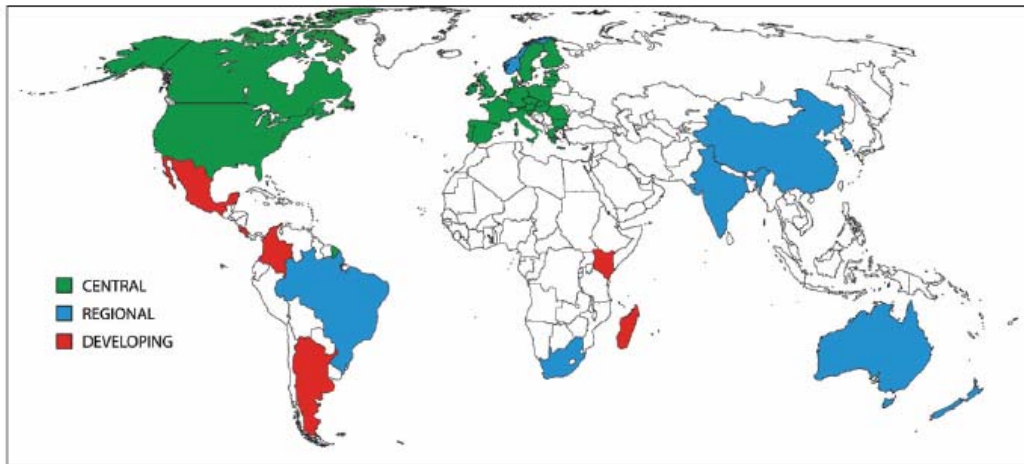
From Robert Hanner, Canadian Barcode of Life Network, University of Guelph






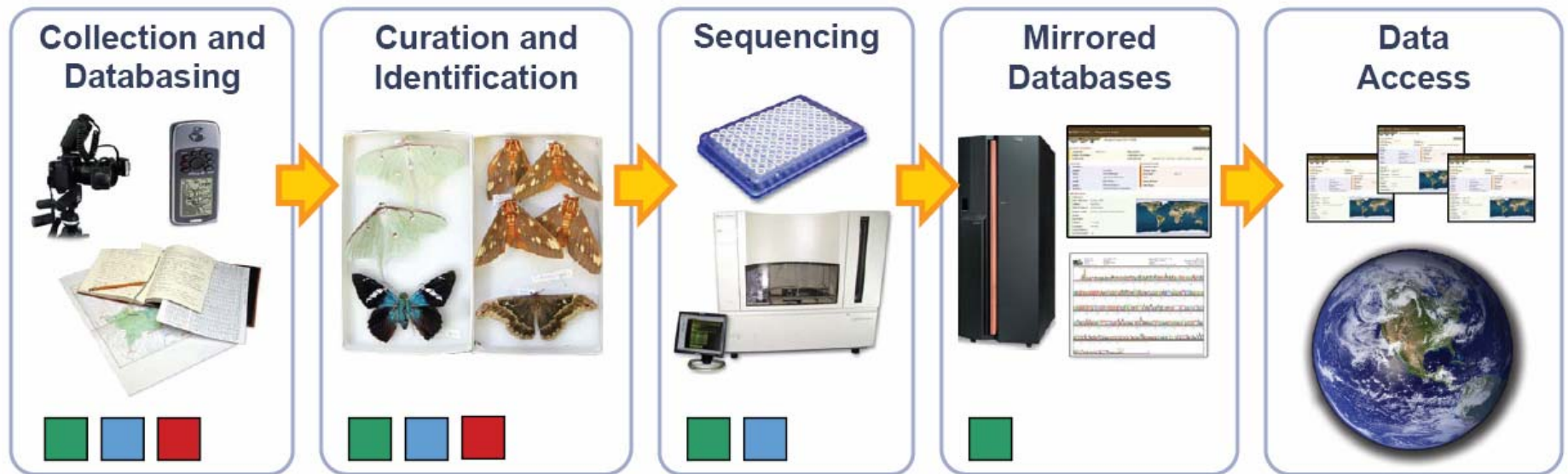
International Barcode of Life Project

**Research Overview
May 2008**

iBOL

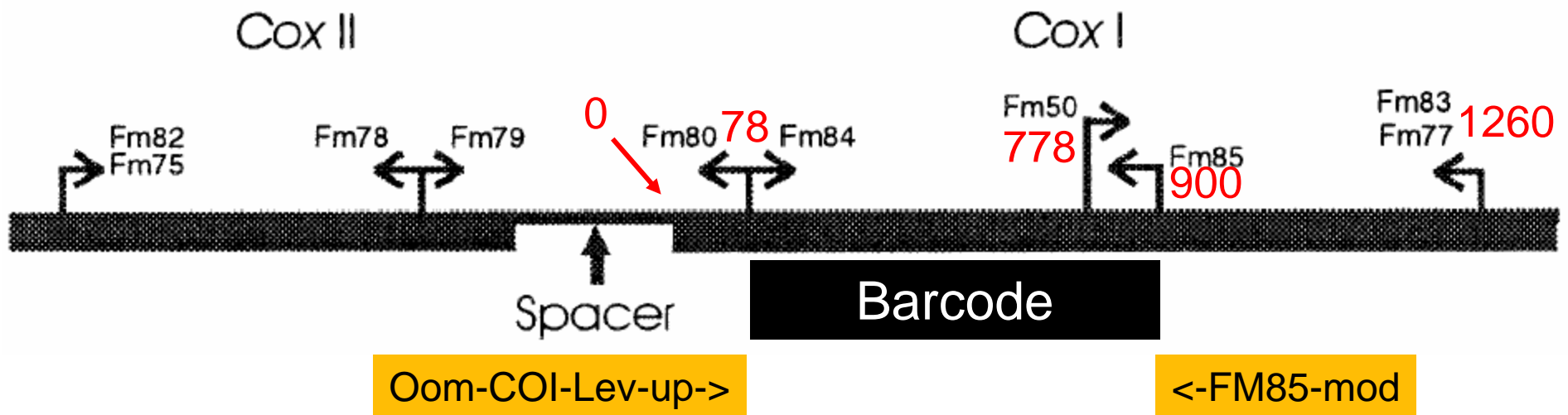


-  **Central Nodes**
-  **Regional Nodes**
-  **Developing Nodes**



Oomycete barcoding

- Cytochrome Oxidase I (*COI*)
- Good primers designed that amplify a 727bp region of *COI*
- No introns in oomycete *COI* (as opposed to true fungi)



Martin, F. N., and P. W. Tooley. 2003. Phylogenetic relationships among *Phytophthora* species inferred from sequence analysis of mitochondrially encoded cytochrome oxidase I and II genes. *Mycologia* **95**:269-284.



Gregg Robideau
Ph.D. Candidate
(since Feb 2008)
Dept. of Biology
Carleton University

- ~1,000 COI & ITS sequences of oomycetes obtained to date
- Over 100 species of *Pythium*, 70 species of *Phytophthora*, and several other genera such as *Achlya*, *Peronophythora*, and *Saprolegnia*

DNA barcoding of oomycetes with the Cytochrome Oxidase I (COI) gene

Robideau, Gregg P.^{1,2}, Smith, Myron L.², and Lévesque, C. André^{2,1}

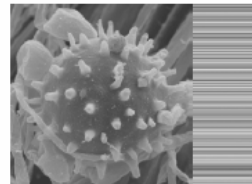
1. Carleton University, Ottawa, ON

2. Agriculture and Agri-Food Canada, ECORC, Ottawa, ON

Introduction

Oomycetes, which include water moulds and downy mildews, are terrestrial and aquatic fungi-like organisms belonging to the kingdom Stramenopila. They include important plant pathogens such as the causal organisms of the potato famine in Ireland (*Phytophthora infestans*), and Sudden Oak Death Syndrome (*Phytophthora ramorum*) devastating California. This group also includes fish pathogens (*Saprolegnia parasitica*) and mammalian pathogens (*Pythium insidiosum*), as well as biological control agents such as *Legidium giganteum*, which infects the larvae of mosquitoes, or the mycoparasite *Pythium oligandrum*. Oomycetes are commonly found in fresh water, oceans, soil, and can also be airborne. Our research to date has focussed on the genera *Pythium* and *Phytophthora*. The current taxonomic groupings of *Pythium* and *Phytophthora* are supplemented significantly by molecular data, specifically from nuclear DNA sequences of the ITS (Internal Transcribed Spacer) and LSU (Large Subunit) regions of rDNA (Lévesque and de Cook 2004). While analyses of these genes and spacers have produced phylogenetic relationships that are generally consistent with morphological species concepts, there are still potential weaknesses in these genes in terms of their usefulness for identification. For example, some species of *Pythium* share the same ITS sequence.

The use of a different gene that may provide resolution to species-level identification is therefore of great interest, especially considering that in *Pythium*, identification to the species level is crucial in determining the ecological role of the organism. Our research to date has focussed on the validity of COI barcode sequencing for oomycete species delimitation.



Materials and Methods

PCR amplification of COI was performed using primers designed to amplify the barcode region of all oomycetes. The forward primer designed was 5'-TCAWCWMGATGGCTTTTTTCAAC-3'. The primary reverse primer used was 5'-RRHWACKTACTDTRATACCAA-3' which was modified from Fm85 (Martin and Tooley 2003). In a few cases an internal reverse, 5'-CYTCHGGRTGWCCRAAAAACCAA-3', was used when the primary reverse did not yield a good PCR product. Sequencing of PCR products was performed with an ABI Prism® 3130XL.

Results and Discussion

COI barcode sequences were easily obtained using the primers designed. The barcode region of the oomycetes sequenced did not contain any introns, and in the vast majority of samples, a 727bp fragment was sequenced as opposed to some samples for which a 680bp fragment was obtained with the internal reverse primer.

The sequences of the barcode region were highly variable, with many species showing intraspecific variation in COI. The barcode region still exhibits ability in species identification however, because conspecific barcode sequences clustered together, as seen in Figure 1.

The phylogenetic relationships created with the barcode region did not match the previously established ones that matched morphological characters in the genus *Pythium* (Lévesque and de Cook 2004), but this was not an unexpected result, as short DNA regions do not generally provide enough variable characters to produce accurate phylogenies.

Resolution of *Pythium* species boundary ambiguities that ITS cannot discriminate was not achieved with COI. In cases where two *Pythium* species shared identical or similar (>95%) ITS sequences, they also had similar or identical COI sequences. In many cases, the classification of an ambiguous pair as separate species was based on subtle and questionable morphological differences. It is possible that with further study and multi-gene analysis, these species with identical barcodes may be conspecific.

Conclusions

Although COI relationships are not congruent with existing phylogenies, our results have provided unique COI sequences for most species of *Pythium* and *Phytophthora* sequenced so far. We have also discovered intraspecific variation of COI sequences in some species studied, including the recently defined species of *Pythium atrantheridium*. Although intraspecific variation exists in COI, the clustering of conspecific COI sequences is reliable for identification. Future investigation will be made into species complexes such as *Pythium irregulare*, and the ability of COI to discriminate among members of such complexes.

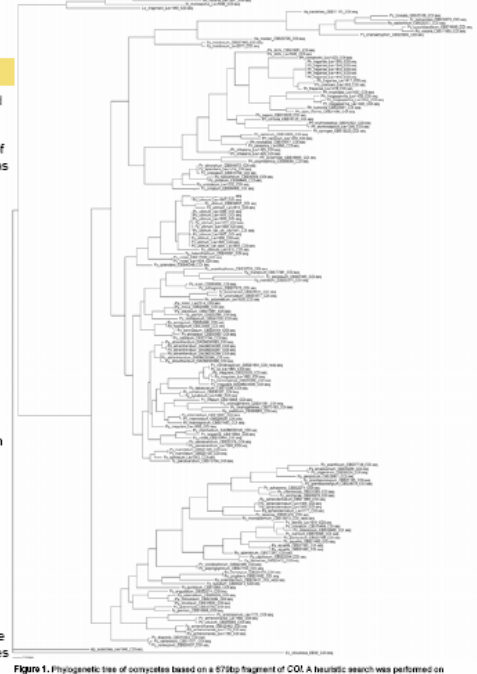


Figure 1. Phylogenetic tree of oomycetes based on a 672bp fragment of COI. A heuristic search was performed on 101 representative sequences out of 700 available using parsimony analysis. A brown algae was used as outgroup. Genus abbreviations: Ac = Achlya, Di = Dictyochus, Ha = Halophytophthora, Pa = Paraphytophthora, Py = Pythium, Ap = Aphanomyces, Ec = Ectosporus, Le = Leptogium, Ph = Phytophthora, Sa = Saprolegnia

Acknowledgements

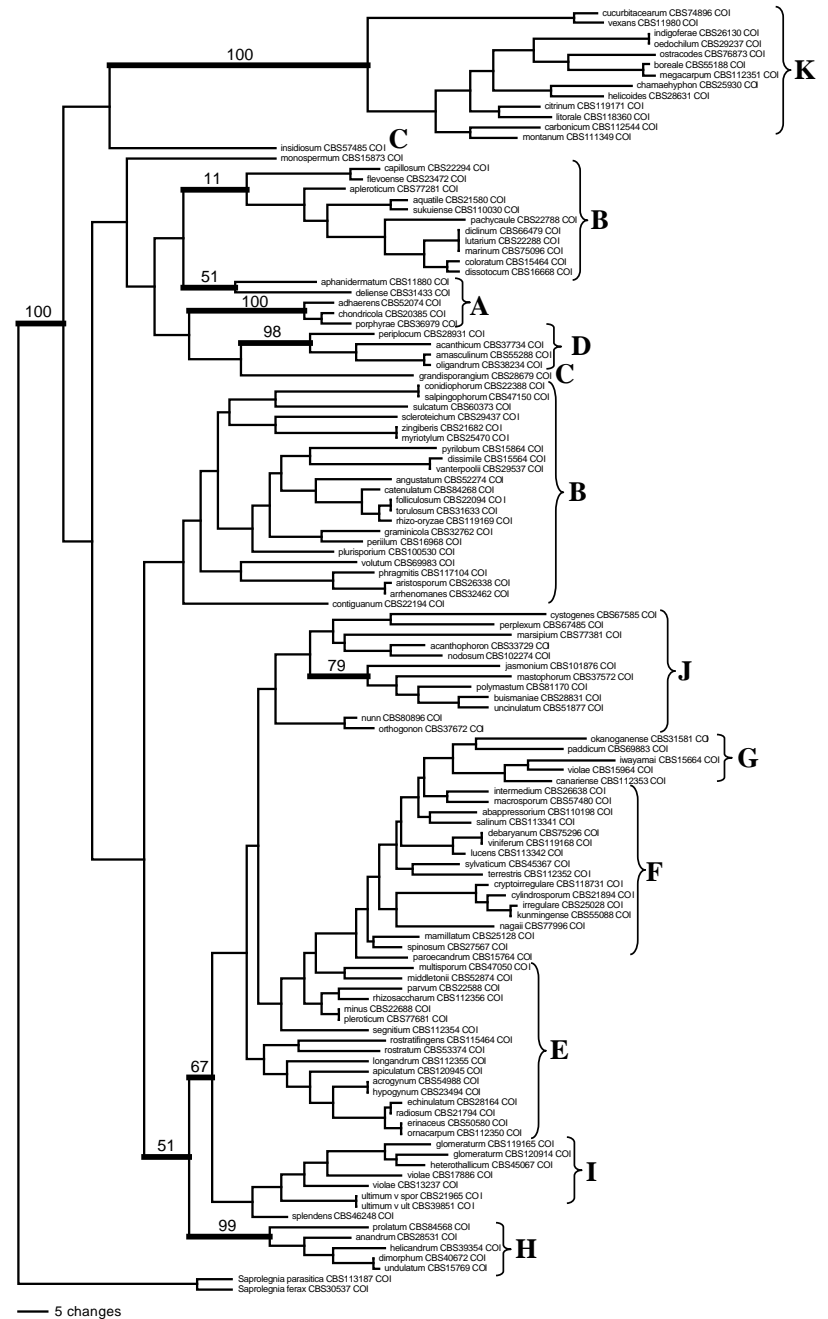
We wish to thank Rafik Assabgui, Julie Chapados, and Nicole Desaulniers for technical assistance.

References

- Lévesque, C. A., and A. W. de Cook. 2004. Molecular phylogeny and taxonomy of the genus *Pythium*. *Mycological Research* 108:1363-1388.
Martin, F.N., and P.W. Tooley. 2003. Phylogenetic relationships among *Phytophthora* species inferred from sequence analysis of mitochondrially encoded cytochrome oxidase I and II genes. *Mycologia* 95 (2):266-284.

COI 5' end of Cytochrome Oxidase I (124 species)

Maximum Parsimony



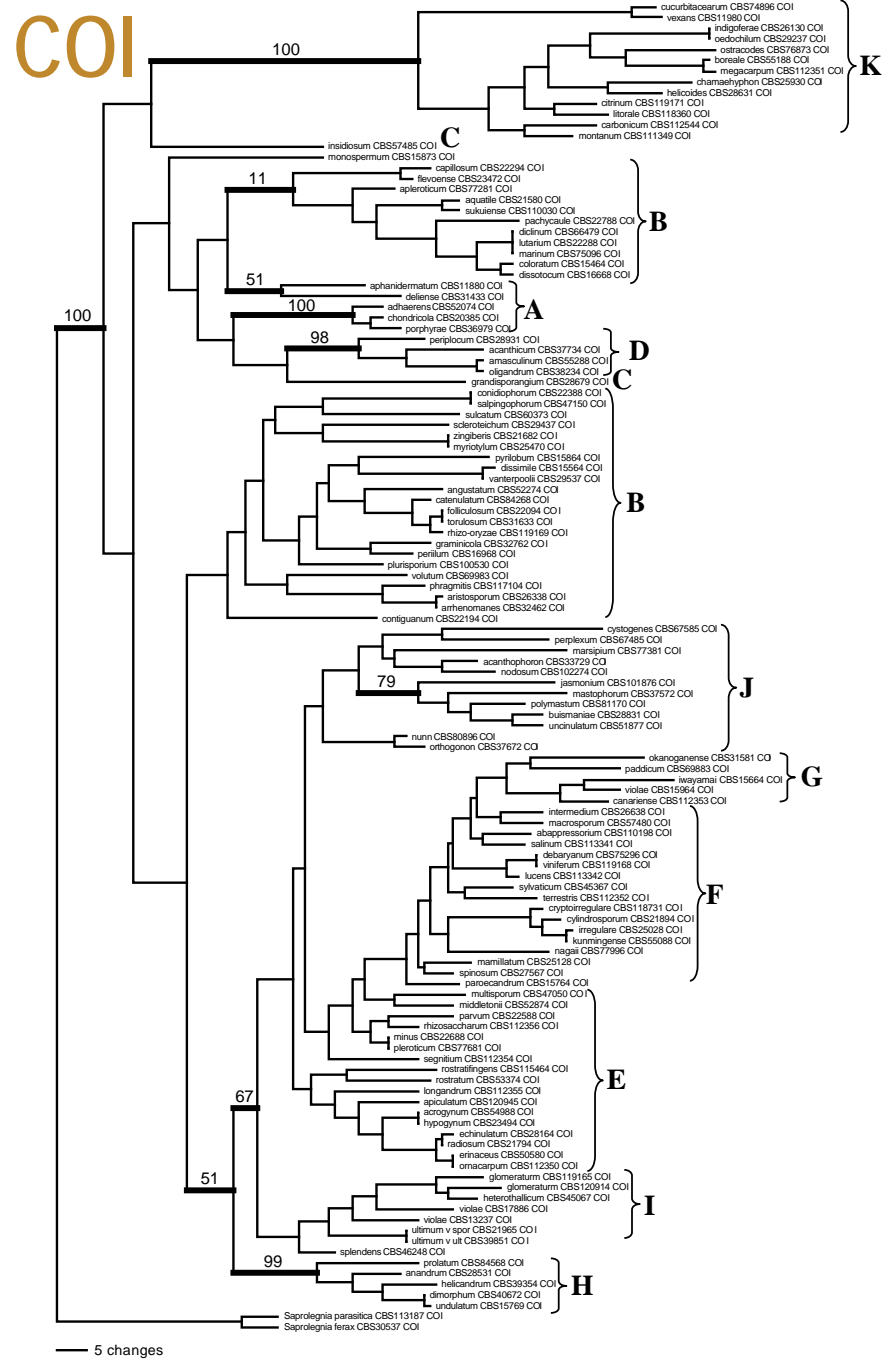
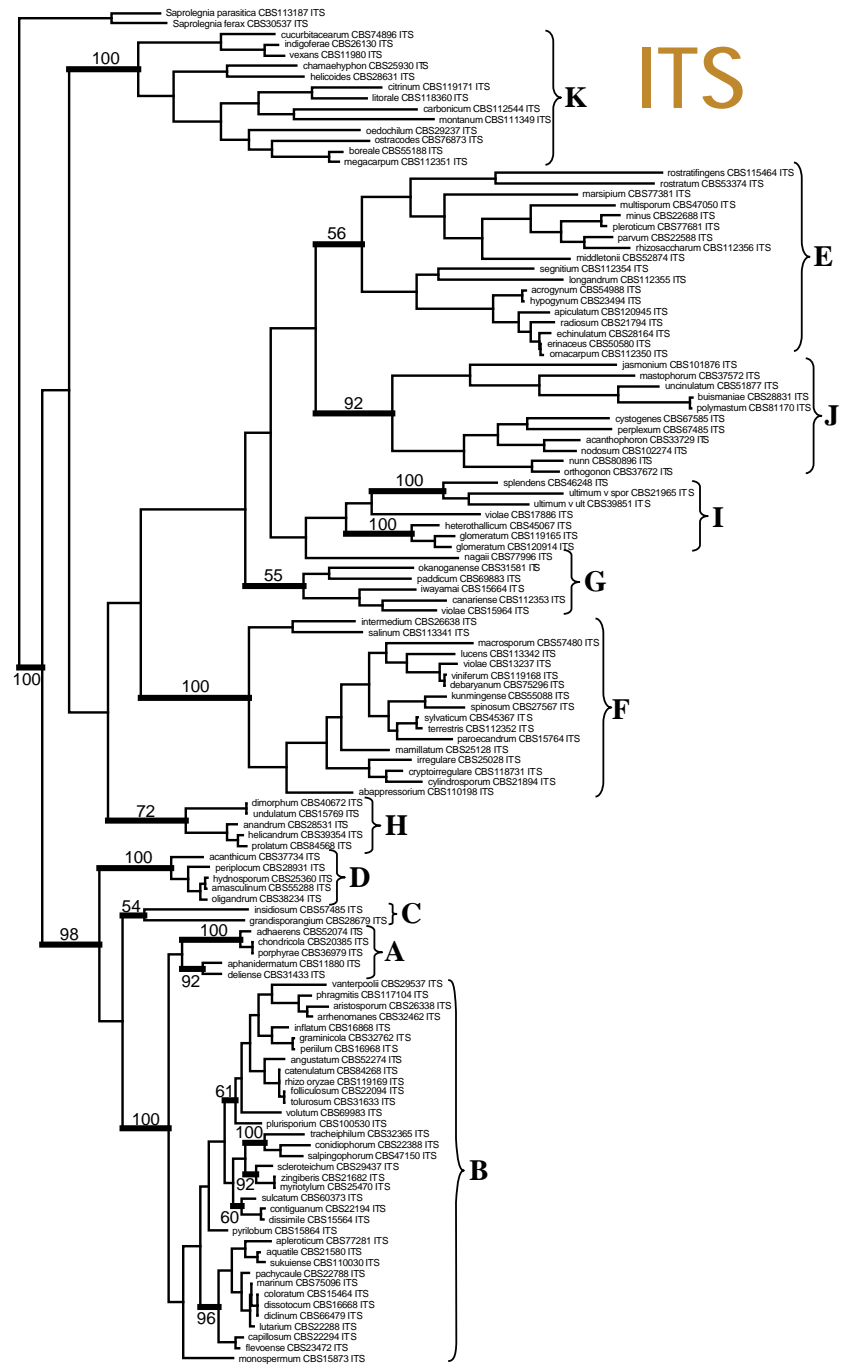
Interspecific divergence for various markers and clades

Interspecific Divergence

Marker	Average Length	Clades A-D			Clades E-J			Clade K		
		Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
LSU	1452	3.8%	0.0%	8.1%	6.0%	0.0%	9.9%	5.8%	0.0%	9.3%
ITS	825	11.4%	0.0%	22.3%	24.8%	0.0%	36.8%	21.0%	1.9%	27.3%
COI	679	7.7%	0.0%	11.5%	6.0%	0.0%	11.2%	6.2%	0.0%	9.9%

Mean Interspecific Divergence per Clade

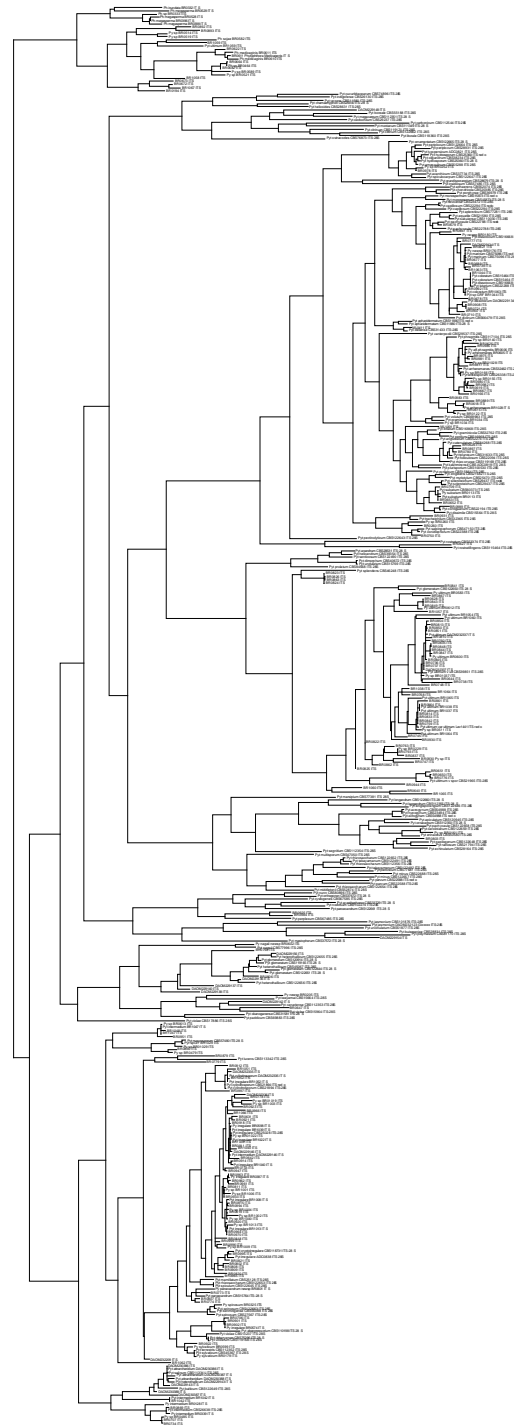
Marker	A	B	D
LSU	2.3%	2.2%	0.4%
ITS	8.7%	8.2%	2.8%
COI	6.2%	6.9%	3.4%



50 changes

5 changes

ITS
444 *Pythium* strains
CBS and DAOM



COI 670 *Pythium*
strains
CBS and DAOM



P. ultimum

5.3%

Intraspecific
("intra complex")
variation based on
the same strains
sequenced for both
COI and ITS

1.4%

ITS - 444 *Pythium* strains

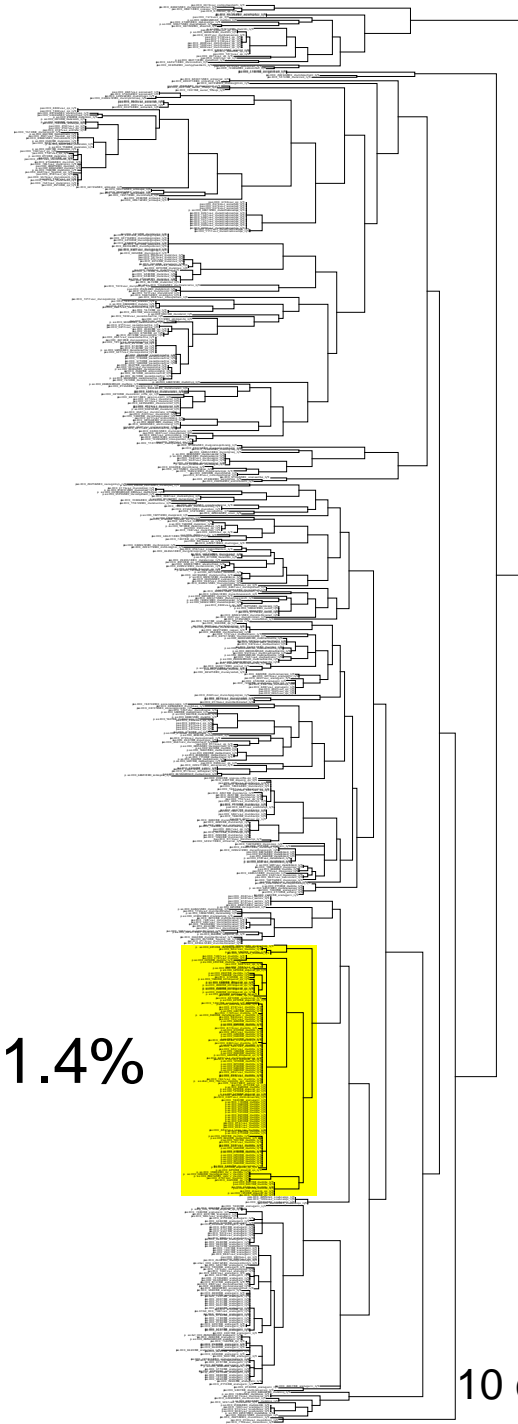
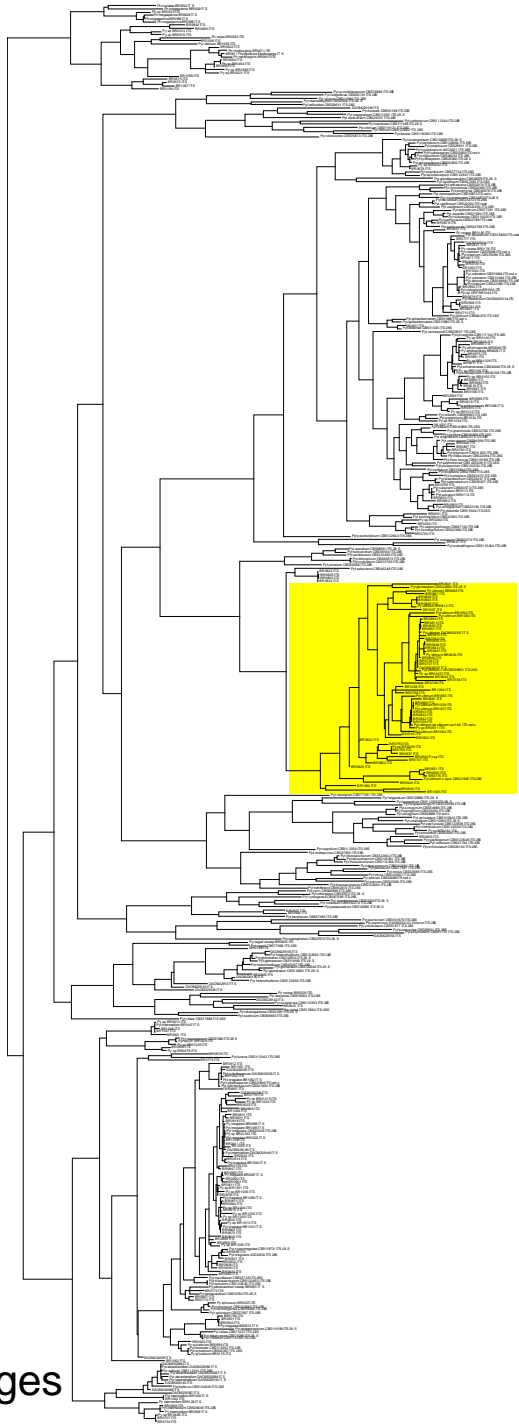
COI - 670 *Pythium* strains

10 changes

10 changes

10 changes

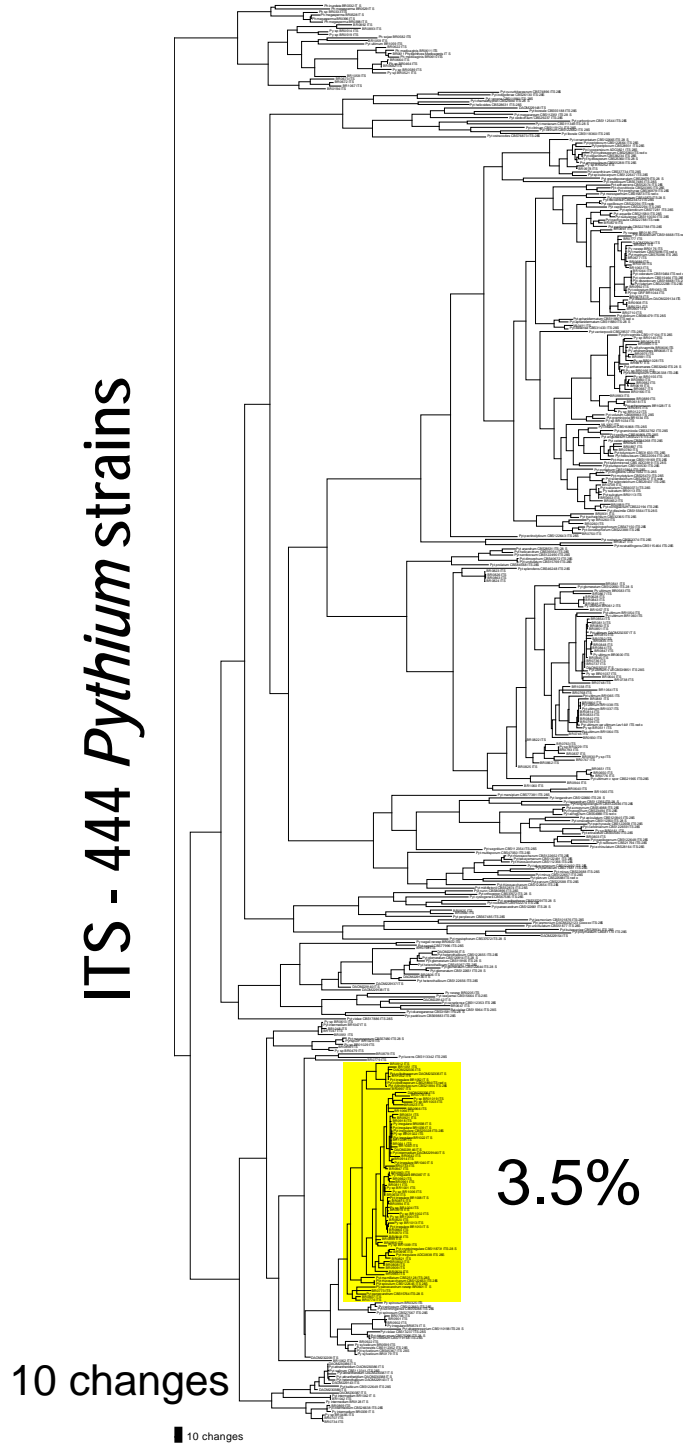
sequence 2



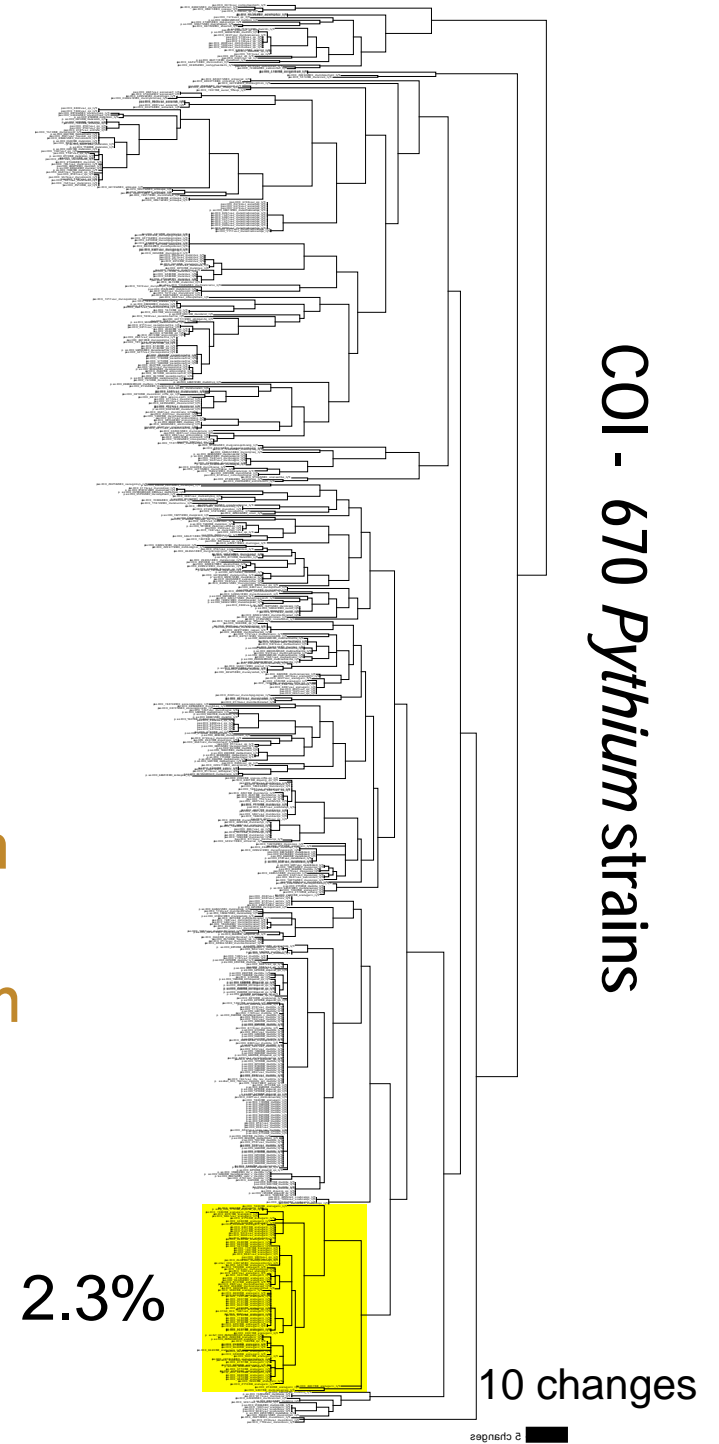
P. irregulare

Intraspecific
("intra complex")
variation based on
the same strains
sequenced for both
COI and ITS


ITS - 444 *Pythium* strains



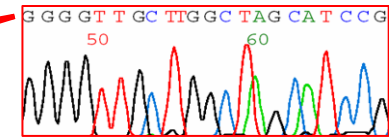
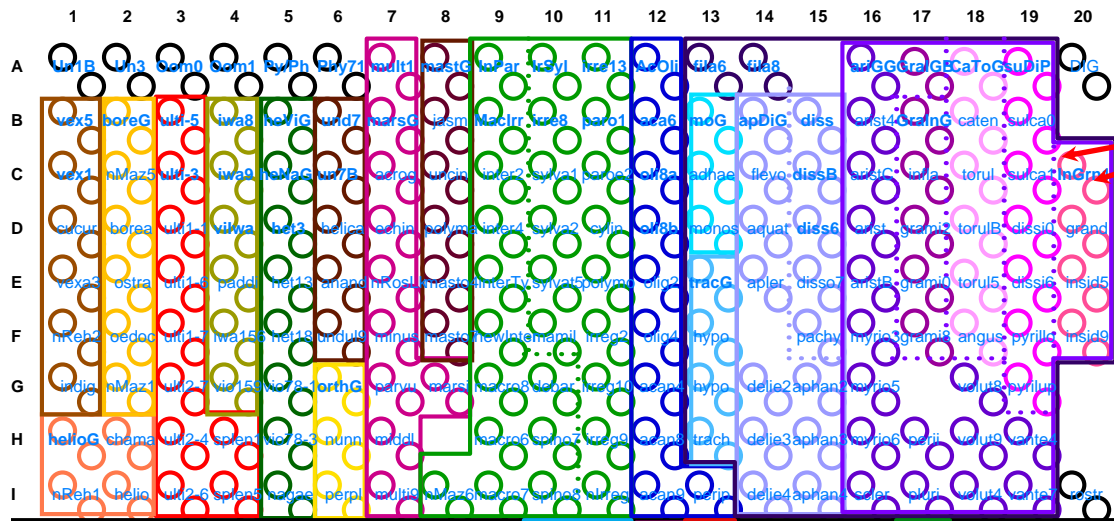
COI - 670 *Pythium* strains



There are also analogies between functional genomics and molecular diagnostics using phylogenetic databases

- Identify Up and Down regulated genes.
some techniques:
EST's
and arrays
 - Quantify most important genes affected
RT-qPCR
- 
- Identify « Up and Down » regulated species.
some techniques:
PCR/cloning/sequencing
and arrays
 - Quantify most important species in pathosystem
(RT)-qPCR

Oligo array for *Pythium* species



Each pair of dots is a «signature» DNA fragment for a species

Pythium species array

Detection with DNA arrays

extract DNA from sample



PCR: amplify-label barcode using oomycete-specific primers



Hybridization: mix PCR product and a DNA Array



read results

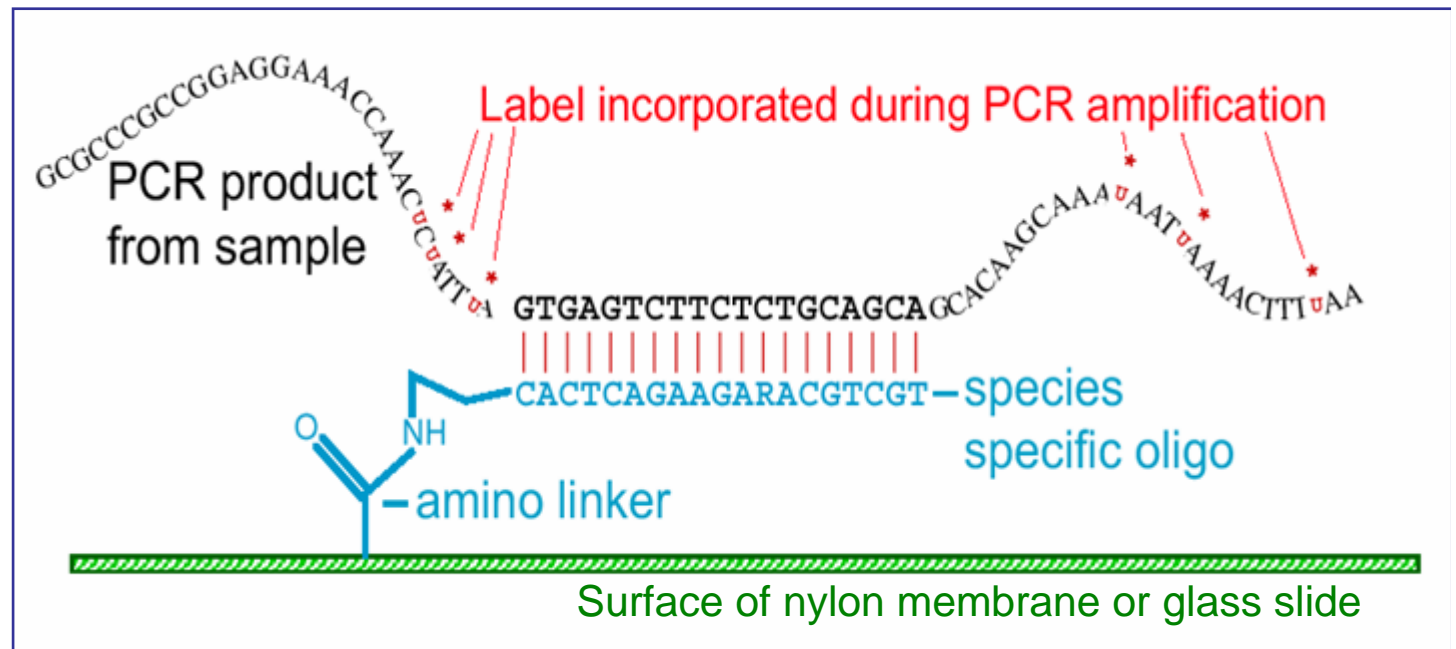


TABLE 1—Continued

Location	Code	Origin(s) of oligonucleotide	Sequence
C16	ari192	<i>aristosporium = arrhenomanes</i>	AGTTAAITCTGTACGCGTGGT
D16	ari193	<i>aristosporium = arrhenomanes</i>	GTTAAITCTGTACGCGTGG
E16	ari194	<i>aristosporium = arrhenomanes</i>	GTTAAITCTGTACGCGTGGT
F16	myr195	<i>nyrtioyilum = zingiberis</i>	GCTCTGCCGAGTGGG
G16	myr196	<i>nyrtioyilum = zingiberis</i>	CTGCTGTTATGGCGGAC
H16	myr197	<i>nyrtioyilum = zingiberis</i>	TGCTGTTATGGCGGACT
I16	scder679	<i>sclerotichium</i>	GTGTAGTAGAACTTTGCTGCTC
A17	Gln210	<i>graminicola, inflatum</i>	CGATGTACTTTTCAAACCCA
B17	Gln211	<i>graminicola, inflatum</i>	CGATGTACTTTTCAAACCCATT
C17	infla	<i>inflatum</i>	GGCGCATGTATGTGTGCTG
D17	gra213	<i>graminicola</i>	TCCACAGACTAATCCAAAT ^b
E17	gra214	<i>graminicola</i>	CTCTCGAGGGTAAAGGAGG
F17	gra215	<i>graminicola</i>	GGCTGCATGTATGTGTAGTC
G17		NS	
H17	perii	New species	GGTGAGGCCGTCAGGTTCT
I17	pluri	<i>plurisporium</i>	CATTTGTTTGGTTCGCGGA
A18	CaTo74	<i>catenulatum, tonulosum</i>	TACATGCACTCTACCTTCGTT ^b
B18	cat222	<i>catenulatum</i>	GGTTTCTGCCGATGACT
C18	tor223	<i>tonulosum</i>	GGTTTTGCCGATGACTT
D18	tor224	<i>tonulosum</i>	GGTTTTGCCGATGACTTT
E18	tor225	<i>tonulosum</i>	CTCTTGAGCCGCTACT
F18	angus	<i>angustatum</i>	CATGTATGTGCGGCTTTGC
G18	vol201	<i>volutum</i>	AATGTAGTTTATCTGTATGCG
H18	vol202	<i>volutum</i>	GTTTATCTGTATGCGCG
I18	vol203	<i>volutum</i>	GTGTTGAGAGAAGTGCTGAC
A19	SuD231	<i>sulcatum-dissimile-pyriobum</i>	GTGGGCCGCTTTATTTGTTG
B19	sul232	<i>sulcatum</i>	AACCGTAATAATCATGTTTTGT
C19	sul233	<i>sulcatum</i>	ACCGTAATAATCATGTTTTGT
D19	dissi234	<i>dissimile</i>	TATTGTGCTTGGCCGATG
E19	dissi235	<i>dissimile</i>	GCTTTATTGTGGCTTGCC
F19	pyri236	<i>pyriobum</i>	GCAGCAACCTCTACTACAC ^b
G19	pyri237	<i>pyriobum</i>	GTGTAGTAGGAGGTTGCTGCT
H19	van204	<i>vanterpoolii</i>	GTGTGACTGTGAACGCATTG
I19	van205	<i>vanterpoolii</i>	AAGGTGGATAGTGGCGTA
A20		SD ^a	
B20		NS	
C20	InGr241	<i>insidiosum, grandisporangium</i>	GCGTTCGAGCATYACACTT ^b
D20	grand	<i>grandisporangium</i>	GTGCTTTGCTGCTGCTGAG
E20	Insi243	<i>insidiosum</i>	CGTTGATCTCTTGTGTCTTA
F20	Insi244	<i>insidiosum</i>	GGCTTGAGGCTGAACGAAG
G20		NS ^b	
H20		NS	
I20	rostr251	<i>rostratum</i>	GAGCAGAGGTGAAGTGTCTC

^a Codes are the first few letters of a species or group from which the oligonucleotides were designed. Oligonucleotides were designed from the positive-sense strand of the ITS sequences, including the 5.8S rRNA gene, except for those indicated.
^b Oligonucleotides designed from the complementary strand of the ITS sequences.



James T. Tambong, A.W. de Cock, N.A. Tinker, and C.A. Lévesque. 2006. Oligonucleotide array for identification and detection of *Pythium* species. Applied & Environmental Microbiology 72:2691-2706

tted on the DNA arrays^a

Sequence	Sequence
TTCTTAACGGAAACAAGCG TGATGTGTGTTGTGGGCG	TTTTGCTGCGTTCATCCG ^b CWAGASATCCRYGYTGAAS GTGTGGTAATGATCCTCCG ^b CGAGCTAGACATCCACTG ^b CATCCACTGCTAAAGTTG ^b AATCCTGCAATTCGCA ^b GTGACCTTTGGCGATGG TTGATTGTGCTGGCGG GAGCATGTTTGGGCTTC GCGTTTTGAGTGTGTGTT GAATTTGTGTTTTGATACCGTC GAGTGTGCTTCCGCAATT ATGTCACAACCGGTTACG ^b CGAGGAAGGCGAGCTATCT AAACAATTCACGTGGAAAG ^b GCCGTTGCTTGTCTTTTGT AGGGCGTTTATTGTGCTGT CCCTTTTTTTTAAACATGAA GCGTGTGCTGCTTTGTGA CCCCCTTTTTTTTATTTTGT CGGGGAGGATGAGCTATC GTGCGTGTCTCTGTTTTG ACCGAAGTCGCAAAA ^b CGCTAGACTTGCTTACAGTT ^b TGCAAGTATGATGGACTAGCT GTGTTTTCTATTTTTGGG CATTTTGGACACTGGAAC CGCAAAATGCTCTATAAC ^b AGATGGAAAATGTGCAGATG GGTGTTTTCATTTTGGAC GTGAAGTCTTACTTACTTTTGC TGAAGTCTTACTTACTTTTGC TGCTGATGTATGCGGG CGTGGTGGCTGTTTATCT GGTGGCGTCTGTCTTCT GTTGAGTGTGTGCTTTTGC GGTGGTCTCGTAATGTAATT GATCTGGTGTTTTGGGATAC CCAGTTCAGCACACAACC ^b GGCGAAAACAGATCCACA ^b ACTGTCAAACCTGTTCTGTG GGTGGTTTTCTTCTTTGAG GTCTCTGTTTTATATATGGG CAATGTGTGTGTCGGGAC AACCCTCAAGTAATAGATTG TACTTGCTGTGCTGCTTTT GGTGGAGTAAAATCTGG GGTCGAGTAAAATCTGGC ACCGTAGGGGTTAATGCTC TCTATTTTTTAAACCCATTCT ATCTATTTTTTAAACCCATTCT ATTCCCAAAGTCGCCG ^b TTGTGCCGTGCTGTTGTC TCTTTCAAACCCATCAATAA GGCTGATCGAAGGTG CAAAGACTTTCCTCTACA ^b TATCGCACTTATTGTGTGTG GTGCGCACTTATTGTGTGTAT GTGGCGTTAGCAAGCATTGTA CGAGAGGATATTGTGATGCA GGAGGATGTGGATGGATGG GTGCTGTATTATATCGTGGC TCTTCTTGAGATGTGTGCG GTACACCTCAAAGCAAACG ^b GCGAGCGAGGAGGAAGA TTTTCTCGATTGCTTTTTAAT CGATTGCCGTTTTAATGA GGGTGTTTTTTCATTTTTG
TGGCGTGCCTTTTGCCT CTTTCGTTCTCACAGTATAATCAGT ^b CGACTACCGGAAGGAAGAA AGGTCGAGTGTCTTTGCT GTCGAGTTGCTTTGCTCT CTGTTGGCTGATTTTGATACTG TGCTGGCTTTGTTGCTGG GAGCTTCATTGTTGGCG TGCGGGTGTCTATTTGAC CTGCTTTGCAATGAATGTG ACACACAACAAATAACGACAG ^b GTTTTTGCATCTTGTGTGTG AGGTCGTTTGTGCTGTGTG GGCTGACTTACTTTTCAA TTGGTATATTTGTTTATGCACA GCGGGTGTGCTGTGCG GCGGGTGTGATGCGGAC AAGTTGTGTGTTGTTATGTG GGTCGTGTGTGCTGTGTG GGTCGTGTGTTGCTGTG GGCTGACTTACTTTTCAA TTGTGTGTCAGTTGAGGGC TAGGTCAGCCGCGCAG ^b TGTTGATGCGCGGCT GAGTGTGTGTGTTGTCGGT CGAGTGTGTGTGTTGTCGGT TGCTATGCGGATGCTCT TCAGCAAATCCGCATC ^b CGTTTCAGCCTCAAATCT ^b GAGTCTGCGTCTATTTTGA TCTGCGCTATTTTGGATG GCTTCTGCAAGACTTG TTGCTGCAAGACTTGA TGTTCTGTGCTGCTGTTGT CTGTGCTGCTGCTGTCAAAA TGTGCCCTGCTTSTTGA GCCTAACATACCGCAA ^b GAACGAAGGTGAGCTGCT TGAGCTGCTGTTATGTTG TATTTTTGTATTGTGCTTGC AGAAGGCACAGAACATAATTT ^b CTGCCGATGCTTTTTCAA GCGATGCTTTATTGCGT CTGCCGATGATTTTTCAAAC GAAGACGAAGCACAGAACATA TGCAAMGTCCGGCCGAA ^b ACAATTAAGCAGSCCACT ^b CGGGAGAGTGAACGAAG TGTTCTGTGCGATCTCCTC CTGTGCTTTTTCTCCTCGG	
ACGAAAGTTTCTGGTTTTAAT CGAAAGTTTCTGGTTTTAAT	
TCTTCTCGGAGAGAGCTG TCTTCTCGGAGAGAGCTGA TGACTGGAGTTGTTTTCTGTT CGCTTAGCTTCGGTTAGA CGAAACAGAGCGTTCAAGA ^b AACGAAAGTTTATGGTTTTAAT ACGAAAGTTTATGGTTTTAAT TTGCAATTTATTTGTAACAA CAAAAACITTCGTTCTCGGA ^b GTGTGACCTCGAATGCGG	

Continued on following page

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Soil processing with *Pythium* ITS array

2700 TAMBONG ET AL.

APPL. ENVIRON. MICROBIOL.

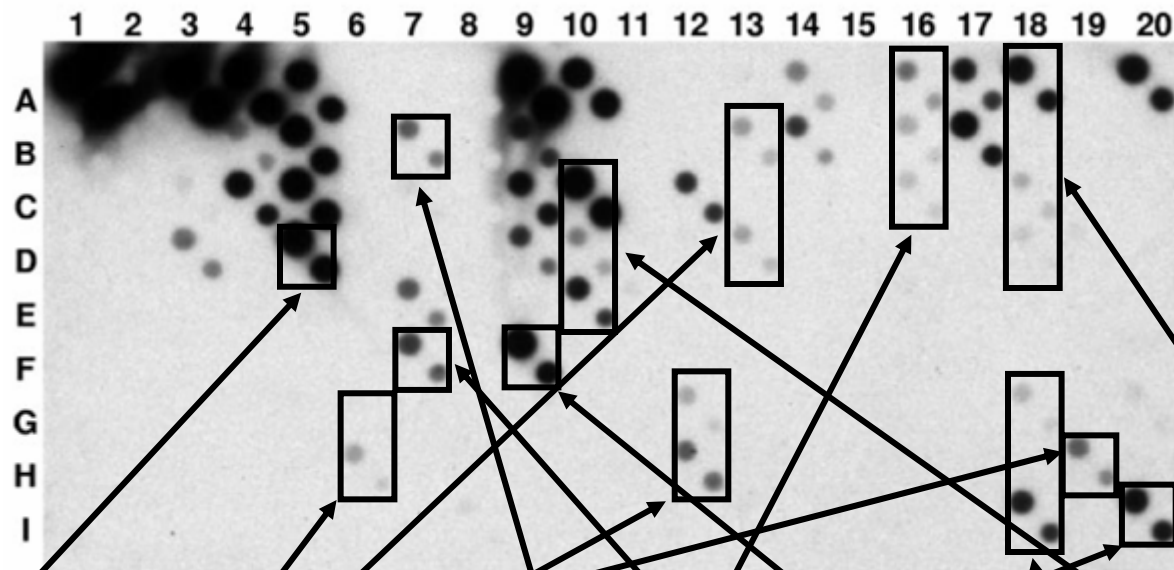
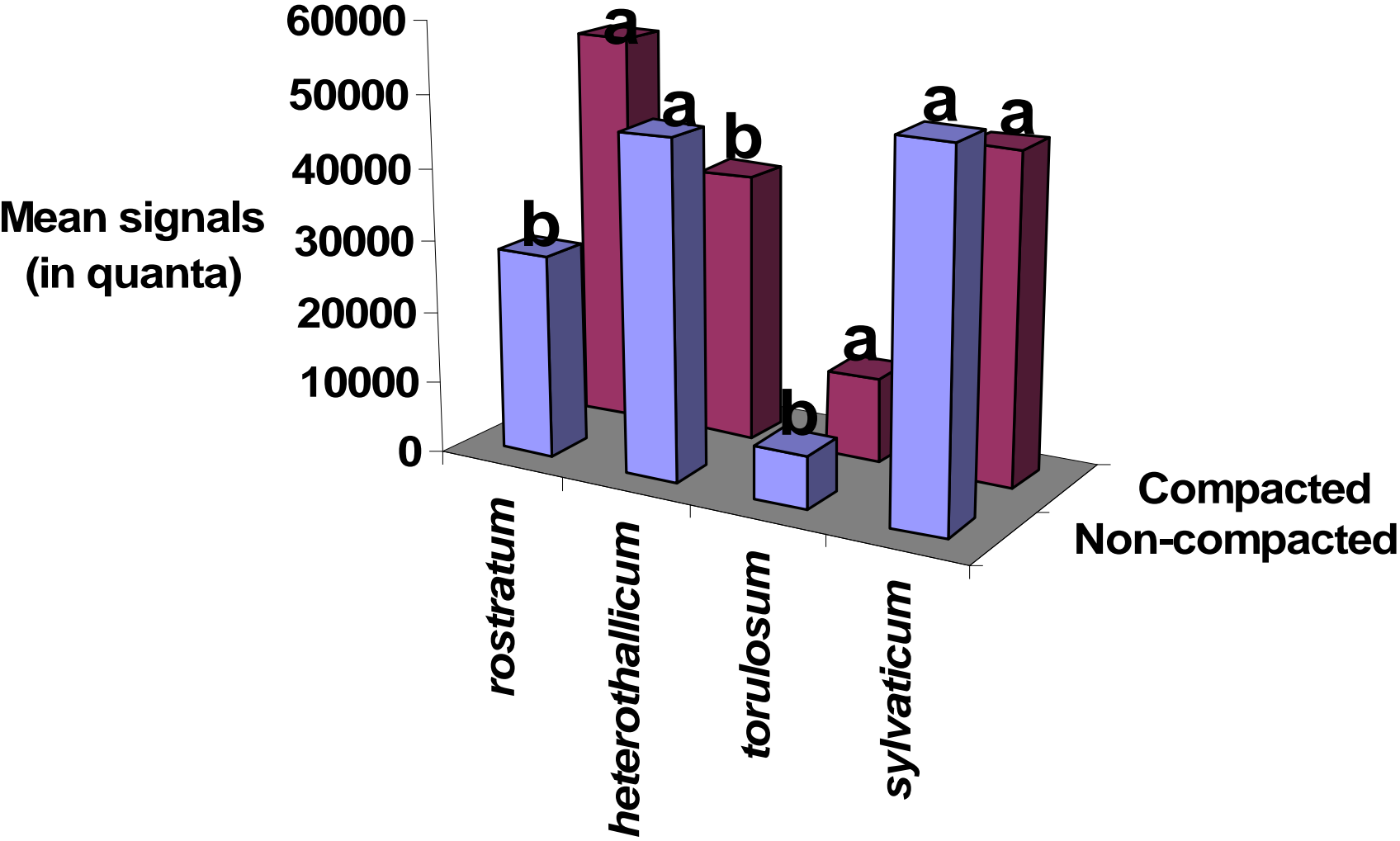


FIG. 3. Hybridization patterns of digoxigenin-labeled PCR amplicons obtained by direct processing of soil. One representative sample out of four replicates that had very similar hybridization patterns and showed the same species complex. Spots A1 to A5 are universal, family- or genus-specific oligonucleotides as described in Table 1. Chemiluminescence patterns were repeated at least twice, and similar patterns were obtained. The species detected in all soil samples were *P. acanthicum* (G12, H12), *P. arrhenomanes* (B16, C16, A16), *P. volutum* (G18, I18), *P. torulosum* (A18, C18, D18), *P. yanterpolii* (H19), *P. monospermum* (B13, D13), *P. acrogynum* (C7), *P. minus* (F7), *P. attrantheridium* (F9), *P. sylvaticum* (C10, D10, E10), *P. heterothallicum* (D5), *P. nunn* (G6, H6), and *P. rostratum* (I20).

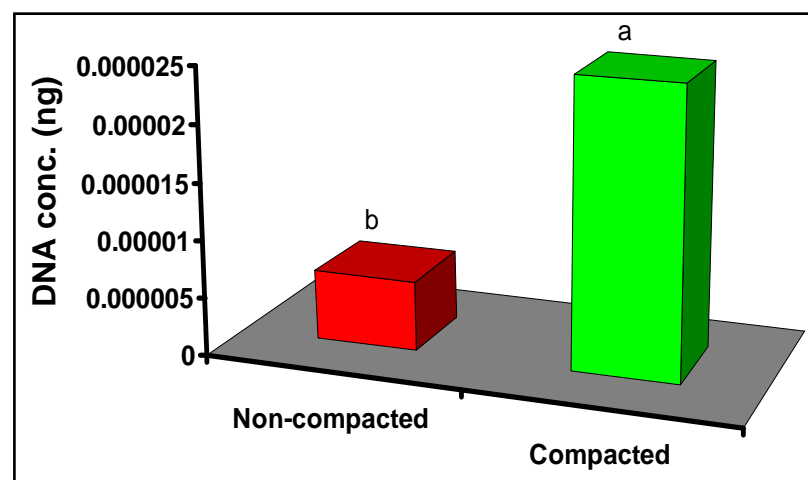
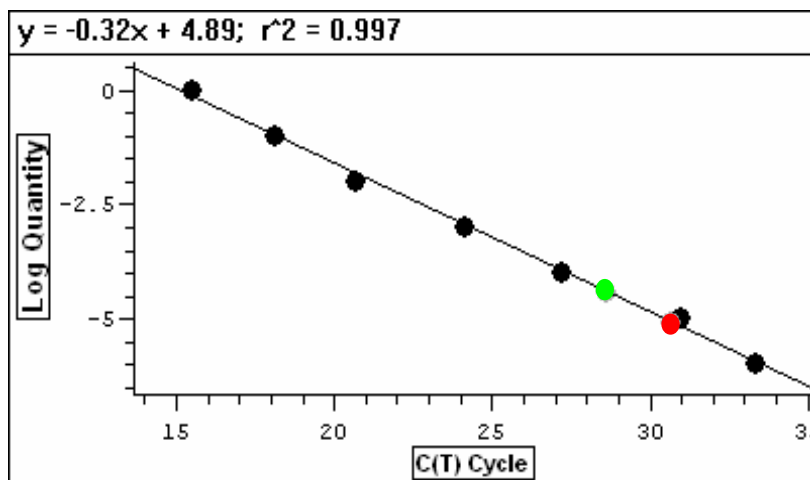
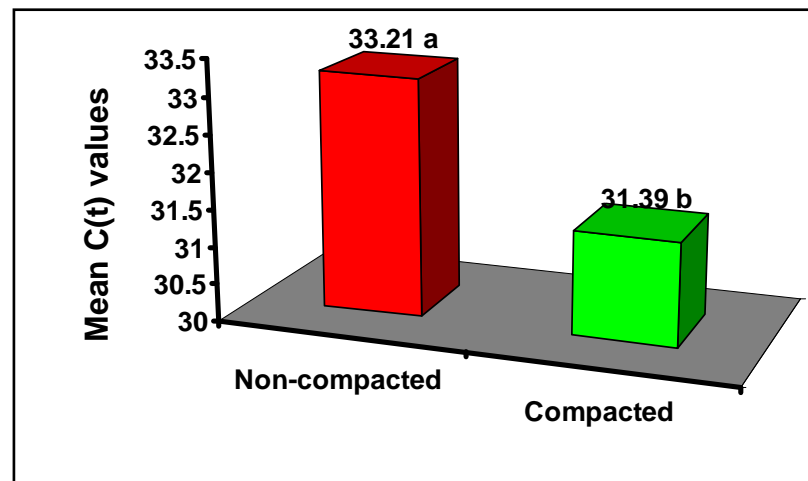
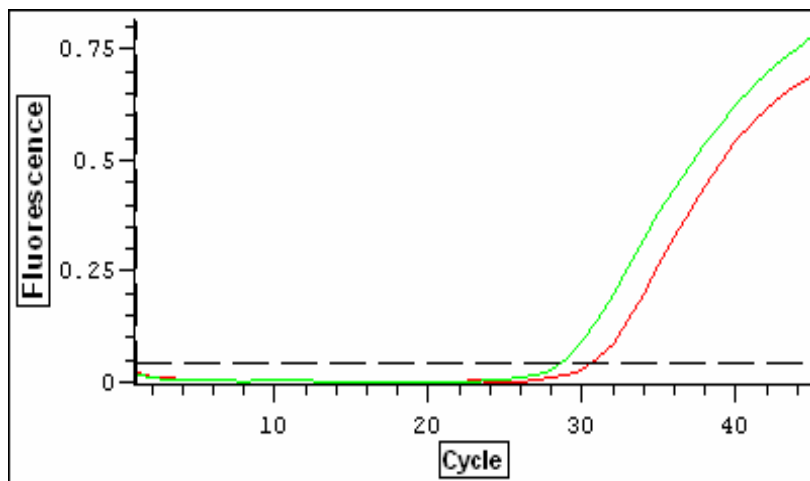
13 *Pythium* species found by array hybridization

Effect of soil compaction on populations of different *Pythium* species

(hybridization signals of species-specific oligonucleotides spotted on DNA array)

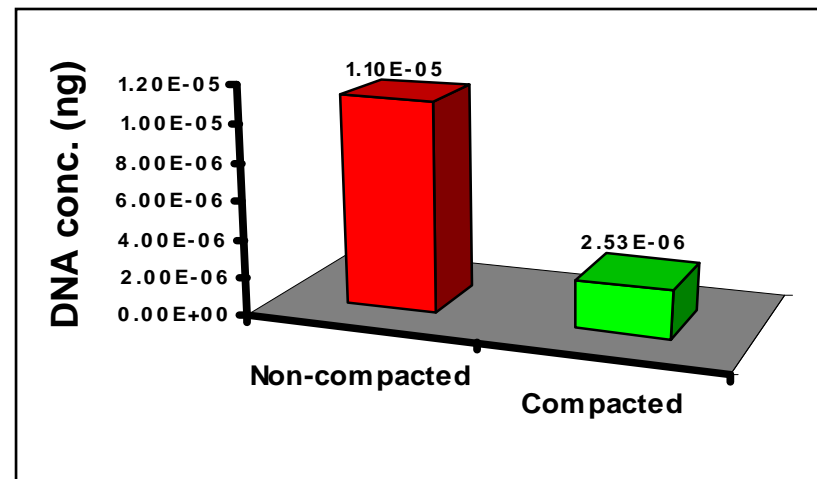
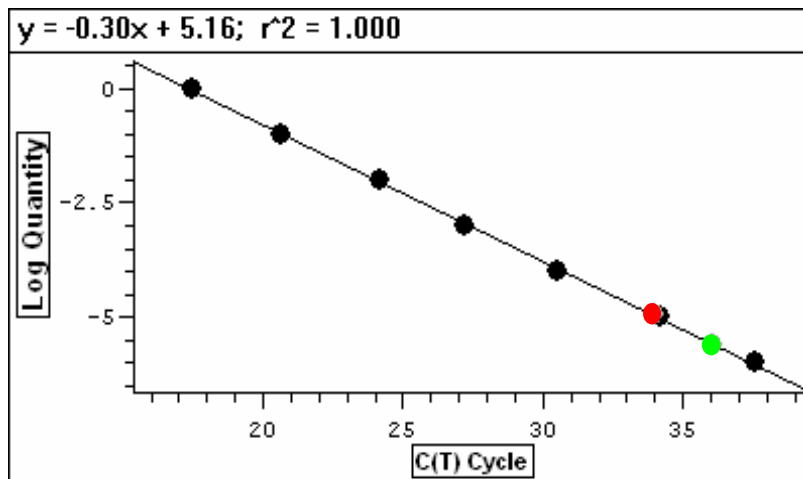
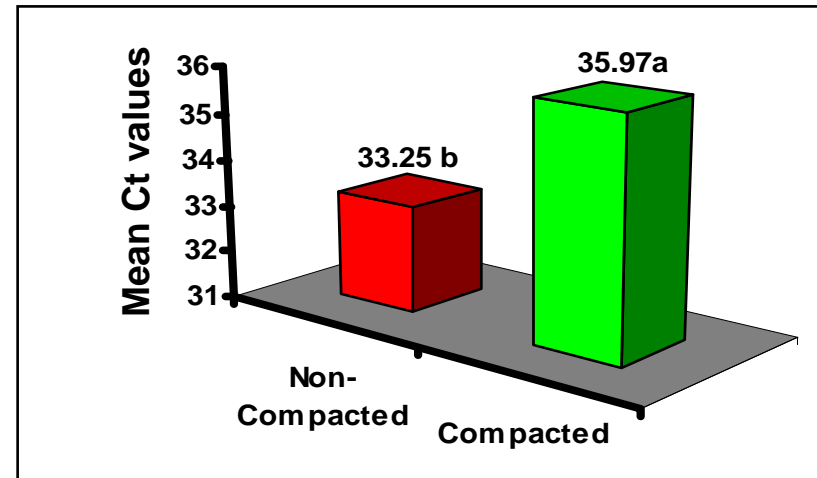
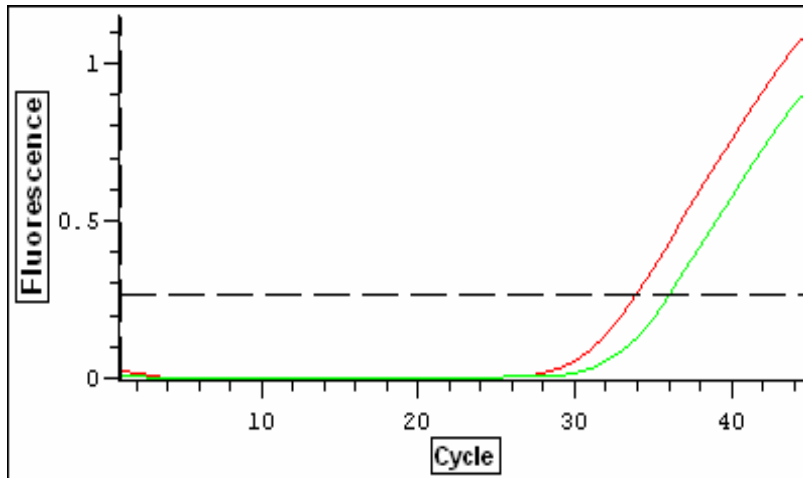


Validation of DNA array quantitative data by real-time PCR using taqman chemistry: *P. rostratum*



Standard curve based on known DNA concentration of *P. rostratum*

Validation of DNA array results for *P. heterothallicum* by Real-time PCR



Standard curve based on known DNA concentration of *P. heterothallicum*



Phylogenomics

LSU (D1-D3)
 Max. Pars. tree
 125 sequences
 96 CBS
 29 GenBank



Hyphochytriales
 Albuginales

Peronospora parasitica
 Peronosporales

Phytophthora infestans
Phytophthora capsici

Phytophthora sojae
Phytophthora ramorum

Pythiales

Pythium ultimum
 Rhipidiales
 Atkinsiellales
 Leptomitales

Saprolegniales

Haliphthorales

“Whole Genome Sequencing of *Pythium ultimum*”
funded by US National Science Foundation & USDA



Colorado State University

Marcello Zerillo Ned Tisserat Jan Leach Jillian Lang



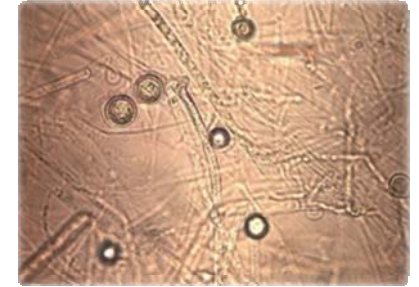
John Hamilton



**Collaboration
between AAFC and
C. Robin Buell,
Michigan State
University
formerly at
The Institute for
Genomic Research
(TIGR)**



Pythium ultimum Transcriptome and Diagnostic Marker Development



Pythium ultimum, J. Lang

- EST Library DAOM BR144 = CBS 805.95
 - Starved and nutrient rich media
 - Sequenced using Sanger (9,578 reads) and 454 technologies (90,664 reads); total = ~10Mb
 - 34,495 unique sequences in hybrid assembly
 - Sanger & 454 were complementary
 - 91% of sequences were similar to other oomycetes
- Testing markers to differentiate *Pythium* from *Phytophthora* based on available EST and genomic sequence
 - First set based on EST derived SSRs – variable results, even strain differentiation
 - Some primers amplified a few *Pythium* sp., but not all
 - Second set based on introns, exons or intergenic regions
 - Most primers are specific to *P. ultimum*

The issue of name (in)consistency

Google Hits

straminipila	1860
straminopile	121
straminipile	87
stramenopile	8010
stremenopile	1
stramenopilous	2
straminopilous	1760
straminopilous	16
stramenopilous	17

Oomycota	129,000
oomycetes	161,000
oomycete	60,800
oomycetous	2,810
peronosporomycetes	5,120
Peronosporomycota	2,240
peronosporomycete	498
peronosporomycetous	9

An identity crisis in the oomycetes?



A more coordinated approach for
assembling the oomycete tree of life is needed

“AOTOL”?

Assembling the mycologists of oomycete for the tree of life "AMOOTOL"

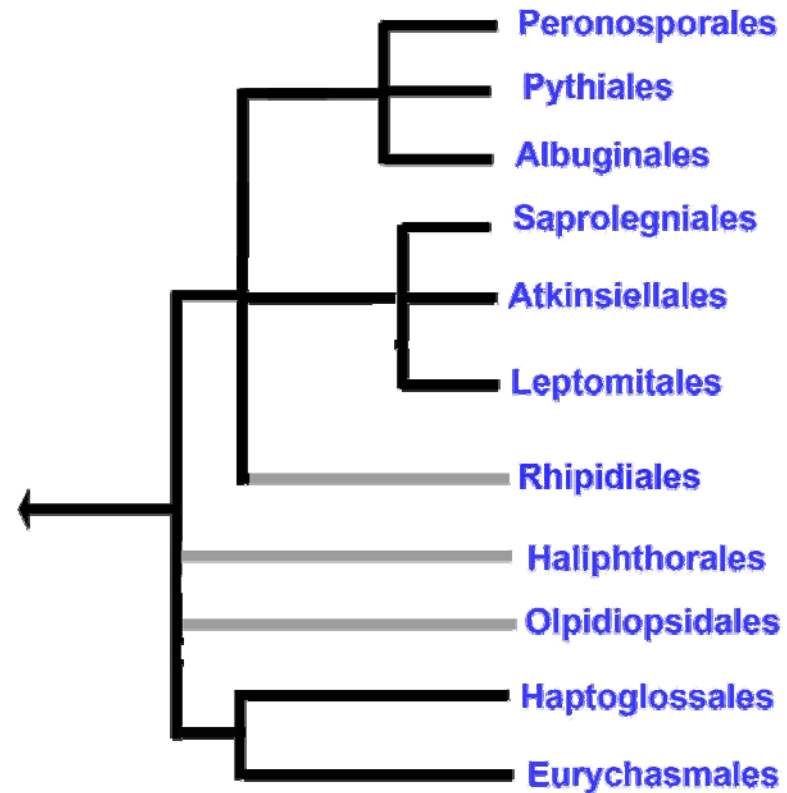


Dr Gordon Beakes

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Protists Tree of Life -
Patrick Keeling



Draft – Order Tree

— These branches may not be monophyletic

Acknowledgements

- Gordon Beakes
- The Barcode of Life Initiative