International Conference on Silvicultural Management for Forests Producers of Edible Mushrooms

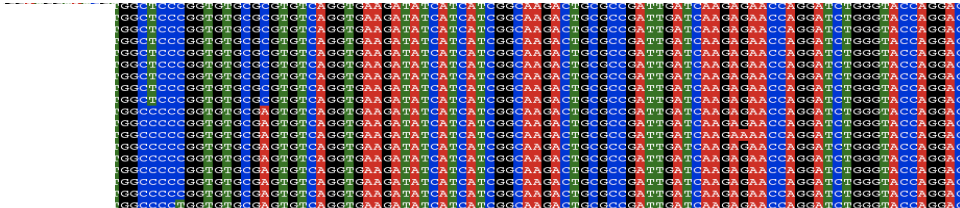
June 9-11, 2011

Sesion 3. Aplication of Molecular Techniques to the research of the Ecology and Diversity of Macromycetes

The revolution of molecular techniques in the fungi research: Explored files and future objectives

María P. Martín
maripaz@rjb.csic.es

Para ver esta película, debe disponer de QuickTime™ y de un descompresor TIFF (sin comprimir).

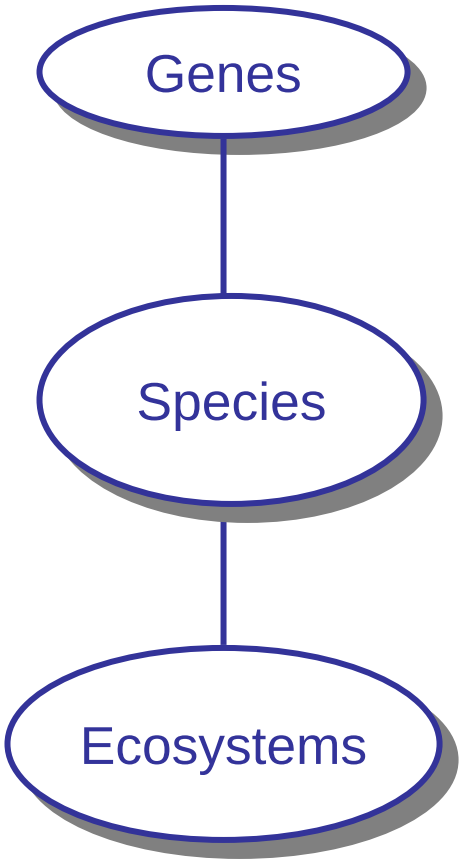


Article 2

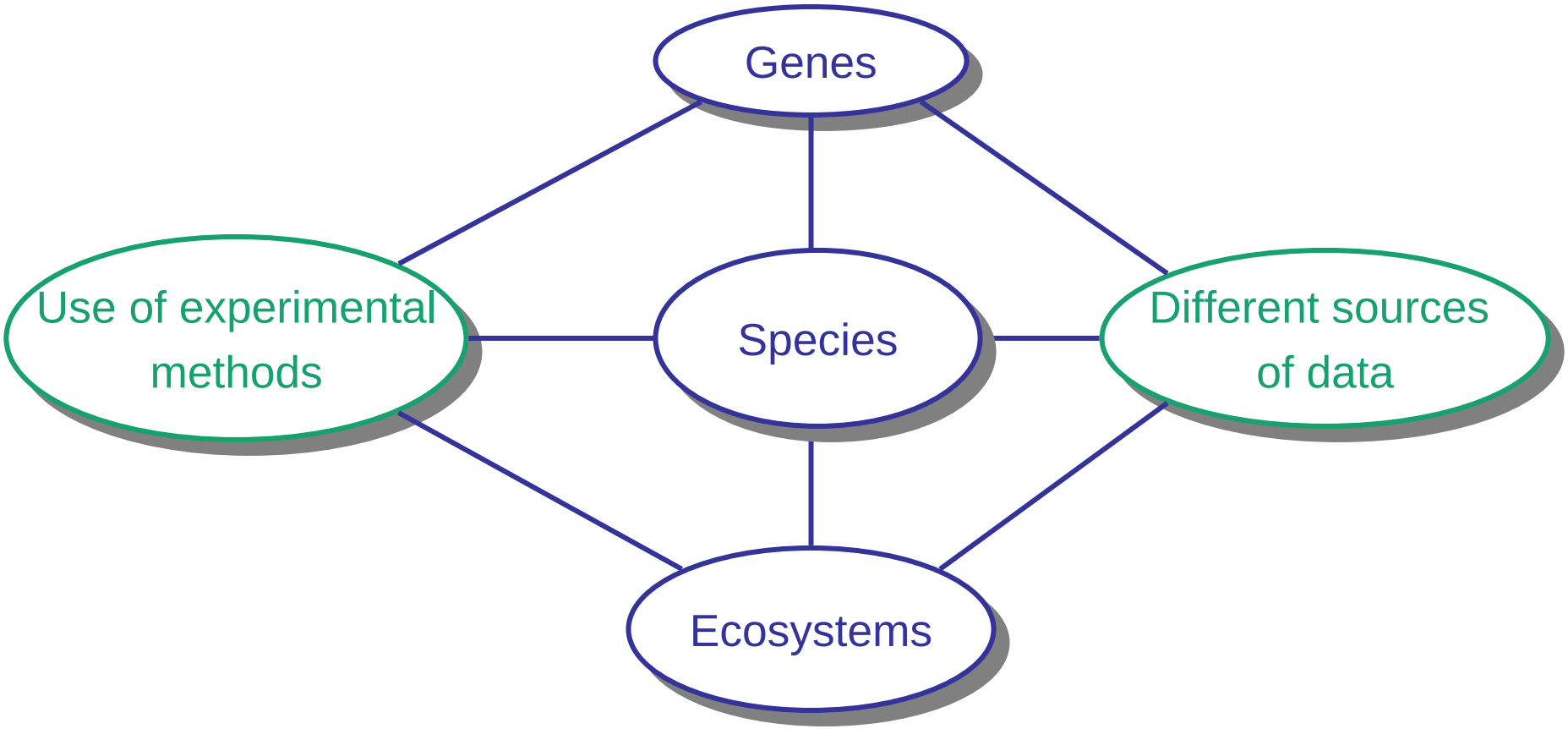
"Biological diversity means the variability among living organisms from all sources including, inter alia, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems".

Convention on Biological Diversity

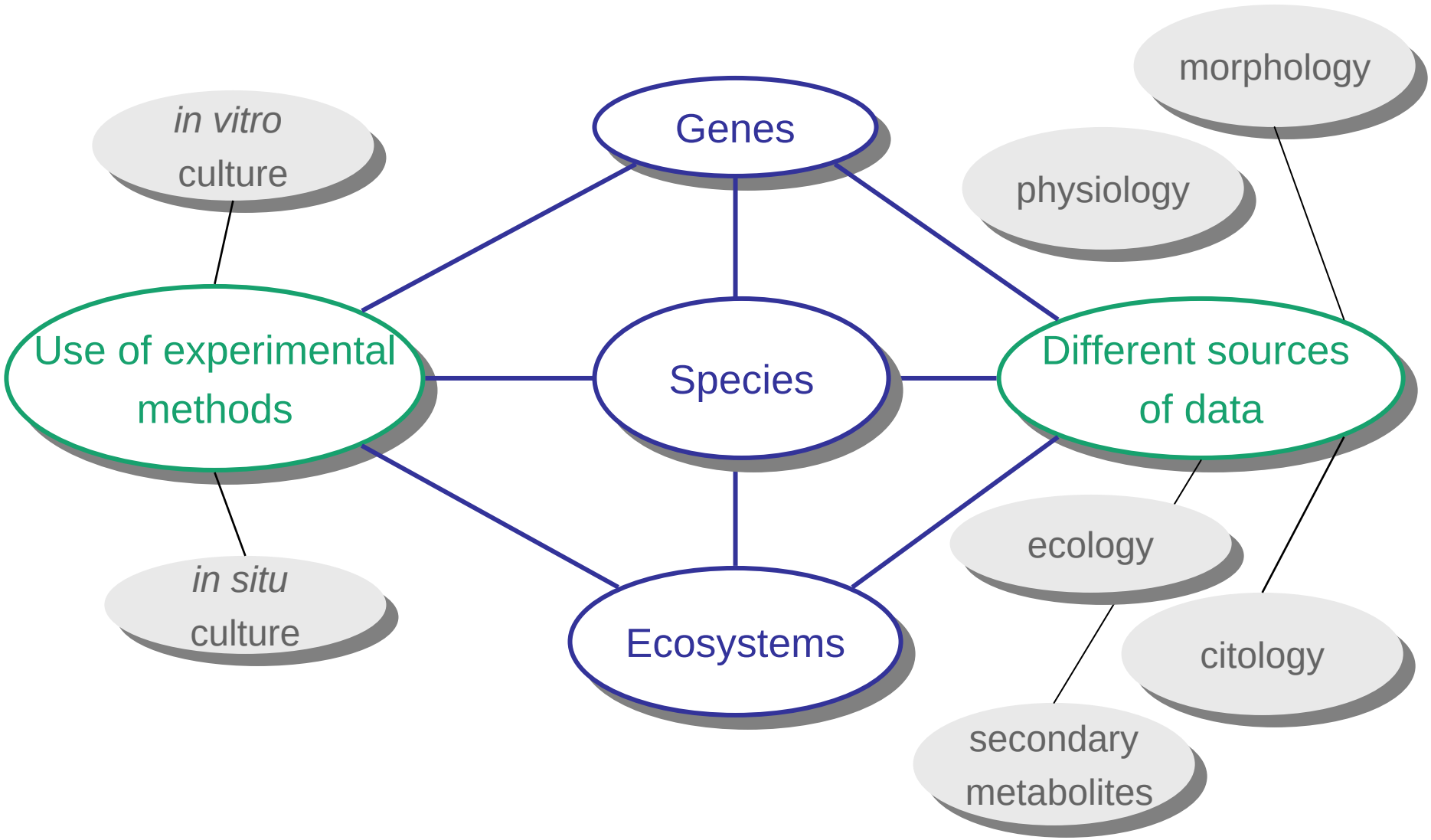
Introduction



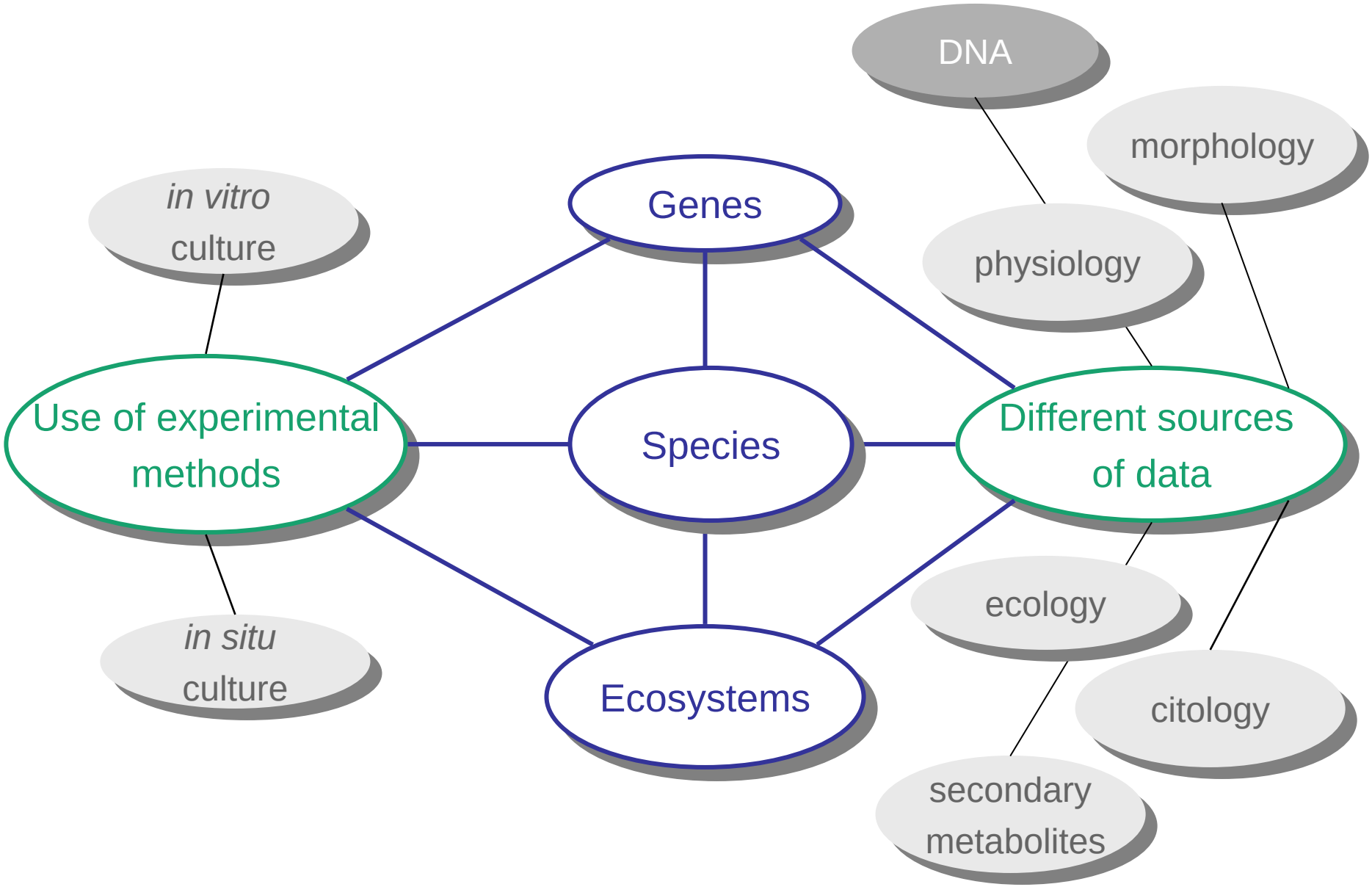
Introduction



Introduction

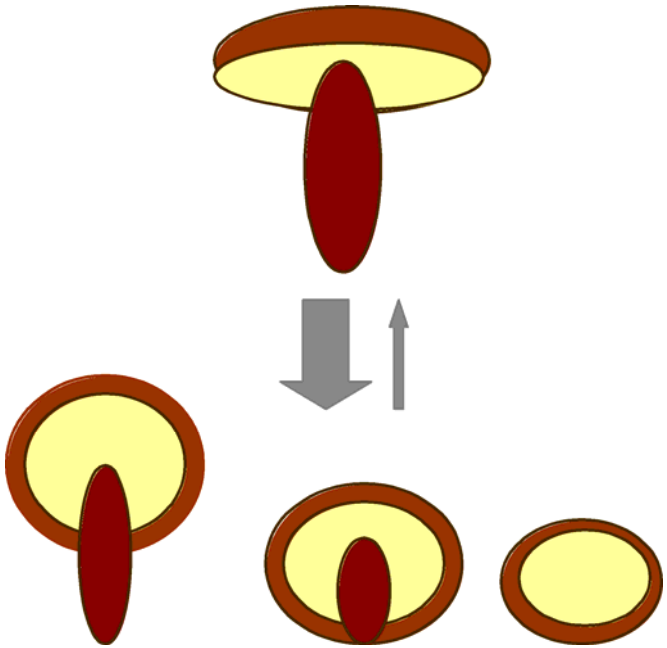


Introduction



Introduction

DNA-based methods have been in long use for identification purpose in mycology.



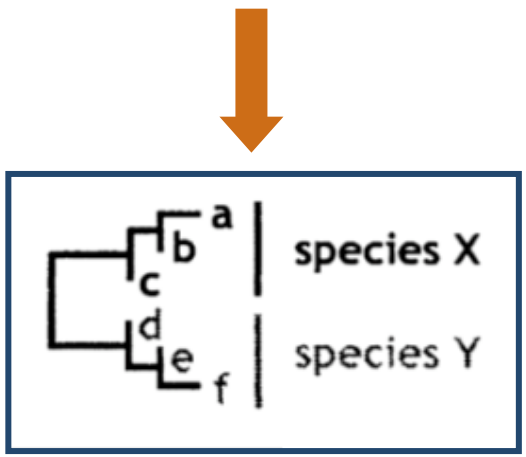
Accelerated evolution of a false-truffle from a mushroom ancestor

Nature
Volume 339, Issue 6220, 1989, Pages 140-142

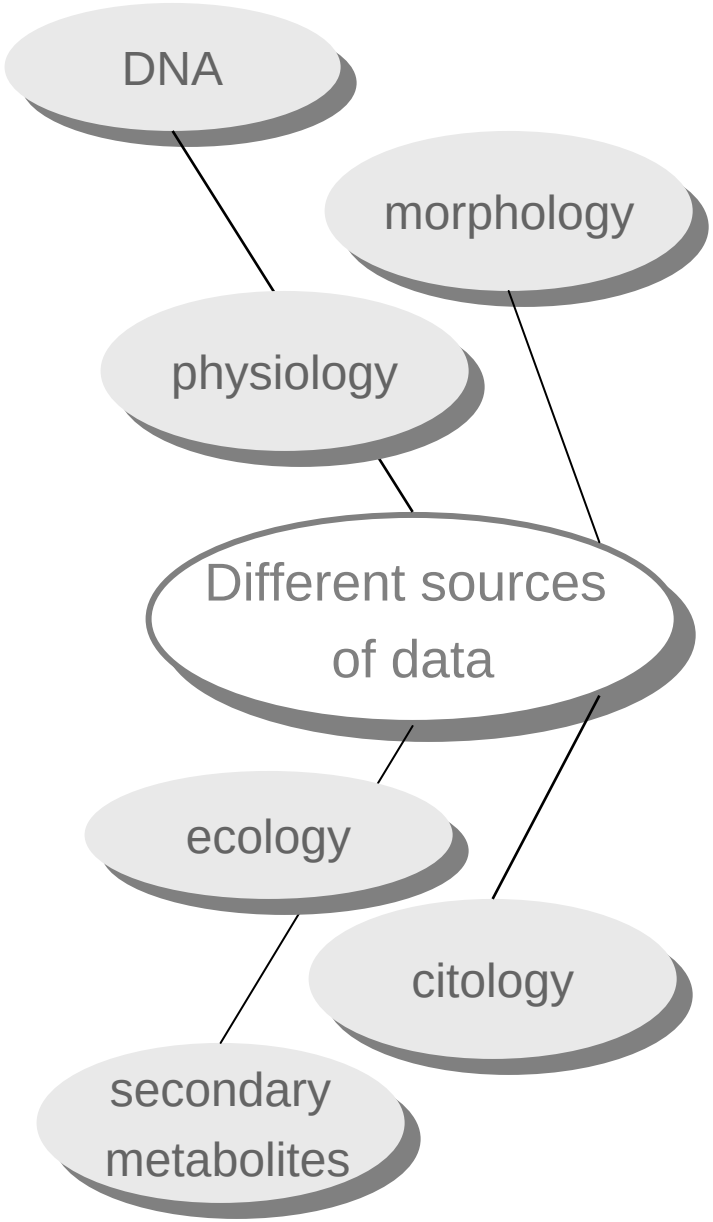
Bruns, T.D.[a], Fogel, R.[b], White, T.J.[c], Palmer, J.D.[b]
[a] Department of Botany, University of California, Berkeley, CA 94720, United States
[b] Department of Biology, University of Michigan, Ann Arbor, MI 48109-1048, United States
[c] Cetus Corporation, 1400 Fifty-third Street, Emeryville, CA 94608, United States

Introduction

Taxonomy is the identification and interpretation of natural group of organisms (taxa) based on different characters.



Phylogenetic reconstruction



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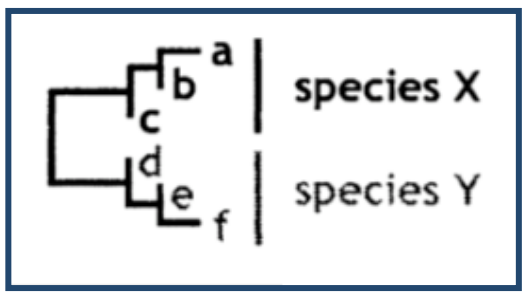
Taxonomy is the identification and interpretation of natural group of organisms (taxa) based on different characters.



Phylogenetic reconstruction

EFFECTS OF GASTEROID FRUITING BODY MORPHOLOGY ON DIVERSIFICATION RATES IN THREE INDEPENDENT CLADES OF FUNGI ESTIMATED USING BINARY STATE SPECIATION AND EXTINCTION ANALYSIS

Andrew W. Wilson,[1,2,3] Manfred Binder,[1,4] and David S. Hibbett[1,5]
[1] Department of Biology, Clark University, Worcester, Massachusetts 01610

Evolution 65-5: 1305–1322

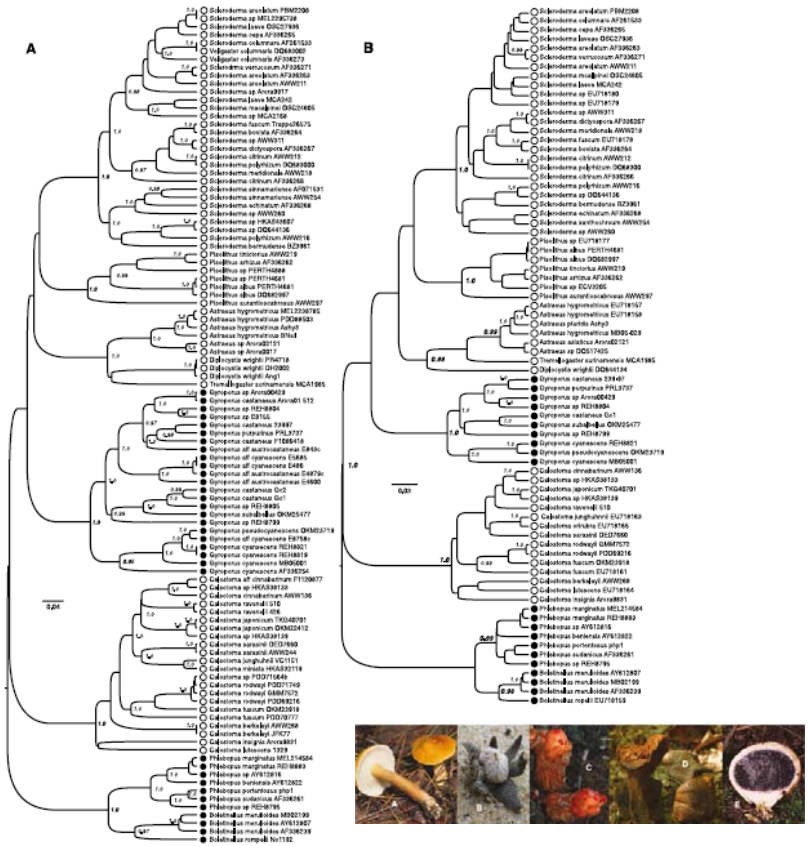


Figure 2. Sclerodermatineae consensus trees of 50 posterior BEAST trees. (A) Sclerodermatineae dataset 1. (B) Sclerodermatineae dataset 2. Closed circles represent nongasteroid forms whereas open circles represent gasteroid forms. Numbers indicate nodes with ≥0.99 posterior probability.

Introduction

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Molecular characterization of fungi (species level)

DNA isolation

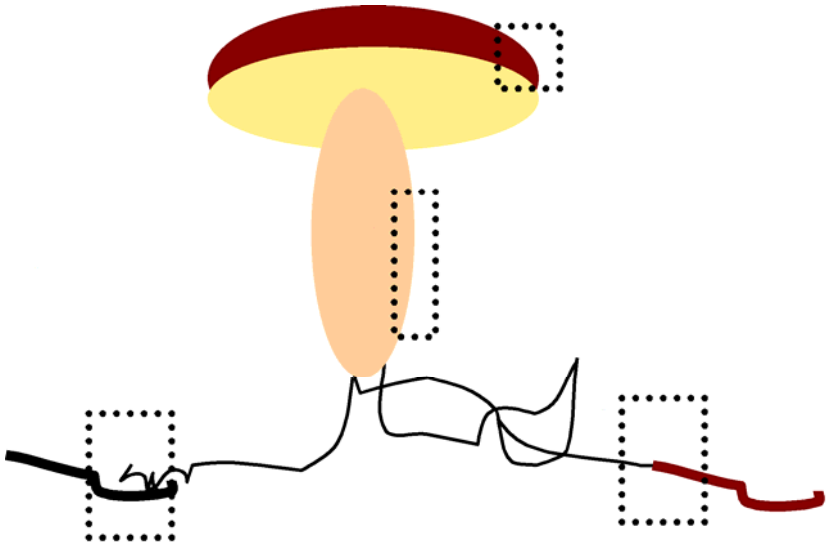
PCR

RFLP analyses

Sequencing

Introduction

The revolution of molecular techniques in the fungi research: Explored files and future objectives



Use of a short standarized genetic marker to provide DNA-based identification of fungi.

Barcoding

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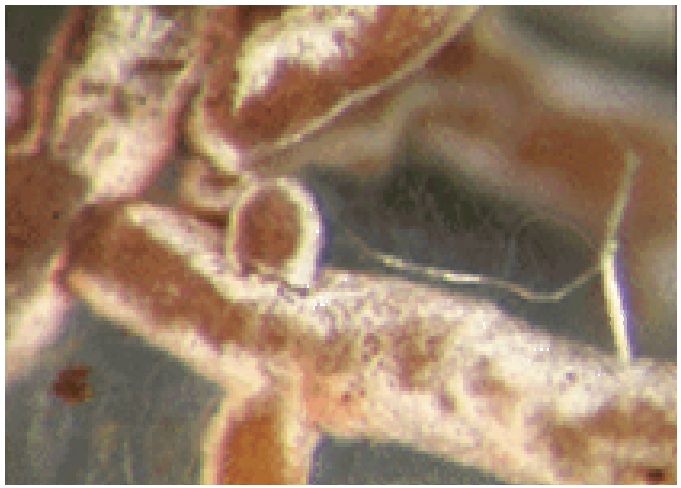
Species



Para ver esta película, debe
disponer de QuickTime™ y de
un descompresor TIFF (sin comprimir).

DNA isolation

Lee, S.B., M.G. Milgroom #

and J.W. Taylor

A rapid, high yield mini-prep method for isolation of total genomic DNA from fungi.

Fungal genetic studies require a rapid method of isolating DNA from a large number of samples for restriction enzyme analysis. Previous methods we have used are limited by relatively low yield of 50 ug DNA/0.1g lyophilized mycelium (Zolan, M.E. and P.J. Pukkila 1986. Mol. Cell. Biol. 6:195-200) or tedious gel exclusion column chromatography (Biel, S.W. and F.W. Parrish 1986. Anal. Biochem. 154:21-25). In addition, these two methods yielded no readily digestable DNA from Phytophthora cinnamomi.

The following method facilitates rapid isolation of large quantities of easily digested total, genomic DNA from several species of Phytophthora, including P. cinnamomi, and several species of Boletus, Chroogomphus vinicolor, Gomphidius glutinosus, Leccinum manzanitae, Magnaporthe grisea, Neurospora crassa, N. tetrasperma, Omphalotus olivascens, and Talaromyces flavus. Yield was increased to 200 ug DNA/0.1 g lyophilized mycelium and isolation of DNA from two to three times as many samples can be achieved using this rapid method (current record is 64 isolates in one day versus 24 using previous methods). DNA has been successfully cut with all restriction enzymes tried to date.

Solutions needed:

1. Lysis buffer: 50 mM Tris-HCl
   50 mM EDTA
   3% SDS
   1% 2-mercaptoethanol (add just before use)
2. Chloroform:phenol (1:1)
3. SEVAG (chloroform:isoamyl alcohol, 24:1)
4. 3 M NaOAc (pH 8.0)
5. Isopropanol
6. Ethanol (100%, -20°C)

Fungal Genet. Newsl. 35: 23-24, 1988

DNA isolation

1. Cell disruption, cell lysis

Para ver esta película, debe disponer de QuickTime™ y de un descompresor TIFF (sin comprimir).

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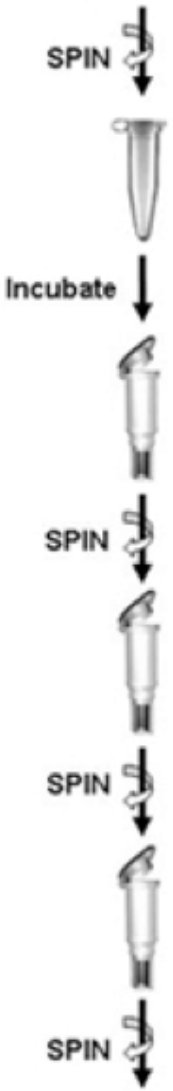

2. DNA purification

3. DNA precipitation

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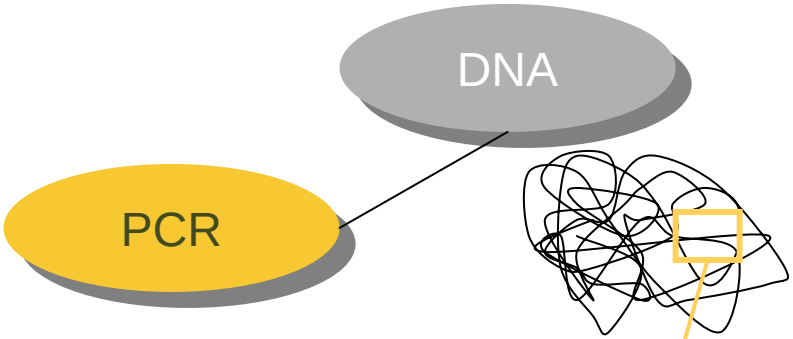
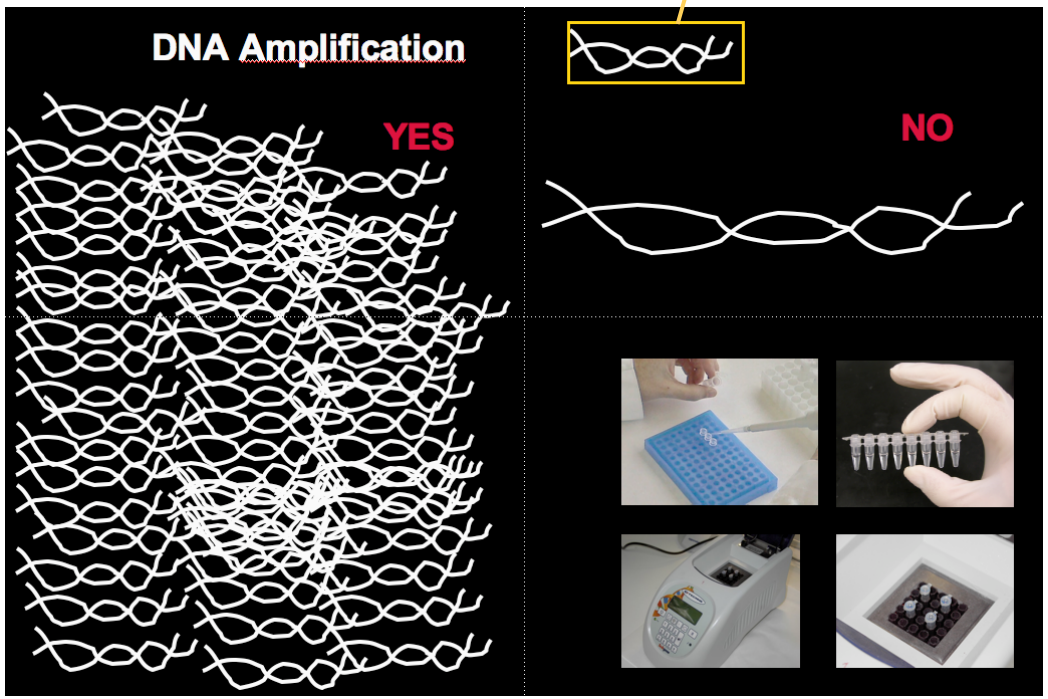
Polymerase Chain Reaction, PCR

DNA expert Kary Mullis — 1993 winner of the Nobel Prize in Chemistry



Para ver esta película, debe disponer de QuickTime™ y de un descompresor TIFF (sin comprimir).



Polymerase Chain Reaction, PCR

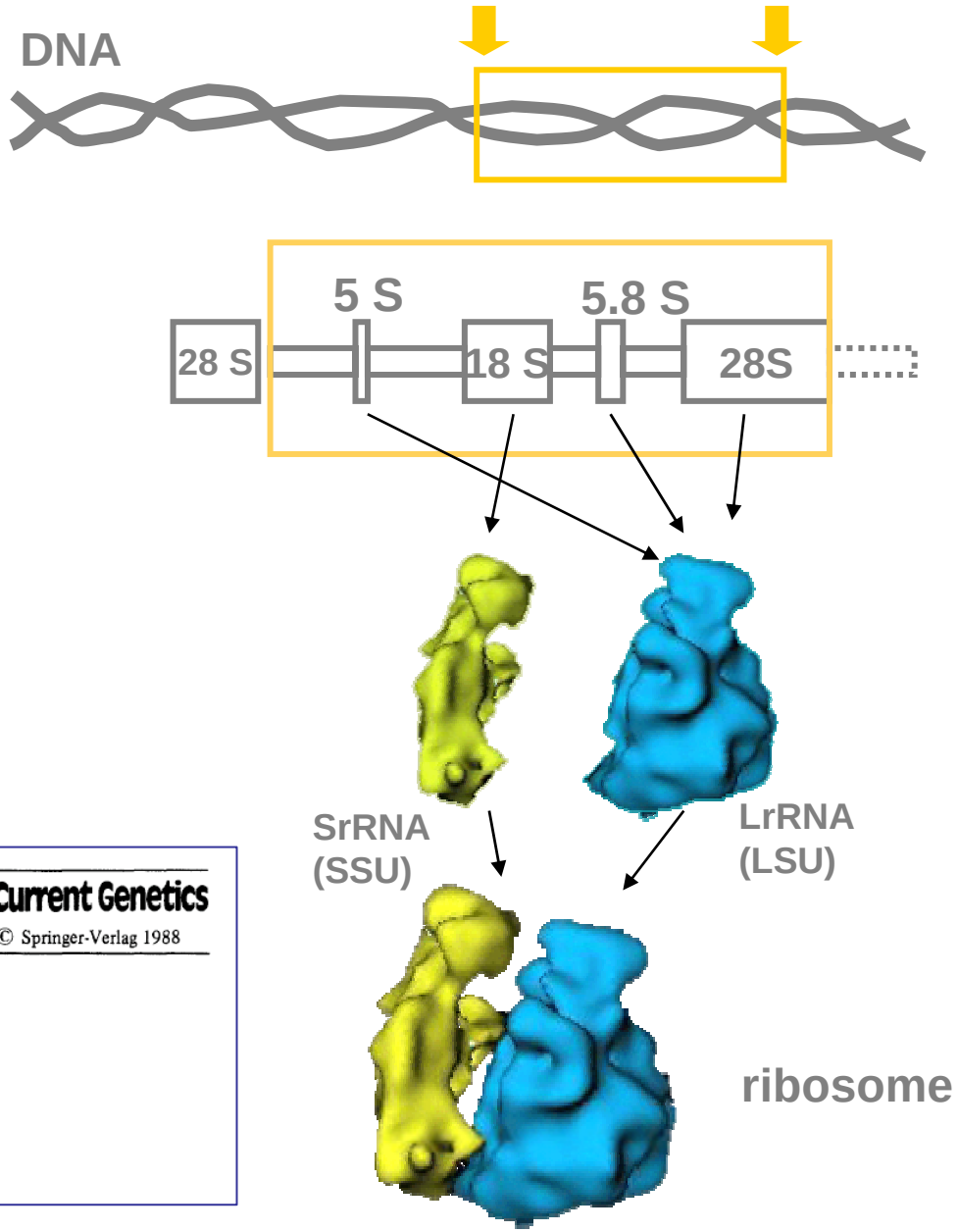
target region

Scopus search:
"PCR" and "fungus"
1989-2011

6.735 articles

Scopus search:
"rDNA" and "fungus"
1989-2011

3.318 articles



Curr Genet (1988) 14:573–582

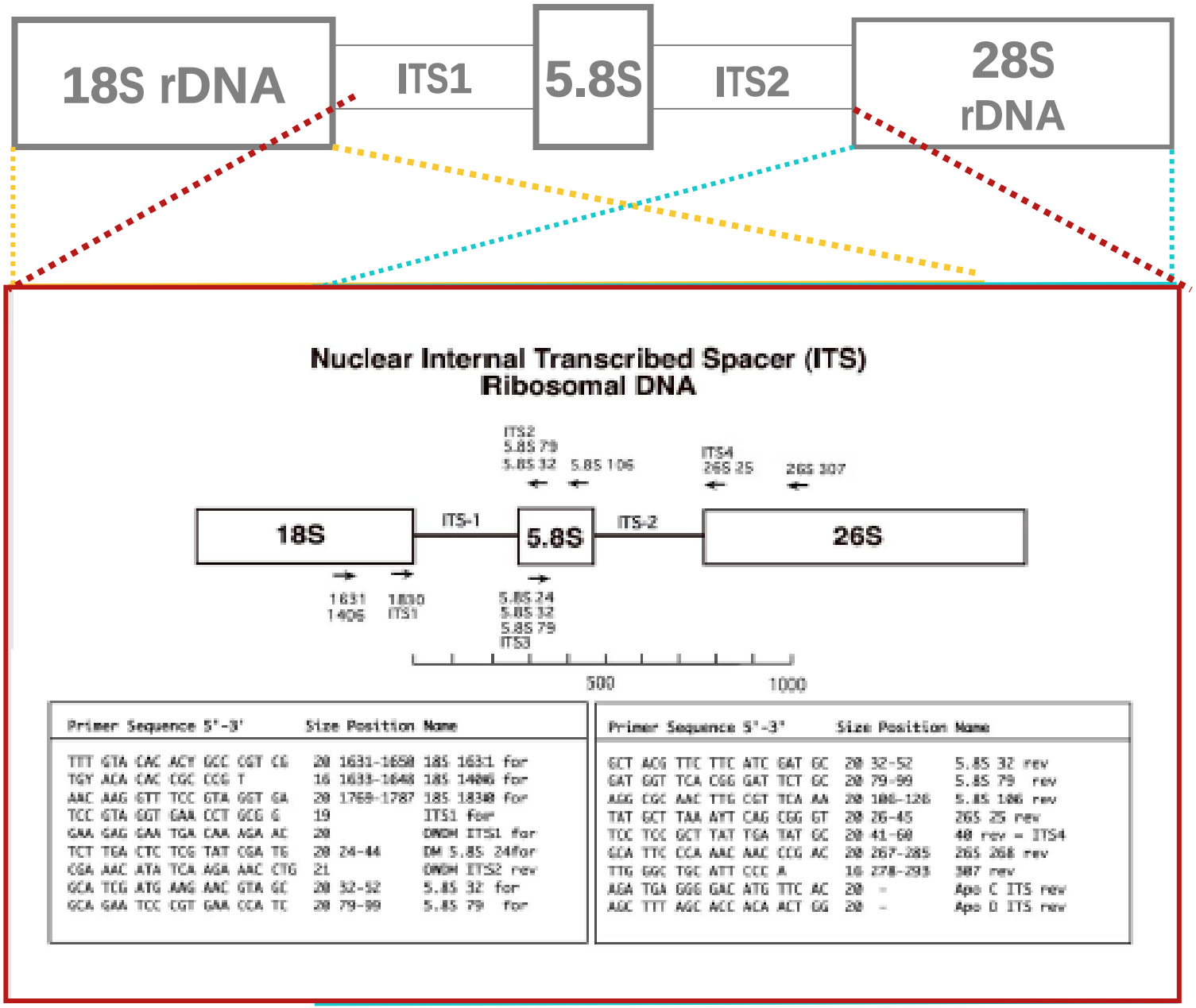
Current Genetics

Organization of ribosomal RNA genes in the fungus *Cochliobolus heterostrophus*

Robert C. Garber[1], B. Gillian Turgeon[2], Eric U. Selker[3], and O. C. Yoder[2]

Polymerase Chain Reaction, PCR

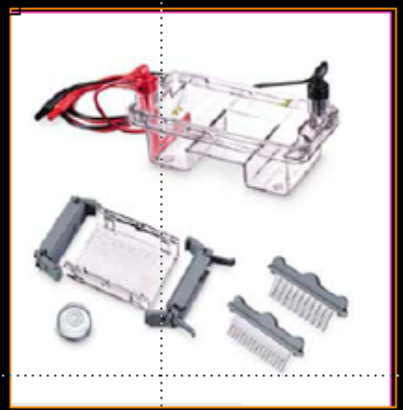
primers



electrophoresis

1

2



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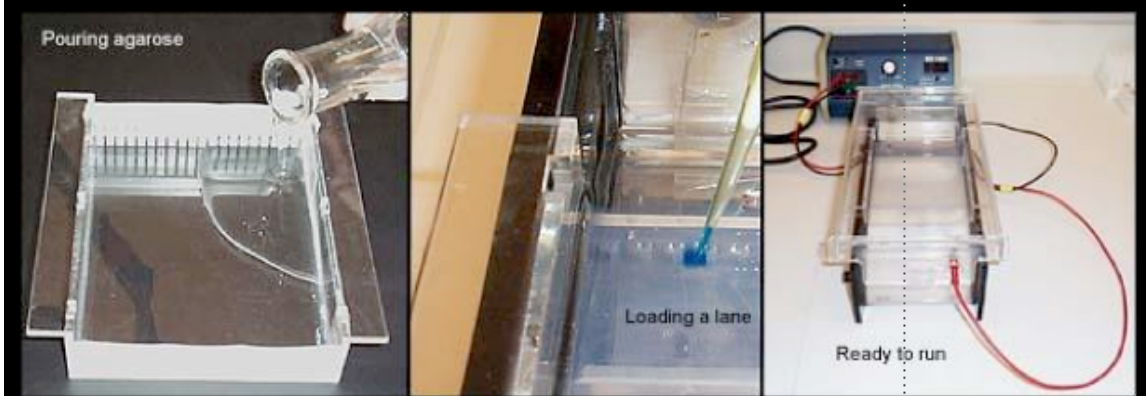

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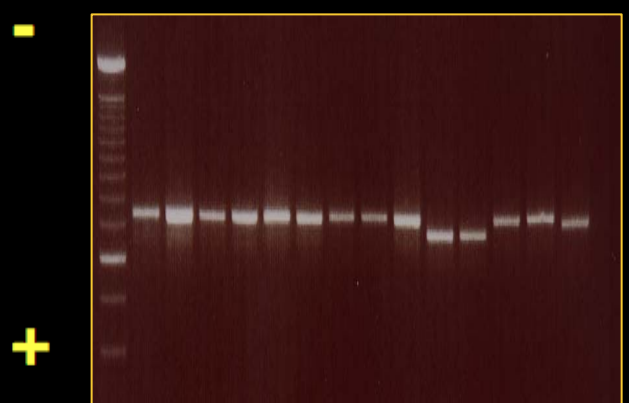
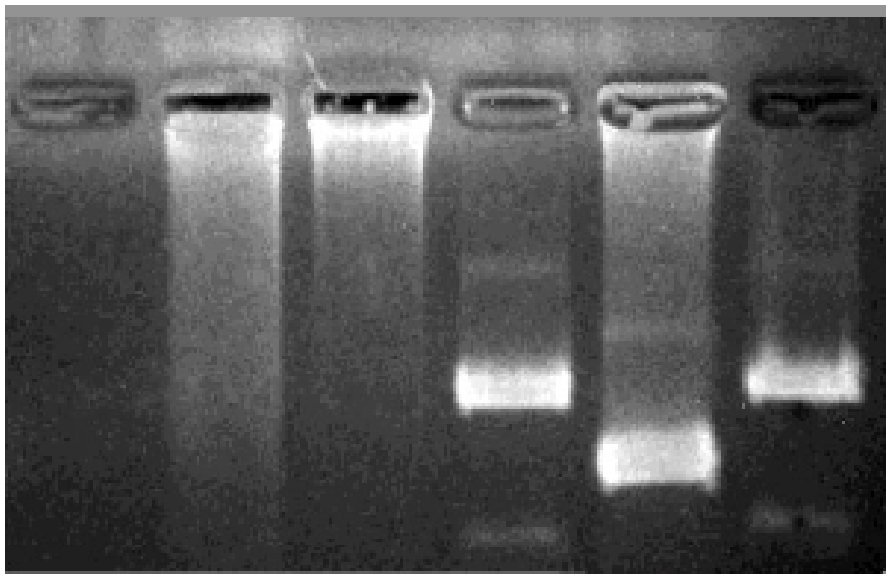
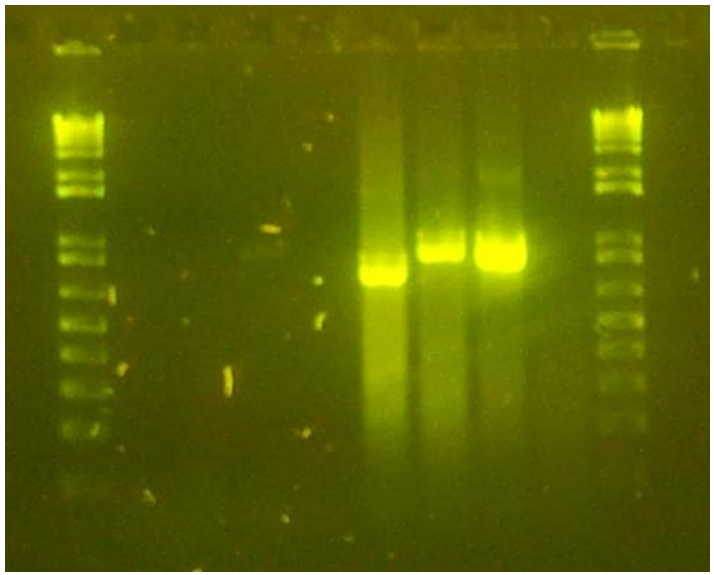


Polymerase Chain Reaction, PCR



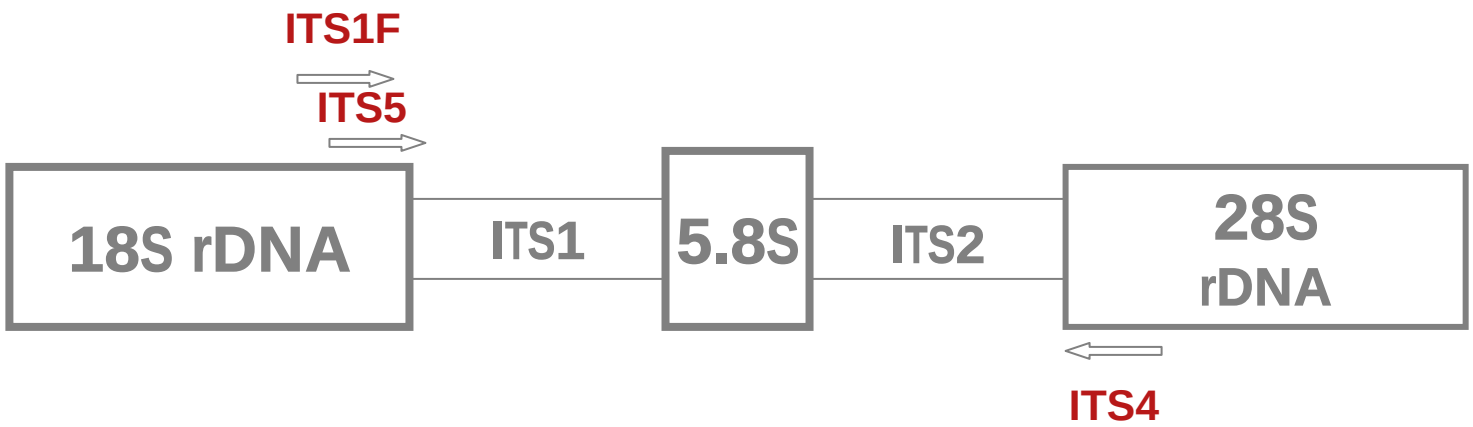


Lichenologist **32**(2): 189–196 (2000)
doi:10.1006/lich.1999.0254
Available online at http://www.idealibrary.com on IDEAL®

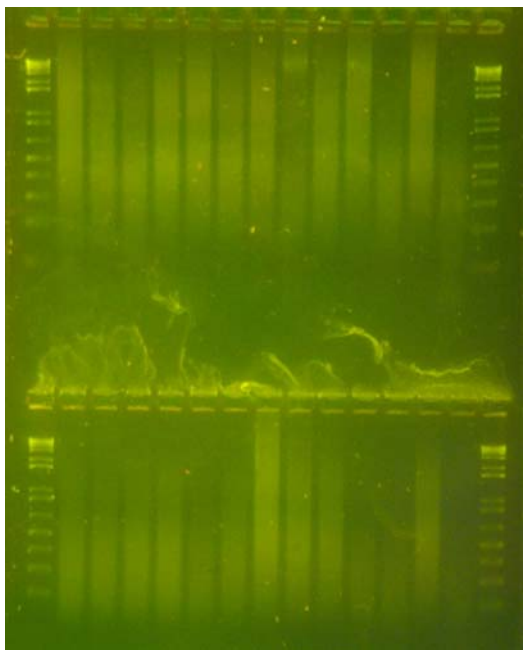
ALTERNATIVE METHODS OF EXTRACTING AND AMPLIFYING DNA FROM LICHENS

María P. MARTÍN* and Katarina WINKA‡

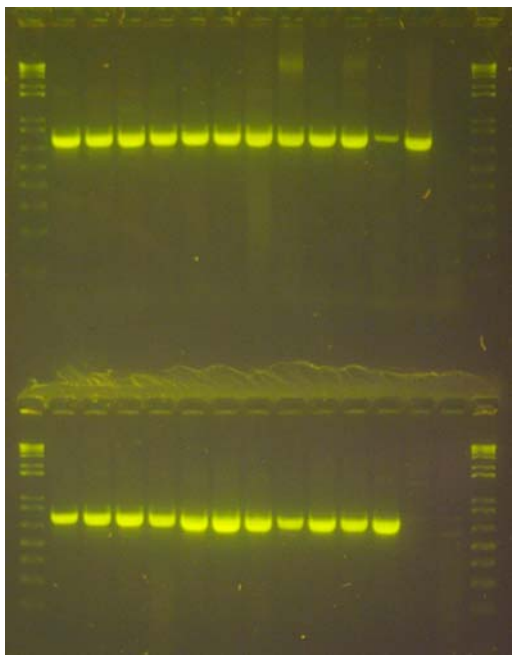
Polymerase Chain Reaction, PCR



ITS1F/ITS4



ITS5/ITS4



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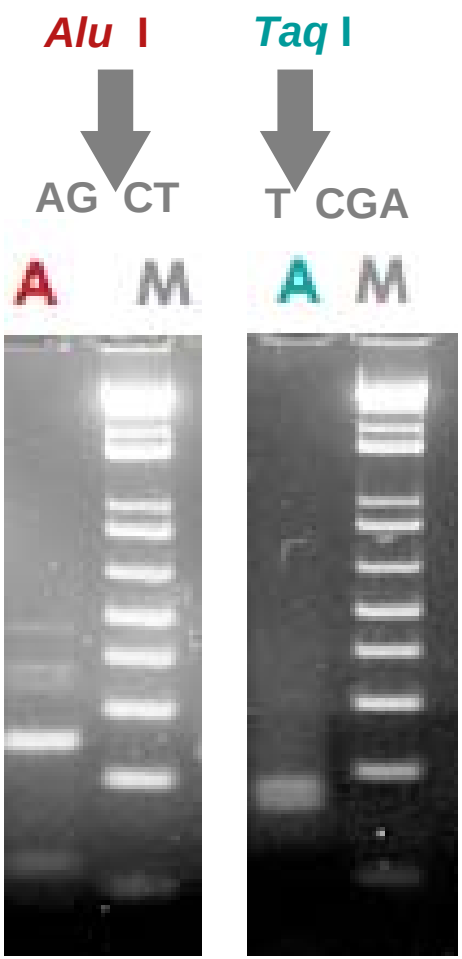
Restriction Fragment Length Polymorphisms, RFLP

Polimorfismo de la Longitud de los Fragmentos de Restricción

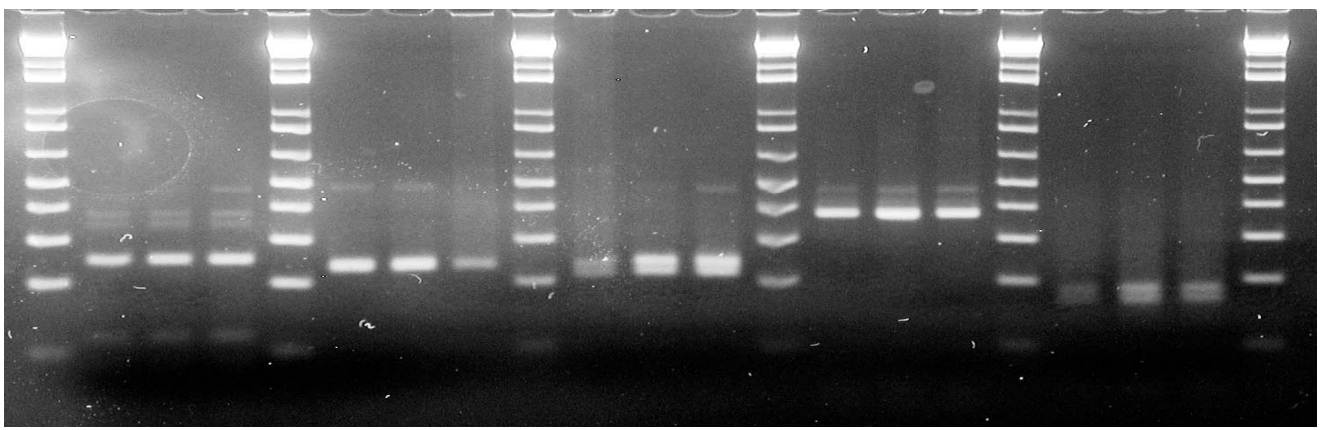


Detection of *Tuber melanosporum* DNA in soil

Laura M. Suz[1], María P. Martín[2], & Carlos Colinas[1]

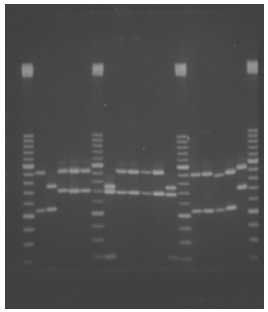
[1]Centre Tecnològic Forestal de Catalunya, Solsona, Spain and [2]Real Jardín Botánico (CSIC), Madrid, Spain

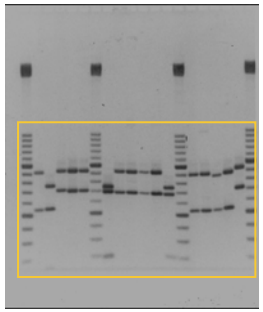
FEMS Microbiol Lett **254** (2006) 251–257

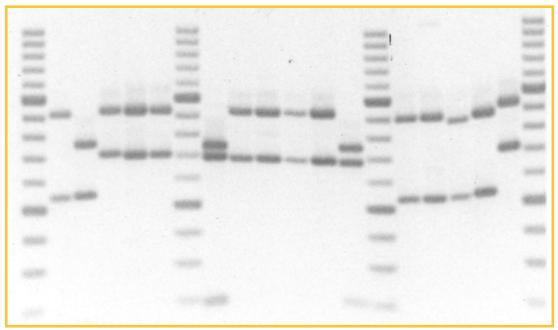


Restriction Fragment Length Polymorphisms, RFLP

1







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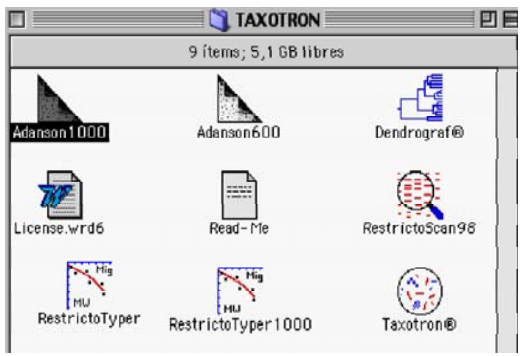

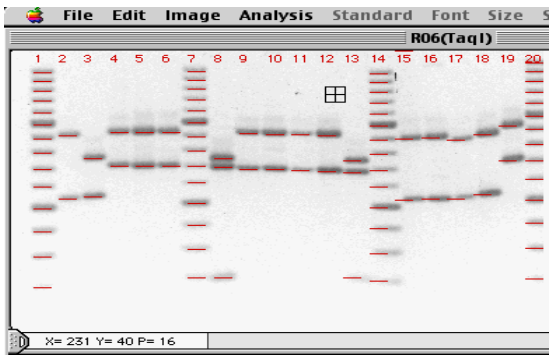

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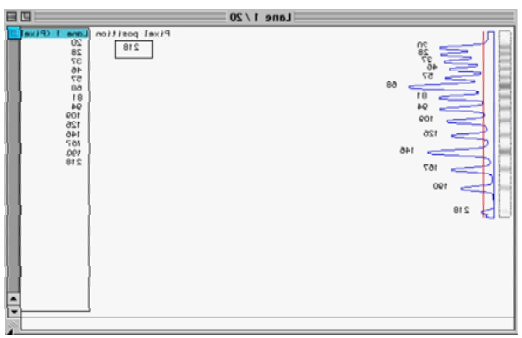

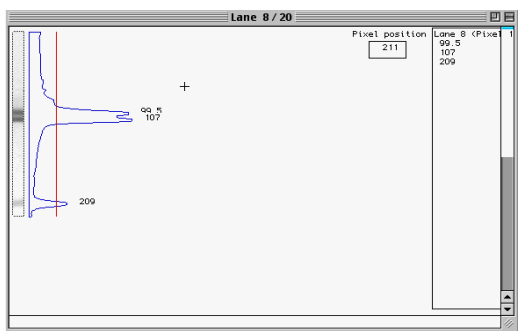

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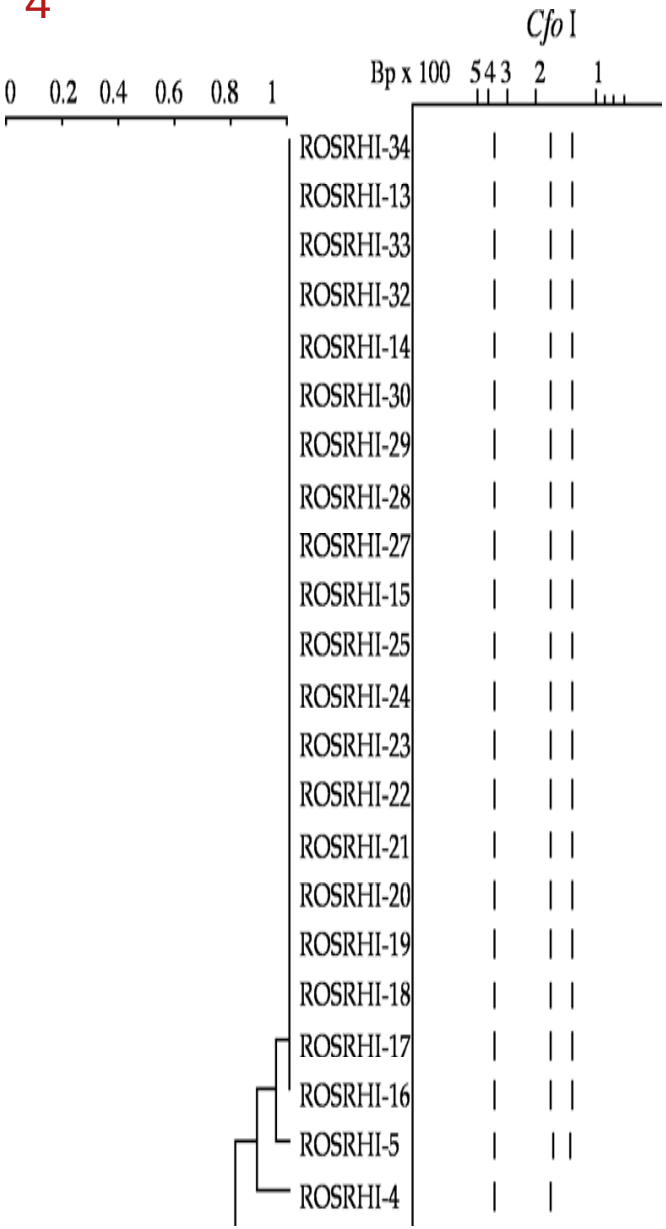

Restriction Fragment Length Polymorphisms, RFLP

Polimorfismo de la Longitud de los Fragmentos de Restricción

Phyton (Austria) Special issue: "Root-soil interactions"	Vol. 40	Fasc. 4	(135)-(141)	25.7.2000

Molecular Ecology of Hypogeous Mycorrhizal Fungi: *Rhizopogon roseolus* (Basidiomycotina)

By

M. P. MARTÍN[1)], O. KÅRÉN[2)] & J.-E. NYLUND[2)]

K e y w o r d s : rDNA, ITS, RFLP, hypogeous fungi, false truffles.

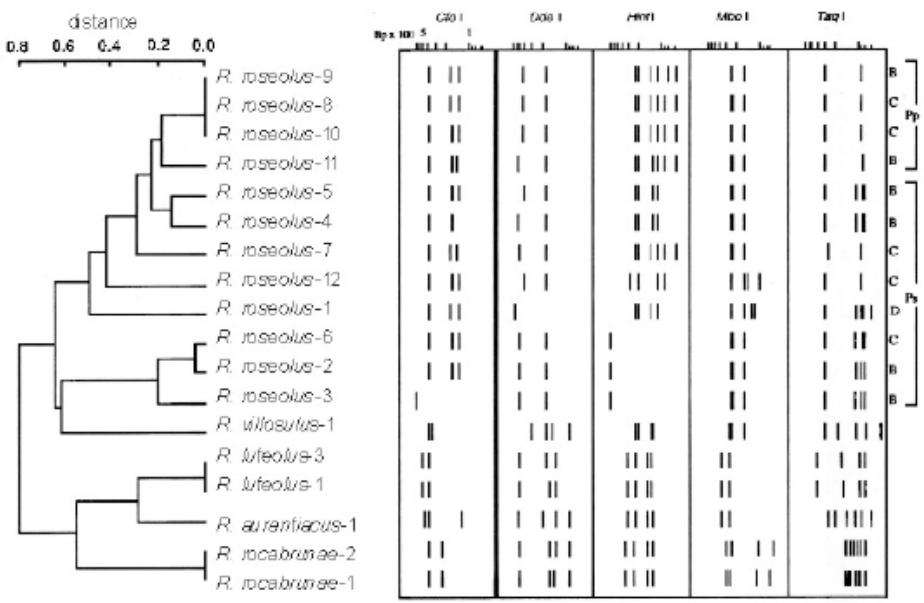

Fig. 1. Phenogram of the UPGMA cluster analysis of the RFLP patterns obtained with the restriction enzymes Cfo I, Dde I, Hinf I, Mbo I and Taq I. The spore volume of *R. roseolus* is indicated according to GROSS & al. 1980 and MARTÍN 1996. Mycorrhizal host: *Pinus pinaster* (Pp) and *Pinus sylvestris* (Ps).

Scopus search:
"RFLP"
1982-2011

26233 articles

Scopus search:
"PCR-RFLP"
1988-2011

10.030 articles

Scopus search:
"PCR-RFLP"
and"fungus" or
"fungi"
1996-2011

336 articles

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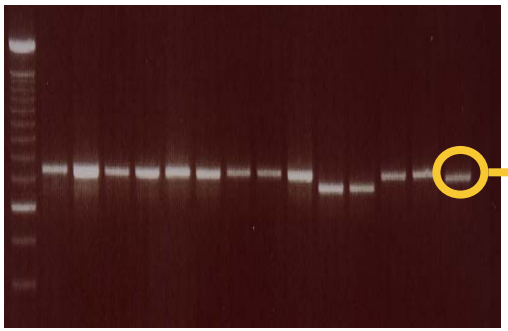
Dr. Fred Sanger
1958 and 1980 winner of the Nobel Prize in Chemistry



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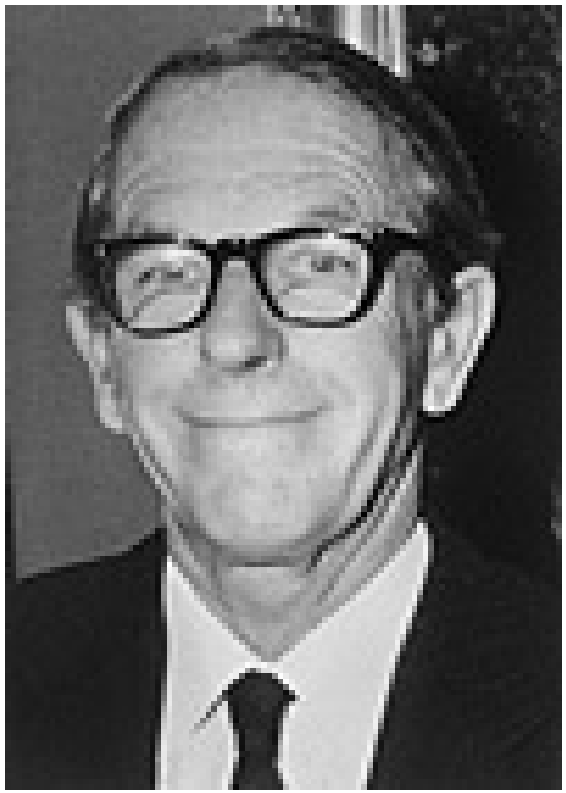


ACCTGAAAGTAGTAGCCGGAGGGAATCCCTATAC

Sequencing

1....ACCTGAAAGTAGTAGCCGGCGGGAATCCATATAC.....
2....ACCTGAAAGTAGTGGCCGGTGGGAATCCGTATAC.....
3....ACCTGAAAGTAGTGGCCGGTGGGAATCCGTAAAC.....

1

2

3

Sequencing


A
AG
AGA
AGAC
AGACC
AGACCT
AGACCTA
AGACCTAG
AGACCTAGG
AGACCTAGGT

Sequencing

Nucleotide BLAST: Search nucleotide databases using a nucleotide query

http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE

UNITE home | NCBI Blast:N...714 letters) | Títulos. Nat...ishing Group | http://www.c...ciacion.pdf | Revistas Cie...electrónica) | Scopus – Basic Search

BLAST — Basic Local Alignment Search Tool

Home | Recent Results | Saved Strategies | Help

My NCBI [Sign In] [Register]

NCBI/ BLAST/ blastn suite

blastn | blastp | blastx | tblastn | tblastx

BLASTN programs search nucleotide databases using a nucleotide query. more...

Reset page | Bookmark

Enter Query Sequence

Enter accession number(s), gi(s), or FASTA sequence(s) — Clear

Query subrange — From — To

Or, upload file — Seleccionar archivo ningún ...ionado

Job Title — Enter a descriptive title for your BLAST search

Align two or more sequences

Choose Search Set

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 — Exclude

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

Exclude (Optional) — Models (XM/XP) — Uncultured/environmental sample sequences

Entrez Query (Optional) — Enter an Entrez query to limit search

Program Selection

Optimize for — Highly similar sequences (megablast) — More dissimilar sequences (discontiguous megablast) — Somewhat similar sequences (blastn)

Choose a BLAST algorithm

BLAST — Search database Nucleotide collection (nr/nt) using Megablast (Optimize for highly similar sequences)

Show results in a new window

Algorithm parameters

Note: Parameter values that differ from the default are highlighted in yellow and marked with ♦ sign

Sequencing

UNITE home

http://unite.ut.ee/

mor – mor –...Bolete</i> IAPT Intern...nt Taxonomy Ciencia y Te...ria sexenios The Interna...– home page Description...search LMU)

unite

A molecular database for the identification of fungi

Home | Run Analysis | Search Pages | Notes and news | Contributors | Acknowledgements

Number of UNITE barcoding sequences: **2629** ITS sequences of **1153** species from **177** genera.
Number of fungal ITS sequences in database (UNITE + INSD): **81884**
How to cite UNITE ?

UNITE is a rDNA sequence database focused on ectomycorrhizal asco- and basidiomycetes. The database initially holds only sequences from the ITS region. The sequences are generated from fruit bodies collected and identified by specialists and deposited in public herbaria; type specimens are used whenever possible. Selected species also have full descriptions and illustrations linked to the sequences.

The purpose of the database is to facilitate identification of environmental samples of fungal DNA. UNITE includes several tools that aid in the identification of unknown sequences. Since unequivocal identification is the main purpose behind UNITE, the implementation of tools that extend beyond simple similarity searches (as offered by BLAST and variations thereof) was an essential part of the database development. This requirement has been met by the development of galaxie, which allows web-based, basic phylogenetic analyses. Galaxie provides maximum parsimony heuristic and neighbour joining analyses under different evolutionary models. To date, two galaxie (galaxieBLAST, galaxieHMM) and one BLAST script have been implemented. We recommend galaxieBLAST as the most appropriate tool for the identification of unknown ITS sequence. Other identification methods will be considered for inclusion in the future. We stress again that the UNITE database is, in its present form, restricted to ITS sequences specific to ECM fungi and as such the input of query sequences from other fungi not covered by the database (i.e. saprotrophic or parasitic fungi) is not recommended for obvious reasons.

UNITE is a relational database built on a MySQL platform running on a Red Hat Linux Apache httpd server. It is accessed through a web interface system written in PHP, Perl, and Python scripting languages.

The development of UNITE is a Nordic-Baltic initiative and a collaboration between several research groups. The project links experts in fungal taxonomy and ecology and also forms part of a student-training network funded by the Nordic Research Council.

INDEXED IN ISI Web of Knowledge™ Current Web Contents

Contact information • How to cite UNITE
Homepage is maintained by
Kessy Abarenkov & R. Henrik Nilsson

Last updated: 8 May, 2008

Powered by MySQL

Blast output

http://unite.ut.ee/cgi-bin/seq_search2.pl

mor – mor –...Bolete</i> IAPT Intern...nt Taxonomy Ciencia y Te...ria sexenios The Interna...– home page Description...search LMU)

Citation

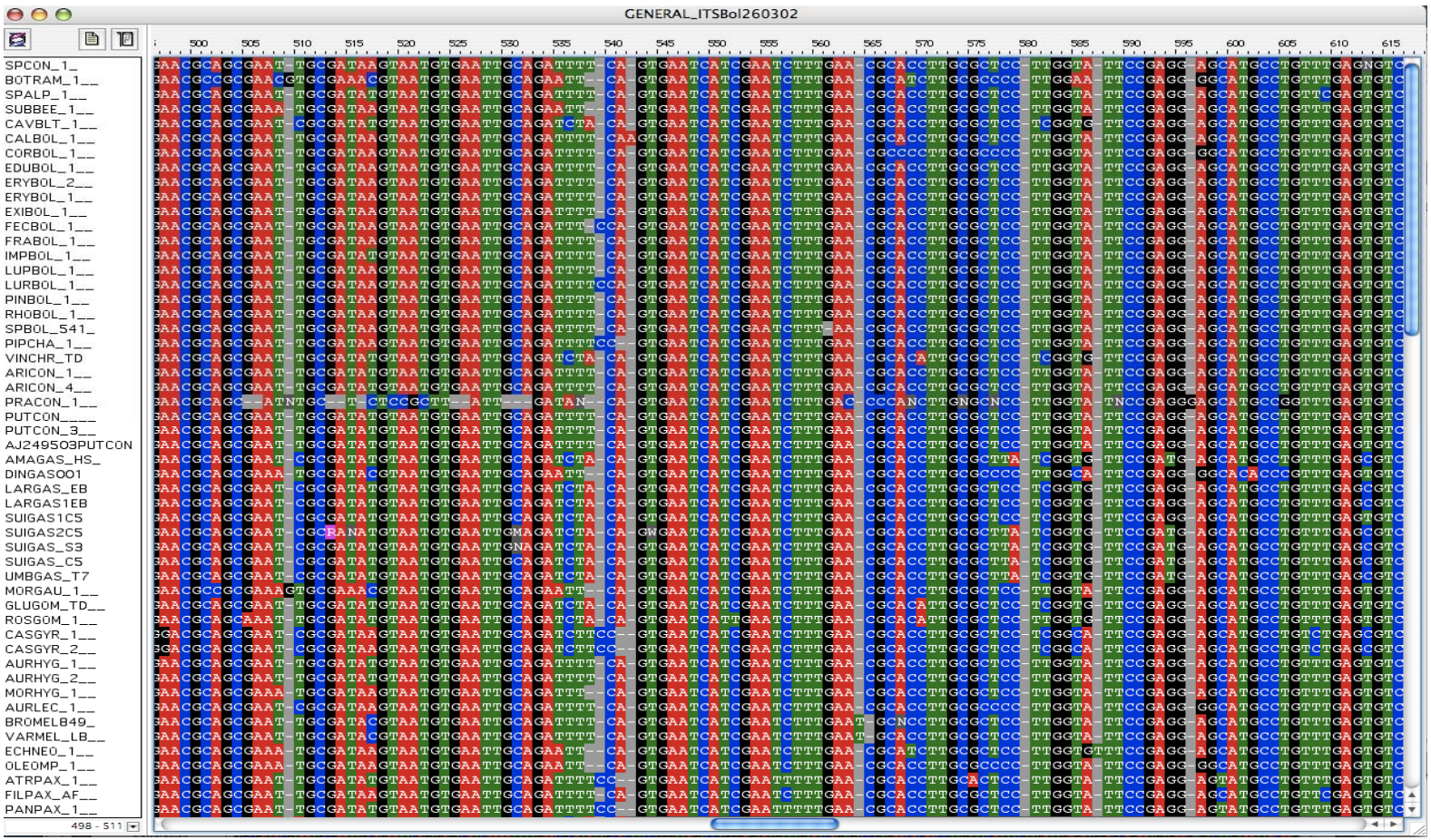
unite

Searching for homologs of sequence NoName
It should take at most 15 seconds. Please wait...

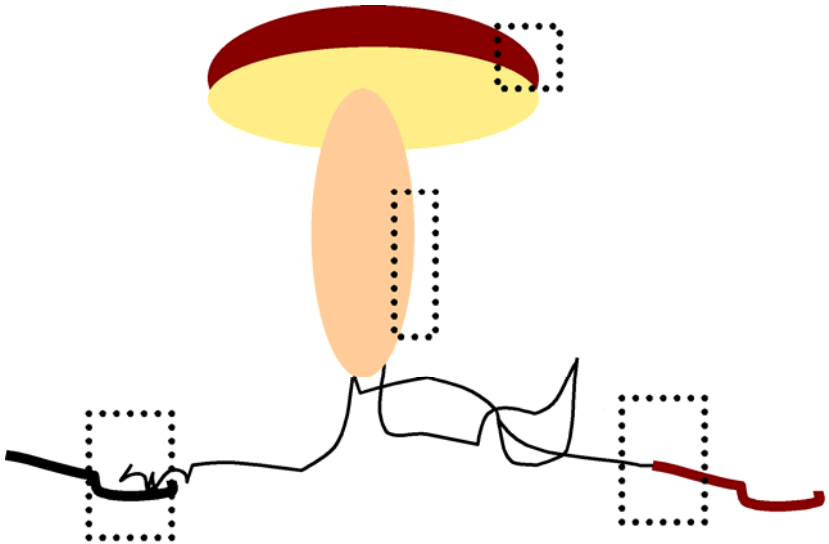
```
Database(s) used: UNITE + INSD ( = GenBank, EMBL, DDBJ)

BLASTN 2.2.6 [Apr-09-2003]


 Reference: Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer,
 Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997),
 "Gapped BLAST and PSI-BLAST: a new generation of protein database search
 programs", Nucleic Acids Res. 25:3389-3402.

 Query= NoName
        (642 letters)

 Database: data/Myco5_fas; data/alla2.fasta
           81,884 sequences; 51,196,936 total letters

 Searching..................................................done

                                                                  Score    E
 Sequences producing significant alignments:                      (bits) Value

DQ375514        Tuber indicum                                      1219   0.0
AF132503        Tuber himalayense                                  1215   0.0
DQ375526        Tuber sinense                                      1187   0.0
DQ329364        Tuber indicum                                      1172   0.0
DQ375527        Tuber sinense                                      1164   0.0
DQ375510        Tuber indicum                                      1164   0.0
DQ375497        Tuber indicum                                      1158   0.0
U89362          Tuber indicum                                      1156   0.0
AF300824        Tuber indicum                                      1148   0.0
AF106881        Tuber indicum                                      1148   0.0
DQ375507        Tuber indicum                                      1140   0.0
U89361          Tuber indicum                                      1132   0.0
DQ375516        Tuber indicum                                      1130   0.0
DQ375498        Tuber indicum                                      1130   0.0
AY773357        Tuber indicum                                      1114   0.0
DQ375518        Tuber indicum                                      1108   0.0
DQ375523        Tuber indicum                                      1096   0.0
DQ375522        Tuber indicum                                      1096   0.0
DQ375521        Tuber indicum                                      1096   0.0
DQ375520        Tuber indicum                                      1096   0.0
DQ375506        Tuber indicum                                      1096   0.0
DQ329376        Tuber sinense                                      1096   0.0
DQ375517        Tuber indicum                                      1094   0.0
AF300822        Tuber indicum                                      1094   0.0
DQ375509        Tuber indicum                                      1092   0.0
DQ375515        Tuber indicum                                      1088   0.0
DQ375524        Tuber indicum                                      1086   0.0
```

Sequencing

Less that 1% of all fungal species have been sequenced for the ITS region, the similarity search often fails to produce a match close enough for reliable species identification.



The revolution of molecular techniques in the fungi research: Explored files and future objectives

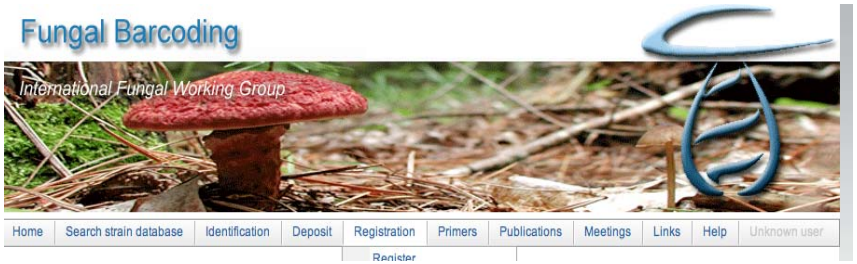


Use of a short standarized genetic marker to provide DNA-based identification of fungi.

Barcoding

DNA Barcoding

In fungi, DNA barcoding is challenging because species concepts are inconsistently applied.



Finding the best gene for Fungi. Participants at workshop, Amsterdam April 16-18, 2011, to finalise a paper declaring a fungal barcode.

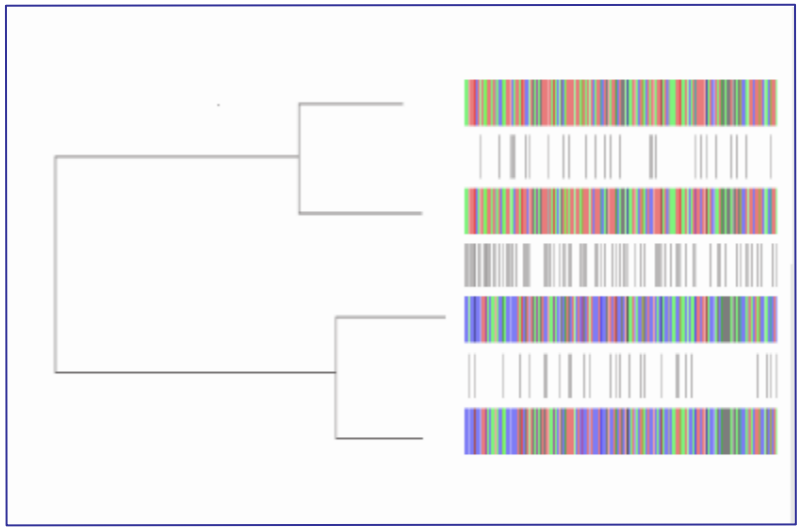
The noncoding ITS region is accepted as the prime barcoding region in fungi.

There is an interest in developing a second barcode:

- SSU nrDNA
- LSU nrDNA

- RPB1
- RPB2
- ATP6
- Others …

DNA barcoding



- Corticiaceous, *Hyphodermella* and *Hypochnicium*

- *Geastrum*

- Boletales, *Rhizopogon*

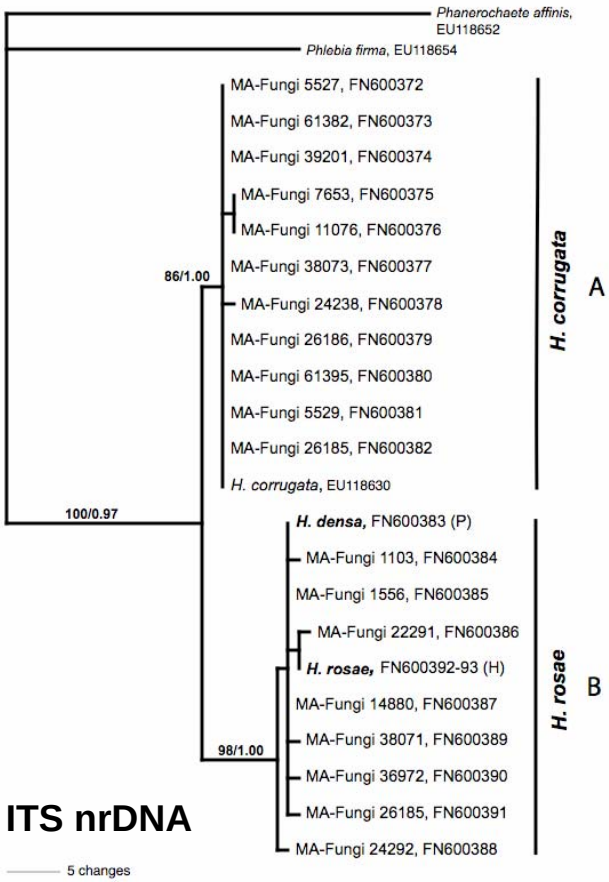
Corticiaceous Fungi - *Hyphodermella*

Mycol Progress (2010) 9:585–596
DOI 10.1007/s11557-010-0666-5

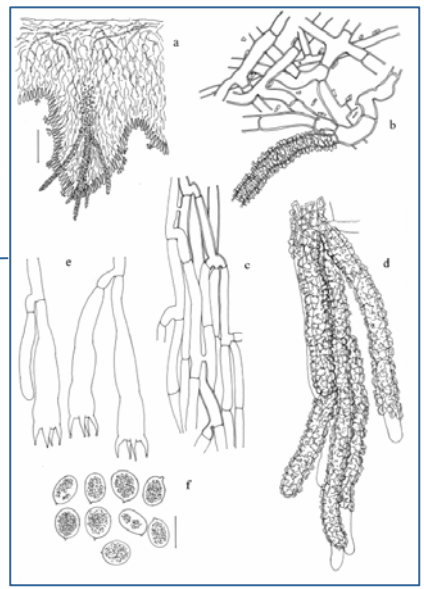
ORIGINAL ARTICLE

Morphological and molecular studies of *Hyphodermella* in the Western Mediterranean area

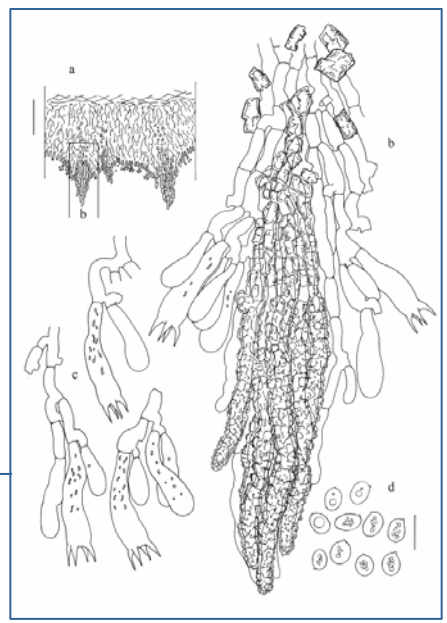
M. Teresa Telleria · Margarita Dueñas · Ireneia Melo · María P. Martín



H. corrugata



H. rosae

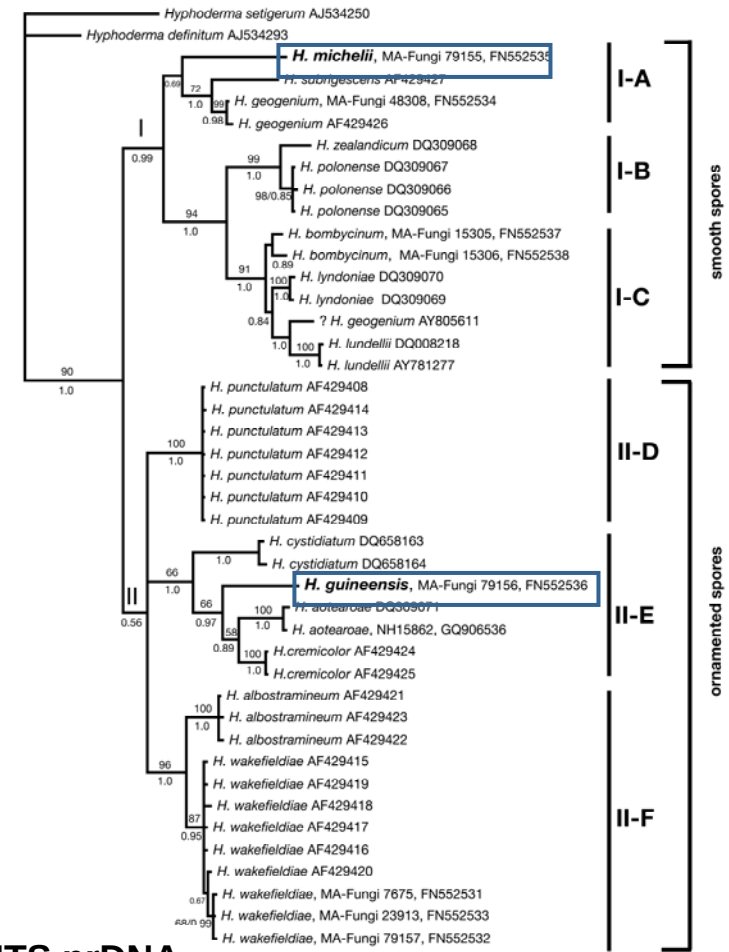


Corticiaceous Fungi - *Hypochnicium*

M. Teresa Telleria[1]
Margarita Dueñas
Departamento de Micología, Real Jardín Botánico, CSIC, Plaza de Murillo, 2. 28014 Madrid, Spain
Ireneia Melo
Jardim Botânico (MNHN), Universidade de Lisboa, CBA/FCUL, Rua da Escola Politécnica 58, 1250-102 Lisboa, Portugal
Nils Hallenberg
Department of Plant and Environmental Sciences, Carl Skottsberg Gata 22b, S-405 30 Göteborg, Sweden
María P. Martín
Departamento de Micología, Real Jardín Botánico, CSIC, Plaza de Murillo, 2. 28014 Madrid, Spain

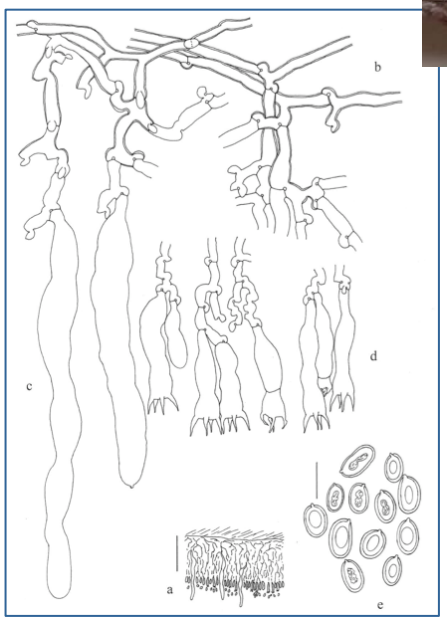
Mycologia, 102(6), 2010, pp. 1426–1436. DOI: 10.3852/09-242

A re-evaluation of *Hypochnicium* (Polyporales) based on morphological and molecular characters

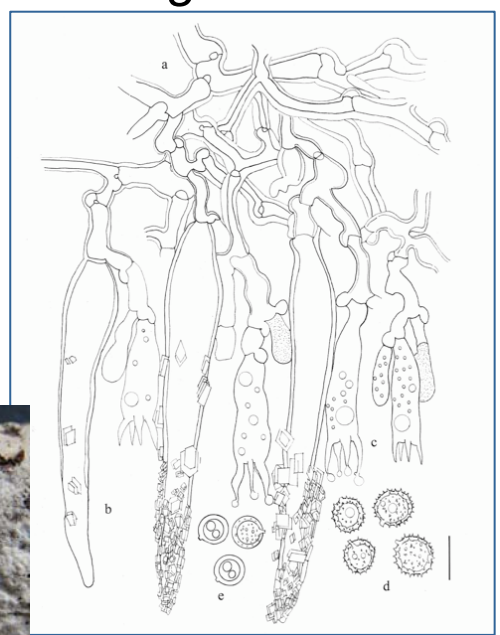


ITS nrDNA

H. michelii



H. guineensis

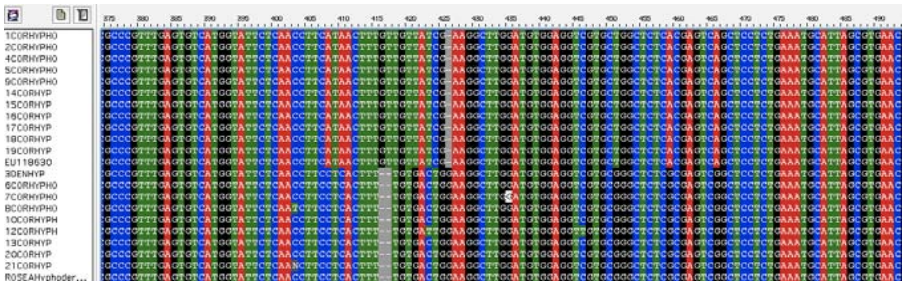


Corticiaceous Fungi

Hyphodermella

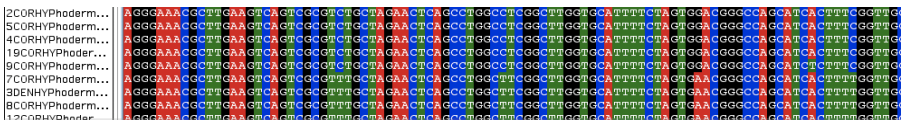
ITS 2

14 bases/230 bp



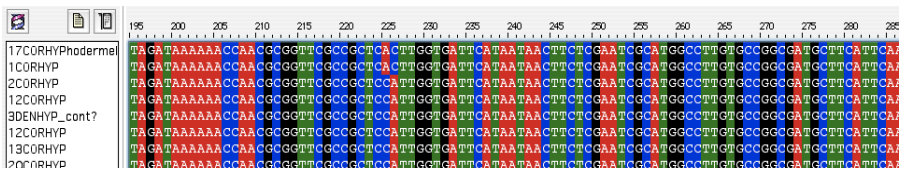
LSU

5 bases/1300 bp

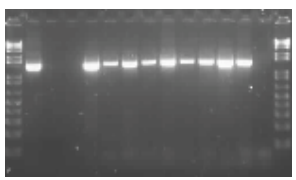


SSU

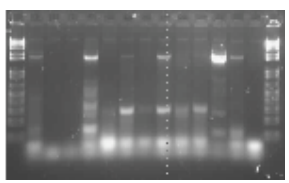
5 bases/1700 bp



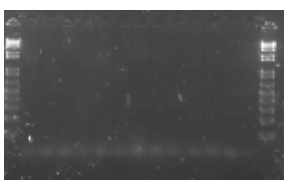
LSU



SSU

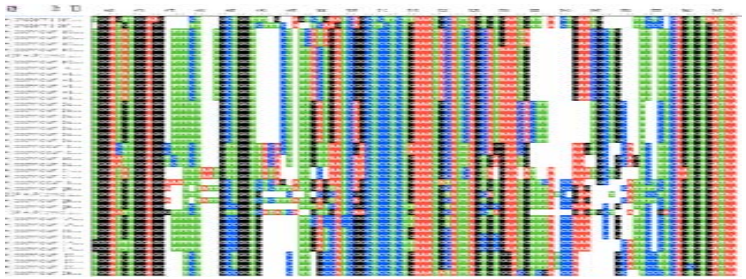


RPB1



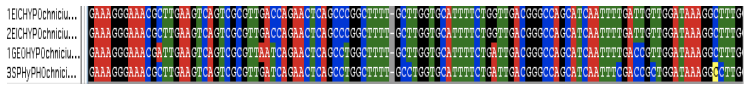
Hypochnicium

ITS 1/ ITS2



LSU

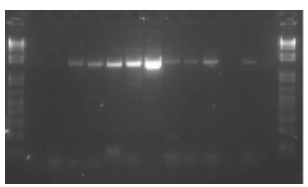
35 bases/1300 bp



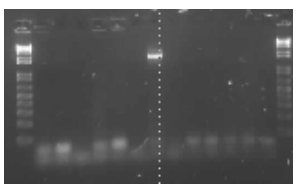
SSU

Only 1 sequence obtained

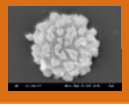
LSU



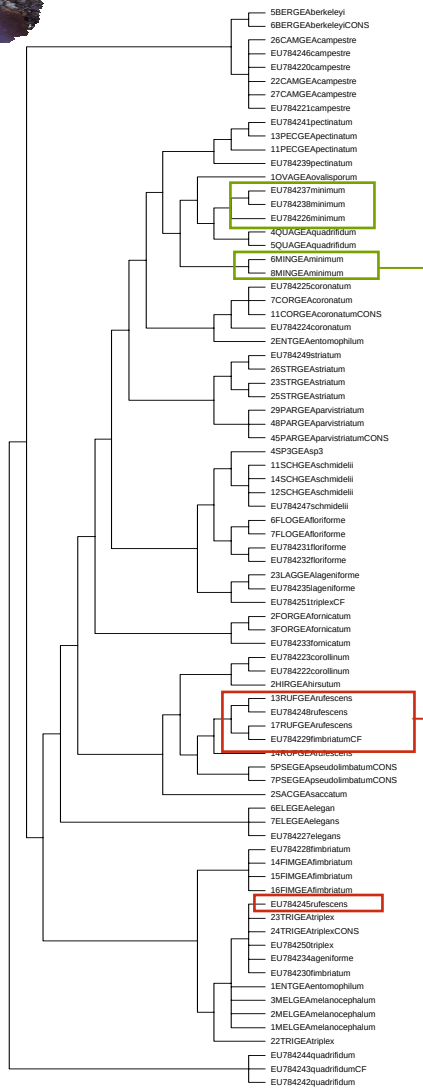
SSU



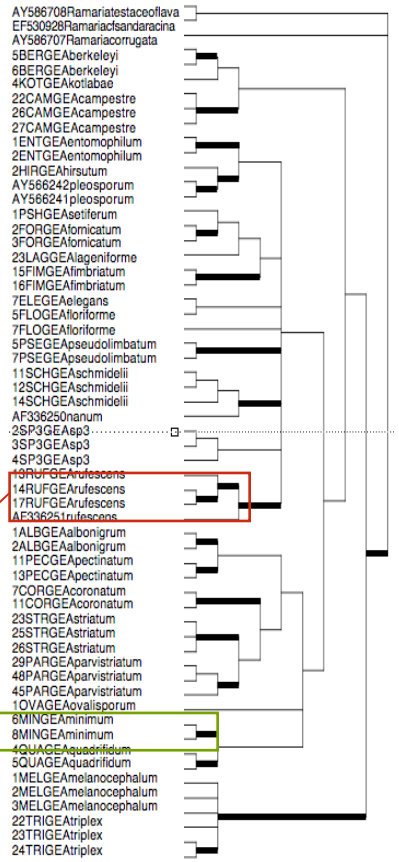
RPB1



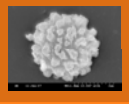
Geastrum



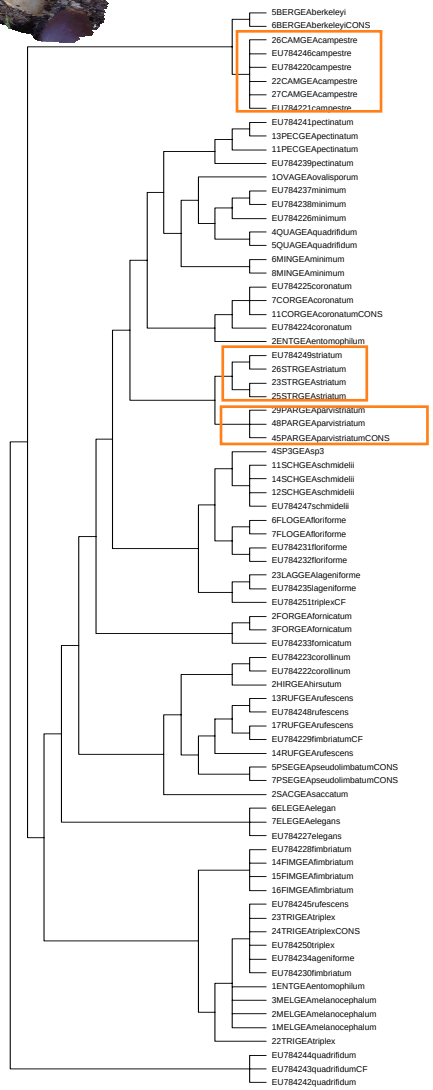
ITS nrDNA



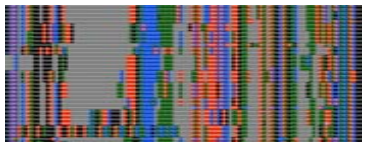
LSU nrDNA



Geastrum



ITS nrDNA



ITS

>100 / 710

LSU

51/ 1002

SSU

2 / 828

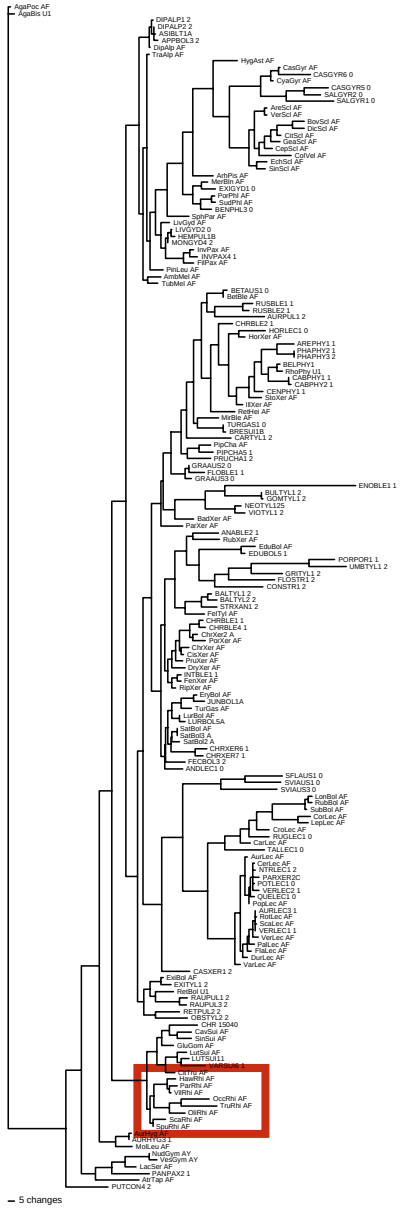
RPB1

80 / 793

ATP6

>100 / 703

Order Boletales - *Rhizopogon*

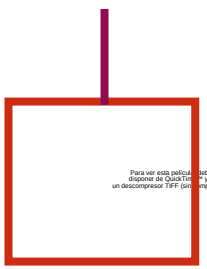


ITS nrDNA

Para ver esta película, debe disponer de QuickTime™ y de un descompresor TIFF (sin comprimir).

Para ver esta película, debe disponer de QuickTime™ y de un descompresor TIFF (sin comprimir).

Rhizopogon



Para ver esta película, debe disponer de QuickTime™ y de un descompresor TIFF (sin comprimir).

Rhizopogon Subg. *Roseoli*

$V=0.5 \times w^2 \times l$

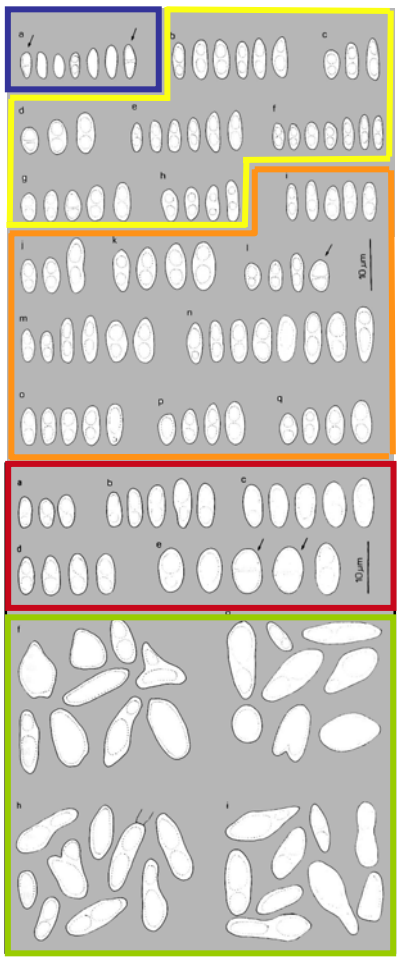
15 *um³*
R. vulgaris* var. *vulgaris

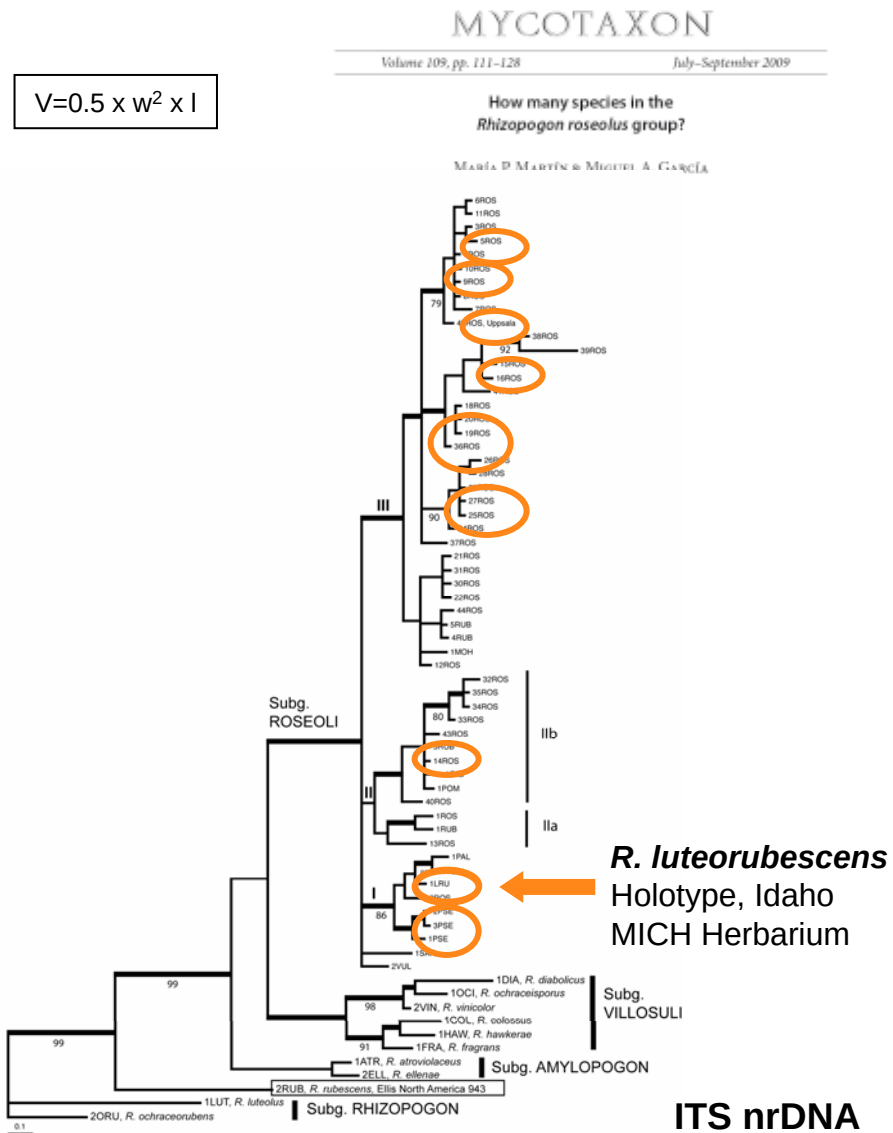
30 *um³*
R. vulgaris* var. *Intermedius

45 *um³*
R. luteorubescens

60 *um³*
R. roseolus
R. rubescens

240-280 *um³*
R. gigasporus
R. hymenogastrosporus
R. roseolus* f. *aberrans
R. ventricisporus

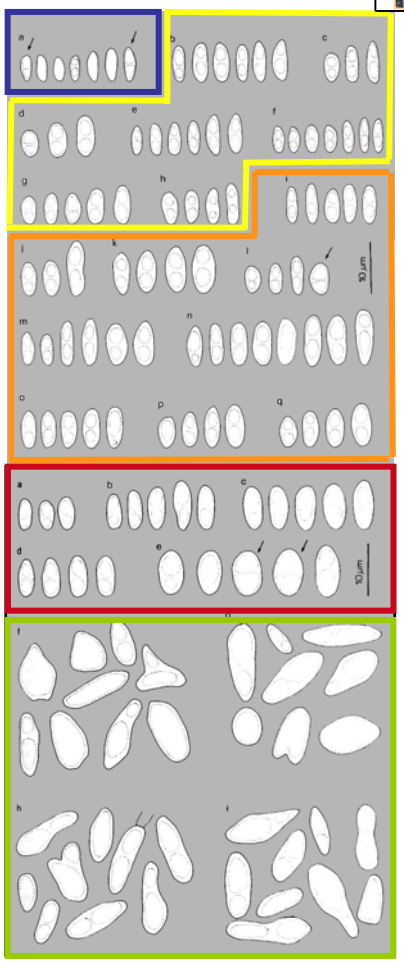




R. luteorubescens
Holotype, Idaho
MICH Herbarium

ITS nrDNA

Rhizopogon Subg. *Roseoli*



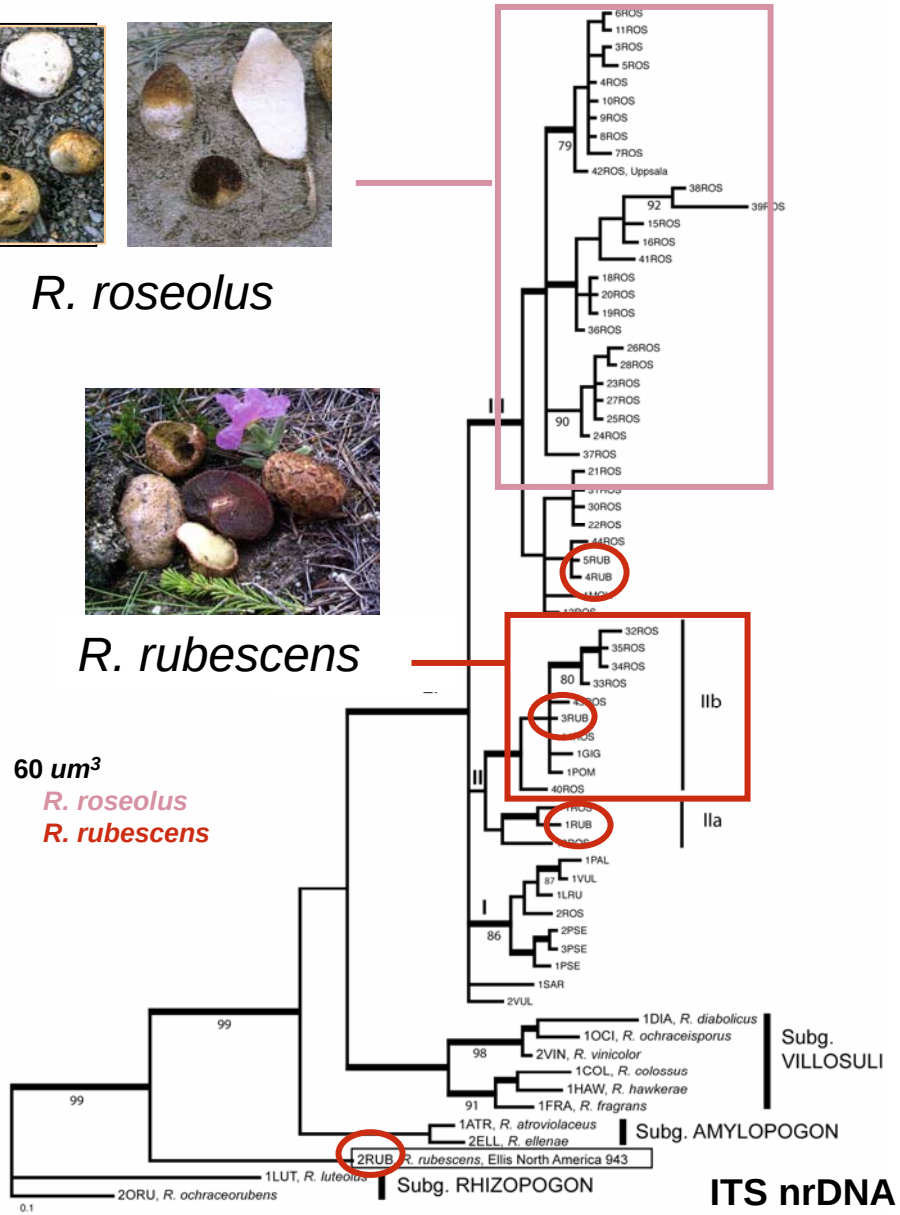
R. roseolus

R. rubescens

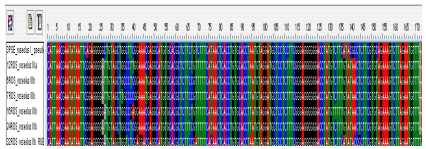
60 *um³*

R. roseolus

R. rubescens



ITS nrDNA



ITS 1

28 / 226

ITS 2

27 / 250

LSU

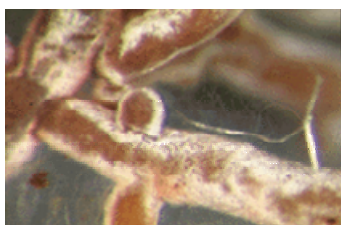
2 / 650

SSU

1 / 800

RPB1

Only 1 good seq.



The revolution of molecular techniques in the fungi research: Explored files and future objectives

The lack of reference sequences from well-identified fungi often poses a challenge to inference of taxonomic affilation of sequences from environmental samples, and many environmental samples are thus unidentified.

Ryberg et al. 2008

Prof. M.Teresa Telleria

Para ver esta película, debe disponer de QuickTime™ y de un descompresor TIFF (sin comprimir).

Para ver esta película, debe disponer de QuickTime™ y de un descompresor TIFF (sin comprimir).

Dr. Margarita Dueñas

Prof. F-D. Calonge

Juan Carlos Zamora

Dr. Miguel A. Garcia

Dr. Laura M. Suz

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