X Convegno SIPaV

Sorrento 1 – 3 ottobre 2003

Patologia Vegetale -Dip. S.A.V.A.
Università degli Studi del Molise
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Consiglio Nazionale delle Ricerche
Bari

MOLECULAR FINGERPRINTING OF ANTAGONIST *AUREOBASIDIUM PULLULANS* ISOLATES BY FLUORESCENT AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (fAFLP)

L. Caputo, F. De Curtis, R. Castoria, G. Lima, G. Stea, V. De Cicco

Biological control agents (BCAs) applications

Soil and root microbiomes

Aerial microbiomes

Postharvest microbiomes

Commercial BCA products

Advantages

Environmentally compatible

Broad an narrow targets depending on organism

Can be site-specific

Less prone to resistance

Integrated control possible, reducing chemical use

Disadvantages

Inconsistent and often low levels of control

Subject to environmental influences

Low persistence

Change of mutation and variation

Poor shelf life

Not cost-effective for certain existing markets

Expensive and more difficult to use

Not practical for large-acreage agronomic crops

Whipps and Lumsden, 2001

Furthermore…

The release of BCA is associated with:

Competitive displacement of no-target organisms

Toxicity to no-target organisms

Pathogenicity to no-target organisms

Human allergens

Therefore…

It needs:

To track and identify the introduced BCA in the environment

To study the modes of action

To monitor the efficacy and stability of the introduced BCA at long-term

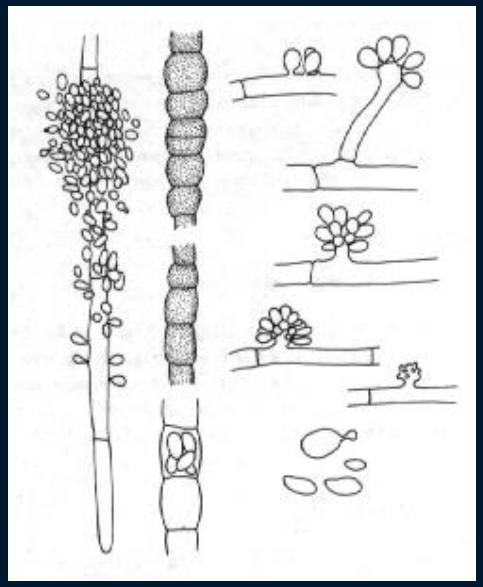
To evaluate the risk assessment of exotic organism

Methods of tracking an individual into the environment

- Morphological and biochemical markers and vegetative compatibility groups (VCGs) — Unstable, subject to reversion
- Allozymes — Large quantity of the introduced clone with unique alleles
- Immunological markers (ELISA) — Cross-reactivity with the antisera
- Karyotype analysis (CLP-PFGE) — Long procedure
- Molecular markers:

RAPD-PCR	→	Easy, small quantities of DNA, low reproducibility
RFLP	→	Large amount of DNA
AFLP	→	Many reliable and reproducible polymorphisms
dsRNA	→	Low fidelity
Introduced molecular marker	→	High fidelity, but questionable technique

Aureobasidium pullulans (de Bary) Arnaud



Superkingdom	*Eukaryota*
Kingdom	*Fungi*
Phylum	*Ascomycota*
Subphylum	*Pezizomycotina*
Class	*Eurotiomycetes*
Family	*Dothioraceae*
Genus	*Aureobasidium*

Principle features

Yeast-like fungus with high pleomorphism

Ubiquitous species, mainly on the phylloplane

Production of cellulosolytic, pectinolytic and ligninolytic enzymes

High tolerance to salt and sugar concentrations

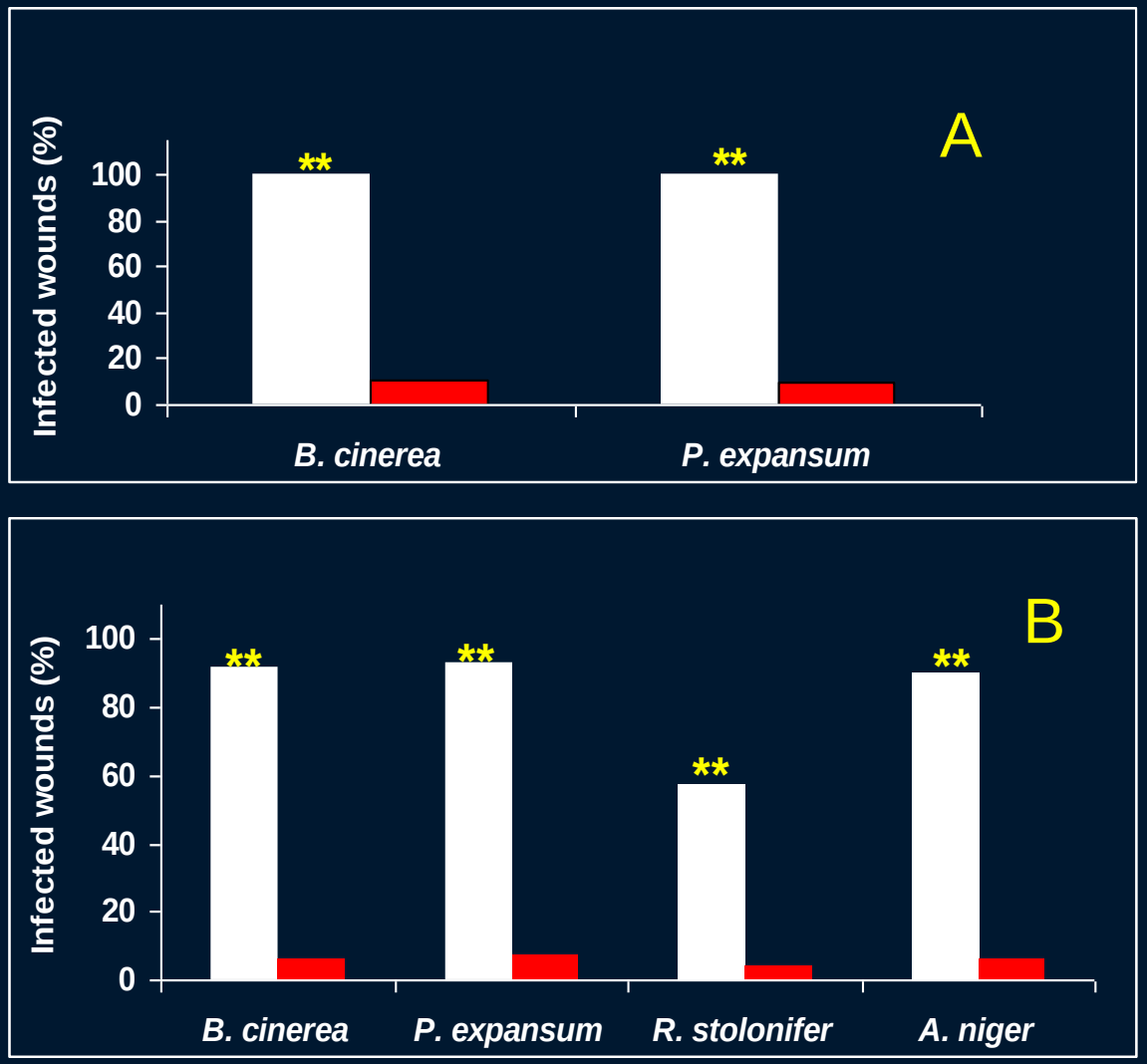
Production of pullulan

Aureobasidium pullulans

Isolate LS30

Biological control on different crops

Biological control against different postharvest pathogens



Statistical significance is given at *P*<0,01 (**) for differences between control and antagonist for each fruit and each pathogen. Controls were treated with water rather than LS-30.

Modes of action of LS30

Competition for space

Lima et al., 1999. J. Industr. Microbiol. 23: 223-229

Competition for nutrients

Castoria et al., 1997. Postharvest Biology and Technology 12: 293-300

Secretion of lytic enzymes (chitinases and glucanases)

No occurrence of antibacterial and antifungal metabolites

Lack of attachment to fungal hyphae

Castoria et al., 2001. Postharvest Biology and Technology 22: 7-17

Relevant *in vitro* resistance to dicarboximides and copper fungicides

Lima et al., 2003. European Journal of Plant Pathology 109: 341–349

Since LS30 was introduced into the field:

How can we track it out of epiphytic population of *A. pullulans* ?

How can we study the efficacy and stability of the introduced LS30 at long-term?

LS30

How can we enhance their modes of action?

How can we evaluate the risk assessment of this exotic organism?

The widespread distribution of *A. pullulans* in the phyllosphere and carposphere of different plant species is a relevant limitation for re-isolation and study of specific biocontrol strains by using classical morphological techniques.

LS200

AU33

AU32/1

LS6

AU53

Fluorescent amplified fragment length polymorphism method (fAFLP)

Multistep procedure

Restriction with rare and frequent cutter (i.e. *Eco*RI and *Mse*I)

Ligation of suitable adaptors

Pre- selective PCR of EcoRI-MseI-ended fragments

Selective PCR with fluorescein labelled and unlabelled primers

Selective PCR primers are with up to a 3-bp extension

Main features

Relatively cheap, easy, fast and reliable technique

No use of radioactive matter

Higher resolution capillary electrophoresis (± 1bp)

Automated procedures of analisys by a Genetic analyzer

Thousands of polymorphisms from the entire genome

Dominant multilocus marker

Typing microorganisms at an isolate level

Disadvantage

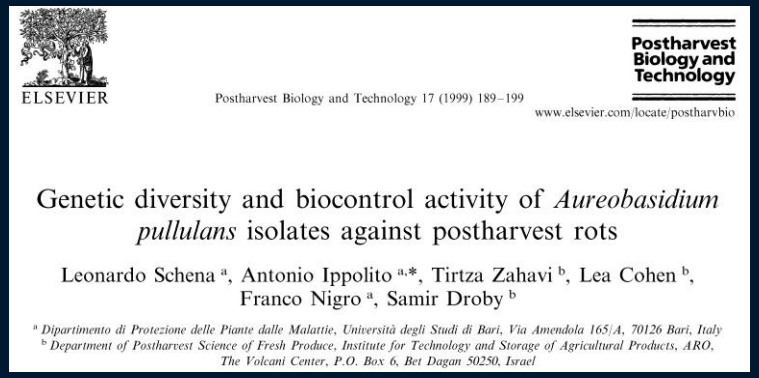
Not suitable for identifying homologous markers (alleles)

Molecular strategies for intra-specific variability of *A. pullulans*

State of the art

- Arbitrarily primed PCR (AP-PCR)
- Random amplified polymorphic DNA (RAPD)
- Sequence characterized amplification region (SCAR) primers
- RNA probes
- Universal primers (UP-PCR)

Amplified restriction length polymorphism (AFLP- simplified protocol)



ELSEVIER

Postharvest Biology and Technology 17 (1999) 189–199

Postharvest Biology and Technology

www.elsevier.com/locate/postharvbio

Genetic diversity and biocontrol activity of *Aureobasidium pullulans* isolates against postharvest rots

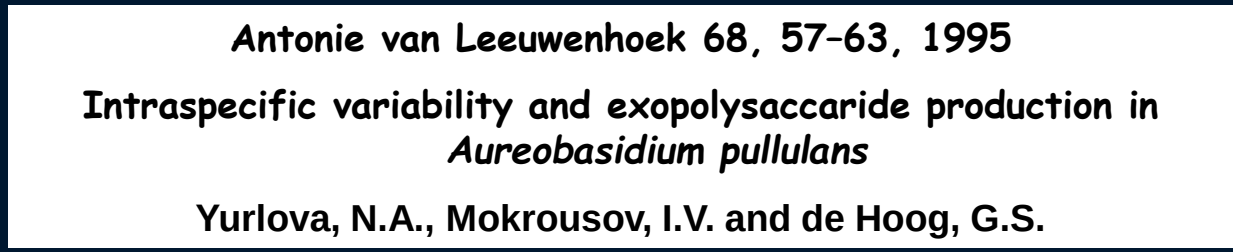
Leonardo Schena [a], Antonio Ippolito [a,*], Tirtza Zahavi [b], Lea Cohen [b], Franco Nigro [a], Samir Droby [b]

[a] *Dipartimento di Protezione delle Piante dalle Malattie, Università degli Studi di Bari, Via Amendola 165/A, 70126 Bari, Italy*
[b] *Department of Postharvest Science of Fresh Produce, Institute for Technology and Storage of Agricultural Products, ARO, The Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel*

Plant Disease / Vol. 86 No. 1 - 2002

Molecular Detection of Strain L47 of *Aureobasidium pullulans*, a Biocontrol Agent of Postharvest Diseases

Leonardo Schena, Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi di Bari; and **Mariella Finetti Sialer** and **Donato Gallitelli**, Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi di Bari and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Via Amendola 165/A, 70126 Bari, Italy



Antonie van Leeuwenhoek 68, 57–63, 1995

Intraspecific variability and exopolysaccaride production in *Aureobasidium pullulans*

Yurlova, N.A., Mokrousov, I.V. and de Hoog, G.S.

European Journal of Plant Pathology **109**: 341–349, 2003.

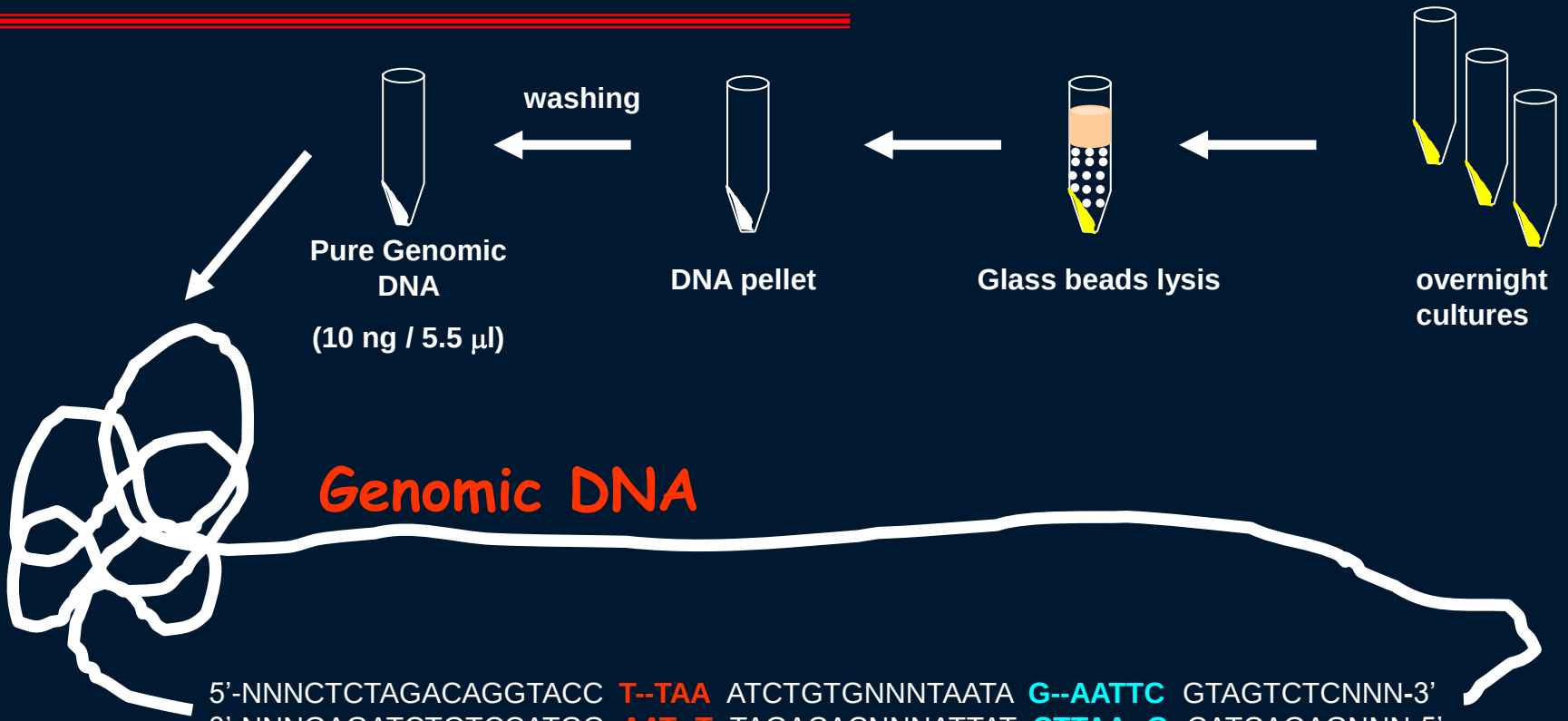
Integrated control of apple postharvest pathogens and survival of biocontrol yeasts in semi-commercial conditions

Giuseppe Lima, Filippo De Curtis, Raffaello Castoria and Vincenzo De Cicco

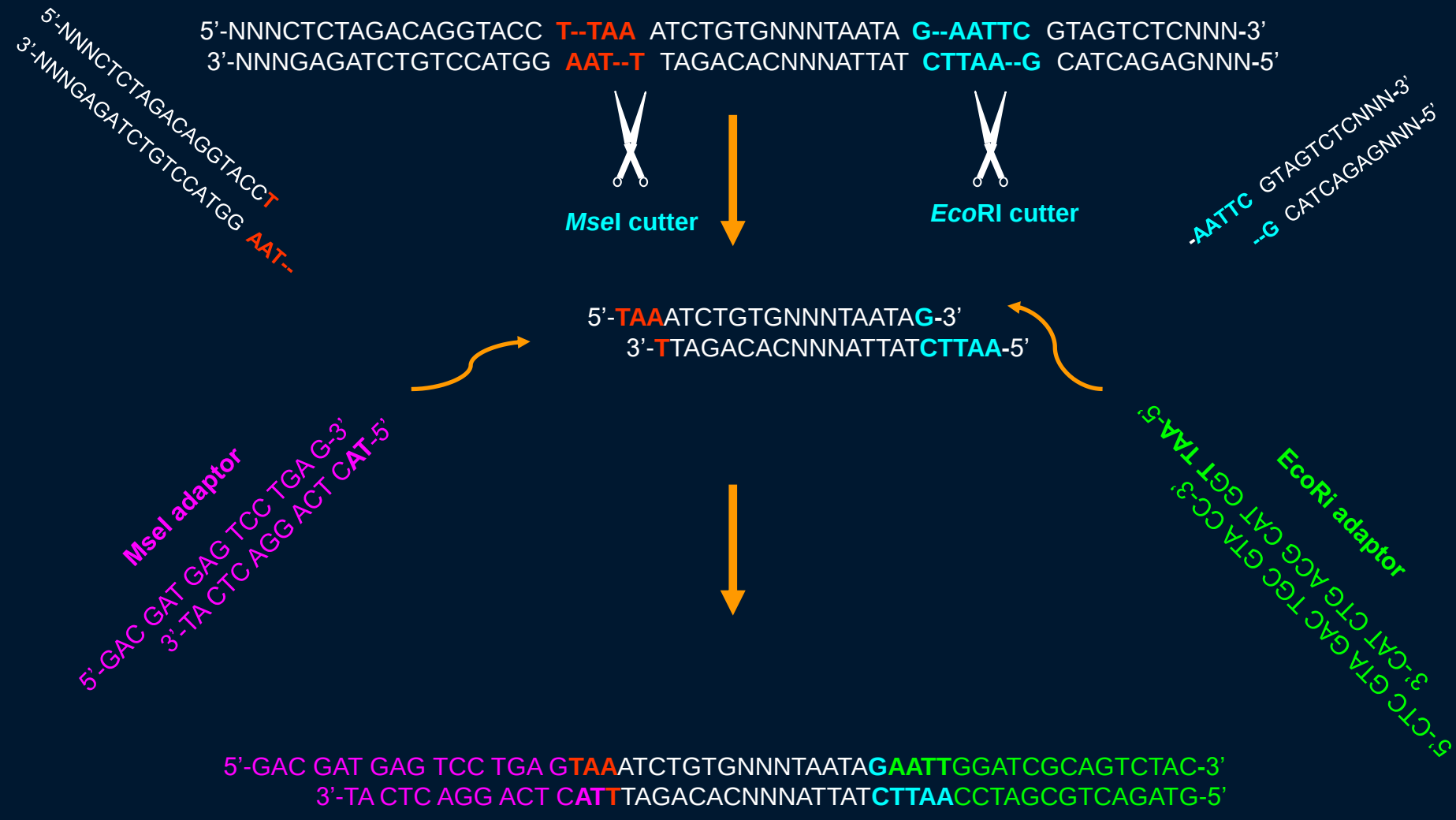
Forty-eight strains were subjected to molecular analysis for comparing their fingerprints

Strains [a]	Host
AU92	Almond tree
AU29, AU66, AU96, AU99, LS30	Apple tree
AU15/2, AU18-2A, AU58	Apricot tree
AU100	Barley
AU23, AU25, AU98	Cherry tree
DSM2404 [b]	Deteriorated army supplies
AU76	Figurative tree
AU20/1, AU28, AU34-2, AU62, AU72, AU74, AU82, AU104, AU111, LS200	Grapevine
AU33, AU69	Lemon tree
LS3, LS6	Mushroom decay
AU31-1, AU42/2	Oak
AU61, AU73, AU80, AU91, AU94, AU95, AU112, AU121	Olive tree
AU32/1	Orange tree
AU53, AU57, AU63	Pear tree
AU17/2, AU45/1, AU68, AU24	Plum tree
AU117	Sugar beet

DNA isolation



RESTRICTION - LIGATION

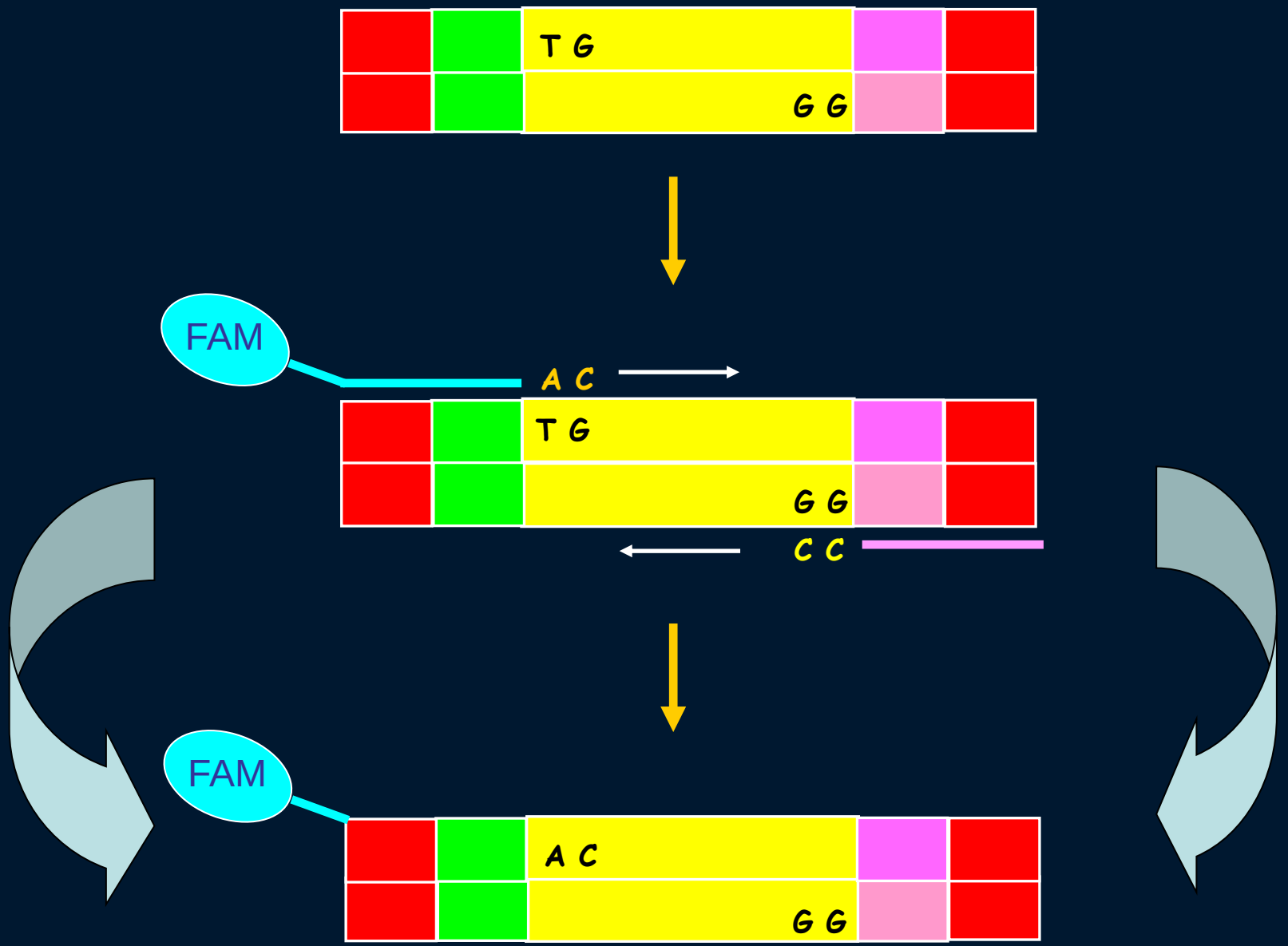


PRE-SELECTIVE PCR

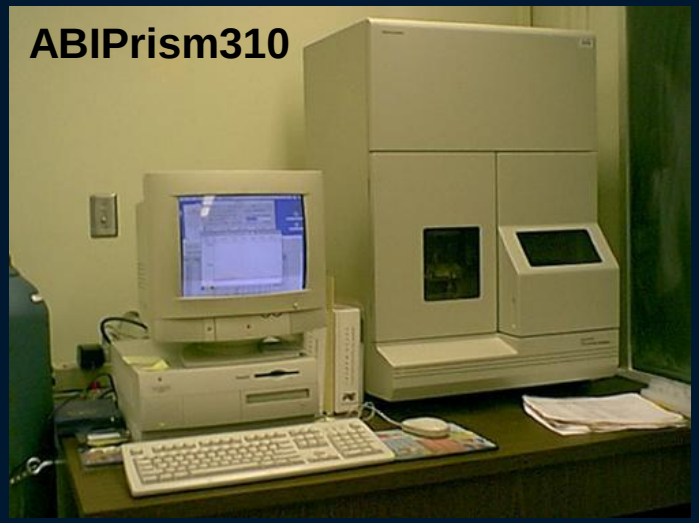
SELECTIVE PCR → *Primers used*

Selective *Eco*RI primers labelled with fluorescein-based fluorophores	Unlabelled selective *Mse*I primers	Sets
FAM 5'-GACTGCTACCAATTC**AC**-3'	5'-GATGAGTCCTGAGTAA**CC**- 3'	AC/CC
NED 5'- GACTGCTACCAATTC**AT**-3'	5'-GATGAGTCCTGAGTAA**CG**- 3'	AT/CG
FAM 5'-GACTGCTACCAATTC**AC**-3'	5'-GATGAGTCCTGAGTAA**CA**- 3'	AC/CA
JOE 5'-GACTGCTACCAATTC**G**-3'	5'-GATGAGTCCTGAGTAA**CT**- 3'	G/CT

SELECTIVE PCR → Highly stringent touchdown PCR

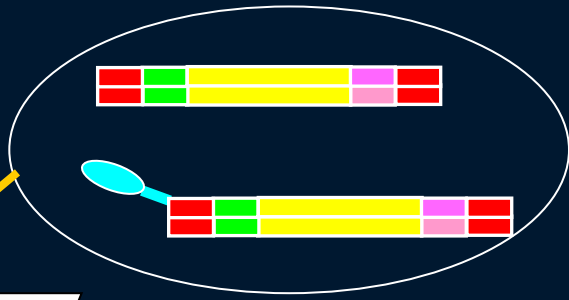


Automated capillary electrophoresis



deionised Formamide

Size standard
(GeneScan500)



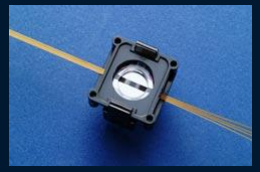
Electrophoresis mixture

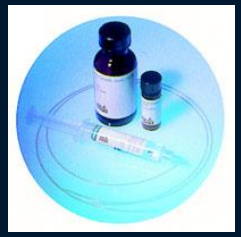
Denaturing

95 °C x 2 min

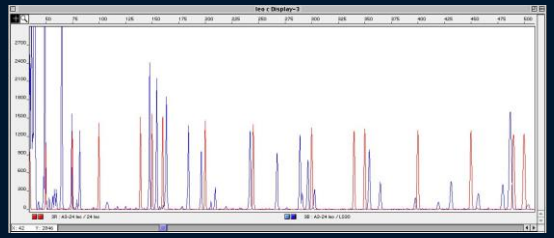
Rapidly cooling

Automated loading of Capillary electrophoresis system
filled with denaturing Performance Optimised Polymer 4 (POP-4)

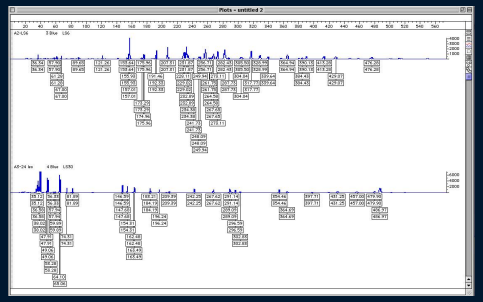




Detection

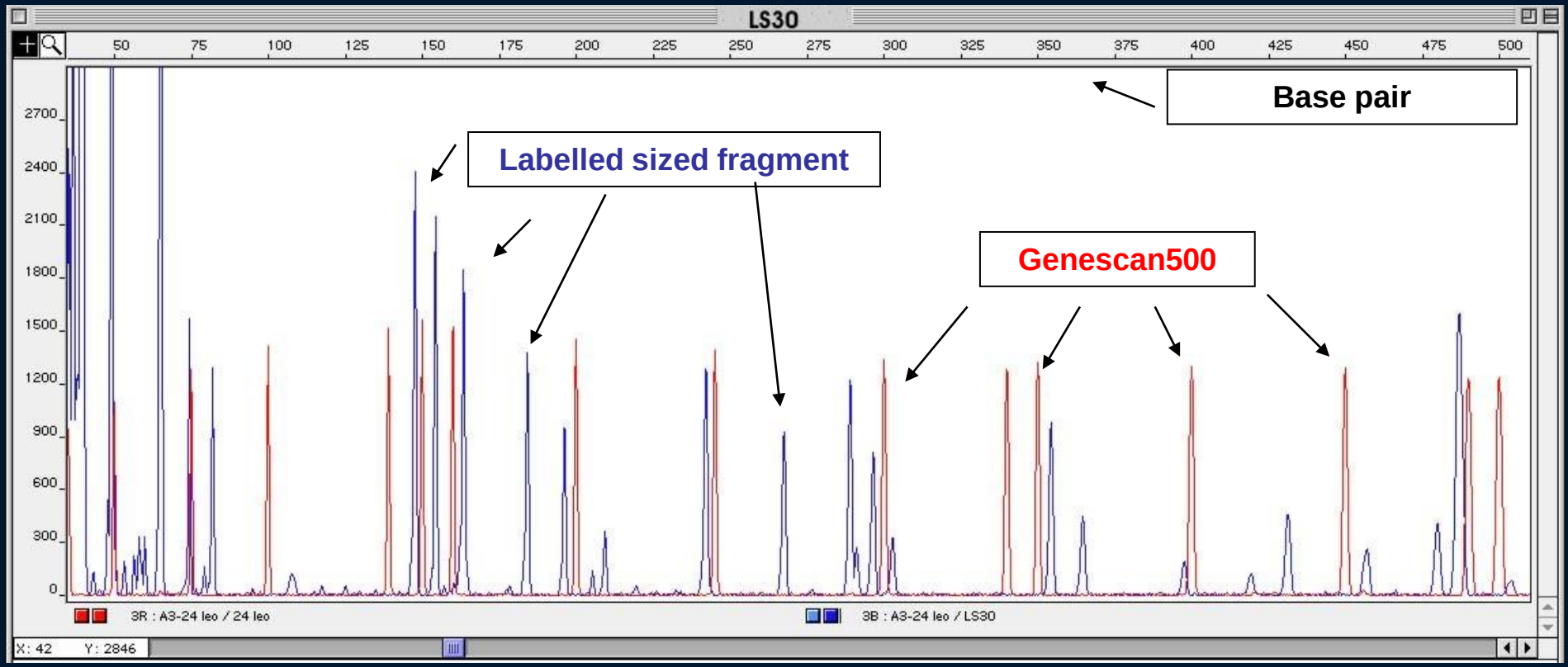


Fragment analysis



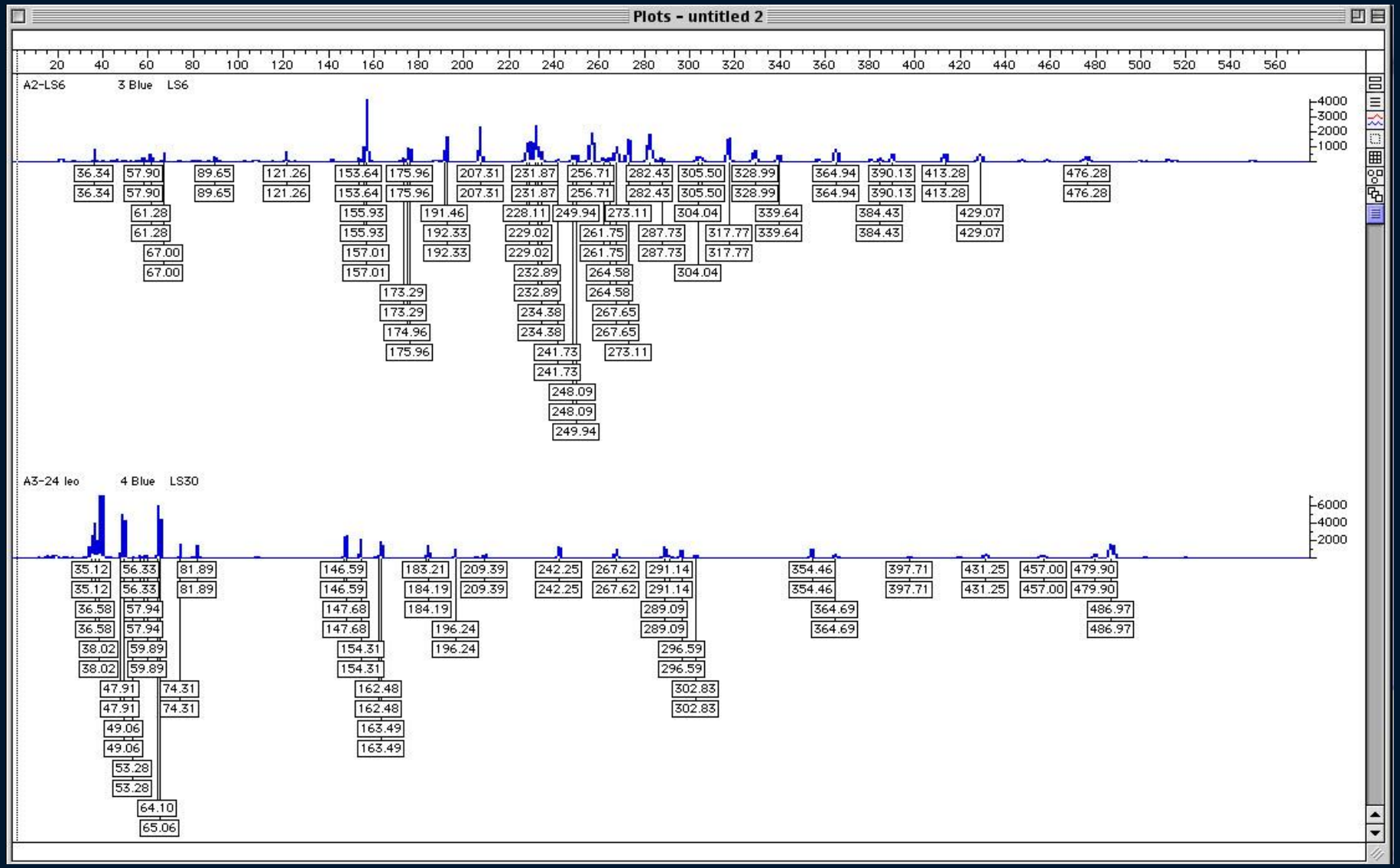
Fragment analysis

Genescan™ output



Data processing

Genotyper™ output



Data processing

0 = fragment absence

1 = fragment presence

Binary matrix

Sample Info	AU33	AU17-2	AU18-2A	DSM2404	LS3	AU54	AU111	AU66	AU73	AU91	AU100	LS30	AU25	AU58
38	0	0	0	0	0	0	1	0	0	0	0	0	0	0
39	0	0	0	0	0	0	0	0	0	0	0	0	1	1
42	1	0	1	1	1	0	0	1	0	0	1	1	0	0
44	0	0	0	1	0	0	0	0	0	0	0	0	0	0
46	0	0	0	0	0	0	0	0	0	0	0	0	0	0
47	0	0	1	0	0	0	0	0	0	0	0	0	0	0
48	1	0	1	1	1	0	0	1	0	1	0	1	0	0
50	0	0	0	0	0	0	0	0	0	1	0	0	0	0
51	1	0	0	1	1	0	0	0	0	0	0	0	0	0
52	0	0	0	0	0	0	0	0	0	0	1	0	0	0
54	1	1	1	1	1	1	1	1	1	1	1	1	1	1
55	0	0	0	0	0	0	1	0	1	1	0	0	1	0
56	1	0	0	0	0	0	1	0	1	1	0	0	1	0
57	0	0	0	0	0	0	1	0	0	0	1	0	0	0
59	0	0	0	0	0	0	1	0	0	0	1	0	0	0
60	0	0	0	0	0	0	1	0	0	0	0	0	0	0
63	0	0	0	0	0	1	0	0	1	1	0	1	0	0
64	0	1	0	0	0	1	1	0	0	0	1	1	0	1
72	1	1	1	1	1	1	1	1	1	1	1	1	1	1
74	0	0	1	0	0	0	0	0	0	0	0	0	1	0
77	1	1	1	1	1	1	1	1	0	0	1	1	1	1
80	0	0	1	0	0	0	0	0	0	0	0	0	0	0
82	0	0	0	0	0	0	0	0	0	0	0	0	0	1
84	0	1	0	0	0	0	0	1	0	0	0	0	1	1
86	0	0	0	0	0	0	0	1	0	0	1	0	0	0
88	1	1	1	1	1	1	1	1	0	0	1	1	1	1
91	0	0	0	0	0	0	0	0	0	1	0	0	0	0
94	0	0	0	0	0	0	0	0	1	0	0	0	0	0
98	0	0	1	0	0	0	0	0	0	0	0	0	0	0
100	0	0	0	0	0	0	0	0	0	0	0	0	0	0
102	1	0	0	0	0	0	0	0	1	1	0	1	0	0

Sizes (bp)

Foglio1 / Foglio2 / Foglio3

Comparison of fAFLP patterns of four strains

Very different

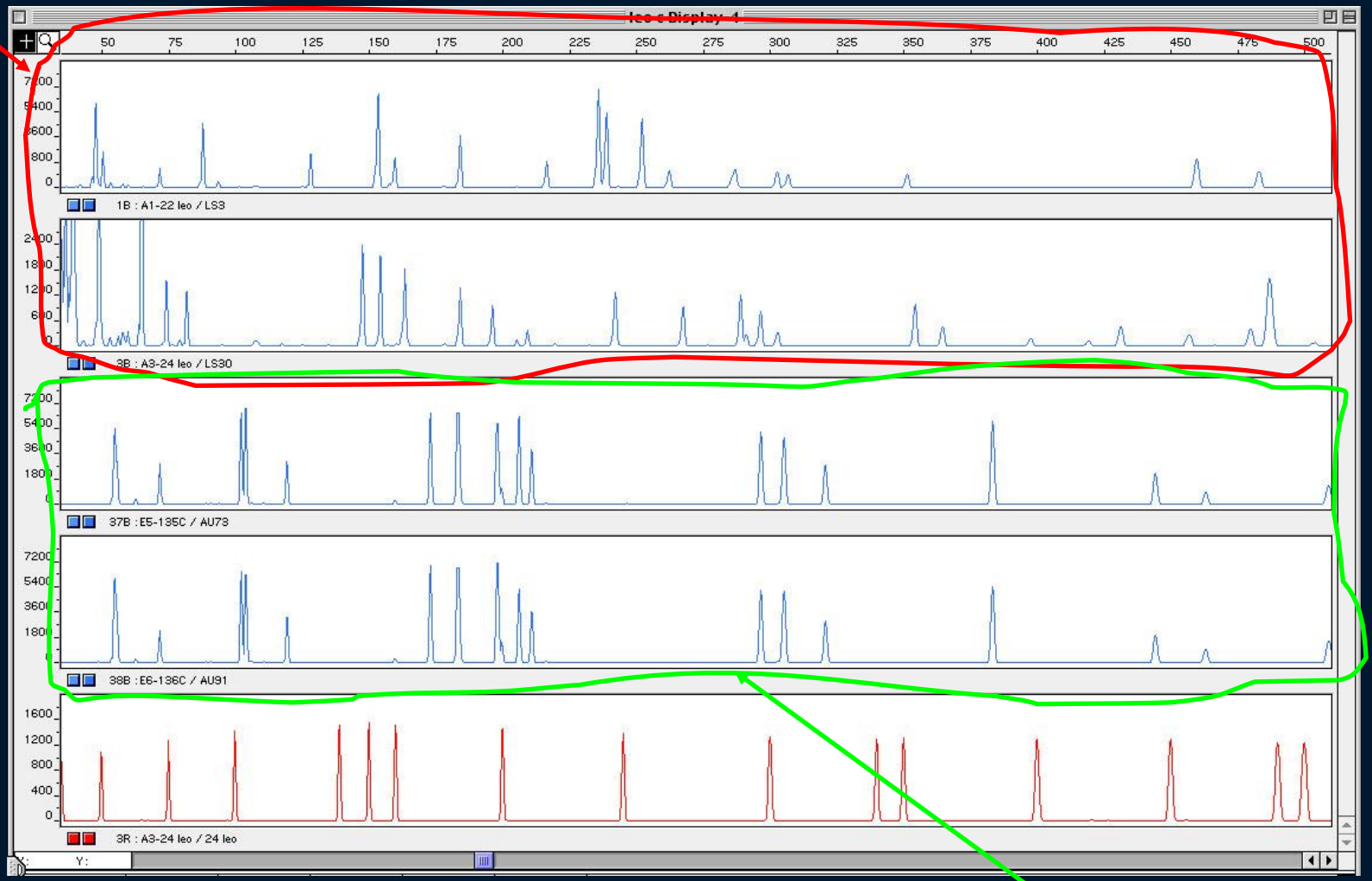
LS3

LS30

AU73

AU91

GeneScan500



Very similar

Table 2. Minimum (Min) and maximum (Max) number of DNA sized fragments yielded from each selective primer combination used in fAFLP analysis on forty-eight isolates of *Aureobasidium pullulans*. Sized fragments were strain-specific (i.e. belonging to only one isolate) or common (i.e. shared by at least two isolates).

	Number of sized fragments		
Primers	Min	Max	Strain-specific	Common
FAM-*EcoR*I–AC and *Mse*I–CC	8	28	38	33
NED-*EcoR*I–AT and *Mse*I–CG	7	35	39	38
FAM-*EcoR*I–AC and *Mse*I–CA	18	44	45	37
JOE-*EcoR*I–G and *Mse*I–CT	5	86	29	42

Table 3. Sizes of strain-specific fluorescent fragments generated by fAFLP analysis with four selective primers on forty-eight *Aureobasidium pullulans* isolates.

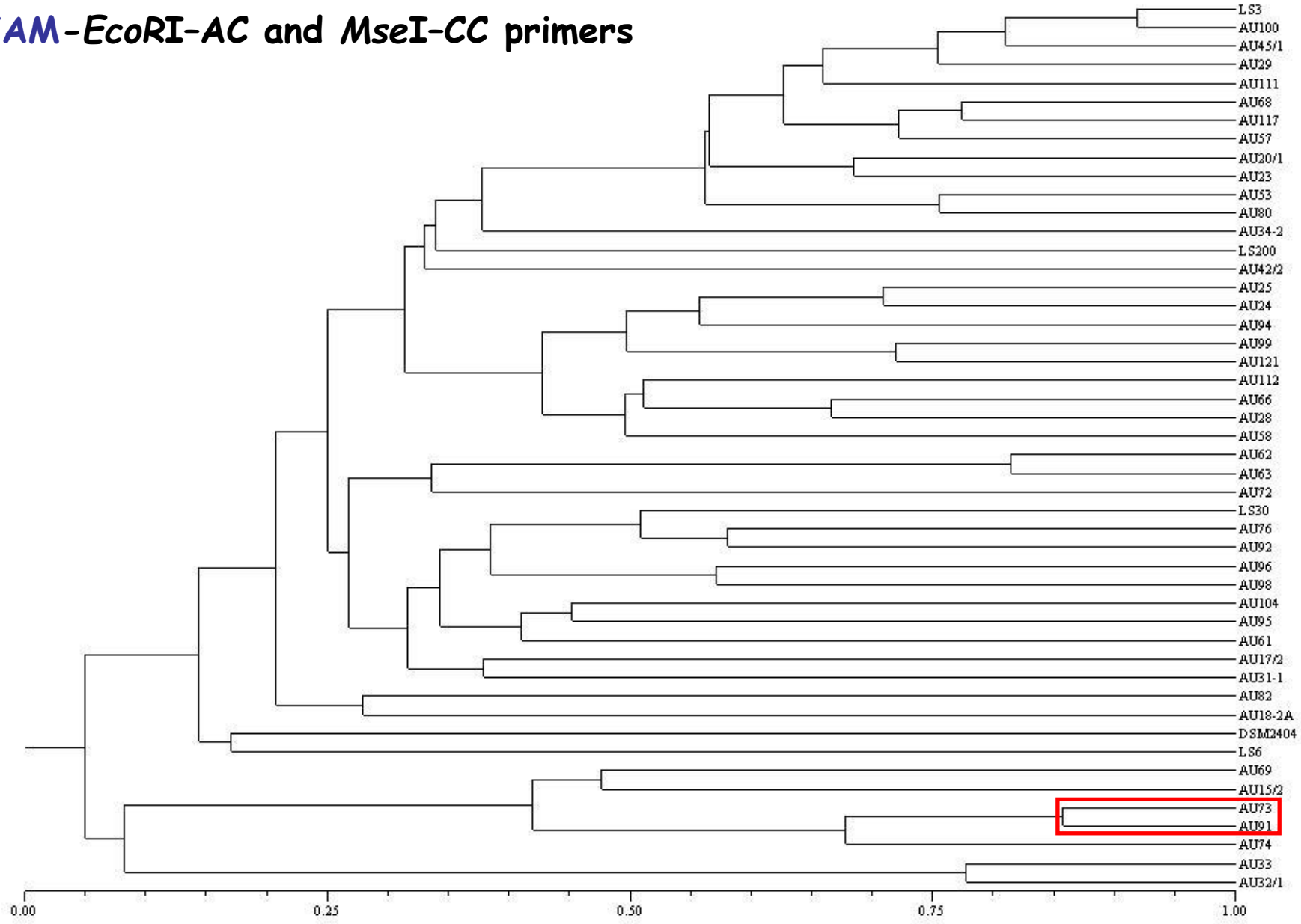
	Strain-specific fragments generated by fAFLP selective primers[a,b]			
Strains	**AC/CC[c]**	**AT/CG**	**AC/CA**	**G/CT**
AU15/2	ND	ND	150, 348, 361, 372, 377, 417	ND
AU17/2	178, 312	483	498	
AU18-2A	381, 403, 420	66, 393	376, 490	226, 403, 496
AU20/1	ND	449	ND	282, 479
AU23	ND	ND	407	ND
AU24	ND	ND	84	139, 159, 324
AU31-1	359	256	ND	237, 408
AU32/1	ND	379	ND	391
AU34-2	314, 374, 491	228	194, 380	ND
AU33	430	ND	201, 280, 328, 387, 425, 492	378
AU42/2	323, 350, 412	103, 396, 485	ND	277
AU45/1	ND	464	342, 356	428
AU53	ND	226, 469	ND	399
AU62	245, 247	ND	ND	ND
AU66	422	ND	ND	339
AU68	251	143	ND	ND
AU69	73, 144, 330	264	165, 277	301

Strain- specific sized fAFLP fragments of LS30

	Strain-specific fragments generated by fAFLP selective primers[a,b]			
Strains	**AC/CC[c]**	**AT/CG**	**AC/CA**	**G/CT**
AU72	103	ND	203	388
AU73	187	ND	ND	ND
AU74	276	ND	105	ND
AU76	70	ND	138	211
AU80	ND	ND	92	ND
AU82	ND	94, 98, 192	ND	38, 314
AU92	ND	437	ND	ND
AU94	101, 196	338, 415, 482	ND	ND
AU95	ND	ND	ND	477
AU98	ND	267	335	ND
AU104	385	178, 235	367	405
AU111	442	223	ND	ND
AU112	100	140	ND	ND
AU117	ND	346	ND	261
DSM2404	83, 213, 287, 292, 407, 464	148, 232, 258, 265, 269, 271, 272, 377, 443, 448	108, 111, 112, 123, 224, 225, 275, 334, 375, 500	235, 327, 471
LS6	299, 340	ND	248	227, 373
LS30	ND	ND	**291, 398, 431, 457**	ND
LS200	106	ND	420	ND

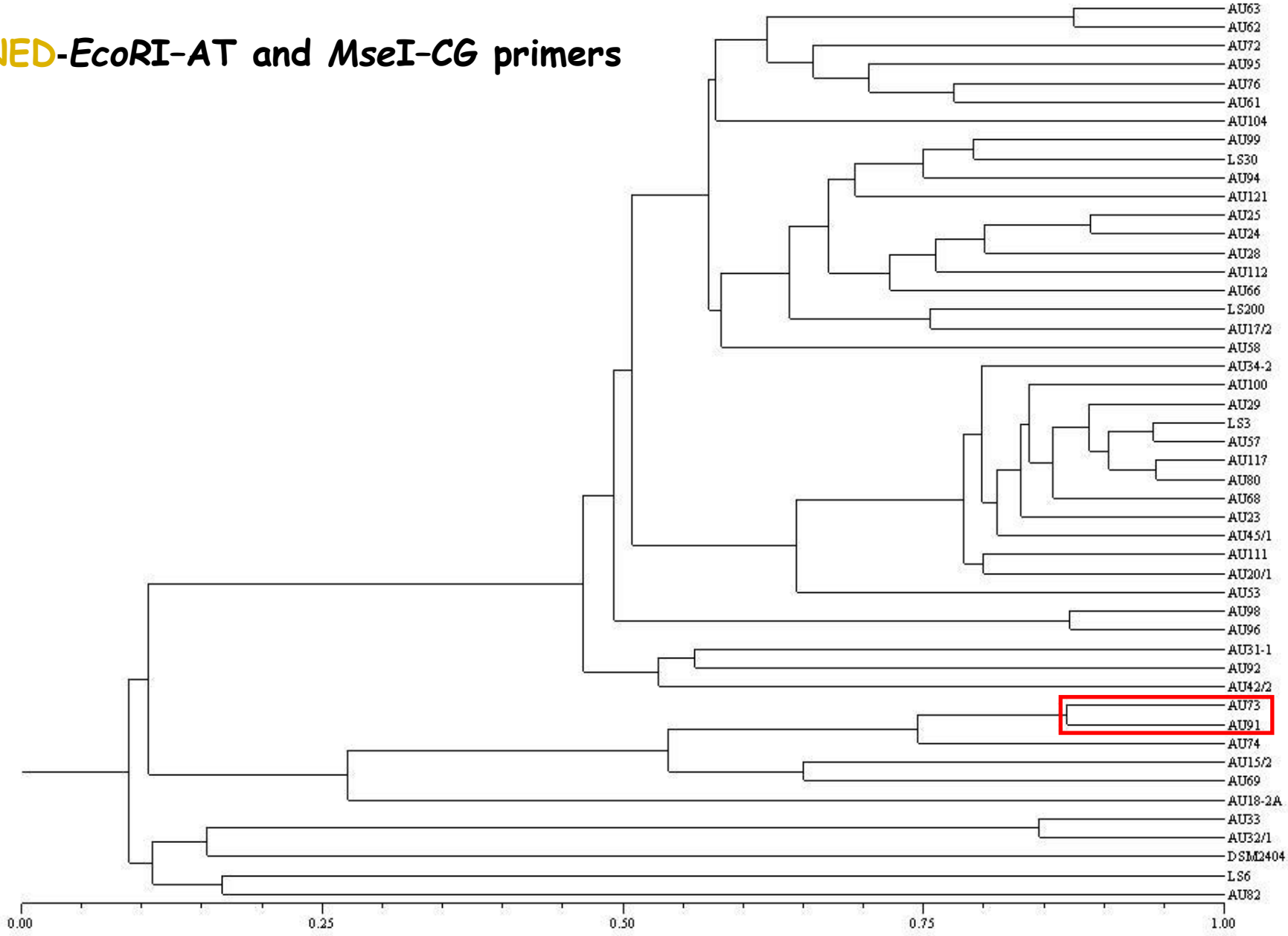
Clustering with the unweighted pair group method by using average (UPGMA) linkages

FAM-*EcoRI-AC* and *MseI-CC* primers

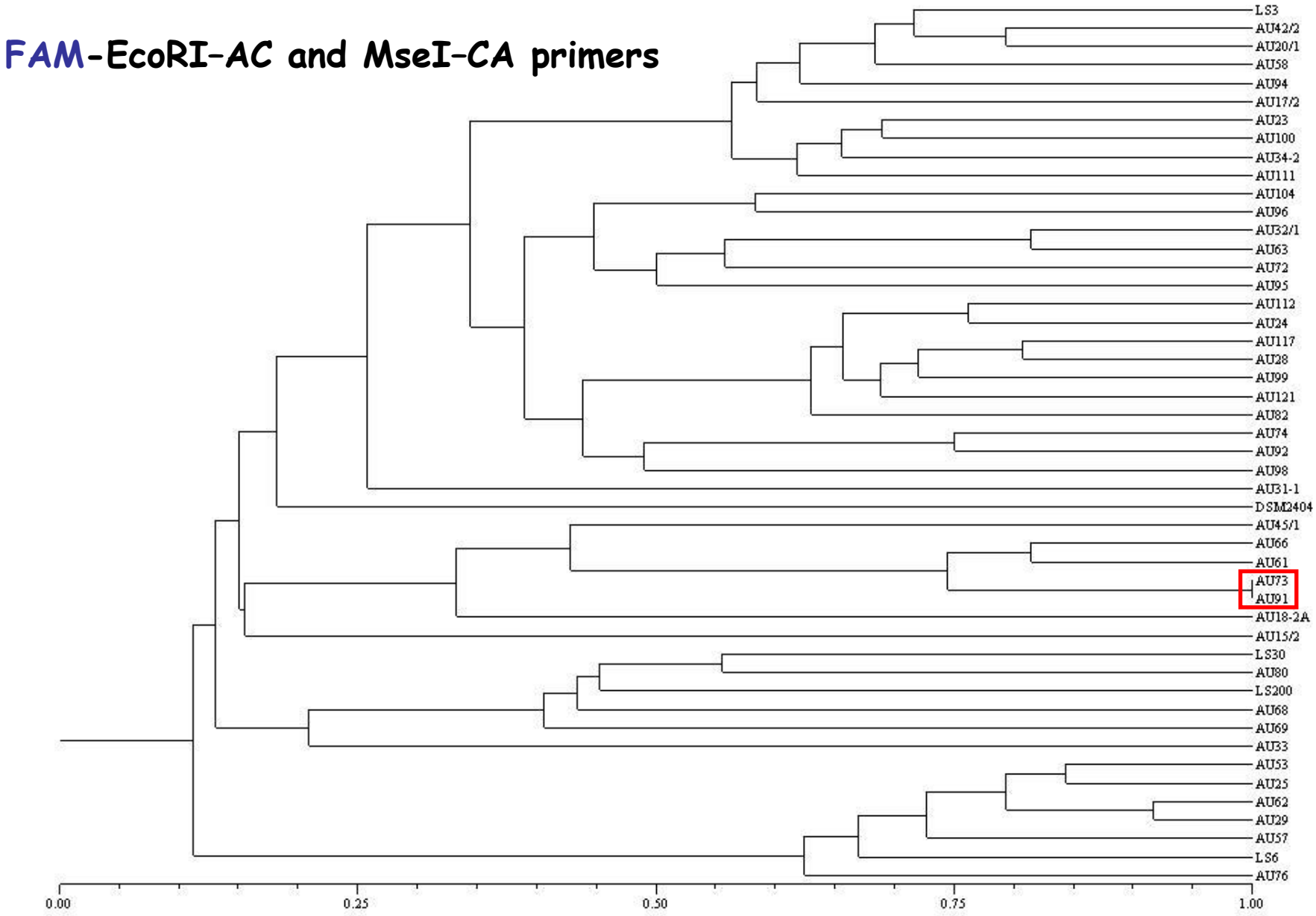


Clustering with the unweighted pair group method by using average (UPGMA) linkages

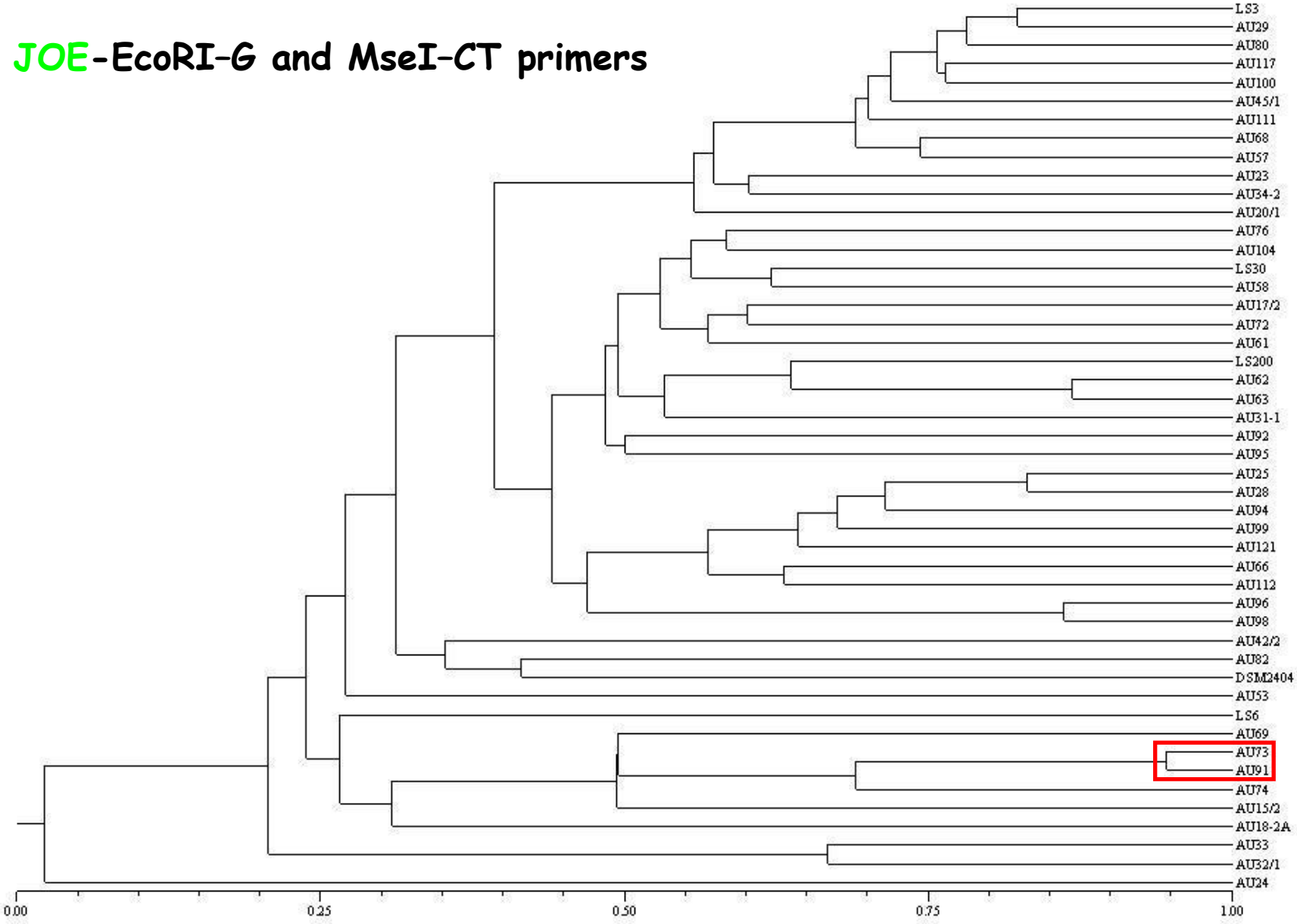
NED-*EcoRI*-AT and *MseI*-CG primers



Clustering with the unweighted pair group method by using average (UPGMA) linkages



Clustering with the unweighted pair group method by using average (UPGMA) linkages



Results

Fragment analysis

More than five thousands highly distinguishable peaks (DNA fragments) were generated by fAFLP of all tested *A. pullulans* isolates.

The separate utilisation of the four primer pairs has allowed the detection of at least one specific fragment for thirty-six out of forty eight isolates (75%).

Selective set of primers *FAM*-EcoRI–AC and MseI–CA produced efficiently forty-five strain-specific fragments from all isolates, whereas *JOE*-EcoRI–G and MseI–CT primers yielded the widest range and the highest number of the shared fragments (forty-two).

No fragment was common to all isolates, whatever the primer pair used in the analyses.

Strain LS30, in particular, displayed four specific fragments only with primers AC/CA, but no specific fragment was detected with the other pairs of primers.

Results

Cluster analysis

The pairwise comparison of fingerprints of different isolates with similar Dice coefficient gave rise to four dendrograms, one for each couple of primers, grouping the isolates according to similarity level.

Each dendrogram showed a high variability degree among all tested strains.

Each dendrogram contained two main clusters with similarity level ranging from 0.02 (primers G/CT) to 0.10 (primers AC/CA).

In all dendrograms, the larger main clusters contained about 90% of the isolates and the pairwise comparison of the different fingerprints scored similarity levels ranging between 0.02 (primers G/CT) and 1 (primers AC/CA).

Only isolates AU73 and AU91 were constantly grouped together according to a similarity level ranging between 0.86 and 1.

Conclusions

The application of automated fAFLP technique allowed us to obtain accurate DNA fingerprints with many highly distinguishable and reliable polymorphisms.

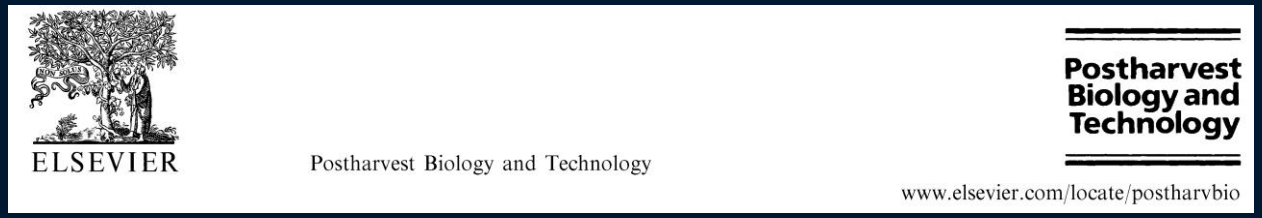
The cluster analysis showed a high variability degree among all tested strains is in agreement with other investigations performed on other strains of *A. pullulans* and with highly eterocaryotic condition of this species

The biocontrol strain LS30 showed a fingerprints with four different strain-specific fragment.

Future goals

Purifying and cloning the fAFLP fragments specific for several *A. pullulans* strains (such as LS30) for developing for developing specific probes or primers for sequence-characterized amplified region (SCAR).

This work was submitted for publication to following journal:

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