Institute of Sciences of Food Production
ISPA-CNR, Bari - Italy

Biodiversity of *Aspergillus* Sect. *Nigri* from grapes in Europe

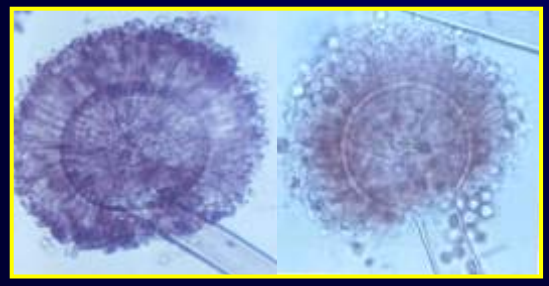
Giancarlo Perrone

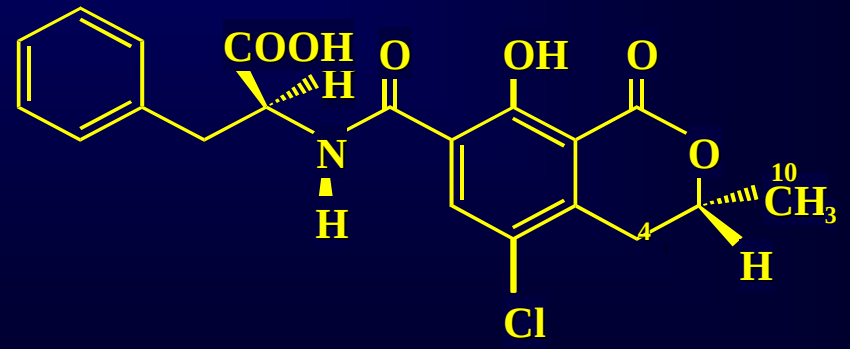
Aspergillus systematics in the genomic era

An international workshop

Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands

12-14 April 2007





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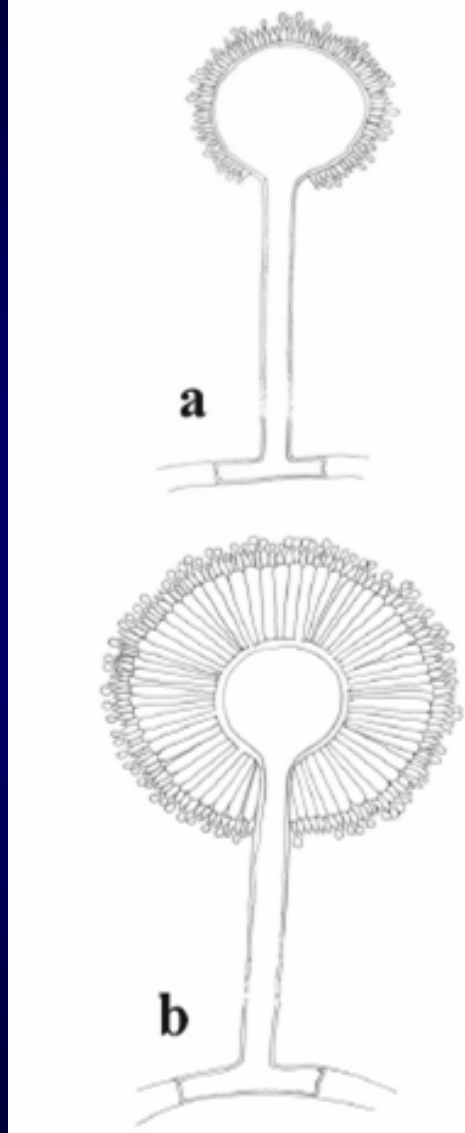
Main common species of the *Nigri* Section

Aspergillus niger is the most frequently reported species in this section.

However, species concepts are uncertain in this complex and otfen the name *A. niger* has been used for any member of the section due to misidentification.

The morphology characters are very similar, and it is difficult to discriminate within the *A. niger* aggregate group.



Species concepts of black aspergilli according to different authors

Raper and Fennell (1965)	Al-Musallam (1980)	Kozakiewicz (1989)	RLFP* analysis	Samson et al. 2004
A. japonicus	*A. japonicus* var. *japonicus*	*A. japonicus*	*A. japonicus*	*A. japonicus*
A. aculeatus	*A. japonicus* var. *aculeatus*	*A. atroviolaceus*	*A. aculeatus*	*A. aculeatus*
A. carbonarius	*A. carbonarius*	*A. carbonarius*	*A. carbonarius*	*A. carbonarius*
		A. fonsecaeus		
A. heteromorphus	*A. heteromorphus*	*A. heteromorphus*	*A. heteromorphus*	*A. heteromorphus*
A. ellipticus	*A. ellipticus*	*A. ellipticus*	*A. ellipticus*	*A. ellipticus*
	A. helicothrix	*A. helicothrix*		
		***A. niger* aggregate:**		
A. niger	*A. niger* var. *niger*	*A. niger* var. *niger*	*A. niger*	*A. niger*
A. tubingensis	*A. niger* var. *niger f. hennebergii*	*A. niger* var. *tubingensis*	*A. tubingensis*	*A. tubingensis*
A. phoenicis	*A. niger* var. *phoenicis*	*A .niger* var. *phoenicis*	*A. foetidus*	*A. foetidus*
A. polverulentus	*A. niger* var. *phoenicis f. polverulentus*	*A.niger* var. *polverulentus*	*A. brasiliensis*	*A. brasiliensis*
A. awamori	*A. niger* var. *awamori*	*A. niger* var. *awamori*		*A. costaricaensis*
A. ficuum	*A. niger* var. *nanus*			*A. homomorphus*
A. foetidus	*A. niger* var. *usamii*	*A. niger* var. *ficuum*		*A. lacticoffeatus*
A. foetidus var. *pallidus*	*A. niger* var. *intermedius*	*A. citrus* var. *citrus*		*A. piperis*
A. foetidus var. *acidus*	*A. foetidus*	*A. acidus*		*A. sclerotioniger*
		A. citrus var. *pallidus*		*A. vadensis*

*Results of various RLFP analysis by different authors: Kusters-van Someren et al. (1991); Megnegneu et al (1993); Varga et al (1993, 1994); Accensi et al. (1999); Parenicova et al (1997, 2001)

Phylogenetic tree of *Aspergillus* sub-genus *Circumdati* based on rDNA sequences

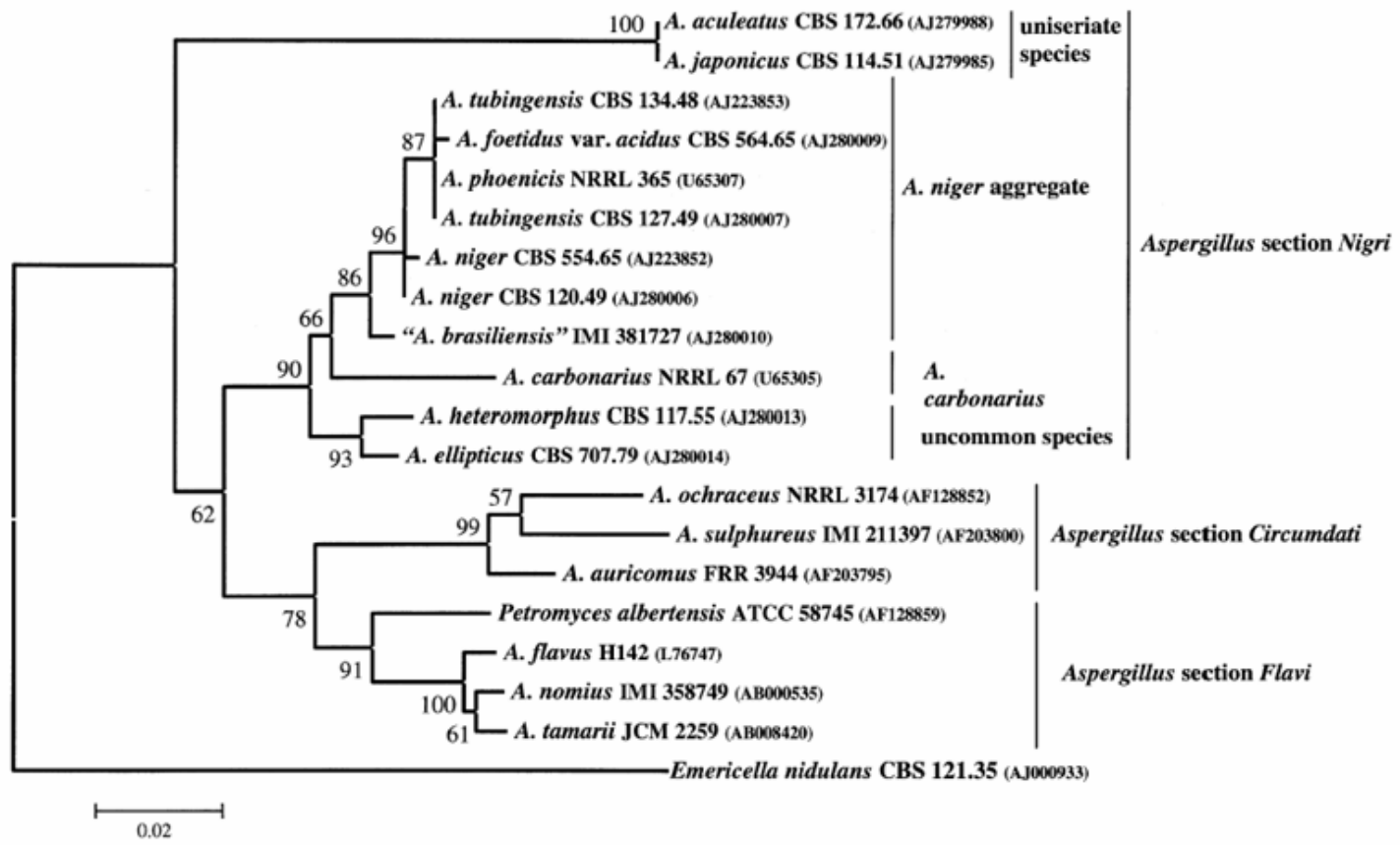
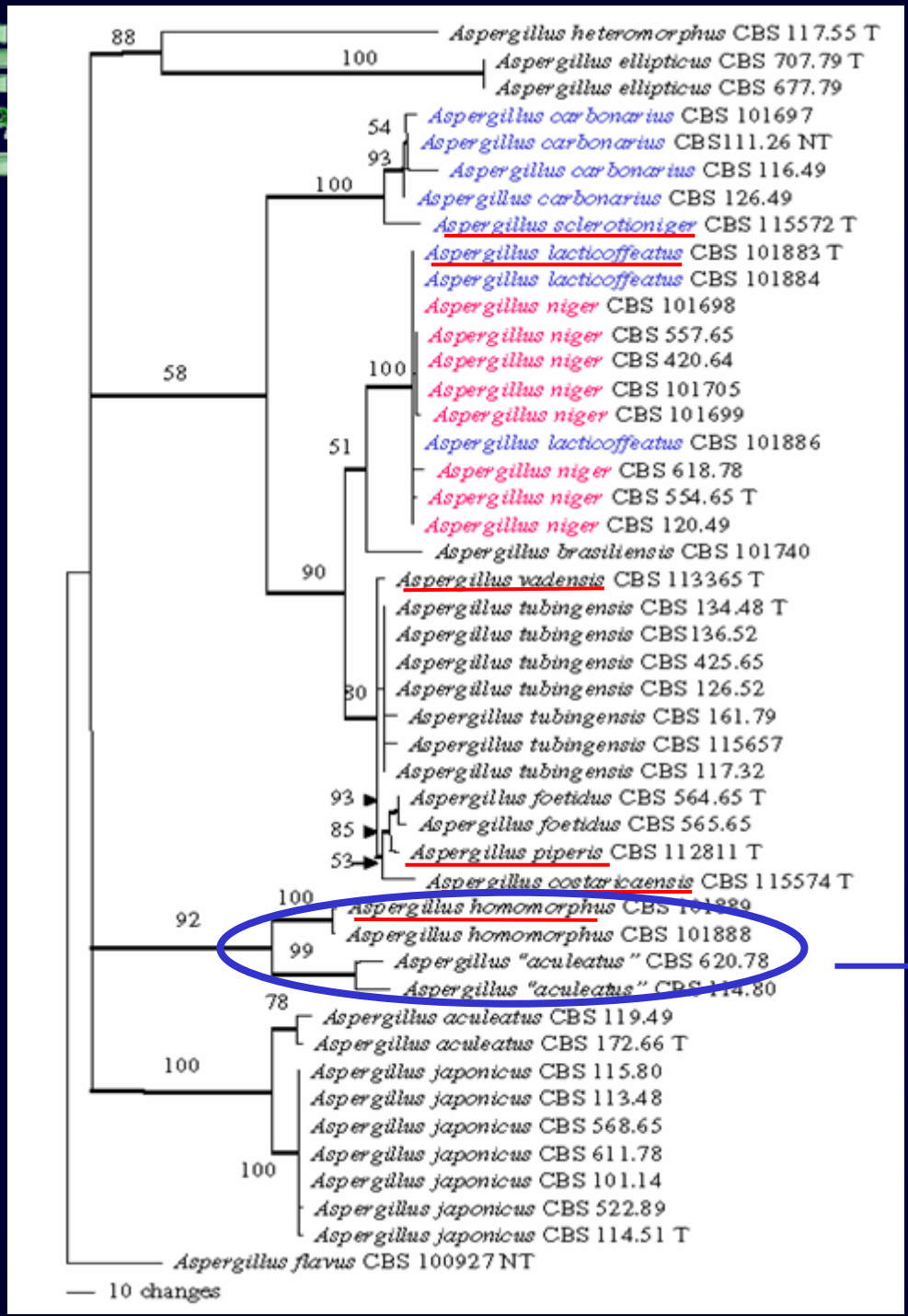


Figure 2. Neighbor-joining tree based on phylogenetic analysis of the ITS1-5.8S rRNA gene-ITS2 sequences. The sequences were aligned with Clustal W (version 1.5) of multiple sequence alignment computer program (Thompson et al. 1994). Adjustments for improvement were made by eye where necessary. Cladistic analyses using the Neighbour-joining method (Saitou and Nei 1987) were performed with the MEGA 2.1 computer program (Kumar et al. 2001) with Kimura 2-parameter model, including transitions and transversions and with pairwise deletion for the treatment of the handling gaps/missing data. Confidence values for individual branches were determined by bootstrap analyses (1000 pseudoreplicates).



Phylogenetic tree obtained by beta-tubulin sequence analysis by recent revision of the black *Aspergilli* species (Samson et al. 2004)

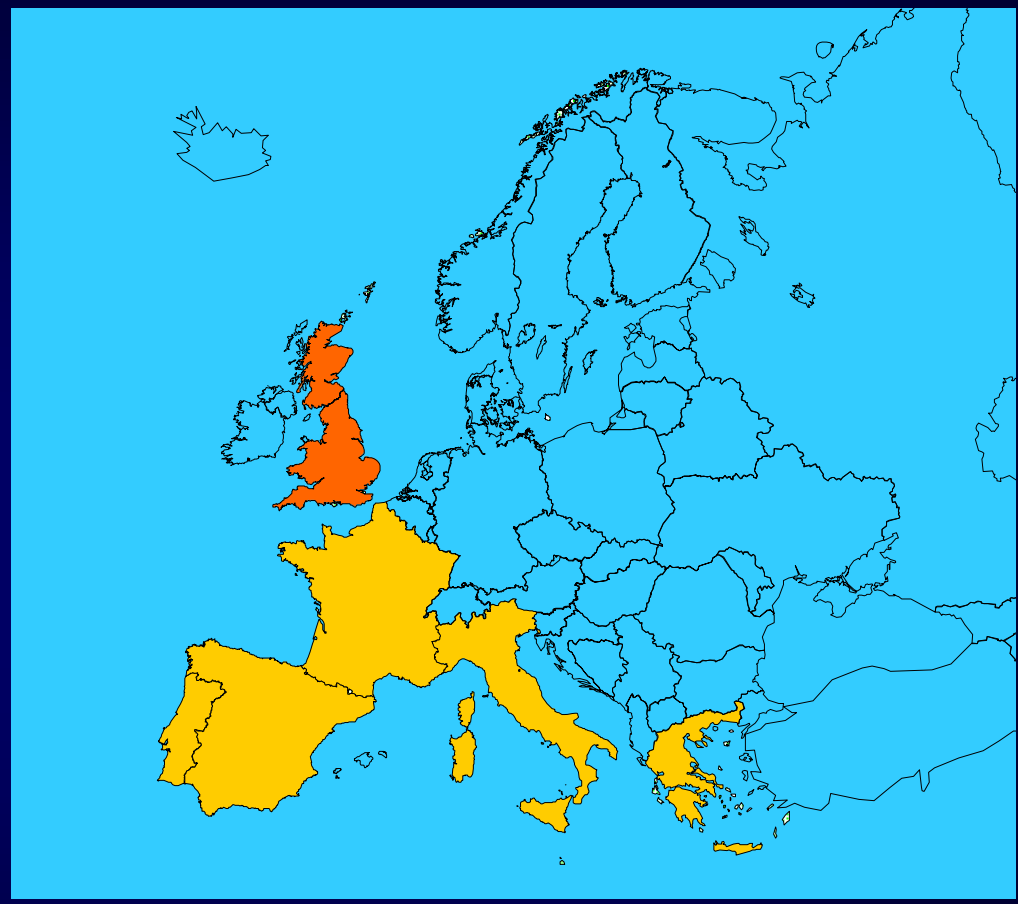
6 new species

blue OTA producer

red possible OTA producer

Atypical clade containing both biseriate and uniseriate species

Risk assessment and intergrated ochratoxin A (OTA) management in grape and wine



WINE-OCHRA RISK

(QLK1-CT-2001-01761)

2001 – 2005

http://www.ochra-wine.com

- 7 Countries
- 12 Partners
- 10 Research Center
- 1 Institute of Applied Research
- 1 Cooperative of wine maker

Evidence of contamination by black aspergilli on grapes

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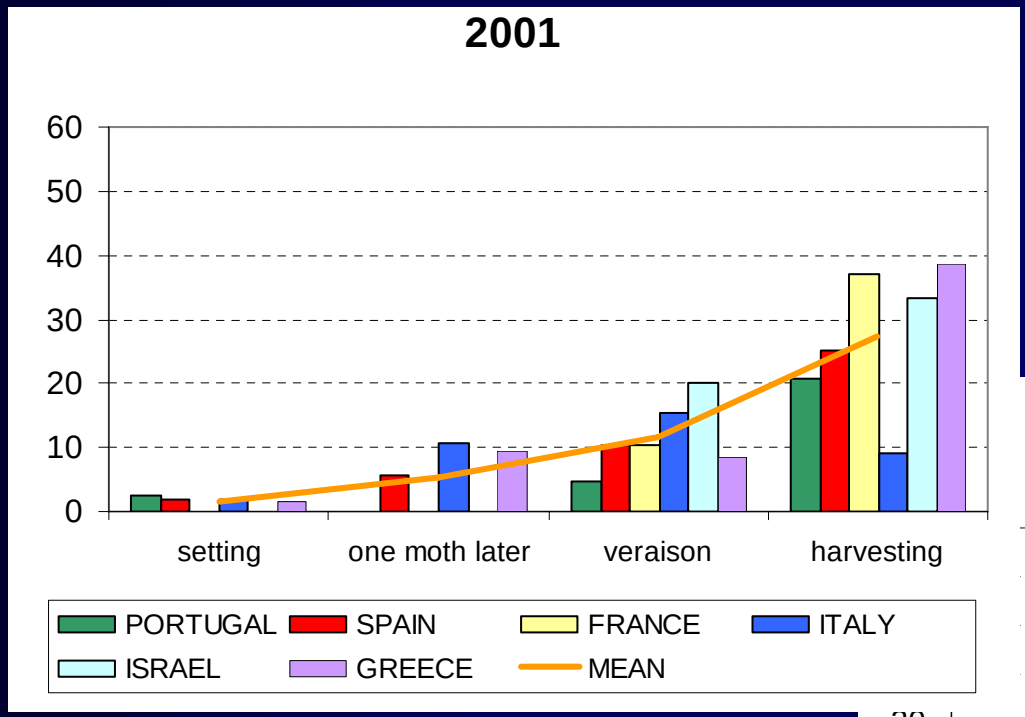
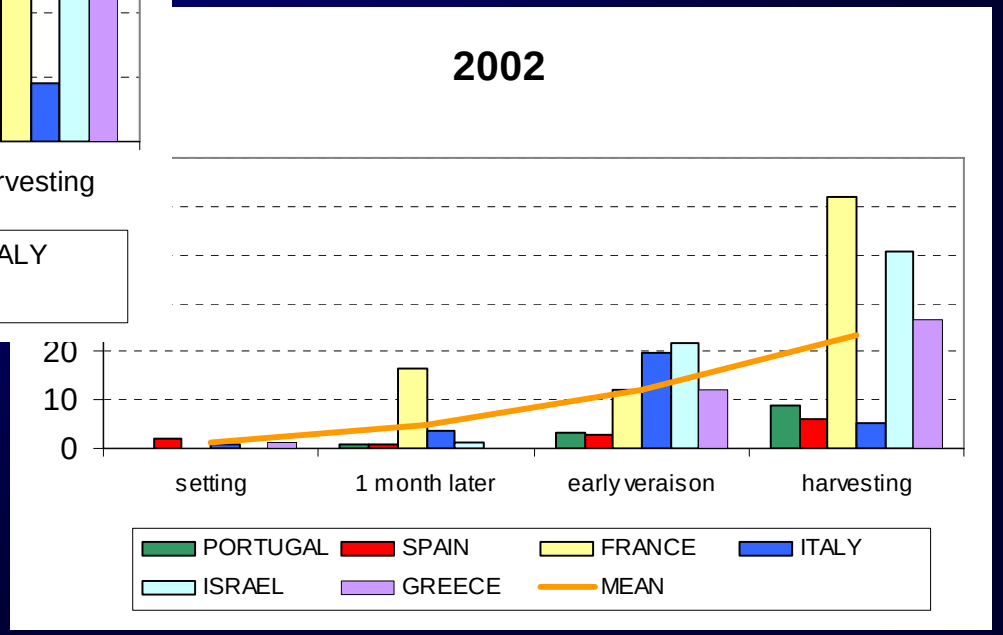
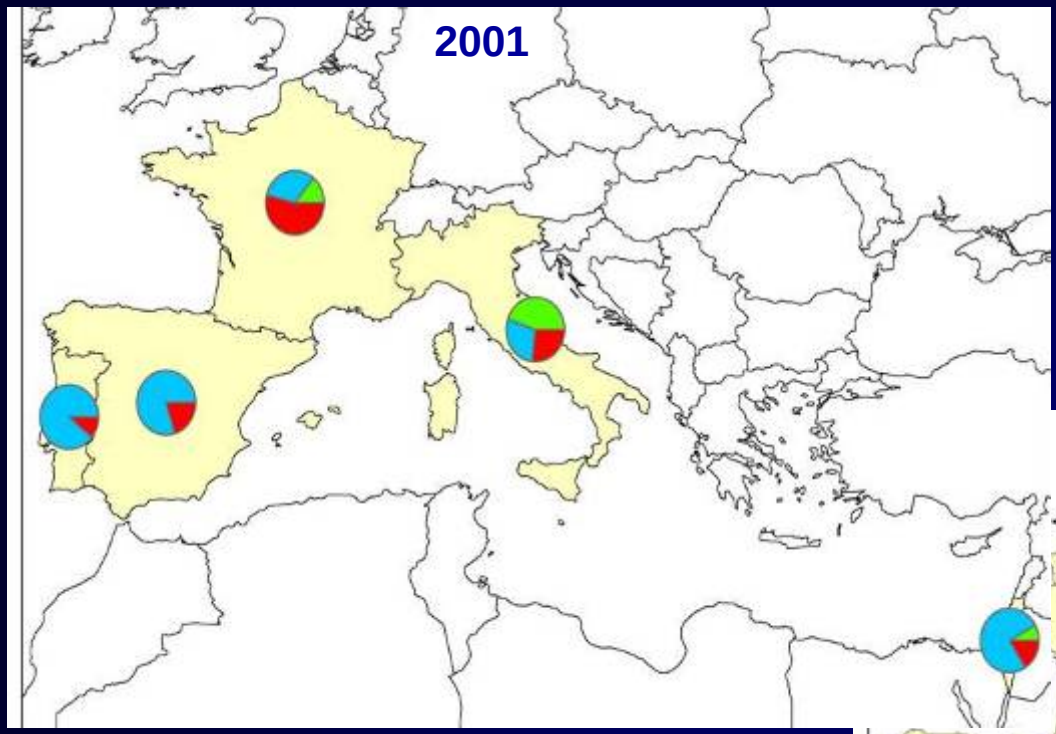
Distribution of *Aspergillus* Sez. *Nigri* species in Europe in various phenologic stages of grapes growing

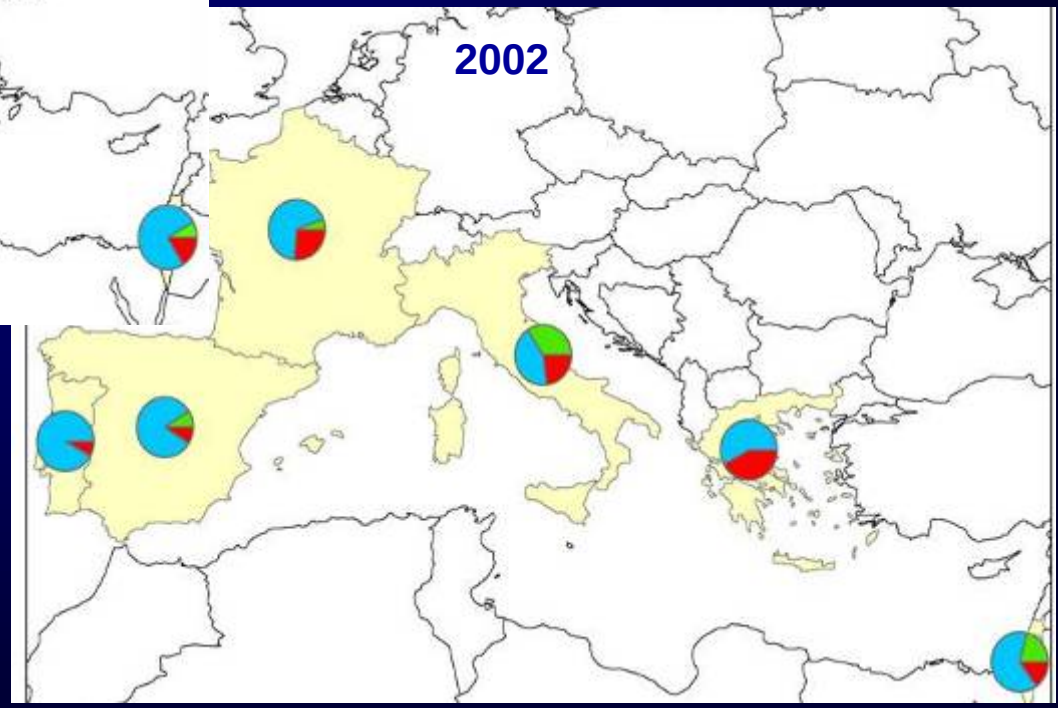




Data from EU project WINE-OCHRA RISK (QLK1-CT-2001-01761)

Distribution in Europe of Sez. *Nigri* strains in 2001-2002





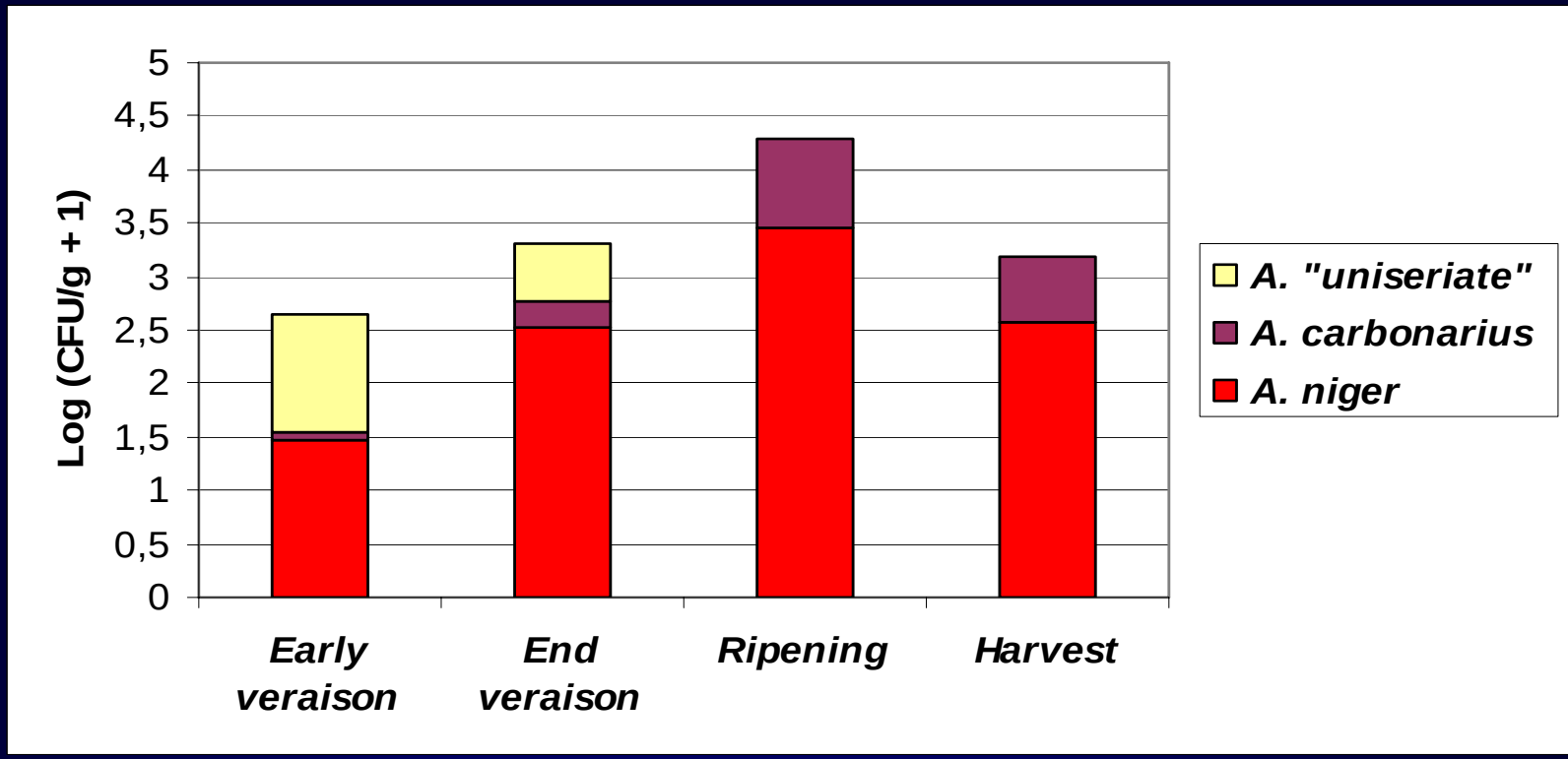
***A. niger* "aggregate"**

A. carbonarius

***Aspergillus* "uniseriate"**

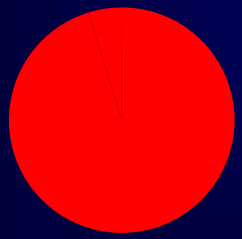
Data from EU project WINE-OCHRA RISK (QLK1-CT-2001-01761)

Distribution of black aspergilli in Apulian vineyards 2004-2006

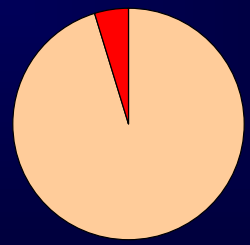


211 *A. carbonarius* strains

360 *A. niger* "aggregate" *strains*



OTA producers



MIN 0.5 ppb **MAX** 7500 ppb

MIN 0.3 ppb **MAX** 459 ppb

Data from Italian Ministerial Project "SIVINA" n 12818

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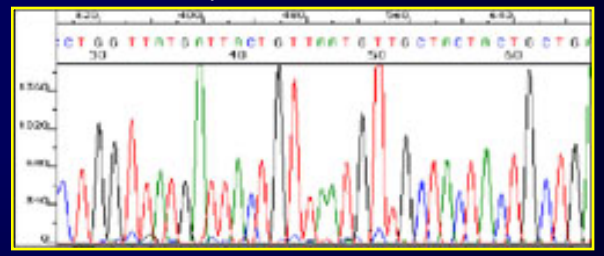
Molecular studies

Members of *Aspergillus* belonging to section *Nigri* resulted the main responsible for the ochratoxin A accumulation in grapes and wine, particularly in Southern Europe.

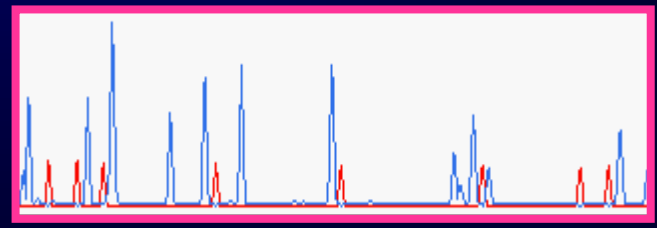
Limited information is available on the species composition and genetic variability of black Aspergilli strains occurring on grapes.

We analyzed more than 300 representative strains from the main wine producing European countries collected in 2001-2002 (Italy, France, Spain, Portugal, Greece and Israel) using amplified fragment length polymorphisms (AFLP) technique and calmodulin sequences.

- **Sequence analyses of different loci: ribosomal DNA regions, calmodulin and β-tubulin gene**



- **fAFLP method**



Black aspergilli strains, isolated from grapes in Europe, and used for molecular studies

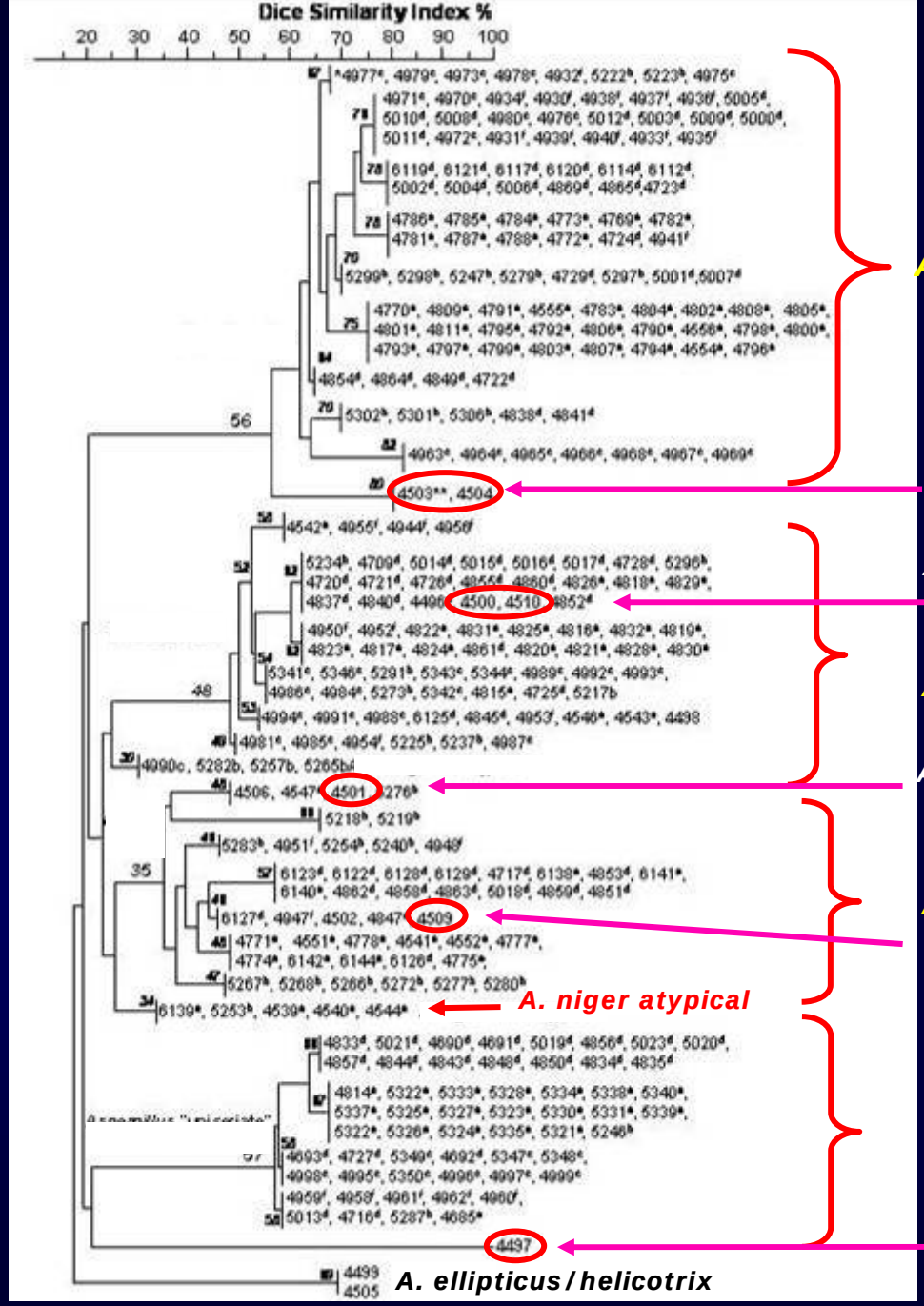
Partners	Total Isolates	*A. niger aggregate*	*A. carbonarius*	*A. japonicus*	*A. ochraceus*
Italy	61	22	20	19	0
Spain	37	14	14	9	0
France	70	18	31	21	0
Portugal	31	17	10	2	2
Greece	93	80	11	2	0
Israel	47	20	18	9	0
Total	339	171	104	62	2

Diagram summarizing the dendrogram of 295 strains *Aspergillus* Sect *Nigri* generated by NTSYS software using cluster UPGMA analysis with Dice Similarity Index

Three groups showed a well defined omogeneous population/species:

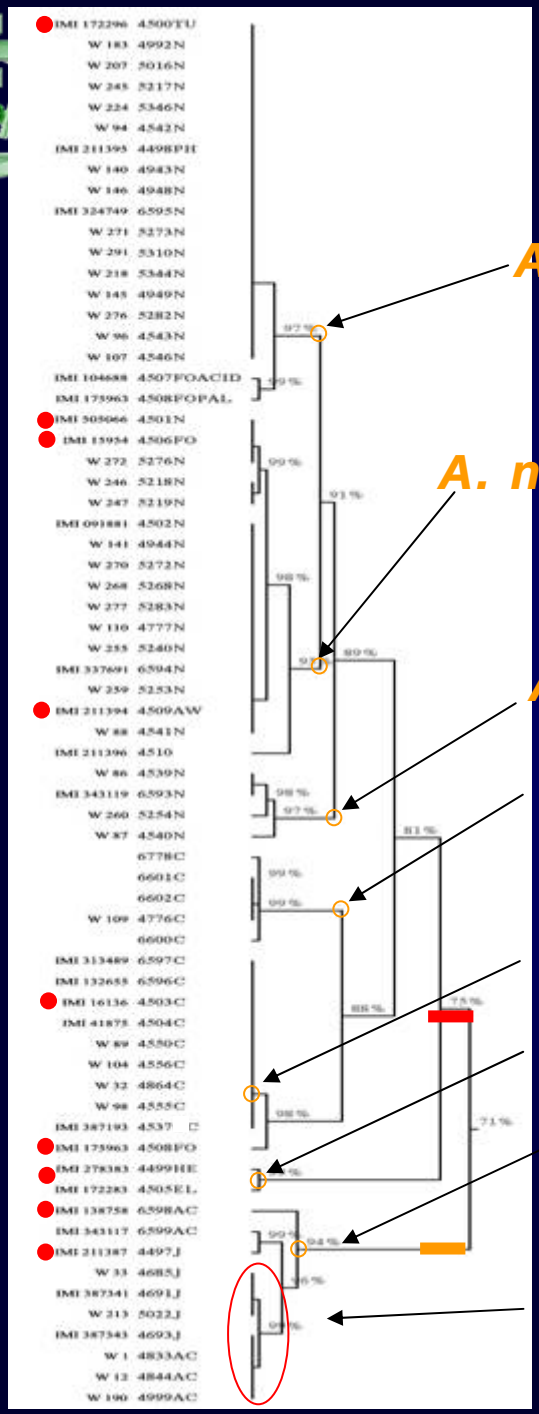
A. carbonarius (105 strains), *A. tubingensis* (69 strains), *Aspergillus* "uniseriate" (56 strains) ≠*A. japonicus* type strain

The *A. niger* cluster (44 strains) showed high variability and supported the possible presence of more than one species.



[Perrone et al. 2006, Int. J. Food Microbiol. 111: S22-S27]

Phylogenetic tree obtained by alignment of sequences from calmodulin gene within the *Aspergillus* Sect. *Nigri*



A. tubingensis

A. niger like

A. niger atypical

A. carbonarius atypical

A. carbonarius

A. ellipticus / helicotrix

A. japonicus / aculeatus

A. uniseriate from grapes

- type strain
- biseriate clade
- uniseriate clade

The calmodulin data confirmed the grouping obtained by AFLP analysis

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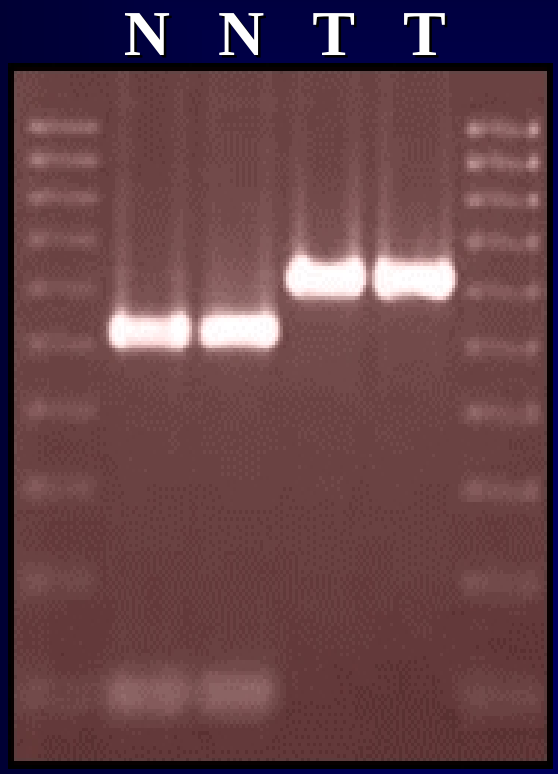
A. niger aggregate: N & T RFLP patterns

(Accensi *et al.*, 1999)



- ✓ Type N: 519-bp / 76-bp (*A. niger* CBS 554.65)
- ✓ Type T: 595-bp (*A. tubingensis* CBS 134.48)

A. niger aggregate/OTA production: "N and T populations from grape"

(Accensi et al. 2001)

Total isolates (n:143)

- N type: 63
- T type: 80
- All OTA +: N type

The production of OTA by *A. niger* aggregate was analyzed by Accensi et al in previous studies, but they didn't found any *A. tubingensis* strains able to produce OTA as in our results.

OTA production and molecular characterization of 94 Italian strains of black aspergilli was studied.

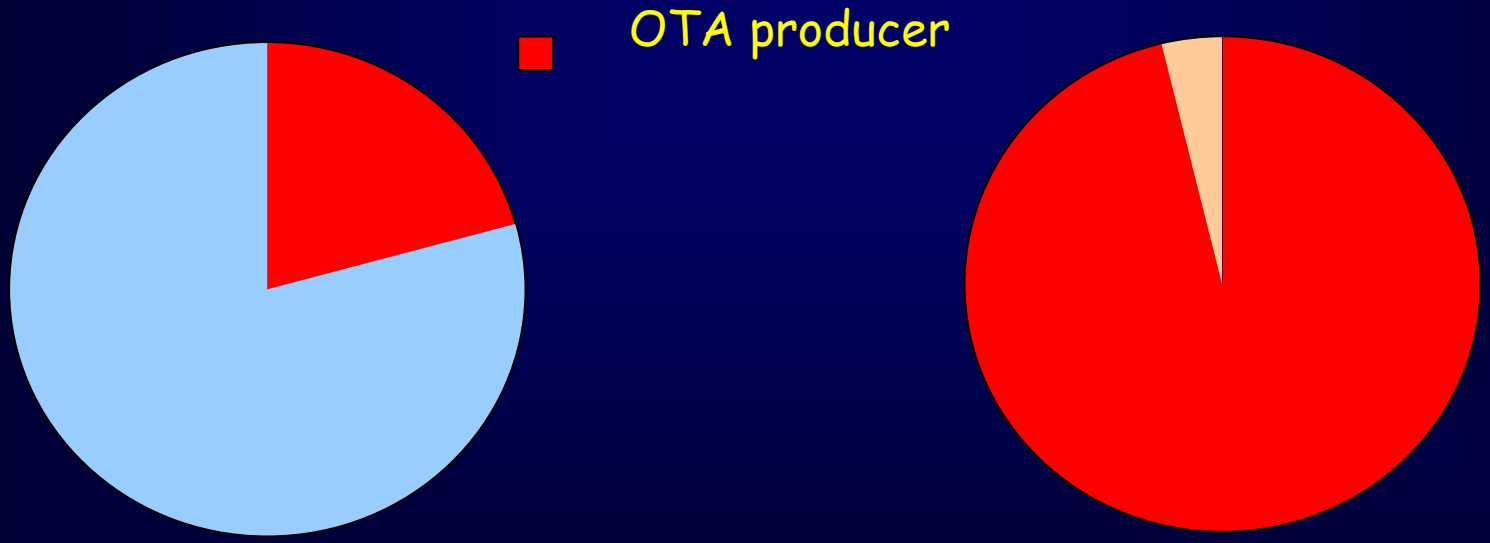
A. niger aggregate
- *A. niger* (3/15)
- *A. tubingensis* (5/20)

A. carbonarius (22/23)

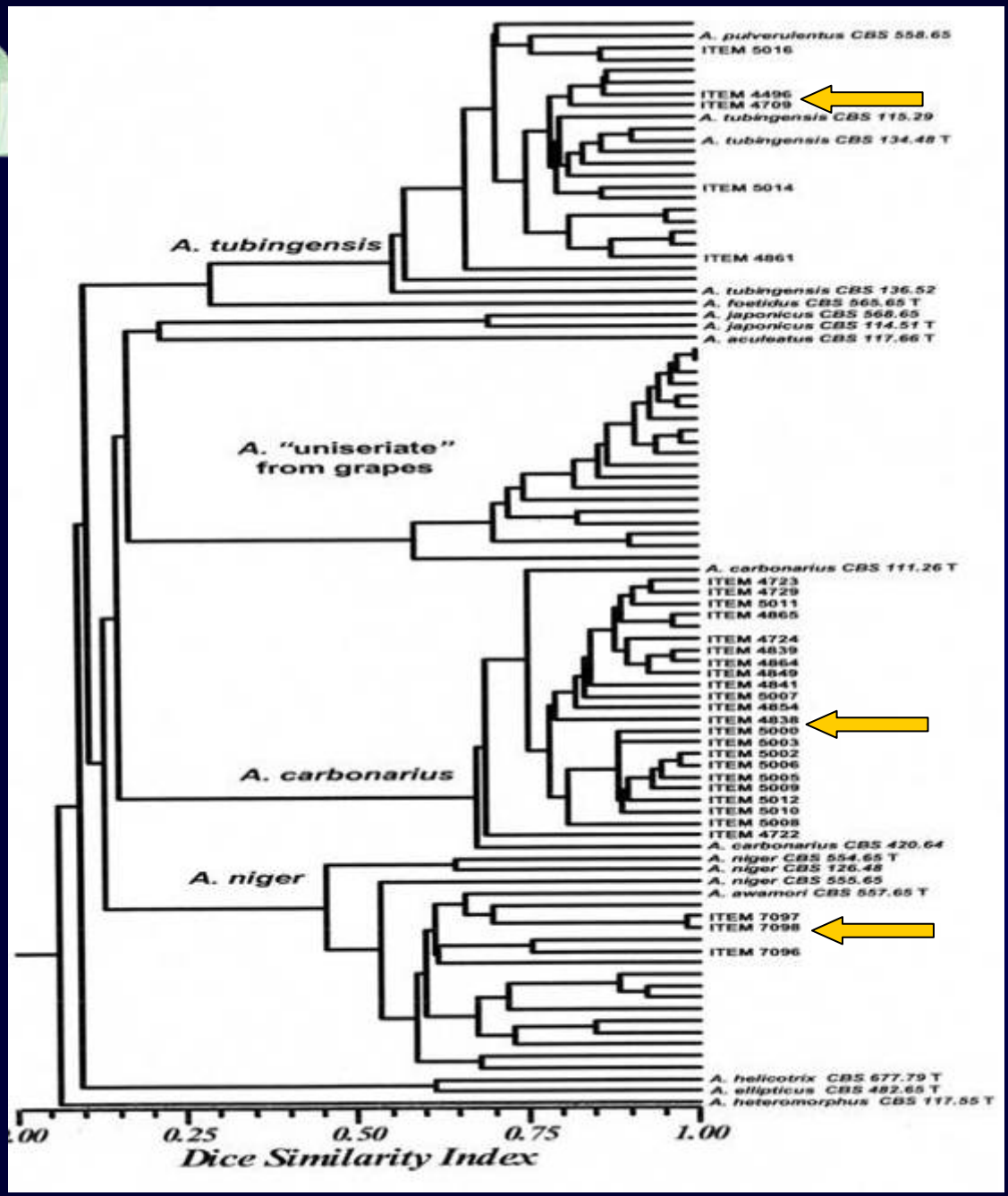
A. niger aggregate MIN	*A. niger* aggregate MAX	*A. carbonarius* MIN	*A. carbonarius* MAX
4.01 ppb	360.2 ppb	6.90 ppb	7,500 ppb

MEAN: 136.25 ppb

MEAN: 555.41 ppb



None of the *A. uniseriate* produced OTA

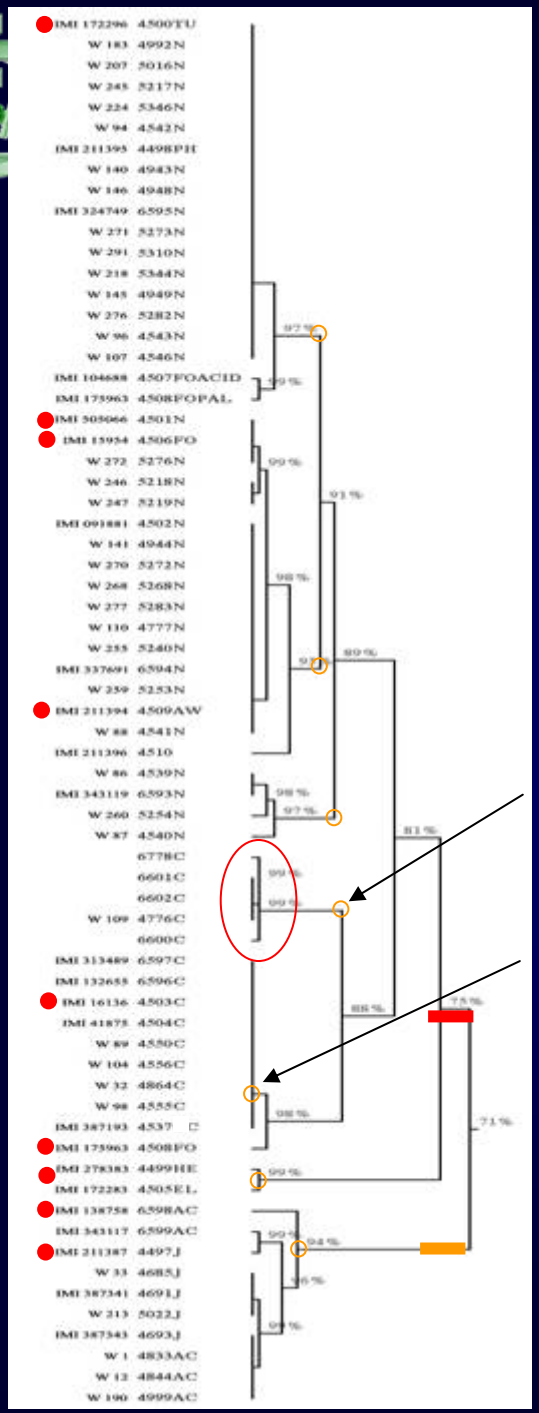


Dendrogram of 94 isolates of *Aspergillus* section *Nigri* based on cluster analysis with the AFLP data obtained with four primer pairs generated by NTSYS software.

Also Medina et al 2005 App. Environ. Microb detected OTA in 14,3 % of *A. tubingensis* strains.

[Perrone et al., 2006 App. Environ. Microb. 72 (1): 680-685]

Studies on atypical *A. carbonarius* strains leads to the identification of a new species close to *A. carbonarius*



- type strain
- biseriate clade
- uniseriate clade

A. carbonarius atypical

A. carbonarius

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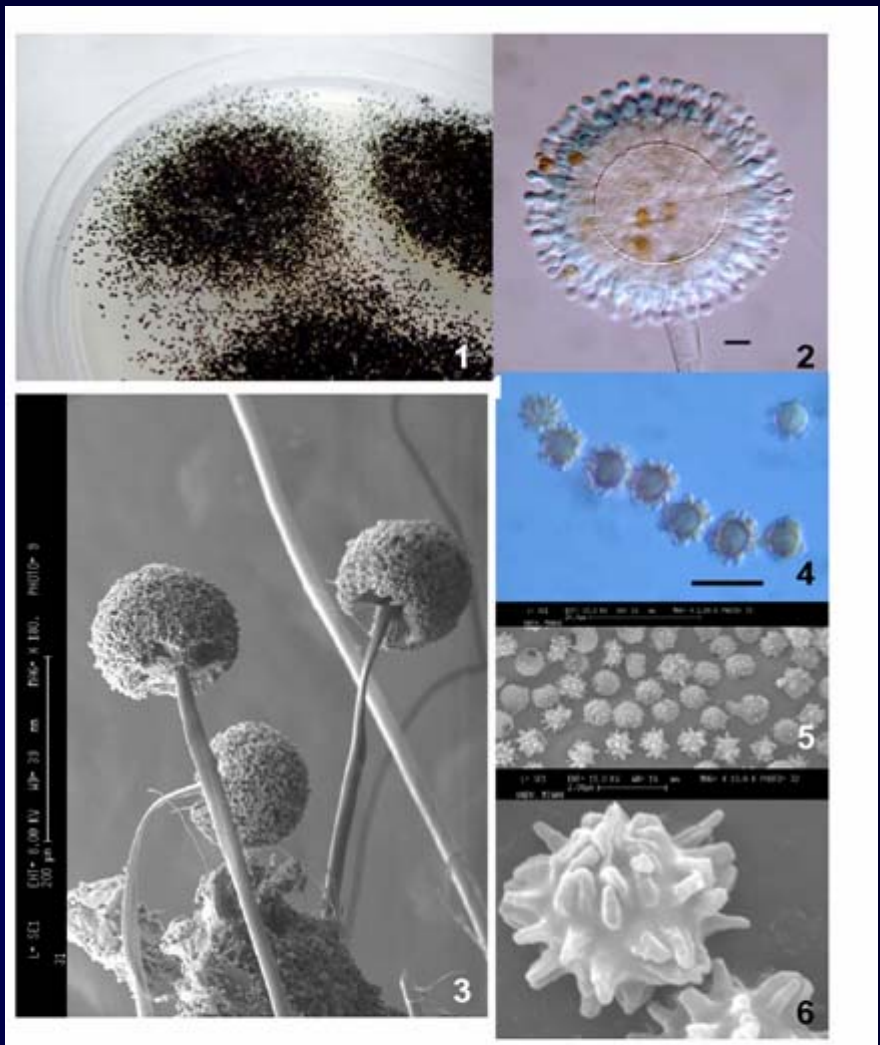
A. ibericus: a new species from grapes

As part of a study on the ochratoxin producing mycoflora of grapes, six *Aspergillus* strains of the section *Nigri* which did not produce detectable amounts of OTA but which had a similar morphology to *A. carbonarius* were isolated from wine grapes and/or dried vine fruit in Portugal and Spain.

These strains, however, have characters that allow morphological distinction from the other species in the section, particularly the spore size (5–7 mm), which allows separation of the species from the two most common biseriate species in section *Nigri*: *A. carbonarius* (7–9 mm) and *A. niger* and its aggregate species (3–5 mm).



Figures 1-6. *Aspergillus ibericus*. 1. Colony grown in CZ (9 days). 2. Biseriate aspergilli of a 4 days old culture in CZ (scale bar = 10 µm). 3. Aspergilli at SEM. 4. Spores seen at Nomarski microscope (scale bar = 10 µm). 5-6. SEM picture of the spores.

[Serra et al., 2006 Mycologia 98 (2):295-306]

Molecular characterization of *A. ibericus* strains (1)

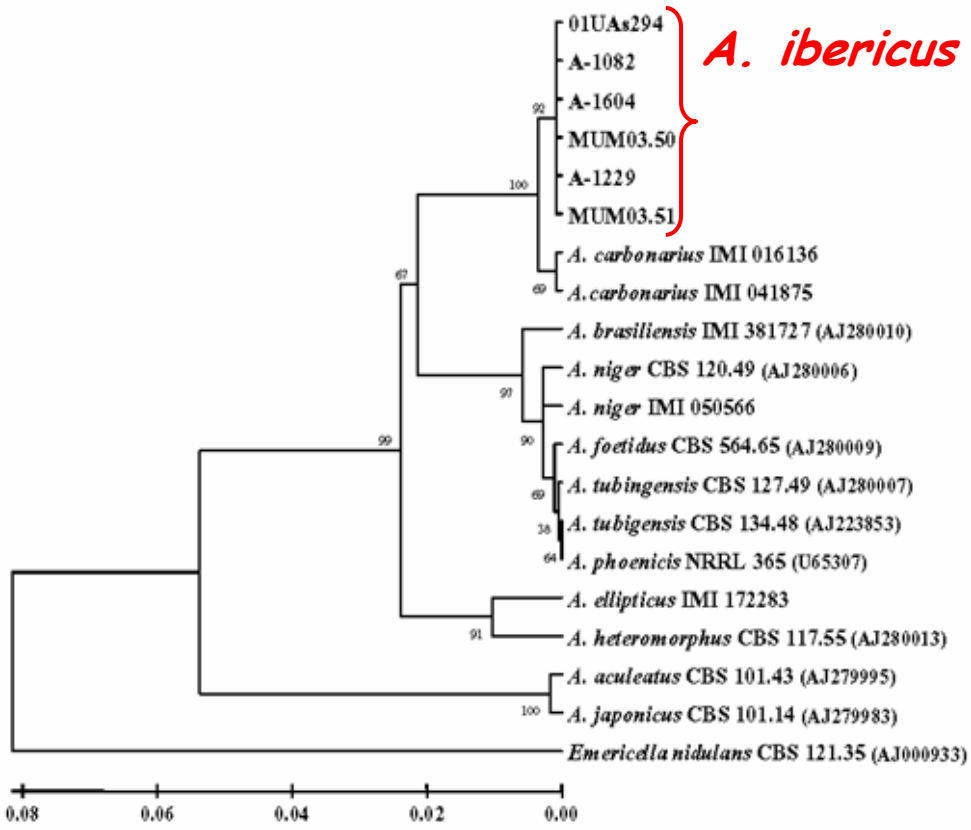
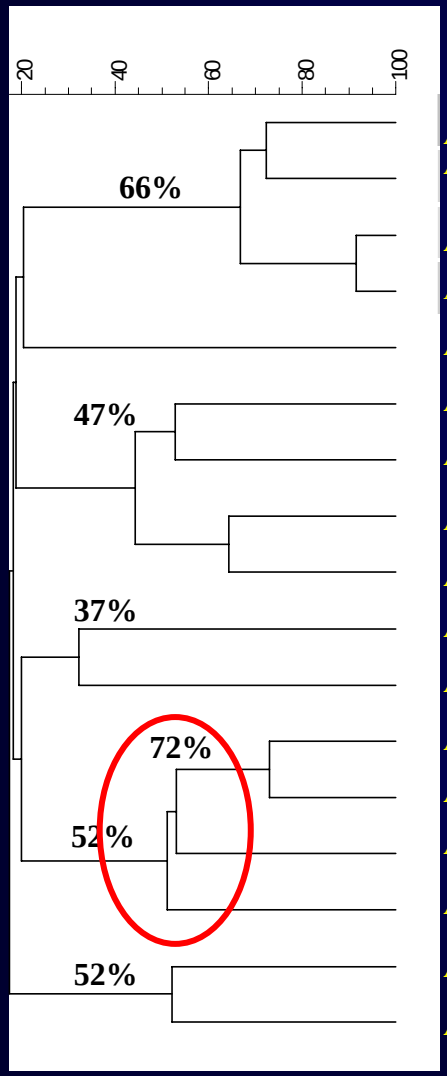


Figure 7. Neighbor-joining tree based on phylogenetic analysis of the ITS1-5.8S rRNA gene-ITS2 sequences. The numbers at branch points are the percentages of 1,000 bootstrapped data sets that supported the specific internal branches. Species with GenBank numbers represent sequences obtained from GenBank.

The validation of this new taxon is supported by analysis of the ITS-5.8S rDNA and calmodulin gene sequences and by analysis of the amplified fragment length polymorphism (AFLP) patterns, which were consistent in separating these strains from other species in the section.

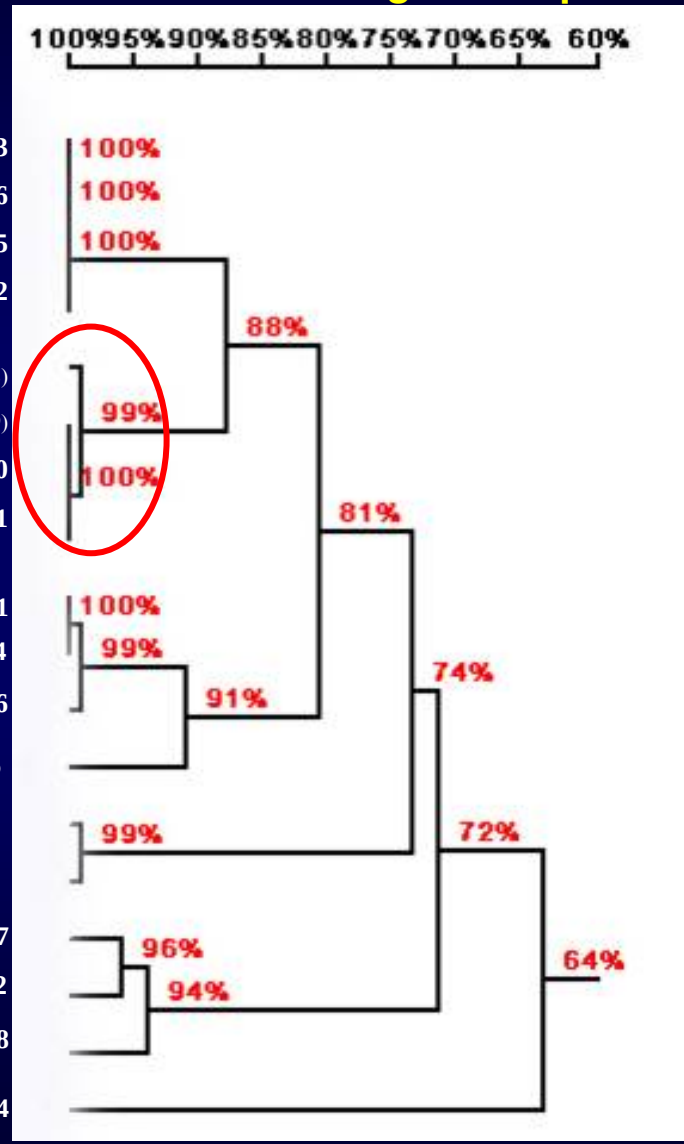
Molecular characterization of *A. ibericus* strains (2)

Dendrogram AFLP analysis



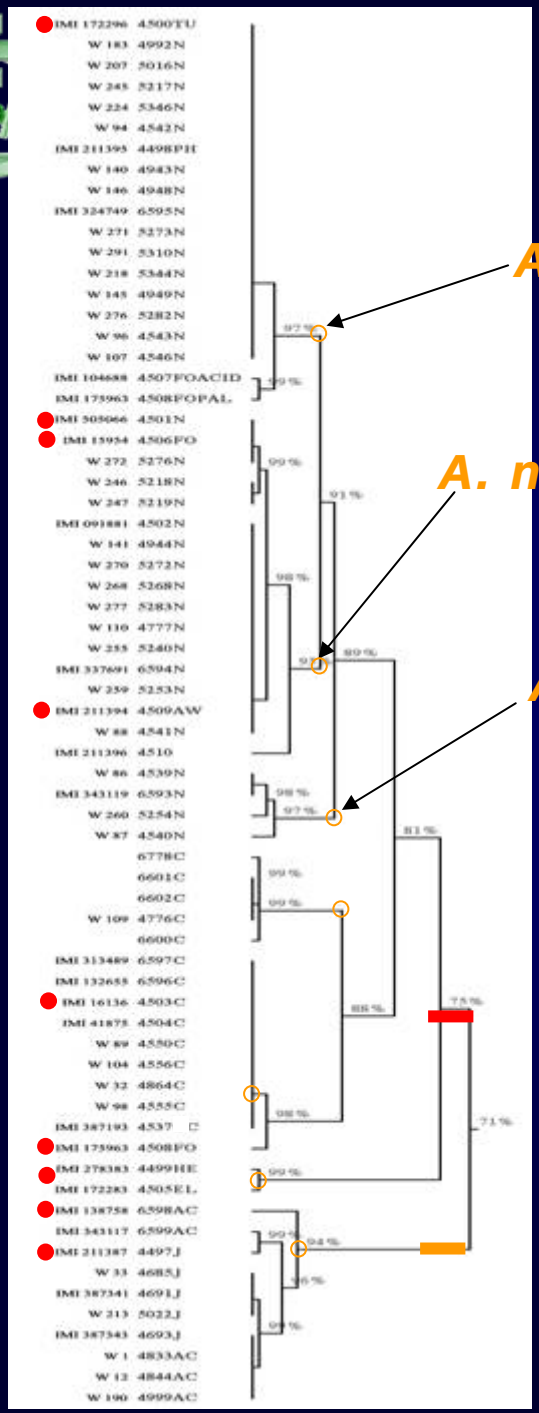
A. carbonarius IMI 016136
A. carbonarius IMI 041875
A. carbonarius IMI 387242
A. carbonarius IMI 387223
A. japonicus IMI 221387
A. niger IMI 091881
A. awamori IMI 211394
A. niger IMI 050566
A. phoetidus IMI 15954
A. phoenicis IMI 211395
A. tubingensis IMI 172296
A. ibericus MUM 03.50
A. ibericus MUM 03.51
A. ibericus MUM 03.49 (IMI 387249)
A. ibericus A-1082 (IMI 391428)
A. helicotrix IMI 278383
A. ellipticus IMI 172283

Homology tree obtained by comparison of partial calmoduline gene sequences



A. carbonarius IMI 387223
A. carbonarius IMI 016136
A. carbonarius IMI 041875
A. carbonarius IMI 387242
A. ibericus A-1082 (IMI 391428)
A. ibericus MUM 03.49 (IMI 387249)
A. ibericus MUM 03.50
A. ibericus MUM 03.51
A. niger IMI 091881
A. awamori IMI 211394
A. niger IMI 050566
A. tubingensis IMI 172296
A. ellipticus IMI 172283
A. helicotrix IMI 278383
A. japonicus IMI 221387
A. aculeatus IMI 388522
A. aculeatus IMI 138758
Fusarium proliferatum NRRL 22944

Characterization of atypical *A. niger* strains by alignment of sequences from calmodulin gene within the *Aspergillus* Sect. *Nigri*



A. tubingensis

A. niger like

A. niger atypical

A. brasiliensis
from grapes

- type strain
- biseriate clade
- uniseriate clade

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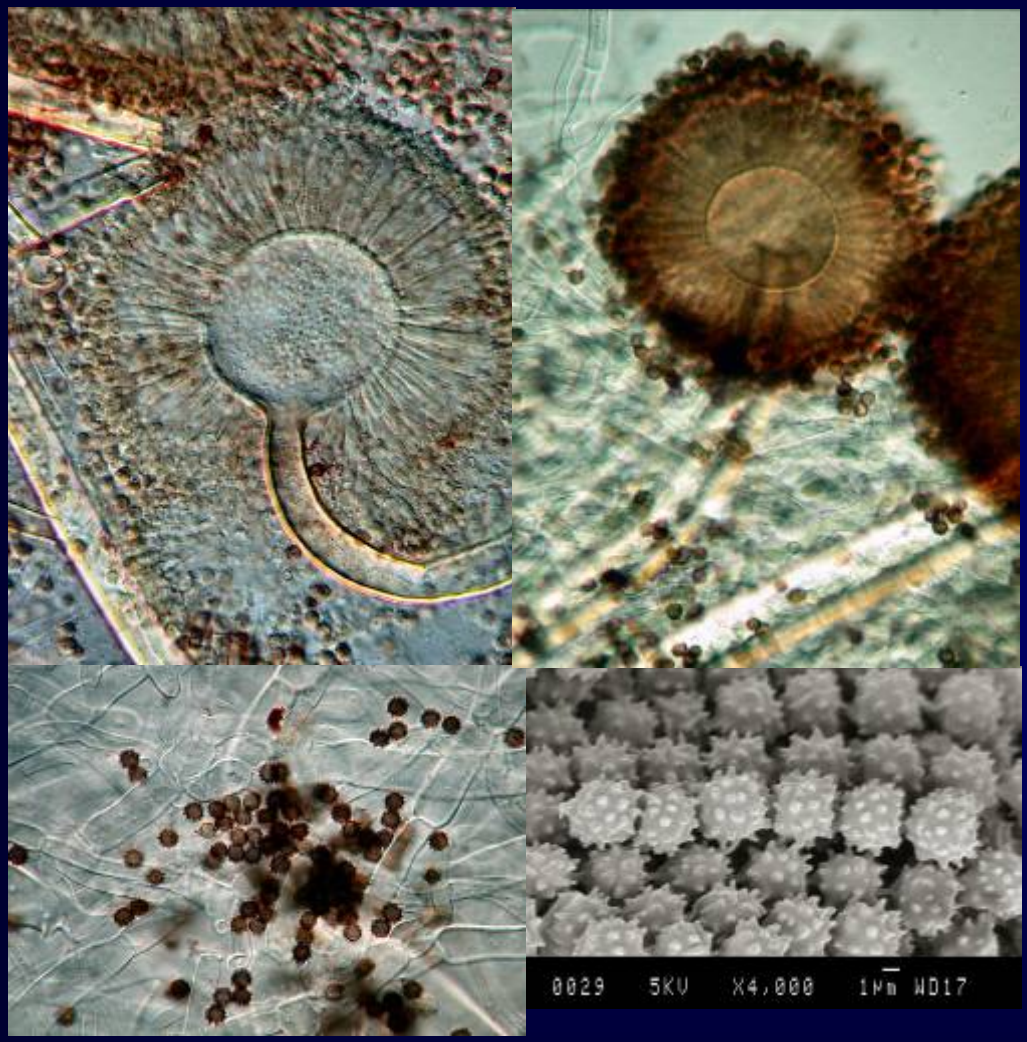
Micromorphology of conidial heads and conidia of *A. brasiliensis* sp. nov.



CYA

MEA

Varga et al., 1994

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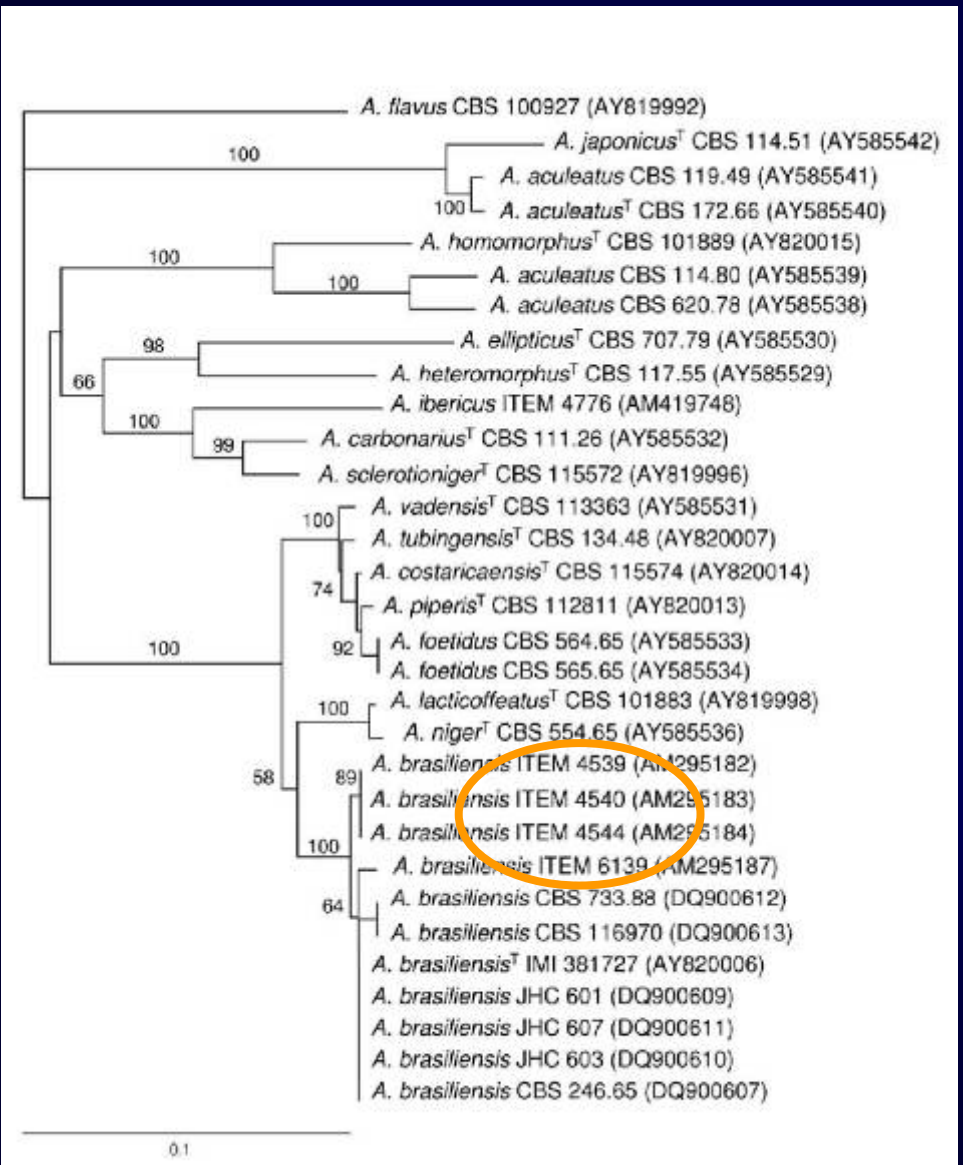
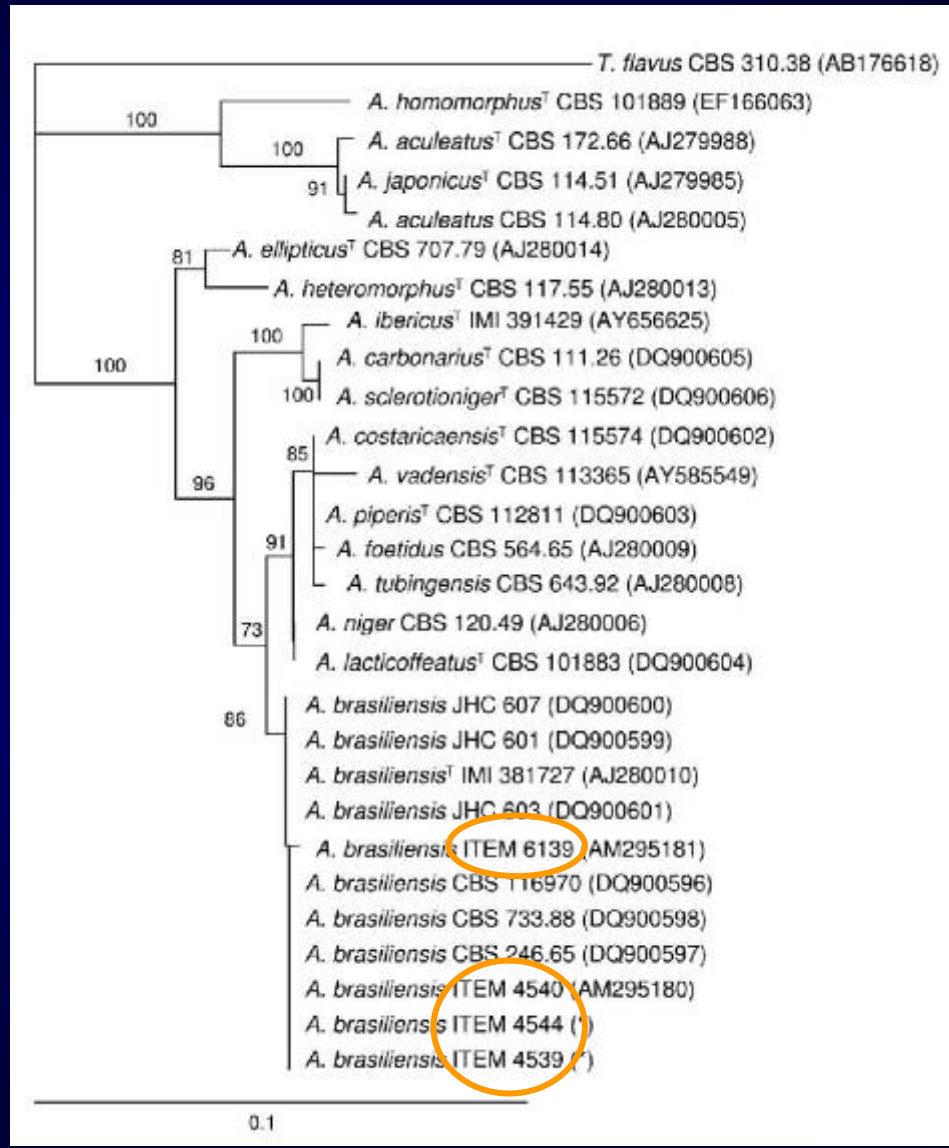
Aspergillus brasiliensis from grapes (1)

ß-tubulin



ITS



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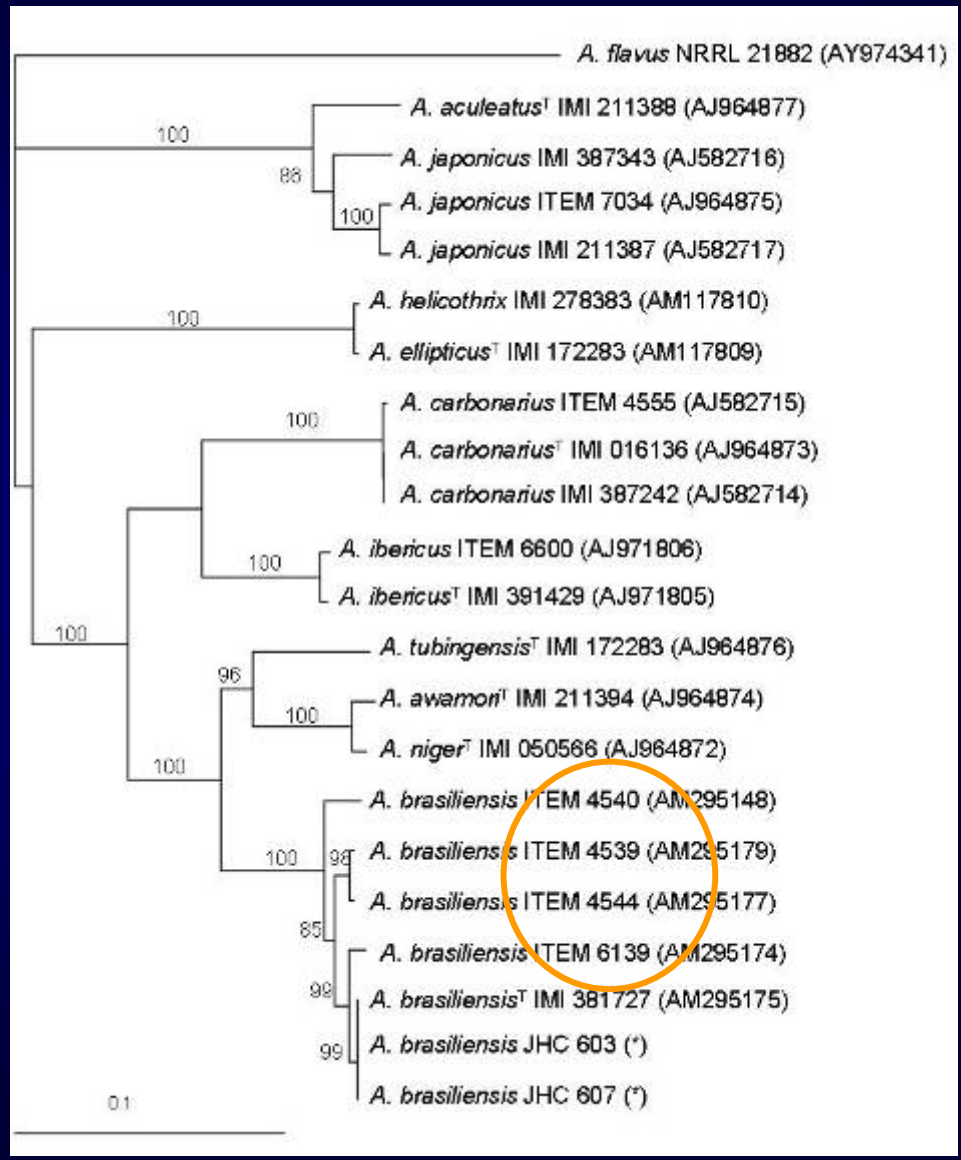
Aspergillus brasiliensis from grapes (2)

Calmodulin

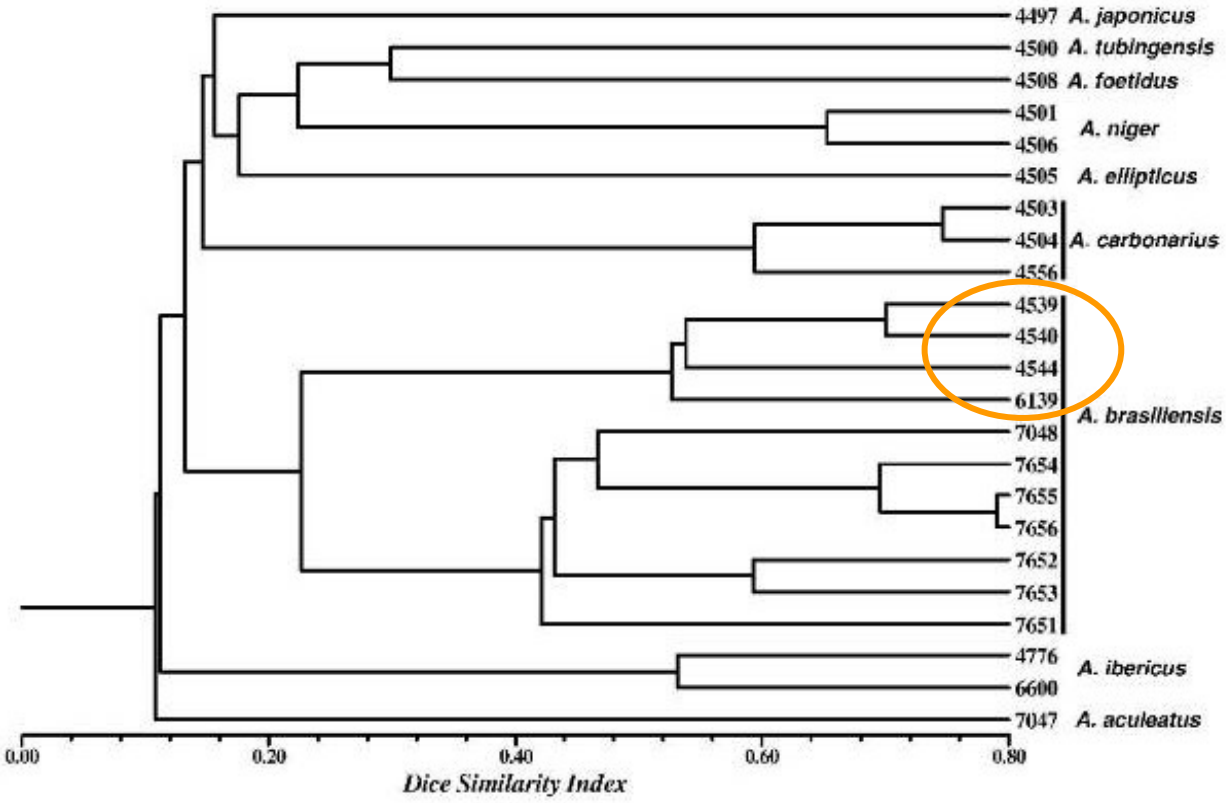
Neighbor-joining tree based on calmodulin sequence data of *Aspergillus* section *Nigri*.



Aspergillus brasiliensis from grapes (3)

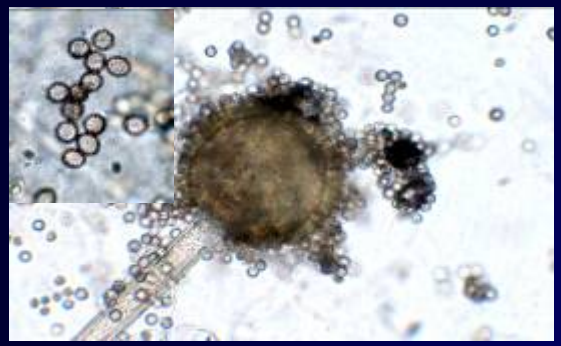
AFLP

Dendrogram of representative black *Aspergillus* isolates together with 11 strains of *A. brasiliensis* based on cluster analysis with the UPGMA method using the Dice genetic distance coefficient on AFLP data obtained with four primer pairs generated by NTSYS software.

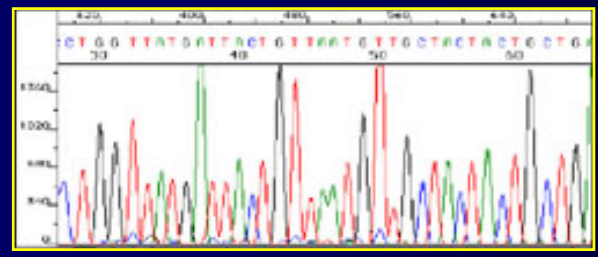


The description of this new species has been recently accepted for publication in IJSEM (Varga et al. 2007).

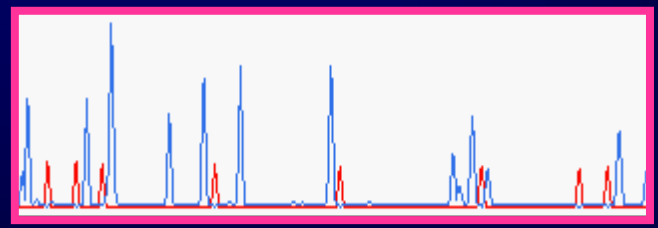
Molecular characterization of *A.* "uniseriate" strains from grapes



- Calmodulin sequences



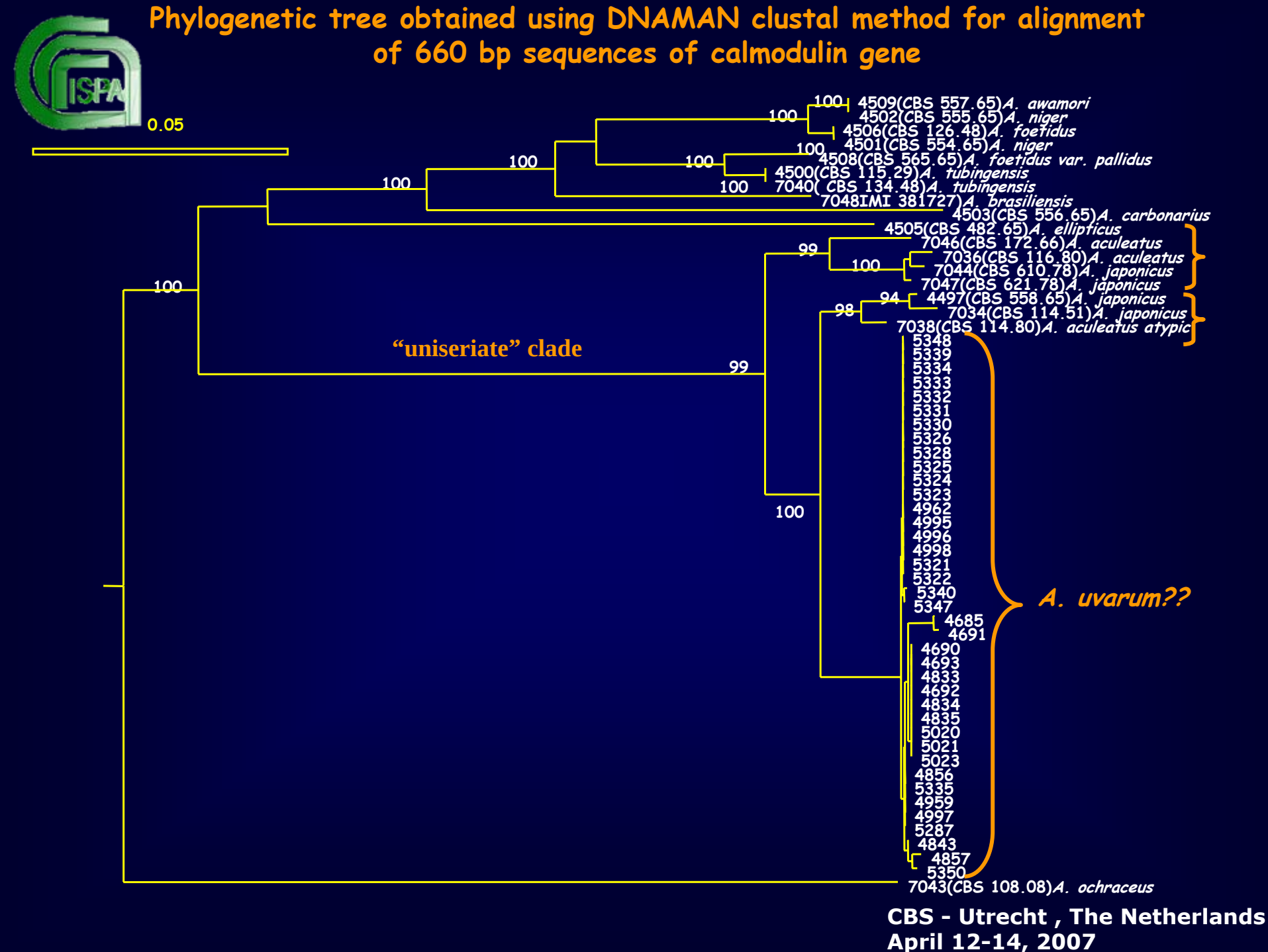
- β-tubulin sequences



- AFLP analysis

Reports of occurence of uniseriate black aspergilli evidence the presence *A. aculeatus* in Australia, and of *A. japonicus* var. *aculeatus* in Brazil/Argentina

Phylogenetic tree obtained using DNAMAN clustal method for alignment of 660 bp sequences of calmodulin gene



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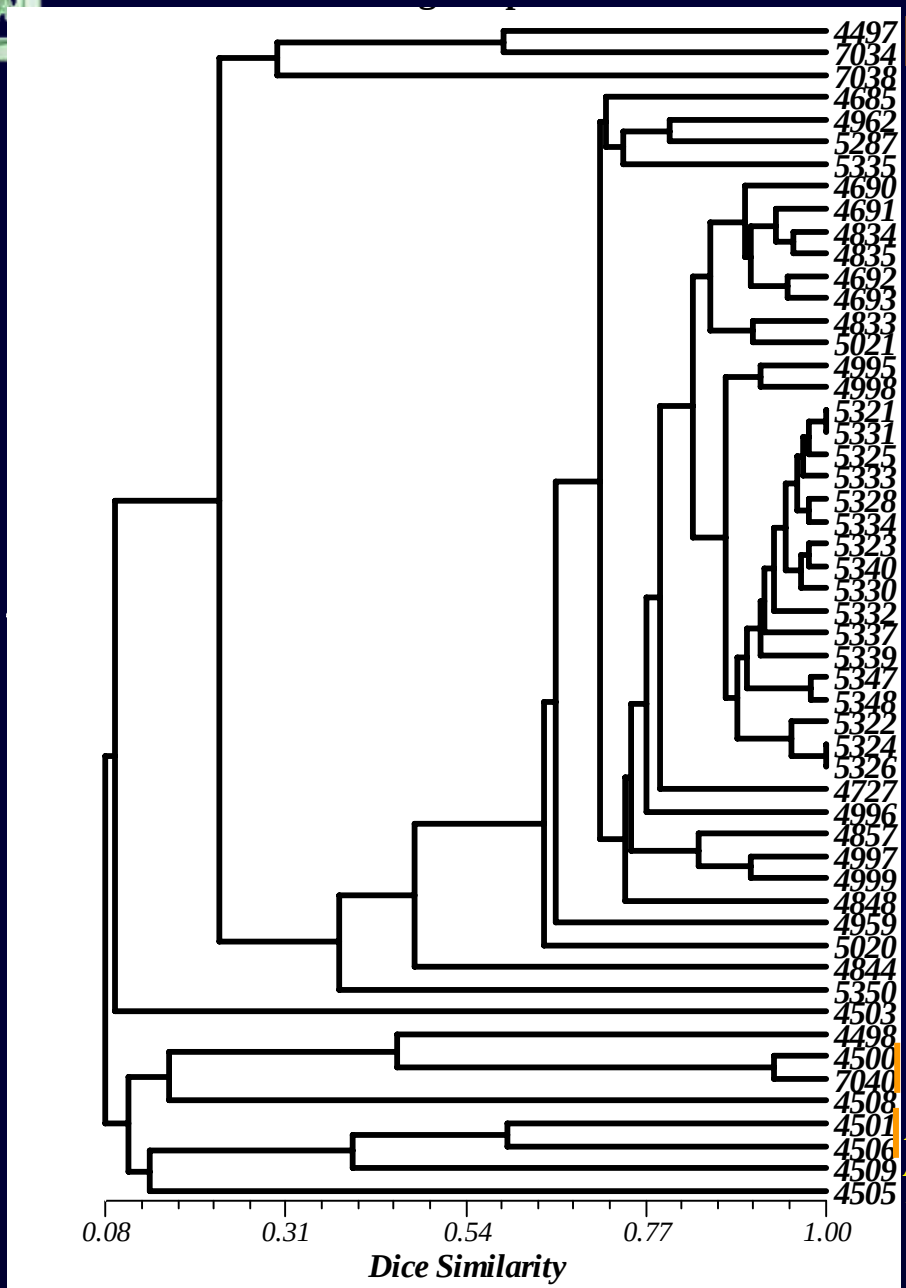
Molecular characterization of *A.* "uniseriate" by AFLP



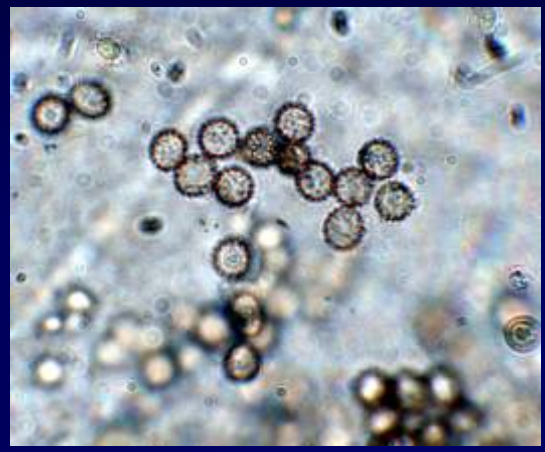
Aspergillus japonicus
Aspergillus aculeatus

A. uvarum??

Aspergillus carbonarius
Aspergillus tubingensis
Aspergillus foetidus
Aspergillus niger
Aspergillus awamori
Aspergillus ellipticus

Aspergillus uvarum

CZ



CYA

SEM picture by R. Samson

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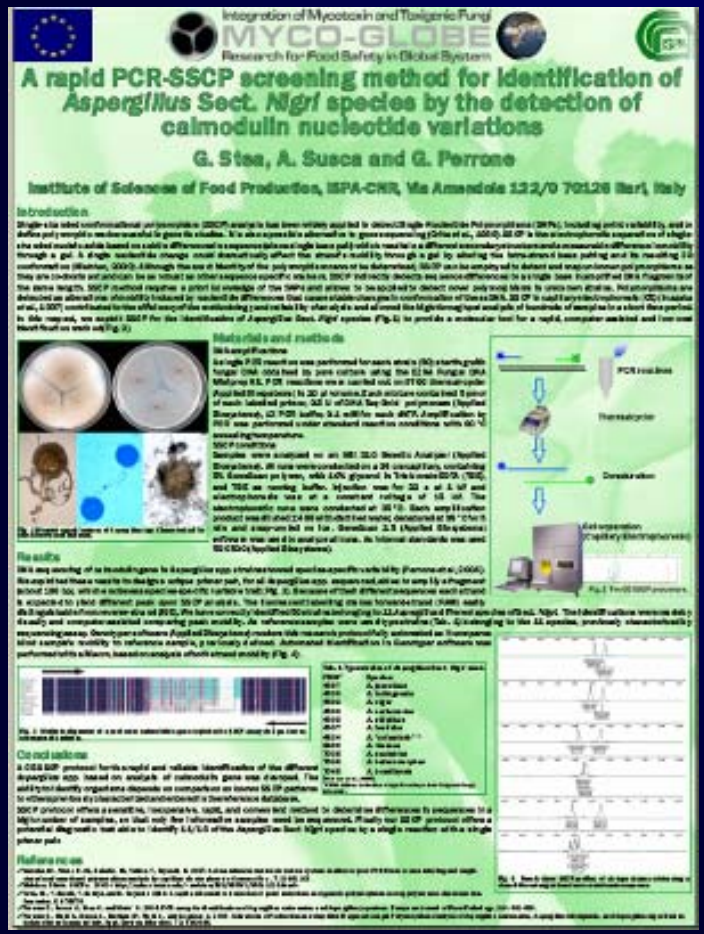
Conclusions

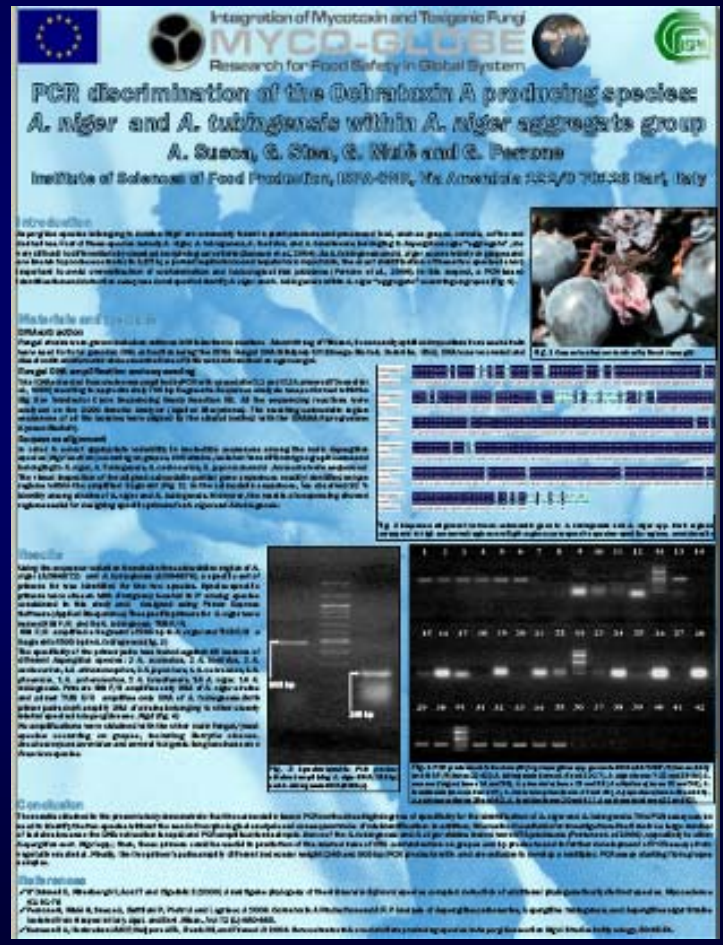
- Black aspergilli represent a various and complex molteplicity of species, with some of these peculiar of grapes;
- 4 main species/population were present on grapes:
  - the well characterized *A. carbonarius* and *A. tubingensis*;
  - the *A. niger* group wich comprises *A. niger (A. awamori), A. foetidus, A. brasiliensis* and others?
  - the *A. "uniseriate"* which could represent a new species peculiar of grapes, molecularly distant from *A. japonicus* and *A. aculeatus;*
- the main responsible of OTA presence in grapes is *A. carbonarius* (99% of strains produce OTA);
- *A. tubingensis*, which has not been previously reported to produce ochratoxin A, together with *A. niger* is also responsible of OTA presence in grapes;
- *A. ibericus* a new described species, isolates from grapes and rarely occurring, is very similar to *A. carbonarius* but not produced OTA

PCR identification in Sect. *Nigri* by DNA-sequencing of calmodulin gene.

A rapid PCR-SSCP screening method for identification of *Aspergillus* Sect. *Nigri* species by the detection of calmodulin nucleotide variations.



PCR discrimination of the Ochratoxin A producing species: *A. niger* and *A. tubingensis* within *A. niger* aggregate group.



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Paola Battilani
University of Piacenza
Italy

Armando Venancio
University of Braga
Portugal

Javier Cabanes
University of Barcelona
Spain

Zofia Kozakiewicz
CABI Institute London
UK

Janos Varga
University of Szeged
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Robert A. Samson
CBS- Utrecht
The Netherlands

Jens C. Frisvad
University of Denmark
Lyngby - Denmark

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