

Biological and epidemiological studies of *P. s. pv. actinidiae* and *P. s. pv. actinidifoliorum* pathogenic on kiwifruit to improve their detection



Photo SBAI Rhône-Alpes 2011

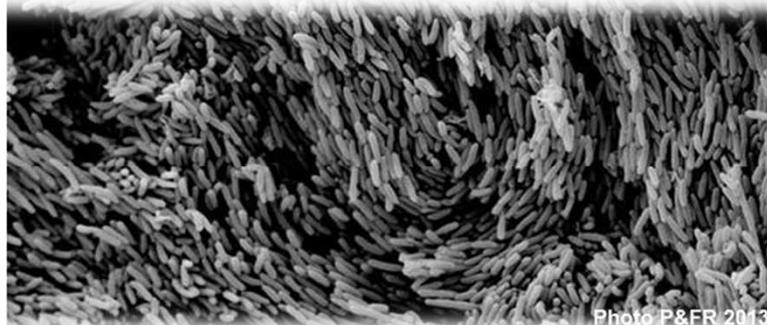


Photo P&ER 2013

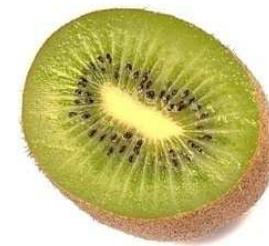
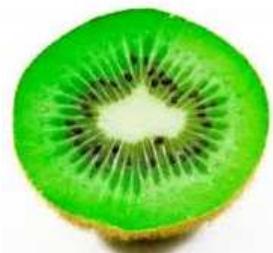


Photo LSV 2012 - Landes

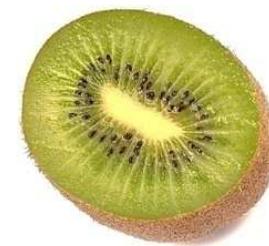
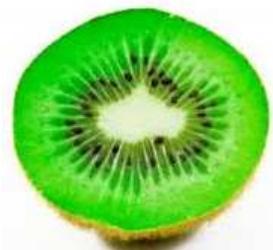
Amandine Cunty^(1,2), Sophie Cesbron⁽²⁾, Françoise Poliakoff⁽¹⁾, Marie-Agnès Jacques⁽²⁾, Charles Manceau⁽¹⁾

⁽¹⁾French agency for food, environmental and occupational health and safety - ANSES - Plant Health Laboratory, Angers - France.

⁽²⁾UMR1345 IRHS – National Institute for Agricultural Research, INRA, Beaucouzé - France.

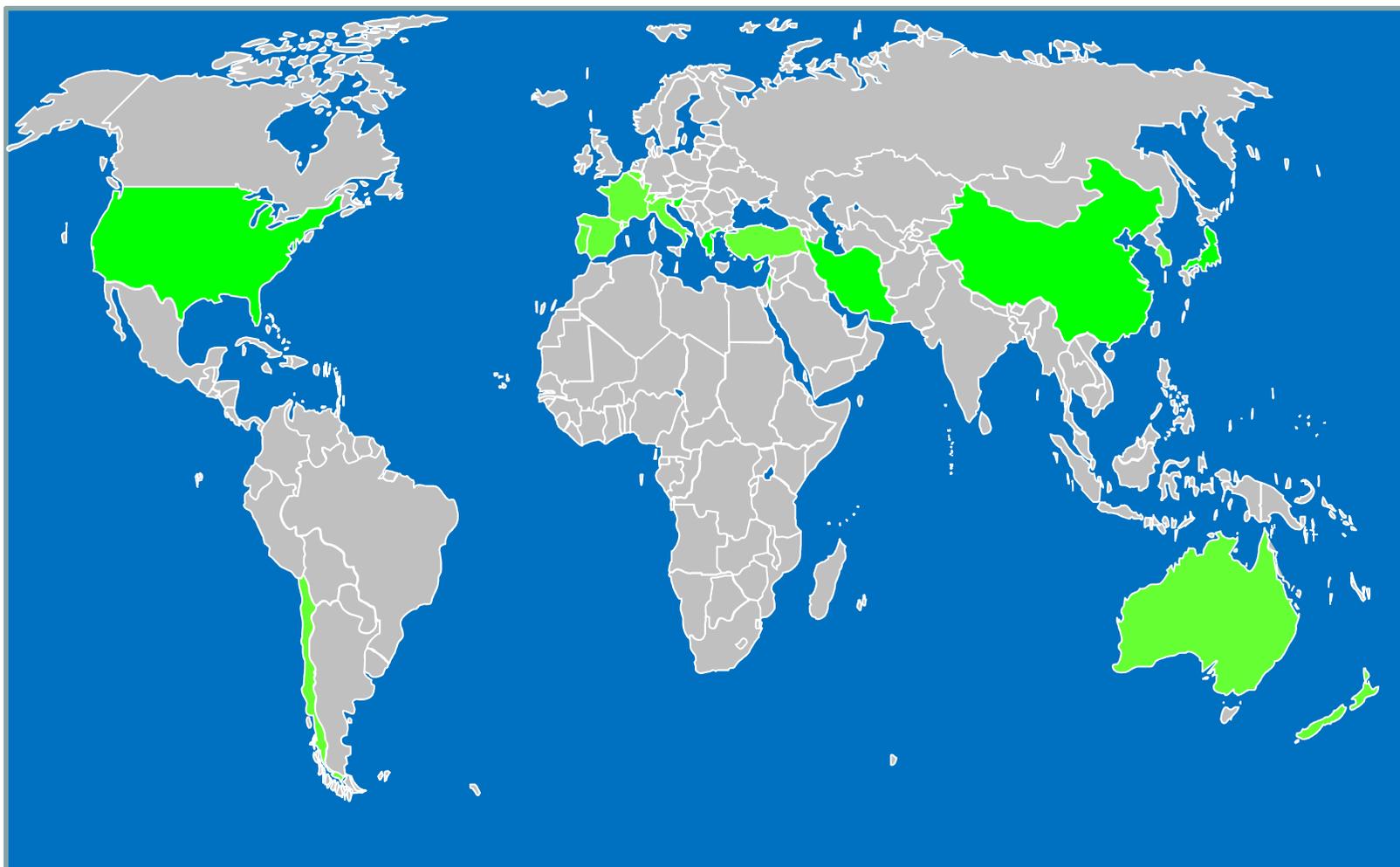


Global overview





Kiwifruit production areas

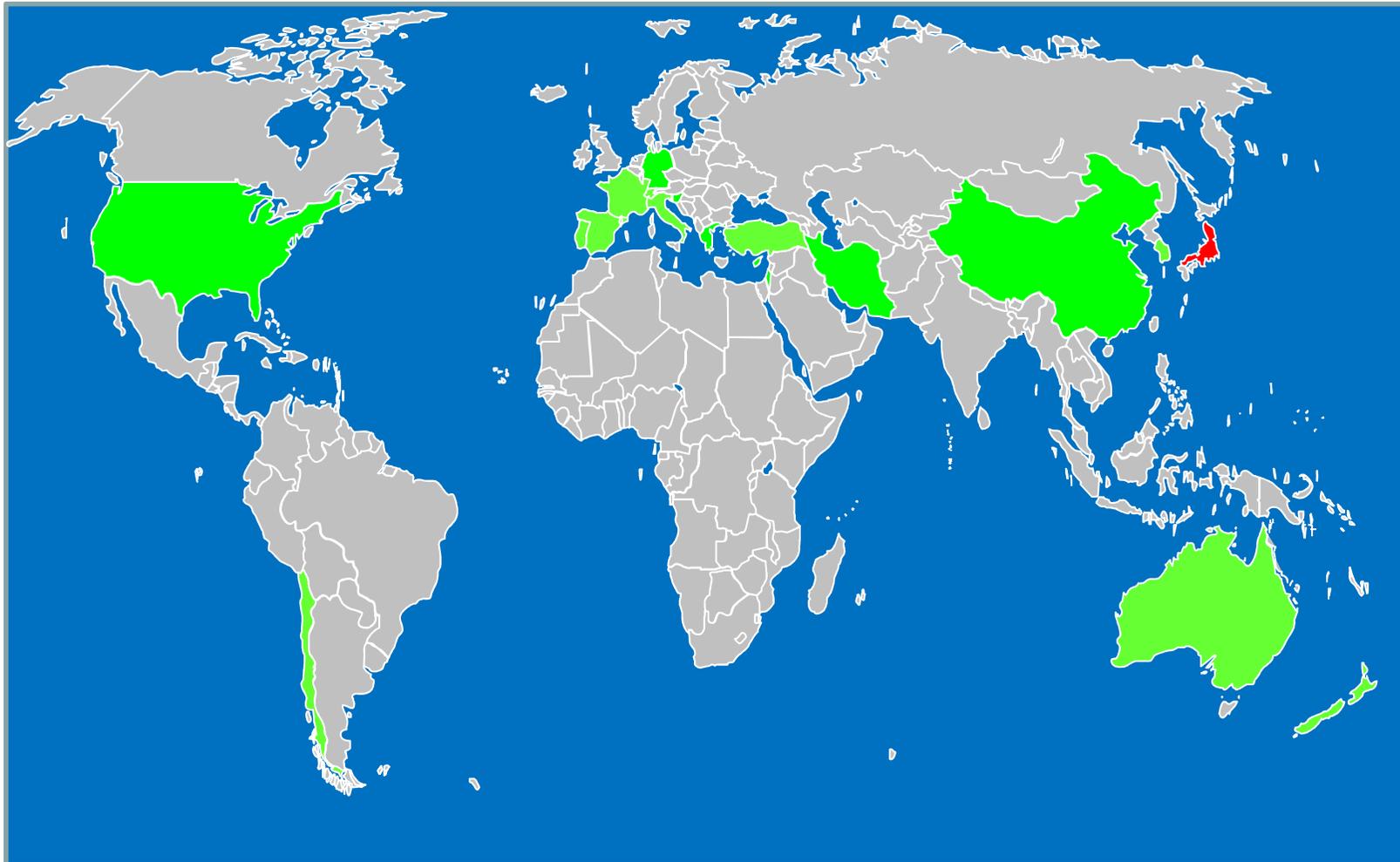


 **Kiwifruit producing countries**



Psa detection

1984: Japan



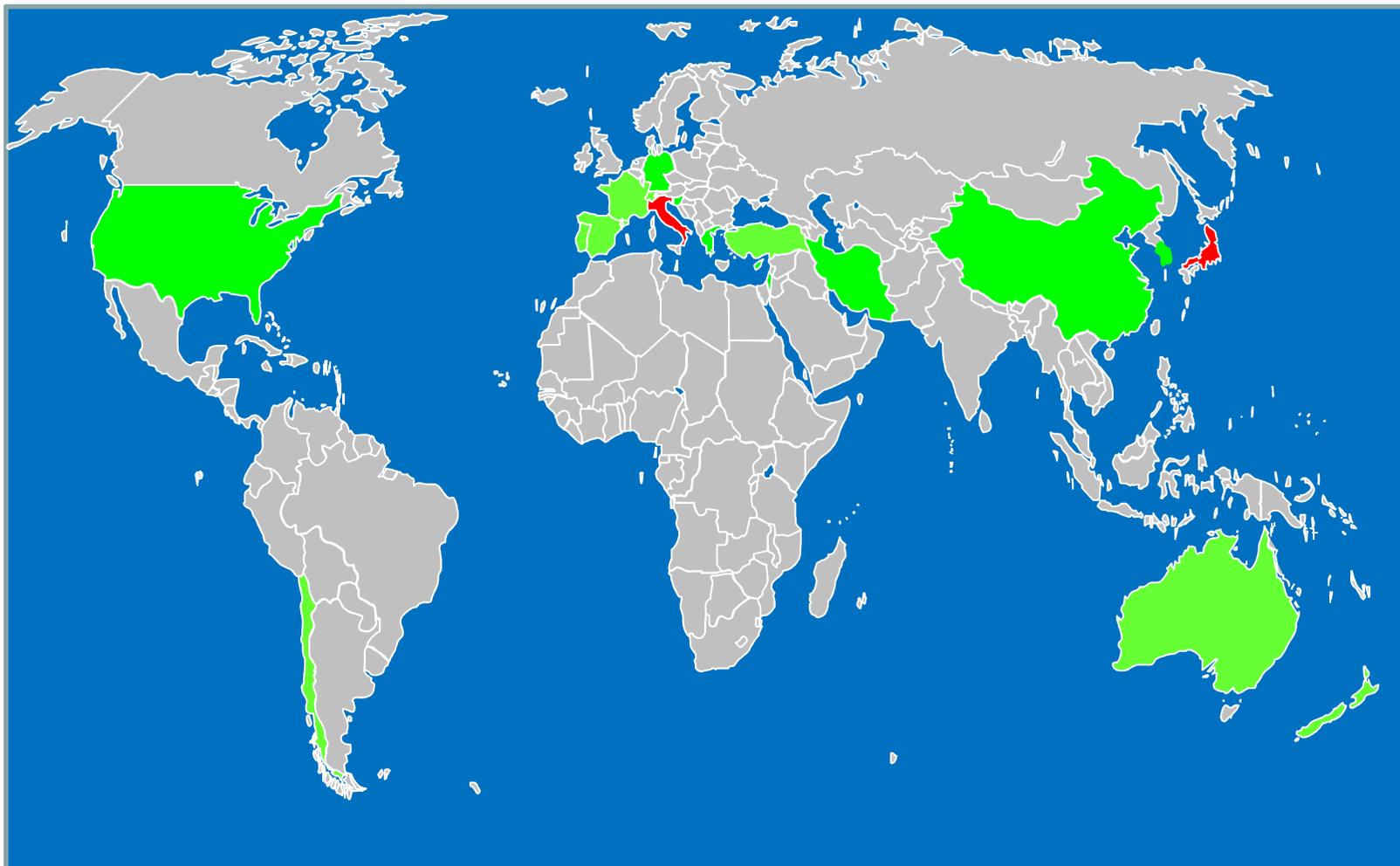
 **Kiwifruit producing countries**

 **Psa detected**



Psa detection

1992: Italy



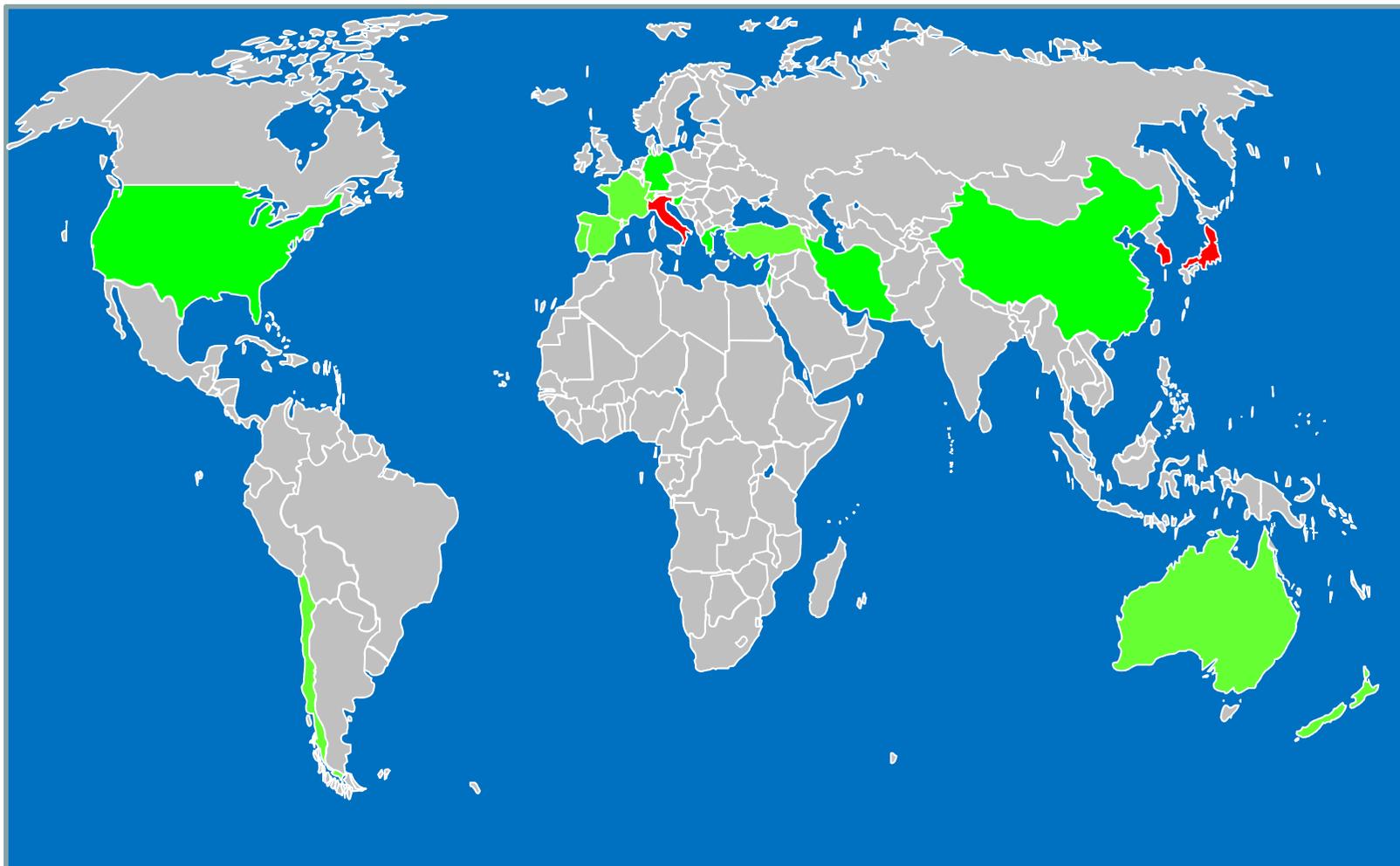
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Psa detection

1994: South Korea



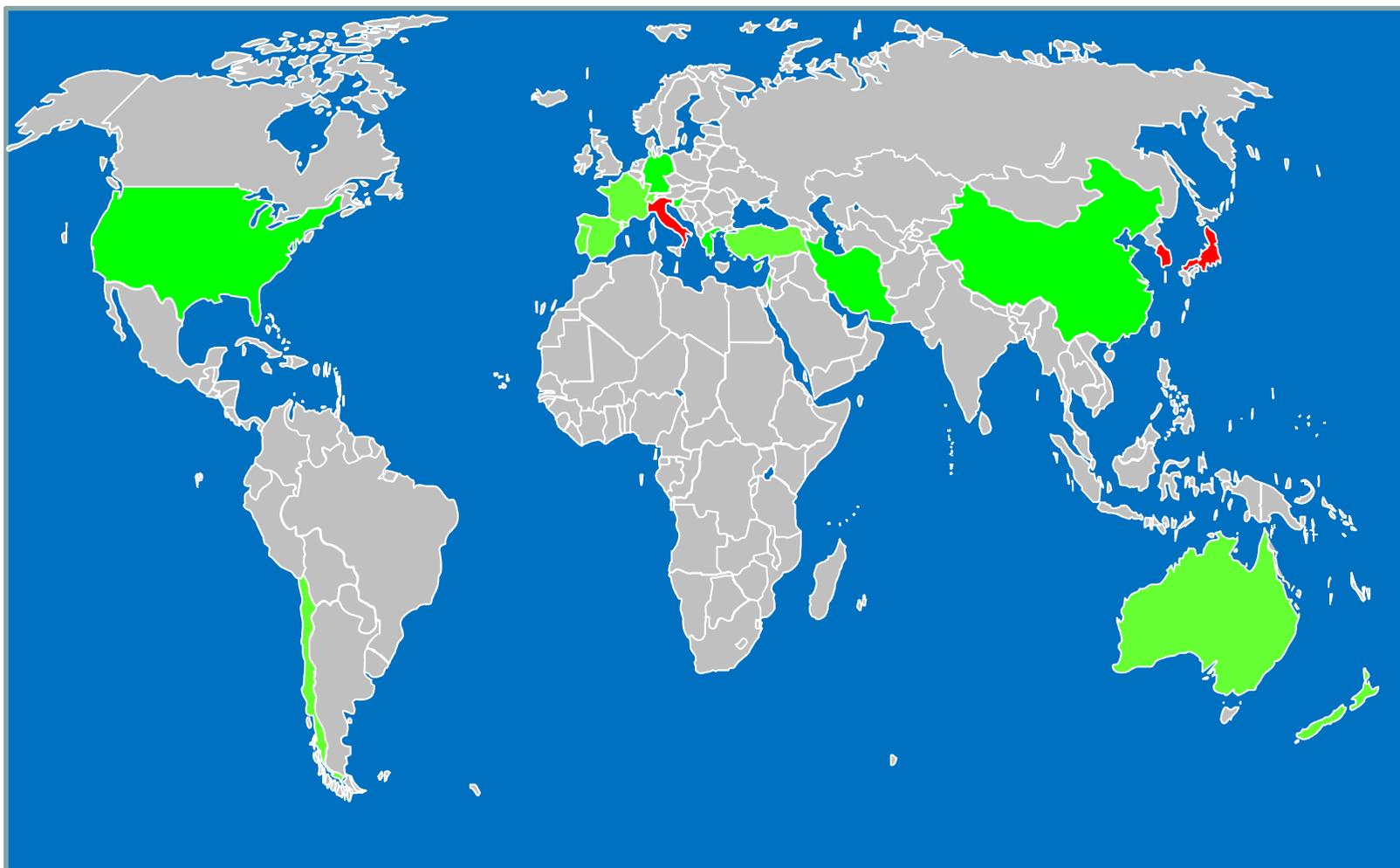
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Psa detection

2008: Italy



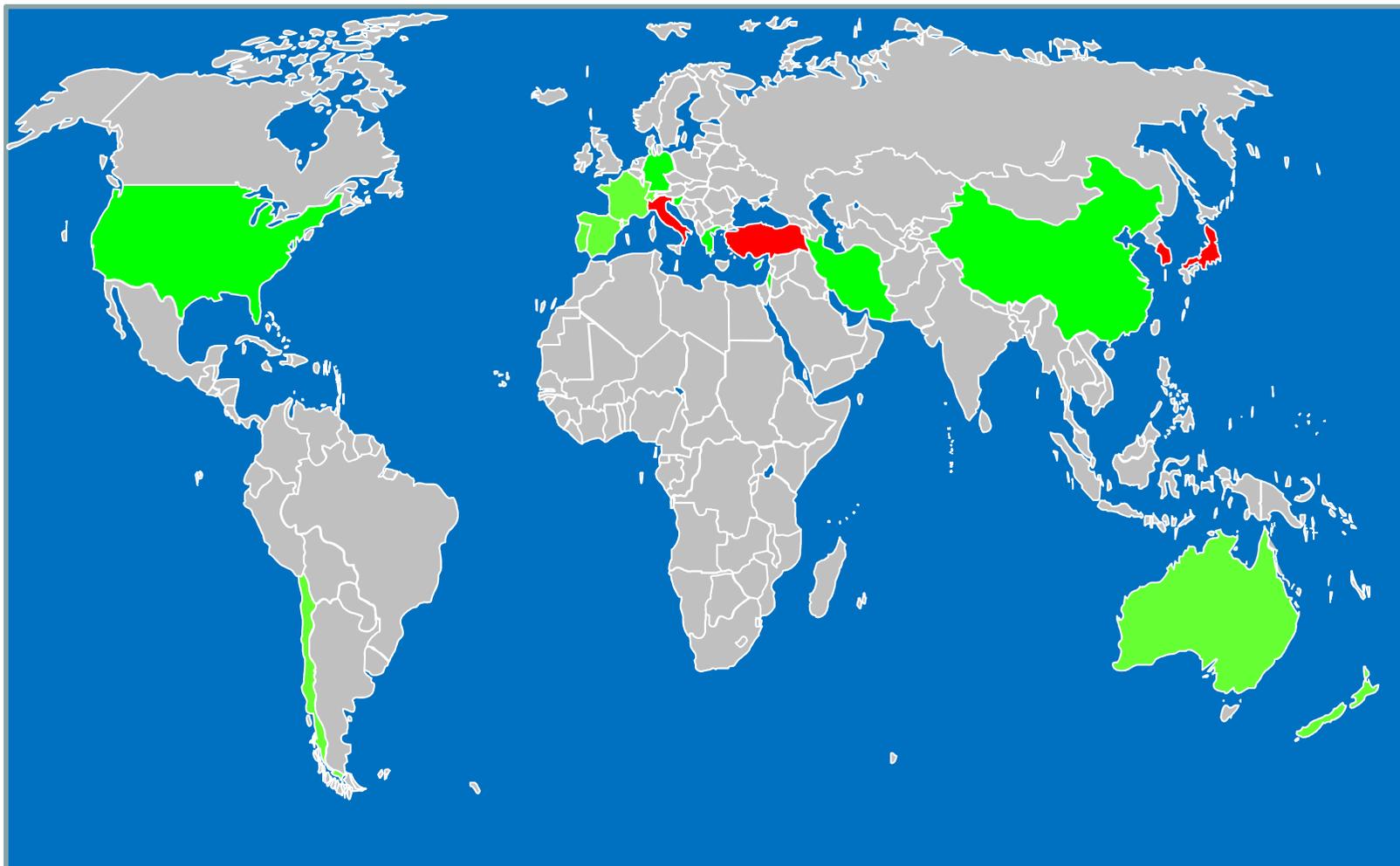
 **Kiwifruit producing countries**

 **Psa detected**



Psa detection

2009: Turkey



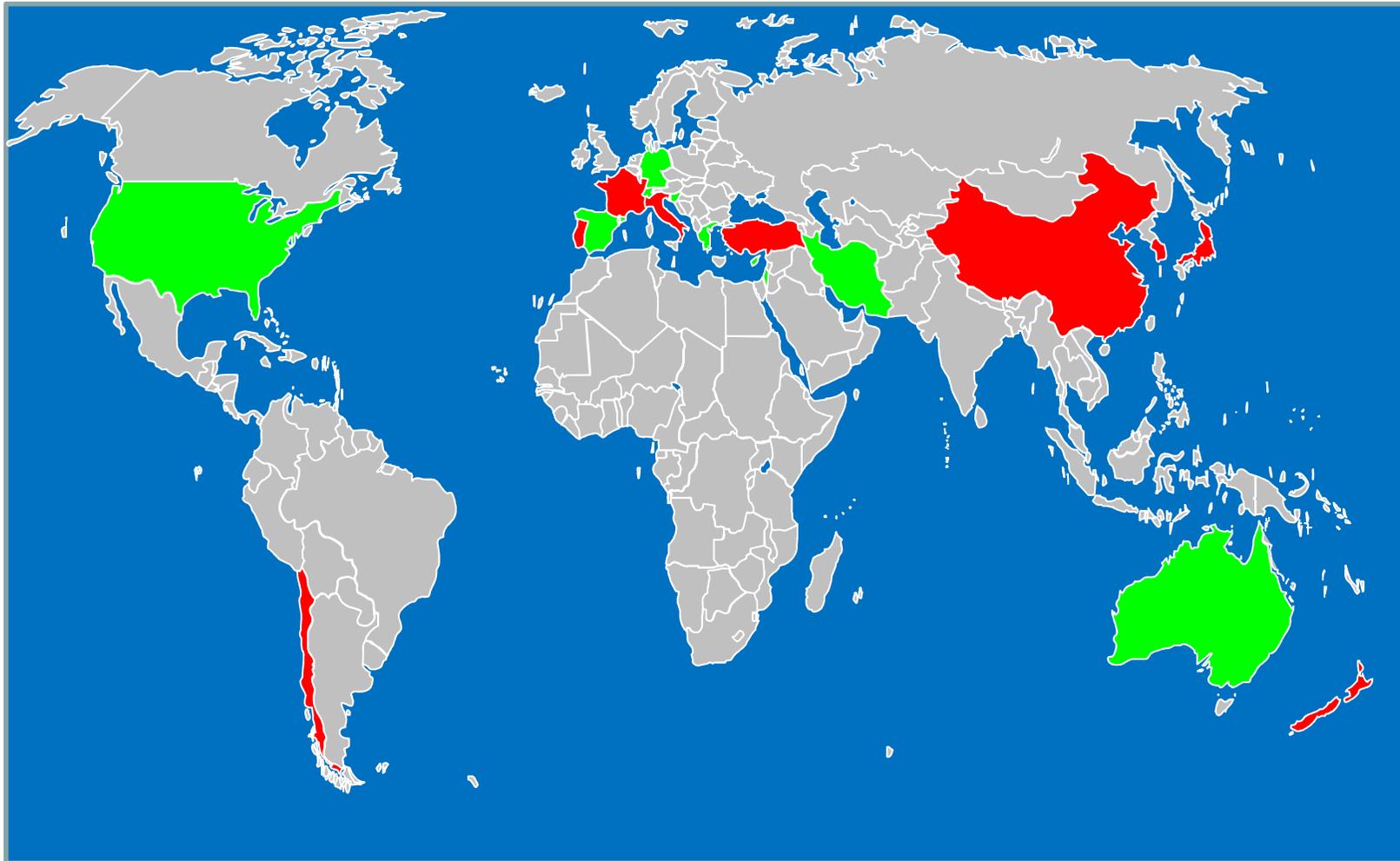
 **Kiwifruit producing countries**

 **Psa detected**



Psa detection

2010: France, Portugal, New Zealand, Chile, China



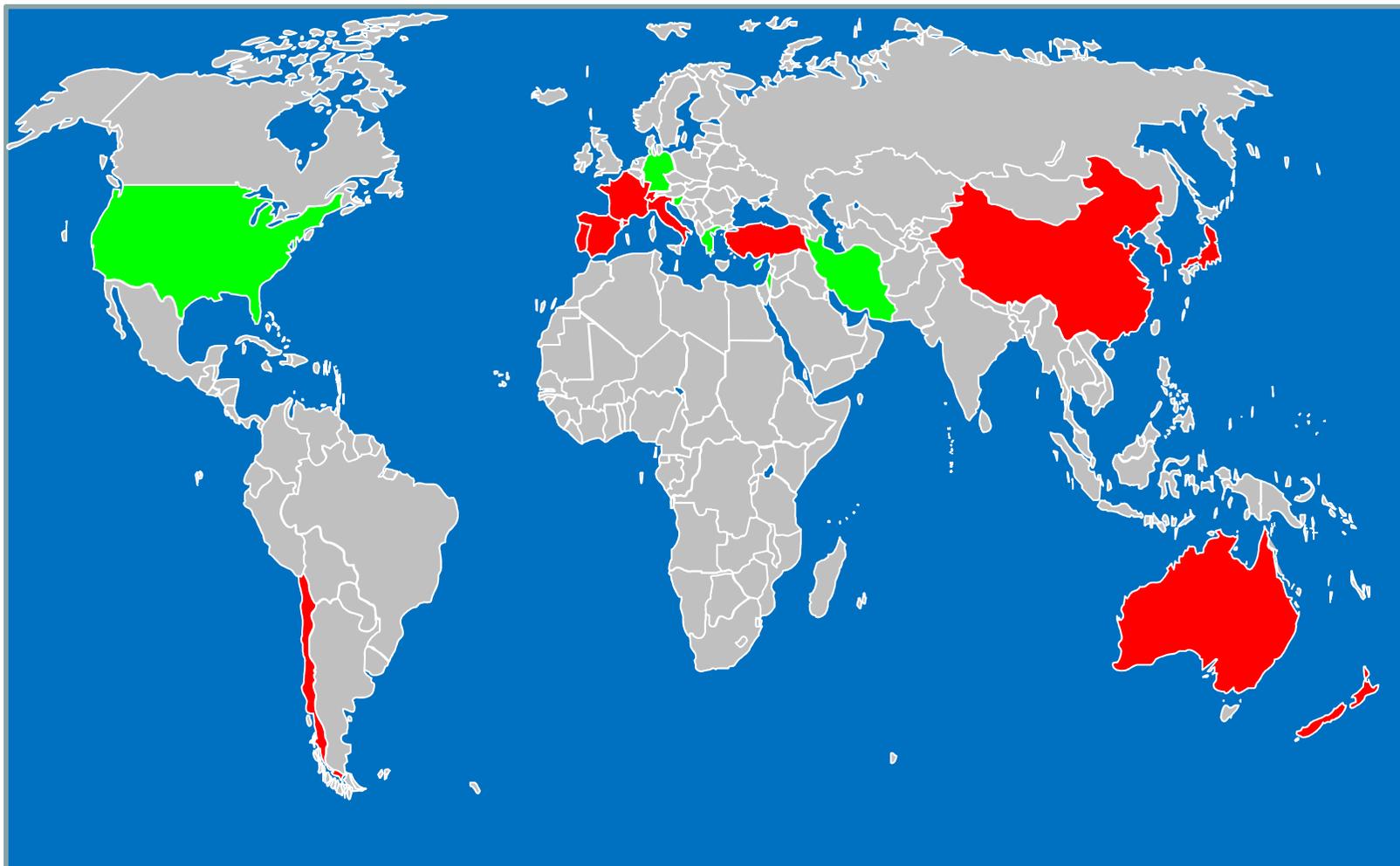
 **Kiwifruit producing countries**

 ***Psa* detected**



Psa detection

2011: Spain, Switzerland, Australia



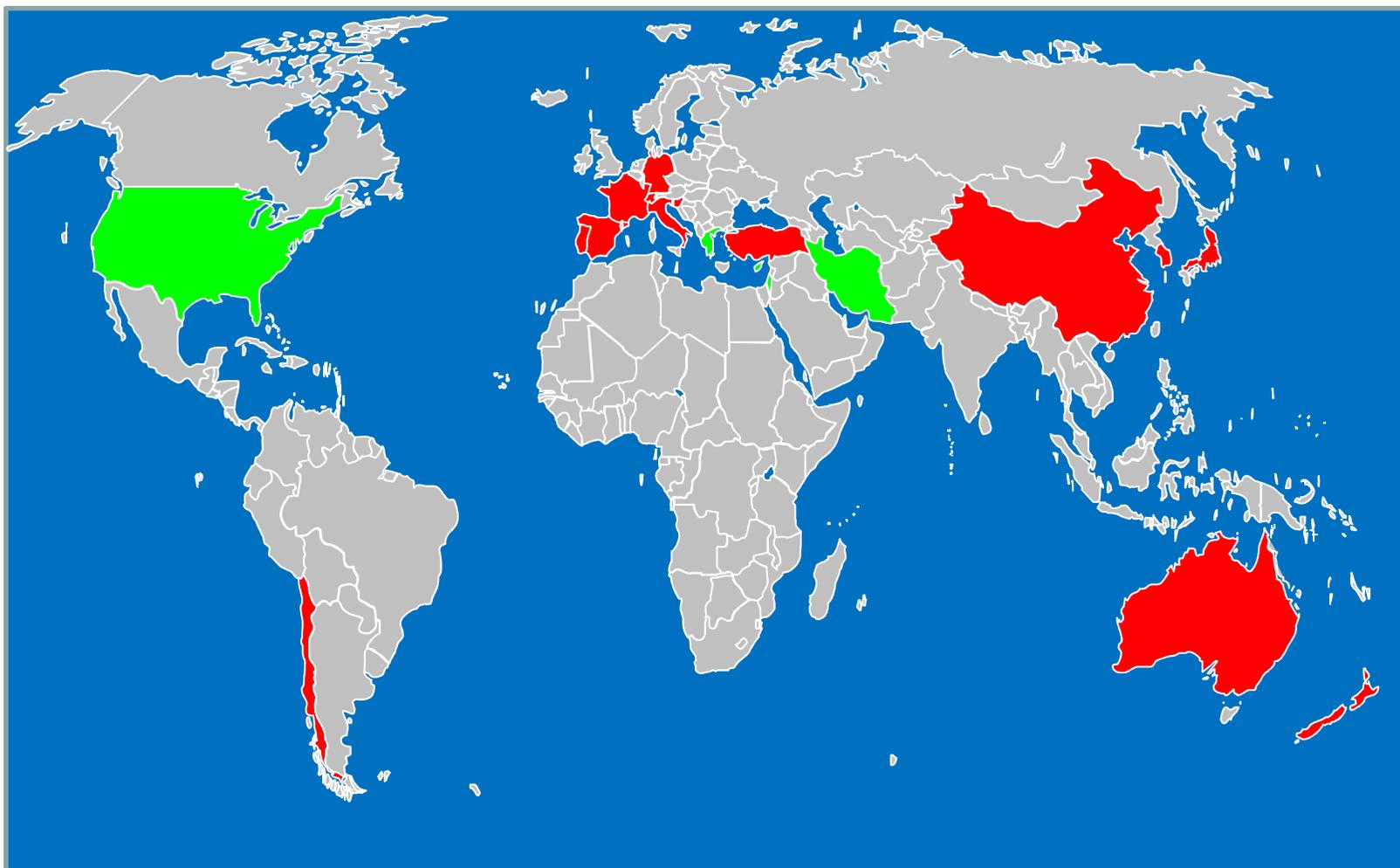
 **Kiwifruit producing countries**

 **Psa detected**



Psa detection

2013: Germany, Slovenia



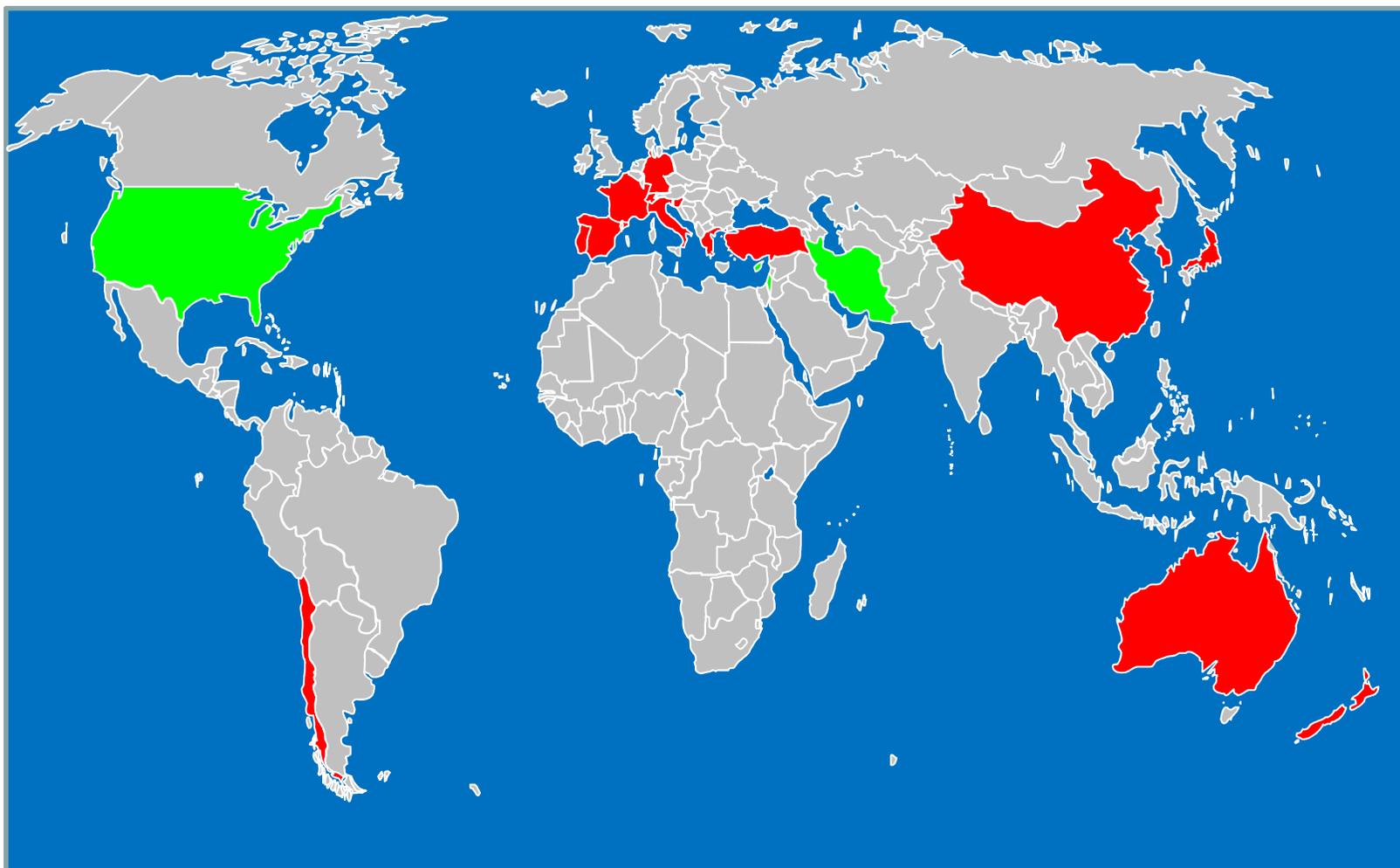
 **Kiwifruit producing countries**

 **Psa detected**



Psa detection

2014: Greece



 **Kiwifruit producing countries**

 **Psa detected**



Symptoms



Photo: SRAL Rhones Alpes

Angular necrotic spots on leaves surrounded or not by a yellow chlorotic halo



Photo LSV 2012 - Landes



Photo LSV 2012 Landes

Red or white ooze exuding from leads or buds



Photo LSV 2012 Landes

Browning and discoloration below the bark



Blossom necrosis



Photo Zespri 2012 - Rhones Alpes

Dieback



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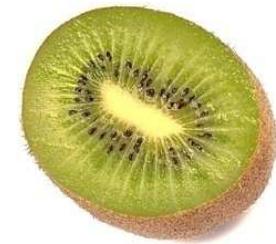
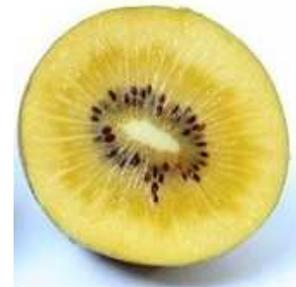
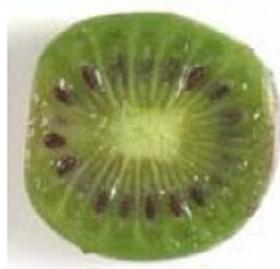
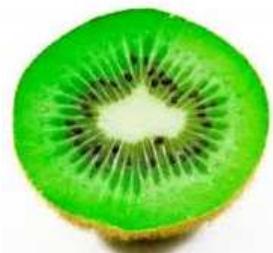
Dieback



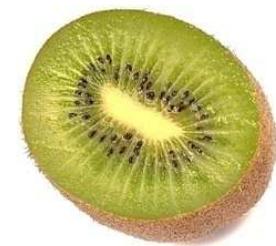
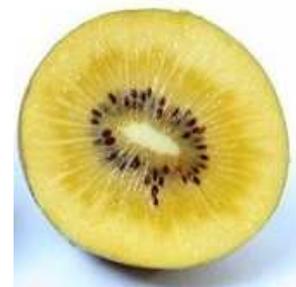
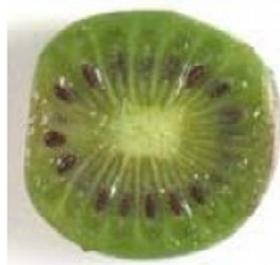
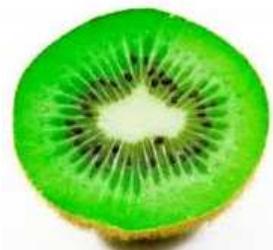
Objectives



- I. What is the **diversity** of *Psa* in France?
- II. What is the **origin** of the epidemic of *Psa* in France?
- III. How to improve the **detection** of *Psa*?



I. What is the diversity of *Psa* in France?





The situation in France

- Detection of *Psa* in France in **2010**



- The French Ministry of Agriculture established a **national monitoring plan** in 2011 to report the infected French orchards
- Since 2010 **280 strains** were isolated from symptomatic kiwifruit samples (LSV, Anses, Angers)



Two kiwifruit pathogens isolated in France

- *P. s. pv actinidiae* (*Psa*)
 - necrotic spot on leaves
 - canker on wood

→ severe yield losses



- *P. s. pv actinidifoliorum* (*Psaf*)
 - necrotic spot on leaves

→ No yield loss





Two kiwifruit pathogens isolated in France

- *P. s. pv actinidiae* (*Psa*)

	Phaseolotoxin	Coronatin	<i>avrD</i>
<i>Psa</i> biovar 1 (<i>Psa</i> b1)	+	-	-
<i>Psa</i> biovar 2 (<i>Psa</i> b2)	-	+	+
<i>Psa</i> biovar 3 (<i>Psa</i> b3)	-	-	+

(Vanneste *et al.*, 2013)



Two kiwifruit pathogens isolated in France

- *P. s. pv actinidiae* (*Psa*)

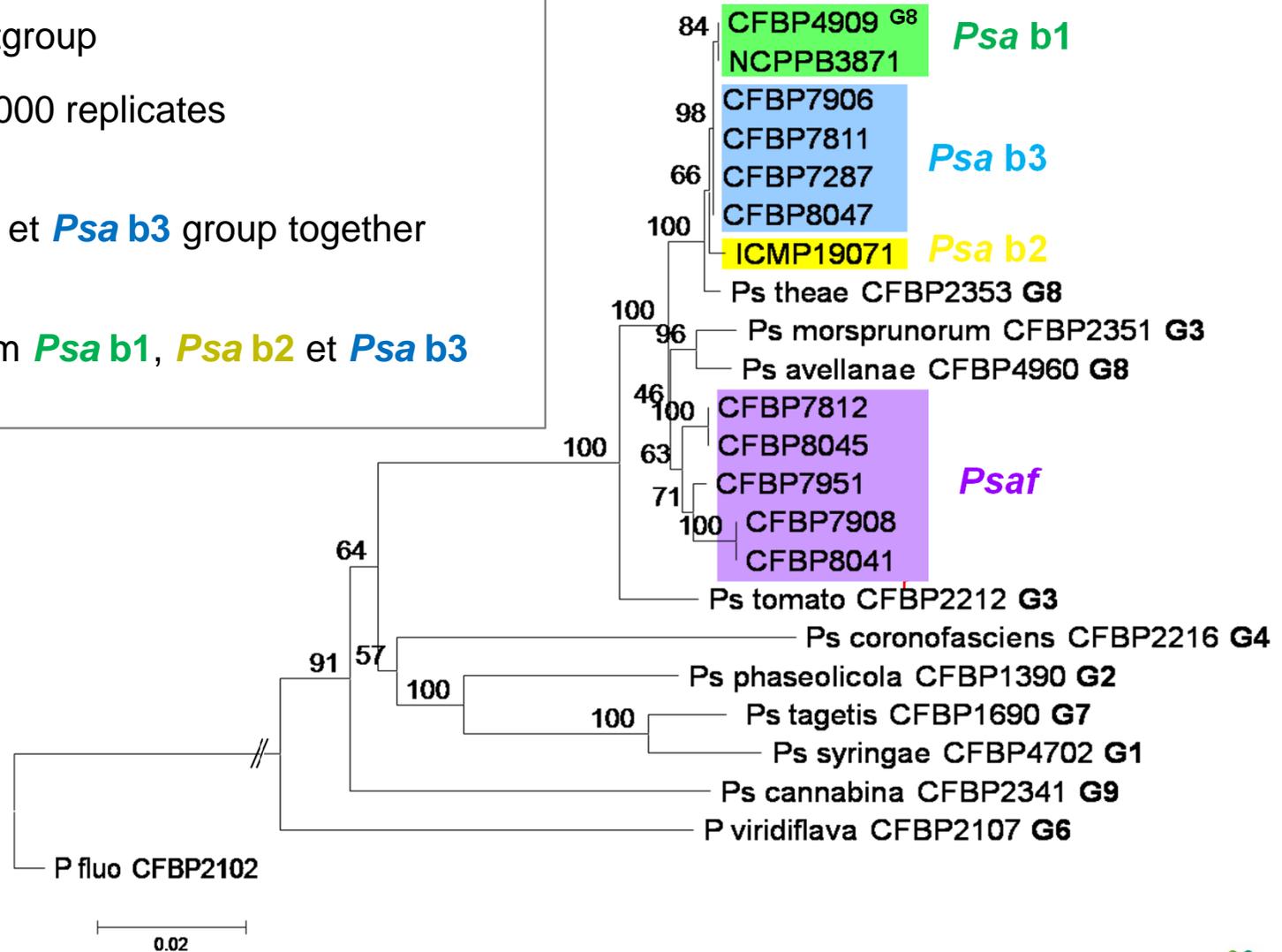
	Phaseolotoxin	Coronatin	<i>avrD</i>
<i>Psa</i> biovar 1 (<i>Psa</i> b1)	+	-	-
<i>Psa</i> biovar 2 (<i>Psa</i> b2)	-	+	+
<i>Psa</i> biovar 3 (<i>Psa</i> b3)	-	-	+

- Among the 280 strains isolated in France:
 - 248 strains → *Psa* b3
 - 32 strains → *Psaf*

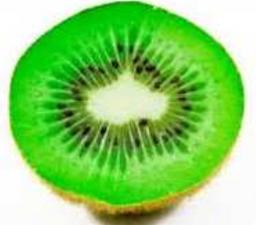


Phylogeny of *Psa* and *Psaf*

- NJ tree: *gapA*, *gltA*, *gyrB* et *rpoD*
 - *P. fluorescens*: outgroup
 - % bootstrap for 1 000 replicates
- *Psa b1*, *Psa b2* et *Psa b3* group together
- *Psaf* distant from *Psa b1*, *Psa b2* et *Psa b3*



Psaf differs from *Psa*



✓ Phenotypic level:

→ *Psaf*: esculine hydrolysis and fluorescent pigment

✓ Phylogenetic level:

→ *Psa* b1, b2 and b3 clustered together

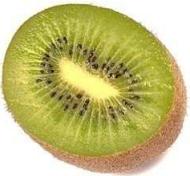
→ *Psaf* distant from *Psa*



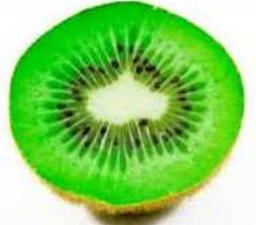
✓ Pathogenic level:

→ *Psa*: leaf symptoms + canker on wood; severe yield losses

→ *Psaf*: leaf symptoms; no yield loss



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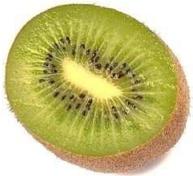
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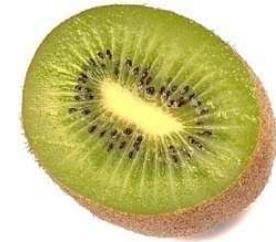
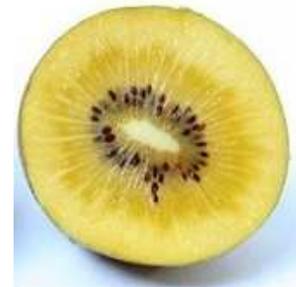
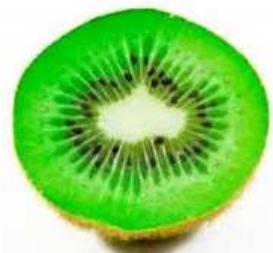


→ Identification of *actinidifoliorum* pathovar was relevant to:

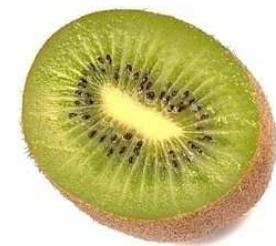
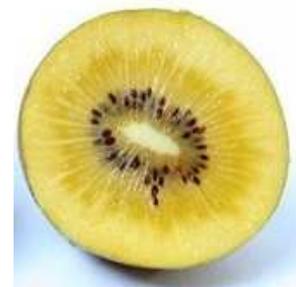
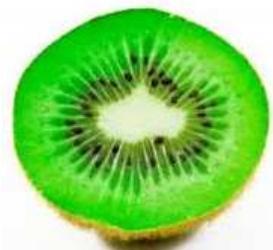
➤ assign to the pathovar *actinidiae* only the strains responsible of the **bacterial canker of kiwifruit**

➤ improve **legislative management options** in order to be only applied to the bacteria responsible for **kiwifruit canker**





II. What is the origin of the *Psa b3* epidemic in France?





The origin of the epidemic of *Psa* b3 in France

→ First *Psa* b3 detection in Europe:

- in Italy: **2008**
- in France: **2010**

→ Plant material exchange, at **the end of 2013**:

- *Psa* registered on the A2 list of EPPO
- The Commission of the European Union: phytosanitary passport required for plant material exchange



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❖ Hypothesis: *Psa* b3 introduced in France from **Italy** through infected plants

→ Study of the genetic structuring of the *Psa* b3 strains isolated in **France** and in **Italy**



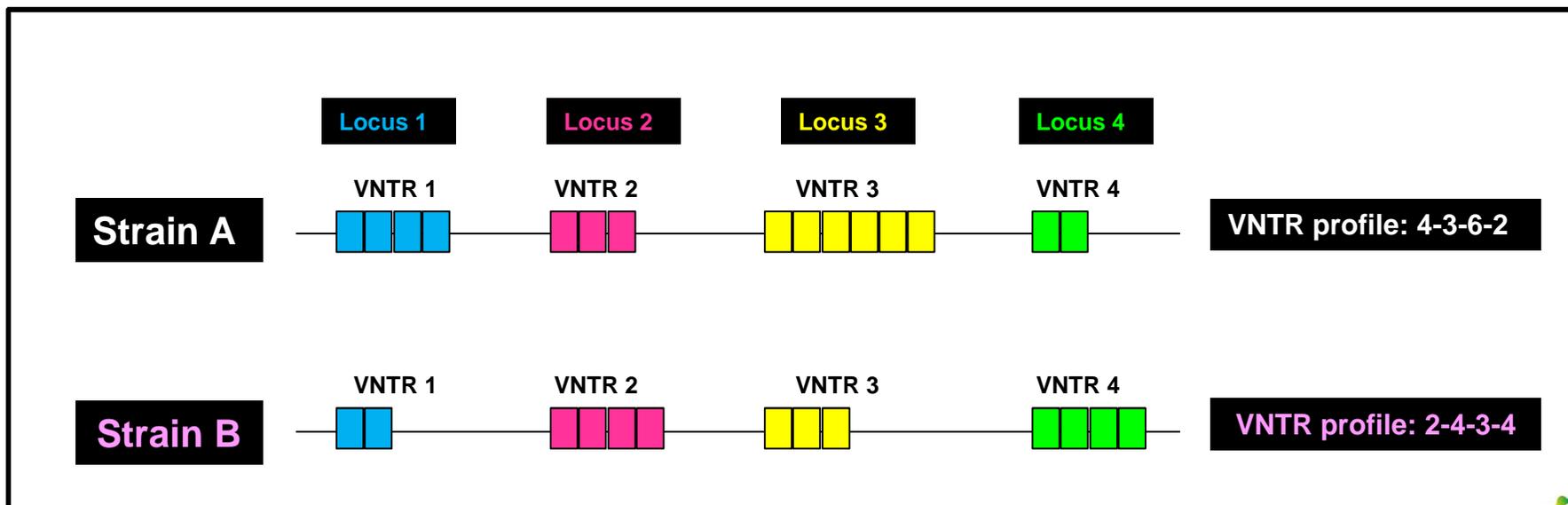
MLVA

- Multilocus Variable-number tandem repeats Analysis (**MLVA**)
- MLVA: powerfull technique used in **epidemiological monitoring** to study **bacterial genetic structure** at **infraspecies** and **infrapathovar** levels



MLVA

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- MLVA: powerful technique used in **epidemiological monitoring** to study **bacterial genetic structure** at **infraspecies** and **intrapathovar** levels
- Aim of MLVA: target variable number of tandem repeats (VNTRs)
- Detection of the TRs by PCR with primers designed on the flanking regions of each TR





MLVA

→ **340** strains of *Psa* b3 :

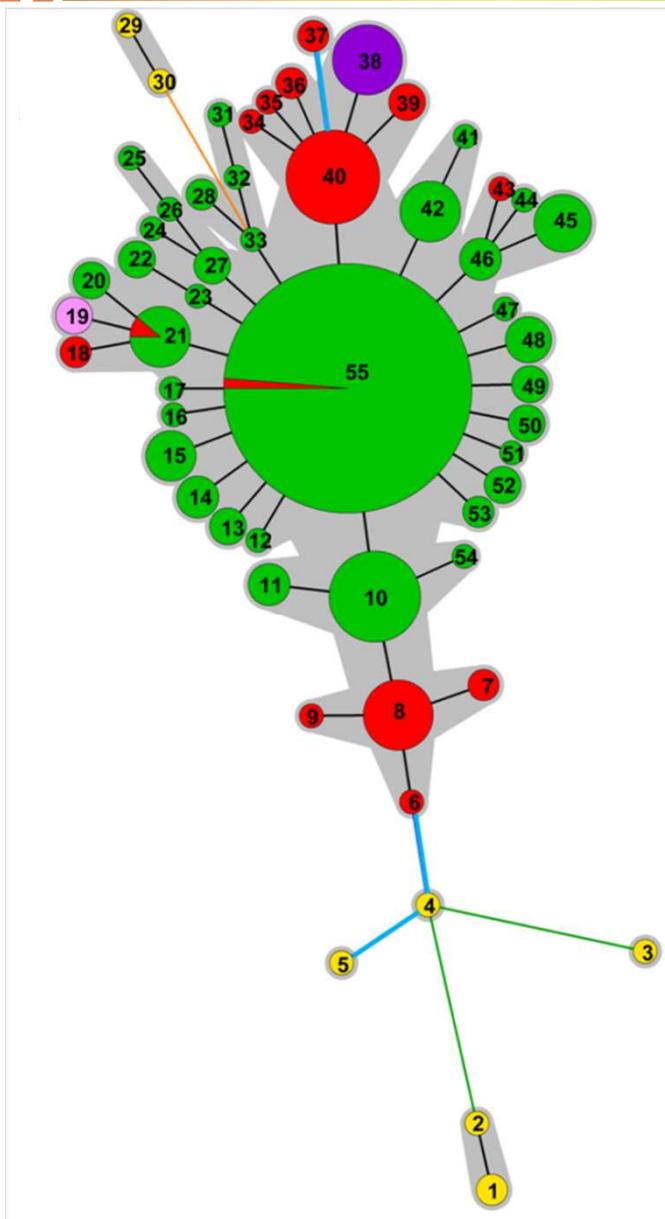
- *Psa* b3 **France:264**
- *Psa* b3 **Italy:53**
- *Psa* b3 **NZ:12**
- *Psa* b3 **China:8**
- *Psa* b3 **Chile:3**

→ **MLVA scheme:**

- Identification of **11 polymorphic VNTR loci**
- Development of primers in the VNTR loci flanking regions



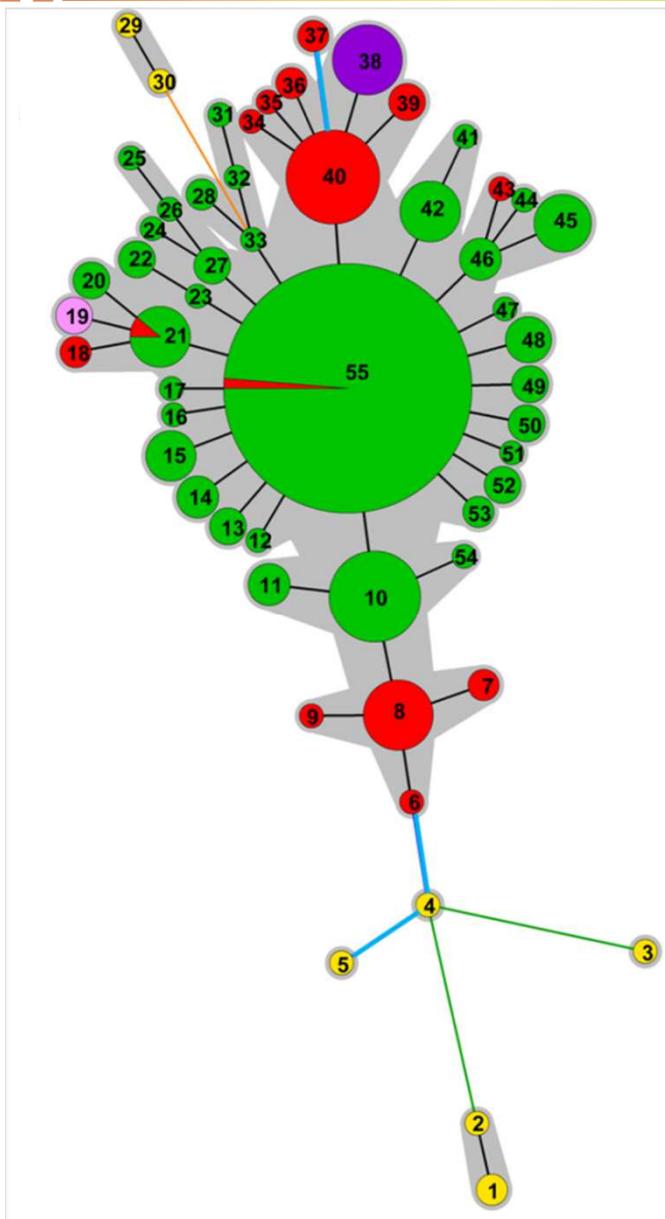
MLVA: *Psa* b3 MST



- 1 different locus
- 2 different loci
- 3 different loci
- 5 different loci
- 6 different loci



MLVA: *Psa* b3 MST



→ *Psa* b3: **55** haplotypes distinguished

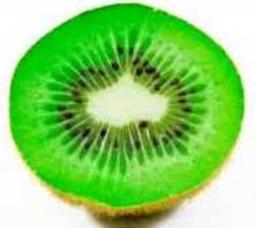
→ strain **geographical origin**:

- China 
- NZ 
- Chile 
- Italy 
- France 

→ Grey areas: clonal complexes including Single Locus Variant (SLV)

-  1 different locus
-  2 different loci
-  3 different loci
-  5 different loci
-  6 different loci

The origin of the epidemic of *Psa* b3 in France



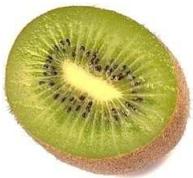
→ MLVA revealed that strains isolated in **France** and in **Italy** were genetically close related



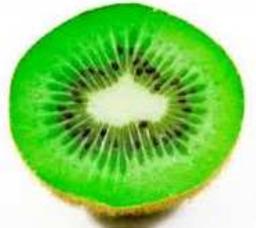
→ Multivariate method (**DAPC**), which allow identifying and describing clusters of genetically related individuals



→ DAPC confirmed MLVA results



The origin of the epidemic of *Psa* b3 in France



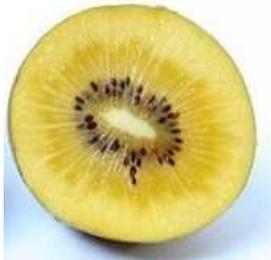
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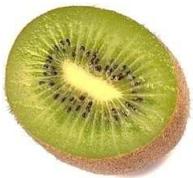
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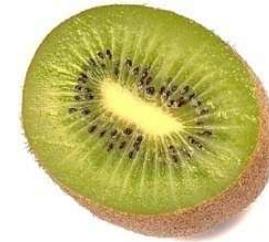
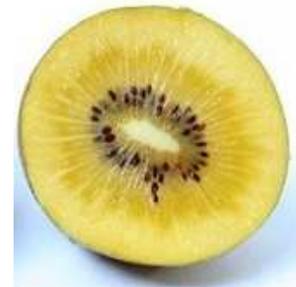
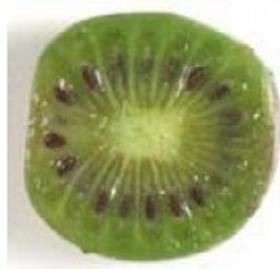
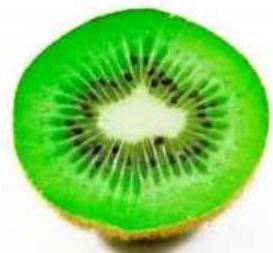


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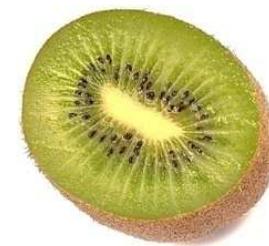
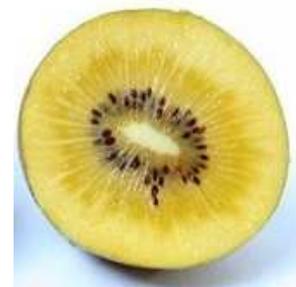
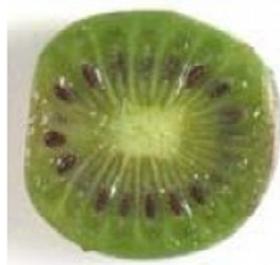
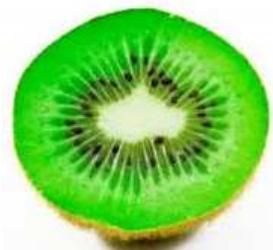


→ Results of MLVA and DAPC supported the hypothesis of a **common origin** (Cunty *et al.*, 2015 AEM)





III. How to improve the detection of *Psa*?





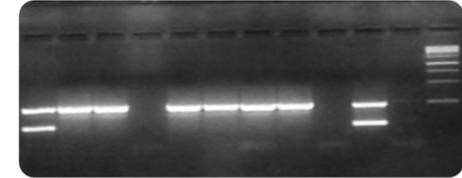
Development of new TaqMan real-time PCRs

Journal of Plant Pathology (2011), 93 (2), 425-435 Edizioni ETS Pisa, 2011

425

GENE SEQUENCE ANALYSIS FOR THE MOLECULAR DETECTION OF *PSEUDOMONAS SYRINGAE* pv. *ACTINIDIAE*: DEVELOPING DIAGNOSTIC PROTOCOLS

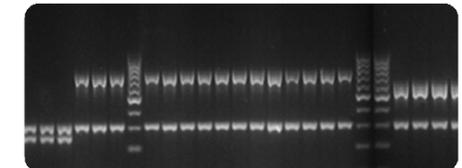
A. Gallelli, A. L'Aurora and S. Loreti



A Multiplex PCR Assay for Detection of *Pseudomonas syringae* pv. *actinidiae* and Differentiation of Populations with Different Geographic Origin

G. M. Balestra, Department of Science and Technologies for Agriculture, Forestry, Nature and Energy, and M. C. Taratufolo, Department of Science and Technologies for Agriculture, Forestry, Nature and Energy, University of Tuscia, Viterbo, Italy; B. A. Vinatzer, Department of Plant Pathology, Physiology, and Weed Science, Virginia Tech, Blacksburg; and A. Mazzaglia, Department of Science and Technologies for Agriculture, Forestry, Nature and Energy, University of Tuscia, Viterbo, Italy

472 Plant Disease / Vol. 97 No. 4

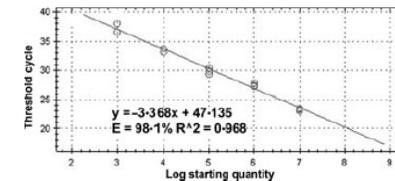


Plant Pathology (2013)

Doi: 10.1111/ppa.12082

Real-time and qualitative PCR for detecting *Pseudomonas syringae* pv. *actinidiae* isolates causing recent outbreaks of kiwifruit bacterial canker

A. Gallelli, S. Talocci, M. Pilotti† and S. Loreti†*

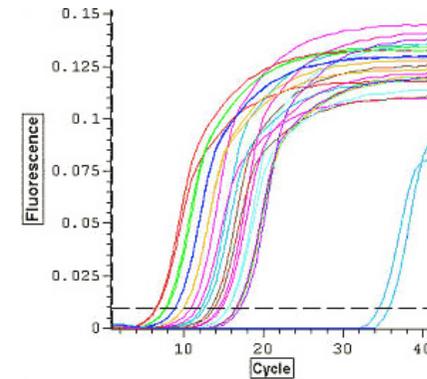




Development of new TaqMan real-time PCRs

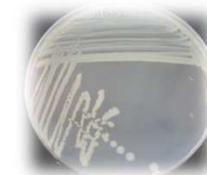
→ Objective develop simplex TaqMan real-time PCRs to detect:

- *Psa* b1
- *Psa* b2
- *Psa* b3
- pathovar *actinidiae* as a whole
- pathovar *actinidifoliorum* as a whole



→ Validation of the method:

- analytical sensitivity (limit of detection)
- analytical specificity (inclusivity and exclusivity)
- specificity





Gene selection, primer and probe design

- Genomic comparison of 49 *Psa* and *Psaf* genomes (44 available on NCBI and 5 *Psaf* genomes sequenced in this study (genome announcement submitted)):
- *Psa*: effector gene
 - *Psa b1*: effector gene
 - *Psa b2*: gene encoding hypothetical protein
 - *Psa b3*: effector gene
 - *Psaf*: gene encoding hypothetical protein



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Specificity validated by *in silico* nucleic BLAST on NCBI



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Development of specific primers and probes



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Development of specific primers and probes



Validation on a small strain collection



Analytical sensitivity

→ asymptomatic wood samples artificially contaminated with 10-fold bacterial series (from 10^7 to 10^3 CFU/mL)



Target organism	CFU/mL	CFU/PCR reaction
<i>Psa</i>	10^3	2
<i>Psa</i> b1	10^3	2
<i>Psa</i> b2	10^3	2
<i>Psa</i> b3	10^3	2
<i>Psaf</i>	10^4	20



Analytical specificity

→ **Inclusivity**: 15 target strains and **exclusivity**: 15 non-target strains



Analytical specificity

→ **Inclusivity:** 15 target strains and **exclusivity:** 15 non-target strains

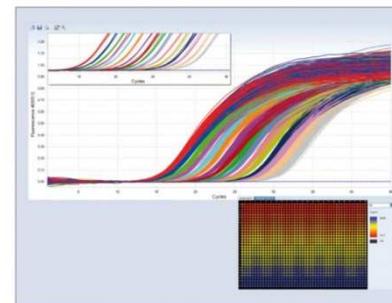
→ Specificity on a **large strain collection (416)**

<i>Pseudomonas</i>					Other genera	
<i>Psa</i> b1	<i>Psa</i> b2	<i>Psa</i> b3	<i>Psaf</i>	<i>P.syringae</i>	<i>Erwinia</i>	<i>Xanthomonas</i>
7	3	342	39	22	1	1

→ PCRs performed on the high-throughput platform IdentityPath of Anses



LightCycler 1536, Roche





Analytical specificity

Target organism	Inclusivity	Exclusivity	Specificity on 416 strains
<i>Psa</i>	100 %	100 %	100 %
<i>Psa b1</i>	100 %	100 %	100 %
<i>Psa b2</i>	100 %	100 %	98 %*
<i>Psa b3</i>	100 %	100 %	100 %
<i>Psaf</i>	100 %	100 %	100 %

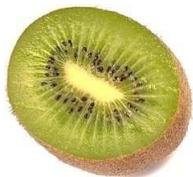
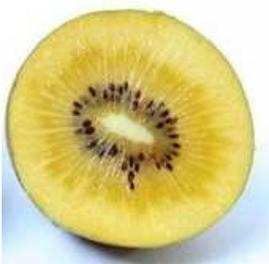
→ * false positives:

- 3 *Psa b3* strains isolated in France
- 3 *Pss* strains isolated from kiwifruit in France (Gaignard, 1984)

Specificity (naturally contaminated samples)

Target organism	12 wood <i>Psa b3</i> 	12 leaves <i>Psa b3</i> 	1 leaf <i>Psaf</i> 
<i>Psa</i>	12/12	12/12	0/1
<i>Psa b1</i>	0/12	0/12	0/1
<i>Psa b2</i>	0/12	0/12	0/1
<i>Psa b3</i>	12/12	12/12	0/1
<i>Psaf</i>	0/12	0/12	1/1

General conclusion

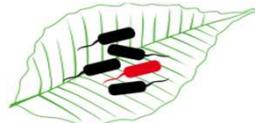


- ✓ The identification of *actinidifoliorum* pathovar was relevant to
 - include in the pathovar *actinidiae* only strains responsible of bacterial canker of kiwifruit
 - improve **legislative management options**
- ✓ MLVA scheme:
 - origin of *Psa* b3 epidemic in France is Italy
 - trace the epidemic routes of *Psa* b3 in **other countries** (New Zealand, Chile, Spain, ...)
- ✓ New TaqMan real-time PCRs:
 - good **sensitivity** and high **specificity**
 - useful for *Psa* and *Psaf* detection
 - perspective: multiplex the real-time PCRs



Investigate, evaluate, protect

Charles Manceau
Françoise Poliakoff
Corinne Audusseau
Sandrine Paillard
Christelle François
Carène Rivoal
Valérie Olivier

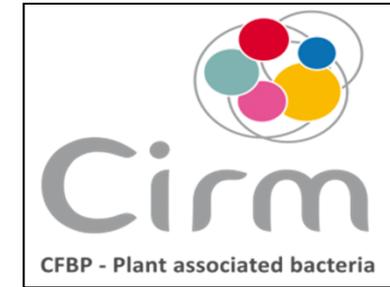


EMERSYS

Sophie Cesbron
Marie-Agnès Jacques
Marion Fisher-Le Saux
Perrine Portier
Martial Briand



Joël Vanneste



IdentityPath

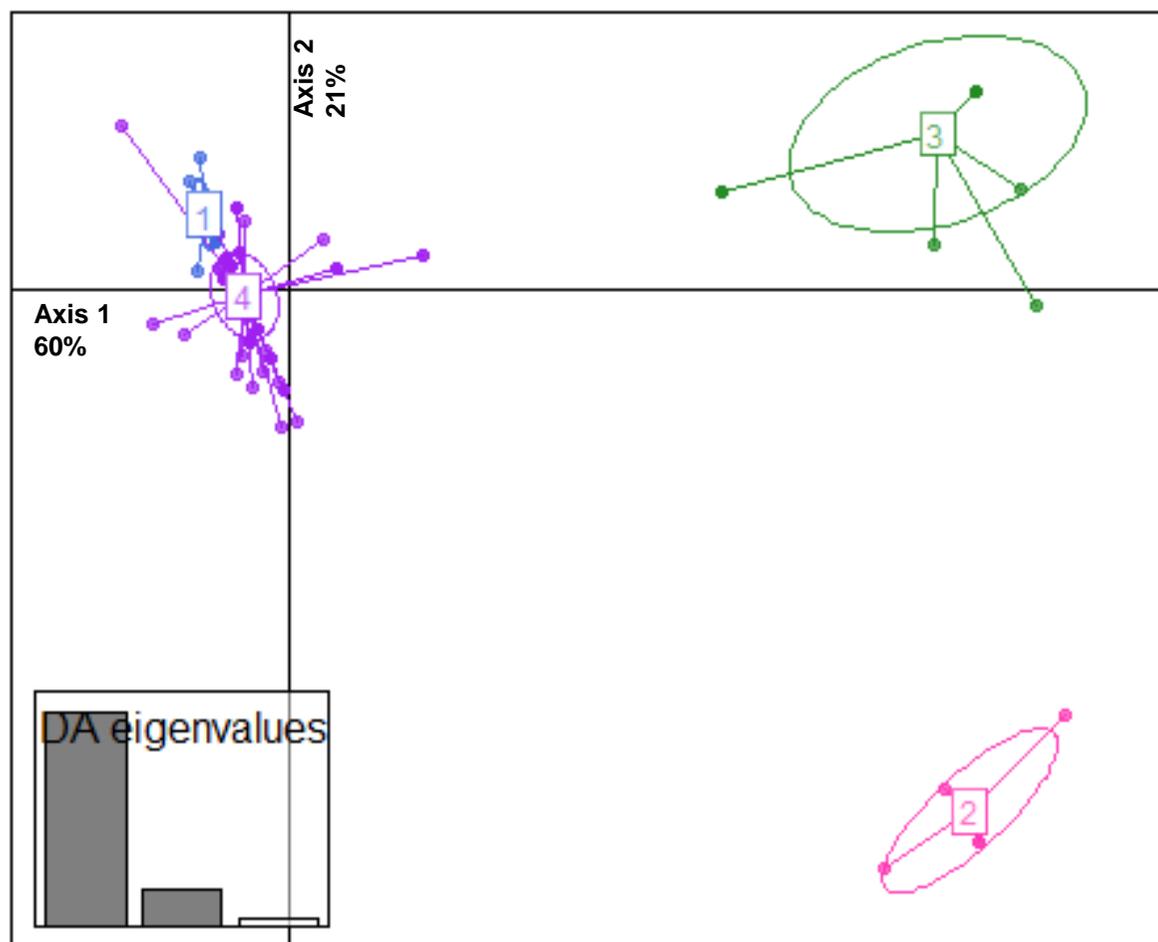


DAPC

→Statistic method determining **population structures**, which are **genetically different** by plotting them in a two-dimensional plan



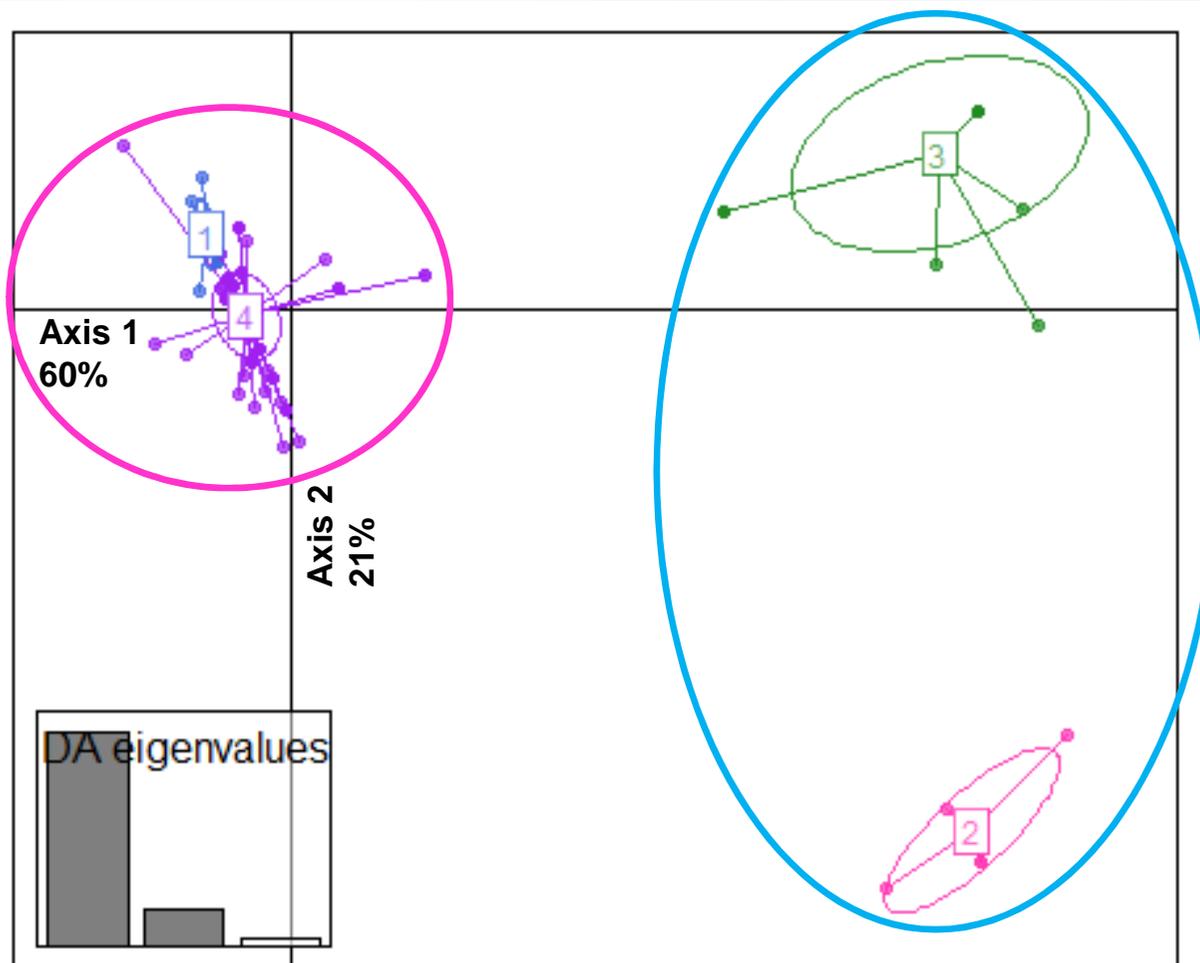
DAPC



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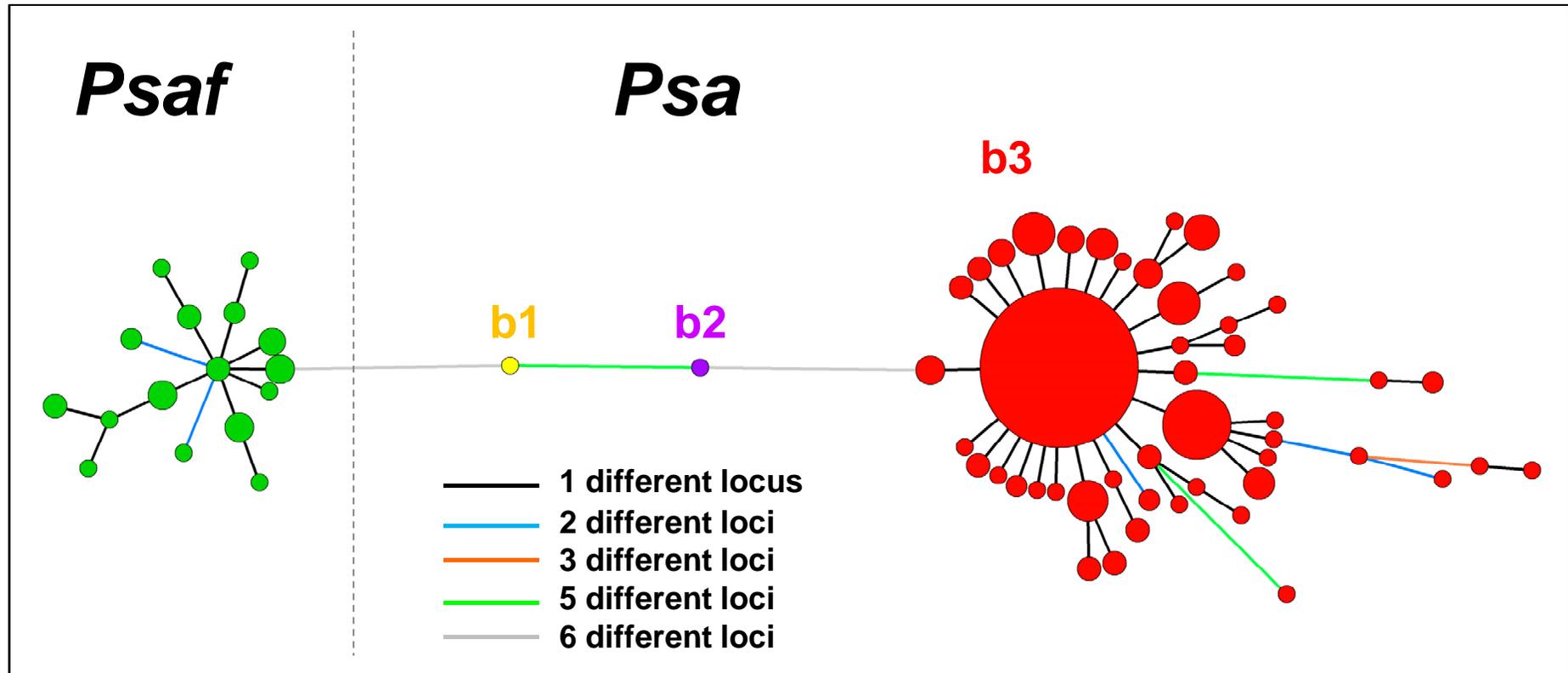
DAPC



- The **horizontal** axis allows the identification of **2 groups**:
- group 1: **France and Italy** (clusters 1 and 4)
 - group 2: **China, New Zealand and Chile** (clusters 2 and 3)



MLVA: Minimum Spanning Tree (MST)



→ Structuring of strains at the pathovar and biovar levels